Comparing CDRH3 diversity captured from secondary lymphoid organs for the generation of recombinant human antibodies

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Abbreviations: AA, amino acid; BCR, B cell receptor; CDR, complementary determining region; CDRH3, CDR3 of the heavy chain of an antibody; ELISA, enzyme-linked immunosorbent assay; hCCL5, chemokine ligand 5 (C-C motif); hIFNγ, human interferon gamma; IFNγ, interferon gamma; IP, intraperitoneal; LN, lymph node; NGS, next generation sequencing; S, spleen; SC, subcutaneous

Introduction

In vitro display and selection technologies involving phage, bacteria, yeast or ribosome are commonly used to generate antibodies for research, diagnostic and therapeutic applications. These approaches allow the isolation of antigen-specific antibody fragments from large libraries of immunoglobulin variable genes. The success of the selection process is dependent on the number, diversity and quality of the sequences present in the library. A direct correlation has been reported between the size of a library and the affinity of the candidates.6-8

Different strategies have been used for the diversification of antibody libraries. Oligonucleotides can be used to randomly diversify defined positions in the complementary-determining regions (CDR) of antibody genes. Alternatively, naturally rearranged variable genes isolated from humans or animals can be assembled to build antibody libraries.11-13 Natural antibody repertoires are an attractive source of diversity because, during B cell maturation, productive gene rearrangements are controlled at several stages. This proofreading mechanism increases the frequency of genes encoding a functional variable domain that are incorporated into the library and, thus, its functionality. In contrast, artificial randomization of CDR sequences lead to a higher proportion of miss-folded and non-functional polypeptides. This limitation is particularly significant when considering the CDR3 of the heavy chain (CDRH3), which can be relatively long and is more difficult to diversify using oligonucleotides while maintaining proper protein folding. On the other hand, natural antibody repertoires contain variable domains that are less-represented or suboptimal from a stability and manufacturing standpoint, and are therefore less well-suited for industrial applications.

To overcome these limitations, we recently described a novel cloning strategy to recover CDRH3 sequences from human or other species. These repertoires are then integrated into human antibody frameworks selected for their representation in human repertoires and for their biochemical stability.14 This approach allows for trapping of CDRH3 sequences from different sources into human antibody frameworks, expanding the diversity that can be exploited to generate human antibodies. For instance, CDRH3 sequences that have been biased against an antigen can be efficiently captured to generate target specific libraries and increase the frequency of isolating specific antibodies. This can...
be achieved by retrieving CDRH3 sequences from either immunized animals or pools of sequences that were enriched against an antigen using in vitro selection approaches such as phage display.14

When B cells encounter an antigen recognized by their B cell receptor (BCR) and encounter T cell help, they undergo affinity maturation and a selection process to produce high affinity antibodies.15 These events occur in the germinal centers of secondary lymphoid tissues such as lymph nodes and spleens. The proportion of B cells in these organs is 25% and 40%, respectively, which are further diversified by somatic hypermutation following immunization.16 B cells from secondary lymphoid organs can be fused to myeloma cells to generate monoclonal antibodies via immunization.

Figure 1. Capture of murine CDRH3 into a library of human scFv. (A) BalbC mice, divided in three groups, were either kept naïve or immunized with hIFNγ or hCCL5. (B) After sacrifice, the spleen and the lymph nodes were kept separated and VH repertoires were recovered by PCR. CDRH3 were then amplified from the VH pool by PCR, along with recognition sites for FokI, a type IIS restriction enzyme, which were added to the inserts. CDR3 are represented in gray, CDRI and 2 in white. (C) Murine inserts were then digested with FokI and (D) ligated to the human acceptor scFv library, itself digested with BsmBI. This second type IIS enzyme permitted the removal of a non-diversified stuffer sequence (“S”) at the location of CDRH3 and the generation of compatible cohesive ends for the incorporation of CDRH3. The acceptor library also contains synthetic diversity at the location of the CDRL3 (“L3”) and tags (“T,” a c-myc and a His tag) at the scFv C-terminal for purification purposes. Libraries featuring mouse CDRH3 were analyzed by NGS, which covered the CDRH3 and part of VH framework 3 (“NGS”). (E) The fusion to gII allowed the expression of scFv at the surface of M13 phage for phage display selection.

