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Single chain antibody fragments with pH dependent binding to FcRn enabled prolonged circulation of therapeutic peptide in vivo

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ABSTRACT

The neonatal Fc receptor for IgG (FcRn) is considered critical for the regulation of endogenous IgG and serum albumin (SA) and their circulation half-life in vivo. Both IgG and SA can bind to FcRn tightly at acidic pH but not so much at neutral pH. Here we reported a few novel single chain antibody fragments (scFv) obtained based on screening of a phage library. FnAb-8 and FnAb-12 can bind to human FcRn with higher affinities than IgG at acidic pH but similar or lower affinities than IgG at pH 7.4. Fusion proteins consisted of the therapeutic peptide, GLP-1 (Glucagon-like peptide-1) connected to the N-terminus of FnAb-8 and FnAb-12, named as G8 and G12, were shown to retain the pH-dependent binding capabilities to FcRn while also bound to the GLP-1 receptor. In vivo efficacy studies in diet induced diabetes mice confirmed the GLP-1 receptor (GLP-1R) agonist activities and sustained blood sugar lowering effect. In vivo pharmacokinetics (PK) studies were performed in nonhuman primates and FnAb-8 was found to have circulation half-life several folds longer than what have been reported for scFvs. G8 may be developed into long acting GLP-1R agonists with great potentials in clinical applications.

1. Introduction

The neonatal Fc receptor (FcRn) is well known for having the functions of transporting IgGs across fetal and neonatal tissue barriers and regulating the rate of IgG and SA degradation throughout life [1–3]. It was firstly identified from the neonatal rodent gut which led to its designation as the neonatal Fc receptor (FcRn) [4,5]. The expression of FcRn was originally believed to be restricted to those sites involved in the transport of maternal IgG from mother to fetus or neonatal [6–8]. However, the presence of FcRn have now been extensively reported in many tissues and cell types including the endothelial cells, epithelial cells, the majority of hematopoietic cells, and some specialized cells such as podocytes, keratinocytes, the blood brain barrier endothelia, and the ocular cells [9–14].

Human FcRn was characterized as a heterodimer of one β2-microglobulin (14 kDa) light chain and one α heavy chain (46 kDa), structurally homologous to the MHC class I molecule [15,16]. The binding of both IgG and SA to FcRn were found to be highly pH dependent, with high affinity at acidic pH and low affinity at neutral pH [17–20]. They were protected from degradation based on a similar mechanism although through different binding sites [21–23]. Specifically, serum proteins including IgG and SA in circulation are taken up by myeloid cells or endothelial cells without FcRn binding near the cell surface when the pH is close to neutral. Entry of IgG and SA into cells is followed by accumulation in early endosomes where the acidic pH is permissive for FcRn binding. Then the early endosomes containing complex of FcRn and IgG/SA are sorted to recycle back to cell surface and IgG/SA released by exocytic processes close to the physiological pH [24–28].

Many studies had used the FcRn binding domains from SA or IgG and made fusion proteins containing these domains to achieve recycling and circulation extension [29–32], there had been also some efforts made to improve drugs' circulation properties by engineering or developing new peptide or protein binding domains of FcRn [33–43]. But most of these attempts including Fc-fusion proteins could not match the endogenous IgGs' long circulation half-life. Higher binding affinity at endosomal pH may not always translate to half-life extension [44,45]. Other factors including the binding sites, pH dependency, and possibly affinity thresholds at different pHs may exist that governed the FcRn mediated recycling process [46].

In this study, we seek to explore whether it is possible to generate small affinity antibody fragments, i.e., single chain variable fragment (scFv Abs), that can interact directly with human FcRn in a pH-

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dependent way. The scFv fragments should have higher binding affinity at pH 6.0 towards FcRn than those of hIgG or SA, but almost no binding at pH 7.4. A specific scFv screening scheme was devised and successfully implemented. The detailed characterizations of the screen results indicated they may have excellent recycling properties and could be used as a long circulation carrier for therapeutical proteins and peptides.

2. Materials and methods

2.1. Materials

Human serum albumin (SA) was purchased from Abcam. Human IgG and HLA-A2 proteins, Lipofectamine2000, 293fectine and AF647 (AlexaFluor647) antibody labeling kit were from Invitrogen. Mouse anti-M13 phage antibody, anti-Poly-histidine tag antibody and GLP-1 were purchased from Genscript. GLP-1R-Fc, β2-microglobulin (β2M) protein, β2M expression vector, FcRn and HLA-A2 cDNAs were obtained from Sinobiological. Anti-hFcRn and anti-HLA-A2 antibodies were from Santa-Cruz. Strepavidin-AF647, anti-mouse IgG-AF647, anti-mouse-IgG-HRP were from Jackson Immunology research. Streptavidin-coupled Dynabeads was from ThermoFisher. Biotin labeling kit was from SoluLink. The luciferase assay system was from Promega. Cell culture medium and reagents were all purchased from GIBCO. Anti-FLAG magnetic beads and other chemicals were from Sigma-Aldrich.

2.2. Protein expression

Soluble extracellular domains of human, cynomolgus and mouse FcRn were cloned, expressed, and purified based on a previous study [47]. Briefly, the cDNA encoding extracellular domains (residues 1 to 297) of hFcRn, cynoFcRn and mFcRn with a c-terminal poly-histidine tag were cloned respectively into pCDNA3.1 vectors. Then the vectors were co-transfected with a β2M expression vector into HEK293 6E cells using 293fectine. After 72 h of incubation with the transfection complex, the supernatants were harvested, and secreted proteins were purified using a HisTrap FF column on AKTA explorer (GE Healthcare). The fractions containing FcRn&β2M heterodimer were pooled and buffer-changed to PBS.

For the production of scFv antibody fragments based on selected phage clone sequences, the genes were obtained by PCR using different sets of primers encompassing parts of the scFv gene and the restriction site EcoRI at the N-terminal or HindIII at the C-terminal. The PCR products were cloned into an expression vector containing a C-terminal FLAG and a poly-histidine tag. Then the vectors were transformed into HEK293 6E cells using 293fectin reagent. scFv fragments were purified from the supernatants using HisTrap FF column followed by affinity purification using anti-FLAG beads. The quality of the scFvs was determined by BCA and SDS-PAGE analysis.

Fusion proteins consisted of the modified GLP-1 sequence (no. 7-36 AA with mutations at A8G, G22E, R36G) connected through a $(G4S)_3$ linker with the selected scFv sequences (FnAb8 or FnAb12) or an unrelated scFv sequence (scFv-n) were synthesized (Genscript) and cloned into the same expression vector as described above. The production and purification of the three fusion proteins were also similar. There were designated as G8, G12, and Gn respectively. The quality of the fusion proteins was determined by BCA and SDS-PAGE analysis.

