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# Known and New Routes to Neutralize HIV-1 with Camelid Single Chain Antibody Fragments

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## Introduction

In spite of all efforts to reduce transmission of HIV-1, 1.7 million persons were infected in 2019 worldwide. Whereas in the developed countries the COVID-19 pandemic is reasonably controlled within 2 years because of the fast and successful development of vaccines, nearly 40 years after the first reported cases of AIDS due to HIV-1 and in spite of tremendous efforts to develop vaccines against HIV-1, there are still no vaccines against the virus. Fortunately, the cART therapy saved the lives of millions of persons, although there are some health problems related with the long term usage of cART, like HIV associated neurocognitive disorders [1]. Moreover, latently infected memory $CD4^+$ T cells pose risks of re-emergence of the HIV-1 infections, if cART has to be interrupted or cannot be used at all. Broadly neutralizing conventional antibodies have been selected from a small percentage of HIV-1 infected humans and clinical trials with these antibodies are promising [2-6]. However, the availability and costs of these antibodies may prevent their usage in developing countries which suffer most of the HIV-1 pandemic.

So, there is still a need for cost effective antibodies that reduce the number of transmissions, to develop immunotherapies, to block re-emergence and to eliminate or at least minimize the number of latently infected memory $CD4^+$T cells.

In this review the development of variable domain heavy chain antibodies (VHH) of *Camelidae* for therapy/prevention against HIV is described. VHH are the smallest high affinity binding domains known and they can be selected from immune, naïve or synthetic libraries. Their often long CDR3 can penetrate the envelope proteins better than conventional antibodies [7], thereby reaching epitopes hidden for conventional antibodies. Their performance can be improved by protein engineering [8] or linking 2 VHH together thereby creating bivalent or bispecific VHH of affinities as low as the best conventional antibodies [9]. These biheads are physically quite stable, can be produced relatively cheap [10] and can be exposed on the surface of liposomes or microorganisms thereby creating nanoparticles decorated with VHH. Probably the most promising development is the display of these proteins on the surface of selected lactic acid bacteria that inhibit transmission of HIV-1 [11].

Due to their single polypeptide nature VHH are the preferred molecules for intrabodies both to study and modulate intramolecular cellular processes. For the study of intracellular processes chimeric molecules consisting of a VHH that recognizes a certain cellular antigen and a fluorescent protein contributed to detailed knowledge of intracellular processes [12]. For therapeutic intrabodies, VHH against HIV-1 specially selected or adapted to the intracellular conditions have large potential.

Finally, the 3D structures of the framework of VHH are well preserved during maturation, which is important to make reliable 3D structures of VHH, starting with just the amino acid sequence. As continuously better Bio-informatic tools are developed to determine structures of more complex proteins

