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RESEARCH ARTICLE

A recombinant dromedary antibody fragment (VHH or nanobody) directed against human Duffy antigen receptor for chemokines

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Abstract Fy blood group antigens are carried by the Duffy antigen receptor for chemokines (DARC), a red cells receptor for *Plasmodium vivax* broadly implicated in human health and diseases. Recombinant VHHs, or nanobodies, the smallest intact antigen binding fragment derivative from the heavy chain-only antibodies present in camelids, were prepared from a dromedary immunized against DARC N-terminal extracellular domain and selected for DARC binding. A described VHH, CA52, does recognize native DARC on cells. It inhibits *P. vivax* invasion of erythrocytes and displaces interleukin-8 bound to DARC. The targeted epitope overlaps the well-defined DARC Fy6 epitope. K_D of CA52–DARC equilibrium is sub-nanomolar, hence ideal to develop diagnostic or therapeutic compounds. Immunocapture by immobilized CA52 yielded highly purified DARC from engineered K562 cells. This first report on a VHH with specificity for a red blood cell protein exemplifies VHHs' potentialities to target, to purify, and to modulate the function of cellular markers.

Keywords Duffy antigen receptor for chemokines · VHH · Atypical chemokine receptor · *Plasmodium vivax* · Immunoaffinity · Cancer · Inflammation · HIV

Abbreviations

DARC	Duffy antigen receptor for chemokines
ECD1	First extracellular domain
CNRGS	Centre National de Reference pour les Groupes Sanguins

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Introduction

DARC or Duffy antigen receptor for chemokines is a protein of the human red cell membrane of paramount importance for human health [1–3]. It is a blood group protein. Two antithetic antigens have been described, Fy^a and Fy^b, corresponding to a single polymorphism in the extracellular part of the molecule [4]. Another antigen named Fy3 is less precisely defined at the molecular level, and may be responsible for human allo-immunization. A fourth important antigen present on both Fy^a and Fy^b allotypes is called

Fy6, it was defined through monoclonal antibodies preparation [5, 6] though no human antiserum exists which recognizes it. Two other genetic conditions have to be mentioned: one relatively rare called $Fy^{b\text{-}weak}$ corresponds to the low level membrane expression of the protein at the red cell surface; the point mutation in reading frame linked to this condition has been elucidated [7]. The other, called Fy-negative, is very widespread among West African blacks (98%) and African Americans (68%) and characterized by the specific extinction of DARC expression on cells of the erythrocytic lineage [1]. People with this genetic property are said to be Duffy- or Fy-negative. This selective extinction is due to a mutation in a GATA box of the DARC promoter region [8]: the GATA transcription factor is driving DARC expression in cells from the erythrocytic lineage while other factors are operating in tissues where DARC is expressed like endothelial cells from the post-capillary venules, Purkinje cells, epithelial cells in the kidney collecting duct [9, 10]. The Fy-negative phenotype has been found in Caucasian people but it is linked to deletion or point mutations in the reading frame of DARC [11].

The linkage of DARC to human pathology was discovered some 40 years ago when it was shown that Fy-negative people were resistant to malaria induced by *Plasmodium vivax* [12]. The role of DARC as a receptor for *P. vivax* merozoite was confirmed by several reports including detailed molecular studies on DARC and *P. vivax* Duffy Binding protein (PvDBP) [13–16]. Furthermore, these studies created hope that vaccination against *P.vivax* malaria might be possible [17, 18]; however, this notion is challenged by recent observations which suggest that resistance of Fy-negative red cells to invasion is not as absolute as originally anticipated [19–21].

Cloning of DARC cDNA [22] and of its gene [23], and the discovery in the early 1990s that DARC is a ligand for chemokines [24], were milestones in DARC studies. Indeed, DARC binds numerous chemokines of both the CC and CXC groups [25]. DARC has the topology characteristic of the 7TM domain GPCR family; however, the intracellular DRY sequence which is necessary for GPCR to interact with G protein and trigger an activation cascade in response to an extracellular stimulus is missing in DARC. Three other 7TM GPCR-like proteins, namely D6, CCXCKR and CXCR7, are known; they also bind several chemokines and are called collectively chemokines interceptors or atypical chemokines receptors [26]. Even though several uncertainties remain regarding their exact physiological role, they are thought to participate in the regulation of the chemokines network. With regard to DARC, today a mass of epidemiological and experimental data, on whole organisms (e.g., genetically modified mice) and also on isolated cells, suggest that DARC is involved in cancer development and its metastatic potential [27–30], in inflammation, and in inflammatory disease [31–38]. Mechanisms which might explain the role of DARC are far from clear in many cases, but there is a growing consensus that chemokine binding is probably central to the pleiotropic effects of DARC [36–38]. Lastly, it should be mentioned that DARC might be involved in HIV binding to red cells, HIV contamination, and AIDS progression, but the ongoing studies have led to somewhat conflicting results [39–45].

Taking into account the certain but complex role of DARC in human diseases, we decided to develop new tools for DARC studies based on VHHs, i.e. the recombinant derivatives of camelid immunoglobulins. Two IgG subclasses of camelid immunoglobulins are composed of heavy chains-only and lack all light chains [46]. The variable region of these heavy chains-only antibodies is easily cloned from lymphocytes from naive or immunized camelids. These fragments, known as VHHs or nanobodies, are the smallest intact antigen-binding fragment derivative endowed with the properties of an authentic antibody. The VHHs are famed for a good expression yield and an excellent solubility compared to most mice or human two chains-derived antibody fragments [review in 47]. They may be used in a range of assays [48], for protein purification [49], and as an aid to crystallization [50]. Multiple therapeutic and diagnostic agents have been or are being developed from VHHs [51, 52].

We immunized a dromedary with the N-terminal extracellular domain of DARC expressed in *E. coli*. This domain (referred to as ECD1) carries the polymorphic site responsible for the Fy^a/Fy^b allotypes and the Fy6 epitope, and participates in the binding of chemokines and PvDBP. We built a VHH library from dromedary's lymphocytes and screened this bank for antigen-specific VHHs also using *E. coli*-produced proteins. Several DARC-specific VHHs were obtained, and in this report we further focus on one of these, referred to as CA52. The linear epitope of CA52 was identified, CA52 is perfectly able to recognize the glycosylated protein present on human cells, although the immunogen was a non-glycosylated protein. Furthermore, CA52 interferes with the interleukin-8 binding to DARC and *P.vivax* infection of red blood cells. Moreover, the CA52 attachment to a gel generates a powerful adsorbent to purify native DARC in a single step from a detergent extract of cells.

Materials and methods

Prokaryotic constructs for immunisation of the dromedary, library screening and preliminary epitope mapping of the VHH

The antigens used for immunization were derived from DARC ECD1. The two allotypic variants of ECD1 with

wild-type sequences, and also ECD1 in which tyrosine 31 and 46 had been mutated to Glu (in order to mimic sulphated tyrosines [15]), were fused at the N-terminus of *Staphylococcus aureus* nuclease. Proteins used for screening contained wild-type ECD1 sequences fused to the C-terminus of GST (pGEX vector; GE Healthcare). Other ECD1-nuclease constructs were prepared in which deletions were introduced in ECD1 to facilitate quick identification of epitope location recognized by VHH. Sequences of all constructs are shown in Table 1.

ECD1 variants fused to *Staphylococus aureus* nuclease were cloned to a plasmid obtained as a much appreciated gift from Prof. D. Engelman from Yale University. It is an early variant of the T7 family of plasmids [53].

All proteins were produced in BL21 *E. coli* cells (Stratagene, La Jolla, CA, USA) grown in autoinduction medium [54]. Whole cell extracts were obtained by passing a bacterial pellet through an Emulsiflex-C5 Avestin (Ottawa, Canada) homogenizer. Nuclease constructs were purified from whole cell extracts by chromatography on Capto-S ion exchanger (GE Healthcare Biosciences, Uppsala, Sweden) equilibrated in 20 mM MES buffer pH 6.0 containing 6 M Urea. Target protein was eluted by a NaCl concentration gradient, then further purified and desalted by RPLC on a 1 cm diameter C4 column (Vydac, Hesperia, CA, USA) operated with trifluoroacetic acid, water, acetonitrile mobile phases.

