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Characteristics of rabbit hapten-specific and germline-based BCR repertoires following repeated immunization

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Abstract

The rabbit is well known for producing diverse antibodies against various antigens including small molecules such as drugs and toxins, due to a robust immune response. Elucidating how hapten repeated immunization shapes the rabbit B cell receptor (BCR) repertoire is crucial to understanding rabbit immune response to small molecules and assisting rare antibody discovery/engineering. In this study, we enriched and sequenced chloramphenicol (CAP)-specific rabbit B cells following repeated immunization, and analyzed both CAP-specific repertoires combined with the structure and affinity features of V1S69/V1S37 germline-based BCRs. The length of rabbit complementarity-determining region 3 of heavy chain (CDRH3) increased after hapten immunization. Repeated immunization significantly reduced the diversity of CAP-specific rabbit BCR clonotypes, and changed the frequency of VDJ usage and the type of V(D)J recombination. The average number of mutations among VL is notably higher than that of VH genes in rabbits, however, they are both not changed along with repeated immunization. Moreover, repeated immunization resulted in an increase surface charge and a decrease in solvent accessible surface area, leading to improvement in the stability of the most abundant V1S69/V1S37 germline-based BCR, along with an affinity increase from an IC_{50} of 898.2 ng mL^{-1} at the 1st immunization to 4.16 ng mL^{-1} at the 6th immunization. The study provides a benchmark for rabbit repertoire-scale analyses and offers a method for antibody discovery of small molecules.

Keywords Rabbit, Hapten-specific BCR repertoires, Germline-based BCR repertoires, Repeated immunization

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Introduction

Rabbit antibodies are very attractive for applications in biomedical research and especially for immunological techniques, such as immunoassay. Rabbits possess unique natural features that make their antibody repertoire attractive for a wide range of applications. Unlike other species, the primary B-cell receptor (BCR) of the rabbit as membrane immunoglobulins (Ig) is modified through the recombination of variable (V), diversity (D), and joining (J) genes during antigen-dependent immune responses and further diversified by both somatic gene conversion (SGC) [1] and somatic hypermutation (SHM). Additionally, the larger body size of a rabbit than mice leads to a higher number of various B cells in the rabbit. Thus, rabbits theoretically and practically elicit strong

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immune responses against a broad range of antigens, including small molecules, when compared to mouse [2]. Hapten-specific monoclonal antibody (mAb) is an important tool for immunoanalytical applications, especially for monitoring environmental contaminants like hormones, as well as mycotoxins and veterinary drug residues in the food industry. Understanding the salient features of the rabbit hapten-specific BCR repertoires could deepen the understanding of the small molecule immune response in the rabbit. However, there have been limited reports on rabbit hapten-specific and germline-based BCR repertoires, which are critical for rare antibody discovery, antibody engineering, and immunoassay establishment.

Earlier studies indicated that more than 200 V gene segments available within the rabbit Ig heavy chain (IGH) locus, over 50% of these segments are "non-functional", and with about 80% to 90% of circulating antibodies are derived from the IGHV1 gene [3]. The rabbit repertoire of VDJ rearrangements is further limited due to a small number of IGHD and IGHJ genes. In the rabbit, the difference from other species was observed such as humans and mice where VH plays a fundamental role in antigen recognition, VL appears to be a major contributor to the rabbit BCR diversity due to more than 50 IGLV functional genes, resulting in a potential larger rabbit VL repertoires [4]. Moreover, the longer complementarity-determining region 3 of light chain (CDRL3) with an average of 12 ± 2 amino acids, compared to 9 ± 1 amino acids in mice and hamans, leading to a potentially larger repertoire of CDRL3 in the rabbit [5].

Comprehensive analysis of BCR repertoires has the potential to provide valuable insights into the dynamics of the B cell responses following antigen stimulation [6]. Wen et al. traced the whole human BCR repertoires during vaccination and found that the repertoire exhibited a high degree of clonal diversity after extensive vaccine boost [7]. Antigen exposure has also been reported to cause a decrease in the length and hydrophobicity of the complementarity-determining region 3 of heavy chain (CDRH3) and to alter the amino acid content of CDRH3 relative to the naive repertoire in humans [8]. Gerald et al. showed that the protein-specific BCR repertoires share common features and also have some distinct characteristics, such as preferential variable gene usage, variable region mutation levels, or lengths of the complementarity-determining region 3 (CDR3) [9]. The stochastic nature of the processes responsible for generating the higher affinity B cells along with the repeated immunization. Thomson et al. indicated that certain germline genes are more frequently used than others in antibodies to particular pathogens in humans [10]. Next-generation sequencing of Ig genes has become an essential tool in immunology but it is known that similar sequences may have markedly different epitope complementarity. Therefore, computer-aided structural analysis of B cell repertoire should be utilized to assist the current gene analysis to provide a clearer understanding of the BCR diversity [11].

In this study, chloramphenicol (CAP) was employed as an analyte, which is widely used for the prevention and treatment of poultry diseases. Due to polluted food posing great health hazards to humans [12, 13], CAP has been banned worldwide. CAP-specific rabbit BCR repertoires were sequenced at multiple time points during a six-time immunization at a 1-month interval compared with native BCR repertoires to supply an insight into the evolution of rabbit BCR. The V1S69/V1S37 germline-based BCR repertoires were exhaustively analyzed, combined with the computer-based structure analysis of the variable region (Fv), and experiment-based evaluation of the single-chain variable fragment (scFv). This study firstly supplies the features of hapten-specific rabbit BCR repertoires and germline-based rabbit BCR, along with the effects of repeated immunizations. It provides a basic understanding of rabbit B cell response to a hapten and proposes an efficient method for rabbit antibody discovery.

Results and discussion

Repeated immunization increases the rabbit antibody response to CAP

To analyze the antibody response induced by CAP-KLH repeated immunization, rabbits were immunized six times at 4-week intervals and repeatedly boosted with CAP-KLH as illustrated in Fig. 1A. The antisera titer and affinity to CAP were monitored at each immunization to reveal the antibody response, which both increased gradually with repeated immunization from the 1st immunization to the 4th immunization, as shown in Fig. 1B and C. The antisera titer reached the highest point, with the average antisera titer of 6.5×10^4 after the 4th immunization. Similarly, the antisera affinity reached the highest point with the average IC_{50} of 0.74 ng mL^{-1} after the 6th immunization (Fig. 1C). The antisera results were tested using Analysis of Variance (ANOVA) and Fisher's exact test. The tests demonstrated that repeated boosting with CAP-KLH could increase the antibody amount and improve the antibody's binding ability to CAP in rabbits. This finding is consistent with previous reports on antigens such as tetramethylenedisulfotetramine in rabbits [14], fluoroacetamide in mouse [15], and heroin vaccine in humans [16].

