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Characterization of nsP-specific nanobodies targeting Chikungunya and Semliki Forest Virus

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Abstract

Characterization of nsP-specific nanobodies targeting Chikungunya and Semliki Forest Virus

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Viral infections are constantly increasing and impose a large threat to the public health. Alphaviruses are responsible for several animal and human diseases and have a large medical importance with few treatments available today. Alphaviruses are small, spherical single stranded RNA viruses, and are most often transmitted by mosquito vectors. Alphaviruses contains a domain of nonstructural proteins that compose the replication machinery. The domain is crucial for viral replication to occur and is therefore an interesting target for antiviral therapy. With the focus on Chikungunya and Semliki Forest Virus this work investigates the events in the cells on molecular level during infections. To do this a panel of Camelid derived single domain antibodies are developed to target the nonstructural proteins of Chikungunya and Semliki Forest Virus. Binding of the produced nanobodies to the viral proteins was investigated by biochemical methods including immunoprecipitations, western blot, and ELISA. Cell lines that express nsP-specific nanobodies in the cytosol were employed for infection- and plaque assays with Semliki Forest Virus in order to determine the antiviral potential of the new nanobodies. Three of the nanobodies proved to bind two different nonstructural proteins of the viruses, providing opportunities for further investigations and a possible use of these nanobodies to identify viral vulnerabilities that could be exploited for antiviral intervention.

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Populärvetenskaplig sammanfattning

Virus tillhör ett av de största hoten mot vår samtid, inte minst har vi fått erfara detta av den pågående pandemin, som började våren 2020, orsakad av Coronaviruset SARS-CoV-2. Den här pandemin har visat oss vilken skada ett virus kan orsaka, både för folkhälsan men också för världsekonomin. Den har även visat det stora, desperata behov som uppstår världen över efter utveckling av medicin och vaccin mot ett snabbt spridande virus.

Coronavirus är bara ett av hundratals kända virus med kapacitet att ställa till stor skada i vårt samhälle. En annan grupp av virus med potential att spridas globalt och eventuellt ställa till liknande, eller värre, skada som SARS-CoV-2 är alphavirus. Alphavirus har samma typ av arvsmassa som Coronaviruset, bestående av positivt strängat RNA. Alphavirus sprids oftast via myggor vilket är fallet med Chikungunya och Semliki Forest Virus som det här arbetet fokuserar på. Precis som Coronavirus har Alphavirus stor potential att infektera både människor och djur och har orsakat flera allvarliga sjukdomar under de senaste åren. En av dessa sjukdomar orsakas av Chikungunyavirus och har fått stor spridning under 2000-talets början. Det finns ett stort behov av nya behandlingar mot Chikungunyavirus som fortsätter härja i främst Asien och Afrika.

Virus består av arvsmassa men saknar en egen ämnesomsättning och förmåga att föröka sig. För att infektera en organism och sprida sig vidare behöver viruset ta sig in i värdcellen och utnyttja dess ämnesomsättning och replikationsmaskineri. Virusets överlevnad hänger alltså på dess mekanismer att ta sig in i värdcellen för att kunna föröka sig där. Kunskap om virusets inträde och replikation i värdcellen kan därför ge ledtrådar till eventuella behandlingssätt. Om ett område av virusets genom visar sig vara nödvändigt för dess replikation kan det vara en lovande måltavla.

Vad gäller Alphavirus, har ett område i dess genom identifierats som extra intressant. Det här området kodar för fyra så kallade icke-strukturella proteiner. Att proteinerna är icke-strukturella innebär att de inte utgör uppbyggnad av virusets partikel, utan istället är inblandade i virusets replikationsmaskineri och därmed ansvariga för virusets spridning i en värdcell.

Alla fyra icke-strukturella proteiner verkar vara nödvändiga för att replikationen överhuvudtaget ska kunna ske. Det här betyder att om någon av dessa proteiner skulle bli inhiberade och därmed inte kunna utföra sina uppgifter kan virusets replikation utebli och virusets spridning i värdcellerna stoppas. I det här arbetet har tio olika antikroppar med affinitet till samtliga icke-strukturella proteiner producerats med förhoppningen att en bindning ska ske med en bromsande effekt på virusets replikation. Cellinjer som uttrycker antikropparna används för att undersöka om en bindning faktiskt bromsar replikationen. I ett sådant fall kan antikropparna peka ut svagheter hos viruset som kan vara användbara i ett potentiellt antiviralt medel.

Table of contents

Abbreviations

BHK-cells	Baby Hamster Kidney fibroblasts
CHIKV	Chikungunya virus
ELISA	Enzyme-linked immunosorbent assay
G3BP	GTPase-activating protein-binding protein
GTP	Guanosine triphosphate
HOS-cells	Human osteosarcoma cells
HRP	Horseradish peroxidase
IFN	Interferon
IP	Immunoprecipitation
MOI	Multiplicity of infection
nsP	Nonstructural protein
SDS-page	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SFV	Semliki Forest Virus

1. Introduction

The spreading of infectious viruses poses a large medical risk all over the world, as currently demonstrated by the SARS CoV-2 pandemic. Viruses are dependent on their host's gene expression machinery in order to replicate, regardless of the origin of the host. When infecting a cell and taking advantage of its functions, the virus is building new genetic material and structural proteins in order to create progeny viruses. The host cell is responding with a restriction of viral expression, imposing a counteraction between virus and host.

Alphaviruses are an example of viruses with potential to spread globally. The alphavirus Chikungunya is a mosquito-borne alphavirus that already has achieved a global spread during recent years, causing disease in millions of individuals. Many other members of the alphavirus family, including Mayaro, O'nyong-nyong, and Ross River viruses, have the potential to spread in a similar manner and therapeutic countermeasures are in demand.

There is a great need to enable the rationale design of therapeutic countermeasures against alphaviral infection, therefore it is critical to better understand the molecular events that occur in cells during infection. This project aims to investigate the interaction between virus and host by characterizing the effect of expressed nanobodies on Chikungunya and Semliki Forest virus replication. The nsP-specific nanobodies will help to better understand the function of the targeted nsPs and may identify vulnerabilities of the virus that lend themselves for pharmaceutical intervention. Cell lines that express the nsP-specific nanobodies in the cytosol under a doxycycline inducible promoter will be employed for plaque and infection assays with Chikungunya and Semliki Forest virus, to determine the antiviral potential of the new nanobodies.

2. Theory

In this section viral and nanobody characteristics are described.

2.1 RNA viruses

RNA viruses are a group of viruses with a genome of ribonucleic acid (RNA). Generally, RNA viruses have a high mutation rate in comparison to DNA viruses and pathogens from other species. There are about 2-3 new human diseases caused by RNA viruses each year (Jácome *et al.*, 2017). The fast mutation rate makes RNA viruses complicated to treat since a possible treatment has to manage with the fast adaptations the RNA viruses are capable of in a short time (Jácome *et al.*, 2017). Studies of possible treatments of RNA viruses are therefore in great need.

RNA viruses are further divided into negative and positive-sense RNA viruses. Negative-sense (3' to 5') RNA viruses have negative-sense single-stranded RNA as its genetic material. Negative viral RNA is complementary to mRNA and is therefore dependent on polymerase to convert to positive sense RNA that can act as viral mRNA and proceed to translation into proteins for further viral production. RNA from a negative-sense RNA-virus is not by itself infectious since it is dependent on polymerase to translate into viral proteins. In contrast, positive-sense (5' to 3') RNA can function as mRNA and allows direct translation into the desired viral proteins (King *et al.*, 2014).

2.2 Viral characteristics of alphaviruses

Alphaviruses are part of the family *Togaviridae,* which is a group of enveloped, positive-sense, single stranded RNA viruses (Bissoyi *et al*, 2017). They are small and spherical with a transmission, for the most part, through a mosquito vector. This group of viruses often cause encephalitis, rash, and arthritis and have caused emergence events in various places in the world (Korsman, 2012). Chikungunya virus cause some of the alphaviruses' more severe illnesses, that are spread by *Aedes* mosquitos primarily in Africa and Asia (Korsman, 2012).

The genome of alphaviruses is divided into two open reading frames: encoding nonstructural proteins and structural proteins. A structural protein is a protein that build the virion structure. The structural protein C compose the protein capsid shell that is covered by glycoprotein spikes made up by the structural proteins E1 and E2 (Figure 1). The alphavirus genome encode capsid proteins that form the nucleocapsid core together with replicated RNA.

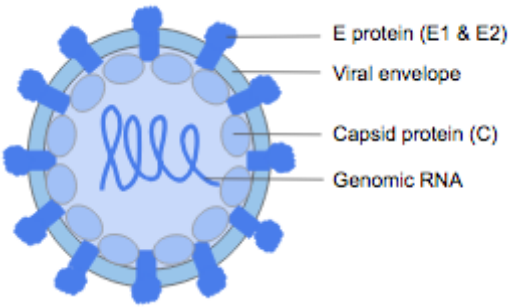

Figure 1. Alphavirus particle structure. *The composition of the alphavirus particle consisting of glycoprotein spikes (composed by the structural proteins E1 and E2), capsid protein (composed by the structural protein C), viral envelope and genomic RNA.*

Entry in host cells is crucial for the virus. The entry requires attachment to the host cell, which is mediated by interactions between the virion and the host cell membrane. When entered in the infected cell the alphavirus particles undergoes disassembly and release of the genomic RNA into the cytoplasm (Sharma & Gupta, 2017). The replication takes place in modified membrane structures called spherules. The membranous structures keep the newly synthesized RNA protected from the host innate immune system (Gottipatti *et al.*, 2020).

2.3 The non-structures of alphaviruses

Alphaviruses have an about 11.6 kb long genome that encodes four nonstructural proteins (nsP1-nsP4), as well as the three structural proteins (Chattopadhyay, 2014; Figure 2). A nonstructural protein is a protein that is encoded by the virus genome but is not part of the virus particle. Instead, the four nonstructural proteins have proved to be fundamental components of the viral replication. The nonstructural proteins are produced as a single polyprotein that is cleaved by the nsP2 in order to perform their respective tasks (Bissoyi *et al*, 2017). When cleaved into four separate proteins, the nonstructural proteins conduct the viral replication machinery. The alphavirus synthesis depends on all four nonstructural proteins (Chattopadhyay, 2014).

