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Characterization of the protection-linked anti-HIV-1 gp41 chimeric simian-human IgG1 monoclonal antibody 61p1E2

BACHELOR'S THESIS

For the degree of

Bachelor of Science (BSc.)

University of Veterinary Medicine, Vienna

Submitted by

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Vienna, July 2016

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Acknowledgements

First of all, I want to thank Prof. Dr. Ruth Ruprecht for giving me this amazing opportunity to work in her laboratory for my bachelor's thesis at the Texas Biomedical Research Institute. It was a wonderful, very educational experience – in many ways, I will never forget it! I truly appreciate having been given the chance join her group and participate in world-class research.

I also want to express my sincere thanks to Prof. Dr. Dieter Klein for encouraging me to travel abroad for my bachelor's thesis and for providing support throughout my internship.

Dr. Hemant Vyas has my deepest appreciation – he is a great supervisor, and I am very thankful for his guidance throughout my project, answering the many scientific questions I had and assisting me with troubleshooting when experiments did not work.

Moreover, I also wish to take this opportunity to acknowledge every member of Dr. Ruprecht's group, namely Dr. Samir Lakhashe, Dr. Sandeep Gupta, Dr. Viraj Kulkarni, Elizabeth Plake, Amanda Gray, Tho Hua, Hanna Scinto, and Siqi Gong. I thank all of them for very interesting scientific discussions, help with my experiments, and making my stay at the Texas Biomedical Research Institute unforgettable.

Special thanks to Juan Esquivel, who was always there for me whenever I needed advice.

Furthermore, I am extremely grateful to Samson Adeniji, Manu Anantpadma and Betina Erhard for their support throughout my stay.

Also, I would like to recognize the Austrian Marshall Plan Foundation for their generous financial support of my stay.

Last but not least, I really thank my family and friends for their strong support in all my life, especially during my stay abroad. Without them, I would not have been able to reach the goals I had set for myself.

Table of contents

1 INTRODUCTION **1**

1.1 The beginning of the AIDS pandemic 1

1.2 Human immunodeficiency virus – HIV 2

1.2.1 Different types of HIV and their origins 2

1.2.2 HIV envelope and its role in virus entry 3

1.3 Animal models in HIV research – and the development of SHIV 4

1.4 Antibodies 6

1.4.1 General information about antibodies 6

1.4.2 IgG 6

1.4.3 Light chain and heavy chain structure 7

1.4.4 Variable region 8

1.4.5 Fragment antigen binding (Fab)-fragment 8

1.5 Rationale and background of thesis 9

1.6 Hypothesis, long-term goals and specific aims of the bachelor thesis 10

1.6.1 Summary of results 10

1.6.2 Significance 10

2 MATERIALS AND METHODS **11**

2.1 List of buffers 11

2.1.1 Buffers used for protein purification of K-CTcc 11

2.1.2 Buffers used for antibody purification 12

2.1.3 Buffers used for ELISA 12

2.1.4 Buffers used for western blot 12

2.2 Plasmid maps 13

2.2.1 Plasmid K-CTcc-pET22b-GFPII encoding bait protein K-CTcc 13

2.2.2 pFUSE-61p1E2-Gamma 14

2.2.3 pFUSE-61p1E2-Lambda 16

2.2.4 Programs used for the construction of the plasmid maps 18

2.3 Plasmid isolation 19

2.3.1 Plasmid K-CTcc-pET22b-GFPII 19

2.3.2 Plasmids for IgG1 mAb 61p1E2 production 20
2.3.3 Glycerol stock preparation 20
2.3.4 Plasmid purification....21
2.3.4.1 Miniprep 21
2.3.4.2 Midiprep 21
2.3.4.3 Measurement of concentration of purified plasmids....21
2.3.5 Quality control: antibiotic resistance....21
2.3.6 Quality control: restriction enzyme digest 21
2.3.6.1 Agarose gel preparation 23
2.3.6.2 Gel pictures....23
2.3.6.3 Fragment lengths 23
2.3.7 Quality control: sequencing....25
2.4 Expression and purification of K-CTcc....26
2.4.1 Buffer exchange and concentrating the protein 26
2.5 Production of mAb 61p1E2 in mammalian cells and purification 27
2.5.1 Maintenance of Expi293F™ cells 27
2.5.2 Transfection of Expi293F™ cells 27
2.5.2.1 ExpiFectamine™ 293 Transfection kit (Thermo Fisher Scientific)....28
2.5.2.2 TransIT-PRO® Transfection Reagent & Kit (Mirus Bio LLC)....28
2.5.2.3 Comparison between transfection reagents 29
2.5.3 Harvest of antibodies 29
2.5.4 Antibody purification by protein A affinity chromatography....30
2.5.4.1 Buffer exchange and concentrating the antibody 30
2.6 Fab fragments of mAb 61p1E2 31
2.6.1 Size exclusion chromatography 31
2.6.2 Papain digestion 31
2.6.3 Buffer exchange and concentration of Fab sample 32
2.7 Characterization of mAb 61p1E2 and Fab-mAb 61p1E2 32
2.7.1 ELISA 33
2.7.2 SDS-PAGE 35
2.7.3 Western blot 35

3 RESULTS **37**
3.1 Recombinant bait protein K-CTcc 37
3.1.1 IPTG induction of BL21 Gen-X™ E. coli 37
3.1.2 Plasmid purification of K-CTcc-pET22b-GFPII 38
3.1.3 Quality control: restriction enzyme digest 39
3.1.4 Quality control: antibiotic resistance 40
3.2 Production of mAb 61p1E2 in Expi293F cells 41
3.2.1 Plasmid purifications 41
3.2.2 pFUSE-61p1E2-Gamma 42
3.2.2.1 Quality control: restriction enzyme digest 42
3.2.2.2 Quality control: sequencing 43
3.2.3 pFUSE-61p1E2-Lambda 44
3.2.3.1 Quality control: restriction enzyme digest 44
3.2.3.2 Quality control: sequencing 45
3.2.4 Transfection of Expi293F cells 46
3.2.5 Standardization of antibody production by Expi293F cells 48
3.2.5.1 TransIT-PRO 49
3.2.5.2 ExpiFectamine™ 293 Transfection kit 51
3.2.6 Purification of mAb 61p1E2 by protein A affinity chromatography 55
3.2.7 Antibody purification profile 56
3.2.8 Verification of antibody production 58
3.2.9 Epitope specify of purified mAb 61p1E2 59
3.3 Fab of mAb 61p1E2 60
3.3.1 Fab production by papain digestion 60
3.3.2 Quality control of Fab-mAb 61p1 E2 61
4 DISCUSSION **63**
4.1 Testing of hypothesis 63
4.1.1 Binding specificity of mAb 61p1E2 63
4.2 Aim 2 – production of Fabs and structural analysis 64
4.2.1 Cloning strategy for Fab production 64
4.3 Conclusion 66

5 ZUSAMMENFASSUNG 67
6 ENGLISH SUMMARY 68
7 REFERENCES 69
8 INDEX OF FIGURES 73
9 INDEX OF TABLES 74
10 INDEX OF ABBREVIATIONS 75

1 INTRODUCTION

1.1 The beginning of the AIDS pandemic

The pandemic of the Acquired Immune Deficiency Syndrome (AIDS), one of the most devastating diseases in world history, officially began in June 1981, when the Centers of Disease Control and Prevention (CDC) reported the occurrence of *Pneumocystis carinii* pneumonia (PCP) in five previously healthy homosexual men. PCP is normally only contracted by severely immunosuppressed people (CDC 1981). Those findings lead to active research into the origins and causes of AIDS.

An infectious retrovirus, now referred to as human immunodeficiency virus type 1 (HIV-1), was identified as the etiological agent of AIDS in 1983 (Barre-Sinoussi, Chermann et al. 1983, Gallo, Salahuddin et al. 1984, Levy, Hoffman et al. 1984). The oldest known viral sequence of HIV-1 goes back to 1959 to the capital city, Kinshasa, Democratic Republic of the Congo (named Léopoldville, Belgian Congo until 1966) (Worobey, Gemmel et al. 2008).

Since the beginning of the AIDS pandemic, almost 71 million people have been infected and about 35 million people have died (WHO-World Health Organization 2016). Recently, the number of AIDS-related deaths has been declining because of better access to antiretroviral therapy (ART) and therefore, HIV-infected people can live longer and better lives. However, the number of HIV-infected people is still high. Globally, 36.9 million people were living with HIV in 2014 and about two million people became newly infected. Sub-Saharan Africa is the most affected region in the world (UNAIDS 2015).

Much effort has been devoted to develop an efficient HIV vaccine, mostly with the HIV-1 envelope (Env) as the primary target, but such vaccines showed limited efficacy. The most successful trial to date was the RV144 trial with 31.2% protection (Rerks-Ngarm, Pitisuttithum et al. 2009).

1.2 Human immunodeficiency virus – HIV

HIV belongs to the family retroviridae and the genus lentivirus (ICTV-International Committee on Taxonomy of Viruses 2016). "*Lenti-*" is the Latin word for "slow" and refers to the long incubation time of lentiviruses. In most cases, HIV leads to AIDS within 10-12 years after infection without medical treatment. AIDS is the end stage of an HIV infection with a CD4+ T cell count below 200 cells per cubic millimeter (mm^3) of blood. An uninfected person usually has 800-1200 CD4+ T cells per mm^3 (NIH-National Institutes of Health 2009). The majority of infected people acquired HIV-1 through exposure at mucosal surfaces, thus AIDS is primarily a sexually transmitted disease (reviewed in Hladik, McElrath 2008).

1.2.1 Different types of HIV and their origins

Two types of HIV exist, HIV-1 and HIV-2. HIV-1 is the causative agent of the AIDS pandemic, whereas HIV-2, which was discovered three years later than HIV-1 in 1986, remains mainly restricted to West Africa. Both HIV-1 and HIV-2 can lead to AIDS; however, these two viruses are only distantly related to each other. Both HIV types originated from cross-species transmissions between monkeys and humans. The simian immunodeficiency virus (SIV) from chimpanzees (SIVcpz) is considered to be the origin of HIV-1 and SIVsm from sooty mangabeys is referred to as the origin of HIV-2 (Sharp, Hahn 2011, Korber, Gaschen et al. 2001).The course of transmission for both viruses was probably through blood from monkeys to human hunters (Taylor, Sobieszczyk et al. 2008).

HIV-1 is divided into four distinct lineages, termed groups M, N, O, and P. Group M ("Main"-group) was discovered first and represents the pandemic form of HIV-1 (Sharp, Hahn 2011). It consists of nine genetic subtypes/ clades (A, B, C, D, F, G, H, J, and K) and more than 40 different circulating recombinant forms (CRFs), which arose when more than one strain of HIV infected the same cell (Taylor, Sobieszczyk et al. 2008). The most prevalent clade of HIV-1 is clade C (HIV-C) accounting for almost 50% of all HIV-1 infections worldwide (Buonaguro, Tornesello et al. 2007).

1.2.2 HIV envelope and its role in virus entry

HIV is about 80 - 120 nm in diameter and is surrounded by a lipoprotein-rich membrane, the HIV Env (Fig.1). HIV infects mainly CD4+ T cells and cells of the monocyte/macrophage lineage. Cell tropism is determined by viral envelope glycoproteins (gp), which are highly glycosylated spikes composed of non-covalently bound heterotrimers of the external surface (SU) gp120 and the transmembrane (TM) spanning gp41. Env glycoproteins are synthesized as a polyprotein precursor, gp160, which is cleaved by cellular proteases to produce the mature SU gp120 and the TM gp41. These gp120/gp41 complexes are essential for the virus entry into the host cell. Gp120 binds to the primary receptor, the CD4 receptor, which is expressed on the cell surface of about 60% of circulating T lymphocytes. This binding triggers a conformational change in gp120, exposing a specific domain in gp120 which can bind to the chemokine co-receptors CCR5 and CXCR4 on the cell membrane. Viruses that use the β-chemokine receptor CCR5 are known as R5-tropic. Those that use the CXCR4 are referred to as X4-tropic. Some viruses can use either CCR5 or CXCR4, and are termed dual tropic or R5X4 viruses. Co-receptor binding causes changes in the structure of gp41 which leads to the insertion of gp41 into the host cell membrane (Checkley, Luttge et al. 2011, lentiviral_vectors_fact_sheet.pdf).

Gp41 triggers fusion of the virus and host cell membranes which allows the HIV viral core to enter the cytosol of the host cell and convert its RNA into double-stranded DNA using the viral enzyme reverse transcriptase (RT). This DNA is translocated across the nuclear pore into the nucleus. There, the viral enzyme integrase integrates the viral DNA into the host chromosomal DNA. Transcription of viral RNAs is carried out by the cellular RNA polymerase in the nucleus. Then, mRNA is transported to the cytoplasm and translated into viral proteins (Checkley, Luttge et al. 2011).

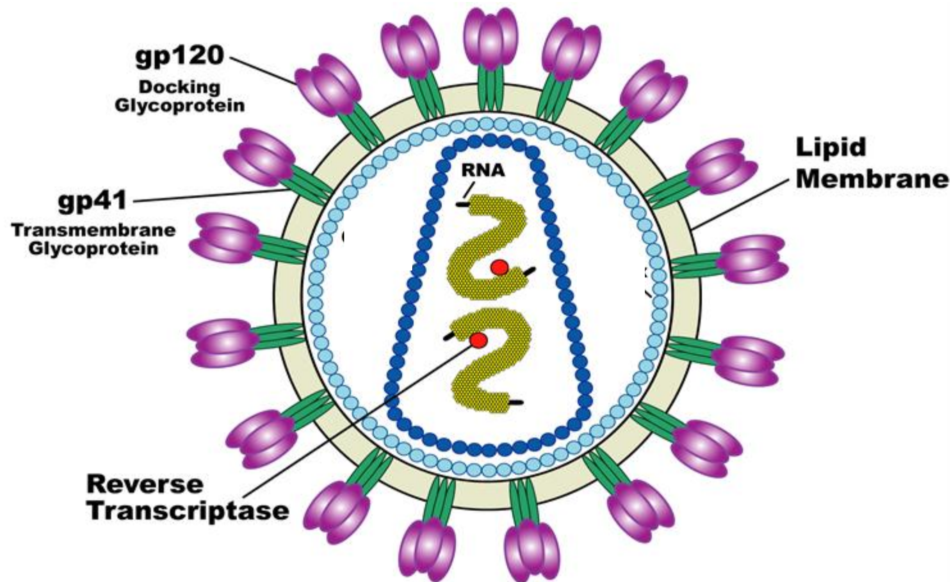

Fig. 1 HIV particle

The structure of an HIV virion is shown. The glycoprotein (gp) 120 is colored violet and the gp41 is shown in green. The conical core, shown in dark blue, contains two copies of viral RNA. Please refer to section (1.2.2) for details (modified after http://www.yalescientific.org/wp-content/uploads/2014/12/HIV-Image-5.png).

1.3 Animal models in HIV research – and the development of SHIV

A suitable animal model for HIV is crucial for HIV vaccine and drug development. However, there were challenges with finding an animal model that showed the same course of disease progression as in humans. HIV can only infect humans and chimpanzees, but since chimpanzees are listed as endangered and they cannot be longer used as an animal model for HIV research (Reardon Nov 18, 2015, reviewed in Hatziioannou, Evans 2012).

SIV is the predecessor of HIV and infects naturally African primate species. However, an SIV infection in those natural hosts does not lead to the development of AIDS. In Asian macaques, who are not natural hosts for primate lentiviruses, SIV can lead to high viral loads, CD4+ T cell depletion and opportunistic infections similar to an HIV infection in humans. The progression to

AIDS occurs within 1-2 years. Thus, those monkeys can be used as a pathogenic model in HIV research. Among them, the rhesus macaque (*Macaca mulatta* (RM)), pig-tailed macaque (*Macaca nemestrina*) and cynomolgus macaque (*Macaca fascicularis*) are most commonly used. However, the envelope of SIV differs significantly from that of HIV. Therefore, SIV cannot be used as challenge virus in vaccine- efficiency studies. Antibodies which can bind to the HIV envelope cannot bind to SIV and vice versa. Neutralizing antibody (nAb) responses raised against the SIV envelope are not the same as against the HIV envelope.

To allow testing of anti-HIV-1 envelope immune responses in primate models, chimeric virus strains, termed simian-human immunodeficiency viruses (SHIVs), were constructed. SHIVs express HIV-1 Env glycoproteins that can bind anti-HIV Env nAbs and can therefore be used to test Env specific vaccines and drugs. Most SHIVs were constructed by replacing some genes of SIV with HIV-1 genes. These initial viruses were not able to replicate efficiently in macaques, but after many animal-to-animal passages, they finally gained the ability to replicate and can cause AIDS (reviewed in Hatziioannou, Evans 2012). Dr. Ruprecht's lab has used multiple SHIV models for passive and active immunization studies (Lakhashe, Wang et al. 2011, Watkins, Sholukh et al. 2013, Ruprecht 2016). One of the SHIVs carrying the HIV-C envelope (SHIV-C) was used to challenge RMs in the vaccine-efficiency study in which the monoclonal antibody (mAb), 61p1E2, was isolated (Lakhashe, Wang et al. 2011, Watkins, Sholukh et al. 2013, Ruprecht 2016).

1.4 Antibodies

1.4.1 General information about antibodies

Five classes (isotypes) of immunoglobulins, termed IgM, IgD, IgG, IgA and IgE, exist. They can be distributed according to their (C) regions and effector functions. All antibodies consist of paired heavy and light polypeptide chains. Since the mAb 61p1E2 is an IgG1 antibody, the IgG class only will be described in detail.

1.4.2 IgG

IgG is the predominant immunoglobulin found in the body. In comparison to other isotypes, IgG has the longest serum half-life. Due to structural, antigenic and functional differences in the constant region of the heavy chain of IgG's, the IgG class can be divided into four different subclasses, IgG1, IgG2, IgG3 and IgG4, which are numbered according to their different abundancy in serum, with IgG1 being the most abundant.

The molecular weight of IgG is about 150 kDa. IgG is composed of two identical heavy chains (H chain) with a molecular weight of 50 kDa each and two identical light chains (L chain) each with a molecular weight of 25 kDa. Every IgG antibody has two identical antigen-binding sites that can bind simultaneously to two identical antigens on a given surface. This gives it high avidity. There exist two types of L chains, lambda and kappa. A given antibody can only have either lambda or kappa chains. There is no functional difference known between antibodies which either have lambda or kappa chains. The antibody mAb 61p1E2, which I characterized, is an antibody with the light chains of type lambda.

1.4.3 Light chain and heavy chain structure

Each light chain consists of a variable light (VL) and a constant light chain domain (CL). Each heavy chain has a variable heavy (VH) and three constant heavy (CH) domains, named CH1, CH2 and CH3, numbered from the amino-terminal end to the carboxy terminus. The VL and VH domains are paired and make up the V region of the antibody which has the ability to bind specific antigens. The region of the immunoglobulin which binds the antigen is also referred to as paratope, and the site on the antigen that is bound is called epitope. CH1 and CL domains are paired and the CH3 domains pair with each other too, but the CH2 domains do not interact. Attached to the CH2 domains are carbohydrate side chains which lie between the two heavy chains.