As the central cell of the adaptive immune system, B cells are responsible for mediating the generation of specific antibodies against antigens. Upon secondary

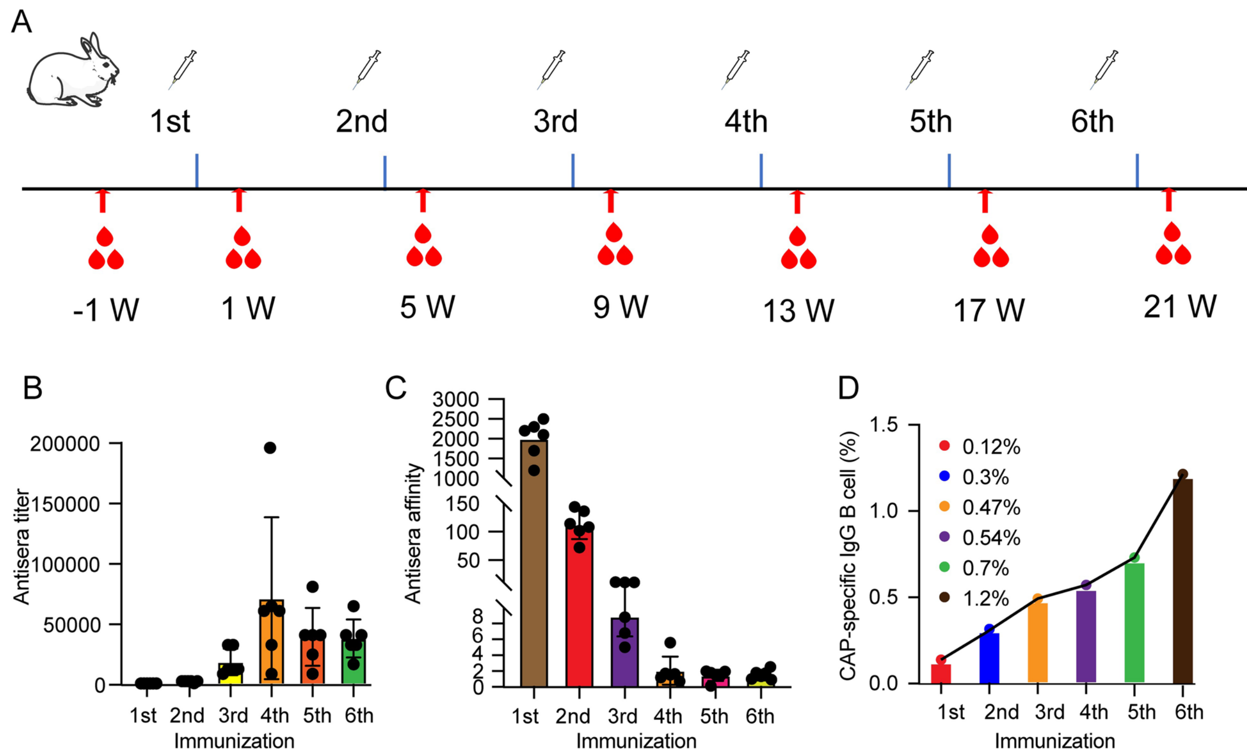

Fig. 1 Antibody response and CAP-specific B cell numbers of repeated immunization in the rabbit. **A** The immunization workflow of rabbits. **B** Antisera titer of the six rabbits during repeated immunization expressed by antisera dilution. **C** Antisera affinity of the six rabbits during repeated immunization expressed by IC_{50} values of CAP in competitive ELISA (ng mL^{-1}). **D** The percentages of CAP-specific IgG B cells during the repeated immunization compared with native PBMCs

encounter with the same antigen, the memory B cells can rapidly produce high-affinity IgG antibodies, leading to a humoral immune response [17]. Liu et al. indicated that the number of hemagglutinin-specific B cells was significantly increased after booster vaccination [18]. To examine the effect of CAP-KLH repeated immunization on the number of CAP-specific IgG B cells, PBMCs from each immunization were collected from the six rabbits. CAP-specific IgG B cells were selected based on their double-positive staining for FITC-CAP-BSA and PE-anti-IgG antibody using FACS (Figure S1). The results showed that the percentage of the CAP-specific IgG B cells in the immunized rabbit PBMCs was gradually increased from 0.12% at the 1st immunization to 1.2% at the 6th immunization. No CAP-specific IgG B cells were observed in the native PBMCs (Figure S1 and Fig. 1D). Thus, repeated immunization could induce the generation of more specific B cells against haptens in rabbits. This is also the main reason why repeated immunization strategy was usually used to induce more B cells to achieve effective protection [19]. Although the number of specific B cells increases with the repeated immunization, it remains unclear how B cell evolution and changes in B cell diversity are affected by hapten repeated immunization. Thus, these key gaps in our understanding require further analysis of BCR repertoires along with repeated immunization.

Repeated immunization declines the diversity of CAP-specific BCR repert

After immunization, naive B cells with lower affinity BCRs enter the germinal centers (GC), where B cell affinity maturation through SGC and SHM in rabbits [20]. High-throughput sequencing of BCR variable region genes could be used to study the B cell repertoire in great depth and shed light on B cell responses. To explore how repeated immunization of CAP-KLH regulate the diversity of CAP-specific BCR repertoires, we prepared and sequenced VH amplicons and VL amplicons of CAP-specific BCRs of each immunization with specific primers (Figure S2 and Table S1). The raw reads and Q20/Q30 analysis were shown in Table S2. We have submitted the sequencing data at NCBI with the accession number of

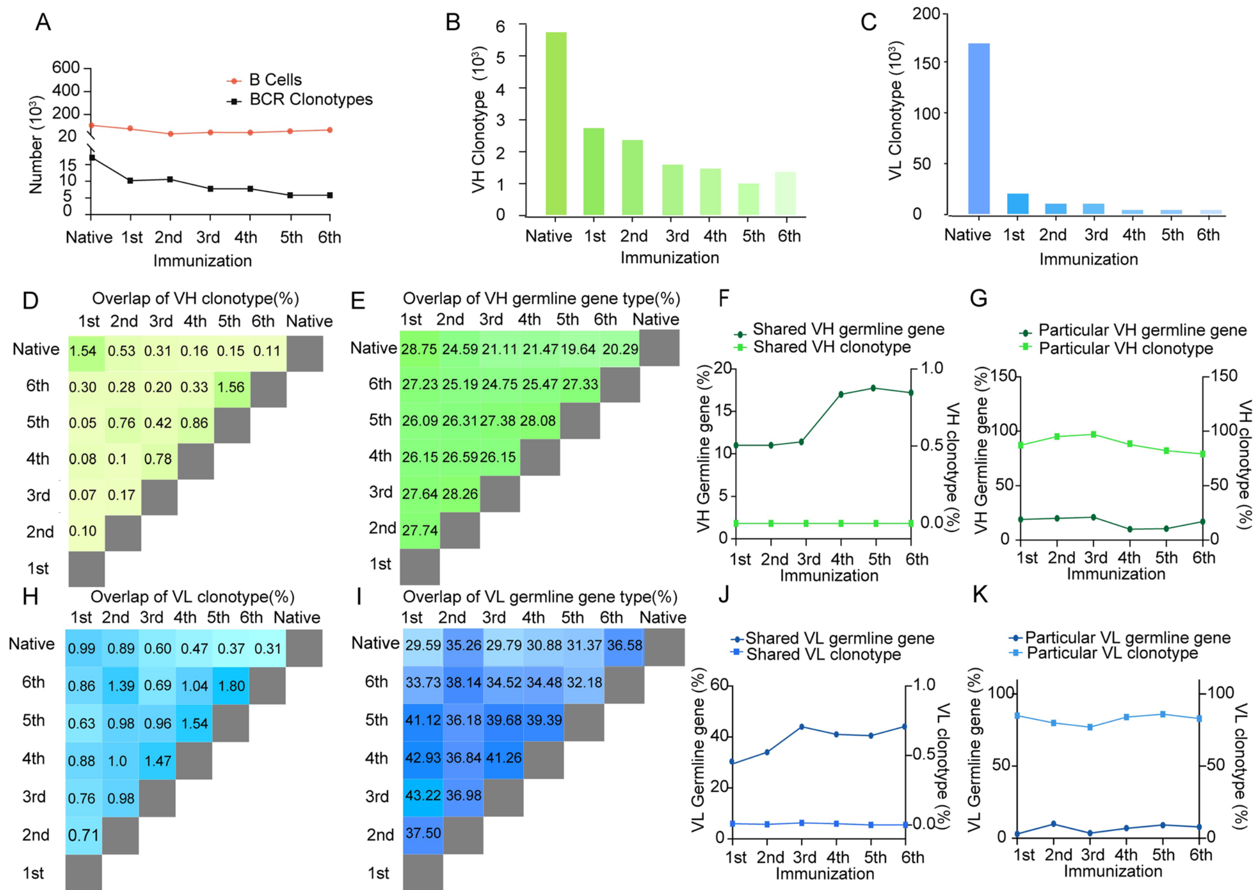

Fig. 2 Analysis of CAP-specific BCR diversity during the repeated immunization. **A** Comparison of the BCR clonotype numbers and B cell numbers. **B** VH clonotype of the CAP-specific BCR repertoires. **C** VL clonotype of the CAP-specific BCR repertoires. **D** Overlap of VH clonotypes. **E** Overlap of VH germline gene type. **F** Shared germline gene and clonotype in VH of the six immunizations. **G** Particular clonotypes and germline gene and clonotype in VH at each immunization. **H** Overlap of VL clonotype. **I** Overlap of VL germline gene type. **J** Shared germline gene and clonotypes in VL of the six immunizations. **K** Particular germline gene and clonotypes in VL at each immunization

