

**Improved Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) of Affinity Maturated and Fc-Engineered Antibodies Directed Against the AML Stem Cell Antigen CD96**

**Dissertation**

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## **DECLARATION**

I declare that I have carried out this thesis by myself and have not used external help except where due reference is made. This thesis was not submitted to any other university and I did not make any earlier attempt to submit this work as a doctoral thesis.

Sahar Mohseni Nodehi

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## LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ABTS	2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt, crystals
Amp	Ampicillin
BM	bone marrow
bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CDR	Complementarity-determining region
cfu	Colony forming units
CH	Constant region of antibody heavy chain
CL	Light chain constant region
CMV	Cytomegalovirus
cp	Coat protein
CSCs	Cancer stem cells
dH <sub>2</sub> O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamine tetraacetate
ELISA	Crystallizable fragment of antibody molecule
Fab	Fragment antigen binding
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcRs	Fc receptors
FCS	Fetal calf serum
FDA	US Food and Drug Administration
g	G force
h	Hour
HRP	Horse radish peroxidase
HSCs	Hematopoietic stem cells
Ig	Immunoglobulin
IPTG	Isopropyl- $\beta$ -D-thiogalactopyranoside

kDa	Kilodalton
L	Liter
LSCs	Leukemia stem cells
M	Molar
mA	Milliampere
MACS	Magnetic cell separation
MFI	Median fluorescence intensity
min	Minutes
mM	Millimolar
NK cells	Natural killer cells
NM	Non-fat dry milk
NTA	Nitrilotriacetic acid
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PMBC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pfu	Plaque-forming unit
PHA	Phytohemagglutinin
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
RNA	Ribonucleic acid
RPM	Rounds per minute
RT	Room temperature
s	Seconds
ScFv	Single-chain variable fragment
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
TB medium	Terrific Broth medium
TEMED	N,N,N',N'tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene (20)-sorbitan-monolaurate
U	Unit
UV	Ultraviolet
V	Volt
VH	Heavy chain variable region
VL	Light chain variable region



v/v	Volume/volume
w/v	Weight/volume
$\beta$ -ME	$\beta$ -Mercaptoethanol
$\mu$ M	Micromolar

## ABSTRACT

Regardless of progress in the therapy of AML, there is no long-term cure for about 70% of AML patients. Leukemic stem cells seem to be an important key for the perpetuation of AML. Most currently used chemotherapeutic agents are not able to eliminate AML-LSCs. The combination of conventional therapies with AML-LSCs-directed therapies may eventually lead to a cure for AML patients. For efficient targeting of AML-LSCs, identification of an appropriate cell surface marker preferentially expressed on AML-LSCs, but absent on normal tissue, especially on normal HSCs is essential. To date only a few potential target antigens have been defined but most of these target antigens are also expressed on normal hematopoietic stem cells or nonhematopoietic tissues. Recently expression of CD96 (TACTILE) was reported on AML-LSCs, while only very low expression levels were found on normal HSCs. CD96 was also detected on the majority of AML blasts, in about 30% of AML patients. Therefore CD96 may be an excellent target antigen for therapeutic antibodies directed against AML stem cells and blasts. TH-111, a CD96 mouse monoclonal antibody was previously generated in our lab. To reduce the immunogenicity of the mouse antibody for clinical use in humans, the V-regions of the CD96 mouse antibody TH-111 were isolated and a functional scFv was generated by phage display. The CD96-scFv retained the antigen specificity of the CD96 parental antibody. CD96-scFv was cloned into a human IgG1-Fc and subjected to ADCC using CD96-positive cells as target cells and human mononuclear effector cells. This mini-antibody mediated ADCC only modestly. To improve the anticancer efficacy of this antibody, CD96-scFv was affinity matured by random mutagenesis through error-prone-PCR and mutated CD96-scFv with high binding affinity to CD96 was selected through phage display and stringent screening process. The mutated variant of scFv-based mini-antibody showed a 4-fold enhanced antigen binding affinity to CD96. Affinity matured variant of the scFv was fused to a wild type IgG1-Fc or a variant with engineered/enhanced effector functions (IgG1-Fc-eng). The mini-antibody with affinity-matured scFv and the engineered IgG1-Fc variant demonstrated the highest lytic capacity, suggesting that affinity to CD96 as well as efficient Fc receptor binding contributed to the observed effects. This recombinant antibody may be potentially useful in different clinical settings, including *ex vivo* purging of AML-LSCs from autologous stem cell preparations or for the targeting of AML-LSCs *in vivo*.

## ZUSAMMENFASSUNG

Bei der akuten myeloischen Leukämie kann nur in ca. 30% der Fälle durch eine intensive Chemotherapie eine anhaltende Remission erreicht werden. Ein Grund für häufige Residive ist in der höheren Resistenz der AML-Stammzellen gegenüber Chemotherapeutika begründet. Daher erscheinen in Zukunft zielgerichtete Therapien aussichtsreich, die primär diese Leukämienstammzellen und auch Leukämie Blasten als Zielzelle erkennen und effektiv eliminieren. Um die AML-Stammzellen zielgerichtet zu attackieren, ist es von entscheidender Bedeutung, Zielmoleküle zu identifizieren und zu validieren, die möglichst spezifisch zwischen entarteter Zielzelle und gesundem Gewebe differenzieren. Nur wenige geeignete Zielstrukturen auf AML-Stammzellen sind bisher beschrieben worden. Diese Zielstrukturen werden jedoch meistens auch auf normalen hämatopoietischen Stammzellen und nicht-hämatopoietischen Zellen exprimiert, was für klinische Anwendungen problematisch ist. Vor kurzem wurde entdeckt, dass AML-Stammzellen das CD96-Antigen (TACTILE) exprimieren. Wichtig dabei ist, dass CD96 nur äußerst gering auf normalen hämatopoetischen Zellen nachzuweisen ist. CD96 wird auf den leukämischen Blasten bei rund 30% aller untersuchten AML Patienten und bei verschiedenen Subtypen der AML membranständig exprimiert. Dadurch könnte CD96 als Zielstruktur für CD96-gerichtete Immuntherapie geeignet sein. Ein CD96 Antikörper TH-111 wurde von unserer Arbeitsgruppe generiert. Um für eine Anwendung im Menschen einen grossen Teil der potentiell immunogenen Mausequenzen des TH-111 Antikörpers zu entfernen, wurden die V-Regionen des murinen Antikörpers unter Verwendung von Phage-Display Technologie isoliert. CD96-scFv behielt die Bindungsspezifität des parentalen Antikörpers. Das isolierte scFv-Fragment wurde zur Klonierung von chimären CD96 Mini-Antikörpern (Fusion aus scFv und humanem IgG1 Fc-Teil) benutzt. Für den ADCC-Assay wurden die CD96-positiven-Zellen als Zielzellen und mononukleären Zellen (MNC) als Effektorzellen verwendet. Der CD96 Mini-Antikörper bewirkte nur eine geringe Effektorzell-vermittelte Lyse. Um hochaffine Antikörpervarianten zu generieren wurde mittels Error-prone-PCR zufällige Punktmutationen in das CD96-scFv cDNA-Fragment eingebracht und Antikörpergenbibliotheken mit hoher Variabilität generiert. Durch stringente Selektionsmethoden wurden Antikörperklone mit verbesserten Bindungseigenschaften zu CD96 isoliert. Der mutierte CD96-scFv zeigte eine ca. 4-5 fache höhere Affinität für CD96, verglichen mit dem Wildtyp-scFv. Die cDNA des

mutierten scFv wurde mit einem Wildtyp sowie einem für eine bessere Fc-Rezeptor Bindung optimierten humanen IgG1-Fc-Teil fusioniert. Der gereinigte mutierte CD96-Mini-Antikörper wurde in ADCC Experimenten mit Wildtyp CD96-Mini-Antikörper verglichen. In funktionellen Analysen (ADCC) war das Affinität-optimierte scFv Molekül (Mutant) mit mutiertem humanen IgG1-Fc-Teil dem Wildtyp scFv mit Wildtyp IgG1-Fc-Teil deutlich überlegen. Aus den Versuchen kann geschlossen werden, dass eine Erhöhung der Affinität zum CD96 und zum Fc Rezeptor eine Steigerung der zytotoxischen Aktivität hervorruft. CD96-Mini-Antikörper könnten zur Eliminierung von Tumorzellen *in vivo* von Interesse sein. Es ist geplant, CD96-Mini-Antikörper *Ex vivo* zum Purgung von Stammzellpräparaten für Autologe Stammzellen weiter zu evaluieren.

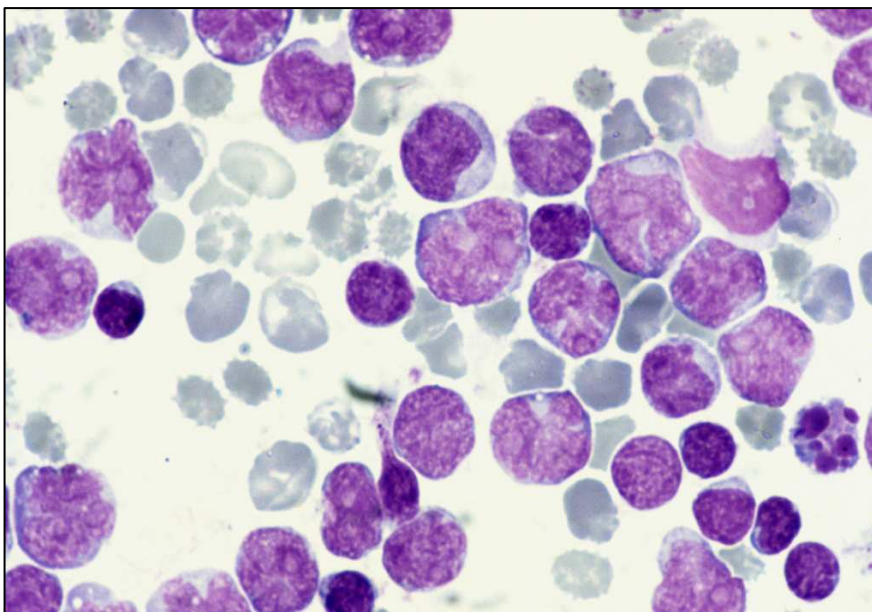
**Chapter 1**

**INTRODUCTION**

### 1. INTRODUCTION

#### 1.1 Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) is a malignant disease of hematopoietic cells characterized by immature cells called blasts (Fig 1.1). AML is the most common acute leukemia among adults and its incidence increases with age (Sievers et al., 2001). 80% of acute leukemia cases in adults and 15-20% in children are AML. The average age of a person with AML is 64 years. About 3600 new cases of AML occur in Germany each year, mostly in older adults. Regardless of progress in the therapy of AML, there is no long-term cure for about 70% of AML patients.



**Fig 1.1 AML blasts formation.** Acute myeloid leukemia or AML is a cancer of the myeloid line of blood cells. In AML, the bone marrow makes many unformed cells called blasts.

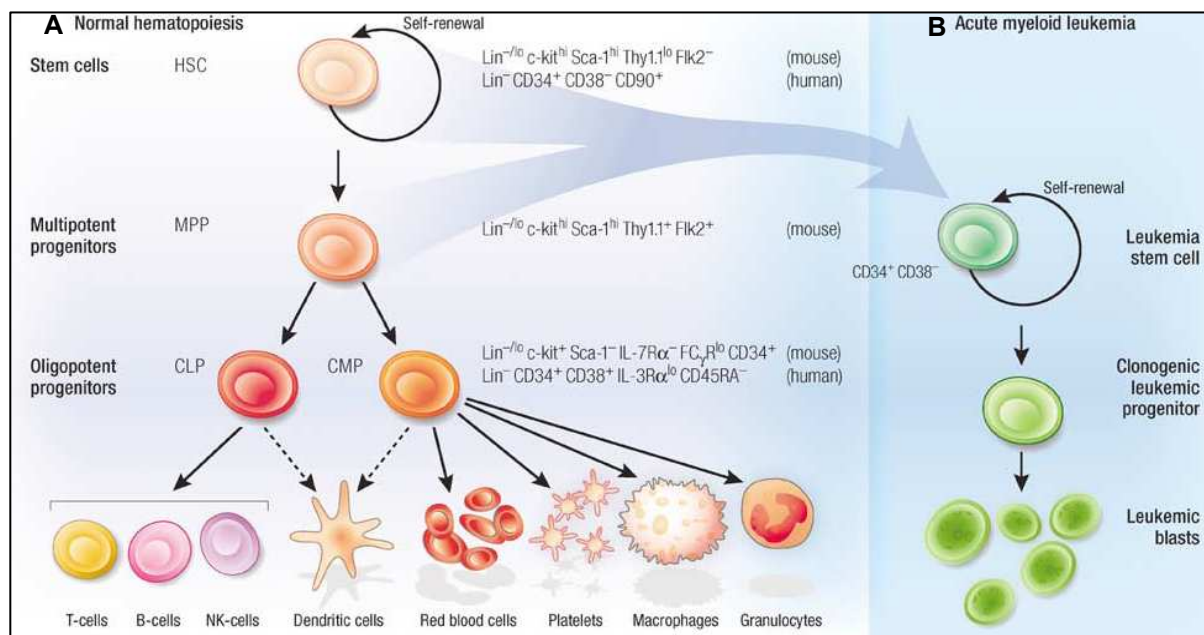
#### 1.1.1 AML classification

AML can be classified into various types. The AML classification is important since not all types of AML are treated in the same way. There are two major classifications for AML: The French-American-British (FAB) which is the first widely accepted classification and the World-Health-Organization (WHO) classification. The FAB classification of AML was established in 1976 and classified AML into eight major types. This classification is based on the degree of maturation, development phase and lineage differentiation of myeloblasts (Bennett et al., 1976; Bennett et al., 1985). The WHO classification of AML was established in 1999 and revised in 2009

(Vardiman et al., 2009). It classified AML according to morphological, immunological, cytogenetic, genetic, and clinical features.

### 1.1.2 Hematopoietic stem cells (HSCs) and leukemia

All blood cells arise from hematopoietic stem cells (HSCs). HSCs are hematopoietic cells with self-renewal potential (Ema and Nakauchi, 2003). HSCs generate a various number of hematopoietic cells by giving rise to multipotent progenitors (MPPs) and progenitors with limited dividing capacity. These progenitor cells are divided to lymphoid and myeloid progenitors, which give rise to various differentiated blood cells (Fig 1.2A) (Tan et al., 2006). Regulation of HSCs development is a critical element in the control of normal hematopoiesis (Ho and Punzel, 2003; Reya, 2003). The stem cell developmental schedule is strongly regulated by intrinsic factors and external stimuli such as soluble cytokines and contact with stroma (Zon, 2001). Dysregulation of stem cell developmental schedule causes abnormal expression of oncogenes, resulting in cancer cells proliferation (Godwin and Smith, 2003). Understanding the regulation factors which regulate this developmental program, and lead to the proliferative diseases such as leukemia, is one of the major challenges in biology (Frederick R. Appelbaum, 2001 ).

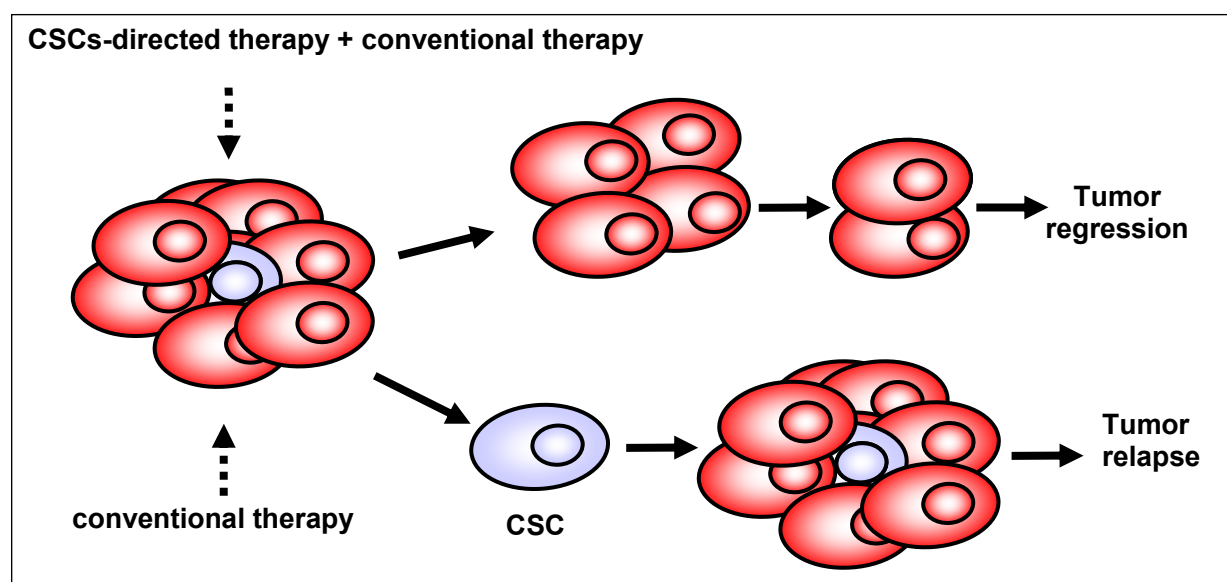


**Fig 1.2 Hematopoiesis and leukemia.** Modified from (Tan et al., 2006). A) Normal hematopoietic stem cells (HSCs) and progenitor cells. HSCs can give rise to multipotent (MPPs) and oligopotent progenitor cells. These progenitor cells generate differentiated blood cells. B) Leukemic stem cells

(LSCs) and progenitor. LSCs arise from immature hematopoietic progenitors or from normal HSCs. LSCs give rise to clonogenic leukemic progenitors that differentiate into differentiated leukemic blasts.

### 1.1.3 Cancer stem cells (CSCs) hypothesis

According to the cancer stem cell hypothesis, these cells are a small subset of cancer cells, which are able to self-renew and recreate the cancer stem cell pool (Hope et al., 2004). The cancer stem cells are also able to differentiate into the heterogeneous nontumorigenic cancer cell types, responsible for the maintenance of the bulk of tumor (Clarke et al., 2006). Cancer stem cells are relatively resistant to conventional therapies which target rapidly dividing cells and their persistence explains the relapse of cancer diseases (Clarke et al., 2006). Therefore, recognition and elimination of the cancer stem cell population is crucial for cure of the cancer patients (Fig 1.3).



**Fig 1.3 Combination of cancer stem cells-directed therapy and conventional therapy.** Conventional therapies generally target the bulk of tumors, resulting in the shrinking the tumors. These therapies are usually unsuccessful in targeting and eliminating the cancer stem cells. Therefore, the therapy can fail and the disease can relapse. According to cancer stem cell hypothesis, novel therapeutics which target and kill CSCs, together with conventional therapy, may help to cure the cancer patients.

### 1.1.4 AML stem cells concept

Acute myeloid leukemia with a hierarchy arrangement is an example of the cancer stem cells hypothesis. The leukemia stem cells (LSCs) are a subpopulation of AML



cells which have long-term repopulating potential and ability to maintain and increase the AML phenotype. The LSCs are able to give rise to clonogenic leukemic progenitors. These progenitor cells differentiate into their differentiated progeny and leukemic blasts (Tan et al., 2006) (Fig 1.2B). The existence of leukemic stem cells has been long challenged (Huntly and Gilliland, 2005). The first supportive data was reported in 1988 using high-speed multiparameter flow cytometry (Spangrude et al., 1988). It was demonstrated that human AML stem cells are found in the CD34<sup>+</sup> CD38<sup>-</sup> cell population, similar to normal human primitive hematopoietic progenitors (Spangrude et al., 1988). Therefore AML stem cells may derive from normal hematopoietic stem cells or immature hematopoietic progenitors. In AML, some cytogenetic anomalies such as translocation t(8;21) occur in normal HSCs (Tan et al., 2006). CD34<sup>+</sup> CD38<sup>-</sup> AML stem cells are able to regenerate human AML cell population in irradiated transplanted nonobese diabetic/severe combined immunodeficient mice, (NOD/SCID), but more committed progenitors which possess CD34<sup>+</sup> CD38<sup>+</sup> cell surface phenotype, do not have such potential (Lapidot et al., 1994; Bonnet and Dick, 1997). *In vitro* studies demonstrated that AML stem cells are more resistant to chemotherapy than the CD34<sup>+</sup> CD38<sup>+</sup> progenitor cells (Costello et al., 1999). Remaining malignant CD34<sup>+</sup> CD38<sup>-</sup> cells after chemotherapy, (minimal residual disease or MRD), cause the relapse of the disease (Feller et al., 2004). Recognition of differences between LSCs and normal hematopoietic stem cells and elimination of LSCs, could result in an effective therapy for AML patients.

### 1.1.5 AML stem cell marker

For an optimal AML stem cell-targeted therapy, it is necessary to recognize an antigen that is especially expressed on these cells. Different potential AML stem cells antigens such as CD123, CD33, CD44, and CD47 are described. But all of these antigens have some disadvantages such as expression on normal stem cells or nonhematopoietic tissues. CD123, the alpha subunit of the interleukin-3 receptor, (IL-3Ra), is expressed on a variety of hematopoietic malignancies as well as LSCs (Jordan et al., 2000; Munoz et al., 2001). High expression of CD123 on CD34<sup>+</sup> CD38<sup>-</sup> non-AML-regenerating bone marrow cells, (RBM), has also been reported (van Rhenen et al., 2007). In addition, CD123 antigen is expressed on normal HSCs. Therefore, CD123 is not an optimal target for AML stem cell-directed therapy. Gemtuzumab ozogamicin ,(GO), which is approved by FDA, is one of the

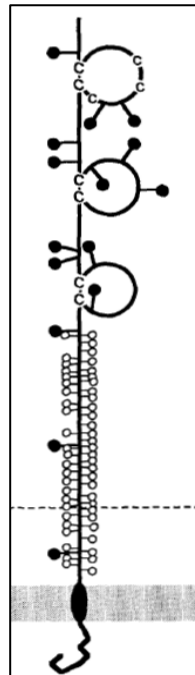
immunological approaches for targeting LSCs and treatment the AML patients (Sievers et al., 1999). GO is an antibody directed against CD33 which is conjugated to a cytotoxic agent. If GO is applied alone, many patients show the relapses (Sievers, 2001), since the majority of leukemic stem cells do not express CD33 (Taussig et al., 2005). In addition, CD33 is highly expressed on normal CD34<sup>+</sup> CD38<sup>-</sup> stem cells. Therefore the anti-CD33 can eliminate normal HSCs as well as LSCs (Taussig et al., 2005; van Rhenen et al., 2007) and can induce cytopenia (Sievers, 2001; Kell et al., 2003). CD44 is another antigen on CD34<sup>+</sup> CD38<sup>-</sup> leukemic stem cells. CD44 antibody ,(H90), can reduce the engraftment in NOD/SCID mice (Jin et al., 2006). But this antigen is also weakly expressed on normal CD34<sup>+</sup> CD38<sup>-</sup>, more differentiated hematopoietic cells (Jin et al., 2006), and many different tissues (Ponta et al., 2003). CD47 is also highly expressed on AML-LSCs, as well as on normal tissues and bone marrow HSCs (Majeti et al., 2009). Expression of CD96 ,(TACTILE), was recently found on AML-LSCs, while only a very low expression level was found on normal HSCs (Hosen et al., 2007).

### **1.1.6 CD96 (Tactile)**

CD96 also known as Tactile is a member of immunoglobulin gene super family. CD96 was previously introduced as a novel cell surface marker on NK cells and an antigen for T cell activation increased late expression ,(Tactile), (Wang et al., 1992; Gramatzki et al., 1998). This antigen has been recently reported as a target antigen on AML-LSCs (Hosen et al., 2007).

#### **1.1.6.1 Structure of CD96**

CD96 or tactile is a glycoprotein with a molecular weight of 160 kDa under reducing condition (Wang et al., 1992). This molecule is composed of a single pass transmembrane domain, a short cytoplasmic domain with 45 residues, and a long extracellular region with three immunoglobulin domains (Fig 1.4), (Wang et al., 1992). The C-terminal of the extracellular domain is extensively glycosylated and is rich in serines, threonines, and pralines but has no cysteines. 2 Ig domains have two cysteines, and the most N-terminal Ig domain has five cysteines. These three extra cysteines are involved in the Ig disulfide bond (Wang et al., 1992).



**Fig 1.4 Schematic structure of the CD96 antigen (Tactile).** Modified from (Wang et al., 1992). Three immunoglobulin domains are displayed as loops. C: cysteine, attached black circles: N-glycosylation sites, attached open circles: serines and threonines in the putative O-glycosylated region, gray bar: plasma membrane.

### 1.1.6.2 Expression of CD96

CD96 is strongly upregulated in activated T and NK cells but has low expression in peripheral T and NK cells (Wang et al., 1992; Gramatzki et al., 1998; Hosen et al., 2007). CD96 is expressed in a variety of T cell leukemia lines such as PEER, HSB, and HUT-78 (Gramatzki et al., 1998; Burger et al., 1999). Non-T cell lines, including fibroblasts and hepatoma cells which express T cell activation antigens, such as VLA-1, VLA-2, and CD26 do not express the CD96 antigen (Wang et al., 1992). CD96 was also detected in ~30% of human AML samples (Gramatzki et al., 1998). Tactile shows no expression on peripheral B cells, granulocytes, monocytes, and RBCs (Wang et al., 1992; Gramatzki et al., 1998). CD96 is expressed in nonhematopoietic tissue such as the mucosal epithelium of the small and large intestines, the convoluted tubular epithelium of the kidney and the vascular endothelium (Gramatzki et al., 1998).

### 1.1.6.3 CD96 monoclonal antibodies

There are two known CD96 monoclonal antibodies, G8.5 and TH-111. The CD96 monoclonal antibody G8.5 was generated by the immunization of mice with an IL-2-dependent, alloantigen-specific human CTL line (Cytotoxic T Lymphocytes) (Wang et al., 1992). The CD96 monoclonal antibody TH-111 was generated by immunizing mice with a T-ALL cell line, HSB-2 cells (Gramatzki et al., 1998).

### 1.1.6.4 CD96 as a stem cell target antigen

CD96 has been recently reported as a specific antigen on AML-LSCs (Hosen et al., 2007). CD96 is expressed on functional AML-LSC. Transplantation of CD34<sup>+</sup> CD38<sup>-</sup> CD96<sup>+</sup> AML cells into irradiated newborn Rag2<sup>-/-</sup> γc<sup>-/-</sup> mice resulted in an engraftment, whereas CD34<sup>+</sup> CD38<sup>-</sup> CD96<sup>-</sup> AML cells did not (Hosen et al., 2007). CD96 shows no expression on the majority of cells in the normal adult BM HSCs-enriched population (Hosen et al., 2007).

### 1.1.6.5 CD96 as a ligand for CD155 (PVR) in NK cells

CD96 expressed on NK cells has been described as a ligand for CD155 or PVR (Polio Virus Receptor), (Fuchs et al., 2004). NK cells distinguish PVR also through the DNAM-1 receptor (Bottino et al., 2003). CD96 binding to CD155 mediates the adhesion of NK cells to the target cells (Fuchs et al., 2004). Human CD96 can be expressed in two splice variants: variant 1 (L-like) or variant 2 (V-like) but the interaction with its ligand, CD155, is mediated through the outermost variant 2 and can be altered by this domain (Meyer et al., 2009). CD155 is a member of nectin and nectin-like family and is highly expressed on some tumors (Masson et al., 2001). Therefore cell-cell interaction between CD96-positive NK cells and tumor cells stimulates the cytotoxicity of these activated NK cells and promotes NK cell-mediated killing activities (Fuchs et al., 2004). This NK cells killing activity in mice is mediated through binding of CD96 to nectin-1 on target cells (Seth et al., 2007). Lacking of nectin-1 in mice mediates microphthalmia (Inagaki et al., 2005) and mutation in nectin-1 in humans mediates cleft lip/palate ectodermal dysplasia syndrome (CLPED) (Suzuki et al., 2000). A mutation in human CD96, or disruption of the genetic locus of CD96, can induce a form of C syndrome (Opitz-Trigonocephaly) (Kaname et al., 2007) since the adhesion activity between CD96 and its ligand, PVR, plays an important role in development processes. CD96 on AML-LSCs may also

show an interaction activity with its ligand on other cells in the bone marrow, possibly niche cells of AML-LSCs. In this case the binding activity of CD96 and its ligand can not mediate a killer function, but may take part in leukemia properties and may play a functional role in LSCs biology (Hosen et al., 2007).

### **1.2 Antibodies (Immunoglobulins)**

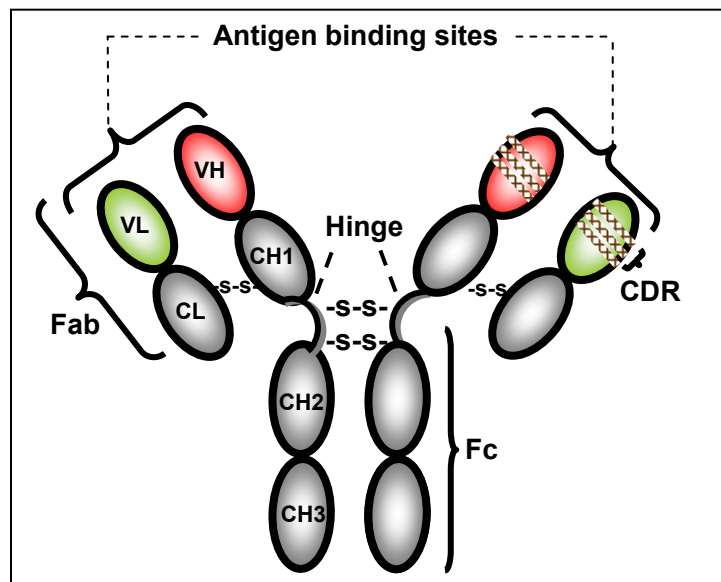
Antibodies are glycoprotein molecules which are generated by B-lymphocytes and plasma cells in response to an antigen. Due to migration with globular proteins in an electrical field, the antibody is also called immunoglobulin.