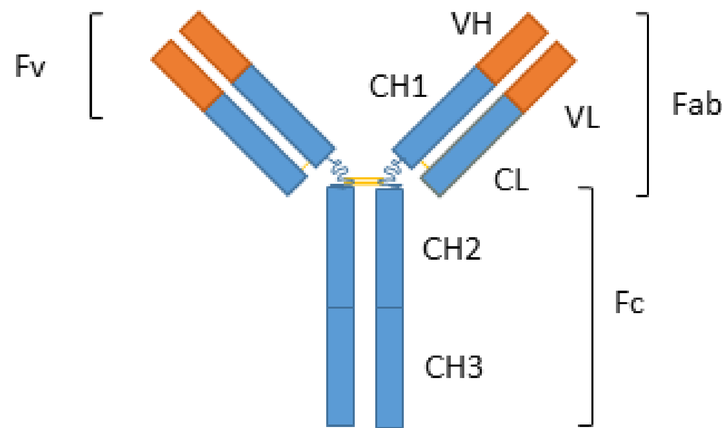

Fig. 2 IgG structure

This figure shows the structure of an IgG. Each light chain consists of a VL domain and a CL domain and each heavy chain consists of a VH and three CH domains, named CH1, CH2 and CH3. The variable region is shown in red and the constant regions are shown in blue. By digestion of an IgG with the enzyme papain it can be separated into a Fragment antigen binding (Fab)-fragment and a single fragment crystallizable region (Fc). For details please refer to the text (section 1.4) (modified after http://www.antibodies-online.com/images/news/Ig-alternatives.png).

1.4.4 Variable region

Every antibody has a different V region, consisting of the VL and VH chain as described above, which determines antigen specificity. The hypervariable regions which bind to the antigen are termed complementarity-determining regions (CDRs). There are six CDRs, three CDRs from the light chain and three CDRs from the heavy chain, numbered CDR1, CDR2, and CDR3. The regions between the CDRs show less variability and are the framework regions (FRs), named FR1, FR2, FR3 and FR4 (Kenneth Murphy 2011, Schroeder, Cavacini 2010).

1.4.5 Fragment antigen binding (Fab)-fragment

A Fragment antigen binding (Fab) fragment binds antigen and contains one entire light chain, the VL and CL domains and the VH and CH1 portions of the heavy chain. Each Fab can be divided into two regions, the variable fragment (Fv) which consists of VL and VH, and the constant fragment, composed of CL and CH1. Fabs can be constructed by digesting an IgG molecule with the enzyme papain which cleaves the antibody molecule into two Fab fragments and a single fragment crystallizable region (Fc). The Fc fragment contains the CH2 and CH3 domains of the heavy chain, has no antigen-binding activity and interacts with effector molecules and cells.

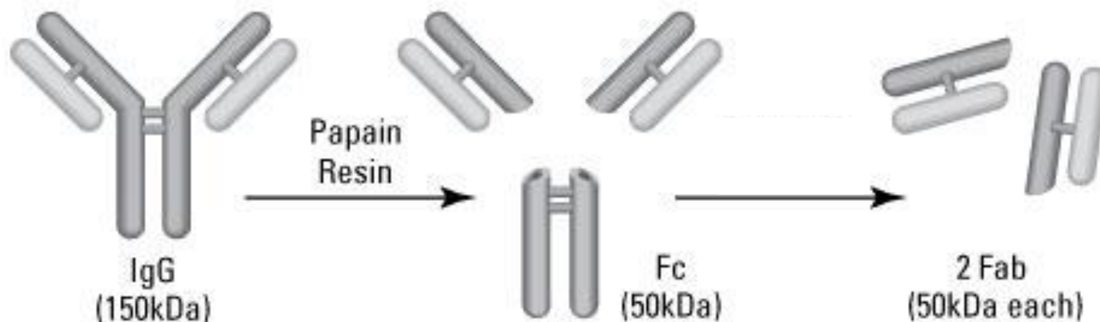

Fig. 3 Fab construction through digestion via papain

The digestion of IgG with the enzyme papain generates two Fab fragments and one Fc fragment. Each fragment has a molecular weight of 50 kDa. Please refer to section (1.4.5) for more details (modified after https://tools.thermofisher.com/content/sfs/gallery/high/44985-Frag-Papain.jpg).

1.5 Rationale and background of thesis

The protection-linked (PL) mAb 61p1E2 has been isolated from a vaccine-protected RM, named RTr-11. This animal was completely protected against multiple live-virus exposures of a SHIV-C strain, SHIV-1157ipEL-p (Siddappa, Watkins et al. 2010). In the vaccine-efficiency study that was carried out by Dr. Ruprecht's lab (Lakhashe, Wang et al. 2011), 12 RMs were vaccinated with a recombinant multicomponent protein vaccine, containing multimeric HIV-C gp160, HIV-1 clade B Tat, and SIV Gag-Pol particles. A control group of 17 RMs received no vaccine. Both the vaccine and control groups were challenged with five low-doses of SHIV-1157ipEL-p. Three weeks after the virus low-dose challenges, 94% of all animals in the control group were infected, whereas in the vaccinated group, one third remained virus-free. The uninfected RMs were rechallenged with one single high dose of SHIV-1157ipEL-p. All controls became infected, whereas in the vaccinated group, two RMs remained aviremic. RTr-11 was one of the latter.

To measure the difference in vaccine-induced antibody responses between protected and unprotected RMs, the Ruprecht group developed a novel epitope selection strategy, "PL biopanning". With this technique, PL-mimotopes of the envelope protein gp41 were isolated. These PL-mimotopes were cloned into a green fluorescent protein (GFP) expression vector to generate fusion proteins. The name of the generated fusion protein is "GFP-gp41 PL-mimotope fusion protein", also referred to as "K-CTcc". Due to confidentiality issues, the exact amino acid sequences cannot be described here, since the work is still ongoing. K-CTcc was used as bait to isolate single memory B cells producing cognate antibodies from vaccine-protected RMs. VH and VL genes were obtained from such single B cells by single-cell reverse transcription-polymerase chain reaction (RT-PCR). The amplified fragments were cloned into vectors of the pFUSE-family, which contain the constant region sequences of human IgG. This cloning strategy yielded chimeric simian-human IgG1 mAbs which bind specific to K-CTcc, PL region of gp41. One such mAb is 61p1E2.

1.6 Hypothesis, long-term goals and specific aims of thesis

My hypothesis is that the mAb 61p1E2 shows the same binding specificity against the PL region of gp41, K-CTcc, as the polyclonal antibodies, which were detected in the sera or plasma samples of vaccine-protected RMs, but not in those of non-protected RMs. If the hypothesis is confirmed, mAb 61p1E2 is therefore representative of an antibody that is linked to vaccine-induced protection, and structural analysis of the mAb-epitope interaction will be important.

Towards this latter goal, Fabs of mAb 61p1E2 will be constructed. These Fabs will be crystallized together with the protein K-CTcc for subsequent X-ray crystallography. The three-dimensional structure will give important information for future vaccine design.

To test my hypothesis and to work towards achieving the long-term goals, my bachelor project has the following Specific Aims:

Aim 1: a) Purification and characterization of sufficient quantities of the bait protein GFP-gp41 PL-mimotope fusion protein, named K-CTcc.

b) Purification and characterization of sufficient quantities of mAb 61p1E2

Aim 2: Generation of Fabs via papain digestion of full-length mAb 61p1E2.

1.6.1 Summary of results

Aim 1: a) A good quantity of the bait fusion protein, K-CTcc, was obtained.

b) 28.23 mg of mAb 61p1E2 was purified and characterized.

Aim 2: Fabs were generated; co-crystallization the bait, K-CTcc, is ongoing.

1.6.2 Significance

The characterization of the binding interaction between the paratope of PL-mAb 61p1E2 and the epitope of the bait, GFP-gp41 PL-mimotope fusion protein will give important information for future vaccine design.

2 MATERIALS AND METHODS

2.1 List of buffers

2.1.1 Buffers used for protein purification of K-CTcc

Diluting Buffer:
50 mM NaH_2HPO_4
0.6 M NaCl
30 mM Imidazole
pH 8.0

Washing Buffer A:
50 mM NaH_2HPO_4
0.3 M NaCl
15 mM Imidazole
pH 8.0

Washing Buffer B:
50 mM NaH_2HPO_4
0.3 M NaCl
25 mM Imidazole
pH 8.0

Elution Buffer:
50 mM NaH_2HPO_4
0.3 M NaCl
200 mM Imidazole
pH 8.0

Table 1 Components of buffers used for protein purification of K-CTcc

Components	Diluting Buffer	Washing Buffer A	Washing Buffer B	Elution Buffer
10x PBS	50 ml	50 ml	50 ml	50 ml
5 M NaCl	45 ml	15 ml	15 ml	15 ml
1 M Imidazole	15 ml	7.5 ml	12.5 ml	100 ml
Endotoxin-free water	390 ml	427.5 ml	422.5 ml	335 ml
Total volume	500 ml	500 ml	500 ml	500 ml

2.1.2 Buffers used for antibody purification

Elution Buffer: 0.1 M Glycine-HCl Buffer, pH 2.7

Glycine (Cat#G7126, Sigma-Aldrich) was dissolved in ultrapure water and adjusted to a pH of 2.7 with HCl.

Neutralization Buffer: 1 M Tris Buffer, pH 12

Trizma® base (Cat#T1503, Sigma-Aldrich) was dissolved in ultrapure water to a concentration of 1 M.

2.1.3 Buffers used for ELISA

Coating Buffer: 0.05 M Carbonate-Bicarbonate Buffer, pH 9.6

Contents of one capsule of Carbonate-Bicarbonate Buffer (Cat#C3041, Sigma-Aldrich) were dissolved in 100 ml of ultrapure water.

Blocking Buffer:

8 ml of Phosphate Buffered Saline (PBS) Casein ELISA Reagent (Cat#ab171532, Abcam) were mixed with 24 ml of distilled water.

PBST: pH 7.4

10x PBS, pH 7.4 (Thermo Fisher Scientific) was mixed with ultrapure water to obtain a concentration of 1x PBS. Tween 20 (Sigma-Aldrich) was added to a concentration of 0.05%.

2.1.4 Buffers used for western blot

Blocking Buffer: 4% Blotting Grade Blocker

Blotting Grade Blocker Non-fat Dry Milk (Cat#1706404XTU, Bio-Rad) was mixed with ultrapure water to a concentration of 4%.

PBST: pH 7.4 (please refer to “Buffers used for ELISA”)

2.2 Plasmid maps

2.2.1 Plasmid K-CTcc-pET22b-GFPII encoding bait protein K-CTcc

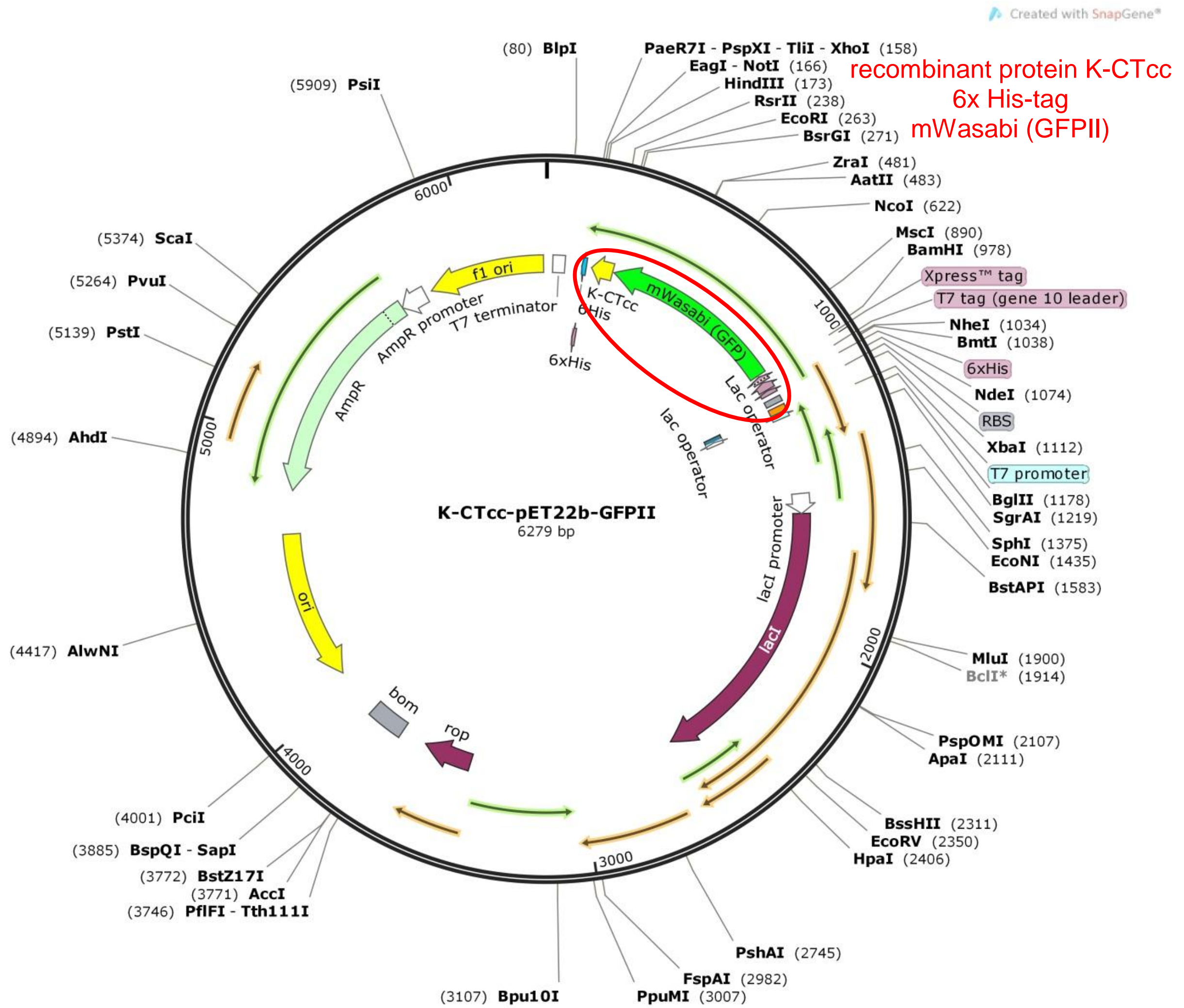

Fig. 4 Plasmid map of K-CTcc-pET22b-GFPII

The sequence of the bait, GFP-gp41 PL-mimotope fusion protein, was obtained by Dr. Ruprecht's lab from PL-mimotopes via phage display (Bachler, Humbert et al. 2013). This sequence was cloned by the Ruprecht lab into a pET-22b(+) vector (Novagen) along with a 6x His-tag and GFPII, also known as mWasabi. Due to confidentiality issues, the sequences of PL-mimotopes cannot be disclosed. For the molecular cloning of the protein K-CTcc, the restriction enzymes HindIII and EcoRI were used.

2.2.2 pFUSE-61p1E2-Gamma

The genes of the variable heavy chain and of the variable light chain of mAb 61p1E2 were obtained from the RM RTr-11 and were cloned into vectors of the pFUSE family (Invivogen) by Dr. Ruprecht's lab. The VH sequence was cloned into the plasmid pFUSE-61p1E2-Gamma and the VL region was cloned into the plasmid pFUSE-61p1E2-Lambda by Dr. Ruprecht's lab.

The plasmid pFUSE-61p1E2-Gamma is meant for the production of the IgG1 heavy chain of chimeric simian-human IgG1 mAbs in human embryonic kidney cells (HEK) cells, Expi293F cells. The vector pFUSE-CHIg-hG1 (#pfuse-hchg1) was purchased from Invivogen. The plasmid expresses the constant region of the human IgG1 heavy chain (CH). The multiple cloning site upstream of the constant region was replaced by the VH region of the RM RTr-11. The details for isolating VH are published (Sholukh, Mukhtar et al. 2012a).The VH was cloned in Dr. Ruprecht's lab into the pFUSE-CHIg-hG1 vector by using the restriction enzymes EcoRI and NheI. VH is 414 bp long and consists of four framework regions and three CDR regions. The plasmids were grown in JM109 cells and can be selected according to their antibiotic resistance against zeocin.

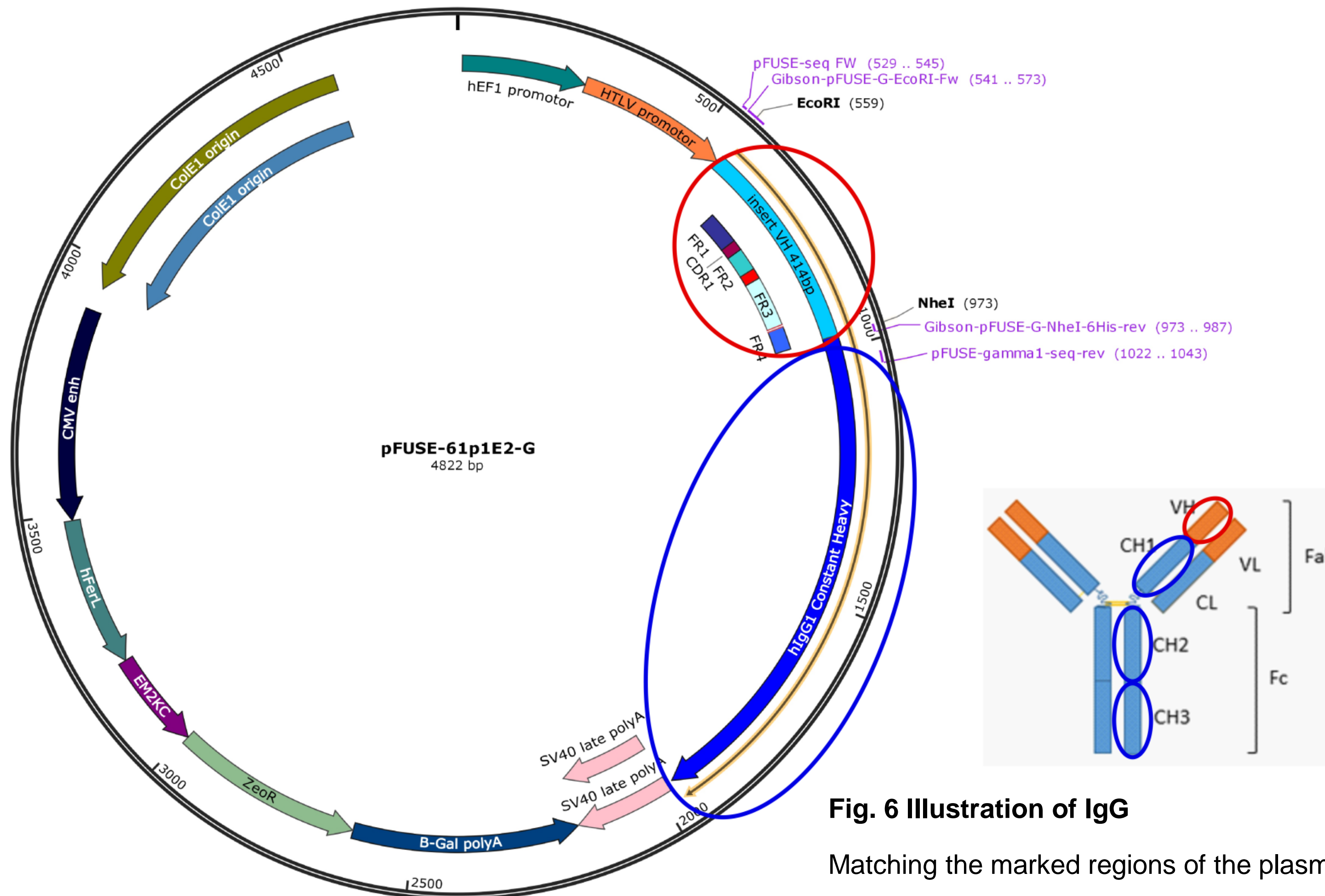

Fig. 5 Plasmid map of pFUSE-61p1E2-Gamma

The VH region of 414 bp, obtained from the RM RTr-11 is denoted with a red oval and the human CH1, CH2 and CH3 regions are marked with a dark blue oval.

