Dual Antigen-Restricted Complementation of a Two-Part Trispecific Antibody for Targeted Immunotherapy of Blood Cancer

Von zwei Antigenen abhängige Komplementierung eines zweiteiligen trispezifischen Antikörpers zur gezielten Immuntherapie von Blutkrebs

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vorgelegt von

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Declaration

I hereby assure that I have carried out the presented study independently and without using any other sources or help, except where stated.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

I further declare that I have not acquired or tried to acquire any other academic degrees, except my diploma degree.

Würzburg, 20 September 2013

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1 Abstract

Cancer cells frequently escape from immune surveillance by down-regulating two important components of the immune defence: antigen-presenting MHC and costimulatory molecules. Therefore several novel anti-tumour compounds that aim to assist the immune system in recognising and fighting cancer are currently under development. Recombinant bispecific antibodies represent one group of such novel therapeutics. They target two different antigens and recruit cytotoxic effector cells to tumour cells. For cancer immunotherapy, bispecific T cell-engaging antibodies are already well characterised. These antibodies target a tumour-associated antigen and CD3ε, the constant molecule of the T cell receptor complex.

On the one hand, this study presents the development of a bispecific antibody targeting CD3ε and the rhabdomyosarcoma-associated fetal acetylcholine receptor. On the other hand, it describes a novel two-part trispecific antibody format for the treatment of leukaemia and other haematological malignancies in the context of haematopoietic stem cell transplantation (HSCT).

For HSCT, an HLA-identical donor is preferred, but very rarely available. In an HLA-mismatched setting, the HLA disparity could be exploited for targeted cancer treatment. In the present study, a two-part trispecific HLA-A2 × CD45 × CD3 antibody was developed for potential cases in which the patient is HLA-A2-positive, but the donor is not. This holds true for about half the cases in Germany, since HLA-A2 is the most common HLA molecule found here. Combinatorial targeting of HLA-A2 and the leucocyte-common antigen CD45 allows for highly specific dual-antigen restricted tumour targeting.

More precisely, two single-chain antibody constructs were developed: i) a single-chain variable fragment (scFv) specific for HLA-A2, and ii) a scFv against CD45, both linked to the V_L and the V_H domain of a CD3ε-specific antibody, respectively. It turned out that, after the concomitant binding of these constructs to the same HLA-A2- and CD45-expressing cell, the unpaired variable domains of a CD3ε-specific antibody assembled to a functional scFv. In a therapeutic situation, this assembly should exclusively occur on the recipient's blood cancer cells, leading to T cell-mediated cancer cell destruction. In this way, a relapse of disease might be prevented, and standard therapy (radiation and chemotherapy) might be omitted.

For both approaches, the antibody constructs were periplasmically expressed in *E. coli*, purified via His tag, and biochemically characterised. Their binding to the respective targets was proven by flow cytometry. The stimulatory properties of the antibodies were assayed by measuring IL-2 release after incubation with T cells and antigen-expressing target cells. Both the bispecific antibody against rhabdomyosarcoma and the

assembled trispecific antibody against blood cancer mediated T-cell activation in a concentration-dependent manner at nanomolar concentrations. For the trispecific antibody, this effect indeed proved to be dual antigen-restricted, as it could be blocked by prior incubation of either HLA-A2- or CD45-specific scFv and did not occur on single-positive ($CD45^+$) or double-negative ($HLA\text{-}A2^-$ $CD45^-$) target cells. Furthermore, antibodies from both approaches recruited T cells for tumour cell destruction *in vitro*.

Kurzbeschreibung

Krebszellen entgehen der Immunüberwachung oftmals dadurch, dass sie zwei wichtige Komponenten der Immunabwehr, nämlich antigenpräsentierende MHC- und kostimulatorische Moleküle, herunter regeln. Zurzeit befindet sich daher eine Reihe neuartiger Anti-Krebs-Substanzen in der Entwicklung, die darauf abzielen, das Immunsystem beim Erkennen und Bekämpfen von Krebs zu unterstützen. Rekombinante bispezifische Antikörper stellen eine Gruppe solch neuartiger Therapeutika dar. Sie erkennen zwei unterschiedliche Antigene und rekrutieren gezielt zytotoxische Effektorzellen zu Tumorzellen. Zur Krebsimmuntherapie sind BiTE-Antikörper (*bispecific T cell engager*) bereits gut untersucht. Diese Antikörper sind gegen ein tumorassoziiertes Antigen sowie gegen CD3ε, das konstante Molekül des T-Zell-Rezeptor-Komplexes, gerichtet.

