Human VH single domains that rival camelid antibodies

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Background: Camelid antibody domains are naturally stable and capable of cleft binding.
Results: Protein engineering can endow such properties onto human antibody domains.
Conclusion: Our strategy does not require undesirable antibody framework changes.
Significance: Robust building blocks for human therapeutic applications.

ABSTRACT

Human VH single domains represent a promising class of antibody fragments with applications as therapeutic modalities. Unfortunately, isolated human VH domains also generally display poor biophysical properties and a propensity to aggregate. This has encouraged the development of non-human antibody domains as alternative means of antigen recognition and, in particular, camelid (VH) domains. Naturally devoid of light chain partners, these domains are characterized by favourable biophysical properties and propensity for cleft binding, a highly desirable characteristic, allowing the targeting of cryptic epitopes. In contrast, previously reported structures of human VH single domains had failed to recapitulate this property. Here we report the engineering and characterization of phage display libraries of stable human VH domains, and the selection of binders against a diverse set of antigens. Unlike ‘camelized’ human domains, the domains do not rely on potentially immunogenic framework mutations and maintain the structure of the VH/VL interface. Structure determination in complex with hen egg-white lysozyme revealed an extended VH binding interface, with complementarity determining region 3 (CDR3) deeply penetrating into the active site cleft, highly reminiscent to what has been observed for cameld domains. Taken together, our results demonstrate that fully human VH domains can be constructed that are not only stable and well-expressed, but also rival the cleft binding properties of camelid antibodies.

Human antibodies are exclusively expressed as paired species, containing both heavy and light chains. In marked contrast to humans, llamas and camels have been shown to also produce non-paired species (1,2). Antigen binding of these heavy chain-only antibodies resides exclusively within a single variable domain (VH) domain (3). Since their discovery in the mid-1990s (1), camelid VH domains have attracted considerable interest and investment, due to their potential as building blocks for therapeutic and biotechnology...
applications (4). The domains are biophysically and structurally well characterized, and have been shown to possess high thermodynamic and colloidal stabilities, together with a preference for binding within pockets of protein structure (high cleft binding propensity) (5,6). These features are achieved by means of an extended CDR3 loop, which has been shown to be capable of protruding deeply into enzyme active sites and otherwise cryptic epitopes of viruses and G-protein coupled receptors (7-9). Residues in the light chain interface differ between in human domains and VHH domains, resulting in a hydrophilic nature of the camelid surface (2). In addition, the extended CDR3 loop of camelid domains is capable of looping backwards, further shielding the V\textsubscript{H}/V\textsubscript{L} interface area through interactions with framework residues (5). However, this feature has also been shown to restrict camelid CDR3 diversity, which is highlighted by randomization studies revealing a strong consensus towards naturally occurring hydrophobic sequence motifs (10).

The favourable properties of camelid domains have inspired attempts to generate binders based on isolated human V\textsubscript{H} domains, although initially with limited success. The main problem holding back the field has not been thermodynamic stability, but rather colloidal stability (aggregation propensity) (11,12). While thermodynamic stability can be achieved by the use of stable human V\textsubscript{H} families (such as VH3) (13), colloidal stability remains difficult to control (14). It results in overall poor biophysical properties, as indicated by low solubility, expression and purification yields, and a lack of heat-refoldability (11,15).

‘Camelization’ strategies, as pioneered by Riechmann and co-workers, have been developed to improve the biophysical properties of human domains (16). These strategies have focused on a set of framework residues, which are hydrophobic in humans, but are largely hydrophilic in camelid domains (V\textsubscript{H} tetrad; positions 37, 44, 45 and 47 (2); all numbering according to Kabat (17)). However, initial attempts at directly transplanting such residues into a human framework were met with mixed success, with the resulting domains suffering from limited stabilities and structural deformations in NMR experiments (18). Structural changes have also been observed upon engineering of human domains through non-native disulfide links (19). Recent camelization attempts have therefore focused on other interface positions (such as 39) and on the introduction of novel substitutions at tetrad positions. A study of human single domains derived from the antibody therapeutic Trastuzumab (Herceptin 4D5) (20) revealed several such novel stabilizing framework mutations (10). The crystal structure of a soluble 4D5 quadruple mutant was reported in the same study (H35G, Q39E, L45E and R50S - clone B1a). Using an elegant display approach on phage (retention of superantigen binding) the authors also demonstrated compatibility of these mutations with CDR3 diversity, suggesting that domains stabilized in this manner may be capable of antigen binding. Indeed, the same group recently reported the structure of a V\textsubscript{H} 4D5 triple mutant in complex with vascular epithelial growth factor (VEGF - V\textsubscript{H} V1a complex) (21).

While such prior camelization approaches have undoubtedly resulted in improvements of biophysical properties, these engineered human domains nevertheless fall short of what would be considered ideal for many applications, and human therapy in particular. For instance, changes of multiple conserved framework residues may well result in the generation of novel B- and T-cell epitopes and an increase of immunogenicity in humans (22). In addition, the introduction of mutations into the V\textsubscript{H}/V\textsubscript{L} interface prevents pairing with light chain, which restricts the developability of the domains and limits their use in antibody bispecifics (23). Previously reported structures of human V\textsubscript{H} single domain in complex with antigen have also largely failed to recapitulate the cleft binding properties of camelid domains (21,24). To overcome these limitations we decided to bypass the mutation of human framework residues altogether, instead exclusively focussing on the engineering of CDR regions. Here we investigate strategies for library design, and report biophysical and structural properties of fully human antibody V\textsubscript{H} domains.