Fig. 6 Illustration of IgG

Matching the marked regions of the plasmid pFUSE-61p1E2-Gamma, the VH region is marked with a red oval and the CH1, CH2 and CH3 regions are denoted with dark blue ovals (modified after http://www.antibodies-online.com/images/news/Ig-alternatives.png).

2.2.3 pFUSE-61p1E2-Lambda

The plasmid pFUSE-61p1E2-Lambda is meant for the production of the IgG1 light chain of chimeric simian-human IgG1 mAbs in HEK cells, Expi293F cells. The vector pFUSE-CLIg-hl2 (#pFUSE-hcll2) was purchased from Invivogen. The plasmid expresses the constant region of the human IgG1 light chain (CL). The multiple cloning site upstream of the constant region was replaced by the VL region obtained from the RM RTr-11. The details for isolating VL has been published (Sholukh, Mukhtar et al. 2012a).The VL was cloned by Dr. Ruprecht's lab into the pFUSE-CHIg-hG1 vector by using the restriction enzymes AgeI and AvrII, also termed BshTI and XmaJI. VL is 393 bp long and consists of four framework regions and three CDR regions. The plasmids were grown in JM109 cells and can be selected according to their antibiotic resistance against blasticidin.

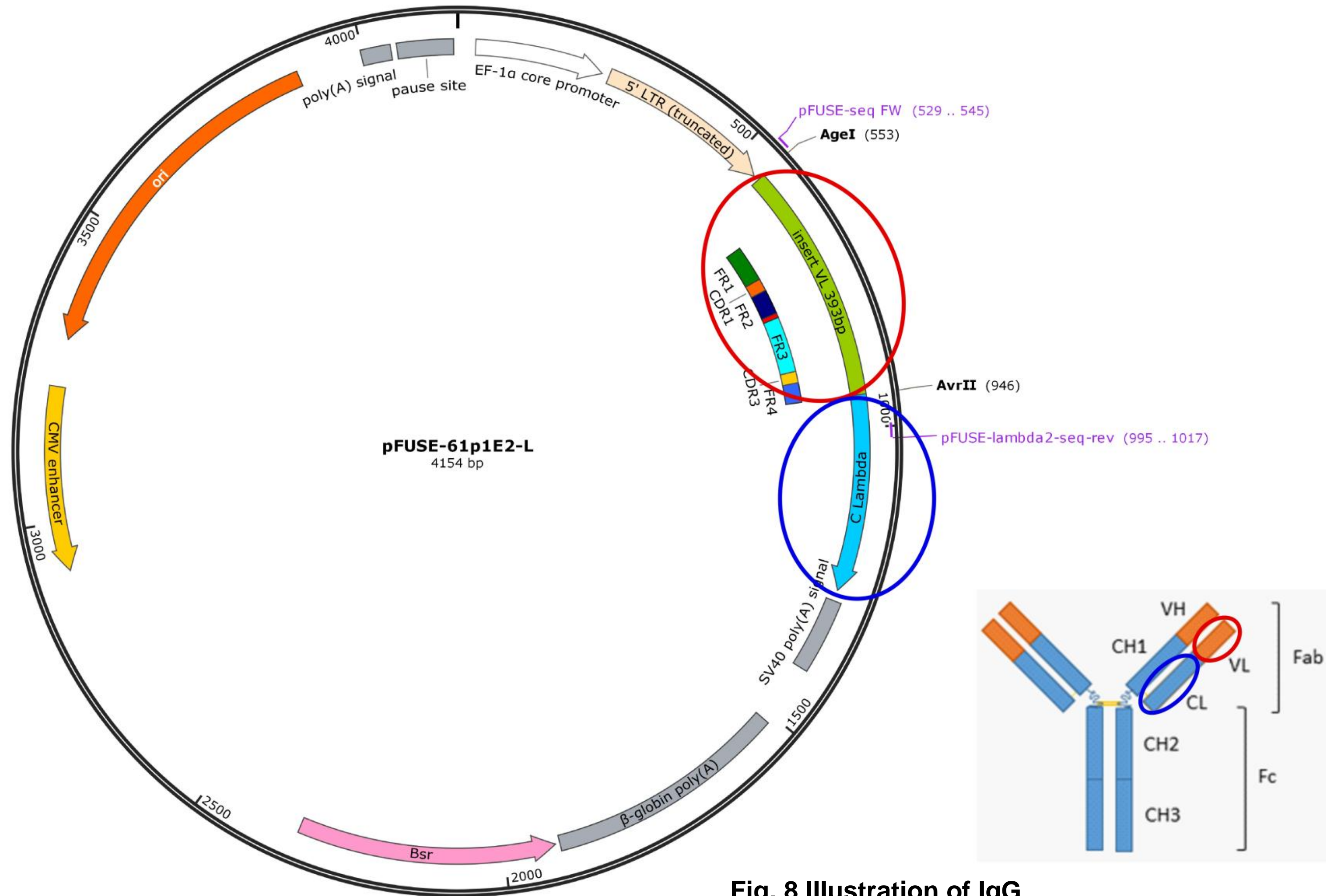

Fig. 7 Plasmid map of pFUSE-61p1E2-Lambda

The VL region obtained from the RM RTr-11 is denoted with a red oval and the human CL region is marked with a dark blue oval.

Fig. 8 Illustration of IgG

VL region of the antibody is marked with a red oval, and the CL region is denoted with a dark blue oval (modified after http://www.antibodies-online.com/images/news/Ig-alternatives.png).

2.2.4 Programs used for the construction of the plasmid maps

For the construction of the plasmid maps, the SnapGene software (from GSL Biotech; available at snapgene.com) was used. The framework- and antibody complementarity-determining regions (FRs and CDRs) of the V regions were determined with the IgBLAST tool (http://www.ncbi.nlm.nih.gov/igblast/).

2.3 Plasmid isolation

2.3.1 Plasmid K-CTcc-pET22b-GFPII

The bacterial glycerol stock of *Escherichia coli* (*E.coli*) cells, termed BL21 Gen-X *E. coli* (Genelantis) transformed with the plasmid K-CTcc-pET22b-GFPII, was kindly provided by Dr. Vyas. The glycerol stock was prepared on 02/22/13 and was stored at -80˚C. Agar plates were prepared with the Lysogeny Broth (LB)-based solid medium supplemented with ampicillin (Fast-Media® Amp Agar, Invivogen) according to manufacturer's instructions. Bacteria were streaked out onto the agar plate and kept in the incubator (Heratherm Incubator, Thermo Scientific) at 37°C overnight. The next morning, four single colonies were selected and each colony was inoculated in 1 ml of LB-based liquid medium supplemented with ampicillin (Fast-Media® Amp LB, Invivogen). These cultures were kept in an incubator (Forma Scientific) at 37°C shaking at 225 rpm on an orbital shaker for eight hours (h). After the incubation, each culture was split into two flasks containing 25 ml Fast-Media® Amp LB (Invivogen). These flasks were incubated in the incubator (Forma Scientific) at 37°C shaking at 225 rpm on an orbital shaker overnight.

The next morning, the optical density (OD) of the cultures was measured with the DU-640 Spectrophotometer (Beckman). To obtain the optimal OD of 0.8-1, Fast-Media® Amp LB was added to each flask. Then, 25 ml of each culture was transferred to a new flask. One culture flask of each colony was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) solution (Thermo Scientific). Both cultures, the IPTG-induced and IPTG-non-induced cultures were kept in the incubator (Forma Scientific) at 37°C shaking at 225 rpm on an orbital shaker for six h. Afterwards, the bacterial cultures were transferred to 50 ml Falcon tubes and centrifuged at 16,128 x g for 20 min at room temperature (Rtemp) in the Avanti™ J-25 Centrifuge, JA-12 Rotor (Beckman). Supernatant was discarded and the fluorescence of the bacterial pellets of IPTG-induced and IPTG non-induced cultures was compared using the Blue View Transilluminator (Vernier Bio-technology). IPTG-induced bacterial pellets were used for the protein purification of K-CTcc and IPTG non-induced cultures were used for the plasmid purification of K-CTcc-pET22b-GFPII. Both culture pellets were frozen at -20°C until the purification steps.

2.3.2 Plasmids for IgG1 mAb 61p1E2 production

The glycerol stocks of *E. coli* cells, termed JM109 (Promega), transformed with either the plasmid pFUSE-61p1E2-Gamma or pFUSE-61p1E2-Lambda were kindly provided by Dr. Vyas. The glycerol stocks were prepared on the 06/12/13 and were kept frozen at -80°C. Agar plates were prepared with the Lysogeny Broth (LB)-based solid medium supplemented with zeocin (Fast-Media® Zeo Agar, Invivogen) and LB-based solid medium supplemented with blasticidin (Fast-Media® Blas Agar, Invivogen) according to manufacturer's instructions. Bacteria transformed with pFUSE-61p1E2-Gamma have a zeocin resistance and were therefore streaked out on a agar plate with Fast-Media® Zeo Agar and bacteria transformed with pFUSE-61p1E2-Lambda have a blasticidin resistance and were streaked out on a agar plate with Fast-Media® Blas Agar. The plates were kept in an incubator (Heratherm Incubator, Thermo Scientific) at 37°C overnight. The next morning, plates were sealed and kept at 4°C. In the afternoon, one single colony was picked from each plate and according to their antibiotic resistance either inoculated in 4 ml of Terrific Broth (TB)-based liquid medium supplemented with blasticidin (Fast-Media® Blas TB, Invivogen) or in 4 ml TB-based liquid medium supplemented with zeocin (Fast-Media® Zeo TB, Invivogen). Tubes were placed on an orbital shaker (Forma Scientific), rotating at 225 rpm at 37°C. The next day, 3.5 ml of culture was taken to purify plasmids via plasmid miniprep using the NucleoSpin® Plasmid QuickPure Kit (Macherey-Nagel GmbH & Co. KG). The quality of the purified plasmids was verified via restriction enzyme digest. (section 2.3.6). After confirmation of good quality of the plasmids, the remaining 500 µl of the bacterial cultures were inoculated in large scale in 35 ml Fast-Media® Zeo TB and 35 ml Fast-Media® Blas TB, respectively. The next day, a Plasmid *Plus* Midi Kit (Qiagen) was performed to purify the plasmids.

2.3.3 Glycerol stock preparation

Glycerol stocks were prepared for all transformed bacterial cultures, for BL21 Gen-X *E. coli* transformed with K-CTcc-pET22b-GFPII and JM109 transformed with pFUSE-61p1E2-Gamma or pFUSE-61p1E2-Lambda. A 2 ml screw cap tube was labelled with a printed label with the name of the cloned bacteria and antibiotic resistance along with date and initials. Glycerol stocks of 30-40% Glycerol concentration were prepared. The bacterial culture was grown overnight, the next morning transferred to the screw cap tube, and Glycerol BioXtra (Sigma-Aldrich) was added up to a final volume of 2 ml.

2.3.4 Plasmid purification

2.3.4.1 Miniprep

Plasmids from 2-4 ml overnight grown bacterial culture were purified using the NucleoSpin® Plasmid QuickPure Kit (Macherey-Nagel GmbH & Co. KG). Manufacturer's instructions were followed.

2.3.4.2 Midiprep

Midipreps were performed for plasmid purifications from 20-35 ml bacterial cultures as per manufactures' instructions using the Plasmid Plus Midi Kit (Qiagen). In short, bacteria were grown 12-16 h by constantly shaking at 225 rpm at 37°C. In the morning, bacterial cultures were pelleted at 6000 x g for 15 min at 4°C with the Avanti™ J-25 Centrifuge, JA-12 Rotor (Beckman).

2.3.4.3 Measurement of concentration of purified plasmids

The concentration of the purified plasmids was measured with the Nanodrop1000 Spectrophotometer (Thermo Scientific). Briefly, 2 µl of ultrapure water was loaded on the device to initialize the spectrometer. Then, 2 µl of "Blank", the buffer in which the sample was eluted, was loaded. After the machine had been calibrated, 2 µl of sample was loaded.

2.3.5 Quality control: antibiotic resistance selection

Transformed bacteria were streaked out on three different agar plates containing either the antibiotic ampicillin, zeocin or blasticidin (Fast-Media® Amp Agar, Fast-Media® Blas Agar, Fast-Media® Zeo Agar; Invivogen). The plates were incubated at 37˚C overnight (Heratherm Incubator, Thermo Scientific).

2.3.6 Quality control: restriction enzyme digest

The volume of each reaction was 20 µl. Manufacturers' instructions were followed. Shortly, the reactions were prepared in sterile 1.5 ml tubes. 10x FastDigest Buffer (Thermo Scientific) was added to a final concentration of 1x. For 2-3 µg of plasmid, 2-3 µl of FastDigest™ enzymes (Thermo Scientific) were used. UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher Scientific) was added to a final volume of 20 µl. Single- and double digests of plasmids were

performed. Samples were kept in a waterbath at 37°C for 30 min. After the waterbath, samples were immediately transferred to a heatblock at 80°C for 10 min to inactivate the enzymes. Then, samples were mixed with 6x DNA Loading Dye (Thermo Scientific) to a final concentration of 1x. Subsequently, samples were loaded on a 1% UltraPure™ Agarose gel. As a negative control, 2-3 µg of undigested plasmids mixed with UltraPure™ DNase/RNase-Free Distilled Water to add up to a volume of 20 µl and 6x DNA Loading Dye were loaded. Gene Ruler 1 kb Plus DNA Ladder (Cat#SM1331, ThermoFisher Scientific) was used as marker for the quality control of plasmid K-CTcc-pET22b-GFPII and Gene Ruler Express DNA Ladder (Cat#SM1553, ThermoFisher Scientific) was used as marker for the gel of the plasmids from the pFUSE family. Gels were run at 80-90 Volts for about 1-1.5 h.

Table 2 List of restriction enzymes

Restriction enzymes	Cleavage site	Catalog number
HindIII	5' A↓AGC T T 3' 3' T TCGA↑A 5'	FD0505
EcoRI	5' G↓AAT T C 3' 3' C TTAA↑G 5'	FD0274
NheI	5' G↓CTA G C 3' 3' C GATC↑G 5'	FD0973
XmaJI, AvrII	5' C↓CTA G G 3' 3' G GATC↑C 5'	FD1564
BshTI, AgeI	5' A↓CCG G T 3' 3' T GGCC↑A 5'	FD1464

2.3.6.1 Agarose gel preparation

An UltraPure™ Agarose gel (Invitrogen) was prepared according to manufacturers' instructions. Briefly, an open, autoclaved flask was filled with 50-150 ml Tris-acetate-EDTA (TAE) buffer. UltraPure™ Agarose was added to a final concentration of 1%. The solution was heated for one min in a microwave. After cooling, SYBRO Safe Gel Stain 10 000x (Cat#S33102, Invitrogen) was added to a final concentration of 1x.

2.3.6.2 Gel pictures

All gel pictures were taken under UV light with the Molecular Imager® Gel Doc XR System (Bio-Rad).

2.3.6.3 Fragment lengths

Table 3 Single digested K-CTcc-pET22b-GFPII

Restriction enzymes	Fragment length
EcoRI, NheI or HindIII	6,279 bp

Table 4 Double digested K-CTcc-pET22b-GFPII

Restriction enzymes	Fragment length	Fragment
EcoRI, NheI	5,508 bp, 771 bp	mWasabi (GFPII)
NheI, HindIII	5,418 bp, 861 bp	K-CTcc and mWasabi
EcoRI, HindIII	6,189 bp, 90 bp	K-CTcc

Table 5 Single digested pFUSE-61p1E2-Gamma

Restriction enzyme	Fragment length
EcoRI	4,822 bp

Table 6 Double digested pFUSE-61p1E2-Gamma

Restriction enzymes	Fragment length	Fragment
EcoRI, NheI	4,408 bp, 414 bp	Variable heavy chain

Table 7 Single digested pFUSE-61p1E2- Lambda

Restriction enzyme	Fragment length
AvrII (XmaJI)	4,154 bp

Table 8 Double digested pFUSE-61p1E2- Lambda

Restriction enzymes	Fragment length	Fragment
AvrII (XmaJI), AgeI (BshTI)	3,761 bp, 393 bp	Variable light chain

2.3.7 Quality control: sequencing

Purified plasmids were sent for Sanger sequencing to the company GENEWIZ. The samples were prepared according to their Sample Submission Guidelines. Briefly, 5 µl of 5 µM primer and 10 µl of 500 ng plasmid sample diluted in UltraPure™ DNase/RNase-Free Distilled Water were mixed in a PCR tube. Two PCR reactions were prepared for each plasmid. In one reaction a reverse primer was added and into the other reaction a forward primer was added. The primers were kindly provided by Dr. Ruprecht's lab.

Table 9 Primers for sequencing of pFUSE-61p1E2-Gamma

Primer name	Sequence
pFUSE-gamma-seq-rev	5'GCCGCTGTGCCCCCAGAGGTGC3'
pFUSE-seq-fw	5'TGTGACCGGCGCCTACC3'

Table 10 Primers for sequencing of pFUSE-61p1E2-Lambda

Primer name	Sequence
pFUSE-lambda-seq-rev	5'TTGTTGGCTTGAAGCTCCTCAGA3'
pFUSE-seq-fw	5'TGTGACCGGCGCCTACC3'

2.4 Expression and purification of K-CTcc

IPTG-induced *E. coli* pellets from 25 ml bacterial culture were stored at -20°C, as described in section 2.3.1. The pellet was resuspended in 10 ml SoluLyse (Genlantis) supplemented with 100x Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Then, a centrifugation step at 16,128 x g for 20 min at Rtemp in the Avanti™ J-25 Centrifuge, JA-12 Rotor (Beckman) was carried out. Supernatant was transferred to a 50 ml Falcon tube and an equal volume of Diluting Buffer was added. This solution was filtered through the Steriflip-HV top filter with 0.22 µm pore size (EMD Millipore). The bacterial pellets were discarded. After the filtration of the supernatant, 100x Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) was added. After that, 2 ml of 1:1 Slurry ProBond™ Nickel-Chelating Resin (Invitrogen) and Washing Buffer A were then added and the solution was kept on an orbital shaker at 4°C for 20 min. Solution was loaded on a 10 ml Pierce™ Column (Thermo Scientific) and set on vacuum manifold. After the lysate drained through the beads, the column was washed with 10 ml of Washing Buffer A and 10 ml of Washing Buffer B. K-CTcc was eluted with 10 ml of Elution Buffer in a 50 ml Falcon™ tube. To restore the column, 20 ml of 50 mM Na-phosphate buffer pH 8.0, 1 M NaCl were added and set on a vacuum manifold. Afterwards, the column was washed with 20 ml of ultrapure water. The column was stored in 0.02% NaN_3.

