Development of novel human antibodies targeting T cells

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Ingeborg Våg

Abstract

Muromonab-CD3, the first therapeutic monoclonal antibody was approved for clinical use by the US Food and Drug Administration in 1986. Since then, several monoclonal antibodies are approved for the treatment of aggressive and metastatic cancers. In addition to targeting cancer cells, blocking co-inhibitory signals in normal lymphocytes with specific antibodies, known as checkpoint inhibitors, transformed the treatment of cancer. However, checkpoint inhibitors such as those targeting CTLA-4 and PD1 are only active in 15-20% of patients and they are also associated with side effects. Hence, there is a need for developing new antibodies that can modulate T cell function. Co-inhibitory receptors those expressions are unregulated or induced in activated/exhausted effector T cells could represent additional targets for immunotherapeutics.

The overall objective of this thesis was the development of human antibodies for modulating T cell function. The use of T cells as an affinity matrix for antibody selection permits the selection of antibodies that recognize natural cell-surface receptors with appropriate post-translational modifications. A semi-synthetic human single-chain (sc) Fv library was affinity selected on activated or resting T cells and several scFv antibody fragments were selected. One of the selected candidates (named S5) was previously fused to the Fc region of human IgG1 (hinge-CH2-CH3 domains) and its effects on T cell activation were investigated in this thesis. Analysis of the S5 antibody binding to T lymphocytes by flow cytometry showed strong binding to all CD4+ T cell population. By contrast, only a fraction of the CD8+ T cell population bound the S5 antibody, and the binding varied between donors. The S5 antibody induced CD4+ T cell aggregation, whereas the effect on CD8+ T cells was minor. The addition of the S5 antibody to allogeneic mixed lymphocyte reactions did not affect IL-2 production. Epitope mapping experiments using random peptide phage libraries failed to identify peptide binders, indicating that the S5 binding site (epitope) cannot be sufficiently mimicked by short peptides. A pool of scFv phage antibodies obtained from round three of selection on activated/exhausted T cells showed strong binding to T cells stimulated with anti-CD3 and anti-CD28 antibodies as compared to resting T cells. This pool may contain binders to different checkpoint inhibitors. In conclusion, the results indicate that human scFv antibodies targeting resting and/or activated T cells can be generated using our selection protocols. Further work should focus on characterizing the phage clones that bind to activated/exhausted T cells and identifying the antibody binding receptors.

Abbreviations

µl	Microliter
µg	Microgram
Ab	Antibody
AP	Alkaline phosphatase
BSA	Bovine serum albumin
CDR	Complementarity-determining region
DC	Dendritic cells
dH_2O	Distilled water
ddH_2O	Double distilled water
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcR	Fc receptor
FDA	The Food and Drug Administration
hu	Human
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
Ig	Immunoglobulin
M	Molar
mAb	Monoclonal antibody
mg	Milligram
ml	Milliliter
MW	Molecular weight
NK	Natural killer

nM	nanomolar
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
pfu	Plaque forming units
pg	picogram
PS	Penicillin-streptomycin
RCF	Relative centrifugal force
RMPI 1640	Roswell Park Memorial Institute 1650
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
scFv	Single-chain variable fragment
Tw	Tween
TBS	Tris-buffered saline
wt	Wild type

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1 Introduction

1.1 The immune system

The immune system is the body´s defence mechanism against abnormal cells and foreign agents with the potential to cause disease, collectively referred to as pathogens (Alberts, 2015). The immune system recognizes antigens, specific molecular structures that provoke immune responses, leading to neutralization or destruction of the infectious agent as well as infected cells displaying these antigens (Weinberg, 2014). The mammalian immune system has two lines of defence; innate immunity and adaptive immunity (**figure 1**).

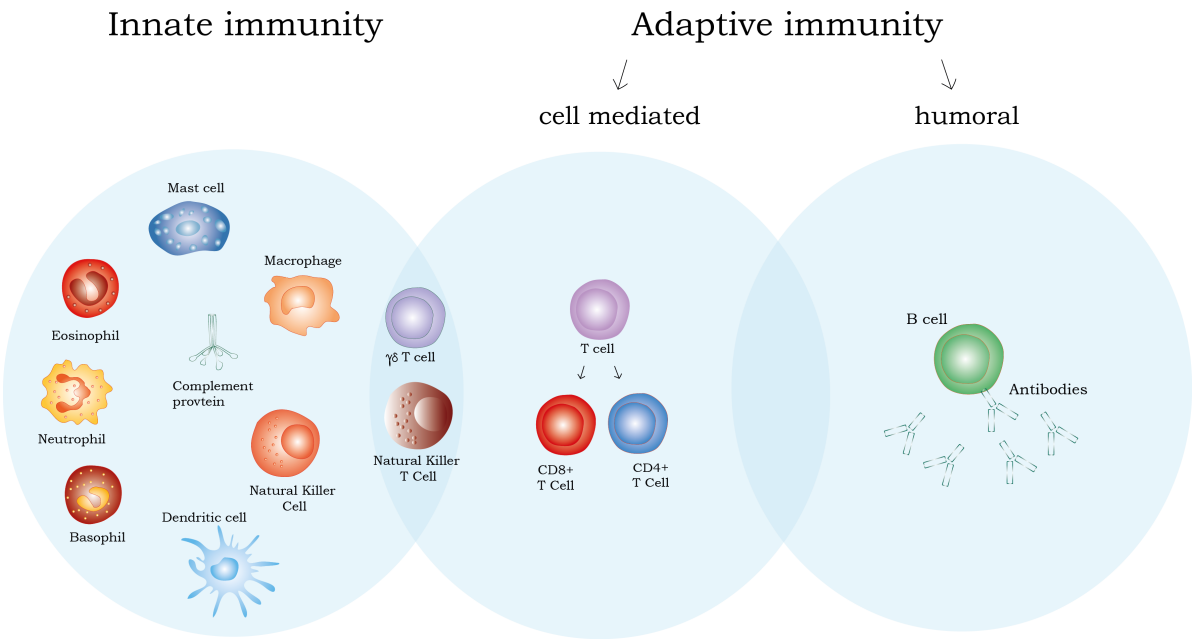

Figure 1. The immune system can be split into innate and adaptive immunity. The rapid innate immune response includes the action of complement proteins, granulocytes (basophils, eosinophils, and neutrophils), mast cells, macrophages, dendritic cells, and natural killer cells. Dendritic cells and macrophages provide an important link to the adaptive immune system by presenting antigens to T cells. The adaptive immune response takes longer to develop but establish increased antigenic specificity and immune memory and comprises B lymphocytes and antibodies in addition to $CD4^+$ and $CD8^+$ T lymphocytes.

1.1.1 Innate immunity

Innate immunity is the instinctive and nonspecific, first-line defence against infection. It comprises both physical barriers like the skin and mucous membranes, chemical barriers like

complement and antimicrobial proteins, and various defensive cell responses (Alberts, 2015). The innate "sensor" cells display invariant pattern recognition receptors (PRRs), which enables them to recognize evolutionary conserved structural motifs on the surface of pathogens and abnormal cells known as pathogen associated molecular patterns (Murphy and Weaver, 2017). Activation of PRRs induces the production of various pro-inflammatory cytokines and chemokines causing an inflammatory response at the site of infection which in turn attracts other immune cells or causes direct elimination of the pathogen through phagocytosis by macrophages or dendritic cells (Alberts, 2015). However, the ability to identify distinct pathogens and to provide the specific protective immunity that prevents reinfection is provided by the antigen-specific lymphocytes of the adaptive immune system.

1.1.2 Adaptive immunity

B and T cells are the two major classes of lymphocytes. Whereas B cells play a major role in humoral immunity by producing antibodies, T cells are involved in cell-mediated responses. The repertoire of B and T cells can recognize an enormous number of potential antigens due to highly diverse cell-surface receptors generated through the genetic recombination of polymorphic gene segments (Parham and Janeway, 2015). The adaptive immune responses are based on the clonal expansion that occurs when the B- and T cells encounter their antigen (Adams et al., 2020). During clonal expansion, B and T lymphocytes proliferate and differentiate into effector or memory cells. Plasma cells are the effector form of B lymphocytes which secrete antibodies with the same antigen specificity as the parent B cell. T lymphocytes are divided into two major classes distinguished by the expression of either a CD4 or CD8 co-receptor (Weinberg, 2014). Upon antigen activation, $CD4^+$ T cells differentiate into different "helper" subtypes, which mediate immune responses through cell-to-cell contact and secretion of various cytokines (Luckheeram et al., 2012). By contrast, $CD8^+$ T cells become cytotoxic effector cells capable of killing virus-infected cells or other intracellular pathogens bearing their cognate antigen (Kaech and Cui, 2012). The memory cells maintain longevity and proliferate and exert effector functions faster and more effectively after a second exposure to their cognate antigen (Alberts, 2015). Thus, they provide long-lasting immunity that follows exposure to disease or vaccination.

1.1.3 T lymphocytes

Most of the human T cell receptors (TCRs) are composed of a transmembrane heterodimer consisting of highly polymorphic α- and β-glycoprotein chains that contain variable and constant regions and are linked by disulphide bonds, resembling the Fab fragment of an antibody (Murphy and Weaver, 2017). A small population of T cells harbours γ and δ-chains instead. T cell clonotypes with unique specificity are generated through the rearrangement of an α and β chain gene segment (Nikolich-Zugich et al., 2004). The heterodimer is non-covalently associated with a group of non-polymorphic signalling chains, called CD3 (Blumberg et al., 1990). Together they form the TCR-CD3 complex which is essential for intracellular signalling and thus T cell activation.

Whereas B cells recognize circulating antigens in their native form, TCRs recognize small peptides derived from degraded proteins (LeBien and Tedder, 2008, Pierce and Weng, 2013). Moreover, the peptide-antigen must be presented by specialized cell-surface glycoproteins called major histocompatibility complex (MHC) proteins. Peptides derived from the cytosolic protein pool are processed and presented by class I MHC proteins on the surface of all nucleated human cells where they are recognized by $CD8^+$ T cells (A Townsend and Bodmer, 1989). In contrast, $CD4^+$ T cells recognize peptides derived from the extracellular environment, which are processed by specialized antigen-presenting cells, such as dendritic cells, macrophages, and B cells, and presented by class II MHC proteins (Alberts, 2015). During T cells development and maturation in the thymus, the T cells undergo positive and negative selection, resulting in the elimination of T cells that react too strongly to self-MHC or self-peptide (Klein et al., 2014). Only those cells that "tolerates" self-antigens as non-immunogenic will leave the thymus and enter the blood and circulate through secondary lymphoid organs.

1.1.4 Antibodies

Antibodies or immunoglobulins (Igs) are relatively large glycoproteins (150kDa) with a characteristic Y-shaped structure composed of two antigen-binding Fab (fragment antigen-binding) arms linked to a single Fc (fragment crystallizable) domain via the hinge region (**figure 2A**). This structural arrangement allows antibodies to act as biological adaptors linking bound antigen to effector functions. The common effector functions are complement-dependent cytotoxicity (CDC), phagocytosis, and antibody-dependent cellular cytotoxicity

(ADCC) (Nimmerjahn and Ravetch, 2008, Meyer et al., 2014). There are five different subclasses of human Igs (IgM, IgD, IgG, IgA, IgE) distinguished by differences in the hinge and constant region (Lu et al., 2018).

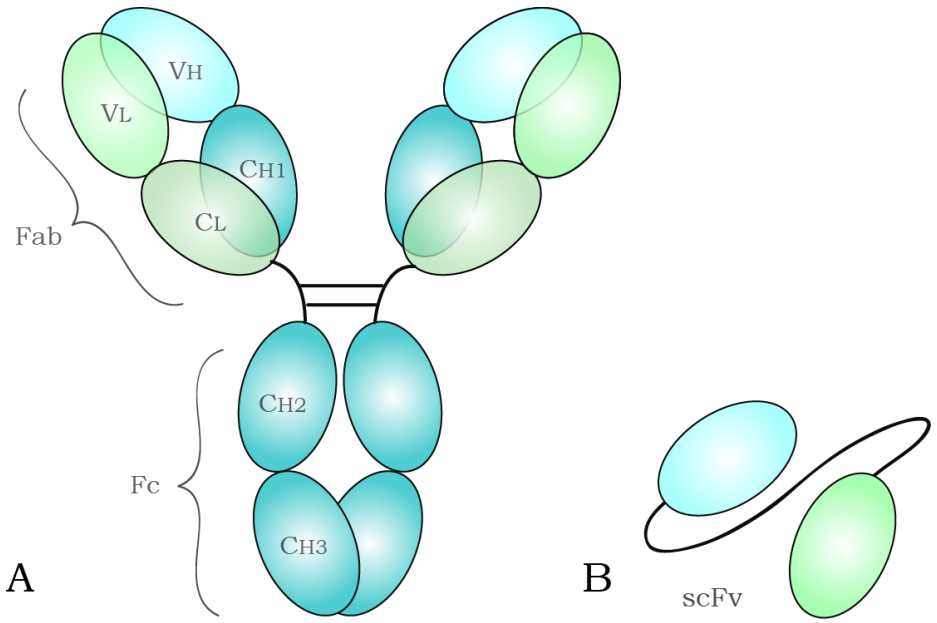

Figure 2. (A) Schematic representation of a human IgG. An antibody consists of two identical heavy chains (blue) and two identical light chains (green) connected via a disulphide bond. Each heavy chain has three constant domains (CH1, CH2, CH3) and one variable domain (VH) and is connected by two disulphide bonds via a hinge region located between CH1 and CH2. Each light chain has one variable (VL) and one constant domain (CL). The variable region of the Fab domain holds the antigen-binding site, whereas the Fc domain confers effector functions by interacting with Fc-binding effector molecules of the immune system. **(B) Schematic representation of a single-chain fragment variable (scFv).** An scFv is a fusion protein consisting of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected by a short, flexible linker peptide of about 12-15 amino acids.

Antibodies recognize and bind to the surface of native antigens of different chemical structures, including proteins and polysaccharides on bacteria and viruses, microbial toxins, and proteins expressed on cancer cells (Murphy and Weaver, 2017). The antigen-binding site or paratope of an antibody is specific for one particular part of an antigen called the epitope (Ahmad et al., 2012). Protein epitopes are generally divided into two categories (Berzofsky, 1985). Conformational epitopes consist of amino acid residues that are widely spaced in the primary sequence, but which are brought together by protein folding. These epitopes are missing in denatured proteins, whereas continuous or linear epitopes are composed of

sequential amino acids in the primary sequence and therefore detectable in denatured proteins (Murphy and Weaver, 2017). The antigen-specificity is acquired by hypervariable regions (short regions with high diversity in amino acid sequence), called the complementarity-determining regions (CDRs) (Parham and Janeway, 2015). There are three CDRs (CDR1, CDR2, CDR3) in each variable region (VL and VH). Following antigen recognition, changes are made in the specificity and effector functions of the antibodies produced by the activated B cell. Antibody affinity and selectivity are increased in a process called somatic hypermutation in which amino acids particularly in the CDRs are mutated (Tonegawa, 1983). Consequently, B cells expressing antibodies with the best fit for the target antigen become subjects for clonal expansion, leading to the destruction of the antigen. The linkage between B cell phenotype and genotype makes the repertoire of immunoglobulins selectable which has been utilized to develop therapeutic antibodies.

1.2 Therapeutic strategies

1.2.1 Monoclonal antibodies

The high specificity of an antibody for a certain epitope makes them useful tools in diagnostics and treatment of diseases. The field of therapeutic antibodies started with the introduction of the hybridoma technology described by Köhler and Milstein (1975). Hybridomas are antibody-secreting cells made by the fusion of lymphocytes from an immunized animal with murine myeloma cells (Parham and Janeway, 2015). The antibodies produced by one hybridoma cell line are identical and therefore called monoclonal antibodies (mAbs). However, the ability of hybridoma-derived mAbs to induce effector responses in humans is modest, and their murine origin can generate human anti-mouse antibody (HAMA) responses, hence limits their clinical applications (Bruhns and Jönsson, 2015, Klimka et al., 2000). These drawbacks have been partially avoided by replacing parts of the murine antibody with human homologous sequences through genetic engineering, giving rise to chimeric (Morrison et al., 1984) and humanized (Jones et al., 1986) antibodies satisfactory for clinical use. Moreover, further advances in recombinant DNA technology and the introduction of the phage display technology and transgenic mice with integrated immunoglobulin genes, enabled generation of fully human antibodies (McCafferty et al., 1990).