In this study, we explored spleen- and lymph node-derived immunoglobulin gene diversity as a source for antibody library construction. We used a CDRH3 diversity trapping approach to generate a panel of human single-chain variable fragment (scFv) libraries using CDRH3 sequences derived from spleens and lymph nodes isolated from the same group of naïve or immunized mice so that these repertoires could be directly compared. These human scFv libraries were characterized by next-generation sequencing (NGS) and their relative performance was evaluated according to their binding properties following phage display selection.

Results

Trapping murine CDRH3 repertoires into human scFv libraries. To create various CDRH3 repertoires, mice were immunized with two different antigens, i.e., human interferon gamma (hIFNγ) or chemokine ligand 5 (C-C motif) (hCCL5). After several booster immunizations, the sera were analyzed. They demonstrated an antigen-specific IgG response and the titers were similar between each animal (data not shown). Three days after a hyperboost, the spleen (S) and lymph nodes (LN) were obtained from the immunized mice and from a control group that did not receive any immunization, i.e., the naïve group. Cells from the spleen and the lymph nodes (4 or 5 mice per group) were separately isolated and mRNA extracted (Fig. 1A). cDNA was synthesized and the murine CDRH3 sequences were randomly selected from each library to homogenize the size of the data sample. Using the NGSAb software, the number of unique CDRH3 sequences inserted in the six libraries were analyzed by NGS, which covered the CDRH3 and part of VH framework 3 (“NGS”). (E) The fusion to gII allowed the expression of scFv at the surface of M13 phage for phage display selection.
sequences, their respective length, and the frequencies of the repeated sequences were determined (Table 1; Figs. 2 and 3).

The CDRH3 length distribution and amino acid (AA) composition found in the lymph nodes libraries (Fig. 2) corresponded to the published characteristics of murine CDRH3 sequences. The highest frequency and overall length of captured naïve CDRH3 sequences (i.e., 12 AA) tended to be shorter than that reported for human CDRH3 (i.e., 14AA described for human) and contained a higher tyrosine content (17% to 20% vs. 14% for human). These features remained present after immunization, albeit with a slight trend toward certain CDRH3 lengths (e.g., 15 and 17AA for the LN/hIFNγ library and 13 and 14 for the LN/hCCL5 library). Similar results were obtained with libraries capturing spleen derived CDRH3, as previously described. These results demonstrate that mouse CDRH3 sequences were captured into human antibody frameworks. The distribution of sequences between the different VH families of the acceptor library was found to be equivalent (Fig. S1), indicating that no significant bias had been introduced during the cloning step.

The analysis of the diversity of CDRH3 sequences in the libraries took into account only those in the correct reading frame (Table 1). The CDRH3 sequences captured from the naïve lymph nodes were more diverse compared with the naïve spleen, i.e., unique CDRH3 sequences comprised 19% vs. 13%, respectively, of the totals (Table 1). The difference in diversity could also be observed by analyzing the frequency of CDRH3 fragments. In the LN/Naïve library, 69% of CDRH3 sequences were represented less than a 100 times while only 31% were more abundant (Fig. 3A). In contrast in the S/Naïve library, 25% of CDRH3 were sequenced less than 100 times while 75% were highly repeated (Fig. 3B). After immunization (Table 1), the diversity of unique CDRH3 sequences dropped from 19% to 4% in the LN libraries, and from 13% to 5% and 9% in the S libraries, indicating that the CDRH3 repertoires were biased in vivo. In line with this, CDRH3 sequences, represented more than 100 times, reached 85% and 86% for LN libraries and 86% and 82% for S libraries (i.e., immunization with hIFNγ and hCCL5, respectively) (Fig. 3). Furthermore, as the CDRH3 sequences are inserted in different VH frameworks and combined with different VL, the scFv sequence diversity is not limited to the CDRH3, and is actually considerably higher, as was confirmed by sequencing a panel of randomly selected clones from each library using the Sanger method (data not shown).

