Antiviral Research 92 (2011) 389–407

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Nanobodies®: New ammunition to battle viruses ☆

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ARTICLE INFO

Article history:
Received 8 July 2011
Revised 30 August 2011
Accepted 6 September 2011
Available online 10 September 2011

Keywords:
Nanobody
Camelidae
Heavy chain-only antibody
VHH
Antiviral

ABSTRACT

In 1989, a new type of antibody was identified, first in the sera of dromedaries and later also in all other species of the *Camelidae* family. These antibodies do not contain a light chain and also lack the first constant heavy domain. Today it is still unclear what the evolutionary advantage of such heavy chain-only antibodies could be. In sharp contrast, the broad applicability of the isolated variable antigen-binding domains (VHH) was rapidly recognized, especially for the development of therapeutic proteins, called Nanobodies®. Here we summarize first some of the unique characteristics and features of VHHs. These will next be described in the context of different experimental therapeutic applications of Nanobodies against different viruses: HIV, Hepatitis B virus, influenza virus, Respiratory Syncytial virus, Rabies virus, FMDV, Poliovirus, Rotavirus, and PERVs. Next, the diagnostic application of VHHs (Vaccinia virus, Marburg virus and plant Tulip virus X), as well as an industrial application (lytic lactococcal 936 phage) will be described. In addition, the described data show that monovalent Nanobodies can possess unique characteristics not observed with conventional antibodies. The straightforward formatting into bivalent, multivalent, and/or multispecific Nanobodies allowed tailoring molecules for potency and cross-reactivity against viral targets with high sequence diversity.

Contents

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0166-3542/$ - see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.antiviral.2011.09.002

1. Introduction

Billions of different antibody molecules are generated by the vertebrate immune system. A specific antibody binding every existing compound is thought to be present in this antibody repertoire. It is this unparalleled high diversity and selectivity that make antibodies attractive and efficient research tools but also therapeutic molecules. Up to 1989, all antibodies were thought to be composed of two heavy chains and two light chains. The two heavy chains are covalently linked by disulfide bonds. The heavy chains of IgGs consist of 1 variable domain (VH) and 3 constant domains called CH1, CH2 and CH3. The light chains consist of a variable (VL) and constant domain (CL) that interact non-covalently with the VH and CH1 domains, respectively (Fig. 1). In 1989, a new type of antibody was identified, first in the sera of dromedaries and later also in all other species of the *Camelidae* family (Hamers-Casterman et al., 1993). These antibodies do not contain a light chain and also lack the first constant heavy domain. Today it is still unclear what the evolutionary advantage of such heavy chain-only antibodies (HcAbs) could be. In sharp contrast, the broad applicability of the isolated variable antigen-binding domains (VHH) was rapidly recognized, especially for the development of therapeutic proteins (for recent reviews see Harmsen and De Haard (2007), Van Bockstaele et al. (2009), Muyldermans et al. (2009), Wesolowski et al. (2009), and Kolkman and Law (2010)). Such therapeutic proteins based on the smallest functional fragments of heavy chain antibodies, naturally occurring in *Camelidae* have been called Nanobodies®. Another type of heavy chain-only antibodies was found in sharks, the socalled immunoglobulin new antigen receptors (IgNARs) (Greenberg et al., 1996; Nuttall et al., 2001). The antigen-binding variable domains of these antibodies (vNARs), as wells as single-domain antibodies (dAbs) derived from human variable heavy domains (VH) and variable light domains (VL) have similar applications (Holliger and Hudson, 2005).

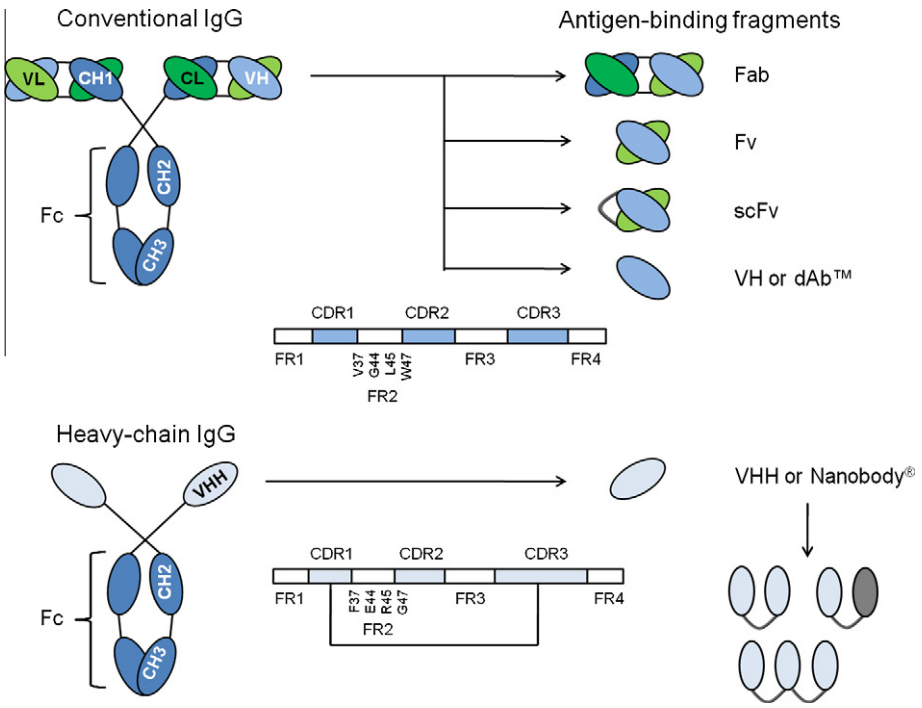

Fig. 1. Distinguishing structural features of conventional antibodies and camelid heavy-chain antibodies. Conventional IgG antibodies comprise of two heavy (H) chains and two light (L) chains, and carry two antigen-binding sites determined by the combination of the variable domains of heavy and light chains (VH and VL). Camelid heavy-chain antibodies lack both constant and variable light chains (CL and VL) and the first heavy chain constant (CH1) domain, and the antigen-binding site is formed only by the heavy chain variable domain (VHH or Nanobody). VHHs are characterized by the presence of hydrophilic amino acid residues in the second framework region (FR2), the socalled hallmark residues (indicated according to Kabat numbering). In many VHHs an additional disulfide bond is present connecting the first (camels) or second (llamas) with the third complementary determining regions (CDRs).

Table 1
Overview of published camelid-derived Nanobodies directed against viruses.

Virus	References	Immunogen	Camelid	Producing host	Mechanism	*In vitro* data	*In vivo* data	Specificity	Observations
Influenza A	Hultberg et al. (2011)	HA (H5N1)	Llama	*E. coli*	Neutralization	Microneutralization IC_{50} 0003–7 nM. Neutralizing pseudotyped MLV(H5) IC_{50}: 1–150 nM		H5N1 (clade 1>clade 2.2>clade 2.5)	
Influenza A	Ibañez et al. 2011)	HA (H5N1)	Llama	*E. coli*	Neutralization		Prophylactic Therapy: 0.5 μg/mouse: lung virus titers below the detection limit. Therapeutic: VHHb 4, 24 or 48 h after challenge, higher body weights and lower lung virus loads	H5N1	
RSV	Hultberg et al. (2011)	Fusion protein	Llama	*E. coli*		IC_{50} bivalent: 0.1 nM; monovalent 250 nM		RSV Long (A) RSV B1 (B). Antigenic site II	
Rabies	Hultberg et al. (2011)	G protein	Llama	*E. coli*		IC_{50} CVS-11 (genotype 1): 7,5–325 nM; EBLV-1 (genotype 5): 012–586 nM		CVS-11 strain (genotype 1), street isolates, 5 EBLV-1 strain; antigenic site IIa	
Poliovirus	Thys et al. (2010)	Type 1 Sabin strain	Drome-dary	*E. coli* WK6	Neutralization	IC_{50} 7–692 nM		Type 1 viruses	
FMDV	Harmsen et al. (2007)	Crude extract of FMDV-infected BHK cells	Llama	Yeast strain VWK 18gal–	Neutralization	VHHs neutralize FMDV O1 Manisa at concentrations below 0.34 mg/ml	Passive transfer of VHH did not protect guinea pigs against FMDV challenge infection.	GH-loop, FMDV type O1 Manisa	
FMDV	Harmsen et al. (2008)	O1 Manisa/Turkey/69 FMDV	Llama	Yeast strain VWK 18gal–	Neutralization	FMDV neutralization titer 1–4 mg/l	3 mg/kg of VHHs (i.m.) reduce viremia and viral shedding but do not prevent the development of FMDV clinical signs or transmission.	GH-loop, FMDV type O1 Manisa	Porcine immunoglobulin (plg) binding VHHs genetically fused to VHHs against FMDV (100-fold increased serum half-life)
FMDV	Harmsen et al. (2009b)	O1 Manisa/Turkey/69FMDV	Llama	Yeast strain VWK 18gal–	Neutralization	FMDV neutralization titer1–5 mg/l	VHHs reduce and delay the development of clinical disease, viraemia and viral shedding; delay FMD transmission.	FMDV O1 Manisa > FMDV A Turkey > FMDV Asia 1 Shamir	
FMDV	Harmsen et al. (2009a)	O1 Manisa/Turkey/69 FMDV	Llama	Yeast strain VWK 18gal–	Neutralization	FMDV neutralization titer 0.008–0016 mg/ml		GH-loop, FMDV type O1 Manisa	Increase the FMDV-neutralizing capacity of two non-glycosylated VHHs by genetic fusion to another VHH that is glycosylated.
Rotavirus	van der Vaart et al. (2006)	G3 strains	Llama	Yeaststrain VWK 18gal1	Neutralization	IC_{50} ~50 ng/ml	50–100 μg significantly reduced the number of days with diarrhea per pup	Rotavirus G3 strains.	A VHH has lost it's neutralizing capacity after production in yeast.

