Selection of Nanobodies that Target Human Neonatal Fc Receptor

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FcRn is a key player in several immunological and non-immunological processes, as it mediates maternal-fetal transfer of IgG, regulates the serum persistence of IgG and albumin, and transports both ligands between different cellular compartments. In addition, FcRn enhances antigen presentation. Thus, there is an intense interest in studies of how FcRn binds and transports its cargo within and across several types of cells, and FcRn detection reagents are in high demand. Here we report on phage display-selected Nanobodies that target human FcRn. The Nanobodies were obtained from a variable-domain repertoire library isolated from a llama immunized with recombinant human FcRn. One candidate, Nb218-H4, was shown to bind FcRn with high affinity at both acidic and neutral pH, without competing ligand binding and interfering with FcRn functions, such as transcytosis of IgG. Thus, Nb218-H4 can be used as a detection probe and as a tracker for visualization of FcRn-mediated cellular transport.
FcRn function is critical for controlling and optimizing pharmacokinetics and bioavailability of monoclonal IgG and IgG Fc-fused therapeutics as well as albumin-fused or conjugated therapeutics. Still, major questions regarding the cellular distribution of FcRn and how it transports both its ligands across or within different cell types remain to be addressed at a molecular and cellular level. To study the biology of FcRn in health and disease, methods to detect its presence and transport are needed, and in particular, detection reagents other than anti-FcRn IgG antibodies, which may interfere with FcRn function, are preferred.

This report describes the generation of FcRn probes from a combinatorial library of light chain devoided IgG variable regions obtained from a llama immunized with recombinant soluble hFcRn using phage display. Such variable HC only (VHH) antibodies are naturally occurring in Camelidae and their entire binding unit is termed Nanobodies® (Nb). We identified Nbs with specificity for folded hFcRn, which do not interfere with pH-dependent binding to IgG or albumin. Importantly, Nb binding to hFcRn was not affected by pH and did not affect FcRn-mediated transcellular transport of IgG. The Nbs could also be directly conjugated to an enzyme or fluorochromes without losing FcRn binding activity. Thus, the Nbs selected are attractive tools for studying FcRn biology.

Figure 1 | Immunization of a llama and selection of anti-hFcRn phage particles. (A) Serum samples collected before and after immunization of a llama with recombinant hFcRn. Serial dilutions of sera were incubated in hFcRn coated ELISA wells and bound antibodies were detected using an anti-llama IgG from mouse followed by an anti-mouse IgG secondary antibody from rabbit. (B) Representative ELISA results showing binding of selected phage clones to hFcRn at pH 6.0 and pH 7.4. The numbers given represent the mean of duplicates.

Figure 2 | Bacterial production of Nbs and binding to hFcRn. (A) Nb candidates were produced in E. coli and subsequently purified and analyzed by 12% SDS-PAGE. Lane 1 shows a molecular weight (MW) standard, lane 2 shows Nb218-H4, lane 3 shows Nb203-G8, lane 4 shows (Nb218-H4)2, and lane 5 (Nb203-G8)2. (B) Binding of GST-tagged hFcRn to the Nb variants captured on an anti-c-Myc antibody at pH 6.0 and pH 7.4. Bound receptor was visualized using an HRP-conjugated anti-GST antibody. (C) Binding of the Nb variants to human β2-microglobulin coated in wells and detected with an anti-c-Myc antibody. An HRP-conjugated anti-human β2-microglobulin antibody was included as a positive control. The numbers given represent the mean of triplicates. (D–E) Anti-hFcRn Nbs bind hFcRn independently of the presence of the ligands. Representative SPR responses showing binding of hFcRn alone or in complex with anti-hFcRn Nbs or a control Nb to (D) human IgG and (E) human albumin immobilized on a CM5 sensor chip. Injections were done at pH 6.0 at 25°C with a flow rate of 50 μl/min. n = 3–4. All data are presented as mean ± s.d.
Immunization of llamas

Results

Immunization of llamas. One llama (L. glama) was immunized with purified soluble recombinant hFcRn over a period of 6 weeks. The induction of llama IgG antibodies directed towards hFcRn was demonstrated by ELISA on sera collected before and 21 days after immunization, (Figure 1A). Subsequently, a cDNA library encoding the in vivo matured variable domain repertoire of HC only antibodies (VHH) were prepared from isolated PBLS and draining lymph nodes biopsies, followed by cloning of the PCR-amplified fragments into a phage display library. The resulting library contained approximately 10^8 independent clones.