Diese Arbeit beschreibt zum einen die Entwicklung eines bispezifischen Antikörpers, der CD3ε und den mit Rhabdomyosarkom assoziierten fetalen Acetylcholinrezeptor erkennt. Zum anderen präsentiert sie ein neues, zweiteiliges trispezifisches Antikörperformat, das zur Behandlung von Leukämie und anderen bösartigen Erkrankungen des blutbildenden Systems im Zusammenhang mit hämatopoetischer Stammzelltransplantation (HSZT) genutzt werden könnte.

Für eine HSZT wird ein HLA-identischer Spender bevorzugt. Dieser steht jedoch nur sehr selten zur Verfügung. In Fällen mit nur einer Unstimmigkeit in den HLA-Merkmalen zwischen Patient und Spender könnte diese HLA-Unstimmigkeit nun zur gezielten Krebsbehandlung ausgenutzt werden. In dieser Arbeit wurde ein trispezifisches HLA-A2 × CD45 × CD3 Antikörperkonstrukt speziell für solche Fälle entwickelt, in denen der Patient HLA-A2-positiv ist, der Spender jedoch nicht. Dies trifft in Deutschland auf ungefähr die Hälfte aller Fälle zu, da HLA-A2 hier als häufigstes HLA-Molekül vorkommt. Mit der Kombination aus HLA-A2 und dem Pan-Leukozytenmarker CD45 (*leucocyte-common antigen*) als Ziel, wird eine hochspezifische, von zwei Antigenen abhängige, zielgerichtete Tumoransteuerung (*tumour targeting*) möglich.

Genauer gesagt wurden zwei Einzelketten-Antikörperkonstrukte entwickelt: i) ein HLA-A2-spezifisches *single-chain variable fragment* (scFv) und ii) ein CD45-spezifisches scFv, jeweils verbunden mit der V_L- bzw. der V_H-Domäne eines CD3ε-spezifischen Antikörpers. Es stellte sich heraus, dass nach gleichzeitiger Bindung der beiden Konstrukte an dieselbe HLA-A2- und CD45-exprimierende Zelle sich die beiden einzelnen, ungepaarten variablen Domänen eines CD3ε-spezifischen Antikörpers zu einem funktionellen scFv zusammenfügen. Dieses Zusammenfügen sollte in einer therapeutischen Situation ausschließlich auf den Blutkrebszellen des Empfängers

geschehen, was zur T-Zell-vermittelten Zerstörung der Krebszellen führen würde. Auf diese Weise könnte ein Rückfall der Erkrankung vermieden und eventuell sogar auf die Standardtherapie (Bestrahlung und Chemotherapie) verzichtet werden.

Für die beiden beschriebenen Ansätze wurden die Antikörperkonstrukte periplasmatisch in *E. coli* exprimiert, über einen His-Tag aufgereinigt und biochemisch charakterisiert. Ihre Bindung an die jeweiligen Zielantigene wurde mittels Durchflusszytometrie nachgewiesen. Die stimulatorischen Eigenschaften der Antikörper wurden durch eine Messung der IL-2-Freisetzung nach Inkubation zusammen mit T-Zellen und antigenexprimierenden Zielzellen untersucht. Sowohl der gegen Rhabdomyosarkom gerichtete BiTE-Antikörper, als auch der zusammengefügte trispezifische Antikörper gegen Blutkrebs vermittelten konzentrationsabhängig eine T-Zellaktivierung bei nanomolaren Konzentrationen. Für den trispezifischen Antikörper erwies sich dieser Effekt tatsächlich als abhängig von zwei Antigenen, da er durch eine vorausgehende Inkubation mit entweder einem HLA-A2- oder einem CD45-spezifischen scFv-Fragment geblockt werden konnte und nicht auf Zellen auftrat, die nur ein Antigen ($CD45^+$) oder keins von beiden ($HLA\text{-}A2^-$ $CD45^-$) tragen. Darüber hinaus rekrutierten die Antikörper beider Ansätze T-Zellen zur Zerstörung von Tumorzellen *in vitro*.