(*continued on next page*)

Table 1 (*continued*)

Virus	References	Immunogen	Camelid	Producing host	Mechanism	*In vitro* data	*In vivo* data	Specificity	Observations
Rotavirus	Garaicoechea et al. (2008)	VP6 protein derived from the bovine rotavirus c486 strain	Llama	*E. coli* TG1	Neutralization	0,2–15,6 μg/ml (concentration that reduces > 80% of focus forming units)	Partial protection against rotavirus diarrhea	Bovine rotavirus C486, IND and B223, Human rotavirus Wa; Equine rotavirus H2.	Broad neutralization activity *in vitro*
Rotavirus	Pant et al. (2006)	G3 RRV strain	Llama	*Lactobacilli paracasei*	Neutralization	60 ng/ml reduce by 60% the number of RRV-infected cells	Lower prevalence, duration and severity of diarrhea	G3 RRV strain	Reconstituted freeze-dried VHH1-anchored lactobacilli are equally as protective as their fresh counterparts. VHH1-secreting lactobacilli do not offer better protection than contransformed lactobacilli
Rotavirus	Martín et al. (2011)	G3 RRV strain	Llama	*L. paracasei* (coding sequence integrated in bacterial genome)	Neutralization		VHHs were shownto reduce the duration and severity of diarrhea	Rotavirus G3 strains	
HIV-1	Forsman et al. (2008)	Envelope protein gp120 from HIV-1CN54 (subtype B/C)	Llama	*E. coli* TG1	Neutralization	IC_{50} 0.003 to 38 μg/ml		HIV-1 primary isolates of subtypesB, C, and CRF07_BC	First description of broadly neutralizing MAbs to HIV-1 envelope which were derived from an immunized animal
HIV-1	Koh et al. (2010)	Envelope protein gp120 from HIV-1CN54 (subtype B/C)	Llama	*E. coli* TG1	Neutralization	IC_{50}: Subtype B: 0,07–0,57 μg/ml; Subtype C: 0,04–0,96 μg/ml		Subtypes B and C	Construction of a Family-specific Phage Display Library
HIV-1	Vercruysse et al. (2010)	Recombinant HIV-Rev protein	Llama	*E. coli*; eucariotic cells	intrabody binds the multimerization domain of Rev and inhibits its oligomerization.	~0,2 μg of plasmid inhibit 50% of p24 Gag amount in the supernatants oftransfected cells		Intrabody-Rev interaction is completely abolished by the K20A and Y23A mutations	First known molecule that destabilizes and prevents the formation of a large organized homoprotein complex required for efficientHIV-1 mRNA export from the nucleus
HIV-1	Bouchet et al. (2011)	Recombinant Nef protein (fragment 57–205)	Llama	*E. coli* K12 strainTG1	VHH binds to HIV-1 Nef and inhibit its critical biologic activities	VHH inhibits virus infectivity in a Nef-dependent mannerand counteracts the positive effect of Nef on virus replication	VHH rescues Nef-mediated thymic CD4 T-cell maturation defect and peripheral CD4 T-cell activation phenotypes of the CD4C/HIV-1Nef Tg mouse model	VHH counteracts most of the HIV-1 nef alleles including Nef proteins from groups M, N, O, and P	
HIV-1	Jähnichen et al. (2010)	CXCR4-expressing HEK293T cells	Llama	*E. coli* TG1	Monovalent VHHs: neutral antagonists, biparatopic VHHs: inverse agonists	IC_{50} Monovalent: 13,6–82 mM; Bivalent: 0,2–0,5 nM	The biparatopic nanobody effectively mobilized CD34-positive stem cells in cynomolgus monkeys	238D4: binds D187,F189, E179, and S178 in ECL2; 238D2: binds F189, N192, W195,P191,V196 and E277 in ECL3. F189, positioned in ECL2, is critical for binding of both VHHs	More than 1000-fold selectivity of 238D2and 238D4 for CXCR4 versus all other GPCRs tested
HBV	Serruys et al. (2009)	*E. coli*-derived nucleocapsids (HBcAg) and plasma-purified HBsAg	Llama	*E. coli* WK6; HepG2 cells		Suppress hbsag particle secretion (80–90%) and increase hbsag accumulation/retention inside the cell.	The concentration of hbv dna in plasma was reduced 10–100-fold. The levels of secreted hbeag were not affected	Envelope protein s	First report of intrabody-mediated inhibition of viral secretion in mammals

HBV	Serruys et al. (2010)	Recombinant HBcAg	Llama	*E. coli* WK6; HepG2 cells	Competition assay: positive result at ± 70 nm. Vhhs targeted to the nucleus: elevated intracellular amount of hbeag and absence of hbcag in lysates	Cross-reativity HBcAg and HBeAg (subtypes subtype ayw and adw)	
Porcine Retrovirus	Dekker et al. (2003)	Purified 60-kDa Gagprotein	Llama	*E. coli* TG1	Intrabody reduces RT activity to approximately 7% of the activity in uninduced state.	PERV-A and PERV-B	
Vaccinia	Goldman et al. (2006)		Naieve llama	*E. coli* Tuner + pRARE	Clones tested in ELISA are vaccinia specific	Vaccinia	
Marburg Virus	Sherwood et al. (2007)		Naieve llama	*E. coli* Tuner + pRARE	The limit of detection 0.1–100 pfu/well.	Nucleoprotein (MARV variants Musoke, Ravn, and Angola)	Diagnostic assays. First recombinant antibody specifically isolated for any MARV protein
Tulip virus X	Beekwil-der et al. (2008)	TuVX particles	Alpaca	*E. coli* TG1	Positive detection of virus particles or diluted tulip leaf extract	Tulip virus X	Diagnostic assay for detection of the plant pathogenic Tulip virus X (TuVX)
Bacterio-phage	De Haard et al. (2005)	*L. lactis* bacterio-phage p2	Llama	*E. coli* and yeast	Neutralization	VHH prevent phage infection, even at concentrationsas low as 2.25 nM	Receptor-binding protein (RBP/ORF18) of Phage sk1, phage p2.
Bacterio-phage	Hultberg et al. (2007)	*L. lactis* bacterio-phage p2	Llama	*L. paracasei*	Neutralization	VHH-secreted neutralise phage p2 by binding to its RBP and inhibiting (86%) its adsorption to the host strain. Surface expressed VHH inhibit phage infection (31%)	

Here we summarize first some of the unique characteristics and features of VHHs. These will next be described in the context of different experimental therapeutic applications of Nanobodies against different viruses: HIV, Hepatitis B virus, influenza virus, Respiratory Syncytial virus, Rabies virus, FMDV, Poliovirus, Rotavirus, and PERVs. Next, the diagnostic application of VHHs (Vaccinia virus, Marburg virus and plant Tulip virus X), as well as an industrial application (lytic lactococcal 936 phage) will be described. All these different applications are summarized in Table 1.

2. Camelid antibodies and Nanobodies

Camelids produce conventional antibodies but they also produce heavy chain-only antibodies (HcAbs). In llama species ∼45% of serum antibodies are HcAbs, while in camelus species this is ∼75% (Hamers-Casterman et al., 1993). In camelids, conventional antibodies are IgG1 isotypes, while IgG2 and IgG3 are HcAbs (Fig. 1). Despite this abundance, which points to a significant role, there is little information on the functions and specificities of these HcAbs in immunity (Daley et al., 2010; Daley-Bauer et al., 2010).

Because of a splice site mutation, heavy chain-only antibodies lack the CH1 domain and also lack the complete light chain that is partially anchored to the CH1 domain (Nguyen et al., 1999; Woolven et al., 1999). The variable heavy-chain domains of HcAbs (VHH) are generated form a V–D–JH gene rearrangement using a separate set of ∼40 V gene segments, all related to the human VH3 gene family (Harmsen et al., 2000; Nguyen et al., 2000). Different VHH subfamilies have been defined, but all share a few crucial substitutions of germline-encoded amino acids Val37 → Phe/Tyr, Gly44 → Glu/Gln, Leu45 → Arg, and Trp47 → Gly/Phe/Leu (Kabat numbering), that increase the hydrophilicity of frame work 2 (FR2), the putative VH–VL interface (Fig. 1). These VHH hall mark residues abrogate a possible interaction with VL domains and contribute to the stability, increased solubility and reduced aggregation tendency of HcAbs and VHHs compared to other single domain antibodies (Hamers-Casterman et al., 1993; Muyldermans et al., 1994; Vu et al., 1997). Remarkably the third complementary determining region CDR3 of VHHs has been shown in many crystal structures to fold back and cover the former VL interface, further contributing to the stability and solubility of VHHs (Desmyter et al., 1996; Muyldermans et al., 2001).

Compared to human VH domains, VHH often display a longer CDR3 loop (Muyldermans et al., 1994; Vu et al., 1997). This leads to an increased surface area and repertoire that can interact with antigens. Such extended CDR3 loops are often stabilized by a disulfide bond between CDR1 and CDR3 or between FR2 and CDR3. Nevertheless, a significant proportion of VHH has a short CDR3 and lack the additional disulfide bond (Vu et al., 1997; Harmsen et al., 2000). Increased binding diversity also results from non-canonical CDR1 and CDR2 loop structures and additional hotspots for somatic hyper mutation in the CDR1 (Nguyen et al., 2000). Besides these VHH domains, VHH-like domains lacking the hall mark residues, the long CDR3 loops and the interloop disulfide bonds are also used in HcAbs (Harmsen and De Haard, 2007). More recently it was suggested that variable genes displaying a high degree of homology to the human VH4 family add to the HcAb Ag-binding diversity (Deschacht et al., 2010).

Because of these unique biophysical and biochemical features of the antigen binding domains of HcAbs, VHH domains have been produced recombinantly as separate entities (VHHs). This created new possibilities and as such several (new) features and applications for VHH have been explored and described.

2.1. VHHs display high affinity

VHHs against many different targets that include haptens, peptides, soluble and transmembrane proteins have been reported. Active immunization of dromedaries or llamas is most often used for proteins and as a result VHHs with affinities in the lower nanomolar or even picomolar range have been reported (reviewed in Harmsen and De Haard (2007), Van Bockstaele et al. (2009), Wesolowski et al. (2009), and Kolkman and Law (2010)). Considering the monomeric nature of VHHs this is remarkable as these affinities are in the same range of what is readily observed for conventional bivalent antibodies. VHHs with nanomolar affinities have also been obtained using naïve or synthetic libraries (Goldman et al., 2006; Groot et al., 2006; Verheesen et al., 2006).

2.2. VHHs can recognize structures not recognized by or inaccessible for conventional antibodies

Structural analysis of conventional antibodies and VHHs, in complex with their antigen has revealed a major difference in the structure of the CDRs. While conventional antibodies typically have a concave or flat antigen binding site, VHHs have a convex conformation with large solvent exposed CDR loops (Desmyter et al., 1996; Muyldermans et al., 2001). The compact shape of VHHs combined with the convex paratope allows binding into clefts or pockets. This was demonstrated for several VHHs that inhibit enzymes like lysozyme and carbonic anhydrase (Desmyter et al., 1996, 2001; Lauwereys et al., 1998; Transue et al., 1998; De Genst et al., 2006; Conrath et al., 2001a, 2009). Besides this unique cavity-penetrating properties, it has also been shown that VHH can be isolated that bind cryptic epitopes on the variant surface glycoproteins of African trypanosome, not accessible for conventional antibodies (Stijlemans et al., 2004, 2011).

2.3. VHH are remarkably stable under different extreme conditions

VHHs display high thermal stability. Tm values between 60 and 80 °C are the rule, not the exception with thermal unfolding often shown to be fully reversible and functional activity sometimes retained at temperatures up to 90 °C (Lauwereys et al., 1998; van der Linden et al., 1999; Pérez et al., 2001; Ewert et al., 2002). VHHs are also exceptionally resistant to high pressure, chemical unfolding with guanidinium chloride and urea, detergents or alkaline and acid pH (Dumoulin et al., 2002; Dolk et al., 2005). Compared to conventional antibodies and antibody-derived fragments, resistance of VHHs to proteases can be improved by *in vitro* selection to generate VHHs that can resist the harsh conditions of the gastro-intestinal tract (Harmsen et al., 2006).

2.4. Enhanced functionality by easy multimerization of VHHs

The high solubility, single domain and single gene nature of VHHs allows rapid and successful generation of multimeric VHHs using genetically encoded amino acid linkers or carrier proteins (Fig. 1). Bivalent, trivalent, pentavalent and even decavalent molecules have been described (Conrath et al., 2001b; Zhang et al., 2004; Groot et al., 2006; Mai et al., 2006; Stewart et al., 2007; Stone et al., 2007a, 2007b; Garaicoechea et al., 2008; Hmila et al., 2010). Multimerization is an easy way to rapidly improve functional potency due to an avidity effect. Fusion of two identical anti-TNFα Nanobodies resulted in a 500-fold increase in TNFα neutralizing activity. The *in vitro* potency of this bivalent Nanobody even exceeded those of clinically used conventional antibodies (Coppieters et al., 2006). Increased potency due to avidity has also been demonstrated for membrane bound receptors using bivalent monospecific and biparatopic VHHs (Roovers et al., 2007). The latter consists of two different VHHs recognizing overlapping or non-overlapping epitopes on the same antigen. Besides improvements in potency, formatting also allows the facile generation of one single molecule capable of binding different molecules (Conrath et al., 2001b; Harmsen et al., 2008; Hmila et al., 2010). An important multispecific application is extension of *in vivo* half-life of therapeutic VHHs. Indeed the molecular weight of monovalent VHHs (~15 kDa) is below the threshold of renal filtration. As a result mono, bi and trimeric VHHs are all rapidly cleared form the blood. By coupling to an VHH that binds an abundant serum protein like albumin or IgG, the half-life of the therapeutic Nanobody becomes similar to that of such proteins (Harmsen et al., 2005; Roovers et al., 2007; Tijink et al., 2008).

