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Master of Science Thesis

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Gothenburg, Sweden, 2013

MASTER THESIS 2013

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Abstract

In Sweden lung cancer is the fifth most common cancer form. One problem with lung cancer is that it often is diagnosed late when the patient already suffers from symptoms, leading to low survival compared to other cancer forms. New and improved screening methods for risk groups could lead to earlier detection of lung cancer and increased survival. Higgins *et al. 2012* presented in the article "Variant Ciz1 is a circulating biomarker for early-stage lung cancer" Ciz1b as a new marker for lung cancer. Ciz1b is an alternative splice variant of the normally occurring Ciz1 protein and has been shown to be a promising biomarker for early-stage lung cancer detection being able to distinguish lung cancer patients from healthy individuals. Higgins *et al.2012* has done this study using Western blot which is a method not suitable for diagnostic purposes. Therefore they suggest that a method generating simplified quantitative data, such as an ELISA, should be established. The problem to be solved is that there are no Ciz1b specific reagents to be used in ELISA format (Higgins *et al*., 2012) and therefore this master thesis project was done.

The aim of this project was to establish, characterize and evaluate antibodies specific for detection of Ciz1b to be used in ELISA format. Ciz1b is an alternative splicing of Ciz1 where eight amino acids have been spliced. To be able to generate antibodies directed toward this small splice site site-specific immunization was used with phage particles.

Filamentous phages were used to express Ciz1/Ciz1b specific peptides on the phage surface by phage display to generate an immune response against specific regions of the Ciz1/Ciz1b proteins, such as the Ciz1b-specific splice site. Phage particles used for immunization were analyzed to verify that the recombinant peptides were located in desired reading frame and that the recombinant pVIII molecules were expressed on the phage surface. The phage particles were immunized in Balb/c female mice several times and blood samples were collected from tail vein. An ELISA was established to analyze anti-Ciz1/Ciz1b titer in blood samples to verify which mice raised an immune response against Ciz1/Ciz1b. Mice with anti-Ciz1/Ciz1b titer were used for hybridoma technology.

Five mice had anti-Ciz1/Ciz1b titer; two were used for hybridoma technology. From the hybridoma technology 15 hybridomas show selectivity towards Ciz1b. Those hybridomas produce antibodies with promising specificity against Ciz1b but further analysis has to be done to verify the specificity.

Keywords: lung cancer, Ciz1b, Ciz1, biomarker, site-specific immunization, phage display, ELISA, hybridoma technology.

PREFACE

The project described in this report is a master thesis report made by Emelie Fransson for degree of Master of Science in Biotechnology at Chalmers University of technology. The master thesis was done from January to May 2013 at the company Fujirebio Diagnostics AB. in Gothenburg.

Fujirebio Diagnostics, AB. is a biotechnology company located in Gothenburg that is a world leader in production of in vitro diagnostics and biomarkers (http://www.fdi.com/). During the master thesis my supervisor at Fujirebio Diagnostics AB. was Maria Lidqvist and my examiner at Chalmers University of technology was Christer Larsson.

Maria Lidqvist has been a great support throughout the project. She has helped, supported and guided me throughout the project. Thus, I would like to thank Maria Lidqvist for all the help and support during my time at Fujirebio Diagnostics AB. I also want to thank Christian Fermér for letting me do the master thesis project at the company.

Of course I would like to thank all the employees at Fujirebio Diagnostics AB. for all the help and support I have gained. Doing my master thesis at Fujirebio Diagnostics AB. has been a huge pleasure and that is all due to the kindness and helpfulness from the employees.

I also want to thank my partner Loui Nydelius, my family and my friends for all the support you given me during the master thesis project and also during my whole time period at Chalmers University of technology. Knowing that you always are there for me is a huge support and something that I always will carry with me, thanks.

Emelie Fransson

Gothenburg May 2013

CONTENTS

1. Introduction

Lung cancer is the fifth most common cancer form in Sweden with 3 500 persons getting the diagnosis every year (Edqvist, 2013). Lung cancer is often diagnosed late, when the patient already suffers from symptoms. The symptoms can also be the same as for other lung diseases leading to problematic diagnosis. When there is a suspicion that the patient suffer from lung cancer the patient's lungs are x-rayed and then the cancer tumor often can be detected. Still there can be hard to find small size cancer tumors and detect recurring disease. Late diagnosis of lung cancer results in late-stage cancer tumors and therefore the chances of survival are low compared to other cancer forms (Edqvist, 2013).

A study made by The National Lung Screening Trial Research Team show that low-dose computed tomography (low-dose CT) detects lung cancer at early-stage in risk groups leading to lower mortality from lung cancer (The National Lung Screening Trail Research Team, 2011). In the study low-dose CT were compared with chest radiography and it was shown that low-dose CT has a higher sensitivity and detect more lung cancer patients than chest radiography. But there was also shown that low-dose CT has lower specificity and generated more false positive results (The National Lung Screening Trail Research Team, 2011). All false positive results from low-dose CT lead to a concern for the patient and a major cost for health care for follow up these. Therefore there is clearly a need for establishing additional methods to detect lung cancer at an early-stage with high sensitivity and high specificity.

In the article "Biomarkörer-nyckeln till nya cancerbehandlingar" written by Ingela Björck the importance of biomarkers for detection of cancer is mentioned. A cancer biomarker is a substance in the body that is in different amount between cancer patients and healthy individuals. Ideally a biomarker for a specific disease should distinguish patients with the disease from healthy individuals and optimally be able to grade the disease (Björck, 2011). Hopefully there will be a biomarker in the future that can distinguish healthy individuals from lung cancer patients with different lung cancer forms, thereby making it possible to use the most optimal treatment and increase the survival rates from lung cancer.

In the study "Variant Ciz1 is a circulating biomarker for early-stage lung cancer" published by Higgins *et al.* in 2012, a new marker for lung cancer was presented. The marker Ciz1b is an alternative splicing of the normally occurring Ciz1 protein, and has only been found in lung cancer patients and not in healthy individuals. Ciz1b has therefore been suggested to be a suitable marker for early-stage lung cancer (Higgins *et al*., 2012).

The research made from Higgins *et al. 2012* describes a promising new approach to detect early-stage lung cancer. Their study has been done using Western blot which is not a method to be used for diagnostic purpose. Therefore they suggest that a method generating simplified quantitative data, such as an ELISA, should be established (Higgins *et* al., 2012). The problem is that there is a lack of usable Ciz1b specific reagents to be used in ELISA format and the current study aims to establish, characterize and evaluate antibodies for specific detection of Ciz1b. Such antibodies could potentially be used for more specific and early-stage detection of lung cancer.

To be able to establish antibodies against Ciz1b filamentous phage site-specific immunization (SSI) were used. In site-specific immunization a specific region of a peptide is expressed on a filamentous phage surface and used to immunize mice. The immune response becomes directed toward the

specific region of the peptide and B-cells from the immunized animal can be used to establish site-specific antibodies (Lidqvist *et al*., 2008). In this project Ciz1/Ciz1b peptides were expressed on the filamentous phage to generate an immune response against specific regions of the Ciz1/Ciz1b peptide, such as the Ciz1b-specific splice site. Phage display has several advantages to generated site-specific immunization. Advantages are that the filamentous phage induces immune response in itself and it is a cost-effective method to express antigens on an immunogenic carrier (Galfre *et al.,* 1996).

1.1. Aim

The aim of the master thesis was to establish, characterize and evaluate antibodies against Ciz1b to be used in ELISA format.

The main goal of the master thesis was to generate antibodies against Ciz1b using site-specific immunization and traditional hybridoma technology. Together with those specific aims:

- Generate antibodies against flanking Ciz1 regions for ELISA construction, using site-specific immunization and traditional hybridoma technology
- Establish methods to measure immune response against Ciz1/Ciz1b in immunized mice
- Find and evaluate different sources of Ciz1b antigen and peptides such as mammalian lung cancer cell lines, recombinant protein and phage display

2. Background

In this section information about Ciz1, Ciz1b and the mammalian lung cancer cell lines used are presented and technologies used: phage display, hybridoma technology and site-specific immunization, explained.

2.1 The nuclear matrix component Ciz1

Ciz1 is a nuclear matrix component that has an important role in DNA replication. In both cell-free and cell-based experiments DNA replication has become stimulated by recombinant Ciz1. The lack of Ciz1 prevent replication of DNA (Ainscough *et al.,* 2007).

2.1.1 Ciz1b as an lung cancer biomarker

Alternative splicing at exon 14 for Ciz1 can give rise to Ciz1b. This alternative splicing generates a loss of eight amino acids from the anchor domain compared to Ciz1. Higgins *et al*. 2012 found that this splice site is a unique junction that could be used as a biomarker for early-stage lung cancer detection. Ciz1b can be detected in blood from lung cancer patients and has been suggested to be a robust biomarker for lung cancer detection in serum samples (Higgins *et al.,* 2012). So far, this detection has only been in Western blot.

2.2 Mammalian lung cancer cell lines

In this study human small cell lung cancer cell lines H345 (ATCC^{R}: HTB-180^{TM}), H69 (ATCC^{R}: HTB-119^{TM}) and H128 (ATCC^{R}: HTB-120^{TM}) were cultivated and analyzed to find a source of native Ciz1b antigen to be used for antibody characterization. All three mammalian lung cancer cell lines grow in suspension.

2.3 Phage display

Bacterial viruses, phages, can be used to express foreign peptides on its surface by a method called phages display (Smith *et al.,* 1997). In this project phage display was used to express Ciz1/Ciz1b peptides on the phage surface to be used for immunization in mice.

Phage is a virus that infects bacterial cells, *Escherichia Coli* (*E.Coli*) used as the standard recombinant DNA host. Foreign DNA sequences can be inserted into the phage genome together with the gene encoding one of the phage coat proteins and when phage particles are being produced in the *E.coli*, the foreign peptide is expressed and displayed on the phage surface (Smith *et al.,* 1997).

Phage display is mostly based on a filamentous phage vector. The filamentous phage is coated with 2700 copies of the major coat protein pVIII encoded from the phage gene VIII (Smith *et al.,* 1997). There are several types of phage display systems and the system used in this project was based on a type 88 vector (Smith *et al.,* 1997). The type 88 vector used was an f88-4 vector (Smith *et al.,* 2006) containing two genes VIII; one being the recombinant gene VIII (into which the foreign DNA can be inserted) and the other the wild-type gene VIII. When *E.coli* replicates the genes VIII the major coat protein pVIII are extruded out from *E.coli* through the cell envelope without killing the cell giving rise

to phage particles called virions. Due to the design of the vector, the virions express a mosaic of major coat protein pVIII on the surface both expressing the recombinant peptide pVIII molecule and the wild-type pVIII molecule. Only a minority of the pVIII copies on the surface will express the recombinant peptide (Smith *et al.,* 1997).

In this project f88-4 vector was used kindly provided by Prof . G. P. Smith (University of Missouri, Columbia, US).The f88-4 vector contains 9234 base-pairs (Smith *et al.,* 2006) and the recombinant gene VIII sequence is shown in figure 1. In the recombinant gene VIII sequence there are two restriction enzyme sites one for *Hind*III and one for *Pst*I. The sequence between the restriction enzyme sites are the stuffer that can be replaced by a foreign DNA sequence (Smith *et al.,* 2006).

tac promoter

TCCCCCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTC

Gene VIII translation initiation region

TTAATGGAAACTTCCTC

Figure 1 Sequence of the recombinant gene VIII of the f88-4 vector. The restriction enzyme sites, for restriction enzyme *Hind*III and *Pst*I, are indicated with fat bodied base pairs and the vertical arrow show were respectively restriction enzyme cleave the sequence. The region between the two restriction enzymes sites are the stuffer that can be replaced by a foreign DNA sequence. The foreign DNA sequence can be amplified and sequenced using: forward primer pVIII, reverse primer pVIII and a f88-4 sequencing primer (indicated with arrows which direction show direction during PCR). The tac promoter, gene VIII translation initiation region, start codon and stop codon are indicated. The red base pairs represent the sequence for the major coat protein pVIII (Modified from (*Smith et al.,* 2006) and (Karlsson, 2005)).

In this project the stuffer was replaced by foreign DNA sequences encoding Ciz1/Ciz1b peptides. Phage clones used in the project were sequenced using an f88-4 sequencing primer and amplified using of a forward primer pVIII and reverse primer pVIII (figure 1).

2.4 Hybridoma technology for antibody production

In this study antibodies were established using classical hybridoma technology (St. Groth *et al.,* 1980). Mice, in this project Balb/c female mice, were immunized with the antigen of interest to produce antibodies against this antigen.

After immunizations mouse serum can be collected and the amount of antibodies against the antigen of interest can be analyzed. When the serum titer is sufficient the B-cells from the spleen can be collected used for fusion (National Research Council (U.S), 1999). B-lymphocytes in spleen produce antibodies (National Research Council (U.S.), 1999) but they have a limited life span in culture. Therefore they are fused with myeloma cells that grow indefinitely in normal growth medium (Alberts *et al.,* 2008).

By growing the newly fused cells in a selective growth medium containing a DNA synthesis inhibitor the myeloma cells not fused with B-lymphocytes will die. B-lymphocytes fused with myeloma cells have the ability to grow in this selective medium. B-lymphocytes not fused have limited life span in culture and will die after a couple of weeks. Therefore only hybridoma cells will be able to survive and proliferate over time (Alberts *et al.,* 2008).