2.4.1 Buffer exchange and concentrating the protein

To replace the Elution Buffer of the eluted protein sample with PBS, pH 7.2 (Thermo Scientific), Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane, 10 kDa (EMD Millipore) were used and three dilution-concentration steps were carried out. Briefly, protein elute was loaded on the Millipore Centrifugal Filter, followed by a centrifugation step at 3000 x g with the Sorvall Legend™ XTR centrifuge, TX-750 rotor (Thermo Scientific) for 10 min. Then, 15 ml of PBS were loaded onto the filter and the centrifugation step at 3000 x g was repeated. This step was done twice. The protein sample was sterilized by filtration through a 0.22 µm filter.

2.5 Production of mAb 61p1E2 in mammalian cells and purification

2.5.1 Maintenance of Expi293F™ cells

Expi293F™ cells (Thermo Fisher Scientific) were cultured in 30-60 ml of Expi293™ Expression Medium (Thermo Fisher Scientific) in Nalgene™ Single-Use PETG Erlenmeyer Flasks (Thermo Scientific). These flasks were kept at 37°C in the Reach-In CO_2 Incubator (Thermo Scientific) with 8% CO_2 constantly shaking at 135 rpm on the MaxQ™ HP Tabletop Orbital Shaker (Thermo Scientific). The cells were passaged twice a week. Briefly, the cells were counted by mixing 40 µl of cell culture and 40 µl of Trypan Blue Stain 0.4% (Cat#15250-061, Life Technologies) together. A volume of 10 µl was transferred into the Countess™ cell counting chamber slide (Invitrogen) and read by the automated cell counter Countess II (Life Technologies). For maintaining the cells, 15-30 million cells were transferred to a 50 ml Falcon tube and centrifuged at 1000 rpm for 10 min with the Sorvall Legend™ centrifuge, TX-750 rotor (Thermo Scientific). The supernatant was discarded and the cell pellet was resuspended in 30-60 ml fresh Expi293™ Expression Medium, prewarmed to 37°C to give a cell density of 0.5 million cells/ml.

2.5.2 Transfection of Expi293F™ cells

Expi293F™ cells (Cat#A14527, Thermo Fisher Scientific) were transfected with the plasmids pFUSE-61p1E2-Gamma and pFUSE-61p1E2-Lambda to produce full length mAb 61p1E2. This 293 cell line is a permanent line established from HEK cells. Two different transfection reagents were used to transfect the Expi293F™ cells. The ExpiFectamine™ 293 Transfection kit (Cat#A14524,Thermo Fisher Scientific) and TransIT-PRO® Transfection Reagent and Kit (Cat#MIR 5700, Mirus Bio LLC). Both reagents form lipid-polymer complexes with the plasmids to allow cellular uptake. First, small-scale transfections of 30 ml Expi293F™ culture were performed and after verifying the successful production of antibodies in the supernatant via SDS-PAGE and ELISA, large-scale transfections of 200 ml Expi293F™ culture were carried out.

2.5.2.1 ExpiFectamine™ 293 Transfection kit (Thermo Fisher Scientific)

Manufacturer's instructions were followed. Briefly, cells were seeded at a density of 2 x 10^6 viable cells/ml on the day before transfection. Therefore, cells were counted with the automated cell counter Countess II (Life Technologies). The number of cells needed was transferred into 50 ml Falcon tubes and centrifuged at 1000 rpm for 10 min with the Sorvall Legend™ centrifuge, TX-750 rotor (Thermo Scientific). The supernatant was discarded and the cell pellet was resuspended in Expi293™ Expression Medium (Thermo Fisher Scientific), which was prewarmed to 37°C in the Isotemp waterbath (Fisher Scientific). Then, the culture was transferred to Nalgene™ Single-Use PETG Erlenmeyer Flasks (Thermo Scientific). Flasks were kept at 37°C in the Reach-In CO_2 Incubator (Thermo Scientific) with 8% CO_2, shaking at 135 rpm overnight. The next day, a cell count was performed and cell cultures were adjusted to a density of 2.5 x 10^6 cells/ml by discarding the calculated amount of cell culture and replacing it with fresh, prewarmed Expi293™ Expression Medium (Thermo Fisher Scientific). Then, flasks were kept back in the incubator and transfection reagent was prepared. In short, for a transfection size of 200 ml cell culture, 102 µg of pFUSE-61p1E2-Gamma and 98 µg pFUSE-61p1E2-Lambda were diluted in 10 ml of Opti-MEM™ I Reduced Serum Medium (Cat#31985-062, Thermo Fisher Scientific) in a sterile 50 ml Falcon tube. In another 50 ml Falcon tube, 534 µl ExpiFectamine™ 293 Reagent was added to 10 ml of Opti-MEM™ I Reduced Serum Medium and incubated at Rtemp for 5 min. Afterwards, both solutions were combined to a total of about 20 ml and incubated at Rtemp for 30 min. After the incubation time, the reaction was transferred to the 200 ml cell culture. The next day, 1 ml of Enhancer 1 and 10 ml of Enhancer 2 were added.

2.5.2.2 TransIT-PRO® Transfection Reagent & Kit (Mirus Bio LLC)

Manufacturer's instructions were followed. In short, cells were seeded to a density of 2 x 10^6 viable cells/ml on the day of transfection and kept back into the incubator. Then, transfection reagent was prepared. Instead of Opti-MEM™ I Reduced Serum Medium, Expi293™ Expression Medium was used. For a transfection size of 200 ml cell culture, 20 ml of Expi293™ Expression Medium were mixed with 200 µg plasmid DNA, which means 101 µg of pFUSE-61p1E2-Gamma and 99 µg of pFUSE-61p1E2-Lambda, and 200 µl of TransIT-PRO® Reagent. Then, the solution was incubated at Rtemp for 20 min. Afterwards, 100 µl of PRO Boost Reagent was added and

solution was incubated at Rtemp for ten more min. After this incubation time, reaction was transferred to the 200 ml of cell culture.

2.5.2.3 Comparison between transfection reagents

To make a side-per-side comparison of these two transfection reagents, four flasks of 200 ml Expi293F™ cells were transfected with the reagent ExpiFectamine™ 293 and five flasks of 200 ml Expi293F™ cells were transfected with the TransIT-PRO® Transfection Kit. After the transfection day, 2 ml of transfected Expi293F™ cell culture was collected every day. Cells were counted with the automated cell counter Countess II (Life Technologies). After the cell count, cell culture was centrifuged at 3000 rpm for 10 min with the Sorvall Legend™ Micro 21 Microcentrifuge (Thermo Scientific). The cell pellet was discarded and supernatant was transferred into a sterile 2 ml tube. All supernatants were kept at 4°C immediately after the collection. Cell viability count in percent was plotted in a graph using GraphPad Prism (version 7.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The quality of produced antibodies was measured via SDS-PAGE and western blot.

2.5.3 Harvest of antibodies

Antibodies were harvested when the cell viability of transfected Expi293F™ cells was reduced to 60% or below. This was usually after six to seven days after the transfection day. To harvest the antibodies from the supernatant of the Expi293F™ cells, the cell culture was split into sterile 50 ml Falcon tubes and centrifuged at 1000 rpm for 10 min at Rtemp with the Sorvall Legend™ centrifuge, TX-750 rotor (Thermo Scientific). The supernatant was transferred to new 50 ml Falcon tubes and the cell pellets were discarded. Another centrifugation step was carried out at 10 000 x g for 10 min at 4C˚ with the Avanti™ J-25 Centrifuge, JA-12 Rotor (Beckman). Afterwards, supernatant was filtered using a vacuum filter system (Corning). Sterile, filtered supernatant was kept at 4°C until antibody purifications via protein A chromatography were performed.

2.5.4 Antibody purification by protein A affinity chromatography

The mAb 61p1E2 was purified from the filtered supernatant of Expi293F™ cells by using the BioLogic LP low-pressure chromatography system (Bio-Rad). Supernatant was slowly passed through a protein A column (GE- Health Care Life Sciences). The Fc portion of mAb 61p1E2 bound to the protein A. Molecules that bound un-specifically bindings were removed by washing the column with PBS, pH 7.2. mAb 61p1E2 was eluted with Elution Buffer, pH 2.7. Eluted antibody was neutralized to a pH of 7 with Neutralization Buffer, pH 12. Afterwards, the system was washed with PBS and stored in 70% ethanol.

2.5.4.1 Buffer exchange and concentrating the antibody

To replace the Elution Buffer with PBS, pH 7.2 and to concentrate the antibody sample, an Amicon Ultra-15 Centrifugal Filter Unit with an Ultracel-50 membrane (EMD Millipore) were used, and three dilution-concentration steps were carried out. Briefly, the antibody eluate was loaded on the Millipore Centrifugal Filter, followed by a centrifugation step at 3000 x g with the Sorvall Legend™ XTR centrifuge, TX-750 rotor (Thermo Scientific) for 10 min. Then, 15 ml of PBS were loaded onto the filter and a centrifugation step at 3000 x g for 20 min was carried out. This step was repeated twice. Purified mAb 61p1E2 in PBS was kept in a sterile 2 ml tube.

2.6 Fab fragments of mAb 61p1E2

Fab fragments of mAb 61p1E2 were produced in order to send them together with the bait protein GFP-gp41 PL-mimotope fusion protein, K-CTcc for X-ray crystallography.

2.6.1 Size exclusion chromatography

A Zeba Spin Desalting Column (Thermo Scientific) was prepared and used for desalting and buffer exchange of the mAb 61p1E2 sample stored in PBS. The Zeba Spin Desalting Column contained a size-exclusion chromatographic resin to separate the IgGs from small molecules. Four mg of mAb 61p1 E2 sample was slowly loaded onto the column. To obtain a final volume of 500 µl, 136 µl of Digestion Buffer was added slowly to the column. The Zeba Spin Desalting Column was centrifuged at 1000 x g for two min with the Sorvall Legend™ centrifuge, TX-750 rotor (Thermo Scientific) to collect the IgGs in the flow through.

2.6.2 Papain digestion

Fabs of mAb 61p1 E2 were generated by using the Thermo Scientific™ Pierce™ Fab Preparation Kit (Cat#44985, Thermo Scientific). The principle of this method is the digestion of the antibody via papain, a non-specific thiol-endopeptidase. The digestion with papain produces two 50 kDa Fab fragments and one Fc fragment. Manufactures' instructions were followed. Briefly, digestion buffer was prepared immediately before use. The pH of the digestion buffer was measured via pH strips. After adding cysteine-HCl, the pH was around 6. To obtain a pH of 7, additional Fab digestion buffer, which has a pH of 10, was added. Then, the spin-column was prepared by adding 250 µl of the immobilized papain (Cat#1851680, Thermo Scientific) to the column. Centrifugation was carried out at 5000 x g for 1 min with the Sorvall Legend™ Micro 21 Microcentrifuge (Thermo Scientific). After that, buffer was discarded and a washing step with 500 µl of digestion buffer was performed. Centrifugation step at 5000 x g for 1 min was repeated. Desalted mAb 61p1E2 was loaded on the spin column containing the immobilized papain. Spin column was placed in an end-over-end mixer located in an incubator (Heratherm Incubator, Thermo Scientific) at 37°C for five h. After the incubation time, the column was centrifuged at 5000 x g for 1 min to separate the digested mAb 61p1E2 from the immobilized papain. A further washing step was carried out with 500 µl of PBS. Then, flow-through fractions were combined and a total volume of 1 ml sample was achieved. This sample contains Fab

fragments and Fc portions of the mAb 61p1E2. To separate Fabs and Fc portions, the NAb Protein A Plus Spin Column (Thermo Scientific) was used. First, column needed to be prepared. NAb Protein A Plus Spin Column was centrifuged Sorvall Legend™ centrifuge, TX-750 rotor (Thermo Scientific) at 1000 x g for one min to remove the storage solution. Two ml Elution Buffer were added before washing the column twice with 2 ml PBS as per protocol to make sure that the capacity of the column was fully given. After column was equilibrated with PBS, 1 ml of digested mAb 61p1E2 was added and column was placed on an end-over-end mixer at Rtemp for 10 min. After the incubation time, column was centrifuged at 1000 x g for 1 min and Fab fragments were collected in the flow-through. The column was washed twice with 1 ml of PBS and wash fractions were combined with the flow-through. The total volume of the Fab fragment sample is about 3 ml. The concentration was measured via a Nanodrop1000 Spectrophotometer (Thermo Scientific) using PBS as blank. Then, the sample was stored at 4°C until concentrating the sample and performing a buffer exchange on the following day. Fc portions and undigested IgGs were eluted by adding three times 1 ml IgG Elution Buffer. Afterwards, the NAb Protein A Plus Spin Column was regenerated.

2.6.3 Buffer exchange and concentration of Fab sample

The buffer PBS was replaced with sodium chloride-Tris-EDTA (STE) Buffer, pH 8.0, (Cat#BP2478500, Fisher BioReagents), because the phosphate in PBS may interfere with the crystallization. Buffer exchange was carried out using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane, 10 kDa (EMD Millipore).

2.7 Characterization of mAb 61p1E2 and Fab-mAb 61p1E2

2.7.1 ELISA

ELISAs were performed to verify the antibody production of mAb 61p1E2 in Expi293F™ cells. Therefore, the supernatant as well as the purified antibody were tested. Furthermore, the antigen specificity of mAb 61p1E2 against envelope proteins of HIV was tested via ELISA. Another ELISA was performed to test the quality of the Fabs after digestion of mAb 61p1E2 via papain.

A Nunc MaxiSorp® flat-bottom 96-well plate (Affymetrix e Biosciece) was coated with the glycoprotein 41 (gp41), gp120, gp140 and gp160 as well as with the "bait" protein K-CTcc. As negative controls were used, the scrambled bait protein Sc-K-CTcc, Coating Buffer and Expi293™ Expression Medium (Cat#A1435101, Thermo Fisher Scientific) (Table 11). All proteins were diluted in Coating Buffer, pH 9.6 to a final concentration of 1 µg/ml. 50 µl of each protein dilution were added per well (50 µl/well). The plate was covered with a plastic foil and kept at 4°C overnight. The next day, the plate was washed three times with PBST using the ELx405™ Microplate Washer (BioTek). Then, 300 µl/well of Blocking Buffer were added. The plate was covered and put into the incubator (Heratherm Incubator, Thermo Scientific) at 37°C for two h. After the incubation time, the plate was washed three times with PBST. Primary antibodies were diluted in PBST to a final concentration of 2 µg/ml and then, 100 µl/well of each primary antibody dilution was added to the plate (Table 12). For testing the supernatant of transfected Expi293F™ cells, 100 µl/well of supernatant was added. The plate was covered and kept back into the incubator at 37°C for one h. Afterwards, plate was washed six times with PBST and then, 100 µl/well of the secondary antibody was added (Table 13). The secondary antibody was diluted to factor 1:5000 in PBST. The plate was incubated for one h at 37°C. After the incubation time, the plate was washed six times with PBST. To develop the plate, 100µl/well of TMB solution (Life Technologies) was added. The reaction was stopped by adding 100 µl/well of 1 N sulfuric acid (H_2SO_4). The optical density (OD) was determined at 450 nm by using a multiwell plate reader (Berthold Technologies).

Table 11 Antigens used for coating

proteins	detailed name	source
gp41 (no glycosylation)	HIV-1 gp41 MN (*E. coli*)	Cat#12027, NIH AIDS Reagent Program
gp120	HIV-1 CN54 gp120	Cat#7749, NIH AIDS Reagent Program
gp140	HIV-1 CN54 gp140	Cat#12064, NIH AIDS Reagent Program
gp160	HIV-1 envelope gp160 1084i	Dr. Ruprecht's lab
K-CTcc	GFP-gp41 PL-mimotope fusion protein	Dr. Ruprecht's lab
Sc-K-CTcc	scrambled GFP-gp41 PL-mimotope fusion protein	Dr. Ruprecht's lab

Table 12 List of primary antibodies

mAb name	mAb specifity	references
mAb 61p1E2		Dr. Ruprecht's lab
Fab- 61p1E2		Dr. Ruprecht's lab
mAb Fm-6	anti-SARS antibody	(Sui, Aird et al. 2008)
mAb F240	anti-HIV-1 gp41 mAb antibody	Cat.#7623 NIH AIDS Reagent Program (Cavacini, Emes et al. 1998)
mAb 33C6	anti-HIV-1 gp120 mAb antibody	(Sholukh, Mukhtar et al. 2012b)

Table 13 List of secondary antibodies

Peroxidase AffiniPure goat anti-human IgG	(Cat#109-035-088, Jackson ImmunoResearch)
Peroxidase AffiniPure goat anti-human IgG, $F(ab')_2$ fragment specific	(Cat#109-035-097, Jackson ImmunoResearch)

2.7.2 SDS-PAGE

SDS-PAGEs were performed to verify the production of the mAb 61p1E2 into the supernatant of Expi293F™ cells, for the assessment of the efficiency of the antibody purification via protein A chromatography, and for the quality control of the Fab fragments after the digestion of mAb 61p1E2 via papain.

Non-reduced samples of supernatant of transfected Expi293F™ cells were prepared by mixing 32 µl of supernatant together with 8 µl Pierce™ Lane Marker Reducing Sample Buffer (Cat#39000,Thermo Fisher Scientific). For details of the procedure how the supernatant was obtained, please refer to (section 2.5.3). Non-reduced samples of purified antibodies and Fab fragments were prepared by diluting 4 µg of antibody or Fabs in PBS to a total volume of 32 µl. Then, 8 µl of Pierce™ Lane Marker Reducing Sample Buffer were added. As positive control, the mAb VRC01, an anti-HIV gp120 mAb that recognizes the CD4 binding site, was used. VRC01 was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (Wu, Yang et al. 2010). To the reduced samples, 2 µl of β-Mercaptoethanol was added in addition. All samples were boiled at 95C˚ for 10 min on a heat block. After boiling, samples were loaded on a 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel (Cat #4561084, Bio-Rad). The marker PageRuler™ Prestained Protein Ladder (Cat #26616,Thermo Scientific) was used. The gel was run at 200 Volts for about 30 min. Afterwards, the gel was washed with ultrapure water and stained with GelCode Blue Stain Reagent (ThermoScientific) overnight. The next day, destaining was carried out with ultrapure water.

2.7.3 Western blot

The samples were prepared as described in the section (2.7.2). After running a SDS-PAGE, the gel was not stained. The proteins on the gel were transferred to a nitrocellulose membrane (iBlot® Transfer Stack, Cat#IB301002, Gibco Life Technologies) using the iBlot® Gel Transfer Device (Gibco Life Technologies) according to the manufacturer's instructions. The membrane was kept in 4% Blocking Buffer for at least two h on a table top shaker. After blocking, washing of the membrane was carried out with PBST and then the secondary antibody was added. For the detection of full length mAb 61p1E2, Peroxidase AffiniPure goat anti-human IgG (Cat#109-035-088, Jackson ImmunoResearch), diluted 1:5000 in PBST, was added and for the detection

of Fab fragments, Peroxidase AffiniPure goat anti-human IgG, $F(ab')_2$ fragment specific (Cat#109-035-097, Jackson ImmunoResearch), diluted 1:5000 in PBST, was added to the membrane. The membrane was incubated with the secondary antibody for one h on a table top shaker. Afterwards, three washing steps with PBST for 10 min each were carried out. The membrane was developed using the Opti-4CN™ Substrate Kit (Bio-Rad).