2.5. Alternative expression of Nanobodies

Classical antibodies are successfully expressed and secreted in mammalian cell lines mainly. Several smaller formats derived from conventional antibodies have been generated: monovalent antibody fragment (Fab), Fab dimer, variable fragment (Fv), single-chain Fv (scFv) and heavy or light chain single domain antibodies (Fig. 1). Although these derivatives can be produced in other host cells, the hydrophobic nature of the VL and VH FR2 interface remains, and is partially responsible for problems in expression yield, solubility, stability and aggregation. In contrast, the single domain nature of VHHs and their increased hydrophilicity enables high production levels in microbial hosts like *Escherichia coli*, *Pichia pastoris* and *Saccharomyces cerevisiae*. In addition VHHs can also be produced and function in different cellular compartments where the formation of disulfide bonds cannot occur. VHHS have been expressed in the ER and in the cytoplasm of cells (Klooster et al., 2009). VHHs have been successfully targeted to the nucleus or mitochondria (Serruys et al., 2010; Van den Abbeele et al., 2010).

3. Therapeutic applications

3.1. Influenza virus Nanobodies

Influenza is an important respiratory disease caused by influenza A and B viruses. In moderate climate zones, influenza typically occurs in epidemics that peak during wintertime. Influenza A viruses can also cause unpredictable pandemic outbreaks, associated with antigenic shift of the viral hemagglutinin. Thanks to intense global monitoring of influenza viruses, currently used vaccines to prevent seasonal influenza have a fairly accurate antigenic composition and protect well in most target groups (Russell et al., 2008). However, pandemic outbreaks remain unpredictable, as illustrated by the 2009 H1N1 virus (also named Mexican flu), which took the world and the vaccine manufactures by surprise. In addition, outbreaks of highly pathogenic avian influenza such as H5N1 have occurred without cessation since 2003, which has resulted in culling of more than a billion birds in the poultry industry, leading to major losses of food and economical income, mainly in south East Asia. Occasional zoonotic infections with these H5N1 viruses and their high propensity to reassort with swine influenza viruses, have earmarked these viruses as a major pandemic threat. The case fatality of zoonotic infections with H5N1 viruses is close to 60% despite intensive care interventions and the use of antiviral drugs such as oseltamivir. In summary, there is a need for novel treatment options against influenza. Therefore, we decided to isolate and evaluate the prophylactic and therapeutic activity of Nanobodies directed against highly pathogenic H5N1 virus. To this end, a llama was immunized with recombinant H5 hemagglutinin and, following phage display and panning against the recombinant antigen, two Nanobodies that neutralized H5N1-pseudotyped

lentiviruses with an IC_{50} of 10 and 30 nM, respectively, were selected (Hultberg et al., 2011). These two Nanobodies blocked the binding of hemagglutinin to sialic acid residues on fetuin, which serves as an *in vitro* surrogate for the natural receptor. Interestingly, by producing bivalent or trivalent Nanobodies by genetic fusion of the coding information of two or three of the neutralizing Nanobodies separated by a glycine–serine (GS) linker of variable length the *in vitro* neutralizing activity against H5N1 pseudotyped lentiviruses or against H5N1 influenza virus increased dramatically. One of the monovalent Nanobodies had an IC_{50} of 7 nM as measured in a microneutralization assay against H5N1 virus, whereas its bivalent and trivalent counterpart displayed an IC_{50} of 3–9 pM in the same assay (Hultberg et al., 2011). This dramatic difference in neutralizing activity between a mono- and a bi- or trivalent VHH can partially most probably be explained by the increased avidity and by a more potent mode of interaction involving intermolecular binding. Ultimately, however, co-crystal structure analysis will be required to help explain the 1000-fold increase in *in vitro* efficacy of these Nanobodies. Remarkably, a number of H5N1 variants which were not or poorly neutralized (IC_{50} > 120–150 nM) by the monovalent Nanobodies, were efficiently neutralized by the bivalent or trivalent Nanobodies (IC_{50} < 10 nM).

We next assessed the *in vivo* efficacy of these Nanobodies in a mouse model for H5N1 influenza (Ibañez et al., 2011). Importantly, we decided to administer the Nanobodies intranasally. The rational for this route of administration was in part to avoid multiple or continuous dosing as systemic administration of Nanobodies leads to rapid clearance from the body. Both prophylactic (up to 48 h before challenge; supporting a long local half life of Nanobodies) and therapeutic (up to 72 h after challenge) significantly reduced virus replication in H5-specific Nanobody treated animals. Interestingly, also *in vivo*, the bivalent neutralizing Nanobody outperformed its monovalent counterpart, by a factor 60. In addition, intranasal administration of bivalent Nanobodies 24 h before a potentially lethal challenge with H5N1 virus, fully protected the animals from death and morbidity. Also in a therapeutic setting, the bivalent Nanobodies were protective and significantly delayed time to death. Finally, we identified the likely site of binding of the Nanobody in the HA, by selecting escape viruses *in vitro*. Both mono- and bi-valent Nanobody selection pressure resulted in escape viruses in which residue Lysine 189 located in antigenic site of hemagglutinin near the receptor-binding site, was changed to glutamic acid. These findings provide proof-of-concept that Nanobodies can protect against H5N1 influenza virus challenge when administered by the intranasal route. In addition, our results favor the design of bi- or trivalent Nanobodies to increase their potency. Additional experiments in another animal model such as the ferret and the isolation of broadly neutralizing Nanobodies will be needed to provide additional proof that the use of Nanobodies is a highly interesting treatment option to prevent and treat influenza virus infection.

3.2. Respiratory Syncytial virus (RSV) Nanobodies

Infections by Respiratory Syncytial virus (RSV) are the leading cause of viral acute lower respiratory tract disease in children worldwide (Hall et al., 2009). In developed countries 1–2% of the RSV infected infants require hospitalization. In this way RSV infections are the most important cause of infant hospitalization. Once hospitalized, there is no effective anti-viral or anti-inflammatory therapy available and treatment is mainly based on supplying oxygen (by mechanically assisted ventilation if required) and rehydration. As RSV infections themselves do not evoke long-living immune protection, RSV infections repeatedly occur throughout life, causing also significant morbidity and mortality in elderly and immune compromised adults (Falsey et al., 1995; Hall, 2001). It has been estimated that annually RSV infects about 64 million people resulting in 160,000 deaths. Next to the acute consequences of infection, severe RSV infections at young age are potentially associated with the development of long-term pulmonary distress. Despite the major clinical importance of RSV, there is neither a vaccine nor any antiviral therapy available.

Although immune protection by natural RSV infections is partial, it correlates with high serum titers of neutralizing antibodies and high serum titers of RSV F specific IgG antibodies (Henderson et al., 1979). Administration of RSV neutralizing serum or antibodies was shown to reduce pulmonary RSV replication in different animal models (Henderson et al., 1979; Taylor et al., 1984; Walsh et al., 1984; Hemming et al., 1985; Prince et al., 1985). These findings suggested that passive immunoprophylaxis with IgG might protect infants from RSV disease. Indeed intravenous administration of human IgG preparations enriched for RSV neutralizing antibodies could partially prevent RSV lower respiratory tract disease in infants (Groothuis et al., 1993; Simoes et al., 1996). Hence in 1996 this therapy was approved by the FDA for the prevention of RSV disease in high-risk infants. Subsequent palivizumab (Synagis), a humanized RSV monoclonal antibody (mAb) directed against the conserved RSV F protein was developed. This mAb could efficiently neutralize a broad range of RSV strains *in vitro* (Johnson et al., 1997). Intramuscular administration of 2.5 mg/kg palivizumab effectively reduced RSV replication in cotton rats. The impact RSV Study revealed that five monthly intramuscular injections of 15 mg/kg could reduce RSV-related hospitalization of infants by 55%. In 1998 the FDA approved monthly intramuscular administration of palivizumab for immunoprophylaxis of RSV induced disease in high-risk infants and children (Wu et al., 2008).

Hultberg et al. aimed at developing RSV F specific Nanobodies with enhanced neutralizing activity (Hultberg et al., 2011). Llamas were immunized 6 times weekly with recombinant membrane anchorless F protein (F_{-TM-}) derived from the RSV Long strain (subtype A) (Calder et al., 2000). Biopanning using F_{-TM-} and competitive elution using excess of palivizumab was used to enrich for RSV neutralizing F-specific Nanobody-phages. The majority of the obtained clones were able to bind to F_{-TM-}. Twelve clones were selected for Nanobody production and purification. From these clones two Nanobodies (RSV-D3 and RSV-C4) could neutralize RSV A subtype Long strain virus *in vitro* and one clone could neutralize RSV B subtype B1 strain virus (RSV-E4). Based on competition experiments with mAbs that specifically recognize well described antigenic sites within the RSV F protein, it was shown that RSV-D3 and RSV-C4, bind to the antigenic site II to which also palivizumab is binding. In contrast, the RSV-E4 Nanobody binds to the antigenic sites IV–VI. Binding of Nanobodies to these specific epitopes was confirmed by the use of specific RSV escape mutants. In order to boost their neutralizing activity, monovalent Nanobodies were fused by GS linkers. Remarkably, linking two identical RSV-D3 Nanobodies improved *in vitro* neutralization by about 4000-fold. As a result bivalent RSV-D3 Nanobodies (IC_{50}: 0.11 nM) could neutralize RSV Long *in vitro* considerably more efficient than palivizumab (IC_{50}: 6.5 nM). In addition bivalent RSV-D3 Nanobodies could also neutralize RSV B1 (subtype B) more efficiently than their monovalent counterparts. Linking two Nanobodies with different epitopes (RSV-D3/RSV-E4) also significantly increased neutralization of RSV Long (subtype A, 50 to100-fold) and RSV B1 (subtype B, 500-fold).

The neutralizing efficiency of palivizumab is about 180-fold enhanced compared to its monovalent Fab fragment. In contrast, linking of two identical monovalent RSV Nanobodies enhanced neutralization efficiency by a much larger extend up to 4000-fold. It is difficult to speculate on the reasons for this difference in increase of neutralization activity. Factors that could attribute to this

difference might include differences in flexibility of the linkers, differences in affinity/avidity interactions, sterical hindrance caused by the palivizumab Fc-tail, differences in size and differences in the length of perturbing CDRs, differences in the relative ability to bind different F proteins within the same trimer or on different trimers (on the same or separate viruses).

