



RESEARCH ARTICLE

In vitro inhibition of HIV-1 replication in autologous CD4⁺ T cells indicates viral containment by multifactorial mechanisms

Ting Tu^{1,2#}, Jianbo Zhan^{3#}, Danlei Mou⁴, Wei Li⁴, Bin Su⁴, Tong Zhang⁴, Tao Li¹, Ning Li⁴, Hao Wu⁴, Cong Jin^{2✉}, Huabiao Chen^{1✉}

1. Jiangsu Key Laboratory of Clinical Laboratory Medicine, Jiangsu University School of Medicine, Zhenjiang 212013, China
2. National HIV/HCV Reference Laboratory, National Center for AIDS/STD Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China
3. Hubei Provincial Center for Disease Control and Prevention, Wuhan 430079, China
4. Beijing You'an Hospital, Capital Medical University, Beijing 100069, China

HIV-1-specific cytotoxic T lymphocytes (CTLs) and neutralizing antibodies (NAbs) are present during chronic infection, but the relative contributions of these effector mechanisms to viral containment remain unclear. Here, using an *in vitro* model involving autologous CD4⁺ T cells, primary HIV-1 isolates, HIV-1-specific CTLs, and neutralizing monoclonal antibodies, we show that b12, a potent and broadly neutralizing monoclonal antibody to HIV-1, was able to block viral infection when preincubated with virus prior to infection, but was much less effective than CTLs at limiting virus replication when added to infected cell cultures. However, the same neutralizing antibody was able to contain viruses by antibody-dependent cell-mediated virus inhibition *in vitro*, which was mediated by natural killer cells (NKs) and dependent on an Fc-Fc receptor interaction. Meanwhile, bulk CTLs from HIV-1 controllers were more effective in suppression of virus replication than those from progressors. These findings indicate that control of HIV-1 replication in activated CD4⁺ T cells is ineffectively mediated by neutralizing antibodies alone, but that both CTLs and antibody-dependent NK-mediated immune mechanisms contribute to viral containment. Our study systemically compared three major players in controlling HIV-1 infection, CTLs, NAbs, and NKs, in an autologous system and highlighted the multifactorial mechanisms for viral containment and vaccine success.

KEYWORDS HIV-1 infection; viral replication; cytotoxic T lymphocyte (CTL); natural killer cells; neutralizing antibody

INTRODUCTION

The host immune response system is thought to be in-

involved in the control of HIV-1 infection. During acute HIV-1 infection, the viral load rises to a peak, which leads to activation of the cellular immune system (Kahn and Walker, 1998). This high-level plasma viremia typically declines dramatically once HIV-1-specific cytotoxic T lymphocyte (CTL)-mediated immune responses are mounted, probably reflecting the strong antiviral activities of these cells during acute infection (Borrow et al., 1994; Koup et al., 1994). The dramatically accelerated progression of simian immunodeficiency virus disease that is noted to occur in rhesus macaques following the depletion of CTLs also provides evidence for the importance of CTLs in the control of viruses (Schmitz et al.,

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These authors contributed equally to this work.

✉ Correspondence:

Huabiao Chen, Phone: +86-511-85038483,

Fax: +86-0511-85038483,

Email: chenhuabiao@hotmail.com

ORCID: 0000-0002-7215-5429

Cong Jin, Phone: +86-10-58900986, Fax: +86-10-58900986,

Email: jincongbj@163.com

ORCID: 0000-0002-5200-5992

1999). Moreover, enhanced ability of HIV-1-specific CTLs to inhibit virus replication in autologous CD4⁺ T cells was demonstrated in a rare subpopulation of HIV-1-infected individuals (Saez-Cirion et al., 2007; Chen et al., 2012), although these cells actually had low responses as measured at baseline by IFN- γ enzyme-linked immunospot (ELISpot) assays (Pereyra et al., 2008). These individuals maintain levels of viremia below the limit of detection by current assays for long periods in the absence of therapy (Cao et al., 1995; Deeks and Walker, 2007) and are referred to here as elite controllers. Similarly, HIV-1-specific neutralizing antibodies (NAbs) are often undetectable or at low titer during acute infection (Moore et al., 1994; Moog et al., 1997) and in elite controllers (Harrer et al., 1996; Bailey et al., 2006; Pereyra et al., 2008), suggesting that antibodies do not play a major role in viral control. These findings constitute a striking contrast to progressive HIV-1 infection, where high levels of virus replication occur in spite of the presence of strong HIV-1-specific CTL and NAb responses. In these progressive patients, HIV-1-specific CTL responses are of higher magnitude and more broadly directed, as measured by the assessment of IFN- γ production (Pereyra et al., 2008), and HIV-1-specific NAbs are at higher titer, as measured by in vitro neutralization of cell-free viruses (Bailey et al., 2006; Pereyra et al., 2008). The ability of these immune responses to inhibit virus replication in autologous CD4⁺ T cells, the ultimate function likely required of protective responses, has rarely been measured.

It has been recently reported that antibody-dependent cell-mediated virus inhibition (ADCVI), a measure of the combined potential of antibody and effector cells dependent on an Fc-Fc receptor (FcR) interaction, was able to contain virus infection rather than cause target cell death (Forthal et al., 2006). Passive infusion of neutralizing monoclonal antibodies has been demonstrated to prevent virus infection in rhesus macaques (Emini et al., 1992; Mascola et al., 2000; Hessel et al., 2007). Furthermore, antibody responses to vaccines have correlated with improved outcomes in macaques after virus challenge (Schmitz et al., 2005; Someya et al., 2005).

Given that a cellular immune response is well documented to contribute to control of HIV-1 infection, it is possible that enhanced antiviral efficacy against HIV-1 could be gained by a combination of NAbs and cell-mediated immunity (Hessel et al., 2007; Walker and Burton, 2008). In this study, we examined the potency of CTLs and natural killer cells (NKs) from HIV-1-infected individuals, in combination with engineered NAbs, to inhibit infectious virus replication in autologous CD4⁺ T cells. Our data indicated that both CTLs and antibody-dependent NK-mediated immunity contribute to viral containment.

