Isolation and characterization of an HIV-1 envelope glycoprotein-specific B-cell from an immortalized human naïve B-cell library

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Abstract

With the recent development of single B-cell cloning techniques, an increasing number of human immunodeficiency virus type 1 (HIV-1)-specific broadly neutralizing antibodies have been isolated since 2009. However, knowledge regarding HIV-1-specific B cells in vivo is limited. In this study, an HIV-1-specific B-cell line was established using healthy PBMC donors by the highly efficient Epstein–Barr virus transformation method to generate immortalized human naïve B-cell libraries. The enrichment of HIV-1 envelope-specific B cells was observed after four rounds of cell panning with the HIV-1 envelope glycoprotein. An HIV-1 envelope-specific stable B-cell line (LCL-P4) was generated. Although this cell line acquired a lymphoblastic phenotype, no expression was observed for activation-induced cytidine deaminase, an enzyme responsible for initiating somatic hypermutation and class switch recombination in B cells. This study describes a method that enables fast isolation of HIV-1-specific B cells, and this approach may extend to isolating other B-cell-specific antigens for further experiments.

INTRODUCTION

Among human immunodeficiency virus type 1 (HIV-1)-infected individuals, approximately 20% generate broadly neutralizing antibodies (bNAbs) for 2–4 years after infection [1]. Since 2009, a large number of bNAbs have been isolated using single B-cell gene cloning and validation by neutralization screening [2]. Additional bNAbs have also been obtained through probe-based B-cell isolation methods [3]. These bNAbs show promising prophylactic and therapeutic values in animal models [4, 5], though one of the bNAbs, VRC01, only slightly delayed viral rebound after stopping antiretroviral therapy in two clinical trials by administering purified antibodies [6]. However, induction of bNAbs has not been achieved by any vaccine candidates so far [7]. Traditional strategies for vaccine development have relied on attenuated or killed virus particles. Protein subunit preparations for a homologous ‘prime-boost’ strategy have also been attempted, which assumed that antigens recognized by naïve B cells in the priming phase would be the same as those recognized by memory B cells during a vaccine boost. Although great successes have been achieved with these strategies to combat infectious diseases in the past century [8, 9], their use in the development of HIV-1 vaccines has failed to elicit effective antibodies.

The unusual characteristics of bNAbs have provided some insights into overcoming these limitations. Extraordinarily high mutation frequencies in the V(D)J fragments (15–35%) are typically observed in bNAbs. These higher-than-average levels occur during the secondary humoral immune response (6%) [10, 11]. This suggests that B-cell activation and affinity maturation may be induced by different antigens [12]. Recent findings also support this hypothesis because putative bNAbs in the germline do not bind recombinant HIV-1 envelopes [13, 14]. In light of this, we have identified five non-HIV-derived polypeptides that bind putative germline bNAbs. Interestingly, these peptides could serve as primary immunogens to initiate humoral immune responses, leading to the discovery of cross-clade HIV-specific neutralizing antibodies produced in rabbits [15]. To investigate whether naïve B cells from healthy individuals stimulated by
non-HIV-1 immunogens can produce bNAbs against HIV-1, we established a B-cell line. An HIV-1 envelope glycoprotein-recognizing B-cell clone was isolated from healthy donors. To establish the B-cell line, a traditional Epstein–Barr virus (EBV) transformation method was optimized to provide higher efficiency. This allowed the establishment of a diversity of human naïve B-cell libraries, which could be panned following stimulation. Next, an HIV-1-specific B-cell line was generated after four rounds of cell panning with the HIV-1 envelope. This cell line, termed LCL-P4, displayed a limited number of mutations in both VH and VL. This naïve B-cell line can be used for future stimulation experiments. Therefore, this study provides a useful method that facilitates rapid isolation of HIV-1-specific B cells.

RESULTS

Generation of a human naïve B-cell library

The procedure for generating HIV-1 naïve B cells from PBMCs from healthy donors is shown in Fig. 1. PBMCs were first isolated from healthy buffy coat donors, and naïve B cells were purified using magnetic beads. Since primary B cells could not survive in long-term culture in vitro, EBV transformation was performed. However, because the transformation efficiency is generally low [16], this method was further optimized using spinfection in the presence of certain cytokines. To construct the naïve B-cell library, 8×10⁷ naïve B cells were isolated from 16 healthy donors. They underwent two rounds of negative selection to remove non-B cells and memory B cells (Fig. 2a). The efficiency was evaluated by assessing lymphoblastoid cell outgrowths on day 21 (Table 1). One colony was regarded as one successful transformation. The transformation efficiency was estimated to be approximately 7.8 %, which was significantly higher than the traditional EBV-based method (approximately 2–3 %). The final size of the naïve B-cell library was estimated to be 5.8×10⁶ based upon counts of cell clumps.

