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Conserved broad HIV-1 Gag-specific responses associated with low viral load and high CD4+ T cell nadir and preserved HAART regimen

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Summary. – Broad human immunodeficiency virus type 1 (HIV-1) Gag-specific cellular responses can control viremia and provide slow progression to Acquired immunodeficiency syndrome (AIDS). In this study, we evaluate multiple HIV-1 Gag-specific lymphoproliferative responses and find their connection with cluster of differentiation 4 (CD4)+ T cell count and viral load from chronically HIV-1-infected patients. We further search for the correlation between multiple Gag-specific lymphoproliferative responses and changes in highly active antiretroviral therapy (HAART) regimen. We found correlation between Gag-specific responses and higher CD4+ T cells nadir and low HIV-1 viral load. Additionally, we observed that HIV-1-infected subjects did not need to change HAART regimen, when multiple Gag responses are present. We concluded that the start of HAART when CD4+ T cell nadir is the highest as possible may promote Gag-specific cellular responses conservation. Multiple Gag responses must be important to suppress HIV-1 replication. Preserved Gag-specific responses reduce HIV-1 viral load and are associated with stability of HAART regimen.

Keywords: HIV-1; Gag; lymphoproliferation; viral load; HAART

Introduction

Cellular mediated immune response plays a critical role in control of viral infections. During acute HIV-1 infection, robust HIV-1-specific immune response has a potential to reduce the level of HIV viremia and to preserve the immune response to a certain degree (Goonetilleke *et al*., 2009). Broad cellular immune response against Gag epitopes has been associated with clinical control of HIV-1 infection (Murakoshi *et al*., 2015). Gag-specific CD8+ T cells are able to recognize infected cells as early as 2 hours post infection (Sacha *et al*., 2007) and persist through the acute HIV infection (Goonetilleke *et al*., 2009) when supported by Gag-specific CD4+ T cells (Lichterfeld *et al*., 2004). The magnitude of the anti-Gag CD4+ and CD8+ T-cell responses correlates with the suppression of viral activity (Harari *et al*., 2004; Murakoshi *et al*., 2015). In agreement, individuals who effectively controlled the HIV-1 infection presented potent and broad cellular response against Gag (Murakoshi *et al*., 2015).

When HIV-1 replication increases, high levels of activated CD4+ T lymphocytes, including Gag-specific CD4+ T cells, become target of the virus (Douek *et al*., 2002). Thus, lymphoproliferative responses are typically reduced (or absent) in chronic HIV-1-infected patients due to continuous loss of HIV-1-specific T cells (Iyasere *et al*., 2003).

To control HIV-1 replication, highly active antiretroviral therapy (HAART) has been successfully used in most cases. Lack of HIV-1 plasma viral load and CD4+ T

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Abbreviations: AIDS = Acquired immunodeficiency syndrome; CD4 = cluster of differentiation 4; CD8 = cluster of differentiation 8; CTL = cytotoxic T lymphocytes; HAART = highly active antiretroviral therapy; HIV-1 = human immunodeficiency virus type 1; HLA = human leukocyte antigen; LPA = lymphoproliferative assay; SI = stimulation index

cell blood count restoration are considered markers of HAART efficacy (Arts and Hazuda, 2012). On the other hand, even when HAART provides the increase of CD4+ T cells counts, Gag-specific cellular responses are difficult to recover (Pitcher *et al*., 1999). Therefore, it is essential to start HAART immediately to conserve HIV-1-specific cellular responses, while CD4+ T cells count is high (McKinnon *et al*., 2010). However, HAART efficacy can be harmed due the drug resistance acquisition – the major cause of treatment failure. HIV-1 evolves continuously under selective pressure due to the HAART regimen (Günthard *et al*., 1999). Drug-resistant strain can emerge in infected patients using HAART because the HIV-1 mutations are constantly accumulating.

The present study set out to analyze multiple HIV-1 Gag-specific cellular immune responses from HIV-1-infected patients under HAART and search for correlations between Gag-specific responses with CD4+ T lymphocytes counts (including nadir) and HIV-1 viral load. We further explore whether multiple Gag-specific response is associated with changes in HAART regimen.

