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Production of Endoglin-specific heavy chain antibody fragments (VHHs)
microarrays for whole-cell SPR imaging

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Abstract

Cell-based Surface Plasmon Resonance imaging (SPRi) with anti-endoglin VHH antibodies microarray on a gold-covered biochip was used to characterize the binding to endoglin expressed on the surface membrane of SC cells. SPRi showed the specific capture of SC cells by the immobilized anti-endoglin VHHs, but not of THP-1 cells that do not express endoglin on their plasmatic membrane. The SPRi reflectivity shifts with SC cells bound by anti-endoglin VHHs ranged from 3.59 to 5.54% (average $4.66 \pm 1.18\%$) and with THP-1 cells from 2.23 to 3.05% (average $2.62 \pm 1.32\%$ ($p < 0.001$)) and the SC cell densities with anti-endoglin VHHs ranged from 417 to 747.7, (average $548 \text{ cells}/2.28 \times 10^5 \mu\text{m}^2 \pm 132$). Anti-endoglin VHHs bind epitopes located in the extracellular domains of endoglin with different avidity as suggested by the differences in SC cell densities. The cell-based SPRi of anti-endoglin VHH microarrays can be used both in studies aimed at a better understanding of endoglin function in living cells and in VHH applications as nanoproboscopes.

1. Introduction

Surface Plasmon Resonance imaging (SPRi) of antibody microarrays enables the label-free functional analysis of living cells in real time by simultaneously measuring changes in reflexivity and monitoring the cellular process imaged with a CCD camera. Cells captured by the antibody on the gold layer surface are detected by SPRi [1], [2], allowing to evaluate the binding affinity and specificity of the antibody to cognate antigen expressed on the surface of cells while occurring and without potential interfering labels. The cell based SPRi with antibodies microarrays allows to study receptor-ligand interaction and the cellular process elicited by the interaction as antibodies and interleukin production by cells of the immune system, [1], [2], [3], and the response of living cells to different stimuli [4]. The main challenge remains the covalent modification and surface immobilization of such large proteins (150 kDa) on microarrays while maintaining their functionality.

VHH is the variable domain of the heavy chain antibody that are naturally produced in camelids [5]. VHHs bind antigens with high specificity and affinity when produced as recombinant proteins in prokaryote and eukaryote expression hosts [6]. The relative small size of VHHs (15 kDa) allows to access to epitopes located under the surface of globular proteins or in the interaction interface of protein subunits, that are not accessible to bulky conventional antibodies (150 kDa). Additionally, VHHs are amenable of modification to improve affinity, specificity, half-life, stability and to add new functions by incorporating sequences encoding signals, effectors or other functional domains [7].

Endoglin is a 180 kDa membrane glycoprotein homodimer with disulfide bonded subunits; each subunit has an N-terminal Orphan Domain (OD), a bi-partite Zona Pellucida (ZP), a transmembrane domain and a cytoplasmic peptide that has 47 or 14 residues sequence producing long and short endoglin isoforms respectively [8], [9]. Endoglin functions as a type III accessory receptor for transforming growth factor β (TGF- β) [10], [11], which plays an important role in

angiogenesis since is highly expressed in the endothelial vasculature during the development of vessels in normal and tumoral tissues [12], [13].

In spite of the great advances in functional mapping of endoglin [14], [15], [16], the regions involved in the pleiotropic cellular effects are not fully unraveled yet. In pursuance of developing new tools to study the structural and functional characteristics of endoglin, we set up a cell-based SPRi with anti-endoglin VHHs microarray on a gold biochip to evaluate the binding to endoglin-expressing cells.

2. Material and Methods

2.1. Cells

SC cells (ATCC® CRL-9855™) were cultured in 90% Iscove's Modified Dulbecco's Medium (IMDM) (ATCC 30-2005™) supplemented with 0.05 mM 2-mercaptoethanol, 0.1 mM hypoxanthine, 0.016 mM thymidine, 1× penicillin/streptomycin and 10% fetal calf serum (FCS). THP-1 cells (ATCC® TIB-202™) were cultured in RPMI medium (ATCC® 30-2001™) supplemented with 1× penicillin/streptomycin and 10% FCS. Cells were maintained in concentrations between 2.5×10^5 and 1×10^6 cells/ml in 85% relative humidity, 5% CO₂ at 37 °C.

2.2. VHH cDNA phage library screening by phage-ELISA

20 µl aliquot of a VHH cDNA library (2.7×10^7 phages/ml) generated from PBMC (peripheral blood mononuclear cells) mRNAs of an alpaca immunized with a culture lysate of the bladder cancer cell line T24 [18] were inoculated into 1 ml of 2xYT medium (Sigma Aldrich) supplemented with 2% glucose and 100 µg/ml ampicillin (named supp 2xYT) and incubated with gentle agitation at 37 °C overnight. 200 µl of culture were plated in supp 2xYT plates and incubated at 37 °C overnight. 375 colonies were randomly picked and inoculated in 100 µl of supp 2xYT in 96-well culture plates and grown with gentle agitation at 30 °C overnight. 2 µl of the culture were inoculated in 248 µl of fresh supp 2xYT in 96-well plate and incubated with shaking 250 rpm at 37 °C until reaching an optical

density at 600 nm (OD_{600}) between 0.5 - 0.6. To each VHH-phage culture were added 3×10^9 of CSV-M13 helper phage in 50 μ l of supp 2xYT and left at 37 °C for 30 min, the plate was centrifuged at 1000 g for 10 min and the precipitate was re-suspended in 150 μ l of supp 2xYT with 100 μ g/ml kanamycin. The plate was incubated with shaking 125 rpm at 30 °C overnight and centrifuged at 1,000 g for 10 minutes, the supernatant was then kept at -20 °C until use.