**EXPERIMENTAL PROCEDURES**

Construction of synthetic human V\textsubscript{H} antibody repertoires - Synthetic libraries were constructed essentially as previously as described (25). For the generation of the Garvan I repertoire, single-
stranded DNA encoding the model V\textsubscript{H} domain HEL4 in the phage vector FdMyc was isolated using a QIAPrep spin M13 kit (Qiagen). Randomization of CDR regions was carried out by combinatorial mutagenesis using partially degenerate oligonucleotides (Table I). For the generation of the Garvan II repertoire, single-stranded DNA encoding DP47 germline (V\textsubscript{3}-23/DP-47 V-segment and J\textsubscript{H}4b J-segment derived from the immunoglobulin heavy chain locus of chromosome 14) in the phagemid vector pHEN1 was isolated using a QIAPrep spin M13 kit (Qiagen). Randomization of CDR1 and CDR2 regions was carried out by Kunkel mutagenesis using reverse-complemented degenerated oligonucleotides. For CDR3 regions of the Garvan II library TRIM codon mutagenesis was utilized and diversity was introduced by splice-overlap extension PCR. After the mutagenesis steps, repertoires were transformed into \textit{E. coli} TG1 bacteria and validated by DNA Sanger sequencing of random clones (>100 clones analyzed). Phages were purified by two precipitations with PEG/NaCl either directly from the culture supernatant in the case of FdMyc, or after a rescue step using KM13 helper phage.

**Phage display selection** - Phage display selections were carried essentially out as described (26). In brief, 100 nM of biotinylated antigen was utilized in rounds 1 and 2, 10 nM in round 3 and 1 nM in round 4, which were immobilized on a neutravidin coated Maxisorp Immunoplate (Nunc) (rounds 1 and 3) or on streptavidin-coated magnetic beads (Dynal – rounds 2 and 4). Plates and beads were blocked with 5% milk powder (w/v) in PBS supplemented with PEG/NaCl either directly from the culture supernatant in the case of FdMyc, or after a rescue step using KM13 helper phage in the case of pHEN1.

**Enzyme-linked immunosorbent assay (ELISA)** - For phage ELISA, a Maxisorp Immunoplate was coated with antigens and blocked with 5% milk powder (w/v) in PBS buffer. Phage supernatant was blocked with MPBST for 30 min and added to wells of the ELISA plate for 1 h. After washes with PBST, bound phage were detected using HRP conjugated anti-M13 antibody (GE Healthcare) and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Becton Dickinson). For soluble ELISA, a Maxisorp Immunoplate was coated, blocked and washed as above. Bound domains were detected using HRP conjugated anti-c-myc antibody (ICL Lab) and TMB substrate.

**Heat refolding on phage** - Aggregation resistance was analyzed by measuring retention of signal after heating in a phage ELISA format utilizing vector FdMyc as described (the pHEN1-based Garvan II library was re-cloned for this purpose) (27). A Maxisorp Immunoplate was coated with \textit{Staphylococcus aureus} protein A (Sigma), and blocked with 5% milk powder (w/v) in PBS buffer. Single colonies were picked from agar plates and grown overnight at 30°C in 2xTY medium supplemented with 15 µg/ml tetracycline. Supernatant was cleared by centrifugation and phages were biotinylated by addition of NHS-PEG\textsubscript{4}-biotin (Pierce) to a final concentration of 50 µM for 2 h. The reaction was then quenched by addition of Tris pH 7.5 to a final concentration of 100 mM and incubated for 1 h. For heat-induced refolding, biotinylated phage supernatant was incubated at 80°C for 10 minutes, followed by incubation at 4°C for 10 minutes. Supernatant was added to wells of the blocked ELISA plate and incubated with the antigen for 1 h. After washes with PBST, bound phage were eluted by addition of Tris pH 7.4, supplemented with 137 mM NaCl and 1 mM CaCl\textsubscript{2} for 1 h. Eluted phages were used to infect mid-log phase \textit{E. coli} TG1 bacteria and plated onto TYE-agar plates supplemented with 4% glucose and 100 µg/ml of ampicillin (pHEN1) or 15 µg/ml of tetracycline (FdMyc).
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Healthcare) using an AKTA Purifier (GE Healthcare) chromatography system and PBS running buffer. Recovery of each variant was determined by measuring the area under the curve after heating, expressed as a percentage of the unheated protein sample.

Protein expression and purification - Expression and purification of human VH domains was performed essentially as described (28). In brief, genes encoding VH domains were recloned from phage or phagemid vectors into the expression vector pET12a (Novagen) and transformed into *E. coli* BL21-Gold (Stratagene). Transformants were used to inoculate 2xTY medium supplemented with 4% glucose, 100 µg/ml ampicillin, 15 µg/ml tetracycline at an OD of 0.05 in baffled flasks, and grown in a shaking incubator at 37°C. At an OD of 0.5, bacteria were pelleted by centrifugation at 3200 x g, resuspended in 2xTY medium supplemented with 1 mM IPTG, 100 µg/ml ampicillin, 15 µg/ml tetracycline and incubated for 36-48 h at 30°C (with a second induction with 1 mM IPTG after 24 h). Supernatant was cleared by centrifugation at 10 000 x g, passed through a 0.45 µm filtration unit and pH adjusted to 7.0 – 8.0 by addition of 1 M HCl. Protein A resin (GE Healthcare) was applied to a gravity flow column (BioRad), and washed successively with 5 column volumes of mQ-H2O and PBS. Supernatant was applied to the column by gravity flow and washed with 1 column volume of PBS. Protein was eluted with 100 mM glycine pH 2.7 and the eluted fractions neutralized with 100 mM Tris pH 8.8. Finally, the fractions were dialysed extensively against PBS and the purified domains concentrated using Amicon-Ultra 10kDa concentration devices (Millipore).

Surface plasmon resonance - Binding kinetics were determined by surface plasmon resonance (SPR) using a Biacore 2000 instrument (GE Healthcare). Biotinylated antigen was immobilized on a streptavidin chip (GE Healthcare) and serial dilutions of purified VH domains in PBS were injected. Association and dissociation curves were fitted using a 1:1 Langmuir binding model and analyzed using BIAevaluation software (GE Healthcare).

Circular dichroism - Thermal unfolding was measured by circular dichroism (CD) using a J-815 spectrometer (Jasco) in a quartz cuvette (2 mm path length). Protein samples at a final concentration of 20 µM in PBS and melting curves were obtained by recording CD signal at 235 nm with a 1 nm bandwidth and 1 s integration time while heating the solutions from 20°C to 80°C at 1°C/min. To measure the reversibility of unfolding, signal was measured during cooling from 80°C to 20°C at 1°C/min.

