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# Highly specific and potently activating Vγ9Vδ2-T cell specific nanobodies for diagnostic and therapeutic applications

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ABSTRACT

Vγ9Vδ2-T cells constitute the predominant subset of γδ-T cells in human peripheral blood and have been shown to play an important role in antimicrobial and antitumor immune responses. Several efforts have been initiated to exploit these cells for cancer immunotherapy, e.g. by using phosphoantigens, adoptive cell transfer, and by a bispecific monoclonal antibody based approach. Here, we report the generation of a novel set of Vγ9Vδ2-T cell specific VHH (or nanobody). VHH have several advantages compared to conventional antibodies related to their small size, stability, ease of generating multispecific molecules and low immunogenicity. With high specificity and affinity, the *anti*-Vγ9Vδ2-T cell receptor VHHs are shown to be useful for FACS, MACS and immunocytochemistry. In addition, some VHH were found to specifically activate Vγ9Vδ2-T cells. Besides being of possible immunotherapeutic value, these single domain antibodies will be of great value in the further study of this important immune effector cell subset.

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## 1. Introduction

Vγ9Vδ2-T cells constitute the predominant subset of peripheral blood γδ-T cells with several unique features that can be valuable for immunotherapeutic approaches. Vγ9Vδ2-T cells become activated by the MHC-independent recognition of non-peptide phosphoantigens (pAg) that are produced upon bacterial infection or are upregulated by malignant cells [1]. Presumably, the type I membrane protein butyrophilin 3A1 (BTN3A1, also known as CD277) obtains an altered conformation and membrane distribution upon the detection of elevated intracellular pAg levels. This extracellular modification of BTN3A1 is consequently sensed by the Vγ9Vδ2-T cell receptor (TCR) resulting in Vγ9Vδ2-T cell activation [2–4]. Stimulation of Vγ9Vδ2-T cells can be enhanced by interaction between the NKG2D receptor expressed on most Vγ9Vδ2-T cells and stress-related MICA, MICB and ULBP molecules, upregulated on infected or transformed cells [5,6]. This allows Vγ9Vδ2-T cells to distinguish normal cells from stressed cells and to consequently produce large amounts of pro-inflammatory cytokines (e.g. IFN-γ, TNF-α and chemokines MIP-1 and RANTES) in addition to cytolytic mediators (perforin, granzyme B) to induce specific lysis of cells with elevated pAg levels [7]. In this way, Vγ9Vδ2-T cells have been shown to be capable of inducing lysis of a broad spectrum of cancer cells [5,8–10]. Furthermore, upon activation Vγ9Vδ2-T cells can stimulate the maturation of immature dendritic cells and can also acquire antigen presenting capacities themselves [11].

Based on these characteristics, several clinical trials were initiated to evaluate whether Vγ9Vδ2-T cells could be exploited for immunotherapy against both hematological and solid malignancies. Trials have included approaches where aminobisphosphonates (to increase endogenous pAg levels) or synthetic pAgs were used alone or in combination with IL-2, and the adoptive transfer of autologous *ex vivo* expanded Vγ9Vδ2-

*Abbreviations:* VHH, variable domain of heavy chain-only Ab; pAg, phosphoantigens; TCR, T cell receptor; PBMC, peripheral blood mononuclear cells; MACS, magnetic-activated cell sorting.

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T cells. In general, these Vγ9Vδ2-T cell-based therapeutic approaches were well tolerated and capable of inducing clinically relevant antitumor responses in several cases. However, results were not consistent [12,13]. A possible explanation could be that these therapies were all aimed at systemic (i.e. not tumor-specific) activation of Vγ9Vδ2-T cells. More recently, a new Vγ9Vδ2-T cell immunotherapy approach was explored through the use of a bispecific antibody. Her2/neu overexpressing pancreatic cells could efficiently be lysed by Vγ9Vδ2-T cells in the presence of a $(Her2)_2$xVγ9 bispecific antibody *in vitro*. When the $(Her2)_2$xVγ9 bispecific antibody was co-administered with Vγ9Vδ2-T cells on a weekly basis to immunocompromised mice inoculated 2 days earlier with a pancreatic cancer cell line, significantly reduced tumor volumes were observed after 4 weeks [14]. This antibody-based approach adds a new dimension to the future of Vγ9Vδ2-T cell-based immunotherapy. However, the development and use of bispecific conventional antibody-based molecules has several limitations, including mispairing of heavy and light chains and instability [15]. Of interest, and in addition to conventional antibodies, camelids (i.e. llamas, camels and dromedaries) also possess a unique antibody class consisting of heavy chain only antibodies which lack the light chain and the CH1 domain of the heavy chain [16]. The variable antigen binding region of these antibodies is called a VHH (or nanobody) and consists of only a single immunoglobulin domain, in contrast to full-length antibodies or single-chain variable fragments (scFv) often used for the design of antibody-based constructs [17]. VHHs can be used as an attractive alternative to conventional antibodies. Since VHHs only need to fold one domain they have increased stability, solubility and ease of refolding. VHHs are ten times smaller than conventional antibodies which allows them to reach clefts in antigen structures and provides enhanced tissue penetration as compared to antibodies [18, 19]. VHHs can easily be made into multispecificity molecules and have a low immunogenicity. Due to the absence of an Fc-domain, VHHs can be produced by *Escherichia coli* or yeast allowing time and cost reduction [15,20].

Based on the above mentioned unique characteristics of VHHs, we set out to generate a novel set of Vγ9Vδ2 T cell specific VHHs and evaluate their use for various applications. Our efforts resulted in the identification of a panel of Vγ9Vδ2-TCR specific VHHs, directed against the Vγ9-chain and/or Vδ2-chain, with activating as well as non-activating properties. These Vγ9- and Vδ2-TCR directed VHHs could successfully be used for flow cytometric analysis, isolation of Vγ9Vδ2-T cells by magnetic bead isolation, immunocytochemistry and have great potential for the development of future Vγ9Vδ2-T cell-based immunotherapies.

## 2. Material & methods

### 2.1. Cell lines

Jurkat and Jurkat/MA were cultured in IMDM + medium, i.e. Iscove's modified Dulbecco's medium (Lonza, catalog #BE12-722F) supplemented with 10% (*v*/*v*) fetal calf serum (FCS) (HyClone GE Healthcare, catalog #SV30160.03), 0.05 mM β-mercaptoethanol (β-ME), 100 IU/mL sodium penicillin, 100 μg/mL streptomycin sulfate and 2.0 mM L-glutamine (Life Technologies, catalog #10378-016). Phoenix-A cells were cultured in Dulbecco's Modified Eagle's Medium (Lonza, catalog #BE12-614F) supplemented with 10% FCS, 0.05 mM β-ME, 100 IU/mL sodium penicillin, 100 μg/mL streptomycin sulfate and 2.0 mM L-glutamine. Cell lines were tested mycoplasma negative.

### 2.2. Flow cytometry and monoclonal antibodies

Antibodies used were FITC-labeled *anti*-TCR Vδ2 (catalog #555738) and PE-labeled pan γδ-TCR (catalog #333141) from BD Biosciences and FITC-labeled *anti*-TCR Vα24 (catalog #IM1589) and PE-labeled *anti*-TCR Vβ11 (catalog #IM2290) from Beckman Coulter. PerCP-labeled *anti*-TCR Vδ2 (catalog #331410), PE-labeled *anti*-TCR Vγ9 (catalog #331308) and APC-labeled *anti*-TCR Vγ9 (catalog #331310) were from Biolegend and FITC-labeled polyclonal swine-*anti*-rabbit antibody (catalog #F0205), RPE-labeled goat-*anti*-mouse F(ab′)2 fragment (catalog #R0480) and Streptavidin/RPE (catalog #R0480) were obtained from Dako. APC-labeled goat-*anti*-mouse F(ab′)2 fragment (catalog #SC-3818) was obtained from Santa Cruz Biotech and Alexa488-conjugated goat-*anti*-rabbit antibody (catalog #A-11008) was from Thermo Fisher Scientific. Rabbit-*anti*-VHH (purified) clone K1216 and rabbit-*anti*-VHH serum clone K976 were obtained from QVQ (on request). Anti-Myc tag antibody clone 4A6 (catalog #05-724) was obtained from Merck Millipore and *anti*-Myc tag antibody clone 9E10 was produced in-house.

All stainings for flow cytometry were performed in PBS supplemented with 0.1% (*w*/*v*) BSA and 0.02% (*w*/*v*) sodium-azide. Stained cells were measured with FACS Calibur or LSRFortessa (BD Biosciences, NJ, USA) and analyzed and analyzed with CellQuest (BD Biosciences) or Kaluza software (Beckman-Coulter).