#### **1.2.1 Human immunoglobulin structure**

Antibodies are Y-shaped macromolecules consist of two light chains with a low molecular weight (23 kDa) and two heavy chains with a higher molecular weight (50-70 kDa) in a monomeric format (Fig 1.5). The N-terminal domains at the tip of the "Y" arms on both the heavy and light chains are variable in amino acid sequence and are known as variable domains. The other domains are called the constant region. The variable region contains the antigen binding site and the constant region determines the isotype and the functional properties of the antibody. Each heavy chain has one variable domain (VH) and three or four constant domains, depending on the isotype of antibody (CH1, CH2, CH3, and CH4). Each light chain has one variable domain (VL) and one constant domain (CL). The region between the CH1 and CH2 domains is called the hinge region because of the flexibility in the molecule at this point. There are different numbers of disulfide bonds in different immunoglobulin molecules. The heavy and light chains and the two heavy chains are bonded together by disulfide bonds (S-S). There are two types of light chains, kappa ( $\kappa$ ) and lambda ( $\lambda$ ), and five types of heavy chain, alpha ( $\alpha$ ), delta ( $\delta$ ), epsilon ( $\epsilon$ ), gamma ( $\gamma$ ), and Mu ( $\mu$ ), according to the constant region. Corresponding to the types of heavy chains, 5 immunoglobulin's isotypes are known: immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin E (IgE), and immunoglobulin D (IgD) (Table 1.1). Human IgG has four subclasses according to number of disulfide bonds and length of the hinge region: IgG1 ( $\gamma$ 1 heavy chains), IgG2 ( $\gamma$ 2 heavy chains), IgG3 ( $\gamma$ 3 heavy chains), and IgG4 ( $\gamma$ 4 heavy chains). IgA is also divided in two subclasses: IgA1 ( $\alpha$ 1 heavy chains) and IgA2 ( $\alpha$ 2 heavy chains) (Table 1.1). There are three regions with a high ratio of different amino acids in variable region of heavy and light

## 1. INTRODUCTION

chains of immunoglobulins which determine their specificity. These are called hypervariable regions (HVR) or complementarity determining regions (CDR): CDR1, CDR2, and CDR3. The remaining regions in the variable region are named the framework regions. There are four framework regions: FR1, FR2, FR3, and FR4.



**Fig 1.5 Schematic drawing of a simple immunoglobulin molecule (IgG).** ■ VL: variable region of light chain, ■ VH = variable region of heavy chain (red), VL + VH = variable region, ■ CL + CH1 + CH2 + CH3 = constant regions, CH2 + CH3 = Fc; VL + CL = light chain, VH + CH1 + CH2 + CH3 = heavy chain, VH + CH1 + L chain = Fab, S-S = disulfide bond, and # CDRs: complementarity determining regions.

**Table 1.1 Overview of human immunoglobulin classes and subclasses.**

Ig isotype	Light chain	Subtype	Heavy chain	Basic structure
IgA	κ or λ κ or λ	IgA1 IgA2	α1 α2	Dimer and Monomer
IgE	κ or λ	None	ε	Monomer
IgD	κ or λ	None	δ	Monomer
IgM	κ or λ	None	μ	Pentamer
IgG	κ or λ κ or λ κ or λ κ or λ	IgG1 IgG2 IgG3 IgG4	γ1 γ2 γ3 γ4	Monomer

Digestion of the immunoglobulin with papain, (a cysteine protease enzyme), breaks the molecule into two equal fragments containing the whole light chain and the VH and CH1 domains of the heavy chain (Fig 1.6). These fragments contain the antigen binding sites of antibody and are called the Fab fragments (fragment antigen-binding). The rest of the two heavy chains after digestion with papain containing two

## 1. INTRODUCTION

or three constant domains are called the Fc region, (fragment crystallizable region), (Fig 1.6). The Fc region can modulate the immune cell activity through binding to the Fc receptors of the immune cells or immune molecules and complement proteins. Digestion of the immunoglobulin with pepsin cleaves the heavy chain after the disulfide bonds and forms a fragment containing both antigen binding sites (Fig 1.7). This fragment is called F(ab')<sub>2</sub>. The F(ab')<sub>2</sub> can bind to antigen but it does not mediate the effector functions of antibodies.

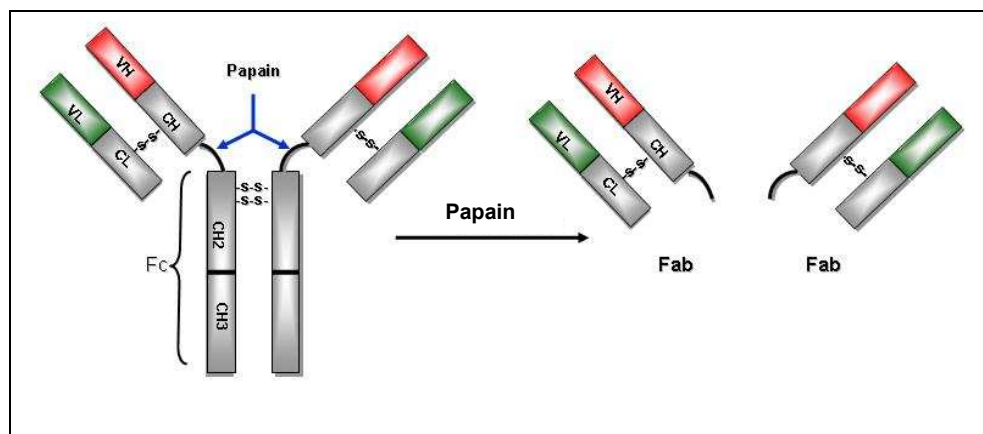


Fig 1.6 Schematic drawing of digestion an immunoglobulin molecule with papain.

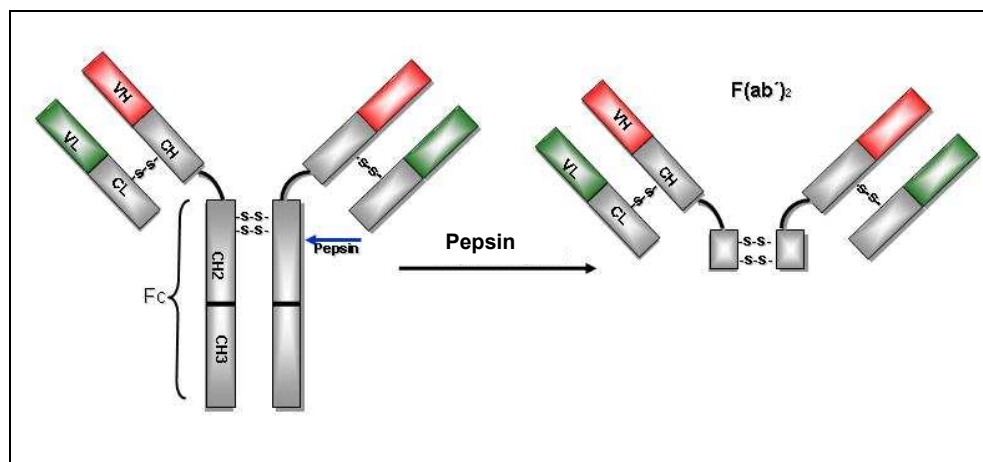


Fig 1.7 Schematic drawing of digestion an immunoglobulin molecule with pepsin.

### 1.2.2 Therapeutic antibodies

#### 1.2.2.1 Murine monoclonal antibodies

The technique to generate monoclonal antibodies was developed by Köhler and Milstein (Kohler and Milstein, 1975) by developing murine hybridoma cells lines, and producing specific monoclonal antibodies targeting specific antigens. In 1986 the

CD3 antibody muromonab ,(Orthoclone OKT3), was approved as a first monoclonal antibody in US (Reichert et al., 2005). Muromonab is an antibody of mouse origin which was used as an immunosuppressant to prevent transplant rejection (Goldstein et al., 1986). Muromonab could also decrease CD3<sup>+</sup> tumor cells in T cell acute lymphoblastic leukemia (Gramatzki et al., 1995). But the dissimilarity of human and murine immune systems led to rapid sensitization with the induction of human anti mouse antibodies (HAMA), which decrease efficacy of the mouse antibody in human immune system. Furthermore, mouse antibodies are not efficient in recruiting the human immune system and human effector functions (Shin, 1991). Therefore, the mouse antibodies were replaced with recombinant antibodies by the process of genetic engineering through chimerization (Morrison et al., 1984) and humanization (Riechmann et al., 1988), to overcome the problem of HAMA and other disadvantages of mouse antibodies. The recombinant antibodies can also be radio-labeled, or conjugated to toxins, for targeting the special tumor cells (Carter, 2006). Several recombinant antibodies are approved in Europe as therapeutics directed against cancer (Deonarain, 2008), and many other antibodies are in various stages of clinical studies (Carter, 2006).

### **1.2.2.2 Chimeric antibodies**

A chimeric antibody consists of the variable region of a mouse antibody combined with constant regions of human immunoglobulin (Fig 1.8). A chimeric antibody consists of approximately 65% human antibody and is named with the suffix “ximab”. Abciximab was the first FDA-approved therapeutic chimeric antibody, which has been directed against the GPIIb/IIIa receptor on platelets and prevents clotting (Simoons et al., 1994). Abciximab was a Fab fragment. In 1997 rituximab was approved as a chimeric antibody, whole monoclonal IgG molecule, for the treatment of non-Hodgkin’s lymphoma ,directed against the CD20 antigen (Anderson et al., 1997). Rituximab is one of the most clinically used antibodies (Peipp and Valerius, 2002).

### **1.2.2.3 Humanized antibodies**

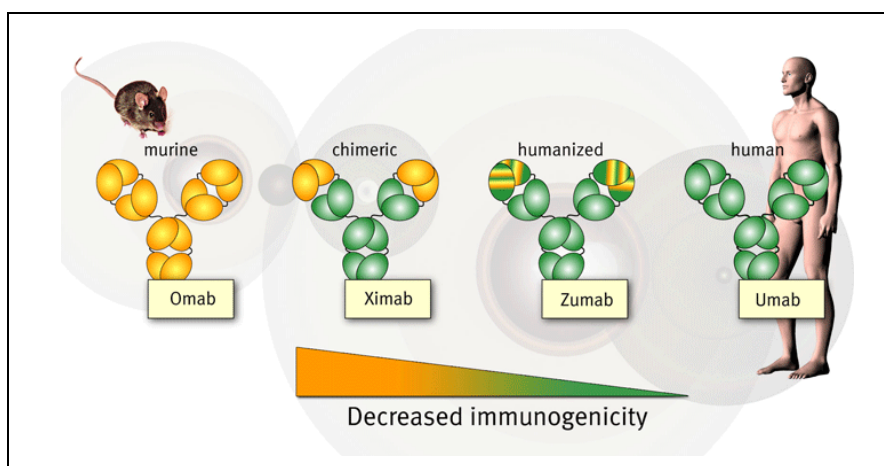
Humanized antibodies have more similarity to human antibodies compared to chimeric antibodies. A humanized antibody is generated by introducing the six hypervariable genes (CDRs) of a mouse antibody into a human framework sequence



and merging with human constant regions (Fig 1.8). This antibody has 90% coding sequences from a human antibody and is named with the suffix “zumab”. Daclizumab (Zenapax®) is the first humanized antibody which was approved in 1997. This antibody is directed against the CD25 antigen for the prevention of acute organ rejection (Ekberg et al., 1999). In 1998 trastuzumab (Herceptin®), a humanized antibody specific for HER2/*neu*, was approved as a first humanized anticancer antibody (Roche and Ingle, 1999).

### 1.2.2.4 Human monoclonal antibodies

Human monoclonal antibodies are developed from display technologies such as yeast, ribosome or phage display (Hoogenboom, 2005) or developed from transgenic mice which express human immunoglobulin genes after vaccination with desired antigen (Lonberg, 2008). Human antibodies are named with the suffix “umab”. Adalimumab (Humira®) was the first fully human monoclonal antibody derived from phage display and was approved in 2002 for the treatment of rheumatoid arthritis targeting TNF- $\alpha$  (Scheinfeld, 2003). Panitumumab (Vectibix®) was the first fully human monoclonal antibody derived from transgenic mice, and was approved in 2006 for the treatment of colon cancer, targeting the epidermal growth factor receptor (EGFR) (Chu, 2006).



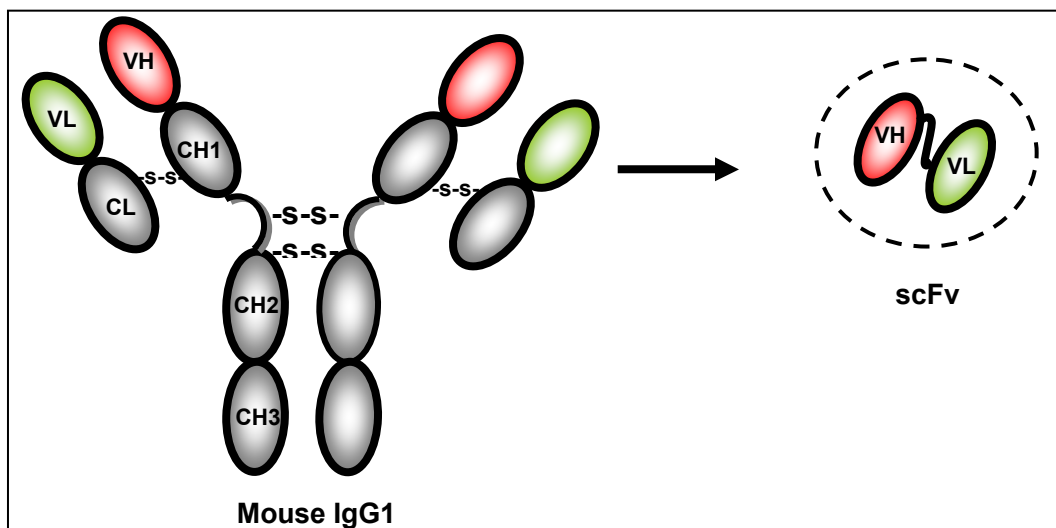
**Fig 1.8 Schematic view of mouse, chimeric, humanized, and human antibodies.** Modified from (Joost Bakker, Genmab). Mouse parts are shown in yellow and human parts in green.

### 1.2.2.5 Single-chain variable fragment (scFv)

Recombinant Fab fragments and single chain variable fragments, are the most commonly used formats of recombinant antibody constructs (Peipp et al., 2004).

## 1. INTRODUCTION

ScFv is a fusion of the variable domains of the light and the heavy chains of immunoglobulins, bonded together with a short peptide linker (Fig 1.9). ScFv has a molecular mass of ~ 25 kDa. The linker includes 10 to 25 amino acids which are mainly glycine for flexibility and serine for solubility of the molecule (Le Gall et al., 2004). ScFv molecules can be generated using antibody engineering and phage display technology (Krebber et al., 1997; Hoogenboom and Chames, 2000; Hoogenboom, 2005). Single chain variable fragment antibodies are generally applied as research and therapeutic reagents (Blažek and Celer, 2003) and are very valuable for the elimination of tumor cells, targeted delivery of drugs, toxins or radionuclides. ScFvs have quicker blood clearance compared to fab, f(ab)<sub>2</sub> and complete IgG molecules (Hudson, 1999). Due to their small size, scFvs show better tissue penetration in comparison to complete immunoglobulin molecules (Milenic et al., 1991; Yokota et al., 1992; Adams et al., 1993). Regardless of the absent constant regions and possessing linker, the scFv molecule retains the binding specificity of the parental immunoglobulin for the target antigens, since scFv includes the complementarity determining regions (CDRs). ScFvs can be expressed in *E. coli* cells.



**Fig 1.9 Schematic drawing of scFv formation.** ScFv is a fusion of ■ VL and ■ VH from IgG1 molecule, connected together with a linker (black line).

### 1.3 The phage display recombinant antibody system

Phage display technology was established in 1985 by George Smith (Smith, 1985). This is a powerful tool to generate a large number of functional peptides and proteins such as single chain variable fragments (Azzazy and Highsmith, 2002). A foreign

DNA fragment can be inserted into a suitable phage coat protein gene using genetic engineering tools. This recombinant gene can be functionally displayed as a fusion protein on the surface of a bacteriophage and the high affinity and specificity antibody fragments can be selected.

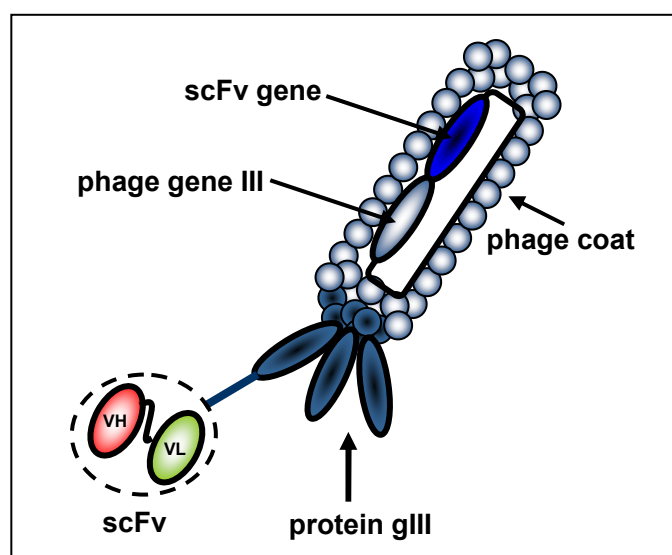
### **1.3.1 Filamentous bacteriophage**

The filamentous phages are mainly used in display technology. They are identified as Ff phages, due to infection of *E. coli* cells through the bacterial F conjugative pilus. There are different strains of filamentous bacteriophages including M13, f1, Fd, and ft (Yacoby and Benhar, 2008). The genomes of these phages have 98% homology and therefore their gene products/proteins are almost the same (Beck and Zink, 1981; Hill and Petersen, 1982; Van de Winkel and Capel, 1993). The most generally used filamentous bacteriophage for displaying scFvs fragments is M13 phage (Manoutcharian et al., 2001). Filamentous bacteriophages M13 utilized in phage display technology are circular and single-stranded DNA viruses (ssDNA) (Manoutcharian et al., 2001). These viruses infect a variety of gram-negative bacteria via their F pili. The DNA of bacteriophage includes 6407 nucleotides, which are wrapped in a long capsid protein cylinder with a diameter of 7 nm and 930 nm in length (Crissman and Smith, 1984). The capsid includes five coat proteins named pVIII, pIII, pVI, pVII, and pIX. The major coat protein, pVIII, with 50 aa, surrounded the phage DNA along its major axis with its 2700 copies. 3 to 5 copies of the proteins pIII and pVI are located in one end of phage, and 3 to 5 copies of the proteins pIX and pVII are located in another end of phage (Webster, 2001). pIII is a 406 aa protein, and mostly used for peptide expression. pIII has two functional domains: 1) N-terminal domain, which is exposed to the surroundings and binds to the F pili of *E. coli* cells, but it is not necessary for phage particle assembly. 2) C-terminal domain, which is located inside of the phage particle, close to the DNA, and is an essential part of the capsid structure. The recombinant fragments are usually expressed as pIII or pVIII fusion proteins and are displayed at the tip of the bacteriophage (Hoogenboom et al., 1991; Willats, 2002).

### **1.3.2 Construction of a phage display library**

Antibody fragments such as scFvs, diabodies, and Fab fragments can be displayed on the surface of a phage (Rader and Barbas, 1997). Gene sequences of

recombinant protein can be inserted into the gene of the coat proteins, pIII or pVIII. Phage DNA is replicated in *E. coli* as a double stranded plasmid, and super infection with the helper phages causes the single-stranded phagemid DNA, which is packaged in phage particles. The bacteriophage replicates its DNA together with foreign DNA (Smith and Petrenko, 1997). The helper phages provide all the proteins which are required for phage assembly, including copies of the wild type coat proteins (Bass et al., 1990) (Fig 1.10). Therefore the yielding phage contains both the wild type coat protein from the helper phages and also the fusion coat proteins from the phagemid which can be displayed as N-terminal fusions to pIII, pVIII (Bass et al., 1990) or pIX (Gao et al., 1999). C-terminal display can be realized with pVI, pIII, and pVIII (Fuh and Sidhu, 2000). A possible difficulty of phage system is the reduction of average number of displayed fusion molecules because of competition with wild type coat proteins (Winter et al., 1994).



**Fig 1.10 Schematic view of phage display.** ScFv gene can be fused to a gene of bacteriophage. ScFv protein can be expressed as pIII, pVIII or pIX fusion protein and displayed at the tip of bacteriophage.

### 1.3.3 Screening

During the cloning of the scFv genes, heavy and light chains are combined randomly and through phage display, each bacteriophage can display a unique antibody with a specific antigen binding site on its surface (Rader and Barbas, 1997; Pini and Bracci, 2000). The genetic information encoding the displayed molecule is contained within the phage coat, thus providing a direct physical link between genotype and phenotype (Rader and Barbas, 1997). This linkage allows the selection and

amplification of a specific clone from a pool with millions phages. During the screening of a phage library, the displayed proteins bind to the specific antigen. The bound phages can be identified with a M13-HRP-conjugated antibody that recognizes the phage coat proteins.

### **1.4 Antibody effector functions**

The effector mechanisms of antibodies can be divided into direct mechanisms and indirect mechanisms (Houghton and Scheinberg, 2000). Direct mechanisms are mediated by the variable regions and indirect mechanisms are triggered by constant domains of antibodies (Peipp et al., 2008a). Antibodies can distinguish exogenous particles such as pathogens and allergens, or endogenous antigens such as tumor cells antigens through two Fab fragments. The Fc domain of antibody is able to induce effector functions, such as the activation of complement system (Casadevall et al., 2004). For most antibodies, a combination of various effector mechanisms is supposed to work together in vivo (Glennie and Van De Winkel, 2003).

#### **1.4.1 Direct mechanisms**

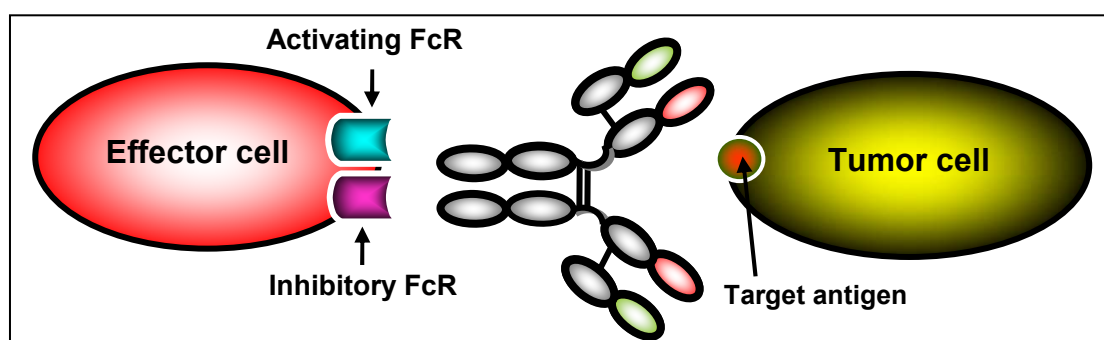
Antibody direct mechanisms include cross-linking of antigen, activation of death receptors, induction of apoptosis or programmed cell death, proliferation inhibition, avoidance of receptor-dimerization, and neutralization of soluble factors (Ludwig et al., 2003; Dübel, 2007). Some therapeutic antibodies can block tumor cells growth by targeting growth factor receptors, such as the epidermal growth factor receptor family, to block the ligand binding, or dimerization of the growth factor receptors which is required for transduction of activating signaling pathways (Sunada et al., 1986; Li et al., 2005). Some antibodies can target the vascular endothelial growth factor to block tumor associated angiogenesis, as well as adhesion molecules to block interaction with stroma cells, and slow down the tumor growth and metastasis (Lafrenie et al., 2007). Some monoclonal antibodies are able to affect cell cycle and induce apoptosis. Apoptosis is a regulated process of programmed cell death in response to specific signals or DNA damage (Lowe and Lin, 2000; Ghobrial et al., 2005). This modulation of regulatory and survival signaling pathways are not enough for most therapeutic antibodies to eradicate tumor cells. Therefore the recruitment of secondary immune effector mechanisms is very important for effective antibody therapy (Nimmerjahn and Ravetch, 2007).

### 1.4.2 Indirect mechanisms

Complement-dependent tumor cell lysis (CDC) and effector cell-mediated tumor killing (ADCC) are two important indirect mechanisms, performed through interactions between the constant regions of the antibodies and effector molecules.

#### 1.4.2.1 Antibody-Dependent Cell Mediated Cytotoxicity (ADCC)

ADCC is an important effector mechanism for most therapeutic anticancer antibodies (Houghton and Scheinberg, 2000). Through ADCC, the antibodies bind to the target cell antigens and recruit the innate immune effector cells, such as natural killer cells (Fig 1.11). The immune effector cells have specific receptors, (Fc receptors), for the constant region of the antibody and can mediate phagocytosis, or release the inflammatory mediators or cytotoxic granules, to eliminate the targeted tumor cells (Ravetch and Kinet, 1991). Natural killer cells are the most common effector cells which mediate ADCC. Subsequent to binding of NK cells to Fc region of antibody, they can destroy the target cells with release the granules which contain perforin and granzyme B, and/or activation of the FAS/FAS ligand apoptosis system in the target cell. Perforin molecules create holes or pores in the membrane of tumor cells, which disturb the osmotic barrier and eliminate the tumor cells by osmotic lysis. Mouse IgG2a, human IgG1, and IgG3 subclasses mediate ADCC effectively (Clynes et al., 2000).



**Fig 1.11 Antibody-Dependent Cell Mediated Cytotoxicity (ADCC).** Through ADCC, the antibodies bind to the target cell antigens and recruit the innate immune effector cells such as natural killer cells (NK). The clinical efficacy of therapeutic antibodies *in vivo* can be enhanced by increasing affinity to activating Fc receptors and by reducing affinity to the inhibitory Fc receptor.

### 1.4.2.2 Complement-dependent cell-mediated cytotoxicity (CDC)

Complement-dependent cell-mediated cytotoxicity is an important effector mechanism for elimination of foreign agents and tumor cells. Through CDC, the antibodies eliminate the targeted cells by triggering the complement cascade on the cell surface. The complement system consists of classical, lectin, and alternative pathways. For the classical pathway, the immunoglobulins are necessary, but the lectin and alternative pathways are activated mainly by microbial elements. For activation of the classical pathway, the first complement component (C1q) binds to the Fc portion of the immunoglobulin. Following this binding, a proteolytic complement cascade is triggered, and a large amount of the main effector molecule of the complement system, (C3b), is generated. C3b molecules act as phagocytosis enhancer, and can bind to the C3 convertase to create a C5 convertase (Cartron et al., 2004). Activation of the complement system finally leads to formation of the membrane attack complex, (MAC), (C5b to C9). This has a function similar to perforin, and forms transmembrane holes or pores that disrupt the osmotic barrier of the membrane and lead the tumor cells to osmotic lysis (Cartron et al., 2004). C3b can also bind to complement receptors (CRs) on effector cells such as granulocytes, macrophages, and NK cells. This induces cell-mediated lysis or phagocytosis, depending on the effector cell complement dependent cellular cytotoxicity (CDCC). Human IgG3 and IgG1 mediate the most effective CDC. IgG2 mediates low CDC, and IgG4 mediates no CDC.

### 1.4.3 Fc receptors (FcRs)

The efficient interaction of the antibody Fc region with cellular Fc receptors, on innate immune effector cells, is a very important factor for antibody activity *in vivo* (Nimmerjahn and Ravetch, 2007). There are specific FcRs for the immunoglobulin classes: FcR for IgA is Fc $\alpha$ R, for IgD is Fc $\delta$ R, for IgE is Fc $\epsilon$ R, for IgG is Fc $\gamma$ R, and for IgM is Fc $\mu$ R. Human Fc $\gamma$  receptors, (F $\gamma$ Rs), for the Fc region of IgG, are main mediators of the adaptive immune response (Sondermann and Oosthuizen, 2002). Human IgG1 with its high ADCC effect is the most suitable therapeutic agent against cancer cells. There are four different classes of Fc $\gamma$ Rs for IgG molecules: Fc $\gamma$ RI (CD64), Fc $\gamma$ RII (CD32), and Fc $\gamma$ RIII (CD16). These FcRs are different in affinity for IgG subtype, cell expression, and receptor structure (Van de Winkel and Capel, 1993; Hulett and Hogarth, 1994). Fc $\gamma$ RI has the highest affinity but Fc $\gamma$ RII and

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Fc $\gamma$ RIII have a low affinity. Fc $\gamma$ Rs are expressed on different immune effector cells such as monocytes, NK cells, and macrophages (Van de Winkel and Capel, 1993). Fc $\gamma$ RI (CD64) is expressed on monocytes and macrophages and can also be expressed on neutrophils (Repp et al., 1991). Fc $\gamma$ RI has four isoforms: Fc $\gamma$ RIa, Fc $\gamma$ RIb1, Fc $\gamma$ RIb2 and Fc $\gamma$ RIc. Fc $\gamma$ RII (CD32) is expressed on several immune cells. Fc $\gamma$ RII can be in Fc $\gamma$ RIIa1, Fc $\gamma$ RIIa2, Fc $\gamma$ RIIb1, Fc $\gamma$ RIIb3, and Fc $\gamma$ RIIc isoforms. Fc $\gamma$ RIII (CD16) is expressed in two isoforms, Fc $\gamma$ RIIIa which is expressed on NK and T cells and Fc $\gamma$ RIIIb which is expressed on neutrophils.

The basic structure of Fc $\gamma$ R consists of an extracellular part, (EC), with two or three immunoglobulin-like domains, a hydrophobic transmembrane, region (TM), and an intracytoplasmic end, (IC), (Qiu et al., 1990). The extracellular domain of Fc $\gamma$ RI has three immunoglobulin-like domains and can be activated by the monomer form of IgG molecule. Fc $\gamma$ RII and Fc $\gamma$ RIII have one extracellular domain less than Fc $\gamma$ RI, and must bind multiple IgG molecules to activate an immune complex. The cytoplasmic domains of Fc $\gamma$ RI, Fc $\gamma$ RIIa/c, and Fc $\gamma$ RIIIa have an immunoreceptor tyrosine based activation motif (ITAM), and are therefore activating receptors. But the Fc $\gamma$ RIIb has the immunoreceptor tyrosine based inhibitory motif (ITIM), and is an inhibitory receptor (Warmerdam et al., 1990). Activation receptors are required, and inhibitory receptors should be blocked, for the efficacy of therapeutic antibodies (Clynes et al., 2000).