Crystal growth, structure solution, refinement and analysis – The VH H04-HEL complex was purified by size exclusion chromatography using 50 mM Tris-HCl pH 7.5 supplemented with 150 mM NaCl as running buffer (as described above). Peak fractions were collected and concentrated using Amicon-Ultra 10kDa concentration devices (Millipore). Initial crystallization hits were obtained with the JCSG-plus crystal screen (Molecular Dimensions) using a Mosquito crystallization robot (TTP Labtech) and 96-well MRC2 sitting drop crystallization plates (Swissci). Crystals were grown at room temperature in a hanging-drop format where 2 µL of VH H04-HEL complex (4.37 mg/mL, in 50 mM Tris - HCl pH 7.5 supplemented with 150 mM NaCl) was combined with an equivalent volume of well solution (100 mM sodium citrate pH 5.4 supplemented with 28% (w/v) PEG1500 for PDB ID: 4PGJ; 300 mM sodium citrate pH 5.5 supplemented with 16% (w/v) PEG3350 for PDB ID: 4U3X). Crystals were snap frozen in liquid nitrogen (100 K) and diffraction data were recorded on the beamline MX2 at the Australian Synchrotron. Reflections were indexed and integrated with iMOSFLM (29), scrutinized for symmetry with POINTLESS (30), scaled with SCALA (30), and imported into the CCP4i software package (31). Reflection data statistics are shown in Table S2. Structures were solved by molecular replacement using PHASER (32). The search models for the VH and HEL components were PDB entry 1OHQ (VH) and PDB entry 1ZVY (HEL), both stripped of side-chain moieties. Two essentially identical VH-HEL complexes were found in the asymmetric unit, where H04 domains are chained A and C, and corresponding HEL molecules are chained B and D. During refinement the two complexes were not averaged using non-crystallographic symmetry but...
were treated independently of each other. Initial rounds of rigid body refinement were followed by B-factor restrained refinement using REFMAC5 (33) and employed translation-libration-screw (TLS) parameterization (34). During later stages of refinement water molecules were modeled into appropriate features of difference density. Models were compared with maps and manipulated in real space using COOT (35). Models were scrutinized using the MOLPROBITY validation server (36). Buried surfaces were calculated with PDBePISA (37). Coordinates for VH H04-HEL structure have been deposited in the PDB as entry 4PGJ and 4U3X respectively.

RESULTS
Generation of the Garvan I human VH phage repertoire - As a starting point for the design of a first generation repertoire of stable VH domains we utilized the HEL4 model protein. This human VH domain had been identified by selection on phage and its structure had been determined by crystallography (27,38). It is characterized by low aggregation propensity (high colloidal stability), heat-refoldability and excellent expression and purification yields (27,39). HEL4 differs from the DP47 germline domain it had been derived from at over twenty CDR and framework positions (Figure 1A), and the specific role of individual changes had largely remained unclear (27,38). We initially focused our attention onto the complementarity determining region 3 (CDR3) (Figure 1B – in blue), which provides the majority of binding energy in antibody/antigen interactions. Using site directed mutagenesis we introduced diversity at residues 95 to 100a/c, predominantly utilizing DVK codons to introduce hydrophilic amino acids into CDR3 (Table 1). The use of such codons has previously been successfully utilized in the generation of paired antibody fragment libraries (40-42). Transformation into E. coli yielded a library of 3 x 10^9 individual clones.

Characterisation of the Garvan I phage repertoire - For initial characterisation, resistance to heat-denaturation was determined using a phage method originally developed by Jespers et al (27). The method is based on the multivalent display of human antibody domains on phage, coupled with heating to 80°C and cooling of the phage particle. Resistant domains can be captured by the conformation dependent superantigen protein A, which binds to folded but not to unfolded or aggregated VH domains (27,43). The phage method is an excellent predictor of solution properties of human antibody VH domains, including heat-refoldability, expression and purification yields (44). Human antibody variable domains, such as DP47 germline, aggregate under the conditions of the phage method, however this is not observed for the HEL4 domain, which resists heat-induced aggregation (Figure 1C).

Initial CDR grafting studies carried out by our group (Figure 1C) (45) and more recently by others (46) had indicated the aggregation determinants of HEL4 largely reside within the CDR1 region. As this region had not been targeted in our randomization strategy (which focused on CDR3 instead), we speculated that the Garvan I library may retain the aggregation resistant properties of the HEL4 domain. Further analyses of randomly selected clones from the naïve Garvan-I repertoire revealed that this was indeed the case, and that the majority of clones displayed considerable resistance against heat-induced aggregation on phage (Figure 1D), with a median level of resistance of 64% compared to 88% for HEL4 and 2% for DP47 germline.

Phage-display selection using the Garvan I phage repertoire - We next decided to assess the potential of the library for the selection of antigen specific VH domains. For this purpose a set of representative antigens was chosen, covering a wide range of molecular weights and structural classes (human tumour necrosis factor alpha, murine interleukin 21, human prolactin receptor, beta-galactosidase) (Figure 1E). The antigens were immobilized on solid supports and single domains selected by phage-display. After three rounds of selection, phage ELISA confirmed that binders had been obtained for all antigens, with 22-57% of all clones displaying antigen binding. After re-cloning into the pET12a expression vector, monoclonal ELISA was performed on soluble protein, and non-redundant binders identified by DNA sequencing. This revealed that multiple unique VH domains had been selected for each target antigen.
Characterisation of a stable human anti-TNFα single domain – For further analyses, clone G07 was expressed in large scale and its biophysical properties and affinity were characterized. Thermal denaturation as monitored by circular dichroism revealed that the domain was fully heat-refoldable (Figure 2A, \( T_m = 63^\circ C \)), similar to what has been observed for HEL4 and camelid domains (but not for other human V\_H domains). Moreover, the domain was also well-expressed in bacteria (at around 5 mg/litre in shaking flasks, compared to 3.3 mg/litre for HEL4 and 0.9 mg/litre for DP47). Affinity measurements by SPR Biacore revealed moderate affinity interactions with the human tumour necrosis factor alpha (TNFα) antigen against which the domain had been selected (\( k_a = 4.8 \times 10^3 \text{ s}^{-1} \text{M}^{-1}, k_d = 8.9 \times 10^{-3} \text{ s}^{-1} \), \( K_D = 1.9 \mu \text{M} \)) (Figure 2B).