2.3. Cell lines

For the characterization of antibody fragments binding to FcRn on cell membrane, we constructed an EGFP-hFcRn stable expression cell line $293T^{EGFP\text{-}hFcRn}$ and a control cell line $293T^{EGFP\text{-}HLA\text{-}A2}$ with stable expression of EGFP-HLA-A2 based on previously published protocols [48]. Specifically, to generate N-terminal EGFP-tagged construct, the pEGFP-N1 (Clontech, 6085–1) vector was modified by inserting the IL2 signal peptide sequence into the downstream of the CMV-IE promotor, between NheI and AgeI; to generate the hFcRn construct, the 5′-coding sequence (966 bp, from AA24 to AA325) was PCR-amplified from hFcRn cDNA using forward (CCT GTA CAA GGC AGA AAG CCA CCT CTC CCT C, BsrGI site underlined) and reverse (TCT AGA CTA CTA CCT CAT CCT TCT CCA, XbaI site underlined) primers. This PCR product was digested with BsrGI and XbaI, and inserted into the downstream of CMV-IE promoter, between BsrGI and XbaI restriction sites and in-frame with the EGFP coding sequence. The HLA-A construct was PCR-amplified from HLA-A2 cDNA using forward (GAG CTG TAC AAG GGC TCT CAC TCC ATG AGG TAT TT, BsrGI site underlined) and reverse (GTC TAG ACT ATC ACA CTT TAC AAG CTG TGA GAG ACA C, XbaI site underlined) primers. The 1030 bp PCR product was digested with BsrGI and XbaI and inserted into the pEGFP-N1 vector as described above. The EGFP-hFcRn and EGFP-HLA-A2 encoding vectors were respectively transfected into 293 T cells together with theβ2M expressing plasmid using Lipofectamine2000. Stably transfected 293 T cells were selected using 400 μg/ml G418 and 2.5 μg/ml Puromycin in 10% FBS-supplemented DMEM. The expression of hFcRn&β2M and HLA-A2&β2M heterodimers in their respective $293T^{EGFP\text{-}hFcRn}$ and $293T^{EGFP\text{-}HLA\text{-}A2}$ cell lines were confirmed using confocal microscopy and FACS analysis with specific antibodies.

The CRE-Luc/GLP-1R HEK293 cell line was obtained from HD Biosciences. HEK293 cells were co-transfected with GLP-1R expression plasmid and plasmid containing cAMP response element (CRE) fused with a Luciferase gene. The colonies expressing both human GLP-1 receptor and CRE-luciferase were selected using a luciferase assay system following treatment with 1 μM of GLP-1 for 24 h in 96-well plates.

2.4. Phage display library screening

The purified hFcRn&β2M heterodimer and β2M monomer were biotin labeled using a biotin protein labeling kit and attached to streptavidin-coupled Dynabeads. A naïve human scFv phage-display library (CP Co. Ltd., Shanghai) was used for subtractive panning against β2M and then to hFcRn&β2M heterodimer at pH 6.0. Briefly, the phage pool solution was adjusted to pH 6.0 and incubated firstly with β2M coated Dynabeads for 1 h at room temperature. These β2M beads were removed and hFcRn&β2M heterodimer Dynabeads were added for panning at pH 6.0 for another 1 h at room temperature. The resulted phage bound beads were then washed extensively with phosphate-buffered saline (PBS; pH 6.0) containing 0.05% Tween-20 and finally PBS (pH 7.4) to collect the released phage pool. The phage pool was amplified by infecting TG1 cells along with helper phage M13KO7 as described previously [49] and the panning was continued for 3 round. The phage clones selected were isolated and tested for binding and characterized by sequencing.

2.5. ELISA analysis of pH dependent binding of selected phages to FcRn

The various phage clones selected by screening were characterized for their pH dependent binding capabilities to FcRn using ELISA. Biotin labeled hFcRn&β2M orβ2M were captured by streptavidin pre-coated ELISA plates and incubated at 37 °C for 1 h. The plates were then washed with pH 6.0 PBST (0.05% tween-20 in PBS) for three times, then 100 μl of phage samples (pH adjusted to pH 6.0) were added, and incubated for 1 h at 37 °C. The plates were washed again with PBST at pH 6.0 for 5 times. Then 100 μl of mouse anti-M13 phage antibody in PBS at pH 6.0 was added to each well, and incubated for 1 h at room temperature, followed by 100 μl HRP-conjugated anti-mouse antibodies with washes between the steps, and detected with 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Pierce) after 5 min development and 2 M HCl stop. All antibodies and buffers were adjusted to pH 6.0. The similar series of protocols were repeated using pH 7.4 buffers to determine the pH dependent binding properties of the various phage clones. The optical absorbance at 450 nm were measured and reported.

2.6. Surface plasmon resonance (SPR) experiments

The various binding characteristics between FcRn and antibody fragments or fusions proteins were examined using surface plasmon resonance measurements. The Biacore T100 instrument (GE Healthcare) and the accompanied CM5 chip sets were used.

The CM5 chips were coated with various FcRn&β2M heterodimers (human, cynomolgus or mouse) or β2M as directed by the manufacturer (GE healthcare). The coupling was done by injecting 10 μg/ml of proteins in 10 mM sodium acetate buffer pH 5.0 followed by a blocking step using ethanolamine. For the SPR measurements, 200 nM of scFv antibodies or GLP1-scFv fusion proteins were flowed over the prepared surface to record the binding process. Bindings at both pH 6.0 and pH 7.4 phosphate buffer solutions were measured.

For the binding kinetics analysis, FnAb-8 and FnAb-12 solutions at serially diluted concentrations (pH 6.0: 1-200 nM; pH 7.4: 78-10,000 nM) were injected with a flow rate of 30 μl/min at 25 °C. The resulting sensorgrams were analyzed using the BIAevaluation 2.0.3 software based on the Langmuir 1:1 binding model.

The competitive binding properties of FnAb-8, FnAb-12 against hIgG or SA were analyzed by introducing hFcRn&β2M solution premixed with the antibody fragments at a constant rate of 30 μl/min over immobilized human IgG or SA chips. A non-related scFv sample was also measured for comparison. The figures were plotted using Prism 5 (GraphPad Software, Inc.) and data analysis done using 2-way ANOVA.

For the binding of GLP1-scFv fusion proteins G8 and G12 with GLP-1R, the soluble GLP1R-Fc proteins were immobilized on CM5 chips. 200 nM of G8, G12 and the original GLP1 peptide were injected. A non-related hIgG was used as Fc control.

2.7. FACS analysis of the various antibody fragments and fusion proteins binding to cell surface FcRns

The $293T^{EGFP\text{-}hFcRn}$ and $293T^{EGFP\text{-}HLA}$ cell lines were established with stable expression and display of hFcRn&β2M or HLA-A2&β2M on cell surface. We used anti-hFcRn or anti-HLA-A2 antibodies to confirm the expression of surface maker. After 45 min incubation at 4 °C, unbound antibodies were washed away and APC conjugated anti-mouse IgG was added for half an hour. Cells were washed, re-suspended in wash buffer plus 0.5% BSA, and analyzed using a FACSCalibur (BD Biosciences).

The binding of hIgG and SA to $293T^{EGFP\text{-}hFcRn}$ and $293T^{EGFP\text{-}HLA}$ cells at various pH conditions were also examined. The cells were incubated with hIgG and SA pre-labeled with AF647 using a fluorescence labeling kit in pH 6.0 or pH 7.4 PBS buffers. They were then washed and resuspended in the same buffer plus 0.5% BSA and analyzed using a FACSCalibur (BD Biosciences).