Phage display selection. To select for hFcRn-specific phages, the phage display library was panned on recombinant hFcRn coated by ELISA wells at pH 5.1 followed by trypsin elution. After two rounds of selection, individual phages were isolated and screened for binding to hFcRn at pH 6.0 and pH 7.1 in ELISA. Phage particles were identified that bound hFcRn at both pH conditions. Binding of four of the selected phage clones is shown in Figure 1B, where the strongest binding response was seen for a phage clone Nb218-C5 followed by Nb216-E6, Nb218-H4 and Nb218-A5. All the clones showed a slightly better response at acidic than at neutral pH.

Binding properties of selected anti-hFcRn Nanobodies. The variable domains from four isolated phage clones were sub-cloned into an expression vector designed for production of recombinant Nbs in E. coli. The cDNAs were cloned upstream of a C-terminal c-Myc tag followed by a His tag. The purified Nbs was analyzed on SDS-PAGE gels and seen as distinct bands of approximately 15–17 kDa, corresponding to their expected molecular weights. A representative SDS-PAGE gel of the purified Nb218-H4 and a control variant with irrelevant specificity (Nb203-G8) is shown in Figure 2A.

To verify that the produced monomeric Nbs bound hFcRn, ELISA was performed with equal amounts of the Nbs captured on an anti-c-Myc mouse IgG1 antibody coated in wells (mlgG1 does not bind hFcRn). Receptor binding was confirmed by adding GST-tagged hFcRn followed by visualization using an HRP-conjugated anti-GST antibody. All four Nbs were shown to bind the receptor at both pH conditions with a slightly better binding at pH 6.0 (Figure 2B). Nb218-C5, Nb216-E6 and Nb218-H4 bound equally well, followed by Nb218-A5, while the negative Nb control did not bind.

FcRn is a heterodimer consisting of a HC that is folded up on β2-microglobulin, which is shared between all MHC class I and related molecules. Thus, to be used as cellular detection probe for hFcRn, the selected Nbs must bind specifically to the HC only. To avoid selection of anti-β2-microglobulin clones, selection steps were performed in the presence of human β2-microglobulin in solution. To confirm that the generated Nbs bound specifically to the HC, we also screened for binding towards human β2-microglobulin by ELISA, and conclusively demonstrated that all four Nbs bound specifically to the hFcRn HC (Figure 2C). Furthermore, we confirmed by SPR that the anti-FcRn Nbs bound hFcRn at both pH conditions (Supplemental Fig. S1).

Selected Nbs bind hFcRn independently of the ligands. To investigate whether or not IgG and albumin bound to hFcRn affected binding of the Nbs at acidic pH, we used SPR with immobilized human IgG1 and albumin on CM5 sensor chips and injected recombinant hFcRn alone or in complex with anti-FcRn Nbs at pH 6.0. None of the Nbs interfered with binding of the ligands to hFcRn, as additive binding to immobilized ligands was observed for all four Nbs, while binding in the presence of Nb control was unaffected (Figure 2D-E).

Monomeric and bivalent Nb218-H4. The binding affinity of monomeric Nb218-H4 was determined by SPR by injection of serial dilutions over immobilized hFcRn at pH 6.0 and pH 7.4. The resulting binding curves were either fitted to a steady state affinity binding model (Figure 3A–B) or a simple 1:1 Langmuir binding model (Figure 3C). Both kinetic binding models gave rise to resulting binding curves were either fitted to a steady state affinity binding model (Figure 3A–B) or a simple 1:1 Langmuir binding model (Figure 3C). Both kinetic binding models gave rise to similar estimated KD values of roughly 20 × 10^-7 M at pH 6.0, and a 2-fold weaker affinity at pH 7.4 (Table 1).