Generation of the Garvan II human V\_H phagemid repertoire – We next investigated strategies to further increase the affinity of the human domains. The first generation library had been constructed in a multivalent phage vector, thereby streamlining analysis of aggregation resistance using the heat/cool method described by Jespers et al. However, it is also evident that multivalent display can promote the enrichment of low affinity clones (through increased avidity, resulting in multiple interactions with antigen immobilized on a solid support) (47). We therefore decided to utilize a phagemid system for the construction of a second-generation library, allowing for monovalent display on the phage surface. In addition to changes to the display system, we also considerably increased the number of CDR positions diversified in the library (Figure 3A), while avoiding positions important for biophysical properties.

Recent work in our laboratory had demonstrated that the introduction of negatively charged amino acids at CDR1 positions significantly improved the colloidal stability of human V\_H domains (aspartate or glutamate at 28, 30, 31, 32, 33 and 35) (48). This is in agreement with the observations for the HEL4 model domain, which carries a negatively charged triad within this region (31-33DED) (Figure 3A). Importantly, introduction of multiple negatively charged amino acids at CDR1 positions is required (two or more) (48). This can be readily observed upon step-wise introduction of mutations into DP47, resulting in considerable increases of heat-refoldability (Figure 3B). In addition to reversible unfolding the introduction of mutations at the above CDR1 positions also significantly increases expression and purification yields (44,48), mimicking important and highly desirable characteristic of camelid domains. We also retained two other CDR mutations (28R, 35G) within the libraries that are present in the HEL4 model domain (Figure 3A). These have been reported to result in modest improvement of V\_H properties (10,27,38,49). In contrast to HEL4 and previously reported camelized domains all of the mutations utilized here are located at CDR positions, fully retaining the human DP47 framework sequence (Figure 3A).

More specifically, negatively charged amino acids were introduced at CDR positions 32 and 33, while extensively randomizing CDR1, CDR2 and CDR3 positions to obtain a maximum degree of repertoire diversity (Figure 3A/C – Table I). Based on our experience with the Garvan I library, we predominantly utilized codons encoding hydrophilic amino acids at CDR1 and CDR2 positions (Table I). For the diversification of CDR3 we utilized tri-phosphoramidite codon mutagenesis (TRIM) (50). This enabled us to encode a high proportion of amino acids commonly observed in the human repertoire (in particular Tyr, Gly, Ala and Ser) with other amino acids at lower defined proportions (Table I) (51). A library of \( 3 \times 10^9 \) individual clones was obtained after transformation into E. coli bacteria (Garvan II library). From the naïve library clones were randomly selected and analysed using the phage heat/cool method (27). This revealed that a large proportion of the library displayed resistance against heat-induced aggregation (with a median resistance of 40%), despite the randomization of up to sixteen positions within three CDR regions (Figure 3D).

Phage-display selections using the Garvan II phagemid repertoire – Antigen specific binders were next selected from the second generation repertoire. As described for the Garvan I library, antigens were immobilized on solid supports, and after three to four rounds of sections binders were
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identified by phagemid ELISA, and verified by soluble ELISA. After selection, a large proportion of clones (14% - 58% depending on the target antigen) displayed antigen binding, with DNA sequencing revealing non-redundant sets of I-6 (unique) clones per target (Figure 3E). Two of the antigens (VEGF and CD25) represent validated therapeutic targets and had been included in the selections for this reason. Biosensor measurements of representative VH domains selected against these targets revealed high affinity interactions with equilibrium binding constants in the mid nanomolar range (VEGF: \(k_a = 5.7 \times 10^4 \text{ s}^{-1}\text{M}^{-1}, k_d = 2.3 \times 10^{-2} \text{ s}^{-1}\), \(K_D = 407 \text{ nM}\) (CD25: \(k_a = 6.6 \times 10^4 \text{ s}^{-1}\text{M}^{-1}, k_d = 1.4 \times 10^{-2} \text{ s}^{-1}\), \(K_D = 208 \text{ nM}\)).

Characterisation of Garvan II VH single domains and epitope binning – In addition to therapeutic targets we had also included the common model antigen hen egg white lysozyme (HEL) in the selections, to streamline comparisons with previously reported structures of camelid VH complexes (52). The selected anti-HEL VH single domains were cloned into the pET12a expression vector, expressed in large scale and further characterized (clones H04, G08, D05, C01 and F05). Surface plasmon resonance was used to determine binding kinetics, revealing equilibrium binding down to the low nanomolar range (Figure 4A/B - 26 to 257 nM). In addition to high affinity antigen binding, all of the selected clones also displayed high levels of resistance against heat-induced aggregation (Figure 4B).

To gain further insight into the epitope distribution of the selected clones, we performed competition assays against three previously reported anti-HEL camelid domains (clones D3L11, D2L19 and D2L24) (52). D3L11 and D2L19 bind to the active site cleft of the HEL antigen (Figure 4C - asterisk). This represents the dominant mode of binding of camelid domains to this antigen, which is well characterized through previously reported co-crystal structures (5,52). In contrast, D2L24 binds to a planar epitope on the back of the molecule, distant from the active site cleft (Figure 4C) (52). Epitope binning experiments were carried out using surface plasmon resonance and one of the human VH single domains selected here (clone H04 characterized by a high affinity and stable SPR baselines). These experiments revealed that H04 competed for binding with D3L11 and D2L19, but not with D2L24 (Figure 4D). Moreover, H04 also competed for binding to antigen with G08, D05, C01 and F05 (Figure 4E). This strongly indicated that all of the human domains selected in this study bind to epitopes within or proximal to the lysozyme active site cleft.