The advantage of phage display compared to the hybridoma technology has had a major impact on the development of therapeutic antibodies, minimizing the immune response associated with nonhuman antibodies when used in human therapy (Almagro et al., 2018). As of today, more of 500 mAbs have been studied in clinical trials around the world, and 79 therapeutic mAbs have been approved by the United States Food and Drug Administration (US FDA) as treatment for diverse human diseases including cancers, autoimmune and infectious diseases (Kaplon and Reichert, 2019, Lu et al., 2020). Therapeutic antibodies are generally designed to block the action of specific signalling molecules or their receptors, to carry toxic molecules targeting specific cells, or to function as signalling molecules (Brekke and Sandlie, 2003).

1.2.2 Phage display

Phage display was first described by George P. Smith who showed that a foreign DNA fragment can be fused to the gene encoding the pIII coat protein of filamentous bacterial viruses called bacteriophages (phage), and expressed as a fusion protein displayed on the phage surface without disrupting phage infectivity (1985). Thus, demonstrating the potential of using modified phages to find interesting ligands for a given target molecule such as antibodies, receptors, and enzymes. Some years later, McCafferty et al. demonstrated phage display of antibody variable domains with preserved antigen-binding function (1990), enabling the generation of human mAbs and other antibody fragments. Since then, phage display technology has become one of the most prominent molecular engineering techniques used in the discovery of peptides and antibodies that may serve as novel therapeutics (Fjell et al., 2011, Nelson et al., 2010).

The general procedure of phage display involves the construction of a phage library comprising a collection of bacteriophages that each display a unique peptide or protein variant, following selection based on affinity to the target of interest, and finally characterization of selected binders by sequencing analysis.

1.2.3 Filamentous phages

M13 and fd filamentous phages are the most commonly used bacteriophages for phage display, although T4, T7, and λ also have been used (Smith and Petrenko, 1997, Efimov et al., 1995, Takakusagi et al., 2008, Sternberg and Hoess, 1995). Like illustrated in **figure 3A**, bacteriophages are a rod-shaped virion consisting of coat proteins assembled around a circular single-stranded DNA (ssDNA) genome encoding the phage proteins (Rakonjac et al., 2011). The most frequently used coat proteins used as a platform for phage display are the minor protein pIII (5 copies) and the major protein pVIII (2700 copies) (Torchilin, 2014). M13 is an attractive vector due to its high capacity for replication and its ability to hold large forging DNA (Sioud, 2019). Moreover, M13 is a non-lytic, male-specific phage that infects host *E. Coli* by binding to the F´-pilus via the pIII N-terminus and does not lyse infected bacteria during their life cycle (**figure 3B**).

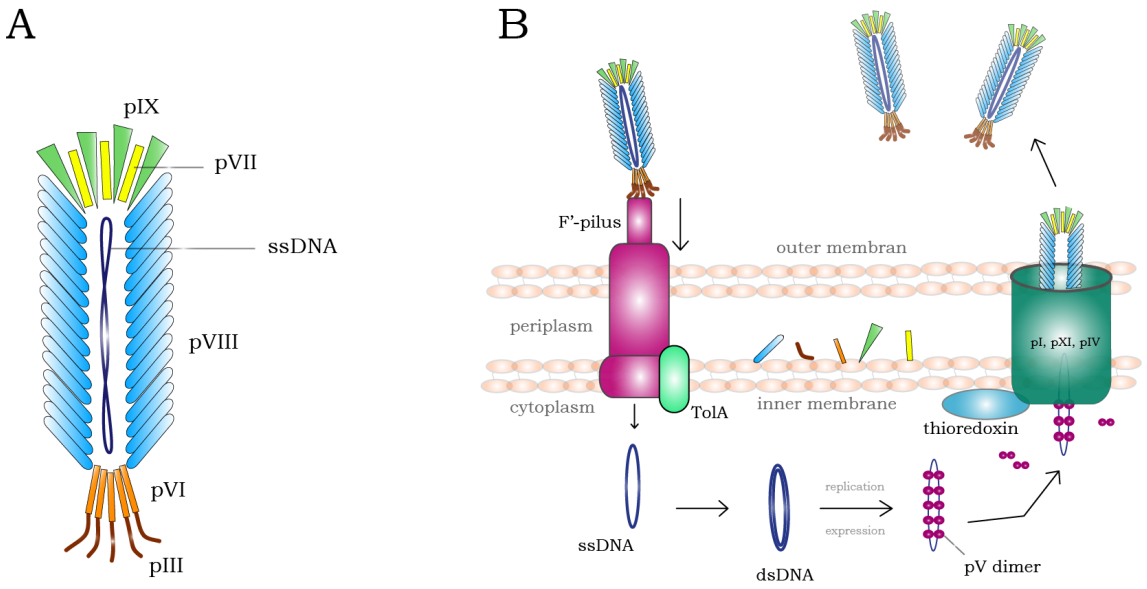

Figure 3. (A) Illustration of filamentous phage M13 (adapted from(Sioud, 2019). (B) Schematic representation of the life cycle of M13. The phage binds to F´-pilus of *E. coli* and subsequently, the host TolA protein depolymerize the phage coat proteins, which remain in the periplasm for recycling, and the ssDNA is brought into the cytoplasm. There, host enzymes convert the ssDNA into double-stranded DNA (ds-DNA) that generate new ssDNA and phage proteins. The newly synthesised ssDNA is coated with pV dimers to prevent conversion into the replicative form, whereas the phage coat proteins are exported to the periplasm. The pI, PXI, and pIV proteins interact to form a channel where pV is replaced by pVIII, facilitated by host thioredoxin in the inner membrane, and then mature phage particles are assembled and released (Russel and Lowman, 2004).

1.2.4 Different types of cloning strategies

For particle assemble reasons, pVIII is limited to displaying short peptide sequences, while pIII can be used to display large insertions of up to 38 amino acids without diminishing particle assemble, though the infectivity may be reduced (Russel and Lowman, 2004). However, this problem can be overcome by using a two-gene system leading to display if both wild-type and modified versions of the coat proteins. There are two types of two-gene systems (Smith, 1993). One involves the use of a plasmid vector, known as a phagemid, which generally contains an antibiotic resistance gene and the fusion gene with a weak promoter. This system needs a helper phage, encoding the phage proteins, to make new phage particles. The other system is a "hybrid system", which utilizes the phage genome, but which contains both the wild-type phage gene and the fusion gene. These systems allow for monovalent display, thus facilitates selection based on pure affinity. In contrast, polyvalent display may prevent identification of the highest-affinity clones because multivalence confers high avidity to weak-binding clones (Huang et al., 2012). Polyvalent display of the modified coat protein is the result of the conventional one-gene systems where the foreign DNA insert is introduced into the natural filamentous phage genome. Hence, which system is the most suitable depends on the desired end-product.

1.2.5 Random peptide libraries

The concept of random peptide phage libraries is based on the insertion of synthetically made DNA oligonucleotides into one of the phage coat proteins, usually the N-terminus of pIII or pVIII. The displayed peptides can vary in length, and they can be constrained by including two flanking cysteine residues that form a disulphide bond, thus mimic discontinuous epitopes (Felici et al., 1993). The diversity of the library is obtained from the oligonucleotides which are encoded by a degenerate codon motif, such as NNK, where K is either G or T, allowing the production of all 12 amino acids and eliminates two stop codons, except the amber stop codon (UAG) (Cwirla et al., 1990). Following synthesis, the random oligonucleotide molecules are converted into a double-stranded (ds) DNA molecule which is digested with the appropriate restriction enzymes to final DNA fragments that can be cloned directly into the desired phage vector.

1.2.6 Generation of antibody libraries

Antibody phage libraries have generally been constructed on single-chain variable fragments (scFvs) or Fabs because of the CDRs located at the variable domains of both VH and VL which determine the binding of antigens (Hudson, 1998, Hudson, 1999). The libraries are usually built in a phagemid vector as fusions to the pIII coat protein. The repertoire of antibody fragments is generated by PCR amplification and random splicing of VH and VL gene repertoires (**figure 4**). The initial V-gene repertoire can have naive (non-immunized) or immunized (antigen-specific) origin, or it can be fully or semi-synthetic. The assembled repertories are subsequently cloned into a phagemid vector, which is electroporated into *E. Coli.*

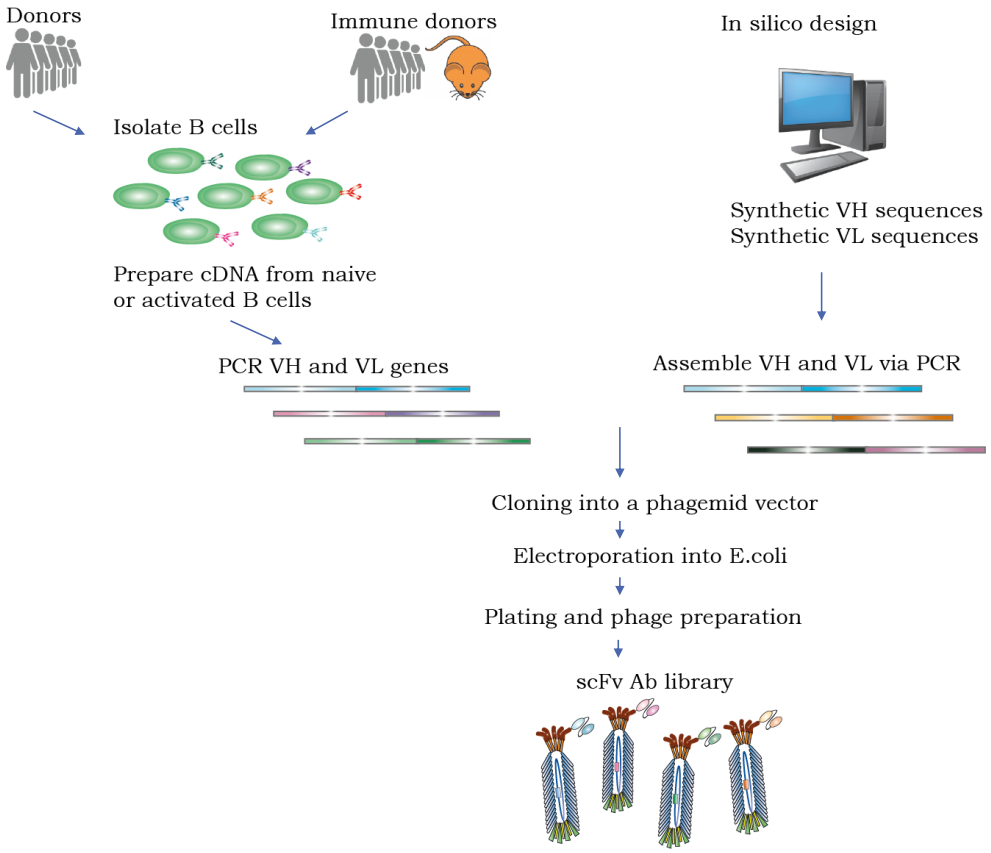

Figure 4: Schematic outline of the construction of antibody libraries (modified from (Sioud, 2019). In the first step of the construction of naive and immune libraries, mRNA or total RNA is isolated from donor B lymphocytes and reversed transcribed into cDNA which serves as a template for PCR amplification of the V gene segments encoding the heavy (VH) and light (VL) chains. The repertoire of synthetic libraries is prepared *in silico* by combining germline gene sequences together with randomized CDRs. The VH and VL genes are randomly paired through PCR assembly to generate diverse repertoires which are subsequently cloned into a phagemid vector and finally electroporated into *E. coli*.

Immune libraries are attractive due to the *in vivo* affinity maturation of the antibodies towards the antigen, which facilitates the selection of high-affinity binders. However, as immune libraries are biased toward the recognition of the antigen used as an immunogen, new libraries are needed for each antigen (Sioud, 2019). In contrast, naive and synthetic libraries allow for screening against all antigens. Semi-synthetic libraries are naive libraries in which the CDR3s of the heavy chains are replaced by random sequences, thus avoiding the use of immunization (Barbas et al., 1992). Additionally, mutations can be introduced within the CDR regions to mimic the process of somatic hypermutation during an immune response following the identification of the desired antibodies.

1.2.7 Affinity selection (biopanning)

The basic method of affinity selection is often referred to as biopanning. In general, a phage library is incubated with the ligand of interest, following multiple rounds of washing to remove adherent non-binders. Bound phage clones are eluted and amplified in bacteria. The rescued phages are typically used in three to five additional rounds to ensure the selection of phage clones with high specificity to the target and to eliminate non-specific binders. After enrichment of high-affinity binding clones from successive rounds of selection, the coding sequences are identified through DNA sequencing. This allows for cost-effective high-throughput screening of millions of phage clones.

1.2,8 Modulation of T cell activation and current strategies promoting effector T cell function

In addition to the interaction between the T cell receptor and the peptide: MHC complex, a complementary signal mediated by a co-stimulatory T cell receptor called CD28 is required for comprehensive T cell activation of naive T cells (Greenwald et al., 2005). CD28 binds to the B7-1/2 co-stimulatory molecule expressed on professional APCs in the presence of infection (Parham and Janeway, 2015). The simultaneous recognition of peptide:MHC complexes and costimulatory ligands by T cells initiate a series of genetic programs resulting in IL-2 production, proliferating and increased survival (Sharpe and Freeman, 2002). Conversely, there are negative regulators of T cell activation that hone the immune response and control hyperactivation. In the context of cancer immunotherapy, two such “break

molecules" have been studied: the cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death 1 (PD1) (Buchbinder and Desai, 2016).

CTLA4 is expressed at a low basal level in naive T cells yet are strongly upregulated following antigen activation. It binds to the same B7 ligands as CD28 though with greater affinity and avidity, thus prevent binding to CD28 and consequently reduces IL-2 secretion and will ultimately stop T cell proliferation (Sharma and Allison, 2015). In contrast, PD1 is upregulated on T cells following persistent antigen exposure and limit T cell activity in peripheral tissues (Stucci et al., 2017). Binding of PD1 to its ligand (PD-L1 or PD-L2) which are expressed on APCs, tumor cells and certain non-hematopoietic cells, result in inhibitory signalling that decreases cytotoxicity and lead to a state of T cell dysfunction called T cell exhaustion (Keir et al., 2008). Tumor cells exploit this mechanism by upregulating the PD1 ligands, thus generating a tumour microenvironment that facilitates tumor growth and invasion (Buchbinder and Desai, 2016). James P. Allison and Tasuku Honjo were awarded the Nobel Prize in Physiology or Medicine in 2018 for revealing that inhibition of CTLA4 and PD1 significantly potentiates the immune responses achieved by T lymphocytes leading to more efficient eradication of cancer cells (Smyth and Teng, 2018). The use of antibodies directed against CTLA4 and PD1 is now known as checkpoint inhibitors and has profoundly altered the outcome for certain groups of patients with advanced cancer (Wolchok et al., 2013).