### 2.3. Generation of donor-derived γδ-T cells

Healthy donor-derived Vγ9Vδ2-T cells were isolated, expanded and cultured as described [26]. In brief, Vγ9Vδ2-T cells were isolated from human peripheral blood mononuclear cells (PBMC) by Magnetic-activated cell sorting (MACS) using FITC-labeled *anti*-TCR Vδ2 or PE-labeled *anti*-TCR Vγ9 antibody in combination with *anti*-mouse IgG MicroBeads (Miltenyi Biotec, catalog #130-048-401). Purified Vγ9Vδ2-T cells were stimulated weekly with irradiated and aminobisphosphonate (100 μM Pamidronate for 3 h, Teva Pharmachemie, catalog #12J08RD) pretreated human mature monocyte derived dendritic cells or an irradiated feeder mixture (PBMC of 2 healthy human donors and Epstein Barr Virus transformed B cells with addition of 50 ng/mL PHA). Vγ9Vδ2-T cells were used for experiments when purity was >90%. Of note, Vγ9Vδ2-T cells used for testing the activation inducing potential of VHHs had a < 40% CD25 expression.

To obtain $V\gamma9^-V\delta2^+$, $V\gamma9^+V\delta2^-$, $V\gamma9^+V\delta2^+$ and $V\gamma9^-V\delta2^-$ γδ-T cell lines, we first isolated a pan γδ-T cell population. Human PBMC were stained with a PE-labeled pan γδ-TCR antibody and purified with MACS using *anti*-mouse IgG MicroBeads. This pan γδ-T cell line was first expanded with feeder mixture and then stained with FITC-labeled *anti*-TCR Vδ2 and PE-labeled *anti*-TCR Vγ9 antibodies to allow flow cytometric cell sorting of 4 separate populations (i.e. $V\gamma9^-V\delta2^+$, $V\gamma9^+V\delta2^-$, $V\gamma9^+V\delta2^+$ and $V\gamma9^-V\delta2^-$ γδ-T cells).

All donor-derived γδ-T cell lines were cultured in Yssels medium [27] supplemented with 1% AB human serum, 50 U/mL rhIL-2 (Proleukin, Novartis), 0.05 mM β-ME, 100 IU/mL sodium penicillin, 100 μg/mL streptomycin sulfate and 2.0 mM L-glutamine at 37 °C with 5% $CO_2$ in a humidified atmosphere. Cell lines were tested mycoplasma negative.

### 2.4. Generation of Vγ9Vδ2-TCR and Vα24Vβ11-TCR transduced cell lines

Jurkat and Jurkat/MA cell lines expressing TCRs of interest were generated as described previously [28]. For the Vγ9Vδ2-TCR, protein sequences of clone G9 Vγ9- and Vδ2-chain [29,30] were used. For the Vα24Vβ11-TCR, protein sequences of clone NKT12 Vα24- and Vβ11-chain [31] were used. Sequences of the individual TCR chains were separated by a picorna virus derived 2 A sequence, codon modified for optimal protein production and synthesized by GeneART (Thermo Fisher Scientific, MA, USA) after which they were cloned into the LZRS vector. After transfection to the Phoenix-A packaging cell line, retroviral supernatants were collected to transduce Jurkat or Jurkat/MA cells in the presence of retronectin (Takara, catalog #T100A) according to the manufacturer's protocol [28]. Purity of the transduced cell lines was obtained by MACS cell separation using *anti*-mouse IgG MicroBeads or by flow cytometric sorting both in combination with FITC-labeled *anti*-TCR Vδ2 and PE-labeled *anti*-TCR Vγ9 antibodies or FITC-labeled *anti*-TCR

Vα24 and PE-labeled *anti*-TCR Vβ11 antibodies for Vγ9Vδ2- and Vα24Vβ11-TCR cell lines, respectively.

### 2.5. Immunization of Lama glama

Two *Lama glamas* were immunized four times each with $1 \times 10^8$ human Vγ9Vδ2-T cells of 4–8 different healthy donors in sterile PBS over a period of six weeks. Blood samples were taken before, at day 28 and at day 43 of the immunization period. To test for serum immune responses against Vγ9Vδ2-T cells, Vγ9Vδ2-T cells were incubated with diluted sera. Bound llama antibodies were detected by sequential incubation with rabbit-*anti*-VHH serum clone K976 and swine-*anti*-rabbit FITC antibody and measured by flow cytometry.

### 2.6. Selection of anti-Vγ9Vδ2-TCR VHH

Phage libraries of immunized llamas were constructed by QVQ (Utrecht, The Netherlands). In short, extracted RNA isolated from llama PBMC was transcribed to cDNA and with specific primers the VH and subsequently VHH region was amplified and ligated into the phagemid vector pUR8100 for display on filamentous phage [32]. This resulted in two libraries with approximately $10^7$ transformants each. Vγ9Vδ2-TCR specific VHH were enriched by two rounds of phage display selections. In the first round, phages from both libraries were incubated for 2 h at 4 °C with Vγ9Vδ2-TCR transduced Jurkat cells (Jurkat-Vγ9Vδ2-TCR cells) in HBSS. After incubation, cells were washed and phages were eluted for 7 min with 100 mM HCl. Eluted phages were harvested and neutralized with Tris-HCl after which they were used to infect *E. coli*. After rescue of the polyclonal pool of selected phages, non-relevant phages were first depleted by two incubations for 1 h at 4 °C with Jurkat cells in HBSS after which unbound phages were used for selection on Jurkat-Vγ9Vδ2-TCR cells. Phages were eluted and used to infect *E. coli* as described for the first round selections. Bacteria were plated on LB-agar plates supplemented with 2% glucose and 100 μg/mL ampicillin (Sigma-Aldrich, catalog #A9518) to generate single bacterial colonies containing selected VHH clones. Periplasmic extracts were made of individual colonies (as described below) and incubated with a 1:1 mixture of Jurkat-Vγ9Vδ2-TCR and Jurkat cells (the latter stained with 40 nM CFSE (Sigma-Aldrich, catalog #21888). VHH specifically binding to Jurkat-Vγ9Vδ2-TCR cells were detected with *anti*-Myc-tag antibody clone 4A6 and an APC-labeled goat-*anti*-mouse $F(ab')_2$ fragment, and selected for production and further characterization.

### 2.7. Production and purification of VHH

VHH DNA from individual clones was cloned into plasmid pMEK219 (a kind gift of Mohamed El Khattabi (QVQ, Utrecht, The Netherlands)), a derivative from pHen1 [33] with addition of a HC-V cassette to enable VHH cloning, a C-terminal Myc- and 6× HIS-tag and deletion of the gene III sequence. TG1 bacteria were transformed with pMEK219-VHH for protein production. Bacteria were grown to log-phase in 2xYT (Serva, catalog #48501.01) plus 100 μg/mL ampicillin and 0.1% glucose. Protein production was induced by addition of a final concentration of 1 mM IPTG (Thermo Fisher Scientific, catalog #R0391). VHH were obtained from the periplasmic space by harvesting the bacteria through centrifugation (4500 rpm, 10′, 4 °C), re-suspending them in PBS and subjecting them to a cycle of freeze-thawing. Bacterial debris were spun down (4500, 30′, 4 °C) and the supernatant was used as periplasmic extract.

VHH were purified from periplasmic extracts by immobilized metal ion affinity chromatography (IMAC) on Talon resin (Clontech, catalog # 635504). VHH were eluted with 150 mM imidazole and dialyzed twice against PBS. Purified VHHs were checked for purity by coomassie stained protein gel. For biotinylation of VHH, VHH were treated with NHS-D-Biotin (Sigma-Aldrich, catalog #H1759) according to the manufacturer's protocol.

### 2.8. Specificity analysis of isolated VHH

To determine the specificity of the *anti*-Vγ9Vδ2-TCR VHHs, $4 * 10^5$ PBMC, $5 * 10^4$ donor-derived γδ-T cells (e.g. $V\gamma9^-V\delta2^+$, $V\gamma9^+V\delta2^-$, $V\gamma9^+V\delta2^+$ or $V\gamma9^-V\delta2^-$) or $5 * 10^4$ (TCR transduced) Jurkat cells were incubated with 100–500 nM purified (biotinylated) VHH. Bound VHH was detected with *anti*-Myc-tag antibody clone 4 A6 and APC-or RPE-labeled goat-*anti*-mouse $F(ab')_2$ fragment or in case of biotinylated VHH, with streptavidin/RPE.

