Presentation of available CTL epitopes that induction of cell-mediated immune response against HIV-1 Koran clade B strain using computational technology

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Objective
Theoretical predicting cytotoxic T lymphocyte (CTL) epitopes are an important tool in vaccine design and CTL therapy for enhancing our understanding of the cellular immune system. We would like to identify available CTL epitopes against HIV-1 Korean clade B. CTL activity was assessed in freshly isolated peripheral blood mononuclear cells from Korean HIV patients in order to assess whether these CTL epitopes induce a cell-mediated immune response (CMI).

Methods
NetCTLpan1.1 software, which is the most popular prediction computer software package, and full atom-based simulation (FABS), which is a 3D modelling system for binding activity between epitopes and human leucocyte antigen (HLA) molecules, were used to predict the peptide-spanning Env region binding to HLA-A*24:02, HLA-A*02:01 and HLA-B*15:01, which are frequently found in the Korean population. Granzyme B and interferon-γ ELISPOT assays were used to determine whether identified CTL epitopes induce CMI.

Results
Three HIV-1 Korean clade B-specific Env CTL epitopes were identified: Gp41-RL and Gp41-RQG are localized within gp41, and Gp120-LLQ within gp120. In in vitro assays using granzyme B ELISPOT, Gp120-LLQ and Gp41-RQG induced epitope-specific CTL responses in HLA-restricted cells. In ex vivo assay using IFN-γ ELISPOT, cell-mediated immune responses to Gp41-RL were present in 50% of HLA-matched patients, and responses to Gp120-LLQ and Gp41-RQG were found in 33% of HLA-matched patients.

Conclusion
In this study, we found that a prediction pipeline for CTL epitopes might be based on the most popular computer prediction software and FABS methods. Our results suggest that these CTL epitopes may provide useful tools and information for the development of a therapeutic vaccine against HIV-1 Korean clade B.

Keywords: cell-mediated immune response (CMI), env CTL epitope, full atom-based simulation (FABS), HIV-1 Korean clade B, therapeutic vaccine

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Introduction
About 33 million people worldwide are living with HIV-1 [1]. Although several antiretroviral therapy regimens have been introduced, these are not able to eradicate HIV from treated patients. Therapeutic vaccination aimed at activating cellular immunity is a promising method for disease prevention. An epitope vaccine could greatly enhance the
Prediction of CTL epitopes against HIV-1 Koran clade B

predefined epitope-specific immune response. In particular, the identification of cytotoxic T lymphocyte (CTL) epitopes provides information allowing evaluation of the effectiveness and the development of peptide-based vaccines [2].

HIV-1-specific CTLs have an important role in the control of HIV-1 replication [3]. HIV infection can elicit a CTL response to viral peptides, which are recognized as epitopes presented by human leucocyte antigen (HLA) class I molecules [4]. The precise identification of epitopes provides information for understanding the host immune response to infection.

HIV-1 envelope (Env), a key viral protein in the process of viral entry into target cells, is first exposed to the host immune system and subsequently induce strong CTL responses in HIV-infected patients [5,6]. HIV-1 subtype B is the predominant subtype circulating globally. Previous studies have demonstrated that HIV-1 Korean Clade B evolved with a distinct phylogenetic cluster compared to foreign HIV-1 strains [7,8]. Therefore, the available Env-focused CTL epitopes are necessary in order to develop an HIV therapeutic vaccine optimized for use in Korea.

Of the large number of peptides that can be derived from a pathogen, only a small minority elicits a CTL response. As a result of this, increasingly epitope prediction algorithms are being used to understand the CTL response. A range of computational algorithms have been developed to predict CTL epitopes in pathogen protein sequences. The algorithms that are used vary in complexity and accuracy [9].

In this study, using NetCTLpan1.1 software (CBS, Lyngby, Denmark), which is the most popular computational prediction software, and unique full atom-based simulation (FABS), we identified three HIV-1 Korean clade B-available CTL epitopes in the Env region and investigated whether these Env CTL epitopes effectively induce a cell-mediated immune response (CMI) against HIV-1-infected Korean patients in vitro and ex vivo.

Methods

Prediction of Env CTL epitope candidates

NetCTLpan1.1 software and FABS were used to predict Env CTL epitopes matched with HLA types frequently found in the Korean population. The full genome sequences of HIV-1 Korean clade B strains that isolated from seven Korean patients were obtained from the Korea National Institute of Health.

Synthetic peptides

Peptides corresponding to Korean clade B consensus Env CTL epitope sequences were synthesized by GL Biochem Ltd. (Shanghai, China) and purified to 90%.