To improve apparent affinity through avid binding, we constructed a dimeric form of Nb218-H4. Two units were genetically fused via a glycine-serine linker. The purified (Nb218-H4)2 variant as well as a dimeric negative control Nb were shown to migrate with the expected molecular sizes on a SDS-PAGE gel (Figure 2A). Then,
equimolar amounts of monomeric and dimeric Nb218-H4 were injected over immobilized hFcRn, which resulted in a considerably increased avidity of the dimer as demonstrated by a very slow dissociation phase compared with the monomer (Figure 3D).

Conjugation and visualization of hFcRn. To validate the utility of these Nb as detection probes, we then tested FcRn binding of Nb218-H4 directly conjugated to an enzyme or fluorochrome. First, HRP-conjugation to Nb218-H4 was shown not to disrupt binding to hFcRn, as the conjugate could be used as a sensitive detection probe in ELISA assays for pH-dependent binding of FcRn to IgG and albumin (Figure 4A–B).

Next, we investigated whether Alexa488-conjugated (Nb218-H4)_2 could be used for visualization of cellularly expressed hFcRn. HeLa cells were transfected with plasmids encoding RFP-fused hFcRn and human β2-microglobulin, and the next day, cells were chased with Alexa488-conjugated (Nb218-H4)_2 or a bivalent Nb control. Only Alexa488-(Nb218-H4)_2 was shown to co-localize with hFcRn-RFP in intracellular compartments (Figure 4C). When Alexa647-conjugated human albumin was added in the presence of Alexa488-(Nb218-H4)_2, RFP-hFcRn was shown to localize with both (Nb218-H4)_2 and albumin (Figure 4D).

Furthermore, HeLa cells transfected with a plasmid encoding only the RFP-fused hFcRn HC showed retention of the HC in the endoplasmatic reticulum, which is in agreement with previous findings demonstrating that proper folding of the FcRn HC is completely dependent on co-expression of β2-microglobulin. Importantly, (Nb218-H4)_2 did not co-stain with RFP-hFcRn in cells transfected with the HC only (Figure 4E). Thus, anti-FcRn (Nb218-H4)_2 binds only to folded heterodimeric hFcRn. In addition, we confirmed that Alexa488-conjugated (Nb218-H4)_2 could be used to stain FcRn positive cells by flow cytometry (Supplemental Fig. S2).

FcRn-mediated transcytosis of IgG. Finally, we addressed whether the presence of Nb218-H4 would affect FcRn-mediated transcytosis of human IgG1 across a monolayer of T84, a polarized human epithelial cell line known to express endogenous hFcRn. Cells were seeded in a Transwell system and grown to high electrical resistance (1000–1500 V/cm²). Samples of human IgG1-WT or IgG1-IHH were added to the apical reservoir. At time 0 h and 4 h, medium samples were collected from the basolateral reservoirs. The amounts of IgG transported across the cellular layer were quantified by ELISA. The same assay was performed with WT IgG1 in the presence of a >15-fold excess amount of Nb218-H4 or a control Nb. The numbers given represent the mean of triplicates. n = 3. All data are presented as mean±s.d.
resistance before recombinantly produced human IgG1 antibodies with specificity for the hapten NIP, a WT variant and a triple mutant (I253A/H310A/H435A; IHH) with reduced FcRn binding, were added to the apical reservoirs. Samples were collected from the basolateral side at time 0 and 4 hours post addition to the apical side. The amounts of IgG1 variants transported across the cellular layers were quantified by ELISA, which demonstrated that IgG1-WT was transported more efficiently than the IHH triple mutant (Figure 5). When the same experiment was performed in the presence of a 15-fold excess amount of Nb218-H4, no difference in transcytosis was observed between the WT IgG1 and the IHH triple mutant (Figure 5). Thus, binding of Nb218-H4 to hFcRn does not interfere with transcellular transport of IgG.