The bindings of the antibody fragments to $293T^{EGFP\text{-}hFcRn}$ or $293T^{EGFP\text{-}HLA}$ cells were analyzed similarly. Gradients of FnAb-8, FnAb-12, or an unrelated scFv were incubated with the cells in pH 6.0 or pH 7.4 PBS binding buffers respectively. Unbound antibodies were washed away with the same buffer. For detection, APC conjugated anti-Histag antibodies were added and incubated for half an hour. Cells were washed and resuspended in the same buffer plus 0.5% BSA and analyzed using a FACSCalibur (BD Biosciences). hIgG and SA labeled with AF647 was used as the positive control.

The competition between FnAb-8 or FnAb-12 and hIgG or SA binding to $293^{EGFP\text{-}hFcRn}$ cells was also examined. The fluorescence labeled $hIgG^{AF647}$ and SA^{AF647} binding to hFcRn at different concentrations were evaluated and plotted using $293^{EGFP\text{-}hFcRn}$ cells. The 80% FcRn bound concentration (EC80) of $hIgG^{AF647}$ and SA^{AF647}, 1 μg/ml and 2.5 μg/ml were selected for scFv competition study. FnAb-8 or FnAb-12 were mixed at different ratios with IgG or SA (final concentration 1 μg/ml and 2.5 μg/ml respectively). The mixtures were added to 5×10^5 $293T^{EGFP\text{-}hFcRn}$ cells and incubated for 30 min at 4 °C. They were then washed 3 times with pH 6.0 buffer. Events were acquired on a FACSCalibur and the binding percentage of labeled hIgG or SA were recorded. All of the FACS data were analyzed with Flowjo software (V10.0.8).

2.8. In vitro characterization of GLP-1R agonist activities of GLP-1-scFv fusion protein G8 and G12

The CRE-Luc/GLP-1R HEK293 cell line was used for biological evaluation of FnAb-8, G8, FnAb-12, G12 and Gn. Briefly, the cells were seeded at a density of 5×10^4 cells/well in 96-well plates and grown overnight. Approximately 24 h later, cells were treated with 10 μl of FnAb-12, G8, FnAb-12, G12 or Gn solutions at various concentrations for 5 h. They were then washed twice with PBS and lysed using 50 μl of 1 × reporter lysis buffer. The luciferase activities were determined using an assay system in an E7031 plate reader (Promega). The activity-concentration data curves were plotted and EC50 values were calculated based on 4-parameters logistic Model using Prism 5 (GraphPad Software, Inc.).

2.9. In vivo characterization of GLP-1R agonist activities of GLP-1-scFv fusion protein G8, G12 and Gn

Male C57BL/6 mice (7 weeks old) were purchased from JOINN Laboratory Inc. (Suzhou, JS). All mice were maintained under a 12-hour light/dark cycle (lights on at 7 AM and off at 7 PM) with free access to food and water, except where noted. Animal handling and experiments were conducted in PegBio (Suzhou) according to the Principles of Laboratory Animal Care and the protocol was approved by the Experimental Animal Management Committee and Experimental Animal Ethics Committee of Shanghai JiaoTong University. The C57BL/6 mice were fed either standard rodent chow (SRC) or a high-fat diet (HFD, 45% kcal from fat; Medicience Inc., Yangzhou, JS) for 18 weeks. Obese mice with 20% weight and 30% blood glucose increase than those SRC fed were randomized to 6 groups and given once subcutaneous (SC) injection of FnAb-8, FnAb-12, G8, G12, Gn or Exenatide (0.1 mg/mouse). Using an Accu-Check Advantage glucometer (Roche), blood glucose were measured at each timepoint (0, 2 h, 4 h, 6 h, 8 h or/and 10 h, 24 h, 32 h and 48 h) and plotted against time point with Prism 5 (GraphPad Software, Inc.) and data analysis done using 2-way ANOVA.

2.10. Pharmacokinetics studies in cynomolgus monkeys

The PK study protocol was approved by the Experimental Animal Management Committee and Experimental Animal Ethics Committee of Shanghai JiaoTong University and conducted at GuangXi Changchun Biotech Co., Ltd. Two healthy female cynomolgus monkeys (age 6.5–7 years; average weight 3.3 kg) were admitted and acclimated in the study room for one week. They were given a single intravenous bolus injection of 1 mg/kg endotoxin-free biotin labeled FnAb-8 protein in PBS, followed by 1 ml sterile PBS flush. Blood samples (~2 ml) were taken at baseline (t0), 0.5, 6, 24, 48, 96, 240, 408, 576, 744, 1008 h after injection. Blood was collected into EDTA containing tubes and centrifuged at 1200 g at 4 °C to separate plasma. Plasmas were stored at −80 °C until analysis. Quantitative ELISA assays were done using streptavidin pre-coated plates to determine the FnAb-8 concentration, using the material for injection as the standard. Data were plotted using ELISA concentration vs. time and analyzed based on the Linear Log Trapezoidal method, plasma (200–202) model (non-compartment model) using WinNonlin (Certara).

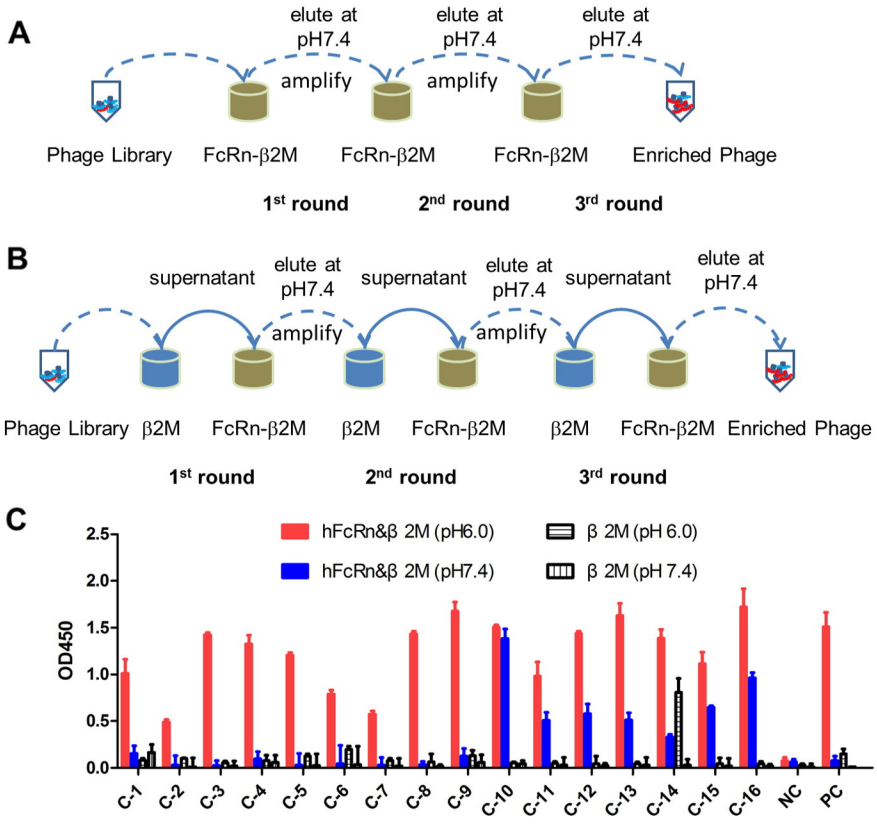


Fig. 1. Phage library panning and binding to hFcRn. (A) Direct panning strategy of phage library. (B) Subtractive panning strategy of phage library. (C) Binding of selected phages to hFcRn&β2M or β2M coated on ELISA plates at acidic or neutral pH. A non-relative phage clone was set as negative control (NC) and hIgG as positive control (PC). Bound phages were visualized using an HRP-conjugated anti-M13 antibody. The numbers given represent the mean of duplicates.