2 Introduction

2.1 Cancer Prevalence and Motivation for the Project

Worldwide, an estimated 12.7 million new cancer cases were diagnosed in 2008 [Jemal *et al.*, 2011]. Being a leading cause of death, cancer accounted for 7.6 million deaths around the world, which corresponds to 13 % of all human deaths, in 2008. In Germany, cancer is the second most common cause of death right after cardiovascular diseases. The latest appropriate data of the so-called cancer atlas of the Federal Republic of Germany provided by the German Cancer Research Centre indicate that of all deceased in 2010, 22 % of women and 29 % of men died due to cancer [Becker and Wahrendorf, 1998; update on internet www.krebsatlas.de]. The most common cancers are principally identical for both sexes. The leading causes of cancer deaths in females were breast cancer (19 %), lung cancer (16 %), and colorectal cancer (10 %). For males, lung cancer (26 %), colorectal cancer (11 %), and prostate cancer (9 %) were the most frequent. Haematological malignancies, including leukaemias, lymphomas, and multiple myelomas, were diagnosed in approximately 35,000 cases, which accounts for 13.6 % of new cancer diagnoses, leading to about 17,600 deaths (8 %) [GEKID atlas on internet www.gekid.de]. On account of the increasing average life expectancy of the population in developed countries, the overall cancer mortality rates will continue to rise. For years, blood cancer and other cancers have been mostly treated with chemotherapy and radiation. Unfortunately, these conventional treatments of cancer can have a negative effect on normal cells. Due to their lacking selectivity for tumour tissue and their mainly cytotoxic and cytostatic manner of acting on proliferating tissues, such treatments often cause severe side effects, thus limiting the administrable dose and, in turn, the therapeutical efficiency. Therefore, targeted therapies are gaining ground. These aim to target the tumour tissue specifically while leaving the healthy tissue unaffected, and ideally induce an immune response against the tumour.

2.2 Targets in Tumour Therapy

Tumour cells can express unique antigens and developmentally down-regulated antigens, which can be recognised by the immune system either by specific immune effector cells and / or by antibodies. Thus, the immune system is able to distinguish between normal and malignant cells. For use as targeted cancer therapy, a number of potential antigens have been identified. Initially, two main categories of such targets have been classified: tumour-specific antigens (TSA) and tumour-associated antigens (TAA). TSA are only expressed on tumour cells. TAA are overexpressed on tumour

cells, but are also found on some healthy cells, albeit to a lesser extent. However, over time many tumour-specific antigens have been found on healthy cells as well, so that TAA, although of heterogeneous nature, are nowadays subdivided into four major groups by their expression pattern: unique antigens (including mutation antigens and viral antigens), cancer/testis antigens, differentiation antigens, and overexpressed antigens [Gires and Seliger, 2009].

One of the major limitations of using tumour cells as targets in tumour therapy is the necessity of the presence of tumour markers which are not expressed on normal tissue. On that account, possible targets should be chosen very carefully.

2.2.1 Fetal Acetylcholine Receptor and Rhabdomyosarcoma

The nicotinic acetylcholine receptor (AChR) is a transmembrane protein, more precisely a cholinergic receptor that forms an ion channel [Silbernagl and Despopoulos, 2003]. It is located at the neuromuscular junctions on the postsynaptic side and is activated by the binding of the neurotransmitter acetylcholine – or nicotine – across the synapse. Binding of acetylcholine causes conformational changes and thus an opening of the pore, which allows Na^+ and K^+ ions to diffuse through the receptor, leading to a depolarisation of the muscle fibre, the so-called end-plate potential. On that account, voltage-gated Na^+ channels are opened, and – when a threshold is reached – an action potential and finally muscular contraction occurs.