PRJNA825090. Sequencing saturation analysis can determine whether the amount of sequencing data meets the requirements to a certain extent, the curve plateaus indicate that sequencing depth is sufficient (Figure S3A and B). As shown in Fig. 2A, the number of the BCR clonotypes is lower than the cell numbers, which indicated the CAP-specific B cells were rapidly expanded due to the encounter of CAP-KLH again. The number of clonotypes containing VH clonotypes and VL clonotypes in the immunized CAP-specific BCR repertoires were significantly lower than that in native BCR repertoires (Fig. 2B and C). Therefore, we assumed that the CAP-specific BCR repertoires were only a small part of the numerous native repertoires, thus, just a small fraction of B cells was exactly activated during antigen immunization in the rabbit. Our finding was similar to that of a vaccine study in humans. Galson et al. previously showed that vaccine-specific BCR repertoires accounted for below 0.1% of the total BCR repertoires, and provided certain stereotypic features in humans [21]. During the repeated immunization, the overlap of VH and VL clonotype both increased between two adjacent immunizations from 0.1% of the VH overlap-clonotype and 0.71% of the VL overlap-clonotype between the 1st and 2nd immunization to 1.56% of VH overlap-clonotype and 1.80% of the VL overlap-clonotype between the 5th and 6th immunization (Fig. 2D, H). This indicated that repeated immunization shapes the CAP-specific BCR repertoire into particular clones. Laserson et al. also showed that certain clones within the global BCR repertoires undergo rapid expansions and contractions in response to vaccination of influenza hepatitis A [22]. However, the overlaps of the germline gene type of both VH and VL were unchanged with the repeated immunization (Fig. 2E and I). Thus, we assumed that the new naive B cell clones with lower affinity to the antigen and that entering the GC after each immunization may derive from the same germline gene. Then the shared VH/VL clonotypes and germline genes

among the six immunizations were also analyzed, and the result showed that the whole shared germline genes of both VH and VL increased from 11.3% and 29.3% in the 1st immunization to the 17.4%–41.2% in the 6th immunization, but there were no shared VH and VL clonotypes among all six immunizations (Fig. 2F and J). This indicates that one CAP-specific B cell clone did not persist throughout the whole repeated immunization. Moreover, as shown in Fig. 2G and K, the particular VH/VL clonotypes and germline genes that emerged in only one immunization did not change along with the repeated immunization, and the percentage of the particular germline genes was significantly higher than that of the shared germline genes. Viant et al. have found that the immune response is mainly shaped by the precursor cells during the HIV-1 antigen vaccines in humans [23]. Thus, we assumed the increased antibody response may mainly due to these newly emerged germline original precursor B cells that matured into high-affinity CAP-specific B cells to produce high-affinity antibodies.

Repeated immunization changes the CDRs length

The diversity of BCR repertoires is generated through recombination of V, D, and J gene segments in the germlines. Further diversity is introduced through the imprecise junction of these gene segments, which can include the addition of P- and N-nucleotides adjacent to the D segment, SGC, and SHM in rabbits [24]. CDR3 is the most hypervariable region in the BCR repertoires due to the junction of V, (D), and J gene segments, and is critical for antibody recognition of antigens. As shown in Fig. 3A, the average CDRH3 region with 15 amino acids in the immunized CAP-specific BCR repertoires is significantly longer than that of 9 amino acids in the native BCR repertoires. However, the length of CDRL3 in the immunized CAP-specific BCR repertoires shows no signifícnat difference, with an average length of 10 amino acids (Fig. 3B). Kodangattil et al. previously reported the CDRH3 and CDRL3 length distributions were similar in native rabbit lymphocytes and peptide immunized lymphocytes. However, we found the hapten-specific BCR repertoires towards longer CDRH3 and sustain the length of CDRL3 in rabbits. It is indicated that traditional mouse antibodies always present cavities for small molecules to bind [25]. Thus, we assumed that longer CDRH3 of rabbits facilitated its binding to small molecules.

Repeated immunization shapes the characteristics of gene usage and recombination

The diversity of the IGLV genes was significantly higher than that of IGLH genes as the percentage of top 20 IGLV genes accounted for only 27% compared with the percentage of top 20 IGHV genes, which was 94% in native rabbit BCR repertoires (Fig. 3C and F). The IGHV mainly used in the study is IGHV1S69, with a frequency of 67%–81% in rabbits immunized with the hapten. However, Kodangattil et al. found that the IGHV1S45 gene was the main IGHV gene in rabbits immunized with a peptide [26], indicating that the use of the dominant gene has a significant bias towards the type of antigen. The usage of the top 10 IGHDs is similar in both the native and CAP-specific BCR repertoires (Fig. 3D). Although there is no difference in the usage of IGHJ genes between the native and CAP-specific BCR repertoires, the IGHJ4 is the most frequently used IGHJ gene, accounting for 75% (Fig. 3E). Likewise, the IGLJ1 is the most frequently used IGLJ gene in both the native and CAP-specific BCR repertoires (Fig. 3G). The above results indicate that the rabbit hapten-specific BCR repertoires have distinct features in V, D, and J gene usage.

The VJ and VDJ recombination are other main factors in the diversity of the rabbit BCR repertoires. Hsu et al. indicated that different V gene segments are used at different frequencies, and certain D genes may be more often recombined with specific J genes in rabbit splenocytes. The IGHV1S40 or IGHV1S45 was indicated to recombine preferentially with IGHJ4 in a rabbit immunized with a 16-mer peptide [26]. As shown in Table S3, the IGHV1S69 mainly recombined with IGHJ4 in CAP-specific rabbit BCR repertoires. The IGHV1S69 differs from the IGHV1S40 by 13 mutations and three deletions; therefore, antibodies encoded by IGHV1S69 and IGHV1S40 should result in quite different antigen-binding sites. Ros et al. indicated that more than 50 functional genes preferentially use one or two IGKJ genes in rabbits [27]. Kodangattil et al. indicated that IGKV1S10、IGKV1S36、IGKV1S1、IGKV1S17 and IGKV1S34 mainly combined with IGKJ1-1 and IGKJ2-1 in peptide immunized rabbits [26]. In this study, we found that numerous recombination events of various IGKVs with IGKJ1-2 in native rabbit BCR repertoires. However, after the 1st immunization, many IGLVs recombined with IGLJ5 and IGLJ6 were merged, and this IGLVs-IGLJs recombination gradually disappeared in CAP-specific BCR repertoires after the 3rd immunization (Table S4). We observed the VJ recombination mainly involving the IGKV1S36-IGKJ1-2 and IGKV1S36-IGKJ1-2 in CAP-specific BCR repertoires. Compared to immunization with peptide antigens, haptens are often highly gene-segment restricted. For example, the anti-NP response in C57BL/6 mice was dominated by B cells utilizing VH1-72 and Vλ1 genes, constituting more than 90% of the response. In this study, the feature of hapten-specific rabbit BCR repertoires was significantly different from that of