To further analyze the captured diversity, the overlap of the CDRH3 unique sequences between libraries was assessed (Fig. 4, Table S1). For the two naïve CDRH3 repertoires, the common sequences represented 5.5% of the LN library and 14.2% for the S library, highlighting that most of the CDRH3 sequences in these organs differed although they had been obtained from the same animals (Fig. 4A). Following immunization, CDRH3 repertoires showed limited overlap with the naïve repertoire (Fig. 4B and C; Table S1), probably resulting from the affinity maturation when generating high affinity antigen-specific antibodies. The overlap observed in the naïve and immunized spleen libraries reached 15.8% (the sum of 6.9 + 8.9%; Figure 4B; Table S1) with hIFNγ immunization and 14.7% (the sum of 7.9 + 6.8%: Figure 4B; Table S1) with hCCL5 immunization. The overlap between the LN/Naïve and each LN/immunized libraries was 6.7% (5.1 + 1.6%) of the LN/hIFNγ sequences and 7.2% (5.5 + 1.7%) of the LN/hCCL5 sequences (Fig. 4B and C; Table S1). These results, which demonstrate a larger overlap in the S libraries, suggest that the immunization induced a stronger bias in the repertoires of lymph nodes compared with the spleen.

Overall, the NGS characterization revealed that the CDRH3 repertoire captured from naïve lymph nodes was the most diverse and that the repertoires isolated from immunized animals had been biased in vivo. This bias was particularly apparent for the repertoires derived from lymph nodes, while only a trend toward a higher redundancy was observed for the spleen repertoires (Fig. 3). Notably, the CDRH3 repertoire from naïve spleens had a profile closer to immune repertoires (Fig. 3).

Relative performance of libraries incorporating CDRH3 repertoires from spleen and lymph nodes. After characterizing the CDRH3 repertoires, the libraries were used in phage display selection against hIFNγ to assess their performance. This was evaluated by their ability to generate specific and potent binders against hIFNγ. Three rounds of selection were performed to enrich scFv repertoires for clones specific for hIFNγ. For each LN library, 528 random clones from the output of selection were tested as soluble scFv by ELISA for specific binding to hIFNγ. The results (Fig. 5) showed an increased frequency of scFv binding to hIFNγ for the LN/hIFNγ library (85%) compared with the LN/Naïve library (54%), while the LN/hCCL5 library was found to perform very poorly against hIFNγ (1% of hits). In addition, the frequency of scFv producing an absorbance of >70% of the positive control was higher in the candidates obtained from the LN/hIFNγ library (63%) compared with those from the LN/Naïve library (29%) (Fig. 5). The same relative performance of the libraries was observed when testing the individual clones by ELISA using scFv expressed at the surface of M13 bacteriophage, which generally leads to an increased signal due to avidity and signal amplification (Fig. S2). Taken together, these results indicate that immune and naïve features of the lymph node derived repertoires were transferred to the libraries via capture of the CDRH3. The same evaluation has been performed with the S libraries and similar results were obtained.

