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Deciphering antigen-responding antibody repertoires by using next-generation sequencing and confirming them through antibody-gene synthesis

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ABSTRACT

Vast diversity and high specificity of antigen recognition by antibodies are hallmarks of the acquired immune system. Although the molecular mechanisms that yield the extremely large antibody repertoires are precisely understood, comprehensive description of the global antibody repertoire generated in individual bodies has been hindered by the lack of powerful measures. To obtain holistic understanding of the antibody-repertoire space, we used next-generation sequencing (NGS) to analyze the deep profiles of naive and antigen-responding repertoires of the IgM, IgG1, and IgG2c classes formed in individual mice. The overall landscapes of naive IgM repertoires were almost the same for each mouse, whereas those of IgG1 and IgG2c differed considerably among naive individuals. Next, we immunized mice with a model antigen, nitrophenol (NP)-hapten linked to chicken γ-globulin (CGG) carrier, and compared the antigen-responding repertoires in individual mice. To extract the complete antigen response, we developed an intelligible method for detecting common components of antigen-responding repertoires. The major responding antibodies were IGHV1-72/IGHD1-1/IGHJ2 for NP-hapten and IGHV9-3/IGHD3-1/IGHJ2 for CGG-carrier protein. The antigen-binding specificities of the identified antibodies were confirmed through ELISA after antibody-gene synthesis and expression of the corresponding NGS reads. Thus, we deciphered antigen-responding antibody repertoires by inclusively analyzing the antibody-repertoire space generated in individual bodies by using NGS, which avoided inadvertent omission of key antibody repertoires.

1. Introduction

The antibody system's potency depends on its vast diversity and fine specificity of antigen recognition. The antibody-producing B-cell repertoire in an individual is generated by VDJ gene recombination in immunoglobulin gene loci [1,2], and its dimension is estimated to be $>10^{15}$ [3]. Immunization with an antigen triggers clonal expansion of antigen-specific B cells pre-formed in the immune system of individuals. To comprehensively understand the protective antibody response against invading pathogens without unconsciously omitting precious antibody repertoires, the overall B-cell repertoires and their dynamic changes in individual immune systems must be precisely described and analyzed. The B-cell repertoires generated in individuals had been regarded as a "black-box" because of the astronomical number of B-cell clones involved; however, the recent advent of next-generation sequencing (NGS) technology has led to a breakthrough in obtaining an overview of B-cell repertoires [4]. Whole antibody repertoires were first analyzed in zebrafish [5], and then in mice [6,7] and humans [6,8,9]. Although this method serves as a powerful tool for studying adaptive immune responses, the commonalities and uniqueness of

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antibody repertoires developed in individuals remain inadequately analyzed.

In this study, we obtained a global view of the common and unique features of antibody repertoires generated in individual mice by using the pyrosequencing technique. We also developed a simple and confirmative method to decipher antigen-responding antibody repertoires by comparing all aspects of B-cell repertoires of individual mice. The accuracy of the detected repertoires was confirmed through gene synthesis and protein expression of the antibody-sequence outputs from NGS.

2. Materials and methods

2.1. Mice and immunizations

We maintained 7–8-week-old C57BL/6J female mice (SLC Japan) in a specific-pathogen-free facility and immunized them intraperitoneally with 100 μg of either 4-hydroxy-3-nitrophenylacetyl $(NP)_{48}$-chicken γ-globulin (CGG) or CGG with alum-adjuvant, and 2 weeks later, collected the spleens for NGS analysis. All animal experiments were performed according to institutional guidelines and with the approval of the National Institute of Infectious Diseases Animal Care and Use Committee.

2.2. RNA preparation, cDNA synthesis, and 5′-RACE PCR amplification

Total RNA was extracted separately from each spleen by using a TRIzol Plus RNA Purification Kit (Thermo Fisher) (Fig. 1A and B), and 1–4 μg of the RNA was used for first-strand cDNA synthesis by SMARTer RACE cDNA Amplication Kit (Clontech) with oligo-dT-containing 5′-RACE CDS Primer A and SMARTer II A Oligonucleotide. Next, cDNAs were amplified by PCR in a 20 μl reaction mixture containing 0.5 μL of unpurified cDNA, 0.4U Phusion High-Fidelity DNA Polymerase, 200 μM each dNTP and 250 nM primers in 1xHF buffer. Universal forward primers of 5′-RACE containing Multiplex Identifier (MID) adaptors (MID9_NUP 5′-TAGTATCAGCAAGCAGTGGTATCAACGCAGAGT-3′, MID11_NUP 5′-TGATACGTCTAAGCAGTGGTATCAACGCAGAGT-3′, MID14_NUP 5′-CGAGAGATACAAGCAGTGGTATCAACGCAGAGT-3′) were used with reverse primers specific for immunoglobulin-constant-region-1 CμH1 (5′-CACCAGATTCTTATCAGACAGGGGGCTCTC-3′), Cγ1H1 (5′-