Crystal structure of a human VH domain in complex with hen-egg white lysozyme - To gain further insights into the interaction of VH H04 with HEL antigen, molecular details were investigated by X-ray crystallography. The VH-antigen complex was isolated by size exclusion chromatography and crystals were obtained by hanging drop vapour diffusion under two distinct conditions (see experimental procedures for details). Diffraction data was collected at the Australian Synchrotron, structures solved by molecular replacement, and refined to 2.60 Å and 2.26 Å resolution (Table II). The structures obtained from both datasets displayed two complexes within the asymmetric unit and overall high levels of structural similarity (with root-mean-square-deviations (RMSD) of 0.38-0.52 Å for 231-246 Cα atoms). Further investigations therefore focused on the 2.26 Å resolution dataset.

Nature of the single domain-antigen interaction and comparative analyses - Initial structural analyses revealed a 1:1 stoichiometry and binding of a single VH H04 domain to a single HEL antigen (Figure 5A). The interaction is centred on the CDR3 region of VH H04, which protrudes deeply into the lysozyme active site cleft (Figure 5B), burying extensive surface area (Table III: 535 Å²). Notably, this interaction surface is achieved within the context of a CDR3 loop of nine residues, considerably shorter than what had been observed for camelid VH and shark VNAR domains raised against HEL antigen (12-18 residues; Table III) (52-54). More specifically, an extensive network of hydrogen bonds is observed between CDR3 residues (Y95, S97, P99, Q100, N100a, H100b) and the HEL cleft surface (Figure 5B). Intriguingly, two prolines (at positions 98 and 99) are observed in the centre of the loop. The presence of sequential prolines in CDR3 is unusual, and results in a distinct bend of backbone orientation, projecting key contact residues...
towards the HEL active site. Complementing CDR3 interactions, minor contacts from CDR1 and CDR2 are observed in the H04-HEL complex (buried surface areas of 20 Å² and 60 Å², respectively). Notably, an aspartate residue at CDR1 position 33, which is a determinant of aggregation resistance (48), participates in a salt bridge with Arg61 of the antigen (Figure 5B). Outside the CDR regions of V_H H04, an additional 168 Å² of single domain surface is buried by the HEL surface. These interactions involve a number of mainly hydrophobic residues (Val37, Leu45 and Trp47) within the light chain interface of the domain, packing against the surface of the HEL antigen (Figure 5C).

In total, a surface area of 783 Å² is buried by V_H H04 in the lysozyme complex, which is comparable to that of a shark V_NAR single domain (755 Å²), but higher than what is observed for a set of camelid domains raised against this antigen (561 – 645 Å²) (Table III). All of the analysed structures display a large degree of shape complementarity between single domains and the HEL antigen (H04: 0.81, V_NAR: 0.77, Camelids: 0.76-0.80). In the majority (5/6) of complexes this high level of complementarity is achieved by means of an extended CDR3 interacting with the active site cleft of the lysozyme antigen. In contrast to H04, shark and camelid domains, previously reported structures of human single domains (V_H9 (24) and V1a (21)) display lower levels of shape complementarity with antigen (0.69 and 0.70). This is reflected by the essentially planar epitope surfaces of their respective antigens (MDM4 and VEGF) (Figure 6). Although both domains bind to antigen predominantly through CDR3 contacts, the loop is not inserted into structural clefts, but rather packs against a hydrophobic helix surface (in the case of V_H9-MDM4) (24), or interfaces with an outer beta strand (in the case of V1a-VEGF) (21).

Structural features of CDR3 and the V_H/V_L interface - In addition to contacts with antigen, the structure of the human H04 single domain also revealed important structural roles of CDR3 residues. As outlined above, a hallmark of camelid domains involves the presence of a hydrophobic pocket formed by CDR3 packing against hydrophobic chains of the V_H framework (5,52,55-57) (as observed in the structure of the D2L24 anti-lysozyme V_H domain - Figure 7A). Although these framework residues are not strictly conserved between human and camelid V_H domains, an analogous core is nonetheless observed in the V_H H04 structure with a tyrosine residue at CDR position 95 packing against a hydrophobic framework core formed by residues at positions 35, 37, 93 and 100d (Figure 7B). Tyrosines are common at Garvan II CDR3 positions and within the human repertoire rendering it likely that similar structural features could be observed in other Garvan II domains. (Table I)(51). This finding is in contrast to the previously reported structure the camelized B1a domain in which the interaction is mediated through a rare CDR tryptophan residue (Figure 7C) (10). Taken together, the formation of a hydrophobic core at the base of H04 CDR3, and the restriction of backbone dihedrals through proline residues, result in the stabilization of CDR conformations, allowing antigen binding through extensive cleft interactions.

The observed hydrophobic core is partially formed by a residue at CDR1 position 35, which is occupied by glycine in H04 and conserved in the Garvan II library. This amino acid is also observed in D2L24 (Figure 7A), B1a (Figure 7C), as well as in HEL4, where results in moderate improvements of gel filtration profiles (but not heat-refoldability) (27,38). This is in agreement with the findings Barthelmy et al. who observed an enrichment of glycine at position 35 when selecting for protein A superantigen binding as a (gentle) proxy of biophysical properties (no heat selection was utilized) (10). In the HEL4 structure, the presence of Gly35 results in a marked conformational shift of Trp47 within the V_H/V_L interface (Figure 5D; in red) resulting in an increase of surface hydrophilicity (38). However, such changes are not observed in the V_H H04 structure reported here with the conformation of Trp47 closely aligned with that observed in representative human Fab antibody structures (Figure 5D). This suggests that the main role of G35 may not be the rearrangement of Trp47 conformations (38), but rather the formation of a hydrophobic pocket centred on positions 35, 37, 93 and 100x which can act as an acceptor for hydrophobic CDR3.
residues (as seen in the H04, B1a and D2L24 structures – Figures 7A, 7B and 7C).

In addition to Trp47 the conformation of the remainder of the H04 VH/VL interface (Figure 8A), formed by residues at positions 37, 44 and 45, is also highly similar to that of native (light chain paired) human antibodies (Figure 8B – PDB identifier 3QOS). In contrast, the H04 interface is noticeably different to that of camelized human domains (Figure 8C) and camelid VH domains (Figure 8D).