Enrichment of HIV-1-specific B cells by panning with gp140YU2 trimers

An antigen-based B-cell enrichment method has been previously developed using magnetic beads [17]. Using this approach, four rounds of cell panning with magnetic beads were performed (Fig. 1). The enrichment of HIV-1-specific B cells was determined following each round of panning using gp140YU2 (Fig. 2b). Biotinylated KLH was used as a negative control to exclude non-specific binding caused by biotinyla-

Characterization of the LCL-P4 antibody

After four rounds of panning with gp140YU2 in vitro, B cells appeared to be a homogenous population based on sequenc-

DISCUSSION

In this study, we report an efficient procedure for isolation of HIV-1-specific naïve B cells from healthy donors. To our knowledge, this is the first HIV-1-specific naïve B-cell line generated that expresses an HIV-1 envelope-recognizing antibody. The isolation of naïve B cells supports the hypoth-

vitro from individuals, which better facilitates the study of B-cell activation and germline antibodies.

One difficulty encountered in this study was the need to increase B-cell immortalization efficiency. Unlike mouse B cells, human B cells were resistant to immortalization using hybridoma technology, and EBV transformation is the only established method for long-term human B-cell immortalization [21]. One study used a Toll-like receptor 9 agonist (CpG DNA), which synergistically increased proliferation and activation of B cells after EBV infection [22]. Furthermore, EBV transformation efficiency was increased to 0.1–2% in the presence of autologous feeder cells. Several human monoclonal antibodies against HIV-1 [23], severe acute respiratory syndrome [19] and influenza virus [23] have also been isolated using this method. In our experiments, the traditional EBV transformation method was optimized to an efficiency of 7.8%, which enabled the generation of B-cell libraries for the subsequent isolation of HIV-1 envelope-specific B cells. In addition, the M-280 beads we used for the isolation of HIV-1-specific cells were 2.8 µm in diameter. This is much larger than the commonly used MACS beads, which are 50 nm in diameter [17]. We found that M-280 beads worked as efficiently as MACS beads for the enrichment of antigen-specific B cells and could be detached easily. The bound cells could be quickly visualized using a light microscope, which then facilitated the process of cell panning.

The cells became a homogeneous population after four rounds of panning and carried limited mutations in VH and VL. LCL-P4 cells bound other strains of gp140 trimers besides the gp140 YU2 trimer. However, LCL-P4 cells did not show any neutralizing activity based on a standardized TZM-bl cell line assay (data not shown). Considering that the neutralizing profile somewhat correlated with antibody affinity maturation, as shown by the bNab b12 [23, 24], it will be interesting to investigate whether antibody affinity maturation can occur in LCL-P4 cells by artificially expressing AID, followed by affinity-based isolation of cells. This may provide further insights into the process of bNAb HIV-1 vaccine design. Since the LCL-P4 cells express high levels of MHCII and other co-stimulators, this cell line might be useful as antigen-presenting cells or as a tool in cell-based vaccine development. In summary, our method to isolate an HIV-1-specific naïve B-cell line provides a model for further study of naïve B-cell activation and maturation in vitro.

![Diagram](https://www.microbiologyresearch.org/static/fig1.png)

**Fig. 1.** Procedure for isolation of HIV-1 envelope-specific naïve B cells from healthy donors. Schematic showing the isolation of naïve B cells from healthy PBMCs transformed using EBV. The library was screened with four cycles of cell panning before testing anti-gp120 activity.
This work may be extended to the isolation of other antigens from pathogens or auto-immune diseases.

**METHODS**

**Preparation of concentrated virus**

Infectious EBV stocks were prepared as previously reported, with minor modifications [16, 25]. Briefly, after inoculation, the EBV-producing marmoset B95.8 cells were seeded at $10^6$ cells ml$^{-1}$ and incubated at 37°C under 5% CO$_2$ for 4 days in Iscove’s modified Dulbecco’s medium (IMDM) complemented with 10% heat-inactivated (w/v) FBS, 1% (v/v) penicillin-streptomycin and 35 ng 12-O-tetradecanoyl-phorbol-13-acetate ml$^{-1}$. After centrifugation at 800 g for 5 min, the supernatant was passed through a 0.45μm filter. The EBV-containing supernatant was loaded into

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Fig. 2. Enrichment of HIV-1-specific intermediate B cells by cell panning. (a) Assessment of purified CD19$^+$CD27$^-$ naive B cells from PBMC of healthy donors by flow cytometry. (b) Enrichment of HIV-1-specific B cells during each round of cell panning (P) was determined by binding to biotinylated YU2 or KLH. (c) Cells obtained by negative selection, positive selection and flow-through washing were visualized with bright-field microscopy. Arrows indicate the M-280 beads with cells bound.
Centricon Plus-70 units (100 000 MW cut-off, Millipore) and centrifuged at 3200 g for 45 min with the minimal retention volume remaining. The EBV-containing concentrate was resuspended with fresh IMDM to 10% of the original volume. Aliquots of 1 ml per vial were stored at −80°C.

Isolation of human naïve B cells

Primary PBMCs were isolated from buffy coats of healthy donors by Ficoll density centrifugation. B cells were isolated using a Human B Cell Isolation Kit (Miltenyi Biotec). Non-B cells were indirectly magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies and removed with microbeads. Memory B cells and naïve B cells were further separated with CD27 microbeads (Miltenyi Biotec). The purity of the cell populations was determined by flow cytometry.