**Table 1.2 Fc $\gamma$  receptors in human.** Modified from (Griggs and Zinkewich-Peotti, 2009). DC = dendritic cell, ITAM = immunoreceptor tyrosine-based activation motif, ITIM = immunoreceptor tyrosine-based inhibitory motif, m $\Phi$  = macrophage, N = neutrophil, B = B cell, NK = natural killer cell.

Receptor	CD	Activatory inhibitory	Relative affinity for IgG	Structural signalling components	Principal effector-cell expression profile <sup>a</sup>				
					m $\Phi$	DC	N	B	NK
<i>Human</i> Fc $\gamma$ RI	CD64	Activatory	High <sup>b</sup>	ITAM (associated $\gamma$ - chain)	●	●	●		
Fc $\gamma$ RIIa	CD32a	Activatory	Low/Mod	ITAM (integral)	●	●	●		
Fc $\gamma$ RIIb	CD32b	Inhibitory	Low/Mod	ITIM (integral)	●	●	●	●	
Fc $\gamma$ RIIc	CD32c	Activatory	Low/Mod	ITAM (integral)	●		●		●
Fc $\gamma$ RIIIa	CD16a	Activatory	Low/Mod	ITAM (associated $\gamma$ - chain)	●	●			●
Fc $\gamma$ RIIIb	CD16b	Activatory	Low/Mod	Non-signalling (gpi-linked)			●		



### **1.5 Optimizing antibody ADCC activity**

ADCC is an important mechanism of therapeutic antibodies in cancer therapy. Therefore to enhance the clinical efficacy and the antitumor potency of antibodies, their ability to mediate cellular cytotoxic effector functions such as ADCC can be improved (Weiner and Carter, 2005).

#### **1.5.1 Optimizing antibody binding affinity to Fc receptors on immune effector cells**

Xenograft studies with some therapeutic antibodies such as rituximab and trastuzumab in FcγR knockout mice, demonstrated that the improvement of the clinical efficacy of these antibodies *in vivo* can be reached by increasing antibody binding affinity to activating Fc receptors, and by reducing binding affinity to the inhibitory Fc receptor (Clynes et al., 2000; Nimmerjahn and Ravetch, 2005) (Fig 1.11). There are two main approaches to optimizing Fc receptor interaction: modulation the mutations in the cDNA of corresponding antibody (protein engineering) to altering the structure of the Fc region (Duncan et al., 1988; Sarmay et al., 1992; Lazar et al., 2006), and modifying the glycosylation profile of antibody (glyco-engineering) (Uma a et al., 1999; Shields et al., 2001; Okazaki et al., 2004; Peipp et al., 2008c).

##### **1.5.1.1 Mutations**

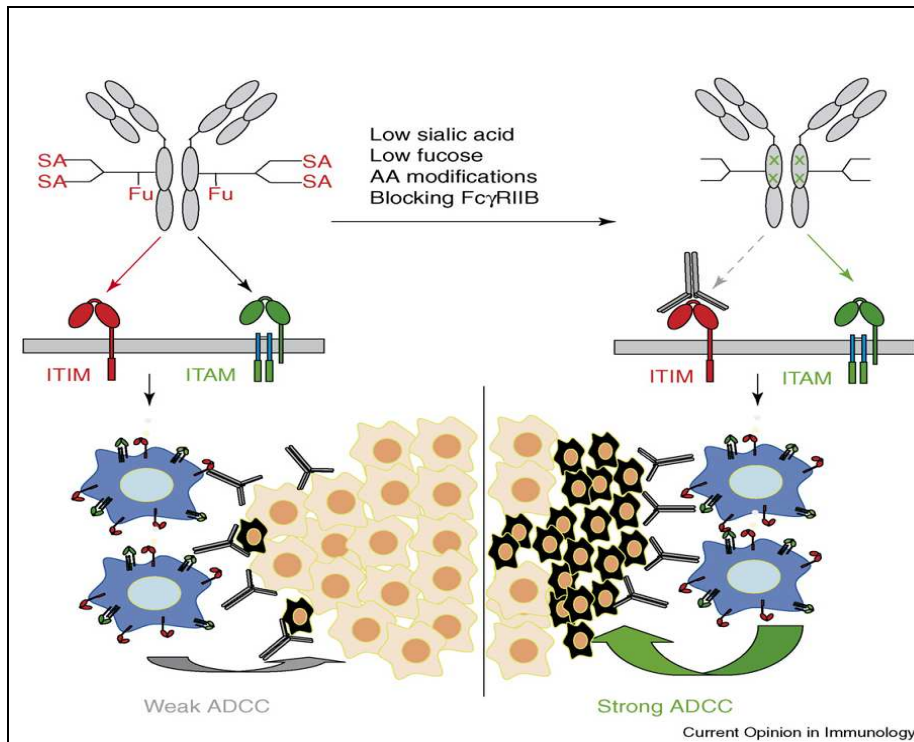
Human IgG1 is the most commonly used antibody isotype for tumor therapy (Ravetch and Bolland, 2001). The clinical efficacy of a human IgG1 antibody is dependent on the binding affinity of Fc portion of antibody to FcγR on effector cells (Cartron et al., 2002). Therefore an IgG1 antibody with optimized FcγR binding represents efficient cytotoxic effects against targeted tumor cells (Cartron et al., 2004). Mutation and change of the amino acid in Fc regions of antibodies to optimizing Fc receptor binding can be performed either by alanin scanning mutagenesis (Shields et al., 2001), or by computer-based calculation methods (computer algorithms) (Lazar et al., 2006).

### **1.5.1.2 Deglycosylation**

Carbohydrate optimization can improve the cytotoxic effector functions of therapeutic antibodies. IgG molecules are heterogeneous, with the fucose attached to the Asn-297 glycosylation site. Deletion of this fucose in the CH2 domain of antibody can increase cytotoxic activity of mouse and all human IgG antibody isotypes (Shinkawa et al., 2003; Niwa et al., 2005). It has also been reported that ADCC efficacy with isolated mononuclear effector cells, is enhanced for low-fucosylated antibodies in comparison with the high-fucosylated variant of the same antibody (Shields et al., 2002; Okazaki et al., 2004; Schuster et al., 2005). Some other studies demonstrated that removal of the fucose at this glycosylation position in some antibodies, enhanced their binding affinity to FcγRIIIa, but binding to some other Fcγ receptors is independent from this modification (Uma a et al., 1999; Shields et al., 2002; Okazaki et al., 2004). Furthermore, the efficacy of glycol-engineered antibodies could significantly be change by effector cell type (Peipp et al., 2008c).

### **1.5.1.3 Deletion of terminal sialic acid residues**

It has been reported that the lack of terminal sialic acid residues, in the antibody sugar side chain, can improve cytotoxic activity efficacy in humans and mice (Rademacher et al., 1994; Kaneko et al., 2006; Scallon et al., 2007). Also an elevated level of sialic acid can decrease antibody binding to both FcγRIIIa and cell surface antigen, (Scallon et al., 2007) and reduce ADCC activity.



**Fig 1.12 Optimizing antibody and Fc receptors interaction.** Modified from (Nimmerjahn and Ravetch, 2007). The efficient anti cancer ADCC activity of antibody can be achieved by optimizing the interaction of the antibody Fc-portion with activating FcRs and by blocking the inhibitory FcR. ITAM: the immunoreceptor tyrosine based activation motif, ITIM: the immunoreceptor tyrosine based inhibitory motif.

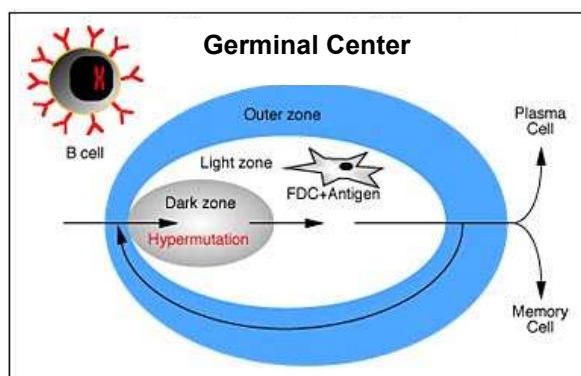
### 1.5.2 Antigen density on target cells

The antigen density on tumor cells is another factor that affects ADCC. It has been reported that target cells with a higher antigen expression level are more competent to be eradicated by an antibody through ADCC (Tang et al., 2007). The low antigen density can even reduce ADCC activity of high affinity IgG antibodies (Tang et al., 2007). This is because more antibody molecules can bind the target cells which express a high level of corresponding antigen, and a stronger ADCC can be performed in comparison to the target cells which express fewer antigens.

### 1.5.3 Antibody binding affinity to target antigen

Human IgG affinity can affect the ability of antibodies to mediate ADCC (Tang et al., 2007). It has been reported that IgG antibodies with higher binding affinities to target antigens, promote more ADCC by human NK cells in comparison to IgG antibodies with a lower affinity to target antigen (Tang et al., 2007). In the human immune

system, the efficient immune response against foreign antigens generally depends on the generation of high affinity antibodies. The antibodies, which are generated in the primary immune response, usually have moderate binding affinities, but these affinities are matured and enhanced in later responses with a process of hypermutation (Kocks and Rajewsky, 1988). The hypermutation occurs in the antibody V-genes of B cells in germinal centers (Berek et al., 1991; Jacob et al., 1991) (Fig 1.13). Antibodies may obtain several mutations, but the most enhancement in binding affinity is achieved by the mutation of a few residues within the antigen binding site (Berek and Milstein, 1987). This immune system characteristic introduced a potent means for the generation of antibodies with high binding affinities *in vitro* (Winter and Milstein, 1991). Recombinant antibodies can be generated on the surface of bacteriophages by the mimicking of primary immune response (Low et al., 1996). The secondary immune response, and antibody affinity maturation can also be mimicked *in vitro* by mutations of antibodies variable genes (Fujii, 2004). The phage antibody mutants can be screened by binding to its antigen, during several rounds of screening, with an increasingly stringent selection condition, to select mutants with enhanced binding affinities.



**Fig 1.13 Hypermutation of antibody genes in germinal centers.** Modified from (Low et al., 1996). During maturation of B cells, before differentiation into plasma cells, the antibodies V-genes undergo somatic hypermutation to generate antibodies with high affinity for their target antigens.

A previous study from Tang and his colleagues demonstrated that only very low concentration of a high affinity scFv-IgG antibody (H3B1 IgG) directed against HER2/*neu* antigen, is required to mediate ADCC in comparison to the antibody variant with lower affinity (G98A IgG) (Tang et al., 2007). It has also been reported that multivalent binding to the tumor antigen mediates ADCC more efficiently in comparison to monovalent binding (Shahied et al., 2004). Affinity Maturation by PCR-

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Mutagenesis is an efficient approach to mimic the process of somatic maturation in the immune system to select the antibodies with a higher affinity to target antigens. PCR-Mutagenesis can be site-directed to introduce diversity at specific locations, usually CDRs, or can be randomly mutated in VL and VH regions. The mutated library has to be very large to express approximately the total immune repertoire and the screening of high affinity target antigen binders (Marks et al., 1991; Vaughan et al., 1996). The mutation frequency, low, medium, or high, can usually be controlled by varying the DNA amount (Table 1.3). Low amounts of DNA undergo more duplications than high amounts of DNA.

**Table 1.3 Mutation frequencies vs. initial target quantities.** A low, medium or high mutation frequency can be produced by adjusting the initial target DNA amounts in the PCR reaction.

<b>Mutation rate</b>	<b>Mutation frequency (mutations/kb)</b>	<b>Initial target amount (ng)</b>	<b>Recommended fold amplification</b>
Low	0-4.5	500-1000	1.5-10
Medium	4.5-9	100-500	10-100
High	9-16	0.1-100	100-10,000

**Chapter 2**

**MATERIALS & METHODS**

## 2. MATERIALS & METHODS

### 2.1 Materials

#### 2.1.1 Mammalian cell lines

Cell line	Cell type	Culture	Medium	source
CEM	human T cell leukemia	suspension	90% Dulbecco's MEM + 10% FBS + 1% Pen/Strep	DSMZ
HSB-2	human T cell leukemia	suspension	90% RPMI 1640 + 10% FBS + 1% Pen/Strep	DSMZ
KG1a	human acute myeloid leukemia	suspension	90% RPMI 1640 + 10% FBS + 1% Pen/Strep (R10 <sup>+</sup> )	DSMZ
TH-111	CD96-hybridoma cells	adherent	90% RPMI 1640 + 10% FBS + 1% Pen/Strep (R10 <sup>+</sup> )	(Gramatzki et al., 1998)
293T	human embryonal kidney	adherent	90% Dulbecco's MEM + 10% FBS + 1% Pen/Strep	DSMZ

#### 2.1.2 Cell culture media and additives

Mammalian cell culture	Source
CryoSure-DMSO	WAK-Chemie Medical
DMEM, Dulbecco's Modified Eagle medium	Invitrogen
Fetal bovine serum (FBS)	Invitrogen
GM-CSF	CellGenix
Hank's medium	PAA
Hygromycin B in PBS, 50mg/ml	Invitrogen
Lipofectamine LTX	Invitrogen
Opti-MEM + glutamine, reduced serum	Invitrogen
Penicillin/streptomycin, 10,000 U/ml Penicillin, 10,000 µg/ml Streptomycin	Invitrogen
Percoll	Biochrom
Phytohemagglutinin-M (PHAM)	Roche
RPMI1640 + GlutaMAX-I medium	Invitrogen
Trypsin/EDTA (1x)	Invitrogen

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### 2.1.3 *Escherichia coli* strains and helper phage

<i>E.coli</i> strains	Genotypes	Source
BL21 (DE3) Electrocompetent	B F <sup>-</sup> <i>ompT hsdS</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>dcm</i> <sup>+</sup> Tet <sup>r</sup> <i>gal</i> λ(DE3) <i>endA Hte</i>	Stratagene
HB2151 Electrocompetent	[Δ( <i>lac-pro</i> ) <i>ara nalr thi/F' proAB LacIq lacZΔM15</i> ]	Dr. G. Winter, MRC Cambridge, UK
Mach1 Chemocompetent	<i>lacZΔM15 hsdR lacX74 recA endA tonA</i>	Invitrogen
TG1 Electrocompetent	<i>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) [F' <i>traD36 proAB lacI<sup>q</sup>ΔM15</i> ]	Stratagene
XL1-Blue Electrocompetent	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI<sup>q</sup>ΔM15 Tn10</i> (Tet <sup>r</sup> )]	Stratagene

Helper phage	Source
M13KO7	New England Biolabs

### 2.1.4 Cultures for bacterial cells

Bacterial culture	Source
Glucose	Carl Roth
IPTG	Carl Roth
LB medium	Carl Roth
PEG 6000	Carl Roth
SOB medium	Carl Roth
SOC medium	SOB + 20% Glucose
2xTY medium	Carl Roth
2xTY-Agar	Carl Roth

### 2.1.5 Antibiotics for bacterial culture

Antibiotics	Stock solution	Working solution
Ampicillin	100 mg/ml	100 µg/ml
Chloramphenicol	30 mg/ml	30 µg/ml
Kanamycin	50 mg/ml	25 µg/ml



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Stock solutions of antibiotics were stored at -20°C. All bacterial culture media were autoclaved. The appropriate antibiotic was added to medium after lowering the temperature to RT and before use.

### 2.1.6 Primers

<b>LB/HB and LF/HF:</b> (Krebber et al., 1997)
<b>Scfor and Scback:</b> (Krebber et al., 1997)
<b>ECD-For:</b> 5'-GATCGCTAGCCACCATGGAGAAAAAATGGAAATACTGTGCTGTC-3' <b>ECD-Rev:</b> 5'-CACAGCGGCCGCCATCTTTGGGCTTATTGACCACAATACC-3'
<b>Mut-Scfor:</b> 5'-GATCGGCCCGAGCCGGCCATGGCGGACTACAAAGAC-3' <b>Mut-Scback:</b> 5'-GATCGGCCCCCGAGGCCGCAGAGACAGTGACCAG-3'

### 2.1.7 Vectors

pAK100	Kindly provided by Dr. Plückthun (Krebber et al., 1997)
pAK400	Kindly provided by Dr. Plückthun (Krebber et al., 1997)
pSec-IgG1-Fc	(Repp et al., 2010)
pSec-IgG1-Fc-eng	(Lazar et al., 2006; Repp et al., 2010) , 3 mutations in CH2 (S239D/I332E/A330L)

### 2.1.8 Enzymes

Enzymes	Source
PeqGOLD PWO DNA Polymerase	Peqlab
PeqGOLD Taq DNA Polymerase	Peqlab
Restriction enzymes	New England Biolabs
SuperScript™ II Reverse Transcriptase	Invitrogen
T4 DNA ligase	Roche

### 2.1.9 DNA and protein markers

Markers	Source
100 bp-DNA Ladder	Carl Roth

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Precision Plus Protein, Dual Color Standard	Bio-Rad
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### 2.1.10 Antibodies and conjugates

Antibodies and conjugates	Source
Anti CD96-PE	eBioscience
Anti-human IgG1-FITC	Beckman-Coulter
Anti-human IgG HRP-conjugated antibody	Sigma-Aldrich
Anti-mouse IgG1-FITC	Sigma- Aldrich
Anti-mouse IgG HRP-conjugated antibody	Sigma-Aldrich
Anti-M13 HRP-conjugated antibody	Amersham pharmacia
Erbitux	Merck
Penta.His Alexa fluor conjugate	Qiagen
Penta-His Antibody	Qiagen

### 2.1.11 Buffers and reagents

Buffer	Source/Components
Coomassie staining solution	20% (w/v) Roti-Blue 5x (Carl Roth) in destaining solution (20% Methanol + 60% H <sub>2</sub> O)
DNA gel loading buffer	Carl Roth
DPBS	Invitrogen
PEG/NaCl	20% (w/v) PEG-6000, 2.5 M NaCl
Periplasmic extraction buffer	100 mM Tris/HCl, 500 mM Sucrose, 1 Mm EDTA, (pH 8.0)
PFA fixation buffer	1% Paraformaldehyd/PBS
SDS loading buffer (5x)	30% Glycerol, 3% SDS, 0.0001% Bromophenole blue; for reducing buffer: + 5% $\beta$ -Mercaptoethanol
SDS-PAGE running buffer	25 mM Tris, 192 mM Glycine , 0.1% w/v SDS, (pH 8.3)
TAE buffer	40 mM Tris-acetate, 1 mM EDTA, (pH 8.0)
TBS buffer	50 mM Tris-HCl, 150 mM NaCl, (pH 7.4)
Western blot transfer buffer	25 mM Tris, 192 mM Glycine, 20% v/v Methanol, (pH 8.3)
2x HBS buffer	50 mM HEPES, 280 mM NaCl, 1.5 mM Na <sub>2</sub> HPO <sub>4</sub> , (pH 7.05)

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### 2.1.12 Protein purification buffers

protein purification	Binding buffer components	Elution buffer components
His-tag, human-IgG1	50 mM NaH <sub>2</sub> PO <sub>4</sub> , 300 mM NaCl, 10 mM Imidazol, (pH 8.0)	50 mM NaH <sub>2</sub> PO <sub>4</sub> , 300 mM NaCl, 250 mM Imidazol, (pH 8.0)
Protein A, human-IgG1	100 mM Tris/HCl, (pH 8.0)	100 mM Glycine, (pH 3.0)
Protein A, mouse IgG1	3 M NaCl, 1.5 M Glycine, (pH 8.9)	100 mM Glycine, (pH 3.0)
Strep-tag	100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, (pH 8.0)	100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 2.5 mM Desthiobiotin, (pH 8.0)

### 2.1.13 Kits

Kit	Source
Annexin V-FITC/7-AAD kit	Beckman Coulter
Cell Proliferation kit I (MTT)	Roche
Experion Pro260 kit	Bio-Rad
GeneMorph II Random Mutagenesis kit	Stratagene
Human NK cell isolation kit	Miltenyi Biotec
NucleoBond® Xtra Maxi	MACHEREY-NAGEL
NucleoSpin® Plasmid QuickPure	MACHEREY-NAGEL
OneStep RT-PCR kit	Invitrogen
QIAquick Gel Extraction kit	Qiagen
RNeasy Midi kit	Qiagen

### 2.1.14 Special laboratory chemicals and equipments

Chemicals/equipments	Source
ABTS	Roche
Agarose for DNA-electrophoresis	Carl Roth
Blotting Grade Blocker Non-Fat-Dry Milk	Bio-Rad
Chloroquine	Sigma-Aldrich
Dialysis membrane	Carl Roth
D-Sorbitol	Sigma-Aldrich
Ethanol	BÜFA

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Extra Thick Blot Paper Protean XL Size	Bio-Rad
Glycine	Carl Roth
HisTrap™ HP Columns (1 ml)	GE Healthcare
HiTrap™ Protein A HP Columns (1 ml)	GE Healthcare
Imidazole	Carl Roth
Immun-blot PVDF membrane	Bio-Rad
Methanol	Merck
Mineral oil	Fulka
N-Butanol	Carl Roth
OptiPhase Supermix	PerkinElmer
Sodium chloride	Carl Roth
Strep-Tactin, Superflow Cartridge (1 ml)	Qiagen
Sucrose	Sigma-Aldrich
SuperSignal West Dura Extended Duration Substrate	Pierce
Triton X-100	Merck
Tween 20	Merck
β-Mercaptoethanol	Sigma-Aldrich
2-Propanol (Isopropanol)	Carl Roth

### 2.1.15 Plastic wares

<b>Plastic wares</b>	<b>Source</b>
ADCC plates: 96-well plate round bottom with lid	Sarstedt
Agar solid media dishes for <i>E. coli</i> culture	NUNC
Cell culture flasks and dishes	Sarstedt
Centrifugal Filtration: Vivaspin 6	Sartorius Stedim
CryoPure tubes	Sarstedt
Electroporation Cuvette: 0.1 and 0.2 cm gap	Bio-Rad
Falcon tubes 15 and 50 ml	Sarstedt
Rotilabo®-syringe filters	Carl Roth
Safe-lock tubes, 1 ml and 1.5 ml	Eppendorf
Stericup® Vacuum filter systems	Millipore

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Sterile Acrodisc Syringe Filters	Pall Coporation
Vacuum filter cups: Stericup & Steriotop	Millipore
96 well plates, flat bottom for ELISA	Costar
96 well sample plate for use with 1450 microbeta	Sartorius Stedim

### 2.1.16 Laboratory apparatuses

<b>Apparatuses</b>	<b>Source</b>
Automated Cell Viability Analyzer: Vi-CELL	Beckman Coulter
Balance: TE 15025	Sartorius
Bio vortexer	Lab 4 you
Camera	Sony
Centrifuge: Biofug Pico (Rotor 3328)	Heraeus
Centrifuge: Megafuge 1.0 R (Rotor 2704)	Heraeus
Centrifuge: Multifuge 4 KR (Rotor LAC-250)	Heraeus
Centrifuge: Sorvall RC-6 (Rotor SS-34)	Thermo Scientific
CO <sub>2</sub> incubator: HERAcell 240	Thermo Scientific
Electrophoresis cell: Mini-PROTEAN 3	Bio-Rad
Electrophoresis power supply: E143	Consort
ELISA reader	TECAN
Experion™ Automated Electrophoresis System	Bio-Rad
Flow cytometer: Cytomics FC 500	Beckman Coulter
Galaxy mini microcentrifuge	VWR
Gel documentation system: E.A.S.Y. 440k	Herolab
Heraeus Function Line Incubators	Thermo Scientific
Incubation Shakers: CERTOMAT® BS-T	Sartorius Stedim
Inverted microscope: Axiovert 40 C	Zeiss
Magnetic stirrer: RCT basic	IKA
Microplate scintillation and luminescence counter: MicroBeta® TriLux	PerkinElmer
Microlitre centrifuge	Heraeus
Microplate reader: TECAN infinite M200	TECAN
MicroPulser Electroporation Apparatus	Bio-Rad

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PH meter: PH 2111 Microprocessor	HANNA
Power supply: PowerPac HC	Bio-Rad
Protein purifier: ÄKTApurifier	GE Healthcare
Scanner: ScanMaker 9800XL	Microtek
Sonicator: Sonopuls	Bandelin
Sterile bench: HERAsafe	Heraeus
Thermocycler: PTC200	MJ Research
Thermomixer comfort	Eppendorf
Trans-Blot SD: Semi-Dry Transfer Cell	Bio-Rad
Tube Roller Mixer: SRT1	Stuart
Upright microscopes: AxioStar plus	Zeiss
Vacuum pump	KNF Neuberger
Vacuum pump: ECOM-P 4153	Eppendorf
Wallac MicroBeta TriLux detector	PerkinElmer
Waterbath	Memmert
Western blot digital imaging system: ChemiDocXRS	Bio-Rad

### 2.1.17 Computer software

Software	Source
Fc 500 CXP	Beckman Coulter
GraphPad Prism 4.0	GraphPad Inc.
Quantity One	Bio-Rad
Vector NTI	Invitrogen

### 2.2 Methods

#### 2.2.1 Cell biological methods

##### 2.2.1.1 Culture of adherent cells

TH-111 hybridoma cells grew adherent. These cells were cultured in RPMI1640 medium containing 10% FBS (heat-inactivated at 56 °C for 30 min) and 1% Pen/Strep at 37 °C with 5% CO<sub>2</sub> in a humidified incubator. The cell density was about 2-3 x 10<sup>6</sup> cells/80 cm<sup>2</sup>. These cells secreted the CD96 monoclonal antibody into the medium. Every two days, the cell flask was rinsed with PBS and incubated with trypsin-EDTA solution until the cells were detached (not more than 5 min). Following the cells detachment, 10 ml prewarmed RPMI1640 medium was added to neutralize the trypsin solution. The cell suspension was centrifuged at 300 x g in a Megafuge 1.0 R (Rotor 2704) for 5 min. A part of cell pellet was resuspended in a new tissue culture flask with prewarmed fresh medium and the rest of cells were frozen. The cell pellet was resuspended in a solution of 10% DMSO/FBS with a concentration of about 2-3 x 10<sup>6</sup> cells/ml. Aliquots of 1 ml were frozen in cryovials and stored first at -80 °C and placed later in liquid nitrogen.

##### 2.2.1.2 Culture of suspension cells

HSB-2 and KG1a cells are suspension cells. These cells were cultured in RPMI1640 medium containing 10% FBS and 1% Pen/Strep at 37 °C with 5% CO<sub>2</sub> in a humidified incubator. The cell density was about 0.3-0.4 x 10<sup>6</sup> cells/ml. The cells were centrifuged at 300 x g in a Megafuge 1.0 R (Rotor 2704) for 10 minutes to spin down the cells every two days and the medium was replaced with fresh prewarmed medium. A part of cell pellet was frozen as described above. The other culture conditions were mentioned above (Table 2.1.1).

##### 2.2.1.3 Isolation of peripheral blood mononuclear cells using density gradient

Citrate-anticoagulated blood, from a healthy volunteer, was layered over a discontinuous gradient consisting of 70% and 62% Percoll/Hank's medium respectively. After brake-off/centrifugation at RT for 20 min, MNCs were collected from the serum/Percoll interface. To remove the platelets, the cell pellet was resuspended in PBS and centrifuged at 300 x g for 10-15 min at 20 °C. The cell pellet was subjected to a shock lysis by mixing it with 45 ml of ice-cold water for 30 s

followed by immediate addition of 5 ml of 10 x PBS. The supernatant was removed and washing was repeated. MNCs from healthy volunteers typically contained about 60% CD3-positive T cells, 10-15% CD56-positive NK cells, and 3-10% CD14-expressing monocytes, as determined by immunofluorescence staining. Viability of the cells tested by Trypan blue exclusion and in an automated cell viability analyzer (Vi-CELL) was higher than 95%.

### **2.2.2 Molecular biological methods**

#### **2.2.2.1 Isolation of RNA**

TH-111 hybridoma cells that express CD96 monoclonal antibody were used as a source of RNA for cloning of CD96 variable region genes. The RNA was isolated with RNeasy Midi kit according to manufacturer's instructions which is summarized below. The appropriate number of cells was centrifuged at 300 x g in a Megafuge 1.0 R (Rotor 2704) for 5 min. All supernatant was carefully removed by aspiration. The cells were disrupted by the addition of 4 ml of RLT buffer and were mixed. 10  $\mu$ l  $\beta$ -ME per 1 ml RLT buffer was added before use. The lysate was then passed at least 5-10 times through a 10-20 gauge needle fitted to a RNase-free syringe. 2-4 ml of 70% ethanol was added to the homogenized lysate and mixed well by pipetting (no centrifugation). The sample was applied onto a RNeasy mini column placed in a 15 ml collection tube and was centrifuged at 3000-5000 x g at 20 °C for 5 min. The flow-through was discarded. 4 ml RW1 buffer was added to the RNeasy column and centrifuged at 3000-5000 x g for an additional 5 min. The flow-through and collection tube were discarded. 2.5 ml RPE buffer was pipetted onto the column and centrifuged at 3000-5000 x g for 2 min to wash the column. The flow-through was discarded again. 2.5 ml buffer RPE was added again to the column and centrifuged at 3000-5000 x g for 5 min to dry the RNeasy silica-gel membrane. The RNeasy column was transferred to a new 15 ml collection tube. 250  $\mu$ l RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and centrifuged at 3000-5000 x g for 1 min. A new RNeasy column was used and the elution step was repeated with a second volume of RNase-free water. The product was immediately used or stored at -20 °C to later use.



### 2.2.2.2 Reverse Transcription of RNA to cDNA

15 µg of total RNA was used as template for reverse transcription to cDNA with SuperScript™ II Reverse Transcriptase, according to manufacturer's instructions that are summarized below. 2 µl oligod T was added to RNA and heated at 70 °C for 10 min. This destabilized mRNA secondary structure. The sample was allowed to cool down to 4 °C. 2 µl RNase inhibitor and 10 µl 5 x RT buffer were added. 7.5 µl dNTP's (10 mM each dATP, dCTP, dGTP, dTTP) were added. 5 µl of 0.1 M DTT and up to 48 µl nuclease-free water were added. 2 µl (200 U) Reverse Transcriptase (SS II RT) was added. The mixture was incubated at RT for 10 min and then at 42 °C for 50 min. The reaction was terminated by incubation at 90 °C for 5 min. The temperature was lowered to 4 °C for 10 min. 1 µl (1 U/µl) of RNase H was added and the mixture was incubated at 37 °C for 20 min. RT product was immediately used or stored at -20 °C to later use. A negative control (without template RNA) was also prepared.