3.3. Rabies virus Nanobodies

Rabies causes 55,000 human deaths per year (Knobel et al., 2005). The virus is present in the saliva of infected animals and transmitted by bites. From the infected wound, the virus travels through the peripheral nerves to the central nervous system and causes lethal brain infection. Once symptoms appear, the case-fatality rate is nearly 100%. Lives can be saved if the person receives post-exposure prophylaxis (PEP) promptly after exposure (WHO Human and animal rabies. Available from URL: http://www.who.int/rabies/vaccines/en/mabs_final_report.pdf). Once the first, often non-specific, symptoms occur, PEP is no longer effective. PEP involves active immunization with vaccine and, for high-risk exposures, immediate passive immunization treatment with anti-rabies antibodies. Most deaths occur in developing countries, where people do not receive appropriate PEP, due to poor availability, high cost or simply ignorance. The antibody products available are polyclonal IgG preparations derived from pooled plasmas of vaccinated humans or horses (Sawyer, 2000). Since large scale production is not feasible, these are very expensive and have limited availability. The World Health Organization urges the development of alternatives. Cocktails of human or (humanized) mouse mAbs are being examined as alternatives for blood-derived antibodies (Goudsmit et al., 2006; Muhamuda et al., 2007; Müller et al., 2009), but VHH-based Nanobodies might be another alternative.

A Nanobody (VHH) phage library was constructed from llamas that had been immunized with inactivated Rabies Vaccine Merieux HCDV (genotype 1, Wistar Pitman Moore Strain, Sanofi Pasteur MSD)(Hultberg et al., 2011). Selections were performed on Elisa strips pre-coated with Rabies G protein (Platelia II Rabies plates, Biorad Libraries). Five Nanobodies were described that neutralized the rabies prototype strain CVS-11 (genotype 1) virus *in vitro,* but also a number of genotype 1 street isolates. IC_{50}s for the CVS-11 strain ranged from 7 to 325 nM. Four of the Nanobodies (Rab-F8, Rab-E8, Rab-E6 and Rab-H7) recognized overlapping epitopes in antigenic site IIa, while the fifth (Rab-C12) recognized a totally different epitope. Despite this overlap their fine specificity was different, as only Rab-H7 and Rab-E8 could neutralize EBLV-1, a genotype 5 strain. As was observed for RSV and H5 influenza virus Nanobodies, improved neutralization potency could be obtained by producing genetically fused bivalent and biparatopic Nanobodies. For example, combining Rab-E6 with Rab-H7 resulted in an IC50 of 140 pM, which represents a ~1672-fold increase when compared to the monovalent Nanobodies. Fusion of Rab-H7 to Rab-F8 resulted in an IC_{50} of 330 pM, which represent a ~782-fold increase when compared to the monovalent counterparts. An improvement of EBLV-1 (genotype 5) neutralization potencies was also observed, even when one partner did not show neutralization as a monovalent Nanobody.

3.4. Poliovirus Nanobodies

Global vaccination programs that are coordinated by the World Health Organization have not yet succeeded in eradicating poliomyelitis (Wassilak and Orenstein, 2010). Therefore, there remains a need for developing antiviral agents against poliovirus, and such antivirals are even simply lacking at this moment. To explore the potential of the VHH technology as a control agent for passive prophylaxis against poliomyelitis, Thys et al. immunized a dromedary with poliovirus type I Sabin strain (Thys et al., 2010). Following cloning of the VHH repertoire as cDNA in a phage display vector and panning, a total of 15 different poliovirus-binding recombinant VHHs were selected for further characterization. These 15 VHHs could be classified into 8 groups, based on the primary sequence of their CDRs. Five of these VHHs, belonging to five different CDR groups, neutralized type 1 Sabin virus (*i.e.* the vaccine strain used for immunization) as well as wild type type 1 Mahoney virus. None of the VHHs was able to neutralize type 2 or type 3 poliovirus, which suggest a high target-specificity. Two of the poliovirus type 1 neutralizing VHHs had *in vitro* IC_{50} values of 9 and 15 nM, respectively, whereas the IC_{50} for the other 3 neutralizing VHHs was at least 20-fold higher. The IC_{50} was defined as the concentration of Nanobodies that inhibit the cytopathic effect of poliovirus type 1 Manhoney vrius by 50%. As a comparison, a conventional type 1 poliovirus neutralizing antibody displayed an IC_{50} of 8 nM in the same assay. It is important to note here that the VHHs were used as monovalent antiviral agents as opposed to the monoclonal IgG antibody that is naturally bivalent. Remarkably, it was not possible to select escape variants with two Nanobodies. The amino acid mutations responsible for escape against the other Nanobodies have not been reported.

The target specificity of the anti-type 1 poliovirus VHHs was diverse: the neutralizing VHHs recognized native poliovirus particle (*i.e.* infectious virions), non-neutralizing VHHs bound to heat-inactivated poliovirus particles and a third set of VHHs bound to 14S subviral particles (Thys et al., 2011). It will be interesting to study the epitope-binding sites of the neutralizing VHHs by crystallography of the poliovirus virions in complex with the Nanobodies. Although the world is now close to the eradication of polio because of intensive vaccination campaigns coordinated by the World Health Organization, the poliovirus neutralizing VHHs may still have clinical relevance, given their ease of production and purification. However, one prerequisite for such applications will be to demonstrate efficacy against poliomyelitis in an animal model.

3.5. Foot-and-mouth disease virus (FMDV) Nanobodies

Another Picornavirus against which neutralizing Nanobodies have been generated and characterized is foot-and-mouth disease virus (FMDV). FMDV is harmless to man, but this highly contagious virus can cause devastating disease and mortality in cloven-hoofed livestock. Because European authorities impose a non-vaccination policy against FMDV, livestock in this continent are particularly susceptible to this virus. Measures to control outbreaks include a ban of animal transport, rapid vaccination of animals in affected regions and culling of affected herds. An effective passive immunotherapy with FMDV neutralizing antibodies (*e.g.* hyperimmune serum) could also be implemented because it would provide more rapid protection and presumably reduce virus transmission earlier compared to vaccination. However, there are some hurdles to overcome to envision such an approach. The therapy should be economical, implying that therapy with FMDV-neutralizing mAbs or convalescent serum would be far too expensive to use in the field. In addition, there are at least 7 serotypes of FMDV and hence a broadly neutralizing serum would be required. Finally, protection against unique antigenic sites is associated with a high risk for escape virus selection, making a mono-selective antiserum ineffective.

Harmsen et al., tried to circumvent some of these consideration by using Nanobodies (Harmsen et al., 2007). These researchers immunized llamas with a mixture of 4 strains of serotype O FMDV, to select high affinity VHH clones by phage display. Interestingly, the authors expressed the candidate FMDV-inhibiting VHHs – 21 unique neutralizing VHHs were isolated – as secreted recombinant Nanobodies in *S. cerevisiae*. A mixture of two different VHHs dis-

playing synergistic *in vitro* FMDV-neutralizing activity turned out to be most effective in a passive prophylaxis setting. However, protection was only partial whereas a convalescent guinea pig serum with a comparable *in vitro* neutralizing titer fully protected the animals against the development of FMDV lesions. Since the Nanobodies had been PEGylated to increase their serum half life, the limited *in vivo* protection was presumably due to the lack of Fc-dependent antiviral effector mechanisms, such as opsonophagocytosis, *i.e.* binding of antibodies to virions followed by phagocytosis of the opsonized virions by macrophages.

In a follow-up study using a swine model for immuno-prophylactic treatment against FMDV challenge, bispecific Nanobodies were engineered (Harmsen et al., 2005, 2008). These bispecific Nanobodies combined one of three VHHs that neutralized FMDV *in vitro* with a second VHH that binds with high affinity to porcine Immunoglobulin G light chain (Harmsen et al., 2005). The rational for this approach was to increase the serum half-life of the Nanobodies, as an alternative for PEGylation. Addionally it was assumed that this VHH would not interfere with Fc-encoded effector functions. Both VHHs, *i.e.* the FMDV neutralizing and the Ig-binding VHHs were separated by a short GGS linker and produced in *S. cerevisiae* in a 100 L fermentor before affinity purification based on a C-terminal poly-histidine tag. The affinity and *in vitro* FMDV neutralizing activity of the bispecific VHHs was comparable with their respective monovalent counterparts with K_D values as low as 0.3–0.5 nM. Likewise the affinities of the bispecific VHHs for swine Ig was comparable to that of the monovalent swine Ig-binding Nanobody (K_D approximately 1 nM). However, when the bispecific VHHs were complexed with pig Ig, the *in vitro* neutralizing activity of two out of tree VHHs increased 4- to 30-fold, presumably as a result of steric hindrance and/or avidity effects. Intramuscular administration of a dose of 3 mg/kg in pigs 24 h prior to challenge with 1000 plaque-forming units FMDV resulted in reduced viremia and virus shedding but did not prevent transmission.

To further increase the neutralizing activity of the anti-FMDV Nanobodies, bispecific VHHs were constructed with two VHH domains directed against the virus and a third VHH directed against swine Ig (Harmsen et al., 2009a,b). This resulted in VHHs with a 5-fold higher neutralizing activity. These molecules, again produced in *S. cerevisae*, were able to reduce clinical disease, viraemia, virus shedding and now also transmission in a pig model when administered (i.v. in the ear) at a dose of 50 mg/kg, 24 h before intradermal inoculation with 10,000 $TCID_{50}$ of FMDV. Finally, the authors also demonstrated that the presence of FMDV-neutralizing VHHs did not interfere with the immune response upon vaccination with conventional FMDV vaccine. This is an important finding because FMDV vaccination of animals that are at risk for being infected is used to try to control outbreaks. In summary, this development of FMDV-neutralizing Nanobodies has provided proof-of-concept that passive immuno-prophylaxis can protect animals against FMDV-induced disease. In particular engineering steps to produce trivalent Nanobodies in which two of the three paratopes have *in vitro* FMDV neutralizing activity and the third paratope allows high affinity binding to circulating immunoglobulin, proved to be effective in preventing disease and transmission. However, it remains to be determined if FMDV-escape viruses would be rapidly selected upon use of such a Nanobody-based intervention, and if a similar approach would also be effective against the other FMDV serotypes.

3.6. Rotavirus Nanobodies

Group A Rotavirus (RV) strains are the most frequent cause of acute gastroenteritis in infants and children under the age of 5. Although fatal outcome of RV infections in developed countries are rare, RV infections cause annually more than 500,000 deaths worldwide (Parashar et al., 2006). RV is a non-enveloped double stranded RNA virus with an outer and inner capsid. The outer capsid is composed of VP4 and VP7 proteins, which are highly variable. Based on the variability of VP7 and VP4, Rotaviruses diverge into 23 G (based on the VP7 glycoprotein) and 31 P (based on the protease-sensitive VP4 protein) serotypes. RV infections induce neutralizing antibodies specific for VP4 and VP7 (Ward, 2009). Upon primary infection these neutralizing antibodies are mainly serotype specific and can hence protect against homosubtypic infections. The inner capsid is composed of VP6 proteins which are immunodominant and highly conserved. Although VP6 antibodies can protect mice from RV infection VP6 antibodies do not neutralize RV *in vitro* (Burns et al., 1996). Their protective capacity can however be explained by polymeric VP6 IgA antibodies that neutralize RV via transcytosis (Corthésy et al., 2006).