MATERIALS AND METHODS

Study subjects

HIV-1-infected individuals were recruited on the basis of viral load from outpatient clinics at local hospitals, following institutional review board approval and written informed consent. Elite controllers were defined as having HIV-1 RNA below the level of detection for the respective available ultrasensitive assay (e.g., < 75 RNA copies/mL by bDNA or < 50 copies by ultrasensitive PCR) without antiretroviral therapy. Untreated chronic progressors were recruited locally on the basis of viral load above 10,000 RNA copies/mL plasma.

Viruses

The primary CXCR4-utilizing HIV-1 strain X4 92HT599 and CCR5-utilizing HIV-1 strain R5 91US056 were obtained from the AIDS Research and Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD, USA). The titers of the virus stocks were 2.57×10^6 TCID₅₀/mL and 2.14×10^6 TCID₅₀/mL, respectively.

Monoclonal antibodies

IgG1 b12 is an engineered monoclonal human antibody that recognizes an epitope overlapping the CD4-binding site of gp120 (Burton et al., 1994; Binley et al., 2004; Nie et al., 2014). LALA, a variant of b12, was created by site-directed mutagenesis (Hezareh et al., 2001). LALA retains antigen binding but binds weakly to human Fc γ receptors, triggers antibody-dependent cellular cytotoxicity (ADCC) with lower efficiency, and binds and activates human complement weakly (Hessel et al., 2007). IgG1 DEN3 is an isotype control monoclonal antibody specific for Dengue virus.

Effector cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of HIV-1-infected individuals by Ficoll-Hypaque density gradient centrifugation. Bulk CD8⁺ T cells and NKs were isolated from PBMCs by negative selection (Dynabeads, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The isolated CD8⁺ T cells and NKs were of up to 98% purity as validated by flow cytometry. The generated CD8⁺ T cells responding to viral proteins were fine-mapped using optimal peptide pools in IFN- γ ELISpot assays.

ELISpot assay

IFN- γ ELISpot assays were performed as described by Addo et al. (2003), using optimally defined epitope peptide pools. To calculate the number of specific spot-forming cells (SFC), the number of spots in the negative control wells was subtracted from the number of spots counted in each well. Negative controls were always 30 SFC per 10^6 input cells or fewer.



Antibody neutralization assay

Virus neutralization was performed using the method described by Trkola *et al.* (1995) with slight modifications. Briefly, CD4⁺ T cells were generated from freshly isolated PBMCs by the addition of CD3: CD8 bispecific monoclonal antibody (Wilson *et al.*, 1995; Wong *et al.*, 1997). After 5–7 days, greater than 98% of these cells coexpressed CD3 and CD4 by flow cytometric analysis (data not shown). Antibodies were adjusted to 200 µg/mL, and eight twofold serial dilutions were prepared. An aliquot (50 µL) of each dilution step was transferred to four replicate wells of a 96-well culture plate. The virus inoculum was adjusted to 2000 TCID₅₀/mL, and a 50-µL (100 TCID₅₀) aliquot was incubated with antibodies (50 µL) for 1 h at 37 °C. The calculated neutralization titers refer to the antibody concentrations present during this preincubation step. The antibody-virus mixture was then diluted twofold by addition of 100 µL of CD4⁺ T cells at a density of 1×10^6 /mL, and the cultures were incubated for 7 days. The cultures were collected and treated with 0.5% Triton, and the p24 antigen concentration was determined by a standard quantitative enzyme-linked immunosorbent assay (HIV-1 p24 ELISA kit, PerkinElmer, Inc., Waltham, Massachusetts, USA). As the antibody-virus inoculum was not washed away from the cells at any stage, control wells containing 50 µL of virus and 150 µL of medium were included to provide an estimate of the amount of input p24 antigen that carried over into the p24 antigen assay for virus production. This value was subtracted from all the test results. The antibody concentrations (µg/mL) causing 50%, 90%, and 99% reduction in p24 antigen production were determined by linear regression analysis; these are the ID₅₀, ID₉₀, and ID₉₉ values.

Antibody-dependent cell-mediated virus inhibition (ADCVI)

Inhibition of viral replication was assessed in a previously established assay system (Yang *et al.*, 2002; Forthal *et al.*, 2006; Hessel *et al.*, 2007) with slight modifications. Briefly, autologous CD4⁺ T cells were infected with designated HIV-1 strains at a multiplicity of infection (MOI) 0.01 for 4 h at 37 °C, washed twice, and plated at 5×10^4 cells per well in a 96-well round-bottom plate. Various dilutions of b12, LALA mutant, or isotype control DEN3 antibodies were added to virally infected cells alone or along with effector cells, either CD8⁺ T cells or NKs, at a ratio of 1:1 or as otherwise specified in a total of 200 µL RPMI 1640 medium containing 50 U/mL interleukin-2 (IL-2). After 7 days of incubation at 37 °C in 5% CO₂, supernatant was collected and p24 antigen was quantified by a standard quantitative enzyme-linked immunosorbent assay (HIV-1 p24 ELISA kit, Dupont, Boston, MA, USA). Virus inhibition was calculated as

follows: Percentage inhibition = $100 \times (1 - \text{p24p}/\text{p24n})$, where p24p and p24n are the concentrations of p24 in supernatant from wells with or without a source of effectors, respectively.

Statistical analyses

Mann-Whitney tests were performed using GraphPad Prism version 4.0a, and *P*-values of *P* < 0.05 were considered significant.

RESULTS

Neutralization of HIV-1 by antibodies

Using two primary HIV-1 isolates, X4 92HT599 and R5 91US056, we first tested the ability of monoclonal antibody b12 and its mutated Fc- and complement-binding variant LALA to neutralize viruses using CD4⁺ T cells from elite controllers as indicator cells. The outgrowth of autologous viruses in CD4⁺ T cells from the elite controllers was markedly delayed, and there were no autologous viruses grown out from the tested subjects during the period of the assay. Representative neutralization titration curves are depicted in Figure 1A.

Mean and median neutralization

The ID₉₀ for both X4 and R5 virus for b12 were in the range of 10 to 25 µg/mL, and LALA was comparable to b12 with an ID₉₀ in the range of 10 to 30 µg/mL. These data showed that both b12 and the LALA variant demonstrated neutralization of cell-free X4 and R5 primary isolates, but for this inoculum of virus a relatively high concentration of antibody was required to achieve 95% neutralization. Moreover, the data showed that both b12 and LALA, which lacks FcγR and complement binding functions compared with b12, mediate comparable neutralization of these primary viruses.