GST constructs were purified on immobilized glutathione purchased from GE Healthcare and operated according to recommendations of the support manufacturer. Product was further purified and desalted by RPLC performed as above. Purified proteins were recovered in dried form using a Speed Vac apparatus (Savant Instruments, Holbrook, NY, USA).

Preparation and screening of the VHH library

The camel immunization protocol followed the animal experimentation guidelines published by the Regional Canary Government and was approved by the Ethic Commission of the Veterinary Faculty, University of Las Palmas. Under the protocol, 1 mg of each antigen (four different proteins, see Table 1) was injected subcutaneously once a week for 5 consecutive weeks. Complete Freund's adjuvant was used for the first injection and incomplete Freund's adjuvant for the following injections. Then a period of 3 weeks without immunization was taken before the animal received two boost injections at a 1-week interval. Blood was taken on EDTA anticoagulating medium 1 week after the last boost.

Construction of the VHH library followed well-established steps [48–52 and references therein]. Lymphocytes were isolated from dromedary's blood and cDNA was prepared. DNA fragments coding for VH (present in conventional antibodies) and VHH (present in heavy chain-only antibodies) were amplified using a suitable primer pair. DNA fragments encoding the VHHs were purified on a preparative agarose gel and re-amplified by nested PCR. The amplicons of the second PCR were ligated into pHEN4 phagemid vector. DNA present in plasmid encodes the VHH followed by a hemagglutinin tag, an amber stop codon, and the g3 protein from M13 phage. Expression in permissive TG1 cells in presence of helper phage M13KO7 produces bacteriophage particles that display the VHH at their tip. In the absence of helper phage, VHH may be recovered from a periplasmic extract of TG1 cells. The VHH library expressed on phage particles was screened for binders to ECD1-GST constructs with wild-type Fy^a and Fy^b sequences coated on ELISA plates. Antigen recognition by VHH from the periplasmic extracts of isolated clones was evaluated by ELISA.

Table 1 Sequences of constructs used in this study

Antigens used for immunization	MATAVHRAELSPSTENSSQLDFEDVWNSSYGVNDSFPDGDYG/DANLEAAAPCHSCNLLDDSALPAAKK-***(nuclease)***
	MATAVHRAELSPSTENSSQLDFEDVWNSS**E**GVNDSFPDGD**E**G/DANLEAAAPCHSCNLLDDSALPAAKK-***(nuclease)***
Antigens for library screening	***(GST)***-GILGNCLHRAELSPSTENSSQLDFEDVWNSSYGVNDSFPDGDYG/DANLEAAAPCHSCNLLDDSASSGRIVTD
Deletion mutant I	MATAV---------ENSSQLDFEDVWNSSYGVNDSFPDGDYGANLEAAAPCHSCNLLDDSALPAAKK-***(nuclease)***
Deletion mutant II	MATAVHRAELSPSTENSS------------GVNDSFPDGDYGANLEAAAPCHSCNLLDDSALPAAKK-***(nuclease)***
Deletion mutant III	MATAVHRAELSPSTENSSQLDFEDV---------SFPDGDYGANLEAAAPCHSCNLLDDSALPAAKK-***(nuclease)***
Deletion mutant IV	MATAVHRAELSPSTENSSQLDFEDVWNSSYGVND------------AAAPCHSCNLLDDSALPAAKK-***(nuclease)***
Deletion mutant V	MATAVHRAELSPSTENSSQLDFEDVWNSSYGVNDSFPDGDYGANLEAAAP----------ALPAAKK-***(nuclease)***

GST Glutathione S-transferase

Residues which belong to the first extracellular domain of DARC are underlined, other residues belong either to the fusion partner (indicated in parentheses) or are flanking residues which were introduced through the construction of the plasmid. A larger font is used to show the allotypic variants (either G or D at position 42 of ECD1). Positions of mutants of Y to E at positions 31 and 40 are shown with a larger and bold font

Subcloning, expression and purification of VHH

VHHs were subcloned in a vector named pHEN6cPm derived from already described plasmids. Translation product contains the PelB-leader sequence, the VHH sequence, HA tag, and finally a six His sequence. Expression of the VHH was done using TB medium (1 l) and fermentation left to proceed from 14 to 20 h at 28°C after isopropyl thiogalactoside induction. Periplasmic extract was prepared by osmotic schock and VHH purified using a 10-ml Nickel-loaded His-select column (Sigma, L'isle d'Abeau, France). The VHH concentration was calculated from the absorbance measurement at 280 nm using an extinction coefficient computed on Expasy website (http://www.expasy.ch/tools/).

SDS-PAGE and western blotting

SDS-PAGE was performed in continuous 15% acrylamide minigels cast in Novex (Invitrogen, Carlsbad, CA, USA) cassettes. Gels were stained either with Coomassie Brilliant Blue or silver nitrate as indicated in legends to figures.

Blotting onto nitrocellulose membranes was performed in the semidry apparatus from Novex. The antibodies used were: (1) CA52 VHH (1 µg/ml), (2) anti-HA antibody (Clone 16B12) purchased as ascitic fluid from Covance (Emeryville, CA, USA) at 1:1,000 dilution; (3) 2C3, an anti Fy6 antibody (1 µg/ml) [6]; (4) an anti-*Staphylococcus aureus* nuclease rabbit antiserum developed in house and diluted 1:2,000; and (5) as needed peroxidase conjugated anti-mouse or anti-rabbit antibodies (antisera from P.A.R.I.S., Compiègne, France) diluted 1/800. Solution for diluting antibodies was Tris buffered saline containing 5% skimmed milk. Incubations with the different antibodies were performed overnight at 4°C or 1 h at 37°C. Washings were in Tris buffered saline supplemented with 0.5% (v/v) Tween 20. The ECL kit (GE Healthcare Biosciences) was used according to the manufacturer's recommendations for chemiluminescent detection of the primary antibodies.

Epitope mapping by western blots of ECD1 deletion constructs and synthetic peptides on plastic pins

Western blot using ECD1 deletion mutants was used as a preliminary and fast specificity screen of VHH. More precise identification of the epitope recognized by CA52 was performed using synthetic peptides grafted at the tip of plastic pins. The epitope scanning kit was purchased from Chiron Mimotopes (Clayton, Victoria, Australia). The syntheses were done by stepwise elongation of the peptides from C- to N-terminus, following the manufacturer's instructions. A set of octapeptides covering the sequence of amino acids 17–30 of the Duffy ECD1 and 95 analogues of pentapeptide FEDVW were synthesized. Binding of purified CA52 VHH to immobilized peptides was determined by an enzyme-linked immunosorbent assay as described earlier [6]. Briefly, the pins arranged according to the format of a 96-well microtiter plate [55] were blocked by immersing in 2% BSA, in TTBS (0.1% Tween-20 in Tris buffered saline) contained in wells of an ELISA plate (200 µl/well), and incubating for 2 h at room temperature. The pins were subsequently incubated in wells containing 150 µl of: CA52 (50 ng/ml in TTBS, overnight, 4°C), anti-HA mouse monoclonal antibody (purified antibody, clone 16B9 Covance, 0.5 µg/ml in TTBS, 1 h, room temperature) and alkaline phosphatase-conjugated rabbit anti-mouse IgG (Dako, 1,500-times diluted in TTBS, 1 h, room temperature). After each step, the pins and the wells were washed five times in TTBS. Color reaction was developed with phosphatase substrate tablets (Sigma). The absorbance was measured at 405 nm in a microtiter plate reader. The peptides, except those for replacement analysis, were synthesized at least in duplicate and the results are mean values of at least two ELISA tests. Reaction with anti-HA antibody and tagged anti-mouse alone was determined as a control and gave low optical density values. Final results (presented in Fig. 2, below) are differences of readings obtained with CA52 followed by anti-HA and tagged anti-mouse antibodies and those obtained with only anti-HA followed by tagged anti-mouse.

In silico studies: proposition of pertinent VHH structural models

Structural templates have been searched with PSI-BLAST software [56]. Two VHH protein structures extracted from the Protein Data Bank [57] were used as templates to build the structural models (PDB codes: 1JT0 [58] and 1OP9 [59]). The protein sequences have been aligned with Clustalw2 software [60] including minor additional manual changes. The comparative modeling has been performed with the Modeller software [61]. The disulfide bond between residue 33 and 107 has been constrained. Two hundred independent structural models were produced and compared after superimposition with ProFit software (http://www.bioinf.org.uk/software/profit/). The mean structural model has been selected based on root mean square deviation values between the structural models. Its topology was checked using ProCheck software [62].

