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running title

VHH against DARC

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ABSTRACT

Fy blood group antigens are carried by 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. 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 :

Duffy Antigen Receptor for Chemokines : DARC

First extracellular domain : ECD1

Centre National de Reference pour les Groupes Sanguins : CNRGS

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 alloimmunization. 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-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 the 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 DARC promoter region [8]: the GATA transcription factor is driving DARC expression in cells from the erythrocytic lineage while other factors are operative 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 forty 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 nineties that DARC is a ligand for chemokines [24] were milestones in DARC studies. Indeed, DARC binds numerous chemokines of both the CC and CXC group [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 galore of epidemiological and experimental data, on whole organisms (e.g.

genetically modified mice) and on isolated cells as well, do 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 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, 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 using also *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 30 and 41 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) grown in autoinduction medium [54]. Whole cell extracts were obtained by passing 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 6M 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) 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)

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. 1 mg of each antigen (4 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 three weeks without immunisation was taken before the animal received two boost injections at one week interval. Blood was taken on EDTA anticoagulating medium one week after the last boost. Construction of the VHH library followed well-established steps [48-52 and references cited 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 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.

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 litre) and fermentation left to proceed from 14 to 20 hours 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 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) 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: (i) CA52 VHH (1 µg/ml) (ii) anti-HA antibody (Clone 16B12) purchased as ascitic fluid from Covance (Emeryville CA) at 1/1000 dilution; (iii) 2C3, an anti Fy6 antibody (1µg/ml) [6]; (iv) an anti *Staphylococcus aureus* nuclease rabbit antiserum developed in house and diluted 1/2000; (v) 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 hour 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 hours at room temperature. The pins were subsequently incubated in wells containing 150μl 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 hour, room temperature) and alkaline phosphatase-conjugated rabbit anti-mouse IgG (Dako, 1500-times diluted in TTBS, 1 hour, room temperature). After each step the pins and the wells were washed 5 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 figure 2) 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: 1JTO [58] and 1OP9 [59]). The protein sequences have been aligned with Clustalw2 software [60] including minor additional manual changes. The comparative modelling has been performed with the Modeller software [61]. The disulfide bond between residue 33 and 107 has been constrained. 200 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 X100 instrument (GE Healthcare). Two kinds of ligands were used, one was wild type ECD1 nuclease fusion construct (table 2), the other was full size recombinant DARC purified from engineered K562 cells (see below details on purification) ; prior to immobilization DARC was dialyzed in Pierce cassettes -Rockford IL- 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 performed also on *in situ* deglysocylated DARC performed as follows. Temperature of the instrument was set to 37°C and flow rate to 5 µl/min 5000 units of N-glycosidase (New England Biolabs, Ipswich, MA) were dissolved in reaction buffer and injected manually onto Fc2 three times consecutively for 1000 seconds 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). Red cells were washed twice in phosphate buffered saline (PBS). After the second wash, the cells were resuspended with either: (i) anti Fy6 murine monoclonal antibody 2C3 (culture supernatant diluted 1:8); (ii) isotype control antimurine IgG (5 µg/ml, Becton Dickinson, Franklin Lakes, NJ); (iii) periplasmic extract of TG1 cells producing CA52 diluted 1:2 in PBS/0.1% BSA; (iv) periplasmic extract of non transformed TG1 cells as control; or (v) purified CA52 (10 µg/ml). Suspension was let at room temperature for one hour. After primary incubation, VHH or periplasmic extract incubated cells were washed twice in wash buffer and incubated for one additional hour in presence of anti-HA monoclonal antibody (clone 16B9 as an ascitic fluid purchased from Covance, diluted 1:1000). A control of red blood cells incubated with anti-HA alone was also prepared. Cells were washed again before one hour incubation in the dark at room temperature with antimurine 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 (v6.1.2), and analyzed using FlowJo (Treestar, Ashland, OR) software v7.2.5.

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

Agglutination studies

In order to perform the agglutination studies, either: (i) crude periplasmic extract diluted 1:2 in PBS; (ii) purified VHH dissolved at 10 µg/ml concentration in PBS and serial two-fold dilutions; or (iii) 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 (Paris, France). Volume of suspension was 40 µl, hematocrit was 4%. After 60 minutes 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 let to proceed for a further 20 minutes. 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 minutes. 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. Inhibitory effect of CA52 on red cells invasion by *P. vivax* was evaluated using short term culture conditions as described before [67]. 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 hours at 37°C at 25µg/ml final concentration to allow saturation binding of CA52 on the red blood cell membrane. At the end of 2nd hour, the excess of 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 hours 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 X oil immersion). Numbers of parasites at various stages are expressed relative to 10^4 erythrocytes (Table 3).

An anti lysozyme VHH (D3L11 [68]) 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 2200 Ci/millimole, Perkin Elmer, Boston, MA) 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) 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 a 85:15 mixture of DC550 silicon oil (Serva ref 35145) and Nujol oil (Alfa Aesar). They were centrifuged at 4000 rpm for 20 seconds. Cell pellets were dissolved in 100 µl dimethylsulfoxide and radioactivity counted in a Packard Tri Carb liquid scintillator. All obtained count numbers were subtracted by the background counts measured for tubes containing cold IL8.

Preparation of immobilized CA52

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 5000 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 ethanolamine in order to de-activate the potentially remaining reactive groups.
The column was then dismantled to allow to recover 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) supplemented with 10% foetal calf serum and 0.8 mg/ml geneticin. A 1 liter Lampire bag (with gas permeable walls and a cap fitted with Luer ports; (Lampire, Pipersville, PA)) was used for cell cultivation. Cell density was maintained between 350 000 and 700 000 cells/ml and 500 ml of cell suspension harvested once a week, and replaced with fresh medium. Cells were recovered by centrifugations, and frozen in aliquots till purification.
Starting material for purification was a frozen pellet corresponding to $150\ 10^6$ cells. 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 X100, and a cocktail of protease inhibitors from Roche, Basel, Switzerland). The suspension was incubated at 4°C, with gentle agitation, for one hour. The tubes were centrifuged for one hour at 72600 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 12000 g for 8 minutes. 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 hours, the cup placed on top of an Eppendorf tube and centrifuged for 2 minutes at 1000 g. Peptide elution was repeated three times but lowering incubation time before centrifugation to 30 minutes. Finally gel was washed twice with buffer without peptide and then treated with 0.1M 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 8 times among these colonies.
Repeated expression of CA52 yielded after purification on the nickel column between 1.0 to 2.5 mg protein per litre of fermentation medium. Figure 1 (panel A) 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 ECD-1 deletion constructs probed with CA52 are shown in Figure 1 (panel B). All deletion constructs are recognised by the CA52, except deletions II and III. This 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 Figure 1B). The lower band (having the nuclease tag) is a proteolysed fragment that deletes the 2C3 and CA52 epitope. From

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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 (Figure 2 panel A). Panels B to 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 section.

In silico experiments of CA52 structure

A pertinent structural model of CA52 VHH has been proposed through a classical comparative modelling approach. The chosen VHH structural templates (1OP9 and 1TO) 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 [70] : likewise CA52 has a pair of cysteines at residues 33 and 107. The second structural template (1JTO) has also 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 4 framework regions of VHH which are structurally very well conserved in all VHHs, and to the disulfide bridge linking CDR1 and CDR3 which strongly constraints 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 colour 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 colour) whilst only extremities of CDR2 are negative (red colour). 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.