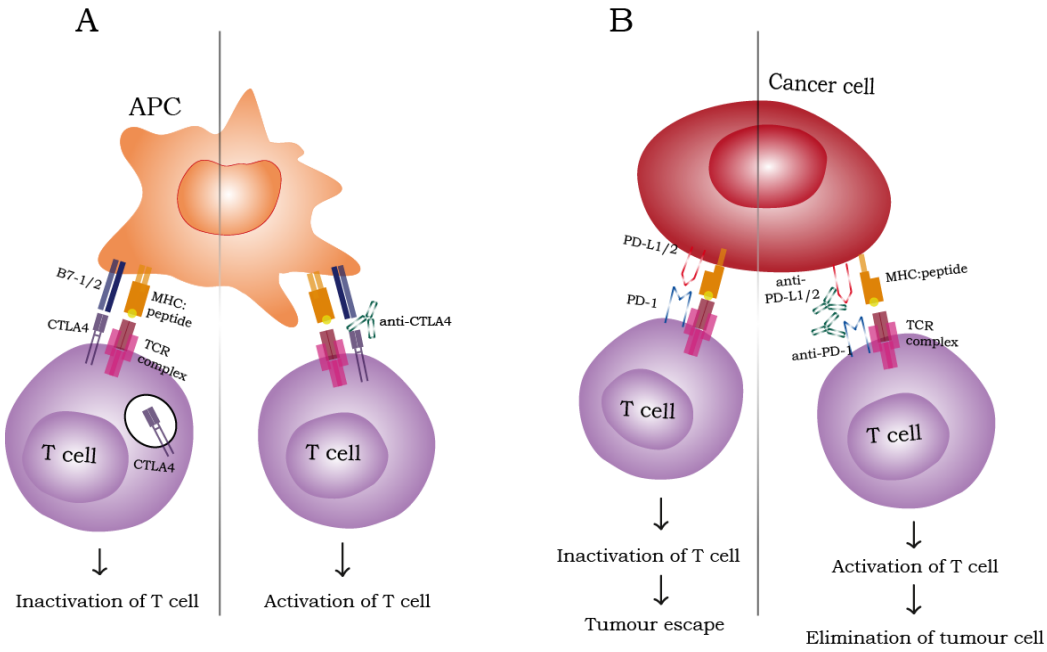

Figure 5. CTLA4 and PD1 are inhibitory receptors that control T cell activation. The interaction between the TCR and peptide: MHC complex must be associated with a second co-stimulatory signal mediated by CD28 to activate T cells. (A) Conversely, the binding of CTLA4 to B7-1/2 provides a regulatory signal that dampens the amplitude of the early stages of T cell activation. (B) PD1 is upregulated on T cells following persistent antigen exposure. When PD1 binds to its ligand, PD-L1/L2, expressed by APCs or tumor cells, the T cell received an inhibitory signal. Antibodies against CTLA4 or PD1/PD-L1 can unleash antitumoral T lymphocyte activity by promoting increased T cell activation and proliferation, by enhancing their effector function and by supporting the formation of memory cells.

2 The rationale for the study

Regulation of the immune system, whether to repress unwanted responses as autoimmunity or to stimulate responses to counteract infectious diseases and cancer, is one of the major goals of immunology research. Given the role played by immune inhibitory receptors in blocking T cell responses against tumours, the identification of antibodies with T cell-binding properties has implications for the development of cancer and/or autoimmunity therapeutics.

The basis for this thesis was the development of affinity screening protocol, in which a semi-synthetic scFv-antibody library was used to isolate scFv antibody fragments that bind and activate T cells. This protocol led to the selection and development of a new human T cell-binding antibody, named S5. In the present study, we aim to further characterize the S5 antibody, with the main focus on the identification of its epitope. Additionally, we aim to isolate new human antibodies specific for cell-surface receptors expressed on activated/exhausted T cells. The identification of new antibodies against checkpoint inhibitors expressed by exhausted T cells has the potential to make a worldwide impact on cancer therapy. The following sub-objectives were defined:

1. Analyse the effects of the S5 antibody on T cells
2. Determine the binding site (epitope) of the S5 antibody using random peptide libraries
3. Isolate antibodies that bind to activated/exhausted T cells and characterise their functions and binding receptors

3 Materials

Table 1: Antibodies

Antibody	Species	Manufacture	Dilution	
			ELISA/ Immunoscreening	Flow cytometry
S5 IgG1 antibody	Human			
Anti-human polyvalent immunoglobulins (α, ɣ, and μ-chain specific) alkaline phosphatase	Goat	Sigma	1:2500	-
Anti-M13 monoclonal antibody	Mouse	Amersham Pharmacia Biotech, Inc.	1:200	-
Anti-Mouse polyvalent immunoglobulins Alkaline Phosphatase conjugate	Mouse	Sigma	1:500	-
Anti-human IgG-FITC	Goat	Sigma	-	1:200
M13 Bacteriophage antibody (biotin)	Mouse	Sino biological Inc	-	1:300
PE-streptavidin	-	BD Pharmingen™	-	1:300
Anti-CD3 (Clone OKT3)	Human	eBioscience		
Anti-CD28 (Clone CD28.2)	Human	eBioscience		

Table 2: Detection substrates

ELISA	BCIP/BT premixed substrate (Sigma)
Immunoscreening	*p*-nitrophenyl phosphate

Table 3: Phage display libraries

Ph.D.™ -7mer Phage Display Peptide library	New England Biolabs
Ph.D.™ -C7C Phage Display Peptide library	New England Biolabs
scFv library	Greg Winter (MRC, Cambridge England)
M13KO7 Helper phage	New England Biolabs (Phage stocks were prepared in the lab)

Table 4: Media, buffer and solutions

2xYT agar	15g Agar, 16 g Bacto-Tryptone, 10g Yeast extract, 5g NaCl (dissolved in 1 L of dH_2O; sterilized by autoclaving)
2xYT medium	16 g Bacto-Tryptone, 10g Yeast extract, 5g NaCl (dissolved in 1 L of dH_2O; sterilized by autoclaving)
Binding buffer	0.1 M sodium phosphate buffer, 0.15M NaCl, pH 7.4
Complete growth media	DMEM/RMPI 1640 supplemented with 10% FBS and penicillin (100 U/ml)- streptomycin (0.1 mg/ml)
DNA loading buffer (6x)	50% (v/v) glycerol, 10% 10xTAE buffer, 0.03% (w/v) bromophenol blue, ddH_2O
Elution buffer	0.2 M Glycine/Hcl, pH 2.5
LB agar	15g Agar, 10g Tryptone, 10g NaCl, 5g yeast extract (dissolved in 1 L of dH_2O; sterilized by autoclaving)*
LB medium	10g Tryptone, 10g NaCl, 5g yeast extract (dissolved in 1 L of dH_2O; sterilized by autoclaving)*
Loading buffer (3x)	0.15M Tris-HCl (pH 6.8), 6% (w/v) SDS, 0,2% (w/v) bromophenol blue, 15% (v/v) glycerol, 0.01% (w/v) DTT
NaCl/PEG	2.5M NaCl/20%PEG-8000
Neutralisation buffer	1M Tris-HCl, pH 9
PBS	137mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4 *
Staining buffer	PBS containing 1% (v/v) FBS/ 1%BSA
TAE (10x)	400mM Tris (pH 6.8) and 10 mM EDTA, pH 8.3
TBS	50mM Tris-HCL, 150mM NaCl, pH 7.5 *
TNT buffer	0.05% (v/v) Tween 20, 10mM Tris Cl (pH 8.0), 150 mM NaCl

* Stock made in our core facility

4 Methods

4.1 Isolation of S5 plasmid, transfection of HEK293T cells and antibody purification through protein G column

Overnight culture was made of S5-transformed ER2738 *E. coli* cells from glycerol stock and S5 plasmid was isolated using NucleoSpin Plasmid kit (Macherey-Nagel, Germany), following the protocol provided by the manufacture.

HEK293T cells were seeded and transfected at 70-90% confluency. First, Lipofectamine 2000 (40µl) and plasmid (20µg) was separately diluted in Optimem and incubated for 5 minutes. The two dilutions were mixed and incubated together for an additional 20min. In the meanwhile, the cells were washed twice with RPMI + PS (no added serum) before 12ml complete DMEM (with low IgG) was added together with the transfection mixture. The transfected cells were incubated at 37°C. Newly produced S5 antibodies are secreted into the medium, hence the medium was harvested after 2-3 days. Possible cells floating in the medium were spun down (2000rmo for 10min) and the supernatant was transferred to a 50ml tube and stored at -20°C until use. Then, new complete medium (low IgG) was added to the cells for further incubation for an additional 2-3 days before harvesting the medium and discarding the cells.

A protein G column was used for antibody purification. To prepare the column, 5 ml elution buffer and 5 ml binding buffer were run through. Afterward, binding buffer (1:2) was added to the thawed medium and then loaded to the column. Unspecific binders were washed out with 10ml binding buffer before the antibodies were eluted in 10 fractions in a total of 3 ml glycine buffer. The strongest fractions from SDS-PAGE analysis were pooled and the concentration measured using NanoDrop® ND-1000 Spectrophotometer (Saveene Werner, Sweden). Aliquots were made and stored at -20°C until use.

4.2 Epitope mapping of S5 antibody using phage display peptide libraries

As illustrated in **figure 6**, a solution-phase panning method exploiting affinity bead capture was used for the epitope mapping experiments of the S5 antibody. Two commercially available random peptide phage display libraries from New England Biolabs

were used; a loop-constrained heptapeptide (c7c) library for mapping of conformational epitopes and a linear heptapeptide (7mer) library to map for linear epitopes. The randomized peptide libraries (~10^{11} pfu) were incubated with 2.8µg/ml S5 in 1xTBS/ 1%BSA/ 0.5% Tween20 (total volume 1 ml) for 2 hours at room temperature with agitation, following a 30 min incubation with protein 0.3 mg/ml Dynabeads protein G (Invitrogen, Thermo fisher Scientific) at 4ºC with agitation. The beads were captured on a magnet and washed 10 times with TBST and two times with 1xTBS to remove unspecific binders. Antibody bound phage particles were eluted from the antibody-bead complex in acidic elution buffer (0.2M glycine-HCl pH 2.2, 1mg/ml BSA). The beads were captured on a magnet and the elution was collected and neutralized with glycine buffer (Tris-Base pH 9.1). Exponential growing *E. coli* host strain ER2738 (in LB medium + tetracycline) were infected with the eluted phages and the phages were amplified the for 4-4.5 hours. Subsequently, the culture was divided into 10 Eppendorf tubes and centrifuged for 10 min at 12000rpm.The phage-containing supernatant was transferred to a new tube and 1/5 2.5M NaCl/20% PEG-8000 was added for phage precipitation overnight. The next day, the phages were spun down at 12000rpm for 20 min, resuspended in 100µl PBS, collected in 1ml, and precipitated again with PEG/NaCl for 30 min on ice, and finally resuspended in 0.5ml PBS. Three to four additional rounds of selection and amplification were repeated to enrich specific binders.

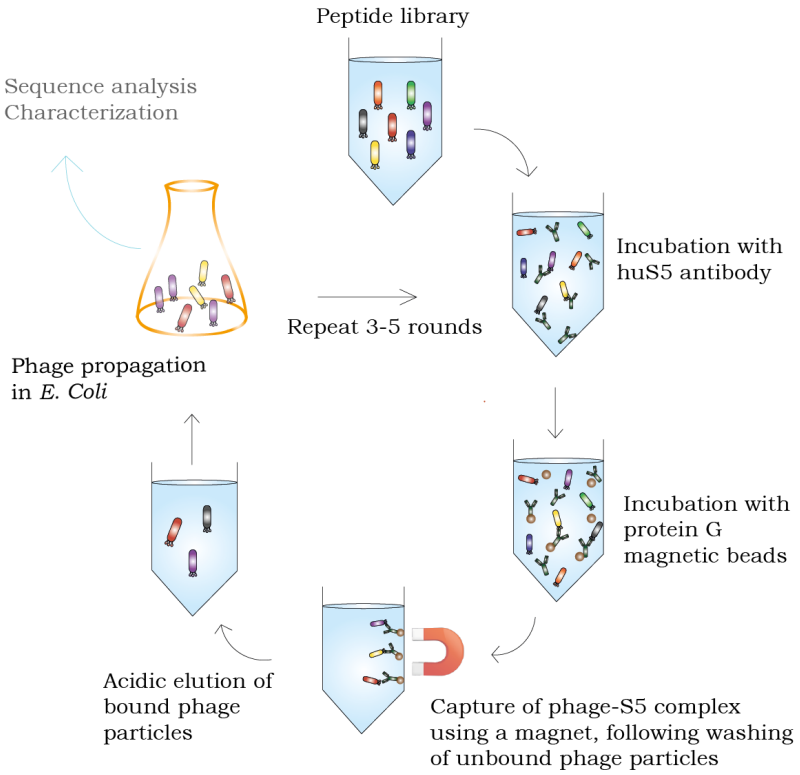

Figure 6. Solution-phase biopanning with affinity bead capture. In this protocol, the peptide phage display library was incubated with the target ligand S5, following incubation with Fc binding magnetic beads. Weak and non-binding phage particles were removed by extensive washing. Bound phages were eluted and amplified in *E. coli*. Enriched phages were used in the next rounds of panning. Three to five rounds were repeated to ensure the selection of phage particles with high specificity to the target.

4.3 Phage ELISA

The binding activity/ target specificity of amplified phage particles was tested by indirect enzyme-linked immune sorbent assay (ELISA) as illustrated by **figure 7**. First, wells of a flat-bottom 96-well microtiter plate were coated with amplified phage (5µl phage in 100µl PBS per well) overnight. The wells were washed with PBS/ 0.05%Tween20 and blocked with 300µl PBS/2%BSA for 1hour at 37°C. After three washes, the wells were incubated with 100µl S5 dilution (0,28µg/ml) for 1 hour at RT. The washing step was repeated before incubation with alkaline phosphatase (AP)-conjugated anti-human IgG (1:2500) for 1hour at room temperature. The wells were washed again before adding *p*-nitrophenyl phosphate as substrate. Finally, the absorbance was measured at 405nm using Sunrise microplate reader (TECAN, Switzerland) and MagellanTM software (TECAN, Switzerland).

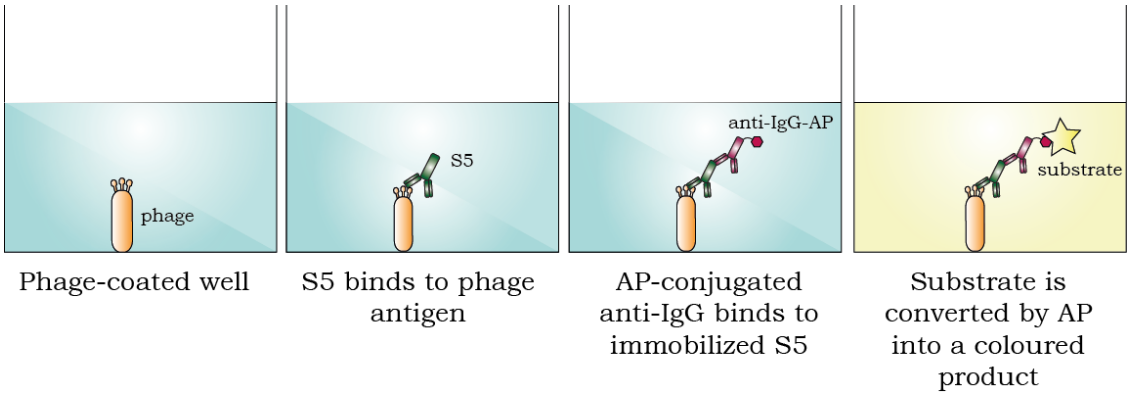

Figure 7. Schematic illustration of indirect ELISA workflow. Wells were coated with phage overnight, washed, and blocked for 1 hour, before adding S5 followed by 1hour incubation. Wells were washed, incubated with anti-human IgG-AP for 1 hour, and washed again before adding *p*-nitrophenyl phosphate as detection substrate. The absorbance was measured at 405nm.

4.4 Immunoscreening

Immunoscreening is a practical screening method that allows direct detection of positive phage particles from successive rounds of biopanning. Exponential growing *E. Coli* ER2738 was infected with phage dilutions for 2-3 minutes. IPTG and X-gal were added for blue colony quantification, and afterward, the infection volume was transferred to Top Agar and poured on pre-heated LB Agar-plates containing tetracycline and incubated at 37°C overnight. The following day, plates with a high percentage of blue plaques were used for immunoscreening. The plaques were transferred from the LB plate to the nitrocellulose membrane by carefully placing the membrane on top of the LB plate. The membrane and plate were marked with corresponding drawings to make it easy to go back and find potential positive clones. Once removed, the membrane was transferred to a clean Petri Dish and washed twice with 1xTNT buffer for 15min and blocked with 20%BSA in TNT buffer for 30min. Afterward, the membrane was incubated with S5 (2,8µg/ml) in 5ml 0.1% BSA/TNT buffer for 2 hours. Following one wash with 0.1% BSA/ 0.1%NP-40/TNT buffer and two washes with 0.1%BSA/TNT buffer (10min each), the membrane was incubated with alkaline phosphatase-conjugated anti-human IgG (1:2500) for 2 hours. Finally, after washing the membrane with TNT buffer (3x5min), 5ml BCIP/NBT premixed substrate (Sigma) was added to detect positive (immunoreactive) phage clones.