The supernatant was assayed by phage-ELISA with 50 ng endoglin (R&D Systems) and 50 ng of unrelated antigen Fas2 [19]. Both antigens were fixed into a microtiter plate (Maxisorb) at 37 °C overnight and washed 5 times in 300 μ l of phage-Elisa washing solution. 100 μ l of blocking solution (4% milk in phosphate buffered saline pH 7.4, PBS) were added to each well, left for 1 h at 37 °C and washed as above. 50 μ l of blocking solution were added and 50 μ l of phage supernatant, incubated at 37 °C for 1 h and washed as described. 200 μ l of the HRP anti-P8 secondary antibody (1:2000 in 2% milk) were added and incubated at 37 °C for 1 h; and then washed. 100 μ l of TMB were added, incubated for 10 minutes and the reaction stopped by adding 50 μ l of 2N H_2SO_4 . The OD_{450} values were measured by a BioTek ELX800 Microplate reader. OD_{450} value 3-times higher than the Fas2 protein (used as an unrelated protein) was the cut-off for selecting phages carrying anti-endoglin VHHs. EngVHH17 [18] was used as a positive phage control. VHH DNA was amplified from phages and sequenced (Macrogen).

2.3. Production of anti-endoglin VHH antibodies

The VHH cDNA amplification proceeded in 50 μ l 1 \times PCR reaction buffer (Invitrogen) containing 10 ng of DNA, 2 mM $MgCl_2$, 0.5 μ M of each primer VHHSfi/*Nco*I and ALLVHHR2/*Not*I [17], 200 μ M of each dNTP and 0.2 units of PlatinumTaq DNA (Invitrogen) with an initial step 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, final cycle at 72 °C for 10 minutes. PCR products were electrophoresed in 1.2% agarose gel, 1 \times TBE at 80 V. VHH cDNA bands were excised and purified with QIAquick Gel Extraction Kit (Qiagen). VHH cDNA and pET22b(+) (Novagen) were digested with *Not*I and *Nco*I and ligated with 1 unit of T4 DNA ligase (New England

Biolabs) at 16 °C overnight. 5 µl of ligation product transformed 100 µl containing about 5×10^{10} cells/ml of *E. coli* BL21 and incubated in LB/amp plate at 37 °C overnight. Some colonies were randomly picked for PCR with T7F or T7R primers that flank pET22b+cloning site to check both insert sizes and also VHHSfi-NcoI and ALLVHHR2-NotI that flank VHH sequences.

A single colony of VHH cloned in *E. coli* was inoculated in 2 ml of LB/Amp and grown at 37 °C with shaking at 250 rpm overnight. 500 µl of culture were added to 48 ml of fresh LB/Amp and incubated at 37 °C with 250 RPM until an OD₆₀₀ value 0.5 - 0.530. IPTG was added to a final concentration of 1 mM, incubated with shaking 250 rpm at 37 °C for 3 h and centrifuged at 7,650 g at 4 °C for 15 minutes. The supernatant was filtered through 0.2 µm pore filter. Recombinant VHHs were purified from supernatant through Ni-NTA columns (Qiagen) and visualized by Coomassie blue stained of 15% SDS-PAGE gels with molecular-weight size markers. Protein concentration was quantified with a Nanodrop 2000 (ThermoFisher Scientific, Wilmington, DE) UV-vis spectro-photometer.

2.4. Endoglin ELISA with VHHs

100 µl of endoglin (0.5 ng/µl) in 0.05 M bicarbonate carbonate buffer pH 9.6 were added to ELISA plates and incubated for 2 h at RT, washed 5 times with 200 µl of washing buffer, shaking gently for 4.5 minutes and then left to stand for 30 s each time. 200 µl of ELISA blocking solution were added, incubated at 37 °C for 1 h, and washed as described. 100 µl of filtered supernatant of the culture of recombinant VHH in *E. coli* and EngVHH17 (20 µg/ml) were added and incubated for 2 h at 37 °C. 100 µl of Penta-His HRP-conjugated antibody (Qiagen) diluted 1:2,000 in blocking solution was added, incubated at 37 °C for 1 h and washed as described. 100 µl of TMB (3,3',5,5'-Tetramethylbenzidine) were added and incubated at RT for 10 minutes. Reactions were stopped with 50 µl of 2 N H₂SO₄. OD₄₅₀ values were measured in triplicate with a BioTek ELX800 Microplate reader. Recombinant VHHs producing OD₄₅₀ values higher than cut-off were set as positives. The cut-off was estimated as the average value, plus 3 times the standard deviation of the OD₄₅₀ values for endoglin-ELISA with anti-Fas2 VHH (negative control).

2.5. Functionalization of the VHHs

The anti-endoglin VHH proteins were concentrated (500 µg/ml) in Phosphate Buffer Saline (PBS, pH 7.4) by centrifugation with 3,000 MWCO (3kDa) VivaSpin minicolumns (Life-Science). The coupling reaction proceeded with addition of 2,5-dioxopyrrolidin-1-yl 11-sulfanylundecanoate linker (Merck) to the VHH solution (10:1 molar ratio), pH 7.4, RT for 1 h [20]. Each VHH coupling reaction was purified with a VivaSpin minicolumn by centrifugation at 15,000 g for 15 min to remove unreacted linker; conjugated VHH was recovered in 200 µl PBS. Conjugated VHHs were stored at 4°C before SAM (Self-Assembling Monolayer) formation on microarrays.