Materials and Methods

Patient enrollment. In this cross-sectional study, forty four chronic HIV-1-infected patients (from Clinical Hospital of University of São Paulo) under HAART regimen were examined (see details in Supplementary Table 1).

Lymphoproliferative assay (LPA). HIV-1-specific immune responses were measured by LPA after stimulation by Gag peptide pools and measured by standard radiolabelled thymidine ([^{3}H]thymidine) incorporation assays. For LPA analysis, fresh heparinized peripheral blood samples (20 ml) from patients were collected and mononuclear cells, such as monocytes, lymphocytes, and macrophages, were isolated by density centrifugation gradient, using Ficoll-Hypaque. Mononuclear cells were stimulated with twelve Gag peptides pools in RPMI medium during six days in culture (2×10^6 cells/ml). The cells were cultured in triplicate. HIV-1 Consensus B Gag Peptide Set including 123 peptides was acquired from National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. Twelve Gag peptides pools comprised 10 peptides with 15-mers overlapping by 10 amino acids and spanned the entire HIV Gag protein. Peptide pools were used at the final concentration 5 µg/ml (for details, see Supplementary Table 2). Twenty four hours before the cell harvesting, 1 µCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Amersham, Buckinghamshire, UK) was added to the culture. The higher the proliferation, the higher was the [^{3}H]thymidine incorporation. LPA was measured by ^{3}H-thymidine incorporation and detected by a scintillation counter (Betaplate, Wallac, Finland). Results of LPA assay were reported as stimulation index (SI). The SI, calculated as the ratio between the mean of ^{3}H-thymidine uptake in stimulated and non-stimulated cultures, was used to determine proliferative response (SI = average ^{3}H-thymidine count in stimulated culture divided by average ^{3}H-thymidine count in the non-stimulated proliferation). SI equal or greater than 3 was considered positive to HIV-1 Gag peptides. LPA details can be found in Fries and Mitsuhashi (1995).

T lymphocyte identification. The lymphocyte subsets (CD4+ and CD8+ T cells) were identified by flow cytometry (FACS; Becton Dickinson Immunocytometry Systems, San Jose, California) using three monoclonal antibodies (BD Tritest CD4-FITC/CD8-PE/CD3-PerCP reagent) according to the manufacturer's instructions. The FACSCaliber was calibrated daily using the BD Calibrite beads and BD-FACSComp software (v 2.0).

HIV RNA detection and sequencing. HIV RNA plasma viral load, quantified by a nucleic acid signal amplification assay bDNA (System 340 bDNA Analyzer; Siemens, Germany). Purified PCR products were sequenced utilizing ABI Prism Big Dye Terminator Ready Reaction Kit version 3.0 (Applied Biosystems™, USA), in accordance with the respective protocol. The reactions were analyzed using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems™, USA). Genotype sequences were performed to define HIV-1 subtypes and drug resistance mutations. Major antiretroviral drug resistance mutations were classified according to Stanford University online HIV-1 Drug Resistance Database. Antiretroviral drugs changes due resistance per patient were counted and correlated with LPA responses.

Ethical approval. The study was approved by the Hospital das Clinical Ethical Board under the number 0483/08. All patients were invited to participate during clinical care, and they signed a consent form.

Results and Discussion

Cytotoxic T lymphocytes (CTL) are able to recognize Gag antigens in infected cells earlier than other HIV-1 antigens, even before proviral DNA integration (Sacha *et al*., 2007). Gag epitopes are markedly recognized and promote a broad HIV-1-specific cellular response (Zuñiga *et al*., 2006; Murakoshi *et al*., 2015). Cellular responses against conserved regions of Gag protein reduce progression to AIDS (Murakoshi *et al*., 2015). Using proliferative assay, we evaluated multiple Gag-specific cellular immune responses and searched for association with HIV-1 viral load and CD4+ T cell counts. LPA is attractive because this *in vitro* method evaluates cell-mediated immune function. This assay is applied to detect T cell responses stimulated by antigens or by peptides able to induce helper T cell response (Iyasere *et al*., 2003). HIV-1-infected subjects that maintained Gag-specific cellular immune responses (based on specific $CD4^+$ and/or $CD8^+$ T cells) had proliferative responses when stimulated with Gag.