**DISCUSSION**

Our study of human VH single domains demonstrates that, unlike what has been reported for camelized human domains, improvements of biophysical properties do not necessarily rely on mutational changes of framework residues. Rather, we demonstrate that CDR composition alone can endow favourable properties onto human single domains. Importantly, we demonstrate that these properties are largely independent of CDR3 diversity, allowing the randomization of this important antigen-binding region. This enabled us to generate a stable first-generation library (capable of refolding after heating to 80°C) and to select antigen binders against a diverse set of antigens.

In a second step, we extensively randomized additional CDR1 and CDR2 positions, while maintaining key determinants of biophysical properties (two or more negatively charged amino acids at Kabat positions 28, 30, 31, 32, 33, 35). This second-generation VH library retained a high proportion of stable VH domains. While we had observed the selection of predominantly low-medium affinity clones for the Garvan I library, affinities in the nanomolar range were observed for the second-generation Garvan II repertoire, comparable to what has been observed for camelid and shark domains (Table III). Moreover, the selected human single domains also displayed structural modes (cleft-binding) and shape complementarities that were highly reminiscent of shark and camelid domains. This is in marked contrast to previously reported structures of human and ‘camelized’ domains, which are characterized the absence of cleft interactions and lower shape complementarities.

In addition to the ‘camelization’ of human VH, strategies for the humanization of camelid single domains have also become available in recent years. For instance, in a detailed study Vinke et al. have reported the generation of a partially humanised domain (h-NbBCI110fGLA) suitable as an acceptor framework for CDR grafting approaches (58). However, the study also highlighted limitations of this strategy, namely the loss of heat-refoldability upon humanization, and the requirement to maintain multiple camelid residues within the framework 2 region (58).

Taken together, our results demonstrate that libraries of human VH single domains can be constructed that rival the stability and cleft binding properties of camelid domains. The availability of fully human domains will minimize potential immunogenicity risks, by removing the necessity to change large conserved antibody framework residues. While the effects of framework changes on the immunogenicity of human antibody therapeutics are unknown, changes to CDR regions are well studied, with the majority of antibodies in clinical practice having been generated through CDR-grafting or CDR affinity maturation approaches (59,60). It is becoming increasingly evident that such changes have little or no detectable effect on immunogenicity in patients (22), further strengthening the rationale for the CDR-only approach outlined here. Moreover, by maintaining the structure of the human VH/VL interface the domains developed in this study retain the potential of light chain pairing; this may allow the modular assembly of bispecifics through native chain interactions. We conclude that the availability of fully human VH domains has the potential to open up new and exciting opportunities for the development of human therapeutics.
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FOOTNOTES

Atomic coordinates (codes 4PGJ and 4U3X) have been deposited in the Protein Data Bank
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### Table I. Oligonucleotides used for library construction

|        | Oligonucleotides used for library construction |
|--------|------------------------------------------------|
| **Garvan I** |                                              |
| CDR3   | 5'-GTATATTATTGCGCAGGTCTDVKVVKDVKDVKDVKCTGCGCTCTGGGCTTTTTGGGTCAGGGAAC-3' |
|        | 5'-GTATATTATTGCGCAGGNNKDVKDVKDVKDVKDVKNNNGCTTTTTGGGTCAGGGAAC-3' |
| **Garvan II** |                                              |
| CDR1   | 5'-CTCTCCCTGCGAGCCCTCAGGATATTGTTTKMTKMTGAGGATAGGGCTGCTG-3' |
| CDR2   | 5'-GAAGGGTCAGAGTGGGTATCAKMTATTKMTTRTSMTGGTKMTAGCAGCATACGAGCAAC-3' |
| CDR3   | 5'-CACCGCGGTTATTATGCGCCGAAXXXXXXXXXXXTTTGACTACTGGGGTCAGGGAACCTG-3' |
|        | 5'-CACCGCGGTTATTATGCGCCGAAAXXXXXXXXXXXTTTGACTACTGGGGTCAGGGAACCTG-3' |

* Diversified positions in bold. Encoded amino acids (codons):

- **DVK**: 16.7% Ser, 11.1% Ala, Gly, Thr, 5.6% Arg, Asn, Asp, Cys, Glu, Lys, Ser, Trp and Tyr
- **KMT**: 25% A, D, S and Y
- **RRT**: 25% D, G, N and S
- **SMT**: 25% A, D, H and P
- **Xxx**: 19.7% Y(TAC), 16.7% G(GGT), 15.2% S(TCT), 6.6% A(GCT), 6.6% D(GAC), 3.9% I(ATT), L(CTG), P(CCG), R(CGT), T(ATT), V(GTT), 1.6% e(GAA), F(TTC), H(CAT), K(AAA), M(ATG), N(AAC), Q(CAG) and W(TGG); all TRIM codons

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TABLE II. V₇ H04 diffraction data, structure refinement and structural features

| Diffraction Data | PDB entry | Diffraction Data | PDB entry |
|------------------|-----------|------------------|-----------|
| Spacegroup       | 4PGJ      | 4U3X             |           |
| Unit cell dimensions: a,b,c (Å); β (°) | 100.1, 40.3, 137.0; 101.2 | 68.1, 39.2, 100.2; 104.2 |
| Wavelength (Å)   | 0.9537    | 0.9537           |           |
| Resolution range (Å) | 49.08 - 2.60 | 97.14 - 2.26 |
| Observed reflections* | 117868 | 162966           |           |
| Unique reflections5 | 16831 | 24456           |           |
| Completeness5* (%) | 99.3 (99.0) | 98.2 (95.5) |
| Multiplicity5* | 7.0 (7.1) | 6.0 (5.8) |           |
| R<sub>merge</sub>5* | 0.152 (0.951) | 0.188 (0.910) |
| Wilson B (Å<sup>2</sup>) | 45.1 | 25.3 |           |

| Refinement | Diffraction Data | PDB entry | Diffraction Data | PDB entry |
|------------|------------------|-----------|------------------|-----------|
| Protein molecules/asu | 2*VH (chains A, C) 2*HEL (chains B, D) | 2*VH (chains A, C) 2*HEL (chains B, D) |
| Atoms modeled/asu | 3380 | 3685 |
| Ramachandran* - favored (%) | 95.07 | 97.47 |
| - outliers (%) | 0 | 0 |
| R | 0.225 | 0.192 |
| R<sub>free</sub> (5% data) | 0.287 | 0.248 |
| RMSD bond lengths (Å) | 0.0153 | 0.0156 |
| RMSD bond angles (°) | 1.5828 | 1.7087 |
| Interface area@ | VH-HEL (chains A-B) (Å<sup>2</sup>) | 785 each chain | 826 each chain |
| VH-HEL (chains C-D) (Å<sup>2</sup>) | 807 each chain | 815 each chain |