Rotarix and Rotateq are two licensed vaccines that have been shown to protect against the main circulating RV strains (G1, G2, G3, G4 and P1A) and are hence applied in childhood vaccination programs. Next to vaccines, oral administration of antibodies against VP7 and VP4 has also been shown to prevent or treat RV infections in children (Sarker et al., 2001). As a more feasible alternative to preparations of conventional antibodies, van der Vaart et al. developed RV neutralizing Nanobodies that are produced in yeast (van der Vaart et al., 2006). A Nanobody (VHH) phage library was constructed from circulating plasma cells derived from a llama that had been immunized five times with whole Rhesus rotavirus (RRV, G3 serotypes). RV binding phages were selected by biopanning, using the RRV strain that was used for immunization. As orally administered anti-RV Nanobodies should be functional in the gut they must resist the acidic conditions of the stomach. Therefore, as part of the selection strategy, the phages were pre-treated at low pH (pH 2.3). The selected Nanobodies were screened and further selected for binding to RRV and a second G3 serotype RV (CK5). Neutralizing Nanobodies and control Nanobodies were recloned for production in *S. cerevisiae*. The yeast produced Nanobodies were tested for CK5 RV *in vitro* neutralization. The most potent Nanobody (2B10) could neutralize CK5 RV in an *in vitro* plaque assay with an IC_{50} of approximately 3 nM. Neutralizing Nanobodies that could be efficiently produced in yeast were also tested in a mouse pup model for rotavirus infection. Daily oral administration of 50 or 100 μg 2B10 Nanobody could either prevent diarrhea or reduce the number of days with diarrhea per pup. These findings illustrate that oral administration of yeast produced Nanobodies could be a feasible strategy for reducing rotavirus induced acute gastroenteritis in infants (van der Linden et al., 1999; van der Vaart et al., 2006). Immunization, selection and *in vivo* testing of the described Nanobodies were all performed with whole G3 serotype rotaviruses. Therefore it is unclear whether the described anti-rotavirus Nanobodies are also effective against other circulating human rotavirus serotypes. As for conventional antibodies, only VP7 or VP4 specific antibodies can neutralize rotavirus *in vitro*, one could conclude that also the neutralizing rotavirus Nanobodies are directed against either VP7 or VP4 and would therefore be specific for G3 serotype rotaviruses. However, as Nanobodies differ considerably from conventional antibodies, the neutralizing Nanobodies might access more conserved neutralizing epitopes within the VP7 of VP4 proteins or within other more conserved rotavirus proteins such as the immune dominant VP6 protein (see later) (De Genst et al., 2006). In the absence of a defined epitope of these Nanobodies it is difficult to speculate on the mechanism by which they neutralize rotavirus. As the described Nanobodies are monovalent it is unlikely that these Nanobodies neutralize rotavirus by cross linking multiple infective viral particles. These findings illustrate that Nanobodies have the potential to be investigated as an oral prophylactic treatment against rotavirus induced gastroenteritis in infants.

As mentioned before the rotavirus inner capsid protein VP6 is highly conserved and highly immunogenic but is not or rarely a target for neutralizing conventional antibodies (Burns et al., 1996; Corthésy et al., 2006). Garaicoechea et al. investigated if this VP6 protein might be a target for neutralizing Nanobodies (Garaicoechea et al., 2008). A llama was immunized 5 times with Sf9 cell extract containing recombinant VP6 protein, derived from the Bovine rotavirus C486 strain. After the final immunization a high amount of rotavirus specific antibody-secreting cells were detected in the blood. In contrast, as expected there was no increase in virus neutralizing antibody titer. From the circulating mononuclear cells a Nanobody phage display library was constructed. RV binding phages were enriched by successive biopanning, using a bovine rotavirus strain (BRV IND). Phage clones with strong specific binding to rotavirus and recombinant VP6 were recloned into a Nanobody expression vector for production in *E. coli*. After purification four selected Nanobodies were all shown to recognize a series of rotaviruses from human and animal origin with different VP6 specificities and different G and P serotypes. Three of the four tested Nanobodies could also neutralize these rotaviruses *in vitro* (IC_{80} ranging from 13 to 1000 nM). Remarkably homo-bivalent formats of these Nanobodies could neutralize these rotaviruses less efficiently. These findings suggest that in contrast to large conventional antibodies of Fab fragments, small monovalent Nanobodies can efficiently access conserved neutralizing epitopes within the inner capsid VP6 protein. As VP6 has been shown to be involved in viral entry via interactions with hsp70 cellular protein, Nanobodies might prevent infection by interfering with the binding between VP6 and hsp70 (Gualtero et al., 2007). The protective potential of these Nanobodies was investigated in a mouse pup model for rotavirus induced diarrhea. Daily, a single dose of 100 μg of monovalent Nanobody was administered intragastrically. On the second day the pups were challenged with either bovine or mouse rotaviruses. Treatment with Nanobody 3B2 could significantly reduce the prevalence of bovine and murine rotavirus induced diarrhea. These findings suggest that VP6 specific Nanobodies could potentially protect against most circulating rotavirus strains. This study has demonstrated that Nanobodies, likely due to their small size and long CDR3, can reach neutralizing epitopes that are inaccessible for conventional antibodies or Fab fragments.

As especially infants from developing countries would benefit from Nanobody based anti-rotavirus therapy, such a therapy should be inexpensive and very easy to distribute, store and apply. To overcome these hurdles Pant et al. investigated the possibility of an anti-rotavirus therapy based on Nanobody expressing lactobacilli, which are normal commensals of the gut (Pant et al., 2006). Recombinant *Lactobacilli paracasei* expressing either surface membrane-anchored (Nanobody fused to the long anchor sequence of the *L. casei* proteinase P gene) or secreted anti-rotavirus 2B10 Nanobody were constructed. When mixed with recombinant lactobacilli, multiple rotaviruses bound to lactobacilli that express membrane anchored Nanobody. Both secreted Nanobodies and lactobacilli (starting from 1000 CFU) that express membrane anchored Nanobody could neutralize G3 serotype rotavirus (RRV) *in vitro*. After oral treatment of mice with Nanobody-anchored lactobacilli, Nanobody expressing lactobacilli could be detected in the murine intestine. It is not clear whether the detected Nanobodies on the surface of lactobacilli in the gut, represent Nanobodies that resisted the environment of the stomach and gut or represent newly synthesized Nanobodies. By surviving these conditions and allowing de novo Nanobody expression, lactobacillus might act as stealth for Nanobody delivery to hard to reach sites. Daily administration of Nanobody-anchored lactobacilli (1.10^8 cfu), starting from 1 day before rotavirus challenge (20 diarrhea $doses_{50}$ RRV) could reduce the rotavirus titer in the small intestine, the prevalence, duration and severity of diarrhea and inflammation of the small intestine in mouse pups. Comparable protection was also observed for reconstituted lyophilized Nanobody-anchored lactobacilli. Importantly, recombinant lactobacilli in which the coding sequence of the membrane-anchored Nanobody was integrated in its chromosome were able to reduce the duration and severity of rotavirus induced diarrhea to comparable extend as lactobacilli that express this Nanobody from plasmids (Martín et al., 2011). In contrast, lactobacilli expressing secreted Nanobodies did not protect against rotavirus induced diarrhea (Pant et al., 2006). This is in line with the observation that monovalent Nanobodies that neutralize rotavirus infection *in vitro* do only reduce rotavirus induced diarrhea in mice when administered in high doses (>10 μg). The authors suggest that the protective activity of Nanobodies anchored to the lactobacillus surface membrane is due to the multivalency of these anchored Nanobodies that allow high avidity interactions. In addition lactobacilli might contribute to protection by killing bound viruses via the production of antiviral molecules such as lactate. Although in this study it was indicated that the described Nanobodies could react with a variety of human strains is was not reported whether they could also neutralize and protect against other human rotavirus serotypes. Ideally, to be protective against most circulating rotavirus strains, lactobacilli should express either a mix of Nanobodies with different specificities at their surface or a single Nanobody that is specific for a strongly conserved neutralizing epitope. This study has illustrated that polypeptide Nanobodies are suitable to be delivered by commensal micro-organisms like lactobacilli.

3.7. Human Immunodeficiency virus (HIV) Nanobodies

Since the start of the HIV pandemic in 1981 over 25 million people have died from acquired immunodeficiency syndrome (AIDS). Current therapy effectively suppresses viral replication, but cannot eradicate the virus and as such does not cure the disease. The therapy has considerable side-effects, is very expensive, lifelong treatment is needed and drug resistance can develop. Approved antiretroviral drugs can be broadly classified by the phase of the retrovirus life-cycle that the drug inhibits and have focused on 5 viral and 1 cellular proteins: reverse-transcriptase, integrase, protease, gp41, GAG and CCR5. Effective preventive methods are another way to control the pandemic. However, the developments of topological microbicides and vaccines, that prevent viral entry and thus could prevent transmission, have proven difficult. HIV entry into target cells is mediated by the trimeric viral envelope protein which consists of gp120 non-covalently bound to the membrane bound gp41 unit (Dalgleish et al., 1984; Klatzmann et al., 1984; Wyatt and Sodroski, 1998). The gp120 binds first CD4 on the target cell and following a conformational change gp120 binds either CCR5 or CXCR4 (Moore et al., 1997). This interaction is normally followed by a gp41-induced fusion of viral and plasma membrane. A very small number of mAbs have been isolated that display broad-neutralizing activity (Binley et al., 2004). Two mAbs, called b12 and 2G12 are directed against gp120. Mab b12 binds an epitope that overlaps a subset of the CD4-binding site, while mAb 2G12 recognizes a carbohydrate motif (Burton et al., 1991, 1994; Barbas et al., 1992; Roben et al., 1994; Zhou et al., 2007). Mabs 4E10 and 2F5 bind gp41 (Buchacher et al., 1994; Trkola et al., 1996; Sanders et al., 2002), while mAb X5 binds to a gp120 epitope exposed after binding to CD4 (Moulard et al., 2002). These antibodies are from individuals infected with HIV-1 subtype B, the dominant subtype in North-America and Europe. So far, immunizations of animals and humans with recombinant gp120 or gp140 have not resulted in successful induction of broadly-neutralizing antibodies and isolation of broadly neutralizing mAbs. Because of the small size of VHH, combined with their

protruding CDR3 loops and their cleft-recognition properties, it was hypothesized that VHH might be able to recognize conserved epitopes.

3.7.1. HIV gp120 Nanobodies

Forsman et al. immunized llamas with recombinant gp120 derived from a subtype B/C virus (CN54) (Forsman et al., 2008). To increase the chance of isolating broad-neutralizing Nanobodies, panning was followed by competitive elution with soluble CD4. Panning on the CN54 gp120 yielded one Nanobody out of 96 tested, that did bind recombinant CN54 gp120 protein. Although this Nanobody neutralized the CN54 virus, it did not neutralize other HIV strains. In a second attempt, recombinant gp120 from another clade B strain (IIIB) was used in the selection effort. This time, 30 clones out of 48 tested were shown to bind IIIB gp120 and 24 of these did neutralize the IIIB virus. From this effort, three Nanobodies (A12, D7 and C8) that were able to neutralize a limited panel of subtype B and C isolates were selected. In a third and final attempt, alternating selections against recombinant gp120 from a subtype A, a subtype C virus and IIIB were performed. Out of 700 clones tested, only 43 did bind gp120 and did neutralize HIV. However, these 43 clones were shown to be identical to the A12 clone isolated already in the second attempt. Further characterization demonstrated that A12 neutralized 42% of the strains tested with IC_{50}s in the range of <0.2–2533 nM. Nanobody D7 neutralized 31% of the virus panel and Nanobody C8 neutralized 35% of the strains. A12 and D7 seemed more potent against subtype B viruses. In comparison, the well known mAb b12 neutralized 54% of the viruses. The three VHH, like mAb b12 did not neutralize strains that belong to clade A, A/G or D. The Nanobodies did bind with affinities between 0.1 and 1 nM to gp120 and blocked binding of CD4 to gp120. The Nanobodies also competed with binding of mAbs known to bind to the CD4 binding site of gp120. Finally it was demonstrated that CD4 inhibited binding of the Nanobodies to gp120 and that the Nanobodies competed with each other for binding to gp120 (Forsman et al., 2008).