2.6. Preparation of biochips

Glass prisms with a 55 nm-thick gold layer were used as biochips (Horiba Scientific-GenOptics, Orsay, France). 24 h before their use, biochips were placed in a low-pressure plasma generator for gold surface cleaning by ionized gas flow at 0.6 mBar (75% oxygen, 25% argon) for 3 minutes at 40 W [21]. Glycerol was added (5 % w/v final concentration) to each functionalized VHH sample (0.1 µg/µl in PBS) before microarraying over the biochips. Each functionalized VHH was manually arrayed on the biochips, by pipetting 0.5 µl of each VHH solution over the gold surface of the biochip. Make sure to avoid pipette tip touching the surface. Each microarray contains one positive and one negative control along the anti-endoglin VHHs, to assess the VHH protein functionality. The VHH microarrays included: 13 anti-endoglin VHHs, EngVHH17, whose affinity toward endoglin has been described elsewhere [18] as positive control, anti-Fas2 VHH [19] as a negative control along with a background control with no VHH (unfunctionalized gold surface). VHH microarrays were placed in a humid chamber for 24 h, to ensure the highest density of self-assembled monolayers. Then, microarrays were rinsed with PBS and kept in solution at 4 °C until use.

2.7. SPRi

One new VHH microarray (single use biochips) was used for each SPRi experiment led with cells. Each VHH microarrays were loaded on the SPR imager and immediately covered with tempered culture medium. 1% BSA (v/v) in the corresponding culture medium was used as a blocking solution, and added on the array at room temperature for 30 minutes before washing three times with the medium before adding the cell suspension. SC and THP-1 cell suspension (1×10^6 cells/ml) in culture media (IMDI when assaying with SC and RPMI with THP-1) were manually loaded on the anti-endoglin VHHs biochip, and incubated, without any flow, at 37 °C. The SPRi signals were monitored with a SPRi Lab™ system that monitored in real-time changes in reflectivity of each ROI (region of interest) (one ROI is corresponding to a VHH sample arrayed as a spot on the microarray), and plotted these responses upon time. Data were collected and processed in spreadsheets.

2.8. SPRi data analysis

SPR images were then processed with the Image J program version FIJI (<http://fiji.sc/Downloads>). Cell counting was done by using the Analyze/Analyze Particles tool of the Image J program and the `im2bw` command of MATLAB program (MathWorks). The particle size threshold was set as 15.4 μm in diameter to approximate average cellular size. SPR biochip images contain 15 VHH spots (ROIs), in each spot a region of 31 x 31 pixels (477.09 μm x 477.09 μm) located approximately at the center of each spot was chosen. Once this area was cropped, the reflectivity value of each pixel was plotted on an 8-bits grayscale, meaning values between 0 and 255 of intensity. A criterion was established to binarize these pixels. To achieve this, the command `BW = im2bw (I,Level)` converted the grayscale image I to binary image BW, by replacing all pixels in the input image with luminance greater than level with the value 1 (white) and replacing all other pixels with the value 0 (black). So, for example at a value 0.15 (15%), the command run in the subsequent manner: `>> lmbw=im2bw (lm, 0.15);` criteria to binarize the area 31 x 31. Then, the command set a lower limit corresponding to the pixel with the lowest luminance value in the area and an upper limit corresponding to the pixel with the highest value. Within this range it only selected the pixels with the value in the highest

15% of luminance and assigned them a value of 1. It then counted how many 1's there are in each region to obtain as a result the number of pixels (particles) per area. Levels of value 0.1, 0.15, 0.2, 0.3, 0.4, 0.6 and 0.8 were used with Matlab program and data was processed for analysis in a spreadsheet. The level that provided a number of particles (cells) per area (cell density) proportional to the SPRi signal (reflectivity variation) in a given time (2 hours) for each VHH was selected for cell counting. Values of the approximation to the apparent association constant of a VHH were estimated as the ratio of SPRi reflectivity after 2 hours of incubation over the initial reflectivity value, measured just after cells were added on the biochip (Table 1).

2.9. VHH sequence analysis

The anti-endoglin VHH sequences were analyzed by BLAST (NCBI), edited using Mega 7 and LALIGN from ExPaSY of the Swiss Institute of Bioinformatics (SIB). VHH sequences were aligned with the MUSCLE program (EMBL-EBI). VHH, homology modeling was performed by the SwissModel ExPasy.

2.10. Ethical issues

The research activities using alpacas were approved by the Committee of ethical use of animals (CIEA) of Universidad Peruana Cayetano Heredia.

3. Results

A VHH cDNA library (2.7×10^7 phages/ml) was screened for endoglin affinity by phage display; 376 phage colonies were randomly picked and evaluated by Phage-ELISA, which resulted in 19 VHH-phages with OD₄₅₀ values over three times higher than the unrelated antigen Fas2 that was used as a control (data not shown). These nineteen VHHs were cloned and expressed in *E. coli*, and the culture supernatant containing the recombinant VHH was evaluated by endoglin-ELISA resulting in 13 recombinant VHHs positive to endoglin (anti-endoglin VHHs) with OD₄₅₀ values higher than 0.096 the cut-off of the assay: VHH1, VHH2, VHH3, VHH7, VHH8, VHH9, VHH11, VHH17, VHH18, VHH20, VHH22, VHH26 and VHH31; 6 VHHs had OD₄₅₀ values lower than the cut-off

(Figure 1). The anti-endoglin VHHs were purified from the culture supernatant by Ni-NTA affinity chromatography resulting in at least 5 mg of each anti-endoglin VHH per 100 ml batches (Figure 2).