### 2.2.2.3 Polymerase chain reaction (PCR) of VH and VL

PCR was performed using peqGOLD Taq DNA polymerase according to manufacturer's instructions (summarized in Table 2.2.1). Appropriate components were applied in a sterile 0.5 ml microcentrifuge tube sitting on ice. VH and VL PCR were achieved in separate tubes. The content of tubes were mixed and incubated in a thermal cycler using the appropriate PCR condition (Krebber et al., 1997) (Table 2.2.2).

**Table 2.2.1 Preparation of a PCR mixture**

Component	PCR/volume	Control/volume
10 x PWO complete reaction buffer	5 µl	5 µl
dNTP's mixture (2.5 mM)	4 µl	4 µl
MgCl <sub>2</sub> (25 mM)	4 µl	4 µl
LB or HB primer (100 µM)	1 µl	1 µl
LF or HF primer (100 µM)	1 µl	1 µl
cDNA	2 µl	0 µl
0.5 µl Pwo (1 U/µl) and 20 µl Taq (5 U/µl) Polymerase mixture	3 µl	3 µl
sterile dH <sub>2</sub> O	up to 50 µl	up to 50 µl
<b>Total volume</b>	50 µl	50 µl

**Table 2.2.2 Thermal cycler conditions**

Number of cycles	temperature	Duration
1	92 °C	5 min
7	92 °C	1 min
7	63 °C	30 s
7	58 °C	50 s
7	72 °C	1 min
23	92 °C	1 min
23	63 °C	1 min
23	72 °C	1 min

### **2.2.2.4 Analysis the amplified products (VL and VH) by electrophoresis**

All products (VL and VH) were run on a 1% agarose gel. Samples were mixed with 1/6 volume of 6 x loading buffer and electrophoresis performed at 115 V for 30 min in a mini-sub cell electrophoresis tank filled with TAE buffer. Size of DNA fragments was identified under UV light by comparison with known DNA size marker (100 bp ladder) loaded in a separate well of gel.

### **2.2.2.5 DNA purification**

DNA fragments were excised from the agarose gel using clean scalpels and the gel pieces were minimized. DNA purification was performed using Qiagen gel extraction kit. 500 µl of QG buffer was added to each sample and samples were incubated at 50 °C for 10 min or until the gel slices were completely dissolved. After this incubation, the colour of mixture must be yellow similar to QG buffer. 150 µl Isopropanol was added to the samples and vortexed. A QIAquick spin column was placed in a provided 2 ml collection tube for every sample. The samples were applied to the column and centrifuged at maximum speed for 1 min. The flow-through was discarded and the column was placed back in the same collection tube. 750 µl PE buffer was added to the column and centrifuged for 1 min. The flow-through was discarded again and the column was centrifuged for an additional 1 min. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. 50 µl of sterile water was added onto the centre of QIAquick membrane and the column was centrifuged for 1 min. All products were run on a 1% agarose gel and were identified by size under UV light. The fragments were purified from the gel using the Qiagen

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extraction kit as described above. The products were immediately used or stored at -20 °C until further use.

### 2.2.2.6 Assembly of scFv by Splice Overlap Extension PCR (SOE-PCR)

ScFvs were assembled in VL-VH orientation with a linker containing 20 amino acids, Glycine-Serine by SOE-PCR (Krebber et al., 1997), using peqGOLD Taq and PWO DNA polymerase according to manufacturer's instructions (summarized in Table 2.2.3). The appropriate components were applied to a sterile 0.5 ml microcentrifuge tube sitting on ice. The contents of the tube were mixed and incubated in a thermal cycler according to appropriate PCR conditions (Table 2.2.4). The PCR products (scFvs) were separated on a 1% agarose gel, and purified from the gel as described above (see 2.2.2.4).

**Table 2.2.3 Preparation of SOE- PCR mixture**

Component	PCR/volume	Control/volume
10 x PWO complete reaction buffer	5 µl	5 µl
2.5 mM dNTP's mixture	4 µl	4 µl
Scfor primer (50 µM)	0.5 µl	0.5 µl
Scback primer (50 µM)	0.5 µl	0.5 µl
0.5 µl Pwo (1 U/µl) and 20 µl Taq (5 U/µl) Polymerase mixture	3 µl	3 µl
VL-PCR product	10 ng	0 ng
VH-PCR product	10 ng	0 ng
sterile dH <sub>2</sub> O	up to 50 µl	up to 50 µl
<b>Total volume</b>	<b>50 µl</b>	<b>50 µl</b>

**Table 2.2.4 Thermal cycler conditions**

Number of cycles	temperature	Duration
7	92 °C	1 min
7	63 °C	30 s
7	58 °C	50 s
7	72 °C	1 min
23	92 °C	1 min
23	63 °C	30 s
23	72 °C	1 min

### 2.2.2.7 Digestion of amplified scFv

The components (Table 2.2.5) were applied and mixed in a 0.5 ml tube. The appropriate restriction enzyme was used, (in this case SfiI). The sample was overlaid with mineral oil to avoid evaporation, and incubated at 50 °C for 3-4 h. The appropriate amount of pAK100 vector was also digested with SfiI restriction enzyme. The digestion products were supplemented with 6 x DNA loading buffer, and run on an agarose gel. The DNA fragments were excised from the gel, and purified from the agarose gel, to utilize for ligation.

**Table 2.2.5 Preparation of restriction mixture**

Component	Volume
10 x enzyme buffer 2	6 µl
100 x BSA	0.6 µl
SfiI (20 U/µl)	1-2 µl
PCR product	50 µl
sterile dH <sub>2</sub> O	up to 60 µl
<b>Total volume</b>	60 µl

### 2.2.2.8 DNA ligation

20 ng SfiI digested scFv gene fragments were ligated into SfiI digested pAK 100 vector (molar ratio vector to insert 3:1). The vector concentration was calculated:  $\text{ng insert} = [(*\text{ng vector}) \times (\text{kb size of insert})] : [(\text{kb size of vector}) \times (\text{molar ratio of insert/vector})]$ . The appropriate components (Table 2.2.6) were mixed in a 0.5 ml tube, and the mixture was incubated overnight at 16 °C.

**Table 2.2.6 Preparation of ligation mixture**

Component	Volume
10 x ligase buffer	1 µl
T4 DNA ligase (1 U/µl)	1 µl
Digested vector	* ng
Digested insert	20 ng
sterile dH <sub>2</sub> O	up to 10 µl
<b>Total volume</b>	10 µl

### 2.2.2.9 Heat-shock bacterial cell transformation

Chemically competent *E. coli* cells, strain Mach1 were generally used for DNA transformation. One vial of *E. coli* was thawed on ice. 1-5 µl of ligation product, (10

pg-100 ng), was added to *E. coli* cells, and mixed gently. The *E. coli* vial was incubated on ice for 30 min. The cells were heat-shocked at 42 °C for 30 s followed by incubation on ice for 2 min. 250 µl of prewarmed SOC medium was added. The vial was sealed tightly and shaken horizontally at 37 °C for 1 h at 225 rpm in a shaking incubator. 25-100 µl of cells was spread on a prewarmed selective agar plate. The plates were inverted and incubated at 37 °C overnight. To verify the ligation, 10 single overnight-grown colonies were inoculated in 3 ml selective media, and shaken at 37 °C for about 4 h. The plasmids were isolated.

### **2.2.2.10 Isolation of plasmid DNA from *E. coli* cells (mini preparation)**

To verify the ligation product, DNA from 10 *E. coli* colonies was isolated using NucleoSpin® Plasmid Quick Pure kit. 1.5 ml of a saturated *E. coli* cells, from overnight-grown colonies, were transferred into a microtube and centrifuged for 3 min at maximum speed. The supernatant was discarded. 250 µl A<sub>1</sub> buffer was added and the cell pellet was resuspended by vigorous vortexing. 250 µl A<sub>2</sub> buffer was added and mixed gently by inverting the tube 6-8 times, and incubated at RT for a maximum of 5 min. 300 µl A<sub>3</sub> buffer was added and mixed gently by inverting the tube 6-8 times. The mixture was centrifuged for 5 min at maximum speed at RT. A NucleoSpin® Plasmid QuickPure column was placed in a 2 ml collecting tube and 700 µl supernatant was loaded onto the column. The column was centrifuged for 1 min at maximum speed and the flow-through was discarded. The column was placed back into the 2 ml collecting tube and 450 µl AQ buffer was added and centrifuged for 1 min. Flow-through was discarded. The column was dried out by centrifugation for 3 min. The column was placed in a 1.5 ml microcentrifuge tube. 50 µl sterile dH<sub>2</sub>O was added and incubated for 1 min at RT. The column was centrifuged for 1 min. The isolated DNA was digested with appropriate enzyme (SfiI) as described above. Digested DNA was supplemented with DNA loading buffer and run on an agarose gel. The size of DNA fragments (insert and vector) were identified under UV light by comparison with DNA size marker.

### **2.2.2.11 Isolation of plasmid DNA from *E. coli* cells (maxi preparation)**

Purification of a large amount of plasmid was utilized using a NucleoBond® Xtra Maxi kit. 200 ml of a saturated *E. coli* cells from the overnight-grown colony was centrifuged at 4 °C. The bacterial cell pellet was resuspended in 12 ml of RES buffer

(RNase was added to RES buffer before use) by pipetting up and down. 12 ml of lysis buffer, LYS was added to the suspension and mixed gently by inverting the tube 5 times. The mixture was incubated at RT for 5 min. A NucleoBond® Xtra column, together with the inserted column filter, was equilibrated with 25 ml of equilibration buffer, EQU. 12 ml of neutralization buffer, NEU, was added to the suspension and the lysate was gently mixed by inverting the tube 10-15 times. The lysate was simultaneously cleared and loaded onto the column. The NucleoBond® Xtra column filter, and NucleoBond® Xtra column, were washed with 15 ml of EQU buffer. The NucleoBond® Xtra column filter was discarded. The NucleoBond® Xtra column was washed with 25 ml of washing buffer, and the plasmid DNA was eluted with 15 ml of elution buffer, ELU. The eluate was collected in a 15 ml or 50 ml centrifuge tube which was appropriated for high speed centrifugation. 10.5 ml of Isopropanol was added to the precipitate of eluted plasmid DNA. The tube was vortexed well and then was incubated at RT for 2 min. The tube was centrifuged at 5000 x g in a Sorvall RC-6 centrifuge (Rotor SS-34) at 4°C for 30 min. The supernatant was carefully discarded. 5 ml of 70% Ethanol was added to the pellet and the tube was centrifuged at 5000 x g for 5 min at RT. The ethanol was carefully removed from the tube and the pellet was air-dried. The DNA pellet was dissolved in 500 µl sterile dH<sub>2</sub>O and concentration of plasmid was determined using a biophotometer.

### **2.2.2.12 Preparation of electro-competent of *E. coli* cells**

A single colony from an *E. coli* plate was inoculated into 10 ml 2xYT medium containing the appropriate antibiotic of the *E. coli* strain (40 µg/ml Tet for XL-1 Blue strain), and incubated at 37 °C overnight in a shaking incubator. 2.5 ml of this overnight culture was added into 0.5 ml 2xYT containing 20% glucose and 5 ml 1M MgCl<sub>2</sub>. The culture was incubated at 37 °C for about 4 h in the shaking incubator until an OD<sub>600</sub> of 0.7-0.8. The *E. coli* cells were chilled on ice for 15 min and then were centrifuged at 4 °C at maximum speed, in a Megafuge 1.0 R (Rotor 2704) for 20 min. The pellet was resuspended in a half volume of pre-chilled 10% glycerol (500 ml for 1 L culture), and was centrifuged at 4 °C for 20 min. The pellet was resuspended again in a half volume of glycerol and centrifuged for an additional 20 min. The pellet was resuspended in 25 ml Glycerol and centrifuged for 15 min. The cell suspension was filled as 50 µl aliquots into eppendorf tubes. The tubes were shock-frozen in liquid Nitrogen. These aliquots were finally stored at -80 °C.

### **2.2.2.13 DNA precipitation**

DNA precipitation was performed to remove the salt from the ligation mixture, to improve the electroporation efficiency and reduce the arcing risk in electroporation cuvettes. The sterile dH<sub>2</sub>O was added to increase the volume of the ligation mixture to 50 µl. 500 µl n-Butanol was added and vortexed for 20 s. The sample was centrifuged at maximum speed in a Microfuge for 15-20 min. The DNA pellet was air dried and subsequently resuspended in 10 µl sterile dH<sub>2</sub>O to use for electroporation.

### **2.2.2.14 Electroporation of *E. coli* cells**

One vial of electrocompetent XL1-Blue *E. coli* was thawed on ice. The ligation mixture was precipitated and desalinated as described above. 1 µl of desalinated ligation mixture was added into a vial of *E. coli* cells and mixed gently. 40-80 µl of mixture was transferred into a pre-chilled electroporation cuvette with 0.1 cm gap. Electroporation was achieved using a MicroPulser electroporation apparatus under the condition of 1800 V. 1 ml of prewarmed SOC medium was immediately added to each vial. The vial was sealed tightly and shaken horizontally at 37 °C for 1 h at 225 rpm in a shaking incubator. 25-100 µl of the electroporation mixture was spread on a prewarmed 2xYT agar plate containing 1% glucose and the appropriate antibiotic. The plate was incubated overnight at 37 °C. The electroporation procedure was repeated for a large library construction.

### **2.2.2.15 Mutagenesis-PCR of scFv**

The first approach to improve the functional characteristics of the scFv was attempted by introducing random mutations over the entire gene coding for the scFv by error-prone PCR, using the GeneMorph II Random Mutagenesis kit as described by manufacturer's instructions (summarized in Table 2.2.7 and 2.2.8). The PCR condition was utilized to obtain 0-4 mutations per 1000 base pairs (Table 2.2.9).

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**Table 2.2.7 Preparation of Mutagenesis-PCR reaction mixture**

Component	PCR/volume	Control/volume
10 x Mutazyme II reaction buffer	5 $\mu$ l	5 $\mu$ l
40 mM dNTP's mixture (10 mM each)	1 $\mu$ l	1 $\mu$ l
Mut-Scfor primer (1 $\mu$ g/ $\mu$ l)	2.5 $\mu$ l	2.5 $\mu$ l
Mut-Scback primer (1 $\mu$ g/ $\mu$ l)	2.5 $\mu$ l	2.5 $\mu$ l
Mutazyme II DNA polymerase (2.5 U/ $\mu$ l)	1 $\mu$ l	1 $\mu$ l
Template DNA	10 $\mu$ l	0 $\mu$ l
sterile dH <sub>2</sub> O	up to 50 $\mu$ l	up to 50 $\mu$ l
<b>Total volume</b>	50 $\mu$ l	50 $\mu$ l

**Table 2.2.8 Thermal cycler conditions**

Number of cycles	temperature	Duration
1	95 °C	2 min
30	95 °C Primer T <sub>m</sub> -5 °C 72 °C	30 s 30 s 1 min
1	72 °C	10 min

**Table 2.2.9 Determine the frequency of mutation**

Mutation rate	Mutation frequency (Mutations/kb)	Initial target amount (ng)	Recommended fold amplification
Low	0-4.5	500-1000	1.5-10
Medium	4.5-9	100-500	10-100
High	9-16	0.1-100	100-10000

### 2.2.2.16 OneStep RT-PCR for CD96-ECD construction

Total RNA was isolated from CD96-positive HSB-2 cells using RNeasy Midi kit according to manufacturer's instructions and as described previously. A master mixture for OneStep RT-PCR was prepared (Table 2.2.10). A negative control, (without template RNA), was also prepared. The master mixture was mixed thoroughly by pipetting and appropriate volumes were dispensed into PCR tubes. The samples were incubated in a thermocycler using the appropriate PCR condition (Table 2.2.11).



**Table 2.2.10 Reaction components for one-step RT-PCR of ECD**

Component	PCR/volume	Control/volume
5 x OneStep RT-PCR buffer	10 $\mu$ l	10 $\mu$ l
dNTP mixture (10 mM each)	2 $\mu$ l	2 $\mu$ l
ECD-For primer (100 $\mu$ M/ $\mu$ l)	1 $\mu$ l	1 $\mu$ l
ECD-Rev primer (100 $\mu$ M/ $\mu$ l)	1 $\mu$ l	1 $\mu$ l
OneStep enzyme mixture	2 $\mu$ l	2 $\mu$ l
Template RNA	1 $\mu$ l	0 $\mu$ l
RNase-free water	up to 50 $\mu$ l	up to 50 $\mu$ l
<b>Total volume</b>	50 $\mu$ l	50 $\mu$ l

**Table 2.2.11 Thermal cycler conditions**

Cycles 25-40	temperature	Duration
Reverse transcription	50 °C	30 min
Initial PCR activation step	95 °C	15 min
Denaturation	94 °C	0.5-1 min
Annealing	55-68 °C	0.5-1 min
Extension	72 °C	1 min
Final extension	72 °C	10 min

The PCR product was run on a 1% agarose gel and was identified by size under UV light. The product was purified from the gel as described before (see 2.2.2.5). CD96-ECD was digested with NheI and NotI restriction enzymes. NheI and NotI digested CD96-ECD was cloned into NheI and NotI digested pSEC-IgG1-Fc vector, resulting pSEC-CD96-ECD-IgG1-Fc. 293T cells were transient and stably transfected with pSEC-CD96-ECD-IgG1-Fc. The supernatant was collected. The purified CD96-ECD-IgG1-Fc protein was analyzed by SDS-PAGE with Coomassie staining and western blot.

### 2.2.3 Phage display recombinant antibody system

#### 2.2.3.1 Infection of *E. coli* cells with helper phages

XL1-Blue colonies were washed off with 2xTY medium from the transformation plates. 100  $\mu$ l of bacterial cell suspension was applied to 100 ml 2xTY medium containing 1% glucose and 30  $\mu$ g/ml chloramphenicol. The suspension was incubated at 37 °C in a shaking incubator until the culture density reached an OD<sub>600</sub>

of 0.7. This OD is equivalent to  $5.6 \times 10^8$  bacteria/ml. The culture was infected with helper phages, M13KO7 ( $10^{12}$  pfu/ml) and incubated at 37 °C without shaking for 30 min. 200 ml of 2xYT medium containing 1% glucose and 30 µg/ml chloramphenicol was added. IPTG was supplied at a final concentration of 0.5 mM. The culture was incubated at 37 °C for 1 h under vigorous shaking. After 1 h, kanamycin was added, (final concentration of 25 µg/ml), and the culture was incubated at 30 °C overnight in the shaking incubator.

### 2.2.3.2 Preparation of phages

The overnight culture was centrifuged at maximum speed in a Megafuge 1.0 R (Rotor 2704) at 4 °C for 15 min. The supernatant was transferred to a new tube. The phages were precipitated with ¼ volume of polyethylene glycol, (20% PEG 6000 + 2.5 M NaCl), on ice for 30 min and were subsequently centrifuged at 10.000 x g in a Sorvall RC-6 (Rotor SS-34) centrifuge at 4 °C for 20 min. The supernatant was removed and the pellet was air-dried. The cell pellet was resuspended in 2 ml PBS buffer and was centrifuged at maximum speed for 5 min. The supernatant was stored at 4 °C until use.

### 2.2.3.3 Estimation of phage titration

50 µl of TG1 *E.coli* cells was added to 25 ml 2xTY medium and incubated at 37 °C for 2 h under vigorous shaking. The phage preparation (from step 2.2.3.2) was diluted ( $10^{-3}$ ,  $10^{-6}$ , and  $10^{-9}$ ) in order to estimate the titration of eluted phages. 1 µl of each dilution was added to 100 µl of *E. coli* cells, and incubated at 37 °C for 30 min without shaking. The aliquot of each tube was spread on three agar plates containing 1% glucose, 30 µg/ml chloramphenicol and incubated at 37 °C overnight. The number of bacterial colonies on each plate was counted. The phage titration was calculated by the multiplication of colonies formed with the dilution factor.

### 2.2.3.4 Screening of library for binders on intact tumor cells

$5 \times 10^6$  CD96-positive cells (HSB-2 cells) were blocked with 500 µl of 4% NM/PBS at RT for 30 min under slow agitation. 500 µl of phage suspension was added and incubated at RT for 1.5-2 h under slow agitation. The HSB-2 cells were rinsed 10 times with 5 ml of 2% NM/PBS and twice with 5 ml PBS. Bound phages were eluted

by incubation with 1.5 ml of 50 mM HCl for 10 min. After neutralization with 500  $\mu$ l of 1 M Tris at pH 7.5, the cells were pelleted by centrifugation. The supernatant was utilized to infect 10 ml growing TG1 cells at 37 °C for 30 min. 20 ml of 2xYT medium containing 1% glucose and 30  $\mu$ g/ml chloramphenicol was added to the culture and the culture was incubated at 37 °C for 2 h under vigorous shaking. The cells were super-infected with 1 ml of  $1 \times 10^{12}$  pfu/ml of helper phages at 37 °C for 30 min without shaking. The IPTG concentration was adjusted to 0.5 mM. 100 ml of 2xYT medium containing 1% glucose and 30  $\mu$ g/ml chloramphenicol was added. 1 h after the infection, kanamycin was added at a final concentration of 25  $\mu$ g/ml. The culture was incubated at 30 °C overnight in a shaking incubator. On the following day, the phages were prepared as described before (see 2.2.3.2) and the screening was repeated 3 times.

### **2.2.3.5 Screening the library for binders on ECD protein**

The scFv product from mutagenesis-PCR was also applied to produce a phage library and a library with about  $1 \times 10^6$  independent colonies was generated. The phages were selected by binding to CD96-ECD protein. Briefly, 10  $\mu$ g/ml of CD96-ECD was utilized to coat the ELISA plates at 4 °C overnight. In each round of selection,  $1 \times 10^{12}$  bacteriophages were used as input. After 2 h of incubation at RT, the plates were rinsed 20 times with PBS supplemented with 1% Triton X-100 and 1% Tween 20. The plates were rinsed also 10 times with PBS. The washing process was optimized to complete elimination of wild type scFv-expressing bacteriophages. Bound phages were eluted and immediately neutralized as described before. After the fifth round of screening, 30 individual colonies were randomly picked and sequenced in comparison to the same number of colonies from the original library.

### **2.2.3.6 Estimation of eluted phage titration**

1  $\mu$ l and 10  $\mu$ l of eluted phages were applied to 100  $\mu$ l of cultivated TG1 *E.coli* cells and incubated for 30 min at 37 °C without shaking. Aliquot of each dilution was spread on agar plates containing 1% glucose and 30  $\mu$ g/ml chloramphenicol. These plates were incubated overnight at 37 °C. The bacterial colonies on each plate were counted. The titre was calculated by multiplication of colonies formed with the dilution factor.

### 2.2.3.7 Whole cell-phage ELISA

Cellular ELISA was performed with CD96-positive cells (HSB-2) to show the binding activity of scFv-expressing bacteriophages to CD96 antigen. CD96-negative cells (CEM) were utilized as negative control.  $1 \times 10^6$  cells per well were blocked with 100  $\mu$ l of 2% NM/PBS in a 96-well plate at RT for 1 h under slow agitation. After 1 h, the cells were centrifuged and resuspended in 50  $\mu$ l phages supernatant supplemented in 1% NM/PBS. The plate was incubated at RT for 1-1.5 h under slow agitation. The cells were rinsed five times with 0.1% NM/PBS and twice with PBS. The cells were incubated with 50  $\mu$ l of 1:2000 of anti-M13 HRP-conjugated antibody/1% NM/PBS per each well at RT for 1 h under slow agitation. The plate was again rinsed three times with 0.1% NM/PBS and twice with PBS. Detection was performed with 80  $\mu$ l of freshly prepared ABTS substrate. The absorbance was scanned at  $A = A(405 \text{ nm}) - A(492 \text{ nm})$ .

### 2.2.3.8 Preparation of monoclonal phage

To identify the ability of single phage colonies to recognize the CD96 antigen, 10 individual colonies were inoculated from the titre plates following last screening into 2 ml 2xTY medium, containing 1% glucose and 30  $\mu$ g/ml chloramphenicol, and incubated at 37 °C for 6-8 h in a shaking incubator. 100  $\mu$ l helper phages were applied into the bacterial culture and incubated at 37 °C for 30 min without shaking. 20 ml of 2xTY medium containing 1% glucose and 30  $\mu$ g/ml chloramphenicol together with 0.5 mM IPTG were added. After 1 h incubation at 37 °C, 4 ml medium containing glucose and chloramphenicol was added. Kanamycin was subsequently supplied at a final concentration of 25  $\mu$ g/ml, and the culture was incubated at 30 °C overnight. The overnight culture was centrifuged at 4 °C for 15 min. The phages were precipitated with  $\frac{1}{4}$  volume polyethylene glycol (20% PEG 6000 + 2.5 M NaCl) on ice for 30 min, and then were centrifuged at 10.000 x g in a Sorvall RC-6 (Rotor SS-34) centrifuge at 4 °C for 20 min. The cell pellet was air-dried and was resuspended in 400  $\mu$ l PBS. The suspension was centrifuged at maximum speed for 5 min. The supernatant was stored at 4 °C until use. Individual phage colonies were tested in ELISA as described above (see 2.2.3.7) to determine of their ability to recognize the CD96 antigen.

### 2.2.4 Protein expression

#### 2.2.4.1 Expression and purification of soluble scFv from HB2151 *E. coli* cells

For soluble expression of scFv fragments in HB2151 *E. coli* cells, bacterial expression vector pAK400 and scFv were digested with SfiI restriction enzyme. The SfiI digested scFv was ligated into SfiI digested pAK400 vector. The plasmids were propagated in HB2151 cells, and the *E. coli* cells were spread on the agar plates containing 1% glucose, 30 µg/ml chloramphenicol, and incubated at 37 °C overnight. A single colony from the *E. coli* plate was inoculated into a 50 ml 2xYT medium containing 1% glucose and 30 µg/ml chloramphenicol and incubated at 37 °C overnight in a shaking incubator. The overnight culture was diluted in a 500 ml 2xYT medium supplemented with 1% glucose and 30 µg/ml chloramphenicol. The cultures were grown at 37 °C to an OD<sub>600</sub> of 0.8 and expression was induced by adding IPTG at a final concentration of 1 mM, and lowering the temperature to 30 °C. After 3 h, the bacteria were collected by centrifugation. The cells pellet was resuspended in 50 ml periplasmic extraction buffer, (without EDTA), and was mixed on a magnetic stirrer at 4 °C for 30 min. The cells were disrupted with an ultrasonic cell disruptor. The supernatant was passed through a cell culture filter, and centrifuged at 5000 x g in a Sorvall RC-6 (Rotor SS-34) centrifuge at 4 °C for 45 min. Purification of 6 x His tagged scFv fragments has been achieved with IMAC affinity chromatography.

#### 2.2.4.2 Expression and purification of soluble scFv from BL21 (DE3) *E. coli* cells

For the soluble expression of scFv fragments in BL21 (DE3) bacteria, expression vector pET27b-STREP-His and scFv were digested with SfiI restriction enzyme. The scFv was ligated into vector pET27b-STREP-His. pET27b-STREP-His-CD96 was propagated in BL21 (DE3) cells. Overnight cultures were diluted in a 500 ml 2xYT medium supplemented with 1% glucose and 30 µg/ml chloramphenicol. The cultures were grown to an OD<sub>600</sub> of 0.8-1 at 37 °C, and protein expression was induced by adding IPTG at a final concentration of 0.2 mM and lowering the temperature to 24 °C. After 20 h, the bacteria were collected by centrifugation. The cells pellet was resuspended in a 50 ml periplasmic extraction buffer and was mixed on a magnetic stirrer at 4 °C for 30 min. The cells were disrupted with an ultrasonic cell disruptor. The cells were centrifuged at 5000 x g in a Sorvall RC-6 (Rotor SS-34) centrifuge at 4 °C for 45 min. The supernatant was passed through a cell culture filter and

purification of Strep-tagged scFv fragments was achieved with Strep-tag affinity chromatography following with IMAC Affinity chromatography. The final elution peaks were concentrated and analyzed by SDS-PAGE, and capillary electrophoresis was used to determine amount and purity of the recombinant protein.

### **2.2.4.3 Production of Fc fusion protein by transient transfection of 293T cells (calcium phosphate transfection)**

The CD96-scFv isolated from the initial phage display library or from the diversified library were digested with SfiI and cloned into pSEC-IgG1-Fc or pSEC-IgG1-Fc-eng vectors resulting pSEC-scFv-IgG1-Fc or pSEC-scFv-IgG1-Fc-eng (carrying a Fc variant optimized for CD16a binding; mutations: S239D/I332E/A330L) (Lazar et al., 2006; Repp et al., 2010). The sequence was confirmed by Sanger sequencing of the final construct to confirm the expected sequence arrangement. 293T cells were transfected with the respective expression vector using the calcium phosphate transfection system, including Chloroquine.  $3-5 \times 10^6$  293T cells were grown in 100 mm tissue culture dishes overnight. After 24 h, the medium was removed and 8 ml prewarmed medium was added. DNA mixture was prepared: 20 µg plasmid DNA in 895 µl sterile water + 100 µl 2.5 M CaCl<sub>2</sub> + 5 µl 100 mM Chloroquine according to established procedures (Kellner et al., 2010). All amounts and volumes are given on a per plate basis. The DNA mixture was added dropwise to the 2 x HBS buffer while simultaneously introducing bubbles via continuous pipetting of air through the solution. The mixture was vortexed shortly and 2 ml of the transfection solution was added dropwise to each tissue culture plate. The plates were incubated 9-10 h at 37 °C and 5-6% CO<sub>2</sub> in a humidified incubator. The transfection medium was changed by prewarmed tissue culture medium. The supernatants were collected every 24 h for 7 days. The collected supernatants were either used directly for immunofluorescence staining or applied for protein purification. The proteins were purified using ÄKTA-purifier by two-step affinity chromatography, using protein A affinity chromatography followed by IMAC Affinity chromatography according the manufacturer's instructions. The final elution peaks were concentrated, dialyzed against PBS buffer, and analyzed by SDS-PAGE to determine amount and purity of the recombinant proteins.