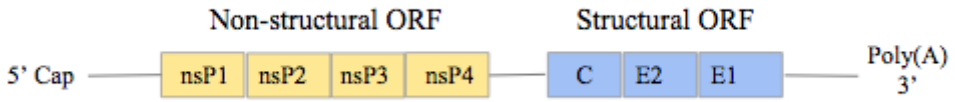

Figure 2. Alphavirus genome. *The alphavirus genome includes a nonstructural open reading frame (ORF), which encodes the nonstructural proteins where the nanobodies are targeted. In a second ORF, the structural proteins of the virus are encoded.*

2.3.1 nsP1, the alphaviral anchor to host cell membranes

nsP1 is a peripheral membrane protein that is responsible for locating and attaching the viral replication complex to the site of replication.

In mammalian cells the alphavirus replication complexes are attached to the plasma membrane, which is possible through the interaction between nsP1 and the lipid bilayers in the host membrane. A disruption of the alphavirus nsP1 binding of membranes has led to diminished replication in host cells in previous experiments, indicating that the nsP1 activity is crucial for the viral growth (Gottipatti *et al.*, 2020).

The nsP1 consists of methyltransferase (MTase) and guanylyltransferase (GTase), whose activities make capping of the newly synthesized RNA possible (Rupp, 2015). The capping initiates by nsP1 methylating GTP and transferring it to the 5' end of the viral RNA to form the cap (Gottipatti *et al.*, 2020). It has further shown to contribute to the downregulation of bone marrow stromal antigen-2 which is expressed from type 1 interferon (IFN) gamma. With all these important functions in the replication machinery and host cell undermination nsP1 is a promising target for antiviral medications.

2.3.2 nsP2, an important suppressor of the host antiviral responses

nsP2 is a protease needed to process the nonstructural polyprotein by cleaving it into four separate proteins (nsP1-nsp4). nsP2 also assists the capping of the nascent RNA with its NTPase and RNA helicase activities (Fros & Pijlman, 2016).

Further nsP2 is an important suppressor of host antiviral pathways (Fros & Pijlman, 2016). For example, it induces degradation of RNA polymerase II, resulting in transcriptional shut-off and cytopathic effects which reduces IFN expression. It also blocks the IFN-induced janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Fros & Pijlman, 2016).

2.3.3 nsP3, the mysterious function of the crucial replication factor

It has been shown that nsP3 is crucial for alphavirus replication, but its specific role in the replication machinery is not clearly understood. It is known that nsP3, like nsP2, is inhibiting the host antiviral pathways, such as the host cell induction of stress granules which instead use the GTPase-activating protein-binding protein (G3BP) to create viral granules that favors viral replication (Fros & Pijlman, 2016). It is also known that nsP3 is highly phosphorylated, which indicates some important enzymatic function.

2.3.4 nsp4, the catalytic core of the alphaviral replication

nsP4 is the major catalytic core for the alphavirus replication complex. It is the first nsP to be cleaved for the polyprotein. When proteolytically cleaved from the polyprotein, nsP4 functions as a RNA-dependent RNA polymerase and produces short-lived replication complexes that produces the negative sense RNA complementary to the alphaviral positive sense genomic RNA. The N-terminal of nsP4 is highly conserved and includes a catalytic triad (Fros & Pijlman, 2016).

2.4 Nanobodies

Antibodies are expressed in all vertebrates' immune systems. They come in different shapes and high diversity, it is thought that there is a specific antibody for every compound existing in the world (Vanlandschoot *et al.*, 2011). Their specificity makes antibodies attractive tools for biotechnology applications. Antibodies usually consists of two heavy chains and two light chains, where the heavy chain is divided into one variable region and three constant regions (Figure 2A).

In *Camelidae* a previously unknown antibody was discovered in 1989 consisting of solely the variable heavy-chain region, completely lacking the light chain (Figure 3; Weiss & Verrips, 2019). These heavy chain-only antibodies got known under the name nanobodies and rapidly became a tool for diagnostics, vaccine design and immunotherapy (Weiss & Verrips, 2019).

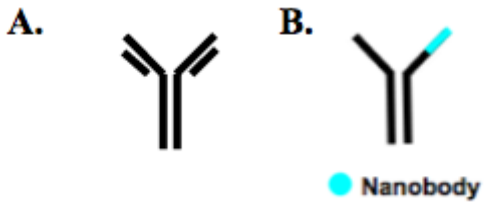

Figure 3. A) Antibody. *The conventional antibody consists of two light chains and two heavy chains. The heavy chain is composed of one variable region and three constant regions.* ***B.) Heavy chain only antibody.*** *In addition to conventional antibody, camelids have heavy chain only antibodies that lack the light chain. The nanobody constitutes only the smallest fraction of the heavy-chain, which is the variable heavy chain region.*

For instance, nanobodies are widely used in virus research. In comparison with conventional antibodies with a molecular weight of ~ 160 kDa nanobodies are much smaller, ~ 15 kDa, and, hence, have a different range of epitopes (Harmsen & De Haard, 2007). The small size enables for instance fitting into small canyons of the viral surface. Nanobodies targeting human immunodeficiency virus (HIV) have proved to fit into canyons in the HIV envelope, that are too small for larger antibodies, and thereby block important viral functions (Weiss & Verrips, 2019). Nanobodies are also, owing to their small size, much more stable than conventional antibodies (Liu *et al.,* 2019).

To obtain the recombinant single variable domains *Camelids* are immunized via injections of the desired protein. This is followed by generation of nanobody libraries the from peripheral blood lymphocytes RNA, and selection by phage display (Muyldermans, 2013). The nanobodies are then easily expressed in large quantity in microorganisms such as bacteria and yeast. A his-tag enables purification by immobilized metal affinity chromatography (IMAC) and gel filtration (Liu *et al.,* 2019).

2.5 Sortase A reaction exchange labels conjugated to the nanobodies

Sortase is a group of membranous prokaryotic transpeptidases that modify and sort proteins on its cell wall. *Staphylococcus aureus,* uses a sortase-recognition motif to perform this task, consisting of Leu-Pro-Xxx-Thr-Gly (LPXTG), where X could be any amino acid (Guimaraes, 2013).

Sortase A cleaves between the threonine and glycine residues in the LPXTG motif to form an acyl-enzyme intermediate. Cysteine in Sortase A reacts with carbonyl of the threonine residue to form an amide bond. In the presence of a nucleophile a nucleophilic attack occurs from the free amino group of the nucleophile. This leads to a breaking of the bond between sortase A and the threonine residue and the formation of a new bond between threonine and the incoming nucleophile. Incubation of sortase, a protein with a LPXTG motif and a nucleophile will therefore lead to an attachment of the nucleophile to the protein of interest (Guimaraes, 2013).

This cell wall sorting reaction is utilized as a post-translational modification of the N or C-termini with an appended label. In this case, the Sortase A reaction is used to replace the nanobody conjugated his-tag for a GGGK-biotin-label in order to use the nanobody in streptavidin-based techniques. GGGK (gly-gly-gly-lys) is a linker to prevent the biotin label from affecting the nanobody activity (Guimaraes, 2013).

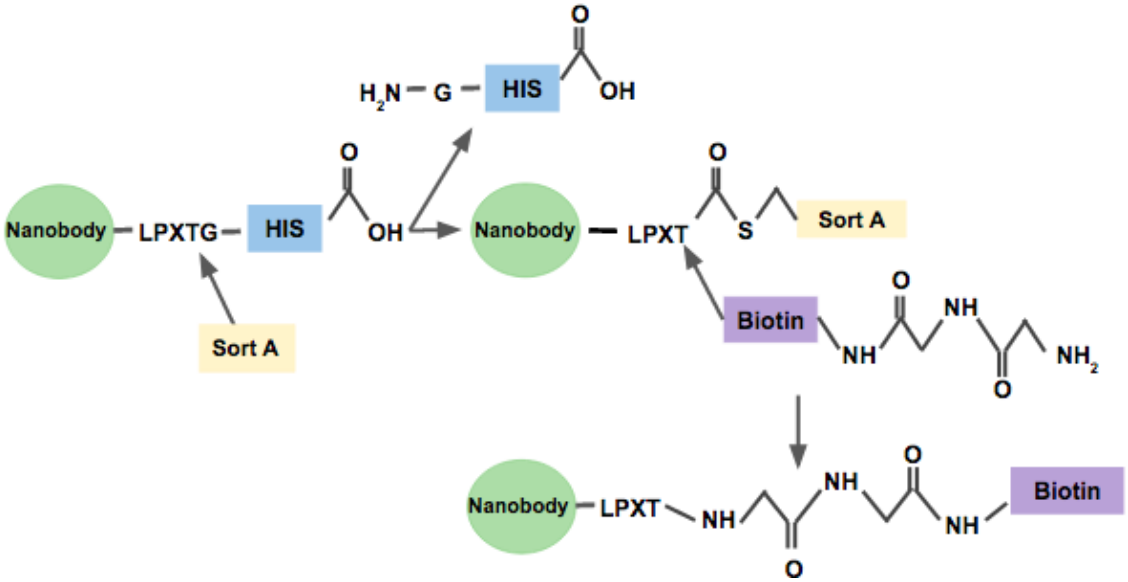

Figure 4. Sortase A reaction. *The exchange of a his-tag to a GGGK-biotin label with the assisting compound Sortase A. This happens through a series of events when incubation occur with a protein of interest conjugated to a LPXTG motif, sortase A and a nucleophile. In this case the protein of interest is a nanobody and the nucleophile is biotin.*

2.6 Plaque assay, a way of quantifying viral titers

Measuring the viral quantity is possible with a plaque assay. The method utilizes the fact that alphaviral infections of mammalian cells results in cell lysis. This means that when infecting mammalian cells with Chikungunya or Semliki Forest Virus infected cells will lyse and leave an area of destroyed cells that are distinguishable from the uninfected cells. Agarose gel are fixing the cells in a sample allowing the virus to only spread to, and lyse, adjacent cells, creating defined areas of infections called plaque forming units (PFU) surrounded by uninfected cells (Kaur *et al.*, 2016).

Plaque assays are performed using a dilution series of the virus stock, since the virus otherwise can grow to too high concentrations in order to distinguish separate plaques. By using samples of different solutions of the virus stock, hopefully at least one sample will contain a dilution of the virus that yields distinct, countable plaques. After counting the plaques, the viral titers can be calculated by multiplying the number of plaques with the dilution factor per milliliter. When investigating a potential antiviral treatment knowledge of the viral titers is important, since they can tell the levels of infectivity in a sample. In this case samples containing infected cells will be compared to samples containing infected cells and the nsP-specific nanobodies, to reveal if the nanobodies have an antiviral effect and, thus, lead to a decreased production of infectious particles (Kaur *et al.*, 2016).