3. Results

3.1. Screening and identification of pH-dependent hFcRn binders from a naïve human scFv phage library

The scFv phage library screening was carried out using soluble extracellular domain of FcRn. Since we are interested only in pH dependent binders, therefore we designed a binding at pH 6.0 and eluting at pH 7.4 panning strategy (Fig. 1A). In addition, because hFcRn naturally forms heterodimer with β2M, we had to use the hFcRn&β2M dimer protein as the panning target and β2M as the control. In order to avoid interferences from β2M binders, we adopted an improved subtractive panning strategy, as shown in Fig. 1B. After three rounds of panning, totally 271 phage clones were identified. They were all tested for binding to hFcRn&β2M at pH 6.0 using ELISA. 182 clones were confirmed positive. DNA sequencing of the positive phages identified 16 clones with unique scFv gene sequences. Fig. 1C plotted the binding activities of all 16 phage clones at pH 6.0 and pH 7.4 to hFcRn&β2M coated ELISA plates. Their bindings to β2M coated plates were also reported.

3.2. Anti-hFcRn scFvs and GLP-1 fusion protein expression and characterizations of their binding properties

The cDNA of 16 positive phage clones and one negative clone were amplified by PCR and inserted into expression vectors for scFv expression in mammalian cells. The expressed scFv fragment contained the antibody variable region followed by a C-terminal FLAG and then a poly-histidine tag. They were purified and designated as FnAb-1 to 16.

All these scFv fragments were tested for binding to $293T^{EGFP\text{-}hFcRn}$ cells which overexpressed hFcRn on cell surfaces. Since the endosome targeting sequence in FcRn was excluded in the expression vector, the expressed EGFP-FcRns were confirmed to be on the cell surface with their respective antibodies (Fig. 2A). All the expressed scFvs were examined for their binding at both pH 6.0 and pH 7.4 buffers to $293T^{EGFP\text{-}hFcRn}$ cells as well as $293^{EGFP\text{-}HLA\text{-}A2}$ cells. The results were plotted in Fig. 2B with also the data from human IgG (PC) and an unrelated scFv control (NC). FnAb-8 and FnAb-12 were identified to have the best pH-preference at pH 6.0 and selected as the candidates for further studies.

The detailed analysis of FnAb-8 and FnAb-12 binding to FcRn at pH 6.0 were examined compared to those of IgG and SA. As shown in Fig. 2C, both FnAb-8 and FnAb-12 had better binding potency than hIgG and SA. In addition, the different binding profiles of FnAb-8 and FnAb-12 at pH 6.0 and pH 7.4, and with $293T^{EGFP\text{-}hFcRn}$ cells as well as $293^{EGFP\text{-}HLA\text{-}A2}$ cells were shown in Fig. 2D indicating clear pH dependency and FcRn specificity.

The pH dependent bindings of FnAb-8 and FnAb-12 were further characterized by SPR. As shown in Fig. 3, both had strong affinity towards FcRn at pH 6.0. But at pH 7.4, FnAb-8 had almost no binding. FnAb-12 and hIgG had similar binding at pH 7.4, which was roughly one tenth of those at pH 6.0 (Fig. 3A).

We also investigated the respective binding affinities of FnAb-8 and FnAb-12 to human FcRn (hFcRn), cynomolgus FcRn (cynoFcRn) and mouse FcRn (mFcRn). As shown in Fig. 3B, since hFcRn and cynoFcRn are highly homologous, FnAb-8 and FnAb-12 bound both with similar kinetics. The mFcRn is quite different, FnAb-8 bound with very low affinity and FnAb-12 with almost no affinity. The binding curves were fitted to a simple 1:1 Langmuir binding model and the estimate KD values were listed in Fig. 3C.

Fusion proteins containing a mutated GLP-1 sequence connected through a $(G4S)_3$ linker with the scFv fragments were expressed in 293E cells, purified, and characterized using SDS-PAGE (Fig. 4A). Both G8 (GLP-1-FnAb8) and G12 (GLP-1-FnAb-12) were studied by SPR for their binding to hFcRn as well as GLP-1R-Fc using SPR. The addition of