* As output by SCALA
* Values in parentheses are of the highest resolution shell
# As calculated by the MOLPROBITY validation server
@ As calculated by PDBePISA
| Framework | Human VH | Camelid VH | Camelid VH | Camelid VH | Camelid VH | Shark VλGκR |
|-----------|----------|------------|------------|------------|------------|---------------|
| PDB ID    | 4U3X V₁₁H₀⁴ | 2VYR V₁₁V₁₀ | 3P9W V₁₁V₁₁ | 1ZVY D₃L₁₁ | 1RI8 D₂L₁⁹ | 1ZV5 D₂L₂⁹ | 1ZVH D₂L₂⁴ | 1SQ2 5A⁷ |
| Clone     | V₁₁H₀⁴   | V₁₁V₁₀     | V₁₁V₁₁     | D₃L₁₁     | D₂L₁⁹     | D₂L₂⁹     | D₂L₂⁴     | 5A⁷       |
| CDR3 length (AA) | 9        | 12         | 12         | 18         | 17         | 12         | 16         | 18        |
| Additional disulfide bonds | 0        | 0          | 0          | 0          | 1          | 1          | 0          | 2         |
| Affinity (nM) | 26       | 44         | 16         | 0.35       | 3          | 10         | 60         | 22        |
| Hydrogen bonds | 21       | 7          | 13         | 17         | 19         | 15         | 9          | 15        |
| Salt bridges | 3        | 0          | 2          | 2          | 1          | 1          | 0          | 2         |
| Shape complementarity | 0.808   | 0.686      | 0.699      | 0.801      | 0.761      | 0.766      | 0.783      | 0.769     |
| Buried surface area (Å²) | 783.3   | 805.3      | 905.6      | 644.8      | 634.0      | 635.3      | 561.0      | 755.3     |
| CDR1      | 20.2     | 74.6       | 9.8        | 70.8       | 0          | 72.7       | 69.9       | 161.9     |
| CDR2      | 60.1     | 0          | 30.3       | 114.6      | 6.4        | 2.0        | 0          | 0         |
| CDR3      | 535.4    | 613.0      | 498.3      | 395.7      | 469.0      | 268.6      | 449.9      | 584.9     |
| Framework | 167.6    | 117.7      | 367.2      | 63.7       | 158.6      | 292.0      | 41.2       | 28.5      |
| Epitope surface | Cleft (HEL) | Planar (p53) | Planar (VEGF) | Cleft (HEL) | Cleft (HEL) | Cleft (HEL) | Planar (HEL) | Cleft (HEL) |

Table III. Structural features of single domains in complex with antigen

aReferences: 1ZVY, 1ZV5, 1ZVH (52); 1RI8 (53); 1SQ2 (54); 2VYR (61); 3P9W (21).
bCalculated by the PISA server (37).
cCalculated by the program SC (62).
**FIGURE 1.** A library of stable human $V_H$ single domains derived from the HEL4 model domain (Garvan I) (A) Amino acid sequence of HEL4 model domain, DP47 germline and Garvan I library with numbering according to Kabat, with CDR1, CDR2 and CDR3 regions highlighted in green, pink and blue respectively. (B) Cartoon representation of human $V_H$ single domain with CDR3 region highlighted. (C) Colloidal stability (aggregation resistance) of HEL4-DP47 chimeras, as measured by retained binding to superantigen after heating to 80ºC (mean, n=3). (D) Colloidal stability of Garvan I library, as measured by retained binding to superantigen after heating to 80ºC. (E) Selection of binders.

**FIGURE 2.** A stable human anti-TNF $V_H$ single domain selected from the Garvan I library. A) Thermal unfolding (red) and refolding (blue) of clone G07 as monitored by circular dichroism. (B) Determination of G07 binding kinetics by surface plasmon resonance.

**FIGURE 3.** A second-generation library of stable human $V_H$ domains (Garvan II). (A) Amino acid sequence of HEL4 model domain, DP47 germline and Garvan II library with numbering according to Kabat, with CDR1, CDR2 and CDR3 regions highlighted in green, pink and blue respectively. (B) Thermal unfolding (red) and refolding (blue) of HEL4, DP47, DP47 CDR1 double and triple mutants as monitored by circular dichroism. (C) Cartoon representation of human $V_H$ single domain with CDR1, CDR2 and CDR3 regions highlighted. D) Colloidal stability of Garvan II library, as measured by retained binding to superantigen after heating to 80ºC. E) Selection of binders.

**FIGURE 4.** Binding kinetics and epitope distribution of human $V_H$ single domains selected from the Garvan II library against hen egg white lysozyme antigen (HEL). (A) Determination of clone H04 binding kinetics by surface plasmon resonance. (B) Kinetic association ($k_a$) and dissociation constants ($k_d$), equilibrium binding constants ($K_D$) and heat refoldability in solution of anti-lysozyme human single domains. (C) Cartoon representation of lysozyme antigen with cleft region (asterisk) and epitopes of D3L11, D2L19 and D2L24 camelid domains highlighted. (D) Epitope binning of camelid domain by surface plasmon resonance. (E) Epitope binning of human domain by surface plasmon resonance.

**FIGURE 5.** Structural basis of cleft recognition by the fully human $V_H$ single domain H04. (A) Structure of the human $V_H$ H04 in complex with hen egg white lysozyme (HEL) antigen (PDB identifier: 4U3X). Cartoon representations with antigen in tan, H04 in grey and CDR1,
CDR2 and CDR3 regions highlighted in green, pink and blue respectively. (B) Hydrogen bonding network with antigen residues in italics. (C) Framework contacts with lysozyme antigen. (D) Conformation of Trp47 residue in V\textsubscript{H}/V\textsubscript{L} interface, with H04 rendered in grey, HEL4 in orange and representative human Fabs in blue (PDB identifiers: 3QOS, 2VXS, 3KDM, 3BN9).