Next, we tested the ability of these antibodies to contain viruses in virally infected CD4⁺ T cells from the elite controllers in whom outgrowth of autologous viruses in CD4⁺ T cells was markedly delayed. CD4⁺ T cells were infected with primary HIV-1 isolates prior to the addition of antibodies. Designated concentrations of b12, LALA mutant, or isotype control DEN3 antibodies were added to virally infected cells. The p24 antigen production in supernatant was evaluated and expressed as the percentage of neutralization (Figure 1B). Both b12 and LALA failed to effectively control viral replication in infected CD4⁺ T cells as the p24 antigen production in supernatant steadily increased during the period of 7 days even with antibody at 100 µg/mL, far in excess of the range estimates for concentration of an antibody produced in vivo. These data suggest that although this antibody is potent in neutralizing free virus, its ability to neutralize virus spread once infection has occurred is

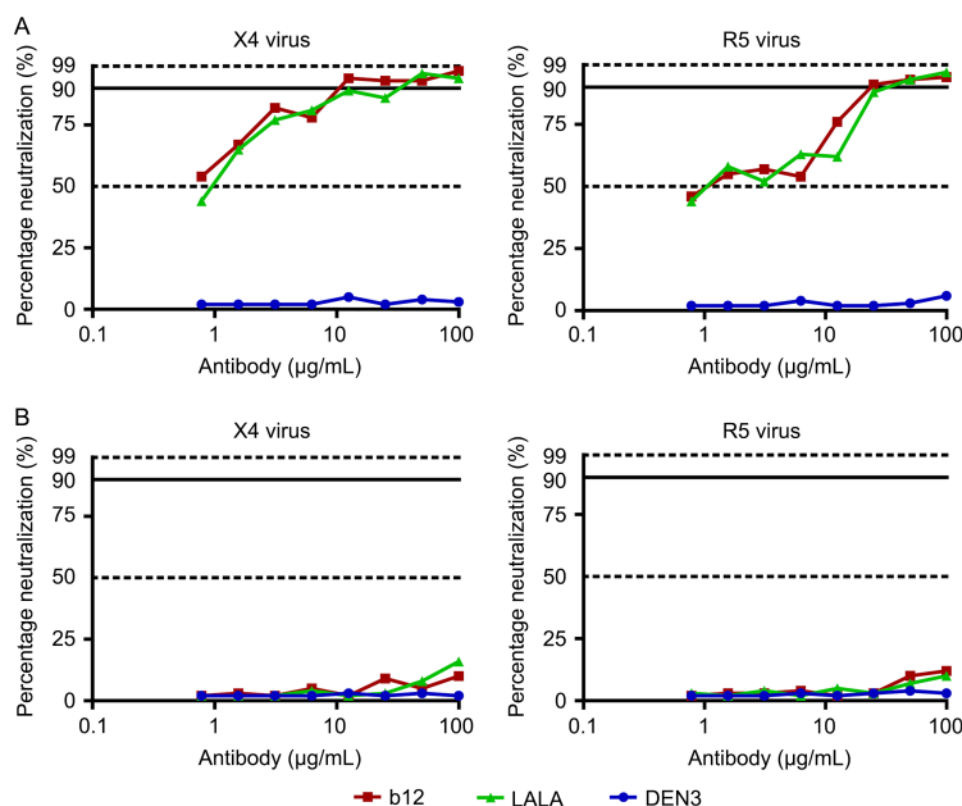


Figure 1. Neutralization of HIV-1 by antibodies. (A) Monoclonal antibodies were serially diluted and incubated with 100 TCID₅₀/mL of the indicated primary viruses for 1 h at 37°C prior to adding to CD4⁺ T cells. (B) Monoclonal antibodies were serially diluted and incubated with CD4⁺ T cells that had been infected with HIV-1 at an MOI of 0.01 4 h earlier. The extent of virus replication was monitored by measurement of p24 antigen production after 7 days. The percentage of neutralization is plotted against antibody concentrations. ID₅₀, ID₉₀, and ID₉₉ values are indicated.

very limited, which is likely due to cell-cell spread of virus in this in vitro culture system, but may also be related to production of high numbers of progeny virions that overwhelm the available antibody binding sites.

Suppression of HIV-1 by CTLs

We next tested the ability of bulk CD8⁺ T cells from HIV-1 elite controllers and chronic progressors to inhibit virus replication in the same cell culture system, using autologous CD4⁺ T cells preincubated for 4 h with the same HIV-1 strains at the same MOI (0.01) at which no effect was observed using the antibodies alone (Chen et al., 2009). For these experiments, we chose elite controllers and chronic progressors in whom outgrowth of autologous viruses in CD4⁺ T cells was markedly delayed, allowing us to use a fixed inoculum of primary viruses in these experiments. As shown as an example in Figure 2A, addition of autologous bulk CD8⁺ T cells from an elite controller to virally infected CD4⁺ T cells at a CD8⁺ to CD4⁺ T cell ratio of 1 to 1 resulted in marked inhibition of virus replication over 7 days in culture, whereas weak inhibition of virus replication was observed in coculture of virally infected CD4⁺ T cells with autologous bulk CD8⁺ T cells from a progressor (Figure 2B).

We next expanded these studies to include 12 elite controllers and 10 chronic progressors, examining the ability of their CTLs to inhibit the same strain of HIV-1

in autologous CD4⁺ T cells. Marked inhibition of virus replication was observed at a CD8⁺ T to CD4⁺ T cell ratio of 1 to 1 for the elite controllers whereas there was weak inhibition of virus replication by bulk CTLs from the chronic progressors (Figure 2C). The difference was highly statistically significant ($P < 0.0001$). The magnitude of HIV-1-specific CTL responses in bulk CTLs was evaluated by optimally defined epitope pools in IFN- γ ELISpot assays (Figure 2D). Although the ability of CTLs to retard virus replication was greater in the elite controllers, the magnitude of HIV-1-specific CTL responses as measured in the elite controllers was actually weaker than those in the progressors (3973 ± 1935 vs. 7324 ± 3691 SFC per million bulk CTLs, $P = 0.0138$). These data indicate that HIV-1-specific CTLs from elite controllers have an enhanced antiviral functional capacity, contributing to the control of virus infection, which is not measured by standard IFN- γ ELISpot assays, suggesting IFN- γ ELISpot approaches may be of limited value in defining CTL functions such as the ability to neutralize virus replication. These data also demonstrate that CTLs from elite controllers can significantly inhibit replication of reference strains of primary isolates in cell culture, whereas a potent NAb, although highly effective when exposed to virus pre-innoculation, is ineffective once initial infection of the cells has occurred.