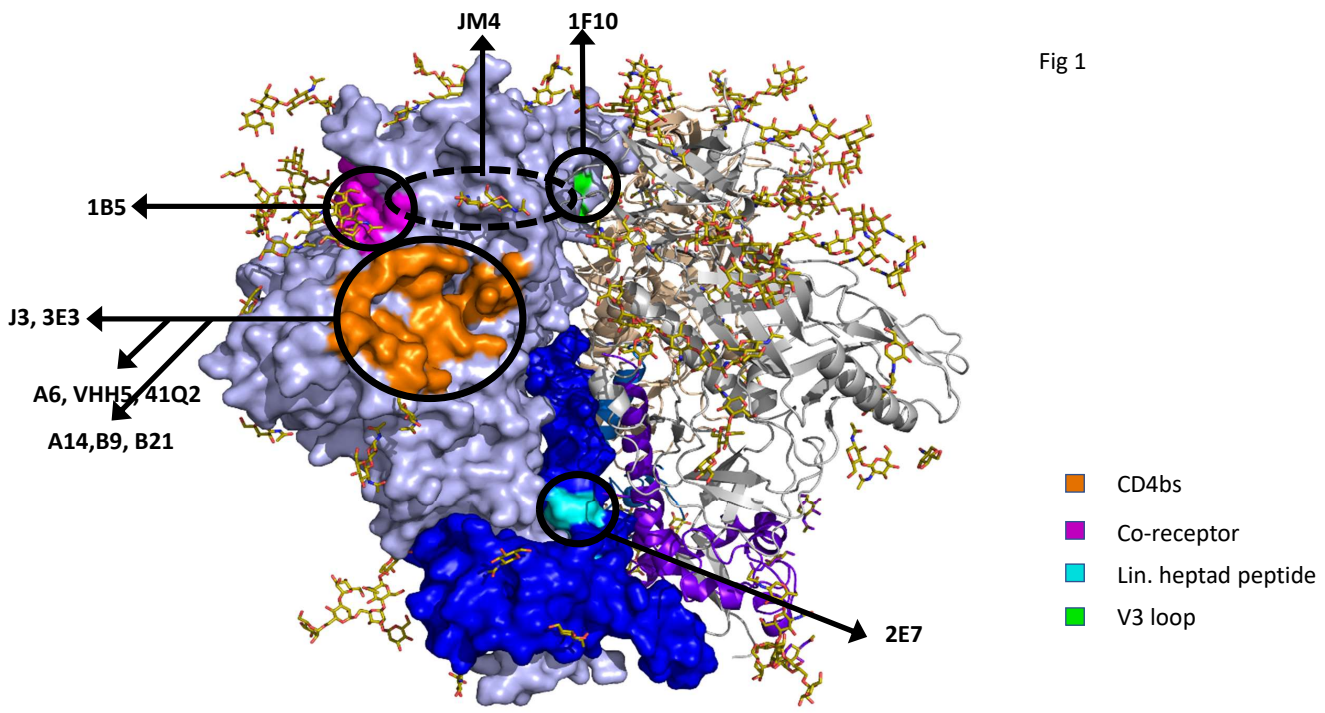


**Figure 1:** Various methods of epitope mapping show that broadly neutralizing VHH recognize at least 5 different epitopes on HIV-1 gp120/140. CD4bs is recognized by 3E3, J3 [13,19]; A14, B9 and B21 [19]; and A6, VHH5 and 41Q2 [20,32,53,54]. The most broadly neutralizing VHH known at present, VHH J3 covers nearly the whole CD4bs, and the well-known cavity on the CD4bs that plays a crucial role in binding of HIV-1 to CD4bs is occupied by CDR3 of VHH J3 as shown by co-structure determination and mutational studies. Moreover, J3 hardly interacts with the amino acids on the periphery of CD4bs. Often these amino acids are prone to mutations. JM4 interacts with part of the CD4bs, the co-receptor side and V3 loop [18]; 1B5 interacts with the coreceptor [9,13], 1F10 interacts with the V3 loop; and 2E7 with the heptad peptide [9,13]. Based on the structure of VHH 5, and the tomographic studies [20], the epitope of VHH5 has been determined. Using I-TASSER and HADDOCK the interaction of 41Q2 with the same envelope protein has been determined (Supplementary Figures 2A and 2B).

on this basis and interactions between antibodies and their cognate antigens, protein engineering will become even more important as it is today. The single polypeptide nature of VHH, combined with the rather conserved frameworks of VHH, the canonical folds of CDR1 and 2 will enable the design of superior VHH.

## VHH as Microbicides

A remarkable high number of neutralizing VHH can be selected from phage libraries constructed from mRNA of lymphocytes obtained after long lasting immunizations with different immunogens. Strokappe [9,13] described that two of such immunizations resulted in the selection of about 100 quite different VHH, encoded by all 4 groups of V-genes [14,15]. These VHH recognize at least 5 different epitopes on the HIV-1 envelope protein (Figure 1), of which 46% percent were neutralizing and 11% can be considered as broadly Neutralizing VHH (bNVHH, defined here as >75% neutralization of Clades A, B, C and Tier 1 and 2). After the first selection of HIV-1 neutralizing VHH [16], more groups have selected VHH against at least 5 different epitopes on the gp140 envelope protein of HIV-1 [17-20] (Table 1). VHH and complexes of VHH/cognate antigens crystallize often quite well enabling determination of their 3D structure, whereas with cryo-EM even for difficult antigens like GPCR, 3D structures have been determined using VHH [21]. More than two dozens of 3D structures of VHH have been determined as well as structures of HIV-1 gp120 and gp140 envelope proteins. These structures enable rather precise location of the VHH on their cognate epitopes. From these studies it became clear that some of the broadest neutralizing VHH recognize nearly the complete CD4bs without interaction with amino acids on the periphery of the CD4bs (Figure 2). More recently, 3D structures of VHH/HIV-1 antigens have been determined. Such studies showed that VHH 2E7 recognizes a linear epitope (res. 582-594 of envelope protein) using mainly CDR3 and a framework residue for the interaction [9], whereas VHH JM4 recognizes a conformational epitope consisting of CD4bs (a.a. 369, 372, 386 interact with CDR3), V3 loop (a.a. 324, 325, 327 interact with CDR2) and bridging sheet (a.a. 422, 425, 434 interact with CDR1 and CDR3) [18]. This clearly shows that with proper immunization and selection protocols a large diversity of HIV-1 neutralizing VHH can be generated and selected. Moreover, it has been shown that bi-specific VHH have even broader neutralization properties and bind epitopes on the envelope protein of HIV-1 with high affinity [9]. Based on their structure and the fact that they do recognize different