Discussion

FcRn fulfills important functions in IgG-mediated immune surveillance and transport of albumin throughout the body. The increasing appreciation of the impact of FcRn in both homeostatic regulation of IgG and FcRn-dependent binding of IgG and albumin to the receptor, which is a prerequisite for the use of such Nbs as FcRn detection probes to examine FcRn expression and distribution without affecting ligand binding and transport. A notable feature of Nb214-H8 is that it only bound to hFcRn, and thus are ideally suited for detection and tracking of hFcRn.

Methods

Production of recombinant hFcRn. Vectors encoding truncated versions of the three ectodomains (N1–N3) of mouse, rat and human FcRn HC. CDNAs genetically fused to the murine/Maackia amurensis S-transferase (GST) have been described. The vectors also contain a cDNA encoding human β2-microglobulin and the Epstein–Barr virus origin of replication (oriP). Similar, truncated forms of FcRn HC CDNAs from monkey and pig were synthesized by Genscript and subcloned into the vector system using the restriction sites Xhol and HindIII. All FcRns variants were produced in adherent human foreskin fibroblasts and the secreted receptor was purified using a GSTrap column as described. An E. coli produced form of refolded hFcRn was produced and purified as previously described.

Immunizations of llamas. Animal experiments were conducted with the approval of the Ethical committee of the Faculty of Veterinary Medicine (University of Ghent, Belgium, EC number is 2006/076, LA number is 1400088). The animal immunization protocol is based upon the guidelines available for Guanaco and Vicuña (llama species) as described in the Ministerial Decree of 05.03.1999 (for zoo animals) and the guidelines for farm animals used as laboratory animals described in Appendix A of the Belgian law. The llama facility was compliant with the basic principles of animal care as specified by the Royal Decree 14/08/1986, Law on the Protection and Welfare of Animals (last amended 19/05/2010). This Decree aims to ensure that all species of animal are protected from harm and neglect and are provided with conditions that meet their welfare needs. One llama was immunized with 3 doses of 50–100 µg E. coli produced hFcRn. Blood was collected 22 days after the last boost. Approximately 1 g of lymph nodes was collected 4 days after boost 6.

Phage library construction. PBLs were purified by a Ficol-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer’s instructions. Next, total RNA was extracted and transcribed into cDNA (Invitrogen superscript III kit). The generated cDNA was used for nested RT-PCR to amplify the Nbs encoding gene fragments as previously described, and cloned into the phagemid pAX50. The size of the library after transformation of E. coli TG1 was determined as 7–8 × 10^6 CFU. 96–100% of the clones contained a correct size insert as evaluated by colony PCR on 24 randomly picked colonies using M13 reverse and a genIII primers. Phages were prepared according to standard methods and stored at 4°C and/or in 20% glycerol at −20°C.

Phage display selections. Refolded hFcRn was used in selections performed at pH 5.1. FcRn (0–10 µg/ml) was immobilized on Maxisorp well microtiter plates (Nunc) in PBS at pH 7.4 overnight at 4°C. After 2 hours blocking with 4% skimmed milk-PBS, the phages were added and incubated at 37°C in 2% skimmed milk-PH 5.1 CPA buffer (10 mM sodium citrate, 10 mM sodium phosphate, 10 mM sodium acetate, 115 mM NaCl, pH 5.1) or in 2% skimmed milk-PBS in the presence of human β2-microglobulin (12–100 µg/ml) (Sigma-Aldrich). After 2 hours, the plates were extensively washed with pH 5.1 CPA buffer – 0.05% Tween20 or with PBS-0.05% Tween20 and bound phages were eluted using trypsin (1 mg/ml) for 15 minutes at 37°C. The eluted phages were amplified in E. coli TG1 and subjected to a second, similar selection. Eluted phages were again rescued in TG1. Individual colonies were picked and grown in 96 well plates. Monoclonal phages were produced by addition of helper phages.

