Methods

Isolation of rabbit single domain antibodies to B7-H3 via protein immunization and phage display

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ABSTRACT

Single domain antibodies have certain advantages including their small size, high stability and excellent tissue penetration, making them attractive drug candidates. Rabbit antibodies can recognize diverse epitopes, including those that are poorly immunogenic in mice and humans. In the present study, we established a method to isolate rabbit VH single domain antibodies for potential cancer therapy. We immunized rabbits with recombinant human B7-H3 (CD276) protein, made a phage-displayed rabbit VH single domain library with a diversity of $7 \times 10^9$, and isolated two binders (A1 and B1; also called RFA1 and RFB1) from phage panning. Both rabbit VH single domains exhibited antigen-dependent binding to B7-H3-positive tumor cell lines but not B7-H3 knockout tumor cell lines. Our study shows that protein immunization followed by phage display screening can be used to isolate rabbit single domain antibodies. The two single domain antibodies reported here may have potential applications in cancer immunotherapy.

Statement of Significance: Rabbit antibodies can recognize diverse epitopes, including those that are poorly immunogenic in mice and humans. We established a method to isolate rabbit VH single domain antibodies to B7-H3 by immunization with recombinant protein followed by phage display screening. The rabbit single domain antibodies specifically bind B7-H3 expressing tumor cells.

KEYWORDS: rabbit monoclonal antibody; single domain antibody; phage display library; B7-H3/CD276; Escherichia coli expression

INTRODUCTION

Single domain antibodies (also commonly called ‘nanobodies’), the smallest antigen-binding fragments of antibodies, have approximately 120 amino acids with a molecular weight of 12–15 kDa [1]. Most single domain antibodies are derived from the variable region of the heavy chain ($V_H$), which naturally occurs in camels (termed $V_H$H) [2] and cartilaginous fishes ($V_NA$R) [3, 4]. They also exist in certain human heavy chain diseases such as immunoproliferative small intestinal disease, Mediterranean lymphoma or Seligmann disease [5, 6]. Due to their small size, high solubility, excellent thermal stability and high tissue penetration as compared to conventional IgG antibodies, single domain antibodies have great potential in medical applications. In 2019, the US FDA approved the first nanobody drug caplacizumab, a bivalent humanized camelid single domain antibody ($V_H$H), for the treatment of acquired thromboocytopenic purpura and thrombosis [7, 8].

In sera of Camelidae, both conventional IgG and heavy chain only IgG (HCAbs) together account for 45–75% of all serum immunoglobulins [1]. The HCAbs consists of the antigen binding $V_H$H fragment and the...
following CH2 and CH3 constant domains. The recombinant VHH domains are the functional entities that are being exploited. Several evolutionarily acquired structural features make the naturally occurring cameld VHH domain highly soluble and stable [9–11]. The first non-VHH mammalian domain antibodies were discovered in 1989 from a cDNA library made from the spleens of mice immunized with lysozyme and keyhole-limpet haemocyanin, with two mouse VH domains showing affinities for lysozyme in the 20 nM range [12]. Human VH single domain antibodies [13–15] have been explored and have potential advantages in clinical applications because of their potential low immunogenicity. Several strategies have been developed to generate human or humanized VH domain antibodies. One strategy involves using phage display on human VH domain libraries [13–15]. Using this approach, we previously reported the human single domain antibody HN3 that targets GPC3, a liver cancer antigen, and showed that the human single domain antibody could inhibit Wnt/Yap signaling by blocking a functional groove on the cell surface protein [13, 16, 17]. Another strategy involves using transgenic animals in which human VH germline genes are introduced in mice [18, 19]. Single domain antibodies based on shark VNARs [4, 20–22] have also been reported. Our laboratory recently described a large phage-displayed shark VNAR library with a size of $1.2 \times 10^{10}$ from six naïve adult nurse sharks and isolated a panel of shark VNAR single domain antibodies to tumor and viral antigens [4]. The diversity of our shark VNAR library was validated by next-generation sequencing of 1.2 million VNAR sequences. Rabbit antibodies recognize diverse epitopes, including those poorly immunogenic in mice and humans. Our previous work using rabbit hybridomas isolated unique rabbit monoclonal antibodies (e.g. YP218) that recognize a very rare epitope in the C-terminal end (Region III) [23] of mesothelin close to the tumor cell surface [24, 25]. Recently, it has been shown that rabbits can be used to generate immunized VH domain antibodies using a low temperature (16°C) phage display method [26]. However, this method resulted in binders that were low in solubility, thermostability and production yield. We raised the question whether it may be possible to select rabbit VH domain antibodies by first immunizing rabbits and then using the phage display method at physiological temperature (e.g. 37°C). In the present project, we performed a proof-of-concept study by immunizing a rabbit with a recombinant protein, followed by phage display screening at 37°C. We chose to focus on B7-H3 based on its emerging potential as a therapeutic target [27,28], and successfully identified two VH binders that displayed high expression levels in Escherichia coli and specifically bound to cell surface B7-H3.