Two strategies can be used for VHH protein arraying on the biochips: 1. either by forming SAMs with thiolated linkers with activated esters, followed by VHH arraying and ethanolamine blocking, or 2. conjugation of thiolated linkers with activated esters on the VHH proteins first and then arraying on the biochip. Due to poorly reproducible results in the formation of activated SAMs (data not shown) and because the second strategy is overall faster, VHH proteins were then conjugated to a thiol moiety and immobilized by monolayer self-assembling on gold covered biochips. Various ratios of linker to VHH proteins were tested to control the functionality of the proteins (data not shown). The best compromise in terms of functionality and efficient engraftment was obtained with a 10:1 molar ratio of 2,5-dioxopyrrolidin-1-yl 11-sulfanylundecanoate linker (Merck) and VHH protein. Thiol-coupling reaction at pH 7.4 favors incorporation of the thiolated linker at N-terminus over ϵ -amine of Lysine residues in the anti-endoglin VHH [22]; each anti-endoglin VHHs varied in Lysine content in the framework region in a range between 3 to 9 Lysine residues. In order to diminish the loss by inconvenient VHH positioning when adsorbed on the biochip, the concentration of anti-endoglin VHH was upped five times, *i.e* from 0.1 μ l to 0.5 μ l at 6.6 μ M (approximately from 0.66 pmoles to 3.33 pmoles) were deposited in each spot. Thiol-functionalized VHHs were manually loaded over a spot to get adsorbed and stabilized by gold-thiol interactions in the biochip [23], [24]. Each array was prepared by loading the 13 anti-endoglin VHHs, anti-endoglin EngVHH17 as positive control and anti-Fas2 VHH as a negative control in a particular arrangement for each assay.

Control experiments with recombinant CD105 proteins flowed over VHHs microarray (250ng/mL) have been carried but showed low consistency and poor reproducibility (data not shown). It seems better results might be obtained with much higher CD105 recombinant protein concentrations, but could not be carried due to the high cost of such controls. For this reason, it was preferred to

run assays with expressing CD105 whole cells, which was also way more relevant on a biological point of view. This would represent an advantage of SPR imaging with CD105-expressing whole cells, which are easier to access than recombinant proteins.

Once the SPR signal was stabilized, a cell suspension (1×10^6 cell/ml) was flowed over the biochip and kept at 37 °C while proceeding the binding assay. All 13 anti-endoglin VHHs showed a reflectivity variation ranged from 3.59 to 5.54% (average $4.66 \pm 1.18\%$) (Figure 3A), while the SPRi signals of VHHs features with THP-1 cells (not expressing endoglin) were not as high as observed with SC cells and close to background level (Figure 3B). The SC cells binding to anti-endoglin VHHs reaches a plateau after 150 minutes (Figure 3A). The drop in reflectivity observed after 4 hours is caused by the removal of the cell suspension by the injection of running buffer (IMDM medium) into the incubation chamber. THP-1 cells are obviously not captured by any of the arrayed anti-endoglin VHHs as shown in the corresponding sensorgrams (Figure 3B). Relative SPR responses recorded during SC cell incubations on VHHs biochip were calculated from the changes in the SPR signal 2 h after the addition of the cells in reference to the initial SPR signal without cells. This value enables the comparison of affinities of arrayed VHHs proteins toward whole cells incubated in culture medium. Indeed, on the contrary to a bi-molecular interaction model, affinity constants cannot be calculated here as a single cell is interacting with a series of VHH proteins arrayed on each spot. The washing step following the cell incubation removes most SC cells captured on VHHs probes, although for some probes, some cellular debris remain bound to the microarray possibly due to different affinities and/or stabilities of the cell/VHH interactions. This phenomenon is not observed for THP-1 cell, which are not interacting with the arrayed VHH proteins.

Interestingly, individual cells captured on VHH spots appeared as individual and countable objects as described elsewhere on similar SPR imaging of captured cells [1]. In order to quantify the number of cells captured on the biochip surface, the grey level of SPR images that provided the cell densities

(cells/area) in order to be proportional to the SPRi signals (reflectivity variation) for the anti-endoglin VHHs was chosen. In the assays, cell counting was performed for each VHH from the differential images captured at 30, 60, 90, 120 and 150 minutes after cell addition using different grey level values ranging from 0 to 1. The 120 minute (2 hours) differential image was selected as it allowed the best cell counting and the cell densities of each VHH were compared with their SPRi signals obtained at the same time of assay. To estimate the cell density for each VHH in the assays with SC cells, grey values of 0.1 and 0.15 were chosen and for the assays with THP-1 cells was 0.3, according to visual comparative analysis and Pearson's coefficient (values equal to or greater than 0.8). Significant differences in SC cell densities were observed among anti-endoglin VHHs arrayed in the biochip (Figure 4A), while images with THP-1 cells appeared as non-specifically interacting the VHH microarray (Figure 4B).