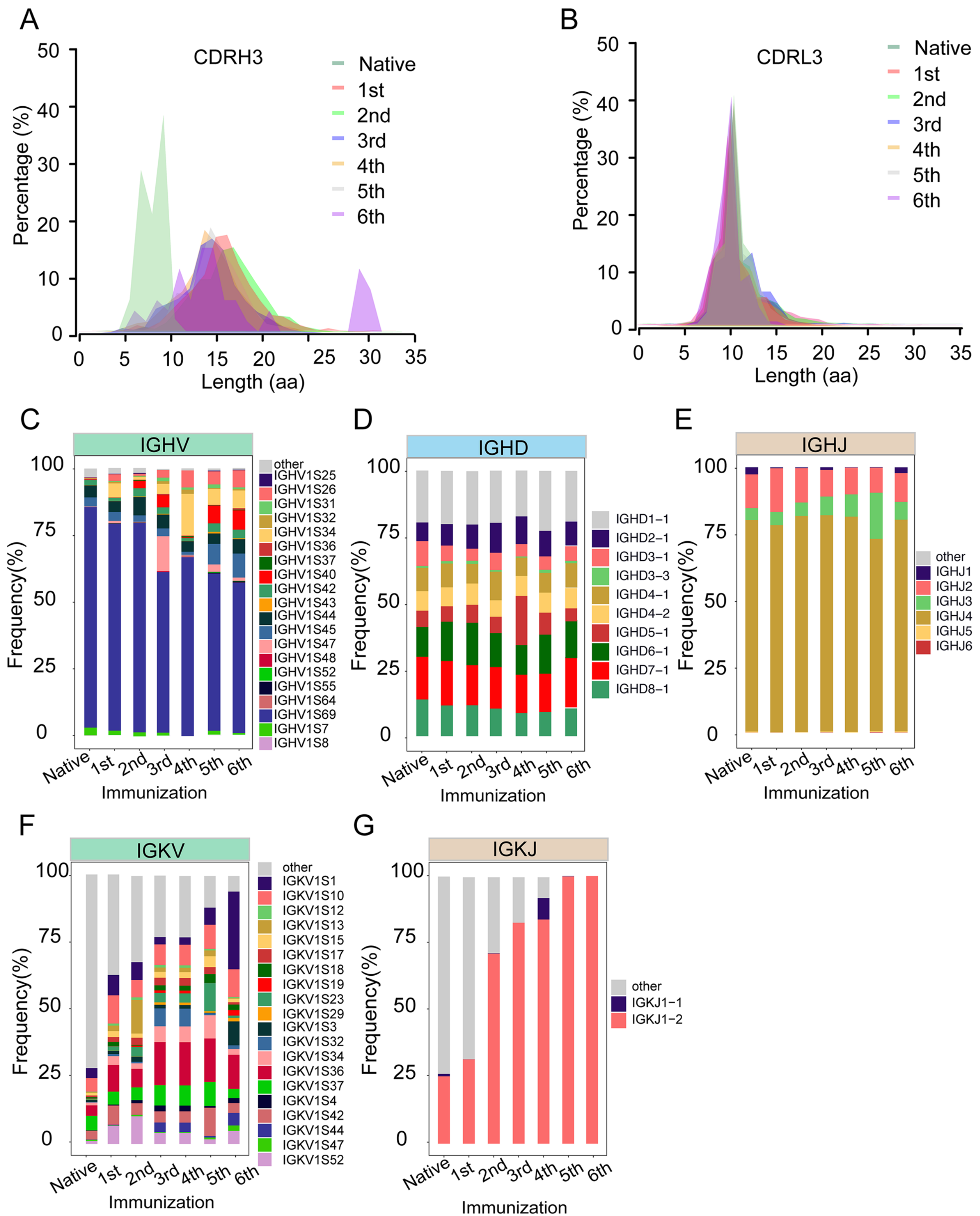

Fig. 3 V, D, J gene usage and recombination with the repeated immunization. **A** the length analysis of CDRH3 in native BCR repertoires and CAP-specific rabbit BCR repertoires. **B** the length analysis of CDRL3 in native BCR repertoires and CAP-specific rabbit BCR repertoires. **C** The usage of the top 20 IGHV genes. **D** The usage of the IGHD genes. **E** The usage of the IGHJ genes. **F** The usage of the top 20 IGKV genes. **G** The usage of the IGKJ genes

peptide-specific rabbit BCR repertoires and hapten-specific mouse BCR repertoires.

Repeated immunization unchanged somatic diversification

The higher diversity of rabbit immune repertoire compared with that of mice and humans is largely dominated by not only SHM but also GSR. Somatic diversification mainly includes V gene mutation, CDR3 mutation containing the junctional site of the V(D)J gene, and J gene mutation [28, 29]. We defined the mutation as the one-base difference and analyzed the mutation frequency in the whole VH repertoire and VL repertoire. Raiees et al. found that the extent of somatic mutation for different mouse antibodies ranged from 14 to 17% in VH and from 9 to 17% in VL chains at the nucleotide level [30]. Our results show that the frequency of V gene mutation in the VL repertoire is higher than that in the VH repertoire in both CAP-specific and native rabbit BCR repertoires, which may also contribute to the high diversity of the VL repertoires compared with that of VH repertoires in rabbits (Fig. 4A). A similar frequency of J gene mutation from the VH repertoire and VL repertoire was observed compared with that of the V gene. More importantly, the frequency of J gene mutation in both VH repertoire and VL repertoire is lower than that of the V gene mutation in CAP-specific and native rabbit BCR repertoires, as J gene mutation only containing SHM compared with VH containing both SGC and SHM in rabbit (Fig. 4B). Among the three CDRs, the CDR3 region is the most variable and is considered a key region for determining antigen binding [31]. The CDR3 mutation in both VH repertoire and VL repertoire of CAP-specific and native rabbit BCR repertoires is the highest, with frequencies exceeding 81% and 74% respectively, compared with the V gene and J gene mutation, owing to the junction of V(D)J recombination (Fig. 4C). Furthermore, the site mutations containing the deletion mutation, insertion mutation, and substitution mutation, were also analyzed to gain a detailed understanding of somatic diversification in CAP-specific and native rabbit BCR repertoires. As shown in Fig. 4D, E, and F, the mutation of somatic diversification in both VH and VL of CAP-specific and native rabbit BCR repertoires mainly relied on the substitution mutations compared with the deletion and insertion mutations. Saunders et al. also found that HIV-specific neutralizing antibodies acquire an abundance of