Binding kinetics of VHH to its ligands

Surface plasmon resonance (SPR) evaluation of kinetic rate constants of binding of VHH to its target on ECD1 has been performed in a Biacore ×100 instrument (GE Healthcare). Two kinds of ligands were used, one was wild-type ECD1

Table 2 Kinetic rate constants measured in the Biacore X100 instrument and the equilibrium dissociation constants derived from them

Ligand	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)
Prokaryotic construct	2.31×10^7	5.49×10^{-3}	2.38×10^{-10}
DARC from K562 cells	5.78×10^6	1.50×10^{-3}	2.59×10^{-10}
DARC from K562 cells after in situ deglycosylation	2.28×10^7	2.39×10^{-3}	1.05×10^{-10}

nuclease fusion construct (Table 2), the other was full size recombinant DARC purified from engineered K562 cells (see below for details on purification); prior to immobilization, DARC was dialyzed in Pierce cassettes (Rockford, IL, USA) to 10 mM HEPES buffer pH 7.4 buffer containing 0.3% $C_{12}E_9$ detergent, (Sigma). Ligands were immobilized on the analysis Fc2 channel of CM5 chips using NHS (N-Hydroxysuccinimide) and EDC (1-[3-(Dimethylamino)-propyl]- 3-ethylcarbodiimide hydrochloride) chemistry according to manufacturer's instructions. Wild-type nuclease has been immobilized on the reference channel when ECD1-nuclease construct was in Fc2 while dextran layer was simply activated and deactivated in the case of analysis with full length DARC.

Amounts of immobilized proteins were kept low in order to be safe from mass transfer limitation artefacts.

Concentrations of analyte (CA52) varied from 0.3 to 10 nM. Langmuir model for a 1:1 interaction was chosen to evaluate the data using manufacturer's software.

To further analyze binding of CA52 to DARC, experiments were also performed on in situ deglysocylated DARC as follows. The temperature of the instrument was set to 37°C and flow rate to 5 µl/min; 5,000 units of N-glycosidase (New England Biolabs, Ipswich, MA, USA) were dissolved in reaction buffer and injected manually onto Fc2 three times consecutively for 1,000 s each time. Deglycosylation was checked using *Sambucus nigra* lectin which is known to be retained by sugars terminated by sialic acid [63, 64]. Lectin was injected at several concentrations from 7 to 70 nM before and after deglycosylation.

Flow cytometry experiments

Erythrocytes of the various phenotypes were obtained from the Centre National de Reference pour les Groupes Sanguins, (CNRGS, Paris, France). Red cells were washed twice in phosphate-buffered saline (PBS). After the second wash, the cells were resuspended with either: (1) anti Fy6 murine monoclonal antibody 2C3 (culture supernatant diluted 1:8); (2) isotype control murine IgG (5 µg/ml; Becton–Dickinson, Franklin Lakes, NJ, USA); (3) periplasmic extract of TG1 cells producing CA52 diluted 1:2 in PBS/0.1% BSA; (4) periplasmic extract of non-transformed TG1 cells as control; or (5) purified CA52 (10 µg/ml). The suspension was left at room temperature for 1 h. After primary incubation, VHH or periplasmic extract incubated cells were washed twice in wash buffer and incubated for one additional hour in the presence of anti-HA monoclonal antibody (clone 16B9 as an ascitic fluid purchased from Covance, diluted 1:1,000). A control of red blood cells incubated with anti-HA alone was also prepared. Cells were washed again before 1 h incubation in the dark at room temperature with anti mouse IgG phycoerythrin(PE)-tagged antibody (Beckman Coulter, Villepinte, France) (5 µg/ml in PBS/0.1% BSA solution). The 2C3-treated and control cells were handled in parallel. After a final wash step in PBS, cells were analyzed by digital high speed analytical flow cytometry. Erythrocytes were identified based on forward and side scatter characteristics using logarithmic amplification. Excitation wavelength was 488 nm, PE signal was collected with a 585/42 band pass filter. Data were acquired by BD FACS Diva software (v.6.1.2), and analyzed using FlowJo (Treestar, Ashland, OR, USA) software v.7.2.5.

Flow cytometry was also used to characterize DARC expressing recombinant K562 cells [66]. Qifikit (Dako, Trappes, France) was used to measure binding sites number per cell.

Agglutination studies

In order to perform the agglutination studies, either: (1) crude periplasmic extract diluted 1:2 in PBS; (2) purified VHH dissolved at 10 µg/ml concentration in PBS and serial two-fold dilutions; or (3) purified 2C3 (10 µg/ml and serial two-fold dilutions) were incubated at room temperature with red blood cells from defined phenotypes obtained from CNRGS. Volume of suspension was 40 µl, hematocrit was 4%. After 60 min of incubation at room temperature, 20 µl of PBS containing anti-HA antibody (ascitic fluid Covance final dilution 1/100) were added to the VHH containing tubes and PBS to the 2C3-containing tubes. Incubation was left to proceed for a further 20 min. Finally, 20 µl of anti mouse IgG, raised in goat (P.A.R.I.S), diluted 1/100 in PBS were added, the suspension was incubated for 20 min. Then, 40 µl of the suspension were loaded on top of the Diamed (Cressier, Switzerland) column, which was then operated as recommended by the manufacturer. Controls with only anti-HA followed by anti mouse IgG, and only anti-mouse IgG, were also prepared and analyzed likewise.

Study of the inhibitory effect of CA52 on red cells invasion by *P. vivax*

This study was approved by the Committee on Human Rights Related to Human Experimentation, Mahidol University, Bangkok, MU-IRB 2009/133.2306. The inhibitory effect of CA52 on red cells invasion by *P. vivax* was evaluated using short-term culture conditions as described before [18]. Red blood cells naturally infected with *P. vivax* parasites were obtained from three volunteers living in Mae Sot, Tak province, Thailand. Informed consent was obtained from each volunteer before the blood collection was performed. Heparin was used as anticoagulant. Leukocytes were removed from the blood by filtration through Plasmodipur® filter (Chemicon, Millipore) and red blood cells were washed with McCoy's medium to remove plasma and heparin. CA52 was incubated with the *P. vivax*-infected blood for 2 h at 37°C at 25 µg/ml final concentration to allow saturation binding of CA52 on the red blood cell membrane. At the end of the second hour, excess CA52 was removed by washing the red blood cells with McCoy's medium twice at room temperature. The red blood cells were reconstituted to 2% cell suspension with McCoy's medium containing 30% heat-inactivated human AB serum and incubated for 24 h under the in vitro malaria culture conditions. Thin blood films were prepared at the end of incubation time and numbers of infected red blood cells at various stages, i.e., ring, amoeboid, schizont, gametocyte and free merozoite, were enumerated under microscope (×100 oil immersion). Numbers of parasites at various stages are expressed relative to 10^4 erythrocytes (Table 3).

An anti-lysozyme VHH (D3L11 [67]) was used and processed in the assay similarly to CA52; moreover, culture medium without VHH addition was used as a control of parasite invasion under the conditions used for the test.

Displacement of interleukin-8 bound to red blood cells by VHH

The interleukin-8 (IL8) binding studies were performed essentially as described [66]. Eppendorf tubes, each containing 10^8 red cells washed in PBS, were incubated with ^{125}I labelled IL8 (specific activity 2,200 Ci/millimole; Perkin Elmer, Boston, MA, USA) at 0.3 nM final concentration in a final volume of 100 µl PBS containing 1% w:v of Bovine Serum Albumin (PBS-BSA). All data points have been obtained in triplicate. Most of collected data points concerned Fy(a+b+) red cells but some were controls with Fy(a−b−) red cells obtained through CNRGS. Three tubes were prepared in which cold IL8 (Peprotech, Rocky Hill, NJ, USA) at 200 nM final concentration had been added together with radioactive cytokine. After incubation, the tube contents were transferred in long and narrow 0.5-ml centrifuge tubes containing 180 µl of an 85:15 mixture of DC550 silicon oil (Serva ref 35145) and Nujol oil (Alfa Aesar). They were centrifuged at 4,000*g* for 20 s. Cell pellets were dissolved in 100 µl dimethylsulfoxide and radioactivity counted in a Packard Tri Carb liquid scintillator. All obtained count numbers were reduced by the level of background counts measured for tubes containing cold IL8.