MATERIALS AND METHODS

Cell lines

Human cancer cell lines (Hep3B, HepG2, IMR32, MC38-B7H3+, A431, IMR32-B7H3 KO and MC38-B7H3 KO) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% L-glutamine and 1% penicillin-streptomycin (Invitrogen) and incubated in 5% CO2 with a balance of air at 37°C. The neuroblastoma cell line NBEB was cultured in RPMI-1640 medium with the same supplements as DMEM. The media were refreshed twice a week. All the cancer cell lines were described in our previous studies [15, 29]. The B7-H3 knockout lines (IMR32-B7H3 KO and MC38-B7H3 KO) were engineered via CRISPR/Cas9 genome editing technology.

Production of recombinant B7-H3 protein

The extracellular domain (ECD) of B7-H3 (GenBank accession number NP_001019907, amino acid 29–466) was fused with a hFc tag. The B7-H3-hFc as well as a hFc tag control, IAB-hFc [23, 30] was expressed in HEK-293F cells. Protein purification was performed using a protein A column (GE Healthcare). A His-Flag was inserted at the C-terminus of the VH sequence. The 6xHis tag was used for Nickel column (GE Healthcare) affinity purification, and the FLAG tag was used to monitor VH binding in ELISAs and flow cytometry.

Rabbit immunization

The rabbit immunization followed our previously published method [24]. Briefly, two female rabbits were intramuscularly immunized with 100 μg of purified recombinant B7-H3 protein mixed with Freund’s complete adjuvant for the priming. Two boosting immunizations were followed by mixing the antigen with Freund’s incomplete adjuvant at the same dose at an interval of 14 days. After the third immunization, the spleens were harvested to make the phage library.

DNA oligos and construction of the rabbit VH phage library

To amplify the rabbit VH cDNA fragment, forward and reverse primers that anneal to the 5'- and 3'-end of VH cDNA were synthesized as described earlier [31]. The primers are listed as follows, with the underlined corresponding to the SfiI restriction enzyme site.

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VH-F1: 5'-GAGGAGTTGGCCCCAGGCAGGCAGCTC
GTTGGAGAGGTCGGG-3'
VH-F2: 5'-GAGGAGTTGGCCCCAGGCAGGCAGCTC
AGTGAAGGGTCGGG-3'
VH-F3: 5'-GAGGAGTTGGCCCCAGGCAGGCAGCTC
GYTGGAGAGGTCGGG-3'
VH-F4: 5'-GAGGAGTTGGCCCCAGGCAGGCAGGA
GCAAGCTGGAGGTGCCGGG-3'
VH-F5: 5'-GAGGAGTTGGCCCCAGAGGCAGGA
GCAAGCTGGAGGTGCCGGG-3'
VH-F6: 5'-GAGGAGTTGGCCCCAGAGGCAGGA
GCAAGCTGGAGGTGCCGGG-3'
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Phage panning

Phage panning was carried out using immobilized B7-H3-human Fc (hFc) protein using our lab protocol [13, 32]. To exclude hFc tag binders, an irrelevant IAB-hFc control was also immobilized in parallel. Ninety six-well ELISA plates were coated with 50 μL/well B7-H3-hFc and IAB-hFc proteins (100 μg/mL in PBS) and incubated at 37°C for 1 h. After removing the coated protein solution, the plate and phage solution were pre-blocked using PBS buffer containing 2% BSA and incubated at 37°C for 30 min. After removing the blocking buffer, pre-blocked phage solution was added to the IAB-hFc plate to deplete hFc binders by incubating at 37°C for 1 h. Thereafter, the unbound phage solution was transferred to the B7-H3-hFc plate and incubated at 37°C for 1 h. B7-H3 specific phage binders were eluted from the plate by pH 2.0 citric acid buffer and were immediately neutralized with pH 8.0 Tris-HCl buffer. The eluted output phage was re-amplified by re-infection of fresh TG1 cells, and the re-amplified phage was used as the input for the next round of panning. After three rounds of panning, single colonies were randomly picked from the output phage infected TG1 cells and monoclonal phage ELISA was conducted to identify B7-H3 specific binders.