Cell density was estimated as the average value from three independent assays with SC cells captured by the anti-endoglin VHH in an area of $2.28 \times 10^5 \mu\text{m}^2$ at the spot and values ranged from 412 to 748 cells/area (Figure 5A): VHH17 (748 cells/area), VHH7 (713 cells/area), VHH31 (621 cells/area), VHH20 (697 cells/area), EngVHH17 (659 cells/area), VHH3 (627 cells/area), VHH2 (620 cells/area), VHH22 (576 cells/area), VHH9 (558 cells/area), VHH8 (550 cells/area), VHH18 (507 cells/area), VHH26 (417 cells/area), VHH1 (412 cells/area), VHH11 (473 cells/area); a linear correlation between counted cell densities and SPRi reflectivity variations was observed (Figure 5B). Assays with THP1 cells showed no correlation between cell density and SPR reflectivity variation (data not shown). Interestingly, we did not observe any reversible release of cells. Such effect might be due to cooperative effects of multiple binding sites present in each arrayed feature along with the recruitment of CD105 biomarker on cell surfaces as described elsewhere on a similar set-up [1].

Cell binding anti-endoglin VHHs can be grouped in two sets based on the density of cells captured on VHH ROIs (Figure 6A): VHH17, VHH7, VHH 31, VHH20, EngVHH17, VHH2, VHH3 with high cell density > 600 cells/area, and

VHH1, VHH22, VHH8, VHH9, VHH11, VHH18, VHH26 with low cell density < 600 cells/area. No correlation was found between cell density and ELISA, neither between SPRi reflectivity variation and ELISA. VHH31 and EngVHH17 showed a high ELISA value (OD450 > 0.3) and high cell count (cell density > 600). A similar distribution is observed with SPRi reflectivity variations (Figure 6B).

Apparent affinity constants (affinities) of the VHHs were estimated from the values of SPRi reflectivity variations 2 hours after the addition of the cells with respect to the initial SPR reflectivity variations (without the cells) of SC cells (Table 1). Such data are describing interactions between microscopic objects (cells), with individual molecular binding motifs immobilized on the surface. For this reason, apparent affinity constant are useful to assess the affinity of a cell type, or cell line with micro-arrayed probes, but cannot be extended to the calculation of kinetic parameters.

4. Discussion

In the present work, the evaluation of 13 anti-endoglin VHHs by cell-based SPRi with SC and THP1 cell lines is presented. SPR images of cells captured by microarrayed VHHs showed that several anti-endoglin VHHs specifically bind SC cells but not THP-1 cells. SC cells showed a binding kinetics that reached a plateau after 1.5 h, as observed on SPR sensorgrams, which might be caused by factors such as mass/volume dependent diffusion of cells, the VHH avidity and cell surface protein depletion as shown on other cell types screened on IgG microarrays [1]. During the assay, SC cells remained bound to anti-endoglin VHHs on the microarray that might allow the study of cellular response of SC cells to endoglin ligands or to other factors.

The SPR images with SC and THP-1 cells led to the conclusion that SC cells captured in the biochip resulted from the specific binding of anti-endoglin VHHs to the extracellular domain of endoglin expressed on SC cell surface. Three lines of evidence support this conclusion: first, SC cells images corresponded to

the spot arrangement of VHHs in the biochip; second, THP-1 images did not exhibit any VHH affinity with SPR responses similar to negative controls; third, a linear correlation between SPR signal and cell density ($R^2= 0.87$) was observed in SC cell assays but not in THP-1 cells.

The variation in SC cell densities estimated by SPRi on each VHH spot suggests differences among the 13 anti-endoglin VHHs in both avidity and accessibility to the epitopes of endoglin in the surface of the plasma membrane of living SC cells, which was not detected by a conventional ELISA. In endoglin-expressing cells, the endoglin protein is surrounded by the plasma membrane containing structural components such as glycocalyx, other peripheral and integral membrane proteins that might not access to epitopes recognized by VHHs.

The SPRi results thus suggested that anti-endoglin VHHs bind to epitopes located in the extracellular domains of endoglin. These are the N-terminal orphan domains that contain the binding site to TGF β ligands such as BMP9 [16], [25], and the bi-partite ZP located adjacent to the membrane, which is involved in oligomerization and interaction to TGF β receptors I and II [16].

The results of cell densities suggested that anti-endoglin VHHs can be distributed in two groups with different abilities to bind membrane-bound endoglin in SC cells, which can result in VHH avidity variation, epitope accessibility or both. Epitope mapping to identify the binding site of the anti-endoglin VHHs and whether the VHH inhibit the binding of BMP9 or TGF β to endoglin or have an effect on angiogenesis is a matter of further investigation.

SPRi results and VHHs microarrays suggested the feasibility to investigate, in real-time and in a label-free process, structural and functional characteristics of endoglin involved in TGF β signaling in living cells. Moreover, interactions between endoglin and cognate ligands could be developed as a high throughput assay with a complex cell population as far cells are kept bound stable to the biochip and cell viability is not affected by the assay conditions [3], **Erreur !**

Source du renvoi introuvable. The cell-based SPRi in conjunction with anti-endoglin VHH microarrays can thus be used both in studies aimed at a better understanding of endoglin function in living cells and in application as nanoprobe for conditions in which endoglin plays an important role.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Supplement material

Video of SC cells being captured by anti-endoglin VHH registered by SPRi.