3 RESULTS

Overall summary: A good amount of the recombinant bait protein K-CTcc was expressed and purified. mAb 61p1E2 was produced in Expi293F cells and purified by protein A chromatography. In total, 28.23 mg of antibody was obtained. To produce Fabs, 12 mg of mAb 61p1E2 were digested with the enzyme papain and afterwards Fabs were sent together with the bait protein for crystallization.

3.1 Recombinant bait protein K-CTcc

The sequence of the recombinant protein K-CTcc, along with a 6x His-tag and GFPII, also known as mWasabi, was cloned by the Ruprecht lab into a pET-22b (+) vector. This protein was obtained by phage display from the vaccine-protected RM RTr-11 (Bachler, Humbert et al. 2013). mAb 61p1E2 binds specific to K-CTcc, a region of gp41. The plasmid K-CTcc-pET22b-GFPII was expressed in BL21 Gen-X™ *E. coli* (Fig. 9).

3.1.1 IPTG induction of BL21 Gen-X™ *E. coli*

BL21 Gen-X™ *E. coli* transformed with plasmid K-CTcc-pET22b-GFPII was induced with 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) solution. This culture was used for the purification of the bait protein K-CTcc. Another culture of BL21 Gen-X™ *E. coli* transformed with K-CTcc-pET22b-GFPII was not induced with IPTG and was used to isolate the plasmid K-CTcc-pET22b-GFPII.

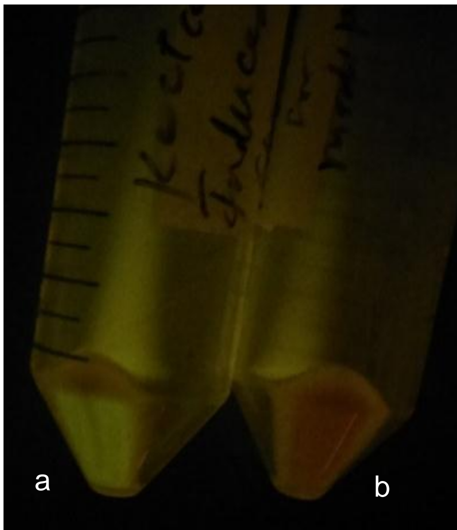

Fig. 9 IPTG induction of BL21 Gen-X™ *E. coli* transformed with K-CTcc-pET22b-GFPII

The picture shows the comparison of the IPTG-induced bacterial cell pellet (Fig. 9a) and the IPTG non-induced pellet (Fig. 9b). The induction of BL21 Gen-X™ *E. coli* transformed with K-CTcc-pET22b-GFPII with IPTG showed a significant difference in the fluorescence in comparison to non-induced bacterial culture under Blue LED light due to the expression of the recombinant protein K-CTcc in the induced culture.

Fig. 10 Lysed IPTG-induced bacterial pellet

The IPTG-induced bacterial pellet of BL21 Gen-X™ *E. coli* transformed with K-CTcc-pET22b-GFPII was lysed with SoluLyse and bright fluorescence was seen under Blue LED light.

3.1.2 Plasmid purification of K-CTcc-pET22b-GFPII

First, small-scale plasmid purifications were performed via miniprep. After verifying the quality of the purified plasmids, larger bacterial cultures were inoculated and plasmid midipreps were performed. On average, the yield of the plasmid miniprep was 150 ng/µl eluted in 50 µl of TE Buffer (10 mM Tris, 1 mM EDTA). The yield of purified plasmids by using the midiprep was about 500 ng/µl in 200 µl Buffer TE (Table 14).

Table 14 Yield of plasmid purifications of K-CTcc-pET22b-GFPII

Method used	Concentration	Volume of elution	Plasmid yield
miniprep	150 ng/µl	50 µl	7.5 µg
midiprep	500 ng/µl	200 µl	100 µg

3.1.3 Quality control: restriction enzyme digest

To verify the quality of the expressed and purified plasmid K-CTcc-pET22b-GFP II, a restriction enzyme digest was carried out. Therefore, the plasmid was cut with the restriction enzymes EcoRI, NheI and HindIII. Undigested plasmid, single-digested and double-digested plasmid K-CTcc-pET22b-GFPII was loaded onto a 1% agarose gel. The size of the excised fragments was as expected. The quality of the purified plasmids was good. No contamination and degradation was detected.

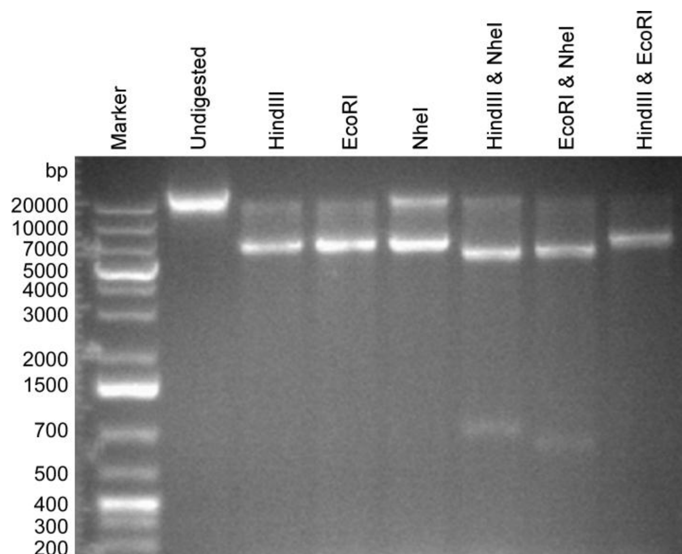

Fig. 11 Restriction enzyme digests of plasmid K-CTcc-pET22b-GFPII

Undigested plasmid, single-digested and double-digested plasmid samples of K-CTcc-pET22b-GFPII were loaded onto a 1% agarose gel. The undigested plasmid sample ("Undigested") can be seen in lane two. Single-digested plasmids were either digested with the restriction enzymes HindIII, EcoRI or NheI. These samples were loaded into lanes three to five. The size of the single-digested plasmid is 6,279 bp. The double-digested plasmid in lane six was digested with HindIII and NheI and shows a fragment of 861 bp. This fragment encodes the recombinant protein K-CTcc and the protein mWasabi (GFPII). The plasmid K-CTcc-pET22b-GFPII in lane seven was digested with the enzymes EcoRI and NheI and shows a fragment of 771 bp, which encodes mWasabi (GFPII). The fragment of 90 bp expected after double-digestion of K-CTcc-pET22b-GFPII with the enzymes HindIII and EcoRI is too small to be detected on a 1% agarose gel. The Gene Ruler 1 kb Plus DNA Ladder (Thermo Scientific) was used as marker. The gel was visualized under UV-light.

3.1.4. Quality control: antibiotic resistance

Table 15 Bacterial growth on antibiotic selection plates

Antibiotic on agar plates	Bacterial growth on agar plates		
	BL21 transformed with K-CTcc-pET22b-GFPII	JM 109 transformed with pFUSE-61p1E2-Lambda	JM 109 transformed with pFUSE-61p1E2-Gamma
Blasticidin	-	+	-
Ampicillin	+	-	-
Zeocin	-	-	+

Antibiotic resistance of transformed bacterial colonies was tested by streaking out bacterial colonies on different selective agar plates. Plates were incubated at 37˚C overnight. The next morning, plates were checked for bacterial growth. The presence or absence of bacterial colonies observed on the plates was marked +/-. Transformed bacterial colonies only grow on agar plates corresponding to the antibiotic selection gene inserted in the plasmid.

3.2 Production of mAb 61p1E2 in Expi293F cells

The anti-HIV gp41 chimeric simian-human IgG1 mAb 61p1E2 was produced in Expi293F cells, a cell line which is derived from human embryonic kidney cells. Thus, Expi293F cells were transfected with plasmid pFUSE-61p1E2-Gamma for the production of the heavy chain of mAb 61p1E2 and with pFUSE-61p1E2-Lambda for the production of the light chain of mAb 61p1E2. First, plasmids were grown JM109 competent cells in small scale and purified via miniprep. Then, quality of plasmids was verified via restriction enzyme digest and Sanger sequencing. If the quality of the plasmids was good, large scale midipreps were performed to purify the plasmids. When a significant amount of plasmids was obtained, Expi293F cells were transfected (section 2.5.2).

3.2.1 Plasmid purifications

Plasmids were grown in JM109 cells and purified via plasmid miniprep and midiprep. The yield by using the plasmid miniprep was approximately 160 ng/µl eluted in 50 µl of Buffer TE. The yield of purified plasmids by using the midiprep was about 3500 ng/µl in 200 µl TE Buffer (Table 16).

Table 16 Yield of plasmid purifications

Method used	Concentration	Volume of elution	Plasmid yield
miniprep	160 ng/µl	50 µl	8 µg
midiprep	3500 ng/µl	200 µl	700 µg

3.2.2 pFUSE-61p1E2-Gamma

3.2.2.1 Quality control: restriction enzyme digest

A restriction enzyme digest was carried out to verify the quality of the purified plasmid pFUSE-61p1E2-Gamma. The plasmids were cut with the enzymes EcoRI and NheI. Undigested, single-digested and double-digested plasmid samples were analyzed by agarose gel electrophoresis. The fragment sizes were as expected and good quality of the purified plasmid sample was confirmed (Fig. 12).

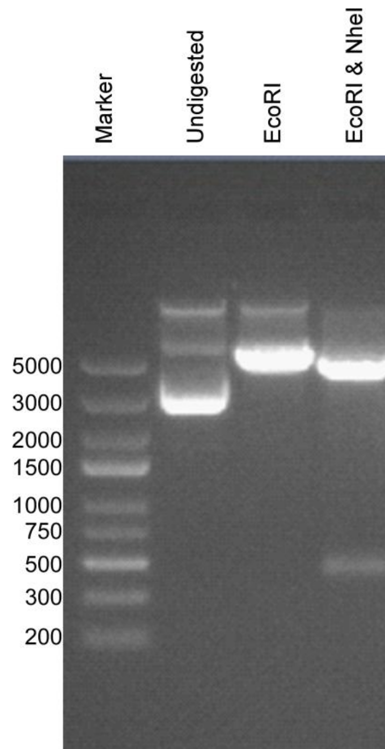

Fig. 12 Restriction enzyme digest of pFUSE-61p1E2-Gamma

Undigested, single-digested and double-digested plasmid samples of pFUSE-61p1E2-Gamma were loaded onto a 1% agarose gel. The second lane ("Undigested") shows the undigested plasmid pFUSE-61p1E2-Gamma. In the next lane, single-digested plasmid that was cut with the restriction enzyme EcoRI, was loaded. The size of the single-digested plasmid is 4,822 bp. The double-digested plasmid was digested with the enzymes EcoRI and NheI. The fragment is seen at 414 bp. This fragment encodes the variable heavy chain. The marker ("Marker") Gene Ruler Express DNA Ladder (Thermo Fisher Scientific) was used. The gel was visualized under UV-light.

3.2.2.2 Quality control: sequencing

The sequence of the VH insert was verified using Sanger sequencing. Thus, purified plasmid was sent to the company GENEWIZ. Samples were prepared according to their sample submission guidelines. Sequencing results were obtained the following day. Sequencing results were as expected and the good quality of the VH insert was confirmed.

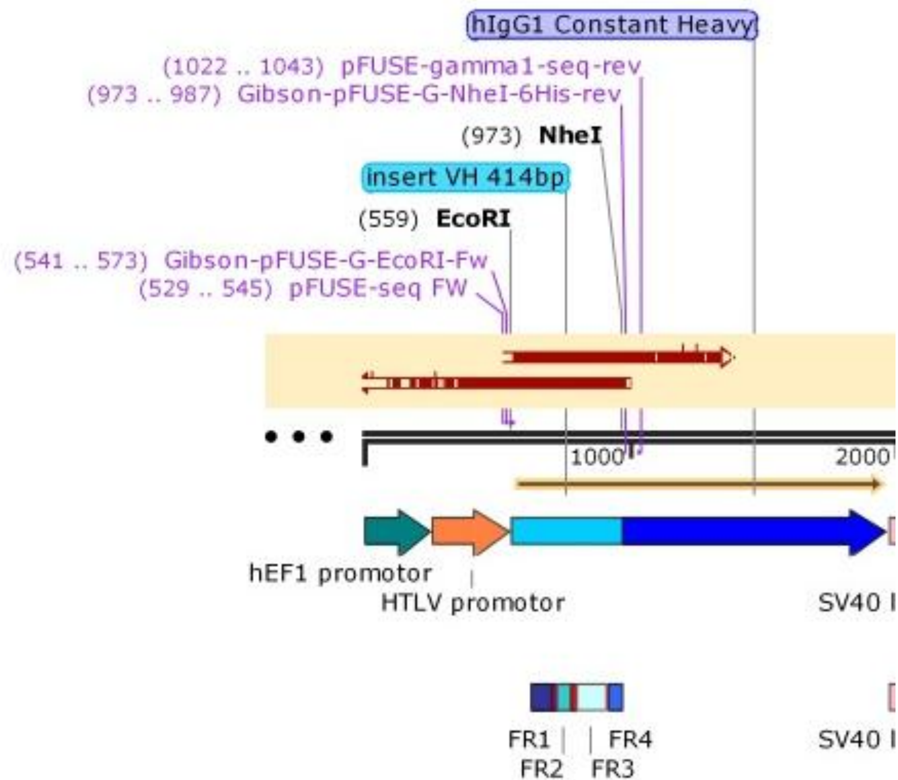

Fig. 13 Sequencing result of purified plasmid pFUSE-61p1E2-Gamma

Two sequencing reactions of plasmid pFUSE-61p1E2-Gamma were prepared, one reaction containing the forward primer, another reaction containing the reverse primer. Samples were sent to the company GENEWIZ. A multiple sequence alignment was performed to the sequence of VH using the SnapGene software (from GSL Biotech; available at snapgene.com). Matching sequences are shown in dark red, and non-matching sequences are seen as white gaps in between.

3.2.3 pFUSE-61p1E2-Lambda

3.2.3.1 Quality control: restriction enzyme digest

A restriction enzyme digest was carried out to verify the quality of the purified plasmid pFUSE-61p1E2-Lambda. Therefore, the plasmid was cut with the restriction enzyme AvrII, also termed XmaJI, and the restriction enzyme AgeI, also referred to as BshTI. Undigested, single-digested and double-digested plasmid samples were analyzed by agarose gel electrophoresis. The sizes of the excised fragments were as expected, thus confirming the good quality of the purified plasmid (Fig 14).

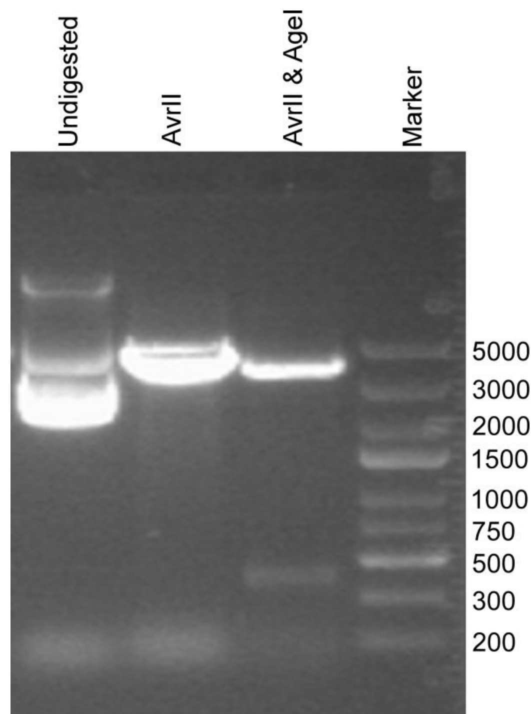

Fig. 14 Restriction enzyme digest of pFUSE-61p1E2-Lambda

Undigested, single-digested and double-digested plasmid samples of pFUSE-61p1E2-Lambda were loaded onto a 1% agarose gel. In the first lane, undigested plasmid pFUSE-61p1E2-Lambda (“Undigested”) was loaded. In the next lane, single-digested plasmid that was digested with the restriction enzyme AvrII is seen at 4,154 bp. The double-digested plasmid at lane three was digested with the enzymes AvrII and AgeI. It shows a fragment size of 393 bp. This fragment encodes for the variable light chain. Gene Ruler Express DNA Ladder (Thermo Fisher Scientific) was used as marker (“Marker”). The gel was visualized under UV-light.

3.2.3.2 Quality control: sequencing

To confirm the quality of the insert VL, the plasmid pFUSE-61p1E2-Lambda was sent for Sanger sequencing to the company GENEWIZ. Samples were prepared according to their sample submission guidelines. Sequencing results were obtained the following day. Sequencing results were as expected, and the good quality of the VL insert was confirmed.

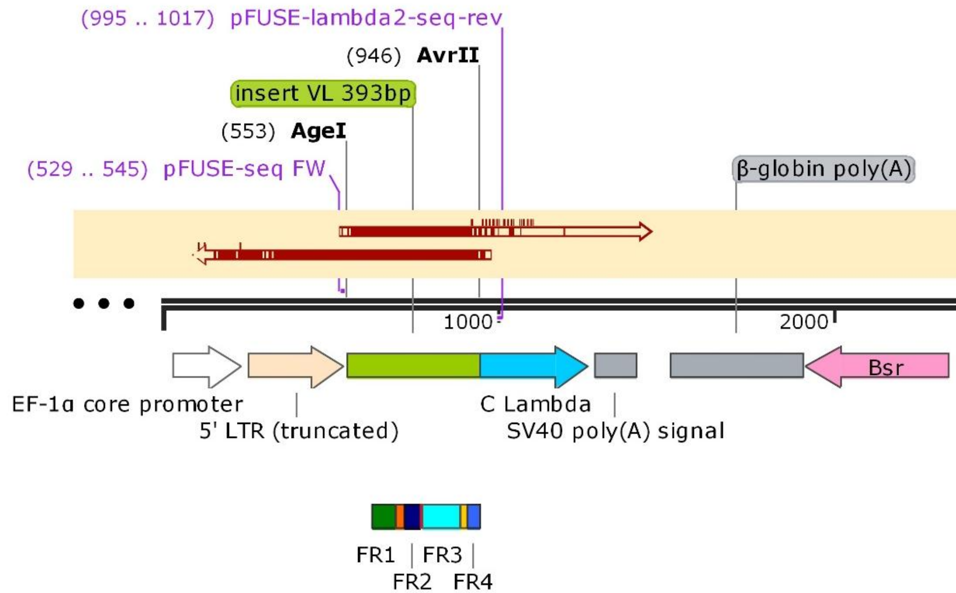

Fig. 15 Sequencing result of purified plasmid pFUSE-61p1E2-Lambda

Two sequencing reactions of plasmid pFUSE-61p1E2-Lambda were prepared, one reaction including the forward primer, another including the reverse primer. Samples were sent to the company GENEWIZ. Sequence alignment was performed using the SnapGene software (from GSL Biotech; available at snapgene.com). Matching sequences are shown in red, and non-matching sequences are shown as white gaps.