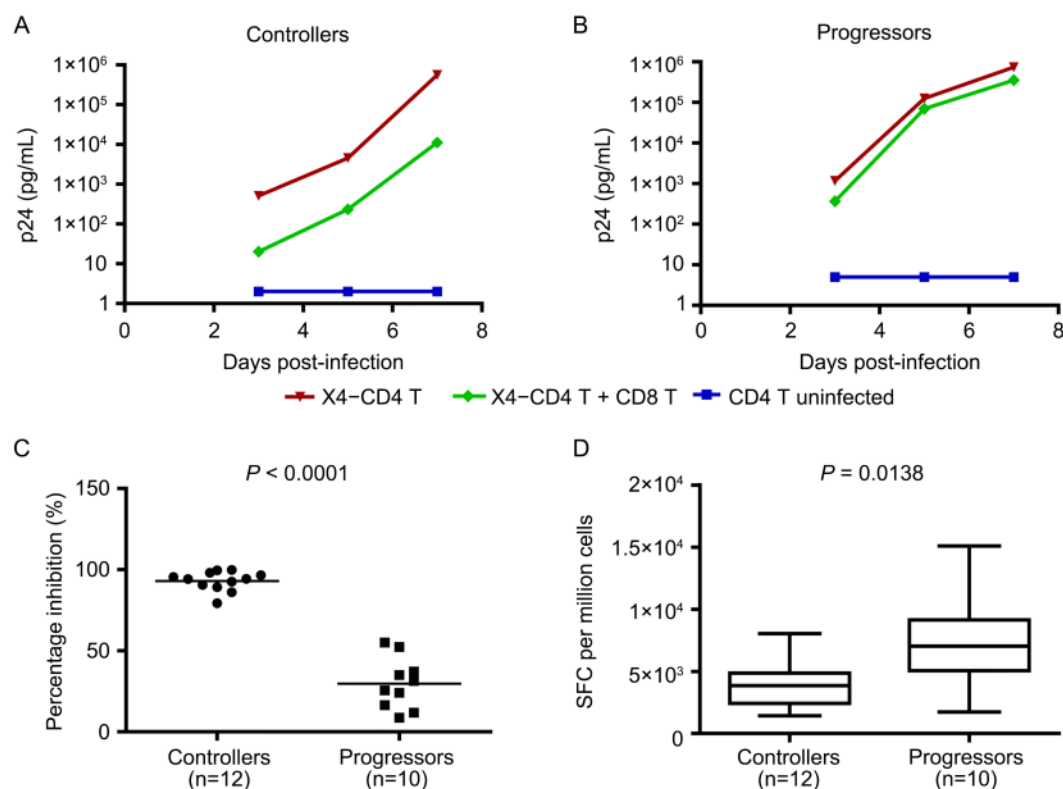


Figure 2. Suppression of HIV-1 by CTLs. The antiviral ability of bulk CD8⁺ T cells was assessed by an *in vitro* virus inhibition assay, by adding CTLs to autologous CD4⁺ cells infected with X4 virus at an MOI of 0.01 4 h earlier. (A) Bulk CD8⁺ T cells from an elite controller effectively inhibited HIV-1 replication at a 1:1 ratio of CD8⁺ to CD4⁺ T cells during a period of 7 days. Results from one experiment are shown as an example. (B) Bulk CD8⁺ T cells from a progressor had weak antiviral efficacy at a 1:1 ratio of CD8⁺ to CD4⁺ T cells. Results from one experiment are shown as an example. The control uninfected CD4⁺ T cells showed that there were no autologous viruses grown out from the tested subjects during the period of the assay. (C) Bulk CD8⁺ T cells from the controllers (n = 12) were more effective than those from the progressors (n = 10) in control of virus replication. Data are expressed as the percentage of inhibition, calculated as $100 \times (1 - \text{p24 with effectors} / \text{p24 without effectors})$ at day 7 in culture. (D) IFN- γ ELISpot study showed HIV-1-specific CTL responses in bulk CD8⁺ T cells from elite controllers (n = 12) were weaker than those from chronic progressors (n = 10). In the box and whisker plot, the line indicates the mean, the box indicates lower quartile and upper quartile, the whisker indicates the minimal and the maximal numbers, Statistical comparisons were made using the Mann-Whitney test.

We next addressed the possibility that enhanced antiviral efficacy could be gained by a combination of CTLs and NAb. In the infected cell cultures, serially diluted concentrations of NAb b12 were added along with CTLs. However, there was no significantly enhanced neutralization observed with added antibodies even at the highest concentration of 100 $\mu\text{g/mL}$ for CTLs from HIV-1 elite controllers (Figure 3A) or chronic progressors (Figure 3B). Given ADCVI is reported to be dependent on Fc-FcR interaction (Forthal et al., 2006; Hessel et al., 2007), we next determined FcR expression at the surface of the HIV-1-specific CTLs by flow cytometry. Little or no expression of various kinds of FcR was detected on the surface of the HIV-1-specific CTLs (data not shown). Using a previously reported assay (Liu et al., 2009), degranulation of intracellular vesicles (CD107a expression) mediated

by Fc-FcR interaction was examined. There was no CD107a upregulation on the CTLs after stimulation with IgG-loaded p815 cells compared with stimulation with p815 cells alone or medium control (data not shown). These data may explain the above finding that enhanced antiviral efficacy against HIV-1 could not be gained by ADCVI mediated by HIV-1-specific CTLs.