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Figures and legends

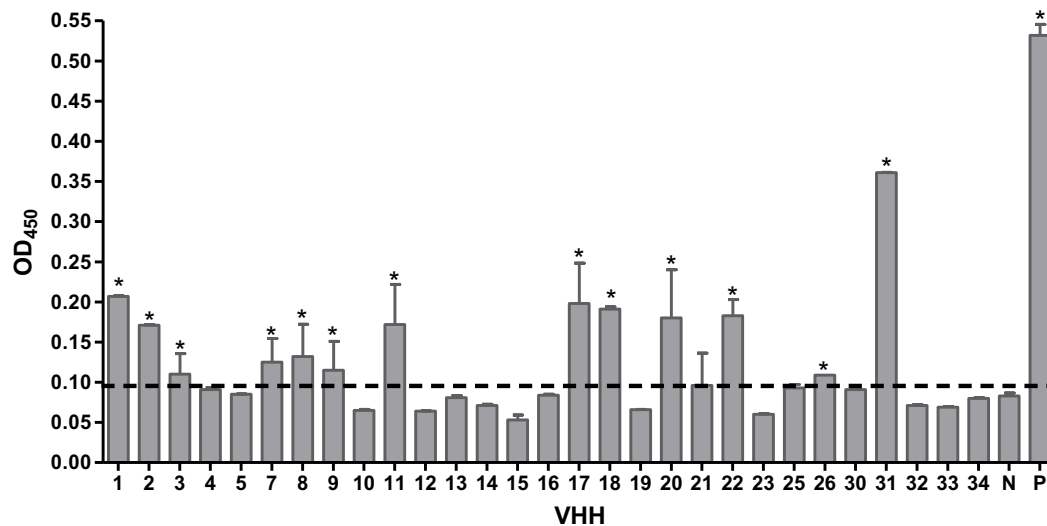


Figure 1. Endoglin-ELISA of VHH antibodies expressed in *E. coli*. 13 VHHs were positive to endoglin ELISA (*) and 6 were negative as OD₄₅₀ values were lower than cut-off (OD₄₅₀= 0.096). N, negative control was anti-Fas2 VHH; P, positive control, EngVHH17. Each OD₄₅₀ value is the average of a triplicate assay, the cut-off value (dotted line) was set as the anti-Fas2 VHH O₄₅₀ mean plus 3 Standard Deviation (SD).

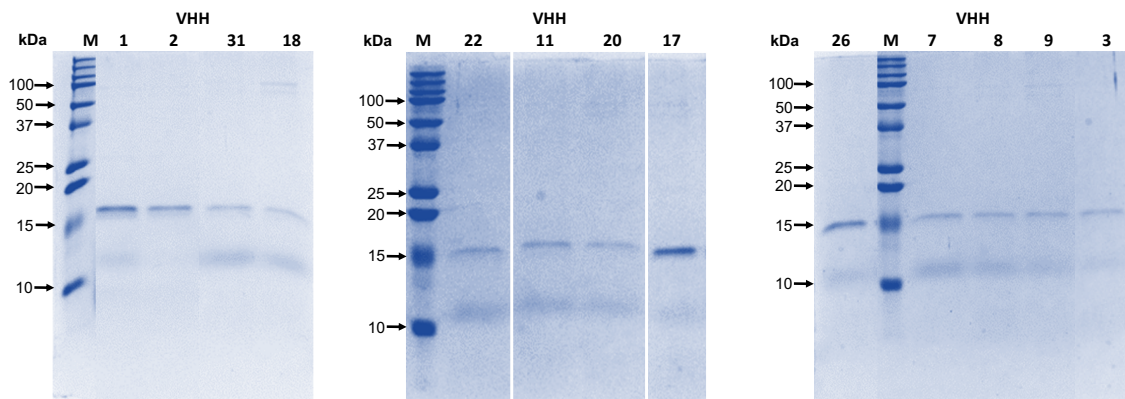


Figure 2. Purified anti-endoglin VHHs. Anti-endoglin VHHs were expressed in *E. coli* and purified by Ni-NTA agarose chromatography. VHHs (~15 kDa) were electrophoresed in 15%-SDS PAGE stained with Coomassie blue. M, Kaleidoscope Prestained Standards (BioRad).

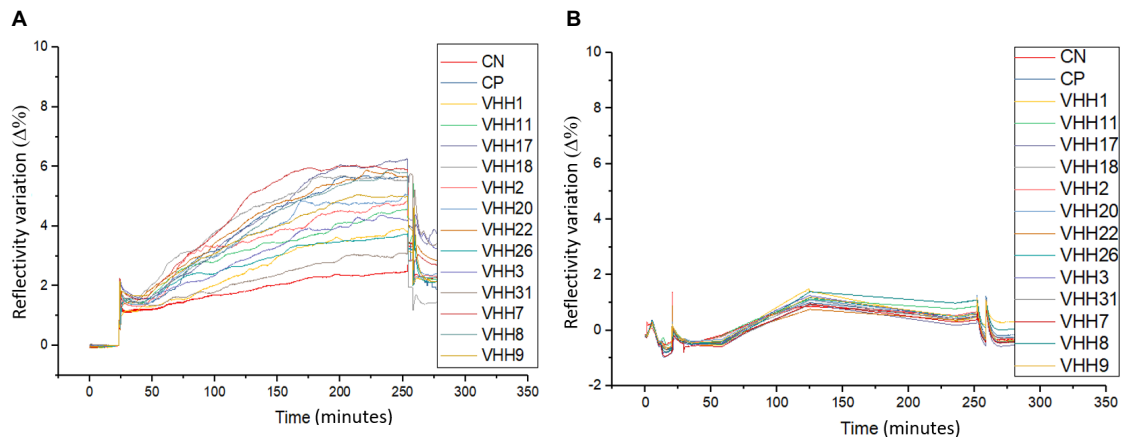


Figure 3. SPR of cells bound by anti-endoglin VHHs in the biochip. (A) SC cells. (B) THP-1 cells. Sensorgrams of cell binding to anti-endoglin VHH to by anti-endoglin VHH on the biochip. Cells were flowed over the sensor surface and binding was followed for 2.5 h before washing with culture media. The assay was performed using as a negative control, anti-Fas2 VHH; as a positive control, anti-endoglin EngVHH17. VHH1, VHH2, VHH3, VHH7, VHH8, VHH9, VHH11, VHH17, VHH18, VHH20, VHH22, VHH26, VHH31.