**Table 1:** Broadly neutralizing VHH (>75 % of neutralization of different clades and tiers)[a].

| Code VHH | Epitope[c] | Characteristics |
|---|---|---|
| J3 [9,13,19] | CD4bs | 96% nearly perfect overlap with HIV binding to CD4 |
| 3E3 [13,19] | CD4bs | 95% nearly perfect overlap with HIV binding to CD4 |
| A6,VHH 5 [20,32] | CD4bs | Highly functional when displayed on LAB [32] |
| JM 4 [17,18][b] | CD4bs and co-receptor | 3D structure with gp120 determined [18] |
| 2E7 [9,13] | gp41 heptad repeat | >80%; 3D structure with epitope determined [9] |
| 41Q2 [53, 54] | CD4bs | The broadest neutralizing VHH selected from dromedaries library |

[a] The neutralization breadth has been determined on different pseudoviruses of subtypes A, B and C and of Tiers 1 and 2.

[b] JM4 even neutralizes pseudoviruses resistant to broadly neutralizing mAb VCR01, PG9 and PGT128 [18].

[c] VHH have also been selected against the co-receptors CXCR4 [52], however they do not have the neutralization breadth of the above given VHH.

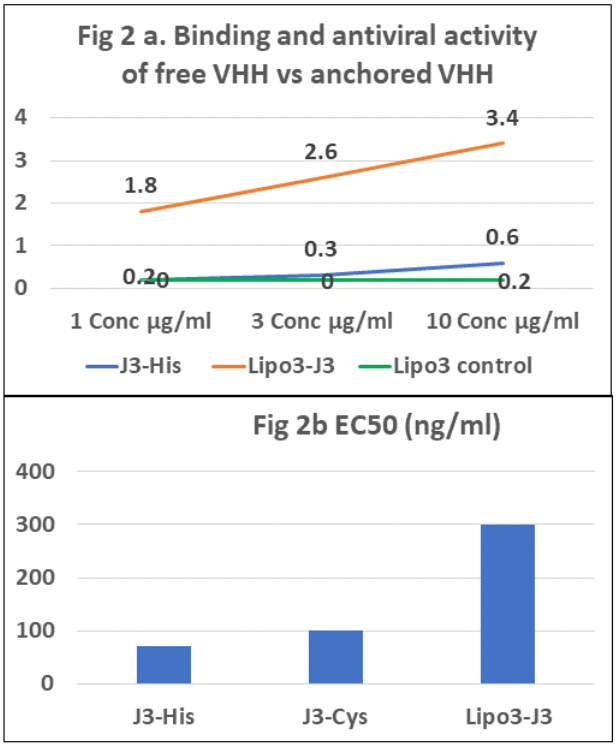


**Figure 2: A.** Comparison of binding of free VHH J3 and VHH J3 covalently coupled to anchors that ensures exposure of VHH J3 on the surface of a liposome. **B.** Comparison of neutralization of free J3 and liposome exposed J3.

epitopes, it can be expected that biheads of JM4-2E7, A6-2E7 and 41Q2-2E7 will cover nearly all HIV-1 strains and may be excellent neutralizers in a cost-effective microbicide.

Shortly after the first results of neutralization of HIV-1 with VHH became available, protection against transmission of HIV-1 by gels containing the reverse transcriptase inhibitor (Tenofovir) were published, showing that microbicides can be effective [22]. It has been determined that VHH present in gels in vaginal rings release active VHH during about 2 weeks. Moreover, these VHH can efficiently pass the epithelial barrier [23], which showed that indeed the small size of these molecules has a significant advantage over conventional antibodies to neutralize HIV-1.

