Journal of Immunological Methods 391 (2013) 60–71

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

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

Research paper

Discovery of diverse and functional antibodies from large human repertoire antibody libraries

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ARTICLE INFO

Article history:
Received 23 October 2012
Received in revised form 12 December 2012
Accepted 21 February 2013
Available online 27 February 2013

Keywords:
Phage display
Antibody discovery
Human antibody repertoire

ABSTRACT

Phage display antibody libraries have a proven track record for the discovery of therapeutic human antibodies, increasing the demand for large and diverse phage antibody libraries for the discovery of new therapeutics. We have constructed naïve antibody phage display libraries in both Fab and scFv formats, with each library having more than 250 billion clones that encompass the human antibody repertoire. These libraries show high fidelity in open reading frame and expression percentages, and their V-gene family distribution, V_H-CDR3 length and amino acid usage mirror the natural diversity of human antibodies. Both the Fab and scFv libraries show robust sequence diversity in target-specific binders and differential V-gene usage for each target tested, supporting the use of libraries that utilize multiple display formats and V-gene utilization to maximize antibody-binding diversity. For each of the targets, clones with picomolar affinities were identified from at least one of the libraries and for the two targets assessed for activity, functional antibodies were identified from both libraries.

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1. Introduction

Monoclonal antibodies are a significant and growing class of therapeutics for a wide range of indications including cancer, metabolic, and inflammatory diseases. Phage display antibody libraries are an important tool for the discovery of human monoclonal antibodies, providing two marketed products, one under review by the FDA, and many more at various stages of clinical trials (Nelson et al., 2010). Specificity and affinity are key components for the successful transition of an antibody from the lab to the clinic. Library size and diversity are extremely important in this endeavor as the larger and more diverse a library, the greater the chance of finding high affinity antibodies with diverse paratopes that bind diverse epitopes (Perelson and Oster, 1979; Perelson, 1989; Griffiths et al., 1994; Vaughan et al., 1996).

The first fully human phage displayed antibody fragment library had 10^7 members and antibody fragments to four proteins were isolated with affinities as low as 86 nM (Marks et al., 1991). Other groups went on to construct larger human libraries: two Fab (6.5×10^{10} and 3.7×10^{10}) (Griffiths et al., 1994; de Haard et al., 1999) and one scFv (1.4×10^{10}) (Vaughan et al., 1996). From each library, antibody fragments with single-digit nanomolar affinities were isolated, and from the scFv library, two fragments were isolated with affinities less than 1 nM. However, Fabs with only moderate affinities (>800 nM) were recovered when selecting from a small portion of the Griffiths library (10^7 clones), supporting the claim that the larger the library, the greater the probability of isolating high affinity antibodies (Griffiths et al., 1994). To

Abbreviations: scFv, single-chain fragment variable; Fab, fragment antigen binding; PBMC, peripheral blood mononuclear cell; V, variable; V_H, variable heavy chain; V_L, variable light chain; V_κ, variable kappa light chain; V_λ, variable lambda light chain; PPE, periplasmic extract; ORF, open reading frame; CDR, complementary determining region; FR, framework region; TIE, tyrosine kinase with immunoglobulin-like and EGF-like domains; β-gal, beta-galactosidase; ANG, angiopoeitin; Ins, insulin; InsR, insulin receptor; RCA, rolling circle amplification; HRP, horseradish peroxidase; PBS, phosphate buffered saline.

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http://dx.doi.org/10.1016/j.jim.2013.02.010

this end, we constructed two phagemid libraries, XFab1 and XscFv2, which display Fab and scFv fragments, respectively, each with more than 2.5×10^{11} members maximizing the potential for isolating high affinity antibodies against any target of interest.

Antibody diversity is influenced by the number of donors, donor tissues used, the types of variable regions from which antibody sequences are amplified and the choice in the utilization of V-gene frameworks. For each of XFab1 and XscFv2, variable regions were amplified from thirty racially-diverse healthy donors using a variety of tissues including bone marrow, PBMCs, spleen and lymph node. The amplification strategy encompasses variable domains derived from IgM, IgG, IgA, IgE and IgD. While other commercial phage display antibody libraries have restricted antibody frameworks to enhance stability or expression of the displayed fragments (Söderlind et al., 2000; Hoet et al., 2005; Rothe et al., 2008), in the XFab1 and XscFv2 libraries, all prominent V-gene families encompassing the human repertoire were utilized to allow increased structural diversity. The performance of a Fab versus a scFv fragment type depends on factors including stability of the variable domains and the expression of the individual molecules (Rothlisberger et al., 2005), as well as the possible differences in the donor pools. Therefore, the performance characteristics of each library will differ, making it advantageous to have a variety of libraries available for selection. Although, fully human naïve Fab and scFv libraries have been made before (Marks et al., 1991; Griffiths et al., 1994; Vaughan et al., 1996; de Haard et al., 1999; Glanville et al., 2009; Lloyd et al., 2009), here we present the first direct comparison between the performances of the two formats. This comparison can be done because these two libraries were constructed using similar donor sources, construction methods and vector backbones, limiting the variability between the libraries.