Study patients

Seven HIV-infected Korean patients (P1–P7) from the Ulsan University Hospital enrolled in this study were treated with highly active antiretroviral treatment (HAART). Six of these patients (not patient P3) showed good compliance for HAART treatment. The study received ethics approval from the Ulsan University Hospital (UUH-IRB-13-085) and Korea Centers for Disease Control and Prevention (2013-11CON-01-P-A). Heparinized blood was collected from each patient. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Hypaque 1.077 (Sigma, St. Louis, MO, USA). HLA genotyping was performed using the AVITA HLA-A and AVITA HLA-B sequenced-based typing kits and Biowithus SBT Analyzer (Biowithus, Seoul, South Korea).

Maturation of dendritic cells (DCs)

Characterized human PBMCs were purchased from Cellular Technology Limited Co. (Shaker Heights, OH, Carlsbad, CA, USA). $1 \times 10^7$ PBMCs were suspended in complete RPMI-1640 medium (Gibco) containing 10% foetal bovine serum and penicillin/streptomycin (Gibco) and incubated for 3 h at 37°C in 5% CO₂. The adherent cells were maintained in complete RPMI-1640 medium containing 50 ng/mL recombinant human granulocyte granulocyte macrophage colony-stimulating factor (rhGM-CSF) and 100 ng/mL interleukin 4 (IL-4) (R&Dsystem, Minneapolis, MN, USA). On days 3 and 6, fresh medium containing rhGM-CSF and IL-4 was added. On Day 7, the immature dendritic cells (DCs) were collected and seeded into a 24-well plate. Immature DCs were treated with 1 μM of each Env CTL epitope and incubated for 2 h at 37°C in 5% CO₂, then washed with PBS. For maturation, immature DCs were cultured in complete RPMI-1640 medium containing rhGM-CSF, IL-4, 1 μg/mL lipopolysaccharide (LPS) (Sigma) and 20 μg/mL polyinosinic-polycytidylic acid [poly(I:C)] (Sigma) at 37°C in 5% CO₂ overnight. Mature DCs were harvested and the maturation markers (CD80, CD83, CD86 and HLA-DR) of DCs were examined by flow cytometry (FACS-verse; BD Bioscience, San Jose, CA, USA).

Generation of HIV-1 Env CTL epitope-specific CTLs

CTLs were isolated using a CD8 T Cell Isolation Kit (Miltenyi Biotec Inc., Bergisch Galdbach, Germany). CTLs were cultured with irradiated mature DCs. Mature DCs were pulsed with 10 μM of each Env CTL epitope in 24-well plates and incubated for 2 h at 37°C in 5% CO₂. CTLs were suspended in complete RPMI-1640 medium containing
12.5 IU/mL IL-2 and 5 ng/mL IL-7 (R&D system) and mixed with Env CTL epitope-loaded DCs. Epitope-specific CTLs were grown at 37°C in 5% CO₂, and IL-2 and IL-7 were both added at Day 2 and Day 5.

**In vitro** Granzyme B (GrB) enzyme-linked immunospot (ELISpot) assay

The GrB ELISPOT (MABTECH, Stockholm, Sweden) assay was performed according to the manufacturer’s protocol. Target cells (DCs) were pulsed with 10 μM Env CTL epitopes for 2 h at 37°C prior to use in the assay. Effector cells (CTLs) were added to triplicate wells at the concentration followed by 2 × 10⁵ target cells per well (effector:target ratio = 10:1). The negative control was nonpulsed target cells in the presence of effector cells. After effector and target cells were incubated for 24 h, the number of spots per well was counted with an ImmunoSpot automated plate counter. GrB responses for Env CTL epitopes were represented as the number of spot-forming units (SFU) per 10⁶ CTLs after the subtraction of negative control GrB secretion.

**Ex vivo** human IFN-γ ELISpot assay

PBMCs from HIV-1-infected patients were incubated in complete RPMI-1640 medium at 37°C in 5% CO₂ overnight prior to use in the ELISpot assay. The IFN-γ ELISpot (MABTECH) assay was performed according to the manufacturer’s protocol. A total of 1 × 10⁶ PBMCs/well was stimulated in triplicate for 24 h at 37°C with medium alone (negative control), anti-CD3 monoclonal antibody (positive control), or 2 μg/mL of each Env CTL epitope. The number of spots per well was counted with an ImmunoSpot automated plate counter. IFN-γ responses were reported as the number of SFU per 10⁶ PBMCs, after subtraction of negative control IFN-γ secretion.