![Table 1](https://example.com/table1.png)

**Table 1. Summary of libraries capturing murine CDRH3 and characterization by NGS**

| CDRH3 Source | Library size | Unique CDRH3 |
|--------------|-------------|--------------|
| Spleen/Naïve | 2.5 × 10⁶ | 70'993 13% 3'094 1% |
| Spleen/hIFNγ | 7.3 × 10⁶ | 29'996 5% 4'279 2% |
| Spleen/hCCL5 | 1.8 × 10⁶ | 72'617 9% 5'357 2% |
| LN/Naïve | 1.1 × 10⁶ | 140'396 19% 7'419 1% |
| LN/hIFNγ | 1.1 × 10⁶ | 38'575 4% 4'215 0.4% |
| LN/hCCL5 | 1.3 × 10⁶ | 35'934 4% 3'309 0.3% |

Analysis on functional sequences. The number of unique CDRH3 is indicated for each library, as well as their frequency relative to the total amount of functional sequences analyzed. The libraries size was determined by colonies titration after electroporation.
To assess the diversity of binders, a selection of scFv specific to hIFNγ was sequenced from each library by the Sanger method, allowing for complete coverage of the VH and VL sequences. With the exception of LN/hCCL5, > 50 clones were analyzed for each library. Only 5 clones of LN/hCCL5 were sequenced because of the very low hit rate obtained with this library. A larger diversity of scFv was obtained from the LN/Naive library compared with the immunized LN libraries (Table 2), and these candidates were different from those retrieved from the spleen derived libraries following the same procedure.\(^\text{34}\)

The candidates identified by screening and defined by sequencing were produced, purified as soluble scFv, and quantified. They were then tested at the same concentrations in a dose-response ELISA for binding to hIFNγ to evaluate their apparent affinity (EC\(_{50}\)) for the target hIFNγ (Table 2). The frequency of the CDRH3 sequences corresponding to each candidate was also retrieved by NGS before selection and after the third round of selection (Table 2). The ranking before and after selection are different, in particular for the immune libraries. This indicates that the most frequent sequences that were integrated in the unselected libraries did not necessarily lead to the generation of a functional and antigen specific binding site when inserted in the novel context of the human antibody frameworks used in this study. The EC\(_{50}\) for clones representing more than 1% of the total sequences after the selection round three are presented in Figure 6. The EC\(_{50}\) values indicate that potent (i.e., < 10 nM) binders of hIFNγ were isolated from the S/hIFNγ and the LN/hIFNγ libraries. In contrast, libraries biased against another target due to the immunization with hCCL5, mainly provided low affinity binders of hIFNγ (20–150 nM). These data indicate that the immune features of murine antibody repertoires were transferred to the human acceptor library. The LN/Naive library, which initially contained the most diverse CDRH3 repertoire, also allowed for the identification of the largest panel of potent (i.e., < 10 nM) candidates (Table 2; Fig. 6).

Specificity of scFv isolated from different repertoires. To assess whether scFv isolated from naïve and immunized CDRH3 repertoires targeted similar epitopes, we compared their binding capacity against IFNγ from human, rhesus, mouse and rabbit using purified scFv in a dose-response ELISA. Only scFv with a frequency over 1% at the selection round three (as defined by NGS) were tested. Cross-reactivity profiles from the LN- and the S-derived libraries are presented in Figure 7 and in Figure S3, respectively. The results indicate that several patterns of cross-reactivity with different species-derived IFNγ could be identified. ScFv derived from the LN/Naive library covered three patterns, i.e., LN/Naive-2 (clone ID) was not cross-reactive, LN/Naive-1 and 4 and 5 were cross-reactive with rhesus only and LN/Naive-3 was cross-reactive with rhesus, mouse and rabbit (Fig. 7A). ScFv capturing LN/hIFNγ CDRH3 displayed two cross-reactivity patterns, i.e., LN/hIFNγ-1 and 4 were cross-reactive with rhesus only and LN/hIFNγ-2 and 3 were cross-reactive with rabbit only (Fig. 7B). For the spleen derived libraries, the S/Naive and the S/hIFNγ scFv presented three and two cross-reactivity patterns, respectively, while the library biased for hCCL5 produced scFv all specific to hIFNγ only. These data indicate that the clones tested bound to different epitopes on hIFNγ, some being conserved between species and others not. The scFv were also tested against streptavidin (Figs. 7 and S3) and other control targets to ensure their specificity (data not shown). These results demonstrate that the diversity of scFv was translated by a diversity of epitopes covered for the naïve and the hIFNγ biased libraries, but not for the S/hCCL5 library biased against another target. Notably, some cross-reactivity patterns were only found in the naïve or the hIFNγ biased libraries, which suggests that these libraries could be complementary for covering a maximum of hIFNγ epitopes.