Screening of hybridoma cells to find which ones that produce antibodies against the antigen of interest can be done by collecting medium from the hybridoma colony and the specificity for the antigen being analyzed. Hybridomas producing antibodies against the antigen of interest are a stable and permanent source of antibodies against the antigen and can be collected and saved (Alberts *et al.,* 2008). In this project enzyme-linked immunosorbent assays (ELISAs) were used as selection method.

2.5 Site-specific immunization

Phage display and hybridoma technology can be used in combination in a method called site-specific immunization. In site-specific immunization a region of interest of a protein is expressed on a filamentous phage surface and immunized into mice to direct the immune system to a specific, predefined region of the protein. The peptide of interest displayed on the phage surface induces a directed immune response in the mice. B-cells from the immunized mice can be used to establish site-specific antibodies by hybridoma technology (Lidqvist, et al., 2008).

In this project Ciz1/Ciz1b peptides were expressed on the filamentous phage to generate an immune response against specific regions of the Ciz1/Ciz1b proteins, such as the Ciz1b-specific splice site. Mice showing Ciz1/Ciz1b titer were used for hybridoma technology to gain site-specific antibodies against Ciz1/Ciz1b.

3. Materials and Methods

Mammalian lung cancer cell lines: H345, H69 and H128, were cultivated and analyzed to find a native source of Ciz1b for future antibody characterization. Both his-tagged proteins and biotinylated peptides were used in ELISAs for serum titer measurement. Recombinant his-tagged Ciz1b protein was expressed. In this study phage display was used to express recombinant Ciz1 peptides on the phage surface to be immunized in Balb/c female mice. Mice were boosted with antigen several times and immunological response analyzed to choose mice for hybridoma technology.

3.1 Sources of Ciz1b antigens

In this project different sources of Ciz1b antigens were used and analyzed. In mammalian lung cancer cell lines the Ciz1/Ciz1b expression was analyzed at mRNA level and protein level by polymerase chain reaction (PCR) and Western blot. Synthesized biotinylated peptides and his-tagged proteins were used in ELISAs for blood serum screening for titer and for selection of hybridomas producing Ciz1b antibodies. His-tagged Ciz1b protein was expressed in *E.coli.*

3.1.1 Human lung cancer cell lines at mRNA level and protein level

Mammalian lung cancer cell line H69 and H128 were cultured with Dulbecco´s Modified Eagle Medium (DMEM, Sigma), 10% fetal bovine serum (FBS, Thermo scientific) and 1% DMEM Supplement (gibco) according to ATCC^{R}. Iscove´s Modified Dulbecco´s medium (17.66 g/l Iscove´s Modified Dulbecco´s medium and 3.02 g/l $NaHCO_3$ in distilled water) supplemented with 5 µg/ml insulin from bovine pancreas (Sigma) and 5% heat treated FBS was used for cell culturing of mammalian lung cancer cell line H345.

3.1.1.1 Ciz1 and Ciz1b at mRNA level

The mRNA expression of Ciz1/Ciz1b was studied in mammalian lung cancer cell lines H69, H128 and H345. The cells were collected by centrifugation at 200xg for eight minutes and washed twice in PBS (0.317 g/l NaH_2PO_4, 1.093 g/l Na_2PO_4, 8.5 g/l NaCl) and re-suspended in PBS and stored in RNAlater^{R} solution (Ambion The RNA Company) according to manufacturer´s instruction. mRNA was extracted using RNeasy midi kit (Qiagen) and cDNA synthesized using Superscript III first strand synthesis system for RT-PCR (Invitrogen).

Amplification of Ciz1 and Ciz1b was done by PCR and the PCR protocol had to be optimized to gain the most optimal conditions for the primers. The splice site was amplified by two different primer pairs: Ciz1 forward primer in combination with Ciz1 splice site primer and Ciz1 forward primer in combination with Ciz1 reverse primer.

3.1.1.1.1 Primer design

All primers were synthesized by Eurofins MWG operon (Germany). In Figure 2 the DNA sequence for Ciz1 is presented. The recognition sites for Ciz1 forward primer, Ciz1 splice site primer and Ciz1 reverse primer are indicated and their direction during PCR marked with an arrow. The splice site generating the Ciz1b variant is marked in green.

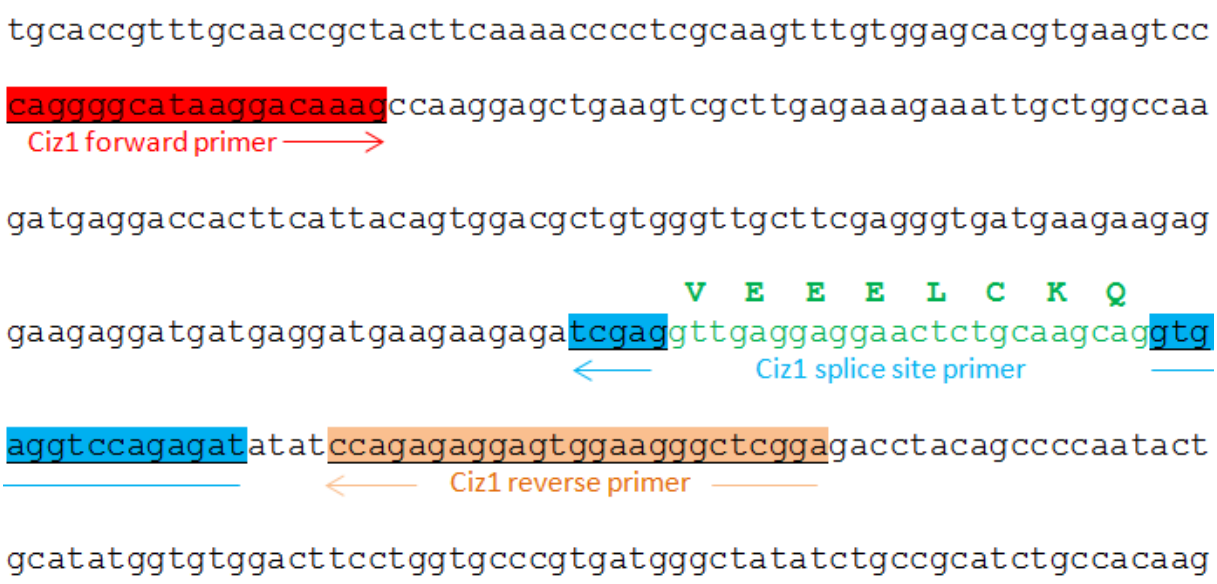

Figure 2 Base pair sequence for Ciz1 indicating position for splice site for Ciz1b, Ciz1 forward primer, Ciz1 splice site primer and Ciz1 reverse primer. The red part of the sequence indicates the recognition for Ciz1 forward primer. The red arrow indicates direction the Ciz1 forward primer amplifies during PCR. The green marked letters indicate the Ciz1 splice site were the small letters represent the base pair sequence and the large letters represent the amino acid sequence. The blue marked sequence represents the recognition for Ciz1 splice site primer and its direction during PCR by the arrow. Ciz1 reverse primer recognizes the sequence indicated by orange color and the direction during PCR are indicated by the arrow.

The Ciz1 forward primer and the Ciz1 reverse primer were constructed according to Higgins *et al.* (Higgins *et al.,* 2012). The PCR using these primers would optimally give products of two different sizes, one being the Ciz1 sequence with 221 base pairs and one the Ciz1b sequence with 197 base pairs. The Ciz1 splice site primer was decided to be Ciz1b specific, not including the spliced sequence. Ciz1 forward primer and Ciz1 splice site primer would therefore only give one product with a length of 168 base pairs if the Ciz1b sequence is present in the sample.

3.1.1.1.1.1 PCR using Ciz1 forward primer and Ciz1 splice site primer

PCR mixture contain 0.5 µM Ciz1 forward primer, 0.5 µM Ciz1 splice site primer, 1 U TAQ DNA polymerase (ABgene), 0.4 mM dNTP, 1x Reaction Buffer IV(ABgene), MQ up to total volume, 1 µl cDNA for each reaction and $MgCl_2$(ABgene). Parameters optimized were: $MgCl_2$ concentration (optimized for 1.5 mM, 1 mM and 0.5 mM) and annealing temperature (optimized for 52^0C to 68^0C with 2^0C increment).

The PCR program was run in a Bio-Rad T100^{TM}Thermal cycler with hot start by 94^0C for three minutes followed by the PCR program. The PCR program was done with 1 cycle initial denaturation 94^0C during one minute followed by 30 cycles: denaturation at 94^0C for 30 seconds, annealing temperature for 30 seconds and extension 72^0C for one minute followed by final extension on cycle at 72^0C under five minutes.

The PCR products were analyzed using 2% agarose gel electrophoresis. The gel contained 2% agarose NA (GE health care) in a TAE buffer (10 mM Tris-HCl (1.2 g/l Tris, 1.8 g/l NaCl and 0.1 g/l NaN3, HCl to pH 7.75), 1.14 ml/l Acetic acid and 0.05 M EDTA pH 8.0) and ethidium bromide solution 0.07% (VWR).

3.1.1.1.1.2 PCR using Ciz1 forward primer and Ciz1 reverse primer

PCR mixture containing 0.5 µM Ciz1 forward primer, 0.5 µM Ciz1 reverse primer, 1 U TAQ DNA polymerase, 0.4 mM dNTP, 1x Reaction Buffer IV, 1.5 mM $MgCl_2$, MQ up to total volume and 1 µl cDNA for each reaction was used.

The PCR program was run in a Bio-Rad T100™Thermal cycler. PCR program with one cycle initial denaturation 94^{0}C during one minute followed by 30 cycles: denaturation at 94^{0}C for 30 seconds, annealing 56^{0}C for 30 seconds and extension 72^{0}C for one minute. Final extension 72^{0}C for one cycle under five minutes.

The PCR products were analyzed as described above.

3.1.1.2 Ciz1 and Ciz1b at protein level

Mammalian lung cancer cell line H69 and H128 were centrifuged at 200xg for eight minutes and cell pellets were re-suspended in cold PBS. Cells were washed three times in cold PBS and centrifuged at 200xg for eight minutes. Finally pellets were stored at -70^{0}C.

Cell pellets were stored at -70^{0}C re-suspended in Ciz1 extraction buffer buffer (10 mM PBS, 100 mM NaCl, 300 mM Sucrose, and 1 mM EDTA) supplemented with 1 mM dithiothreitol (DTT), 1% Triton x100, 1 mM $MgCl_2$, phosSTOP (Roch) and Complete mini EDTA-free (Roch) and incubated on ice for 20 minutes. The lysates were centrifuged at 2000xg for two minutes and supernatant collected to 1x NuPage LDS Sample buffer 4X (life technology). 75 mM DTT was added and the samples were stored at -20^{0}C until Western blot analysis.

3.1.1.2.1 Western blot of Ciz1 and Ciz1b protein extracted from mammalian lung cancer cell lines

The samples prepared as described above were separated on a NuPage 10% Bis-Tris gel (life technologies) together with a ladder Magic MarkTM XP Western standard (Invitrogen) at 200V for 35 minutes and transferred to PVDF membrane (Bio-rad) according to manufacturer´s instruction. The membrane was blocked overnight in 5% non-fat dry milk in 4^{0}C. pAb anti-Ciz1 Antibody (Novus biological), recognizing Nov4 at the Ciz1 C-terminal, was diluted in 1/1000 in 5% non-fat dry milk, pre-blocked 1 hour and then added to the membrane and incubated 1 hour. The membrane was washed three times during 20 minutes with TBST 0.2% Tween 20 (3 g/l Tris, 8.75 g/l NaCl, 2 g/l Tween20, HCl to pH 7.3) and Polyclonal Swine Anti-Rabbit immunoglobulins/horseradish peroxidase (HRP, DakoCytomation) diluted in 1/3000 in 5% non-fat milk pre-blocked 1 hour added to the membrane incubated 1 hour. Wash step was repeated and protein detection using chemiluminescece was performed (ECL+, GE Healthcare) according to manufacturer's instruction.

3.1.2 Recombinant his-tagged protein

His-tagged protein was prepared by Cizzle biotech and used to screen blood samples from immunized mice to detect anti-Ciz1/Ciz1b titer. The proteins were expressed in *E.coli* from pTrcHisA vector (Invitrogen). The protein contains the 6HIS-tag, a linker region and the C-terminal sequence of Ciz1 region (either the Ciz1b region or Ciz1 region) amino acid number 678 to amino acid 898. The last 40 amino acids at the C-terminal region, marked in green in the figure below, is the Nov4 detected by pAb anti-Ciz1 Antibody (Novus biological). The amino acids marked red in the sequence below is the eight amino acids that are spliced in the Ciz1b variant, further mentioned his-tagged Ciz1b protein. His-tagged protein containing the red amino acids is the Ciz1 further mentioned his-tagged Ciz1 protein.

6XHIS –linkerMGGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS-
IAKQSLRPFCTVCNRYFKTPRKFVEHVKSQGHKDKAKELKSLEKEIAGQDEDHFITVDAVGCFEGDEEEEEDDEDEEE
IEVEEELCKQVRSRDISREEWKGSETYSPNTAYGVDFLVPVMGYICRICHKFYHSNSGAQLSHCKSLGHFENLQKYKA
AKNPSPTTRPVSRRCAINARNALTALFTSSGRPPSQPNTQDKTPSKVTARPSQPPLPRRSTRLKT

Expression optimization of his-tagged Ciz1b protein is described in section 3.2.