Both XFab1 and XscFv2 were assessed for multiple qualification parameters, including percentage of open reading frame (%ORF), expression levels, V-gene family distribution, V_H-CDR3 length, and germline occurrence. Our libraries have been used for selections against seven targets and the resulting clones analyzed to determine unique hit rate, V-gene usage, and affinity. These parameters have allowed us to validate and compare the libraries and demonstrate their utility as potential sources for high affinity, functional therapeutic antibodies.

2. Materials and methods

2.1. Source material, cell lines, enzymes

The source RNA and cDNA used to amplify the V-genes was purchased from AllCells and Cureline. The *E. coli* strain TG1 (Lucigen) was used for all molecular cloning, phage production, and expression assays. Restriction endonucleases and T4-DNA ligase were purchased from New England Biolabs. KOD Hot Start DNA Polymerase and associated 10× buffer, dNTP mix, and $MgSO_4$ (EMD Biosciences), were used for all PCR reactions. Some PCR reactions also included betaine (Sigma-Aldrich) and/or DMSO (Sigma-Aldrich). PCR primers were purchased from Elim Biosciences or IDT. ArrayScript™ Reverse Transcriptase (Ambion) with Random primers (NEB) was used to make cDNA libraries from RNA samples. All media and solutions were purchased from Teknova.

For the CHO cells expressing TIE2 and InsR used for screening, mammalian expression vectors encoding TIE2 and InsR were each transfected into CHO-K1 cells using a PEI transfection reagent (JetPEI®, Polyplus). Individual G-418-resistant clones were screened by FACS using commercially available antibodies to TIE2 or InsR.

2.2. Library construction

2.2.1. XFab1

XFab1 used cDNA generated from 15 PBMC samples and 15 bone marrow samples. The variable regions were amplified from cDNA using primers designed based on sequences in V-Base to amplify each family of $V_\lambda 1$–$V_\lambda 10$, $V_\kappa 1$–$V_\kappa 6$, and $V_H 1$–$V_H 6$ individually with forward primers annealing to the V segment and reverse primers annealing in the C_λ or C_κ for V_λ and V_κ and in the V_HJ region for V_H (Table S1). Secondary PCRs (Table S2) were performed to add restriction enzyme sites 5′ and 3′ to each V-gene for cloning into pXHMV-US2-L-Fab or pXHMV-US2-K-Fab vectors (Fig. S1). The PCR products for each variable region were pooled according to the natural distribution as described on V-Base. The light chain variable regions were cloned first using restriction digest with *Sfi*I and *Avr*II for V_λ and *Sfi*I and *Bsi*WI for V_κ and transformed into electrocompetent TG1 cells (48 μg DNA in 48 200 μL transformations for V_κ and 65 μg DNA in 65 200 μL transformations for V_λ). Transformations were spread on 2xYT medium with 2% glucose and 100 μg/mL carbenicillin, which were incubated overnight at 30 °C. The following morning the bacteria were scraped from the plates, combined and plasmid DNA purified with the GenElute™ HP Maxiprep Kit (Sigma-Aldrich). The resulting DNA was prepared for cloning V_H with *Nco*I-HF and *Nhe*I-HF. The ligated DNA was cleaned with the Wizard® SV Gel and PCR Clean-up system (Promega) and transformed into electrocompetent TG1 cells (66 μg DNA in 66 200 μL transformations for V_κ and 100 μg DNA in 100 200 μL transformations for V_λ). Transformations were spread on 2xYT medium with 2% glucose and 100 μg/mL carbenicillin, which were incubated overnight at 30 °C. The following morning the bacteria were scraped from the plates, combined, and stored in 15% glycerol 2xYT at −80 °C.

2.2.2. XscFv2

The scFv library was constructed similarly to the above described Fab library with the following changes. Primer sequences are listed in Tables S3 and S4. cDNA from 20 PBMC samples, 8 bone marrow samples, 1 lymph node sample, and 1 spleen sample were used. The reverse secondary PCR primers for VH and forward secondary primers for V_κ and V_λ had complementary extensions for an AST(G_4S)3 linker and the forward secondary PCR primers for V_H and reverse secondary primers for V_κ and V_λ had sequences to add flanking *Sfi*I restriction sites. A tertiary PCR step was then done to assemble the full length scFv fragment, which was next cloned into pXHMV-scFv (Fig. S1) using the *Sfi*I sites. The ligated DNA was transformed into electrocompetent TG1 cells (147 μg DNA in 120 200 μL transformations for V_κ and 44 μg DNA in 40 200 μL transformations for V_λ). Transformations were spread on 2xYT medium with 2% glucose and

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