3. Material & Method

Characterization of the nsP-specific nanobodies was performed first by using biochemical techniques to confirm the binding of the nanobodies to the alphavirus non-structural proteins, and later by assessing viral replication efficiency in the presence by means of infection and plaque assays.

3.1 Producing nanobodies

Eleven different nanobodies were produced in this project, 10 of them targeting towards a certain nonstructural protein of Chikungunya and Semliki Forest Virus. The nanobodies were named after the sequences of the nonstructural proteins they were targeted towards. A negative control nanobody was also produced, which targets Influenza A nucleoprotein NP.

Table 1. Ten nanobodies produced to bind to the different nonstructural proteins of Chikungunya and Semliki Forest Virus and one nanobody targeting influenza, produced as a negative control.

Influenza	nsP1	nsP2	nsP3	nsP4
52	B4	C2	D3	A3
	F2	E12		A12
		H10		D2
				D8

3.1.1 Transformation of E.coli with heat shock

Escherichia Coli (*E.Coli)* BL21-D1 cells were used for transformation with a pHen plasmid containing a gene encoding the desired nanobody. The cells were thawed and mixed with 2 µL of the plasmid. The mix were flicked to gently mix the content and placed on ice for 2 minutes. Upon this, the mix was heat shocked in 42 °C for exactly 30 seconds. The transformed cells were then put on ice again for 2 minutes and transferred to 50 mL TB + 1:1000 Ampicillin. The starter culture was put on a shaker at 37 °C overnight.

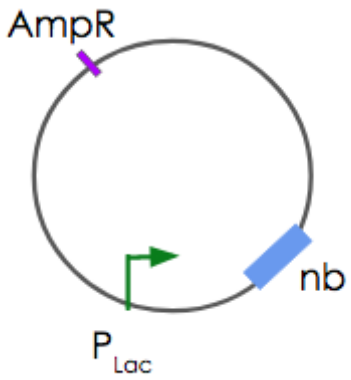

Figure 5. Plasmid for nanobody expression. *The plasmid contains nb, a gene encoding nanobodies targeting a certain nonstructural protein of Chikungunya or Semliki Forest Virus. It also carries P_{Lac}, a Lac-promoter inducible with IPTG and AmpR, a gene encoding Ampicillin resistance.*

The next day 25 mL of the starter culture were transferred to 1 L of TB + 1:1000 Ampicillin. The mix was left shaking in 37 °C until OD was ~0.8. Then 1 mL IPTG was added to the culture to induce protein expression. The cells were left for expression in 30 °C overnight.

The following day the culture was centrifuged at 6000 rpm for 10 minutes. The supernatant was removed and the pellet, containing the protein, was resuspended in 10 mL 1x TES buffer. It was left rotating at 4 °C for 2 hours and was further diluted in 0,25x TES buffer and left rotating overnight.

3.1.2 Purification of nanobodies

The next day the newly produced nanobodies were centrifuged at 18000 rpm for 20 minutes. The supernatant was then transferred to a new tube and loaded onto an equilibrated Ni-NTA column. The column was washed with 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM imidazole. To elute the protein 50 mM Tris pH 7.5, 150 mM NaCl, 250 mM imidazole was added to the column. The eluted nanobody was at last purified by size-exclusion. A chromatogram from a size-exclusion run in the ÄKTA system can be found in Supplementary S2.

3.2 Sortase A reaction to label nanobodies

The newly produced nanobodies have a C-terminal his-tag, that allows purification on the nickel beads. When proceeding with the nanobodies it was desired to exchange the his-tag for a biotin label. A biotin label has affinity for streptavidin and will therefore bind to the magnetic beads containing streptavidin when performing an immunoprecipitation (IP) in order to create nanobody - virus complexes.

To exchange the his-tag conjugated to the nanobody for a GGGK-biotin label 10 mM CaCl2, 200 µM biotin, 10 µM Sortase A, 30 - 100 µM nanobody and 10% TBS-T were mixed. The samples were then incubated in 25 °C for 2 hours. To remove the sortase and unreacted nanobody, the samples were purified on nickel NTA beads. The purified samples were concentrated using 3000 kDa concentrating tubes.

3.3 Infection assay to produce virus lysate for immunoprecipitation

Baby Hamster Kidney fibroblasts (BHK cells) were used for infection. The cells were split regularly according to the cell culture protocol in Supplementary S1. A T75 flask confluent with BHK cells was washed with PBS(-). 1 mL trypsin 5% was added and incubated for 5 minutes at 37 °C to detach the cells from the flask. The cells were then resuspended with 9 mL GMEM and mixed thoroughly. 9.5 mL of the resuspended cells was removed from the flask and added to a T300 flask with 35 mL GMEM. The remaining 0.5 mL in the T75 was mixed with 10 mL GMEM. Both flasks were incubated overnight at 37 °C.

The following day a virus inoculum was prepared by mixing viral cells, according to Table 2, and infection media (DMEM, 0.2 % BSA, 10 mL HEPES). Before adding the virus inoculum the cell media was removed and the cells were washed in PBS(+). Thereafter the virus inoculum was added and incubated for 1 hour in 37 °C. The virus inoculum was then removed and 40 mL GMEM was added. The cells were incubated for ~8 hours.

Table 2. Virus inoculum for Chikungunya virus lysate production.

MOI	10
Number of cells	3.4×10^7 cells
Viral titer	1.25×10^{10} pfu/mL
Virus inoculum (µL)	$\frac{Number\ of\ cells\ \times\ MOI}{Viral\ titer} \times 1000$
Infection media (mL)	$20\ mL\ -\ Virus\ inoculum$

The next day the media was removed and the cells were washed in PBS(-). 1 mL 10% trypsin was added and incubated for 5 minutes at 37 °C to detach the cells. The cells were then resuspended in 10 mL GMEM media and transferred to a 50 mL tube. Thereafter they were centrifuged for 5 minutes in 4 °C. The supernatant was discarded and the cells were washed in 50 mL PBS(-). Upon this the cells were resuspended in 4 mL lysis buffer. The cells were divided in Eppendorf tubes and sonicated for 15 minutes on ice, followed by 20 min incubation on rotation at 4 °C. The cells were centrifuged at 10000 rpm for 10 minutes at 4 °C. The supernatant was transferred to new tubes and stored at -20°C.

3.4 Determining nanobody specificity

Nanobody specificity was determined with biochemical analysis including immunoprecipitations, western blot and ELISA. The techniques were performed using the produced nanobodies and viral nonstructural proteins, either purified from Semliki Forest virus or from Chikungunya lysate.

3.4.1 Immunoprecipitation to determine nanobody specificity

An immunoprecipitation was performed to confirm the target and specificity of the nanobodies. 20 µL M280 streptavidin beads per sample were washed in TBST 2 times and diluted in TBST to a final volume of 500 µL. 5-10 µg nanobody was added and left on rotation for 10 minutes. The samples were washed 1 time with TBST. Upon this 100-300 µL virus lysate or 10 µg purified viral protein was added and left on rotation for 2 hours in room temperature, alternatively in 4 °C overnight. The samples were washed 3 times with TBST and eluted in 40 µL 0,2 M glycine buffer pH 2.2. To enable SDS-page the samples had 10 µL LDS sample buffer, 8 µL 1 M Tris pH 9.1 and 2 µL DTT added to them and was boiled for 5 minutes.

3.4.2 Western blot to detect immunoprecipitated proteins

The samples were separated by SDS-page at 150 v, 400 A for 1 hour. A transfer apparatus was used to transfer the gel to a polyvinylidene difluoride membrane according to the manufacturer's protocols (BioRad, 2020). It was incubated for 1 hour and then blocked with 5% skim milk in TBST (10 mM Tris, ph 8.0, 150 mM NaCl, 0.5% Tween 20) for 1 hour. The membrane was then incubated for 1 hour with rabbit primary antibody against the nsP of the desired nanobody: nsP1 (1:2000), nsP2 (1:1000), nsP3 (1:2000), nsP4 (1:2000). After incubation the membrane was washed 3 times in TBST for 5 minutes, followed by another incubation of the membrane with the secondary antibody, a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit antibody for 1 hour. The membrane was then washed 3 times in TBST for 5 minutes and developed by adding luminol.

3.4.3 ELISA to verify nanobody specificity

The wells of the plates were coated with 100 µL antigen diluted in TBST in 4 °C overnight. 8 different antigens were used: Uninfected 'mock' lysate, Chikungunya lysate lysed with cristea buffer in small volume 20 µL, Chikungunya lysate lysed with cristea buffer in large volume 200 µL, Chikungunya lysate lysed with EE buffer in large volume 200 µL, purified nsP1 protein from Semliki Forest Virus, purified nsP2 protein from Chikungunya virus, purified nsP3 protein from Chikungunya virus and purified nsP4 protein from Chikungunya virus.

The next day the wells were blocked with 200 µL 5% skim milk in TBST in room temperature. They were then washed 3 times in TBST and 100 µL primary antibody, the nanobodies, were added. The samples were incubated for 1 hour in room temperature. After the incubation the plate was washed in TBST, 3 times and 100 µL secondary antibody, Streptavidin conjugated to HRP, was added in a 1:5000 dilution. The plate was then washed 3 times in TBST. Detection was performed by adding 50 µL chromogenic substrate (TMB) to visualize the reactions. The reactions were stopped with 100 µL H_2SO_4 and the absorbance at 450 nm was measured with an ELISA plate reader.

3.5 Infection assay of HOS-cells with Semliki Forest Virus

Human osteosarcoma cells (HOS-cells) were used for infection. A T75 flask confluent with HOS-cells expressing nsP1-F2 was washed with PBS(-). 1 mL 5 % trypsin was added and the cells were incubated for 5 minutes in 37 °C. The cells were then resuspended in 9 mL DMEM and transferred to a 15 mL tube. 20 µL of the cell suspension was collected and mixed with 20 µL trypan blue. 10 µL of the mix was added to a counting slide. The cells were then counted with trypan blue staining in a cell counting chamber. 0,3 x 10^6 cells/well was seeded for 7 wells (2,1 x 10^6 cells in 14 mL DMEM). The cells were divided into two tubes, 7 mL/tube. One of the tubes were supplemented with doxycycline in a 1:1000 dilution. 2 mL of the cell suspension was added to each well and the cells were incubated at 37 °C overnight.