### **2.2.4.4 Generation of stably transfected 293T cells for expression of Fc fusion protein**

293T cells were stably transfected to expression Fc fusion protein. All amounts and volumes are given on a well basis. The day before transfection,  $5 \times 10^5$  cells were grown in 3 ml D10<sup>+</sup> per well in a 6-well plate. After 24 h, the medium was replaced with 3 ml prewarmed fresh medium. For each well 2-3  $\mu\text{g}$  of DNA was diluted in 500  $\mu\text{l}$  of Opti-MEM medium. 6.5  $\mu\text{l}$  of Lipofectamine LTX reagent was added into the above diluted DNA solution and mixed gently and incubated at RT for 30 min to form DNA-Lipofectamine LTX reagent complexes. After a 30 min incubation, the solution was added directly to each well and mixed gently. The cells were incubated at 37 °C in a humidified incubator for 48 h. After incubation time, the growth medium was replaced with fresh medium containing the selection medium, 100  $\mu\text{g}/\text{ml}$  of Hygromycin B. The selection was continued for about 1 week until mid-sized colonies grew out. The cells were then trypsinized and cultivated through screening single cells by limiting dilution in a 96-well plate (1 cell/well) for 2 weeks. The supernatant of tissue cultures from every well were tested by ELISA to select the positive colonies.

### **2.2.4.5 Single cell screening using ELISA**

A 96-well flat bottom plate was coated with 25  $\mu\text{l}/\text{well}$  of 5  $\mu\text{g}/\text{ml}$  anti-His or anti-myc (9E10) antibodies in PBS, and incubated at 4 °C overnight. On the following day, the reactions were blocked with 100  $\mu\text{l}$  3% BSA/PBS per well at RT for 1 h under slow agitation. The wells were washed three times with 125  $\mu\text{l}$  PBS/well. To detect the Fc portion of scFv-Fc protein, anti-Human IgG HRP conjugated antibody was added at a dilution of 1:5000 in 1% BSA/PBS at RT for 1 h under slow agitation. The wells were washed three times with 125  $\mu\text{l}$  PBS/well. The ELISA was developed with 50  $\mu\text{l}/\text{well}$  ABTS solution and OD was measured at  $A = A(405 \text{ nm}) - A(492 \text{ nm})$ . The colonies with highest signals were selected and cultivated in a small tissue culture flask.

## **2.2.5 Biochemical methods**

### **2.2.5.1 Tris/Glycine SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)**

The protein electrophoresis was performed as described by Laemmli (Laemmli, 1970). The concentration of separating gel varies from 6 to 15% according to the molecular weight of proteins, while the concentration of the stacking gel is usually

## 2. MATERIALS & METHODS

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3.6-5%. The appropriate components for separating gel were prepared (Table 2.2.12). After pouring the separating gel up  $\frac{3}{4}$  of plates, ethanol was added on the top of the separating gel to produce a smooth surface. Before adding the stacking gel, ethanol was poured off and excess ethanol was removed from the surfaces. The stacking gel (Table 2.2.13) was poured to the top of separating gel and the comb was inserted between the plates. The comb was removed after 20 min. The samples were diluted 1:4 in 5 x SDS-PAGE loading buffer and heated at 95 °C for 3 min. The serial dilutions of BSA were used as control to determine the concentration of appreciated molecule. The electrophoresis was carried out at 100 V in stacking gel, and 150 V in separating gel for approx. 1 h in SDS-PAGE running buffer.

**Table 2.2.12 Separating gel**

Component	6%	8%	10%	12%	15%
TEMED	0.008 ml	0.006 ml	0.004 ml	0.004 ml	0.004 ml
10% APS	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
1.5 M Tris (pH 8.8)	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
30% Acrylamide mixture	2 ml	2.7 ml	3.3 ml	4 ml	5 ml
dH <sub>2</sub> O	5.3 µl	4.6 µl	4 µl	3.3 µl	2.3 µl
<b>Total volume</b>	10 ml	10 ml	10 ml	10 ml	10 ml

**Table 2.2.13 Stacking gel**

Component	Volume
TEMED	0.003 ml
10% APS	0.03 ml
10% SDS	0.03 ml
0.5 M Tris (pH 8.8)	0.76 ml
30% Acrylamide mixture	0.5 ml
dH <sub>2</sub> O	1.34 µl
<b>Total volume</b>	3 ml

### 2.2.5.2 Western blot of scFv-IgG1-Fc, mouse-IgG1-Fc, and ECD-IgG1-Fc

Following SDS-PAGE, the gels were electroblotted to PVDF membrane as described by Towbin (Towbin et al., 1979). The membrane was incubated in methanol followed by incubation in western blot transfer buffer for 5 min. The proteins were electro-transferred to a PVDF membrane using Semi-Dry Transfer Cell with the current of 50



mA or 15 V for 60 min. The membrane was blocked with 5% NM/TBS buffer at 37 °C for 1 h. For scFv-IgG1-Fc and ECD-IgG1-Fc, the PVDF membranes were incubated in 1:5000 of anti-human IgG HRP conjugated antibody in blocking solution at RT for 1 h. For mouse-IgG1-Fc protein, anti-mouse IgG HRP-conjugated antibody was utilized in same dilution at RT for 1 h. The membranes were rinsed with 0.1% Tween 20/TBS, 3 times for 10 min. The proteins were detected with SuperSignal West Dura substrate and analyzed by a digital imaging system.

### **2.2.5.3 Western blot of scFv fragments**

The protein was electro-transferred from the gel to the PVDF membrane as described above. The membrane was blocked with 3% BSA/TBS buffer at 37 °C for 1 h. The membrane was incubated in Penta-His antibody which was diluted 1:1000 in blocking solution at RT for 1 h. The membrane was washed 3 times for 10 min with wash buffer (0.05% Tween 20/0.2% Triton X-100/TBS). The PVDF membrane was incubated with 1:5000 of anti-mouse IgG HRP-conjugated antibody in 10% NM/TBS at RT for 1 h. The membrane was washed again 3 times for 10 min. The protein was detected with SuperSignal West Dura substrate and analyzed by a digital imaging system.

### **2.2.5.4 Analysis of CD96-ECD-IgG1-Fc by ELISA**

A 96-well ELISA plate was coated with 50 µl of the CD96 parental antibody (TH-111) or an irrelevant antibody (TH-69) (50 µg/ml in PBS) at 37 °C for 1 h. The wells were blocked with 150 µl of 3% BSA/PBS for 1 h at 37 °C. Blocking solution was replaced by 50 µl of 10 µg/ml soluble CD96-ECD-IgG1-Fc in blocking buffer and incubated at RT for 1 h. The wells were washed 3 times with 150 µl of 1% BSA/PBS and incubated with a goat anti-Human IgG HRP conjugated antibody. The ECD protein was detected with 50 µl of ABTS substrate and was analyzed in an ELISA reader. Results were expressed as  $A = A(405 \text{ nm}) - A(492 \text{ nm})$ .

### 2.2.6 Antibody effector mechanisms

#### 2.2.6.1 Flow cytometry

To monitor binding of antibody-derivatives to antigen-positive cells,  $5 \times 10^5$  cells were incubated with serial dilutions of the appropriate antibody. The unbound antibody was removed by washing twice with 1% BSA/PBS. The cells were stained with 1:20 appropriate FITC-labeled antibody at 4 °C for 30 min. After 30 min, the cells were washed once with 1% BSA/PBS. The cells were resuspended in PFA fixation buffer and analyzed on a Cytomics FC500 flow cytometer using the CXP software. Ten thousand events were collected for each sample.

#### 2.2.6.2 Binding competition of the CD96 recombinant mini-antibody and the CD96 parental antibody (TH-111) to the CD96 antigen

To investigate competitive binding of recombinant CD96-scFv-IgG1-Fc antibody-derivative and the parental murine monoclonal antibody (TH-111),  $5 \times 10^5$  cells were incubated with the antibody-derivative in the presence of a 50-fold molar excess of either TH-111, or an irrelevant monoclonal antibody (TH-69) at RT for 30 min. The cells were rinsed once with PBA and the cell pellet was resuspended in 20  $\mu$ l of 1:20 diluted of the anti-human-IgG1-FITC antibody, and incubated on ice. After 30 min, unbound antibodies were removed by washing with 1% BSA/PBS and the cells were resuspended in PFA fixation buffer and analyzed on a Cytomics FC500 flow cytometer using CXP software. Ten thousand events were collected for each sample.

#### 2.2.6.3 Antibody-Dependent Cell Mediated Cytotoxicity assay (ADCC)

To study CD96-induced ADCC, target cells were labelled with 100  $\mu$ Ci of  $^{51}\text{Cr}$  per  $1 \times 10^6$  cells at 37 °C in a humidified  $\text{CO}_2$  incubator for 2 h. After this incubation period, the cells were rinsed three times with  $\text{R10}^+$  medium and incubated with serial dilutions of appropriate antibodies for 30 min at RT. The cells were washed once with 10 ml  $\text{R10}^+$  medium and then adjusted to  $1 \times 10^5$  cells/ml and added to the 96 well plates (50  $\mu$ l/well) filled with effector cells resulting in a final volume of 200  $\mu$ l. After 3 h incubation at 37 °C in the humidified  $\text{CO}_2$  incubator, the assays were stopped by centrifugation. 25  $\mu$ l supernatant from each well was applied to 150  $\mu$ l OptiPhase Supermix in a 96-well flexible plate for evaluation in a microplate scintillation and luminescence counter. The plate was sealed and shaken vigorously for 15 min.  $^{51}\text{Cr}$

release from triplicates was then counted on a MicroBeta counter and measured in counts per minute (cpm). Maximal  $^{51}\text{Cr}$  release was determined by adding Triton X-100 (1% final concentration) to target cells and spontaneous release was measured in the absence of effector cells. The percentage of specific lysis of labeled target cells was calculated using the formula:

$$\% \text{ specific lysis} = 100 \times \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}}$$

### 2.2.7 Data processing and statistical analyses

Data is displayed graphically and statistical analyses were performed using GraphPad Prism 4.0 software. Dose response curves were generated. The  $\text{EC}_{50}$  value (antibody concentration inducing half-maximal lysis) was calculated for each curve. P-values were calculated using the student's t-test and the null hypothesis was rejected for  $p < 0.05$ .

### 2.2.8 Homology modeling of CD96-scFv

The three-dimensional model of the wild type CD96-scFv (CD96-wt-scFv) was calculated, using the Rosetta server/algorithm (Sircar et al., 2009). The surface display model was generated, using the Accelrys DS Viewer software.

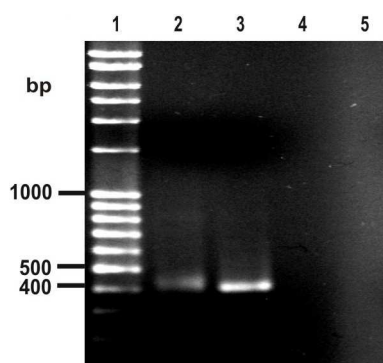
**Chapter 3**  
**RESULTS**

## 3. RESULTS

### 3.1 Construction of CD96-scFv

#### 3.1.1 Amplification of variable domains of CD96

The total RNA was isolated from  $1 \times 10^7$  TH-111 hybridoma cells. The isolated RNA was reverse transcribed into cDNA. The VH and VL fragments were amplified by PCR using the first strand cDNA as a template. The RT-PCR products were run on a 1 % agarose gel. One prominent band of about 400 bp for the heavy and the light chains was visible, and no visible band was detected when H<sub>2</sub>O as control was utilized (Fig 3.1). The amplified VH and VL fragments were purified from the agarose gel, and the amounts of the purified fragments were calculated by agarose gel quantification. These fragments were applied to the assembly of the scFv gene in the next experiment.

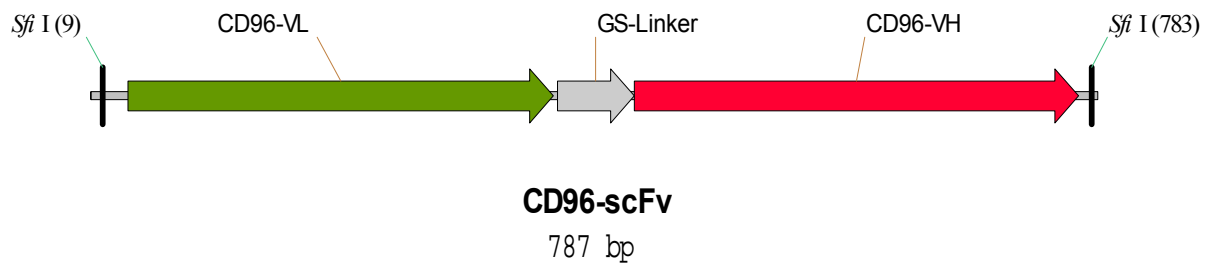


**Fig 3.1 Amplification of CD96-VL and -VH regions.** The PCR reactions were separated on a 1% agarose gel. Fragment sizes are indicated in base pairs (bp). Lane 1: 100 bp-DNA Ladder, lane 2: VL chain, lane 3: VH chain and lanes 4, 5: negative controls. One representative experiment out of four is indicated.

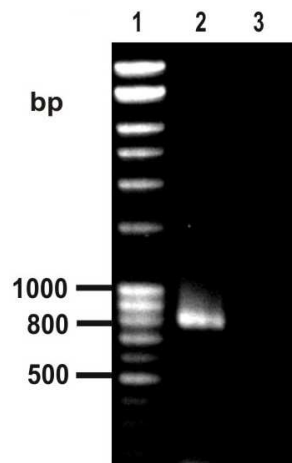
#### 3.1.2 Assembly of scFv gene by Splice Overlap Extension, SOE-PCR

The VH and VL DNA fragments were connected together with a (Gly<sub>4</sub> Ser)<sub>3</sub> linker by SOE-PCR (Fig 3.2). The amplified PCR product introduced the expected size of about 800 bp on an agarose gel, and no visible band was observed for H<sub>2</sub>O as negative control (Fig 3.3).

### 3. RESULTS



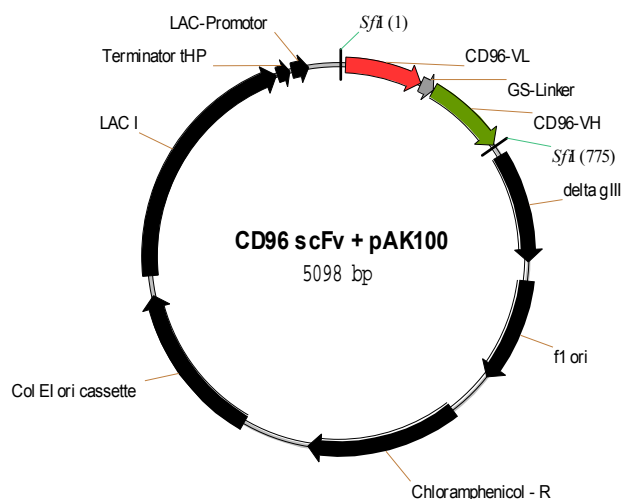
**Fig 3.2 Schematic view of CD96 single chain variable fragment (CD96-scFv).** ■ VL, ■ VH, and ■ linker.



**Fig 3.3 ScFv fragments generated by SOE-PCR.** The PCR reactions were separated on a 1% agarose gel. Fragment sizes are indicated in base pairs (bp). Lane 1: 100 bp-DNA Ladder, lane 2: scFv fragment, and lane 3: negative control. One representative experiment out of four is indicated.

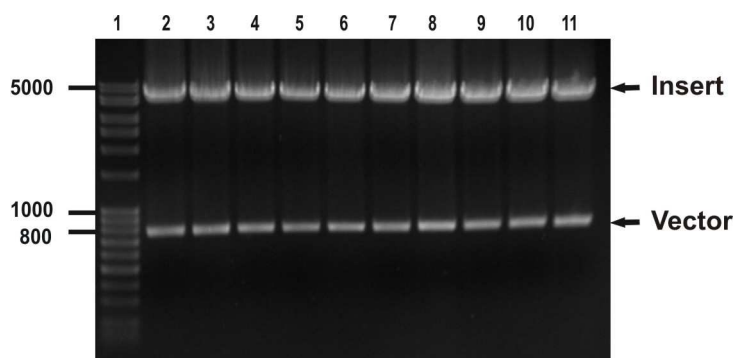
#### 3.1.3 Cloning of CD96-scFv into pAK100 phagemid vector

The amplified scFv fragments were purified from the agarose gel. The purified fragments were digested with *Sfi*I restriction enzyme. The digested scFv fragments were cloned into *Sfi*I digested pAK100 phagemid vector (Fig 3.4) and transformed into chemically competent *E. coli* cells, strain Mach1.



**Fig 3.4 Cloning of CD96-scFv into pAK100.** ■ VL, ■ VH, and ■ linker.

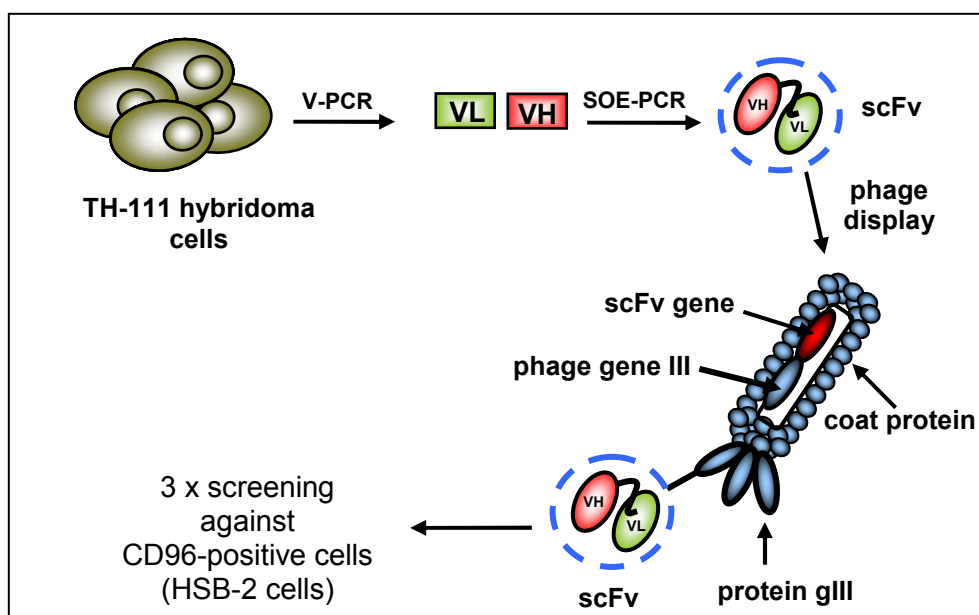
To verify the presence of the insert and the vector, 10 clones from the library were randomly selected and DNA isolation (mini preparations) was performed. The DNA fragments were digested with *Sfi*I restriction enzyme. The digested fragments were pooled in an agarose gel. All the clones presented the recombinant insert and vector with expected sizes of about 800 bp and 4000 respectively (Fig 3.5).



**Fig 3.5 Agarose gel electrophoresis of CD96-scFv + pAK100 phagemid vector mini preparations.** Fragment sizes are indicated in base pairs (bp). Lane 1: 100 bp-DNA Ladder, lanes 2-11: 10 randomly selected clones from the library which were digested with *Sfi*I restriction enzyme. One representative experiment out of three is indicated.

### 3.1.4 Construction of the CD96-scFv phage display library

The CD96-scFv + pAK100 ligated mixture was precipitated with n-Butanol, and transformed into electrocompetent *E. coli* cells, strain XL1-Blue, to prepare a library containing scFv genes. As the hybridoma cells may carry non-functional RNA therefore the scFv fragments were subjected to the phage display and screened against CD96-positive HSB-2 cells (Fig 3.6).

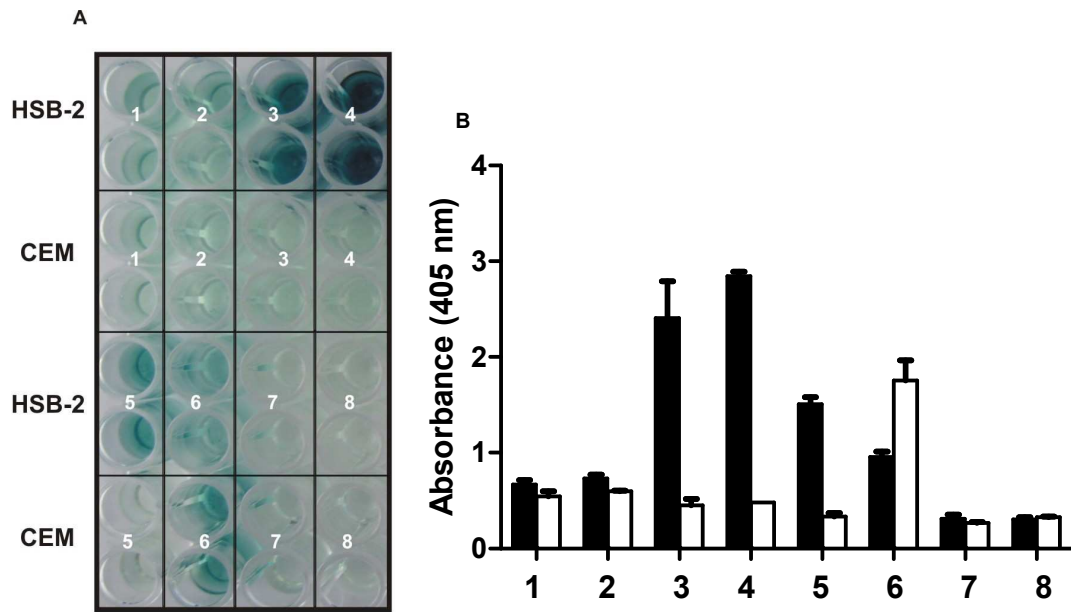


**Fig 3.6 Schematic view of scFv formation from TH-111 hybridoma cells, and selection of functional CD96-scFv through phage display.** VH: variable domain of the antibody heavy chain, VL: variable domain of the antibody light chain, and scFv: single chain variable fragment.

### 3.1.5 Whole cell-phage ELISA

Polyclonal ELISA was carried out with original library, and sub-libraries of all 3 screening rounds, to evaluate their binding specificity to the CD96 antigen and the enrichment of sub-libraries after screening. ELISA was performed using CD96-positive/CD7-negative HSB-2, and CD96-negative/CD7-positive CEM cells. The absorbance or optical density (OD) was read at  $A = A(405 \text{ nm}) - A(492 \text{ nm})$ . The most pronounced enrichment was observed after round 2 and the highest OD was read at round 3 (Fig 3.7). M13 helper phages and PBS, which were used as negative control, presented no binding activity to CD96. CD96 (TH-111) and CD7 (TH-69) monoclonal antibodies which were utilized as positive control presented binding activity to the CD96 antigen. In the case of CEM cells only TH-69 presented binding activity to the CD7 antigen. Therefore bacteriophages expressing CD96-scFv specifically bound to the CD96 antigen, and the process of screening could remove the non-functional scFvs and also scFvs with weak binding activity to CD96.





**Fig 3.7 Polyclonal cell-phage ELISA.** A) The ELISA plate was coated with HSB-2, (first and third rows), and CEM cells, (second and fourth rows). B) ■ coating with HSB-2 cells and □ coating with CEM cells. The cells were incubated with bacteriophages from 1: original library, 2: first round, 3: second round, 4: third round of screening, and also with 5: TH-111 monoclonal antibody, 6: TH-69 monoclonal antibody, 7: helper phages, and 8: PBS. The reactions were developed using an anti-M13 HRP-conjugated antibody. One representative experiment out of three is indicated.

### 3.1.6 Sequence alignment of CD96 variable light and heavy chains

10 single clones were picked from the last round of screening, and were sequenced. The sequence alignment of all CD96-scFv clones indicated expected sequence arrangements of variable heavy and light chains of murine IgG (Fig 3.8).

### 3. RESULTS

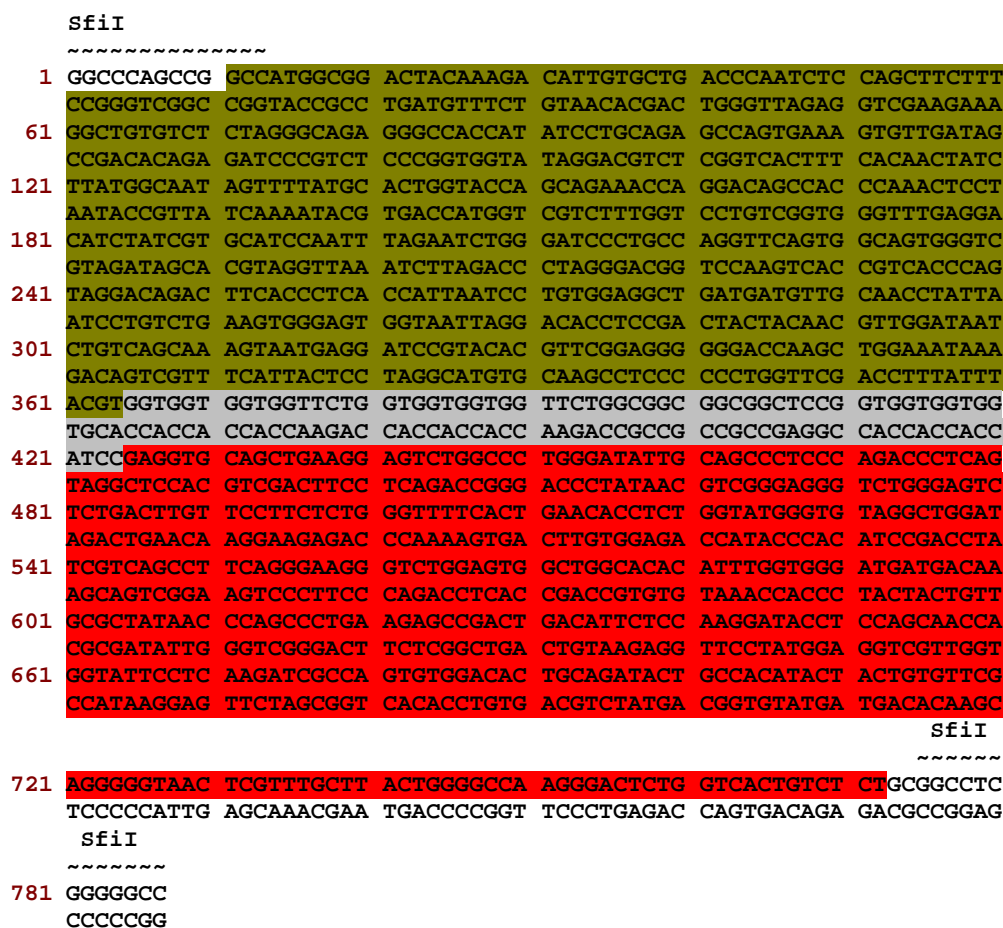
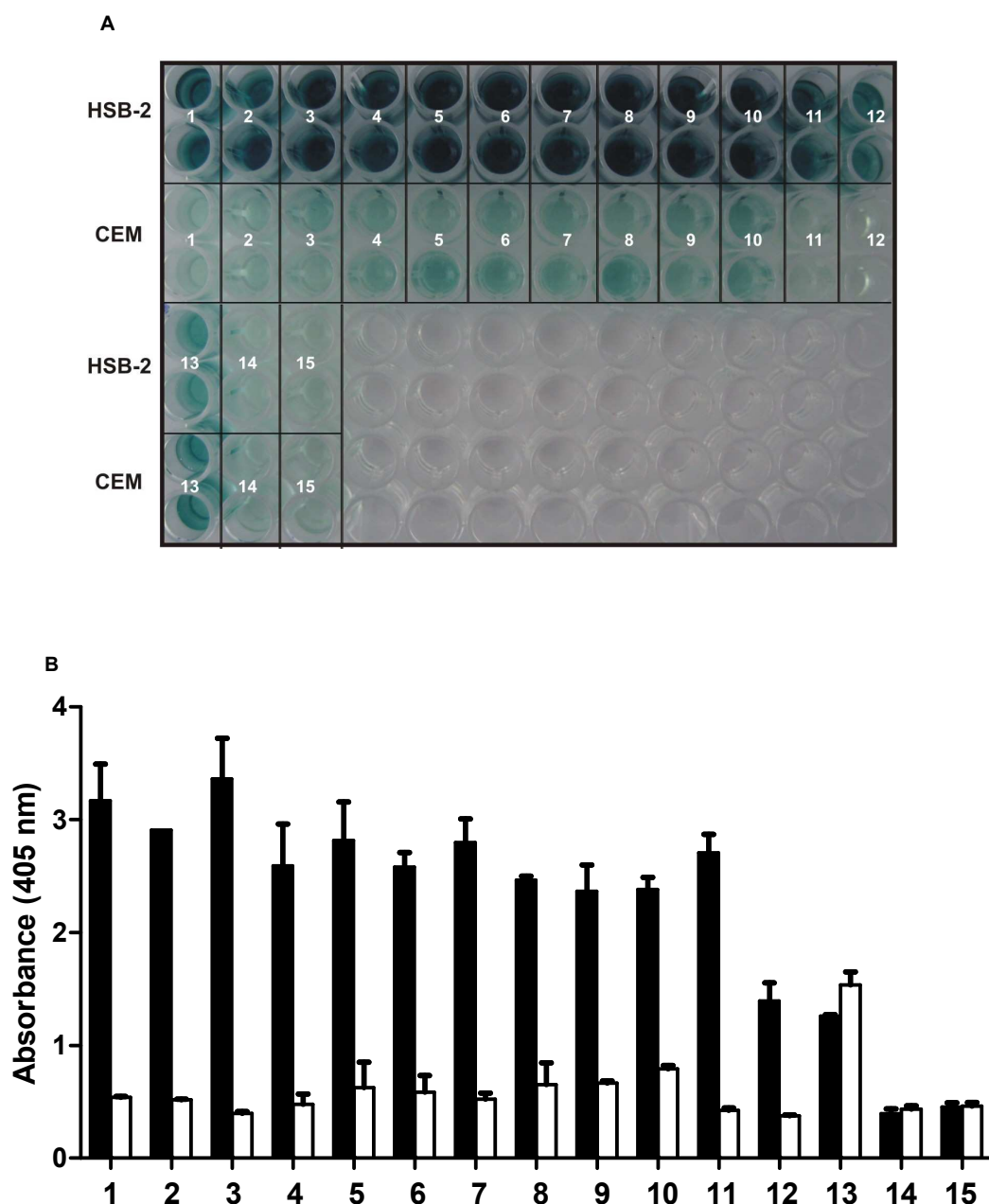


Fig 3.8 Sequence alignment of CD96-scFv. ■ VL, ■ VH, and ■ linker.