### 2.9. Activation of Vγ9Vδ2-TCR transduced Jurkat/MA cells by Vγ9Vδ2-TCR specific VHH

To determine the capacity of the Vγ9Vδ2-TCR VHHs to induce activation of Vγ9Vδ2-TCR, an assay based on the Jurkat/MA luciferase system was used as described previously [34]. A 96-well flat bottom culture plate was coated overnight with 4 μg/mL *anti*-Myc-tag clone 9E10 antibody in PBS. In a separate plate, $10^5$ HeLa cells were seeded. The following day, the *anti*-Myc coated plate was washed, blocked with 4% (*w*/*v*) BSA in PBS and incubated with 100 nM VHH in PBS after which excess VHH was washed away. To the plate seeded with HeLa cells, 0 or 50 μM of the aminobiphosphonate Pamidronate was added for 3 h at 37⁰C after which wells were carefully washed. To both plates, $10^5$ Jurkat/MA cells carrying a luciferase gene under NFAT transcriptional control [21] and transduced with a Vγ9Vδ2-TCR (Jurkat/MA-Vγ9Vδ2-TCR cells), were added in IMDM + medium and incubated for 24 h at 37⁰C. The next day, 50 ng/mL PMA and 500 ng/mL ionomycin was added for 4 h at 37⁰C to the positive control conditions. To stop the assay, all wells were washed with PBS after which Jurkat/MA-Vγ9Vδ2-TCR cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega, catalog #E1531). Luminescence was measured by addition of Luciferase assay reagent (Promega, catalog #E1483) according to the manufacturer's protocol. Luminescence was measured on a Lumat LB 9507 luminometer (EG and G Berthold, Bad Wildbad, Germany).

### 2.10. Magnetic-activated cell sorting with VHH

$45–90 * 10^6$ human PBMC were incubated with 0.5 μg biotinylated VHH to isolate Vγ9Vδ2-T cells from PBMC via positive selection with *anti*-Biotin MicroBeads (Miltenyi Biotec, catalog #130-090-485) according to the manufacturer's protocol.

### 2.11. Immunocytochemistry with VHHs

VHH used for immunocytochemistry were produced in a similar way as described above, with some adjustments. VHH was cloned to plasmid pMEK220 (kindly provided by Mohamed El Khattabi (QVQ, Utrecht, The Netherlands)), a variant of pMEK219 with the absence of a C-terminal Myc- and 6× HIS-tag. Produced VHH was purified using a HITrap protein A HP column (catalog #17-0402-01) and desalted using a HITrap Desalting column (catalog #29-0486-84) on an ÄKTAxpress (all from GE Healthcare, Zeist, The Netherlands) using the manufacturer's protocol.

Cytospins were made by spinning Jurkat, Jurkat-Vγ9Vδ2-TCR, healthy donor-derived Vγ9Vδ2-T cells or Vα24β11-T cells on Starfrost slides (Knittel) and allowing them to dry overnight. Cells were fixed with 4% PFA in 0.1 M PHEM buffer pH 6.9 and 100 mM ammonium chloride in PBS was used for quenching. Blocking was performed with blocking buffer (Elly van Donselaar, personal communication, manuscript in preparation): 0.5% (*w*/*v*) gelatin from cold water fish skin (Sigma-Aldrich, catalog #G7765) plus 0.1% (*v*/*v*) BSA-c (Aurion, catalog # 900.099) followed by incubation with 500 nM VHH in the same buffer. Bound VHH was detected with rabbit-*anti*-VHH (clone K1216) and

Alexa488-conjugated goat-*anti*-rabbit antibody in blocking buffer. Cells were co-stained with DAPI to visualize nuclei.

For cryosections, 50 ∗ $10^6$ Jurkat or Jurkat-Vγ9Vδ2-TCR cells were pelleted and resuspended in Tissue Tek OCT Compound (Sakura, catalog #4583). The tube with pelleted cells was snap frozen in liquid nitrogen and stored at −80 °C. A cryostat (CM 1850 UV, Leica, Wetzlar, Germany) was used to cut 5 μM sections which were transferred to Starfrost slides and stained as described above for cytospin slides.

Coverslips were mounted with ProLong Gold (Thermo Fisher Scientific, catalog #P36930) and images were made by wide field fluorescent microscopy.

### 2.12. Statistical analyses

Statistical analyses were performed in GraphPad Prism version 5 (La Jolla, CA, USA) using paired Student t-tests. Findings were considered significant when *p*-values were $<0.05$.

## 3. Results

### 3.1. Induction of an anti-Vγ9Vδ2-T cell immune response in Lama glama

In order to generate VHH specific for the human Vγ9Vδ2-TCR, 2 llamas (named llama A and llama B) were subcutaneously immunized with $10^8$ purified human healthy donor-derived Vγ9Vδ2-T cells for four times over a period of six weeks. The induction of a humoral immune response was followed by testing sera of llama A and B previous to, midway (day 28) and post immunization (day 42). To this end, purified Vγ9Vδ2-T cells were incubated with diluted sera and bound llama antibodies were detected by flow cytometry. Both immunized llamas display an increasing humoral immune response over the course of the immunization against Vγ9Vδ2-T cells (Fig. 1).

### 3.2. Selection of Vγ9Vδ2-TCR specific VHH

Phage VHH repertoires were synthesized by RT-PCR from peripheral blood lymphocytes obtained from immunized llama A and B. Vγ9Vδ2-TCR specific VHHs were selected by means of phage-display using sequential positive and negative selection on a Jurkat cell line genetically modified to express the Vγ9Vδ2-TCR (Jurkat-Vγ9Vδ2-TCR) and a non-transduced Jurkat cell line, respectively (Fig. 2A). After this selection, 190 bacterial periplasmic extracts of single monoclonal VHH clones were randomly selected and screened for their ability to specifically bind the Vγ9Vδ2-TCR. For this purpose, the periplasmic extracts were incubated with a 1:1 mixture of CFSE-labeled Jurkat cells and non-labeled Jurkat-Vγ9Vδ2-TCR cells and specific binding was determined by flow cytometry. This screening resulted in the identification of 42 individual VHH clones that bound to Jurkat-Vγ9Vδ2-TCR cells but not to the control Jurkat cell line (Fig. 2B), suggesting specificity to either the Vγ9Vδ2-TCR or to CD3 co-expressed in the TCR-complex. After elimination of poor protein producing clones and sequencing of the remaining selected clones to exclude duplicates, a set of 20 individual VHH clones remained; 9 derived from llama A and 11 derived from llama B. These 20 clones were subsequently tested for binding to CD3 or αβ-TCR using Jurkat cells genetically modified to express the invariant Vα24Vβ11 TCR of CD1d-restricted iNKT cells (Jurkat-Vα24Vβ11-TCR cells). Importantly, whereas all 20 clones were confirmed to bind to Jurkat-Vγ9Vδ2-TCR cells, none of the clones showed binding to αβ-TCR expressing Jurkat cells indicating that the selected VHHs were indeed specific for the Vγ9Vδ2-TCR and did not cross-react with the αβ-TCR or CD3 (Fig. 2C). However, these data do not exclude binding of the selected VHHs to conserved constant γδ-TCR domains. The 20 VHH clones were purified for further characterization studies.

### 3.3. Characterization of Vγ9Vδ2-TCR specific VHHs

To determine whether the *anti*-Vγ9Vδ2-TCR VHHs were specific for the TCR Vδ2- and/or Vγ9- chain, pan γδ-T cells were first purified from healthy donor-derived PBMC by Magnetic-activated cell sorting (MACS). After an initial round of expansion, the resulting population of pan γδ-T cells (98% purity) was separated by flow cytometric sorting into 4 distinct populations: $V\gamma9^-V\delta2^+$, $V\gamma9^+V\delta2^-$, $V\gamma9^+V\delta2^+$ and $V\gamma9^-V\delta2^-$ γδ-T cells (Fig. 3A). Binding of the 20 previously selected and purified putative Vγ9Vδ2-TCR specific VHHs to these 4 γδ-T cell populations was then determined and defined as non-binding, weak, moderate or strong binding to each population according the mean fluorescence index (Fig. 3B). As is depicted in Table 1, all selected VHHs demonstrated binding to $V\gamma9^+V\delta2^+$ γδ-T cells but not to $V\gamma9^-V\delta2^-$ γδ-T cells (i.e. γδ-T cells expressing other γ- or δ-TCR chains). This indicated that none of the VHHs bound to the constant γδ-TCR region but that they all bound to the variable region of the TCR. The majority of the tested VHHs were specific for the Vδ2-TCR chain, with 5 VHHs showing preferential or exclusive binding to the Vγ9-TCR chain. Clones 5C1, 6H3, and 6C4 showed reactivity to both the Vγ9- and the Vδ2-chain, although in all cases there was a preference for binding to one of the chains.