3.1.3 Biotinylated peptide

The biotinylated Ciz1 and Ciz1b peptides were synthesized by Innovagen. Below the biotinylated Ciz1 amino acid sequence are shown. The amino acids marked in red are the eight amino acids that are spliced away when generating Ciz1b. Biotinylated Ciz1 peptide includes all the amino acids shown in the sequence and biotinylated Ciz1b peptide include the amino acids not marked in red.

Biotin-Ahx-DEDEEEIEVEEELCKQVRSRDISR

3.2 Recombinant his-tagged Ciz1b protein expression

Constructs for expression of the his-tagged Ciz1b proteins were prepared by Cizzle Biotech and used for his-tagged Ciz1 protein expression. The constructs were transformed into *E.coli* TOP10 strain (Invitrogen) to be used for recombinant his-tagged Ciz1b protein expression. His-tagged protein expression were evaluated for: with or without glucose and lysis buffer (8 M Urea, 10 mM Tris pH 8.0, 1 mM DTT, phosSTOP and Complete mini EDTA-free) with LB medium(0.5% NaCl, 0.5% yeast extract and 1% tryptone) or SOB medium (20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl pH 7 and 1 mM $MgCl_2$). Lysis and protein purification was evaluated with or without lysis buffer.

3.2.1 Transformation of pTrcHisA vectors including his-tagged Ciz1b protein into *E.coli* TOP 10

5 ml LB medium were inoculated with one *E.coli* TOP 10 colony growing at 37^0C with vigorous shaking overnight. Overnight culture were inoculated in LB medium 1:100 growing with vigorous shaking at 37^0C to OD_{600nm}=0.5. At OD_{600nm}=0.5 the culture was incubated 10 minutes on ice and then cells were collected by centrifugation at 3000xg for 10 minutes at 4^0C. To get $CaCl_2$- competent *E.coli* the pellet was re-suspended in ice-cold 0.1M $CaCl_2$ on ice. Cells collected by centrifugation at 13000xg, 10 minutes at 4^0C and re-suspended in 0.1 M ice cold $CaCl_2$ stored on ice overnight.

Next day pTrcHisA constructs were inoculated 1:200 in the $CaCl_2$ competent cells incubated 30 minutes on ice, heat chocked at 42^0C for 90 seconds and placed on ice for one minute. Followed by addition of 1 ml LB medium and incubation at 37^0C for 60 minutes. 200 µl culture was added to an ampicillin agar plate (LB medium, 0.1 g/l Ampicillin and 15 g/l agar) and incubated at 37^0C overnight.

3.2.1.1 Optimization of his-tagged Ciz1b protein

5 ml LB medium with 50 µg/ml ampicillin was inoculated with one transformed *E.coli* TOP 10 colony growing overnight at 37^0C with vigorous shaking. LB medium and 50 µg/ml ampicillin were inoculated 1:100 with overnight culture to two culture flasks. With 20 mM glucose or without glucose. The cultures were grown at 37^0C with vigorous shaking to OD_{600nm}= 0.6 and OD_{600nm}=0.8. 1 ml sample taken as a zero sample. To induce protein expression 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the cultures grown at 37^0C with vigorous shaking, taking 1ml samples each hour during four hours for expression analysis.

The 1ml samples were centrifuged at 14000xg for one minute, and supernatant collected to -20^{0}C. The pellets were re-suspended in 50 mM Tris-HCl supplemented with Complete mini EDTA-free and 0.1mg lysozyme (GE Healthcare) and placed on ice until viscous then stored at -20^{0}C overnight.

Cell lysates were thawed and 5 µg/ml DNase1 stock solution (DNase1 (Sigma), 10 mM Tris-HCl pH7.75, 50 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$ and 50% (w/v) Glycerol) supplemented with 12 mM $MgCl_2$ was added. The samples were incubated on ice during 30 minutes with shaking and then centrifuged at 28000xg, 4^{0}C for 20 minutes. Then cell lysate and cell pellets were stored at -20^{0}C.

3.2.1.2 His-tagged Ciz1b protein extraction

His-tagged Ciz1b protein extraction with lysis buffer was evaluated on cultures grown in LB medium or SOB medium.

2 ml medium was inoculated with 50 µg/ml ampicillin together with one clone *E.coli* TOP10 containing pTrcHisA vector growing overnight at 37^{0}C with vigorous shaking. 1:100 overnight culture inoculated with medium and 50 µg/ml ampicillin and grown at 37^{0}C with vigorous shaking to OD_{600nm}= 0.8.

At OD_{600nm}=0.8 1 ml sample were collected and 1 mM IPTG added to rest of culture and continued growth during three hours were 1ml samples collected each hour. After three hours the rest culture were centrifuged at 4600xg, five minutes at 20^{0}C and pellet re-suspended in lysis buffer placed overnight in a rotary shaker in room temperature. 1ml samples centrifuged at 14000xg, 10 minutes and pellet re-suspended in lysis buffer placed in a rotary shaker in room temperature overnight. Next day 1ml samples were centrifuged at 12000xg, one minute and supernatant stored in -20^{0}C. Rest of the culture centrifuged at 3000xg for 15 minutes and supernatant stored in -20^{0}C.

3.2.1.3 ELISA screening of his-tagged Ciz1b protein expression

Cell lysate diluted 1/100 in Blocker™ Casein in PBS (Nordic biolabs) were screened in Ni-NTA HisSorb™ strips (Qiagen) together with his-tagged Ciz1b protein and his-tagged Ciz1 protein from Cizzle Biotech as controls. Cell lysate were added to the Ni-NTA HisSorb™ plate incubated one hour at room temperature with shaking. Plates were washed three times and addition of pAb anti-Ciz1 Antibody (Novus biological) diluted 1/1000 in Blocker™ Casein in PBS incubated one hour at room temperature with shaking. After three washes, Polyclonal Swine Anti-Rabbit immunoglobulins/HRP (Dako) diluted 1/1000 in Blocker™ Casein in PBS was added and incubated 45 minutes at room temperature with shaking. The plate was washed six times and HRP substrate Enhanced K-blue (Neogen Corp.) added and OD_{620nm} measured after 30 minutes.

All wash steps were performed in an automatic plate wash with WASH solution (1.25 g/l Tween 20, 25 g/l Germall II, 225 g/l NaCl, 15.14 g/l Tris, pH 7.75).

3.3 Establishment of Ciz1 antibodies

Ciz1b specific reagents were to be established by immunization of Balb/c female mice. Balb/c female mice were immunized with phage particles displaying Ciz1/Ciz1b peptides on its surface and mice generating anti-Ciz1/Ciz1b titer were used for traditional hybridoma technology.

3.3.1 Production of phage particles for immunization

Two types of peptides were chosen to be displayed on the phage surface: one to direct the immune system to the Ciz1b splice site junction and one to direct the immune response to nearby flanking

regions of Ciz1 upstream and downstream the Ciz1b splice site. The idea was to generate antibodies directed to the Ciz1b splice site junction and to the flanking regions to be used in a pair in ELISA. To direct immune system to the Ciz1b splice site the recombinant peptides named 44 and 76 were used. To direct the immune system toward the flanking regions recombinant peptides named 719 and 777 were used.

Peptide 44 and 76 cover the Ciz1b splice site junction, peptide 44 containing four amino acids upstream and four amino acids downstream the splice site, 4+4, while peptide 76 containing seven amino acids upstream and six amino acids downstream the Ciz1b splice site, 7+6. Peptide 719 encodes a 20 amino acid long peptide on the N-terminal side of the splice site of Ciz1b, amino acid number 719 to 738. While peptide 777 contains amino acid sequence C-terminal of the Ciz1b splice site, corresponding amino acid 777 to 796 (Figure 3).

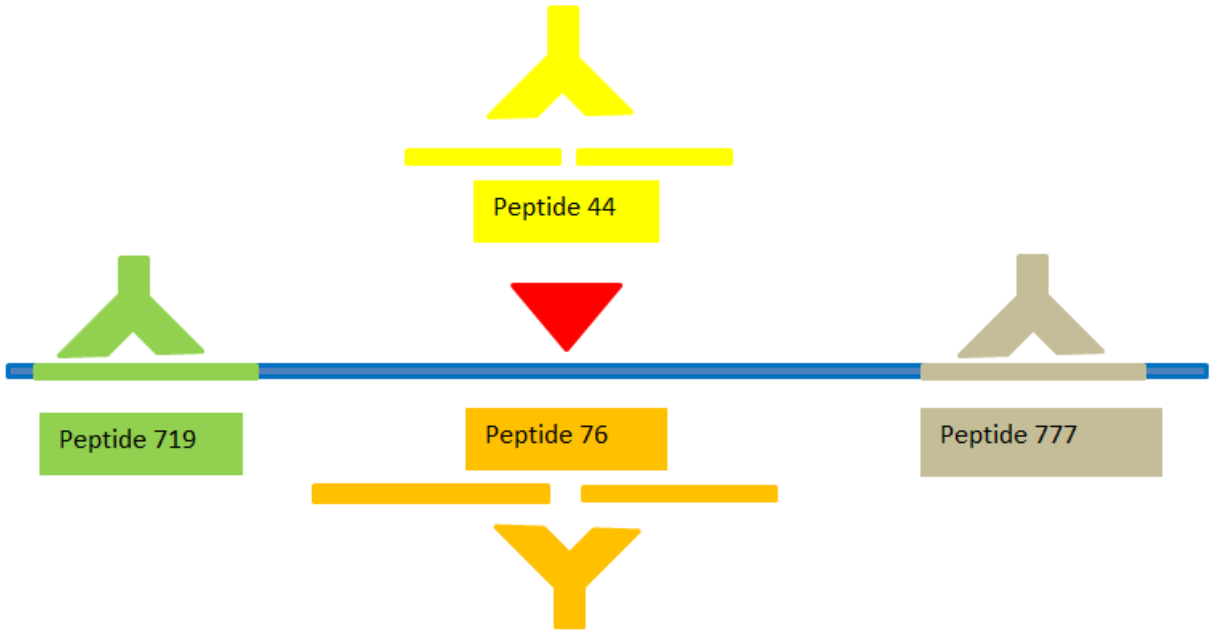

Figure 3 The Ciz1 sequence with the peptides immunized into mice for production of antibodies. The green sequence represent the recombinant peptide sequence for peptide 719 and the inverted green "Y" represent the antibodies raised as an immune response toward peptide 719. The same is for the peptide 777 indicated with gray sequence on the downstream side of Ciz1b splice site. The yellow sequences represent peptide 44 with four amino acids covering the Ciz1b splice site. The same is for peptide 76 indicated with orange sequences flanking the Ciz1b splice site regions with seven respectively six amino acids.

Before the master thesis begun, f88-4 phage display vector (kindly provided by prof G.P. Smith) was cleaved with *Hind*III and *Pst*I restriction enzyme exchanging the stuffer, figure 1, with DNA sequence coding for the Ciz1/Ciz1b peptides constructed as shown in figure 3.

To ensure that the phage clones contained each of the DNA inserts the phage clones were screened using PCR and gel electrophoresis. Selected clones were sequenced to ensure that insert were correct and located in the desired reading frame. The major coat pVIII molecules of the phage clones were analyzed by gel electrophoresis and silver staining to ensure that recombinant pVIII molecule was expressed on the phage surface. For experimental details about these analyses see following sections. The recombinant phage particles were prepared by double PEG precipitation and used to immunize mice.

3.3.1.1 Polymerase chain reaction of phage clones amplifying f88-4 inserts

All phage clones were screened by PCR to control the DNA inserted into the f88-4 vector. The PCR mixture contained 25 pmol of forward primer pVIII (cybergene), 25 pmol reverse primer pVIII(cybergene), 0.4 mM dNTP, 1x Reaction Buffer IV, 1.5 mM $MgCl_2$ and 1 U Taq DNA polymerase to a final volume of 50 µl with MQ. The PCR program done in a Bio-Rad T100™ Thermal cycler with five minutes denaturation at 95°C, one-minute incubation at 95°C, 55°C and 72°C in 30 cycles. Ending with five minutes at 72°C followed by 4°C.

PCR products were separated on a 2% agarose gel together with a control (f88-4 vector with stuffer fragment) and a low DNA mass ladder (Invitrogen). The gel contained 2% agarose NA in a TAE buffer and ethidium bromide solution 0.07%.

3.3.1.2 Purification of phage DNA for sequencing

Phage clones were sequenced to verify that the recombinant amino acids sequence were correct and located in the desired reading frame.

Phage clones were grown overnight in 5 ml LB medium and 20 µg/ml tetracycline with vigorous shaking at 37^0C. Overnight culture was diluted 1:50 in LB medium with 12.5 µg/ml tetracycline and incubated with vigorous shaking at 37^0C during five hours. Over day culture used to purify single stranded phage DNA according to manual "Ph.D.-7™ Phage Display Peptide Library Kit"(New England Biolabs, Beverly, MA, USA).Purified phage DNA pellet re-suspended in 5 mM pH8 Tris-HCl. f88-4 sequence primer pVIII (figure 1) 1:7 with purified DNA were sequenced by Eurofins MWG operon (Germany). Sequence results analyzed using Biology WorkBench (http://seqtool.sdsc.edu/CGI/BW.cgi) and CLUSTALW. Sequence verified clones were used to prepare phage particles for immunization.