Inhibition of HIV-1 by ADCVI

NKs have been reported to serve as main effector cells and in combination with NAb to mediate ADCVI as described previously (Forthal et al., 2006; Hessel et al., 2007; Liu et al., 2009; Asmal et al., 2011). We next evaluated ADCVI of NKs from virally infected individuals in combination with NAb. Autologous CD4⁺ T cells were infected with primary X4 virus and cocultured

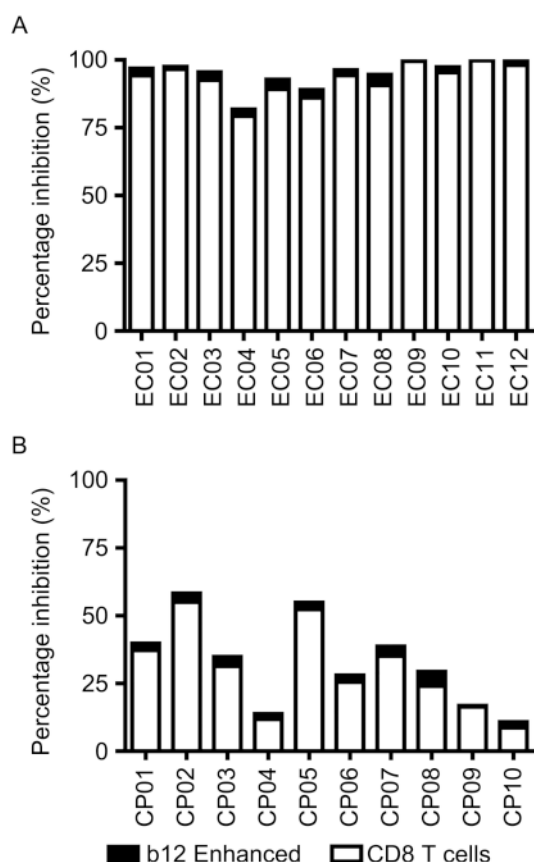


Figure 3. Inhibition of HIV-1 by CTLs in the presence of NABs. Autologous CD4⁺ cells infected with X4 virus at an MOI of 0.01 for 4 h. NAb b12 at 100 µg/mL was added along with bulk CD8⁺ T cells from (A) elite controllers (EC, *n* = 12) or (B) chronic progressors (CP, *n* = 10) at a 1:1 ratio of CD8⁺ to CD4⁺ T cells during a period of 7 days of culture. Data are expressed as the percentage of inhibition. There was no significantly enhanced inhibition to the bulk CD8⁺ T cells with added NAb b12.

with NKs alone or along with designated concentrations of antibodies. As shown as an example in Figure 4A, addition of autologous NKs to virally infected CD4⁺ T cells resulted in significantly enhanced inhibition of virus replication over 7 days in culture containing 50 U/mL IL-2 and this enhanced inhibition was mediated by antibody b12 but not by LALA or the isotype control monoclonal antibody DEN3. From the study of the 12 elite controllers and 10 chronic progressors, weak inhibition of primary X4 virus was observed by NKs alone from the controllers (Figure 4B) or progressors (Figure 4C), which may be mediated by direct killing of NKs through activation of receptors on NKs that interact with major histocompatibility complex class I molecules on HIV-1-infected CD4⁺ T cells (Bonaparte and Barker,

2004; Vieillard et al., 2005). However, significantly enhanced inhibition ($P < 0.01$) was gained by addition of antibody b12 at a concentration of 100 µg/mL to NKs for both the controllers (Figure 4B) and progressors (Figure 4C) whereas there was no statistical difference in the antiviral activity of NKs alone ($P = 0.5704$) or antibody-mediated enhanced inhibition ($P = 0.8336$) between the controllers and progressors. Moreover, this impact by ADCVI was mediated by antibody b12 but not by LALA, further demonstrating that antiviral potency by ADCVI is dependent on Fc-FcR interaction.

DISCUSSION

The relative contributions of various immune mechanisms to control of ongoing viral replication remain elusive. These data, employing a functional assay that measures the ability of effector cells, neutralizing monoclonal antibodies, and the combined power of these effectors to inhibit viral replication of primary HIV-1 isolates in autologous CD4⁺ T cells, indicate that NAb b12 is highly potent in neutralizing free viruses but is limited in containment of cell-cell spread of viruses once infection has occurred. In contrast, bulk CTLs from HIV-1 controllers are effective in containment of viruses under conditions where little or no effect is seen from NABs. Moreover, bulk CTLs from HIV-1 controllers are more effective in suppression of virus replication than those from progressors. However, the same b12 NAb is able to contain viruses by ADCVI. Importantly, these data demonstrate that ADCVI is mediated by NKs and dependent on an Fc-FcR interaction, suggesting innate immune mechanisms such as NKs or monocytes/macrophages that generally express FcRs may contribute to control of ongoing viral replication by ADCVI in the presence of functional antibodies. However, these data and previous findings that the high-level plasma viremia declines dramatically once HIV-1-specific CTL responses are mounted (Borrow et al., 1994; Koup et al., 1994) probably reflect that the HIV-1-specific CTLs are the major effector cells in containment of viruses whereas the mounted NK response during acute HIV-1 infection is not enough to contain viruses.

The goal of HIV-1 vaccination strategies is to have an immunologic barrier in place that will prevent infection or limit virus replication once infection occurs, thereby limiting symptoms of disease caused by virus infection (Wang et al., 2016). A priority in HIV-1 vaccine development has been the generation of NABs, ideally to provide sterilizing immunity, but this has remained an elusive goal. Only one antibody-based AIDS vaccine has been taken through efficacy trials thus far, using the gp120 subunit protein as an immunogen, and this