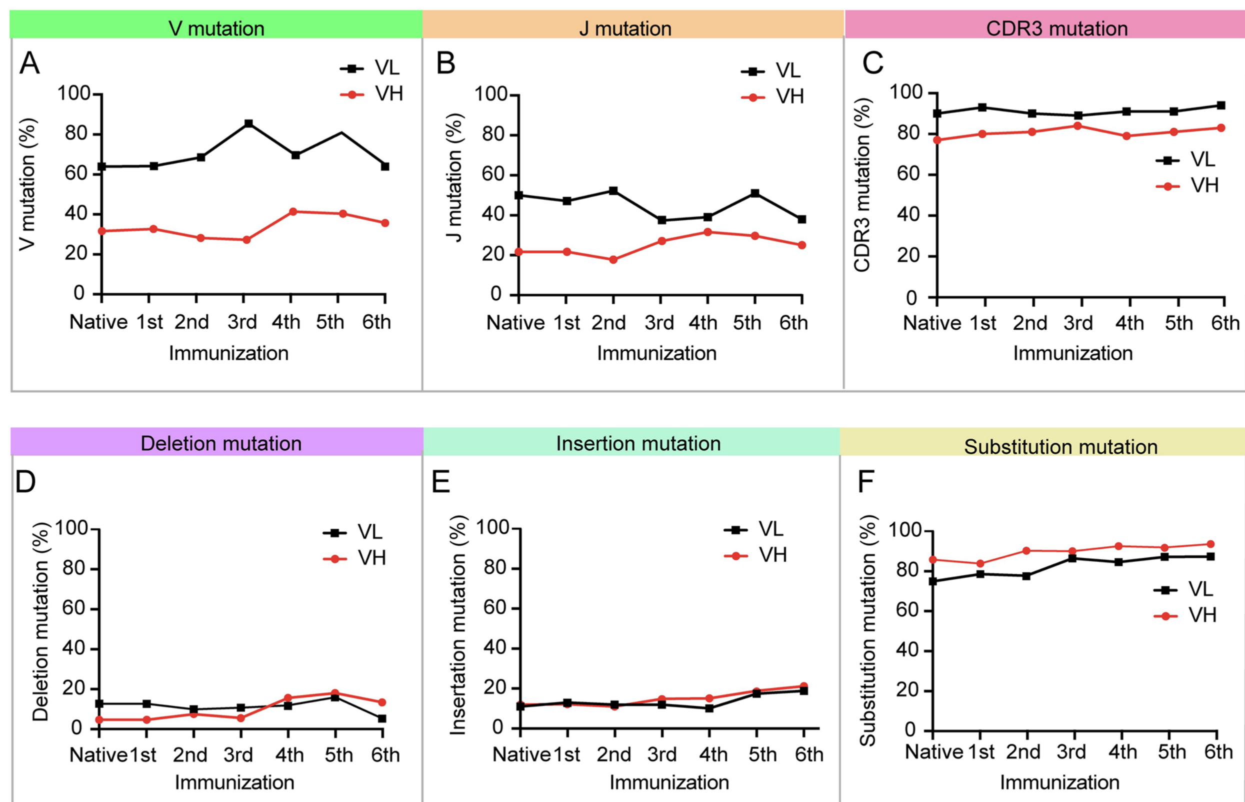

Fig. 4 Mutation frequency in VH and VL of native BCR repertoires and CAP-specific rabbit BCR repertoires. **A** The mutation frequency of V gene mutation. **B** The mutation frequency of J gene mutation. **C** The mutation frequency of CDR3 mutation. **D** The mutation frequency of deletion mutation in VH and VL. **E** The mutation frequency of insertion mutation in VH and VL. **F** The frequency of substitution mutation in VH and VL

improbable substitutions at variable region sequences for antibody mutation [32]. However, all mutations shown in Fig. 4 were unchanged along with the repeated immunization in rabbits, which is corresponding to the previous study, indicating that somatic mutation is unchanged in the BCR repertoires after SARS-CoV-2 infection and vaccination in humans [33]. Galson et al. also found the Hepatitis B vaccine-specific BCR repertoires had similar mutation levels to the native BCR repertoires [21]. This may owe to the mutated memory B cells out from GCs could not re-entry into germinal centers after repeated immunization and the most (>90%) B cells in the secondary GCs have no prior GC experience of somatic diversification [34]. Thus, the results indicate that the somatic diversification in rabbit BCR repertoires to hapten mainly derived from VL via the substitution mutation and the repeated immunization would not change the somatic diversification in rabbits due to the addition of new B cells without GC experience.

Repeated immunization changes germline-based BCR repertoires

It has been reported that naive B cells with lower affinity antibodies can migrate into the GCs, undergo proliferation, and mature soon after immunization, to generate high-affinity antibodies [35]. The diversity of the naive B cells is primarily due to the V(D)J recombination before they enter the GCs. Corsiero et al. noted that the same germline-derived B cells could produce diverse native B cells during the immune response [36]. Brooks et al. and Hargreaves et al. identified some antibodies to one antigen that were derived from the same set of germline genes [37, 38]. To investigate the extent of the germline-based BCR repertoires during the repeated hapten immunization, we extracted the germline-based BCR information from native and CAP-specific BCR repertoires aligning with one CAP-specific rabbit mAb prepared in our laboratory with the VH derived from IGHV1S69/ IGHD7-1/ IGHJ1 and VL derived from IGKV1S34/ IGKJ1-2. To gain insight into the dynamics of IGHV1S69/IGKV1S34 germline-based BCR repertoires, both the genes and structures information was analyzed to provide the snapshots into how the rabbit immune system untilizes the same set of inherited germline gene segments to generate multiple possible diversity.

As shown in Fig. 5A and B, the average length of CDRH3 in the CAP-specific IGHV1S69/ IGKV1S34 germline-based BCR repertoires with 15 amino acids is longer than that of CDRH3 with 9 amino acids in the native IGHV1S69/IGKV1S34 BCR repertoires. The length of CDRL3 did not exhibit any significant difference between CAP-specific and native IGHV1S69/ IGKV1S34 germline-based repertoires with an average length of 15 amino acids, whereas, is higher than the average length of CDRL3 with 10 amino acids in the whole CAP-specific rabbit BCR repertoires. In addition, CDRL3 is taken as an antibody molecule's most critical component in conferring binding activity and specificity. Therefore, we hypothesized that different CAP-specific germline-based BCR with different CDR lengths may form diverse antibody-antigen bindings leading to significantly different affinities. The clonotype of the VL is notably higher than VH in the IGHV1S69/ IGKV1S34 germline-based BCR repertoires, both of which were decreased with the repeated immunization (Fig. 5C). These results indicated that certain germline-based CAP-specific clones within the germline-based BCR repertoires undergo rapid expansion in rabbits during the repeated immunization. The sequences of VH and VL with the highest abundance from native and each immunized IGHV1S69/ IGKV1S34 germline-based BCR repertoires were aligned with the similarity of 79% and 84%, respectively (Fig. 5D and E), this finding which is consistent with the previous study showed that the antibody derived from the same germline genes with higher similar sequence [39]. Then the Fv structures of the most abundant V1S69/V1S37 germline-based BCRs in each immunization were further homology-modelled to reveal the structural dynamics during the repeated immunization. The Ramachandran plot and Profile-3D analysis indicated that all of these IGHV1S69/IGKV1S34 germline-based BCR Fv models were reasonable (Figures S4 and S5). The whole Fv structures of the seven IGHV1S69/IGKV1S34 germline-based BCRs from native and each immunization showed a high degree of similarity (Fig. 5F). As shown in Fig. 5G and H, the differences between these IGHV1S69/IGKV1S34 germline-based BCR structures of VH and VL are mainly located in the CDRs. Specially, the CDRL3 shows higher diversity when compared to CDRL1 and CDRL2. The CDRL3 of the immunized IGHV1S69/IGKV1S34 germline-based BCR formed a larger loop, compared with the CDRL3 of the native IGHV1S69/IGKV1S34 germline-based BCR, and remained without significant changes along with the repeated immunization (Fig. 5I). The CDRH2 and CDRH3 showed higher diversity than CDRH1, and the length of CDRH1 in native IGHV1S69/ IGKV1S34 germline-based BCR was shortened after the immunization (Fig. 5L). Clark et al. found that SARS-CoV-2 neutralizing antibodies derived from IGHV3-53/3–66 with different sequences and conformation have essentially identical surface properties, which affect the stability and affinity of germline-based BCRs to the virus. Thus, the repeated immunization changed the sequence and structure of the germline-based BCRs, which might affect their binding to the hapten.