The structure of D7 resembles known llama VHH structures, contains two canonical CDR1 and CDR2 conformations and a long 18 residue CDR3 with a non-canonical conformation (Hinz et al., 2010). The structure revealed that the tip of the long CDR3 is highly mobile and suggest that this conformational flexibility might be important for gp120 recognition. A comparison with the CDR3 loops of antibodies that bind to CD4 site on gp120 did not reveal any significant structural homology, indicating differences in binding mode. Mutational analysis identified 3 CDR3 residues that make crucial contributions to the interaction with IIIB gp120. One of these key residues is part of the flexible tip, further emphasizing the importance of the CDR3 flexibility in binding IIIB gp120. The same mutations that lead to this decreased interactions with IIIB gp120, resulted in weaker neutralization potencies. Comparison of the D7 and A12 sequence demonstrated differences in CDR1, CDR2 and CDR3, which could account for the higher neutralization potency of A12. Indeed, introduction of the A12 CDR3 residues YYD into D7, resulted in a 10-fold improved affinity and 5-fold improved neutralization.

In a follow up study, Koh et al. reported a new approach to isolate Nanobodies closely related to A12 and D7 (Koh et al., 2010). They constructed an A12/D7 family specific phage display library, using a degenerate primer that recognizes the C-terminal stretch of nucleotides in the CDR3 loops and the first 4 conserved amino acids of framework 4 of A12/D7. Together with a primer to a highly conserved frame work 1 region RNA was amplified by PCR and a phage display library constructed. From this library 49 unique VHH amino acid sequences were isolated with high homology to A12 and D7. Variations in the frame work regions as well as in the CDRs were observed. Of these, 15 were tested and shown to inhibit binding of sCD4 to gp120. Thirty-one clones, including the 15 tested for CD4 inhibition, were evaluated in HIV neutralization assays against 3 subtype B and 3 subtype C viruses. While all Nanobodies showed identical neutralization profiles against the B strains, three different neutralization profiles (Broad A12-like, Intermediate and Narrow D7-like potency) could be distinguished for the C type strains. To understand the underlying molecular basis of these differences, the amino acid sequences of the CDRs were studied. Interestingly, a triple amino acid motif YYD at the C-terminal end of the CDR3 was suggested to be crucial for the broad neutralizing potency of A12/D7 family members. Mutations in this triple motif changed the neutralization phenotype from Broad to Narrow and vice versa, demonstrating that this YYD motif is indeed responsible for the broad potency against subtype C viruses. Finally, it was demonstrated that Nanobodies with affinities <1 nM for IIIB gp120 all carried the YYD motif. All Nanobodies with affinities >1 nM were without this motif.

Overall this work on the anti-gp120 Nanobodies has demonstrated for the first time that broadly neutralizing antibodies can be obtained upon immunization. It also suggests that such Nanobodies can be considered for applications as microbicide development. In addition this work demonstrates that Nanobodies might be very useful tools to define broadly-neutralizing epitopes in order to rationally design HIV-1 vaccines.

3.7.2. HIV Rev Nanobodies

HIV RNAs are exported from the nucleus to the cytoplasm (Pollard and Malim, 1998). Cellular mechanisms export fully spliced viral mRNA, but to transport unspliced viral RNAs, the Rev protein is essential and exploits the CRM1-mediated cellular machinery (Fornerod et al., 1997; Fukuda et al., 1997; Neville et al., 1997). The Rev protein recognizes the Rev responsive element (RRE, a secondary structured RNA element) present in the (partially) unspliced viral mRNAs. Rev consists of 116 amino acids. A stretch of 10 arginine residues serves both as a nuclear localization signal (NLS) and an RNA binding domain. This basic stretch is flanked on both sides by sequences that contribute to Rev oligomerization on the RRE (Malim et al., 1989). A leucine-rich nuclear export signal (NES) binds CRM1 and mediates nuclear export (Fischer et al., 1995) (Daelemans et al., 2005). The essential role of Rev in HIV replication makes this protein an important therapeutic target. Candidate Rev inhibitors all target the Rev-RRE or the Rev-CRM1 interaction. Rev multimerization, which is crucial for efficient viral replication, has not been targeted. Rev specific Nanobodies were isolated from llamas immunized with recombinant Rev (Vercruysse et al., 2010, 2011). After 3 rounds of selection on immobilized Rev protein, 12 different Nanobodies that interacted with Rev protein were selected. An *in vitro* multimerization assay based on fluorescence resonance energy transfer (FRET) was designed to identify Nanobodies that inhibit Rev multimerization. Only one Nanobody, Nb190 was shown to inhibit the Rev protein–protein interaction. This Nanobody not only inhibited multimerization of Rev but it could also disassemble existing multimers of Rev, confirming the dynamic nature of the Rev-Rev interaction. Nb190 complexed with Rev still interacted with the RRE, but prevented further Rev assembly on the RRE, causing an accumulation of Rev dimers on the RNA. Rev residues critical for the interactions with Nb190 were shown to be Lys20 and Tyr23 in the N-terminal alpha-helix. To study whether inhibition of Rev multimerization could also interfere with Rev-mediated functions, Nb190 was expressed as an intrabody in mammalian cells. Expression of Rev in HeLa cells localized primarily to the nucleoli while Nb190 was found in cytoplasm and nucleus. Upon co-expression of Rev and Nb190, both proteins co-localized in the cytoplasm. Using inhibitors of nuclear export and disruption of the NES, it was demonstrated that Nb190 does not prohibit shuffling of Rev between

nucleus and cytoplasm. It was further demonstrated that Nb190 inhibits Rev protein–protein interactions and inhibited the Rev dependent expression of a RRE reporter system. Finally, it was demonstrated that cytoplasmic expression of Nb190 dose-dependent inhibited HIV production. Moreover, it was demonstrated that in the presence Nb190 the late viral unspliced and partially RNA species were no longer detectable. Overall this data demonstrated that Nb190 is the first molecule that prevents the formation of a large protein complex required for HIV mRNA export to the cytoplasm. It also demonstrates that interfering with Rev multimerization is a valid approach to inhibit HIV replication.

3.7.3. HIV Nef Nanobodies

The Nef protein is a multifunctional non-structural HIV protein. Nef is necessary for full HIV-1 virulence and has been defined as a pathogenic factor because disease progression is lacking in patients infected with Nef deleted viruses. Whether Nef is directly pathogenic remains to be dissolved (Foster and Garcia, 2008). *Nef* is a small myristoylated protein of 200–215 amino acids. It is found in the cytoplasm, mainly localized in the paranuclear region. Four *in vitro Nef* activities have been documented and each of these could contribute to the Nef-induced pathologies: down regulation of CD4, down regulation of MHC-I molecules, cellular signaling and activation and infectivity enhancement of viral particles by CD4-independent mechanism. Different motifs located in different locations of Nef are involved in the different Nef-mediated actions. Given its central role, targeting Nef might prevent or delay pathogenesis, yet only a few Nef-inhibitors have been described (reviewed in Foster and Garcia, 2008). A Nef Nanobody, sdAb19, was isolated from a llama immunized with a recombinant Nef fragment (aa 57–205) (Bouchet et al., 2011). It was identified in an ELISA on immobilized Nef and has a calculated K_D of 2 nM as determined by surface plasmon resonance. A cytoplasmic and nuclear distribution was observed upon expression of sdAb19 in cells. Upon expression of sdAb19 in Nef-GFP producing cells, both proteins co-localized in cytoplasmic dotted structures, concentrated in the perinuclear region. Co-localized association was confirmed by immune precipitation experiments. sdAb19 did not bind a N-terminal deletion mutant (aa1–61), core deletion mutant (aa 58–189) and C-terminal deletion mutant (aa 160–206). This suggests that this Nanobody recognizes a conformational core domain structure. sdAb19 was able to cross-react with a broad panel of Nef proteins derived from different HIV-1 groups. Upon co-expression of Nef and sdAb19, the Nef-induced down regulation of CD4 was dose-dependently inhibited. This probably resulted from inhibition of the Nef-mediated CD4 internalization likely through interference with AP complex machinery. The Nanobody failed to inhibit Nef-induced down regulation of MHC-I cell surface expression. Expression of sdAb19 reversed the inhibitory effect of Nef on anti-CD3 induced actin remodeling in T cells, probably by inhibition of Pak2 and subsequently cofilin phosphorylation. When sdAb19 was expressed during production of GFP reporter viruses, the Nanobody was incorporated into the viral particles. The incorporation was Nef dependent. This presence of the Nanobody reduced viral infectivity of GFP reporter viruses capable of a single round infection only. A similar reduction in viral infectivity was observed when replication competent viruses were produced first in the presence of sdAb19. Finally, it was demonstrated that sdAb19 inhibited Nef-mediated activities *in vivo*, using transgenic mice in which expression of the Nef gene is driven by the CD4 regulatory sequences. In these mice Nef is expressed in CD4 T cells and cells of the monocyte/macrophage lineage (Hanna et al., 1998). In such animals, CD4 cell surface down regulation, altered thymic CD4 T cell development and peripheral CD4 T cell depletion has been demonstrated. Transplantation of such mice with Nef transgenic fetal liver cells, first infected with a retrovirus encoding the sdAb19 Nanobody, demonstrated reversal of the thymic maturation defect. Moreover there was also a reversal of the CD4 down regulation in these thymic CD4 T cells. This reversal was not due to absence of Nef. sdAb19 also reduced the number of activated (effector/memory) peripheral CD4 T cells, but did not prevent their depletion. Overall this data demonstrate that Nanobodies against Nef are excellent tools to study the role of Nef in viral replication and disease progression. The work also demonstrates that it is possible to interfere with important functions of Nef with a single agent.