Preparation of immobilized CA52

An amount of 10 mg CA52 was brought to 0.2 M sodium carbonate pH 8.5 containing 0.5 M NaCl using a Millipore-Amicon ULTRA-15 centrifugal device fitted with a 5,000-Da molecular weight cut-off membrane. The VHH solution was then recirculated overnight in the cold room into a Hitrap 1 ml N-hydroxy succinimide activated column (GE Healthcare) prepared according to the manufacturer's recommendation. After that, the column was rinsed with

Table 3 In vitro merozoite infection assay

Sample code	VHH/controls	Rings[a]	Amoeboids[a]	Schizonts[a]	Gametocytes[a]	Free merozoites[a]	Total parasites[b]
PV1	CA52	1.5	0	0	0.5	1.0	2
	D3L11	5	1.5	1	0	1	7.5
	Complete medium (control)	5.8	0.5	0.3	0.8	0.3	7.4
PV2	CA52	0	0.5	0.5	0	1	1
	D3L11	0.5	0.5	4.0	0.5	0	5.5
	Complete medium (control)	1.0	0.5	2.5	0	0.5	4
PV3	CA52	0	0	0	0	0	0
	D3L11	0	0	0.5	1.5	0	1.5
	Complete medium (control)	0.8	0.3	0.3	0.8	0	2.2

[a] Total number of parasites forms in 10^4 red blood cells

[b] Free merozoites are excluded

ethanolamine in order to de-activate the potentially remaining reactive groups.

The column was then dismantled to allow the recovery of the gel which was pushed out with a syringe into a test tube.

Purification of DARC on immobilized VHH

Transfected K562 cells expressing DARC wild-type sequence have been described [66]. They were grown at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium-Glutamax I (GIBCO; Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal calf serum and 0.8 mg/ml geneticin. A 1-l Lampire bag (with gas permeable walls and a cap fitted with Luer ports; Lampire, Pipersville, PA, USA) was used for cell cultivation. Cell density was maintained between 350,000 and 700,000 cells/ml, and 500 ml of cell suspension was harvested once a week, and replaced with fresh medium. Cells were recovered by centrifuging, and frozen in aliquots until purification.

Starting material for purification was a frozen pellet corresponding to 150×10^6 cells. The pellet was thawed and suspended in 40 ml of lysis buffer (20 mM Tris–HCl pH 8.0, 0.15 M NaCl, 5 mM EDTA, 1% Triton ×100, and a cocktail of protease inhibitors from Roche, Basel, Switzerland). The suspension was incubated at 4°C, with gentle agitation, for 1 h. The tubes were centrifuged for 1 h at 72,600*g* in a Beckman 25.5 rotor.

The affinity gel (200 µl) with the immobilized CA52, prepared as described above, was equilibrated with the lysis buffer. Gel and cell lysate supernatant were mixed and incubated overnight in the cold room, with gentle agitation. The suspension was centrifuged at 12,000*g* for 8 min. The gel was washed by centrifugation once with the lysis buffer and then twice with wash buffer (150 mM NaCl, 10 mM Tris 20 mM NaCl 5 mM EDTA (pH 7.6) containing 0.3% octaethylene glycol monododecyl ether ($C_{12}E_8$). The gel was transferred to an Amicon Ultrafree MC 500 µl filter unit (Millipore, Saint Quentin en Yvelines, France) fitted with a 0.5-µm pore size membrane. Then, 100 µl of wash buffer plus 1% glycerol and 2.5 mg of DFEDVW synthetic peptide (Polypeptide Laboratories, Strasbourg, France) was added to the sedimented gel in the filtration cup. The suspension was gently agitated at room temperature for 4 h, the cup placed on top of an Eppendorf tube and centrifuged for 2 min at 1,000*g*. Peptide elution was repeated three times but lowering incubation time before centrifugation to 30 min. Finally, the gel was washed twice with buffer without peptide and then treated with 0.1 M Glycine pH 2.8 buffer, recovering filtrates by centrifugation as above. All recovered fractions were analyzed by SDS PAGE and western blotting. Purified material was also digested by N-glycosidase according to recommendations of the supplier of the enzyme (New England Biolabs).

Results

Construction of VHH library, retrieval and purification of VHH-CA52

The VHHs from circulating lymphocytes of the dromedary immunized with ECD1 fusion protein were cloned in a library of 7.5×10^8 independent colonies of which 80% contains an insert in the phagemid with a size that corresponds to that of a VHH. After phage display and panning on ECD1 GST, 28 VHHs that recognized the antigen specifically were identified. The VHH referred to as CA52 which is the subject of this report was found eight times among the positive clones.

Repeated expression of CA52 yielded after purification on the nickel column between 1.0 and 2.5 mg protein per litre of fermentation medium. Figure 1a illustrates the purity of the final product on SDS-PAGE and western blot.

Epitope mapping of CA52 by western blots of ECD1 deletion constructs and synthetic peptides on plastic pins

Western blots of the ECD1 deletion constructs probed with CA52 are shown in Fig. 1b. All deletion constructs are recognised by the CA52, except deletions II and III. This

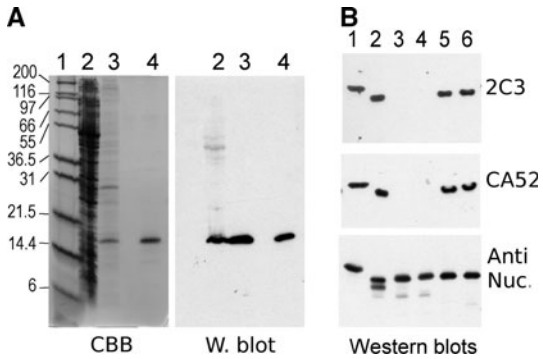

Fig. 1 VHH purification and antigen-specificity screening. **a** Expression and purification of CA52 VHH. The Coomassie Blue stained gel (*CBB*) contains molecular mass standards (*lane 1*, size in kDa are indicated), lysed bacterial pellet taken at the end of the fermentation (*lane 2*); the periplasmic extract from harvested bacteria (*lane 3*) and an aliquot of the purified material (*lane 4*). Western blot was probed with anti-HA antibody. *Lanes 2*, *3* and *4* were loaded with same material as used for the CBB stained gel. **b** Three *western blots* using *2C3*, *CA52*, and *Antinuc* (antinuclease antiserum) as primary antibody. Samples in *lanes 1* contain wild-type ECD1-nuclease fusion while *lanes 2–6* contain the deletion mutants (I to V, see Table 1 for sequences of the deletion mutants)

result is similar to that obtained for the monoclonal anti-Fy6 murine antibody 2C3. The control with antinuclease antiserum demonstrates that all lanes have been loaded with the same amount of antigen. The antinuclease antiserum reveals two bands in the deletion I preparation (lane 2 of Fig. 1b). The lower band (having the nuclease tag) is a proteolysed fragment that deletes the 2C3 and CA52 epitope. From all these data, we propose that the CA52 epitope should encompass the ECD1 sequence QLDFEDVWNSSYGVND.

Peptides synthesized on plastic pins were used to identify the amino acids of ECD1 that are important for CA52 recognition. The octapeptides of the ECD1 sequence that react with CA52 exhibit a very similar reactivity to those described for 2C3: the FEDVW peptide sequence is the linear epitope of CA52. Lengthening this sequence to either end does not change the reactivity to a great extent (Fig. 2a). Figure 2b–f show the effect of individual substitutions for each amino acid of the sequence by the 19 other amino acids. Again, one may appreciate the close similarity between the CA52 and the 2C3 association with the various peptides. We will elaborate more on the minor differences between the CA52 and murine 2C3 in the "Discussion".

In silico experiments of CA52 structure

A pertinent structural model of CA52 VHH has been proposed through a classical comparative modeling approach. The chosen VHH structural templates (1OP9 and 1JTO) share 75% of sequence identity with CA52. As VHH specificities are mainly determined by the three complementarity determining regions (CDR1, CDR2 and CDR3), these regions received particular attention. The first structural template (1OP9) has been selected since it possesses a good compatibility with CA52 for its length and amino acid composition within CDR1 and CDR2. Moreover, in addition to the characteristic disulfide bond present in immunoglobulin domains linking B and F β-sheets, 1OP9 has an extra disulfide bond linking CDR1 and CDR3 frequently present in dromedaries' VHHs [69]; likewise CA52 has a pair of cysteines at residues 33 and 107. The second structural template (1JTO) also has a good similarity for CDR2 and CDR3, but it lacks the interloop disulfide bond. Figure 3a shows the alignment of the amino acid sequences of CA52, and the 1JTO or 1OP9 VHHs; the CDRs and the disulfide bonds are also indicated.