**FIGURE 6. Antigen recognition by V\textsubscript{H} H04 and other human V\textsubscript{H} domains.** (A) V\textsubscript{H} H04 in complex with hen egg white lysozyme (HEL) antigen, with structural cleft indicated (asterisk) (PDB identifier: 4U3X). (B) V\textsubscript{H} 9 in complex with MDM2 (PDB identifier: 2VYR). (C) V\textsubscript{H} V1a in complex with vascular endothelial growth factor (VEGF) (PDB identifier: 3P9W).

**FIGURE 7. Interaction of CDR3 with framework residues at positions 35, 37, 93 and 100x.** (A) Camelid V\textsubscript{H} D2L24 (PDB identifier: 1ZVH). (B) Human V\textsubscript{H} H04 (PDB identifier: 1ZVH). (C) Camelized V\textsubscript{H} B1a (PDB identifier: 3P9W).

**FIGURE 8. Structural features of the V\textsubscript{H}/V\textsubscript{L} interface.** (A) Human V\textsubscript{H} H04 (PDB identifier: 4U3X). B) Human DP47 Fab (PDB identifier: 3QOS). (C) Camelized V\textsubscript{H} B1a (PDB identifier: 3P9W). (D) Camelid V\textsubscript{H} D2L24 (PDB identifier: 1ZVH).
Figure 1

A

| HEL4 | EVQLLESGGGLVQPGGSLRLSCAAS\text{GFRISDEDMG}WVRQAPGK\text{LEWVS}SIY\text{GST} |
| DP47 | EVQLLESGGGLVQPGGSLRLSCAAS\text{GFTSSYAMSW}WVRQAPGK\text{LEWVS}AISGSG\text{GST} |
| LIB1 | EVQLLESGGGLVQPGGSLRLSCAAS\text{GFRISDEDMG}WVRQAPGK\text{LEWVS}SIY\text{GST} |

| HEL4 | YYADSVKGRFTISRDN\text{SKNTLYQMNS}LRAEDTA\text{YYCAS ALEPLSEP} LGFWGQGTLVTVSS |
| DP47 | YYADSVKGRFTISRDN\text{SKNTLYQMNS}LRAEDTA\text{YYCAS SYGA} FDYWGQGTLVTVSS |
| LIB1 | YYADSVKGRFTISRDN\text{SKNTLYQMNS}LRAEDTA\text{YYCAS (X)7/9} LGFWGQGTLVTVSS |

B

C

D

E

| Antigen | Binders (phage) | Binders (soluble nr) |
|---------|-----------------|----------------------|
| Human tumour necrosis factor (TNF-alpha) | 121/584 (22%) | 13/57 (23%) |
| Murine interleukin 21 (mIL21) | 27/47 (57%) | 3/5 (60%) |
| Human prolactin receptor (hPRLR) | 23/47 (49%) | 9/10 (90%) |
| Beta-galactosidase | 14/47% (30%) | 2/13 (15%) |
Figure 2

A

B

- G07
- Anti-TNFα

Temperature (°C)

- Folded (%)

Heat

Cool

Time (s)

- Resonance Units

2μM

1μM

500nM

G07

Anti-TNFα
A

HEL4  EVQLLESGGGLVQPGSSLRLSCAASGFRISDEDMGWRQAPGKGLEWVSQSYGPGSTYYADSVKGRFTI
DP47  EVQLLESGGGLVQPGSSLRLSCAASGFTFSSYAMSWRQAPGKGLEWSASIGSGSTYYADSVKGRFTI
LIB2  EVQLLESGGGLVQPGSSLRLSCAASGFRFXXEDMGWRQAPGKGLEWVSXIXXXSTYYADSVKGRFTI

HEL4  SRDNSKNTLYQMNSLRAEDTAVYVYCAS ALEPLSEP  LGFWGQGTIVLTVSS
DP47  SRDNSKNTLYQMNSLRAEDTAVYVYCAK SYGA FDYWQGQTLTVSS
LIB2  SRDNSKNTLYQMNSLRAEDTAVYVYCAK (X)_{7/9}  FDYWQGQTLTVSS

B

Figure 3

Antigen                          Binders (phage) Binders (soluble nr)
Vascular endothelial growth factor (VEGF) 35/98 (57%) 4/192 (2%)
Interleukin-2 receptor alpha (CD25) 17/96 (18%) 6/192 (3%)
Hen egg white lysozyme 26/45 (58%) 5/45 (11%)
Amino peptidase P (AMPP) 13/92 (58%) 5/45 (11%)
Human prolactin receptor (hPRLR) 13/92 (14%) 1/40 (2%)
Figure 4

A. Graph showing resonance units over time for different concentrations of Anti-HEL.

B. Table showing parameters for different clones:

| Clone | $k_+$ (M$^{-1}$s$^{-1}$) | $k_-$ (s$^{-1}$) | $K_d$ (nM) | Heat refolding |
|-------|-------------------------|-----------------|-----------|---------------|
| H04   | $1.0 \times 10^4$       | $2.6 \times 10^3$ | 26        | 111%          |
| G09   | $1.9 \times 10^4$       | $1.1 \times 10^3$ | 59        | 73%           |
| F05   | $3.8 \times 10^4$       | $8.0 \times 10^3$ | 210       | 85%           |
| D05   | $2.1 \times 10^4$       | $5.4 \times 10^3$ | 257       | 88%           |
| C01   | $1.4 \times 10^4$       | $2.6 \times 10^3$ | 101       | 121%          |

C. 3D model of HEL with regions labeled.

D. Graph showing resonance units over time for different clones.

E. Graph showing resonance units over time for different clones.
Figure 5

A

HEL

V_{H} H04

B

N103
S97
P98
R112
N100a
Q100
P99
Y95
D101
H100b
T47
R61
D33

C

L45
V37
W47

W47 alternative

W47 native
Figure 7
Figure 8
Fully human VH single domains that rival the stability and cleft recognition of camelid antibodies

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