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Kinetic binding analysis of CA52 ECD-1 interaction

A SPR sensorgram acquired using ECD1-Nuclease construct as ligand is shown in Figure 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 ka and kd 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 (Figure 5) clearly proves that CA52 recognizes Duffy antigen positive red cells independently of the actual allotypes present on the red blood cell surface (panels D to F) and fails to interact with the Fy(a-b-) cells (panel C). Additional experiments show that the anti hemagglutin antibody - used as an auxiliary antibody for CA52 detection - does not react appreciably with red cells (panel G). As expected 2C3 reacts with Fy(a+b+) cells (panel B) and not with Fy(a-b-)(panel A).

Panels H and I in Figure 5 show 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 (panel I) while similar periplasmic extract of non transformed TG1 bacteria fails to react with red cells (panel H).

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

1. 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.

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. The2C3 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. 35000 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 visualised 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 (i) western blot probed with 2C3, (ii) 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 Figure 7 panel A), and an unidentified protein (arrow 2 on Figure 7 panel A).

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 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 [69] 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 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 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 favourable for future diagnostic or therapeutic applications. Worth of notice, 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 value similar to that of the *E. coli* produced ligand. It is interesting to note that enzymatic modification of 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 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 [71]. 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 [72-73]. 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 of quick, reliable, sensitive techniques for diagnostic of malaria causative species (ideally applicable also in remote and poor areas [74]). 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 role of DARC in disease. Our results showing the effective displacement of IL8 from DARC by CA52 are in this regard highly relevant. Worth to notice chemokines and chemokines receptors, despite complexities and redundancies, are targets for present days' pharmaceutical research and some successes have been met [reviews in 75-76].

Similar to what has been observed with *P.vivax* infection, it will be interesting to investigate the possibility of CA52 to 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 relation 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 to DARC function (at least in other cells than red blood cells) [35]. Presence of oligomers of membrane proteins on SDS gels is in fact rather common and it was shown in several occasions that multimers formation might be correlated to concentration in sample [77], 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 to the success [78]. 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 [79].

Immunoaffinity chromatography on a high affinity antibody provides a powerful and elegant method for the facile 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 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 [80-82]. 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: (i) to immunize a camelid with (easily expressed) *E. coli* recombinant proteins containing extra membranous loops of the target protein, (ii) to prepare and to screen the derived library to obtain VHHs (iii) finally, 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 to be a proof of concept, is interesting since it should facilitate collecting data on this blood group protein, important in many devastating human diseases.

making available phenotyped human blood, Dr. Christophe Tournamille for the recombinant K562 cells, Prof. Donald Engelman for the plasmid used for some of the *E. coli* protein productions.

References

1. Pogo, AO. and Chaudhuri, A. (2000) The Duffy protein: a malarial and chemokine receptor. Semin. Hematol. 37, 122-129.

2. Rot, A. (2005) Contribution of Duffy antigen to chemokine function. Cytokine Growth Factor Rev. 6, 687-94.

3. Rot, A. and Horuk, R. (2009) The duffy antigen receptor for chemokines. Methods Enzymol. 461, 191-206.

4. Tournamille, C., Le Van Kim, C., Gane, P., Cartron, JP., and Colin, Y. (1995) Molecular basis and PCR-DNA typing of the Fya/fyb blood group polymorphism. Hum Genet. 95, 407-410.

5. Nichols, ME., Rubinstein., P, Barnwell, J., Rodriguez de Cordoba, S., and Rosenfield, RE. (1987) A new human Duffy blood group specificity defined by a murine monoclonal antibody. Immunogenetics and association with susceptibility to Plasmodium vivax. J Exp Med. 166, 776-785.

6. Wasniowska, K., Petit-LeRoux, Y., Tournamille, C., Le van Kim, C., Cartron, JP., Colin, Y., Lisowska, E., and Blanchard, D. (2002) Structural characterization of the epitope recognized by the new anti-Fy6 monoclonal antibody NaM 185-2C3. Transfus. Med. 12, 205-211

7. Tournamille, C., Le Van Kim, C., Gane, P., Le Pennec, PY., Roubinet, F., Babinet, J., Cartron, JP, and Colin Y. (1998) Arg89Cys substitution results in very low membrane expression of the Duffy antigen/receptor for chemokines in Fy(x) individuals. Blood. 92, 2147-2156.

8. Tournamille, C., Colin, Y., Cartron, J.P., and Le Van Kim, C. (1995) Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals, Nat. Genet. 10, 224-228.

9. Peiper, S.C., Wang, Z.X., Neote, K., Martin, A.W., Showell, H.J., Conklyn, M.J., Ogborne, K., Hadley, T.J., Lu, Z.H., Hesselgesser, J., and Horuk, R. (1995) The Duffy antigen/receptor for chemokines (DARC) is expressed in endothelial cells of Duffy negative individuals who lack the erythrocyte receptor. J. Exp. Med. 181, 1311-1317.

10. Horuk, R., Martin, A., Hesselgesser, J., Hadley, T., Lu, ZH., Wang, ZX., and Peiper, SC. (1996) The Duffy antigen receptor for chemokines: structural analysis and expression in the brain. J. Leukoc. Biol. 59, 29-38.

11. Rios, M., Chaudhuri, A., Mallinson, G., Sausais, L., Gomensoro-Garcia, AE., Hannon, J., Rosenberger, S., Poole, J., Burgess, G., Pogo, O., and Reid, M. (2000) New genotypes in Fy(a-b-) individuals: nonsense mutations (Trp to stop) in the coding sequence of either FY A or FY B. Br J Haematol. 108,448-454.

12. Miller, L.H., Mason, S.J., Clyde, D.F., and McGinniss, M.H. (1976) The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy, N Engl J Med 295,302-304

13. Tournamille, C., Filipe, A., Badaut, C., Riottot, M.M., Longacre, S., Cartron, J.P., Le Van Kim, C., and Colin, Y. (2005) Fine mapping of the Duffy antigen binding site for the Plasmodium vivax Duffy-binding protein, Mol Biochem Parasitol 144, 100-103.

14. Hans, D., Pattnaik, P., Bhattacharyya, A., Shakri, A.R., Yazdani, S.S., Sharma, M., Choe, H., Farzan, M., and Chitnis, C.E. (2005) Mapping binding residues in the Plasmodium vivax domain that binds Duffy antigen during red cell invasion, Mol Microbiol 55,1423-1434.

15. Choe, H., Moore, MJ., Owens, CM., Wright, PL., Vasilieva, N., Li W., Singh, AP., Shakri, R., Chitnis, CE., and Farzan, M. (2005) Sulphated tyrosines mediate association of chemokines and Plasmodium vivax Duffy binding protein with the Duffy antigen/receptor for chemokines (DARC). Mol Microbiol. 55,1413-1422

16. Singh, SK., Hora, R., Belrhali, H., Chitnis, CE., and Sharma, A. (2006) Structural basis for Duffy recognition by the malaria parasite Duffy-binding-like domain. Nature. 439,741-744.