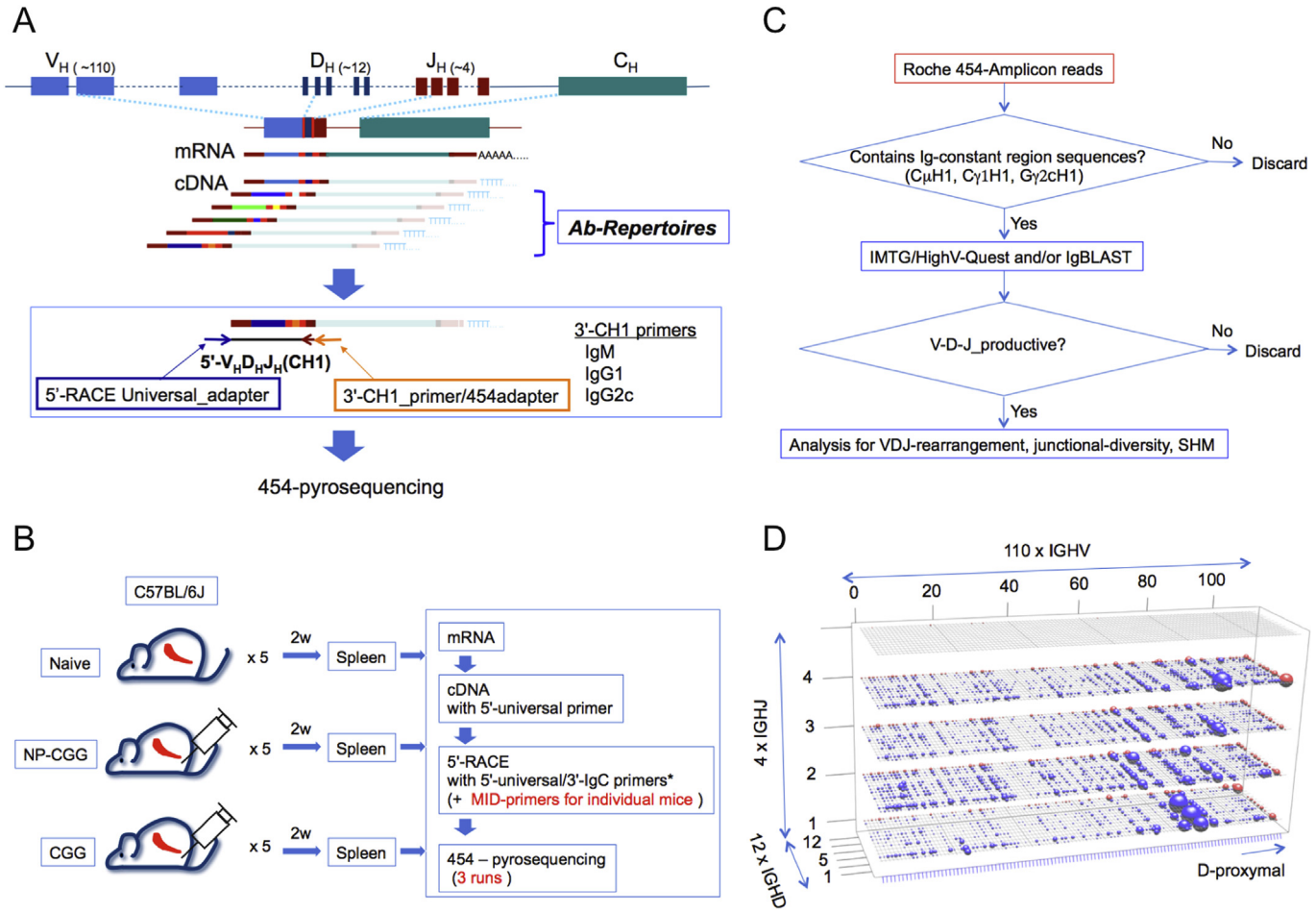

Fig. 1. Schematic of sequencing strategy, immunization protocol, data-processing flowchart, and data visualization for analyzing antibody repertoires in individual mice. (A) Spleen total RNA was reverse-transcribed and fragments containing V_H-D_H-J_H and partial CH1 were PCR-amplified using 5′-RACE universal primer and 3′-CH1 primer specific for CμH1, Cγ1H1, or Cγ2cH1. These PCR products representing IgM, IgG1, and IgG2c repertoires were equally mixed and pyrosequenced. (B) C57BL/6 mice (5 each) were not immunized (Naive group), immunized with NP-CGG (NP-CGG group), or immunized with CGG (CGG group), and after 2 weeks, spleen total RNA from each mouse was purified and pyrosequenced. (C) Amplicon reads obtained after pyrosequencing were processed as follows: (1) read sequences were translated in 6 reading-frames and checked for a defined sequence of CμH1, Cγ1H1, or Cγ2cH1; (2) sequences were examined using IMGT/HighV-Quest and IgBLAST; (3) purified sequences containing a productive VDJ junction were collected; and (4) these sequences were further analyzed. (D) To visualize the overall antibody-repertoire landscape, reads were arrayed on 3D-VDJ-plots in which the x-axis represents 110x IGHV genes and the y-axis represents 12x IGHD genes and 4x IGHJ genes. The gene order in the mesh is the same order as on the chromosome. Each sphere's volume represents the number of reads classified on the node. Red spheres: un-annotated V, D, and J genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)