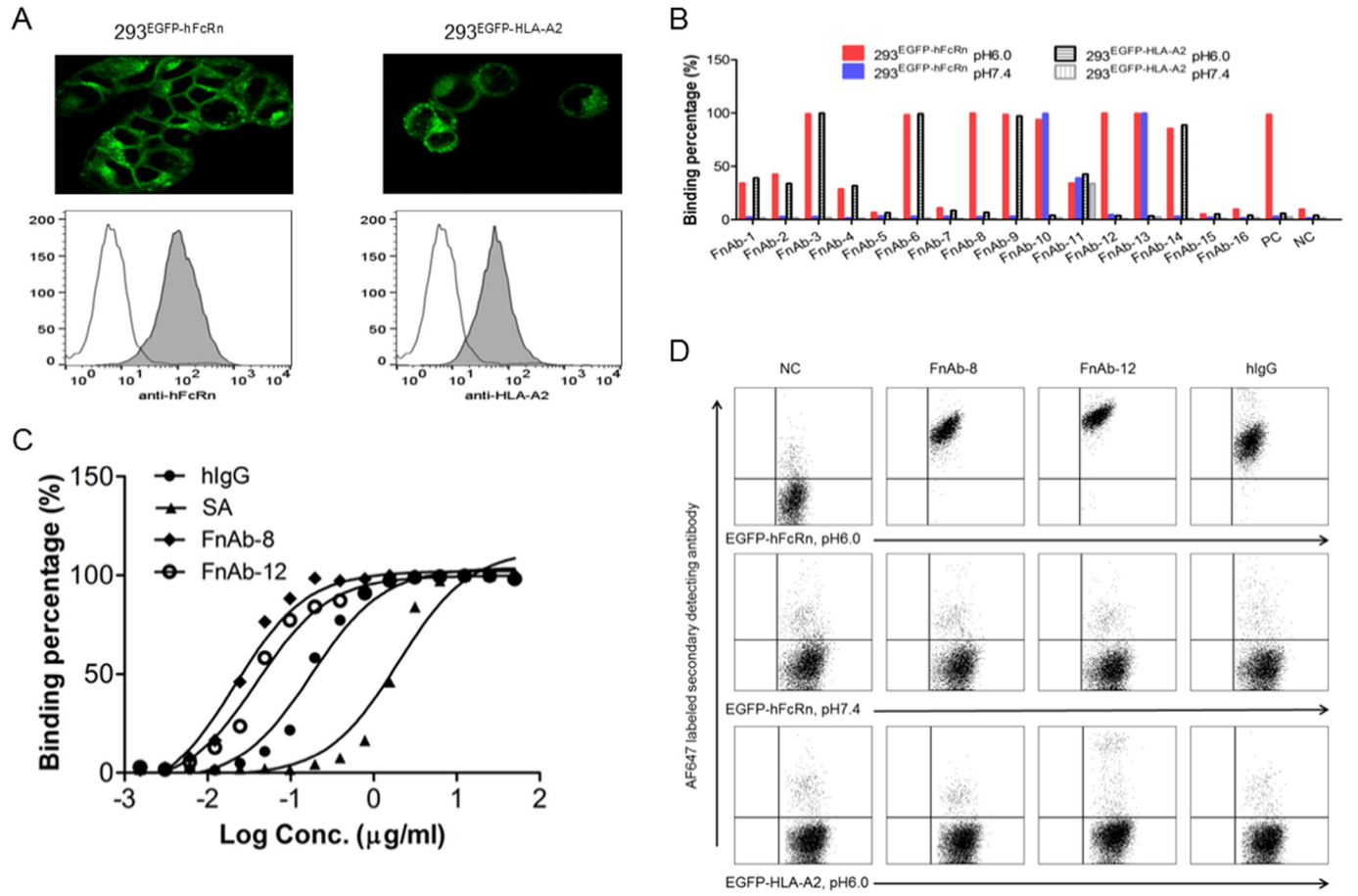


Fig. 2. Purified scFv antibodies binding to FcRn displaying cells. (A) 293T cells stably transfected with plasmid encoding the EGFP-fused hFcRn (left) or EGFP-fused HLA-A2 (right) were inspected by confocal microscopy. The $293T^{EGFP\text{-}hFcRn}$ cells were stained with anti-hFcRn antibody (the gray filled, bottom-left) and the $293^{EGFP\text{-}HLA\text{-}A2}$ cells were stained by anti-HLA-A2 antibody (the gray filled, bottom-right). (B) Purified scFv antibodies were examined for binding to $293^{EGFP\text{-}hFcRn}$ and $293^{EGFP\text{-}HLA\text{-}A2}$ cells at pH 6.0 and pH 7.4. The binding percentage is the population of scFv antibodies binding to those EGFP gated cells. (C) The dose dependent binding analysis of FnAb-8 and FnAb-12 with hFcRn on cell surface compared to hIgG and SA. (D) Flow cytometry analysis of FnAb-8 and FnAb-12 binding to $293^{EGFP\text{-}hFcRn}$ cells. $293T^{EGFP\text{-}hFcRn}$ and $293T^{EGFP\text{-}HLA\text{-}A2}$ cells were incubated with unrelated scFv control (NC), FnAb-8, FnAb-12 and hIgG at pH 6.0 or pH 7.4, followed by staining with secondary detecting antibody labeled with AF647 fluorescence. Data are representative of at least three experiments.

GLP-1 sequence to the scFvs didn't change their FcRn binding capabilities and pH dependency at all (Fig. 4B). The fusion proteins also retained their binding to GLP1R-Fc (Fig. 4C).

3.3. The interference of IgG/SA binding to scFv fragment interactions with FcRn

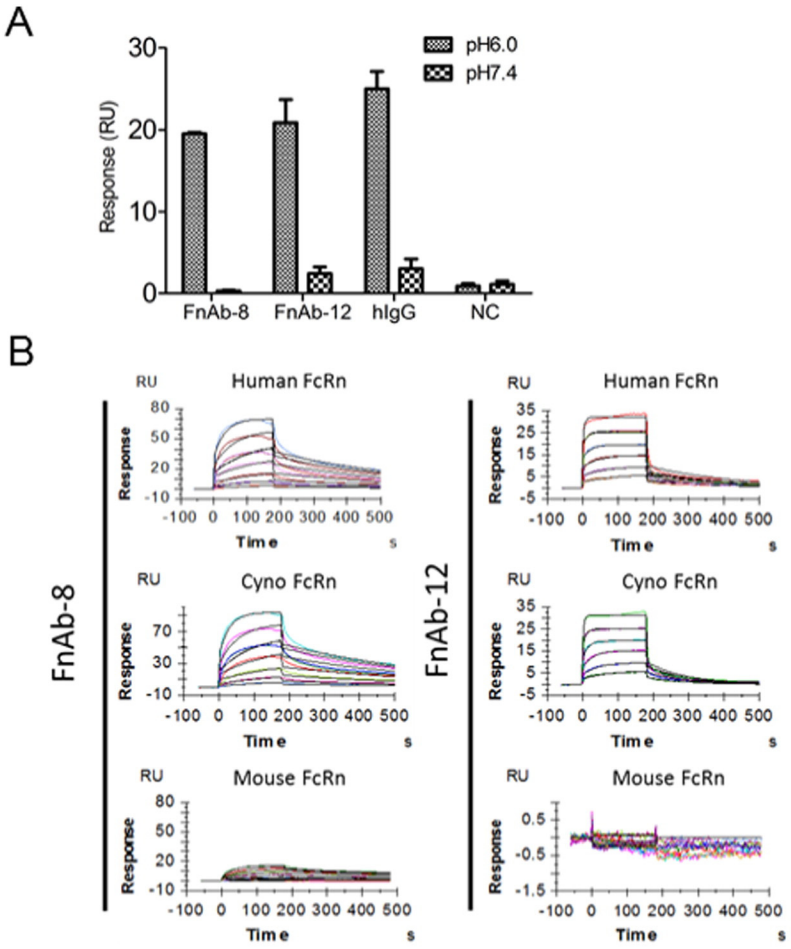
Since both endogenous IgG and SA can bind to FcRn although at different binding sites, we have to investigate the binding of scFvs to hFcRn under the context of hIgG or SA binding as well. We immobilized hIgG and SA on CM5 sensor chips and injected soluble hFcRn pre-mixed with anti-hFcRn scFvs at pH 6.0. As shown in Fig. 5A, SA had no effect whatsoever to scFv-FcRn interactions, suggesting SA bind at a region distinct from scFvs or has a much lower affinity to FcRn. FnAb-8 interfered with IgG binding to FcRn slightly, suggesting there may be some binding site overlapping. FnAb-12, on the other hand, was shown to result in even higher signals based on FcRn binding to IgG. It may indicate that FcRn can bind to IgG and FnAb-12 simultaneously or the binding of FnAb-12 could even induce FcRn conformation change to facilitate IgG binding.

Similarly, the binding of FnAb-8 and FnAb-12 to $293^{hFcRn\text{-}GFP}$ cells were also examined in the presence of IgG or SA. The concentrations of IgG and SA in the studies were set at 1 μg/ml $hIgG^{AF647}$ or 2.5 μg/ml SA^{AF647} respectively when about 80% of the cell surface FcRns were estimated to be occupied. The FACS analysis confirmed the interference of scFv antibodies to hIgG and SA interaction with hFcRn on $293T^{EGFP\text{-}hFcRn}$ cells. The results also demonstrated a different binding pattern of FnAb-8 and FnAb-12 (Fig. 5B), confirming the SPR assay data.