3.7.4. CXCR4 Nanobodies

Until recently, approved antiviral drugs were always directed against viral proteins. Maravoric, which was approved in 2007, is probably the first anti-viral drug that targets a cellular protein. Maravoric binds CCR5, preventing the interaction of HIV gp120 with this co-receptor for viral entry. Besides CCR5, HIV can also use CXCR4 as a co-receptor for viral entry (Moore et al., 1997). Viruses using CXCR4 are typically associated with development of AIDS. AMD3100, a CXCR4 antagonist, which is now approved for stem cell mobilization, was originally developed for blocking HIV entry. The chemokine receptors CCR5 and CXCR4 are members of the large family of G protein coupled receptors (GPCRs). These represent the largest family of drug target proteins to date and they are mostly targeted by small molecules. GPCRs appear to be difficult targets for antibody-based therapeutics. To investigate the potential of Nanobodies to target GPCRs, Jähnichen et al., selected CXCR4. Llamas were immunized with HEK293T cells transiently transfected with human CXCR4 (Jähnichen et al., 2010). In the first round of phage selections cell membranes of CHO cells, over-expressing CXCR4 were used. Counter selection with non-transfected CHO cell membranes was performed to deplete non-CXCR4 specific phages. In a second round of selection, membranes from CXCR4-expressing COS-7 cells were used. A total of only 180 Nanobodies were selected and periplasmatic fractions were screened for competition of the natural CXCR4 ligand SDF-1 (CXCL12). Two Nanobodies, 238D2 and 238D4, were shown to inhibit bind of radiolabeled SDF-1 to CXCR4. Following sequencing and purification of these Nanobodies, the binding characteristics were determined. Both Nanobodies fully displaced binding of SDF-1 showing potencies in the low nM range. Both Nanobodies specifically competed each other for binding to CXCR4. AMD3100 displaced binding of 238D2 and 238D4. A well known mAb 12G5 also inhibited binding of the Nanobodies to CXCR4. Using a shotgun mutagenesis approach the epitope of 238D2 and 238D4 was mapped (Jähnichen et al., 2010). Both Nanobodies focus on the second extracellular loop, but different amino acids are involved. Critical residues for binding of 238D4 are D187, F189, E179 and S178. Critical residues for binding of 238D2 are F189, N192, W195, P191, V196 and also E277, a residue located in the third extracellular loop of CXCR4. Importantly, F189 appears critical for binding of both Nanobodies. 238D2 and 238D4 are highly selective for human CXCR4, as no binding to mouse CXCR4 was detected. In addition, the Nanobodies did not bind or alter the agonist-induced activity of 11 other GPCRs tested. 238D2 and 238D4 were shown to inhibit SDF-1 induced signaling and SDF-1 induced cellular chemotaxis of Jurkat leukemia T cells. Astonishingly, two biparatopic Nanobodies, obtained by short peptide linkage of 238D2 to 238D4, resulted in a significantly increased affinity for CXCR4. SDF-1 displacement and inhibition of chemotaxis now reached picomolar potencies. Interestingly, only these biparatopic Nanobodies were capable of reducing the high basal signaling activity of a constitutive active CXCR4 mutant. The monovalent 238D2 and 238D4 Nanobodies were finally shown to display anti-HIV-1 activity. Nanomolar (~10–100 nM IC_{50}) inhibition was observed against the CXCR4-using lab strain (NL4.3) but also for the HE strain which can use both CXCR4 and CCR5. Viral infection mediated by CCR5 using the BAL strain was

not inhibited, again demonstrating the high selectivity of 238D2 and 238D4 for CXCR4. The anti-HIV activity of the Nanobodies was independent of the types of cells (cell lines or PBMC). When the biparatopic 238D2–238D4 Nanobodies were tested again a significantly increased inhibitory potency was obtained. The IC_{50} for the CXCR4-specifc NL4.3 virus reached ~100–250 pM. A more potent inhibition (~2 nM IC_{50}) was also observed for the HE strain, while infection of the BAL strain was still not affected. In conclusion, in this study the isolation and rapid generation of the most potent CXCR4 antagonist and HIV entry blockers were reported.

3.8. Hepatitis B virus (HBV) Nanobodies

3.8.1. HBV S domain Nanobodies

Hepatitis B virus infections represent a global health problem. With ~400 million people infected and ~500,000–700,000 deaths per year, the virus can be considered a destructive major health burden (Lavanchy, 2008). Effective prophylactic vaccines are available, but therapy with interferons and synthetic nucleos(t)ide reverse transcriptase inhibitors are the only approved treatments. Interferons are only effective in 20–40% of patients and cause numerous side effects. Appearance of drug resistant mutants is a recurrent problem with the reverse transcriptase blockers (Hilleman, 2003; Lavanchy, 2005). A number of novel strategies to combat chronic HBV infections are being explored. One approach is the use of intrabodies. Using Nanobodies, Serruys et al. demonstrated for the first time intrabody-mediated inhibition of viral replication *in vivo* (Serruys et al., 2009). A late step in the HBV replication cycles, namely secretion of viral particles (virions and non-infectious viral like particles), was targeted. Nanobodies used recognized the HBV S domain (HBsAg) present in the three viral membrane proteins (S, M and L) (Serruys et al., 2009). These three proteins all share 226 C-terminal amino acids S domain. The M and L proteins have N-terminal amino acid extensions. The S protein is the most abundant viral membrane protein. Llamas were immunized with serum derived non-infectious viral-like particles. These contain S, M and L proteins. To isolate S domain specific Nanobodies, panning was performed on recombinant VLPs that only contained S protein. Five Nanobodies were selected that were shown to bind S protein with different affinities. To express these Nanobodies in eukaryotic cells, their coding sequence was cloned into an expression vector in frame with an ER-targeting sequence and the SEKDEL ER-retention signal. Co-transfection of the HepG2 hepatoma cell line with these plasmids together with an HBV-expressing plasmid was performed to study the effect on secretion of viral particles. Confocal microscopy revealed the presence of Nanobodies in the ER, co-localizing with the S domains. Interestingly, more positive cells with a more intense S domain staining were observed, only when S-specific Nanobodies were co-expressed. This suggested a Nanobody-mediated intracellular accumulation of the S domains. Indeed, the levels in cell supernatant of secreted S domains dropped by more than 80–90%, while there was an accumulation detected inside the cells. There was a selective retention of the S domains, because the secretion of another HBV protein (HBeAg) was not affected. Whether the S domain specific Nanobodies can reduce secretion of HBV viral particles *in vivo* was tested in the hydrodynamics-based HBV mouse model (Yang et al., 2002). The Nanobody and HBV expressing plasmids were co-injected intravenously in immune deficient Scid mice (Serruys et al., 2009). Immunohistochemistry demonstrated expression of the five different Nanobodies in hepatocytes at day 1 after injection of the plasmids. By day 7 very little Nanobody was detected. The presence of the HBV nucleocapsid protein was clearly detected from day 1 to day 7. However, only at day 4 after plasmid injections, the intracellular presence of the S proteins in hepatocytes was observed. Importantly, this was only observed when an S domain specific Nanobody was expressed. This suggested that the S domain specific Nanobodies prevented secretion of S molecules and were capable of retaining S molecules inside the hepatocytes. Indeed, the concentration of the VLPs and more importantly HBV virions in the blood were reduced significantly (10- to >100-fold). In addition an increased amount of intracellular S proteins was demonstrated further confirming the intracellular retention of viral envelope proteins. The secretion of another HBV protein (HBeAg) was not affected, demonstrating that the S domain specific Nanobodies had no inhibitory effect on protein secretion in general. No evidence for Nanobody induced liver damage (hepatocyte apoptosis and kuppfer cell activation) was observed. Overall, these reports provided proof of principle for the use of Nanobody-based intrabodies to inhibit viral replication by interference with viral secretion *in vivo*.

3.8.2. HBV nucleocapsid Nanobodies

HBV nucleocapsids are formed in the cytoplasm by 180–240 monomeric core proteins. During this multimerization process one copy of viral pre-genomic RNA (pgRNA) is encapsidated (Bruss, 2007). Nucleocapsids can either move to the ER where they interact with the cytoplasmic loops of the viral membrane proteins. This leads to budding of virions into the secretion pathway. The cytoplasmic nucleocapsids can also be transported to the nucleus, where they disintegrate and release the viral genome (Rabe et al., 2003; Kann et al., 2007). This causes persistence of viral infection of the cells. The biological role of the nucleocapsids in the nucleus is unclear, although it has been suggested they function as interferon antagonists. The HBV nucleocapsid is an attractive new therapeutic candidate, and several small molecule antivirals have shown to inhibit viral replication (Feld et al., 2003; Xu et al., 2003; Zoulim, 2011). Nanobodies binding the nucleocapsid were obtained by immunization of llamas with recombinant protein (Serruys et al., 2010). Following two rounds of panning 6 Nanobodies were selected and their binding properties characterized. The binding affinity of three Nanobodies (C2, C4 and C6) to HBcAg was comparable with that of a reference mAb. Two Nanobodies (C4 and C6) also recognized the HBeAg antigen. This secreted protein shares a large part of its amino acid sequence with HBcAg. Despite difference in conformation and sequence, HBeAg and HBcAg share indeed some antigenic epitopes. Nanobodies C2, C4 and C6 were also shown to recognize two HBcAg variants. Because the nucleocapsids have different functions in different cellular compartments, the coding sequences of C2, C4 and C6 were cloned expressed either in the cytoplasm or targeted to the nucleus. The latter was obtained by adding a triple nuclear localization signal (NLS) form the SV40 large T antigen. Confocal microscopy demonstrated indeed that the Nanobodies without this NLS were found in the cytoplasm. Nanobodies with an NLS were detected in the nucleus. Co-expression of cytoplasmic Nanobodies with HBV had no visible effect on the cellular distribution of nucleocapsids. A diffuse cytoplasmic staining was observed. Unexpectedly, co-expression of HBV with the nuclear Nanobodies caused a more intense speckled nucleocapsid staining in the cytoplasm. No nucleocapsid staining was observed in the nucleus. The cytoplasmic or nuclear presence of Nanobodies did not reduce or enhance secretion of S proteins and HBeAg. However an increase in intracellular HBeAg was observed when Nanobodies C2 and C6 were present in the nucleus. Even more surprisingly was the observation that only in lysates of cells expressing the cytoplasmic Nanobodies, nucleocapsids were detected by ELISA. Nucleocapsids were no longer detected when the nuclear Nanobodies were present. While in this study it was not demonstrate that nucleocapsid Nanobodies inhibited viral replication, these did reveal that targeting these Nanobodies to the nucleus have an effect on the expression and intracellular trafficking of nucleocapsids and HBeAg.

3.8.3. Porcine endogenous retrovirus (PERV) Nanobodies

To solve the shortage of human organs for transplantation, the use of organs from other species is considered. The preferred donor species is the pig (*Sus scrofa*) due to the anatomical and physiological similarities. Also the lower risk of transmissible infectious diseases when compared to primates is a widely used argument. While specific pathogen-free breeding might solve this problem for known pathogens, this is less obvious for the in the pig genome encoded endogenous retroviruses (PERV). There are ~50 proviral integration sites in the genome, but elimination by knock out or breeding is impossible. Three classes of PERVs (A, B and C) have been identified (Le Tissier et al., 1997; Takeuchi et al., 1998; Patience et al., 2001). PERV-A and PERV-B are able to infect human cells *in vitro*. PERVs can infect mouse cells *in vivo*, but long term infection of humans after transplantation has not been demonstrated (Patience et al., 1997; Takeuchi et al., 1998; Paradis et al., 1999; Czauderna et al., 2000; Deng et al., 2000; van der Laan et al., 2000; Specke et al., 2001). Nevertheless, the potential risk for infection and spread of infectious PERVs beyond the xenotransplant recipient cannot be excluded (reviewed in (Denner, 2011). The GAG and POL genes, but not the envelope genes, from the A, B and C PERVs show high homology (Takeuchi et al., 1998). For this reason llamas were immunized with a recombinant 60 kDa GAG protein produced in *E. coli* (Dekker et al., 2003). This GAG protein was derived from a B type PERV from the porcine PK15 cell line. Sera recognized the 60 kDa precursor but also all intermediate and mature forms of the GAG proteins: major capsid (p27), matrix (p15), inner coat and nucleocapsid protein. Following selection and screening on purified GAG protein, eight Nanobodies were obtained. These Nanobodies not only recognized the recombinant GAG protein, but also GAG protein form porcine cell lysates and virus. Two Nanobodies were shown to bind p27, while the other 6 recognized the p15 protein. To test the capacity of the Nanobodies to reduce viral replication, three Nanobodies were stably expressed in the cytoplasm of PK15 cell line using the TET-on expression system. In these cells, the GAG protein is detected in a punctuate staining at the plasma membrane when no Nanobodies are present. In the presence of two Nanobodies A5 and E11, this (punctuate) staining was not detected. Western blotting analysis demonstrated the rapid disappearance of the precursor GAG and p27 proteins. The activity of the Reverse transcriptase was also reduced strongly only when A5 and E11 were expressed. Expression of the third Nanobody D2, which binds p27 did not reduce GAG and RT activity. Finally it was demonstrated that production of cell free PERV-A and PERV-B viral particles was reduced in the presence of the A5 Nanobody.