Phage ELISA

A 96-well ELISA plate was coated with B7-H3-hFc and IAB-hFc tag control. After blocking with PBS buffer containing 2% BSA, 50 μL of pre-blocked phage solution was added to the plate and incubated at 37°C for 30 min. After the plate was washed twice with PBS buffer containing 0.05% Tween 20, phage binding was detected by HRP-conjugated anti-M13 antibody (Sino biological, Cat. #11973-MM05T-H).

For antibody binding ELISA, various concentrations of antibody (1:2 serial dilutions starting from 100 μg/mL) were incubated on the B7-H3-hFc coated plate as mentioned above. Antibody binding was detected by HRP-conjugated anti-FLAG mouse monoclonal antibody M2 (Sigma, Cat. #A8592).

Flow cytometry

Cells were harvested by detaching with trypsin-EDTA (Thermo Fisher, Cat. #25200114), centrifuged to form pellet, resuspended in ice-cold PBS at a density of 10^6 cells/mL and incubated with 10 μg/mL of B7-H3 domain antibodies. The antibody binding was detected by allophycocyanin (APC)-conjugated anti-FLAG mouse monoclonal antibody (Biolegend, Cat. #637308). The fluorescence associated with the live cells was measured using a FACS Calibur (BD Biosciences, Franklin Lakes, NJ).

Protein structure modeling

Structure modeling of rabbit single domain antibodies is visualized using the web tool SWISS-MODEL (https://swissmodel.expasy.org/). Auto-models and default parameters were used to generate modeling. Models were built based on the target-template alignment using ProMod3 Version 1.3.0 algorithm. The SWISS-MODEL template library (SMTL version 2019-04-11, PDB release 2019-04-05) was searched with BLAST. Overall 16 597 templates were found, and the models were built on the template 5DUB, which had a high sequence identity of 78.76 for A1 binder and 78.26 for B1 binder. The GMQE scores were 0.96 for both binders, and the QMEAN scores were 0.74 and 2.09 for A1 and B1 binders, respectively.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).
RESULTS

Schematic of the rabbit VH single domain antibody production

To generate rabbit single domain antibodies, as shown in Fig. 1, we immunized rabbits with the recombinant ECD of B7-H3. After the rabbits developed immune responses, mRNA was isolated from lymphocytes, and a cDNA library of variable heavy chain domains was created by reverse transcription. The cDNA was then used to express VH as fusions containing the phage coat proteins (phage display) and the VH binders that were enriched by three rounds of panning against the immobilized antigen. The selected binders were expressed in E. coli with a C-terminal hexahistidine tag to allow purification by nickel-nitrilotriacetic acid affinity chromatography, and contained a N-terminal secretion signal sequence to direct the expressed protein to the periplasm for disulfide bond formation.

Expression and purification of recombinant B7-H3-hFc and immunization of rabbits with recombinant B7-H3-hFc

The ECD of human B7-H3 (NCBI Reference Sequence: NP_001019907, amino acids 29–466) was fused with human IgG1 Fc and expressed in HEK293 cells. After purification on protein A columns, the purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under both reducing and non-reducing conditions (Fig. 2A). The theoretical size of the reduced B7-H3-hFc is about 75 kDa, with an apparent migration position of about 100 kDa on the gel, likely due to glycosylation since B7-H3 contains six N-glycosylation sites. Two female rabbits were intramuscularly immunized with 100 micrograms of B7-H3-hFc in PBS buffer mixed with an equal volume of Freund’s adjuvant. After three immunizations with an interval of 14 days between doses, the titer of anti-B7-H3-hFc antibodies was measured at about 1:10 000 by ELISA, using hFc tagged irrelevant antigen IAB-hFc [23, 30] as a control (Fig. 2B). The serum from the second (M2) and third (M3) immunization showed increased binding to B7-H3-hFc compared to IAB-hFc, although both M2 and M3 showed weak hFc tag binding. Cell binding activity of the polyclonal serum was assessed by flow cytometry (Fig. 2C). The polyclonal serum of the final immunization showed clear cell binding to hepatocellular carcinoma cell lines Hep3B and HepG2 [33, 34]. The results of both ELISA and flow cytometry indicated that B7-H3 is immunogenic in rabbits, at least in the hFc-fusion format, even though this protein is highly conserved across species. Rabbit and human B7-H3 share 90.94% amino acid identity while the overall similarity of mouse, rabbit and human B7-H3 is 98.6%.