The next day virus inoculum was prepared according to Table 3. The cell media was removed and the cells were washed in PBS(+). The virus inoculum was then added to the wells and the cells were incubated for 1 h at 37 °C. 200 µL of supernatant was collected and stored at -80 °C. The remaining supernatant was removed and the cells were washed in ice-cold PBS(+). 300 µL Cristea lysis buffer was added and the cells were incubated for 10 minutes. Cells were scraped, collected in an Eppendorf tube and sonicated for 10 minutes. They were then incubated at 4 °C with rotation for 20 minutes. Samples were centrifuged 10 minutes at 10000 rpm at 4 °C. Supernatant was transferred to a new tube. 50 µL of cleared supernatant was added to a tube with 12,5 µL of LDS sample buffer. Samples were boiled for 10 minutes at 95 °C. Both samples were stored at -20 °C and analyzed with western blot using four different primary antibodies: Ms α GAPDH (1:1000), Rb α SFV-nsP1 (1:3000), Rb α SFV-nsP3 (1:3000) and Ms α HA (1:500).

Table 3. Virus inoculum for infection assay.

MOI	10
Number of cells	1.2×10^6 cells
Viral titer	2.7×10^9 pfu/mL
Virus inoculum (µL)	$\frac{Number\ of\ cells\ \times\ MOI}{Viral\ titer} \times 1000$
Infection media (mL)	$750\ \mu L/well\ -\ Virus\ inoculum$

3.6 Plaque assay to measure viral titers

BHK cell lines that express nsP-specific nanobodies under a doxycycline induced promoter were used. The cell media in a T75 flask with BHK cells was removed and washed in PBS (-). 1 mL trypsin was added and incubated at 37 °C for 5 minutes. The cells were then resuspended in 9 mL cell media and transferred to a 15 mL tube. 20 µL of the cell suspension was collected and mixed with 20 µL trypan blue. 10 µL of the mix was added to a counting slide and counted with trypan blue staining in a cell counting chamber. The volume of cells needed was calculated. Cells were seeded on 2 plates with 12-wells and incubated overnight at 37 °C.

A dilution series was prepared. Cell media was removed, cells were washed in PBS (+) and 200 µL virus inoculum was added for infection. The samples were arranged as described in Table 4. The infected cells were incubated at 37 °C and were shaken every 15 minutes. An agarose overlay was prepared by boiling agarose in plaque assay media. When it had cooled down DMEM was added and mixed for immediate use or stored in a water bath at 42 °C. The virus inoculum was removed and washed in PBS (+). 1 mL agarose media was added per well and the cells were left for incubation for 36 hours at 37 °C.

The cells were fixed in 10% formaldehyde overnight at RT. The agarose was removed and cells were washed in running water. 1 mL of crystal violet was added and incubated for 10 minutes at RT. The crystal violet was removed and samples were washed in running water. The samples were then air dried, plaques were counted and titer calculated.

Table 4. Arrangement of samples on the 12-well plate for the plaque assay.

SFV 10^{-1}	SFV 10^{-4}	SFV + dox 10^{-1}	SFV + dox 10^{-4}
SFV 10^{-2}	SFV 10^{-5}	SFV + dox 10^{-2}	SFV + dox 10^{-5}
SFV 10^{-3}	SFV 10^{-6}	SFV + dox 10^{-3}	SFV + dox 10^{-6}

4. Results

This section describes the most important results in chronological order. From producing virus lysate and labelling nanobodies to determining nanobody specificity and their possible effect on viral replication.

4.1 Sortase A labelling of nanobodies

To verify correct labelling of nanobodies with biotin in the sortase reaction, the proteins were analyzed with SDS-page, coomassie staining and western blot. This allowed to visualize the process of removing the his-tag and adding the GGGK-biotin label (Figure 6). The control was made for 2 of the 11 produced nanobodies: nsP1-F2 and the control nanobody 52 targeting influenza A nucleoprotein NP. Three samples of each nanobody were taken at different timepoints: **unreacted**, when the sample only contained a his-tagged nanobody, **intermediate**, when the sortase A reaction had occurred but no purification had been made and **pure**, when the biotinylated nanobody had been purified.

The coomassie in Figure 6A. shows that the sample with the unreacted nanobody only contained the nanobody ~15 kDa. In the intermediate sample, where Sortase A has been added, both the nanobody and the sortase A are visible. When the nanobody was purified and biotinylated there is a small shift compared to the unreacted nanobody (Figure 6A). The shift occurs since GGGK-biotin has been covalently attached to the nanobody while the His-tag has been removed.

Figure 6B. shows the result of a western blot screening for biotin. The biotin should be present in the intermediate sample and in the pure product when the nanobody is biotinylated. It should not be present in the unreacted sample. This corresponds to the achieved result. See Figure 6B.

In Figure 6C. the western blot screened for the his-tag which should occur in the unreacted sample, where the nanobody still was carrying a his-tag. It should also be present in the intermediate sample, which was confirmed. See Figure 6C.

The control of the sortase A reaction indicated that the labelling of the nanobodies F2 and 52 worked. Based on these results, we assumed that the biotin labelling also worked for the other 9 nanobodies.

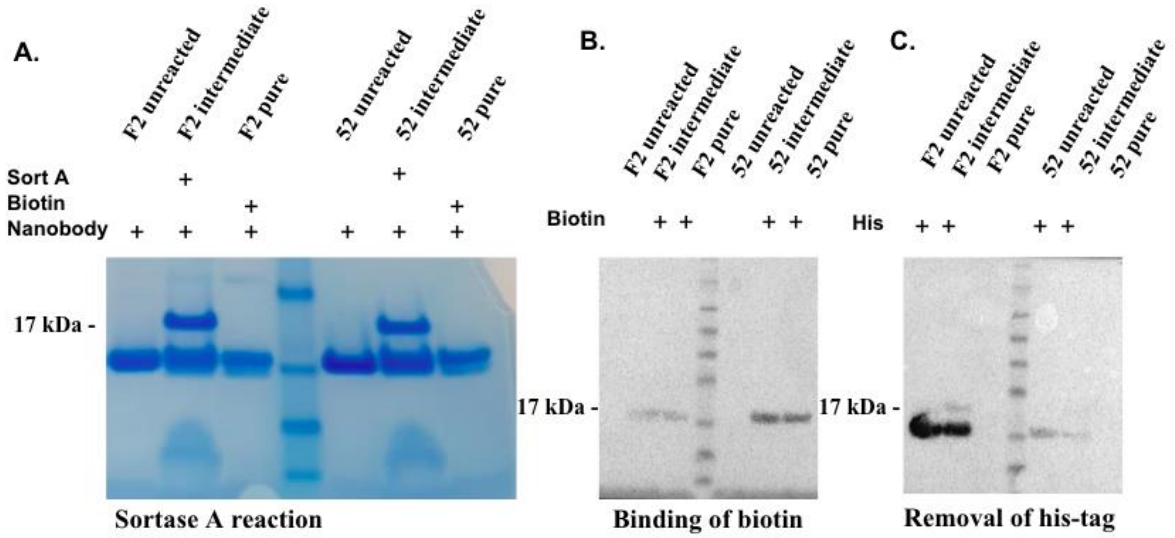

Figure 6. Nanobody biotinylation using Sortase A. *A control to make sure that the sortase A reaction had occurred. This control was made with 2 of the 11 produced nanobodies: nsP1-F2 and 52 in 3 states, unreacted, intermediate (where sortase A was added) and pure (where the reaction was finished and the nanobody was biotinylated).* ***A)*** *In the unreacted sample only the nanobody is present in the sample, visible at ~ 15 kDa. In the intermediate sample the nanobody is present at ~ 15 kDa and sortase A with a weight of ~21 kDa is added. In the pure sample there is a small shift indicating a loss of His-tag and the addition of a GGGK-biotin label to the nanobody.* ***B) Binding of biotin.*** *The biotin is visible in the intermediate and pure sample.* ***C) Removal of his-tag.*** *The his-tag is present and visible in the unreacted sample and the intermediate sample.*

4.2 Determining nanobody specificity

The nanobody specificity was determined with biochemical methods including ELISA, immunoprecipitations and western blots.

4.2.1 Determining nanobody specificity by ELISA

The antigens based on chikungunya lysate had weak signals for nanobody binding to the antigens, indicating that no binding had occurred between the nanobodies and the nsPs in the Chikungunya virus lysates, see Supplementary S3. The ELISA indicated binding between the purified nsP1, of Semliki Forest Virus, and nsP1-F2 and nsP1-B4. The nsP2-targeting nanobodies indicated binding between nsP2-E12 and nsP2-H10 and nsP2 with weak signals.

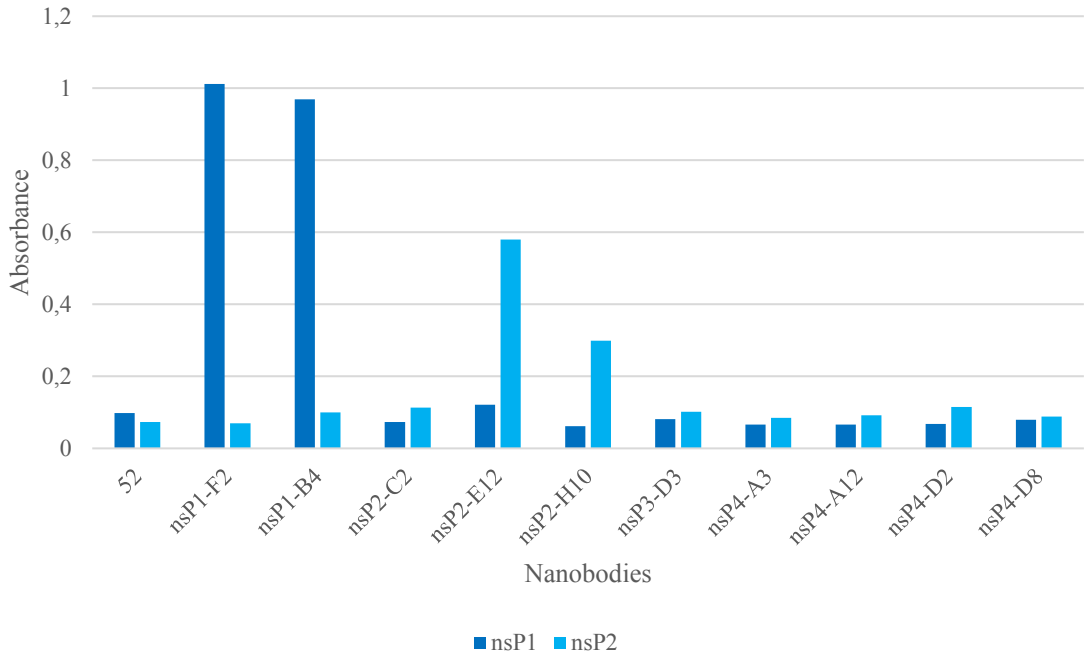

Figure 7. ELISA, *confirming nanobody specificities to viral proteins. This confirmed binding of nsP1-F2 and nsP1-B4 to Semliki Forest Virus. It also indicated a binding between nsP1-F2 and nsP1-B4 to nsP1 of Semliki Forest Virus. nsP2-E12 and H10 in contrast, specifically recognizes CHIKV nsP2.*

4.2.2 Determining nanobody specificities by immunoprecipitations

Immunoprecipitations were performed to determine nanobody specificity. Western blot and/or coomassie staining were used for detection. Chikungunya virus lysate was used as a positive control, while influenza NP-targeting nanobody 52 and/or G3BP-targeting nanobody G1D7 were used as negative controls.