17. King, C.L., Michon, P., Shakri, A.R., Marcotty, A., Stanisic, D., Zimmerman, P.A., Cole-Tobian, J.L., Mueller, I., and Chitnis, C.E. (2008) Naturally acquired Duffy-binding protein-specific binding inhibitory antibodies confer protection from blood-stage Plasmodium vivax infection, Proc Natl Acad Sci U S A. 105,8363-8836

18. Grimberg, B.T., Udomsangpetch, R., Xainli, J., McHenry, A., Panichakul, T., Sattabongkot, J., Cui, L., Bockarie, M., Chitnis, C., Adams, J., Zimmerman, P.A., and King, C.L. (2007) Plasmodium vivax invasion of human erythrocytes inhibited by antibodies directed against the Duffy binding protein, PLoS Med 4,e337

19. Rosenberg, R. (2007) Plasmodium vivax in Africa: hidden in plain sight?, Trends Parasitol 23,193-196.

20. Cavasini, CE, de Mattos, LC, Couto, AA, Couto, VS, Gollino, Y, Moretti, LJ,Bonini-Domingos, CR, Rossi,t AR, Castilho, L, and Machado, RL. (2007) Duffy blood group gene polymorphisms among malaria vivax patients in four areas of the Brazilian Amazon region. Malar J. 6, 167-175

21. Menard, D., Barnadas, C., Bouchier, C., Henry-Halldin, C., Gray, L., Ratsimbasoa, A., Thonier, V, Carod, J-F., Domarle, O., Colin, Y., Bertrand, O., Picot, J., King, C., Grimberg, B., Mercereau-Puijalon, O., and Zimmerman, P. Plasmodium vivax Clinical Malaria is Commonly Observed in Duffy-negative Malagasy People Proc Natl Acad Sci U S A. in press

22. Chaudhuri, A., Polyakova., J, Zbrzezna, V., Williams, K., Gulati, S., and Pogo, AO. (1993) Cloning of glycoprotein D cDNA, which encodes the major subunit of the Duffy blood group system and the receptor for the Plasmodium vivax malaria parasite. Proc Natl Acad Sci U S A. 90, 10793-10797.

23. Iwamoto, S., Omi, T., Kajii, E., and Ikemoto, S. (1995) Genomic organization of the glycoprotein D gene: Duffy blood group Fya/Fyb alloantigen system is associated with a polymorphism at the 44-amino acid residue. Blood. 85, 622-626

24. Horuk, R., Chitnis, C.E., Darbonne, W.C., Colby, T.J., Rybicki, A., Hadley, T.J., and Miller, L.H., (1993) A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor, Science 261, 1182-1184.

25. Szabo, M.C., Soo, K.S., Zlotnik, A., and Schall, T.J. (1995) Chemokine class differences in binding to the Duffy antigen-erythrocyte chemokine receptor, J Biol Chem 270, 25348-25351.

26. Graham, GJ. (2009) D6 and the atypical chemokine receptor family: novel regulators of immune and inflammatory processes. Eur J Immunol. 39, 342-351

27. Lentsch, A.B. (2002) The Duffy antigen/receptor for chemokines (DARC) and prostate cancer. A role as clear as black and white?, FASEB J 16, 1093-1095.

28. Addison, C.L., Belperio, J.A., Burdick, M.D., and Strieter, R.M. (2004) Overexpression of the duffy antigen receptor for chemokines (DARC) by NSCLC tumor cells results in increased tumor necrosis, BMC Cancer 4, 1-28.

29. Wang, J., Ou, Z.L., Hou, Y.F., Luo, J.M., Shen, Z.Z., Ding, J., and Shao, Z.M. (2006) Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential, Oncogene 25, 7201-7211.

30. Vandercappellen, J., Van Damme, J., and Struyf, S. (2008) The role of CXC chemokines and their receptors in cancer, Cancer Lett. 267, 226-244.

31. Gardner, L., Wilson, C., Patterson, A.M., Bresnihan, B., FitzGerald, O., Stone, M.A., Ashton, B.A., and Middleton, J., (2006) Temporal expression pattern of Duffy antigen in rheumatoid arthritis: up-regulation in early disease, Arthritis Rheum 54, 2022-2026.

32. Murdoch, C., and Finn, A. (2000) Chemokine receptors and their role in inflammation and infectious diseases, Blood 95, 3032-3043.

33. Segerer, S., Regele, H., Mac, K.M., Kain, R., Cartron, J.P., Colin, Y., Kerjaschk, D., and Schlondorff, D. (2000) The Duffy antigen receptor for chemokines is up-regulated during acute renal transplant rejection and crescentic glomerulonephritis, Kidney Int 58:1546-1556.

34. Zarbock, A, Bishop, J, Müller, H, Schmolke, M, Buschmann, K, Van Aken, H, and Singbartl, K. (2010) Chemokine homeostasis vs. chemokine presentation during severe acute lung injury, the other side of the Duffy antigen receptor for chemokines. Am J Physiol Lung Cell Mol Physiol. 298, L462-71

35. Chakera, A., Seeber, R.M., John, A.E., Eidne, K.A., and Greaves, D.R. (2008) The duffy antigen/receptor for chemokines exists in an oligomeric form in living cells and functionally antagonizes CCR5 signaling through hetero-oligomerization, Mol Pharmacol. 73, 1362-1370.

36. Comerford, I., and Nibbs, R.J. (2005) Post-translational control of chemokines, a role for decoy receptors ?, Immunol Lett. 4, 163-174.

37. Rot, A. (2005) Contribution of Duffy antigen to chemokine function. Cytokine Growth Factor Rev. 16, 687-694.

38. Pruenster, M., and Rot, A. (2006) Throwing light on DARC, Biochem Soc Trans 34 1005-1008.

39. He, W., Neil, S., Kulkarni, H., Wright, E., Agan, B.K., Marconi, V.C., Dolan, M.J., Weiss, R.A., and Ahuja, S.K. (2008) Duffy antigen receptor for chemokines mediates trans-infection of HIV-1 from red blood cells to target cells and affects HIV-AIDS susceptibility, Cell Host Microbe. 4, 52-62.

40. Horne, KC., Li, X., Jacobson, LP., Palella, F., Jamieson, BD., Margolick, JB., Martinson, J., Turkozu, V., Visvanathan, K., and Woolley, IJ. (2009) Duffy antigen polymorphisms do not alter progression of HIV in African Americans in the MACS cohort. Cell Host Microbe. 5, 415-417

41. Julg, B, Reddy, S., van der Stok, M., Kulkarni, S., Qi, Y, Bass, S., Gold, B., Nalls, MA., Nelson, GW., Walker, BD., Carrington, M., and Ndung'u, T. (2009) Lack of Duffy antigen receptor for chemokines, no influence on HIV disease progression in an African treatment-naïve population. Cell Host Microbe. 2009 May 8 5(5), 413-415

42. Winkler, CA, An, P, Johnson, R, Nelson, GW, and Kirk, G. (2009) Expression of Duffy antigen receptor for chemokines (DARC) has no effect on HIV-1 acquisition or progression to AIDS in African Americans. Cell Host Microbe. 2009 May 8 5(5), 411-413

43. Walley, NM, Julg, B, Dickson, SP, Fellay, J, Ge, D, Walker, BD, Carrington, M, Cohen, MS, de Bakker, PI, Goldstein, DB, Shianna, KV, Haynes, BF, Letvin, NL, McMichael, AJ, Michael, NL, and Weintrob, AC. (2009) The Duffy antigen receptor for chemokines null promoter variant does not influence HIV-1 acquisition or disease progression. Cell Host Microbe. 2009 May 8 5(5), 408-410

44. Beck, Z., Brown, BK., Wieczorek, L., Peachman, KK., Matyas., GR., Polonis, VR., Rao, M., and Alving CR. (2009) Human erythrocytes selectively bind and enrich infectious HIV-1 virions. PLoS One. 4, e8297.

45. Kulkarni, H., Marconi, VC., He, W., Landrum, ML., Okulicz, JF., Delmar, J., Kazandjian, D., Castiblanco, J., Ahuja SS., Wright, EJ., Weiss, RA., Clark, RA, Dolan, MJ, and Ahuja, SK. (2009) The Duffy-null state is associated with a survival advantage in leukopenic HIV-infected persons of African ancestry. Blood. 114, 2783-2792.