Differences between the generated structural models are limited (average value of root mean square deviations between models is 0.45 Å, its maximum goes to 2.5 Å). This peculiarly limited variability is mainly due to the four framework regions of VHH which are structurally very well conserved in all VHHs, and to the disulfide bridge linking CDR1 and CDR3 which strongly constrains the CDRs. Figure 3b shows two representations of the selected structural model, each seen from two view angles: left as cartoons and right with a color-coded electrostatics surface. The first representation gives a good view of the position of CDRs forming a continuous surface at one side of the VHH. The second representation illustrates the charge distribution on the surface. The CDR1 and CDR3 and the central part of CDR2 present a strong positive charge (blue color) whilst only extremities of CDR2 are negative (red color). This peculiar charge distribution observed on CA52 surface is absent on the two structural templates. From crystal structures of other VHHs in complex with their cognate antigens, it is known that CDR3 is the major contributor for the antigen recognition [44]. The presence of positive charges in this area and the presence of negative charges in the linear target peptide are suggestive for important ionic interactions.

Kinetic binding analysis of CA52–ECD-1 interaction

A SPR sensorgram acquired using ECD1-nuclease construct as ligand is shown in Fig. 4. The fitted kinetic data derived from it are given in Table 2. The kinetic analysis reveals that indeed the affinity of CA52 is very high with a computed K_D of 2.38×10^{-10} M. This high affinity is attained largely by an extremely fast association rate constant of 2.31×10^7 $M^{-1}s^{-1}$. With DARC purified from eukaryotic cells, instead of recombinant prokaryotic ECD1-nuclease construct, a similar K_D was obtained (2.59×10^{-10} M) but k_a and k_d were somewhat lower (Table 2). Deglycosylation of immobilized DARC brought kinetic constants close to those observed with prokaryotic construct. Efficient deglycosylation through N-glycosidase was suggested by a baseline signal drop after enzymatic treatment. It was proven by the absence of binding of *Sambucus nigra* lectin after deglycosylation contrasting to definite binding before deglycosylation.

Flow cytometry and agglutination studies

We assessed the possible recognition of the native DARC epitope on red blood cells by CA52 by flow cytometry and agglutination tests. The results of the flow cytometry (Fig. 5) clearly proves that CA52 recognizes Duffy antigen positive red cells independently of the actual allotypes present on the red blood cell surface (Fig. 5d–f) and fails to interact with the Fy(a−b−) cells (Fig. 5c). Additional experiments show that the anti hemagglutin antibody—used as an auxiliary antibody for CA52 detection—does not react appreciably with red cells (Fig. 5g). As expected 2C3 reacts with Fy(a+b+) cells (Fig. 5b) and not with Fy(a−b−) (Fig. 5a).

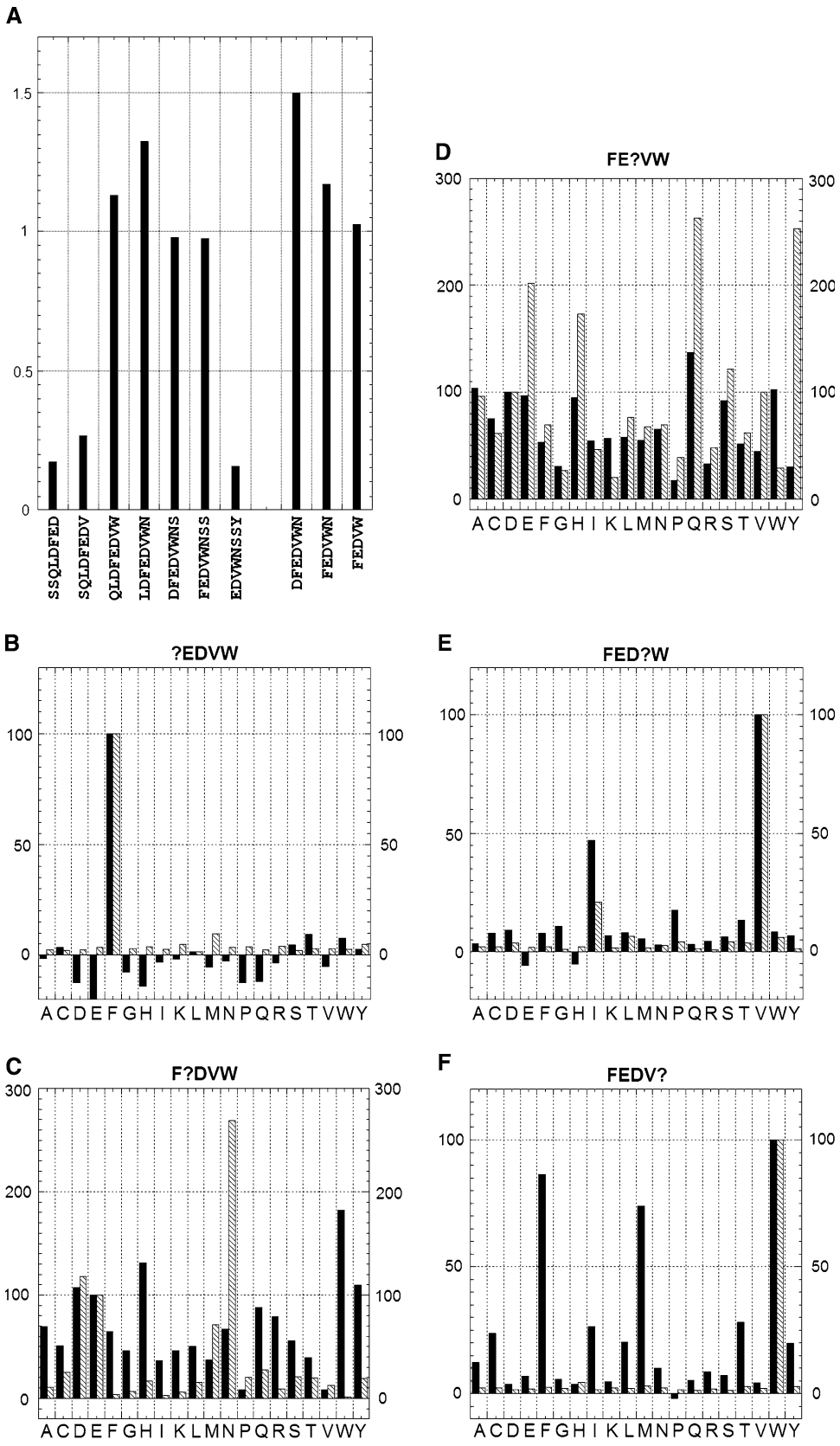

Fig. 2 Pepscan analysis of CA52 reactivity. **a** Binding of CA52 to octapeptides overlapping the DARC ECD-1 sequence from Ser 17 to Tyr 31. Binding of CA52 was detected using anti-HA antibody as described in "Materials and methods" and the final plotted value (measured after incubation with successively CA52, anti-HA and peroxydase tagged anti-mouse antibody) was substracted by the signal value observed in a separate experiment with only anti-HA and peroxydase tagged anti-mouse antibody. **b–f** The results of replacement analysis. Analogs of the pentapeptide FEDVW were used in which each amino acid was replaced with 19 other residues. They were tested for binding to CA52 (*solid bars*). Position of changed aminoacid in the pentapeptide sequence is shown by the question mark at *top* of each histogram. Optical density values have been corrected for background signals from reaction values observed with only anti-HA and peroxydase tagged anti mouse antibody. The 100% value is the absorbance value obtained with the wild-type sequence. For comparison purposes, values observed with anti Fy6 murine antibody 2C3 [5] are shown also on the histograms (*hatched bars*)