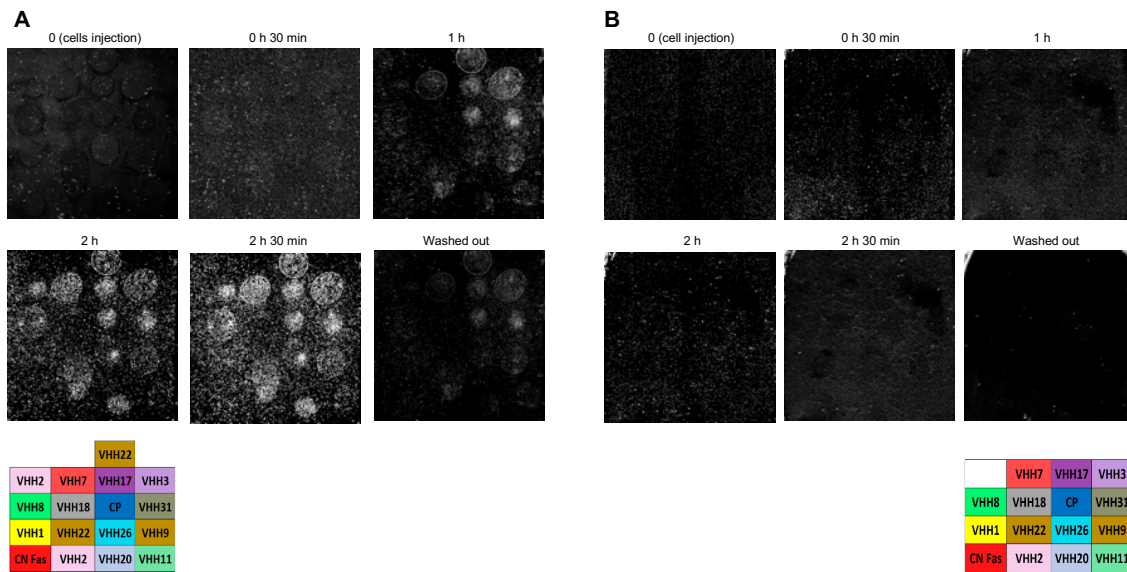


Figure 4. SPRi of the time course of cells binding to anti-endoglin VHHs on the biochip. (A) SPR Images of SC cells incubated on the anti-endoglin VHHs microarray during the course of the assay (2.5 hours). (B) SPR Images of THP1 cells incubated on the anti-endoglin VHHs microarray during the course of the assay (2.5 hours). The assay was performed using as a negative control, anti-Fas2 VHH; positive control anti-endoglin EngVHH17 and VHH1, VHH2, VHH3, VHH7, VHH8, VHH9, VHH11, VHH17, VHH18, VHH20, VHH22, VHH26, VHH31. VHHs were arrayed on the biochip as shown in the colored boxes. SPR Images were taken at 0, 30 m, 1 h, 2 h, 2 h 30 m, and after the washing out SC cells. Grey level was 0.15.