3.2.4 Transfection of Expi293F cells

Expi293F cells were transfected with plasmids pFUSE-61p1E2-Gamma and pFUSE-61p1E2-Lambda for the production of mAb 61p1E2 in the supernatant. The principle of this transfection system is based on the formation of lipid-polymer complexes together with the plasmids, which facilitates plasmid entry into the cell. First, the transfection reagent TransIT-PRO® was used. To test if mAb 61p1E2 was produced, SDS-PAGE and western blot analysis were carried out (Fig. 16 and Fig. 20). The production of mAb 61p1E2 was successful. To test if the antibody yield could be increased, another transfection reagent, termed ExpiFectamine™, was used (section 2.5.2). After confirming the presence of mAb 61p1E2 in Expi293F cell supernatant, mAb 61p1E2 was purified via protein A chromatography (section 2.5.4).

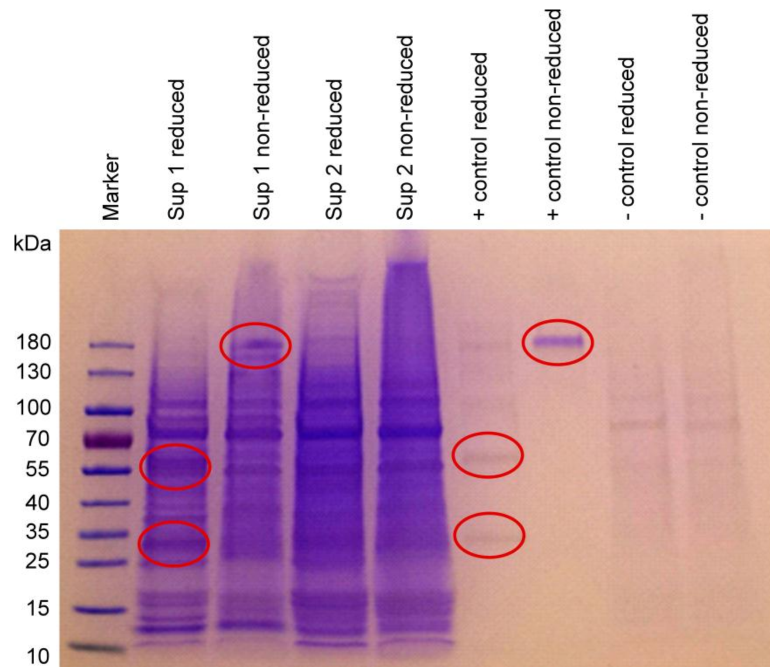

Fig. 16 SDS-PAGE of supernatant of transfected Expi293F cells

On day 7 after transfection, the supernatant of transfected Expi293F cells was harvested and tested for mAb 61p1E2 by SDS-PAGE. Supernatants from two cell cultures (Sup 1 and Sup 2) were mixed with the Pierce™ Lane Marker Reducing Sample Buffer, boiled and loaded on the gel. These two samples are referred to as "Sup 1 non-reduced" and "Sup 2 non-reduced." The mAb 61p1E2 can be seen at around 150 kDa-170 kDa in lane three ("Sup 1 non-reduced") and is marked with a red circle. In addition, β-mercaptoethanol was added to the samples (lanes "Sup 1 reduced" and "Sup 2 reduced"). The reduced antibodies were separated in their light and heavy chains and can be detected at around 25 kDa and 50 kDa. These bands can be seen in lane two ("Sup 1 reduced") and are marked with red circles. Due to excess of other cellular proteins in Sup 2, it cannot be distinguished between the heavy and light chain. Therefore, the supernatant was tested again on ELISA (Fig. 23). As positive control ("+ control"), the mAb VRC01 was used. The full-length mAb is denoted with a red circle in the lane "+ control non-reduced" and the bands of the light and heavy chains of the "+ control reduced" are marked with red circles as well. As negative control ("- control"), the supernatant of non-transfected Expi293F cells was used.

3.2.5 Standardization of antibody production by Expi293F cells

After confirming the successful production of mAb 61p1E2 by Expi293F cells using the transfection reagent TransIT-PRO® (Fig. 16), the question arose whether mAb yield could be increased. Therefore, another transfection reagent, termed ExpiFectamine™, was tested. The principle of both reagents is the formation of lipid-polymer complexes with the plasmids.

I sought to test whether there was an advantage of using one transfection reagent over the other. Therefore, to compare these two reagents, cell cultures were transfected and cell viability counts were performed daily (Table 17 and Table 18; Fig. 17 and Fig. 18). Additionally, 2 ml of cell culture supernatant was collected from each flask daily to assess mAb 61p1E2 production by western blot analysis (Figs. 20A and 20B). One day post-transfection, no antibodies were detected in the supernatants. On day 2, TransIT PRO-transfected cell supernatant showed a slight band of mAb 61p1E2 at 150-170 kDa (Fig. 20B); on day 2 (no sample was available for the ExpiFectamine™ 293-transfected cell culture (Fig. 20A)). Day 3 to day 6 show an increase in the amount of mAb 61p1E2 in the supernatants for both reagents.

Although differences in cell viability were seen and it seemed that mAb production using the reagent ExpiFectamine™ 293 was higher in the early days post-transfection (Fig. 20A), no difference was seen in the mAb yield after purification via protein A chromatography. Therefore, both reagents were used for the production of mAb 61p1E2.

3.2.5.1 TransIT-PRO

Table 17 Cell count data of transfection reagent TransIT-PRO®

Days	Culture A viability (%)	Culture B viability (%)	Culture C viability (%)	Culture D viability (%)	Culture E viability (%)
0	100	100	100	98	98
1	84	89	98	84	74
2	87	78	82	89	89
3	91	89	85	84	88
4	n/a	n/a	n/a	90	87
5	88	92	89	n/a	n/a
6	68	77	68	n/a	n/a
7	5	47	7	62	67

Table 17 shows the cell count data of five 200 ml Expi293F cell cultures transfected with the plasmids pFUSE-61p1E2-Gamma and pFUSE-61p1E2-Lambda using the reagent TransIT-PRO®, named “Culture A” to “Culture E”. The first column shows on which day the cell count was taken. “Day 0” is referred to as the day of transfection, when plasmids pFUSE-61p1E2-Gamma and pFUSE-61p1E2-Lambda were mixed with the TransIT-PRO® reagent and added to the Expi293F cells. Cell counts were performed using an automated cell counter. n/a, cell count data not available. On day 7 post-transfection, the cell cultures were terminated and mAb 61p1E2 was harvested.

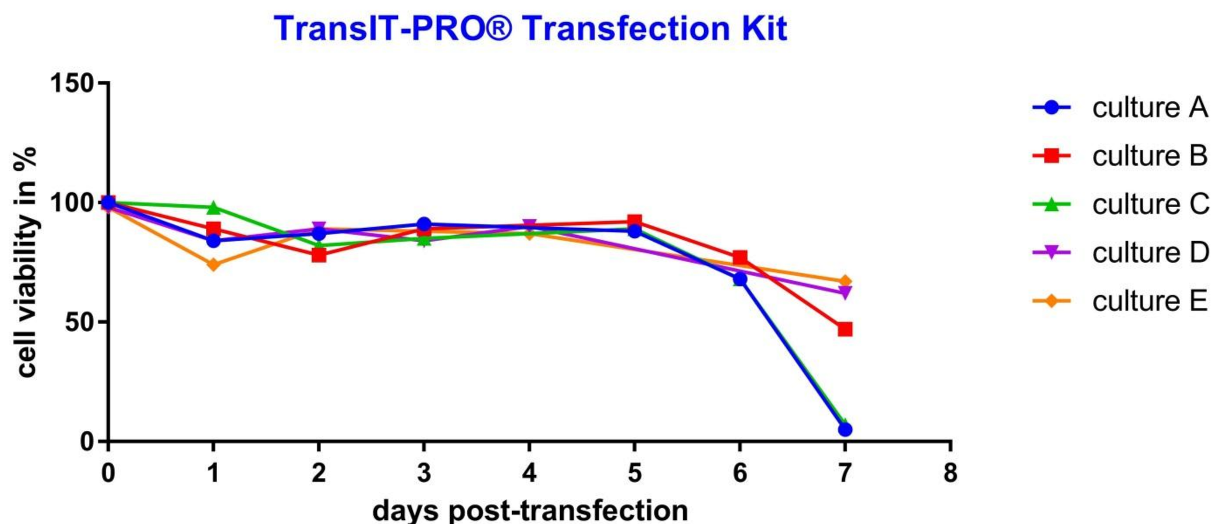

Fig. 17 Cell counts after transfection with the TransIT-PRO® reagent

The percent of viable cells (please see Table 18) was plotted using GraphPad Prism (version 7.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Day 0, day of transfection. Day 7, when cell viability was reduced to about 60% or lower, mAb 61p1E2 was harvested. Cell counts were not available for day 4 for the cell cultures A, B and C and day 5 and 6 for cell cultures D and E.

3.2.5.2 ExpiFectamine™ 293 Transfection kit

Table 18 Cell count data of transfection reagent ExpiFectamine™ 293

Days	Culture 1 viability (%)	Culture 2 viability (%)	Culture 3 viability (%)	Culture 4 viability (%)
0	100	100	100	100
1	98	98	96	91
2	96	95	86	83
3	96	98	n/a	n/a
4	91	77	n/a	n/a
5	52	62	36	46
6	5	9	21	29

Table 18 shows the cell counts of four 200 ml Expi293F cell cultures transfected with the plasmids pFUSE-61p1E2-Gamma and pFUSE-61p1E2-Lambda using the reagent ExpiFectamine™ 293, termed “Culture 1” to “Culture 4”. Day 0, day of transfection when plasmids pFUSE-61p1E2-Gamma and pFUSE-61p1E2-Lambda were mixed with the ExpiFectamine™ 293 reagent and added to Expi293F cells. Cell counts were performed using an automated cell counter. n/a, data not available. Day 6, termination of the cultures and harvest of mAb 61p1E2.

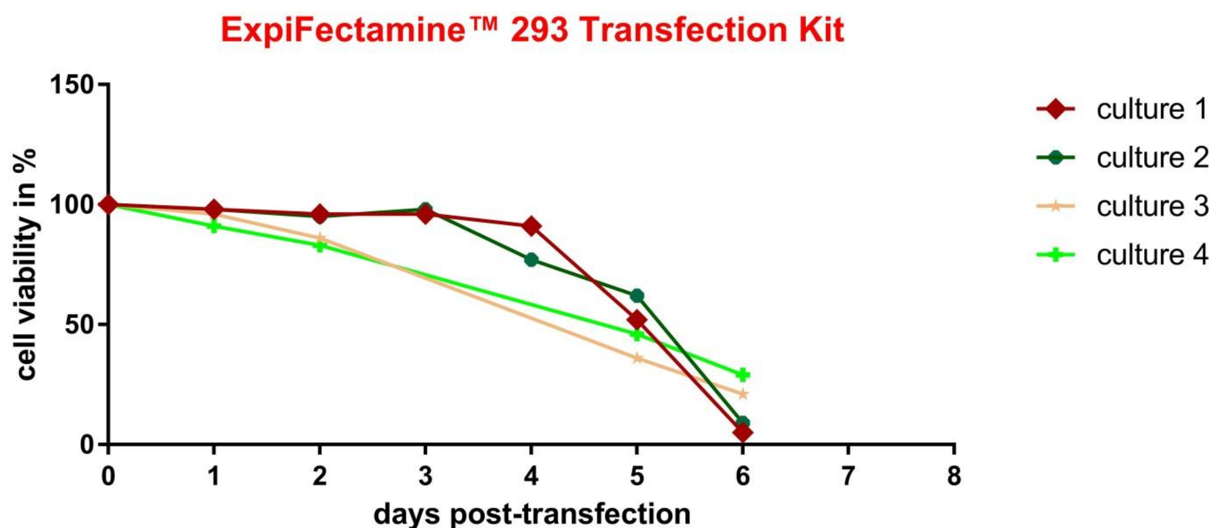

Fig. 18 Cell counts after transfection with the ExpiFectamine™ 293 reagent

The percent of viable cells (please see Table 18) was plotted using GraphPad Prism (version 7.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Day 0, day of transfection. On day 6, when the cell viability was reduced to 60% or lower, mAb 61p1E2 was harvested. Transfected cell cultures are named “culture 1” to “culture 4”. Cell counts of “culture 3” and “culture 4” were not available on days 3 and 4.

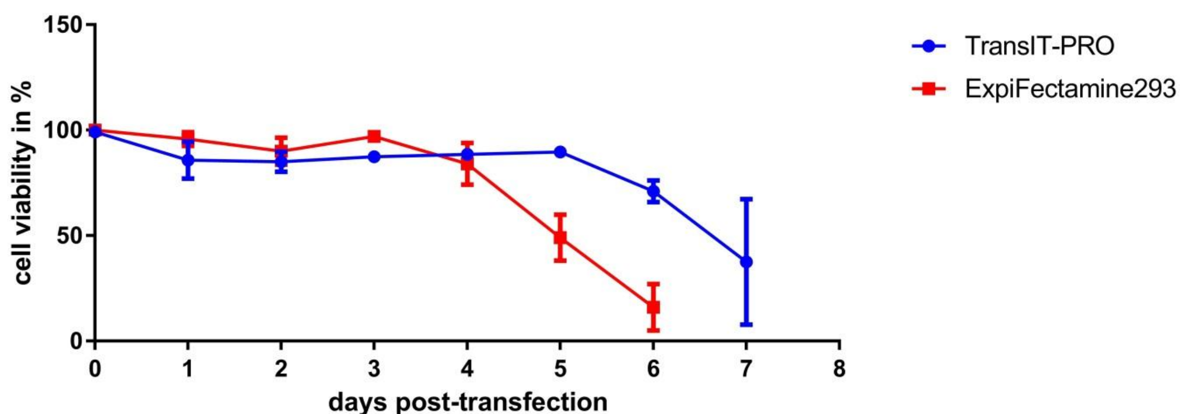

Fig. 19 Comparison of cell count data of both transfection reagents

The percent of viable cells transfected using either TransIT-PRO® or ExpiFectamine™ 293 was plotted using GraphPad Prism (version 7.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Cell viability data are from Tables 17 and 18 (see also Fig. 17 and Fig. 18).

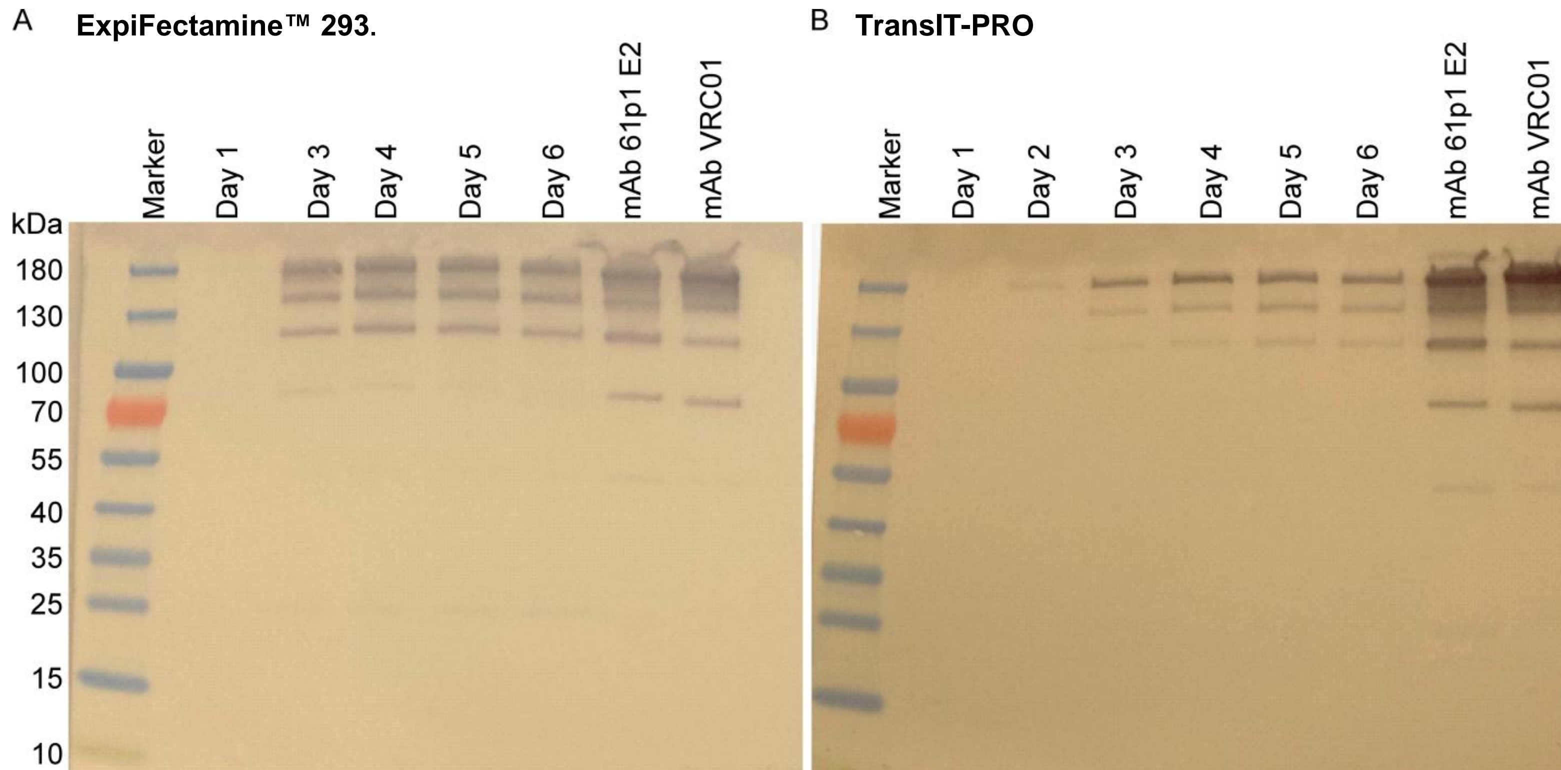

Fig. 20 Western blots: comparison of two different transfection methods (A, ExpiFectamine™ 293; B, TransIT-PRO) Supernatants of transfected Expi293F cells were tested daily by western blot. On day 2 post-transfection, no supernatant sample was available for ExpiFectamine™ 293-transfected cells (Fig. 20A). As positive controls, the purified mAb 61p1E2 and the mAb VRC01 were used. PageRuler™ Prestained Protein Ladder (Thermo Scientific) was used as marker.

3.2.6 Purification of mAb 61p1E2 by protein A affinity chromatography

In total, 5 liters of Expi293F cells were transfected with the plasmids pFUSE-61p1E2-Gamma and pFUSE-61p1E2-Lambda for the production of mAb 61p1E2 into the supernatant. After verification via SDS-PAGE (Fig.16) and ELISA (Fig.23) that mAb 61p1E2 was successful produced, antibodies were purified by protein A chromatography. Six purifications were performed and a total amount of 28.23 mg mAb 61p1E2 was obtained. The efficiency of the antibody purification was approximately 5.65 µg/ml of supernatant (Table 19).

Table 19 Purification efficiency of mAb 61p1E2

	Supernatant collected (ml)	mAb 61p1E2 yield (mg)	Concentration of mAb 61p1E2 (mg/ml)	Volume of mAb 61p1E2 sample (ml)
Purification 1	490	2.8	13.8	0.20
Purification 2	1,900	10.6	18.9	0.56
Purification 3	70	0.4	2.0	0.20
Purification 4	980	5.5	11.0	0.50
Purification 5	372	2.1	4.7	0.44
Purification 6	1,200	6.9	8.0	0.86
Total	**5,010**	**28.3**	**10.2**	**2.76**

Table 19 shows the results of six mAb 61p1E2 purification runs. Column on the right, final volume of purified mAb after buffer exchange and concentration (section 2.5.4.1). All mAb 61p1E2 samples of all purifications were filter sterilized, pooled, split into multiple 200 µl aliquots and stored a 4°C.