In order to gain more knowledge about the characteristics of the specific VHHs, we determined the affinity of these VHHs for the Vγ9Vδ2-TCR. Jurkat-Vγ9Vδ2-TCR cells were incubated with different concentrations of each VHH and the concentration at which half of the maximum fluorescence intensity was reached by flow cytometry analysis, was determined. Most VHHs had an affinity of 1–50 nM with the

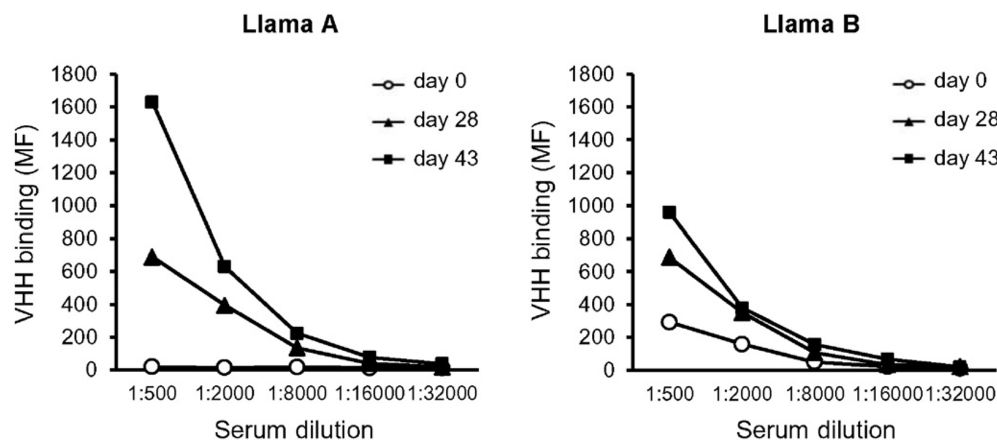


**Fig. 1.** Immunization of *Lama glama* with human healthy donor-derived Vγ9Vδ2-T cells induces a strong immune response. Vγ9Vδ2-T cells were incubated with diluted pre-immune (day 0: *open circles*) or immune sera (day 28: *triangles* and day 43: *squares*) of immunized llamas A and B. VHH binding was assessed by flow cytometry. Abbreviations: mean fluorescence intensity (MF).

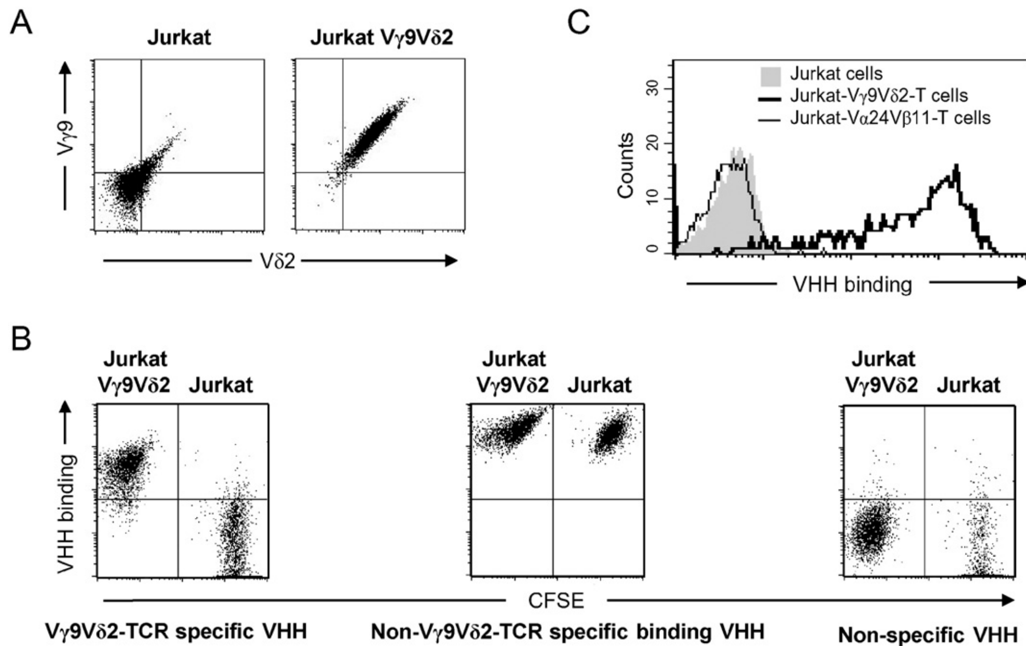


**Fig. 2.** Screening of bacterial periplasmic extracts of Vγ9Vδ2-TCR specific VHH clones. A) Dot-plots illustrating Vγ9Vδ2-TCR expression of non-transduced (left) and Vγ9Vδ2-TCR transduced (right) Jurkat cell lines for the selection of *anti*-Vγ9Vδ2-TCR VHH. B) After positive and negative selection, VHH clones were screened for binding to Jurkat-Vγ9Vδ2-TCR and CFSE-labeled Jurkat cells using flow cytometry. Representative dot-plots of binding of the selected VHHs to Vγ9Vδ2-TCR transduced Jurkat cells versus non-transduced Jurkat cells. The three depicted dot-plots show examples of a Vγ9Vδ2-TCR specific VHH (left dot-plot; binding to Vγ9Vδ2-TCR transduced Jurkat, but not to Jurkat), a non-Vγ9Vδ2-TCR specific binding VHH (middle dot-plot; binding to both Vγ9Vδ2-TCR transduced Jurkat as well as to Jurkat, i.e. not specific for the Vγ9Vδ2-TCR), and a non-specific VHH (right dot-plot; no binding to either Vγ9Vδ2-TCR transduced Jurkat or Jurkat).

exception of VHH 5C7, 5B11, 6E3 and 6G3 which had an affinity of 200–350 nM (data not shown).

Since Vγ9Vδ2-T cells are promising with respect to cancer immunotherapy [1], we explored whether the Vγ9Vδ2-TCR specific VHHs were capable of activating Vγ9Vδ2-T cells. For this purpose, Jurkat/MA cells carrying a luciferase gene under the control of an NFAT-regulated promoter [21], were retrovirally transduced to express the Vγ9Vδ2-TCR. The established Jurkat/MA-Vγ9Vδ2-TCR cell line produces levels of

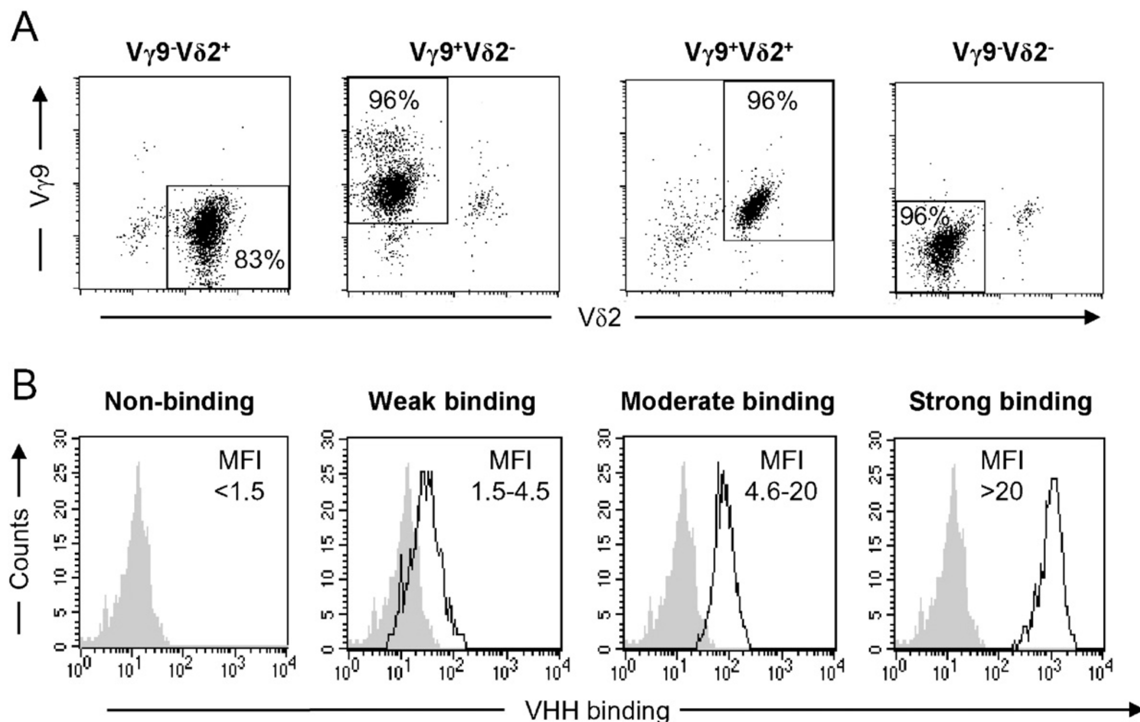


**Fig. 3.** Assessment of the chain specificity and binding strength of *anti*-Vγ9Vδ2-TCR VHHs. A) A pan γδ-T cell line was split into $V\gamma9^-V\delta2^+$, $V\gamma9^+V\delta2^-$, $V\gamma9^+V\delta2^+$ and $V\gamma9^-V\delta2^-$ γδ-T cell populations by flow cytometric sorting. Indicated is the purity of the cell lines after sorting. B) Representative histograms illustrating how VHH binding intensity to $V\gamma9^-V\delta2^+$, $V\gamma9^+V\delta2^-$, $V\gamma9^+V\delta2^+$ and $V\gamma9^-V\delta2^-$ γδ-T cell populations was defined. The mean fluorescence index (MFI) of each VHH to an individual γδ-T cell population was calculated by determining the ratio between the mean fluorescence intensity of a VHH with that of a negative control VHH. VHH binding intensity was then defined as non-binding (−, MFI < 1.5), weak binding (±, MFI 1.5–4.5), moderate binding (+, MFI 4.6–20) or strong binding (++, MFI > 20).