The viral protein nsP1 of Chikungunya was immunoprecipitated with nsP1-targeting nanobodies in Figure 8. A clear band for nsP1-F2 appears, indicating binding. For nsP1-B4 there is no band, indicating no binding to nsP1 of Chikungunya.

Immunoprecipitations of nsP2-targeting nanobodies and Chikungunya nsP2 shows binding between nsP2-C2 and nsP2 (Figure 9). There are no bands for nsP2-E12 and nsP2-H10, indicating no binding to nsP2 of Chikungunya.

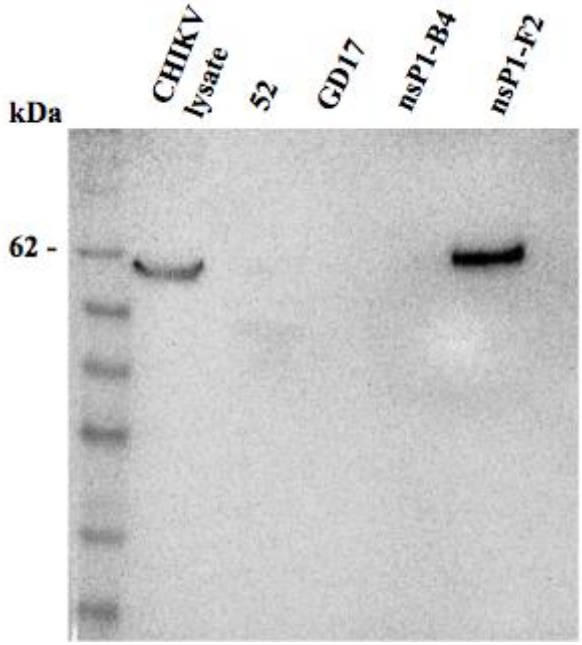

Figure 8. Immunoprecipitation of chikungunya virus nsP1 from infected cells using nanobodies. *Biotinylated nanobodies were immobilized on streptavidin beads and incubated with lysate from chikungunya virus infected cells. Bound protein was detected by western blot using nsP1-specific antibodies. The binding between nsP1-F2 and the virus is shown with a clear band, while there is no band indicating binding for nsP1-B4.*

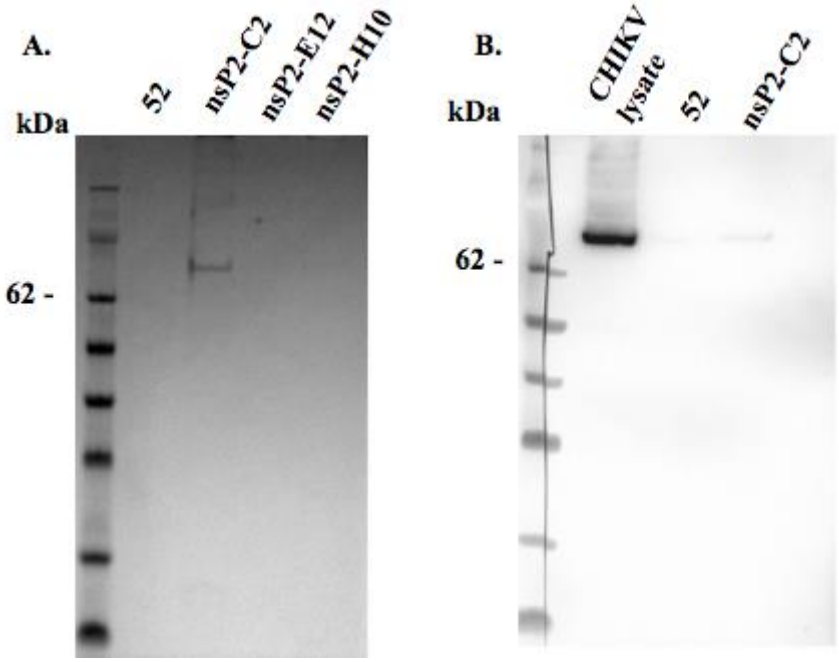

Figure 9. Immunoprecipitation of nsP2 from infected cell lysate and nsP2-nanobodies. *Biotinylated nanobodies were immobilized on streptavidin beads and incubated with lysate from chikungunya virus infected cells. Bound protein was detected by coomassie staining and western blot using nsP2-specific antibodies.* ***A) Coomassie staining,*** *with a strong band for nsP2-C2 at 62 kDa and no bands for nsP2-E2 and nsP2-H10.* ***B) Western blot,*** *indicating binding for nsP2-C2 with a weak band.*

4.3 Screening for ideal conditions to immunoprecipitate alphavirus nsP1

The outcome of an immunoprecipitation depends on several factors such as binding kinetics, the amount of beads, amount of nanobodies, amount of lysate, time for binding, the temperature during binding and the lysis buffer used to lyse the infected cells. Some factors have more influence on an IP than others. One factor that proved to affect the outcome heavily was the type of lysis buffer to produce the virus lysate.

4.3.1 The importance of lysis buffer composition for nsP1 recognition by the nanobodies

We compared three different kinds of lysate buffers, concluded in Table 5. When changing lysis buffer from cristea buffer to EE buffer the results of the IP started to differ. When performing an IP with viral proteins from virus lysate lysed with cristea buffer a distinct band for nsP1-F2 is detected, indicating binding between nsP1-F2 and nsP1 of Chikungunya virus (Figure 10A). When repeating the experiment under the same conditions but using another lysate, lysed with EE buffer, no band appears indicating no binding between nsP1-F2 and nsP1 of Chikungunya (Figure 10B). The control with the pure lysate yields a clear band, indicating a functional lysate and blot.

Table 5. Lysis buffers used in the experiment.

Lysis buffer	Date of production	Components
Cristea buffer	191129	500 mM NaCl, potassium acetate, magnesium chloride, tween-20, triton X-100, sodium deoxycholate
EE buffer	200206	150 mM NaCl
EE buffer	191104	150 mM NaCl

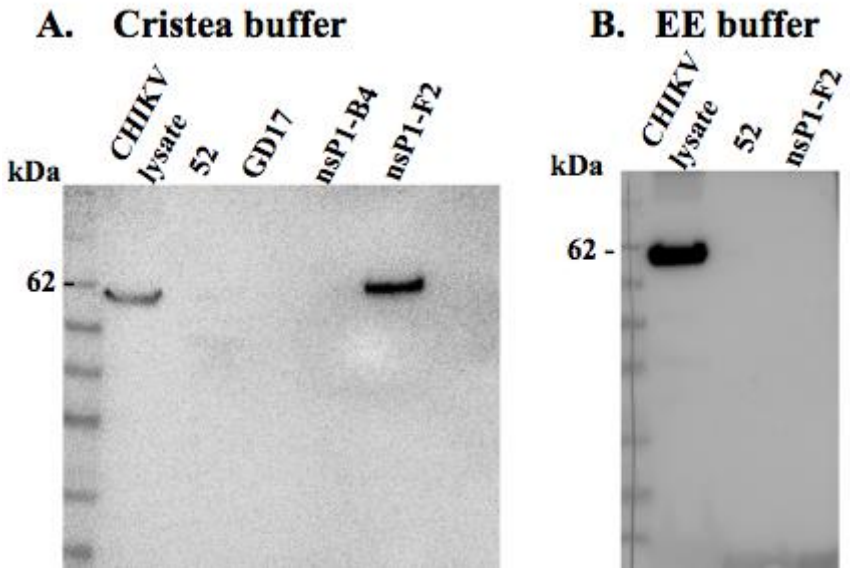

Figure 10. Immunoprecipitation of CHIKV nsP1 from differentially lysed cells. *Nanobodies were immobilized on streptavidin beads and incubated with virus lysate generated with two different lysis buffers.* ***A) Cristea buffer****, results in a clear band indicating successful binding between nsP1 of Chikungunya virus and nsP1-F2.* ***B) EE buffer,*** *with the exact same conditions except from the lysis buffer used to lyse the BHK-cells infected with Chikungunya virus. No band is visible for nsP1-F2 but with a clear band for the pure lysate, indicating a functional lysate. This implies that something is lacking in the EE buffer to make the IP possible.*

When a deviation between the lysis buffers was detected an experiment to compare the three available lysates in Table 5 was initiated. We incubated nsP1-F2 with virus lysates generated from the three different lysis buffers. nsP1-F2 was used as a positive control since we previously confirmed its specificity to Chikungunya virus nsP1 (Figure 8; Figure 10). The IP:ed proteins were detected by western blot. Figure 11 shows a clear band for the lysate lysed with cristea buffer and no band for the lysates lysed with EE buffer, confirming the results in Figure 10.

To test this hypothesis, we added cristea buffer to a lysate originally prepared with EE buffer. With this trick we could rescue binding of nsP1-F2 to nsP1 and confirm that components in the cristea buffer are necessary for this binding (Figure 11).