Phage screening of B7-H3 specific binders

After confirming a successful immunization, the spleens of the two immunized rabbits were harvested separately, and the VH gene fragments from each rabbit were amplified using degenerate primers [31] and ligated into the phage display vector pComb3x. Ten micrograms of the ligations were used to transform TG1 competent cells by electroporation, resulting in a VH library containing $7 \times 10^9$ clones. Phage panning was carried out at 37°C.

The panning was performed on immobilized B7-H3-hFc. We randomly picked 96 clones and 41 were found to specifically bind B7-H3, but not hFc alone. Sequencing analysis identified two highly enriched binders, A1 and B1 (also called RFA1 and RFB1) (Fig. 3A). These two binders shared very similar germline sequences that were also similar to a VH from rabbit anti-hypusine mAb (deposited in GeneBank structure database, protein data bank (PDB) #5DUB).

Structure modeling of A1, B1 using SWISS-MODEL, showed that A1 and B1 share similar CDR1 and CDR2 loop conformations, with anti-hypusine VH (PDB# 5DUB), but the CDR3 loops of A1 and B1 were apparently different (Fig. 3B).

Binding properties of the B7-H3 VH single domains

The VH domain of A1 and B1 were fused with a tandem His-FLAG tag at their C-termini, expressed in E.coli, and soluble VH domains were purified by Nickel affinity chromatography [4]. The purification yields for A1 and B1 was 2 mg/L and 10 mg/L, respectively, while the rabbit single domain antibodies previously selected at a low temperature had extremely low expression levels (0.003 and 0.08 mg/L) [26]. The purity was high as shown by SDS-PAGE (Fig. 4A). The theoretical size of a reduced rabbit VH single domain is about 15 kDa, with an apparent migration position of a single protein band for A1 or B1 under the non-reducing condition. Biophysics analysis using size exclusion chromatography and nano-differential scanning fluorimetry (nanoDSF) may be useful to more accurately check the molecular weight (or oligomerization state) and thermostability of rabbit single domain antibodies in the future when developing rabbit single domains as drugs. Protein binding was validated by ELISA (Fig. 4B). Next,
Figure 2. SDS-PAGE analysis of purified B7-H3-hFc, titer of B7-H3-hFc immunized rabbit sera and confirmation of its cell binding. (A) One or five micrograms of purified B7-H3-hFc, non-reduced (Non.) or reduced (Red.) by β-mercaptoethanol were separated on 8% SDS-PAGE gel. Protein bands were visualized by Coomassie Blue R-250 staining. (B) Titration of B7-H3-hFc immunized rabbit sera by protein binding ELISA. IAB-hFc, which is derived from an N-terminal fragment of mesothelin, served as a hFc tag control. R31M0, R31M1, R31M2 and R31M3 represented the sera of pre-immunization, 1st, 2nd and 3rd immunization. (C) Cell binding assay of the immunized sera. Shaded area represents cells stained with fluorescein isothiocyanate (FITC)-conjugated 2nd (goat anti-rabbit) antibody only; blue curve represents cells stained with pre-immunization sera; red curve represents cells stained with immunized sera R31M3.

Flow cytometry was performed to assess cell binding capability (Fig. 4C). In this assay, A1 and B1 specifically bound B7-H3-positive human neuroblastoma cell lines IMR32 and NBEB, murine colon adenocarcinoma MC38B7H3+ and epidermoid carcinoma A431, but not their B7-H3 knockout counterparts IMR32-B7H3 KO and MC38-B7H3 KO. Therefore, we were able to successfully isolate two rabbit VH domain antibodies that bind cell surface B7-H3 in multiple cancer lines.