3.4. In-vitro and in-vivo activities of GLP1-scFv fusion proteins

The in vitro activities of GLP-1-scFv fusion proteins in parallel with their parental scFvs was examined in CRE-luc/GLP-1R co-transfected HEK293 cells (Fig. 6A). The EC50 of both G8 and G12 was found to be around 0.5 nM, which is comparable to GLP-1 analogs reported using similar assays [50,51]. In contrast, the parental scFvs FnAb-8 and FnAb-12 showed no agonistic effect to GLP-1R.

We next examined whether G8, G12 and Gn can in fact reduce glucose excursion in mice with high fat diet induced obesity and diabetes. After 18 weeks of feeding, the high fat diet fed (HFD) mice were over-weight and blood glucose concentrations ~30% above normal level. A single bolus injection of both G8, G12 as well as Gn resulted in significantly reduced blood glucose concentrations for several hours after injection (Fig. 6B-D). Fig. 6B indicated that such effects were due to the GLP-1 analog fusion, because both FnAb-8 and FnAb-12 had no activity. Fig. 6C showed that the duration of activities by G8 and G12 were both significantly longer than the GLP-1 analog Exenatide ($P < 0.01$). Most importantly, as shown in Fig. 6D, the effects of G8 and G12 also lasted slightly longer than Gn which had roughly the same molecular weight but no interaction with mouse FcRn at all ($P < 0.01$). Such differences were thought to be due to the residue binding affinities of



C

Binding kinetics of scFvs

Analyte	Ligand	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
FnAb-8	**hFcRn**	6.14E+05	2.65E-03	4.32E-09
FnAb-8	**cyFcRn**	9.34E+05	4.16E-03	4.45E-09
FnAb-12	**hFcRn**	2.37E+06	1.02E-01	4.32E-08
FnAb-12	**cyFcRn**	1.06E+06	1.55E-01	1.46E-07

Fig. 3. Binding of scFvs to FcRn examined by SPR. (A) Soluble hFcRn were immobilized on CM5 chip and binding of scFvs and hIgG at pH 6.0 and pH 7.4 were plotted. (B) Kinetics study of purified scFv fragments to human, cyno and mouse FcRn at pH 6.0. (C) The binding parameters of scFvs to human and cyno FcRn at pH 6.0.

FnAb-8 and FnAb-12 with mouse FcRn, although they may be much lower than their affinities towards human FcRn.

3.5. In vivo pharmacokinetics study of FnAb-8 in cynomolgus monkeys

Since cyno FcRn is highly homologous to human FcRn, we studied the PK properties of FnAb-8 in two cynomolgus macaques. FnAb-8 was administered as a single bolus injection. Its plasma-concentration following the injection was monitored for 1000 h and plotted in Fig. 7A. There were some discrepancies between the two data sets from two animals, we analyzed them individually and reported the calculated PK parameters in Fig. 7B.

4. Discussion

A major challenge for the therapeutic use of many peptides and proteins is their short blood half-life. Their fast clearance from circulation is mainly based on two mechanisms: renal filtration and lysosome degradation. There are in general two strategies to address these problems for prolongation of the drug's half-life: (i) to increase molecular size and its hydrodynamic volume by coupling polymers, carbohydrates or other proteins [52–54] and (ii) to take advantage of the recycling mechanism mediated by FcRn and avoid rapid degradation [55–57]. Both strategies have been pursued extensively and proven to be successful. Especially based on the later strategy, the Fc part of IgG, SA and some Fc, SA

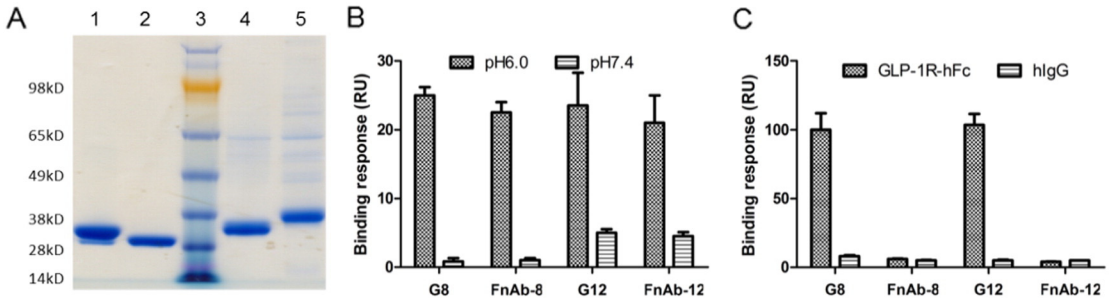


Fig. 4. Mammalian cell production of scFvs and GLP-1 fusion proteins and their binding to hFcRn and GLP-1R. (A) FnAb-8, FnAb-12, G8 and G12 were produced in 293E cell and purified and analyzed by 4–12% SDS-PAGE. Lane 1: G8, Lane 2: FnAb-8, Lane 3: molecular weight (MW) markers, Lane 4: FnAb-12, Lane 5: G12. (B) Binding to soluble hFcRn by SPR. (C) Binding to soluble GLP-1R-hFc by SPR. Injections were done at pH 6.0 at 25 °C with a flow rate of 30 μl/min. n = 3–4.

binding peptides/proteins had been developed and used to fuse with biotherapeutics for longer half-life in vivo. A comprehensive review by Sockolosky & Szoka was published recently [58]. FcRn was regarded as an important target for drug delivery, with applications going beyond half-life extension.

There are some limitations using the endogenous Fc fragment or SA as the carrier for other drugs. One concern is that Fc has other targets besides FcRn that can lead to other pharmacological effects [47]. Also, both are quite big and add to the manufacture costs. So it is highly desirable to develop new structures targeting to FcRn that can achieve even better pharmacokinetic performance in clinical applications. Ying et al. reported a short FcRn binding motif derived from Fc CH3 domain that exhibited improved FcRn binding [33]. Seijsing et al. described a detailed study using an Affibody library to screen for small affinity