To test this, one of the broadest VHH, J3 (coverage >96 %) described until now [9,13,16,19] was produced in a semi-industrial yeast fermentation process that showed that production of this VHH was economically feasible [10]. Subsequently, this VHH was used in a small macaque challenge test by the group of R. LeGrand at CEA, Paris. The results were promising. Eight macaques that were treated

with a gel containing VHH J3 remained non- infected during the ten weeks of trial, whereas six of the eight macaques treated with the same gel without VHH J3 became infected. In lymph node samples from non-infected macaques, the viral DNA copy number remained below detection level (10 copies/million cells) [24]. However, the stability of VHH in these gels or the release of VHH from the vaginal ring were still not optimal for a low-cost product, so either better delivery systems should be developed and/or the production process has to become cheaper. An alternative approach can be the display of VHH on biological or chemical nanoparticles. One of the approaches to improve display and thereby functionality was the integration of anchored VHH into liposomes. As it can be seen from Figure 2, this resulted in better binding to gp140CN52 and neutralization was shown using TZM-bl cells. By loading these liposomes with the reverse transcriptase inhibitor dapivirine, a potent microbicide was generated [25]. However, the production of such decorated and filled liposomes at low cost will be a challenge.

Lactic acid bacteria, mainly *Lactobacillus* spp., are commensals in the human vagina [26] and have at least two properties that contribute to the reduction of transmission of HIV-1, notably they create a low pH and form an additional layer on the mucosal layer thereby creating a larger physical barrier [27]. Moreover, certain lactic acid bacteria produce anti-inflammatory molecules that result in lower HIV transmission [28]. Consequently, research had been carried out to select health promoting lactic acid bacteria, even taking ethnical and regional differences into account [29]. Early in the development of VHH as therapeutic agents, an EU project team advocated the development of lactic acid bacteria as producer of single domain antibodies against pathogens in the G/I tract [11]. Already in 2006, Pant et al. reported that such a system provided protection against rotavirus [30]. For the development of this route, lactic acid bacteria, which have the General Recognized As Safe (GRAS) status, have been selected that are adapted to the conditions of the G/I tract or the vagina.

Two delivery modes can be designed using lactobacilli. Either the metabolically active lactobacilli secrete VHH continuously or they carry VHH as immobilized binding entities on their surfaces. As shown for the neutralization of *C. difficile* tox B, the latter mode may probably be more effective [31]. Subsequently, similar systems have been developed for HIV. Recently a study was published to show the potential of lactobacilli decorated with VHH to reduce transmission [32]. From a phage library constructed after immunization of dromedaries, potent VHH targeting the envelope protein have been selected, with VHH A6 as best performer [20], although later on from the same library VHH 41Q2 has been selected that is even a broader neutralizer and for most pseudoviruses tested, more effective (Supplementary Figures 2A and 2B). All the selected VHH from the library derived from immunization of dromedaries have -besides the normal Cys22-Cys92 bridge- a second Cys33-CysCDR3 bridge that creates a cavity in the CDR3 loop. Such cavities are often present in VHH generated by dromedaries and camels, whereas only one of the 4 groups of V-genes encoding VHH of Llamas and alpacas have often an additional Cys50-CysCDR3 when produced in eukaryotic cells. However, prokaryotes like lactic acid bacteria do not have the sophisticated ER folding machinery of eukaryotes. Statistically there are 6 options for S-S bridge formation in lactic acid bacteria that may result in incorrect folding of these VHH in lactic acid bacteria. However, VHH A6 has been expressed quite well either as free VHH or as VHH genetically coupled to cell wall proteins of *Lactobacillus rhamnosus,* previously isolated from the vagina. In an infectivity depletion test using 8 pseudovirus subtypes of clade B and C of tiers 1B and 2, the half maximum inhibitory concentrations ($IOD_{50}$) of the decorated LAB were between 0,27 and 2,54 (equivalent to 0,08 and 0,8 nmol VHH) showing once more the potential of this approach. The inhibitory values are comparable to those of the free VHH A6. Moreover, *E. coli* produced soluble VHH A6 protected nine out of 9 humanized mice, whereas when treated with PBS or a control VHH, five out of 9 mice were infected [32]. As most VHH genes encoding anti HIV VHH are selected from Llamas and alpacas, it is worth to discuss another aspect of the anti HIV VHH raised in dromedaries. Due to the presence of a double S-S bridge there is a cavity in CDRs, but simultaneously the adjacent amino acids are more exposed. This has significant consequences on neutralization as shown by Dietrich's group [32]. Two VHH (encoded VHH5 and 41Q2) are over 96% identical, but differ in a mutation in CDR3, notably amino acid sequence CGGLDD (VHH5) is replaced by CGGLHD (41Q2). Detailed 3D modelling showed a clear difference between the two paratopes, resulting in a much stronger electrostatic interaction of the CDR3 loop of 41Q2 with the envelope protein (Supplementary file). This resulted in a much more powerful neutralization compared to VHH5 (Table S1, Supplementary file). With the advent of much better modelling tools, more precise determination of the epitope/paratope interactions will be possible and consequently more rational design of VHH with improved binding and neutralization capacities (Supplementary file).