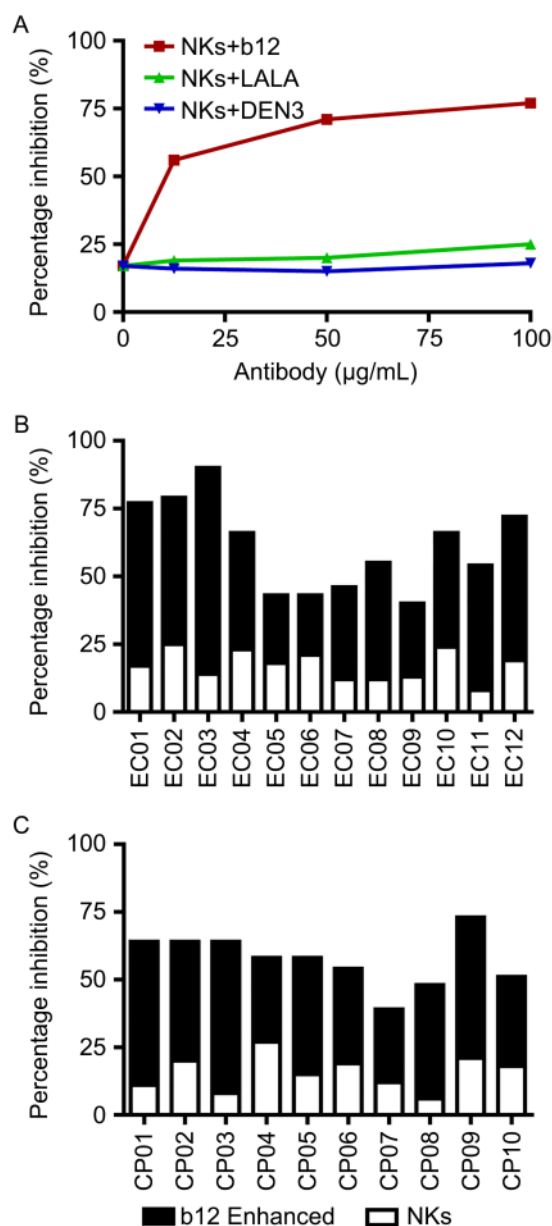


Figure 4. Inhibition of HIV-1 by ADCVI. Autologous CD4⁺ T cells were infected with primary X4 virus at an MOI of 0.01 for 4 h and then incubated with NKs alone or along with designated concentrations of b12, LALA mutant, or isotype control DEN3 antibodies. The extent of virus replication was monitored by measurement of p24 antigen production after 7 days. (A) The percentage of inhibition of an example experiment was plotted against antibody concentration. (B) ADCVI was mediated by NKs from elite controllers (EC, *n* = 12) in the presence of NAb b12 at 100 μg/mL. (C) ADCVI was mediated by NKs from chronic progressors (CP, *n* = 10) in the presence of NAb b12 at 100 μg/mL.

vaccine candidate did not elicit antibodies neutralizing field isolates of HIV-1, did not prevent infection, and did

not affect subsequent viral load (Flynn, 2005). Although the recent RV144 Thai trial showed moderate protection against viral acquisition (Gilbert *et al.*, 2011), the protection mechanisms remain unclear (Kim *et al.*, 2015). The most important second line of defense is the cellular immune response, including NKs and CTLs as the presumed major effector arms. Together with previous findings (Yang *et al.*, 1996; Yang *et al.*, 1997; Yang *et al.*, 2003; Chen *et al.*, 2009), our studies have demonstrated that both CTLs alone and NKs in the presence of functional antibodies can suppress HIV-1 replication *in vitro*. However, not all CTL responses created in natural HIV-1 infection are equally potent in terms of the ability to inhibit virus replication. The limitation of this study is that all these antiviral factors were assessed *in vitro*. It is critical to define *in vivo* HIV-1-specific CTL responses for viral control during natural infection and vaccine protection against HIV-1.

There is a remarkable subset of HIV-1-infected individuals, here named elite controllers, who are able to maintain a state of apparent durable control of HIV-1 replication without the need for antiviral therapy, providing clear evidence that durable containment of HIV-1 in the absence of therapy is possible. Some evidence for the involvement of CTL responses (Migueles *et al.*, 2002; Zimmerli *et al.*, 2005; Betts *et al.*, 2006) was documented for the mechanisms of virus control in these cases. In this study, a virus suppression assay was used to assess the antiviral potential of CTL responses from these subjects versus chronic progressors. As compared with chronic progressors, bulk CTLs from elite controllers had higher capacities to inhibit virus replication in infected autologous CD4⁺ T cells, which is consistent with the finding by Saez-Cirion and his colleagues that HIV-1 controllers rather than progressors exhibited potent CTL capacity to suppress HIV-1 infection *ex vivo* (Saez-Cirion *et al.*, 2007). Factors that potentially modulate protective CTL responses include, among others, polyfunctionality, antigen sensitivity or functional avidity, proliferative capacity, loading of lytic granules, *ex vivo* expression of perforin, T cell antigen receptor (TCR) clonotypes, and properties of the interactions among TCRs, viral peptide, and the major histocompatibility complex (Chen *et al.*, 2012). Collectively, this evidence strongly argues that potent CTL responses directed at HIV-1 are causally related to HIV-1 control in at least some controllers. However, many of these studies are by necessity correlative in nature, making it difficult to rule out the possibility that the preservation of potent HIV-1-specific CTL responses is a consequence rather than a cause of HIV-1 control. Although NKs might exert innate antiviral immunity in acute HIV-1 infection, the mounted NK cell response during acute HIV-1 infection does not appear to be enough to contain viruses. The role of NKs in chronic HIV-1 infection

in elite controllers and progressors remains unclear. Unraveling the mechanisms of HIV-1 control in these cases should be a high priority because this may facilitate the design of immunogens able to elicit the corresponding protective responses.

It has been reported that certain engineered monoclonal antibodies and primary antibodies from HIV-1-infected individuals, in the presence of effector cells, were able to inhibit HIV-1 replication *in vitro* (Forthal et al., 2001; Forthal et al., 2006) and modulate viremia *in vivo* dependent on Fc-FcR interactions (Hessell et al., 2007). This control of virus replication involving not only cytolytic but also noncytolytic mechanisms was distinguished from ADCC and termed ADCVI by Forthal and his colleagues (Forthal et al., 2006). Given that HIV-1-specific CTLs tested in this study do not express FcRs (data not shown), they would be unlikely to mediate ADCVI, although they showed highly potent ability to inhibit virus replication and limit cell-cell spread of viruses. NKs were reported to serve as the effector cells in ADCVI (Forthal et al., 2001; Forthal et al., 2005; Forthal et al., 2006) because NKs express various kinds of FcRs and can therefore interact with antibodies to modulate antiviral activity. It is to be expected that there are other subsets of cells, such as monocytes/macrophages, which express FcRs, involved in ADCVI and that contribute to viral control. ADCVI also allows the possibility that antibodies, passively transferred via a viral vector, could be competent to control viruses in the presence of effector cells, overcoming the barrier that antibodies that neutralize HIV-1 are often absent or at low titer in natural HIV-1 infection. The protection mechanisms for HIV-1 containment need to be further investigated with broader CTLs, NABs, NKs, or other factors in the context of acute infection.