**Table 1**
Vγ9Vδ2-TCR specific VHH with indicated TCR chain specificity[a].

| VHH | Binds γδ-T cell population | | | |
|---|---|---|---|---|
| | Vγ9⁻Vδ2⁺ | Vγ9⁺Vδ2⁻ | Vγ9⁺Vδ2⁺ | Vγ9⁻Vδ2⁻ |
| 5C1 | ± | ++ | ++ | − |
| 5D3 | ++ | − | ++ | − |
| 5E3 | − | ++ | ++ | − |
| 5G3 | − | ++ | ++ | − |
| 5F5 | ++ | − | ++ | − |
| 5C7 | ± | − | ± | − |
| 5D7 | ++ | − | ++ | − |
| 5E7 | ++ | − | ++ | − |
| 5C8 | ++ | − | ++ | − |
| 5B11 | − | − | + | − |
| 6A1 | ++ | − | ++ | − |
| 6C1 | ++ | − | ++ | − |
| 6H1 | − | ++ | ++ | − |
| 6E3 | ++ | − | ++ | − |
| 6G3 | ++ | − | ++ | − |
| 6H3 | ++ | ± | ++ | − |
| 6C4 | ± | ++ | ++ | − |
| 6E4 | ++ | − | ++ | − |
| 6H4 | ++ | − | ++ | − |
| 6F6 | ++ | − | ++ | − |

[a] Distinct γδ-T cell populations were obtained by flow cytometric sorting of a donor-derived pan γδ-T cell line. The mean fluorescence index (MFI) of each VHH to an individual γδ-T cell population was calculated by determining the ratio between the mean fluorescence intensity of a VHH with that of a negative control VHH studied by flow cytometry ($n = 3$). VHH binding intensity was then defined as non-binding (−, MFI < 1.5), weak binding (±, MFI 1.5–4.5), moderate binding (+, MFI 4.6–20) or strong binding (++, MFI > 20).

luciferase that correlate to TCR activation. Indeed, stimuli known to activate Vγ9Vδ2-T cells (positive controls: PMA/ionomycin or aminobisphosphonate pretreated HeLa cells that express enhanced levels of pAg) showed enhanced luciferase activity, while stimuli expected not to activate Vγ9Vδ2-T cells (negative controls: medium, HeLa cells or control VHH) showed baseline levels of luciferase activity indicating the established Jurkat/MA-Vγ9Vδ2-TCR luciferase system to be a reliable method to test Vγ9Vδ2-T cell activation (Fig. 4). In order to test the activating potential of individual Vγ9Vδ2-TCR specific VHHs, VHHs were captured to culture plates and cultured for 24 h with Jurkat/MA-Vγ9Vδ2-TCR cells after which luciferase activity in

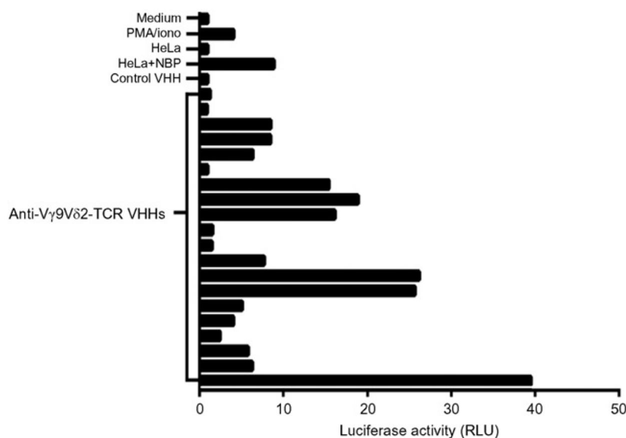


**Fig. 4.** Differential activation of individual Vγ9Vδ2-TCR specific VHHs in an NFAT regulated luciferase reporter system. Jurkat/MA were retrovirally transduced to express Vγ9Vδ2-TCR. Jurkat/MA-Vγ9Vδ2-TCR cells were incubated for 24 h with indicated positive and negative control conditions or plate-bound Vγ9Vδ2-TCR specific VHHs. As a positive control, Jurkat/MA-Vγ9Vδ2-TCR cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 4 h. Luciferase activity was calculated relative to measured luminescence induced by control VHH and expressed as relative luminescence units (RLU) and set to one. Abbreviations: PMA/iono (PMA and ionomycin); HeLa (HeLa untreated cells); HeLa + NBP (aminobisphosphonate pretreated HeLa cells); control VHH (non-Vγ9Vδ2-TCR specific VHH).

Jurkat/MA-Vγ9Vδ2-TCR cell lysates was measured. The capacity to induce activation of Jurkat/MA-Vγ9Vδ2-TCR cells varied considerably among the different Vγ9Vδ2-TCR specific VHHs (Fig. 4). Some clones were not able to trigger Jurkat/MA-Vγ9Vδ2-TCR cell activation, while others were substantially more potent than established positive controls. We confirmed that the activating Vγ9Vδ2-TCR specific VHHs were also capable of inducing activation, degranulation and cytokine production of Vγ9Vδ2-T cell lines obtained from healthy volunteers (data not shown) indicating the potential of these VHHs to be further developed for e.g. cancer immunotherapeutic approaches (R.C.G. de Bruin et al., manuscript in preparation).

### *3.4. Vγ9Vδ2-TCR specific VHH are able to identify specific T-cell subsets in cytometry*

Since we found such a broad panel of Vδ2- and/ or Vγ9-chain specific VHHs with both Vγ9Vδ2-T cell activating and non-activating properties, we evaluated their use for several research and diagnostic applications as these novel reagents could be of great value for the scientific and health care community interested in Vγ9Vδ2-T cell biology.

First, we tested whether the Vγ9Vδ2-TCR specific VHHs could be used for flow cytometry. For this purpose, we selected two VHHs from our panel with the highest affinity for the Vδ2-chain (VHH 6H4, 3.3 nM) and for the Vγ9-chain (VHH 6H1, 2.8 nM). These VHHs were biotinylated for straightforward detection using fluorescently labeled streptavidin. A donor-derived Vγ9Vδ2-T cell line was stained with two commercially available Vδ2- and Vγ9-TCR specific antibodies to confirm its purity (Fig. 5A). In separate stainings, either the Vδ2-TCR specific or the Vγ9-TCR specific commercially available antibody was replaced by the biotinylated Vδ2-specific VHH 6H4 or the Vγ9-specific VHH 6H1, respectively. Staining of a healthy donor-derived Vγ9Vδ2-T cell line with either the Vδ2-TCR specific VHH or the Vγ9-TCR-specific VHH resulted in a similar staining pattern and similar Vγ9Vδ2-T cell frequencies as obtained with the commercially available antibodies (Fig. 5A). To further substantiate the usefulness of these VHHs for flow cytometry, human peripheral blood mononuclear cells (PBMC) were also stained with Vδ2- and Vγ9-TCR specific VHHs. The staining of PBMC with either commercially available *anti*-Vδ2 and *anti*-Vγ9 antibodies or the Vδ2- and Vγ9-TCR specific VHHs showed exactly the same frequency of the Vγ9Vδ2-T cell population (1.18%) (Fig. 5B). Based on differences in mean fluorescence intensity, the discriminatory potential of VHH 6H4 appeared to be superior compared to that of the commercially available *anti*-Vδ2 monoclonal antibody (Fig. 5B). Taken together, these data indicate that Vδ2-TCR specific VHH 6H4 and Vγ9-TCR specific VHH 6H1 are suitable tools for use in flow cytometry .