#### 3.1.7 Monoclonal cell-phage ELISA

The 10 single clones were also tested for specific binding to CD96 using ELISA. All clones bound specifically to HSB-2 cells. Helper phages and PBS which were used as negative control presented no binding activity for the CD96 antigen. Monoclonal CD96 (TH-111) and CD7 (TH-69) antibodies which were used as positive control presented binding activity to the CD96 antigen. In the case of CEM cells, only TH-69 presented the binding activity to CD7. One clone (number 8) with high absorbance was selected (Fig 3.9).



**Fig 3.9 Monoclonal cell-phase ELISA.** A) The ELISA plate was coated with HSB-2 (first and third rows) and CEM cells (second and fourth rows). B) ■ coating with HSB-2 cells and □ coating with CEM cells. The cells were incubated with bacteriophages from 1-10: 10 single clones, 11: first round of screening, and also with 12: TH-111 monoclonal antibody, 13: TH-69 monoclonal antibody, 14: helper phages, and 15: PBS. The reactions were developed using anti-M13 HRP-conjugated antibody. One representative experiment out of three is indicated.

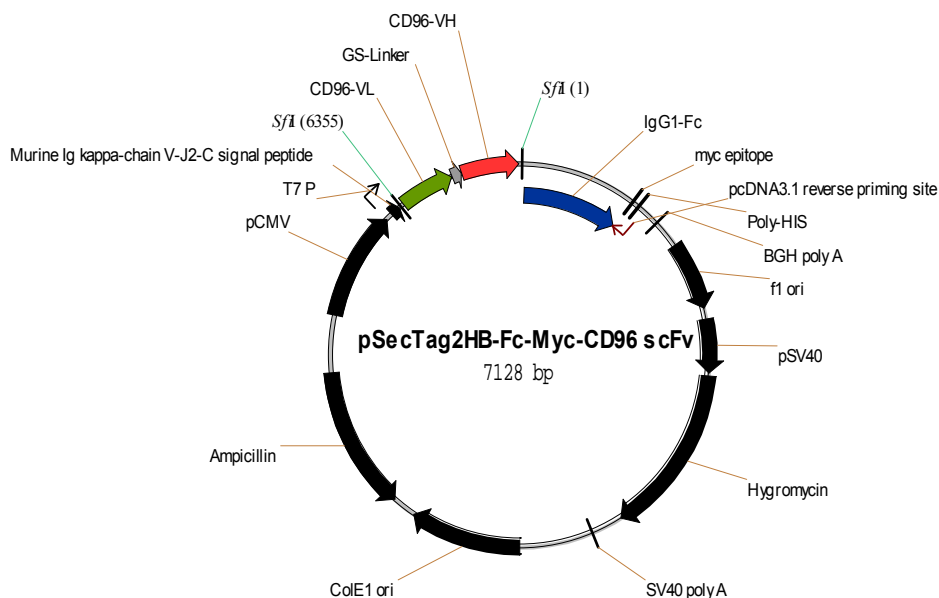
### 3.2 Generation and characterization of soluble CD96-scFv-IgG1-Fc

#### 3.2.1 Cloning of CD96-scFv into human IgG1-Fc

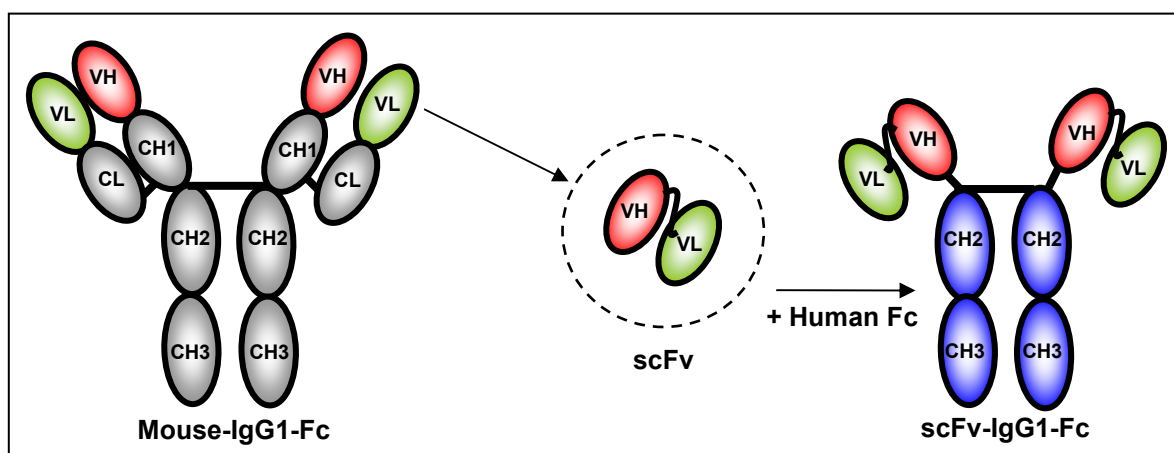
For functional characterization of CD96-scFv, the selected CD96-scFv was genetically fused to a human IgG1-Fc part (Fig 3.10). This format of recombinant

### 3. RESULTS

antibody is constructed of the scFv from mouse, and the Fc region from human antibody (Fig 3.11).



**Fig 3.10.** Schematic view of scFv cloning into the pSec-IgG1-Fc vector. ■ VL, ■ VH, ■ linker, and ■ IgG-Fc.



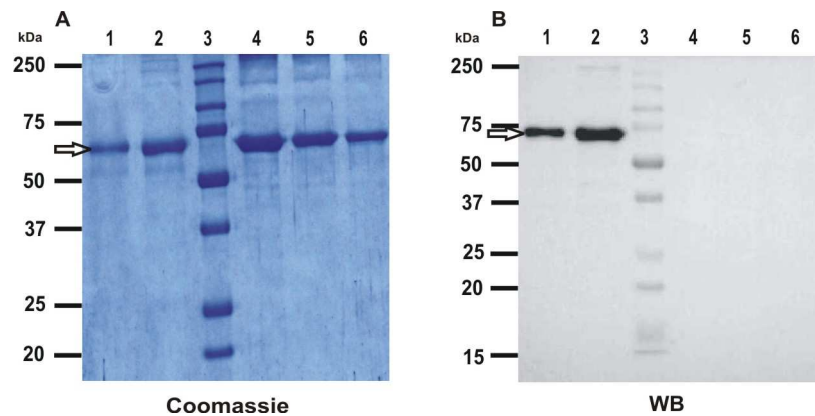
**Fig 3.11** Construction of the scFv-IgG1-Fc mini-antibody. The scFv from the CD96 monoclonal mouse antibody was cloned into human IgG1-Fc part.

#### 3.2.2 Expression and purification of the CD96-scFv-IgG1-Fc mini-antibody

293T cells were transient and stably transfected with the CD96-scFv-IgG1-Fc molecule. The supernatant was purified using protein A and IMAC affinity chromatography. Integrity and purity of the purified CD96-scFv-IgG1-Fc antibody was confirmed by SDS-PAGE and Coomassie-staining. One prominent band of about 60 kDa was visible on reducing SDS-gel (Fig 3.12A), roughly corresponding with

### 3. RESULTS

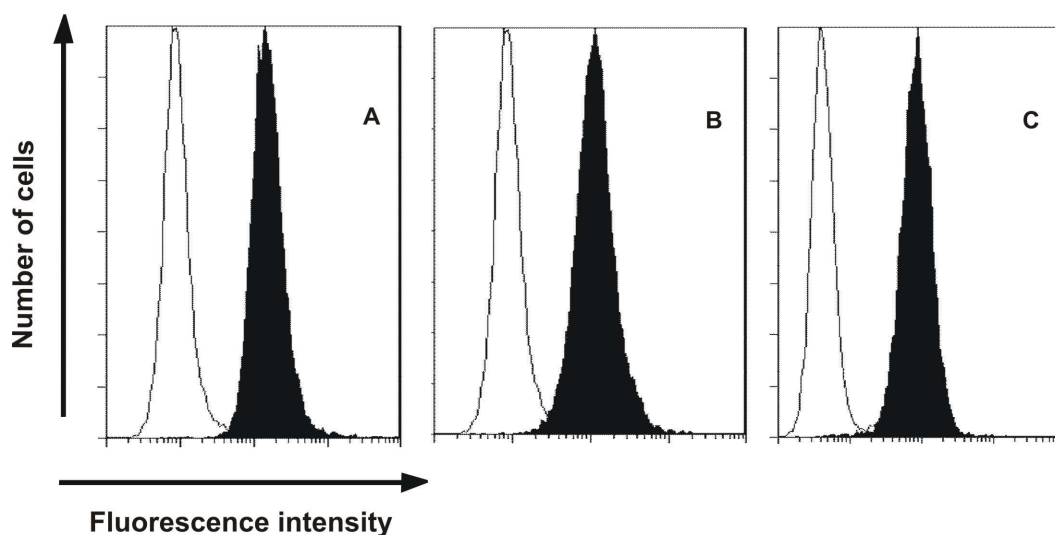
calculated molecular weight of 56.6 kDa. Under non-reducing condition, CD96-scFv-IgG1-Fc, demonstrated a band of about 110-120 kDa (Data not shown), indicating the expected dimer formation of the recombinant molecule. Western blot analysis further confirmed the identity of the purified protein (Fig 3.12B).



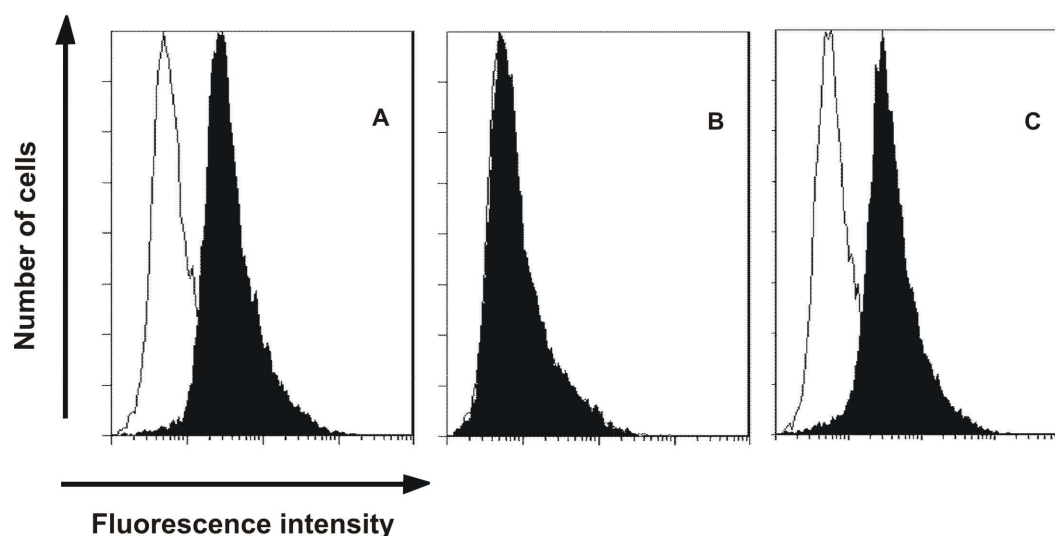
**Fig 3.12 Purification and analysis of the CD96-scFv-IgG1-Fc mini-antibody.** The protein sizes are indicated in kDa. A) Coomassie blue stained SDS-PAGE gel. B) Western blot. Lanes 1 and 2: purified CD96-scFv-IgG1-Fc protein, lane 3: size marker, lanes 4-6: calibration with 5, 2.5 and 1.25 µg of BSA. Data shown are representative of five independent experiments.

#### 3.2.3 Binding specificity of the CD96-scFv based mini-antibody to CD96

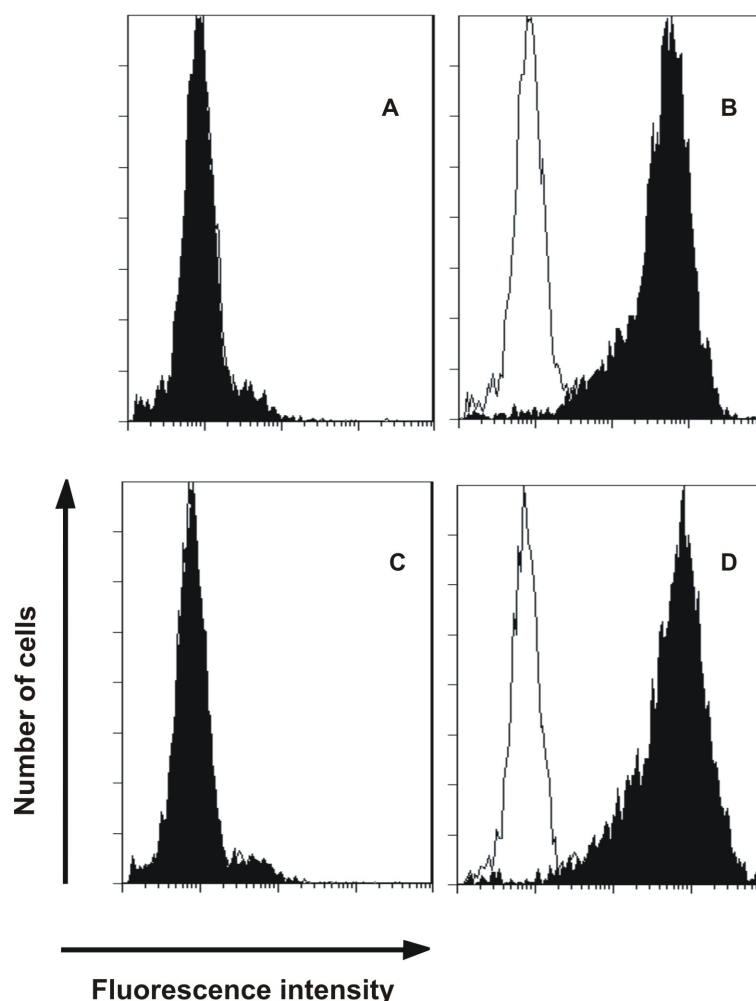
The binding activity and specificity of the CD96-scFv-IgG1-Fc mini-antibody was analyzed by flow cytometry using CD96-positive HSB-2 (Fig 3.13) and KG1a cells (Fig 3.14). CD96-negative CEM cells were used as negative control (Fig 3.15). Antigen binding was detected using Penta.His Alexa fluor conjugate. CD96-scFv-IgG1-Fc antibody confirmed the CD96 antigen specific binding, but no binding activity to CEM cells was observed. Therefore CD96-scFv-IgG1-Fc could specifically bind to CD96.



**Fig 3.13** Flow cytometry analysis of the recombinant CD96-scFv-IgG1-Fc mini-antibody using HSB-2 cells (CD96<sup>+</sup> and CD7<sup>+</sup>). The cells were incubated with A) CD96-scFv-IgG1-Fc, B) CD7-scFv-IgG1-Fc, and C) TH-111. The CD7 antibody was used as control. One representative experiment out of three is indicated.



**Fig 3.14** Flow cytometry analysis of the recombinant CD96-scFv-IgG1-Fc mini-antibody using KG1a cells (CD96<sup>+</sup> and CD7<sup>+</sup>). The cells were incubated with A) CD96-scFv-IgG1-Fc, B) CD7-scFv-IgG1-Fc, and C) TH-111. The CD7 antibody was used as control. One representative experiment out of three is indicated.



**Fig 3.15** Flow cytometry analysis of the recombinant CD96-scFv-IgG1-Fc mini-antibody using CEM cells. The cells were incubated with A) CD96-scFv-IgG1-Fc, B) CD7-scFv-IgG1-Fc, C) TH-111, and D) TH-69. The CD7 antibody was used as control. One representative experiment out of three is indicated.

### 3.2.4 Binding competition of the CD96-scFv based mini-antibody and the CD96 monoclonal antibody TH-111 to CD96

To further demonstrate that during the construction process of scFv fragment, the binding specificity of CD96-scFv was not altered, the binding competition of the CD96 recombinant mini-antibody, and the CD96 parental antibody (TH-111) to the CD96 antigen, was considered (Fig 3.16). High concentrations of the TH-111 antibody inhibited the binding of the CD96 mini-antibody to CD96 (Fig 3.17B), while an irrelevant antibody, (TH-69), used at the same concentration, presented no inhibitory effect (Fig 3.17C). In conclusion, CD96-scFv retained the antigen specificity of the parental murine antibody.

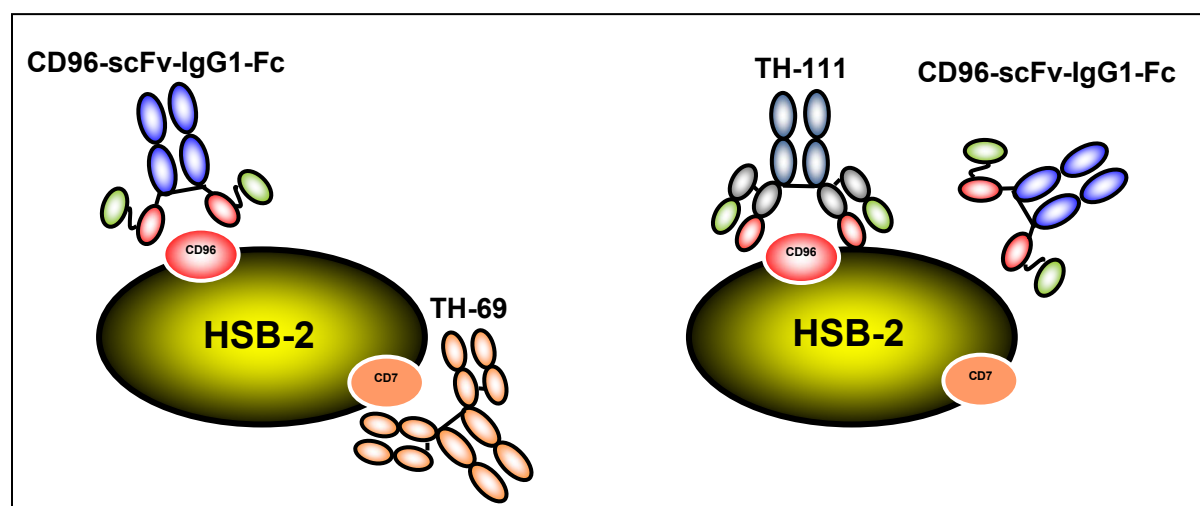


Fig 3.16 Schematic view of CD96-scFv-IgG1-Fc binding competition with the TH-111 monoclonal antibody to the CD96 antigen.

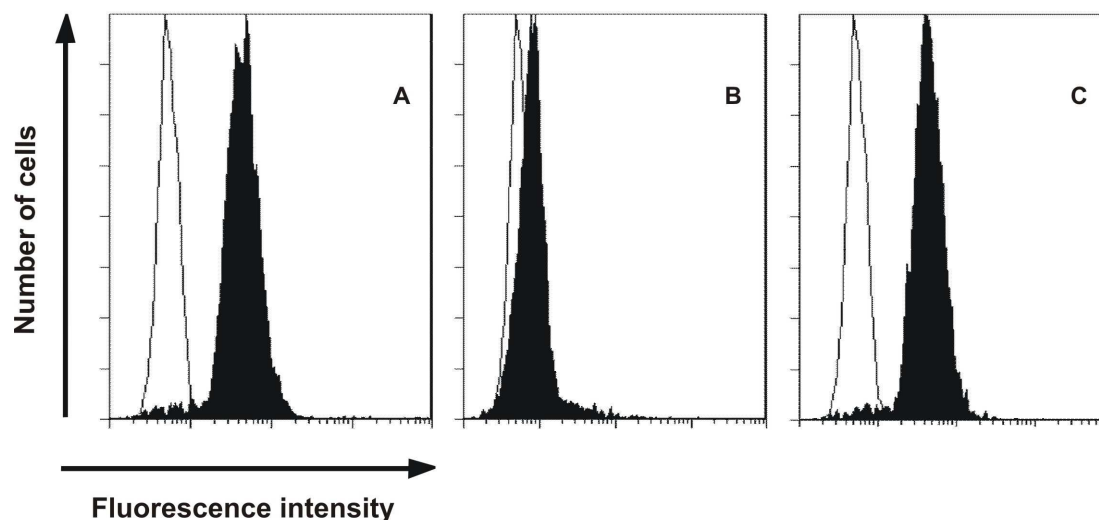
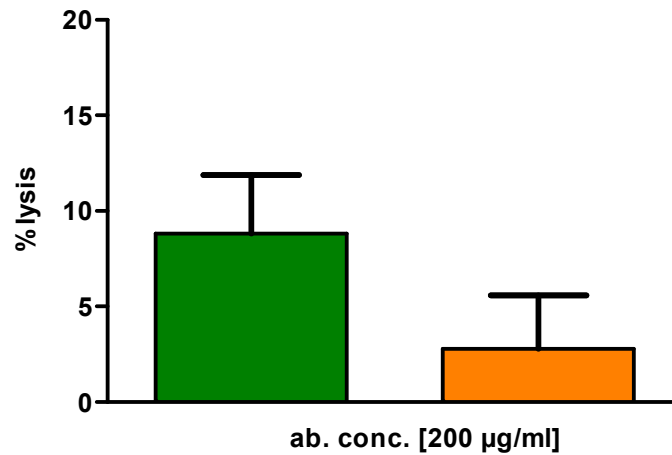


Fig 3.17 Binding competition of the CD96-scFv based mini-antibody and TH-111 to CD96. HSB-2 cells were incubated with A) CD96-scFv-IgG1-Fc, B) CD96-scFv-IgG1-Fc + TH-111, C) CD96-scFv-IgG1-Fc + TH-69. One representative experiment out of three is indicated.

### 3.2.5 Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) by the CD96-scFv based mini-antibody

To indicate the cytolytic activity of the recombinant CD96-scFv-IgG1-Fc mini-antibody, ADCC assay was performed using  $^{51}\text{Cr}$  labelled HSB-2 cells as target cells. The CD96 monoclonal antibody, TH-111, was used as control. The cells were incubated with antibodies and MNCs from healthy volunteers were used to trigger the CD96- positive cells. After 3 h incubation, the CD96-scFv-IgG1-Fc protein mediated low ADCC, and the CD96 mouse antibody presented very weak/no ADCC (Fig 3.18).



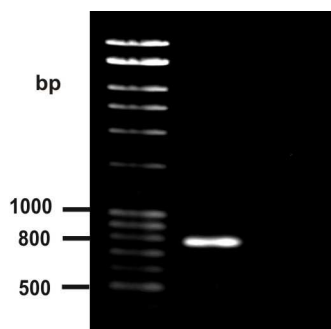


**Fig 3.18 ADCC analysis of the CD96-scFv-IgG1-Fc mini-antibody and the CD96 mouse antibody TH-111.** ■ CD96-scFv-IgG1-Fc and ■ TH-111. HSB-2 cells were used as target and MNCs as effector cells. E/T ratio (80:1). Data are presented as mean values +/- SEM from five independent experiments.

### 3.3 Generation of CD96-scFv with improved binding affinity to CD96

#### 3.3.1 Construction of a mutated CD96-scFv library

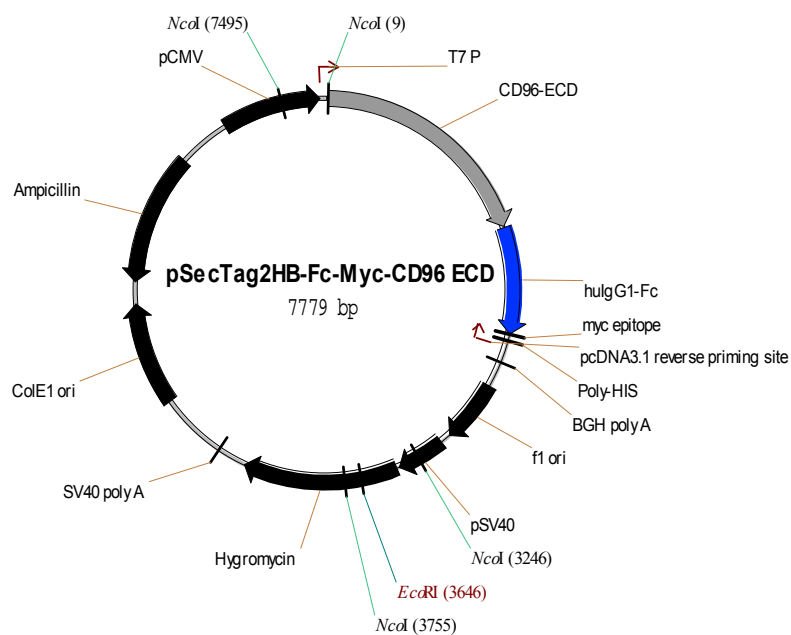
To optimize the cytolytic potential of the CD96-directed mini-antibody, the binding affinity of CD96-scFv to CD96 was enhanced. The coding sequence of the CD96-specific scFv fragment was amplified, using error-prone PCR, thereby introducing random mutations with low frequency (Fig 3.19). Lower PCR mutation frequencies could be achieved by using higher DNA template concentrations to limit the number of target duplications. In this test an initial target amount of 1000 ng is used to achieve low mutation frequencies of 0-4.5 mutations/kb. The amplified product was used to generate a “diversified” phage display library, and a library with  $1 \times 10^6$  independent clones was constructed.



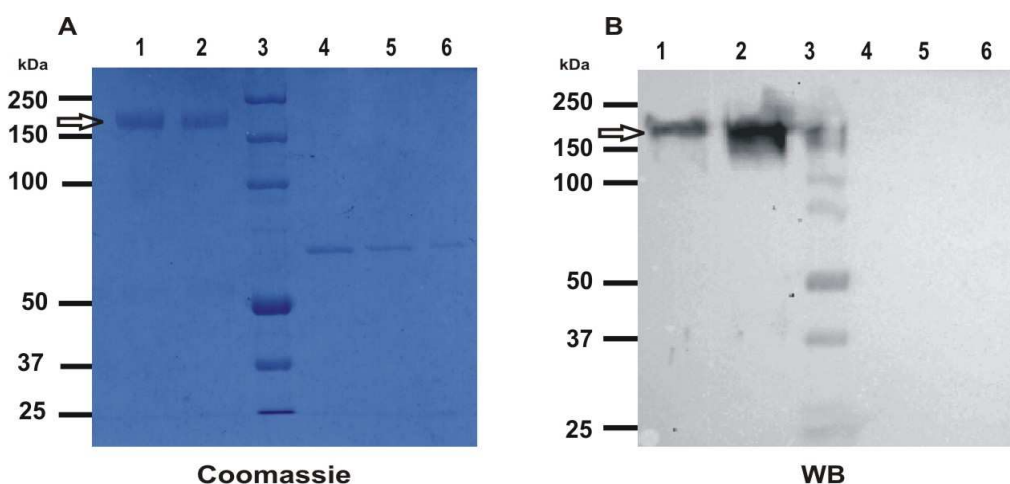
**Fig 3.19 Agarose gel electrophoresis of the mutagenesis-PCR of CD96-scFv.** Fragment sizes are indicated in base pairs (bp). Lane 1: 100 bp-DNA Ladder, lane 2: mutated-scFv, and lane 3: H<sub>2</sub>O as negative control. One representative experiment out of three is indicated.

#### **3.3.2 Generation, expression and purification of the CD96-ECD protein**

For the panning of “diversified” phage display library under stringent washing-selection conditions and remove the wild type CD96-scFv from the library, CD96-ECD was generated. Total RNA was isolated from CD96-positive HSB-2 cells, and the CD96-ECD was amplified by OneStep RT-PCR. CD96-ECD was cloned into a human IgG-Fc portion to facilitate of protein purification (Fig 3.20). 293T cells were transient and stably transfected with CD96-ECD-IgG1-Fc. The integrity and purity of the purified protein was confirmed by SDS-PAGE and Coomassie staining. One prominent band of about 200 kDa was visible on Coomassie-stained reducing SDS-gel (Fig 3.21A). Western blot analysis further confirmed the identity of the purified protein (Fig 3.21B).



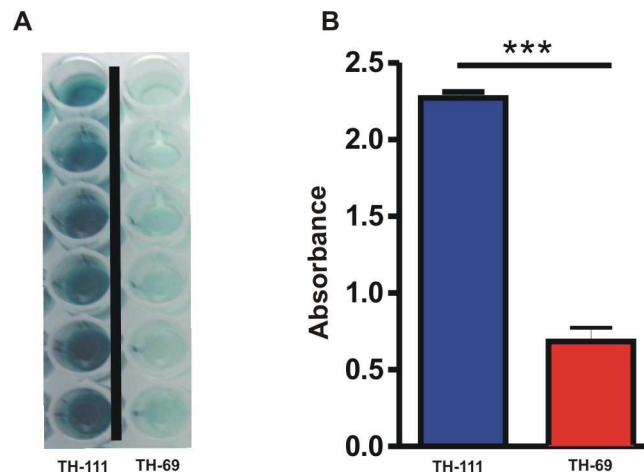
**Fig 3.20** Schematic view of CD96-ECD cloning into the pSec-IgG1-Fc vector. ■ CD96-ECD and ■ IgG1-Fc.



**Fig 3.21** Purification and analysis of the CD96-ECD-IgG1-Fc protein. The protein sizes are indicated in kDa. A) Coomassie blue stained SDS-PAGE gel. B) Western blot. Lanes 1 and 2: purified CD96-ECD-IgG1-Fc protein, lane 3: size marker and lanes 4-6: calibration with 1.25, 0.65 and 0.31  $\mu$ g of BSA. Data shown are representative of four independent experiments.

### 3.3.3 Binding specificity of the CD96 antibody to CD96-ECD

To test proper native protein folding a capture ELISA assay was established. The ELISA plate was coated with the CD96-ECD protein. The plate was incubated with the TH-111 or TH-69 irrelevant antibody. TH-111 bound to CD96-ECD while TH-69 presented no binding activity (Fig 3.22). Therefore recombinant CD96-ECD protein is correctly folded.