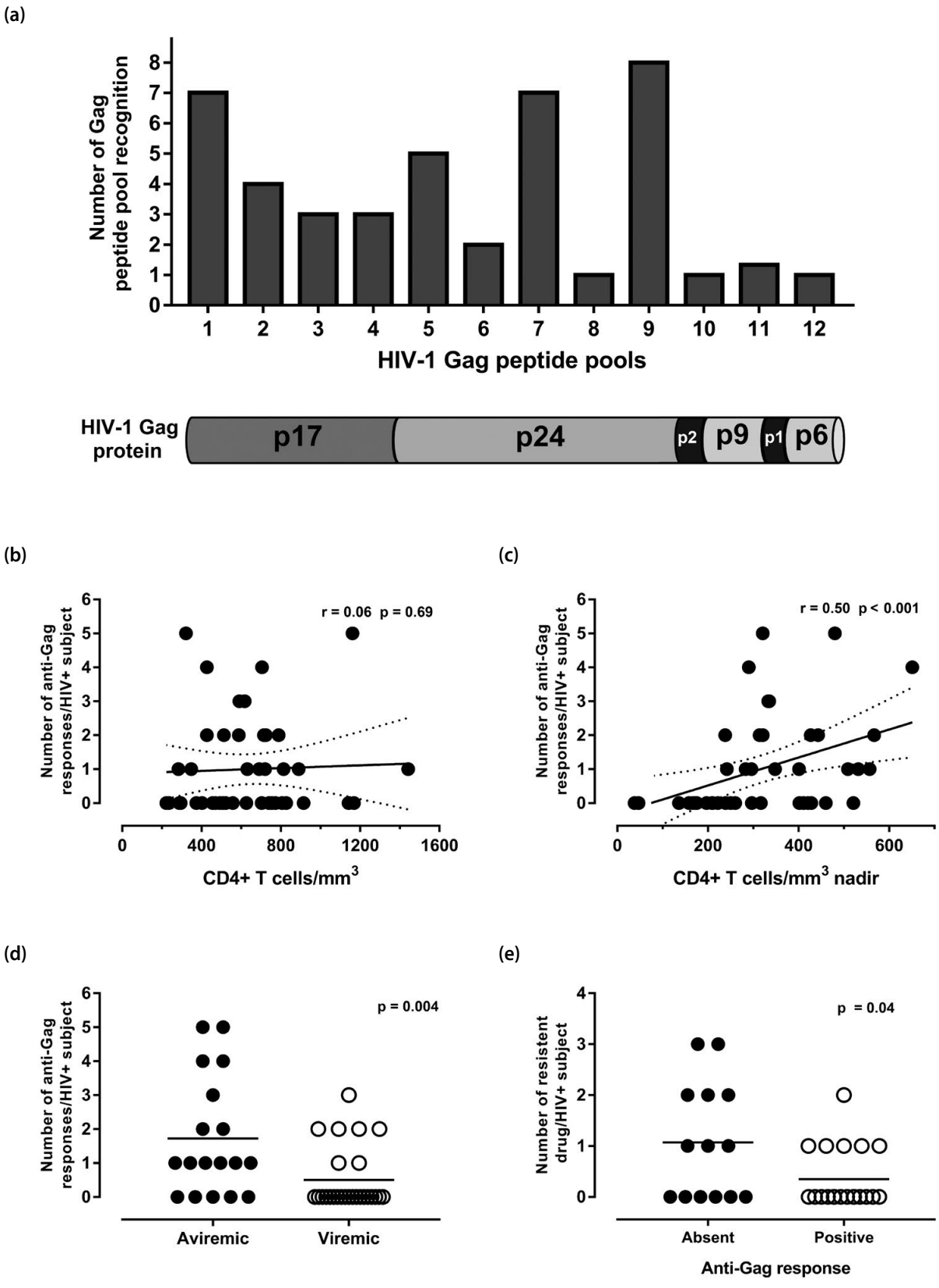
(a)
Number of Gag peptide pool recognition
HIV-1 Gag peptide pools
HIV-1 Gag protein
p17
p24
p2
p9
p1
p6
(b)
r = 0.06 p = 0.69
Number of anti-Gag responses/HIV+ subject
CD4+ T cells/mm3
(c)
r = 0.50 p < 0.001
Number of anti-Gag responses/HIV+ subject
CD4+ T cells/mm3 nadir
(d)
p = 0.004
Number of anti-Gag responses/HIV+ subject
Aviremic
Viremic
(e)
p = 0.04
Number of resistent drug/HIV+ subject
Absent
Positive
Anti-Gag response

