Methods 65 (2014) 57–67

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Retrocyte Display® technology: Generation and screening of a high diversity cellular antibody library

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ARTICLE INFO

Article history:
Available online 12 September 2013

Keywords:
Mammalian cell surface antibody display
Fully human antibody
Flow cytometry
Retroviral transduction

ABSTRACT

Over the last nearly three decades *in vitro* display technologies have played an important role in the discovery and optimization of antibodies and other proteins for therapeutic applications. Here we describe the use of retroviral expression technology for the display of full-length IgG on B lineage cells *in vitro* with a hallmark of a tight and stable genotype to phenotype coupling. We describe the creation of a high-diversity (>1.0E09 different heavy- and light-chain combinations) cell displayed fully human antibody library from healthy donor-derived heavy- and light-chain gene libraries, and demonstrate the recovery of high affinity target-specific antibodies from this library by staining of cells with a labeled target antigen and their magnetic- and flow cytometry-based cell sorting. The present technology represents a further evolution in the discovery of full-length, fully human antibodies using mammalian display, and is termed Retrocyte Display® (*Retro*viral B lympho*cyte Display*).

1. Introduction – mammalian cell surface antibody display

In vitro display technologies are designed to allow the construction of large libraries of biomolecules displayed on a suitable carrier that couples the phenotype of the expressed protein (or protein fragment) to the genotype of the carrier, and subsequently allows rapid isolation of genes encoding proteins (or protein fragments) with desired properties. Bacteriophage-based *in vitro* display technologies have now become mainstream antibody and protein engineering and discovery platforms [1]. However, prokaryotic expression-based display technologies have limitations, in particular for the expression of large multi-chain vertebrate proteins, such as full-length antibodies. Due to the general inability to express normally glycosylated and folded full-length antibodies, phage-display technologies have resorted to displaying minimal antigen-binding regions of antibodies, such as single-chain fragment variable or Fab fragments. This can pose challenges if the final clinical use of intended product is a full-length glycosylated antibody expressed in a mammalian host cell, as the cellular expression machinery of mammalian cells is very different from *Escherichia coli*.

B lineage cells in the immune system select antibody heavy- and light-chain combinations based on their ability to productively fold and pair [2]. On the level of heavy-chains this is quality controlled early in B lymphopoiesis by testing nascent heavy-chain for its pairing capability with the pre-B cell specific and invariant surrogate light-chain complex composed of λ_5 and V_{preB} proteins that tests heavy-chains for the expression of a pre-B cell receptor, which then generates a specific signal allowing B lymphopoiesis to proceed *in vivo* [3,4]. The subsequent association of conventional light-chains with heavy-chains is a carefully controlled process, and light-chains only pair with heavy-chains if they are able to displace the heavy-chain chaperone immunoglobulin heavy chain binding protein (BiP) that binds to the CH1 domain of immunoglobulin heavy-chains. Displacement of BiP induces proper folding of the CH1 domain and eventually results in the formation of a stable antibody with two identical heavy-chains and two identical light-chains [5–7]. Not every heavy-/light-chain association will result in a stable antibody molecule, and B lineage cells that fail to express a matching heavy-/light-chain combination will fail to

Abbreviations: A-MuLV, Abelson murine leukemia virus; BCR, B cell receptor; BiP, binding immunoglobulin protein; CDR, complementarity determining region; DR6, death receptor 6; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; IRES, internal ribosome entry site; MACS, magnetic activated cell sorting; Retrocyte Display®, *Retro*viral B lympho*cyte Display*; TNF-α, tumor necrosis factor-alpha; VH, heavy chain; Vκ, kappa light-chain.

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1046-2023/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymeth.2013.09.003

mature [8]. In absence of this intricate cellular proofing system, binders identified from prokaryotic expression systems based on forced expression of random combinations of heavy- and light-chain fragments may not always yield antibodies with favorable biophysical properties upon re-formatting into full-length IgG and subsequent expression in mammalian cells.

In an effort to remedy this limitation, various *in vitro* approaches have been developed in recent years in which antibodies are displayed as full-length IgG on the surface of lower eukaryotic or mammalian cells, with the aim to render discovery of therapeutic monoclonal antibodies with favorable biophysical properties more effective [9–14]. To achieve this, different mammalian expression systems have been employed including for instance, transient expression using non-replicating plasmid vectors, episomally replicating plasmids or viral vector systems such as Sindbis virus or vaccinia virus, or stable expression using Flp recombinase [10,14,15]. However, many of these technologies are either limited by the size of the antibody libraries that can be expressed by these systems, or by the lack of a stable genotype to phenotype coupling in mammalian cells. We have developed Retrocyte Display® (*Retro*viral B lympho*cyte Display*) technology with the aim to display full-length fully human antibodies in their natural B-cell environment, in order to mimic as closely as possible the natural heavy- and light-chain pairing and folding process *in vivo*. We report here the construction of a high diversity antibody display library in murine pre-B cells, and the screening of this library for isolation of high affinity antibodies to two different targets. This powerful technology represents a further evolution in the use of mammalian display for the discovery of fully human antibodies.