In humans, the AChR occurs in two isoforms: in a fetal isoform (fAChR) or in an adult isoform [Mishina *et al.*, 1986]. The AChR is a glycosylated protein of 290 kDa with a total length of about 16 nm. It is composed of five separate but structurally related subunits: two α, one β, one δ, and one ε (adult) or γ (fetal) subunit [Unwin, 2000; 2005] (Fig. 2.1). Its pentameric structure is further divided into three domains: a large N-terminal extracellular ligand-binding domain, a membrane-spanning pore, and a smaller intracellular domain [Unwin, 2005]. In the course of the development of the neuromuscular junction, the γ subunit of the fetal AChR ($\alpha_2\beta\gamma\delta$) is exchanged by an ε subunit, resulting in the mature AChR subtype ($\alpha_2\beta\varepsilon\delta$) [Mishina *et al.*, 1986; Witzemann *et al.*, 1987]. This gradual transition is genetically controlled [Missias *et al.*, 1996]. After the first postnatal week, the mature AChR is found in nearly all innervated muscles, while the fAChR is exclusively expressed into adulthood on a few tissues, including extraocular muscle [Horton *et al.*, 1993; Kaminski *et al.*, 1996] and thymic myoid cells [Schluep *et al.*, 1987; Marx *et al.*, 1989]. This normal expression aside, a high expression of fAChR was found on the vast majority of human rhabdomyosarcomas [Gattenloehner *et al.*, 1998], making fAChR a promising tumour target for immunotherapy.

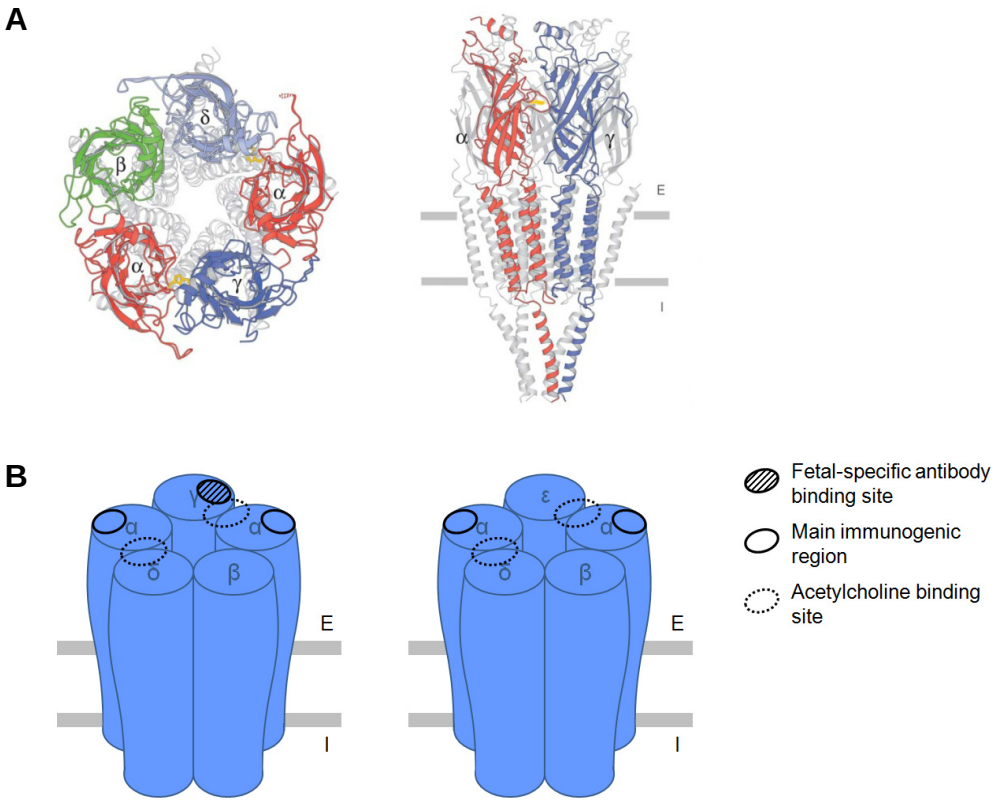


Fig. 2.1: **The nicotinic acetylcholine receptor (AChR). A)** The crystal structure of AChR in top and side view. The membrane is indicated by grey horizontal bars. Reprinted from Unwin [2005] with permission from Elsevier. **B)** The AChR is of pentameric structure and composed of either $\alpha_2\beta\gamma\delta$ (fetal form) or $\alpha_2\beta\varepsilon\delta$ (adult form). Each of the subunits consists of an extracellular, a transmembrane, and a cytoplasmic domain. Antibodies found in most patients with myasthenia gravis bind to the main immunogenic region, which is different from the acetylcholine-binding site. The fetal-specific antibodies that cause arthrogryposis multiplex congenita bind close to the acetylcholine binding site and block the function of the fetal AChR. *E*, extracellular; *I*, intracellular. Adapted from Vincent [2002].