**Results**

Identification of Env CTL epitopes against HIV-1 Korean clade B

The full genome sequences of seven Korean clade B strains were used to identify the CTL epitopes based on HIV-1 Korean clade B. The NetCTLpan1.1 algorithm (Fig. 1a) was used to predict binding of HLA class I molecules with the Env sequence, and allows prediction of 9-mer peptides [10]. NetCTLpan1.1 carried out the protease cleavage, peptide transporter protein (TAP) binding and MHC class I binding with peptides. Thus, the 122 CTL epitope candidates identified by the NetCTLpan1.1 software were selected. Eight of these CTL epitope candidates were reselected as common CTL epitopes that existed in more than four Korean clade B strains, and which matched the major HLA class I types present in Koreans (HLA-A*02:01, A*24:02, B*15:01). Subsequently, the FABS program (AmberTools15) was used to select the most stable and powerful CTL epitopes [11] (Fig. 1b). To enhance prediction of epitopes, molecular dynamic simulation was performed in which atomic stability was simulated between MHC class I (HLA-A*02:01, A*24:02, B*15:01) and peptides. Finally, three available Env CTL epitopes were selected that had the lowest energy of interaction with HLA class I molecules. Gp4-RYL interacted with HLA-A*02:01, HLA-A*24:02 and HLA-B*15:01 molecules. Gp120-LLQ and Gp41-RQG interacted with HLA-A*02:01 and HLA-B*15:01 molecules. Gp41-RYL and Gp41-RQG were localized within the gp41 site of HIV-1 and Gp120-LLQ was localized in the gp120 site of HIV-1 (Fig. 1c).

**In vitro and ex vivo** CMI induced with three Env CTL epitopes

Number of SFU/millions was obtained by substracting the value of the control groups from each group. GrB and IFN-γ ELISpot assay were used to determine whether CTL epitopes induced CMI. Detection of GrB provides a direct measure of cell-mediated cytotoxicity because GrB is a key mediator of target cell death induced by the granule-mediated lytic pathways [12]. One of the major mechanisms of cell-mediated cytotoxicity is IFN-γ secretion from CTLs in HIV-infected patients.

In order to investigate epitope- and HLA type-specific GrB secretion induced by Env CTL epitopes, CTL reactivity against DCs loaded with Env CTL epitopes was measured by the GrB ELISPOT assay. DCs and CTLs were isolated from the commercial HLA-A*02:01/HLA-B*15:01- and HLA-A*02:01/HLA-A*24:02-restricted healthy donors. The increased number of spots per million CTLs against HLA-A*02:01/HLA-B*15:01-positive DCs loaded with Gp41-RYL, Gp120-LLQ and Gp41-RQG were 366.7, 384.4 and 316.7, respectively (Fig. 2a, upper panels). The increased number of spots induced by Gp41-RYL, Gp120-LLQ and Gp41-RQG in HLA-A*02:01/HLA-A*24:02-positive DCs were 16.7, 133.3 and 16.7, respectively (Fig. 2a, lower panels). Gp41-RYL-pulsed CTLs showed CMI induction with all Env CTL epitope-loaded HLA-A*02:01/HLA-B*15:01-restricted DCs, but no induction by all Env CTL epitope-loaded HLA-A*02:01/HLA-A*24:02-restricted DCs. Gp120-LLQ-pulsed CTLs showed CMI induction with all Env CTL epitope-loaded DCs, but Gp41-RQG-pulsed
CTLs showed higher CTL induction only with Gp41-RQG-loaded HLA-A*02:01/HLA-B*15:01-restricted DCs.

IFN-γ secretion by PBMCs from seven HIV-1-infected patients (Fig. 2b) was measured by IFN-γ ELISPOT assays to clarify the ex vivo CMI against Env CTL epitopes. Figure 2c shows that Env CTL epitopes induced IFN-γ secretion that means these epitopes leads to CTL responses in HIV-1-infected patients. CMI to the Gp41-RYL was present in 50% (two of four subjects), and to Gp120-LLQ and Gp41-RQG in 33% (one of three subjects) of HLA class I-matched patients.

**Discussion**

Cell-mediated vaccines for HIV-1 therapy have been developed on the principle that HIV-specific T cell responses may provide protection from infection [13]. Designing T cell-mediated vaccines requires careful planning because of the need to generate immune responses in the context of most HLA alleles. However, HLA alleles can be grouped into supertypes, which are clusters of HLA molecules sharing overlapping peptide-binding specificity. Identifying these supertypes is an important task with clear implications for the development of epitope-based vaccines [14,15]. Therefore, we carried out the prediction of epitopes with the most popular software and FABS methods based on the HLA types.