4.5 Amplification of single phage

LB medium (3ml /single colony) was inoculated with tetracycline (1µg/ml) and *E. coli* ER2738 from a plate and placed at 37°C with shaking for 1-2 hours (until O.D600 0.01-0.05). Single blue colonies were transferred to a culture tube containing 3 ml bacteria culture using a pipette tip and amplified at 37°C with shaking for 4-4.5 hours. After amplification, half of the volume was transferred to the Eppendorf tube (the other half was stored at 4°C for later use) and centrifuged at 12000rcf for 10 min. The supernatant containing the phage particles were transferred to a clean Eppendorf tube and added PEG (1/6 of the phage-volume) following precipitation overnight at 4°C. The next day, the precipitated phage particles were spun down by centrifugation at 12000rcf for 20 min, the supernatant was discarded while the phage pellet was resuspended in 100µl TBS. To get rid of potential residual cell components, the sample was centrifuged for 1min at 12000rcf. The supernatant containing the amplified phage particles were transferred to a clean Eppendorf tube and stored at 4°C.

4.6 Purification of sequencing template (ssDNA)

Relevant single phage clones were amplified in 3 ml ER2738 culture as described earlier, divided into two Eppendorf tubes, and centrifuged for 15 min at 12000rpm. The supernatant of one sample was added 1/6 PEG and precipitated overnight following ELISA analysis, while the other sample was used to purify phage ssDNA using the QIAprep Spin M13 Kit. After treating the supernatant with M13 precipitation buffer for 5 min at room temperature, the samples were added to QIAprep spin columns where intact phage particles stick to the silica-gel membrane. To purify the phage ssDNA, the phage particles were lysed by adding M13 lysis and binding buffer which contains high salt concentration and makes the ssDNA absorb to the membrane. The column was then washed with Buffer PE containing ethanol and ssDNA was eluted in 50µl of buffer EB. The samples were analysed by agarose gel electrophoresis. A 1% agarose gel was run in 1xTAE buffer at 100V for 20 minutes. The ssDNA concentrations were measured using the NanoDrop® ND-1000 Spectrophotometer.

4.7 Mixed lymphocyte reaction and human IL-2 ELISA

Mixed lymphocyte reaction (MLR) can be used to study how T lymphocytes from one individual respond to cells from a different individual that possesses allogeneic (genetic

different due to polymorphic genes) MHC molecules. The foreign MHC molecules serve as activating stimuli to the responding T lymphocytes, similar to the recognition of peptide epitopes presented by self-MHC molecules (L A Sherman and Chattopadhyay, 1993). In this study, an MLR assay was used as an *in vitro* model for T cell activation and proliferation to check if incubation with S5 would influence the production of interleukin 2 (IL-2).

The assay was performed by seeding $1x10^5$ responder PBMCs into the wells of a round-bottomed 96-well plate. Subsequently, $1x10^5$ allogeneic PBMCs were added to each well in a final volume of 300µl lymphocyte medium. Two concentrations of S5 antibody were tested (1.5 µg/ml and 3µg/ml) as well as an additional IgG FC control (1.5 µg/ml). Two PBMC responder donors were tested against three different PBMC donors and every group set had three replicates. The cells were incubated at 37ºC for 3 days. Then, the cells were spun down and the supernatant was analysed by using Human IL2 DuoSet ELISA (R&D Systems, USA) according to the manufacture protocol.

4.8 Isolation of peripheral blood mononuclear cells (PBMCs), CD4+ T cells and CD8+ T cells

PBMCs were isolated by gradient centrifugation of buffy coats using the SepMate™ procedure as described in **figure 8**. $CD4^+$ and $CD8^+$ T cells were purified from PBMCs by using either the Dynal CD4 or CD8 Positive Isolation Kit, following the manufacturer's instructions (Invitrogen Dynal AS, Oslo, Norway).

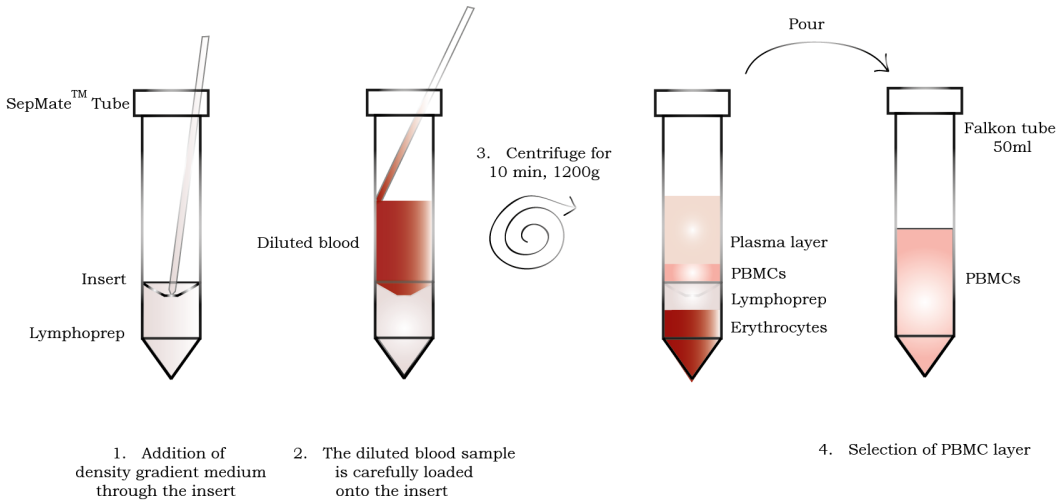

Figure 8. Illustration of the SepMate™ workflow for isolation of PBMCs from buffy coat. First, 15ml density gradient medium (Lymphoprep) is pipetted directly through the central hole in the insert

of the SepMate™ tube. The blood is diluted with PBS containing 2% FBS (1:1) and subsequently pipetted down the side of the tube. Next, the samples are centrifuged at 1200g for 10 minutes at room temperature with the brake on. Erythrocytes and granulocytes pellet to the bottom of the tube, while PBMCs remain above the insert. The PBMCs are harvested by pouring into a fresh tube in one quick motion, washed twice, and then ready to use in downstream applications.

4.9 Biopanning of antibody library on activated T cells

A 25cm^2 flask was incubated with anti-CD3 (5µg/ml) in 3ml PBS for a minimum of 1 hour at 37°C. Then, PBMCs (20-30 million) and anti-CD28 (1µg/ml) in were added to the anti-CD3 coated flask and incubated at 37°C for 3 days for T cell activation (**Figure 9**).

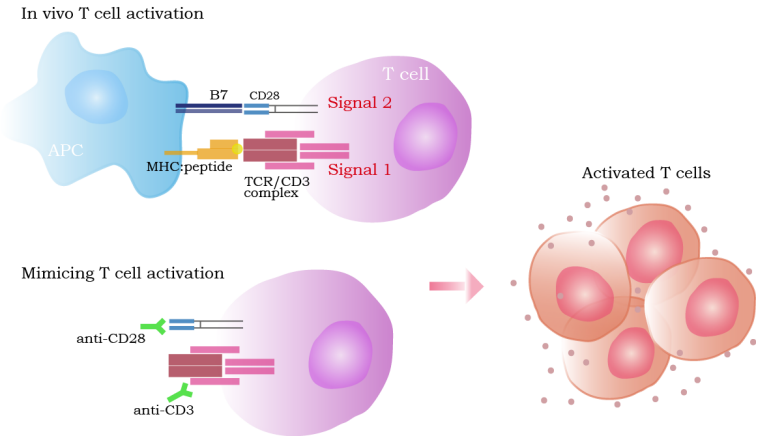

Figure 9. Schematic illustration of T cell activation. *In vivo*, MHC molecules of antigen-presenting cells (APCs) present its peptide antigen to the TCR on the T cell surface. Binding of co-stimulatory protein CD28 is required for the activation and proliferation of the T cells. Anti-CD3 and CD28 monoclonal antibodies mimic the effects of APCs, stimulating cultured T cells to proliferate.

On the third day, the cells were spun down and resuspended in PBS containing 2%FCS following incubation with the antibody phage display library (~10^{9}pfu) for 2 hours at 4°C with agitation. Following incubation, the cells were washed 10 times in 2%FCS- PBS and incubated with anti-CD3 magnetic beads (Dynabeads® CD3, Invitrogen™) for 30min at 4°C with agitation. The CD3$^+$ T cells were captured on a magnet and washed 5 times with 2%FCS- PBS. Then, bound phage particles were eluted in elution buffer. The elution was immediately neutralized (Tris-Base pH 9.1) and incubated with 1.5ml exponential growing TG1 cells for 30 minutes at 37°C. The infected TG1 cells were streaked on two 2xYT plates containing 100µg amp and 1% glucose and incubated overnight at 30°C.

The next day, 2ml 2xYT medium was added to the plates. The cells were scraped and 200µl of the scraped cells were added to 50-100ml 2xYT medium + amp and grown for 1-2 hours at 37°C. Afterward, 25ml of the culture was infected with 100µl helper phage and incubated at 37°C without shaking for 30min. To remove excessive helper phage, the cells were centrifuged at 4000rpm for 5 min and resuspended in 50ml 2xTY medium + 100µg amp and 50µg kanamycin and incubated at 30°C with shaking overnight. The overnight culture was divided into 10 Eppendorf tubes and centrifuged for 10min at 12000rpm. The phage containing supernatant was transferred to a new tube and PEG/NaCl precipitated as described earlier. Three rounds of selection and amplification were repeated and the binding of selected phages to CD3/CD28 stimulated PBMC and untreated PBMC were analysed by Flow Cytometry.

4.10 Flow cytometry

Binding of polyclonal and monoclonal scFv phage to CD3 and CD28 stimulated PBMCs (2-3 days) and untreated PBMCs were analysed by using flow cytometry. The cells were spun down at 500xg for 5 min (~300 000 cells per sample), washed with 400µl staining buffer (1%BSA in PBS), and incubated with 10µl phage for 1h at 4°C. The cells were spun down and washed twice, and then incubated with biotin-conjugated anti-M13 mAb for 40 min at 4°C. After washing, the cells were incubated with phycoerythrin (PE)-conjugated streptavidin for 20min in the dark. The cells were then washed, resuspended in 300µl staining buffer, and finally analysed on FacsCanto using FACSDiva software. Data analysis was performed using FlowJo software (FlowJo LLC, USA).

Furthermore, flow cytometry was used for studying the binding of S5 to PBMCs, CD4+, and CD8+ T cells following the same protocol. Though, the cells were incubated with S5 for 60min at 4°C, washed and incubated with FITC-conjugated anti-human IgG for 20 minutes in the dark.

4.11 Amplification of single phage

Exponential growing TG1 bacteria were infected with dilutions of phage from positive rounds of affinity selection and streaked on 2xYT plates containing 100µg/ml ampicillin + 1%

glucose and incubated overnight at 37°C to make single colonies. Ten to twenty single colonies were picked and grown in 3ml 2xYT medium + ampicillin in 15ml culture tubes for 1-2h at 37°C. Half of the culture (was transferred into an Eppendorf tube and infected with helper phage (1µl) and incubated at 37°C for 30min. The remaining TG1 culture was saved at 4°C for phagemid isolation of potential positive clones. After incubation, the cells were spun down at 4000rpm for 5 min (to remove excess helper phage) and resuspend in 2xYT medium containing 100µg/ml ampicillin and 50µg/ml kanamycin and grown overnight at 30°C with shaking. The next day, the cells were spun down in Eppendorf tubes (10min, 12000rpm) and the phages were precipitated in PEG/NaCl overnight. Finally, phages were rescued and resuspend in 100µl. All clones were tested for binding to PBMC by Flow cytometry and phagemid from positive phage clones were isolated using NucleoSpin Plasmid kit (Macherey-Nagel, Germany) following the protocol provided by the manufacture.

The purified phagemid was examined by agarose gel electrophoresis and the concentration was measured using NanoDrop® ND-1000 Spectrophotometer (Saveene Werner, Sweden) before sequencing.

5 Results

5.1 Selection of the S5 antibody

Previously, an affinity selection protocol was established in the lab to facilitate the selection of scFv antibodies capable of binding to human T cells and inducing cell aggregation as illustrated in **Figure 10**. Briefly, in this protocol the library was incubated with resting $CD4^+$ T cells at 37ºC for 2 hours, then the cells were shortly spun down (30s, 500g) to separate T cell aggregates from single cells. After centrifugation, supernatants were removed, and cell pellets were washed 10 times to remove unspecific binders. Subsequently, T cell-bound phages were eluted, amplified in *E. Coli*, and used in successive rounds of affinity selection. After three rounds of selection, single phage antibody clones were amplified and tested for binding to purified peripheral blood $CD4^+$ T cells. Around 90% (27 out of 30) of the phage clones showed binding to T cells. Sequence analysis showed that all the positive clones are identical. The sequences of a representative clone, referred to as scFv S5, was fused to theFc domain of human IgG1 and expressed in HEK-293T cells as described below.

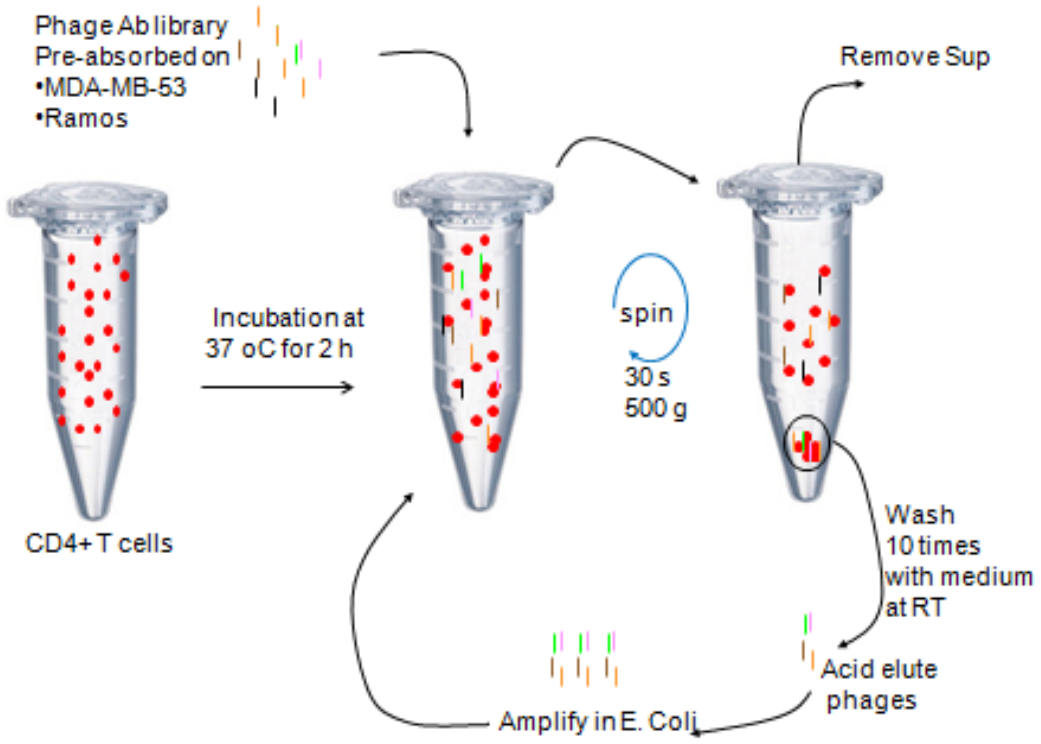

Figure 10. Illustration of the biopanning protocol that led to the discovery of S5. The illustartion was made by Mouldy Sioud.