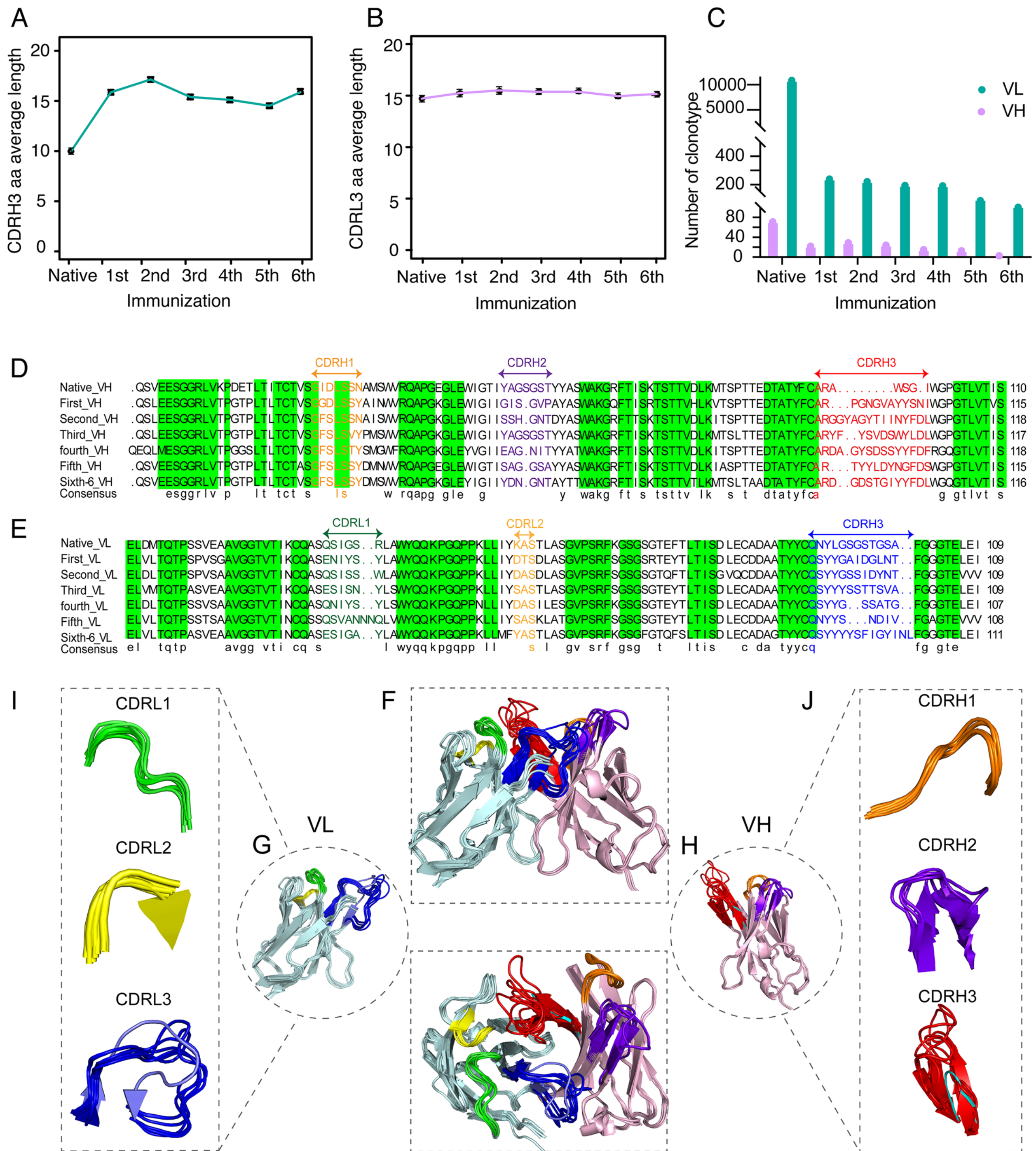

Fig. 5 Characteristics of the IGHV1S69/ IGKV1S34 germline-based repertoires. **A** The average length of CDRH3 from native immunization to 6th immunization. **B** The average length of CDRL3 from native immunization to 6th immunization. **C** The number of the VH and VL clonotypes from native immunization to 6th immunization. **D** and **E** Sequence alignment of VH and VL with the highest abundance of native and each immunized IGHV1S69/ IGKV1S34 germline-based repertoires. **F** The structure alignment of Fv with the highest abundance of native and each immunized IGHV1S69/ IGKV1S34 germline-based repertoires. **G** The structure alignment of VL. **H** The structure alignment of VH. **I** The structure alignment of CDRL1, CDRL2, and CDRL3. **J** The structure alignment of CDRH1, CDRH2, and CDRH3

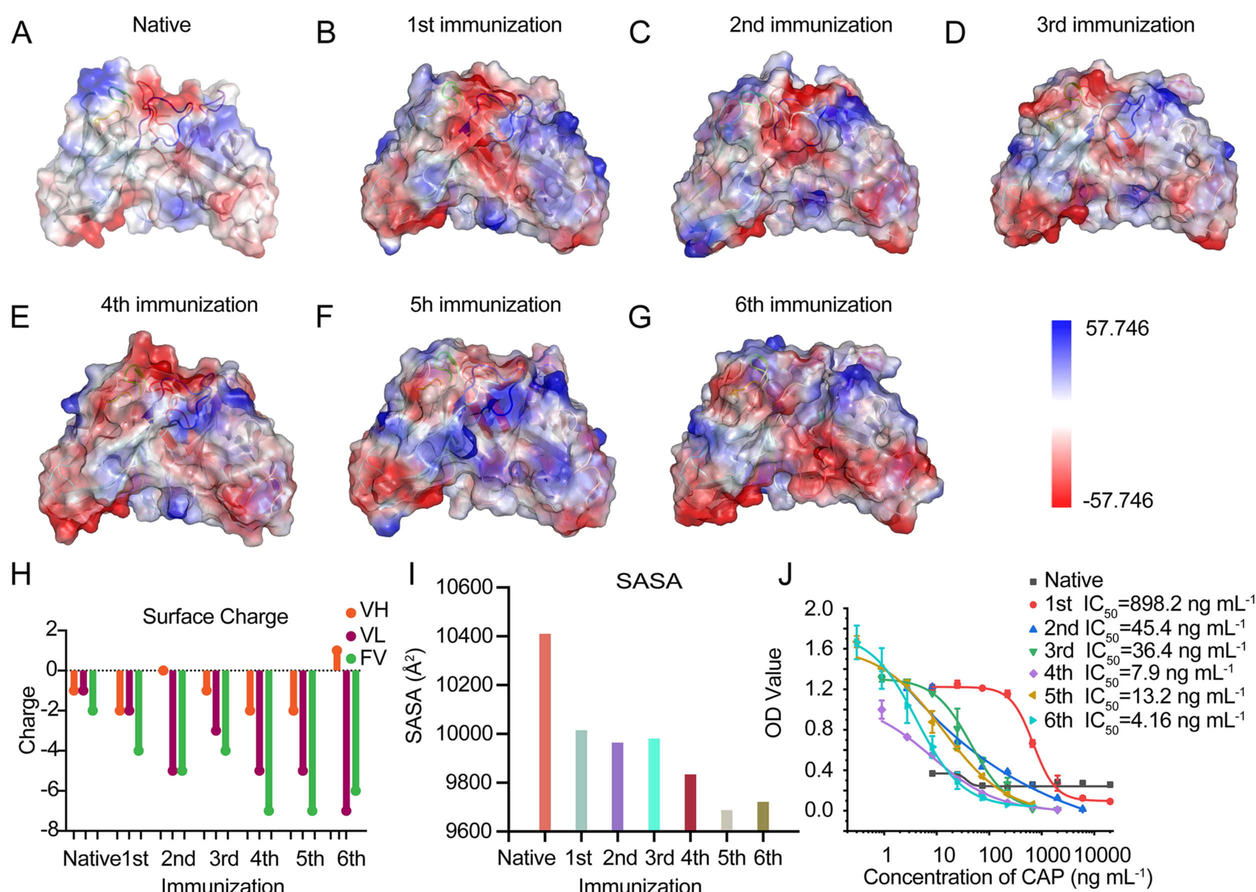

Fig. 6 Analysis of physicochemical properties and affinity of the number one abundance V1S69/V1S37 germline-based BCR in each immunization. **A**–**G** Surface charge analysis of IGHV1S69/ IGKV1S34 germline-based Fvs of native BCR repertoire and CAP-specific BCR repertoires from each immunization. **H** The dynamics of surface charge of IGHV1S69/ IGKV1S34 germline-based VH, VL and Fvs along with the repeated immunization. **I** The diversity of SASA of IGHV1S69/ IGKV1S34 germline-based Fvs along with the repeated immunization. **J** The affinity analysis of IGHV1S69/ IGKV1S34 germline-based scFvs along with the repeated immunization