3.3.1.3 Phage preparation for immunization in mice

One phage colony for each phage clone was inoculated in 5 ml LB medium and 20 µg/ml tetracycline and cultured overnight with vigorous shaking at 37°C. Overnight culture was inoculated 1:100 with LB medium in 12.5 µg/ml tetracycline and was grown over day with vigorous shaking at 37^0C. The culture then diluted 1:125 in LB medium and grown overnight in 12.5 µg/ml tetracycline and 1 mM IPTG with vigorous shaking at 30^0C.

The overnight culture was centrifuged at 9000xg, 4^0C for 15 minutes and the phage containing supernatant transferred to PEG/NaCl (20% Polyethylene glycol 8000 in 2.5 M NaCl) incubated on ice during one hour followed by centrifugation at 9000xg, 4^0C for 30 minutes. Phage pellet was re-suspended in TBS (0.05 M Tris, 0.15 M NaCl, pH 7.5) and collected by centrifugation at 9000xg, 4^0C for 10 minutes. The phages in the supernatant were precipitated with PEG/NaCl incubated on ice one hour and phages collected by centrifugation at 9000xg, 4^0C for 30 minutes and re-suspended in TBS and sterile filtered with 0.8/0.2 µm PALL® Acrodisc® PF (PALL life sciences) and absorbance measured at wavelength 269 nm and 320 nm. All phage particles were stored at 4^0C until immunization.

Phage concentration was calculated using equation 1 (Smith *et al.,* 2006):

$$\frac{\text{Virons}}{\text{ml}} = \frac{(\text{Abs}_{269} - \text{Abs}_{320}) * 6 * 10^{16}}{\text{genom size}} \qquad (1)$$

3.3.1.4 Analysis of the major coat protein of filamentous phage ensuring coating with recombinant pVIII peptides

Phage clones were separated by gel electrophoresis to verify that the phages contain both the wild-type pVIII molecule and recombinant pVIII molecule on the surface.

Phage preparations were diluted fivefold with DTT. 1X NuPage LDS Sample Buffer was added and the samples denatured at 70^0C for 10 minutes followed by incubation on ice for 10 minutes. Phage proteins separated on a NuPAGE 12% Bis-Tris gel (life technologies) with MES SDS Running Buffer at 200 voltages for 40 minutes. The gel stained with a ProteoSilver™Silver Stain Kit (Sigma).

See Blue Pre-Stained Standard (Invitrogen) was used as ladder and wild-type f88-4 phage used as control.

3.3.2 Balb/c female mice immunization with phage clones

A total of 30 eight to ten weeks old Balb/c female mice (Charles River) divided into five groups with six mice each were used. Each group was immunized with one of the following phage clones: 44, 719, 777 and 76. The groups immunized with phage clone 44, 719 and 777 were immunized with $5*10^{11}$ virions each immunization. To verify the risk of allergic reaction against the phage preparations two groups were immunized with different virion concentration of phage clone 76. 76: low immunized with $1*10^{11}$ virions and 76: high immunized with $5*10^{11}$ virions for the first immunization. No allergic response was seen after the first immunization and therefore 76: low were immunized with $5*10^{11}$ virions for all following immunizations. The mice were immunized with a 200 µl dose intraperioneally containing 100 µl Sigma Adjuvant system® (Sigma). Immunization was done at day 0, 21, 42, 63, 84 and 105.

3.3.2.1 Establish ELISAs for analysis of antibody response in immunized mice

To measure the immune response in mice two different ELISAs were established: one using biotinylated peptides and one using his-tagged proteins. The protocols were optimized regarding solution to dilute the components with. Solutions tested were: 0.2% BSA in PBS, 1% BSA in PBS and Blocker™ Casein in PBS.

His-tagged Ciz1b protein and his-tagged Ciz1 protein described in section 3.1.2 were used to analyze mice immunized with antigen 719 and 777 while biotinylated Ciz1b peptides and biotinylated Ciz1 peptides described in section 3.1.3 were used to analyze serum from mice immunized with antigen 44 and 76.

To follow the immune response blood samples were collected at day 0, 49, 70 and 91 from tail vein. The blood was diluted in PBS and stored at -20^0C. Blood samples were screened for titer using ELISA. Mice with significant titer were used for hybridoma technology. All mice for all groups were analyzed in ELISA for Ciz1/Ciz1b titer. The mice generated titers were used for hybridoma technology.

3.3.2.1.1 Serum analysis with his-tagged proteins

The following protocol were optimized for dilution solution: 0.2% BSA in PBS, 1% BSA in PBS and Blocker™ Casein in PBS. The protocol was then used for his-tagged proteins to measure immune response from mice immunized with antigen 719 and antigen 777.

Ni-NTA HisSorb™ was used to immobilize the his-tagged proteins. The wells were incubated with his-tagged Ciz1b protein diluted 1/100 in dilution solution overnight in moisture chamber at 4^0C. The

wells were washed three times and normal mouse sera diluted 1/250 and 1/500 in dilution solution incubated two hours with shaking. The wells were washed three times and Polyclonal Rabbit anti mouse immunoglobulins/HRP (Nordic biolabs) diluted 1/1000 in dilution solution incubated 60 minutes at room temperature with shaking. Wells washed six times and HRP substrate Enhanced K-blue added and incubated 30 minutes. For the protocol optimization for dilution solution OD_{620nm} were measured. When study immune response in mice STOP solution (0.12 M 32% HCL in distilled water) was added and OD_{450nm} measured.

All wash steps performed in an automatic plate wash with WASH solution.

As a positive control for his-tagged proteins pAb anti-Ciz1 Antibody (Novus biological) recognizing Nov4 for the Ciz1- C terminal was used. Control experiment with normal mouse sera and wells without any antigen were included.

3.3.2.1.2 Serum analysis with biotinylated peptides

The dilution solution chosen from section 3.3.2.1.1 was used for ELISA protocol studying mice titer against Ciz1b in mice immunized with antigen 44 and 76.

Maxisorp strip (Nunc) coated with streptavidin washed once and biotinylated peptides diluted to concentration 1µg/ml with Blocker™ Casein in PBS immobilized during 30 minutes at room temperature with shaking. Wells washed three times and mouse sera diluted 1/250 and 1/500 in Blocker™ Casein in PBS incubated at room temperature during 60 minutes with shaking. Wells washed three times and Polyclonal Rabbit anti mouse immunoglobulins/HRP diluted 1/1000 in Blocker™ Casein in PBS were incubated 60 minutes at room temperature with shaking. Wells washed six times and HRP substrate Enhanced K-blue incubated during 30 minutes at room temperature with shaking and OD_{620nm} measured.

Positive control for biotinylated Ciz1b peptide was antibody TB (from Cizzle biotech) recognizing Ciz1b splice site and negative control was normal mice sera and wells without any antigen. All wash steps performed in an automatic plate wash with WASH solution.

3.3.3 Traditionally hybridoma technology by fusion of spleen cells and myeloma cells

Spleen cells from mice with high anti-Ciz1b/Ciz1 serum titer were used to establish antibodies, as described in the review article “Production of monoclonal antibodies: Strategy and tactics” (St. Groth *et al*., 1980). The mice were sacrificed and the spleen collected sterile. 10^6 spleen cells were fused with 10^7 newly harvested P3X63Ag8.653 myeloma cells (ATCC®: CRL-1580™) and the newly fused cells were grown in 96 well culturing trays in Iscove´s Modified Dulbecco´s Medium supplemented with: 10% FBS, 1% DMEM supplement, 1% gentamicin, 1X HAT supplement (gibco), 5% supernatant from cell line 5637 at 37^0C and 8% CO_2.

3.3.3.1 ELISA for selection of Ciz1/Ciz1b antibody producing hybridomas

Ciz1b/Ciz1 antibody producing hybridomas were selected using ELISA. Two ELISA screening methods were used in a two-step manner.

ELISA screen number one were done seven days after fusion using Affini Pure Goat Anti-Mouse IgG+ IgM (H+L) (Jackson ImmunoResearch) immobilized maxisorp strips. The wells washed twice, antibody containing growth media added and incubated in a moisture chamber at room temperature overnight. Wells washed twice and 1 µg/ml biotinylated Ciz1b peptide diluted in 1% BSA in PBS

incubated during two hours in moisture chamber at room temperature. Wells washed twice and ExtrAvidinR-peroxidase (Sigma) diluted 1/1000 in 1% BSA in PBS incubated one hour in moisture chamber at room temperature. Wells washed twice and OPD substrate (Citrate buffer pH 5, o-Phenylenediamine dihydrocloride (OPD, Sigma) and H_2O_2) added and OD_{450nm} measured after 60 minutes incubation at room temperature with shaking.

All wash steps performed in an automated plate wash with WASH solution. Hybridomas generating OD_{450nm} over 0.2 were continued growing in six well culturing trays with Iscove´s Modified Dulbecco´s medium supplemented with: 10% FBS, 1% DMEM supplement, 1% gentamicin, 1x HT supplement (gibco) and 5% 5637-supernatant.

Hybridomas showing selectivity for Ciz1b in ELISA screen number one were chosen to be frozen for storage. Hybridoma cells were collected by centrifugation 200xg at eight minutes and pellet re-suspended in cold freezing solution containing 10% Dimethyl Sulphoxide (DMSO) Hybri-Max^R (Sigma) and 90% FBS and distributed in cryo tubes (Nunc) placed at 4^0C in 30 minutes, -70^0C overnight and then stored at -170^0C.

Medium from the hybridoma cultures were collected to 0.01% MIT to be screened in ELISA screen number two. ELISA screen number two was done against antigen immobilized in plate with streptavidin coated maxisorp strips. Plates were washed once and 1 µg/ml biotinylated Ciz1b peptide, 1 µg/ml biotinylated Ciz1 peptide and without antigen were diluted with Blocker^{TM} Casein in PBS immobilized one hour at room temperature with shaking. Wells washed twice and medium from hybridoma colony were immobilized two hours at room temperature with shaking. The wells were washed twice and Polyclonal Rabbit anti mouse immunoglobulins/HRP diluted 1/1000 in Blocker^{TM} Casein in PBS were added for one hour at room temperature with shaking. Wells washed four times and incubated with OPD substrate during 60 minutes then OD_{450nm} were measured.

Positive control was antibody TB and negative control culturing medium without antibodies.

All steps preformed with an automatic plate wash with WASH solution.

4. Results and discussion

In this section the results are presented, interpreted and discussed. Results for Ciz1/Ciz1b expression at mRNA level and protein level are first presented followed by results for his-tagged Ciz1b protein expression. Results from analysis of phage are presented together with ELISA optimization, blood serum analysis and screening of hybridomas.

4.1 Ciz1 and Ciz1b at mRNA level

To explore whether the mammalian lung cancer cell lines analyzed express Ciz1 and Ciz1b at mRNA level two sets of primers were used. In the PCR protocol using Ciz1 forward primer and Ciz1 splice site primer one fragment of approximately 168 base pairs should be amplified if the Ciz1b variant was present. Ciz1 splice site primer was designed not to recognize the Ciz1 sequence.

The PCR protocol using Ciz1 forward primer and Ciz1 reverse primer amplify both Ciz1 and Ciz1b if present. If both Ciz1 and Ciz1b are present in the mammalian lung cancer cell lines two fragments with different lengths are expected to be seen on the gel. A fragment with a size of 221 base pairs amplified from Ciz1 (Figure 2, green base pairs included) and a fragment with 197 base pairs amplified from Ciz1b (Figure 2, green base pairs excluded).

To be able to use Ciz1 forward primer and Ciz1 splice site primer the PCR protocol had to be optimized reducing unspecific binding of Ciz1 splice site primer to Ciz1 DNA. The optimization was done for $MgCl_2$ concentration and for annealing temperature. $MgCl_2$ concentration of 0.5 mM generated lowest unspecific binding for the Ciz1 splice site primer towards Ciz1 DNA. Higher $MgCl_2$ concentration, 1mM and 1.5mM, made Ciz1 splice site primer unspecific amplifying Ciz1 DNA.

Annealing temperature was evaluated and the optimal annealing temperature was 62^0C when using 0.5 mM $MgCl_2$. An annealing temperature higher than 62^0C generated no detection of DNA when using 0.5 mM $MgCl_2$.

Using 0.5 mM $MgCl_2$ and annealing temperature 62^0C for the mammalian lung cancer cell lines (H128, H69 and H345) there were seen that all three mammalian lung cancer cell lines possibly containing Ciz1b on mRNA level. In figure 4 PCR products using Ciz1 forward primer and Ciz1 splice site primer separated on 2% agarose gel are shown. All three mammalian lung cancer cell lines: H69, H128 and H345 show one distinct band with a length of approximately 170 base pairs. Therefore indicating that they might contain Ciz1b on mRNA level. One thing to have in mind is that the Ciz1 splice site primer maybe is not specific enough and may amplify Ciz1 DNA as well. Therefore there is hard to say with certainty that those mammalian lung cancer cell lines contain Ciz1b on mRNA level.