**Discussion**

Our aim was to investigate novel diversification strategies when building human immunoglobulin libraries by exploiting CDRH3
specialized in clearing bacteria from the blood and driving the humoral response against bacteria capsular polysaccharides. Indeed, splenectomy results in increased susceptibility to infection by this class of pathogens.

In addition, the spleen and the lymph nodes are characterized by different anatomical structures that may further affect the B cell repertoires. Thus, we compared the performance of human antibody libraries featuring CDRH3 repertoires isolated from murine spleen and lymph nodes with, and prior to, immunization.

Figure 3. Evaluation of CDRH3 redundancy. Evaluation by NGS of CDRH3 repartition in the context of human scFv libraries. The percentage of total CDRH3 is represented in function of their frequency according to a color code. For each library, one million clones were analyzed. The values on the histograms are the number of unique CDRH3, i.e., the number of CDRH3 with a different amino acids sequence, corresponding to each section. (A) represents the level of redundancy of lymph nodes derived libraries while (B) represents the repartition of CDRH3 from spleens derived libraries.

Figure 4. Overlap between CDRH3 repertoires. Evaluation of the unique CDRH3 sequences in common between libraries. The analysis included one million clones for each library (NGS). Diversity of unique CDRH3 (i.e., all CDRH3 with a different amino acids sequence) is represented by circles which size is proportional to the size of the sample. Naïve libraries are represented in red, libraries biased against hIFNγ in blue and libraries biased against hCCL5 in green. For each library, the number of unique CDRH3 is described with the same color code, U refers to “unique sequences.” Unique sequences in common between libraries are symbolized by the overlap between circles. Numbers in black represent the number of unique sequences corresponding to each section determined by a black line. (A) represents the overlap between both naïve libraries, (B) represents the overlap between the spleen derived libraries, and (C) represents the overlap between the spleen derived libraries.

combinatorial designs. To achieve this, we explored natural repertoires extracted from different organ types to define the most appropriate source for the generation of such libraries. Secondary lymphoid tissues contain a high percentage of B cells and, thus represent good sources of naturally rearranged immunoglobulin genes. As the spleen and the lymph nodes drain different body fluids, i.e., blood and lymph, respectively, B cells present in these organs are preferentially exposed to different types of pathogens, potentially biasing the repertoire. The spleen is specialized in clearing bacteria from the blood and driving the humoral response against bacteria capsular polysaccharides. Indeed, splenectomy results in increased susceptibility to infection by this class of pathogens. In addition, the spleen and the lymph nodes are characterized by different anatomical structures that may further affect the B cell repertoires. Thus, we compared the performance of human antibody libraries featuring CDRH3 repertoires isolated from murine spleen and lymph nodes with, and prior to, immunization.
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A library of phagemid vectors was generated encoding human scFv sequences. This library included 7 human VH and 7 human VL framework sequences of which CDR1 and 2 were kept germline while synthetic diversity was added at the level of the CDRL3. At the location of the CDRH3, a non-diversified “stuffer” sequence that could be digested off via type IIS restriction enzyme, BsmBI, was added, thereby allowing the integration of diversity in only one cloning step. This phagemid library was electroporated into TG1 E. coli cells, generating a diversity of 2.2 x 10^9 transformants. The library with the ability to accept CDRH3 sequences after BsmBI digestion was referred as the acceptor library.