Rhabdomyosarcoma comprises a heterogeneous group of the single most soft-tissue malignancies in children and adolescents [Newton *et al.*, 1995]. The two major forms of rhabdomyosarcoma in children are alveolar and embryonal rhabdomyosarcoma. The alveolar form, which typically shows more aggressive clinical behaviour and a poor prognosis, can be classified by gene expression profiling [Davicioni *et al.*, 2009]. For patients with rhabdomyosarcoma, risk-adapted multimodality therapy is used, including surgery, chemotherapy, and usually radiotherapy, depending on the correct pathologic diagnosis, histologic subtype, primary site, extent of disease, and extent of resection [Gosiengfiao *et al.*, 2012]. Systemic chemotherapy is required in almost all cases, though. Due to the new multimodality therapy, rhabdomyosarcoma is now curable in most patients with localised disease with more than 70 % surviving five years after diagnosis [Walterhouse and Watson, 2007]. However, the prognosis remains poor for patients with metastatic or recurrent diseases, resulting in five-year survival rates of 20 – 30 %. Children over 10 years of age or those with bone and / or bone marrow

metastases have an even lower survival rate of about 5 % [Carli *et al.*, 2004; Koscielniak *et al.*, 2002]. Hence, there is an urgent need for new therapeutic approaches, especially for those with the poorest prognoses. A possible novel approach could be a fAChR-targeting immunotherapy. Knowledge of the existence of anti-AChR and anti-fAChR antibodies was gained from myasthenia gravis, an autoimmune neuromuscular disease most commonly caused by the appearance of anti-AChR antibodies [Lindstrom *et al.*, 1976; Vincent, 2002]. In a few cases of pregnant women with myasthenia gravis, anti-fAChR antibodies can emerge and pass through the placenta to their unborn babies. These antibodies cause arthrogryposis multiplex congenita in the unborn babies by blocking the function of the fetal AChR, leading to a paralysis of the unborn baby [Riemersma *et al.*, 1996]. As the antibodies do not bind adult AChR (see Fig. 2.1), some of these women were entirely unaffected. On account of these findings, the use of a fAChR-targeting approach can be assumed to be safe; *i.e.* it is unlikely to induce serious side effects. Additionally, they show that the γ subunit of the fAChR bears immunologically available epitopes. Finally, the thymus of the above mentioned women appeared to be a beneficial source for the successful isolation of human antibodies highly specific for fAChR [Matthews *et al.*, 2002]. These antibodies, or more precisely Fabs (Fab, fragment antigen-binding), were isolated from a thymic combinatorial cDNA library from a myasthenia gravis patient with high levels of AChRγ-specific autoantibodies. One of those Fabs, namely Fab35, was converted into the single-chain variable fragment (scFv) format in order to generate a chimeric antigen receptor (CAR), which is also known as chimeric T-cell receptor [Gattenlöhner *et al.*, 2006]. This AChRγ-specific CAR was shown to kill rhabdomyosarcoma cell lines that express fAChR *in vitro* and *in vivo*, but with suboptimal results, as it needed an additive blockade of survivin, an antiapoptotic molecule [Simon-Keller *et al.*, 2013]. However, among other obstacles, a routine application of such an adoptive T-cell therapy in the clinic is mainly limited by the need of *ex vivo* culture and sophisticated techniques [Neoptolemos *et al.*, 2010]. Alternatively, an immunotoxin generated by fusing the same scFv to an exotoxin A (scFv35-ETA) has already been generated and tested *in vitro* and *in vivo* [Gattenlöhner *et al.*, 2010].