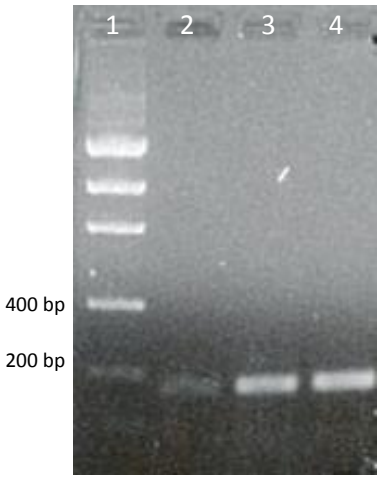

Figure 4 Results for PCR products using Ciz1 forward primer and Ciz1 splice site primer separated on a 2% agarose gel. From left: Lane 1 Low DNA mass ladder, Lane 2 H69, Lane 3 H128 and Line 4 H345. The mammalian lung cancer cell lines give one distinct band at an approximately size of 170 base pairs that may represent Ciz1b (Figure 2).

The PCR protocol using Ciz1 forward primer and Ciz1 reverse primer did not need any optimization. In figure 5 PCR products using Ciz1 forward primer and Ciz1 reverse primer separated on a 2% agarose gel are shown. The three mammalian lung cancer cell lines generated a distinct band with more than 200 base pairs, approximately 220 base pairs, as expected and were concluded to represent Ciz1. The size of this fragment is the same as the Ciz1 DNA control in lane 5. Mammalian lung cancer cell line H345 also show a band with lower intensity precisely under the distinct band with less than 200 base pairs, approximately 195 base pairs, representing Ciz1b.

The Ciz1 DNA and Ciz1b DNA, lane 5 and lane 6 in figure 5, generated one band each with more than 200 base pairs representing Ciz1 and one band each with less than 200 base pairs representing Ciz1b. There should only be one band for each of them representing Ciz1 respectively Ciz1b. In figure 5 there are seen two bands for both the Ciz1 DNA and Ciz1b DNA which might be explained by impurities in the sample.

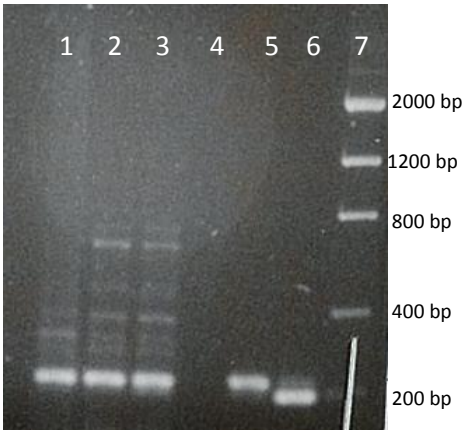

Figure 5 Results for PCR products using Ciz1 forward primer and Ciz1 reverse primer separated on a 2% agarose gel. From left: Lane 1 H69, Lane 2 128, Lane 3 H345, Lane 4 no DNA, Lane 5 Ciz1 DNA, Lane 6 Ciz1b DNA and Lane 7 low DNA mass ladder. Mammalian lung cancer cell lines H69 and H128 show bands with more than 200base pairs representing Ciz1 on mRNA level. Mammalian lung cancer cell line H345 show one band with an approximated size of 200 base pairs and one band with slightly lower than 200 base pairs representing Ciz1 respectively Ciz1b on mRNA level.

When comparing the results generated from PCR protocol using Ciz1 forward primer and Ciz1 splice site primer with the results from PCR protocol using Ciz1 forward primer and Ciz1 reverse primer it can be concluded that mammalian lung cancer cell line H345 contain both Ciz1 and Ciz1b on mRNA level. The mammalian lung cancer cell lines H69 and H128 generated different results in the two PCR protocols. The difference in results for those two PCR protocols may be explained by unspecific binding of Ciz1 splice site primer to Ciz1 DNA as well. Another explanation can be that there is more Ciz1 fragment in those mammalian lung cancer cell lines being amplified using Ciz1 forward primer and Ciz1 reverse primer and therefore there will be no band visualized for Ciz1b fragment.

The conclusion drawn from the two PCR protocols is that mammalian lung cancer cell line H345 contains both Ciz1 and Ciz1b on mRNA level. Mammalian lung cancer cell lines H69 and H128 contain Ciz1 but it is hard to say with certainty whether they contain Ciz1b or not.

4.2 Ciz1 and Ciz1b at protein level

Ciz1 and Ciz1b protein expression in mammalian lung cancer cell lines H69 and H128 was verified by Western blot. Due to the lack of cells from mammalian lung cancer cell line H345 the Ciz1 and Ciz1b expression on protein level were not analyzed for this cell line.

In section 3.1.2 the amino acid sequence for his-tagged Ciz1b protein is given. His-tagged Ciz1b protein contain 267 amino acids (section 3.1.2, red amino acids excluded) representing approximately 30 kDa. The pAb anti-Ciz1 Antibody bind to the C-terminal end of Ciz1b/Ciz1, green amino acid sequence marked in the figure of section 3.1.2. If Ciz1 and Ciz1b protein were present in the samples two bands with full length Ciz1/Ciz1b molecular weights should be generated. Were Ciz1b is having the smaller molecular weight and Ciz1 representing the larger molecular weight. The molecular weight difference between those is approximately 1kDa not possible to be separated on this gel. Therefore only one band should have been generated representing: Ciz1, Ciz1b or both when using pAb anti-Ciz1 Antibody for detection. To generate specific detection of Ciz1b the detection has had to be done with a Ciz1b specific antibody (e.g. TB antibody).

In figure 6 Western blot results after 30 seconds exposure are shown. Mammalian lung cancer cell lines H69 and H128 do not show any visualized bands. His-tagged Ciz1b protein, lane 6, show one distinct band with a correct size of approximately 30kDa indicating that the Western blot protocol has worked. There are also three other less distinct bands visualized that may come from the Magic Mark^{TM} XP western standard, lane 7.

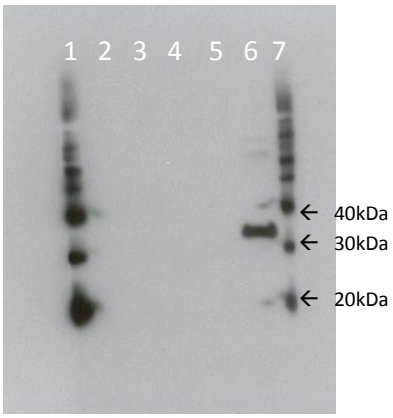

Figure 6 Western blot results after 30 seconds exposure. From left: Lane 1 Magic Mark XP Western Standard, Lane 2 H69 5µl, Lane 3 H69 10µl, Lane 4 H128 5µl, Lane 5 H128 10µl, Lane 6 His-tagged Ciz1b protein and Lane 7 Magic Mark XP Western Standard. Mammalian lung cancer cell lines H69 and H128 generated no visible bands on the gel. His-tagged Ciz1b protein gives one distinct band with a correct size, approximately 30 kDa, representing Ciz1b.

No bands were detected by Western blot for the mammalian lung cancer cell lines H69 and H128. Either the mammalian lung cancer cell lines do not express Ciz1 or Ciz1b on protein level, the Ciz1 and Ciz1b protein were not purified out from the cell or the level of Ciz1 and Ciz1b protein were to low to be detected in this experiment.

4.3 Recombinant his-tagged Ciz1b protein expression

His-tagged Ciz1b protein, described in section 3.1.2, expression was evaluated to generate antigen for analysis of titer in blood serum from mice immunized with antigen 719 and 777. His-tagged Ciz1b protein expression was evaluated with and without glucose in growth media and by inducing protein expression at different cell densities (OD=0.6 and OD=0.8) as well as different growth media compositions (LB medium and SOB medium). His-tagged Ciz1b protein was controlled under a *lac* operon. When glucose is available the gene under the *lac* operon are only expressed in a basal level (Alberts *et al.,* 2008). Cizzle Biotech has seen that the his-tagged Ciz1b protein can be toxic to the cells and therefore glucose were added during cell growth to minimize the toxic effect. Urea in the lysis buffer was used to denature the protein to see if the protein were located in inclusions bodies. Protein extraction was evaluated and none of the protocols generated his-tagged Ciz1b protein expression.

The conclusion drawn from those optimizations is that neither of them has been successful for his-tagged Ciz1b protein expression. The protocols have to be further optimized regarding both expression and protein extraction to hopefully be able to generate his-tagged Ciz1b protein.

4.4 Polymerase chain reaction of phage clones amplifying f88-4 insert

Phage constructs were analyzed by PCR to verify that they contain the sequence encoding peptide 44, 76, 719 or 777 in the f88-4 vector before being used for immunization in mice. If the phage clones exchanged the stuffer (figure 1, 21 base pairs) with peptide 719 and peptide 777 (Section 3.3.1, 20 amino acids representing 60 base pairs) the phage clones are expected to generate one band on the gel 39 base pairs longer than the control. If phage clones exchanged stuffer with peptide 44 (section 3.3.1, eight amino acids representing 24 base pairs) and peptide 76 (section 3.3.1, 13 amino acids representing 39 base pairs) one band with an increase of three base pairs respectively 18 base pairs compared to the control are expected to be generated on the gel.

In figure 7 six phage clones expressing peptide 719 and five phage clones expressing peptide 777 are separated together with a DNA mass ladder and the control (f88-4 vector containing the stuffer). All phage clones except clone number three expressing peptide 777 generated one distinct band on the gel with increased size of approximately 40 base pairs compared to the control, line 2. Indicating that the stuffer has been exchanged with the foreign DNA sequence. In phage clone number three expressing peptide 777 three bands were generated on the gel with different sizes. Indicating that phage clone number three expressing peptide 777 contain a mix of fragment and are not to be used for further analyses.

Figure 7 Separation of PCR products. Upper part, left: Lane 1 Low DNA mass ladder, Lane 2 control f88-4 vector containing stuffer, Lane 3- Lane 8 phage clones expressing peptide719, Lane 9 – lane 13 phage clones expressing peptide 777, Lane 14 low DNA mass ladder. All phage clones except phage clone number three expressing peptide 777, lane 11, generated one distinct band approximately 40 base pairs longer than the control; indicating that the stuffer has been exchanged with sequence encoding peptide 719 or peptide 777. Phage clone number three expressing peptide 777, lane 11, contains several bands indicating a mixture of fragments.

The same results were generated for phage clones expressing peptide 44 or peptide 76.

4.5 Sequence result for phage clones

Sequence result verified that the correct DNA sequences were inserted into correct reading frame together with gene pVIII for the four phage clones. Sequence results for the four phage clones are shown in Appendix A.

4.6 Phage preparation for immunization in mice

Phage particle concentration was calculated from equation 1. Concentrations (virions per milliliter) for the four phage clones are shown in table 1 together with absorbance values at 269 nm and 320 nm. The samples were diluted 1:20 and that dilution factor was taken into account when calculating the concentrations.

Table 1 Absorbance values at 269nm and 320nm and calculated concentrations (virions per milliliter) for the four phage clones.

Phage clone	Abs_{269nm}	Abs_{320nm}	Concentration (virions/milliliter)
44	0.455	0.017	$5.7*10^{13}$
76	0.390	0.015	$4.8*10^{13}$
719	0.06	0.004	$7.2*10^{12}$
777	0.171	0.008	$2.1*10^{13}$

4.7 Analysis of the major coat protein of filamentous phage ensuring coating with recombinant pVIII peptides

Before immunizing mice with the phage preparations we wanted to ensure that the recombinant peptide was displayed on the phage surface as the recombinant pVIII molecule.

The wild-type pVIII molecule has a molecular weight of approximately 5.5 kDa (Barbas III *et al.,* 2001). Recombinant pVIII molecule containing peptide 719 and peptide 777 has an increase of 20 amino acids (Section 3.3.1) representing an increase of approximately 2.5 kDa compared to the wild-type pVIII molecule. Recombinant pVIII molecules containing peptide 44 and peptide 76 has an increase of eight amino acids respectively 13 amino acids (Section 3.3.1) representing approximately 1kDa respectively 1.5kDa increase compared to the wild-type pVIII molecule. If the phage clones contain both wild-type pVIII molecule and recombinant pVIII molecule two bands should be separated on the gel.

Both phage preparations have strong bands of approximately 5-5.5 kDa representing the wild-type pVIII molecule.

Another band with a larger molecular weight compared to the wild-type pVIII molecule was also seen in both phage preparations. The molecular weights for those bands are estimated to be approximately 7 kDa. A molecular weight increase from approximately 5 kDa to 7 kDa represent approximately 2.5 kDa increase indicating that phage clones also express the recombinant pVIII molecule.