3.2.7 Antibody purification profile

To assess the efficiency of the mAb purification via protein A chromatography, samples were collected after each step in the purification procedure and tested by SDS-PAGE and western blot (Fig. 21 and Fig. 22). A mAb 61p1E2 band is seen at around 150 kDa-170 kDa in unfractionated supernatant of transfected Expi293F cells ("Supernatant", Fig. 21). No mAb was detected in the "Flow-Through" after loading the supernatant onto the protein A column. The purification of mAb 61p1 E2 was very efficient by using the protein A chromatography.

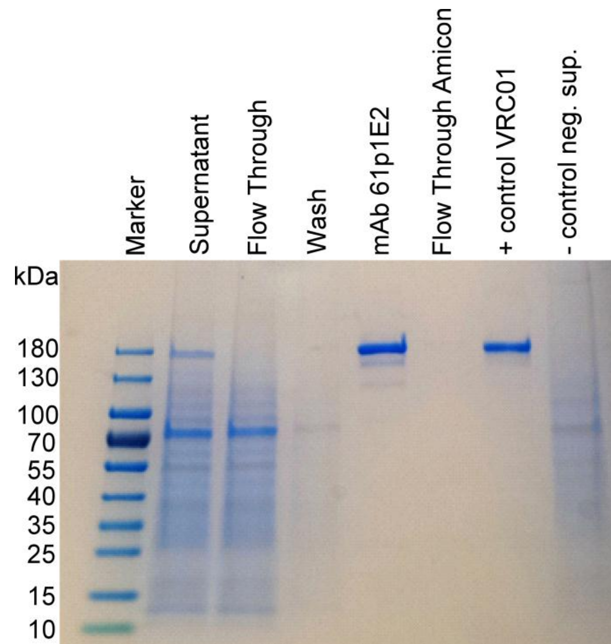

Fig. 21 SDS-PAGE of purification profile of mAb 61p1E2

Lane 1, Marker (PageRuler™ Prestained Protein Ladder, Thermo Scientific); lane 2, supernatant before purification; lane 3 ("Flow Through") collected after passing the supernatant through the protein A column. Lane 4 ("Wash"), obtained by washing the column with PBS in order to remove proteins binding non-specifically. Lane 5 ("mAb 61p1E2"), purified mAb used as positive control; Lane 6 ("Flow Through Amicon"), flow-through of the Millipore centrifugal filters that were used to replace the Elution Buffer and to concentrate the mAb 61p1E2 sample; lane 7 ("+ control VRC01"), a human IgG1 mAb directed against the CD4 binding site; lane 8 ("- control neg. sup."), supernatant of non-transfected Expi293F cells.

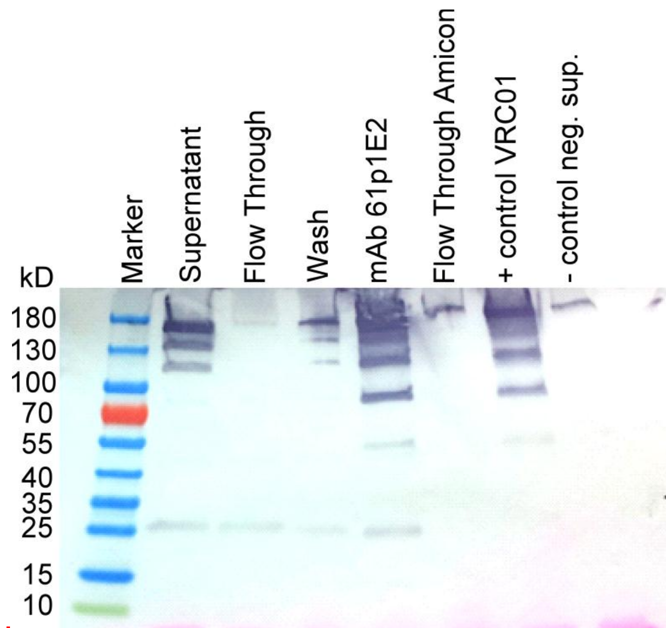

Fig. 22 Western blot of purification profile of mAb 61p1E2

To determine the efficiency of the mAb 61p1E2 purification, a western blot in addition to the SDS-PAGE (Fig. 21) was performed. Supernatant samples collected after each purification step as seen in the SDS-PAGE (Fig. 21) were loaded. Lane 1, Marker (PageRuler™ Prestained Protein Ladder, Thermo Scientific); lane 2, supernatant before purification; lane 3 ("Flow Through") collected after passing the supernatant through the protein A column. Lane 4 ("Wash"), obtained by washing the column with PBS in order to remove proteins binding non-specifically. Lane 5 ("mAb 61p1E2"), purified mAb used as positive control; Lane 6 ("Flow Through Amicon"), flow-through of the Millipore centrifugal filters that were used to replace the Elution Buffer and to concentrate the mAb 61p1E2 sample. Lane 7 ("+ control VRC01"), a human IgG1 mAb directed against the CD4 binding site; lane 8 ("- control neg. sup."), supernatant of non-transfected Expi293F cells. A spill-over of sample is seen in lanes of "Flow-Through", "Wash", "Flow Through Amicon" and the "– control neg. supernatant". Peroxidase AffiniPure goat anti-human IgG was used as secondary antibody.

3.2.8 Verification of mAb production

After the mAb 61p1E2 was purified from supernatants of the transfected Expi293F cells by protein A chromatography, its binding was tested by ELISA. These mAb preps were purified from three different batches. Furthermore, before performing the next purification steps, transfected supernatant was tested. For this ELISA, supernatant were no mAb 61p1E2 was detected by SDS-PAGE (Fig. 16), was tested again. mAb 61p1E2 was detected in the Expi293F supernatant by ELISA and the binding of the purified mAb 61p1E2 against the bait K-CTcc was as expected. The mAb 61p1E2 in the supernatant of transfected Expi293F cells and the purified mAb 61p1E2 showed specific binding against the bait protein K-CTcc.

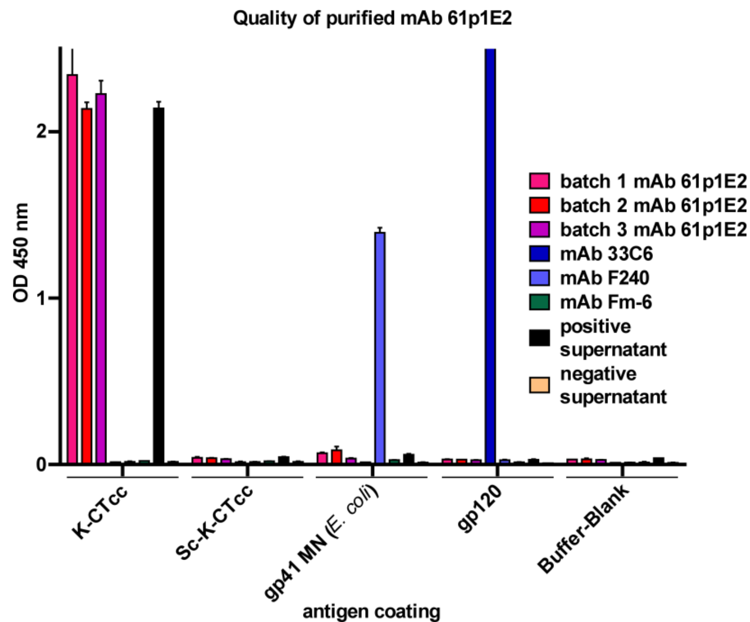

Fig. 23 ELISA to test binding of purified mAb 61p1E2 and transfected supernatant

A 96-well Nunc Maxisorp plate was coated with different HIV-envelope proteins, HIV-1 gp41 MN (*E.coli*), HIV-1 CN54 gp120 and the recombinant protein K-CTcc. As negative controls, scrambled K-CTcc (Sc-K-CTcc) and Coating Buffer were loaded on the plate. The next day, the plate was blocked and primary antibodies were added. mAb F240, a specific anti-gp41 antibody and mAb 33C6, specific to the V3 loop of HIV gp120, were used as positive controls. As negative isotype control, mAb Fm-6, an anti-SARS antibody, was used. The supernatant of non-transfected Expi293F cells served as negative control for the mAb 61p1E2 in the supernatant of transfected Expi293F cells.

3.2.9 Epitope specify of purified mAb 61p1E2

To assess if the binding of mAb 61p1E2 is specific to HIV envelope proteins gp41, gp160 and to K-CTcc, mAb 61p1E2 was tested against different HIV antigens, including gp160 HIV-1 1084i, a component of the recombinant multicomponent protein vaccine, which was used for the immunization of the RM RTr-11 (Lakhashe et al., 2011). The mAb 61p1E2 had been isolated from this vaccine-protected animal (please refer to section 1.5 for more details). The purified mAb 61p1E2 showed specific binding against the bait protein K-CTcc and gp160 1084i (Fig. 24).

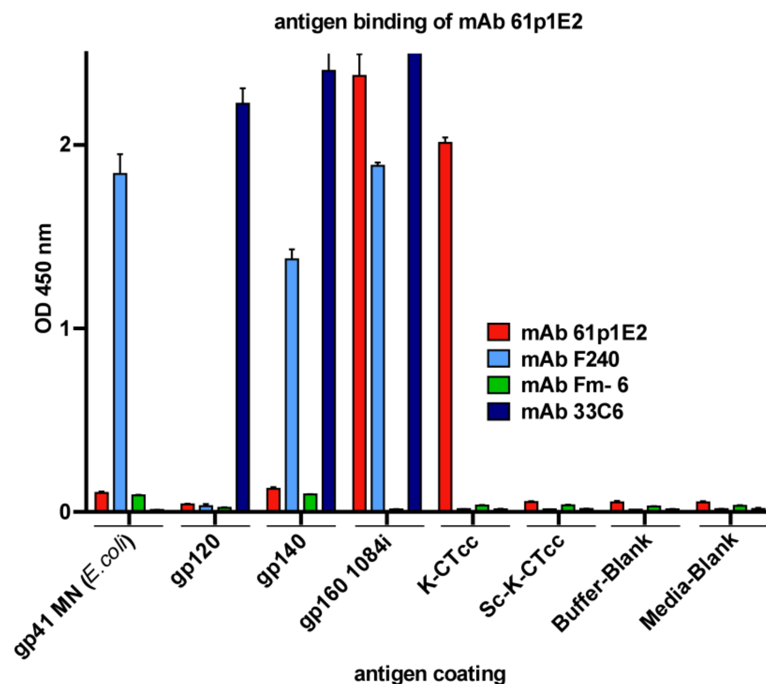

Fig. 24 ELISA to test binding of mAb 61p1E2 against different HIV envelope proteins

A 96-well Nunc Maxisorp plate was coated with different HIV-envelope proteins, HIV-1 gp41 MN (produced in *E. coli*), HIV-1 CN54 gp120, HIV-1 CN54 gp140, HIV-1 gp160 1084i and the bait protein K-CTcc. As negative controls, scrambled K-CTcc (Sc-K-CTcc), Coating Buffer and Expi293™ Expression Medium were loaded onto the plate. The next day, the plate was blocked and primary antibodies were added. mAb F240, a specific anti-gp41 antibody, and mAb 33C6, a specific anti-gp120 antibody, were used as positive controls. The mAb Fm-6, an anti-SARS antibody, served as negative control.

3.3 Fab of mAb 61p1E2

To get more insight in the binding interaction between the bait protein K-CTcc and the paratope of mAb 61p1E2, Fab fragments of mAb 61p1E2 were generated. After purification, these Fabs were sent together with K-CTcc for crystallization.

To produce Fabs of mAb 61p1E2, purified mAb 61p1E2 was digested with the enzyme papain (Fig. 25).

3.3.1 Fab production by papain digestion

A good amount of 28.23 mg of mAb 61p1E2 was produced by Expi293F cells and purified by protein A chromatography (Table 19). Thus, mAb 61p1E2 was available in sufficient quantities for the production of Fabs by digestion with the enzyme papain. In total, 12 mg of mAb 61p1E2 were digested and about 5 mg of Fabs were obtained (Fig. 25).

Fabs were characterized by SDS-PAGE (Fig. 26) and ELISA (Fig. 27). Then, Fabs were sent together with the bait protein K-CTcc for crystallization.

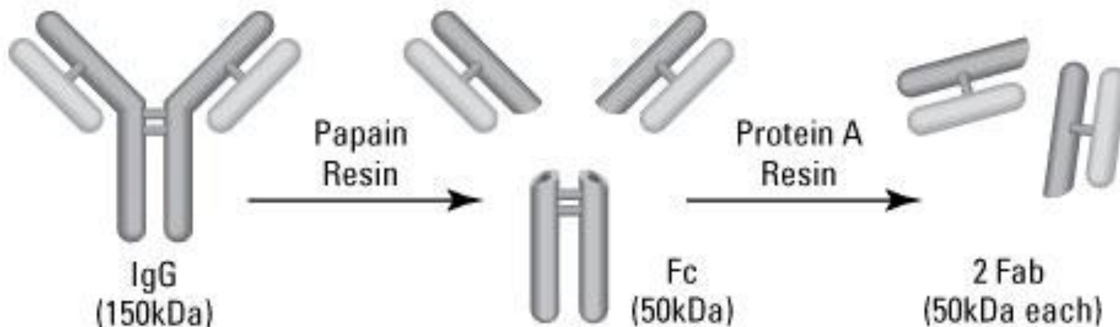

Fig. 25 Papain digestion of mAb 61p1E2: generation of Fabs (see also Fig. 3)

Purified mAb 61p1E2 was digested with the enzyme papain. Fabs and Fc portions were separated by using a protein A column, where the Fc are retained. The Fabs of mAb 61p1E2 were collected in the flow through. Each Fab has a molecular weight of 50 kDa (https://tools.thermofisher.com/content/sfs/gallery/high/44985-Frag-Papain.jpg).

3.3.2 Quality control of Fab-mAb 61p1 E2

To characterize the Fabs, we used SDS-PAGE (Fig. 26) and ELISA (Fig. 27). For the SDS-PAGE, samples were boiled before loading. Therefore, two bands of Fabs are visible on the gel. The molecular weight of reduced Fabs is seen at 25-30 kDa and non-reduced Fabs are seen at around 50 kDa. The elute of the protein A column contains the Fc portion of mAb 61p1E2. The reduced Fc portion can be seen at a molecular weight of 28-30 kDa, the non-reduced Fc portion can be seen at around 50 kDa. The gel demonstrated the purity of the Fab prep. The Fabs of mAb 61p1E2 showed specific binding against the bait protein K-CTcc (Fig. 27).

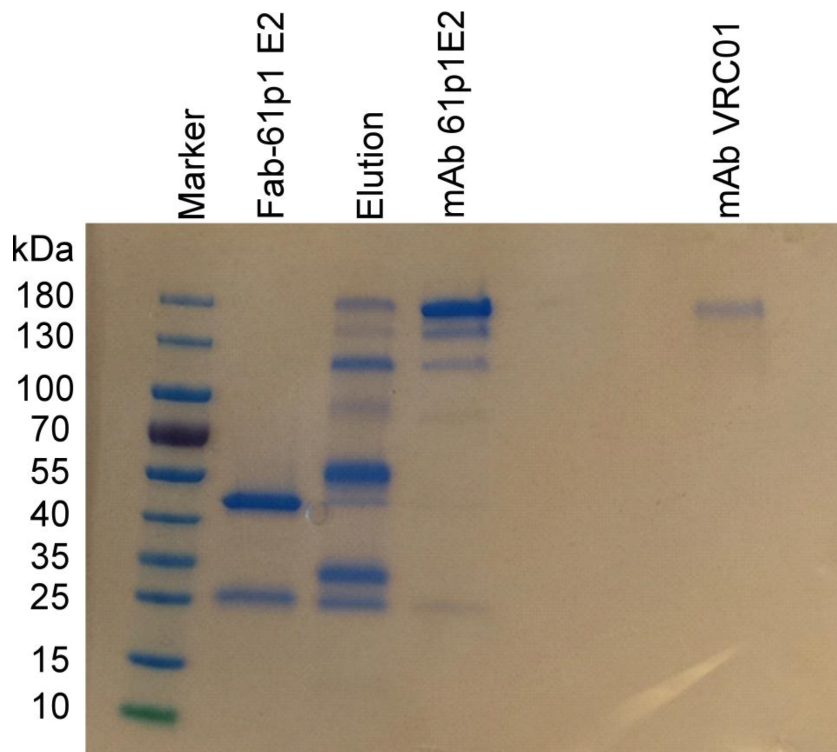

Fig. 26 SDS-PAGE of Fab-mAb 61p1E2

Lane 1, Marker (PageRuler™ Prestained Protein Ladder, Thermo Scientific); lane 2, Fab fragments of mAb 61p1E2; lane 3 (“Elution”) elute of the protein A column; lane 4, purified mAb 61p1E2 was used as positive control and in lane 7, the positive control mAb VRC01 was loaded.

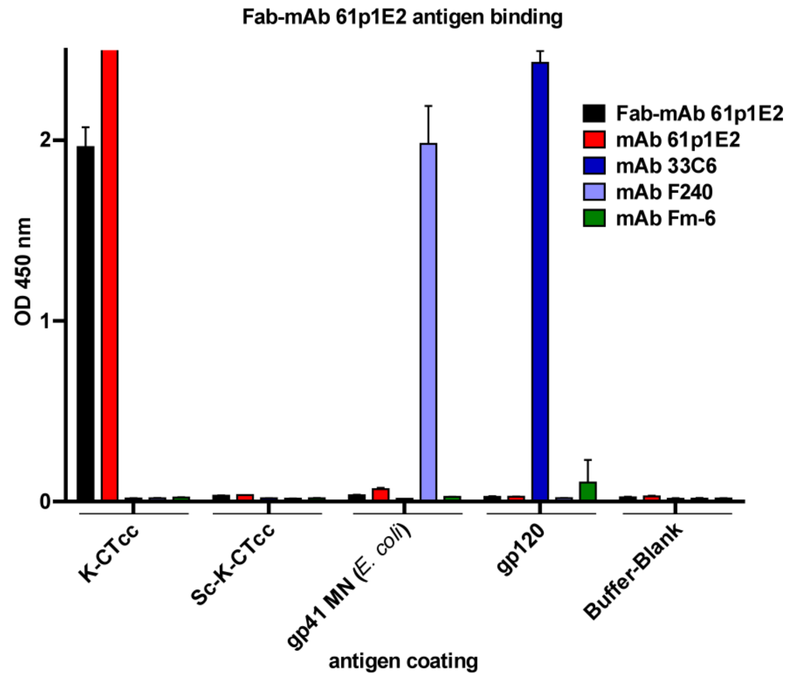

Fig. 27 ELISA to test binding of Fab-mAb 61p1E2

A 96-well Nunc Maxisorp plate was coated with different HIV-1 envelope proteins, HIV-1 gp41 MN (produced in *E. coli*), HIV-1 CN54 gp120 and the recombinant protein K-CTcc. As negative controls, scrambled K-CTcc (Sc-K-CTcc) and Coating Buffer were loaded onto the plate. The next day, the plate was blocked and primary antibodies were added. The purified mAb 61p1E2; mAb F240, a specific anti-gp41 antibody and mAb 33C6, a specific anti-gp120 antibody were used as positive controls. As negative control was mAb Fm-6, an anti-SARS antibody, used.