2.2.2 HLA-A2 and the Function of MHC Molecules

HLA-A2 is a serotype of the human leucocyte antigen (HLA), the human version of the major histocompatibility complex (MHC). MHC molecules are cell-surface glycoproteins with a peptide-binding cleft that can bind a wide variety of different peptides and they are encoded in a large cluster of genes, known as the MHC, located in humans on chromosome 6. MHC molecules are mainly responsible for antigen presentation, which is important for the induction and regulation of adaptive immune responses. More

precisely, the MHC molecules are involved in the T-cell activation through the T-cell receptor (TCR) (see Section 2.4.2) by presenting short peptide fragments of foreign proteins (antigens) to TCRs. A combination of polygeny and an extensive polymorphism of the MHC leads to a great diversity of MHC molecules and thus to an extended range of peptides that can be presented to T cells by each individual and the population at large. There are two classes of MHC molecules, which are of similar structure (Fig. 2.2), but different in the origin of the peptides they capture and transport to the cell surface for the purpose of antigen presentation. MHC class I molecules are composed of two polypeptide chains, a membrane-inserted heavy chain α (44 kDa) folding into three domains, and β_2-microglobulin (β_2m, 12 kDa), which associates noncovalently and is monomorphic, so it does not vary in sequence between individuals. MHC class II molecules are based on a noncovalent complex of two chains of similar size, α (34 kDa) and β (29 kDa). The length of MHC molecules is approximately 7 nm.

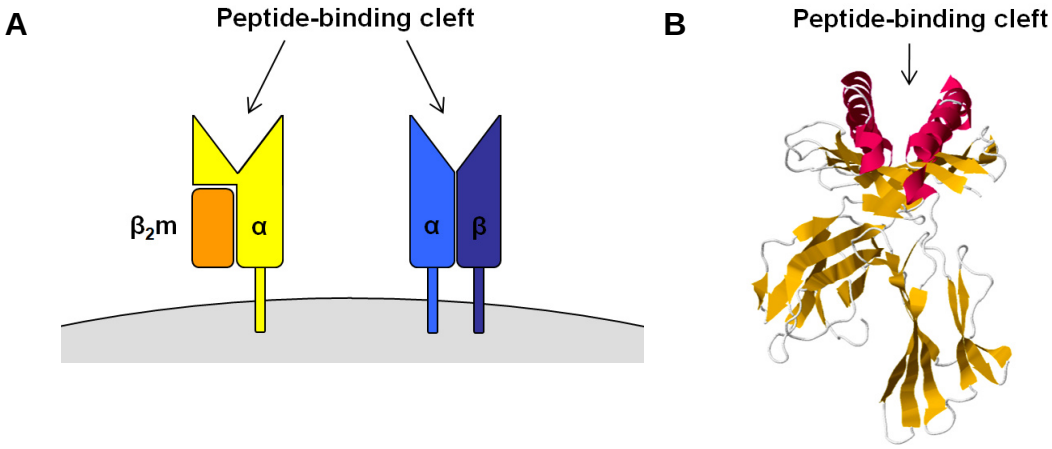


Fig. 2.2: Schematic structure of MHC molecules. A) MHC class I (left) and MHC class II (right). **B)** Crystal structure of the human MHC class I molecule, HLA-A2, without the transmembrane region. Image from the RCSB Protein Data Bank (www.pdb.org, Berman *et al.*, 2000) of PDB ID 3HLA [Saper *et al.*, 1991]. *β_2m*, β_2-microglobulin.

Their most striking feature is a cleft formed by the two outermost domains, which characterises their peptide-binding specificity (Fig. 2.3). Peptide binding, in turn, stabilises the molecule's whole structure. Peptides, so-called T cell epitopes, presented by the MHC class I molecules are usually 8 – 10 amino acids (aa) long and derive from proteins degraded in the cytosol by the proteasome, a multicatalytic protease complex. MHC class II molecules bind peptides from proteins that are degraded in endosomes and about 13 – 17 aa long, but the length is not constrained here.