In this study, we showed that identified available CTL epitope against HIV-1 Korean clade B using computational technology and that these epitopes induced CMI in vitro and ex vivo. NetCTLpan1.1 and FABS were used to predict CTL epitopes. NetCTLpan1.1 performs predic-
Fig. 2 In vitro and ex vivo cell-mediated immune responses (CMI) induced by three Env cytotoxic T lymphocyte (CTL) epitopes using ELISPOT assay. (a) In vitro CTL responses induced by Env CTL epitopes using GrB ELISPOT assay. The number of spot-forming units (SFU) per $1 \times 10^6$ CTLs was elicited by incubation with Env CTL epitope-loaded dendritic cells from HLA-A*02:01/HLA-B*15:01- (upper) and HLA-A*02:01/HLA-A*24:02-restricted healthy donors (lower) for 24 h. (b) Ex vivo CMI induced by Env CTL epitopes using human peripheral blood mononuclear cells (PBMCs) purified from HIV-1-infected Korean patients. (c) Induction of ex vivo CMI response was measured by IFN-γ ELISPOT assay. PBMCs obtained from seven HIV-1-infected patients were cultured for 24 h with Env CTL epitopes Gp41-RYL (left), Gp120-LLQ, (middle) and Gp41-RQG (right). CMI responses were reported as the number of SFU per $10^6$ cells, after subtraction of negative control.
tions for binding of HLA class I molecules with the known protein sequences [10]. CTL epitope candidates were the 122 based on the Env protein sequences identified with NetCTLpan1.1. Eight epitope candidates of them were reselected by matching with the most common HLA class I types in Koreans. [16,17]. FABS software, which can be helpful with epitope prediction, was used to improve the accuracy of epitopes binding to HLA molecules. Amber is the collective name for a suite of programs that allows users to carry out and analyse simulated molecular dynamics [18,19]. Three epitopes from them were finally reselected simulated through the 10 thousand motion with the FABS software. Gp120-LLQ and Gp41-RQG were matched with HLA-A*02:01 and HLA-B*15:01; Gp41-RYL was matched with HLA-A*24:02, HLA-A*02:01 and HLA-B*15:01.

In order to test the effect of CTL-specific epitopes, we investigated whether these epitopes could induce epitope-specific CTL responses by ELISPOT assay in vitro and ex vivo. In vitro CTL induction was measured by Grb ELISPOT assay using epitope-loaded DCs and epitope-pulsed CTLs. One of the major mechanisms of cell-mediated cytotoxicity is exocytosis of cytoplasmic granules from the effectors toward the target cells. We isolated DCs and CTLs from commercial PBMCs expressing HLA-A*02:01/HLA-B*15:01 and HLA-A*02:01/HLA-A*24:02, because Env CTL epitopes were matched with HLA-A*02:01, HLA-A*24:02 and HLA-B*15:01. Although these epitopes showed different levels of CTL induction, they induced more epitope-specific CTL responses in PBMCs expressing HLA-A*02:01/HLA-B*15:01 compared with PBMCs expressing HLA-A*02:01/HLA-A*24:02.

The use of IFN-γ ELISPOT assays has gained increasing popularity as a surrogate measure of CTL activity. It has been determined to be one of the most useful assays for evaluating immune responses to various diseases. Therefore, ex vivo epitope-specific CMI activity was investigated by IFN-γ ELISPOT assay using HIV-1 Korean clade B-infected patients. Gp41-RLY induced higher levels of CMI in HIV-1-infected patients P1, P3, P5, P6 and P7. Gp120-LLQ induced CMI in patients P1, P2, P3, P5 and P6, and Gp41-RQG induced CMI in patients P1, P2 and P3.

Similarly, in both the ex vivo and in vitro assays, Env CTL epitopes induced epitope-specific CTL responses in both HLA-matched and -mismatched cells. This phenomenon can be explained by HLA cross-reactivity. HLA class I molecules can share similar immunogenic molecular epitopes and can be immunologically cross-reactive [20].

Env CTL epitope candidates predicted with the most popular software and the FABS methods induced CMI in HIV-1 Korean clade B-infected patients. This suggests that the identified available CTL epitopes could give some indicators helpful to the development of a country-specific therapeutic vaccine against HIV-1 Korean clade B.

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