4 DISCUSSION

4.1 Testing of hypothesis

In order to proof my hypothesis that the mAb 61p1E2 shows the same binding specificity against the PL region of gp41, K-CTcc, as the polyclonal antibodies, which were detected in the sera or plasma samples of vaccine-protected RMs, but not in those of non-protected RMs, characterization of mAb 61p1E2 was carried out. A large amount mAb 61p1E2 was produced, and this mAb was tested for its binding specificity against the bait, K-CTcc, a GFP-gp41 PL-mimotope fusion protein and other HIV envelope proteins. The purified mAb 61p1E2 showed specific binding to K-CTcc; importantly, this mAb did not bind to the scrambled form of the bait protein K-CTcc. Thus, epitope binding was specific and confirmed my hypothesis. I conclude that mAb 61p1E2 may be representative of an antibody that is linked to vaccine-induced protection.

4.1.1 Binding specificity of mAb 61p1E2

An interesting aspect of mAb 61p1E2 is that it showed binding to the immunogen, gp160 HIV-C 1084i. This trimeric Env gp160 was part of a multi-component recombinant protein vaccine used in a vaccine-efficiency study (Lakhashe, Wang et al. 2011), in which RM RTr-11 was protected against multiple live-virus exposures to a SHIV-C strain, SHIV-1157ipEL-p (Siddappa, Watkins et al. 2010).

Surprisingly, mAb 61p1E2 showed no binding against Env of the challenge virus SHIV-1157ipEL-p (Hemant Vyas, unpublished data). Thus, the binding of mAb 61p1E2 seems to be specific to a certain epitope present in the gp160 HIV-C 1084i immunogen but not in the heterologous challenge virus particles. As such, mAb 61p1E2 is one of the vaccine-induced antibodies. At this point in time, it is unclear how mAb 61p1E2 protected against the challenge virus. It is possible that the 61p1E2 epitope is only transiently accessible during challenge virus replication. Perhaps, the epitope is displayed on infected cells.

Interestingly, mAb 61p1E2 binds specifically to the gp41 region represented by K-CTcc, but it did not show any binding to HIV-1 gp41 MN, which was produced in *E. coli*. The reasons could be

the lack of the glycosylation of (g)p41 MN, since it was produced in bacteria. Alternatively, the K-CTcc epitope was not present or was hidden on this particular (g)p41.

On the other hand, mAb 61p1E2 bound specifically to trimeric gp160 of HIV-C 1084i. Since the latter had been produced in eukaryotic cells, it was glycosylated. Therefore, the reason for the binding could be either the glycosylation pattern or – since it contains the full-length sequence of gp41 and gp120, the epitope of K-CTcc is maybe shown. Furthermore, mAb 61p1E2 was tested against gp140, which was also produced in eukaryotic cells, but it did not show any binding. The exact sequence of this gp140 has not been given by the company. Therefore, maybe K-CTcc does not contain the specific epitope of K-CTcc.

4.2 Aim 2 – production of Fabs and structural analysis

Since mAb 61p1E2 is representative of a vaccine-induced antibody isolated from a protected RM that had never shown viremia after the multiple exposures to the challenge virus, structural analysis of the mAb interaction with epitope represented by the “bait” protein is important. Therefore, the second aim of my thesis was the production of Fab fragments of mAb 61p1E2. Fabs were successful produced by the digestion of mAb 61p1E2 with the enzyme papain and sent for co-crystallization with the bait protein K-CTcc. These experiments are ongoing.

4.2.1 Cloning strategy for Fab production

If the ongoing attempts at crystallization fail, the Ruprecht lab will need to produce larger Fab amounts. For such future experiments, production of Fabs by molecular cloning and direct expression of the fragments would be an alternative approach. Production of Fabs in Expi293F cells would be a faster and more efficient way to get a good amount of Fabs for crystallization than mAb production followed by papain cleavage. Therefore, recloning of the pFUSE-61p1E2-Gamma vector has been attempted. Another advantage of this production method is that the insert encoding Fab-mAb 61p1E2 could be replaced by the sequences encoding Fabs from other mAbs.

Recloning of pFUSE-61p1E2-Gamma could be accomplished by either Gibson assembly or the traditional molecular cloning strategy using restriction enzymes. Since pFUSE-61p1E2-Gamma was used for the heavy chain production in Expi293F cells, it contains the VH, CH1, CH2 and

CH3 regions of mAb 61p1E2. However, the heavy chain portion of Fab consists only of VH and CH1. Therefore, a stop codon will need to be inserted in front of the CH2 and CH3 regions, also referred to as Fc portion. In addition to the sequence of the stop codon, a HRV3C cleavage site and a 6x His-tag will be needed. Since Fabs cannot bind to protein A to purify them out of the supernatant of Expi293F cells, the sequence of a 6x His-tag needs to be inserted. The latter can be used to purify Fabs by nickel affinity chromatography. After Fab purification, the 6x His-tag can be cleaved off with a HRV3C protease. This new cloning vector, named Fab-pFUSE-61p1E2-G (Fig. 28), and the vector pFUSE-61p1E2-Lambda, which contains the sequences encoding the VL and CL regions, could then be used for the Fab production in Expi293F cells.

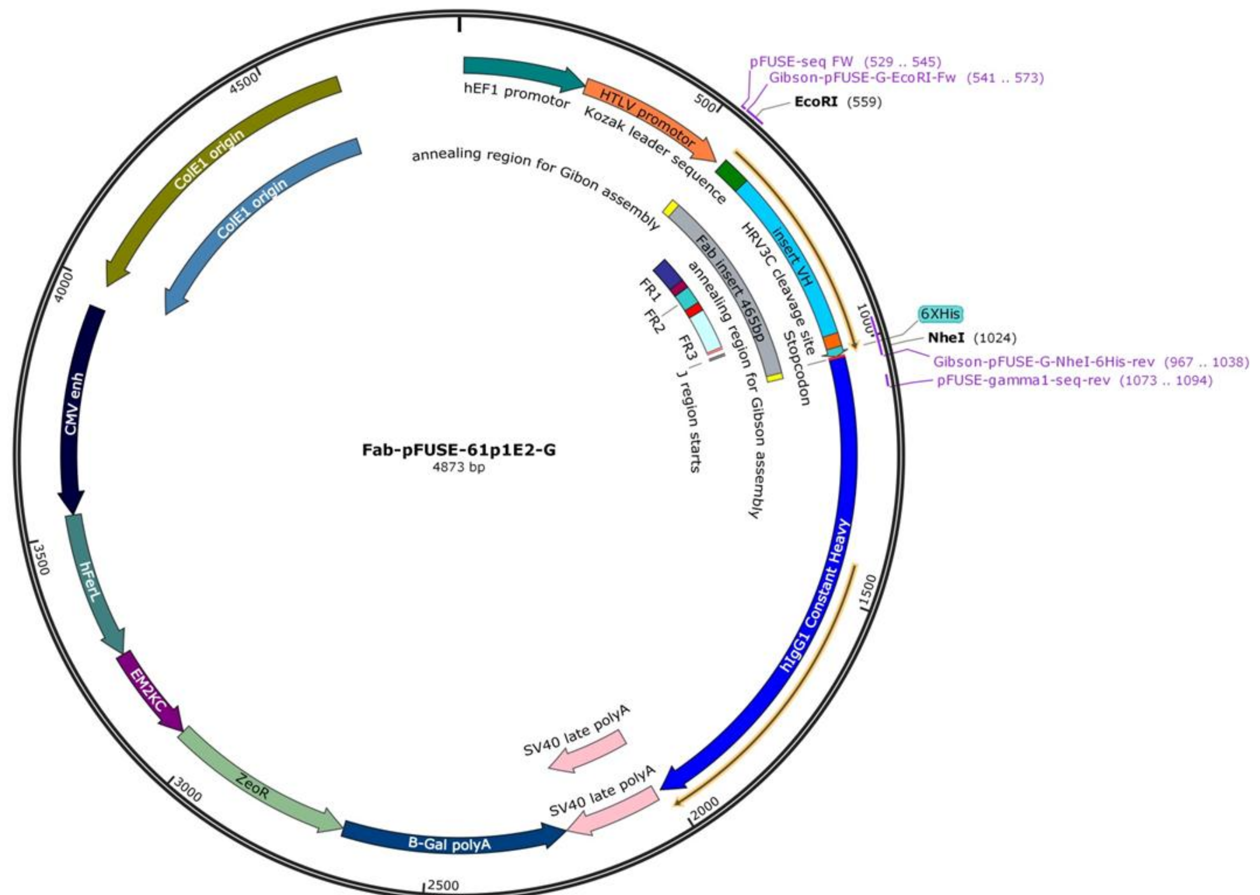

Fig. 28 Plasmid map of Fab-pFUSE-61p1E2-G

The plasmid map of Fab-pFUSE-61p1E2-G is shown. It can be constructed by using two different cloning techniques, Gibson assembly or traditional molecular cloning using restriction enzymes. The insert of Fab consists of the VH chain, HRV3C cleavage site, 6x His-tag and a stop codon. This plasmid can be used together with the vector pFUSE-61p1-E2-Lambda for the Fab production in Expi293F cells.

4.3 Conclusion

First, my hypothesis was confirmed: mAb 61p1E2 showed the same binding specificity as the polyclonal antibodies, which had been detected in the sera or plasma samples of vaccine-protected RMs but not in those of non-protected RMs. Therefore, we proceeded with experiments aimed at determining the binding interaction between the mAb and its bait protein, K-CTcc, a mimic of protection-linked region of HIV gp41. We generated Fabs from the mAb and are currently attempting co-crystallization of the latter with the bait protein. Such information will be important for future vaccine design.

5 ZUSAMMENFASSUNG

In einer SHIV-Vakzinstudie, die durch das Forschungsteam von Frau Dr. Ruprecht durchgeführt wurde, sind vakzinierte Rhesusaffen trotz mehrfacher Gabe des Simianen-Humanen Immundefizienz Virus, SHIV-1157ipEL-p, nicht infiziert worden. Insgesamt wurden zwölf Rhesusaffen geimpft, von denen zwei Tiere komplette Immunität aufwiesen. Im Plasma dieser zwei immunen Affen wurden polyklonale Antikörper, die spezifisch an eine Region des Glykoproteins (Gp) 41 binden, gefunden. Diese Antikörper wurden nur von den immunen Tieren gebildet. Die Sequenz dieser Region von Gp 41 wurde in einen GFP-Expressions Vektor geklont, um ein rekombinantes GFP- Fusionsprotein, K-CTcc, zu generieren. Dieses rekombinante Protein wurde zur Isolierung von B-Gedächtniszellen aus dem Blutplasma der immunen Affen verwendet. Eine dieser B- Gedächtniszellen produzierte den Antikörper 61p1E2. Ein wichtiger Teil meines Projekts war die Herstellung des chimären Simianen-Humanen monoklonalen Antikörpers 61p1E2 mittels menschlicher embryonaler Nierenzellen um anschließend zu beweisen, dass dieser Antikörper dieselbe Bindungsspezifität wie die polyklonalen Antikörper im Blutplasma des immunen Tieres, RTr-11, aufweist. Um dieser Frage nachzugehen, wurde der generierte monoklonale Antikörper 61p1E2 mittels SDS-PAGE, Western Blot und ELISA charakterisiert. Der Antikörper wies dieselbe Bindungsspezifität gegen K-CTcc auf. Interessanterweise bindet der Antikörper 61p1E2 ebenfalls spezifisch das Gp 160, eine Komponente des Impfstoffes, welcher zu Immunisierung verwendet wurde. Da dieselbe Spezifität des generierten monoklonalen Antikörpers 61p1 E2 bestätigt wurde, ist die strukturelle Analyse der Interaktion zwischen dem Epitop von K-CTcc und dem Paratop des Antikörpers essentiell. Darum wurden Fab-Fragmente des Antikörpers 61p1E2 mithilfe der enzymatischen Spaltung durch Papain generiert. Diese wurden zusammen mit dem Protein K-CTcc zur Kristallisierung zu Kollaboratoren der Forschungsgruppe von Frau Dr. Ruprecht geschickt. Die Röntgenstrukturanalyse wird Aufschluss über die genaue Bindungsinteraktion zwischen dem monoklonalen Antikörper 61p1E2 und dem Protein K-CTcc geben. Dieses Wissen könnte eine wichtige Rolle in der zukünftigen Impfstoffentwicklung spielen.

6 ENGLISH SUMMARY

In a vaccine efficiency study, which was carried out by Dr. Ruprecht's group, two rhesus macaques (RMs) out of twelve were completely protected against multiple live-virus exposures to a SHIV-C strain, SHIV-1157ipEL-p. In the sera of these vaccine-protected RMs but not animals with vaccine failure, polyclonal antibodies against a specific region of gp41 were detected. This protection-linked (PL) gp41 region was cloned into a green fluorescent protein (GFP) expression vector to generate the GFP-gp41 PL-mimotope fusion protein, K-CTcc. This recombinant protein was used as bait to isolate single memory B cells that were producing cognate antibodies from the vaccine-protected RM, named RTr-11. One such antibody was the mAb 61p1E2. One important part of my project was to test if this mAb shows the same binding specificity against the recombinant K-CTcc as the polyclonal antibodies in the vaccine-protected animal RTr-11. Therefore, mAb 61p1E2 was produced in Expi293F cells, a cell line established from human embryonic kidney cells. A large amount of mAb 61p1E2 was produced and purified. The antibody was characterized by SDS-PAGE, western blot and ELISA. The mAb 61p1E2 showed specific binding against K-CTcc as well as to the trimeric HIV gp160 immunogen, a component of the vaccine. Since the mAb's specific binding against K-CTcc was confirmed, structural analysis of the paratope-epitope interaction by X-ray crystallography will be important. Towards this goal, Fabs of mAb 61p1E2 were generated by papain digestion of the mAb and sent together with the bait protein K-CTcc for crystallization. If crystals are formed, X-ray crystallography will be performed to gain insight into the binding interaction between mAb 61p1E2 and the bait protein K-CTcc. Such an analysis could give important information for future vaccine design.

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8 INDEX OF FIGURES

Fig. 1 HIV particle ... 4
Fig. 2 IgG structure ... 7
Fig. 3 Fab construction through digestion via papain ... 8
Fig. 4 Plasmid map of K-CTcc-pET22b-GFPII ... 13
Fig. 5 Plasmid map of pFUSE-61p1E2-Gamma ... 15
Fig. 6 Illustration of IgG ... 15
Fig. 7 Plasmid map of pFUSE-61p1E2-Lambda ... 17
Fig. 8 Illustration of IgG ... 17
Fig. 9 IPTG induction of BL21 *E. coli* transformed with K-CTcc-pET22b-GFPII ... 37
Fig. 10 Lysed IPTG-induced bacterial pellet ... 38
Fig. 11 Restriction enzyme digests of plasmid K-CTcc-pET22b-GFPII ... 39
Fig. 12 Restriction enzyme digest of pFUSE-61p1E2-Gamma ... 42
Fig. 13 Sequencing result of purified plasmid pFUSE-61p1E2-Gamma ... 43
Fig. 14 Restriction enzyme digest of pFUSE-61p1E2-Lambda ... 44
Fig. 15 Sequencing result of purified plasmid pFUSE-61p1E2-Lambda. ... 45
Fig. 16 SDS-PAGE of supernatant of transfected Expi293F cells ... 47
Fig. 17 Cell counts after transfection with the TransIT-PRO® reagent ... 50
Fig. 18 Cell counts after transfection with the ExpiFectamine™ 293 reagent ... 52
Fig. 19 Comparison of cell count data of both transfection reagents ... 53
Fig. 20 Western blots: comparison of two different transfection methods ... 54
Fig. 21 SDS-PAGE of purification profile of mAb 61p1E2 ... 56
Fig. 22 Western blot of purification profile of mAb 61p1E2 ... 57
Fig. 23 ELISA to test binding of purified mAb 61p1E2 and transfected supernatant ... 58
Fig. 24 ELISA to test binding of mAb 61p1E2 against different HIV envelope proteins ... 59
Fig. 25 Papain digestion of mAb 61p1E2: generation of Fabs ... 60
Fig. 26 SDS-PAGE of Fab-mAb 61p1E2 ... 61
Fig. 27 ELISA to test binding of Fab-mAb 61p1E2 ... 62
Fig. 28 Plasmid map of Fab-pFUSE-61p1E2-G ... 65

9 INDEX OF TABLES

Table 1 Components of buffers used for protein purification of K-CTcc 11
Table 2 List of restriction enzymes 22
Table 3 Single digested K-CTcc-pET22b-GFPII 23
Table 4 Double digested K-CTcc-pET22b-GFPII 23
Table 5 Single digested pFUSE-61p1E2-Gamma 24
Table 6 Double digested pFUSE-61p1E2-Gamma 24
Table 7 Single digested pFUSE-61p1E2- Lambda 24
Table 8 Double digested pFUSE-61p1E2- Lambda 24
Table 9 Primers for sequencing of pFUSE-61p1E2-Gamma 25
Table 10 Primers for sequencing of pFUSE-61p1E2-Lambda 25
Table 11 Antigens used for coating 34
Table 12 List of primary antibodies 34
Table 13 List of secondary antibodies 34
Table 14 Yield of plasmid purifications of K-CTcc-pET22b-GFPII 38
Table 15 Bacterial growth on antibiotic selection plates. 40
Table 16 Yield of plasmid purifications 41
Table 17 Cell count data of transfection reagent TransIT-PRO® 49
Table 18 Cell count data of transfection reagent ExpiFectamine™ 293 51
Table 19 Purification efficiency of mAb 61p1E2 55

10 INDEX OF ABBREVIATIONS

6x His-tag	polyhistidine-tag
AIDS	Acquired Immune Deficiency Syndrome
ART	antiretroviral therapy
bp	base pair
C region	constant region
CDC	Centers of Disease Control and Prevention
CDR	complementarity-determining region
CH	constant heavy chain
CL	constant light chain
CRF	circulating recombinant form
E. coli	*Escherichia coli*
Env	envelope
Fab	Fragment antigen binding
Fc	fragment crystallizable region
FR	framework region
Fv	variable fragment
GFP	green fluorescent protein
gp	glycoprotein
h	hour(s)
H chain	heavy chain
HEK	human embryonic kidney cells
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HRV3C	Human rhinovirus 3C protease
IgG	immunoglobulin G
IPTG	isopropyl β-D-1-thiogalactopyranoside
K-CTcc	GFP-gp41 protection-linked mimotope fusion protein
L chain	light chain
LB	Lysogeny Broth
mAb	monoclonal antibody
min	minute(s)
nAb	neutralizing antibody
OD	optical density
PBS	phosphate-buffered saline
PCP	*Pneumocystis carinii* pneumonia

PL	protection-linked
R5	CCR5 tropic
R5X4	dual tropic (CXCR4 and CCR5 tropic)
RM	rhesus macaque
RT	reverse transcriptase
Rtemp	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus
SIVcpz	SIV from chimpanzees
SIVsm	SIV from sooty mangabeys
SU	external surface
TAE	Tris-acetate-EDTA
TB	Terrific Broth
TM	transmembrane
V region	variable region
VH	variable heavy chain
VL	variable light chain
X4	CXCR4 topic