To avoid hyper acute rejection of the transplanted organ, pigs have been obtained in which the α-1,3 galactosyltransferase gene has been knocked out (Dai et al., 2002; Lai et al., 2002; Phelps et al., 2003). In addition pigs transgenic for human complement regulatory proteins have been generated (Platt, 2002). While reducing hyper acute rejection, these modifications also potentially increase the risk for xenozoonosis caused by PERVs. The transgenic intracellular expression of the inhibitory GAG-specific Nanobodies in pigs might represent an elegant solution of the PERV safety problem.

4. Diagnostic applications

4.1. Vaccinia virus and Marburg virus VHHs

The remarkable heat stability and refolding capacity of VHHs has spurred the idea to use VHHs in rugged diagnostic assays to be used in resource-poor and remote settings where reliable cold chains are lacking. This was considered to be of importance to detect bio-threat targets and/or emerging viruses like smallpox virus and filoviruses. To isolate VHHs binding vaccinia virus, a smallpox virus surrogate, a highly diverse library was generated starting from a 10^6 member non-immune library (Goldman et al., 2006). Error prone PCR was followed by PCR segmentation and fragment reshuffling. This resulted in a library of 10^9 individual VHH members. Selections were performed on coated purified vaccinia virus (strain Western Reserve). After panning and screening 7 unique VHHs binding vaccinia virus were identified. Two VHHS, called G and D showed significant binding to vaccinia virus in ELISA and did not cross-react with SARS and an influenza virus. This demonstrated the selectivity of the two VHHs for vaccinia virus. After a 5 min incubation of the VHHs at a temperature between 95 and 100 °C, the two VHHS retained their binding capacity. In comparison, the binding ability of conventional antibodies and scFv was rapidly lost even after incubation at 60 or 75 °C. Even after incubation at 95 °C for 40 min, clone G retained 40% of its activity. Only 3% of the activity was retained after incubation for 80 min.

Marburg virus and Ebola virus belong to the filovirus family and are causative agents of hemorrhagic fever often resulting in a fatal outcome in humans. These viruses cause sporadic outbreaks in Sub-Saharan Africa (Peterson et al., 2004). Highly sensitive, specific and rapid diagnostics are crucial for an adequate response to viral outbreaks of this nature. Using phage display, Sherwood and colleagues were able to select Marburg specific Nanobodies, not cross-reactive to Ebola virus, from a semi-synthetic library (Sherwood et al., 2007). In addition, they developed a Marburg virus specific antigen capture assay and this all in less than 3 weeks in a biosafety level 4 (BSL4) environment. The rapid assay development was made possible by the use of a synthetic library, phage display and purification of polyhistidine tagged Nanobodies from prokaryotic expression systems which bypassed the need for time consuming immunization and hybridoma production typically associated with mAb generation. The most sensitive assay using chemiluminescence and phage displayed Nanobodies could detect virus as low as 0.1–1 pfu/ml which was superior over RT-PCR. As the initial library contained heat stable Nanobodies the authors developed a second assay using labeled purified Nanobodies for virus detection. Such reagents are considered essential for diagnosis in remote settings for which a cold chain may not be feasible.

In conclusion, Nanobodies are promising reagents for viral diagnosis and have clear advantages in difficult environments such as BSL4 and field testing without the need for reagent refrigeration.

4.2. Plant virus VHHs

Tulip virus X (TuVX), a flexuous filamentous particles, is a positive-stranded RNA virus belonging to the family of flexiviridae. It is a mechanically transmissible virus that causes chlorotic and necrotic lesions in leaves and streaks of intensified pigmentation in tepals of tulip plants. Several governments consider this virus as a pathogen of potential quarantine concern on flower bulb imports; therefore it is necessary to have tools that allow its reliable and fast identification to test the phytosanitary status of large volumes of plant material for trade. Traditionally, immunological tests for detection of plant viruses have involved the use of antiserum raised in rabbit, chicken or mouse. However, Beekwilder and colleagues developed a novel immunological detection tool, base on VHHs, to identify TuVX-infected tulip leaf (Beekwilder et al., 2008). They immunized alpacas with TuVX particles and selected 28 positive clones by phage display, which were subsequently grouped in five sequence groups by variations in the CDR regions. The specificity of these clones to TuVX was investigated using an

ELISA test, they compared the binding properties of VHHs to different members of the flexiviridae family such as Potato virus S, Potato virus X, Kalanchoe mosaic virus, Pepino mosaic virus, and the carlavirus Chrysanthemum virus B. They were able to confirm that VHHs were specific for TuVX. They also tested one of the VHHs in a double antibody sandwich ELISA tests (DAS-ELISA), a routinely used technique. Virus particles and leaf extract were tested in different dilutions, significant signals were observed with both kind of samples and the detection limit was comparable to that of other serological virus tests.

5. Industrial applications

5.1. Lactococcus bacteriophage VHH

Lactic acid bacteria such as *Lactococcus lactis* have been used for centuries for the industrial production of fermented dairy products. A major and critical factor in this process is the risk of lysis of the milk-fermenting lactic acid bacteria by bacteriophages, an event that leads to delays in the milk fermentation process or even failure. Lactococcal phages have a head structure, containing the double-stranded DNA genome, and a tail, which is involved in host recognition. Based on DNA sequence homology, 3 groups of lactococcal phages are discerned: 936-, c2- and P335-like phages (Moineau, 1999). One theoretical way to reduce infection of the milk-fermenting lactococci by lytic bacteriophages would be the use of neutralizing agents that can prevent binding of phages to their receptors expressed on the bacterial surface. However, a practical concern is that these neutralizing agents, apart from being highly effective, should be applicable at the vast scale of industrial milk fermentors and be cost-effective. For such an application heavy-chain antibodies appeared to be very well suited since they can easily be produced in gram amounts in gram-positive and -negative bacteria or in lower eukaryotes such as *S. cerevisiae* (a GRAS organism), ensuring a low production cost.

The lactococcal bacteriophage p2 (group 936 phage) was selected to isolate and functionally characterize such VHHs (Ledeboer et al., 2002; De Haard et al., 2005). A llama was immunized with purified p2 bacteriophages for the generation of a phage M13 library to display the VHH-repertoire of the animal. By combining conventional biopanning against immobilized p2 phage with a p2 phage neutralization assay, three neutralizing and four non-neutralizing VHH antibody producing clones were isolated (De Haard et al., 2005). The neutralizing VHH fragments had a similar sequence and therefore presumably recognized the same epitope in p2, whereas the non-neutralizing VHH fragments were more divergent. Both types of VHHs were subsequently produced in *S. cerevisiae* and purified for further characterization. One of the VHHs (VHH5) neutralized the infectivity of p2 phage applied at a load of 10^3–10^5 plaque-forming units/ml, at a concentration as low as 2.25 nM. This value agreed well with the affinity of 1.4 nM (K_D, as measured by surface plasmon resonance). Using immuno-electron microscopy analysis, De Haard and colleagues demonstrated that VHH5 recognized the tip to the p2 phage tail, whereas a non-neutralizing VHH2 bound to ORF11, the major structural protein of the phage head. The tip of head-and-tail bacteriophages is typically involved in bacterial host cell recognition and hence this recognition agrees well with the neutralizing activity of VHH5. The target epitope of this neutralizing VHH was further characterized by Western blot analysis and characterization of purified recombinant p2 phage ORF18, a protein located at the tip of the tail. In fact the neutralizing Nanobodies were instrumental in elucidating the role of ORF18 in 936-type bacteriophage biology (Tremblay et al., 2006). The structure of the p2 receptor binding protein was finally solved and to show similarity proteins form other bacterial and mammalian viruses. The structure of the receptor binding site on p2 was identified by solving the structure of p2, complexed with VVH5 (Spinelli et al., 2006).

In a follow up study, VHH2 and VHH5 were produced in *L. paracasei*, a Gram-positive bacterium that is also used in the milk fermentation industry, *e.g.* for the production of cheese (Hultberg et al., 2007). VHH5 secreted by the recombinant *L. paracasei* retained its capacity to neutralize bacteriophage p2. Interestingly, also the non-neutralizing VHH2 could inhibit the infectivity of p2, by expressing this VHH on the surface of *L. paracasei*. Most likely, phage p2 particles are trapped by the surface-anchored VHH2 on *L. paracasei* cells, as was demonstrated by scanning electron microscopy (Hultberg et al., 2007). This result demonstrated that also VHHs, not recognizing the receptor binding protein, could be applied to prevent infection milk-fermenting lactic with bacteriophages.

Taken together, bacteriophage neutralizing VHH can be used in industrial milk-fermentation processes such as cheese production, to protect the microbial starter culture against lysis by bacteriophages. Such VHHs can be produced in GRAS organisms, including milk-fermenting bacteria, either as secreted antibody-fragments or as surface anchored baits.

6. Conclusion

While VHH share the high selectivity, specificity and affinities of conventional antibodies, their smaller size, heightened stability, solubility and modularity give them unparalleled advantages for drug development and other applications. Although heavy chain-only antibodies in *Camelidae* were discovered more than 20 years ago, it was not until 2002 before the first VHHs directed against a virus (bacteriophage) were reported. One year later the first Nanobody directed against a mammalian virus (PERV) was described. Since then, the list of viruses and viral proteins for which Nanobodies have been raised is growing steadily. However, form the work published one might indeed think of Nanobodies as "new ammunition to battle viruses". Antiviral Nanobodies with nanomolar neutralization potencies are isolated after immunization. However, picomolar neutralization potencies are easily obtained by simple genetic multmerization of Nanobodies. Multimerization can also be used to generate broadly neutralizing Nanobodies which is important when dealing with highly variable viruses like HIV and influenza. The fusion of Nanobodies that recognize different neutralizing epitopes might even reduce the change of viral escape during treatment. The Nanobodies can be delivered into the pulmonary and gastro-intestinal tract instead of being injected peripherally. This allows very high dosing in the relevant infected organ or tissue. The advantage of Nanobodies as antiviral intrabodies has been recognized very early on. In fact, the first successful intrabody-mediated inhibition of viral replication *in vivo* was obtained with a Nanobody. However the successful application of intra-Nanobodies in patients requires more efficient and safe delivery tools. Viral delivery seems to be the most advanced methodology.

Several Nanobodies have entered clinical trials the last few years. An RSV neutralizing Nanobody is expected to enter phase I clinical trial during the course of 2011. Of note, this Nanobody is planned to be delivered via the lungs (see www.Ablynx.com). A cause for concern for the use of Nanobodies is of course their llama origin. However, to reduce the risk of immunogenicity, Nanobodies can be humanized (Vincke et al., 2009). This is a straightforward procedure because Nanobodies already display relatively high sequence homology to human heavy chain variable domains. However, similarly to conventional antibodies, immunogenicity needs to be assessed on a case by case basis in the clinical setting.

Disclosure statement

P.V., C.S., E.D. are employees of ABlynx NV. E.B. is a former employee of Ablynx. P.V., C.S., E.D., E.B. own Ablynx stocks or warrants. P.V., C.S., E.D., E.B., X.S. and B.S. are inventors on patent applications owned by Ablynx NV.

Acknowledgements

The authors would like to thank all the colleagues at Ablynx and our academic collaborators who have contributed to the antiviral Nanobody work mentioned in this paper. Part of the work performed by Ablynx and our collaborators was supported by the Institute for the Promotion of Innovation by Science and technology in Flanders Ghent (IWT70050) from the Flemish government.

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