Immunization. As described earlier, BalbC mice were either immunized or kept naïve to allow comparison of murine CDRH3 repertoires, focused or not, in the context of human scFv sequences. Five mice were kept naïve, and 2 groups of 4 mice each were immunized with hIFNγ or hCCL5 administered in RIBI via intraperitoneal (IP) and subcutaneous (SC) injection (mice were immunized both IP and IV along each boost); both targets were produced at NovImmune SA. After sacrifice, the spleen and lymph nodes (popliteal, inguinal, brachio, axillary, cervical) were recovered and kept separated for RNA extraction.

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GE Healthcare) and nested PCR for the recovery of the CDRH3 sequences. In order to avoid cross contamination between the repertoires, mRNA isolation was performed on different days for the different groups of animals. In addition, rigorous cleaning of the equipment was performed between extractions and only disposable precast gels (Invitrogen) were used for DNA purification. During the last amplification, recognition sites for FokI type IIS restriction enzymes were also added using biotinylated primers. The acceptor library and the CDRH3 inserts were digested with BsmBI and FokI (both from New England Biolabs), respectively, which generated compatible cohesive ends, and purified with Chroma Spin T1000 columns (Clontech) and Dynabeads (M280 Streptavidin from Invitrogen), respectively. CDRH3 were then ligated into the acceptor library pool with Rapid DNA Ligation Kit (Roche) and electroporated into TG1 E. coli cells. Each library reached around 10^8 transformants (Table 1).

**Sequencing.** A selection of clones was sequenced with the Sanger method (service provided by Fasteris SA). TG1 colonies were separately cultured overnight at 37°C in 2 mL of LB AG and vectors were purified with the Qiaprep Spin Miniprep kit (Qiagen).

Libraries and selection rounds 3 were also analyzed by NGS via the HiSeq Illumina platform (service provided by Fasteris SA) and 87% to 92% of the bases had a Phred score ≥ 30. Sequencing primers, designed in VH J region, could cover CDRH3 and part of VH framework 3, allowing identification of families using DNA signatures. Analysis of large data sets was performed with the NGSab software developed at NovImmune. For each library, the analysis was performed from a pool of one million random clones to ensure samples were of comparable size.

**Selection against hIFNγ.** Phage particles displaying scFv fragments were produced and purified. Libraries electroporated into TG1 cells were cultured at 37°C and 240 rpm in 2xTY AG until OD_{600} 0.5. Cells were then infected with M13K07 helper phage (MOI of 10) 1 h at 37°C and 100 rpm. The medium was replaced by 2xTY AK to select cells that incorporated a helper phage vector. Cells were incubated overnight at 30°C with shaking at 280 rpm for robust production of phage. Viral particles were purified from supernatants by two consecutive PEG precipitations (using 1/3 v/v of 20% PEG-8000/2.5 M NaCl). Purified phage were used to infect fresh TG1 cells, which were then titrated. Phage preparations were thus evaluated from 5 × 10^13 to 1 × 10^14 pfu/mL.

Phage from each library were used in three rounds of phage display selection against the target hIFNγ (biotinylated, produced at NovImmune SA). For each library, 10^10 viral particles were blocked in skimmed milk 3% (w/v) and deselected twice via incubation for 1 h on Dynabeads (M-280 Invitrogen) coated with streptavidin. Phage remaining in the supernatant were discarded at NovImmune (defined by ELISA). None of the binders found from the library LN/hCCL5 having a frequency above 1%, the library is not represented in this graph.