Optimized B-cell immortalization method by EBV infection

Purified naïve B cells were suspended in 3 ml IMDM supplemented with 10% (w/v) heat-inactivated fetal bovine medium, 100 units penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 0.66 µg cyclosporine ml⁻¹, 2 µg CpG2006 ml⁻¹, 25 ng B-cell activating factor (BAFF) ml⁻¹, 2 µg Lipopolysaccharide (LPS) ml⁻¹ and 5 ng IL-2 ml⁻¹. An equal volume of concentrated EBV-containing supernatant was added to the cell suspension and mixed well. The sample was centrifuged at 500 g for 60 min at room temperature and then resuspended at a concentration of 10⁶ cells ml⁻¹. Cells were seeded into 96-well U-bottom plates at a volume of 200 µl per well. Plates were incubated at 37°C under 5% CO₂. They underwent a half-media change every 7 days. After 3 weeks of culture, cells were transferred to a T75 flask for further growth.

Enrichment of gp140YU2-based B cells

Cells were washed with MACS buffer [1× PBS, 2% (v/v) FCS, 2 mM EDTA] twice before cell panning, which included one negative selection followed by one positive selection. For negative selection, cells were directly incubated with Dynabeads M-280 Streptavidin (Life Technologies) at 4°C for 30 min with gentle rotation. The tube was placed in a magnet for 3 min to harvest the cell suspension. For positive selection, cells were incubated with biotinylated gp140YU2 at 4°C for 60 min. Cells were washed with MACS buffer twice and incubated with Dynabeads at 4°C for 30 min. The tube was placed in a magnet for 3 min and washed 5–10 times with MACS buffer. The cells were resuspended in IMDM growth

<table>
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<th>Sequence ID</th>
<th>V-GENE and allele</th>
<th>Functionality</th>
<th>V-REGION identity % (nt)</th>
<th>J-GENE and allele</th>
<th>D-GENE and allele</th>
<th>D-REGION reading frame</th>
<th>AA JUNCTION JUNCTION frame</th>
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<td>Productive 1345</td>
<td>96.53% (278/289 nt)</td>
<td>Homasp IGH J6-13*01 F</td>
<td>Homasp IGHD6-13*01 F</td>
<td>1</td>
<td>[8.8.18] CVKDTSSVYA DFFYYGVDW in-frame</td>
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Fig. 3. Analysis of LCL-P4 antibody sequence. (a) DNA sequence alignment of the variable region of the heavy and light chains of LCL-P4 with their germlines by IMGT/V-QUEST. (b) Mutations occurred in both CDRs and FWRs of LCL-P4 cells in the VH and VK genes.

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Table 1. Cell densities and success rates for the establishment of LCLs

Fig. 4. LCL-P4 antibody preferentially bound gp140 trimer rather than gp120 or gp41 monomer. (a) Immunoglobulin subtypes in supernatants after each round of panning were detected by indirect ELISA. (b) ELISA for gp140_{YU2}. (c) ELISA for gp140_{SF162}. (d) ELISA for BSA. (e) ELISA for gp140_{YU2} using the supernatant-purified IgG. (f) ELISA for gp140_{SF162} using the supernatant-purified IgG.
medium for subculturing. The envelope trimers gp140\textsubscript{YU2} and gp140\textsubscript{SF162} were kindly provided by Peter Kwong (National Institutes of Health).

**Flow cytometry to assess gp140\textsubscript{YU2} binding and perform cell phenotyping**

Flow cytometry was performed using a FACScalibur (BD Sciences). For the gp140\textsubscript{YU2} binding assay, cells were incubated with biotinylated gp140\textsubscript{YU2} or biotinylated KLH at different concentrations and then stained with streptavidin-PE (BD Sciences) at 4°C for 30 min before analysis. For phenotyping, cells were stained with antibodies against human CD19, CD21, CD27, CD38, CD83, CD86, CD138, IgD, IgM, IgG, IgA and HLA-DR (Biolegend) at 4°C for 30 min. Data analysis was performed using FlowJo software.

**ELISA**

To quantify the concentrations of IgG, IgM and IgA in the supernatant, goat anti-human IgG (Fcγ fragment specific), goat anti-human IgM (Fcζ fragment specific) and goat anti-human IgA (α-chain specific) antibodies were used for ELISA plate coating, respectively. HRP-conjugated goat anti-human IgG F(ab′)\textsubscript{2} fragment-specific antibody was used as the secondary detection antibody for these ELISA assays. All antibodies were obtained from Jackson ImmunoResearch.

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**Fig. 5.** LCL-P4 characterization and phenotype. (a) Titration of biotinylated gp140\textsubscript{YU2} trimer binding to LCL-P4 at different concentrations. Binding was detected by streptavidin-PE. (b) LCL-P4 phenotyping by flow cytometry. Grey filled histograms show background staining with isotype. (c) Detection of AID expression in LCL-P4 cells by Western blot.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The Ethical Committee of The University of Hong Kong, where the work was done, has approved it. The subjects gave informed consent to the work.

References

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