2. Materials and methods

2.1. Antibody gene libraries

2.1.1. Heavy-chain library

cliniMACS (Miltenyi Biotec) separated $CD19^+$ B cells of leukapheresis material from six healthy donors were obtained from University hospital Erlangen. Total mRNA was prepared from 3.0E8 B cells and cDNA was synthesized (RevertAid First Strand cDNA Synthesis Kit, Fermentas). Library construction was performed separately per heavy-chain (VH) family in order to achieve the desired high complexity of at least 1.0E9 colony-forming units (an estimated 5–10-fold over-representation of the original VH diversity present in the donor mRNA pool). The variable regions of VH1–6 were PCR amplified with family-specific primers, digested with restriction enzymes and ligated into the VH retroviral expression vector (Fig. 1A). *E. coli* DH10B were transformed with the ligated VH-chain library and plated on LB (Amp) agar plates. Bacteria colonies were collected and plasmid DNA was purified. Finally, DNA preparations of all six VH families were pooled. The resultant VH-chain library was quality controlled by performing 454-sequencing (see Section 2.6). 15212 sequences were obtained indicating faithful capture of the natural repertoire. Relative representation of VH families as percent of total genes sequenced is shown in Fig. 1B. VH-chain complementarity determining region (CDR) 3 length distributions are shown in Fig. 1C.

Empty retroviral heavy- and light-chain expression constructs contain the coding information for either chain constant region and a restriction enzyme-flanked non-coding stuffer sequence upstream that can be replaced by cloned variable VH- and κ- or λ-chain coding regions from human or synthetic origin (Fig. 1A). These constructs can be used to clone single VH and kappa light-chain (Vκ) coding regions from selected antibodies in order to generate cells expressing a specific recombinant monoclonal antibody on the cell surface, or they can be used for the cloning of diverse repertoires of heavy- and light-chain regions, e.g. isolated by RT-PCR from human B cell sources using heavy- and light-chain specific primers, or generated by gene synthesis. Detailed information on retroviral expression vector construction, restriction enzymes used, PCR primers and PCR conditions for Sections 2.1.1 and 2.1.2 can be found in [16].

2.1.2. Light-chain mini library

91 Vκ-chains with 100% V-region homology to germline Vκ sequences were cloned in the Vκ expression vector (Fig. 1A). The resultant Vκ-gene family distribution was composed as follows: 48 Vκ1, 13 Vκ2, 20 Vκ1, 4 Vκ4 and 6 Vκ5 chains.

2.2. Cells

For all experiments described in this report we used 1624–5 cells, an Ig-negative Abelson murine leukemia virus (A-MuLV) transformed pre-B cell line derived from a triple-surrogate light-chain knock-out mice [17] in order to eliminate interference of murine surrogate light-chain components (encoded by λ_5, V_{preB1}, or V_{preB2}) with the expression of cognate human heavy- and light-chain pairs. 1624–5 cells were cultured in SF-IMDM media (Amimed) supplemented with 3% fetal calf serum (FCS; Amimed) and 0.1% β-mercaptoethanol in Erlenmeyer flasks (80 rpm in a Multitron Standard incubator; INFORS HT) or filter cap cell culture flasks (Cellstar) at 37 °C under 10% CO_2. CHO-S (Invitrogen) cells were grown in SF-IMDM media supplemented with 2% FCS (PAA) in filter cap cell culture flasks (Cellstar) at 37 °C under 10% CO_2.

2.3. Retroviral particle production

Heavy- and light-chain gene libraries were packaged separately into replication-incompetent retroviral particles in HEK 293 cells (ATCC) expressing gag-pol and env genes (pVPack vector system, Stratagene) using FuGENE 6 Transfection Reagent (Roche Applied Science) according to manufacturer instructions. The resulting retroviral supernatants were harvested three days later and were kept frozen at −80 °C. 1624–5 pre-B cells were spin-transduced with retroviral supernatants for 3 h at 30 °C as previously described [18]. Heavy- and light-chain gene libraries were transduced separately and sequentially (Fig. 2). Transduction was done in 6-well plates (Cellstar) with 1.5E08 cells per plate and in Eppendorf centrifuges. Prior to experiments, retroviral supernatants were titrated to give <5% transduction as measured by expression of the CD4 membrane marker. Following transduction, cells were transferred in Erlenmeyer flasks, with cell concentration of 3.0E06 per 1 mL, and allowed to expand for 24 h.

2.4. Antigens

Death receptor 6 (DR6)-Fc, tumor necrosis factor-alpha (TNF-α) and CD28-Fc were from R&D Systems. Antigens were labeled using EZ-Link Sulfo-NHS-LC-Biotin (Pierce), and LYNX Rapid PE or APC conjugation (AbD Serotec) kits according to the manufacturer's instructions.

2.5. Cell sorting

Magnetic activated cell sorting (MACS) was done according to the manufacturers instructions (Miltenyi Biotec). Cells were labeled with biotinylated anti-human Ig κ antibody (BD Biosciences), DR6 or TNF-α (20 ng per 1.0E6 cells) for 30 min at 4 °C followed by labeling with anti-biotin microbeads for 15 min, and subsequent positive cell selection using an LS column. Prior to this, Fc receptor block (eBioscience) was added to cells for 10 min at 4 °C at a concentration