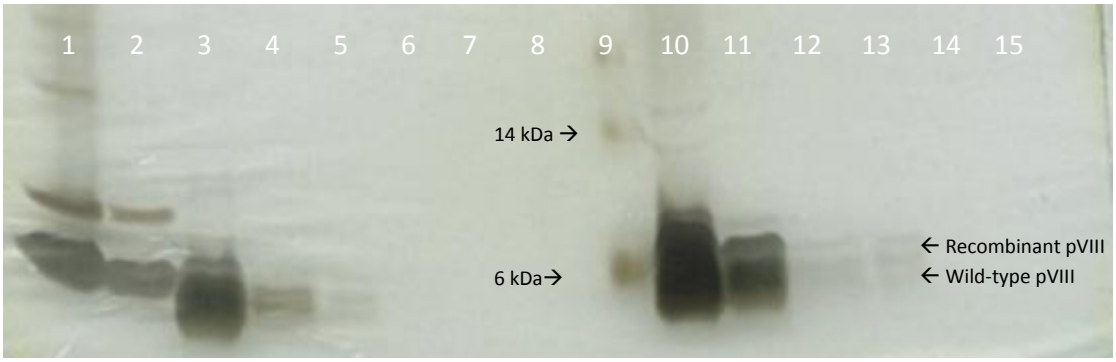

Figure 8 Gel picture with separated phages: f88-4, phage clones expressing peptide 719 or peptide 777 together with the SeeBlue Plus2 Pre-Stained Standard ladder. Lane 1-Lane 2 f88-4 (fivefold dilution starting with $1.0*10^{11}$ phages), lane 3-Lane 8 phage clone expressing peptide 719 (fivefold dilution starting with $4*10^{10}$ phages),Lane 9 See Blue Plus2 Pre-Stained Standard, Lane 10-Lane 15 phage clone expressing peptide 777 (fivefold dilutions starting with $1*10^{11}$ phages).Both phage clones generate one band with an molecular weight of 5-5.5 kDa and one band with molecular weight 7kDa representing wild-type pVIII molecule respectively recombinant pVIII molecule.

Results for phage clones expressing peptide 44 or peptide 76 indicated that they express both wild-type and recombinant pVIII molecule (Appendix B). Therefore all phage clones can be used for site-specific immunization in mice to be able to gain an immune response toward the selected peptides.

Fivefold dilution was done for the phage clones to estimate the fraction of wild-type pVIII molecules on the phage surface compared to the recombinant pVIII molecule. However, the separation on the gel was not good enough to allow conclusions regarding amount of recombinant pVIII molecule and wild-type pVIII molecule. But since the experiment shows that phages express the recombinant pVIII molecule they were used for site-specific immunization.

4.8 Establish ELISAs for analysis of antibody response in immunized mice

To follow the immune response in the immunized mice an ELISA set up has to be optimized for which diluent to be used. Diluents analyzed were: 0.2% BSA in PBS, 1% BSA in PBS and Blocker™ Casein in

PBS. To be able to select mice that have generated a titer against Ciz1/Ciz1b blood serum were screened using ELISA. A titer is seen when the specific signal for the mouse sera decrease with increased dilution and when the signal increase after immunization boosts.

Two types of ELISAs were used to measure the serum titer in immunized mice. One using his-tagged protein (described in section 3.1.2) immobilized as described in section 3.3.2.1.1. His-tagged proteins were used to analyze mice immunized with antigen 719 and antigen 777. The other ELISA set up immobilized with biotinylated peptides (described in section 3.1.3) as described in section 3.3.2.1.2. The biotinylated peptides were used for mice immunized with antigen 44 and antigen 76. Mice immunized with antigen 44 and antigen 76 was analyzed to measure titer against Ciz1b. Since the biotinylated peptide only contain Ciz1b splice site those peptides were used instead of his-tagged proteins that also contain the flanking regions for those mice that should have gained anti-Ciz1b titer.

4.8.1 Serum analysis with his-tagged proteins

The ELISA protocol to be used for the blood serum screen had to be optimized to generate low unspecific binding to the well. The ELISA protocol was optimized with three diluents. Diluents evaluate were: 0.2% BSA in PBS, 1% BSA in PBS and Blocker™ Casein in PBS. An ideal diluent should generate low unspecific binding of normal mouse sera toward his-tagged Ciz1b protein without affecting the specific signal.

When using 0.2% BSA in PBS high OD_{450nm} values for normal mouse sera was generated that indicating high unspecific binding to his-tagged Ciz1b protein. Therefore 0.2% BSA in PBS was not decided to be used as diluent (Figure 9). 1%BSA in PBS generated lower unspecific binding to his-tagged Ciz1b protein for normal mouse sera compared to 0.2% BSA in PBS but still high unspecificity. Blocker™ Casein in PBS generated the lowest OD_{450nm} values representing the lowest unspecific binding of normal mouse sera toward his-tagged Ciz1b protein and was further used as diluent.

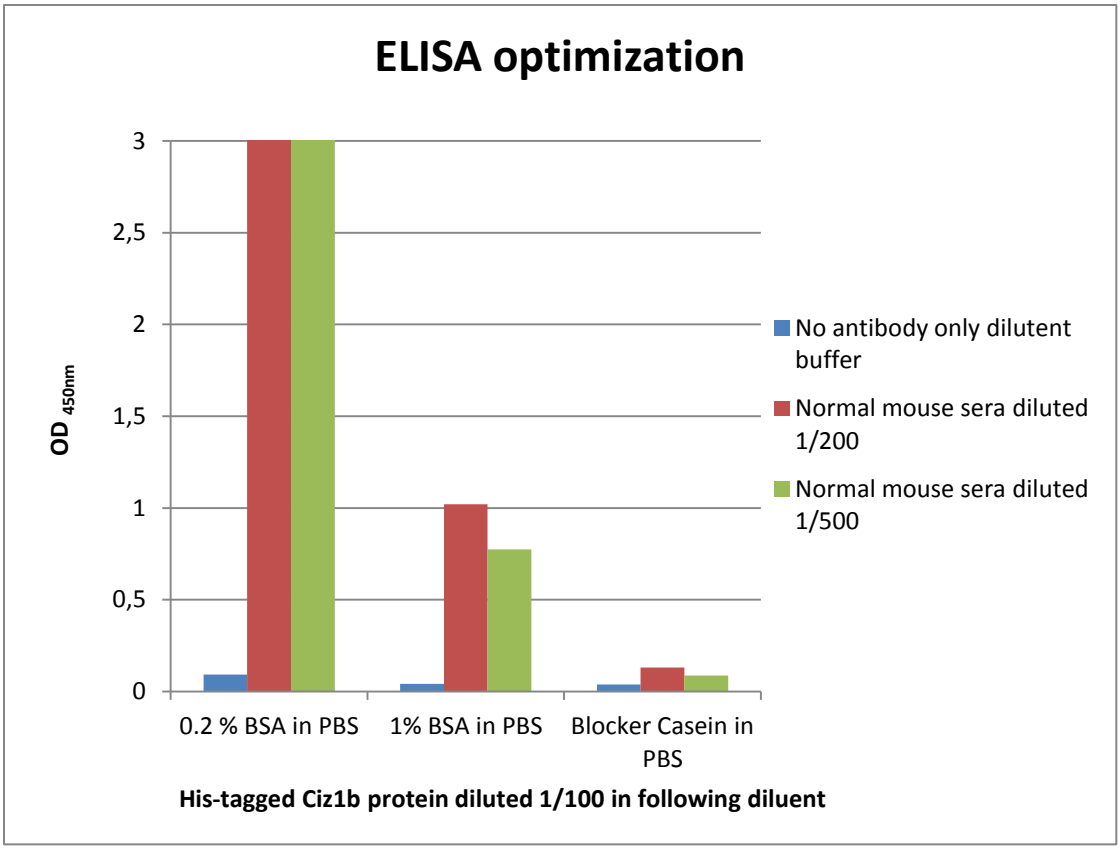

Figure 9 Evaluation of the unspecific binding in ELISA using the three diluents: 0.2% BSA in PBS, 1% BSA in PBS and Blocker™ Casein in PBS. 0.2% BSA in PBS and 1% BSA in PBS generated high unspecific binding for the normal mouse sera toward his-tagged Ciz1b protein. 0.2% BSA in PBS generated higher OD_{450nm} values than three which is the detection limit. Blocker™ Casein in PBS generated almost no unspecific binding of normal mouse sera toward his-tagged Ciz1b protein and was decided to be used as diluent.

Blood samples from mice groups immunized with antigen 719 and antigen 777 were analyzed for anti-Ciz1 titer using his-tagged protein as antigen.

In figure 10 blood sample from mouse number one immunized with antigen 719 is presented for bleeding number four, bleeding at day 91. There is seen that increased dilution for the blood sample generate a decrease in signal for the his-tagged Ciz1 protein indicating specific signal. The reactivity against Ciz1 has increased compared to the zero sample. Therefore it was concluded that mouse number one immunized with antigen 719 had generated a titer against Ciz1.

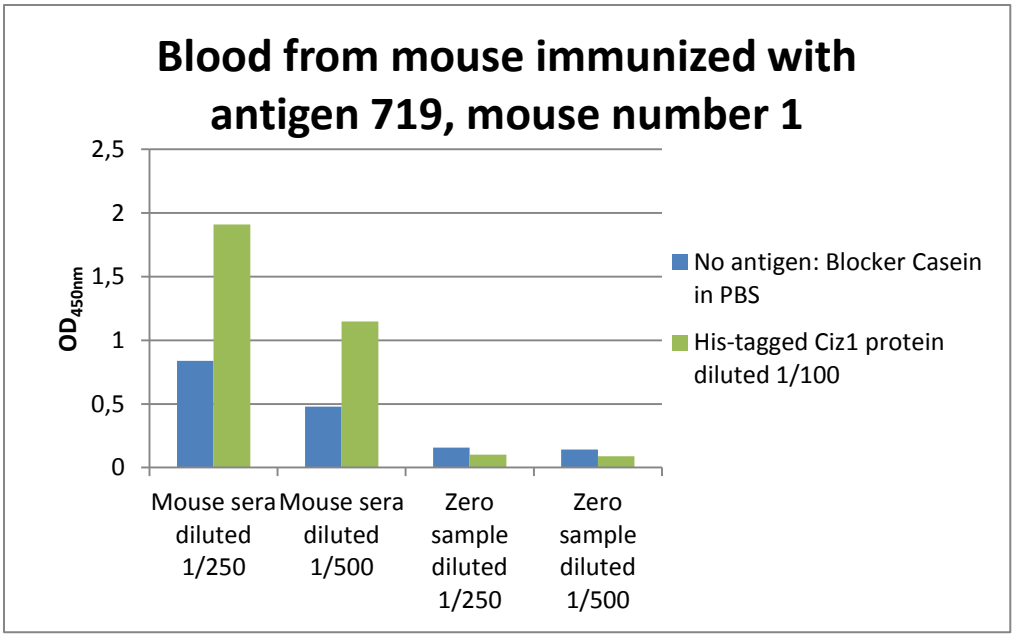

Figure 10 OD_{450nm} values for mouse number one immunized with antigen 719 for blood serum after forth blood collection (after immunization number six) for two dilutions, 1/250 and 1/500. The specific signal for Ciz1 is high and the signal decrease with increased dilution. The signal for Ciz1 has increased compared to the zero sample. Therefore mouse number one immunized with antigen 719 have a significant titer against Ciz1.

Mouse number four immunized with antigen 719 and mouse number three immunized with antigen 777 show similar result as mouse number one immunized with antigen 719 (Appendix C).

These mice generated titer towards Ciz1 and can be used for hybridoma technology. Since the titer for those mice were seen late in the master thesis project the fusion of spleen cell with myeloma cells from these mice will not be included in this master thesis project.

4.8.2 Serum analysis with biotinylated peptides

Since Blocker™ Casein in PBS shown low unspecificity in section 4.8.1 it were also used as diluent for ELISA protocol analyzing blood samples from mice immunized with antigen 44 and antigen 76 without any optimization.

In figure 11 results for blood samples from mouse number four immunized with antigen 44 is presented for bleeding number one and number two. The signal for Ciz1b and Ciz1 decrease with increased dilution and increase after immunization boost verifying specific signal against Ciz1b/Ciz1. The specificity against Ciz1/Ciz1 has also increased compared to the zero sample. Therefore it is concluded that mouse number four immunized with antigen 44 has generated a titer against Ciz1b and was used for hybridoma technology.