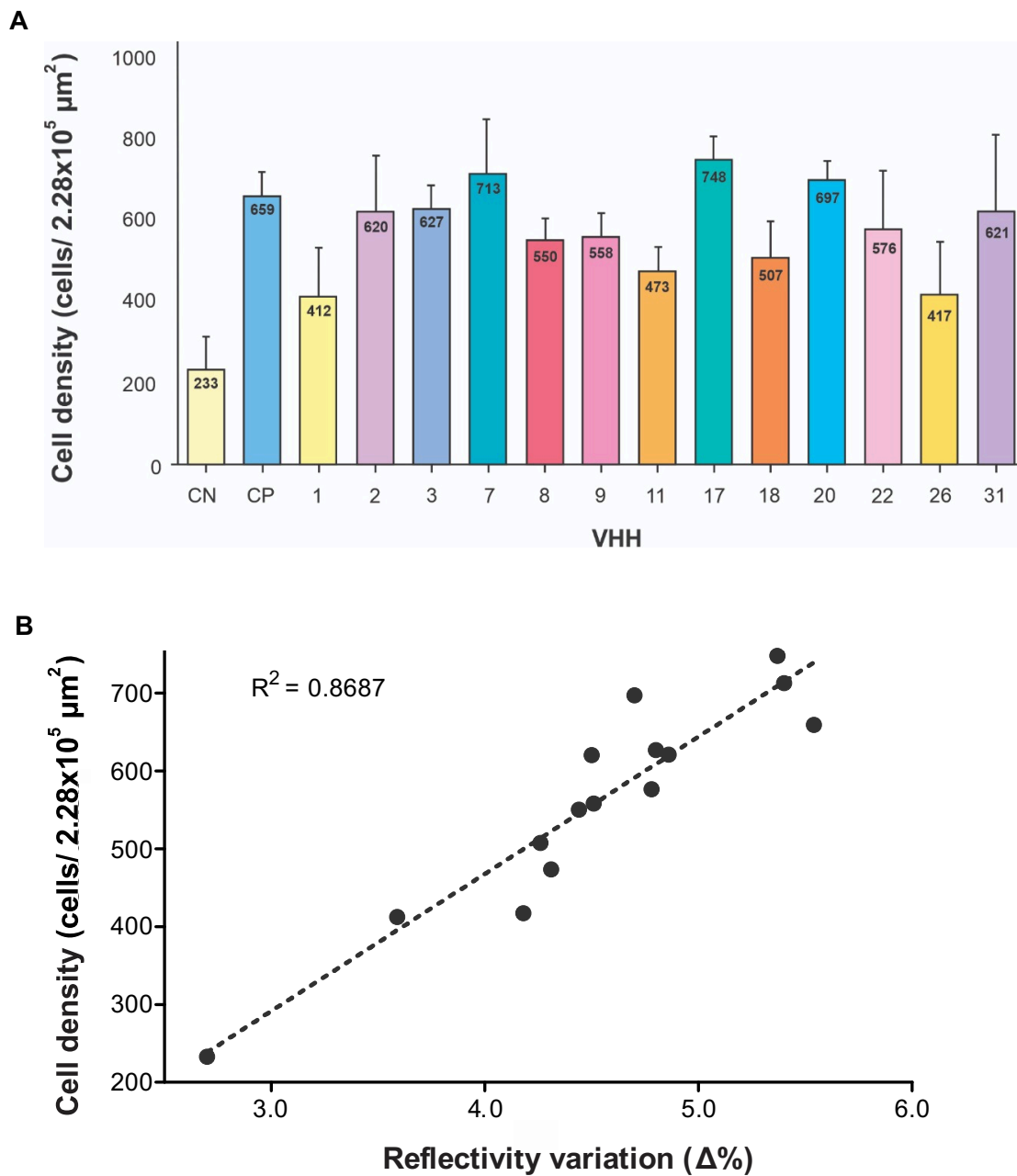


Figure 5. Cell density of SC cells bound by anti-endoglin VHH on the biochip. (A) Cell density for each VHH was estimated as the average number of cells bound by the anti-endoglin VHH in an area of $2.28 \times 10^5 \mu\text{m}^2$ at the spot. Values were estimated from the average of three independent assays using as a negative control (CN), anti-Fas2 VHH; positive control (CP), EngVHH17. Standard deviations (SD) are shown as bars over the columns (B) Linear correlation Cell density vs Response Unit.

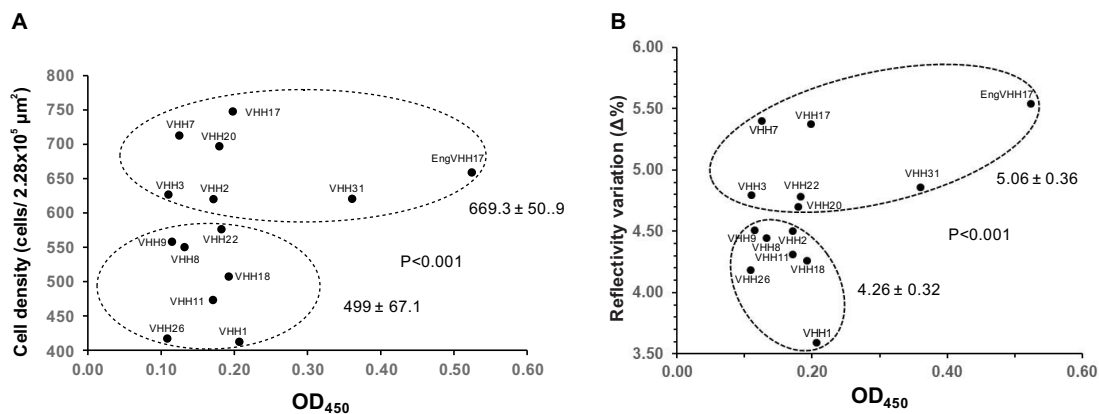


Figure 6. Cell density and anti-endoglin ELISA of VHHs. (A) VHHs grouped in two sets according to capacity of binding SC cells on the biochip: VHH17, VHH7, VHH20, EngVHH17, VHH3, VHH2, VHH31 with cell densities higher than 600 cells/area; VHH22, VHH9, VHH8, VHH18, VHH11, VHH26, VHH1 with cell density lower than 500 cells/area. $P < 0.001$, Mann Whitney test. Cell density was estimated as the average value from three independent assays with SC cells captured by the anti-endoglin VHH in an area of $2.28 \times 10^5 \mu\text{m}^2$ at the spot. (B) VHHs were grouped in two sets according to RU values: VHH17, VHH7, VHH20, EngVHH17, VHH3, VHH22, VHH31 with RU values in the range 5 ± 0.36 ; VHH2, VHH9, VHH8, VHH18, VHH11, VHH26, VHH1 with RU values in the range 4.26 ± 0.32 . $P < 0.001$, Mann Whitney test. OD₄₅₀ ELISA values of each anti-endoglin VHH were estimated as previously described in Fig. 1.

Table 1. Relative SPR response after a two-hours SC cell incubation on the VHHs biochip.

VHH	Relative SPR response
EngVHH17	1.1170
VHH17	1.1171
VHH7	1.1227
VHH20	1.1003
VHH3	1.1017
VHH31	1.1006
VHH2	1.1014
VHH22	1.1074
VHH9	1.0968
VHH8	1.1043
VH18	1.1019
VHH11	1.0922
VHH26	1.0922
VHH1	1.0863