Taken together, our study, for the first time to our knowledge, provides a systemic comparison of three major players, NABs, CTLs, and NKs, in an autologous system to assess neutralization, cytotoxicity, and ADCVI on HIV-1 replication in CD4⁺ T cells, and shows that no single arm thus far can be defined as the correlate of complete protection of HIV-1 infection. Alternative factors or combinations of multiple factors must be pursued. Our findings provide an important consideration for HIV-1 control and vaccine success that may be crucially dependent on the combination of antibody and cell-mediated immunity, as well as the innate and adaptive immunity.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors declare that they have no conflict of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee.

AUTHOR CONTRIBUTIONS

HBC and CJ designed and supervised the experiments, TT, JBZ, TL, DM, WL, BS, and TZ collected samples and carried out the experiments, HBC, CJ, TT, and JBZ analyzed the data and wrote the paper. NL and HW provided helpful suggestions about the study. All authors read and approved the final manuscript.

REFERENCES

- Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, Strick D, Johnston MN, Corcoran C, Wurcel AG, Fitzpatrick CA, et al. 2003. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J Virol*, 77: 2081–2092.
- Asmal M, Sun Y, Lane S, Yeh W, Schmidt SD, Mascola JR, Letvin NL. 2011. Antibody-dependent cell-mediated viral inhibition emerges after simian immunodeficiency virus SIVmac251 infection of rhesus monkeys coincident with gp140-binding antibodies and is effective against neutralization-resistant viruses. *J Virol*, 85: 5465–5475.
- Bailey JR, Lassen KG, Yang HC, Quinn TC, Ray SC, Blankson JN, Siliciano RF. 2006. Neutralizing Antibodies Do Not Mediate Suppression of Human Immunodeficiency Virus Type 1 in Elite Suppressors or Selection of Plasma Virus Variants in Patients on Highly Active Antiretroviral Therapy. *J Virol*, 80: 4758–4770.
- Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, Lederman MM, Benito JM, Goepfert PA, Connors M, Roederer M, Koup RA. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8⁺ T cells. *Blood*, 107: 4781–4789.
- Binley JM, Wrinn T, Korber B, Zwick MB, Wang M, Chappey C, Stiegler G, Kunert R, Zolla-Pazner S, Katinger H, Petropoulos CJ, Burton DR. 2004. Comprehensive Cross-Clade Neutralization Analysis of a Panel of Anti-Human Immunodeficiency Virus Type 1 Monoclonal Antibodies. *J Virol*, 78: 13232–13252.
- Bonaparte MI, Barker E. 2004. Killing of human immunodeficiency virus-infected cells by CD8⁺ T cells. *J Virol*, 78: 13253–13262.



- ciency virus-infected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules. *Blood*, 104: 2087–2094.
- Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. 1994. Virus-specific CD8⁺ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*, 68: 6103–6110.
- Burton DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, Parren PW, Sawyer LS, Hendry RM, Dunlop N, Nara PL, et al. 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. *Science*, 266: 1024–1027.
- Cao Y, Qin L, Zhang L, Safrit J, Ho DD. 1995. Virologic and Immunologic Characterization of Long-Term Survivors of Human Immunodeficiency Virus Type 1 Infection. *N Engl J Med*, 332: 201–208.
- Chen H, Ndhlovu ZM, Liu D, Porter LC, Fang JW, Darko S, Brockman MA, Miura T, Brumme ZL, Schneidewind A, et al. 2012. TCR clonotypes modulate the protective effect of HLA class I molecules in HIV-1 infection. *Nat Immunol*, 13: 691–700.
- Chen H, Piechocka-Trocha A, Miura T, Brockman MA, Julg BD, Baker BM, Rothchild AC, Block BL, Schneidewind A, Koibuchi T, Pereyra F, Allen TM, Walker BD. 2009. Differential Neutralization of Human Immunodeficiency Virus (HIV) Replication in Autologous CD4 T Cells by HIV-Specific Cytotoxic T Lymphocytes. *J Virol*, 83: 3138–3149.
- Deeks SG, Walker BD. 2007. Human Immunodeficiency Virus Controllers: Mechanisms of Durable Virus Control in the Absence of Antiretroviral Therapy. *Immunity*, 27: 406–416.
- Emini EA, Schleif WA, Nunberg JH, Conley AJ, Eda Y, Tokiyoshi S, Putney SD, Matsushita S, Cobb KE, Jett CM, Eichberg JW, Murthy KK. 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature*, 355: 728–730.
- Flynn NM FD, Harro CD, Judson FN, Mayer KH, Para MF; The rgp120 HIV Vaccine Study Group. 2005. Placebo-Controlled Phase 3 Trial of a Recombinant Glycoprotein 120 Vaccine to Prevent HIV-1 Infection. *J Infect Dis*, 191: 654–665.
- Forthal DN, Landucci G, Cole KS, Marthas M, Becerra JC, Van Rompay K. 2006. Rhesus macaque polyclonal and monoclonal antibodies inhibit simian immunodeficiency virus in the presence of human or autologous rhesus effector cells. *J Virol*, 80: 9217–9225.
- Forthal DN, Landucci G, Daar ES. 2001. Antibody from Patients with Acute Human Immunodeficiency Virus (HIV) Infection Inhibits Primary Strains of HIV Type 1 in the Presence of Natural-Killer Effector Cells. *J Virol*, 75: 6953–6961.
- Forthal DN, Landucci G, Phan TB, Becerra J. 2005. Interactions between Natural Killer Cells and Antibody Fc Result in Enhanced Antibody Neutralization of Human Immunodeficiency Virus Type 1. *J Virol*, 79: 2042–2049.
- Gilbert PB, Berger JO, Stablein D, Becker S, Essex M, Hammer SM, Kim JH, Degruittola VG. 2011. Statistical interpretation of the RV144 HIV vaccine efficacy trial in Thailand: a case study for statistical issues in efficacy trials. *J Infect Dis*, 203: 969–975.
- Harrer T, Harrer E, Kalams SA, Elbeik T, Staprans SI, Feinberg MB, Cao Y, Ho DD, Yilma T, Caliendo AM, Johnson RP, Buchbinder SP, Walker BD. 1996. Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Res Hum Retroviruses*, 12: 585–592.
- Hessell AJ, Hangartner L, Hunter M, Havenith CEG, Beurskens FJ, Bakker JM, Lanigan CMS, Landucci G, Forthal DN, Parren PW, Marx PA, Burton DR. 2007. Fc receptor but not complement binding is important in antibody protection against HIV. *Nature*, 449: 101–104.
- Hezareh M, Hessell AJ, Jensen RC, van de Winkel JGJ, Parren PW. 2001. Effector Function Activities of a Panel of Mutants of a Broadly Neutralizing Antibody against Human Immunodeficiency Virus Type 1. *J Virol*, 75: 12161–12168.
- Kahn JO, Walker BD. 1998. Acute Human Immunodeficiency Virus Type 1 Infection. *N Engl J Med*, 339: 33–39.
- Kim JH, Excler JL, Michael NL. 2015. Lessons from the RV144 Thai phase III HIV-1 vaccine trial and the search for correlates of protection. *Annu Rev Med*, 66: 423–437.
- Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol*, 68: 4650–4655.
- Liu Q, Sun Y, Rihn S, Nolting A, Tsoukas PN, Jost S, Cohen K, Walker B, Alter G. 2009. Matrix Metalloprotease inhibitors restore impaired NK cell mediated Antibody dependent cellular cytotoxicity in human immunodeficiency virus-1 infection. *J Virol*, 83: 8705–8712.
- Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, Lewis MG. 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med*, 6: 207–210.
- Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, Van Baarle D, Kostense S, Miedema F, McLaughlin M, Ehler L, Metcalf J, Liu S, Connors M. 2002. HIV-specific CD8⁺ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol*, 3: 1061–1068.
- Moog C, Fleury HJ, Pellegrin I, Kirn A, Aubertin AM. 1997. Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficiency virus type 1-infected individuals. *J Virol*, 71: 3734–3741.
- Moore JP, Cao Y, Ho DD, Koup RA. 1994. Development of the anti-gp120 antibody response during seroconversion to human immunodeficiency virus type 1. *J Virol*, 68: 5142–5155.
- Nie J, Zhao J, Chen Q, Huang W, Wang Y. 2014. Three amino acid residues in the envelope of human immunodeficiency virus type 1 CRF07_BC regulate viral neutralization susceptibility to the human monoclonal neutralizing antibody IgG1b12. *Virol Sin*, 29: 299–307.
- Pereyra F, Addo MM, Kaufmann DE, Liu Y, Miura T, Rathod A, Baker B, Trocha A, Rosenberg R, Mackey E, Ueda P, Lu Z, Cohen D, Wrinn T, Petropoulos CJ, Rosenberg ES, Walker BD. 2008. Genetic and Immunologic Heterogeneity among Persons Who Control HIV Infection in the Absence of Therapy. *J Infect Dis*, 197: 563–571.
- Saez-Cirion A, Lacabartz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, Barre-Sinoussi F, Delfraissy JF, Sinet M, Pancino G, Venet A. 2007. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. *Proc Natl Acad Sci U S A*, 104: 6776–6781.
- Schmitz JE, Johnson RP, McClure HM, Manson KH, Wyand MS, Kuroda MJ, Lifton MA, Khunkhun RS, McEvers KJ, Gillis J, et al. 2005. Effect of CD8⁺ Lymphocyte Depletion on Virus Containment after Simian Immunodeficiency Virus SIVmac251 Challenge of Live Attenuated SIVmac239{Delta}3-Vaccinated Rhesus Macaques. *J Virol*, 79: 8131–8141.
- Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA,

- Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallan BJ, Ghayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL, Reimann KA. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science*, 283: 857–860.
- Someya K, Cecilia D, Ami Y, Nakasone T, Matsuo K, Burda S, Yamamoto H, Yoshino N, Kaizu M, Ando S, Okuda K, Zolla-Pazner S, Yamazaki S, Yamamoto N, Honda M. 2005. Vaccination of Rhesus Macaques with Recombinant Mycobacterium bovis Bacillus Calmette-Guerin Env V3 Elicits Neutralizing Antibody-Mediated Protection against Simian-Human Immunodeficiency Virus with a Homologous but Not a Heterologous V3 Motif. *J Virol*, 79: 1452–1462.
- Trkola A, Pomaes AB, Yuan H, Korber B, Maddon PJ, Allaway GP, Katinger H, Barbas CF, 3rd, Burton DR, Ho DD. 1995. Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG. *J Virol*, 69: 6609–6617.
- Vieillard V, Strominger JL, Debre P. 2005. NK cytotoxicity against CD4+ T cells during HIV-1 infection: a gp41 peptide induces the expression of an NKp44 ligand. *Proc Natl Acad Sci U S A*, 102: 10981–10986.
- Walker BD, Burton DR. 2008. Toward an AIDS Vaccine. *Science*, 320: 760–764.
- Wang Z, Qin C, Hu J, Guo X, Yin J. 2016. Recent advances in synthetic carbohydrate-based human immunodeficiency virus vaccines. *Virol Sin*, 31: 110–117.
- Wilson CC, Wong JT, Girard DD, Merrill DP, Dynan M, An DD, Kalams SA, Johnson RP, Hirsch MS, D'Aquila RT, et al. 1995. Ex vivo expansion of CD4 lymphocytes from human immunodeficiency virus type 1-infected persons in the presence of combination antiretroviral agents. *J Infect Dis*, 172: 88–96.
- Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science*, 278: 1291–1295.
- Yang OO, Kalams SA, Rosenzweig M, Trocha A, Jones N, Koziel M, Walker BD, Johnson RP. 1996. Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. *J Virol*, 70: 5799–5806.
- Yang OO, Kalams SA, Trocha A, Cao H, Luster A, Johnson RP, Walker BD. 1997. Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J Virol*, 71: 3120–3128.
- Yang OO, Nguyen PT, Kalams SA, Dorfman T, Gottlinger HG, Stewart S, Chen IS, Threlkeld S, Walker BD. 2002. Nef-mediated resistance of human immunodeficiency virus type 1 to anti-viral cytotoxic T lymphocytes. *J Virol*, 76: 1626–1631.
- Yang OO, Sarkis PT, Trocha A, Kalams SA, Johnson RP, Walker BD. 2003. Impacts of avidity and specificity on the antiviral efficiency of HIV-1-specific CTL. *J Immunol*, 171: 3718–3724.
- Zimmerli SC, Harari A, Cellera C, Vallelian F, Bart PA, Pantaleo G. 2005. HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc Natl Acad Sci U S A*, 102: 7239–7244.