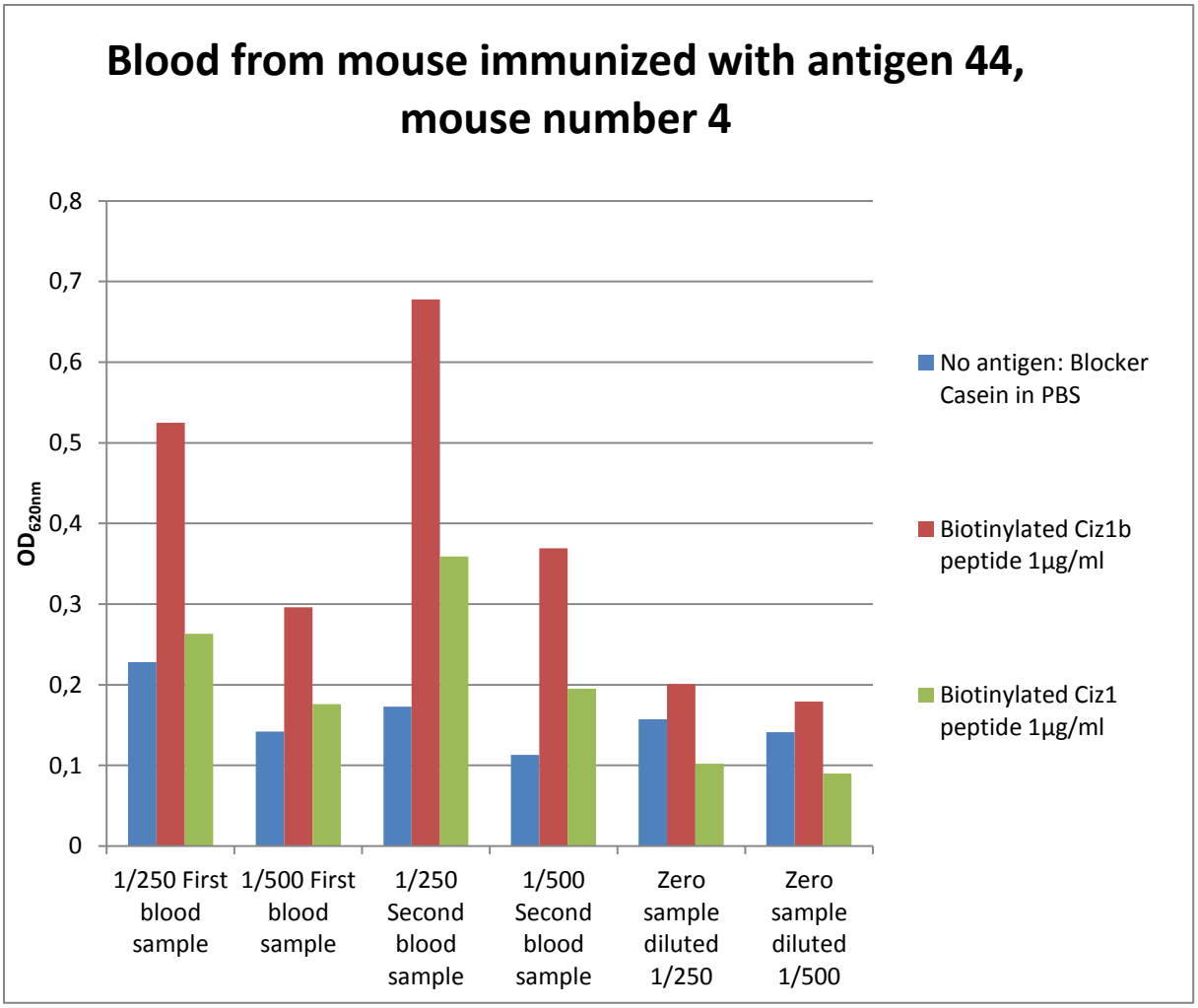

Figure 11 OD_{620} values for mouse number 4 immunized with antigen 44 for blood serum at first and second bleeding for two dilutions, 1/250 and 1/500. The specific signal for Ciz1b are always higher than for the Ciz1 and the signals always decrease when the dilution increase. Specify against Ciz1/Ciz1b are higher after immunization compared to the zero sample indicating that the immune system has produced antibodies directed against Ciz1b. Therefore mouse number four immunized with antigen 44 has a anti-Ciz1b titer.

Mouse number two immunized with antigen 76: high generated similar results as mouse number four immunized with antigen 44 (Appendix D).

Mouse number four immunized with antigen 44 and mouse number two immunized with antigen 76: high shown titer against Ciz1b and were decided to be used for hybridoma technology.

4.9 ELISA for selection of Ciz1/Ciz1b antibody producing hybridomas

Within this project, two fusions were done, each on one mouse, resulting in approximately 1000 hybridomas each.

To select hybridomas with highest specify against Ciz1b, Ciz1-antibody producing hybridomas were selected in a two-step wise manner using two ELISA screening methods. Hybridomas generated from fusion of spleen cells form mouse number four immunized with antigen 44 will further be mentioned Ciz1-. Hybridomas generated from fusion of spleen cells from mouse number two immunized with antigen 76: high will further be mentioned Ciz2-.

In ELISA screen number one hybridomas generating OD_{450nm} values above 0.2 were selected. This resulted in a large number of positive hybridomas: 56 out of 968 Ciz1- hybridomas and 38 out of 824 Ciz2-hybridomas. ELISA screen number two generating OD_{450nm} values above 0.2 were selected. For Ciz1- hybridomas 11 out of the 56 hybridomas were selected and for Ciz2- hybridomas 4 out of 38 hybridomas were selected.

In figure 12 ELISA result from screen number two for the selected Ciz1- hybridomas and Ciz2-hybridomas with an OD_{450nm} above 0.2 are shown.

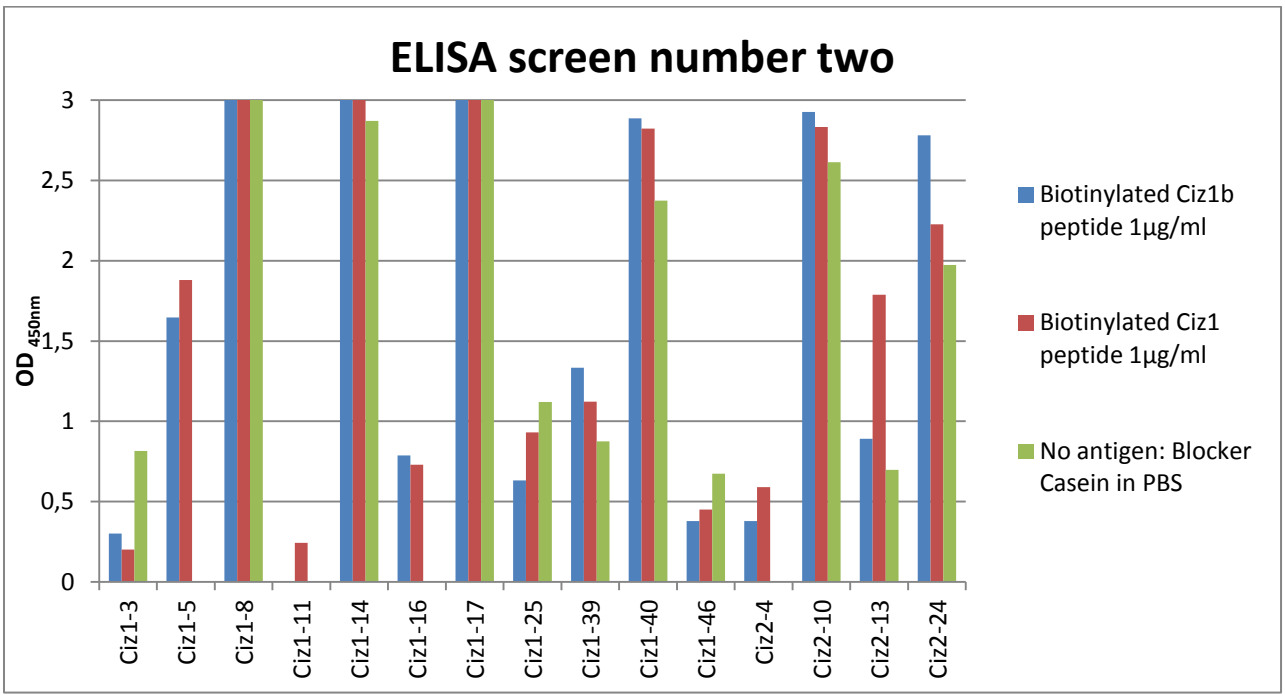

Figure 12 OD_{450nm} values for hybridomas with OD_{450nm} above 0.2. Signal for Ciz1- and Ciz2- hybridomas are plotted against biotinylated Ciz1b peptide and biotinylated Ciz1 peptide. Hybridomas Ciz1-5, Ciz1-16 and Ciz2-4 show specificity against Ciz1b and Ciz1 but not for the wells without antigen. Hybridoma Ciz1-11 only shows specificity against Ciz1. Ciz1-8, Ciz1-14 and Ciz1-17 hybridomas generated higher OD_{450nm} values than three which is the detection limit. All hybridomas showing specificity against Ciz1b and Ciz1 are interesting to further analyze for identify which having highest specificity against Ciz1b.

Hybridomas Ciz1-5, Ciz1-16 and Ciz2-4 generate signal both against Ciz1b and Ciz1 but not against wells without antigen.

Hybridoma Ciz1-11 only shows specificity for Ciz1. Maybe there is also specificity for Ciz1b but that are not visualized due to lower concentration. Analysis of different dilutions of this hybridoma may lead to visualization of specificity against Ciz1b as well.

Hybridomas Ciz1-8, Ciz1-14 and Ciz1-17 generated too high signals and have to be diluted and analyzed further.

Hybridomas generating signal against both Ciz1 and Ciz1b should be studied further to decide whether they recognize Ciz1b and/or Ciz1. Difference in signal between hybridomas can be due to a difference in concentration of the antibodies in the medium or the affinity of the antibody against Ciz1b/Ciz1. Therefore further screening and selection of those hybridomas are important to select

the hybridomas producing antibodies with highest specificity and sensitivity against Ciz1b. All hybridomas shown in figure 12 generated signals for Ciz1b and/or Ciz1 and are therefore important for further analysis to identify one or several hybridomas with highest specificity and sensitivity against Ciz1b.

It should be kept in mind that the studied hybridomas might be a mix of different clones expressing antibodies with different specificities against Ciz1b and different isotypes. The background seen in some of the samples might be due to hybridomas producing antibodies against irrelevant antigens.

5. Conclusions

- Mammalian lung cancer cell line H345 was concluded to contain Ciz1b and Ciz1 on mRNA level. Mammalian lung cancer cell lines H69 and H128 contain Ciz1 on mRNA level but it is hard to say with certainty whether they contain Ciz1b on mRNA level or not. The two PCR protocols generated different results for H69 and H128 whether they contain Ciz1b on mRNA level or not. This can be due to that the Ciz1 splice site primer is not specific enough and amplify Ciz1 as well. Therefore the PCR protocol using Ciz1 forward primer and Ciz1 splice site primer has to be further optimized to generate specific binding to Ciz1b. Another explanation can be that when using Ciz1 forward primer and Ciz1 reverse primer there maybe are more Ciz1 fragments amplified leading to no visualization of Ciz1b fragment on the gel.
- Ciz1b and Ciz1 were not identified at protein level for mammalian lung cancer cell lines H69 and H128 using Western blot. This indicates that the Ciz1 and Ciz1b proteins maybe are not expressed or that they were not purified out from the cell in a detectable level. Higgins *et al*. has seen that both Ciz1 and Ciz1b are located in the nucleus (Higgins *et al*., 2012) and if that's the case those proteins can be hard to purify out from the nucleus and therefore not being detected in this analysis.
- For all the protocols used for his-tagged Ciz1b protein expression none were successful in the way they were performed in this report. Therefore further optimization has to be done.
- The phage clones used for immunization had the recombinant sequence inserted correctly into the reading frame and the recombinant pVIII molecules were displayed on phage surface.
- All four phage clones raised an immune response in respectively mice group.
    - Mouse number one and four immunized with antigen 719 and mouse number three immunized with antigen 777 generated a titer against Ciz1. Those mice can in the future be used for hybridoma technology.
    - A anti-Ciz1b titer was seen for mouse number four immunized with antigen 44 and mouse number two immunized with antigen 76: high. Those mice were used for hybridoma technology.
- Fusion of spleen cells from mouse number four immunized with antigen 44 and mouse number two immunized with antigen 76: high resulted in 11 respectively four hybridomas producing promising antibodies against Ciz1b. To verify the specificity of these antibodies further analysis has to be done.

6. Future perspectives

- To further verify that mammalian lung cancer cell lines H69, H128 and H345 contain Ciz1b on mRNA level or not it is suggested that the PCR protocols should be optimized further.
- The Western blot protocol used for protein analysis of Ciz1b and Ciz1 protein expression in mammalian lung cancer cell line H69 and H128 worked well. The protocol used for Ciz1b and Ciz1 protein purification from the lung cancer cell lines need to be optimized to be able to verify Ciz1b and Ciz1 protein expression. It is suggested to study protein expression of Ciz1 and Ciz1b for mammalian lung cancer cell line H345.
- To increase his-tagged Ciz1b protein yield the protocols have to be further optimized. For examples continue to study if his-tagged Ciz1b protein is in inclusion bodies.
- Mouse number one and four immunized with antigen 719 and mouse number three immunized with antigen 777 show titer against Ciz1 and can be used for hybridoma technology.
- Hybridomas producing promising antibodies against Ciz1b from fusion made from mouse number four immunized with antigen 44 and mouse number two immunized with antigen 76: high need to be further analyzed to select which hybridomas that produce antibodies with highest specificity against Ciz1b.

7. Acknowledgements

Thanks to Cizzle biotech that has been assisting me with protocols, Ciz1b specific antibodies and his-tagged protein.

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Appendix

Appendix A:

Sequence result for the four phage clones using f88-4 sequencing primer. The blue marked sequence represents the recognition site for forward primer pVIII (figure 1). The under lined base pairs represent the recognition site for *Hind*III (Figure 1). The green marked base pairs represent the base pair sequence for the representing recombinant peptide.