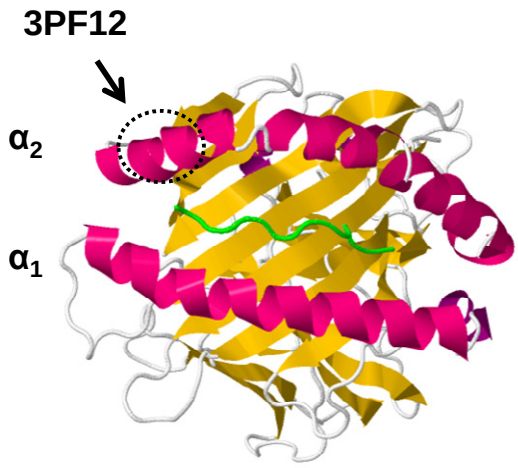


Fig. 2.3: Peptide-binding cleft of human MHC class I molecule. Depicted in complex with an antigenic nonapeptide in top view. Image from the RCSB Protein Data Bank (www.pdb.org, Berman *et al.*, 2000) of PDB ID 1B0G [Zhao *et al.*, 1999] slightly modified. Assumed binding site of the antibody fragment 3PF12 is encircled on the α2 domain of the HLA class I molecule HLA-A2 according to Watkins *et al.* [2000].

Having reached the cell surface, the MHC-peptide complexes are recognised by different functional classes of T cell by means of their TCRs (see Section 2.4.2, p. 19). The two classes of T cell vary in their co-receptors they express, which play an important role in the interaction between the MHC-peptide complex and TCR (see Fig. 2.7, p. 19), and therefore in the activation process of T cells. MHC class I molecules, which carry peptides from viruses or other intracellular pathogens, are recognised by cytotoxic T cells bearing the co-receptor CD8, which are specialised to kill any infected cell that displays the same foreign peptide. MHC class II-peptide complexes are recognised by T helper cells, which express the co-receptor CD4. T helper cells are specialised to activate other effector cells of the immune system: Macrophages, for example, are activated to kill the intravesicular pathogens they harbour, and B cells to secrete immunglobulins against foreign molecules. Due to their different functions, MHC class I molecules are expressed on most nucleated cells, whereas MHC class II molecules are only expressed on immunologically active cells, such as dendritic cells, macrophages, or B cells.

MHC molecules determine the compatibility between donor and recipient for allogeneic transplantation. Thus, they are the major reason for graft rejections and graft-versus-host disease [Kanda *et al.*, 2003]. With the aid of MHC molecules, cancer cells may be recognised and eliminated by the immune system. On the other hand, a lack of MHC-antigen presentation (due to down-regulation by a viral infection) sometimes enables cancer cells to escape from immune surveillance. Furthermore, the MHC locus is associated with a myriad of autoimmune diseases, such as multiple sclerosis or rheumatoid arthritis [Fernando *et al.*, 2008].

Since HLA-A2 belongs to MHC class I molecules, it is expressed on almost all nucleated cells, although to a variable extent. While an abundant expression of MHC class I molecules is found on immune cells, low expression is found on cells of the liver, kidney, and brain [Janeway *et al.*, 2005]. Moreover, HLA-A2 is expressed at a high frequency in most population groups (especially Caucasoids) and is the most frequently occurring HLA class I allele in Germany (50 %) [Gonzalez-Galarza *et al.*, 2011]. The high resolution crystal structure of HLA-A2 in complex with an unknown peptide was revealed by Bjorkman *et al.* [1987] and its peptide binding properties are also known [Falk *et al.*, 1991]. The fact that HLA-A2 is expressed either at high amounts (in HLA-A2-positive individuals) or not at all (in HLA-A2-negative individuals) makes it a suitable candidate as a target for combinatorial dual-targeting approaches in a transplantation context (see Section 2.6, p. 24). Human anti-HLA antibodies may arise during pregnancy, after solid organ transplantation, and after the transfusion of HLA-mismatched platelet concentrates or blood [Watkins *et al.*, 2000]. The human HLA-A2-specific antibody fragment 3PF12 used in this study was isolated from a patient alloimmunised by blood transfusion, selected by V gene phage display technology [Watkins *et al.*, 2000].

2.2.3 CD45 – Leucocyte-Common Antigen

CD45 is exclusively expressed on all nucleated haematopoietic cells [Trowbridge and Thomas, 1994]. Eight isoforms of CD45 may exist due to alternative splicing of three exons encoding the regions A, B, and C, but only five are expressed at significant levels: CD45RABC, CD45RAB, CD45RBC, CD45RB, and CD45R0 [Fukuhara *et al.*, 2002]. The isoforms are differentially expressed on leucocytes and are regulated in a cell type and activation state-dependent manner. For example, CD45RABC is expressed on B cells, CD45RA and other large isoforms are found on naive T cells, and CD45R0 (lacking the exons A, B, and C) is located on memory T cells. Upon activation, naive T cells undergo a switch to CD45R0. Myeloid lineage cells generally express CD45R0 until activated, when they switch to CD45RA [Hermiston *et al.*, 2009]. However, more than one isoform can be expressed on a single leucocyte type.