46. Hamers-Casterman，C.，Atarhouch，T.，Muyldermans，S.，Robinson，G.，Hamers，C, Songa，EB, Bendahman，N, and Hamers，R. (1993) Naturally occurring antibodies devoid of light chains. Nature. 363, 446-448.

47. Muyldermans, S., Baral, TN., Retamozzo, VC., De Baetselier, P., De Genst, E., Kinne, J., Leonhardt, H., Magez ,S., Nguyen, VK., Revets H., Rothbauer, U., Stijlemans, B., Tillib, S.,

Wernery, U., Wyns, L., Hassanzadeh-Ghassabeh, G., and Saerens D. (2009) Camelid immunoglobulins and nanobody technology. Vet Immunol Immunopathol. 128, 178-183.

48. Saerens, D., Frederix, F., Reekmans, G., Conrath, K., Jans, K., Brys, L., Huang, L., Bosmans E., Maes G., Borghs G., and Muyldermans S. (2005) Engineering camel single-domain antibodies and immobilization chemistry for human prostate-specific antigen sensing. Anal Chem. 77, 7547-7555.

49. Conrath, K., Pereira, AS., Martins, CE., Timóteo, CG., Tavares, P., Spinelli, S., Kinne, J., Flaudrops, C., Cambillau, C., Muyldermans, S., Moura, I., Moura, JJ., Tegoni M., and Desmyter, A. (2009) Camelid nanobodies raised against an integral membrane enzyme, nitric oxide reductase. Protein Sci. 18, 619-628.

50. Tereshko, V., Uysal, S., Koide A., Margalef, K., Koide, S., and Kossiakoff, AA. (2008) Toward chaperone-assisted crystallography, protein engineering enhancement of crystal packing and X-ray phasing capabilities of a camelid single-domain antibody (VHH) scaffold. Protein Sci. 17, 1175-1187.

51. Baral, TN., Magez, S., Stijlemans, B., Conrath, K., Vanhollebeke, B., Pays, E., Muyldermans, S., and De Baetselier,P. (2006) Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. Nat Med. 12, 580-584

52. Abderrazek, RB., Hmila, I., Vincke, C., Benlasfar, Z., Pellis, M., Dabbek, H., Saerens, D., El Ayeb, M., Muyldermans, S., and Bouhaouala-Zahar, B. (2009) Identification of potent nanobodies to neutralize the most poisonous polypeptide from scorpion venom. Biochem J.424, 263-272.

53. Studier, FW., Rosenberg, AH., Dunn, JJ., and Dubendorff, JW. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185, 60-89.

54. Studier, FW. (2005) Protein production by auto-induction in high density shaking cultures. Protein Expr Purif. 41, 207-234

55. Geysen, HM., Rodda, SJ., Mason, TJ., Tribbick, G., and Schoofs, PG, (1987) Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods 102, 259-274

56. Altschul, SF., Madden, TL., Schäffer, AA., Zhang, J., Zhang, Z., Miller, W., and Lipman, DJ. (1997). Gapped BLAST and PSI-BLAST, a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402..

57. Berman, HM., Westbrook, J., Feng, Z., Gilliland, G., Bhat, TN., Weissig, H., Shindyalov, IN., and Bourne, PE. (2000) The Protein Data Bank. Nucleic Acids Res. 28, 235-242.

58. Decanniere, K., Transue,TR., Desmyter, A., Maes, D., Muyldermans, S., and Wyns, L. (2001) Degenerate interfaces in antigen-antibody complexes. J Mol Biol.313, 473-478.

59. Dumoulin, M., Last, AM., Desmyter, A., Decanniere, K., Canet, D., Larsson, G., Spencer, A., Archer, DB., Sasse, J., Muyldermans, S., Wyns, L., Redfield, C., Matagne, A., Robinson, CV.,

and Dobson, CM.. (2003) A camelid antibody fragment inhibits the formation of amyloid fibrils by human lysozyme. Nature. 424, 783-788.

60. Thompson, JD., Gibson, TJ., Plewniak, F., Jeanmougin, F., and Higgins, DG. (1997) The CLUSTAL_X windows interface, flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.

61. Sali, A., and Blundell, TL. (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol. 234, 779-815.

62. Laskowski, RA., Moss, DS., and Thornton, JM. (1993) Main-chain bond lengths and bond angles in protein structures. J Mol Biol. 231, 1049-1067.

63. Shibuya, N., Goldstein, IJ., Broekaert, WF., Nsimba-Lubaki, M., Peeters, B., and Peumans, WJ. (1987) The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. J Biol Chem. 262, 1596-601.

64. Grodecka, M., Czerwiński, M., Duk, M., Lisowska, E., and Waśniowska K. (2010) Analysis of recombinant Duffy protein-linked N-glycans using lectins and glycosidases. Acta. Biochim Pol. 57,49-53.

65. Czerwinski ,M., Kern, J., Grodecka, M., Paprocka, M., Krop-Watorek, A., and Wasniowska, K. (2007) Mutational analysis of the N-glycosylation sites of Duffy antigen/receptor for chemokines, Biochem Biophys Res Commun.356, 816-821

66. Tournamille, C., Filipe, A., Wasniowska, K., Gane, P., Lisowska, E., Cartron, J.P., Colin, Y., and Le Van Kim, C. (2003) Structure-function analysis of the extracellular domains of the Duffy antigen/receptor for chemokines, characterization of antibody and chemokine binding sites, Br J Haematol 122, 1014-1023

67. Grimberg, B.T., Udomsangpetch, R., Xainli, J., McHenry, A, Panichakul, T, Sattabongkot, J, Cui, L, Bockarie, M, Chitnis, C, Adams, J, Zimmerman, PA, and King, CL. (2007) Plasmodium vivax invasion of human erythrocytes inhibited by antibodies directed against the Duffy binding protein, PLoS Med 4, e337.

68. De Genst, E., Silence, K., Decanniere, K., Conrath, K., Loris, R., Kinne, J., Muyldermans, S., and Wyns, L. (2006) Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. Proc Natl Acad Sci U S A.103, 4586-4591.

69. Gallivan, JP., and Dougherty, DA. (1999) Cation-pi interactions in structural biology. Proc Natl Acad Sci U S A. 96, 9459-9464.

70. Nguyen, VK., Hamers, R., Wyns, L., and Muyldermans., S. (2000) Camel heavy-chain antibodies : diverse germline V(H)H and specific mechanisms enlarge the antigen-binding repertoire. EMBO J. 19, 921-930.

71. Handel, TM., and Horuk, R. (2010) Duffy antigen inhibitors, useful therapeutics for malaria? Trends Parasitol. In press : doi:10.1016/j.pt.2010.03.010

72. Kasehagen, LJ., Mueller, I., Kiniboro, B., Bockarie, MJ., Reeder, JC., Kazura, JW., Kastens, W., McNamara, DT., King, C., Whalen, CC., and Zimmerman, PA. (2007) Reduced Plasmodium vivax erythrocyte infection in PNG Duffy-negative heterozygotes. PLoS One. 2, e336.

73. Albuquerque, SR., de Oliveira Cavalcante, F., Sanguino, EC., Tezza, L., Chacon, F., Castilho, L., and Dos Santos, MC. (2010) FY polymorphisms and vivax malaria in inhabitants of Amazonas State, Brazil. Parasitol Res. Inpress

74. Mariette, N., Barnadas, C., Bouchier, C., Tichit, M., and Ménard D. (2008) Country-wide assessment of the genetic polymorphism in Plasmodium falciparum and Plasmodium vivax antigens detected with rapid diagnostic tests for malaria. Malar J. 7,219-227.