### *3.5. Vγ9Vδ2-TCR specific VHH are powerful tools for Vγ9Vδ2-T cell isolation*

Since VHH 6H4 and VHH 6H1 bind Vγ9Vδ2-T cells with high specificity, we reasoned that these VHHs might also be excellent tools for cell purification purposes. A common method used in the field to separate and purify various cell populations is Magnetic-activated cell sorting (MACS). With this method, magnetic beads are coupled with an antibody that binds to an antigen presented on the cell membrane of the cell population of interest. To assess if our VHHs could be used for this method, biotinylated Vδ2-specific VHH 6H4 and biotinylated Vγ9-specific VHH 6H1 were used in combination with *anti*-biotin MicroBeads to generate an enriched Vγ9Vδ2-T cell population from PBMC (a representative example of Vδ2-enrichment is shown in Fig. 6A). Percentages of Vγ9Vδ2-T cells are generally low in human PBMC (0.5–5%), but could be strikingly enriched by VHH-based MACS purification. Using VHH 6H4, cell populations were significantly enriched from 1.6 ± 1.1% Vδ2$^+$ cells in the PBMC fraction to 91 ± 10% Vδ2$^+$ cells ($n = 15$, $p < 0.0001$) in the fraction after MACS with a yield of 49 ± 21%, similar to purification with a commercially available *anti*-Vδ2 monoclonal antibody (data not shown). For Vγ9$^+$ cells enrichment,

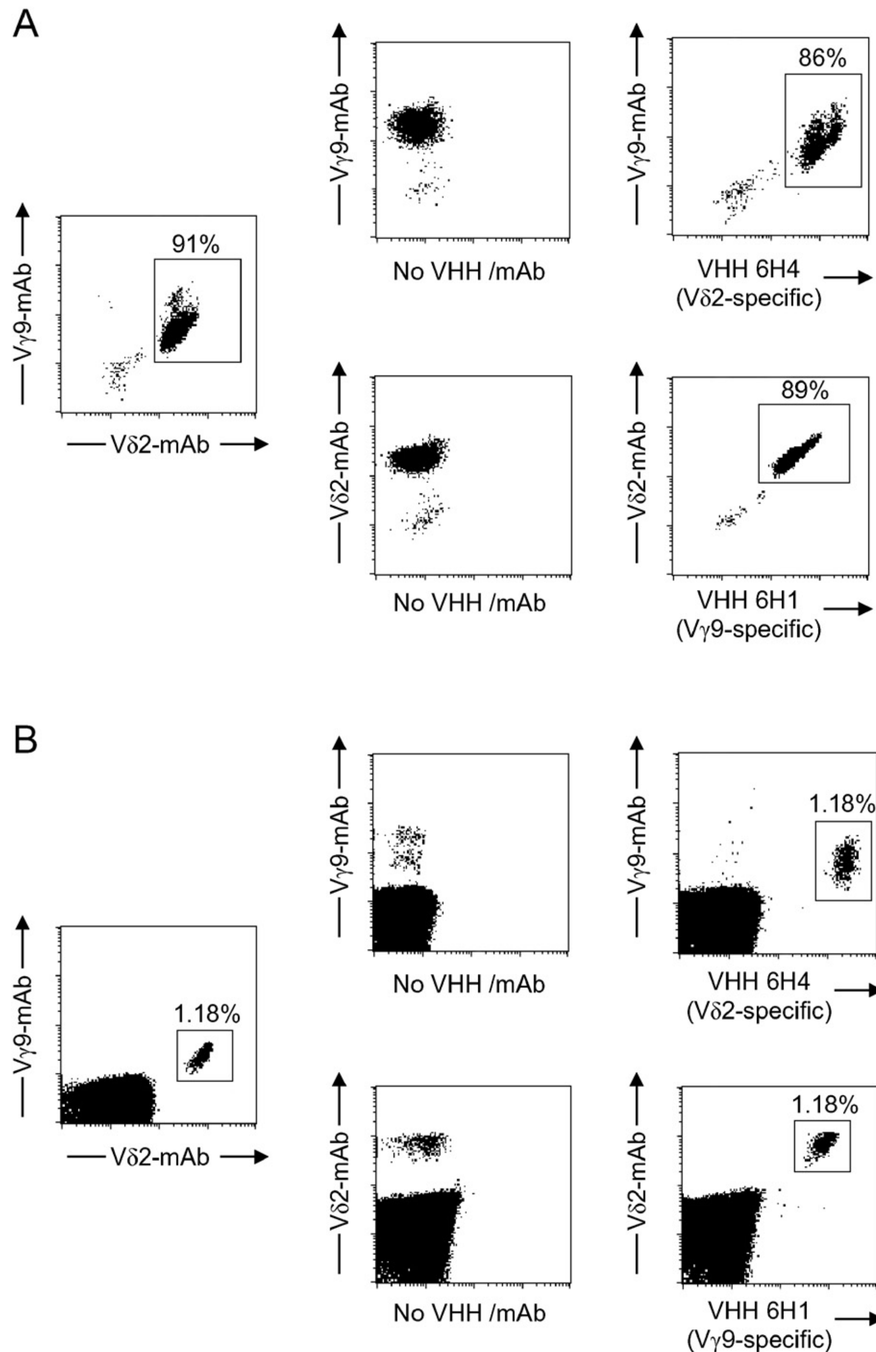


**Fig. 5.** Vδ2-specific and Vγ9-specific VHHs are suitable tools for use in flow cytometry. Frequency of $V\gamma9^+V\delta2^+$ T cells within a population was determined using a fixed number of a donor-derived Vγ9Vδ2-T cell line (A) or PBMC from the same donor (B). Frequency of Vγ9Vδ2-T cells within the population as determined using commercially available PE-conjugated *anti*-Vγ9 (5 μg/mL) or FITC-conjugated *anti*-Vδ2 (5 μg/mL) antibodies for flow cytometry (left). The *anti*-Vγ9 (top) or *anti*-Vδ2 (bottom) antibodies are replaced by biotinylated Vγ9-specific VHH 6H1 (1.5 μg/mL) or biotinylated Vδ2-specific VHH 6H4 (1.5 μg/mL), respectively and detected with streptavidin-APC.

populations were significantly enriched from 1.6 $\pm$ 0.8% $V\gamma9^+$ cells in the PBMC fraction to as high as 95 $\pm$ 5% $V\gamma9^+$ cells ($n = 15$, $p < 0.0001$) in the fraction after MACS with a yield of 70 $\pm$ 22% when using the VHH 6H1 (see Fig. 6B and Table 2). For 3 random PBMC donors, the cells were cultured after purification for at least 3 weeks by a weekly stimulation with feeder mixture plus PHA and IL-2. In one week, VHH MACS isolated Vγ9Vδ2-T cells expanded with an average of 5.4 $\pm$ 2.3 fold for cell populations enriched by Vδ2-specific VHH 6H4 and with an average of 5.5 $\pm$ 2.1 fold for cell populations enriched by Vγ9-specific VHH 6H1 (mean $\pm$ SD, $n = 3$, data not shown). These results show that both the Vδ2-specific VHH 6H4 and the Vγ9-specific VHH 6H1 can be used to isolate $V\delta2^+$ and $V\gamma9^+$-T cells from PBMC with high purity and yield and that these isolated cells can be maintained and expanded in cell culture.

### *3.6. Vγ9Vδ2-TCR specific VHH are reactive in immunocytochemistry*

Currently, there is a limited number of Vδ2- and Vγ9-TCR specific antibodies available for the detection of Vγ9Vδ2-T cells in immunocytochemistry. To this end, we examined if the Vδ2- and Vγ9 specific VHHs could be used to detect Vγ9Vδ2-T cells on cytospins and cryosections. For cytospin slides, Jurkat and Jurkat-Vγ9Vδ2-TCR (Fig. 7A) as well as healthy donor-derived Vγ9Vδ2-T and Vα24Vβ11-T cells (Fig. 7B) were spun on glass slides and stained with either Vδ2-specific VHH