5.2 Antibody purification through protein G column

The S5 antibody, S5 scFv fused to the Fc region of human IgG (hinge-CH2 and CH3 domains), was expressed in HEK-293T cells and then purified using protein G/A affinity chromatography as described in section 4.1. Ten fractions (300µl/fraction) were collected, pH adjusted to 7.5 and then analysed by 10% SDS-PAGE followed by Coomassie staining. Fractions 2,3, and 4 were collected and stored at -20 ºC until use.

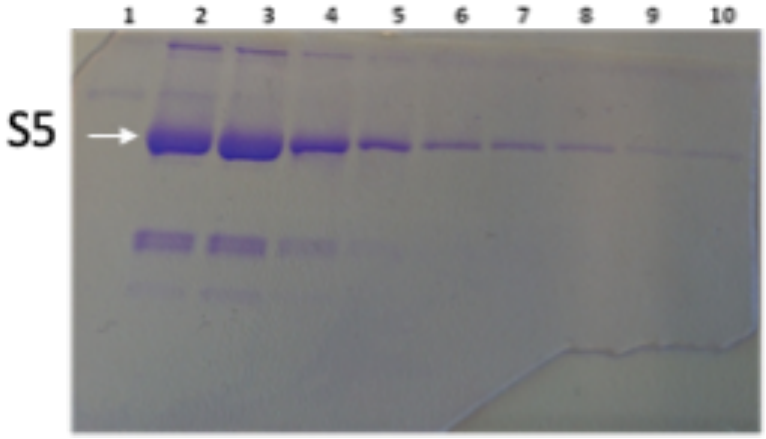

Figure 11. A representative example of SDS-PAGE analysis of eluted fractions from a mini protein G/A column. After purification, 10µl from each fraction was analysed **by** SDS-PAGE anf then the gel was stained with Coomassie dye. of fraction. The MW of the S5 antibody dimer is around 110 kDa, whereas the monomer is approximately 55kDa.

5.3 Binding of the S5 antibody to T cells

Next, we have investigated the binding of the antibody to PBMC lymphocyte populations using flow cytometry. As shown in **figure 12**, the binding of S5 antibody to PBMC lymphocyte population varied between donors from 30-80%. **Figure 13** shows the binding of the S5 antibody to purified $CD4^+$ T cells and $CD8^+$ T cells. Whereas the antibody binds to all $CD4^+$ T cells, variations are found between different donors with regard to $CD8^+$ T cells, thus explaining the variations between PBMC donors. Further analysis showed that the S5 antibody bind to only $CD45RA^+$ $CD8^+$ T cells (naive) and not $CD45RO^+CD8^+$T cells.

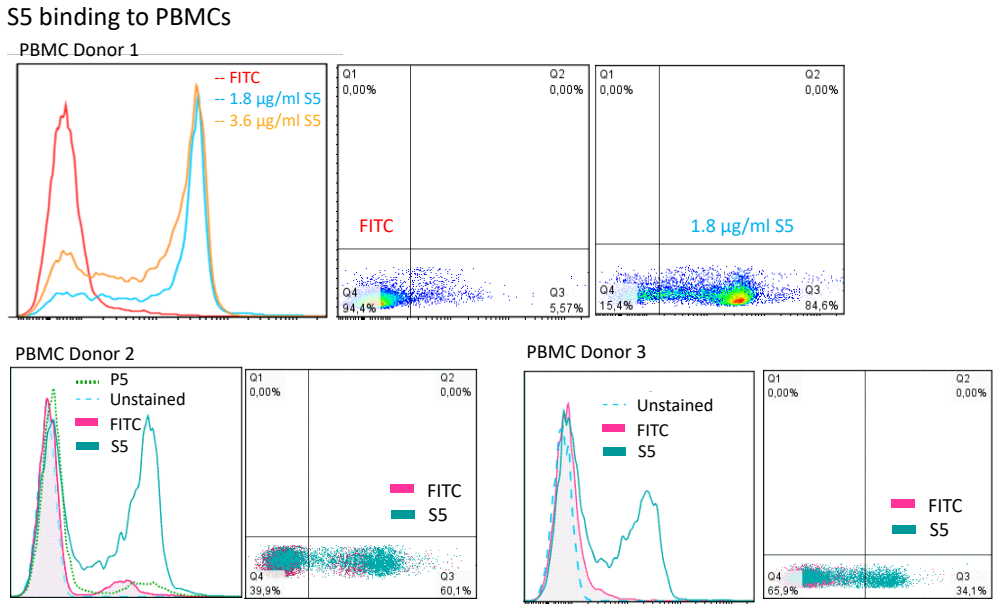

Figure 12. Binding of S5 to PBMC lymphocyte population. Representative examples of binding of the S5 antibody to PBMCs analysed by flow cytometry. Red histograms represent cells stained with only FITC-conjugated anti-huIgG antibody. Dashed histograms represent unstained cells, and dotted line (PBMC donor 2) represent cells stained with the isotype matched IgG1 antibody P5. Only lymphocyte populations were gated and analysed.

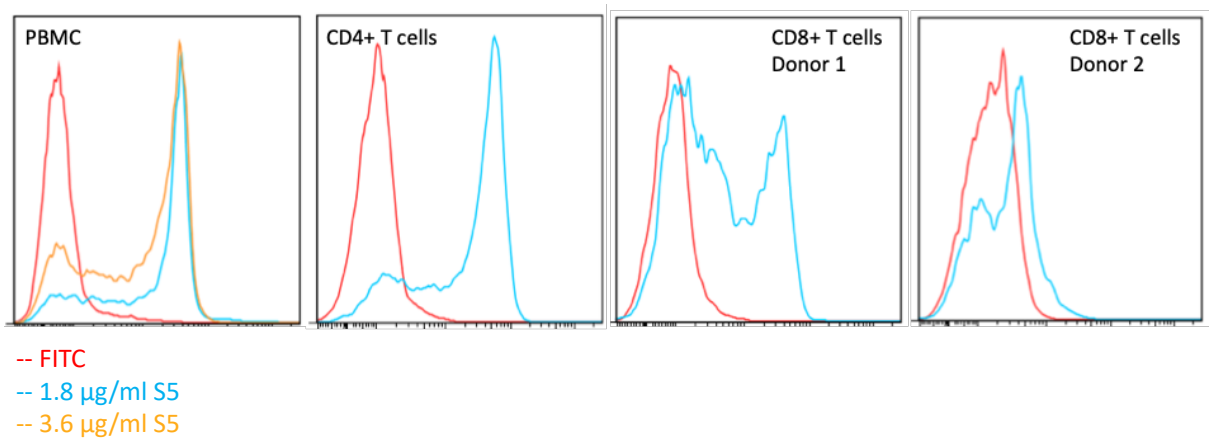

Figure 13. Binding profiles of the S5 antibody. Representative examples of the antibody binding to purified $CD4^+$ and $CD8^+$ T cells. The cells were purified from PBMCs using magnetic beads, stained with the S5 antibody and then analysed by flow cytometry. Red histograms represent cells stained with only FITC-conjugated anti-huIgG antibody.

5.4 S5 induce cell aggregation of T cells

To study the function of the S5 antibody, T cells were cultured in the presence or absence of the antibody or isotype matched control antibody, named P5. Cell aggregation was observed under inverted microscope (**figure 14-15**).

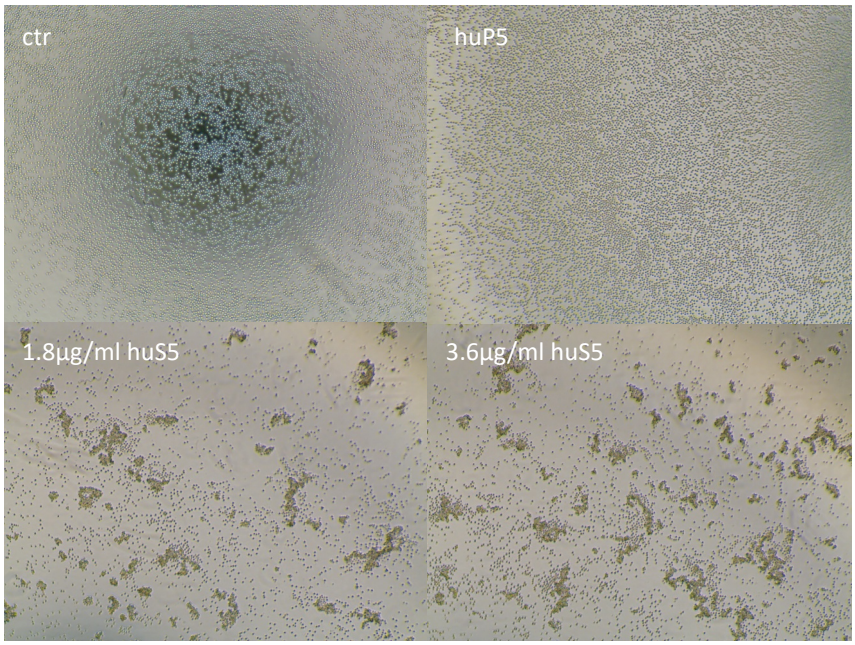

Figure 14 Microscope pictures showing CD4$^+$ T cells phenotype after incubation with S5. 1x10^5 CD4$^+$ T cells in 100µl medium were seeded into the wells of a flat-bottomed 96-well plate and incubated with two concentrations of S5 (1.8µg/ml and 3.6µg/ml) at 37ºC for 3 days. Incubation with antibody huP5 (5.7µg/ml) was included as an isotope control in addition to one negative control (ctr). Each group set had three replicates.

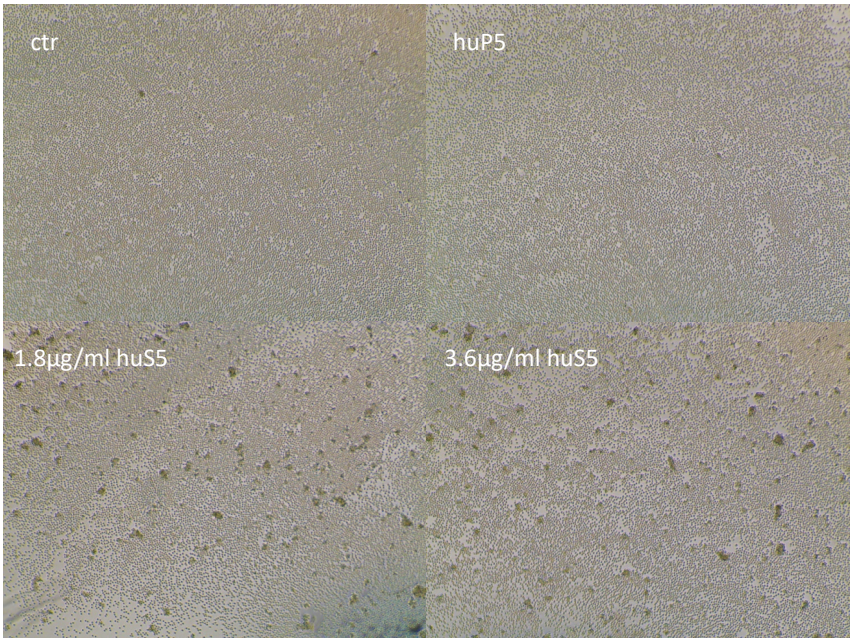

Figure 15. Microscope pictures showing CD8$^+$ T cells phenotypes after incubation with S5. 1x10^5 CD8$^+$ T cells were seeded into the wells of a flat-bottomed 96-well plate and incubated with two concentrations of S5 (1.8µg/ml and 3.6µg/ml) at 37ºC for 3 days. Incubation with antibody huP5 (5.7µg/ml) was included as an isotype control in addition to one negative control (ctr). Each group set had three replicates.

In accordance with the screening protocol, the S5 antibody induced $CD4^+$ T cell aggregation. In contrast, the isotype-matched control antibody did not induce $CD4^+$ T cell aggregation. Of note, cell aggregation occurred about 1 hour after incubation at 37ºC. And the aggregation was maintained up to 5 days. In contrast to $CD4^+$ T cell, $CD8^+$ T cell showed low degree of aggregation that showed inter-individual variations. This observation seems to correlate with the flow data.

5.5 Analysis of the S5 antibody effects on IL-2 expression

The presence of IL-2 is a sensitive measure of T cell proliferation/activation as it is produced at an early stage of immune activation (Zhou et al., 2014). Therefore, we have analysed the expression of IL-2 by PBMCs in response to the S5 antibody. We have used a mixed lymphocyte reaction as *in vitro* model for T cell activation and proliferation. Although some increase in IL-2 production was observed in some experiments, there was no significant tendency that S5 antibody treatment affected IL-2 production (**figure 16**).

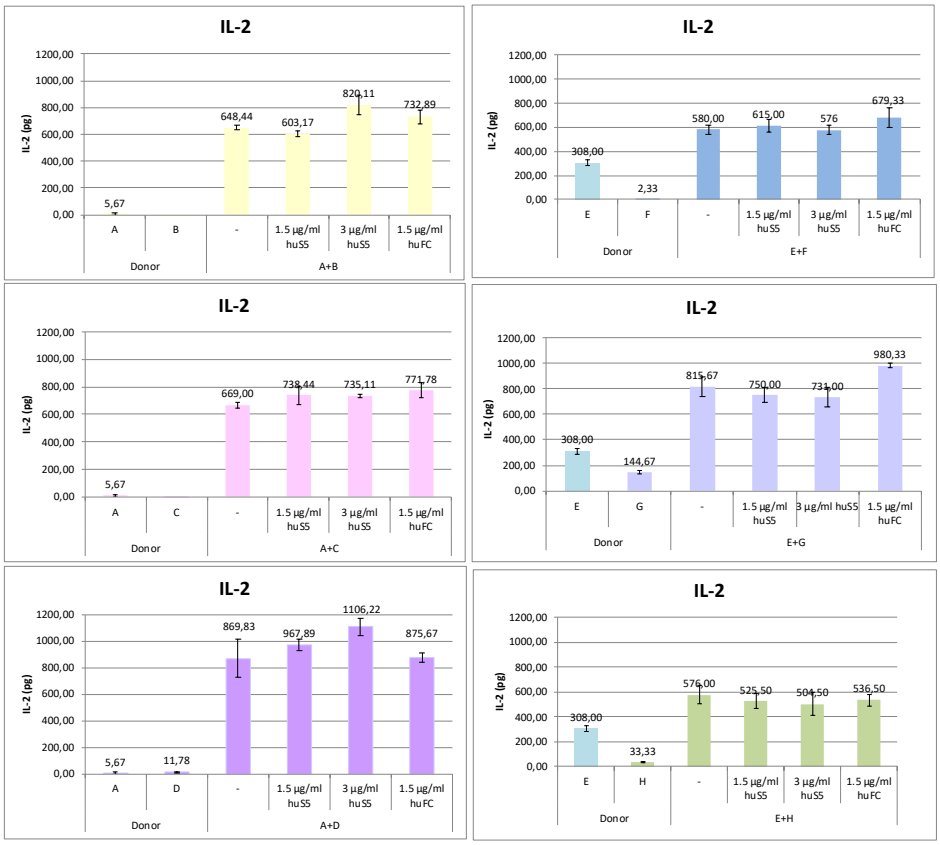

Figure 16. Human IL-2 ELISA results obtained with medium from mix lymphocyte reaction (MLR) experiments. The IL-2 concentrations are the mean of three independent determinations, calculated based on the standard curve (Appendix). The error bars show the standard deviation for each data set.

5.5 Epitope mapping of the S5 antibody using random peptide libraries

Multiple biopanning experiments (see section 4.2) with the S5 antibody and peptide libraries were performed, and affinity enrichment were tested by ELISA. The phage ELISA protocol used in this study will not determine whether the selcted phages bind S5 with high or low affinity, but is sufficient for determining affinity enrichemnt between successive rounds of selection.