Fig. 1

We showed that all regions of Gag protein are recognized and stimulate lymphoproliferative responses (Fig. 1a). The magnitude of proliferative responses were shown in the Supplementary Fig. 1. All Gag peptides pools were recognized at least one time. Pools #1–4 included entire Gag p17, while p24-derived peptides were included in pools #5–9. Proliferative responses against p17 were discrete. Pool #1 had 7 recognized responses, while pool #2, containing SL9 (SLYNTVATL) peptide, was recognized by four individuals. Results from previous study showed that HIV-1 chronic infected individuals had SL9 immunodominant response (Brander *et al*., 1998), differently from our results. SL9-specific CTL-based response is restricted to HLA-A*0201 (Brander *et al*., 1998), but the frequency of HLA-A*02 allele detected in Brazilian samples is not superior than 20% (Lima *et al*., 2019). Furthermore, HLA-I downregulation by Nef can affect SL9-specific CTL-based response (Balamurugan *et al*., 2013). We also show that specific responses against p24 were more frequent, similarly to previous study (Zuñiga *et al*., 2006). Our results indicated proliferative responses against p24 peptides from pool #9 as the most frequent - eight HIV-1 infected individuals elaborated specific responses.

We reported low cellular proliferative responses against Gag. HIV-1-specific proliferative responses may be impaired by disruption of cytokine secretion, such as IL-2 and IFN-γ (Iyasere *et al*., 2003; Harari *et al*., 2004). Additionally, in chronic persistent infections, CD8+ T cells become progressively exhausted (Streeck and Nixon, 2010). Furthermore, absent and poor Gag-specific proliferative responses may have occurred because HIV-1 infects preferentially HIV-1-specific CD4+ T cells (Douek *et al*., 2002) and also induces apoptosis in these cells (Yue *et al*., 2005).

The wide age range between HIV-1 infected patients could be a limitation to our analysis, considering immune response might vary according to the life stages. However, we found no correlation between the age and viral load from HIV-1 infected subjects, neither between age and CD4+ T cell counts (Supplementary Fig. 2).

Despite CD4+ T cell counts being considered an important marker for clinical follow up, studies reported weak association or lack of association between CD4+ T cell counts and HIV-1-specific responses (Zuñiga *et al*., 2006). Here, we did not see association between CD4+ T cell counts and HIV-1 Gag-specific responses (r = 0.06; p = 0.69) (Fig. 1b). Patients treated with HAART did probably not restore strong and persistent HIV-1-specific CD4+ T cell responses. HAART provides elevation of CD4+ T cell counts, but the numerical recovery of CD4+ T cells is not associated with T cell competence and repertoire regeneration (Pontesilli *et al*., 1999).

Conservation of HIV-1-specific cellular responses is linked with early treatment during acute infection. Starting HAART when CD4+T cell counts are elevated can promote a broad immune response (McKinnon *et al*., 2010). Here we showed that subjects with higher $CD4^+$ T cell nadir had a broader anti-Gag response, while those with low nadir counts had reduced responses (Fig. 1c). Our results indicated a positive correlation between higher CD4+ T cell nadir and multiple Gag-specific responses (r = 0.50 and p <0.001). The higher the $CD4^+$ T cell nadir counts, the better is the recognition of Gag peptide pool. HIV-1 infected individuals with low nadir had compromised proliferative response against Gag peptides. As we showed, patients with high CD4+ T cell nadir can conserve broad specific CD4+ T cells, which is in agreement with literature (Siddique *et al*., 2006).