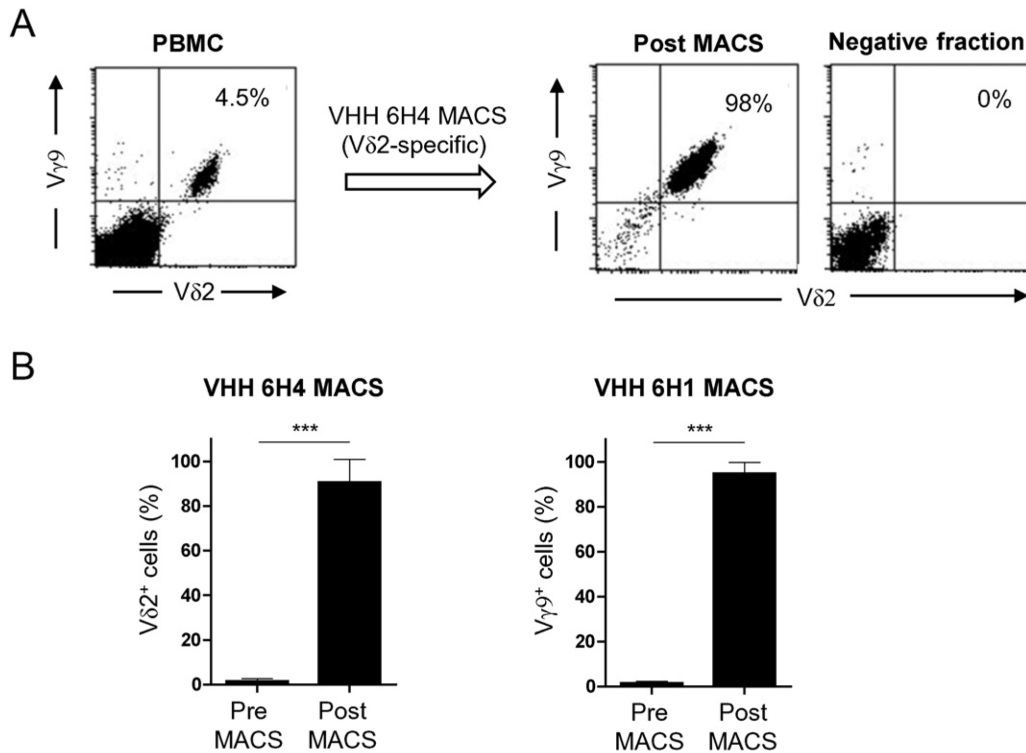


**Fig. 6.** Vδ2-specific and Vγ9-specific VHHs can be used for Vγ9Vδ2-T cell purification. Human PBMC were incubated with either biotinylated Vδ2-specific VHH 6H4 or biotinylated Vγ9-specific VHH 6H1 to allow cell separation by MACS using *anti*-biotin MicroBeads. A) Representative figure of Vδ2$^+$ isolated cells from PBMC using VHH 6H4. Percentages of Vγ9$^+$Vδ2$^+$-T cells in the population are shown before MACS (left) and of the two cell populations after MACS; positive fraction (post MACS, middle dot-plot) and negative fraction (right dot-plot). B) Percentage of Vδ2$^+$ or Vγ9$^+$-T cells in the cell population before (pre MACS) and after MACS (post MACS) using VHH 6H4 or VHH 6H1, respectively. Shown are mean ± SD of $n$ = 15. $p < 0.0001$.

6H4, Vγ9-specific VHH 6H1 or no VHH as negative control. For both the Jurkat-Vγ9Vδ2-TCR cells (TCR expression 92%) as well as the healthy donor-derived Vγ9Vδ2-T cells (TCR expression 97%) that were stained with either the Vγ9-or the Vδ2-TCR specific VHH, nearly 100% of the cells stained positively. As expected with staining a cell surface receptor, the staining pattern of the TCR was restricted to the cell membrane (Fig. 7A and B). The observed staining was specific for the Vγ9Vδ2-TCR as neither non-transduced Jurkat cells (not expressing a TCR), nor T-cells expressing an αβ-TCR (TCR expression 96%), nor slides not incubated with VHH (negative control) showed staining with the VHHs.

Next, we explored if the VHHs could also be used for immunocytochemistry staining of Vγ9Vδ2-T cells in cryosections. To this end, 5 μM sections were made of snap frozen Jurkat or Jurkat-Vγ9Vδ2-TCR cells and incubated with Vδ2-specific VHH 6H4, Vγ9-specific VHH 6H1 or no VHH as negative control. Staining of Vγ9Vδ2-T cells in cryosections showed a similar result as for the staining of Vγ9Vδ2-T cells in cytospins; VHH 6H4 and VHH 6H1 showed specific staining of nearly all Jurkat-Vγ9Vδ2-TCR cells (TCR expression 89%) but did not show staining of Jurkat cells without TCR expression or cryosections not incubated with VHH (Fig. 7C). These observations indicate that Vδ2-specific VHH 6H4 and Vγ9-specific VHH 6H1 can be used to detect Vγ9Vδ2-T cells in cytospins of cell suspensions and cryosections containing human Vγ9Vδ2-T cells.

## 4. Discussion

Vγ9Vδ2-T cells have the ability to distinguish "normal" cells from "altered" cells by the MHC-independent recognition of elevated pAg levels due to bacterial infection or malignancy. When activated, Vγ9Vδ2-T cells can produce large amounts of cytokines, chemokines and cytotoxic mediators to efficiently kill their targets [7]. In addition, activated Vγ9Vδ2-T cells can act as antigen presenting cells for αβ-T cells, inducing a predominantly pro-inflammatory cytokine profile and they are able to stimulate the maturation of immature dendritic cells [11,22]. These functions make Vγ9Vδ2-T cells attractive for use in immunotherapy.

Here, we set out to generate VHH antibody fragments against the Vγ9Vδ2-TCR. VHH are smaller than conventional antibodies or scFvs and consist of a single domain. Moreover, VHH do not require the presence of a light chain and are characterized by high stability, good solubility, strong ability to easily refold and low immunogenicity. Also, multispecific molecules can be easily generated by DNA cloning and production can be performed in *E. coli* or yeast reducing production costs [15,18,20]. Their small size allows enhanced tissue penetration but also rapid clearing by the kidneys *in vivo*. However, the latter can be circumvented by fusion to an *anti*-albumin VHH which prolongs the *in vivo* half-life [19,23].

**Table 2**
MACS cell seperation using anti-Vγ9Vδ2-TCR VHHs[a].

| | Pre MACS (purity %) | Post MACS (purity %) | Yield (%) |
|---|---|---|---|
| VHH 6H4 (Vδ2-specific) | 1.6 ± 1.1 (0.5–4.6) | 91 ± 10 (65–100) | 49 ± 21 (12–88) |
| VHH 6H1 (Vγ9-specific) | 1.6 ± 0.8 (0.6–3.1) | 95 ± 5 (83–100) | 70 ± 22 (11–94) |

[a] Vδ2$^+$ or Vγ9$^+$-T cells were isolated from human PBMC using either biotinylated Vδ2-specific VHH 6H4 or biotinylated Vγ9-specific VHH 6H1 and anti-biotin MicroBeads. Presence of Vδ2$^+$ or Vγ9$^+$-T cells within the population are shown before (pre MACS) and after MACS (post MACS). Yield is calculated by setting the amount of Vδ2$^+$ or Vγ9$^+$-T cells in PBMC before MACS to 100%. $n$ = 15, shown are mean ± SD and the range in parentheses.