75. Johnson, Z., Schwarz, M., Power, CA., Wells, TN., and Proudfoot, AE. (2005) Multi-faceted strategies to combat disease by interference with the chemokine system. Trends Immunol. 26(5), 268-274.

76. Wells, TN., Power, CA., Shaw, JP., and Proudfoot, AE. (2006) Chemokine blockers--therapeutics in the making? Trends Pharmacol Sci. 27, 41-47

77. Furthmayr, H., and Marchesi, VT. (1976) Subunit structure of human erythrocyte glycophorin A. Biochemistry. 15, 1137-44.

78. Grisshammer, R. (2009) Purification of recombinant G-protein-coupled receptors.Methods Enzymol. 463, 631-645.

79. Blackburn, P.E.(2004).Purification and biochemical characterization of the D6 chemokine receptor. Biochem. J. 379, 263–272.

80. Field, J., Nikawa, J., Broekv D., MacDonaldv B., Rodgers, L., Wilson, IA., Lerner, RA., and Wigler, M. (1988) Purification of a RAS-responsive adenylyl cyclase complex from Saccharomyces cerevisiae by use of an epitope addition method. Mol Cell Biol. 8, 2159-2165

81. Chelikani, P., Reeves, PJ., Rajbhandaryv UL., and Khorana, HG. (2006) The synthesis and high-level expression of a beta2-adrenergic receptor gene in a tetracycline-inducible stable mammalian cell line. Protein Sci. 15(6), 1433-40.

82. Mouro-Chanteloup, I., Cochet, C., Chami, M., Genetet, S., Zidi-Yahiaoui, N., Engel, A., Colin, Y., Bertrand, O., and Ripoche, P (2010) Functional Reconstitution into Liposomes of Purified Human RhCG Ammonia Channel PLoS One 5, e8921.

Table 1, Sequences of constructs used in this study.

Antigens used for immunization	MATAV<u>HRAELSPSTENSSQLDFEDVWNSSYGVNDSFPDGDY</u>G/DANLEAAAPCHSCNLLDDSALPAAKK- *(nuclease)*
	MATAV<u>HRAELSPSTENSSQLDFEDVWNSS**E**GVNDSFPDGD**E**</u>G/DANLEAAAPCHSCNLLDDSALPAAKK- *(nuclease)*
Antigens for library screening	*(GST)*-GILGNC<u>LHRAELSPSTENSSQLDFEDVWNSSYGVNDSFPDGDYG/DANLEAAAPCHSCNLLDDSASS</u>GRIVTD
Deletion mutant I	MATAV---------E<u>NSSQLDFEDVWNSSYGVNDSFPDGDYGANLEAAAPCHSCNLLDDSALP</u>AAKK- *(nuclease)*
Deletion mutant II	MATAV<u>HRAELSPSTENSS</u>-----------<u>GVNDSFPDGDYGANLEAAAPCHSCNLLDDSALP</u>AAKK- *(nuclease)*
Deletion mutant III	MATAV<u>HRAELSPSTENSSQLDFEDV</u>---------<u>SFPDGDYGANLEAAAPCHSCNLLDDSALP</u>AAKK- *(nuclease)*
Deletion mutant IV	MATAV<u>HRAELSPSTENSSQLDFEDVWNSSYGVND</u>-----------<u>AAAPCHSCNLLDDSALP</u>AAKK- *(nuclease)*
Deletion mutant V	MATAV<u>HRAELSPSTENSSQLDFEDVWNSSYGVNDSFPDGDYGANLEAAAP</u>---------<u>ALP</u>AAKK- *(nuclease)*

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 30 and 41 are shown with a larger and bold font. (*(GST)* is the abbreviation for Glutathione S-transferase)

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

Ligand	ka (M^{-1}s^{-1})	kd (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}

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
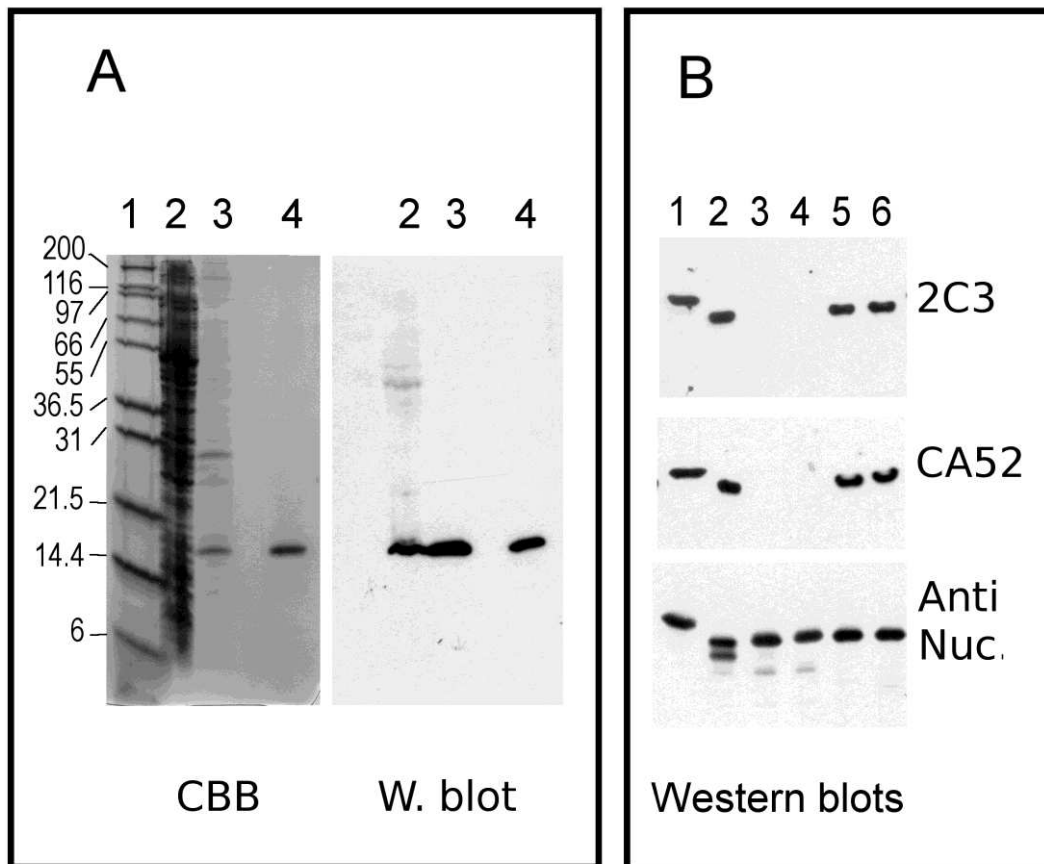
Figure 1

Figure 1

VHH purification and antigen-specificity screening

Panel 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.