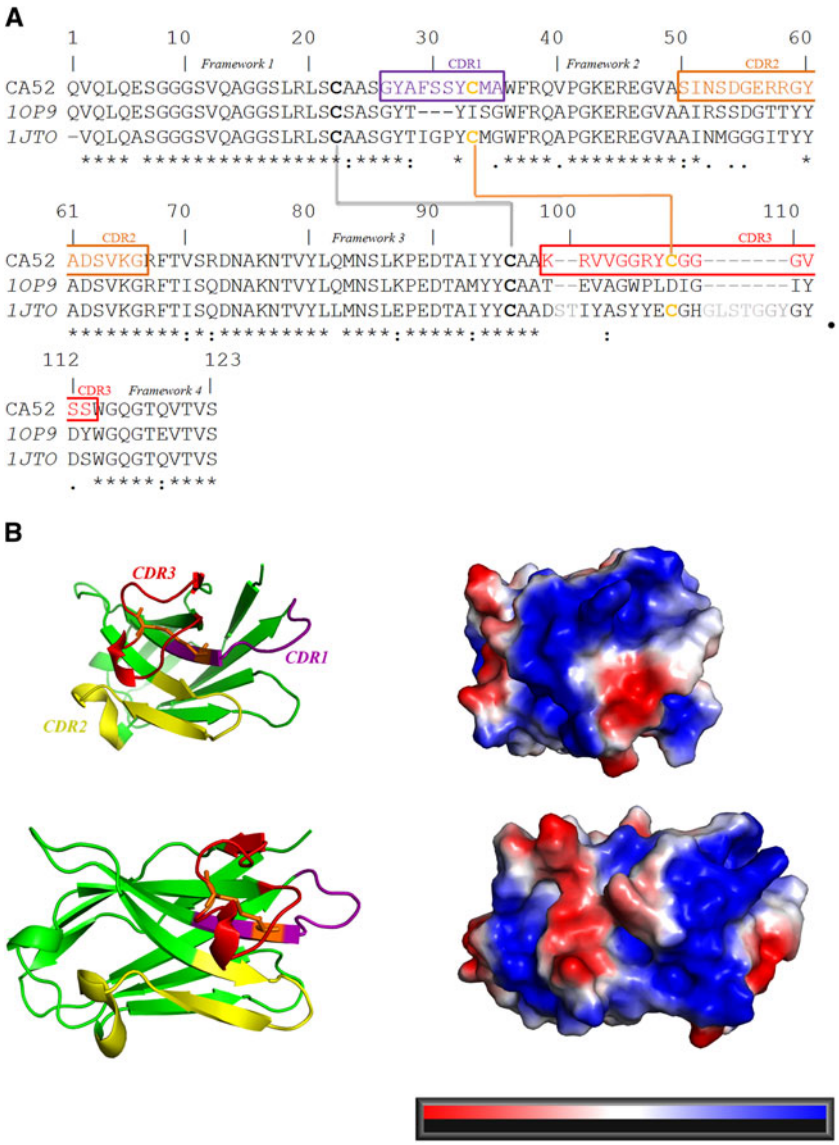

Fig. 3 Homology modelling of CA52. **a** Alignment of CA52 with the two templates used for comparative modelling. Identity is indicated by *stars* and conservative replacements by *colons* under the sequences. Complementarity determining regions are *boxed* and disulfide bonds are indicated by *gray* and *orange lines* (*gray* is the disulfide bond common to all immunoglobulin domains, *orange* is the extra SS bond found frequently in dromedaries' VHHs, linking CDR1 and CDR3). **b** Selected model viewed from two different viewpoints, the *top* representation is from an antigen-eye view, while for the *lower* one, we rotated the molecule by 90° over a vertical axis. *Left* is a cartoon representation of the model, *right* shows the electrostatic surface distribution with basic area (*blue*) concentrated at the three CDRs

Figure 5h, i shows that flow cytometry can be used to detect the presence of cell-specific VHH in a periplasmic extract prepared in the conditions used for library screening (Fig. 5i) while similar periplasmic extract of non transformed TG1 bacteria fails to react with red cells (Fig. 5h).

Agglutination experiments performed with purified CA52 at concentrations of 10 and 5 µg/ml exhibited a positive reaction (+++ according to the Diamed scoring criteria), provided that anti-HA antibody and anti-mouse IgG were present in the assay. Negative reactions were obtained for higher dilutions of CA52.

Agglutination was also observed (++) with crude periplasmic extract from CA52-transformed cells but not from non-transformed cells. As controls, agglutination was also performed with 2C3: murine 2C3 monoclonal in combination with an anti-mouse IgG shows a zone effect in the agglutination of red blood cells with no agglutination at 10 µg/ml, ++ agglutination at 5 µg/ml, +++ reaction from 2.5 to 0.16 µg/ml and ++ at 0.08 µg/ml.

Modulation of DARC function by CA52

CA52 prevents Plasmodium vivax red blood cell invasion

Inhibitory activity of CA52 on the merozoite invasion of red blood cells was determined using three clinical *P.vivax* isolates. The parasites co-cultivated with CA52 showed significant reduction of the number of infected red blood cells compared with those in the cultivation without VHH or with control VHH, i.e., an anti-lysozyme VHH. CA52 does inhibit strongly merozoite invasion in all three isolates (see Table 3). Obviously these results should be confirmed on a larger number of isolates taken at several places of endemicity.

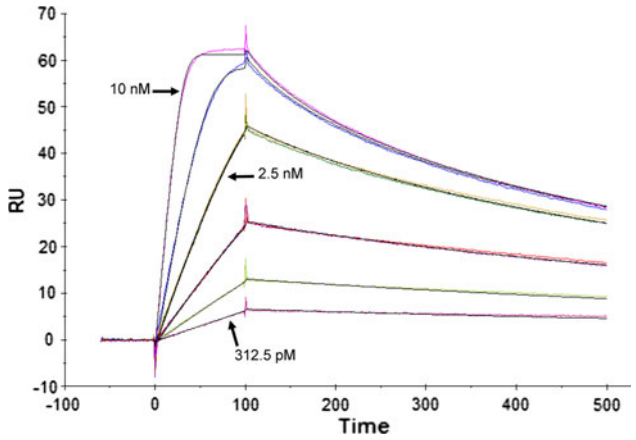

Fig. 4 Surface Plasmon Resonance analysis of CA52 interaction with the ECD1-nuclease construct. Sensorgrams obtained with the Biacore X100 apparatus using CA52 as analyte. ECD1 fused to nuclease was immobilised on Fc2, the reference channel (Fc1) contained the nuclease. The highest concentration of CA52 was 10 nM and four twofold serial dilutions were also injected. *Colored curves* are experimental and *black lines* correspond to the fitted kinetic values (Table 2)

Displacement of IL8 bound to Fy(a+b+) red cells by CA52

The interference of CA52 with IL8 binding on DARC was evaluated in competition experiments. Figure 6 reveals that CA52, even at low nanomolar concentrations, competes effectively with IL8 for binding to Fy(a+b+) red blood cells. The 2C3 is also effective under these conditions whereas controls including anti-lysozyme VHH and non-relevant IgG are without any effect. No binding of IL8 to Fy(a−b−) red blood cells is observed, as expected.

Immunoaffinity purification of DARC on immobilized CA52

Preliminary tests indicated that CA52 (in presence of anti-HA antibody) was able to immunoprecipitate DARC from red cells membrane extract. This suggested that CA52 might be used to immunopurify DARC. We used transfected K562 cells as starting material. DARC is expressed at a density of ca. 35,000 sites/cell. Figure 7 demonstrates that immobilized CA52 is indeed able to immunocapture DARC satisfactorily and that this antigen is efficiently eluted by a synthetic hexapeptide containing the epitope sequence. As expected, DARC appears on the silver nitrate-stained gel as a smear from 45 to 55 kDa. This corresponds quite well to what is usually observed in red cell lysate after western blotting. A prominent band is also visualized at ca. 90 kDa which is obviously a dimer of DARC, and bands which barely migrate into the gel are probably higher oligomers. The DARC-nature of all these bands is confirmed by (1) western blot probed with 2C3, and (2) the lowered apparent molecular mass of monomer, dimer and oligomers after N-glycosidase treatment. Two extra bands are also discerned on the silver nitrate stained gels: one corresponding to the VHH that leaks from the immunoaffinity support (arrow 1 on Fig. 7a), and an unidentified protein (arrow 2 on Fig. 7a).

Discussion

To the best of our knowledge, this report is the first description of the preparation of recombinant antibodies derived from an immunized dromedary and specific for a blood group protein. It is a further proof that VHHs provide powerful tools for purification of membrane proteins [49] and for the modulation of the biological function of their targets in the absence of the Fc effector function.