Phage binding ELISA. hFcRn or human β2-microglobulin (0.5 µg/ml) were immobilized on Maxisorp ELISA plates (Nunc) at 4°C over night, followed by 2 hours blocking using 4% skimmed milk-PBS. After washing with pH 5.1 or pH 7.4 CPA buffer, 10 µl of the different phages diluted in 100 µl of 2% skimmed milk-ph 5.1 or pH 7.4 CPA buffer and allowed to bind to the immobilized antigen. After 2 hours incubation and washing with pH 5.1 or pH 7.4 CPA buffer, 100 µl of a horse reddish peroxidase (HRP)-conjugated monoclonal-anti-M13 antibody (GE Healthcare, 1:10000 dilutions) in 1% skimmed milk/ph 5.1 or pH 7.4 CPA buffer was added to the wells for 1 hour. Phage binding was detected by adding 100 µl of 3,3’,5,5’-Tetramethylbenzidine (TMB) substrate (Pierce). The reaction was stopped after 15 minutes and absorbance was measured at 450 nm using a TECAN Sunrise spectrophotometer (TECAN, Männedorf, Switzerland). Binding specificity was determined based on OD values compared to controls wells having received an irrelevant phage clone or no phage.

Generation of bivalent Nanobodies. Bivalent Nb fusions consisting of two copies of the Nb218-H4 or a Nb with irrelevant specificity (Nb203-G8) linked via a 20 amino acid linker (repetitive (GGGSG) sequence) were constructed by separate PCR reactions (one for the N-terminal and one for the C-terminal), using different sets of primers encompassing parts of the linker and restriction sites MfeI in the N-terminal, or BstEII in the C-terminal. The resulting PCR products were digested with the...
correspondent enzymes and sub-cloned into an expression vector containing C-terminal c-Myc and His6 tags.

**Nanobody production and purification.** Exponentially growing *E. coli* cultures (50–1000 ml) were induced with 1 mM isopropyl-D-thiogalactopyranoside for 4 hours at 37°C. Bacterial periplasmic space was extracted by centrifugation, freeze/thaw of the pellet at −20°C and subsequent centrifugation. Nbs purified from the supernatants i) using TALON beads (Clontech Laboratories, Mountain View, CA) according to the manufacturer recommendation, and dialyzing against PBS, or ii) by using His Trap™ FF chromatography columns (GE Healthcare), followed by gel filtration using a HiLoad 16/10 Superdex 75 prep grade column (GE Healthcare). Purity and concentration of the purified Nbs were determined by SDS-PAGE analysis.

**Nanobody HRP conjugation.** Purified Nbs were conjugated to HRP using the EZ-Link Plus Activated Peroxidase kit (Pierce). Conjugates were purified using a Sephadex G-75 column. 0.5 ml fractions were collected and size-separated by SDS-PAGE followed by Coomassie staining. The HRP activity of the fractions was determined by ELISA where Nb was captured in an immobilized anti-c-Myc tag antibody (monoclonal, mlgG1; Serotec) and detection was carried out using TMB substrate.

**Alexa labeling.** Purified Nbs were adjusted to pH 8.3 with 1/10 volume 1 M NaHCO3 and directly labeled with amine-reactive AlexaFluor667 or with AlexaFluor488 (Invitrogen). A 10-fold M excess of dye over protein was used. Labeling reaction was quenched using 1/10 volume 1.5 M Tris-HCl. Desalting was performed on PD MiniTRAP G25 spin columns (GE Healthcare). Human albumin (Sigma-Aldrich) was conjugated to AlexaFluor488 using Alexa Fluor 487 Protein Labeling Kit (Invitrogen). The concentration of the conjugated proteins was estimated both spectrophotometrically (Nanodrop, Thermo Scientific) and by densitometry analyses of scanned Coomassie-stained SDS-PAGE.