Panel B are three western blots using 2C3, CA52, and antinuclease antiserum as primary antibody. Samples in lanes 1 contain wild type ECD-1 nuclease fusion while lanes 2 to 6 contain the deletion mutants (I to V, see Table 1 for sequences of the deletion mutants).
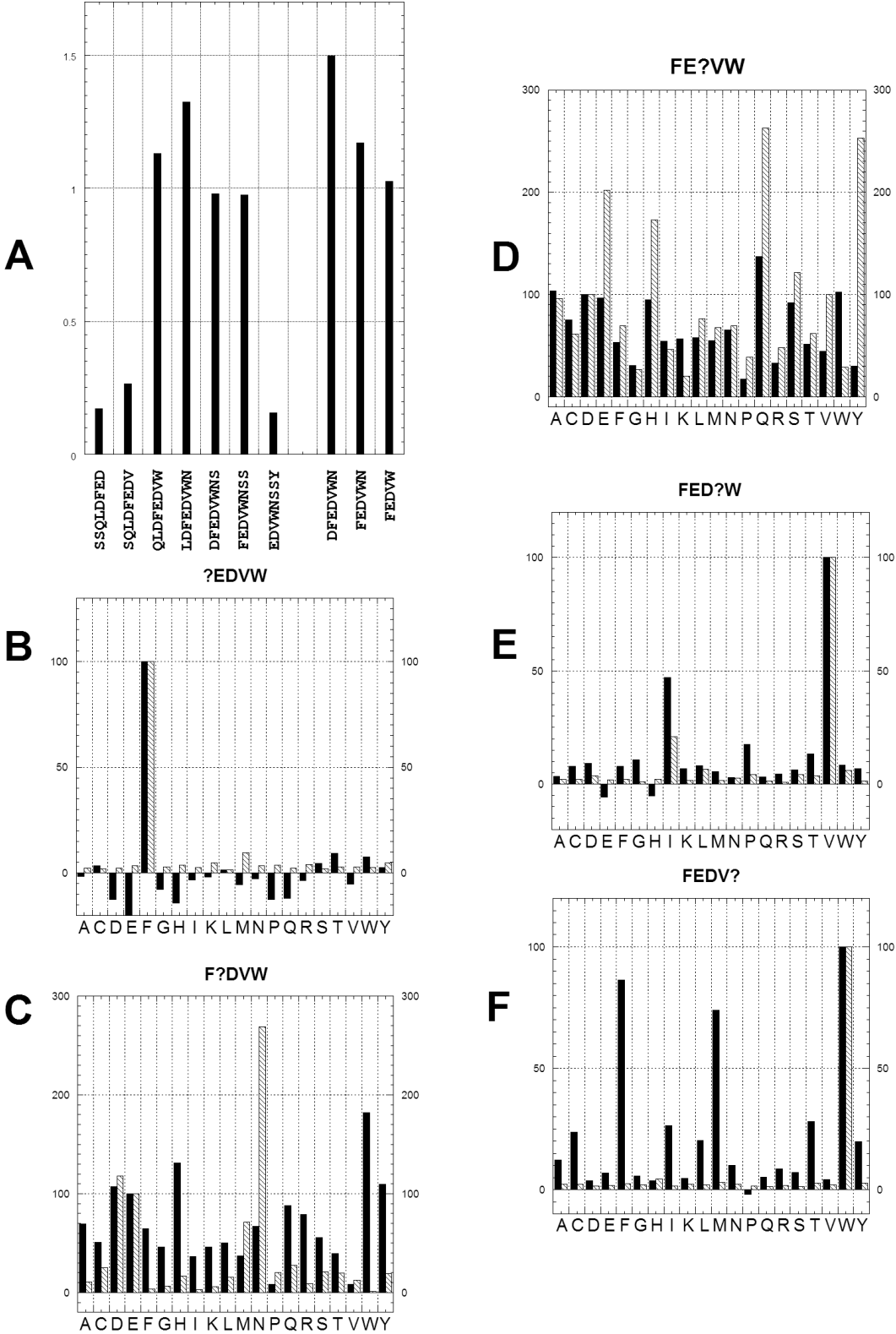
Figure 2

Figure 2,

Pepscan analysis of CA52 reactivity.

Panel A shows binding of CA52 to octapeptides overlapping the DARC ECD-1 sequence from Ser 17 to Tyr 30. Binding of CA52 was detected using anti-HA antibody as described in 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.

Panels B to F show 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 panel. 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).
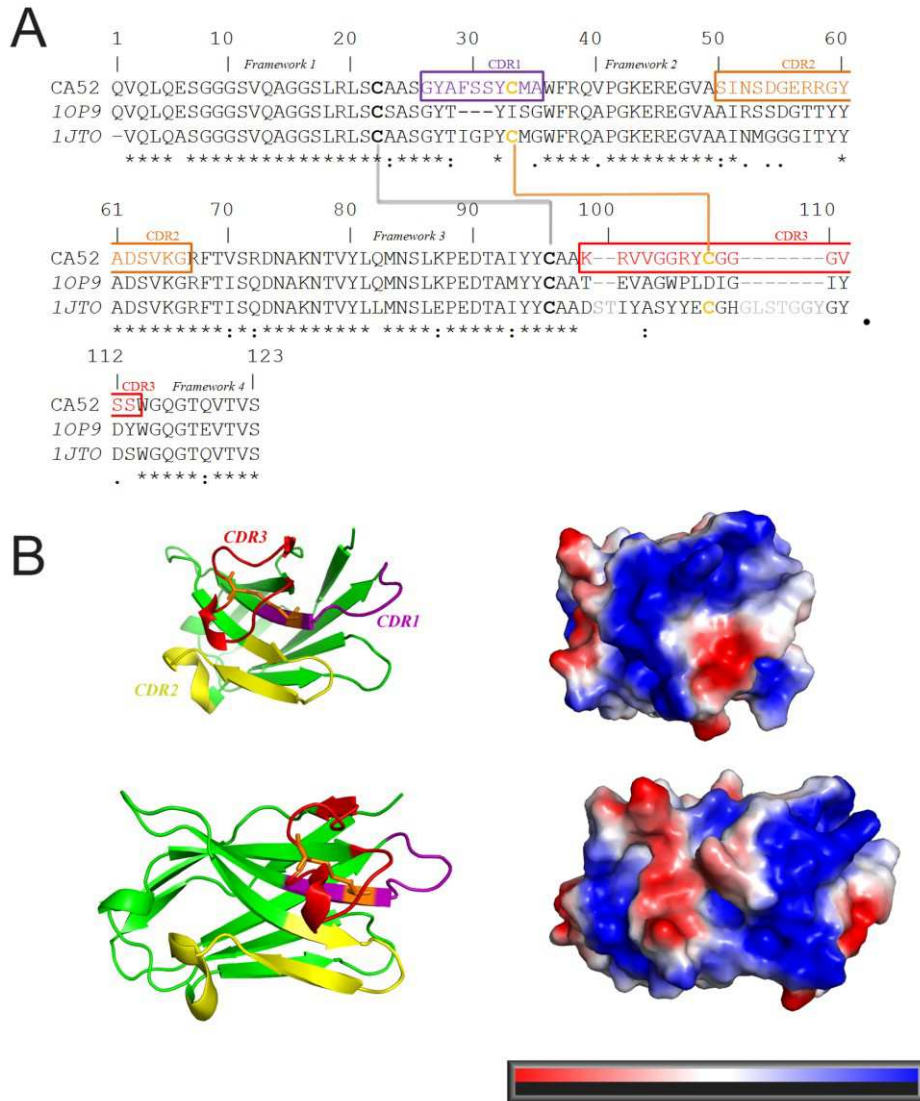
Figure 3

A

```
       1        10        20        30        40        50        60
       |         |         |         |         |         |         |
            Framework 1  |          CDR1   |    Framework 2        CDR2
CA52 QVQLQESGGGSVQAGGSLRLSCAAS GYAFSSYCMA WFRQVPGKEREGVA SINSDGERRGY
1OP9 QVQLQESGGGSVQAGGSLRLSCSASGYT---YISGWFRQAPGKEREGVAAIRSSDGTTYY
1JTO -VQLQASGGGSVQAGGSLRLSCAASGYTIGPYCMGWFRQAPGKEREGVAAINMGGGITYY
      **** ***************:****:   *  .****.*********:*. ..     *

       61        70        80        90       100       110
        |         |         |         |         |         |
     CDR2               Framework 3                      CDR3
CA52 ADSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAIYYCAA K--RVVGGRYCGG-------GV
1OP9 ADSVKGRFTISQDNAKNTVYLQMNSLKPEDTAMYYCAAT--EVAGWPLDIG-------IY
1JTO ADSVKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTIYASYYECGHGLSTGGYGY
      *********:*:*********  ****:*****:*****                :        .

      112       123
        |         |
     CDR3  Framework 4 |
CA52 SSWGQGTQVTVS
1OP9 DYWGQGTEVTVS
1JTO DSWGQGTQVTVS
      .  *****:****
```

B

Figure 3

Homology modelling of CA52

Panel A is the 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 grey and orange lines (grey is for the disulfide bond common to all immunoglobulin domains, orange is for the extra SS bond found frequently in dromedaries' VHHs, linking CDR1 and CDR3).