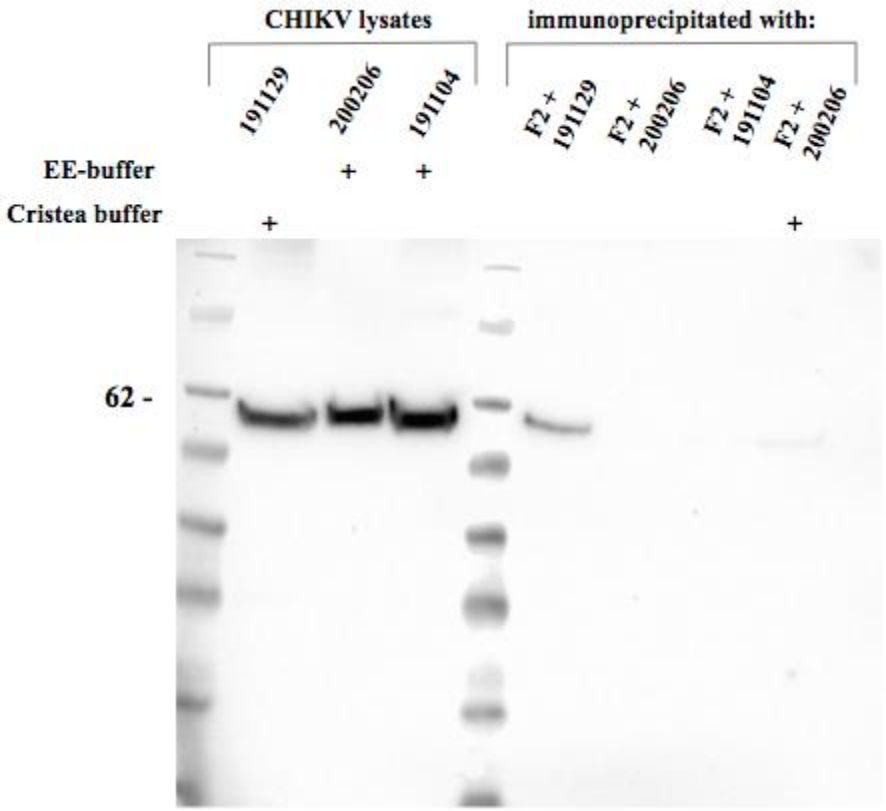

Figure 11. Immunoprecipitation with nsP1-F2 specific nanobody and nsP1 of Chikungunya from 3 virus lysates lysed with different lysis buffers. *The Chikungunya virus lysate from 191129 was lysed with cristea buffer while the lysates from 200206 and 191104 was lysed with EE buffer. nsP1-F2 was incubated on streptavidin beads with the different lysates. There is a clear band for nsP1-F2 that was immunoprecipitated with the nsP1 of Chikungunya virus, lysed with cristea buffer from 191129. For the lysates lysed with EE buffer there is no band at all for the immunoprecipitation with nsP1-F2. When adding cristea buffer to the lysate lysed with EE buffer there is a weak band appearing for the IP with nsP1-F2. This indicated that cristea buffer was needed to make an immunoprecipitation detectable.*

4.3.2 Determining the ideal immunoprecipitation conditions

Other factors that could influence an immunoprecipitation are the amount of beads, nanobody and lysate. To know if this could affect the signal two immunoprecipitations were prepared using nsP1-F2 (Figure 12). The first sample of nsP1-F2 contained 20 µL magnetic beads, 5 µL nanobody and 150 µL lysate while nsP1-F2 x2 contained the double: 40 µL magnetic beads, 10 µL nanobody and 300 µL lysate. nsP1-F2 x2 results in an increased signal (Figure 12).

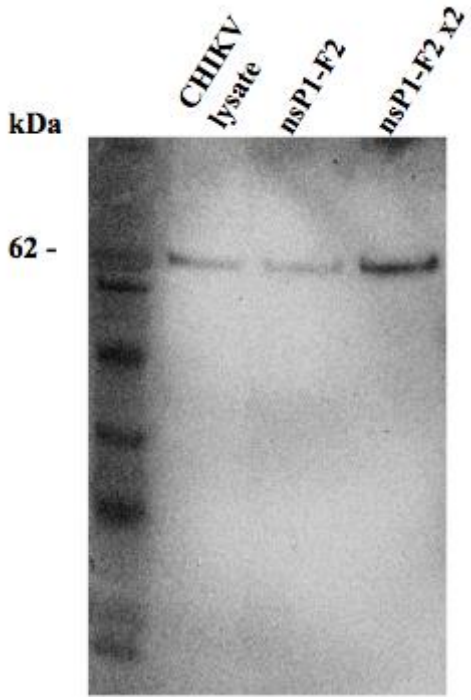

Figure 12. Two immunoprecipitations with chikungunya viral protein nsP1 and nsP1-F2 with increasing amounts of reagents. *Two samples of nsP1-F2 were incubated on streptavidin beads with Chikungunya virus lysate. The IP with the double amount of magnetic beads, nanobody and lysate results in a stronger signal.*

4.4 Infection assay to reveal the nanobody effect on virus replication

With binding of nsP1-F2 to nsP1 confirmed, we next wanted to find out if the nanobody has an effect on virus replication if expressed inside a cell.

We performed an infection assay with HOS-cells expressing nsP1-F2 under a doxycycline inducible promoter was detected with Western blot. To detect nanobody and viral proteins we employed western blot. The assay examined 4 different samples: **mock**, containing uninfected cells, **mock + dox,** containing uninfected cells and induced nanobodies, **SFV,** containing cells infected by Semliki Forest Virus and **SFV + dox**, containing cells infected by Semliki Forest Virus and induced nanobodies. We stained the western blot with SFV-nsP3 to detect virus replication. GAPDH as a loading control and HA-tag to visualize nanobody expression. SFV-nsP3 ~ 60 kDa shows in infected cells since only those would contain the viral protein. GAPDH ~ 38 kDa, an enzyme involved in glycolysis, was screened for as a control to see that all samples contained cells. The anti-HA-tag antibody was used to detect the HA-tagged nanobody (~ 15 kDa).

This proved that the infection was successful: In all lysates from infected cell, we could detect the viral protein nsP3, while the GAPDH signal was similar in all lanes. The experiment also confirmed efficient nanobody expression after addition of doxycycline (Figure 13).

By comparing the bands for SFV-nsP3 between the cell lysate with and without doxycycline the effect of nsP1-F2 on the viral replication could be demonstrated. For this blot the difference in signal between the two samples is not much (Figure 13), indicating that the nanobody binding to the virus had little effect on the viral replication or that a possible difference in viral replication could not be detected.

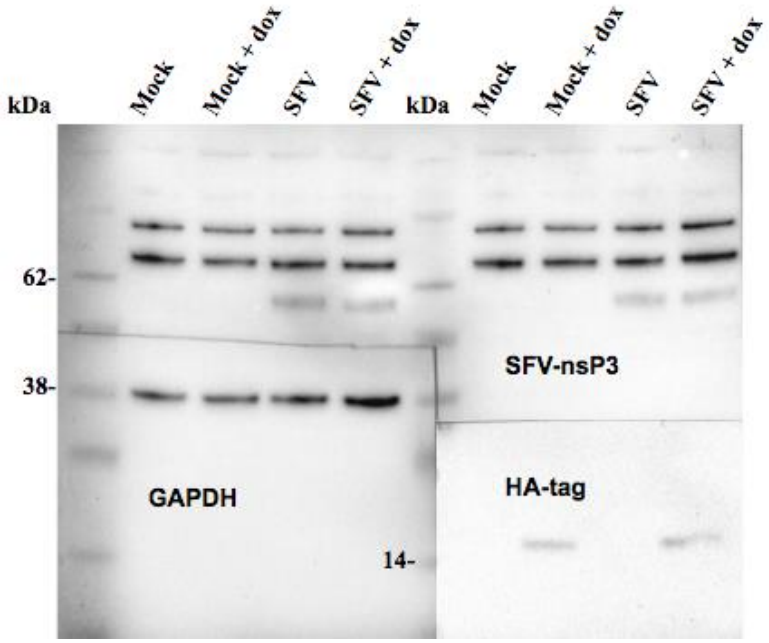

Figure 13. Infection assay detected with western blot. *HOS-cells expressing nsP1-F2 under a doxycycline induced promoter were infected with Semliki Forest Virus and analyzed with Western blot. Half of the samples had doxycycline added to them, inducing nsP1-F2 expression. The upper half screens for SFV-nsP3, the bands around 60 kDa indicates presence of Semliki Forest Virus in the infected samples. The left corner screens for GAPDH, an enzyme involved in glycolysis ~ 38 kDa, showing that the cells are present in the samples. The right corner screens for a HA-tag bound to the nanobodies, ~ 15 kDa, and shows samples containing nanobodies induced by doxycycline.*

4.5 Plaque assay to quantify the viral titers

With no detectable change of SFV in the infected cells, we next wanted to see if the presence of the nanobody decreased virus production of the infected cells. Quantification of the viral titers were possible by counting the plaques in the plaque assay. To minimize errors only the wells with distinct areas of viral infections were considered. Plate 1 has distinct plaques in the 10^{-3} dilution, 5 plaques are counted in the sample with doxycycline while only 1 plaque is counted in the sample with doxycycline (Figure 14). Plate 2 also has distinct plaques in the 10^{-3} dilution, 4 plaques are counted in the sample with doxycycline while 2 plaques are counted in the sample with doxycycline (Figure 15).

Calculation of the viral titers (pfu/mL) were calculated by multiplying the dilution factor and the number of plaques per milliliter. The viral titers in the 10^{-3} dilutions were concluded in Table 6.

Table 6. Two virus stocks were prepared from HOS-cells infected with Semliki Forest Virus, either expressing nsP1-F2 or not. BHK-cells were exposed to the stocks and the viral titers are calculated and compared.