DISCUSSION

Rabbits are a unique source for generating monoclonal antibodies that can be used as research tools, diagnostics and therapeutics [35]. There are several major advantages of using rabbit antibodies. First, rabbits are phylogenetically more distant from humans than mice, and therefore those conserved proteins that are poorly immunogenic in mice may evoke better immunogenicity in rabbits [24, 36]. We previously isolated a unique high affinity rabbit monoclonal antibody (YP218) from almost 8000 rabbit hybridoma clones, which binds a poorly immunogenic site in the C-terminal region of mesothelin [24]. Second, rabbit monoclonal antibodies generally have high affinity and specificity, with the affinity range of 20–200 pM [35, 37]. Third, our previous work showed that rabbit monoclonal antibodies can be successfully humanized without losing their affinity and specificity [25], therefore immunogenicity should not be a barrier for therapeutic applications. Despite their superiority, unlike mouse monoclonal antibodies, only a few rabbit monoclonal antibodies have been investigated for clinical applications likely due to the lag in development of rabbit hybridoma technology [38] and rabbit antibody phage display libraries [39] at major institutes and companies.
Figure 3. Sequences and structural modeling of the B7-H3 single domain antibodies. (A) Sequence alignment of the B7-H3 binders, along with a similar VH from a rabbit anti-hypusine mAb (PDB# 5DUB). CDR regions were defined by IMGT delineating system. (B) Structure modeling of A1 and B1 binders using online software SWISS-MODEL (https://swissmodel.expasy.org/). The crystal structure of 5DUB is also shown for comparison.

With the advantages of small size, high solubility and stability, single domain antibodies are garnering increased attention. New therapeutic applications for V_{HH}s are continuously being identified in many diseases. A recently study demonstrated that high affinity rabbit VH domain antibodies could be generated by a low temperature (16°C) phage display method [26]. However, the low temperature phage display method tends to enrich binders that are unstable and difficult to express, bolstering the need for additional efforts to improve the physicochemical properties of the binders, especially their expression [10]. In the present study, we sought to test the possibility of screening well-expressed binders using a physiological temperature phage display method.

As a proof-of-concept, we chose B7-H3 as a target, because it is overexpressed in many cancer types, inhibits T-cell activation and is regarded as an important immune check point member of the B7 and CD28 families [28, 40]. It is also overexpressed in many solid tumors, making it an attractive therapeutic target [27]. Here, we were able to successfully express the ECD of B7-H3 in HEK-293 cells. We then used a physiological temperature (37°C) phage display method to make the rabbit VH phage library particles and performed phage panning. We finally obtained two representative binders that bound B7-H3 protein and B7-H3 positive cancer cells. Importantly, the rabbit single domain antibodies previously isolated by using a low temperature phage panning had extremely low expression (less than 0.1 mg/L) in E. coli; in contrast, the purification yields for the A1 and B1 rabbit single domain antibodies isolated in the physiological temperature (37°C) were 10–100 fold higher (2–10 mg/L). Furthermore, the A1 and B1 binders had positive cell binding for B7-H3-positive human tumor cells, but not B7-H3 knockout tumor cells.

Phage panning is an artificial selection system. The fact that we can isolate rabbit single domain antibodies by phage display does not establish whether rabbits naturally can have heavy chain antibodies. It has been reported that some humans with certain heavy chain diseases may naturally produce heavy chain antibodies [5, 6].

Our study demonstrates that rabbit VH domain antibodies can be generated by phage display. Given the important role of B7-H3 in regulating T-cell functions, the two B7-H3 domain antibodies generated here may have some unique potential applications, especially for cancer immunotherapy where single domain antibodies can be used for development of CAR-T cells or bispecific antibodies.
Figure 4. Binding properties of the B7-H3 single domain antibodies. (A) SDS-PAGE analysis of the purified A1 and B1 binders (VH-His-FLAG fusion) from *E. coli*. Two micrograms of purified rabbit VH single domain antibody (A1 and B1), non-reduced (Non.) or reduced (Red.) by β-mercaptoethanol were separated on 8% SDS-PAGE gel. Protein bands were visualized by Coomassie Blue R-250 staining. (B) Antigen binding by ELISA. Five micrograms of B7-H3-hFc were coated on the ELISA plate, and different concentrations of rabbit single domain antibodies were incubated. Binding was detected by HRP conjugated anti-FLAG mouse monoclonal antibody M2. The binding curves were plotted using the software GraphPad Prism and the calculated EC_{50} values were determined by the software's algorithm of hyperbola one site binding. (C) Cell binding determination by flow cytometry. Ten microgram per mL of rabbit single domain antibodies was co-incubated with 1 million cells. Antibody binding was visualized by APC conjugated anti-FLAG monoclonal antibody. Red curve represented cells stained with 2nd antibody (goat anti-rabbit) only; Blue curve represented cells stained with A1 or B1 rabbit single domain antibody.

Conflict of interest statement. None declared.

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