In the first biopanning experiment, the S5 antibody was separately incubated with disulphide-constrained 7-mer (Cx_7C) or 7mer linear peptide library (New England, Biolabs), while in later experiments an equal amount of the two libraries were mixed as the binding peptides can be distinguished upon sequencing. Each library consists of approximately 10^9 electroporated sequences amplified once to yield approximately 100 copies of each sequence in 10µl of the supplied phage. Libraries with Cx_nC structure (cyclic peptides) are expected to yield peptides with high binding affinity (Sioud, 2019). **Figure 17** shows the results from phage ELISA performed with amplified phages from the 4th and 5th round of the first biopanning experiment. No affinity enrichment is observed between the two rounds for either the 7mer or c7c-library. Although S5 seem to bind stronger to the phages then anti-M13, one would expect a higher difference (3-fold increase in signal) for selected phages with high affinity.

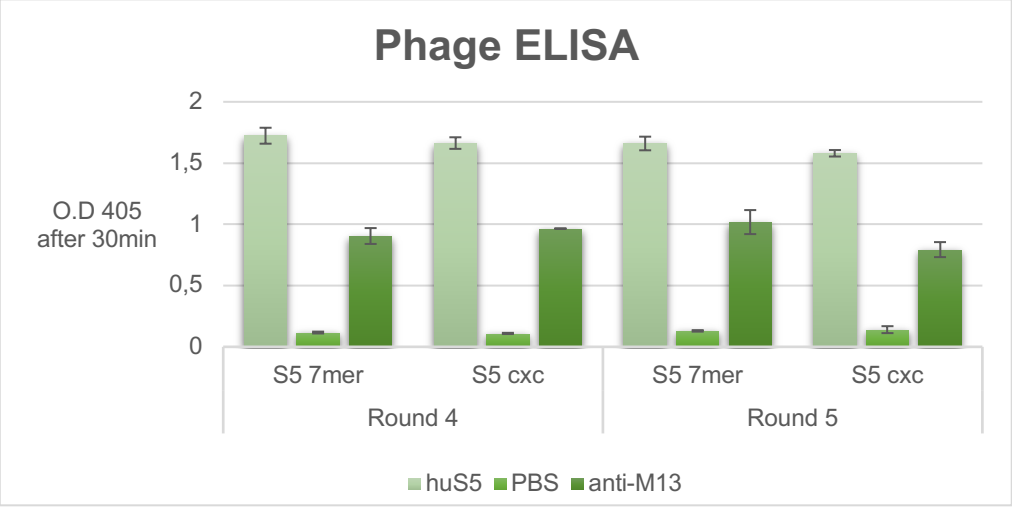

Figure 17. Phage ELISA of phages from biopanning experiment 1. The binding of S5 to phages from the 4th and 5th round of biopanning. Absorbance values are the mean of triplicates in one experiment, and error bars show the standard deviation for each data set. Negative control wells (PBS) were included for each sample to check for cross-reactivity with the secondary antibody, and specificity was assessed by using an anti-M13 antibody (anti-M13).

5.6 Immunoscreening

As mentioned earlier, immunoscreening can be used to monitor the enrichment of specific phage-displaying peptides after successive rounds of biopanning as well as assess the specificity of selected phages (Sioud and Hansen, 2001, Chikaev et al., 2015). Therefore, one would expect a high number of clearly distinct positive clones compared to the total number of clones after the third round of selection in the case of successful biopanning. In immunoscreening, the peptide specificity is assessed by the signal intensity of the spots. No positive clones were detected after immunoscreening with the S5 antibody on phage particles derived from the 4th and 5th round of biopanning (**figure 18A**). Moreover, no clones were detected on the membrane whatsoever. To control the immunoscreening procedure, the peptide phage library was used in a parallel biopanning experiment using human serum from a breast cancer patient as target ligand (see Appendix). Biopanning of peptide libraries on patient serum antibodies have identified high affinity binders (Hansen et al., 2001). Affinity selected phage clones from the 4th round was used for immunoscreening with serum, and despite high background staining, some positive clones were clearly visible (**figure 18B).** Although there seems a problem transferring the phage clones onto nitrocellulose membranes, the protocol worked with patient serum.

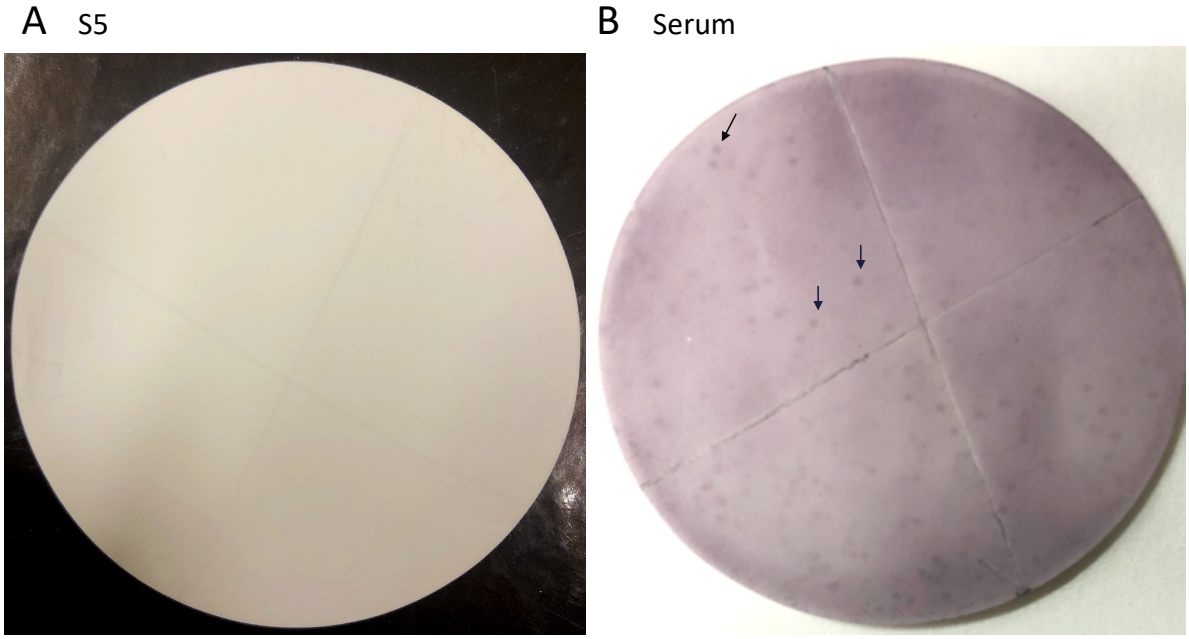

Figure 18. (A) Representative example of immunoscreening of phage-displayed c7c-peptides derived from the 5th round of affinity selection with S5. Detection of immunoreactive clones was done with alkaline phosphatase-conjugated anti-human IgG and BCIP/NBT substrate. Nitrocellulose membrane from immunoscreening with human serum **(B)** was included for comparison and as positive control for the immunoscreening procedure. Some positive clones are indicated by arrows.

5.7 Amplification of single phage

Although the phage ELISA and immunoscreening experiments suggest poor enrichment of S5 specific phages, we decided to test a number of random blue colonies from Xgal/IPTG titer plates (peptides expressed in-frame with β galactosidase). Twenty-six blue colonies from a plate holding ER2738 infected with phages from the 5th round of biopanning with the c7c-library were amplified and tested by ELISA. Ten of the best binders, based on the ELISA experiment, were tested further (**figure 19**), and ssDNA from phage clone named 1, 3, 4, 5, and 17 were extracted and sent for sequencing. Phages 1 and 3 displayed the same sequence, CHSAPPNLC, while phages 4, 5, and 7, displayed the sequence CAIRLLATC. Although there was enrichment of these two sequences, no significant reactivity was detected. Further analysis showed that the reactivity seen in ELISA when compared to PBS, was due to the S5 antibody binding to the ELISA plate. This unspecific binding masked the antibody specificity reactivity. Different blocking conditions and incubation times may overcome this problem.

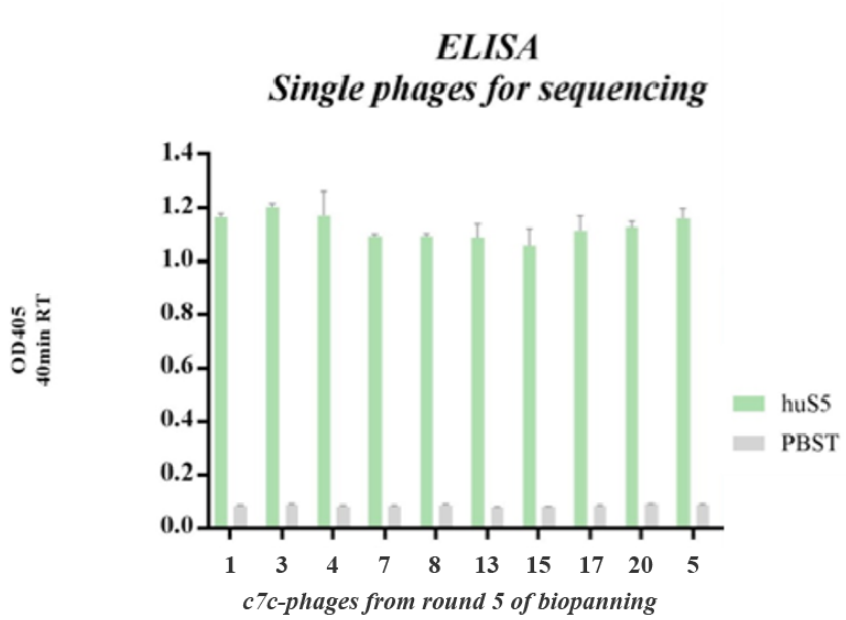

Figure 19. Phage-ELISA results obtained with monoclonal phage clones from the 5th round of biopanning with the c7c-library. Absorbance values are the mean of triplicates in one experiment, and error bars show the standard deviation for each data set. The ssDNA from phage clones numbered 1, 3, 4, 5, and 17 were selected for sequencing.

New efforts of biopanning experiments were performed. Tween20 may contribute to non-specific background binding in phage ELISA (Hakami et al., 2015). Therefore, in the last try, two pararell biopanning experiments were run with and without (w/o) the use of Tween20 in the washing buffer/ phage diluents. **Figure 20** show the result of phage ELISA after three rounds of biopanning. No differences between the two experiments were found. In addition,

the resluts show no increase of S5 binding-specificity after three rounds of biopannig. To test S5 background binding, wells were also coated with BSA and tested against S5 and anti-M13. The result revealed relative strong binidng of S5 to BSA or potatially the plastic wells.

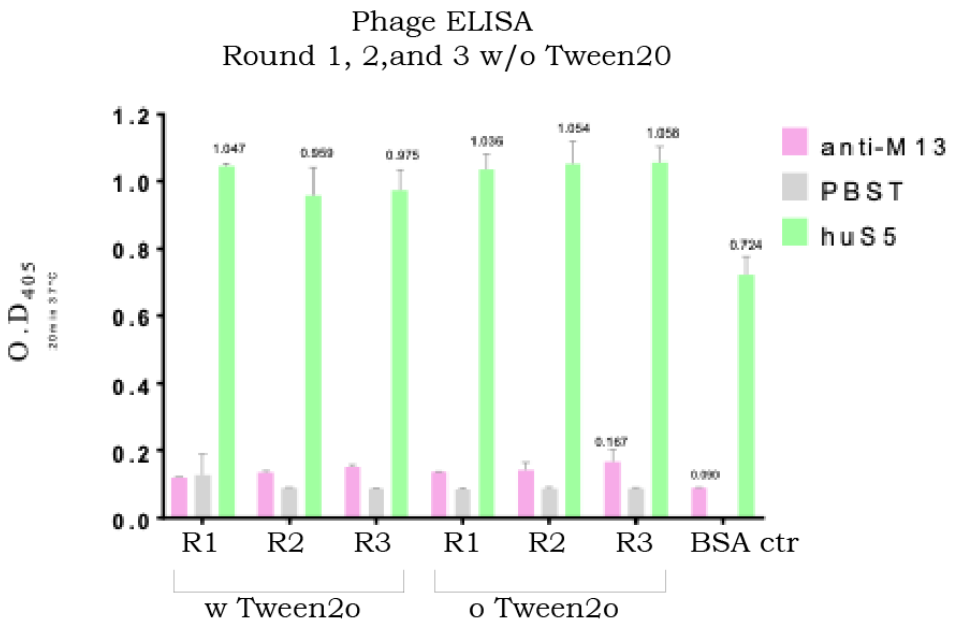

Figure 20. Binding of S5 to amplified phage from 3 rounds of biopanning with and without the use of Tween20. Absorbance values are the mean of triplicates in one experiment, and error bars show the standard deviation for each data set. Negative control wells (PBS) were included for each sample to check for cross-reactivity with the secondary antibody, and specificity was assessed by using an anti-M13 antibody (anti-M13).

5.8 Affinity selection of T cell-binding scFv antibodies

As indicated above, despite the successful use of checkpoint inhibitors to treat cancer patients, only a minority of patients responded to this treatment. Therefore, the need remains for the identification of more effective therapeutic antibodies. Accordingly, identifying new antibodies targeting inhibitory molecules expressed by activated/exhausted T cells will likely yield better treatments. In these experiments we aim to select antibodies on activated/exhausted human T cells to potentially generate a pool of scFvs against cell-surface receptors including inhibitory receptors expressed on these cells. PBMCs were stimulated with anti-CD3 and anti-CD28 for 3 days, and then the cells were used in the biopanning experiments as described in section 4.9. After activation, T cells were purified using anti-CD3-conjugated magnetic beads and the bound phages were rescued, amplified, and used for the next round of selection. The binding of amplified polyclonal scFv-phage antibodies to

untreated PBMCs and CD3/CD28 stimulated PBMCs were analysed after three rounds of biopanning using flow cytometry (**figure 21**). The binding profiles show that the enrichment of specific scFv-phages after three rounds has been successful. Furthermore, the phages seem to bind more to stimulated PBMC lymphocytes compared to untreated, suggesting that the scFv binding receptors are upregulated in some cell populations in response to CD3/CD8 antibody stimulation. Thus, our selection protocol has worked.

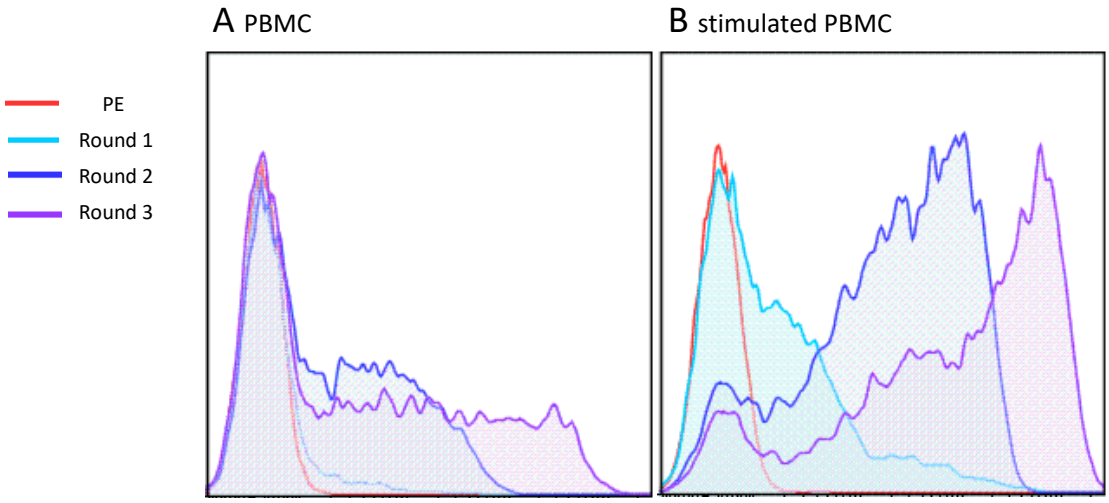

Figure 21. Binding of amplified scFv-phages from the 1st ,2nd and 3rd round of biopanning to untreated PBMCs (A) and CD3/28 stimulated PBMCs (B). The cells were stained with 10µl phage (around 10^9 transduction units/ml) and analysed by flow cytometry. Red histograms represent cells stained with only PE-conjugated streptavidin. The lymphocyte population was gated and analysed as shown in **figure 23**.