	SFV (pfu/mL)	SFV + dox (pfu/mL)
Plate 1, 10^{-3}	2.5×10^{4}	5×10^{3}
Plate 2, 10^{-3}	2×10^{4}	1×10^{4}
Average	$\mathbf{2.25\times10^{4}}$	$\mathbf{7.5\times10^{3}}$

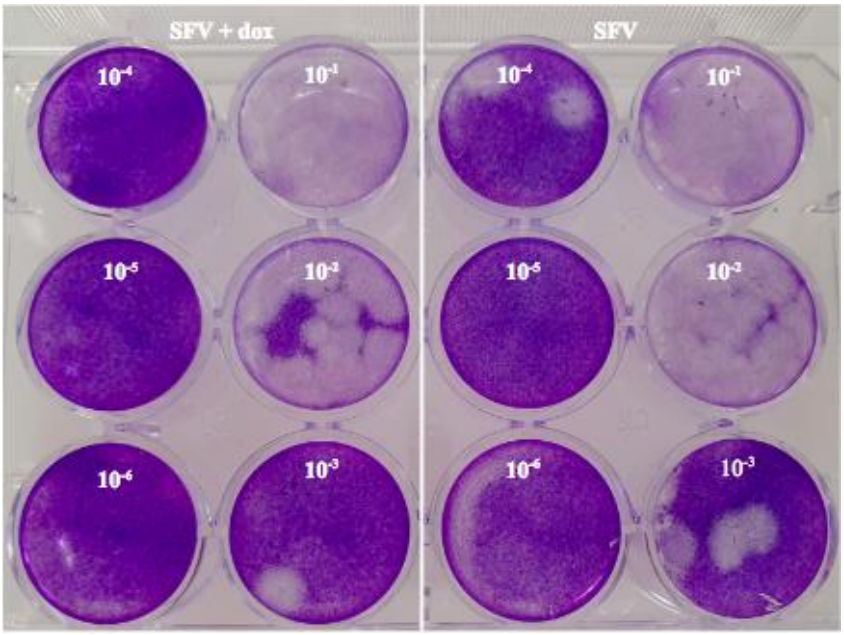

Figure 14. Plaque assay, plate 1. *BHK cells were exposed to 10-fold dilutions of culture supernatants from infected HOS-cells either expressing nsP1-F2 (left) or not (right). The variation in plaque counts is a result of the viral dilutions. In the 10^{-3} dilution 1 plaque is detected for the cells expressing nsP1-F2 specific nanobodies while 5 plaques are detected for the cells with no nsP1-F2 expression.*

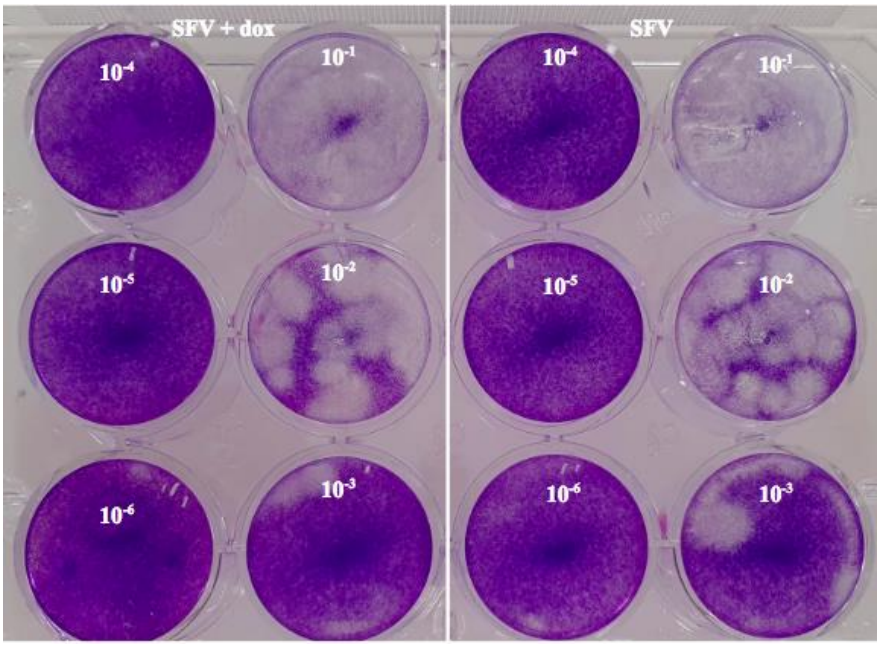

Figure 15. Plaque assay, plate 2. *BHK cells were exposed to 10-fold dilutions of culture supernatants from infected HOS-cells either expressing nsP1-F2 (left) or not (right). The variation in plaque counts is a result of the different viral dilutions. In the 10^{-3} dilution 1 plaque is detected for the cells expressing nsP1-F2 specific nanobodies while 5 plaques are detected for the cells with no nsP1-F2 expression.*

5. Discussion

The goal of this project was to confirm nanobody specificity to alphavirus nonstructural proteins and examine their possible function on the viral replication.

The nanobodies tested for specificity against Chikungunya or Semliki Forest Virus proteins are summarized in Table 7. nsP1-F2 indicated binding to nsP1 of both Chikungunya and Semliki Forest Virus. nsP1-B4 indicated binding to nsP1 of Semliki Forest Virus. We confirmed binding of nsP2-C2 to nsP2 of Chikungunya virus. We could not confirm binding of nanobody D3 to nsP3.

Table 7. Expressed nanobodies and their binding abilities to the nsP:s. *B4 and F2 targeting nsP1 could be confirmed to bind nsP1. The nsP2-targeting nanobody C2 is confirmed to bind nsP2.*

Influenza	nsP1	nsP2	nsP3	nsP4
52	B4	C2	D3	A3
	F2	E12		A12
		H10		D2
				D8

5.1 Confirmed nanobody specificities

The three nanobodies that were confirmed to bind the nonstructural proteins of Chikungunya and/or Semliki Forest Virus, nsP1-F2, nsP1-B4 and nsP2-C2, proved binding in more than one experiment. nsP1-F2 proves binding to nsP1 of Chikungunya, in both immunoprecipitations and in the ELISA (Figure 7; Figure 8). It also proves to bind nsP1 of Semliki Forest Virus (Figure 7). nsP1-B4 proves binding to the purified nsP1 of Semliki Forest Virus in immunoprecipitations and in the ELISA (Figure 7).

nsP2-C2 proves binding to nsP2 of Chikungunya in immunoprecipitations (Figure 9). Although it did not prove binding in the ELISA it was considered as confirmed to bind nsP2 of Chikungunya since it proved that in several IPs. Binding for nsP2-E12 and nsP2-H10 could not be confirmed in an IP, but they have signals in the ELISA, indicating binding.

5.2 Nanobodies do not detect their target in ELISA using cell lysate from CHIKV infected cells

The nanobodies with confirmed specificity could not detect their target in ELISA coated with lysates from infected cells (Supplementary S3, Table 1). A reason for that could be that the nonstructural proteins obtained from the lysate were not stable enough for the ELISA process.

ELISA is highly dependent on correct sample preparation and some step in preparing the lysates could have been destructive for the target viral protein. A common issue with ELISA is that the lysis buffer used to lyse the infected cells contains detergents with a denaturing effect in the target protein, making it impossible for the antibodies to recognize it in the ELISA (Thermo Fisher, 2020). Cristea buffer contains several non-denaturing detergents such as sodium deoxycholate, triton X-100 and tween-20, which should make it suitable for ELISA. EE buffer does not contain any non-denaturing detergents which could have had a denaturing effect on the target protein and could also explain its inability in the IP. Another reason for the lack of binding between the nsPs in the lysate and the targeting nanobody could be that the concentration of the target protein in the lysate was too low to bind enough nanobodies in order to yield a strong signal.

5.3 Factors influencing an immunoprecipitation

Immunoprecipitation was the main method to test binding of the nanobodies to the viral proteins. An optimization of the technique was made when it was realized that the outcome of the technique varied heavily. The choice of lysis buffer seemed to have the biggest effect on the immunoprecipitation outcome and cristea buffer was identified as a functional buffer for an immunoprecipitation between nsP1-F2 and nsP1 of Chikungunya virus. nsP1-F2 was the only nanobody the conditions were examined on and it is possible that the result could differ among the nanobodies. Other factors that were suspected to have an effect on the outcome were amount of beads, nanobodies, lysate and the time the reagents were left for binding. In Table 8 the optimal immunoprecipitation is summarized.

5.3.1 The choice of lysis buffer is crucial for binding of nanobodies to their antigens

Figure 10 portraits how important the choice of lysis buffer is for binding nanobodies to the viral proteins in an immunoprecipitation. When lysing cells infected with Chikungunya virus with EE buffer there is no binding for nsP1-F2. With the exact same conditions, except from instead using cristea buffer as lysis buffer, there is a clear band for nsP1-F2 indicating binding between the nanobody and nsP1 of Chikungunya virus.

The cristea buffer contains several detergents, while EE buffer only contain NaCl, which in addition is in a smaller concentration than in the cristea buffer. The detergents make the target proteins in the lysate soluble and available for the nanobodies in the IP. The detergents of the lysis buffers are concluded in Table 5. Probably the combination of all the detergents in the cristea buffer is needed to properly solve the target protein in the lysate in order to be available for the nanobody in the IP.

Table 8. Conditions for an optimal immunoprecipitation. *This is considered the optimal conditions for an immunoprecipitation with nsP-specific nanobodies and Chikungunya viral proteins.*

Amount of beads (µL)	Amount of nanobody (µg)	Amount of lysate (µL)	Lysis buffer	Time for binding (h)
40	10	200	Cristea	2 h room temp. alt. O/N 4 °C

5.4 Nanobody effect on viral replication

If a nanobody binds the virus there are several possible outcomes. The hope is that the binding will have a disadvantageous effect on the viral replication and thereby expose a vulnerability of the virus, but there are also possibilities that the binding of a nanobody does not affect the replication at all or could even possibly increase the viral replication.

To get an idea of the effect the nanobody has on viral replication, an infection assay was carried out. To detect virus replication, we stained for the viral nsP3 in western blot (Figure 13). When staining for the viral protein nsP3 there is no detectable difference in signal between the sample containing nsP1-F2 specific nanobodies and the sample without nanobodies. This implies that the presence of the nanobody does not make a difference on production of viral proteins in infected cells, or that the difference is not detectable in this experiment.

To analyze if the nanobody has effect on the production of progeny virus, we performed plaque assays (Figure 14; Figure 15). Figure 14 & 15 show that the number of plaques is lower in the samples containing nsP1-F2 specific nanobodies. The viral titers are calculated from the number of plaques and resulted in higher titers for the samples lacking the nsP1 specific nanobody, indicating that the binding of the nanobody to nsP1decreased viral replication (Table 6). Thus, nsP1-F2 reveals a vulnerability of the Semliki Forest Virus. Further studies of the vulnerability of the alphaviral nonstructural proteins are in demand and could contribute to a treatment for diseases caused by alphaviruses.

The cell lines used for the infection and plaque assays produced nsP1-F2 specific nanobodies under a doxycycline inducible promoter. The other two nanobodies that were confirmed binding to the viruses were not tested in a quantitative method. Further investigations could reveal the effect of nsP1-B4 and nsP2-C2 on viral replication.