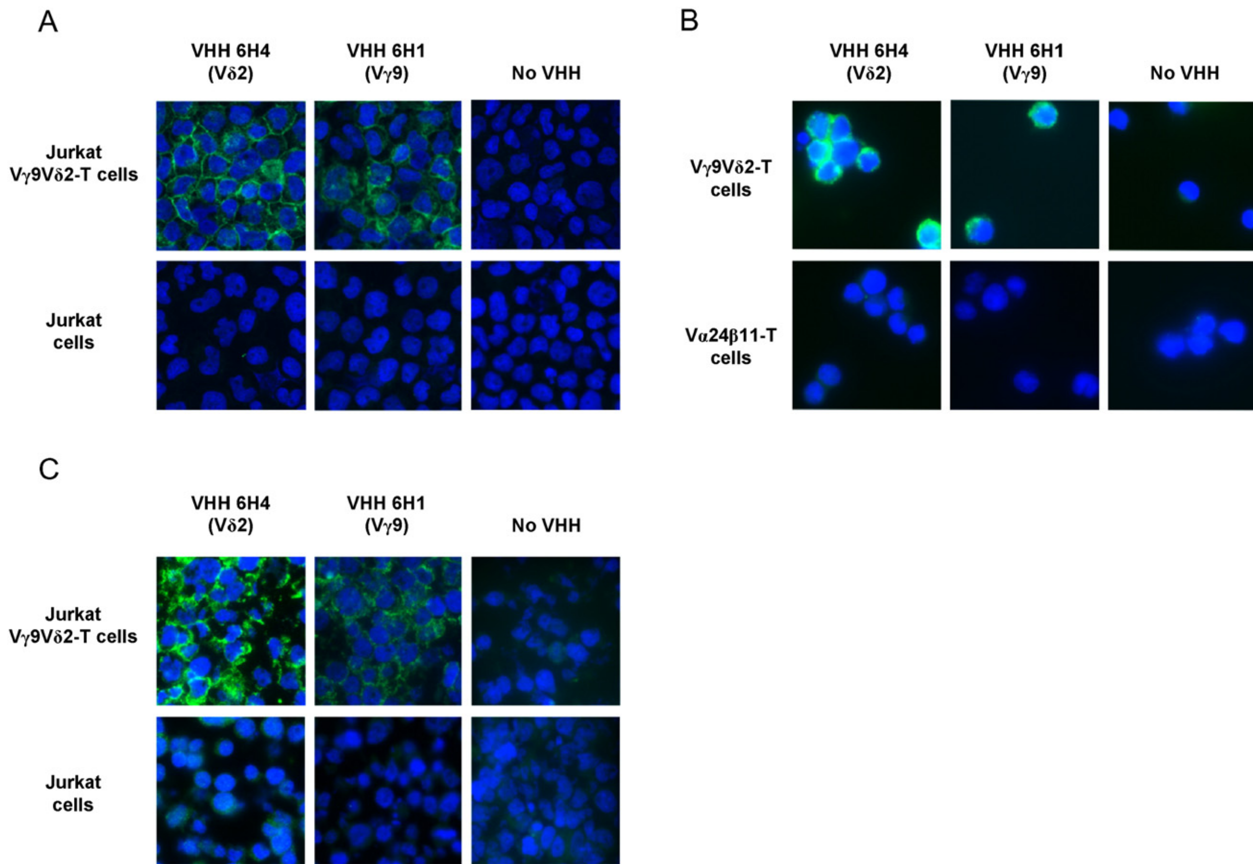


**Fig. 7.** Immunocytochemistry staining of Vγ9Vδ2-TCR using VHHs. Jurkat and Jurkat-Vγ9Vδ2-TCR cells (A, C) or healthy donor-derived Vγ9Vδ2-T and Vα24Vβ11-T cells (B) were incubated with Vδ2-specific VHH 6H4, Vγ9-specific VHH 6H1 or no VHH. Bound VHH was detected with a rabbit-*anti*-VHH antibody and an Alexa488-conjugated goat-*anti*-rabbit antibody and imaged by wide field fluorescent microscopy. A–B) Cytospin slides stained with VHH. C) Cryosections stained with VHH.

Through immunization of llamas with human Vγ9Vδ2-T cells, phage-display and screenings, we obtained a panel of 20 diverse VHHs that did not cross-react with CD3 (co-expressed in the TCR-complex) or other TCRs. We have found that all VHHs bind to the variable region of the γδ-TCR and have specificity for either the Vγ9- or the Vδ2-chain. Surprisingly, we also found some VHHs which recognize both chains, but with a preference for one chain over the other chain. Presumably the latter group of VHHs recognizes an epitope that is composed of a region of both chains where these are in close proximity.

From the broad panel of *anti*-Vγ9Vδ2-TCR VHHs, we decided to further explore possible applications of our strongest Vδ2 binding VHH (VHH 6H4) and strongest Vγ9-chain binding VHH (VHH 6H1). Both these VHHs were able to specifically detect γδ-T cell populations expressing only the chain to which the VHH was directed in flow cytometry; Vδ2$^+$ populations for VHH 6H4 and Vγ9$^+$ populations for VHH 6H1. These VHHs were successfully applied both in staining Vγ9Vδ2-T cells of cultures with a dominant presence of Vγ9Vδ2-T cells as well as for the identification of Vγ9Vδ2-T cells within a large pool of other cells, such as PBMC where Vγ9Vδ2-T cells are only present in percentages as low as 1–5%. This indicates that these VHHs have high specificity with negligible non-specific binding to other cells. Furthermore, the mean fluorescence intensity obtained by Vδ2-TCR specific VHH 6H4 stained in flow cytometry, appears to be higher compared to the staining with a commercial *anti*-Vδ2 monoclonal antibody. The highly discriminating capacity of these VHHs would also make them very good tools for flow cytometric sorting.

The highly specific staining of Vγ9Vδ2-T cells with VHH 6H4 and VHH 6H1 allows these VHH also to be used for Vγ9Vδ2-T cell enrichment from PBMC by MACS. Both VHH 6H4 as well as VHH 6H1 provide high enrichment and yield of Vδ2$^+$ or Vγ9$^+$ populations, respectively, when used for their isolation from PBMC. Furthermore, isolated populations can be successfully cultured for at least 3 weeks by a weekly stimulation of feeder mixture plus PHA and IL-2. This could provide an excellent method for the culturing of Vγ9Vδ2-T cell populations for research purposes or even adoptive transfer therapies.

Both the Vδ2-specific VHH 6H4 and the Vγ9-specific VHH 6H1 could specifically stain Jurkat-Vγ9Vδ2-TCR cells as well as healthy donor-derived Vγ9Vδ2-T cells on cytospin slides. Also, we found that the Vδ2-specific VHH 6H4 and the Vγ9-specific VHH 6H1 could be used to detect Vγ9Vδ2-TCR expressing cells in cryosections with similar success. The stainings were strictly Vγ9Vδ2-TCR specific as Jurkat cells that did not express a TCR, or Jurkat cells not incubated with VHH, did not show a signal. Though some Vδ2- and Vγ9-specific antibodies are currently on the market for the use in immunocytochemistry, not all antibodies perform well in this application and the choice is limited. Our data indicate that VHH 6H4 and VHH 6H1 could expand the set of antibody (−fragments) available for the detection of Vδ2$^+$ and Vγ9$^+$-T cells in immunocytochemistry. The detection of Vγ9Vδ2-T cells with immunocytochemistry provides opportunities for both research and diagnostics applications that focus on detecting Vδ2$^+$ or Vγ9$^+$-T cells in e.g. healthy or diseased tissue, tumors as well as PBMC.

Among the panel of Vγ9Vδ2-TCR VHHs, we found both activating and non-activating Vγ9Vδ2-T cell VHHs. Activating VHHs could be of interest for the development of cancer immunotherapeutics in which an enhanced Vγ9Vδ2-T cell function is desired. Recently, a bispecific $(Her2)_2$xVγ9 antibody construct has been developed to direct Vγ9Vδ2-T cells more specifically to Her2 overexpressing tumor cells in order to enhance tumor cell lysis. This approach was exploited for pancreatic cancer cells both *in vitro* and *in vivo* with success [14]. However,

since antibody-based constructs require both the presence of heavy and light chain domains which come with several limitations, the use of VHHs would be a preferential alternative [15,24].

With respect to the non-activating Vγ9Vδ2-T cell VHHs, it would be interesting to investigate if these VHHs could also provide neutralization of the Vγ9Vδ2-TCR in the presence of elevated pAg levels. Patients treated with aminobisphophonates for osteoporosis or prevention of fractures due to bone metastasis, present with elevated levels of pAg as a side-effect of this treatment [11]. The elevated pAg levels induce a systemic activation of Vγ9Vδ2-T cells that can lead to bothersome acute phase reaction symptoms like fever, muscle ache and shivering. To date, multiple clinical studies have attempted to reduce the systemic activation of Vγ9Vδ2-T cells in this setting but so far without success [25]. When identified in our panel of VHHs, a neutralizing *anti*-Vγ9Vδ2-TCR VHH could constitute a novel and promising approach to dampen these side-effects of aminobisphosphonate treatment.

Taken together, the data reported here show that the obtained *anti*-Vγ9Vδ2-TCR VHHs bind Vγ9Vδ2-T cells with high specificity making them valuable tools for the detection and enrichment of Vγ9Vδ2-T cells using flow cytometry, MACS and immunocytochemistry, which is relevant both for research and diagnostic purposes. Furthermore, these VHHs can be promising for the development of future immunotherapeutics either aimed at the activation or possibly neutralization of Vγ9Vδ2-T cells.

## Authorship contributions

Renée C.G. de Bruin: performed research, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript.

Sinéad M. Lougheed: performed research, analyzed and interpreted data and wrote the manuscript.

Liza van der Kruk: performed research, analyzed and interpreted data and revised the manuscript critically.

Anita G. Stam: performed research, analyzed and interpreted data and revised the manuscript critically.

Erik Hooijberg: designed research and revised the manuscript critically.

Rob C. Roovers: designed research and wrote the manuscript.

Paul M.P. van Bergen en Henegouwen: designed research and revised the manuscript critically.

Henk M. Verheul: designed research and revised the manuscript critically.

Tanja D. de Gruijl: designed research, analyzed and interpreted data, and wrote the manuscript.

Hans J. van der Vliet: designed research, analyzed and interpreted data, and wrote the manuscript.

All authors approved with the final version to be published.

## Conflict of interest statement

All authors have no conflicts of interest to declare.

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