The antigen we used for the immunization of the dromedary and screening of the library was a recombinant derivative of the target protein produced in *E. coli*. Only part of the molecule, i.e., DARC ECD1 (fused with nuclease), was injected into the animal, and numerous antibodies were elicited. We have not yet studied the detailed specificities towards DARC of all the retrieved VHHs but several were shown to react specifically with Fy(a+b+) red blood cells. This point is of importance since DARC is known to be N-glycosylated on the three available sites on ECD1 (N16, N27 and N33) [65], so that epitopes on the non-glycosylated *E.coli* produced recombinant protein might be cryptic in the glycosylated DARC present on red blood cells. Reactivity of our VHH CA52 with red blood cells was evidenced by flow cytometry as well as by agglutination techniques. An equal reactivity of CA52 was observed for cells expressing either Fy^a or Fy^b allotypes.

The CA52 recognizes the same linear FEDVW epitope as the murine 2C3 monoclonal, a reference antibody for the Fy6 antigen [6]. Nevertheless, there are some notable differences regarding the detailed specificities of 2C3 and CA52. In the FEDVW oligopeptide, F cannot be replaced by any other residue for both CA52 and 2C3, whereas E can be substituted by several amino acids without abolishing the binding of CA52. Surprisingly, E can be replaced in the peptide by W, Y, and H to give a binding of, respectively, 180, 100, and 130% to the level observed with E. By contrast, for 2C3, E can be replaced by D and N only. That E might be exchanged by an aromatic residue in the epitope recognized by CA52 suggests that the paratope is probably engaged in either a purely ionic interaction or in cation–Pi interactions [68] when challenged in the synthetic peptide with an aromatic amino acid at this position. For CA52, the D in the epitope can be freely substituted by nearly any amino acid except by P; likewise, 2C3 is also

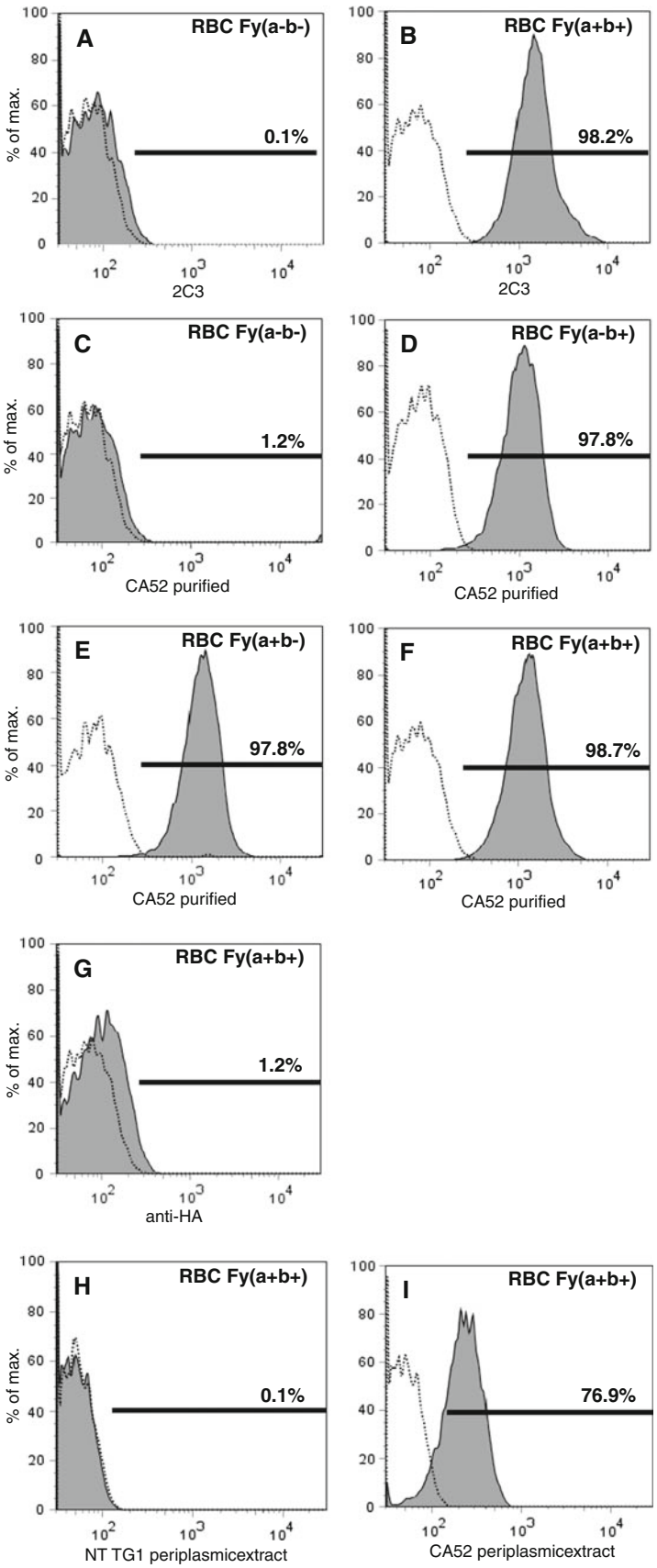

Fig. 5 Flow cytometry of red blood cells. The red blood cells with phenotype as indicated (*upper right corner* of each graph) were incubated with the 2C3 monoclonal (**a**,**b**), or with the purified CA52 (**c**–**f**), or with the crude CA52 extract (**i**). The *gray* areas under the lines (AULs) (**a**–**g**) correspond to results obtained with primary antibodies as indicated at the *bottom* of each graph. Clear AULs show the signals obtained using non-relevant murine IgG and phycoerythrin-tagged anti-murine IgG (**a**,**b**), or only the secondary anti-HA antibody and phycoerythrin-tagged anti-murine IgG (**c**–**f**). **g** A control from cells stained with anti-HA antibody and phycoerythrin-tagged anti-murine IgG (*gray* AUL), *clear* AUL in (**g**) shows results obtained with non-relevant murine IgG and phycoerythrin-tagged anti-murine IgG. **h**, **i** The results from cells stained with proteins from the periplasmic extract of mock transformed cells (NT TG1), or of cells expressing CA52 respectively; *gray* AUL is the specific signal while the clear AUL was obtained with anti-HA and phycoerythrin tagged anti-murine IgG. Numbers above the *horizontal bars* indicate the percentage of cells of *gray* graphs which are reactive with the indicated antibody

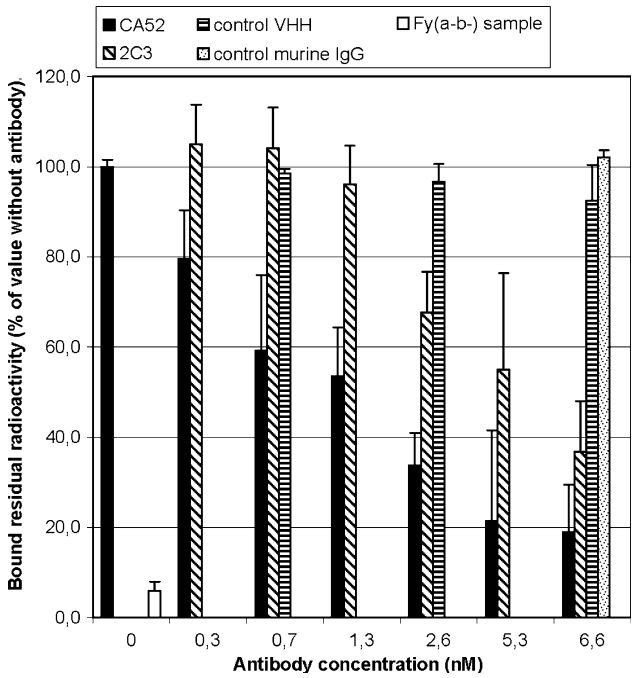

Fig. 6 Displacement of interleukin-8 from red cells by CA52. Histograms showing that CA52 and 2C3 compete with radioactive interleukin-8 for binding to Fy(a+b+) red cells. Each *column* is the mean of three determinations; *error bars* correspond to one standard deviation. Also shown are results obtained with controls: (1) neither anti-lysozyme VHH D3L11 nor irrelevant murine IgG interfere with interleukin-8 binding to Duffy positive cells, and (2) Duffy negative cells do not bind appreciably interleukin-8