6. Conclusion

Three nanobodies proved to bind two of the nonstructural proteins under optimized conditions with immunoprecipitation, western blot and ELISA as its premier analytical methods. One of the nanobodies, nsP1-F2, was further investigated with infection and plaque assays. The plaque assay proved nsP1-F2 to have a decelerated effect on the viral replication and therefore contributed to a revelation of a viral vulnerability.

7. Acknowledgements

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References

BioRad, 2020. General Protocol for Western Blotting, Retrieved 2020, 20 April. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf

Bissoyi, A., Pattanayak, S.K., Bit, A., Patel, A., Singh, A.K., Behera, S.S., Satpathy. 2017. Alphavirus Nonstructural Proteases and Their Inhibitors, *Viral Proteases and Their Inhibitors,* 77-104. https://doi.org/10.1016/B978-0-12-809712-0.00004-6

Bonifacino, J.S., Gershlick, D.C., Dell'Angelica, E.C. 2016. Immunoprecipitation, *Current Protocols,* 71(1), https://doi-org.ezproxy.its.uu.se/10.1002/cpcb.3

Cytvia, 2013. ÄKTA Start, Chromatography Systems, Retrieved 2020, 1 May. https://cdn.gelifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=16705

Fros, J.J., Pijlman, G.P. 2016. Alphavirus Infection: Host Cell Shut-Off and Inhibition of Antiviral Responses, *Viruses.* 8(6):166 https://doi.org/10.3390/v8060166

Gottipati, K., Woodson, M., H.Choi, K. 2020. Membrane binding and rearrangement by chikungunya virus capping enzyme nsP1, *Virology,* 544:31-41 https://doi.org/10.1016/j.virol.2020.02.006

Guimaraes, C.P., Witte, MD., Theile CS., Bozkurt, G., Kundrat, L., Blom A., Ploegh, H.L. 2013. Site-specific C-terminal and internal loop labeling of proteins using sortase-mediated reactions. *Nature Protocols,* 8:1787-1799. https://doi-org.ezproxy.its.uu.se/10.1038/nprot.2013.101

Harmsen, M.M., De Haard, H.J. 2007. Properties, production, and applications of camelid single-domain antibody fragments. *Appl Microbiol Biotechnol,* 77:13–22. https://doi.org/10.1007/s00253-007-1142-2

Hnasko R. 2015. Direct ELISA, *ELISA Methods and Protocols*, 1318:61-67. https://doi.org/10.1007/978-1-4939-2742-5

Jácome, R. López. Vidal, Y. Ponce de Léon, S. 2017. Are RNA Viruses Candidate Agents for the Next Global Pandemic? *ILAR Journal,* 58(3):343-358. https://doi.org/10.1093/ilar/ilx026

Kaur P., Lee R.C.H., Chu J.J.H. 2016 Infectious Viral Quantification of Chikungunya Virus—Virus Plaque Assay. *Methods in Molecular Biology,* 1426:93-103. https://doi-org.ezproxy.its.uu.se/10.1007/978-1-4939-3618-2_9

Kim B. 2017. Western blot techniques, *Molecular Profiling. Methods in Molecular Biology,* 1606:133-139. https://doi-org.ezproxy.its.uu.se/10.1007/978-1-4939-6990-6_9

King, R.C., Mulligan P.K., Stansfield W. 2014. Baltimore classification of viruses, *A Dictionary of Genetics*, 8.

Korsman, S., van Zyl, G.U., Nutt, L., Andersson, M.I., Preiser, W. 2012. Togaviruses, *Virology,* 98-99. https://doi.org/10.1016/B978-0-443-07367-0.00042-2

Liu, J.L., Shriver-Lake, L.O., Zabetakis, D., Anderson, G.P., Goldman, E.R. 2019. Selection and characterixation of protective anti-chikungunya virus single domain antibodies, *Molecular Immunology.* 105:190-197. https://doi.org/10.1016/j.molimm.2018.11.016

Muyldermans S. 2013. Nanobodies: Natural Single-Domain Antibodies, *Annual Review of Biochemistry,* 82:775-797. https://doi.org/10.1146/annurev-biochem-063011-092449

Sharma, A., Gupta, S.P. 2017. Fundamentals of Viruses and Their Proteases, *Viral Proteases and Their Inhibitors*. https://doi.org/10.1016/B978-0-12-809712-0.00001-0

The European Molecular Biology Laboratory, 2020. Protein Purification - Extracting and purification core facility. Retrieved 2020, 20 April from https://www.embl.de/pepcore/pepcore_services/protein_purification/extraction_clarification/lysis_buffer_additives/index.html

Thermo Fisher, 2020. Detergents for Cell Lysis and Protein Extraction. Retrieved 2020, 20 April from https://www.thermofisher.com/se/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/detergents-cell-lysis-protein-extraction.html

Vanlandschoot P, Stortelers C, Beirnaert E, Ibanez L.I, Schepens B, Depla E, Saelens X, 2011. Nanobodies®: New ammunition to battle viruses, *Antiviral Research,* 92(3):389-407 https://doi.org/10.1016/j.antiviral.2011.09.002

Weiss, R.A. Verrips, C.T. 2019. Nanobodies that Neutralize HIV, *Vaccines* 7(3). https://doi.org/10.3390/vaccines7030077

8. Supplementary

S1. Cell culturing

To maintain and keep the cells alive between experiments the cells were split and transferred to new, fresh growth medium regularly.

Split cells
Heat cell media (~ 20 mL) in water bath (35-37 °C), alternatively make new one.
Remove all media, the cells are attached to the surface.
Add 10 mL PBS -
Add 1 mL trypsin (1:10). Make sure the trypsin is covering the whole surface.
Incubate in 37°C for 4-5 minutes
Add 9 mL media and work it up and down & in all corners
Transfer all liquid to an empty falcon tube and remove bubbles
Add 9 mL media
Add 1 mL of the cells from the empty falcon tube
Incubate for 2 days

Growth medium
50 mL TBP
50 mL FBS
10 mL Hepes
5 mL Penicillin Streptomycin

S2. Protein purification with gel chromatography

The protein purification of the nanobodies were done with size-exclusion chromatography with ÄKTA start purification system, following the manufacturer's protocol (Cytvia, 2013). The purified nanobody in the sample in Figure 1 was nsP1-F2 and was visible in the peak of the chromatogram.

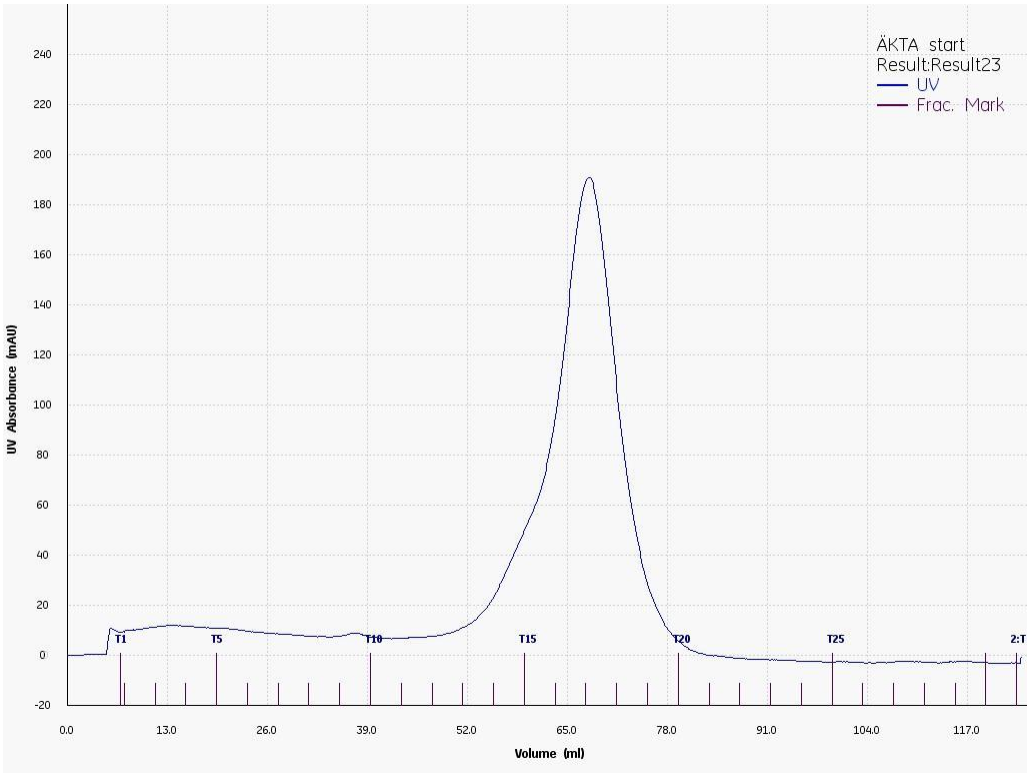

Figure 1. Protein purification of nsP1-F2 with ÄKTA Start system, gel chromatography.

S3. Result of the ELISA

To know if binding had occurred between the nanobodies and the viral proteins the absorbance at 450 nm was measured with an ELISA plate reader. The results are found in Table S1.

Table S1. The result of the ELISA. *For the samples with Chikungunya virus lysate as antigen there were no binding at all.*

	52	nsP1-F2	nsP1-B4	nsP2-C2	nsP2-E12	nsP2-H10	nsP3-D3	nsP4-A3	nsP4-A12	nsP4-D2	nsP4-D8
Uninfected	0,08	0,084	0,102	0,091	0,088	0,059	0,125	0,107	0,092	0,088	0,09
Chik lysate cris (20 µL)	0,08	0,084	0,097	0,081	0,07	0,065	0,099	0,079	0,076	0,071	0,078
Chik lysate cris (200 µL)	0,07	0,075	0,088	0,073	0,089	0,063	0,098	0,082	0,082	0,068	0,076
Chik lysate EE	0,07	0,071	0,068	0,066	0,058	0,058	0,092	0,074	0,074	0,086	0,09
nsP1 purified	0,1	1,012	0,969	0,073	0,121	0,062	0,081	0,066	0,066	0,068	0,079
nsP2 purified	0,07	0,07	0,1	0,113	0,58	0,299	0,102	0,085	0,092	0,115	0,088
nsP3 purified	0,07	0,077	0,081	0,107	0,267	0,054	0,13	0,348	0,086	0,1	0,11
nsP4 purified	0,07	1,454	0,212	0,452	0,188	0,062	2,677	0,323	0,57	2,828	2,797