Phage clone containing sequence 44:

TTTCTTAATGGAAACTTCCTCATGAAAAAGTCTTTAGTTCTTAAAGCATC

TGTTGCTGTTGCGANTCTTGTTCCTATGCTAAGCTTTGCCGAAATTGAAG

TGCGTAGCCGTGATGCA

Phage clone containing sequence 76:

CTTCCTCATGAAAAAGTCTTTAGTTCTTAAAGCATCTGTTGCTGTTGCGA

CTCTTGTTCCTATGNTAAGCTTTGCCGATGAAGAAGAAATTGAAGTGCGT

AGCCGTGATATTTNNGCN

Phage clone containing sequence 719:

TGTGGAATTGTGAGCGGATAACAATTTCTTAATGGAAACTTCCTCATGAA

AAAGTCTTTAGTTCTTAAAGCATCTGTTGCTGTTGCGACTCTTGTTCCTA

TGCTAAGCTTTGCCCTGGAAAAANAAANTGCGGGCCAGGATGAAGATCAT

TTTATTACCGNTGGATGCCGNGGGTNCN

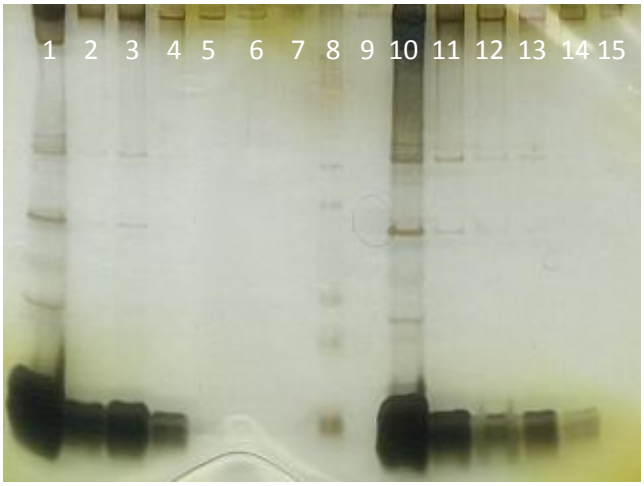
Phage clone containing sequence 777:

ACTTCCTCATGAAAAAGTCTTTAGTTCTTAAAGCATCTGTTGCTGTTGCG

ACTCTTGTTCCTATGCTAAGCTTTGCCAAAGGCAGCGAAACCTATAGCCC

GAACACCGCGTATGGCGTGGATTTTCTGGTGCCGGTTGCA

Appendix B:

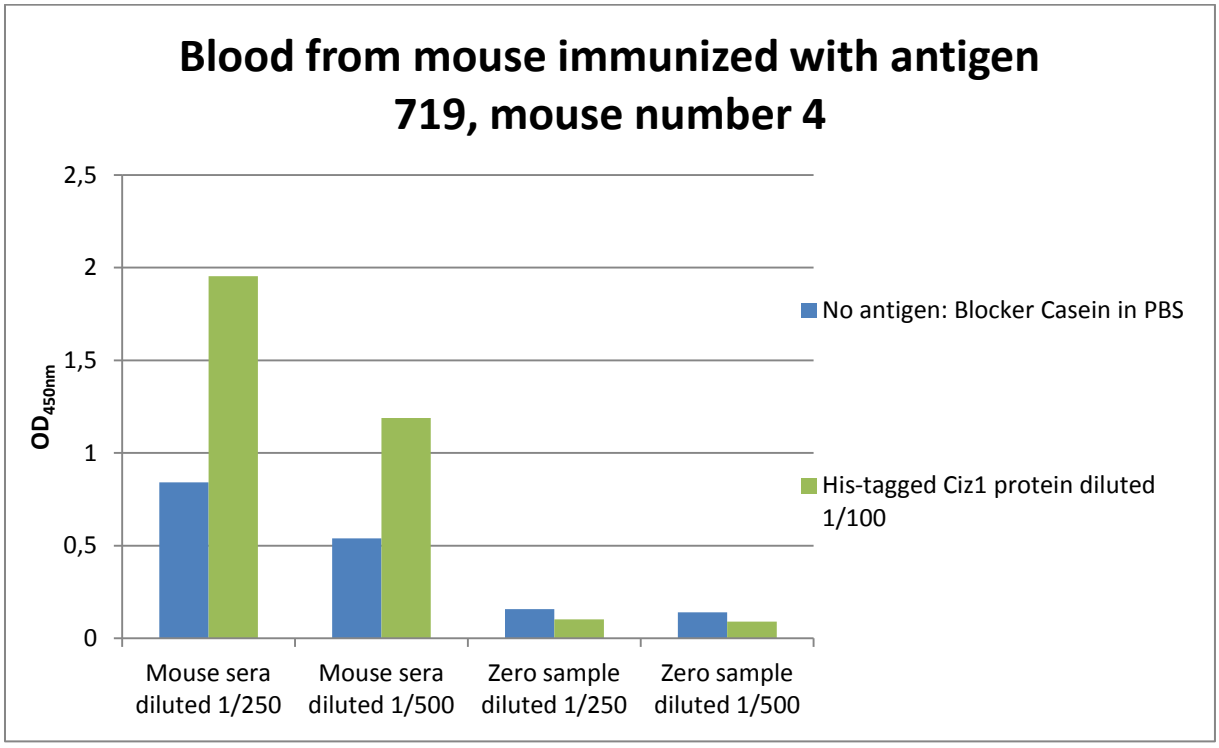


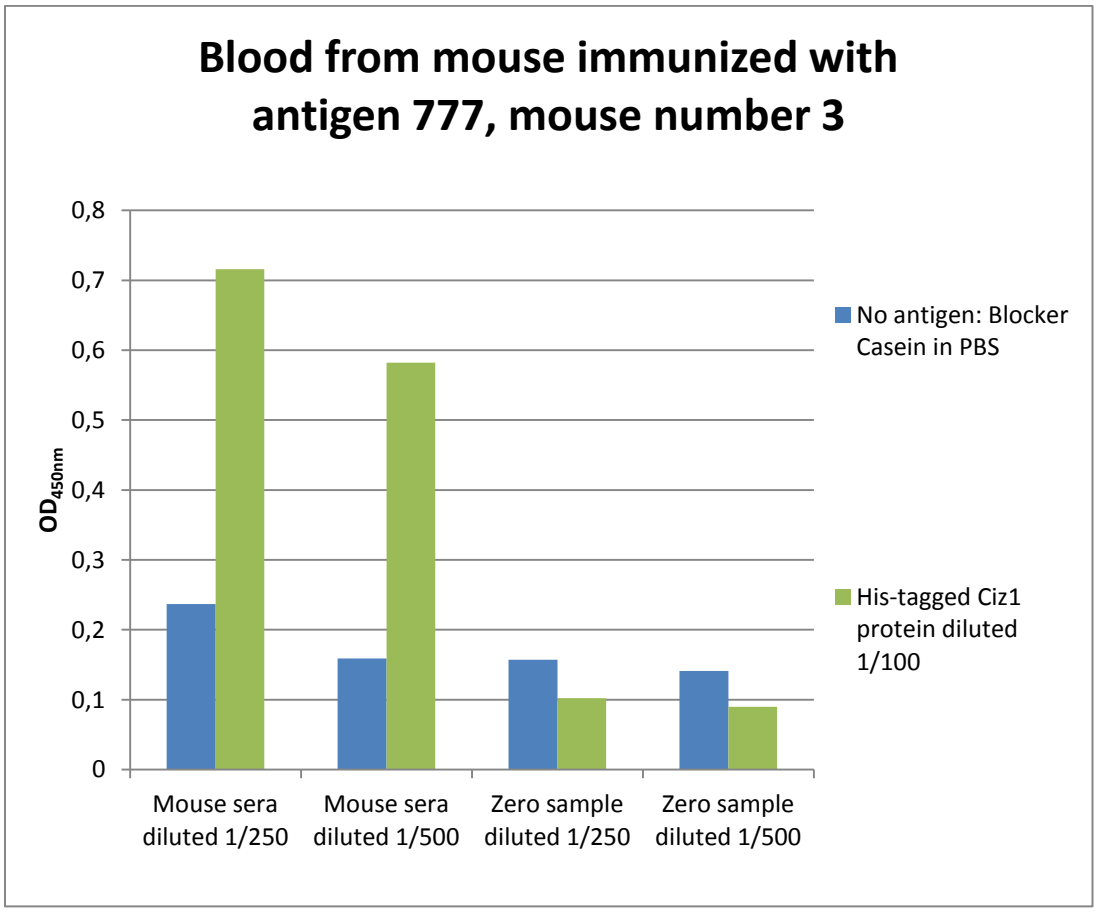
Gel picture with separated phages expressing peptide 44 or peptide 76 together with the SeeBlue Plus2 Pre-Stained Standard ladder. From left: Lane 1 – Lane 6 phage clone expressing peptide 44 (fivefold dilution starting with $3*10^{11}$ phages), Lane 7 empty well, Lane 8 See Blue Plus2 Pre-Stained Standard, Line 9 empty well, Line 10 – Lane 15 phage clone expressing peptide 76 (fivefold dilution starting with $3*10^{11}$ phages).

Appendix C:

ELISA results from blood serum analysis of mouse number four immunized with antigen 719 and mouse number three immunized with antigen 777.

OD_{450nm} plotted against mouse sera for blood serum after forth blood collection for two dilutions, 1/250 and 1/500. The specific signal is high against Ciz1. The signal decrease with increased dilution indicating that those mice have titer and can be used for hybridoma technology.

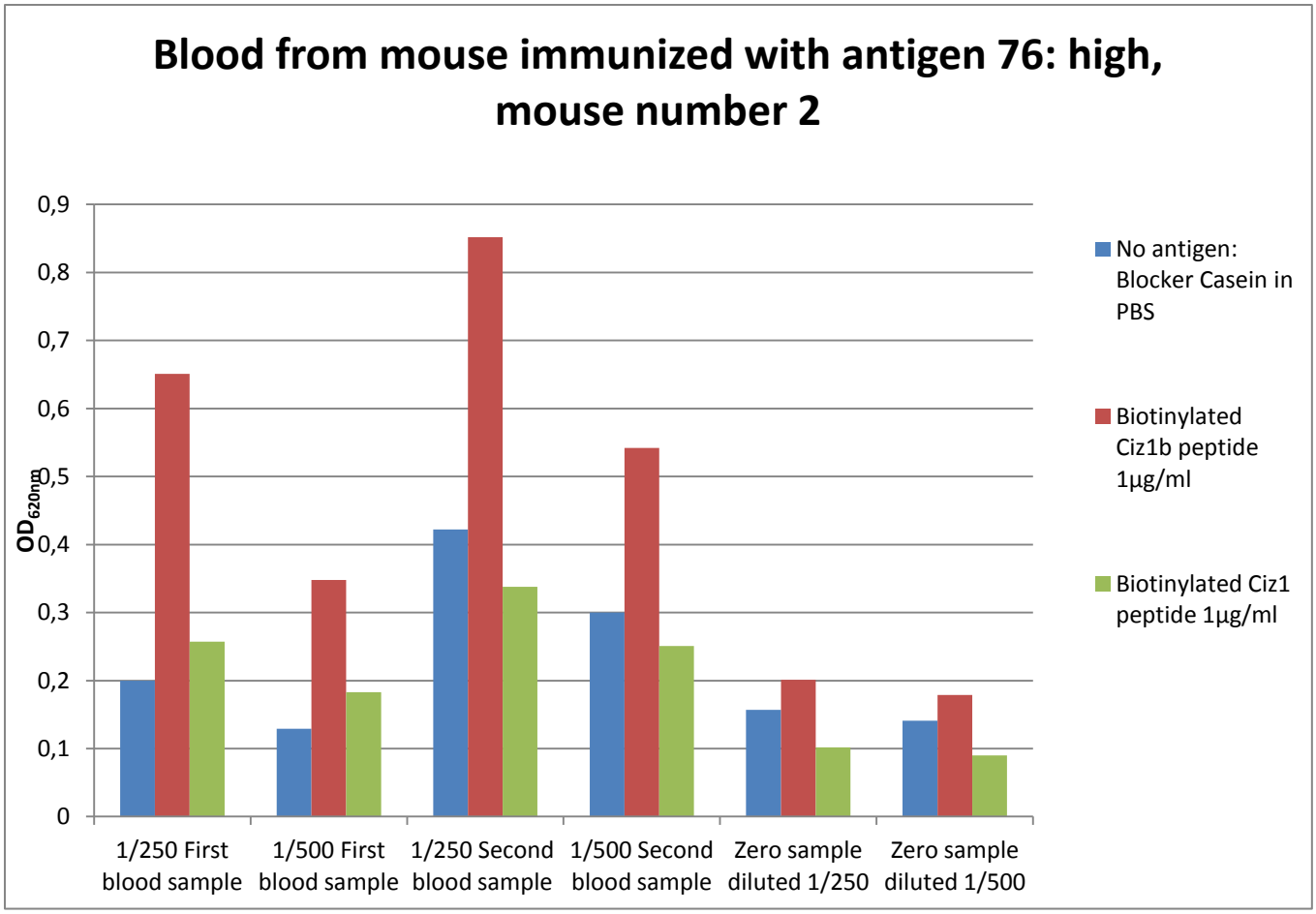




Appendix D:

ELISA results from blood serum analysis of mouse number two immunized with antigen 76: high.

OD_{620nm} value for mouse number two immunized with antigen 76:high for blood serum at first and second bleeding for two dilutions, 1/250 and 1/500. The specific signal for Ciz1b is high and the signal increase after immunization boost and decrease with increased dilution. Therefore it was concluded that mouse number two immunized with antigen 76: high produce antibodies against Ciz1b.


Blood from mouse immunized with antigen 76: high, mouse number 2
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
OD620nm
1/250 First blood sample
1/500 First blood sample
1/250 Second blood sample
1/500 Second blood sample
Zero sample diluted 1/250
Zero sample diluted 1/500
No antigen: Blocker Casein in PBS
Biotinylated Ciz1b peptide 1µg/ml
Biotinylated Ciz1 peptide 1µg/ml