**Fig 3.22 Binding specificity of the CD96 antibody and the CD96-ECD.** ELISA was performed with ■ TH-111 and ■ TH-69 antibodies. (\*): significant difference in binding activity. One representative experiment out of three is displayed.

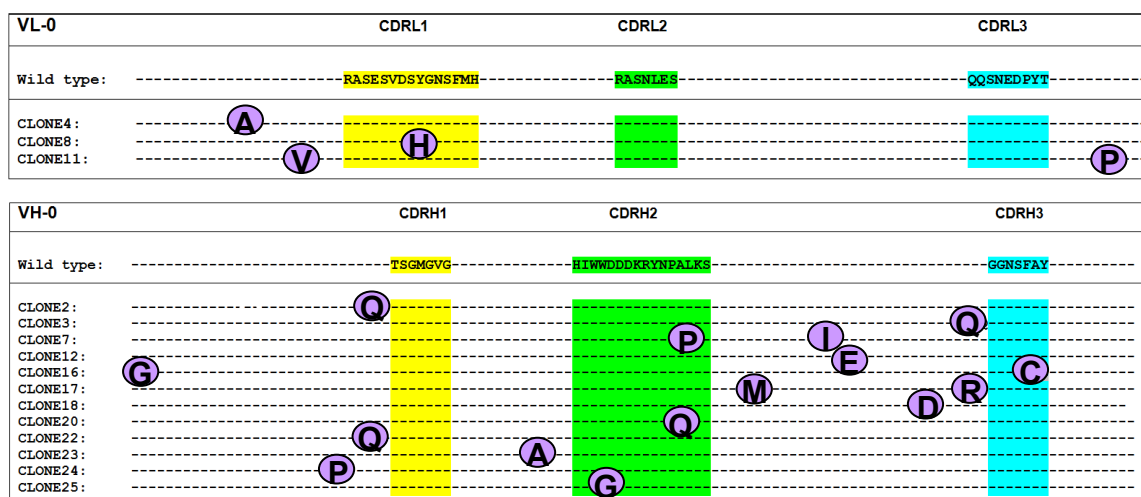
### 3.3.4 Selection of the CD96-scFv mutant library with enhanced binding affinity

The “diversified” phage display library was screened in five rounds using the recombinantly expressed CD96-ECD as antigen under stringent washing conditions to eliminate wild type CD96-scFv. 30 randomly selected clones from the last screening of the “diversified” phage display library were sequenced and compared to sequences obtained from the original library.

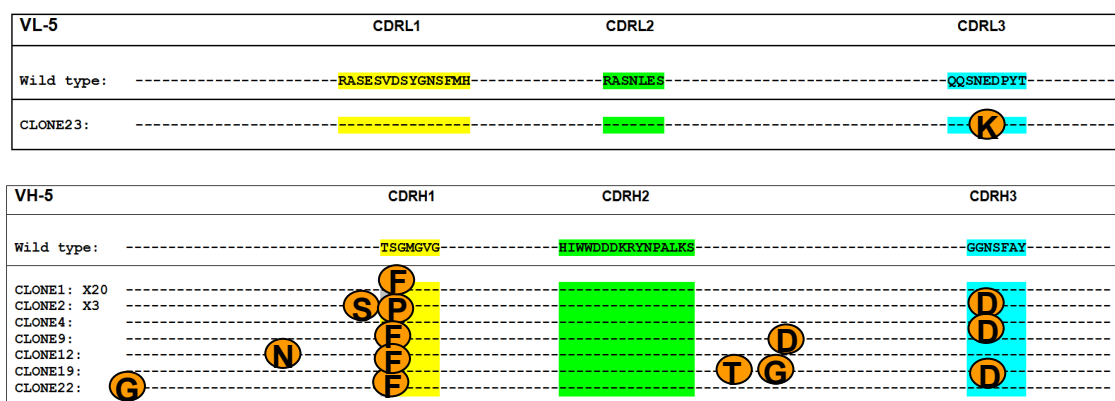
### 3.3.5 Sequence analysis of variable regions of mutated CD96-scFv

Sequence analysis of clones obtained from the original library revealed that 0-4 mutations were introduced in each clone that resulted in amino acid exchanges and that mutation sites were randomly distributed in both VH and VL genes (Fig 3.23).

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**Fig 3.23** Sequence alignment of VL and VH domains of mutated CD96-scFv from the original library. VL-0: variable light chain of clones from the original library. VH-0: variable heavy chain of clones from the original library. The CDRs are defined according the Kabat database. ■ CDR1, ■ CDR2, ■ CDR3, and ■ mutation position. The clones with same mutations were displayed only once.



**Fig 3.24** Sequence alignment of the VL and VH domains of mutated CD96-scFv from the fifth sub-library. VL-5: variable light chain of clones from the fifth sub-library. VH-5: variable heavy chain of clones from the fifth sub-library. The CDRs are defined according the Kabat database. ■ CDR1, ■ CDR2, ■ CDR3, and ■ mutation position. The clones with same mutations were displayed only once.

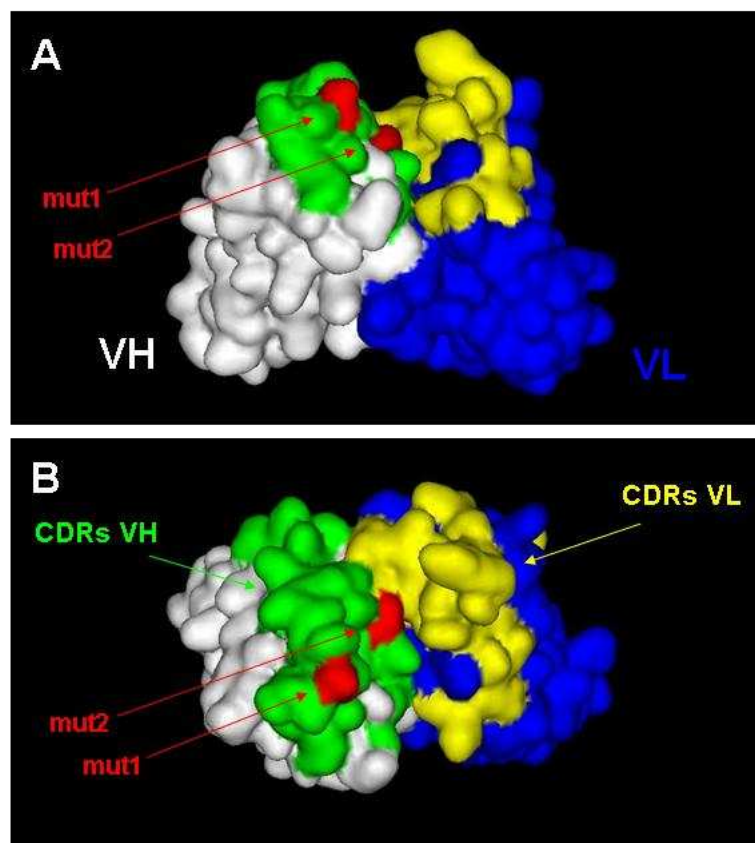
After five round of screening, 29 of 30 clones showed amino acid exchanges (Fig 3.24), while only one wild type clone was picked. This data indicated that wild type scFvs were successfully eliminated during the selection procedure. Most amino acid exchanges were found in VH, in the CDRH1 domain while only one clone with a mutation in the VL domain was found. The most striking mutation was detected at amino acid position H32 (Ser<sup>H32</sup>), which resulted in a serine to phenylalanine substitution (Ser<sup>H32</sup> → Phe<sup>H32</sup>) (S32F). A missense mutation resulting in a serine to proline exchange (Ser<sup>H32</sup> → Pro<sup>H32</sup>) (S32P) was the second most abundant mutation

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at the same amino acid position. In 5 clones, an amino acid exchange in the CDRH3 domain was observed, resulting in an asparagine to aspartic acid substitution ( $\text{Asn}^{\text{H97}} \rightarrow \text{Asp}^{\text{H97}}$ ) (N97D) (Fig 3.24).

#### 3.3.6 Calculated homology model of wild type CD96-scFv

To obtain first indications whether the identified positions in the VH domain may directly contribute to CD96 binding, a homology model of wild type CD96-scFv was calculated (Fig 3.25). This model would predict that two most common positions found to be altered in CDRH1 and CDRH3 are surface exposed, and therefore may directly contribute to CD96 binding. Clone 1, (S32F or mut1), carrying mutation identically found in 19 other clones including the most abundantly detected CDRH1 mutation was chosen for further analysis.

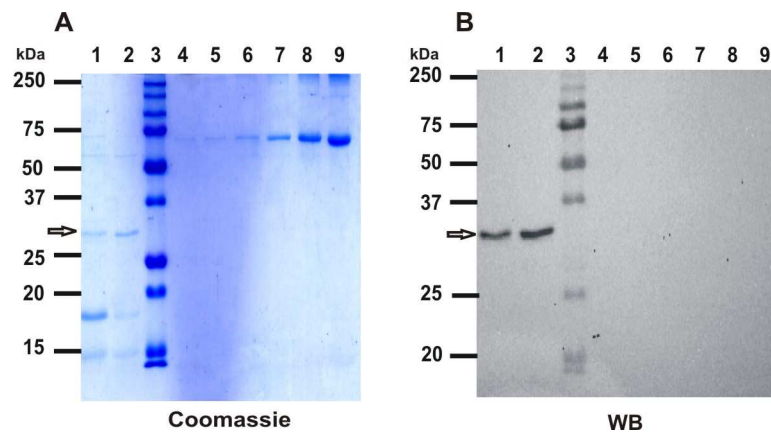


**Fig 3.25** Calculated homology model of wild type CD96-scFv. A) A homology model of wild type CD96-scFv was calculated using the Rosetta server/algorithm (Sircar et al., 2009) and displayed as a surface model using the Accelrys DS Viewer software. B) Top view of the model displayed in (A). The 6 loops defining the CDRs were assigned according to the Kabat numbering scheme. ■ Heavy chain CDRs, ■ light chain CDRs, □ heavy chain frameworks, ■ light chain frameworks, and ■ positions found mutated after screening; mut1 = S32F and mut2 = N97D.

### 3.4 Generation and characterization of monovalent CD96-scFv

#### 3.4.1 Expression and purification of monovalent CD96-scFv

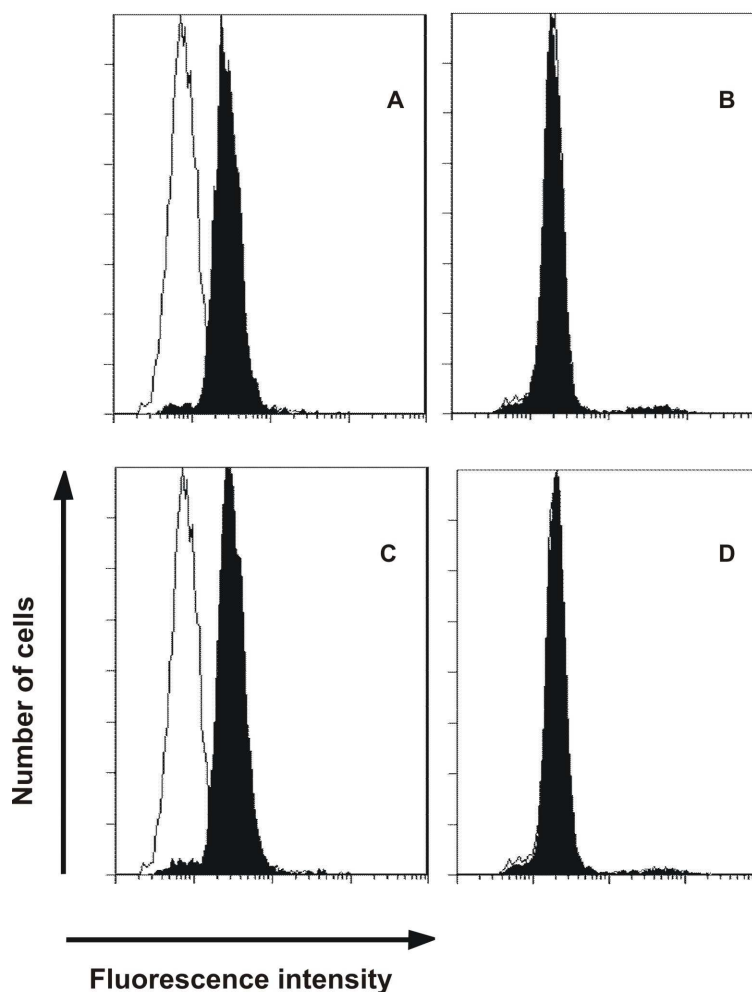
To analyze whether the detected amino acid exchanges resulted in altered/stronger antigen binding, monovalent CD96-scFv, the wild type (CD96-wt-scFv) and the mutated variant (CD96-S32F-scFv) were expressed in *E. coli* cells. Coomassie-stained gel of the purified CD96-scFvs presented the size of proteins corresponded with the expected molecular weights of about 30 kDa (Fig 3.26A). Western blot analysis confirmed the identity of the purified scFvs (Fig 3.26B).



**Fig 3.26 Purification and analysis of monovalent CD96-scFv.** The protein sizes are indicated in kDa. A) Coomassie blue stained SDS-PAGE. B) Western blot. Lane 1: CD96-S32F-scFv, lane 2: CD96-wt-scFv, lane 3: size marker and lanes 4-9: calibration with 0.08, 0.15, 0.3, 0.65, 1.25 and 2.5  $\mu$ g of BSA. Data shown are representative of three independent experiments.

#### 3.4.2 Binding specificity of monovalent CD96-scFv to CD96

The binding activity and specificity of CD96-scFv was analyzed by flow cytometry using CD96-positive HSB-2 cells, and CD96-negative CEM cells as negative control (Fig 3.27). The antigen binding was detected using Penta.His Alexa fluor conjugate. CD96-scFv wild type and mutated variant confirmed the CD96 antigen specific binding and no binding activity to CEM cells was presented. Therefore the wild type and the mutated variant of CD96-scFv could bind specifically to CD96 (Fig 3.27).

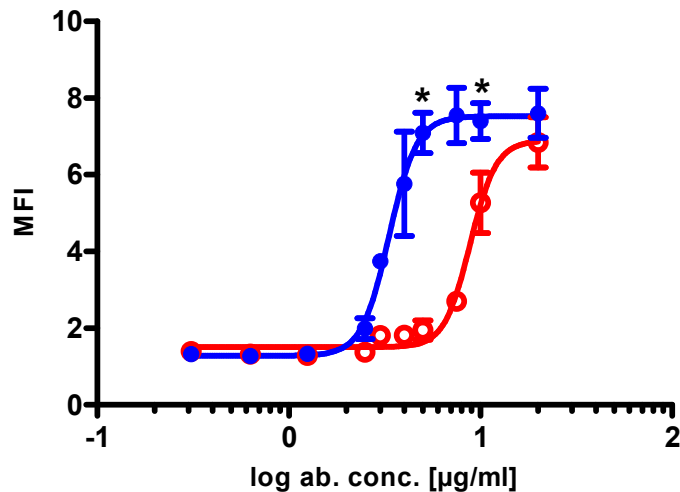


**Fig 3.27** Flow cytometry analysis of monovalent CD96-scFv using HSB-2 and CEM cells. HSB-2 cells were incubated with A) wild type CD96-scFv, C) mutated CD96-scFv and CEM cells were incubated with B) wild type CD96-scFv, D) mutated CD96-scFv. One representative experiment out of three is indicated.

### 3.4.3 Affinity comparison of the monovalent CD96-scFv wild type and the mutated variant

To indicate the comparative binding affinity of the CD96-scFv wild type and the mutated variant to CD96, the HSB-2 cells were incubated with serial dilutions of CD96-wt-scFv and CD96-S32F-scFv and dose-dependent binding of these two antibodies was analyzed. The CD96-S32F-scFv demonstrated about 3-fold enhanced binding ( $EC_{50} = 3.3 \mu\text{g/ml}$ ; 95% confidence interval = 3.0-3.7) compared to CD96-wt-scFvs ( $EC_{50} = 8.9 \mu\text{g/ml}$ ; 95% confidence interval = 8.0-9.8) (Fig 3.28). Therefore mutation of CD96-scFv improved the binding affinity of this antibody to the CD96 antigen.



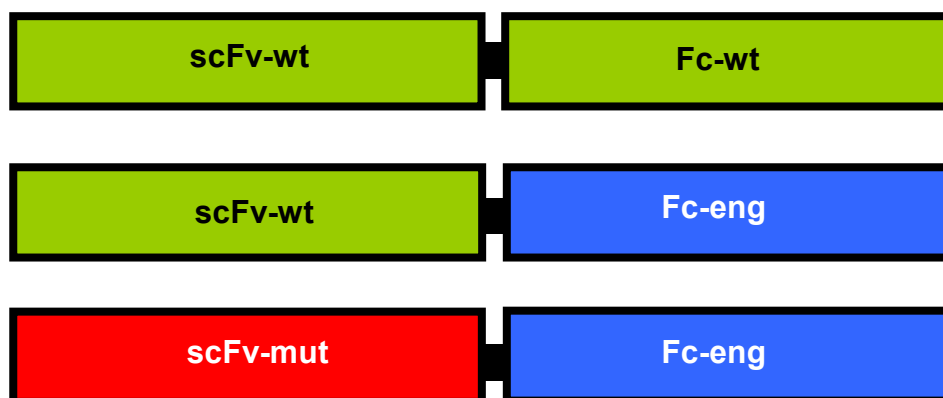


**Fig 3.28** Dose dependent binding analysis of CD96-scFvs to CD96 by flow cytometry using HSB-2 cells. ○ CD96-wt-scFv, ● CD96-S32F-scFv. (\*): significant differences in binding at the indicated dose level. Data are presented as mean values  $\pm$  SEM from three independent experiments.

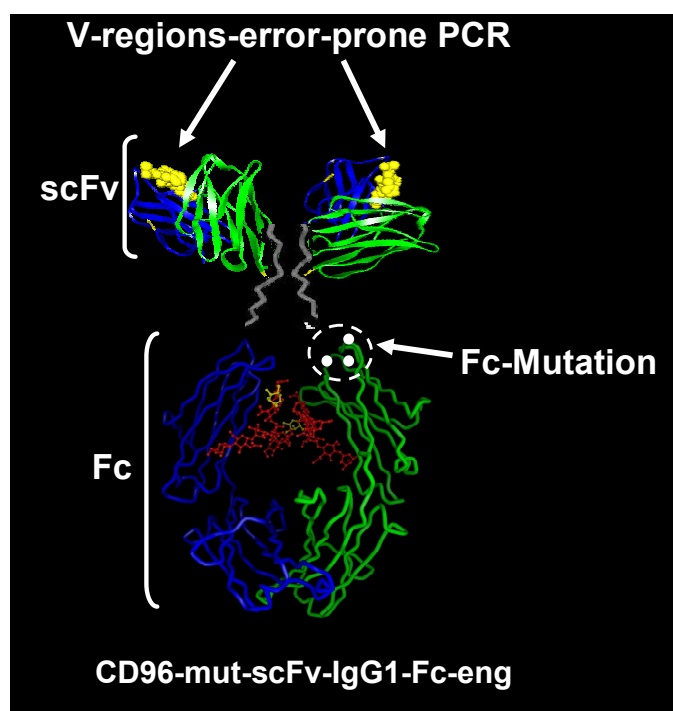
### 3.5 Construction and characterization of bivalent CD96-scFv-IgG1-Fc mini-antibodies

#### 3.5.1 Cloning of CD96-scFv into the engineered IgG1-Fc region (IgG1-Fc-eng)

To increase the ADCC effect of the CD96-scFv-IgG1-Fc antibody, the variable genes of this antibody were mutated to enhance binding affinity of CD96-scFv to CD96 (Fig 3.30). The second approach to increase the ADCC effect of this recombinant antibody was Fc-engineering which enhanced Fc $\gamma$ R binding affinity, leading to improved Fc $\gamma$ R-dependent effector cell function, ADCC (Fig 3.29). The wild type and mutated variant of CD96-scFv were cloned into IgG1-Fc-eng which has three mutation in CH2 and was approved for improved ADCC (Lazar et al., 2006; Repp et al., 2010), resulting CD96-wt-scFv-IgG1-Fc-eng and CD96-S32F-scFv-IgG1-Fc-eng (Fig 3.30).



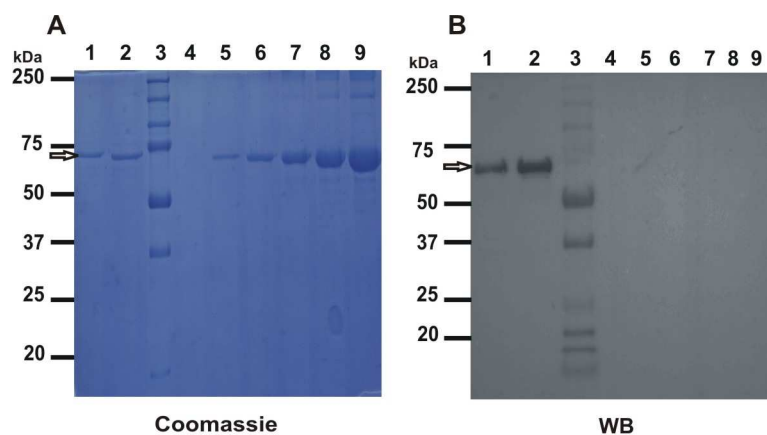
**Fig 3.29 Schematic view of different formats of the CD96-scFv based mini-antibody.** The scFv-wild type and the mutated variant of CD96-scFv were cloned into IgG1-Fc-eng.



**Fig 3.30 Optimizing of ADCC activity of the CD96-scFv-IgG1-Fc antibody.** To increase ADCC activity 1) CD96 variable genes were subjected to error-prone PCR. 2) CD96-scFv was cloned into engineered IgG1-Fc with three mutations in CH2, (S239D/I332E/A330L).

### 3.5.2 Expression and purification of optimized CD96 mini-antibodies

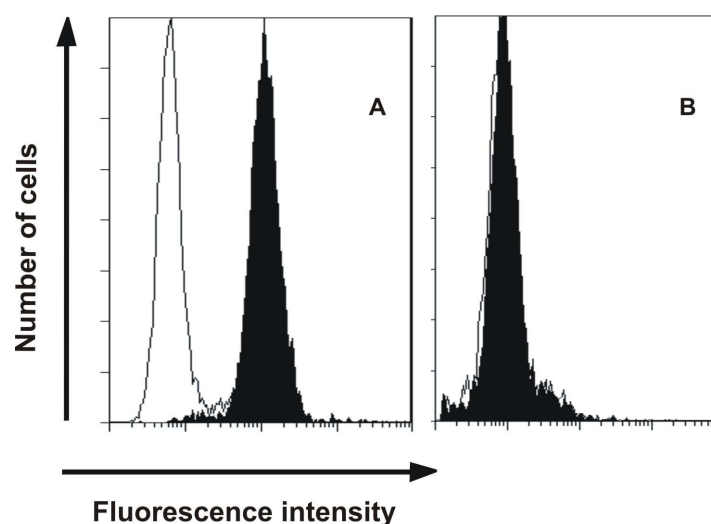
293T cells were transient and stably transfected with CD96-wt-scFv-IgG1-Fc-eng and CD96-S32F-scFv-IgG1-Fc-eng mini-antibodies. Coomassie-stained gel of both purified antibodies presented one prominent band of about 60 kDa roughly corresponding with the calculated molecular weight of 56.6 kDa (Fig 3.31A). Western blot analysis confirmed the identity of the purified scFvs (Fig 3.31B).



**Fig 3.31 Purification and analysis of CD96-scFv-IgG1-Fc.** The protein sizes are indicated in kDa. A) Coomassie blue stained SDS-PAGE gel. B) Western blot. Lane 1: CD96-S32F-scFv-IgG1-Fc-eng, lane 2: CD96-wt-scFv-IgG1-Fc-eng, lane 3: size marker and lanes 4-9: calibration with 0.15, 0.31, 0.65, 1.25, 2.5 and 5  $\mu$ g of BSA. Data shown are representative of five independent experiments.

### 3.5.3 Binding specificity of the mutated CD96-scFv based mini-antibody (CD96-S32F-scFv-IgG1-Fc) to CD96

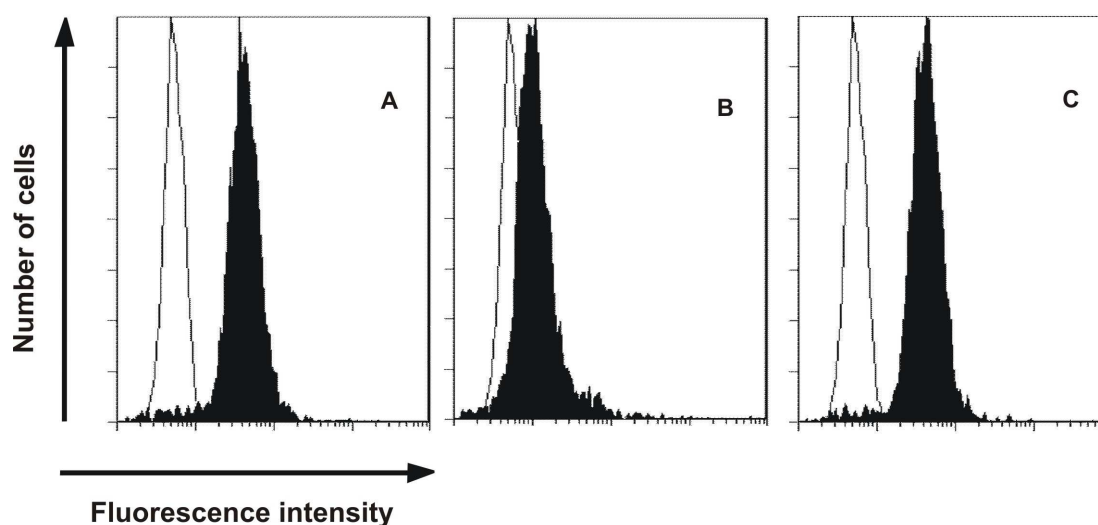
The binding activity and specificity of the CD96-scFv based mini-antibody (CD96-S32F-scFv-IgG1-Fc) was analyzed by flow cytometry using CD96-positive HSB-2 cells (Fig 3.32A), and CD96-negative CEM cells (Fig 3.32B) as negative control. Antigen binding was detected using Penta.His Alexa fluor conjugate. The CD96-scFv-IgG1-Fc antibody confirmed the CD96 antigen specific binding, and no binding activity to CEM cells was observed. Therefore CD96-S32F-scFv-IgG1-Fc could specifically bind to CD96.



**Fig 3.32** Flow cytometry analysis of the recombinant mutated CD96-scFv (CD96-S32F) based mini antibody. A) The HSB-2 cells were incubated with CD96-S32F-scFv-IgG1-Fc and B) The CEM cells were incubated with CD96-S32F-scFv-IgG1-Fc. One representative experiment out of three is indicated.

#### 3.5.4 Binding competition of the mutated CD96-scFv based mini-antibody (CD96-S32F-scFv) and the CD96 monoclonal antibody TH-111 to CD96

To further demonstrate, that during the construction process of optimized-CD96-scFv, the binding specificity of scFv was not altered, the binding competition of CD96-S32F-scFv-IgG1-Fc and the CD96 parental antibody TH-111 to the CD96 antigen was considered. High concentration of the TH-111 antibody inhibited the binding of CD96-S32F-scFv-IgG1-Fc to CD96 (Fig 3.33B), while the TH-69 irrelevant antibody utilized at the same concentration displayed no inhibitory effect (Fig 3.33C).



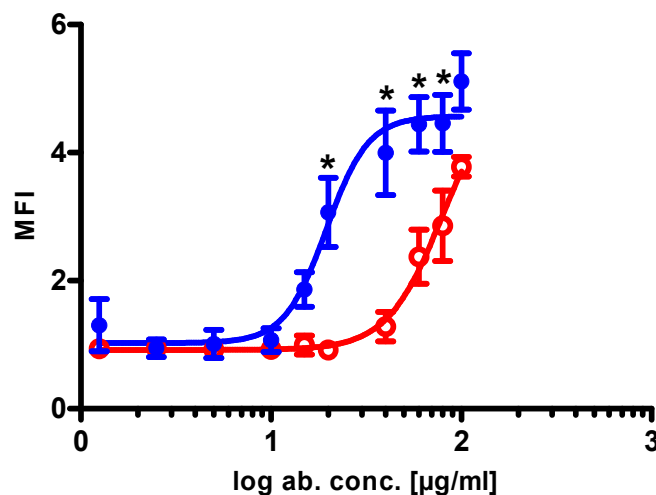
**Fig 3.33** Binding competition of the mutated CD96-scFv (CD96-S32F) based mini antibody and TH-111 to CD96. HSB-2 cells were incubated with A) CD96-S32F-scFv-IgG1-Fc, B) CD96-S32F-

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scFv-IgG1-Fc + TH-111, C) CD96-S32F-scFv-IgG1-Fc + TH-69. One representative experiment out of three is indicated.

#### 3.5.5 Binding comparison of wild type and mutated CD96-scFv-IgG1-Fc mini-antibodies to CD96

To evaluate dose dependent binding of bivalent mini-antibody constructs, wild type and mutated CD96-scFv-IgG1-Fc were compared. HSB-2 cells were incubated with a serial dilution of these antibodies and were analyzed on a flow cytometer. A 4-fold improvement in binding of mutated CD96-scFv-based mini-antibody to CD96 was detected compared to wild type variant (Fig 3.34). CD96-S32F-scFv-IgG1-Fc-eng and CD96-wt-scFv-IgG1-Fc-eng demonstrated EC50-values of 19.5  $\mu\text{g/ml}$  (95% CI = 15.7-24.3) and 79.9  $\mu\text{g/ml}$  (95% CI= 32.8-194.7) respectively, indicating that no avidity effect was observed.