## Anti HIV-1 VHH as Intrabodies

Already decades ago, scientists tried to modulate cellular processes with (fragments of) antibodies, with limited success. Aggregation of the single domain intrabody (scFv) was one of the main problems [33,34]. Nevertheless, intrabodies against a large number of mammalian and plant viruses have been developed that often function quite well (Table S2, Supplementary file). As VHH are single chain proteins, their genes are much simpler to integrate into vectors or genomes of mammalian, insect and plant cells. Moreover, VHH are less prone for aggregation as they do not have the rather hydrophobic interface for VH VL interaction present in scFv which have often been used as intrabody (Table S2, Supplementary file). Consequently, VHH sometimes also equipped with NLS sequences have been expressed intracellularly. In spite that the redox potential in

the cytoplasm is not optimal for the formation of S-S bridges, the formation of which provides stability of VHH [35,36] the folding of VHH in cells was often correct, may be due to induced folding of the VHH by its cognate antigen. Moreover effective procedures have been developed to select from large libraries of VHH that recognize an intracellular antigen, those VHH that proved to be functional intrabodies [35,36]. An elegant example of such approach is the yeast two hybrid system enabling the selection of VHH that recognize Vpr of HIV-1 and interfere with its localization to the nucleus [37]. Further work resulted in the development of a protein engineering approach to convert single domain antibodies derived from conventional antibodies that aggregate in the cytoplasm into stable single domain functional antibodies [38]. Protein engineering methods were applied to VHH in order to ensure that intrabodies have the optimal hydrophobic core and the correct intramolecular electrostatic interaction between the β strands covering the hydrophobic core [39]. Moreover VHH have to be modified to ensure that the pI is just below the pH of the cytoplasm [38].

Also intrabodies based on VHH have been applied successfully to combat HIV-1 [40-44] (Table 2). Against representatives of three groups of proteins that have crucial roles in the life cycle of HIV, reverse transcriptase, integrase and nuclear im- and export, VHH have been selected and expressed in cells.

In a recent review [45] the two routes to transport either for genes encoding VHH or VHH as such were discussed. The way VHH can block the reproduction cycle of HIV-1 is depicted in Figure 3. Although various methods for gene delivery are developed, like AAV mediated gene transfer which is

**Table 2:** Various intrabodies with their cognate targets that showed inhibition of steps in the life cycle of HIV-1.

| References | Target[a] |
|---|---|
| Vercruysse et al [44] | VHH recognizing N-terminal α-helix of multimerization domain |
| Boons et al [41] | VHH recognizing Rev |
| Matz et al [17] | VHH recognizing Vpr |
| Bouchet et al [42] | VHH recognizing Nef and chimer of VHH and SH3 domain against Nef |
| Luif et al [43] | VHH against Nef; 3D analysis of interaction between VHH and Nef |

[a] The intracellular antigens of these VHH or steps in synthesis of HIV-1 blocked by these VHH are indicated. Although proteases play an important role in the life cycle of HIV and small molecular protease inhibitors are present in certain cART therapeutics, until now no studies have been published that show inhibition of proteases with intrabodies.