Panel B represents the 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. The left is a cartoon representation of the model, the right shows the electrostatic surface distribution with basic area (blue) concentrated at the three CDR's.
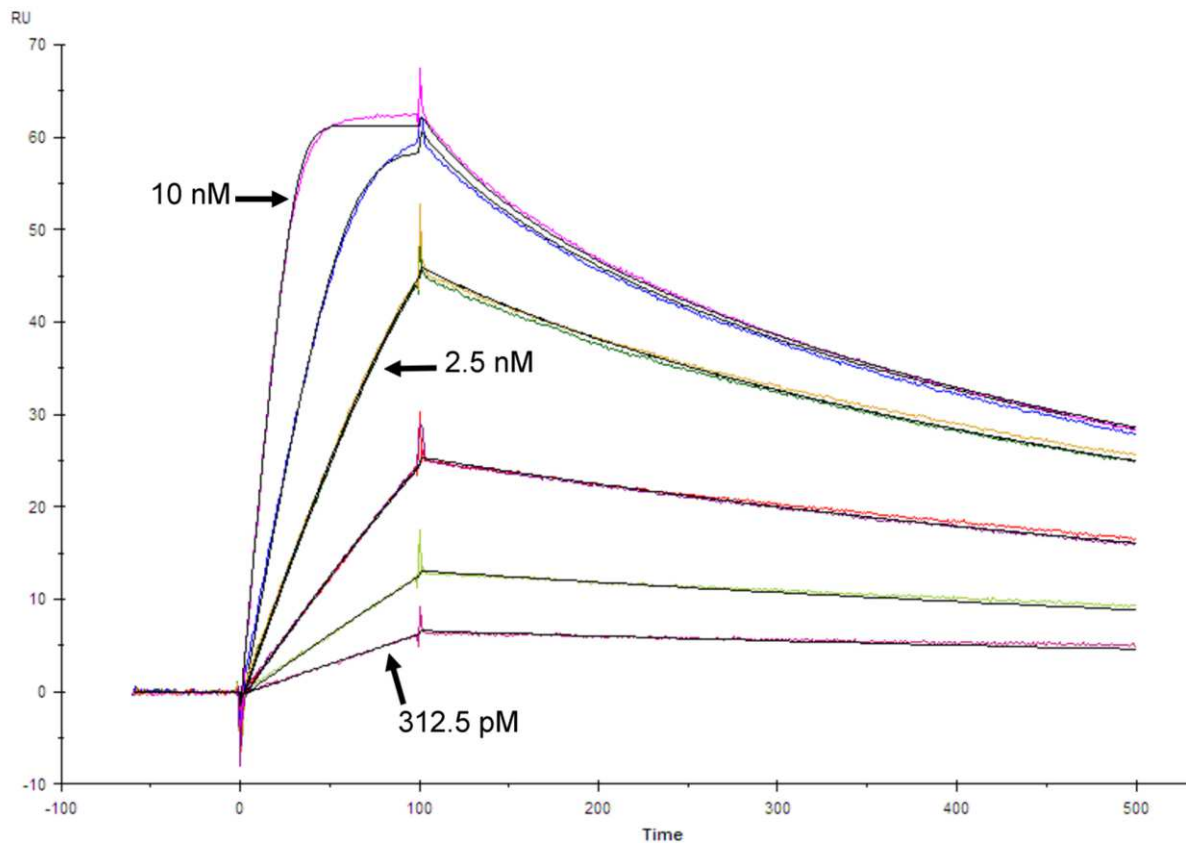
Figure 4

Figure 4

Surface Plasmon Resonance analysis of CA52 interaction with the ECD-1 nuclease construct

Sensorgrams obtained with the Biacore X100 apparatus using CA52 as analyte. ECD-1 fused to nuclease was immobilised on Fc2, the reference channel (Fc1) contained the nuclease. The highest concentration of CA52 was 10 nM and 4 two-fold serial dilutions were also injected. Coloured curves are experimental and black lines correspond to the fitted kinetic values (Table 2).
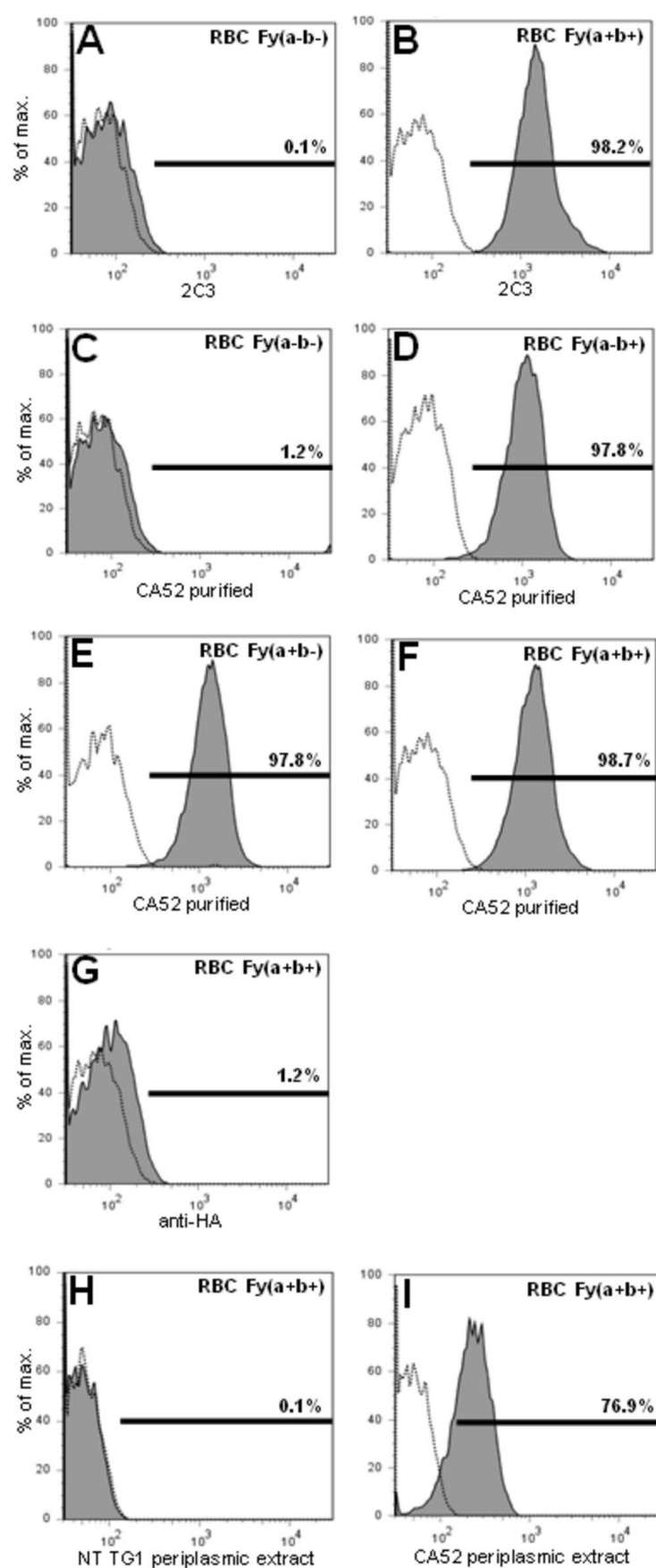
Figure 5

Figure 5

Flow cytometry of red blood cells.