The physicochemical properties of the IGHV1S69/ IGKV1S34 germline-based BCR Fv, including surface charge and solvent accessible surface area (SASA) which are tightly linked to the antibody stability and binding surface [40], were analyzed. Native and immunized IGHV1S69/IGKV1S34 germline-based BCRs Fv displayed a more negative charge (Fig. 6A–G) which increased from the 1st immunization to the 6th immunization. The negative charge of VL is higher than that of VH in IGHV1S69/IGKV1S34 germline-based BCRs (Fig. 6H). Proteins with negatively charged surfaces are generally known to be halotolerant proteins [41]. Rabbit mAb with IGHV1S69/IGKV1S34 germline was found to be an extremely halophilic antibody. As shown in Fig. 6I, the rabbit mAb with IGHV1S69/IGKV1S34 germline was found to be an extremely halophilic antibody, the SASA of the immunized IGHV1S69/IGKV1S34 germline-based BCRs was smaller than that of the native IGHV1S69/IGKV1S34 germline-based BCRs Fv, decreasing from 9963.816 Å^2 to 9686.832 Å^2 after repeated immunization. Thus, the result indicated that the antibodies derived from the same germline gene had similar physicochemical character and that repeated immunization could enhance the stability of the germline-based BCRs. The difference in physicochemical properties of IGHV1S69/ IGKV1S34 germline-based BCR Fv may significantly affect the interaction interface related to its affinity to the hapten. Angelo et al. indicated that different clones of anti-CDK2 antibody shared 91.6%–97.8% homology, with the same germline-based VH (5–51, D2-08, and J3) and VL (IGLV3-21 and IGLJ1), showed the affinities spanned a tenfold range (from 30.1 to 352.5 nM) [42]. As shown in Fig. 6J, the affinity of IGHV1S69/ IGKV1S34 germline-based BCR scFvs to CAP was highest at the 6th immunization with an IC_{50} value of 4.16 ng mL^{-1}, higher than those at the 1st immunization of 898.2 ng mL^{-1}. The scFv of the native

IGHV1S69/IGKV1S34 germline-based BCR showed no significant binding with CAP at 2000 ng mL^{-1}. Therefore, repeated immunization could significantly improve the affinity of the germline-based BCRs in rabbits and guarantee the discovery of high-quality antibody. This is why multi-stage immunization strategies are essential for efficient immune response and high-affinity antibody discovery.

Conclusions

This study systematically analyzed the dynamics of hapten-specific rabbit BCR repertoires compared with native BCR repertoires, and evaluated how repeated immunization of CAP-KLH shapes the rabbit BCR repertoires, combing with the gene and structure analysis of the V1S69/V1S37 germline-based BCR. We found that the length of CDRH3 got longer after the 1st immunization of hapten in rabbits, which is different from that of protein in rabbits. Besides, repeated immunization increased the surface charge and decreased SASA of V1S69/V1S37 germline-based BCR Fv to improve the stability and affinity of the rabbit BCR repertoires. Therefore, all the above study provides a fundamental understanding of the characteristics of hapten-specific rabbit BCR repertoires.

Materials and methods

Materials and apparatus

Chloramphenicol (CAP), hemocyanin-keyhole limpet (KLH), bovine serum albumin (BSA), fluorescein Isothiocyanate (FITC), and red blood cell lysis buffer were supplied by Sigma-Aldrich (St. Louis, MO, USA). RNeasy Mini Kit and SuperScript™ III cDNA synthesis kit were supplied by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Rabbit peripheral blood lymphocyte separation kit and carbonate buffer solution (CBS) were obtained from Solarbio Life Sciences, Inc. (Beijing, China). The horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG and phycoerythrin (PE)-anti-rabbit IgG antibody were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). White polystyrene microtiter plates, confocal laser microscope plates, 96-well plates, 6-well plates, and falcon tubes were obtained from Costar, Inc. (Milpitas, CA, USA).

The optical density (OD) values were measured via a PerkinElmer Envision plate reader (Waltham, MA, USA). Cells were counted by Count star (Shanghai, China). Antigen-specific B cells were enriched by FACSAria II (Franklin Lakes, NJ, USA). BCR repertoires were sequenced by Illumina HiSeq 2500 sequencing system (San Diego, CA, USA). Specific pathogen-free, female New Zealand rabbits used in all experiments were acquired from Beijing Vital River Laboratory Animal Technology Company (Beijing, China).

Animal immunization and CAP-specific IgG B cell enrichment

All animal procedures of six 2-month-old female New Zealand white rabbits were approved by the Animal Ethics Committee of China Agricultural University and strictly conducted by Chinese laws and guidelines. The Immunogen of CAP-KLH prepared by our previous work was dissolved in 0.6 mL of PBS emulsified with Freund's adjuvant for the first immunization and Freund's incomplete adjuvant for further boosts [43]. Then, the water-in-oil mixture was subcutaneously injected into rabbits at multiple sites. The rabbit was injected six times at 4-week intervals. The peripheral blood was collected from ear veins before immunization and after 7 days of each immunization. Antisera were assayed by enzyme-linked immunosorbent assay (ELISA) and indirect competitive enzyme-linked immunosorbent assay (icELISA) with the coating of CAP-BSA. The antisera titer was represented by antisera dilution. The antisera affinity was represented by the half-maximal inhibitory concentration (IC_{50}) values from the standard curves of the icELISA based on antisera, which were constructed by OriginPro 8.0 (Originlab Corp., Northampton, MA, USA), and data was fitted to the following four-parameter logistic equation according to the Eq. 1.

$$Y = (A - B)/[1 + (X/C)^\wedge B] + D \quad (1)$$

where A represents the responses at high asymptotes of the curve, B acts as the slope factor, C is the IC_{50} of the curve, D is the responses at low asymptotes of the curve, and X is the calibration concentration.

FITC was conjugated to CAP-BSA via an active ester method [44]. 15 mL of peripheral blood from each rabbit was collected into vacuum tubes containing acid citrate dextrose. Peripheral blood was mixed with an equal volume of PBS, layered onto 15 mL of lymphocyte separation medium, and centrifuged at 800 g (no brake) for 30 min. Peripheral blood lymphocytes (PBMCs) were collected, washed with PBS, and resuspended in flow cytometry cell sorting (FACS) buffer (0.5% BSA, 2 mM EDTA, and 96.6% HBSS) with the concentration of 1×10^7 cells mL^{-1}, incubated with 10 μg mL^{-1} PE-anti-rabbit IgG antibody and 10 μg mL^{-1} CAP-BSA-FITC in dark at 4 ℃ for 30 min and then washed twice in the buffer before being finally resuspended in the buffer for FACS sorting. With the absence of a general B cell marker, the rabbit system was restricted to positively staining with antigen (labeled with fluorophores) and IgG only. FACS sorting was carried out on a BD FACS ARIA III, using a 100 μm sort nozzle at no greater than 2000 events per second. A gate was drawn around the double-positive

of CAP-specific and IgG-specific populations. Then CAP-specific IgG B cells from each immunization were sorted by FACS and collected in a 1.5 mL tube with lysis buffer.