![Figure 6. Relative performance of most amplified binders. Representation of all the scFv defined as binders to hIFNγ from each library and with a frequency defined by deep sequencing above 1% at the selection round 3. Clones are grouped according to their library of origin and represented by their EC_{50} in nM (defined by ELISA). None of the binders found from each library LN/hCCL5 having a frequency above 1%, the library is not represented in this graph.](image-url)
Clones were tested in ELISA for their specificity for hIFNγ after being produced as soluble scFv or as scFv displayed on phage. Screening ELISA was first performed in the scFv format (6 plates tested per library, i.e., 528 individual clones). Clones were cultured in 2xTY AG for 6 h at 37°C, followed by IPTG addition (1 mM final) and incubated overnight at 30°C and 150 rpm for scFv production. A positive control clone encoding a scFv with high potency against hIFNγ was also included in each plate. Streptavidin plates (Greiner Bio-one) were coated with 50 μL of biotinylated hIFNγ (1 μg/mL) for 1 h. After washing, 50 μL of cell culture supernatants were added to plates that were incubated for 2 h at room temperature. After washing, remaining scFv were revealed via a mouse anti-c-myc (produced at NovImmune SA) and then a goat anti-mouse Fcγ HRP (Jackson). Then, TMB (Sigma) was added, followed by H2SO4 (2 N) for blocking. In the final step, absorbance was read at 450 nm (Synergy HT, BioTek).

The screening ELISA was also performed using phage particles displaying the scFv at their surface (1 plate tested per library, i.e., 88 individual clones). Clones were cultured in 2xTY AG for 6 h at 37°C and then infected with the helper phage (MOI of 10) for 1 h. The medium was replaced by 2xTY AK and the cells incubated overnight at 30°C for phage production. Phage particles excreted in the supernatant were then used as the input of the next selection round.

**ELISA screening.** Individual TG1 clones from the output of the third selection rounds were cultured separately in 96-wells plates, in 2xTY AG overnight at 30°C and 150 rpm. These master plates were supplemented with glycerol to 17% for storage at −80°C and used as a source for the various screenings. Clones were tested in ELISA for their specificity for hIFNγ after being produced as soluble scFv or as scFv displayed on phage.

**Figure 7.** Evaluation of the cross specificity of clones. A selection of clones from each LN library was tested for their cross reactivity on IFNγ. Purified scFv were tested at four concentrations (1100, 110, 11 and 1.1 nM, n = 2) for their specificity to a panel of IFNγ from different species, i.e., human, rhesus monkey, mouse and rabbit, and streptavidin as a negative control. Revelation was performed via HRP and read at 450 nm. All clones above 1% frequency at the selection round 3 were tested.
The ELISA procedure was similar to the one described for scFv, except that the first detection antibody was a mouse anti-M13 P8 (Abcam).

**Dose-response ELISA using purified scFv.** A selection of clones were produced as soluble scFv and purified via their His tag with Ni-NTA agarose chromatography (Qiagen). Purified scFv were quantified using a Nanodrop ND 1000 spectrophotometer (Witec AG). Serial dilutions of scFv were applied in duplicates to streptavidin plates (Greiner Bio-one) coated with biotinylated hIFNγ (coating with 50 μL of hIFNγ at 1 μg/mL) for 2 h at room temperature (as described for the screening ELISA). After washing, specific scFv were detected using a mouse anti-c-myc antibody and a goat anti-mouse Fcγ HRP antibody, followed by the addition of TMB and H2SO4 (2 N). Absorbance was then read at 450 nm (Synergy HT from Bio TeK).

**Cross-specific ELISA.** A selection of clones, including those most amplified from each library, were tested in dose-response ELISA against a panel of IFNγ from different species (i.e., human, rhesus, mouse and rabbit) in duplicates. Targets produced (NovImmune SA, biotinylated and with a His tag) were coated for 30 min at 1 μg/mL in 50 μL of PBS BSA 1% on microplates precoated with streptavidin (Greiner Bio-one). Purified scFv were tested at 1100 nM, 110 nM, 11 nM and 1.1 nM in 50 μL PBS BSA 1%. Plates were revealed using a mouse anti-c-myc and goat anti-mouse Fcγ HRP as described in the dose-response ELISA section.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Materials**

Supplemental materials may be found here: www.landesbioscience.com/journals/mabs/article/25592

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