The red blood cells with phenotype as indicated (upper right corner of each panel) were incubated with the 2C3 monoclonal (panels A and B), or with the purified CA52 (panels C-F), or with crude CA52 extract (panel I). The gray histograms (panels A to G) correspond to results obtained with primary antibodies as indicated at the bottom of each panel. Clear histograms show the signals obtained using non relevant murine IgG and phycoerythrin tagged anti murine IgG (panels A and B), or only the secondary anti-HA antibody and phycoerythrin tagged anti murine IgG (panels C to F).

Panel G is a control from cells stained with anti-HA antibody and phycoerythrin tagged anti murine IgG (gray histogram), blank histogram in panel G show results obtained with non relevant murine IgG and phycoerythrin tagged anti murine IgG.

Panel H and I are the results from cells stained with proteins from the periplasmic extract of cells expressing CA52 or mock transformed cells (NT TG1), respectively. Gray histogram is the specific signal while clear histogram was obtained with anti-HA and phycoerythrin tagged anti murine IgG. Numbers above the horizontal bars indicate the percentage of cells of greyed histograms which are reactive with the indicated antibody.
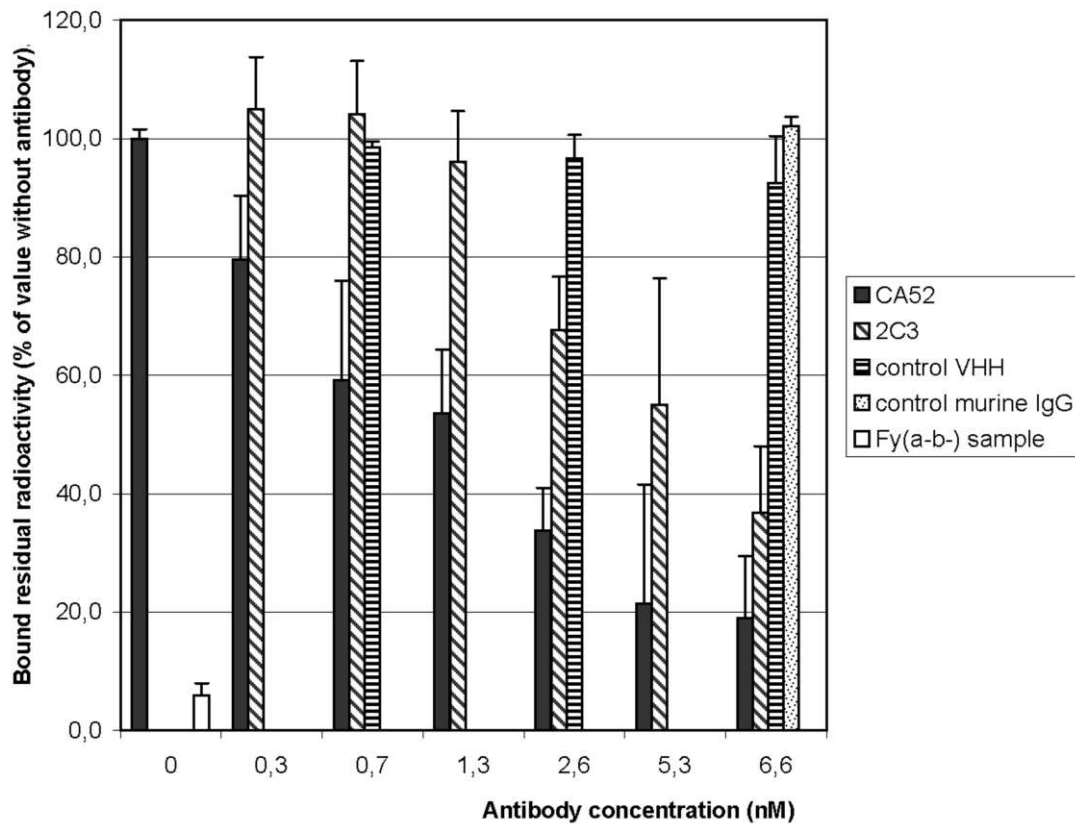
Figure 6

Figure 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 bar is the mean of three determinations; error bars correspond to one standard deviation. Shown also are results obtained with controls : (i) neither anti-lysozyme VHH D3L11 nor irrelevant murine IgG interfere with interleukin-8 binding to Duffy positive cells (ii) Duffy negative cells do not bind appreciably interleukin-8.
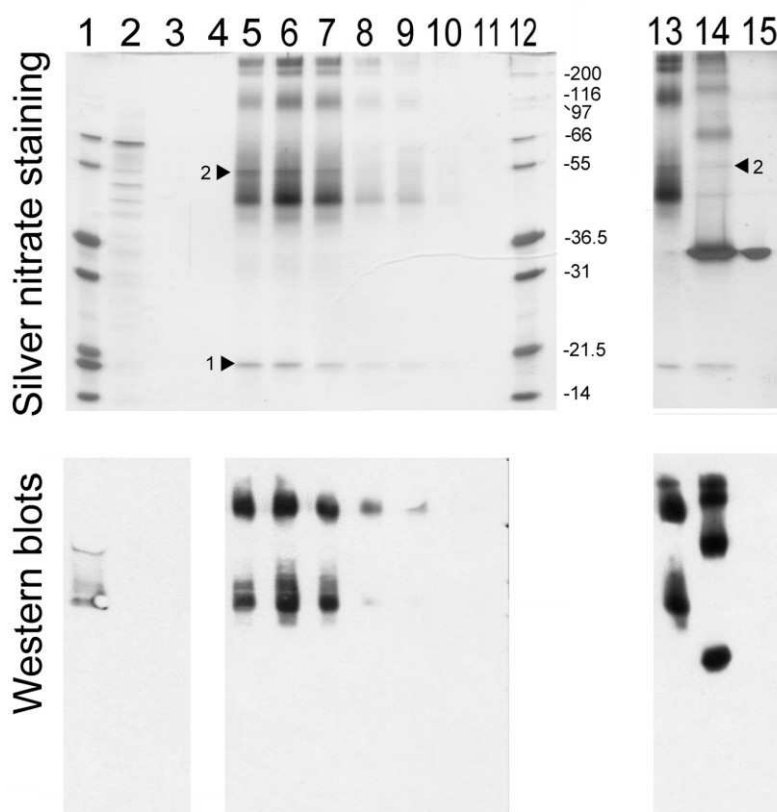
Figure 7

Figure 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 to 4 contain aliquots of washings of the support performed after the sample loading. Lanes 5 to 7 contain aliquots of material eluted with a solution of a hexapeptide containing the epitope sequence, whereas lanes 8 to 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. Arrowheads 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.