**FcRn binding ELISA.** Wells were coated with 100 μl of an anti-Myc antibody (Serotec) at 1.0 μg/ml, and incubated overnight at 4°C. They were then blocked for 1 hour with 4% skimmed milk (Acumedia) and washed four times with PBS/0.005% Tween 20 (PBST)/pH 7.4. Nb candidates (2 μg/ml) in 4% skimmed milk/PBS/T were added to the wells. After incubation for 1 hour, the wells were washed four times with PBS/T pH 7.4. GST-conjugated c-Myc antibody (mIgG1, Sigma-Aldrich) was pre-incubated with a GST-conjugated polyclonal anti-GST antibody from goat (1:5000; GE Healthcare) in 4% skimmed milk/PBS/T pH 7.4 was added to each well and incubated for 1 hour. Following four washes with PBS/T pH 7.4, 100 μl of ABTS/His2 (Sigma-Aldrich) was added and absorbance at 405 nm was measured using a TECAN Sunrise plate reader.

**Flow cytometry.** Human monocytic cell line THP-1 (ATCC) was cultured in RPMI 1640 (BioWhittaker, Belgium) supplemented with 4 mM L-glutamine, 100 U/ml penicillin (Integro, The Netherlands), 0.1 mM nonessential amino acids and 10% foetal calf serum (FCS) (Integro). 1 X 106 cells were washed three times with PBS followed by centrifugation at 800 rpm, re-suspended and added to 96-well plates (Nunc). Cells were fixed with 3% paraformaldehyde (PFA) (Sigma-Aldrich) for 30 min, permeabilized with 0.1% saponin and then blocked with 2% bovine serum albumin (BSA)/PBS (Sigma-Aldrich) for 1 hour. Following three washes with PBS/BSA, Alexa488 or Alexa647 conjugated (Nb218-H4) or a bivalent control Nb (1 μg/ml) diluted in PBS/BSA were added. Following 1 hour staining at 4°C, cells were again washed as described above and analyzed by flow cytometry.

**Immunofluorescence and confocal laser scanning microscopy.** Hela cells were grown on coverslips to 70% confluence in complete DMEM and transfected with vectors encoding hFcRn-RFP and human β2-microglobulin or hFcRn-RFP alone, using lipofectamine 2020 transfection reagent according to the recommendations from the manufacturer (Roche). One day post-transfection, cells were incubated with 2 μg/ml fluorescently conjugated (Nb218-H4) (Alexa 488), an Nb control (Alexa 488), or human albumin (Alexa 647, all fluorochromes from Invitrogen) for 1 hour. Cells were then washed in PBS, fixed in 3% PFA and mounted onto object-glasses with Mowiol (Calbiochem). Confocal images were acquired on an Olympus Fluoview 1000 microscope equipped with a PlanApo 0.11/1.0 objective (Olympus, Hamburg, Germany). Fluorochromes were exited with 488 nm Argon, 543 nm and 647 nm HeNe lasers. All image acquisition was done by sequential line scanning to eliminate bleed-through. Images were processed with ImageJ (NIH, USA) and assembled in Adobe Illustrator (Adobe systems Inc., CA, USA).

**Transepithelial IgG transport.** The human T84 cell line (ATCC) was maintained in DMEM and HAMS F-12 medium (1:1) supplied with 10% FCS (Intebro b.v.), 2.5 mM L-glutamine, 25 μg/ml streptomycin and 25 μM penicillin (all from Bio-Whittaker). 3 X 105 cells were seeded on Transwell filters with 0.4 μm pore size (Corning, NY, MA, USA) and grown for 48 h at 37°C. Culture medium was replaced every 24 h. For binding experiments, samples were collected from the basolateral reservoirs, and the amounts of IgG present were quantified by ELISA. The same assay was performed in the presence of 1 μM of the Nb218-H4 or an Nb with irrelevant specificity, both added to the apical side 20 minutes before the IgG antibodies were provided.

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Additional information

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