CAP-specific rabbit BCR repertoires preparation and sequencing

The enriched CAP-specific IgG B cells from each immunization and whole native PBMCs were lysed with the cell number of 10^4 and the mRNA was reverse transcribed to cDNA. The mRNA was isolated from CAP-specific IgG B cells from 1st immunization to 6th immunization. Due to the little to no CAP-specific IgG B cells in native PBMCs, the mRNA of the native repertoire is from bulk PBMCs. Then, the variable region of the heavy chain (VH) and the variable region of light chain (VL) of B cells were amplificated with the specific primers by PCR (95 °C 5 min; 95 °C 20 s, 55 °C 40 s, 72 °C 2 min, 30 cycles; 72 °C 10 min). The CAP-specific BCR repertoire is the sum of CAP-specific B cell clones in each immunization. As shown in Table S1, the forward primer of VH/VL(Vκ/Vλ) is specific for the FR1 region with mixed codon usage, we have set four forward primers of VH specific for all VHs of IgG, two forward primers of Vκ specific for all Vκs of IgG, and one forward primer of Vλ specific for all Vλs of IgG in the rabbit. the reverse primer of VH/Vκ/Vλ was placed within the J regions with mixed codon usage. The PCR was carried out using FastPfu Fly DNA Polymerase mix according to the manufacturer's instructions. The VL and VH regions were amplified in separate reactions. PCR-primers (0.2 μM per reaction) mixed with 100 nM cDNA, 5μL FastPfu Fly DNA Polymerase mix, and added water up to 10 μL. The amplified transcripts of VH and VL were sequenced using a HiSeq 4000 sequencing system. BCR repertoires libraries were sequenced to a mean depth of 147 million reads with the Phred quality of 30 and 20 both over 90%. The raw output files after quality filtering were processed to generate FASTA files for further analysis. The FASTA files were uploaded to the IMGT HighVQuest website (http://imgt.org/HighV-QUEST/home.action). The definition of clonotypes was performed by MiXCR software. The clone diversity between native BCR repertoires and repeated immunized BCR repertoires was analyzed by ChaoE by normalizing the amount of clone data [45]. The overlap of germline genetype, shared germline gene type, particular germline gene by VDJtools. Mean percentages of gene usage were compared using two-way ANOVA. All correlations were performed using Spearman's correlation. The enrichment of V-J combinations in BCRs with high mutation rates was tested by a one-sided Fisher exact test. Statistical analysis was performed by R packet with ggplot2 for graphing.

V1S69/V1S37 germline-based BCR analysis

V1S69/V1S37 antibody was a CAP-specific rabbit antibody produced in our laboratory. V1S69/V1S37 germline-based BCR repertoires were analyzed by IgBLAST alignments. Seven Fvs of V1S69/V1S37 germline-based BCR with the highest frequency in native BCR repertoires and each immunized BCR repertoires were homology modeled using the Discovery Studio 2019 software [46] to reveal the structural features of germline-based BCRs during the repeated immunization. A protein–protein BLAST search in the PDB database was performed to find a suitable homologous sequence (template) with the Fvs of V1S69/V1S37 germline-based BCRs. Five antibody crystal structures were selected as the templates with the identity and similarity both higher than 80%. The three-dimensional (3D) structure of the V1S69/V1S37 germline-based BCR Fvs was then constructed by aligning to the heavy and light chains of these templates with Fvs to determine the relative spatial orientation of the VH and VL. The highest quality model with the lowest probability density function energy [47] and higher discrete optimized protein energy [48], was selected to optimize the complementarity determining regions (CDRs) by aligning with the published crystal structures through the IMGT/V-QUEST database (http://www.imgt.org). Ramachandran plot and Profile 3D analysis were applied to evaluate the homology models [49]. Protein minimization has been performed in Flare by Minimize tool by using Normal calculation methods [50].

The scFv genes of V1S69/V1S37 germline-based BCR with the highest frequency in native BCR repertoires and each immunized BCR repertoire were constructed to the recombinant expression vector pJB33. *E. coli* RV308 was transformed with vector pJB33–scFvs. The single bacterial colony was incubated in a 2.5 mL 2×YT medium containing chloramphenicol overnight (37 °C, 200 g). Then, a volume of the bacteria solution was diluted 100-fold and cultured in a 250 mL 2×YT medium. When the OD_{600} value reached 0.6–0.8, IPTG was added to *E. coli* suspension to induce scFvs expressing at 24 °C. The affinity was extensively assessed and compared by using icELISA [44]. In this study, antibody affinity was defined as the IC_{50} value of scFvs based on the icELISA.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44280-023-00013-z.

Additional file 1: Figure S1. The selection of the CAP-specific IgG B cells in PBMCs from 1st immunization to 6th immunization. (A) and (B): Control. (C) and (D): 1st immunized CAP-specific IgG B cells. (E) and (F): 2nd immunized CAP-specific IgG B cells. (G) and (H): 3rd immunized CAP-specific IgG B cells. (I) and (J): 4th immunized CAP-specific IgG B cells. (K) and (L): 5th immunized CAP-specific IgG B cells. (M) and (N): 6th immunized CAP-specific IgG B cells. **Figure S2.** The agarose gel electrophoresis analysis of the VH and VL of CAP-specific BCRs from 1st immunization to 6th immunization. **Figure S3.** Rarefaction curves for diversity. (A): Rarefaction analysis of VH. (B): Rarefaction analysis of VL. **Figure S4.** The Ramachandran plot analysis of IGHV1S69/ IGKV1S34 germline-based Fvs homology models. (A): native IGHV1S69/ IGKV1S34 germline-based Fv. (B): 1st immunized IGHV1S69/ IGKV1S34 germline-based Fv. (C): 2nd immunized IGHV1S69/ IGKV1S34 germline-based Fv. (D): 3rd immunized IGHV1S69/ IGKV1S34 germline-based Fv. (E): 4th immunized IGHV1S69/ IGKV1S34 germline-based Fv. (F): 5th immunized IGHV1S69/ IGKV1S34 germline-based Fv. (G): 6th immunized IGHV1S69/ IGKV1S34 germline-based Fv. **Figure S5.** Profile-3D analysis of IGHV1S69/ IGKV1S34 germline-based Fvs homology models. (A): Native IGHV1S69/ IGKV1S34 germline-based Fv. (B): 1st immunized IGHV1S69/ IGKV1S34 germline-based Fv. (C): 2nd immunized IGHV1S69/ IGKV1S34 germline-based Fv. (D): 3rd immunized IGHV1S69/ IGKV1S34 germline-based Fv. (E): 4th immunized IGHV1S69/ IGKV1S34 germline-based Fv. (F): 5th immunized IGHV1S69/ IGKV1S34 germline-based Fv. (G): 6th immunized IGHV1S69/ IGKV1S34 germline-based Fv. **Table S1.** Primers of rabbit VH and VL. **Table S2.** The raw reads and quality analysis. **Table S3.** VDJ recombination of VH. **Table S4.** VJ recombination of VL.

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Authors' contributions
Y.L. contributed to the methodology and original draft preparation. Y.K. handled resource allocation. W.Y. was responsible for creating visualizations. K.W. assisted with resource management. X.Y. and J.S. were in charge of project administration. Z.W. contributed to the methodology and editing of the writing. The authors read and approved the final manuscript.

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Availability of data and materials
Data will be shared upon request by the readers.

Declarations

Ethics approval and consent to participate
The animal study protocol was approved by the Animal Ethics Committee of China Agricultural University and strictly conducted by Chinese laws and guidelines (AW32602202-2-2).

Consent for publication
Not applicable.

Competing interests
Prof. Jianzhong Shen is Editor-in-Chief and Prof. Zhanhui Wang is Science Editor of One Health Advances. They were not involved in the journal's review and decisions related to this manuscript.

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