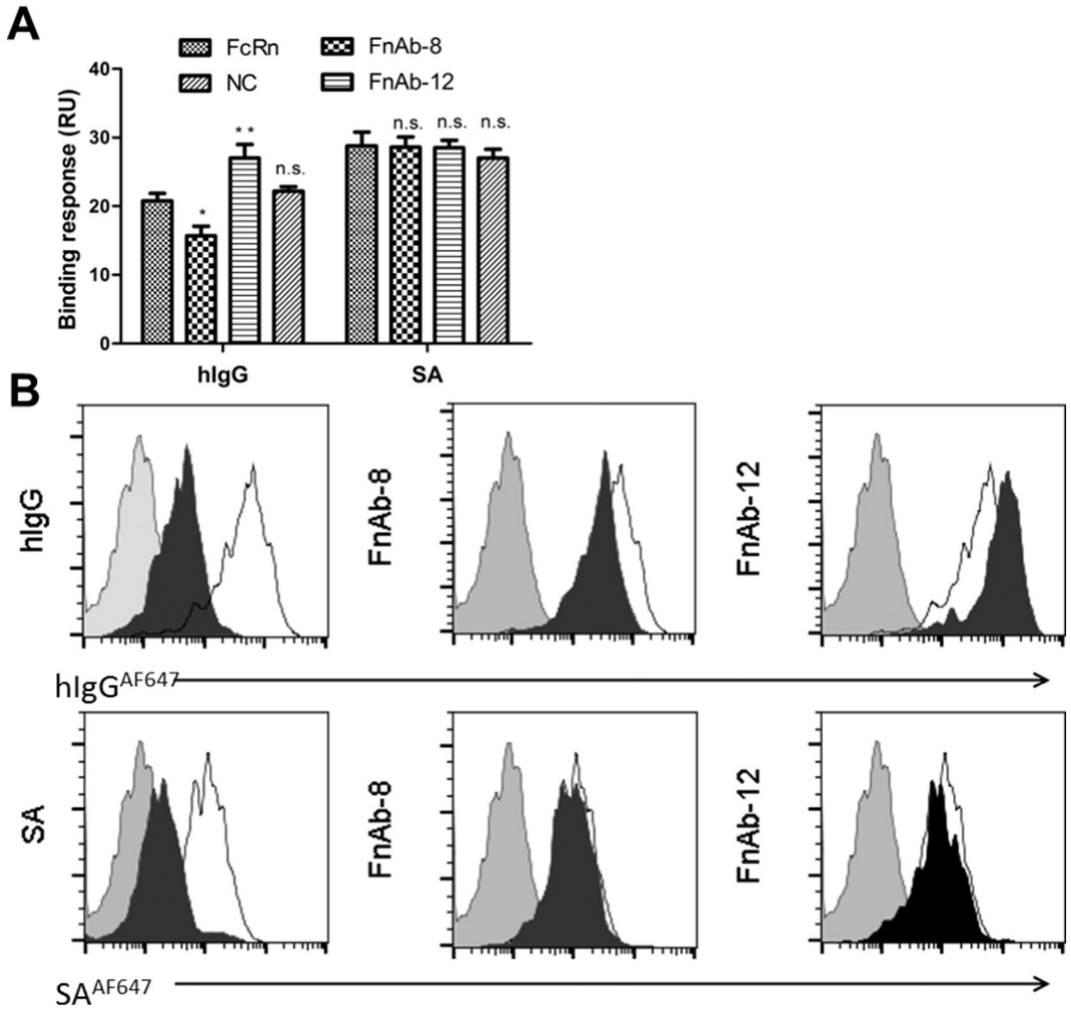


Fig. 5. Interference of IgG/SA binding to scFv fragment interactions with FcRn. (A) SPR signals based on hFcRN binding to immobilized hIgG or SA chips affected by anti-hFcRn scFvs. Injections were done at pH 6.0 at 25 °C with a flow rate of 30 μl/min. n = 3. All data were reported as mean ± s.d. and analyzed using two-way ANOVA, n.s., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$. (B) Binding of fluoresce labeled $hIgG^{AF647}$ or SA^{AF647} to hFcRn expressing cells affected by anti-hFcRn scFvs or unlabeled IgG/SA. The gray filled peaks are cells without binding. The blank peaks are $hIgG^{AF647}$ or SA^{AF647} bound cells at 1 μg/ml $hIgG^{AF647}$ or 2.5 μg/ml SA^{AF647} respectively. The black filled peaks are $hIgG^{AF647}$ or SA^{AF647} bound cells in the presence of unlabeled IgG/SA, FnAb-8, and FnAb-12. Data were representative at least two experiments.

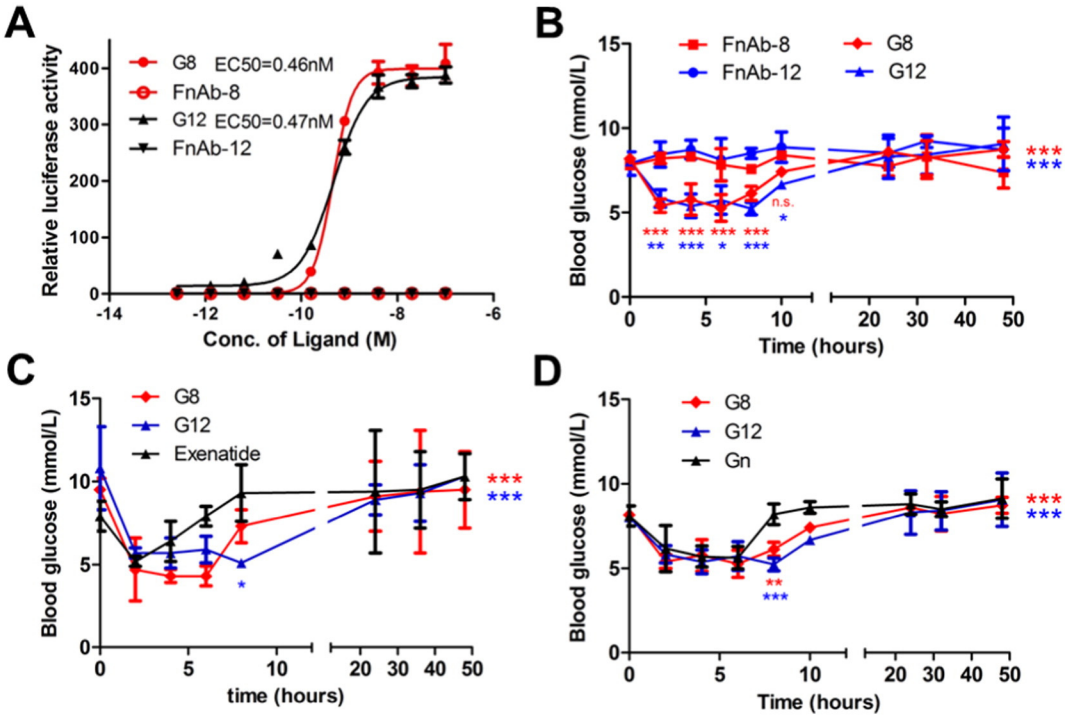
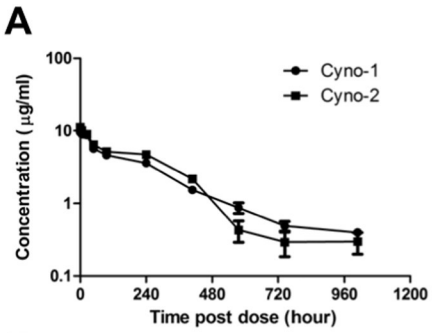


Fig. 6. Biological activity of scFv-GLP-1 fusion proteins in-vitro and in-vivo. (A) Evaluation of FnAb-8, G8, FnAb-12 and G12 activities as human GLP-1 receptor agonists using a luciferase report assay in a human GLP-1 receptor/CRE-Luciferase-co-expressed HEK293 cell line. (B) FnAb-8 (red square), G8 (red diamond), FnAb-12 (blue diamond) and G12 (blue triangle) activities for lowering blood glucose levels of HFD mice, as competed to FnAb-8 (red square) and FnAb-12 (Blue dot) respectively. (C) G8 (red line) and G12 (blue line) activities for lowering blood glucose levels of HFD mice, as compared to that of Exenatide (black line). (D) G8 (red line) and G12 (blue line) activities for lowering blood glucose levels of HFD mice, as compared to Gn (black line). All of the data are presented as means ± s.d. n = 3; analyzed using two-way ANOVA. n.s., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The red stars at each time point indicated the statistical analysis between data from G8 and Exenatide/Gn. The blue stars at each time point indicated the statistical analysis between data from G12 and Exenatide/Gn and represented the significance between G8/G12 and Exenatide or Gn separately. The red and blue stars at the end of the curves indicate the significance of difference of the whole time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proteins (consisting of only 58 amino acids) [34]. Sockolosky et al. used a short peptide sequence that could compete with human IgG for FcRn [35]. Andersen et al. identified a nanobody from a variable-domain repertoire library isolated from a llama immunized with human FcRn [36]. Nixon et al. selected potent inhibitors of IgG binding to human-FcRn at pH 7.4 from a phage library [45].