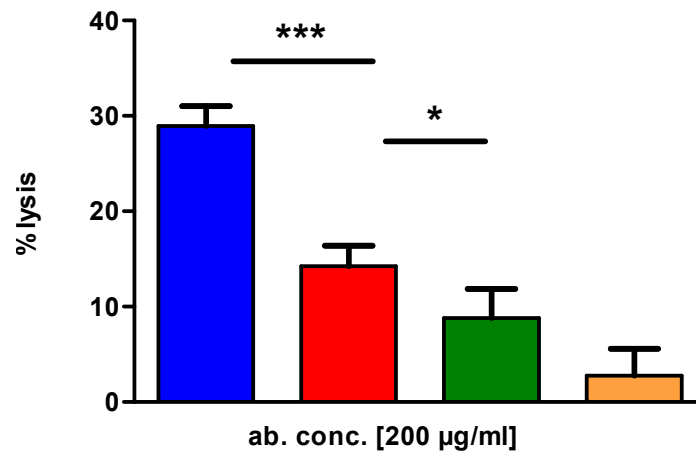
**Fig 3.34** Dose dependent binding analysis of mini-antibodies by flow cytometry using HSB-2 cells.  $\circ$  CD96-wt-scFv-IgG1-Fc,  $\bullet$  CD96-S32F-scFv-IgG1-Fc. (\*): significant differences in binding at the indicated dose level. Data are presented as mean values  $\pm$  SEM from five independent experiments.

#### 3.5.6 Comparison of ADCC activity of CD96 antibodies

The cytolytic properties of recombinant CD96 mini-antibodies were investigated by chromium release cytotoxicity assay with CD96-positive HSB-2 cells and isolated MNCs. The double-engineered CD96 mini-antibody (affinity-maturated scFv and optimized Fc part, CD96-S32F-scFv-IgG1-Fc-eng) proved to be significantly better in mediating tumor cell lysis in comparison to its non-affinity matured counterpart at saturating concentrations, demonstrating that the gain in affinity translated in

### 3. RESULTS

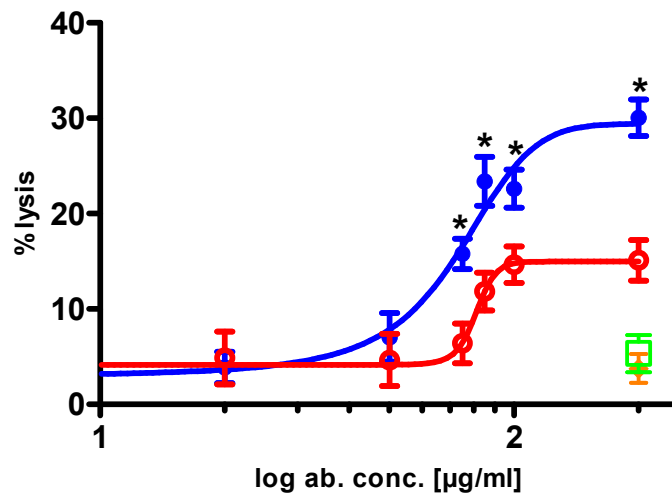
improved ADCC potency. CD96-wt-scFv-IgG1-Fc-eng also performed significantly higher ADCC compared to CD96-wt-scFv-IgG1-Fc. Therefore Fc-engineering alone, leading to enhanced FcγR binding affinity, already improved cytolytic ADCC. The TH-111 monoclonal antibody showed only extremely weak/no ADCC. Briefly, the three variants of the CD96-specific mini-antibody demonstrated cytolytic activity to CD96-positive cells with CD96-S32F-scFv-IgG1-Fc-eng > CD96-wt-scFv-IgG1-Fc-eng > CD96-wt-scFv-IgG1-Fc > TH-111 (Fig 3.35).



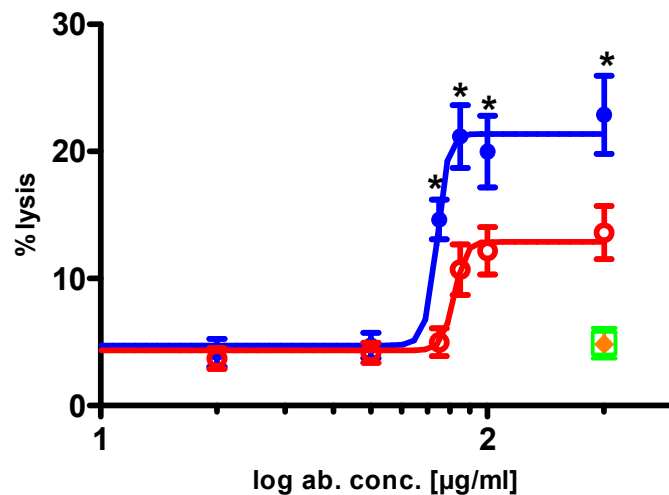
**Fig 3.35 Comparison of ADCC activity of CD96 antibodies.** ADCC experiments with HSB-2 cells were performed using mononuclear cells as effector cells. ■ CD96-S32F-scFv-IgG1-Fc-eng, ■ CD96-wt-scFv-IgG1-Fc-eng, ■ CD96-wt-scFv-IgG1-Fc, and ■ TH-111. (\*): significant differences in ADCC activity. E/T ratio (80:1). Antibody concentration 200 µg/ml. Data are presented as mean values +/- SEM from five independent experiments.

#### 3.5.7 Comparison of dose-dependent ADCC activity of CD96 antibodies

The CD96-positive HSB-2 (Fig 3.36) and KG1a cells (Fig 3.37) were used to compare dose-dependent ADCC activity of CD96 mini-antibodies. The CD96 mini-antibody with mutated scFv, and also engineered Fc (CD96-S32F-scFv-IgG1-Fc-eng), presented better ADCC compared to the antibody format with wild type scFv but the engineered-Fc part (CD96-wt-scFv-IgG1-Fc-eng).



**Fig 3.36 Dose dependent killing of Fc-engineered CD96-mini-antibodies with HSB-2 cells.** ○ CD96-wt-scFv-IgG1-Fc-eng, ● CD96-S32F-scFv-IgG1-Fc-eng, □ CD20-scFv-IgG1-Fc-eng, and ◆ TH-111. (\*): significant differences in ADCC activity. E/T ratio (80:1). Data are presented as mean values +/- SEM from five independent experiments.



**Fig 3.37 Dose dependent killing of Fc-engineered CD96-mini-antibodies with KG1a cells.** ○ CD96-wt-scFv-IgG1-Fc-eng, ● CD96-S32F-scFv-IgG1-Fc-eng, □ CD20-scFv-IgG1-Fc-eng, and ◆ TH-111. (\*): significant differences in ADCC activity. E/T ratio (80:1). Data are presented as mean values +/- SEM from four independent experiments.

**Chapter 4**  
**DISCUSSION**



### 4. DISCUSSION

#### 4.1 CD96 mini-antibody

In the present study, the functional variable regions of a CD96 monoclonal antibody were isolated and a CD96-scFv molecule was generated. To study the effector mechanisms of CD96-scFv, this molecule was cloned into an IgG1-Fc portion and a chimeric CD96-scFv-IgG1-Fc mini-antibody (CD96-scFv-IgG1-Fc) was generated. This mini-antibody bound to CD96 antigen specifically. In addition, the CD96-scFv-based mini-antibody binding to CD96 antigen was inhibited, in the presence of excess monoclonal CD96 antibody. Accordingly the CD96-scFv-IgG1-Fc mini-antibody retained the binding specificity of parental monoclonal antibody. The CD96-scFv-based mini-antibody mediated ADCC only weakly. Consequently *in vitro* affinity maturation and Fc engineering of this antibody showed enhanced ADCC activity. The engineered CD96-scFv mini-antibodies were tested for their ability to elicit ADCC *in vitro*, against two tumor cell lines. The molecule with engineered-Fc and mutated-scFv showed the most effective ADCC. These results demonstrated that, both antigen binding affinity and Fc receptor binding, regulate ADCC activity and obviously persuade the efficacy of ADCC.

#### 4.2 ScFv-Fc characteristics

The CD96 monoclonal mouse antibody, TH-111, was previously generated in our lab. For clinical use of mouse antibodies in humans, the immunogenicity of these antibodies has to be reduced. Furthermore, mouse antibodies are not efficient in recruiting the human immune system and effector functions. Therefore, the CD96 monoclonal mouse antibody was replaced by a recombinant antibody. Recombinant single chain variable fragment (scFvs) is one of the most commonly used formats of recombinant antibodies (Peipp et al., 2004). ScFv and scFv-based antibodies are promising therapeutic and diagnostic alternative to monoclonal antibodies in the treatment of cancer, autoimmune, inflammatory and chronic viral diseases (Weisser and Hall, 2009). The production and purification of these molecules is uncomplicated. They can be expressed in large scales in both prokaryotic and eukaryotic expression systems. The scFv-based mini-antibody displays functional characteristics similar to that of a complete antibody and can mediate effector mechanisms of whole IgG1 molecule. The scFv-Fc fusion protein has a lower molecular weight in comparison to the whole IgG1 molecule. The molecular weight is reduced by roughly one third,

probably shows improved tumor penetrating characteristics. The use of recombinant antibody technology to design scFv-based antibody derivatives may allow further optimization of these therapeutically interesting molecules (Winter and Milstein, 1991). Fc-engineering strategies such as fucose removal and amino acid substitutions, which improve effector mechanisms of complete IgG1 molecules, could enhance the effector mechanisms of mini-antibodies.

In the current study a CD96-scFv-based mini-antibody, which represents one promising new molecule format of therapeutic antibodies, was generated.

### **4.3 Improvement of ADCC by engineering of the CD96-scFv-based mini-antibody**

It has been proposed that tumor cells show resistance to therapeutic antibodies, through different mechanisms. This reduces the efficacy of antibodies for tumor cells lysis (Smith, 2003). To overcome this resistance, the effector functions of therapeutic antibodies can be optimized. Several strategies are known to increase the antitumor efficacy of therapeutic antibodies by enhancing ADCC activity (Smith, 2003; Weiner, 2005; Weiner and Carter, 2005). Consequently *in vitro* affinity maturation of antibody V-genes to improve binding affinity, to target antigens and Fc engineering, to enhance binding affinity to Fc receptors on effector cells have been described, as technologies suitable for enhancing ADCC. In addition to antibody binding affinity, the density of antigen on the tumor cell surface is also an important factor for ADCC efficacy (Tang et al., 2007). A previous study showed that ADCC mediated by trastuzumab, an antibody directed against HER2/*neu*, is associated with the antigen expression levels (Mimura et al., 2005). But high affinity antibodies can mediate efficient ADCC even with cells that express target antigen in relatively low levels.

In the present study, the engineered CD96-scFv-based mini-antibody performed ADCC effectively, even with cells that express CD96 in quite low levels.

#### **4.3.1 Random mutagenesis of CD96 V-genes**

Antibodies which are generated in a primary immune response usually have moderate binding affinities. These affinities are matured/enhanced in a later response, which occurs in antibody V-genes of B cells in germinal centers. This process is known as somatic hypermutation. Hypermutation process introduces point

#### 4. DISCUSSION

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mutations into the variable regions of the heavy and light chain genes, giving rise to mutant B cell receptors on the surface of B cells (Kocks and Rajewsky, 1989). Some of these mutant immunoglobulin molecules can bind to antigen, better than the original B cell receptors. B cells, which express these mutant immunoglobulin molecules, are preferentially selected to mature into antibody secreting cells. This *in vivo* process can be mimicked *in vitro* by random mutation of antibody V-genes (Chowdhury and Pastan, 1999). The selection of mutated molecules with high binding affinity can be performed under stringent selection conditions, through phage display. The most important advantage of random mutagenesis is that, a large number of mutant clones can be generated. A main disadvantage is that, only a few of mutant clones will maintain their antigen binding potential (Miyazaki et al., 1999). In the current study, the entire CD96-scFv was subjected to a random mutagenesis with low mutation rate, through an error-prone PCR. Mutation with high frequency is likely to yield a large number of non-functional clones (Daugherty et al., 2000), therefore the condition for mutation with a low frequency was used. The final phage display library was  $1 \times 10^6$  in size, which had scFvs with different mutations. To select strong binding scFvs and remove wild type scFvs, phage clones were selected by stringent selections through binding to purified CD96-ECD protein and sequential washing process. These washing conditions were optimized for complete removal of wild type scFv-expressing bacteriophages. 30 randomly selected phage clones from the last screening round of the mutated phage display library were sequenced, and compared to sequences obtained from the original library. The clones from the original library had 0-4 mutations in variable genes that resulted in amino acid exchanges and the mutation positions were randomly distributed in both VH and VL genes. After five rounds of screening, 29 of 30 clones had amino acid exchanges, and only one wild type clone was observed. Therefore all wild type scFvs were successfully removed through the selection procedure.

Most amino acid exchanges were in CDRH1 at amino acid position H32 (Ser<sup>H32</sup>), a serine to phenylalanine substitution. Only one clone with a mutation in the VL domain, in CDRL3 was found. These data may indicate that mutations in the VL region resulted in scFv variants with diminished or similar binding characteristics in the range of the wild type scFv and were therefore eliminated during the stringent screening process. In 5 clones, an amino acid exchange in the CDRH3 region was

#### 4. DISCUSSION

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observed, resulting in an asparagine to aspartic acid substitution at position H97 (Asn<sup>H97</sup>).

It seems likely, that exchanges in positions H32 and H97 are important for antibody binding to CD96 antigen. Some previous studies demonstrated that even a single amino acid substitution can dramatically change antigen binding affinity (Sharon, 1990; Xiang et al., 1993). To confirm the importance of these amino acid exchanges, a homology model of wild type CD96-scFv was calculated. This model demonstrated that the two most common positions, which are altered in CDRH1 and CDRH3, are surface exposed and consequently may represent direct interaction sites of antibody and antigen.

In different previous studies, mutations were subjected only in CDRs using different mutagenesis methods. These studies demonstrated the importance of several residues in the CDR loop, which are responsible for improved binding affinity (Webster et al., 1994; Yang et al., 1995; Yelton et al., 1995; Low et al., 1996; Aburatani et al., 2002; Dong et al., 2003; Yuan et al., 2006; Kobayashi et al., 2010). Affinity maturation of the human anti-HER2/neu (c-erbB-2) scFv ,C6.5, produced by site-directed mutagenesis in CDRH3, showed a 320-fold higher affinity to the antigen in comparison to the wild type antibody (Adams et al., 1998). Adams and his colleagues reported the first examination of the affinity effects on tumor targeting by antibody based molecules that bind to the same antigen. They showed that, the degree and specificity of tumor localization in mice, were increased significantly with increasing antigen binding affinity (Adams et al., 1998). Yoon and his colleagues generated, AKA, an anti-TAG-72 humanized antibody. The tumor-associated glycoprotein, (TAG)-72, is expressed in the majority of human adenocarcinomas. This humanized antibody showed an approximate 2-fold lower affinity in comparison to the original murine monoclonal antibody. The affinity of this antibody was increased by random mutagenesis of the CDRH3 domain. The highest affinity variant, 3E8, showed approximate a 22-fold higher affinity and 2-fold improved tumor targeting in comparison to AKA. The antibody with improved affinity showed a 1.7-fold improvement in tumor-specific growth delay and 50% survival rate in comparison to the control in athymic mice bearing human colon adenocarcinoma xenografts (Yoon et al., 2006).

In the current study, random mutations were subjected to V-genes completely. After stringent washing processes, the clones with mutations in CDRs remained. It

confirmed the importance of the CDRs in the antigen binding affinity. The flow cytometry experiments with the most abundantly detected mutant, S32F (Ser<sup>H32</sup> to Phe<sup>H32</sup>), showed an approximate 3-4 fold improved binding to CD96. This improvement in binding affinity of the S32F mutant to CD96 was due to a substitution of a polar amino acid (Ser) to a non-polar amino acid (Phe) in V-regions. This substitution demonstrated that, a hydrophilic surface was replaced with a hydrophobic surface. It was previously explained that hydrophobic interactions are the major driving force for protein-protein binding interactions (Wang et al., 2009). A previous study also showed that increasing buried hydrophobic surface and reducing buried hydrophilic surface is an efficient approach to improve the protein-protein binding affinity (Sammond et al., 2007). Sundberg and his colleagues reported a strong relation between a reduction in buried nonpolar accessible surface and an increase of the free binding energy which induces instability of protein-protein binding (Sundberg et al., 2000). Therefore an enhancement in nonpolar surface area reduces the free energy of binding and improves protein-protein binding stability.

### 4.3.2 Fc-engineering

ADCC of IgG antibodies are mediated by engagement of the Fc region of the antibody with FcγRs of immune effector cells (Lazar et al., 2006). *In vivo* studies with some clinically approved antibodies in γ-chain knock-out mice, which leak activating FcγRs expression, showed no therapeutic activity in such animal models (Clynes et al., 2000). In addition, some clinical studies showed a relation between FcγRs polymorphisms and enhanced FcγR-dependent effector functions, such as ADCC. This relation was particularly defined in cancer patients homozygous for the FcγRIIIa-V158 allele treated with therapeutic antibodies such as rituximab (Weng and Levy, 2003) and trastuzumab (Musolino et al., 2008). FcγRIIIa, (CD16a), is a main ADCC triggering receptor in NK cells and FcγRIIIa-V158 allele is the high affinity allotype of FcγRIIIa for IgG1-Fc portion. The relation between this polymorphism and the clinical responses in some other diseases, such as Crohn's disease patients treated with anti-TNF-α infliximab and pregnant women with fetal hemolytic disease treated with anti-RhD, was also confirmed (Louis et al., 2004; Miescher et al., 2004). Therefore Fc-mediated ADCC enhancement technology seems to play an important role in the development of therapeutic antibodies (Natsume et al., 2009).

ADCC can be improved by engineering the coding of amino acid sequences of the constant regions of antibodies (Shields et al., 2001; Aburatani et al., 2002; Okazaki et al., 2004; Lazar et al., 2006) or by altering the glycosylation profile of antibodies (Shields et al., 2002; Okazaki et al., 2004; Peipp et al., 2008b), which improves binding affinity to FcγRIIIa in NK cells.

Different research groups produced optimized-Fc variants with enhanced binding affinity to FcγRIIIa. The Xencor group, generated Fc variants with strong FcγRIIIa binding and enhanced cytotoxic efficacy in cynomolgus monkeys, using a computational design algorithm (Lazar et al., 2006). The Genentech group, developed Fc variants, including up to three mutations which showed enhanced ADCC, using a comprehensive alanine scanning method (Shields et al., 2001). The MacroGenics group, produced Fc mutants with enhanced ADCC, using a yeast display system (Stavenhagen et al., 2007; Stavenhagen et al., 2008).

In the present study a protein-engineered IgG1-Fc variant was utilized to improve NK cell mediated killing of scFv-based mini-antibodies. This protein-engineered IgG1-Fc variant, with three mutations (S239D/I332E/A330L) in CH2 of the Fc region, was previously reported as a variant with high binding affinity to FcγRIIIa in NK cells and improved ADCC performance (Lazar et al., 2006; Repp et al., 2010). The CD96-scFv-based mini-antibody with protein-engineered IgG1-Fc variant, performed a higher ADCC compared to mini-antibody with a non-engineered IgG1-Fc variant, but the CD96-scFv mini-antibody, with double engineering, optimized-Fc and mutated-scFv, showed the most effective ADCC. In conclusion, the ADCC activity of the Fc-engineered mini-antibody was further improved when the affinity matured scFv was utilized. This confirmed other studies which demonstrated that ADCC activity correlates with antibody binding affinity (Tang et al., 2007).

### 5. OUTLOOK

#### 5.1 CD96 mini-antibody in clinical trials

CD96 antibody could be an excellent antibody-based therapeutic agent against AML stem cells and AML blasts. As CD96 is expressed on normal hematopoietic stem cells in a very low level, the CD96 antibody could be used for *ex vivo* purging or FACS selection of AML stem cells from autologous stem cell preparations. This antibody could be used also for *in vivo* targeting of AML stem cells and blasts in AML patients (Hosen et al., 2007).

#### 5.2 CD96 mini-antibody ADCC activity *in vivo*

A further analysis of a large number of AML samples will be required to fully clarify the ADCC activity of CD96 mini-antibodies with freshly prepared CD96<sup>+</sup> AML cells from AML patients. Antibody activity *in vitro* can not predict activity *in vivo*. In addition, the response to antibody in different patients is depended to various factors such as antigen density on target cells, levels of circulating Ig and the function of the effector cells. Some antibodies perform effective ADCC *in vitro* but are not efficient in clinical trials. Rituximab performs ADCC and CDC *in vitro*, while the main determinants of its clinical efficacy have not been defined (Eccles, 2000). The xenotransplantation experiments could reveal *in vivo* anti-tumor effect of CD96 mini-antibodies.

#### 5.3 CD96 expression in other hematopoietic cells or nonhematopoietic tissues

CD96 is not expressed in RBCs, monocytes, granulocytes, and B cells (Wang et al., 1992; Gramatzki et al., 1998). CD96 is only weakly expressed in peripheral T cells, and this expression increases in activated T cells (Wang et al., 1992). This antigen was found on a major subset of T-cell acute lymphoblastic leukemia (T-ALL) (Gramatzki et al., 1998; Burger et al., 1999). CD96 has been reported to be expressed in peripheral NK cells at a expression level even lower than peripheral T cells (Wang et al., 1992). CD96 expressed on NK cells has been indicated as a ligand for CD155 (polio virus receptor), and mediates adhesion of NK cells to tumor cells, thereby it modulates the effector function of NK cells (Fuchs et al., 2004). For *in vivo* use of the CD96 antibody, the expression of CD96 in T and NK cells has to be considered.

CD96 is expressed on AML-LSCs (Hosen et al., 2007) and on the majority of blasts in about 30% of patients (Gramatzki et al., 1998). To date only a few potential target antigens for AML stem cells have been determined. These include CD33 (Bernstein et al., 1992), CD123 (Sievers et al., 1999; Jordan et al., 2000), CD44 (Jin et al., 2006) and CD47 (Majeti et al., 2009). Most of these target antigens have some disadvantages, such as expression on normal hematopoietic stem cells. But CD96 is expressed in very low levels in normal hematopoietic stem cell. For clinical uses of CD96 mini-antibodies, a further analysis such as *ex vivo* purging, colony assays and AML stem cells transplantation in mice will be required to completely demonstrate that these antibodies do not impair normal hematopoietic cells.

In nonhematopoietic tissues, CD96 is expressed in the convoluted tubular epithelium of the kidney, in the mucosal epithelium of the small intestine, in the mucosal epithelium and the vascular endothelium of the large intestine (Gramatzki et al., 1998). Expression of CD96 on the surface of vascular endothelium maybe problematic for clinical use, potentially leading to vascular damage, causing capillary leak syndrome. The expression on mucosal epithelium maybe less problematic because of the inaccessibility of antibodies to the luminal side of the intestine. In order to determine the potential disadvantages of CD96 mini-antibodies in clinical uses and to show the expression level of CD96 in other potential targets, immunohistochemical staining of different tissues is essential.

In addition, human CD96 is expressed in two splice variants, variant 1 and variant 2. Variant 2 predominates in all the CD96<sup>+</sup> cells and tissues, but some AML tumor samples express variant 1 of CD96 (Meyer et al., 2009). The variant of CD96<sup>+</sup> AML stem cells should also be determined. If the AML stem cells express variant 1, an antibody specific against variant 1 can be produced and used for efficient targeting of AML stem cells.

### **5.4 Humanized antibody**

The chimeric antibodies possess a fully human Fc portion. Therefore, these antibodies are significantly less immunogenic in humans and are able to interact with human effector cells and the complement cascade. The use of chimeric antibodies significantly reduced the HAMA responses of mouse antibodies but did not eliminate them completely. Therefore the probability of HAMA responses, shown in multiple clinical trials, must be considered (Kuus-Reichel et al., 1994; Pimm, 1994). In



## 5. OUTLOOK

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addition, for certain targets, fully humanized antibodies are necessary to achieve maximal efficacy.

### **CONCLUSION**

CD96 was reported as an AML stem cell marker. It was also detected on the majority of leukemic blasts in about 30% of AML patients. Therefore, CD96 could be an efficient antigen to target AML stem cells and blasts. The CD96 mouse monoclonal antibody, (TH-111), was previously generated in our lab. To reduce the disadvantages of mouse antibody, the variable regions of this antibody were isolated and a CD96-scFv was generated by phage display. For functional analysis of this antibody, CD96-scFv was cloned into a human Fc-IgG1 portion. The CD96-scFv-based mini-antibody (CD96-scFv-IgG1-Fc) mediated ADCC only weakly. Two approaches were used to enhance the ADCC efficacy: random mutagenesis of CD96-V-genes, and Fc-engineering. The CD96 mini-antibody with mutated-scFv, and an engineered-IgG1-Fc variant (CD96-S32F-scFv-Fc-IgG1-eng), proved to be more effective in mediating tumor cell lysis, compared to other formats of this antibody. The CD96 mini-antibody with wild type scFv and an engineered-IgG1-Fc variant (CD96-wt-scFv-IgG1-Fc-eng), also showed significantly higher ADCC activity compared to the CD96 mini-antibody with wild type scFv, and wild type IgG1-Fc (CD96-wt-scFv-IgG1-Fc). In conclusion, these data proved that ADCC could be triggered by CD96-specific antibodies. This triggering was improved by antibody engineering. Further experiments can demonstrate whether CD96 mini-antibodies are able to eliminate AML leukemic stem cells.

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## CURRICULUM VITAE

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### Personal information

Place of birth: Noshahr; Iran



### Education and employment

2007-2010 PhD student in cell biology, Division of Stem Cell Transplantation and Immunotherapy, University Hospital, Kiel, Germany  
Supervisors: Prof. Dr. Dr. h.c. Thomas Bosch and Priv.-Doz. Dr. Roland Repp

Title of PhD thesis: Improved Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) of Affinity Maturated and Fc-Engineered Antibodies Directed Against the AML Stem Cell Antigen CD96.

2004-2006 Institute of Hematopathology, University Hospital, Kiel, Germany  
Supervisor: Prof. Dr. Dr. h.c. Reza Parwaresch (†)

2001-2004 Instructor of cell biology, Azad University, Tehran, Iran

1999-2001 Master of science in Developmental Biology, Azad University, Tehran, Iran

Title of master thesis: Morphological and Histopathological Effects of Morphine on Development of Prenatal Rat.

Grade: very good

1995-1999 Bachelor of Science in Zoology, Shahid Beheshti University, Tehran, Iran

Grade: very good

### **Scholarships**

- 2008-2010: FAZIT foundation, Frankfurt am Main, Germany
- 2006-2007: University Hospital, Schleswig-Holstein, Kiel, Germany
- 2005-2006: KAAD foundation (Catholic academic foreigners service), Bonn, Germany

### **Publications**

- **Mohseni Nodehi, S.**, Kellner, C., Bräutigam, J., Staudinger, M., Sheiding, A., Repp, R., Peipp, M., Gramatzki, M., Humpe, A. Enhanced ADCC of Affinity Maturated and Fc-Engineered Mini-Antibodies Directed against the AML Stem Cell Antigen CD96. (in preparation)
- Repp, R., Kellner, C., Muskulus, A., Staudinger, M., **Mohseni Nodehi, S.**, Glorius, P., Akramiene, D., Dechant, M., Fey, G.H., van Berkel, P., van de Winkel, J.G.J., Parren, P.W.H.I., Gramatzki, M., Peipp, M. Combined Fc-protein- and Fc-Glyco-Engineering of Single-Chain Mini-Antibodies Synergistically Enhances CD16a Binding but does not Further Enhance NK-Cell Mediated ADCC. (submitted to the Journal of Biological Chemistry)
- Peipp, M., Ehlert, C., Staudinger, M., Muskulus, A., Kellner, C., **Mohseni Nodehi, S.**, Glorius, P., Dechant, M., Burger, M., Brünke, J., Fey, G.H., Gramatzki, M., Repp, R. In vitro Characterization of a Novel Recombinant Bispecific Antibody Targeting HM1.24 on Multiple Myeloma. (in Revision by Mabs)
- Kellner, C., Hallack, D., Glorius, P., Staudinger, M., **Mohseni Nodehi, S.**, de Weers, M., van de Winkel, J.G.J., Parren, P.W.H.I., Valerius, T., Repp, R., Humpe, A., Gramatzki, M., Peipp, M. Fusion Proteins between Ligands for NKG2D and CD20-Directed Single Chain Variable Fragments Sensitize Lymphoma Cells for Natural Killer Cell-Mediated Lysis. (submitted to Blood)

### **Book chapter**

- Kellner, C., **Mohseni Nodehi, S** and Peipp, M., 2010, Mouse Immune Libraries for the Generation of ScFv Fragments Directed Against Human Cell Surface Antigens. Antibody Engineering, 47-63.



### **Oral presentations**

**Mohseni Nodehi, S.**, Staudinger, M., Kellner, C., von Below, B., Repp, R., Peipp, M., Gramatzki, M., Humpe, A. Effektormechanismen affinitätsgereifter und Fc-optimierter Mini-Antikörper gegen das AML Stammzellantigen CD96. Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaften für Hämatologie und Onkologie, October 2010, Berlin, Germany.

**Mohseni Nodehi, S;** Azarnia, M; Minaee Zangi, B; Sahraie, H. Morphine Consumption Induces Sever Morphological and Quantitative Changes in the Rat Embryo. 14<sup>th</sup> international congress of geographic medicine and 15<sup>th</sup> Iranian congress of physiology and pharmacology, November 2001, Shiraz, Iran.

### **Posters**

- Fusion Proteins of Ligands for NKG2D and CD20-Directed ScFvs Sensitize Lymphomas for NK Cell-Mediated Lysis, December 2010, Orlando, USA.
- A Novel Recombinant Bispecific Antibody Targeting HM1.24 Multiple Myeloma, 14<sup>th</sup> congress of the EHA, June 2009, Berlin, Germany.
- Investigation on the Teratogenic Effects in Development of Rat Embryos by using Scanning Electron Microscope. 5<sup>th</sup> international Iranian congress of anatomical sciences, November 2001, Tehran, Iran.
- The study of the Effects of Morphine on the Liver Histomorphological Changes in Rat Embryo. Eurotox 2001, September 2001, Istanbul, Turkey.
- The study of the Oral Morphine Consumption on Fetal Growth and Development in Addicted Rats. Eurotox 2001, September 2001, Istanbul, Turkey.
- The Effects of Male Addiction to Morphine on the Embryo Development in Rats. Eurotox 2001, September 2001, Istanbul, Turkey.

"First they ignore you, then they laugh at you, then they fight you, then you win."

Mahatma Gandhi