5.9 Analysis of single phage antibody clones

In the next experiments, single clones were prepared and their binding to activated and resting PBMCs was investigated by flow cytometry. 15 random single phage particles were picked from a plate of single colonies, amplified, and analysed by flow cytometry. The phages were first analysed in groups of five to eliminate negative phages (**figure 22**).

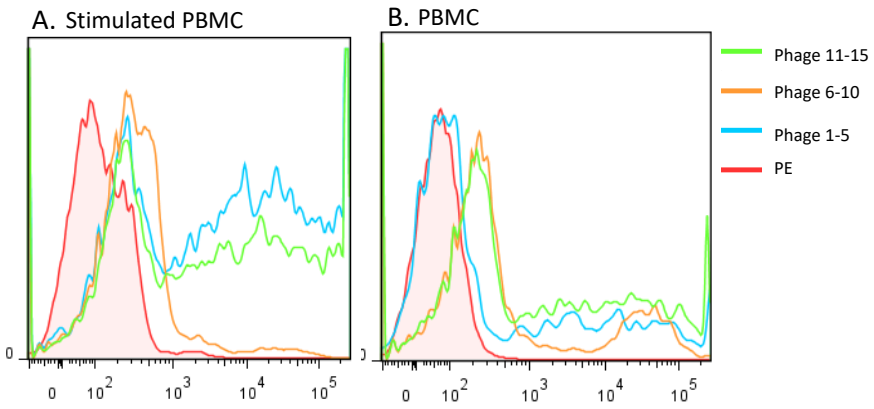

Figure 22. Group screening of 15 new phage particles. (A) Phage binding profiles to stimulated PBMCs. The cells were stained with 10µl phage (around 10^9 transduction units/ml) and analysed by flow cytometry. (B) Phage binding profiles to untreated PBMCs. The lymphocyte population was gated and analysed as shown in **figure 23**.

As shown in **figure 22**, scFv-phages in pool 1 and 3 showed strong binding when compared to those in pool 2. Finally, two phage clones (phage 1 and 11) which showed strong binding were selected for further characterization. **Figure 23** shows their binding profiles to resting and activated PBMCs.

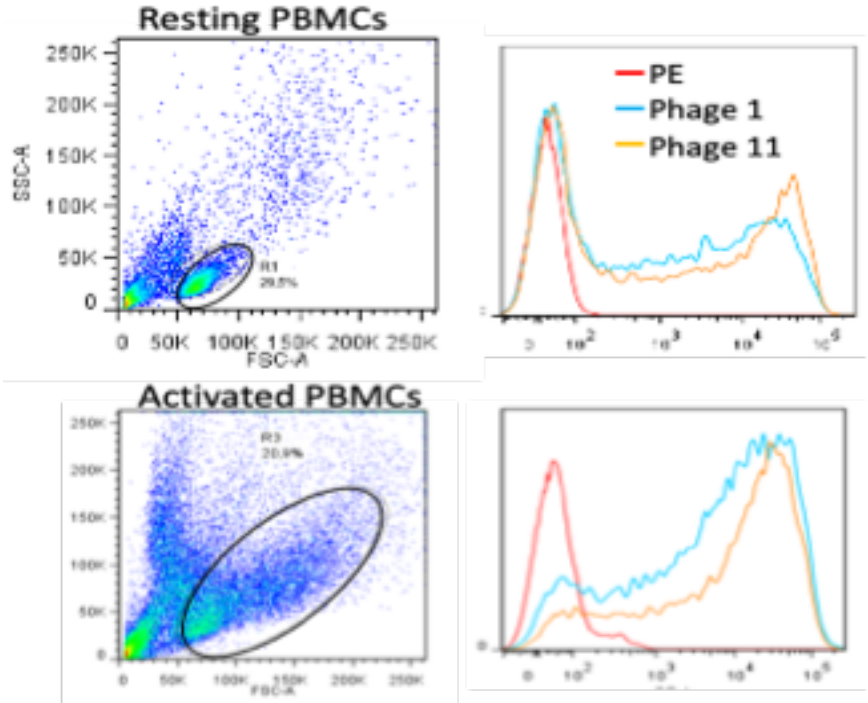

Figure 23. Unstimulated and CD3/CD28-stimulated cells were stained with phage antibody 1 and 11 (around 10^9 TU/ml each) and then analysed by flow cytometry. Gated lymphocyte population is indicated.

Altogether, we have demonstrated that T-cell binding scFv antibodies can be selected. The binding receptors seem to be differentially expressed between resting and activated T cells. Due to the coronavirus pandemic I was not able to characterise the selected phage antibodies.

6 Discussion

Soon after monoclonal antibody generation was reported in 1975 by Köhler and Milstein, the potential use of antibodies in therapies and diagnosis became clear and many antibodies are now used in the clinic. Today, cancer immunotherapy based on blocking immune checkpoints is a part of modern cancer treatment. Antibodies against PD1, CTLA-4 and PDL-1/2 showed significant anti-tumor effects. However, only a fraction of the immunotherapy treated patients respond to this treatment. Moreover, the treatment is not effective in highly common cancer types such as breast, prostate and colorectal cancers. Therefore, further progress in cancer immunotherapy could be made by the development of new immunomodulatory antibody targeting human T cells.

6.1 The S5 antibody

In the first part of this thesis, the main focus has been to determine the specificity of the S5 antibody using peptide phage libraries in hopes of predicting the parental antigen. The determination of the antibody specificity can give important implications for further studies as specific and high-affinity antibodies are desired as well as required for many therapies. Once a peptide is selected, certain amino acids in the peptide can be altered to further improve its binding affinity and specificity. Additionally, other properties like solubility, stability, serum half-life, cross-reactivity against human orthologs, and expression yield in manufacturing cells, are important considerations (Almagro et al., 2018). Such developable antibodies may require lower doses, which may directly impact the cost of treatments.

Peptide libraries facilitate the detection of specific peptides for any given antibody without prior knowledge of its specificity (Scott and Smith, 1990, Cwirla et al., 1990, Devlin et al., 1990). During affinity selection the antibody home in on target-specific interactions in the competitive presence of other potential partners and select peptides with a reasonable affinity for its paratope (Böttger and Böttger, 2009). In the search for the S5 epitope, a solution-phase panning method, was performed using both a linear 7mer and a constrained (c7c) peptide library. Compared to panning against a target immobilized on a surface, solution-phase biopanning requires less target per experiment and may improve the availability of the ligand-binding site to the phage-displayed peptides. Additionally, this method avoids denaturation of the target on a plastic surface. Following selection of potential peptide-partners, consensus

motives deduced from multiple peptide sequences can ideally reveal critical components of the antibody epitope when used in homology searches. However, the antibody may select its ideal peptides and consequently, some selected peptides may fit the antibody perfectly although the peptides may not have any homologous sequence to the parental antigen (Bonnycastle et al., 1996). Moreover, the use of different libraries can result in the findings of unrelated motifs (McConnell et al., 1994b). Hence, sequences identified from homology searches should be considered only as leads for further analyses. Conversely, high-affinity peptides that only mimic the epitopes, so-called mimotopes, have proven useful as diagnostic and prognostic tools and can be useful as vaccines, especially if not the parental antigen can be used (Riemer and Jensen-Jarolim, 2007). Unfortunately, epitope mapping experiments with the S5 antibody failed to identify potential partners.

Multiple factors may affect the selection of ligands from phage libraries, including the confirmation of the displayed peptides, the complexity of the ligand as well as the diversity of the library. Though we know from flow cytometry analysis and microscope observations that the S5 antibody binds to $CD4^+$ T cells and naive $CD8^+$ T cells as well as trigger proliferation and cell aggregation, no physical information is known about the interactions between the S5 antibody and its ligand Human IL-2 ELISA carried out with supernatant derived from MLR assays was performed to examine the effect of the S5 antibody on IL-2 production, however no correlation between IL-2 production and the S5 antibody was found. As an additional experiment, it could be interesting to quantify the number of S5-activated cells through lymphocyte proliferation assays, such as the H-thymidine incorporation assay, and compare with proliferation triggered by CD3 and CD28 stimulation.

The lack of structural information of the S5 epitope makes it difficult to predict what kind of library that will yield the most productive ligands. Structurally constrained libraries are useful for targets whose native ligands are in conjunction with surface loops, such as antibodies with structural epitopes (McConnell et al., 1994a). However, in our case, none of the phage displayed peptides rescued after five rounds of biopanning with the constrained C_7C-peptide library showed any significant reactivity towards the S5 antibody in ELISA or immunoscreening experiments. The S5 epitope may require more than the window of seven residues of the 7-mer libraries for efficient binding. Hence, other libraries, e.g., a 12-mer library, displaying longer peptides might yield the desired target-specific sequences.

A more intuitive aspect of phage display libraries is the relationship between library size and the chance of selecting the desired peptide. The larger the library, the higher the probability is of isolating more distinct and higher affinity clones, thus the higher the chance of selecting the desired protein. Accordingly, the larger the library, the higher the likelihood that an antibody will specifically bind a random epitope with higher affinity. During the successive rounds of selection and amplification, phages may be lost and consequently the library diversity may be reduced. In the absence of any strong binders the most "fit" peptide in the library will not bind the S5 antibody with high enough affinity to be selected.

Phage display technology is a well-established method, yet the outcome can vary due to multiple factor and technical issues. The phage pellet obtained from PEG/NaCl precipitation, indicated no technical problem in phage production and rescue. However, we encountered problems with fluctuating titration results. Plates made with the same dilution varied from showing just a couple of plaques to hundreds of plaques. Infected bacteria emerge as turbid plaque on LB plates caused by diminish cell growth rather than cell lysis and can therefore be difficult to see. Plating on Xgal/IPTG media facilitates visualisation of the plaques as well as helps distinguish the plaques caused by wild type phage (white plaques) and recombinant phage (blue plaques). An increase of phage titers after each round of biopanning can be a first indicator of successful affinity enrichment though are not decisive for successful selection of a desired target antigen. As mentioned, M13 is a male-specific phage hence loss of the F factor inhibits infection of the bacteria, which might have happened in this case. However, a fresh ER2738 *E. Coli* stock were made and only blue plaques from a plate holding ER2738 infected with phages from the 5th round of biopanning with the c7c-library were selected for sequencing. Though two sequences were uncovered from the five sequenced phages, indicating enrichment of those two sequences, no significant reactivity was detected in ELISA.

Absence of a strong target preference for the peptide sequences in the library can lead to selection of unspecific peptides that are usually rich in aromatic residues (Phe, Tyr, Trp, His), so-called "plastic-binders". These peptides typically give high background signals in ELISA experiments. Conversely, reactivity towards the S5 antibody seen in ELISA experiments when compared to negative PBS controls, proved to be unspecific binding of the S5 antibody to the ELISA plate rather than a consequence of the selection of unspecific plastic-binders. Furthermore, no immunoreactive clones were detected in the immunoscreening experiments,

i.e., no S5 specific phage clones were selected from the successive rounds of biopanning, conforming the assumption drawn from the ELISA experiments. Hopefully, this problem can be avoided through further experimentation with blocking conditions and incubation times.

6.2 Affinity selection of T cell-binding scFv-antibodies

Progress in the knowledge about the cellular and molecular mechanisms that orchestrate the interplay between the different components of the immune system provide the rational for the development of immunotherapeutic strategies. The innate arm of the immune system with its cytokines, complement proteins, and phagocytes, interact with the adaptive arm consisting of the B and T lymphocytes and their somatically generated repertoire of antigen receptors. The advantages of mobilizing T cells for cancer treatment became clear with the unravelling of the complexity of T cell activation and the discovery of inhibitory mediators. As described earlier, the immunosuppressive actions of CTLA-4 and PD-1 downregulate T-cell activation to preserve peripheral tolerance and can be exploited by tumors to facilitate tumor growth and development (Sharma and Allison, 2015).

Mobilizing T cells for cancer therapy have several benefits such as the TCRs specificity which are not limited to peptide antigens derived from cell surface molecules, and the generation of long-lived memory T cells, which provide rapid secondary responses if the antigen returns. Unleashing the T cells by blocking immune checkpoints like shown with CTLA-4 and PD-1 or PD-L1/2 can re-establish antitumor immune responses and provide long-term benefits in a significant proportion of treated patients. A primary advantage of the phage display technology is that affinity-based interactions can be discovered in native biological systems. Screening on intact cells preserves the original conformation of the cell surface proteins with proper post-translational modification and protein-protein interactions that could be relevant *in vivo* (Sioud, 2019). Activated T cells may express new checkpoint inhibitors and using such cells as an affinity matrix may lead to the selection of new antibodies that can be used to block inhibitory signals in T cells. The pool of scFv phages obtained from the third round of affinity selection on activated/exhausted T cells in this study may contain binders to different checkpoint inhibitors. Phage 1 and phage 11 showed strong binding to CD3/CD28-stimulated T cells when compared to resting T cells. Unfortunately, we do not have data describing the effects of the newly isolated scFv phages on T cells.

7 Conclusions

This thesis describes the use of two different biopanning protocols utilizing commercial phage display libraries, i.e., epitope mapping of the S5 antibody and hole cell biopanning on exhausted T cells.

Binding profiles obtained from flow cytometry analysis showed that of the S5 antibody bound to all $CD4^+$ T cells. By contrast, only a fraction of the $CD8^+$ T cells population bound the antibody, moreover the binding varied between donors. The S5 antibody induced $CD4^+$ T cell aggregation, but not IL-2 production. Unfortunately, epitope mapping experiments using random peptide phage libraries (both linear and constrained) failed to identify peptide binders.

Selection of new human antibodies using CD3/CD28-stimulated T cells as an affinity matrix resulted in the isolation of two scFv-phages (phage 1 and phage 11) which showed strong binding to activated T cell when compared to resting T cells. The phages are partially characterized through sequencing, but we do not have data describing the effect of these phages on T cells. Although further work is needed, the pool of scFv-phage antibodies isolated form the third round of selection in this experiment may contain binders to different checkpoint inhibitors.

8 References

A TOWNSEND, A. & BODMER, H. 1989. Antigen Recognition by Class I-Restricted T Lymphocytes. *Annual Review of Immunology,* 7**,** 601-624.

ADAMS, N. M., GRASSMANN, S. & SUN, J. C. 2020. Clonal expansion of innate and adaptive lymphocytes. *Nature Reviews Immunology*.

AHMAD, Z. A., YEAP, S. K., ALI, A. M., HO, W. Y., ALITHEEN, N. B. M. & HAMID, M. 2012. scFv antibody: principles and clinical application. *Clinical & developmental immunology,* 2012**,** 980250-980250.

ALBERTS, B. 2015. *Molecular biology of the cell*.

ALMAGRO, J. C., DANIELS-WELLS, T. R., PEREZ-TAPIA, S. M. & PENICHET, M. L. 2018. Progress and Challenges in the Design and Clinical Development of Antibodies for Cancer Therapy. *Frontiers in Immunology,* 8.

BARBAS, C. F., 3RD, BAIN, J. D., HOEKSTRA, D. M. & LERNER, R. A. 1992. Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. *Proceedings of the National Academy of Sciences of the United States of America,* 89**,** 4457-4461.

BERZOFSKY, J. A. 1985. Intrinsic and extrinsic factors in protein antigenic structure. *Science,* 229**,** 932-40.

BLUMBERG, R. S., LEY, S., SANCHO, J., LONBERG, N., LACY, E., MCDERMOTT, F., SCHAD, V., GREENSTEIN, J. L. & TERHORST, C. 1990. Structure of the T-cell antigen receptor: evidence for two CD3 epsilon subunits in the T-cell receptor-CD3 complex. *Proceedings of the National Academy of Sciences,* 87**,** 7220-7224.

BONNYCASTLE, L. L. C., MEHROKE, J. S., RASHED, M., GONG, X. & SCOTT, J. K. 1996. Probing the Basis of Antibody Reactivity with a Panel of Constrained Peptide Libraries Displayed by Filamentous Phage. *Journal of Molecular Biology,* 258**,** 747-762.

BREKKE, O. H. & SANDLIE, I. 2003. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nature Reviews Drug Discovery,* 2**,** 52-62.