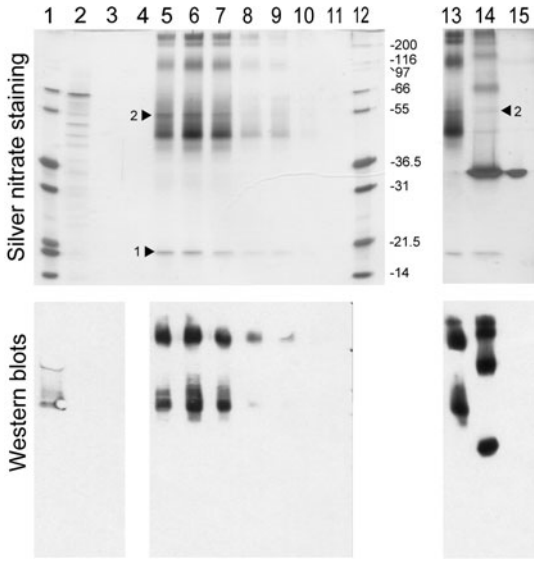

Fig. 7 Purification of DARC from transfected K562 by CA52 affinity chromatography. A lysate of the transfected K562 cells expressing DARC was loaded on a CA52 immuno adsorbent. *Upper panel* shows silver nitrate stained SDS-gels. *Lanes 1* and *12* contain molecular weight standards (purified CA52 has been added). *Lanes 2–4* contain aliquots of washings of the support performed after the sample loading. *Lanes 5–7* contain aliquots of material eluted with a solution of a hexapeptide containing the epitope sequence, whereas *lanes 8–10* contain aliquots of washings made after the elution with peptide. *Lane 11* contains an aliquot of acidic eluate from immobilized CA52 gel. *Lane 13* contains an aliquot of purified DARC, *lane 14* contains an aliquot of purified DARC after treatment with N-glycosidase, and *lane 15* contains the N-glycosidase, as a control. *Arrows 1* and *2* indicate the positions of CA52 leaked from the adsorbent and an unknown protein, respectively. The *lower panel* shows the western blot revealed with 2C3 as primary antibody. The material loaded in the lanes is identical to that in the silver nitrate stained gel except that *lane 1* contains an aliquot of red blood cells membrane lysate

tolerant to substitutions at this position. The V residue can be replaced by hydrophobic aliphatic residue I with retention of CA52 interaction, while it cannot be replaced by any other residue in the case of 2C3. The W26 is mandatory for recognition by 2C3 whereas this residue in the peptide may be changed to an F or hydrophobic M in the case of CA52.

These CA52 binding results on target peptide variants are in line with the current structural model of CA52. The structural model reveals the presence of a large patch of basic side chains on the paratope face of the VHH which are likely to participate in interactions with acidic and aromatic residues on the epitope.

The SPR results of affinity measurements reveal a subnanomolar equilibrium dissociation constant for CA52 on DARC, a value that is most favorable for future diagnostic or therapeutic applications. Worthy of note, glycosylation of DARC exerts some influence on kinetic constants, since k_a and k_d measured on glycosylated DARC are lower compared to values with prokaryotic construct as ligand (but K_D with both ligands, *E. coli*-expressed ECD1 and K562-expressed DARC, is in the 10^{-10} range). Enzymatic deglycosylation of DARC, does increase k_a to a value similar to that of the *E. coli*-produced ligand. It is interesting to note that enzymatic modification of the ligand could be easily performed in the Biacore instrument.

Importantly, CA52 inhibits two well-identified biological functions of DARC, the IL8 binding, and the invasion of red blood cells by *P. vivax* merozoites. DARC plays a critical role in a wide range of diseases, including malaria where DARC has been identified as the main receptor for the parasite, even though its precise molecular interactions with *Plasmodium vivax* Duffy binding protein (PvDBP) remain elusive. Our results establish that CA52 inhibits *P. vivax* merozoite red blood cell invasion. It is surmised that the inhibitory effect of CA52 is caused by a steric hindrance: binding of CA52 to its cognate ECD1 target precludes binding of PvDBP at the same or overlapping site. Previous studies using ECD1-GST fusions and PvDBP confirmed that the sequence recognized by 2C3 (and now by CA52 as well) were important to PvDBP binding [13].

An obvious suggestion would be to design a therapeutic agent, taking the CA52 as a lead, to prevent the invasion of

human red blood cells by *P.vivax*, indeed it has been already suggested that Duffy antigen inhibitors might be useful therapeutic agents for *P. vivax* malaria [70]. However, recent data suggest that *P.vivax* has shortcut its absolute dependency on DARC to invade red blood cells [19–21], even if it is still believed to be the main entry route, as suggested by the inverse correlation of *P. vivax* malaria severity with expression level of DARC on red cells [71, 72]. One can observe mixed infections with more than one species of *Plasmodium* in one single individual; coexistence of diverse *Plasmodium* species infecting populations living in the same area is also frequently encountered. All this pinpoints the need for quick, reliable, sensitive techniques for diagnostic of malaria-causative species (ideally also applicable in remote and poor areas [73]). Lastly, if a drug targeting DARC was designed, it should be carefully checked that it does not interfere with the normal functions of DARC which presently are not yet fully understood. In this regard, it may be noted that DARC plays a role in the equilibrium of the chemokine–chemokine receptor network implicated in many diseases, including cancer and inflammatory diseases [27–35]. CA52 might help to understand the role of DARC in disease. Our results showing the effective displacement of IL8 from DARC by CA52 are in this regard highly relevant. Worthy of note, chemokines and chemokines receptors, despite complexities and redundancies, are targets for present day pharmaceutical research and some successes have been achieved [reviews in 74–75].

Similar to what has been observed with *P.vivax* infection, it will be interesting to investigate the possibility that CA52 might preclude binding of HIV to DARC-positive red blood cells [39], keeping in mind that, presently, experimental data on binding of HIV to red cells and the relationship of this binding with HIV infection and AIDS progression seems to be complex and controversial [39–45].

The CA52 is indeed an invaluable tool for DARC purification. At present, our results involve only small-scale experiments, but apparent yield and purity are sufficiently promising to switch to more ambitious studies, e.g., aiming to crystallize DARC. In this regard, it should be mentioned that VHHs have been proposed as a versatile tool to aid the crystallization of proteins [50]. The CA52-purified DARC appears as multimers on our gels, while it is usually present as a monomer when analyzed through western blot in red blood cells membrane lysate. Indeed, it has been indicated that, in cell lines, multimer formation was likely to occur, and this form might even participate in DARC function (at least in cells other than red blood cells) [35]. The presence of oligomers of membrane proteins on SDS gels is in fact rather common, and it was shown on several occasions that multimers formation might be correlated to concentration in the sample [76], definitely higher in purified material compared to that present in red cells.

Purification of membrane proteins is notoriously difficult. Expression level of target protein in starting material is indeed an important factor in the success [77]. DARC expression level in our recombinant K562 cells is far from the one reached by others: e.g., poly-His-tagged D6 was purified from engineered cells efficiently on immobilized metal but copy number was more than 20-fold higher than ours [78].

Immunoaffinity chromatography on a high affinity antibody provides a powerful and elegant method for the easy recovery of membrane proteins even when they are present at a relatively low concentration in starting material. Common practice when using affinity chromatography is to use harsh eluting solutions like pH jump or "deforming" mobile phases which may denature the target protein. Elution through epitope peptide is likely to maintain native conformation. Tagging proteins with short linear epitopes has been used as a shortcut to immunopurify membrane proteins when no convenient antibody was available [79–81]. However, introduction of a foreign peptide tag may be deleterious to proper expression and/or function of the protein. Hence, as a (possibly) generally applicable strategy for purification of membrane proteins we propose: (1) to immunize a camelid with (easily expressed) *E. coli* recombinant proteins containing extra membranous loops of the target protein, (2) to prepare and to screen the derived library to obtain VHHs, and, finally, (3) to prepare from selected VHHs suitable immunoaffinity supports for purification of the target protein.

The appeal of this strategy relies entirely on the peculiarly favorable properties of VHHs as stable, cheap, easily produced reagents. Its application to DARC, besides being a proof of concept, is interesting since it should facilitate collecting data on this blood group protein, important in many devastating human diseases.

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