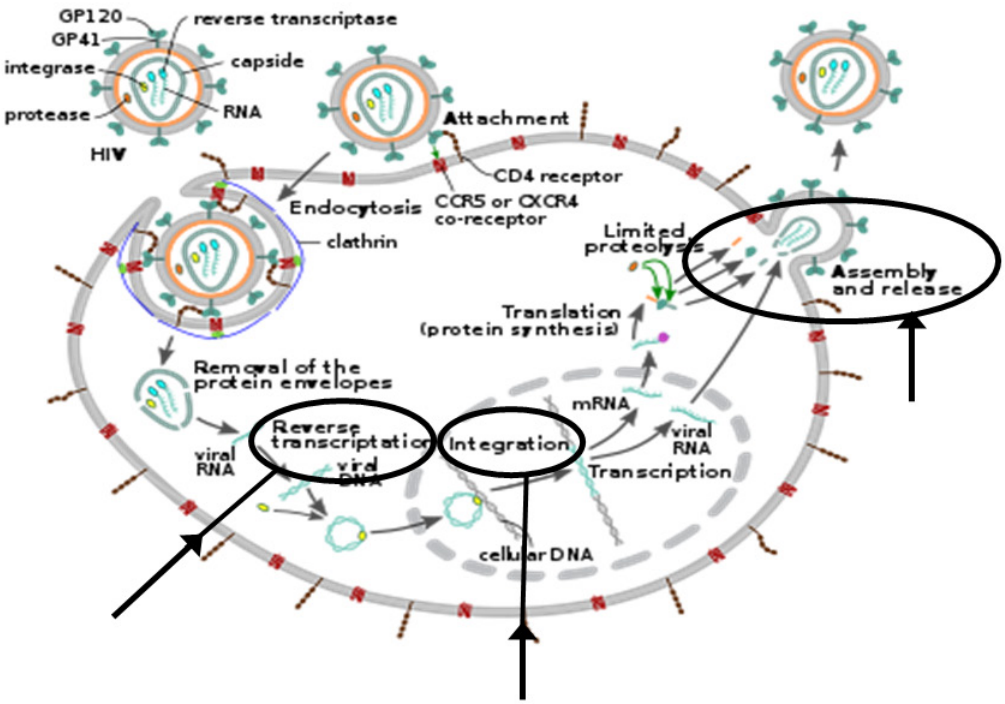


**Figure 3:** A scheme showing transmission of HIV-1 to $CD4^+$ T cells due to interaction with CD4bs and CXCR4 and crucial steps in the life cycle of HIV-1 that have been blocked by intrabodies, either scFv's or VHH [nanobodies] (indicated with arrows).

promising, the transport of VHH using protein transduction domains, cell penetrating peptides, virus like particles and liposomes/micelles may even be more suitable for getting intrabodies just in the target cells. However more efficient and specific delivery routes are necessary to develop therapies based on intrabodies. Based on the results until now and the further improvement of delivery systems to latently infected $CD4^+$ T cells, there are good prospects to eliminate re-emergence of HIV-1 in these cells in patients that cannot be treated with cART.

## Final Remarks

About 30 years ago the discovery of heavy chain antibodies devoid of light chains [46] was considered as a curiosity. At present nearly all pharmaceutical companies are working with nanobodies (VHH) with Sanofi/Ablynx [47] in the lead and their nanobody (VHH) Capiacizumab (ALX0681) is the first FDA approved nanobody. Recently the potential of mono- and biheads of VHH to block transmission of HIV-1 has been reviewed [48], therefore we focussed here on displayed VHH. *Lactobacillus* delivery systems would have the advantage of combining the specificity of the VHH antibody with the general beneficial properties of the lactobacilli. As the use of genetically engineered lactobacilli for medical purposes must guarantee their stability and safety, we are developing systems for chromosomal integration of the VHH expression cassette and containment within the host [49]. VHH may become the preferred option for intrabodies that block the Life Cycle of HIV-1. Their simple structure enabled modifications into mono- or bispecific VHH that have better or new functionalities. An example of the latter turned out to provide a complete new potential therapeutic route to clear HIV-1. These bispecific VHH consist of a VHH recognizing CD4bs or another conserved region of the envelope protein of HIV-1, whereas the other VHH target the bispecific VHH - loaded with the virus - to cells not belonging to the immune system, providing a clearance route independent of the immune system [50]. This route may offer a therapy for immunocompromised persons, in particular HIV-1 infected persons that for other health reasons cannot use cART. Also, to deal with latently infected $CD4^+$ T-cells VHH may have a role to play. Various groups have shown that due to their small sizes and the ease to label VHH with dyes [12], effective photo dynamic treatments can be developed [51]. Using labelled bispecific VHH, one recognizing rare markers of infected cells and the other a viral protein present on the cell surface of infected cells may create sufficient difference between healthy and infected $CD4^+$ T cells to treat just infected cells with intrabodies.

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