BRUHNS, P. & JÖNSSON, F. 2015. Mouse and human FcR effector functions. *Immunological Reviews,* 268**,** 25-51.

BUCHBINDER, E. I. & DESAI, A. 2016. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *American journal of clinical oncology,* 39**,** 98-106.

BÖTTGER, V. & BÖTTGER, A. 2009. Epitope Mapping Using Phage Display Peptide Libraries. *In:* SCHUTKOWSKI, M. & REINEKE, U. (eds.) *Epitope Mapping Protocols: Second Edition.* Totowa, NJ: Humana Press.

CHIKAEV, A. N., BAKULINA, A. Y., BURDICK, R. C., KARPENKO, L. I., PATHAK, V. K. & ILYICHEV, A. A. 2015. Selection of peptide mimics of HIV-1 epitope recognized by neutralizing antibody VRC01. *PloS one,* 10**,** e0120847-e0120847.

CWIRLA, S. E., PETERS, E. A., BARRETT, R. W. & DOWER, W. J. 1990. Peptides on phage: a vast library of peptides for identifying ligands. *Proceedings of the National Academy of Sciences,* 87**,** 6378-6382.

DEVLIN, J., PANGANIBAN, L. & DEVLIN, P. 1990. Random peptide libraries: a source of specific protein binding molecules. *Science,* 249**,** 404-406.

EFIMOV, V. P., NEPLUEV, I. V. & MESYANZHINOV, V. V. 1995. Bacteriophage T4 as a surface display vector. *Virus Genes,* 10**,** 173-7.

FELICI, F., LUZZAGO, A., FOLGORI, A. & CORTESE, R. 1993. Mimicking of discontinuous epitopes by phage-displayed peptides, II. Selection of clones recognized by a protective monoclonal antibody against the Bordetella pertussis toxin from phage peptide libraries. *Gene,* 128**,** 21-27.

FJELL, C. D., HISS, J. A., HANCOCK, R. E. & SCHNEIDER, G. 2011. Designing antimicrobial peptides: form follows function. *Nat Rev Drug Discov,* 11**,** 37-51.

GREENWALD, R. J., FREEMAN, G. J. & SHARPE, A. H. 2005. THE B7 FAMILY REVISITED. *Annual Review of Immunology,* 23**,** 515-548.

HAKAMI, A., BALL, J. & TARR, A. 2015. Non-ionic detergents facilitate non-specific binding of M13 bacteriophage to polystyrene surfaces. *Journal of Virological Methods,* 221.

HANSEN, M. H., OSTENSTAD, B. & SIOUD, M. 2001. Antigen-specific IgG antibodies in stage IV long-time survival breast cancer patients. *Mol Med,* 7**,** 230-9.

HUANG, J. X., BISHOP-HURLEY, S. L. & COOPER, M. A. 2012. Development of Anti-Infectives Using Phage Display: Biological Agents against Bacteria, Viruses, and Parasites. *Antimicrobial Agents and Chemotherapy,* 56**,** 4569-4582.

HUDSON, P. J. 1998. Recombinant antibody fragments. *Current Opinion in Biotechnology,* 9**,** 395-402.

HUDSON, P. J. 1999. Recombinant antibody constructs in cancer therapy. *Current Opinion in Immunology,* 11**,** 548-557.

JONES, P. T., DEAR, P. H., FOOTE, J., NEUBERGER, M. S. & WINTER, G. 1986. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature,* 321**,** 522-525.

KAECH, S. M. & CUI, W. 2012. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nature reviews. Immunology,* 12**,** 749-761.

KAPLON, H. & REICHERT, J. M. 2019. Antibodies to watch in 2019. *mAbs,* 11**,** 219-238.

KEIR, M. E., BUTTE, M. J., FREEMAN, G. J. & SHARPE, A. H. 2008. PD-1 and Its Ligands in Tolerance and Immunity. *Annual Review of Immunology,* 26**,** 677-704.

KLEIN, L., KYEWSKI, B., ALLEN, P. M. & HOGQUIST, K. A. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature reviews. Immunology,* 14**,** 377-391.

KLIMKA, A., MATTHEY, B., ROOVERS, R. C., BARTH, S., ARENDS, J. W., ENGERT, A. & HOOGENBOOM, H. R. 2000. Human anti-CD30 recombinant antibodies by guided phage antibody selection using cell panning. *British journal of cancer,* 83**,** 252-260.

KÖHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* 256**,** 495-497.

L A SHERMAN, A. & CHATTOPADHYAY, S. 1993. The Molecular Basis of Allorecognition. *Annual Review of Immunology,* 11**,** 385-402.

LEBIEN, T. W. & TEDDER, T. F. 2008. B lymphocytes: how they develop and function. *Blood,* 112**,** 1570-1580.

LU, L. L., SUSCOVICH, T. J., FORTUNE, S. M. & ALTER, G. 2018. Beyond binding: antibody effector functions in infectious diseases. *Nature Reviews Immunology,* 18**,** 46-61.

LU, R.-M., HWANG, Y.-C., LIU, I. J., LEE, C.-C., TSAI, H.-Z., LI, H.-J. & WU, H.-C. 2020. Development of therapeutic antibodies for the treatment of diseases. *Journal of Biomedical Science,* 27**,** 1.

LUCKHEERAM, R. V., ZHOU, R., VERMA, A. D. & XIA, B. 2012. $CD4^+$T cells: differentiation and functions. *Clinical & developmental immunology,* 2012**,** 925135-925135.

MCCAFFERTY, J., GRIFFITHS, A. D., WINTER, G. & CHISWELL, D. J. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature,* 348**,** 552.

MCCONNELL, S. J., KENDALL, M. L., REILLY, T. M. & HOESS, R. H. 1994a. Constrained peptide libraries as a tool for finding mimotopes. *Gene,* 151**,** 115-118.

MCCONNELL, S. J., KENDALL, M. L., REILLY, T. M. & HOESS, R. H. 1994b. Constrained peptide libraries as a tool for finding mimotopes. *Gene,* 151**,** 115-118.

MEYER, S., LEUSEN, J. H. W. & BOROSS, P. 2014. Regulation of complement and modulation of its activity in monoclonal antibody therapy of cancer. *mAbs,* 6**,** 1133-1144.

MORRISON, S. L., JOHNSON, M. J., HERZENBERG, L. A. & OI, V. T. 1984. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proceedings of the National Academy of Sciences,* 81**,** 6851-6855.

MURPHY, K. & WEAVER, C. 2017. *Janeway's immunobiology*.

NELSON, A. L., DHIMOLEA, E. & REICHERT, J. M. 2010. Development trends for human monoclonal antibody therapeutics. *Nat Rev Drug Discov,* 9**,** 767-74.

NIKOLICH-ZUGICH, J., SLIFKA, M. K. & MESSAOUDI, I. 2004. The many important facets of T-cell repertoire diversity. *Nat Rev Immunol,* 4**,** 123-32.

NIMMERJAHN, F. & RAVETCH, J. V. 2008. Fcγ receptors as regulators of immune responses. *Nature Reviews Immunology,* 8**,** 34-47.

PARHAM, P. & JANEWAY, C. 2015. *The immune system*.

PIERCE, B. G. & WENG, Z. 2013. A flexible docking approach for prediction of T cell receptor-peptide-MHC complexes. *Protein science : a publication of the Protein Society,* 22**,** 35-46.

RAKONJAC, J., BENNETT, N. J., SPAGNUOLO, J., GAGIC, D. & RUSSEL, M. 2011. Filamentous bacteriophage: biology, phage display and nanotechnology applications. *Curr Issues Mol Biol,* 13**,** 51-76.

RIEMER, A. B. & JENSEN-JAROLIM, E. 2007. Mimotope vaccines: epitope mimics induce anti-cancer antibodies. *Immunology letters,* 113**,** 1-5.

RUSSEL, M. & LOWMAN, H. B. 2004. Introduction to phage biology and phage display. *Phage Display: A practical approach***,** 1-26.

SCOTT, J. & SMITH, G. 1990. Searching for peptide ligands with an epitope library. *Science,* 249**,** 386-390.

SHARMA, P. & ALLISON, J. P. 2015. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell,* 161**,** 205-214.

SHARPE, A. H. & FREEMAN, G. J. 2002. The B7–CD28 superfamily. *Nature Reviews Immunology,* 2**,** 116-126.

SIOUD, M. 2019. Phage display libraries: From binders to targeted drug delivery and human therapeutics. *Mol. Biotechnol.,* 61**,** 286.

SIOUD, M. & HANSEN, M. H. 2001. Profiling the immune response in patients with breast cancer by phage-displayed cDNA libraries. *Eur J Immunol,* 31**,** 716-25.

SMITH, G. P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science,* 228**,** 1315.

SMITH, G. P. 1993. Surface display and peptide libraries. *GENE-AMSTERDAM-,* 128**,** 1-1.

SMITH, G. P. & PETRENKO, V. A. 1997. Phage display. *Chem. Rev.,* 97**,** 391.

SMYTH, M. J. & TENG, M. W. 2018. 2018 Nobel Prize in physiology or medicine. *Clinical & translational immunology,* 7**,** e1041-e1041.

STERNBERG, N. & HOESS, R. H. 1995. Display of peptides and proteins on the surface of bacteriophage lambda. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 1609-1613.

STUCCI, S., PALMIROTTA, R., PASSARELLI, A., SILVESTRIS, E., ARGENTIERO, A., LANOTTE, L., ACQUAFREDDA, S., TODISCO, A. & SILVESTRIS, F. 2017. Immune-related adverse events during anticancer immunotherapy: Pathogenesis and management (Review). *Oncology Letters*.

TAKAKUSAGI, Y., KURAMOCHI, K., TAKAGI, M., KUSAYANAGI, T., MANITA, D., OZAWA, H., IWAKIRI, K., TAKAKUSAGI, K., MIYANO, Y., NAKAZAKI, A., KOBAYASHI, S., SUGAWARA, F. & SAKAGUCHI, K. 2008. Efficient one-cycle affinity selection of binding proteins or peptides specific for a small-molecule using a T7 phage display pool. *Bioorg Med Chem,* 16**,** 9837-46.

TONEGAWA, S. 1983. Somatic generation of antibody diversity. *Nature,* 302**,** 575-581.

TORCHILIN, V. 2014. *Handbook of Nanobiomedical Research: Fundamentals, Applications, and Recent Developments*, World Scientific Publishing Company.

WEINBERG, R. A. 2014. *The biology of cancer*.

WOLCHOK, J. D., KLUGER, H., CALLAHAN, M. K., POSTOW, M. A., RIZVI, N. A., LESOKHIN, A. M., SEGAL, N. H., ARIYAN, C. E., GORDON, R.-A., REED, K., BURKE, M. M., CALDWELL, A., KRONENBERG, S. A., AGUNWAMBA, B. U., ZHANG, X., LOWY, I., INZUNZA, H. D., FEELY, W., HORAK, C. E., HONG, Q., KORMAN, A. J., WIGGINTON, J. M., GUPTA, A. & SZNOL, M. 2013. Nivolumab plus Ipilimumab in Advanced Melanoma. *New England Journal of Medicine,* 369**,** 122-133.

ZHOU, J., HE, W., LUO, G. & WU, J. 2014. Mixed lymphocyte reaction induced by multiple alloantigens and the role for IL-10 in proliferation inhibition. *Burns & trauma,* 2**,** 24-28.

9 Appendix

9.1 Biopanning of human serum from a breast cancer patient

Biopanning of human serum a patient with breast cancer was preformed using random peptide phage libraries as desscribed in section 4.2. Single phages from the fourth was picked from LB + tetracycline plates (containing XGal/IPTG), amplified and the reactivity towards sthe patient serum was tested by ELISA. Phage clone 6 showed promesing reactivity compared to the rest and will be sent for sequencing.

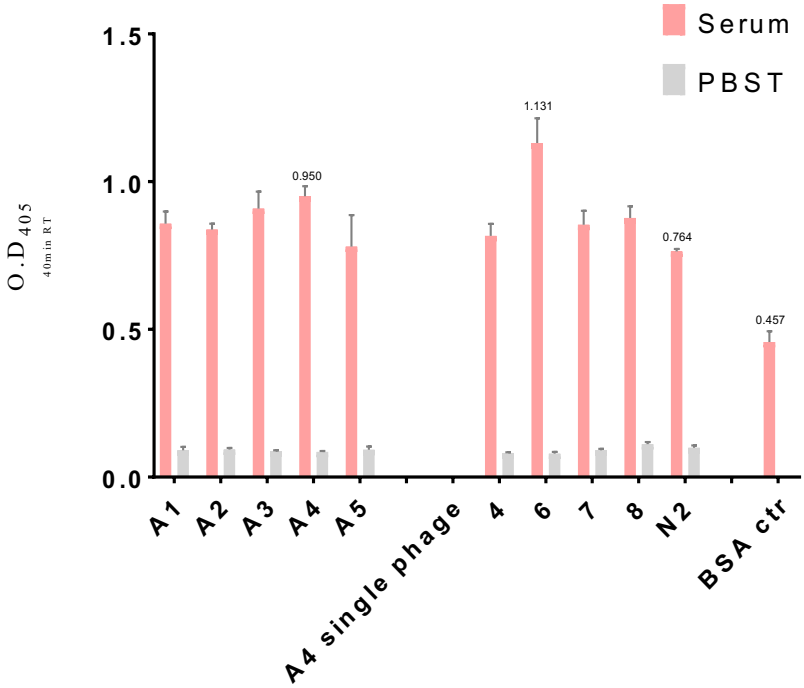

Figure 24. ELISA results indicating the reactivity of rescued polyclonal (A1-A5 = Biopanning round 1-5) and monoclonal (A4 single phages: 4, 6, 7, 8, N2) pahges to serum from a breast cancer patient.

9.2 Standard curve: Human IL-2 ELISA

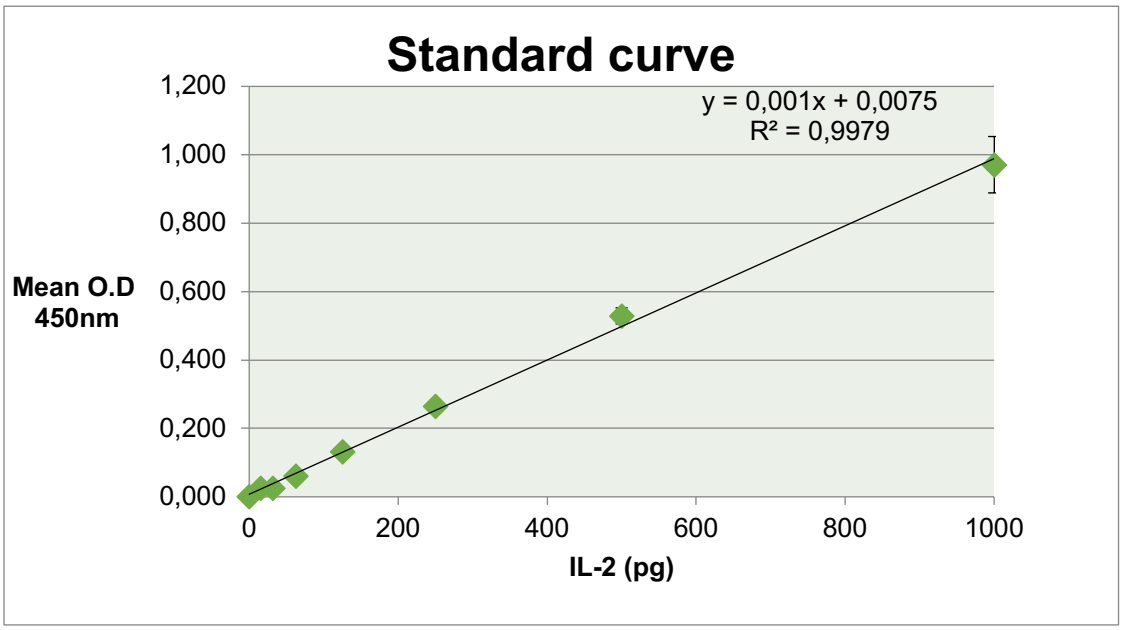

Figure 25. IL-2 Standard curve