B

Pharmacokinetic study of FnAb-8 in cynomolgus macaque

ID	β phase $T_{1/2}$(hour)	AUC_{inf} (ug*hour/ml)	Cl_F (ml/hour/kg)	MRT (hour)
Cyno-1	204	2084	0.48	273
Cyno-2	98	2200	0.45	204

β phase $T_{1/2}$ (hour), elimination half-life; AUC, area under the plasma concentration curve; CL, total body clearance; MRT, mean residence time.

Fig. 7. Pharmacokinetics study of FnAb-8 in cynomolgus monkeys. (A) Measurements of FnAb-8 blood concentration over time. (B) Analysis of in vivo PK data.

While most studies have done comprehensive characterizations of FcRn binding in various in vitro and cell models, in vivo studies examining the circulation half-lives have been hard to compare because of different species preferences and model selections. Ying et al. described an FcRn binding motif derived from the Fc CH3 domain which had an elimination half-life at about 44.1 h in cynomolgus macaques [33]. The pH-independent FcRn antibodies selected by Nixon et al. had only 5 to 13 h half-life in cynomolgus monkey [45]. Compared to these numbers, our preliminary data as shown in Fig. 7 seemed to be superior.

Indeed, we initiated this study with the hypothesis that scFv antibodies that specifically recognize hFcRn with high affinity and superior pH preference at acidic pH may be optimal as carrier scaffolds for biopharmaceutics. The IgG Fc and SA interactions with FcRn at pH 6 had been shown to have KDs in the hundreds nM range [17,20]. But since the IgG binding to FcRn was found to have a 1:2 stoichiometry [66,67], the apparent binding affinity between IgG and FcRn would be higher than that of SA. As shown in Fig. 2C, our two scFvs had higher affinities than both IgG and SA, although their bindings should be based on a 1:1 stoichiometry. The KD value was estimated to be in the 5-50 nM range at pH 6.0, similar to the 1G3 antibody previously reported [59].

But based on the FcRn mediated IgG/SA recycling mechanism, the pH depend binding property may be even more important than the binding affinity per se [44,46]. Although FcRn can be found both at the cell surface and in intracellular compartments, studies based on FcRn knockout mice suggested that it's the interactions at acidic pH that determines the recycling activities [27]. In endothelial and other cell types, FcRn mainly resides in the endosomal compartments. FcRn binds to IgG and SA that were nonspecifically endocytosed and salvages them from lysosomal degradation. So all the screening assays as well as the most initial characterizations were done at pH 6.0. But we also incorporated a special elusion step at pH 7.4 in the phage screening scheme (Fig. 1). It is based on the biological fact that the ligand-FcRn complex would have to dissociate after being transported back to the plasma membrane via recycling endosomes [24–28]. In fact, it's the differences between bindings at these two pHs that was considered as the major screening criteria for the selection of final candidates.

Furthermore, our screening scheme also included a subtractive panning strategy which was important for the successful panning (Fig. 1). Since FcRn is stable in the heterodimer form with β2m, we found in preliminary studies that there might be many hits bound to β2m. The subtractive panning step was used to successfully rule out those binders. In later studies when we verify the selected phages or scFvs, we always included a HLA control which is also in a heterodimer form with β2m.

With all these considerations, the screening effort was quite successful. FnAb-8 and FnAb-12 were selected as the final candidates. They were validated as hFcRn specific binders with pH dependency. Both have significantly improved hFcRn binding affinity (about 10 to 100-folds higher than hIgG) at pH 6.0 but maintained weak binding at neutral pH. However, unlike what was reported by Seijsing et al., FnAb-8 and FnAb-12 had narrower specificity towards only human and cynomolgus FcRn, with only minimum affinity towards mouse FcRns. It may due to different binding epitopes in hFcRn and mFcRn, just like the dramatic cross-species differences in IgG and albumin binding [20]. Therefore, we had to examine the PK profile only in primate models. The calculated elimination half-life was between 100 and 200 h, which is about two times longer than the 44.1 h mean number reported by Ying et al. [33]. It is very encouraging considering the overall small molecular weight of scFvs. Other scFv or scFv fusion proteins usually had in vivo circulation half-lives of minutes to hours [60,61] without the Fc domain and the recycling mechanism. Clearly, the binding of FnAb-8 and FnAb-12 to FcRn at pH 6.0 made huge differences. They can be highly valuable as carriers to help prolonging the serum half-life of other therapeutic proteins.

Finally, we explored the activities of a peptide fusion protein with the selected scFvs. GLP-1 is a typical peptide hormone which is highly potent but not suitable for therapy due to its very short circulation life [62]. Native GLP-1 is rapidly inactivated with a half-life of 1–2 min [63]. GLP-1 (7–36) was the active metabolite and several amino acid substitutions (A8G, G22E, R36G) were suggested to render the molecule resistant to DPP-4 and chemically more stable. A fusion protein containing a modified GLP-1 connected to a SA has been approved for clinical use with human half-life around 5 days [64,65]. We constructed G8 and G12 based on the FnAb-8 and FnAb-12 sequence and examined their activities in vitro and in vivo. In addition, the fusion of an unrelated ScFv with GLP-1 designated as Gn was also tested for comparison. As shown in Fig. 4, G8 and G12 retained their affinity and pH-dependent binding to FcRn. They were all active in cell assays demonstrating GLP-1R agonist activities (Fig. 6A). In HFD mouse studies, G8, G12, as well as Gn were able to reduce blood glucose levels with similar potency as the GLP-1 analog peptide Exenatide. Their activity durations were also longer than that of Exenatide because of the higher MWs (Fig. 6C, D). However, as shown in Fig. 6D, G8 and G12's activities lasted slightly (yet significantly) longer than that of Gn, suggesting there were still residue interactions between G8 and G12 with mouse FcRn, despite that both FnAb-8 and FnAb-12 were selected for human FcRn and only very poor binding with mouse FcRn might exist.

The data shown in Fig. 7 was based on a preliminary evaluation of the half-life extension effects of FnAb-8 based on pH dependent binding to FcRn. The results were highly encouraging as discussed earlier. Further studies examining the activities and PK properties of fusion proteins such as G8 in primate models would be necessary to fully evaluate the clinical potentials of the ScFv scaffolds developed in this study.

5. Conclusion

In this study, we identified two candidate scFv structures that can bind to human FcRn with great specificity, high affinity at pH 6.0, and low to almost no binding at pH 7.4. They were shown to have extended circulation half-lives in cynomolgus monkeys. They can be used as a carrier protein to be fuse with the therapeutic peptide GLP-1 to improve its stability and activity duration in vivo.

Acknowledgments

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