Direct and indirect effects of active viral replication are CD4+ T cell depletion and impaired HIV-1-specific lymphoproliferative response (Douek *et al*., 2002; Iyasere *et al*., 2003; Yue *et al*., 2005). Suppression of HIV-1 replication by HAART avoids massive CD4+ T cell depletion and conserves HIV-1-specific cellular responses (Harari *et al*., 2004). We showed that HIV-1-infected subjects with aviremic status presented broader HIV-1 Gag-specific lymphoproliferative response than individuals with detected HIV-1 viral load (p = 0.004) (Fig. 1d). Suppressed HIV-1 plasma levels enable conservation of multiple Gag-specific responses. Multiple Gag-specific lymphoproliferative responses are associated with *in vivo* controlled HIV-1 replication. Certainly, suppression of the plasma viral load by antiretroviral therapies is highly efficient in

Fig. 1

Gag-specific lymphoproliferative responses

(a) Positive Gag-specific lymphoproliferative responses (when stimulation index is ≥3) clustered by peptide pools stimulation. Every HIV-1 infected patient was sampled for lymphoproliferative responses stimulated by twelve HIV-1 Gag peptides pools (in X-axis). The Y-axis shows the number of HIV-1 infected subjects who conserved lymphoproliferative response for each Gag peptide pool. **(b)** Correlation analysis (with linear regression and 95% confidence intervals) between the number of positive Gag lymphoproliferative responses with CD4+ T cell counts and **(c)** CD4+ T cell nadir. Both analyses (**b** and **c**) were made using Spearman correlation test. **(d)** Comparison of the number of positive Gag-specific lymphoproliferative responses from aviremic and viremic HIV-1 infected subjects. **(e)** Number of antiretroviral drug resistance occurrences from HIV-1-infected subjects, whose Gag-specific lymphoproliferative response is absent compared with infected subjects with conserved Gag-specific proliferative responses (or positive). Both analyses (**d** and **e**) were made using the t test. Resistance to antiretroviral drugs were determined based on genotyping and mutation analysis using Stanford online HIV Drug Resistance Database. Graphical presentation and statistical analysis were performed by GraphPad Prism 7.0 software.

reducing or even inhibiting HIV-1 replication. However, HIV-1 generates resistant strains when antiretroviral drug targets accumulate mutations (Arts and Hazuda, 2012). While Gag protein is conserved, HIV-1 *Env* and *Pol* genes bearing multiple mutations that confer escape from immune responses and antiretroviral drug resistance (Arts and Hazuda, 2012). Escape mutations in Gag are expensive for HIV-1 and compromise viral fitness (Martinez-Picado *et al*., 2006). Thus, it is expected that cellular immune responses against multiple epitopes of Gag maintain a reduced HIV-1 replication independently of drug resistance status. In agreement, we show that HIV-1-infected individuals who conserved proliferative responses against Gag had reduced occurrence of antiretroviral drug resistance in comparison with those, whose Gag-specific responses are absent ($p = 0.04$) (Fig. 1e). Thus, broad Gag-specific cellular response plays an important role in suppressing viral replication, recognizing infected cells even when infected with HAART resistant strain.

In summary, cellular immune response against HIV-1 Gag must be preserved by starting HAART earlier, suppressing HIV-1 viral load, while CD4+ T cell nadir is elevated. Preserved Gag-specific T cells reduce HIV-1 replication and are associated with stability of HAART regimen. Considering that HIV-1 Gag elicits a broad cellular mediated immune response, enables to decrease the viral replication, and it is highly conserved, Gag protein (or peptides) should be an essential component of a multiepitope vaccine strategy (therapeutic or prophylactic) to promote a broad and protective response against HIV-1. Furthermore, specific cellular responses to Gag epitopes should be considered in anti-HIV clinical therapy decisions.

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Supplementary information is available in the online version of the paper.

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