CD45 belongs to the protein tyrosine phosphatase family (PTPase) [Charbonneau *et al.*, 1988] and is also known as PTPRC, protein tyrosine phosphatase, receptor type C. CD45 is a type I transmembrane glycoprotein of 180 – 240 kDa, depending on the isoform, and consists of a large extracellular domain, a single transmembrane domain, and an extensive cytoplasmic tail (Fig. 2.4) [Thomas, 1989]. The extracellular domain consists of the alternatively spliced exons with multiple sites for O-linked glycosylation, followed by a cysteine-rich domain and three fibronectin type III domains, both comprising multiple sites for N-glycosylation. While the extracellular domain is

highly variable, the cytoplasmic part is highly conserved in all mammalian species and includes intrinsic PTPase activity.

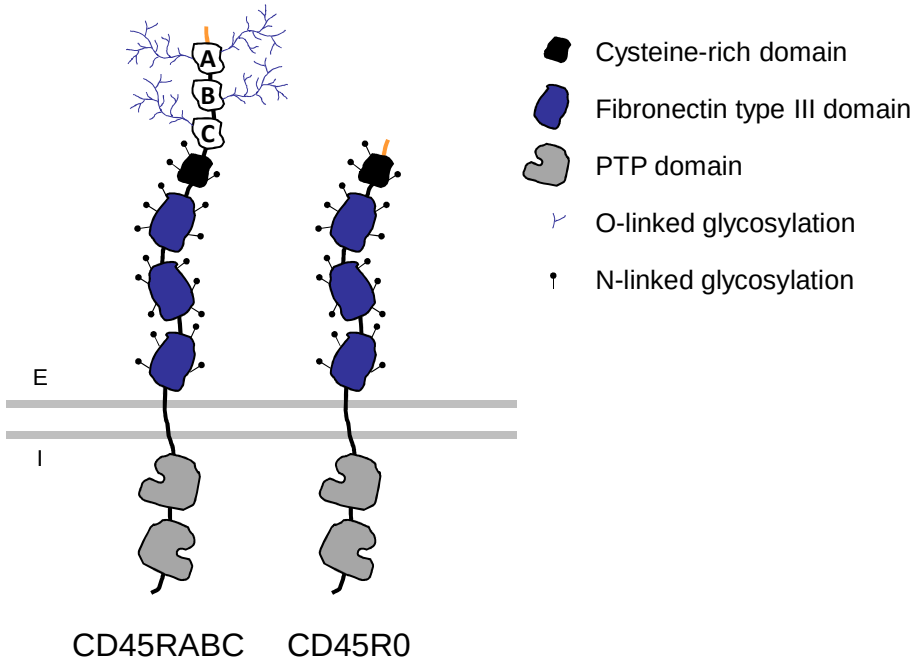


Fig. 2.4: Schematic structure of CD45 molecules. Multiple isoforms of CD45 are found due to alternative splicing of three exons encoding the regions A, B, and C, which are part of the extracellular domain. Only two isoforms are shown, CD45RABC and CD45R0. The cytoplasmic portion includes intrinsic protein tyrosine phosphatase (PTPase) activity. The membrane, key functional domains, and glycosylation sites (O- and N-linked) of CD45 are indicated. The membrane is indicated by grey horizontal bars. *E*, extracellular; *I*, intracellular; *PTP*, protein tyrosine phosphatase. Adapted from Hermiston *et al.* [2009].

The function of the different isoforms has remained elusive yet. What is known is that CD45 is required for receptor signalling in immune cells. CD45 is thought to modulate signalling networks mediated by Src-family kinases (SFKs). It can remove the phosphate from phosphotyrosines, especially from the inhibitory tyrosine residue of SFKs, which, in turn, are associated with antigen receptors and phosphorylate the tyrosines in immunoreceptor tyrosine-based activation motifs (ITAMs) [Janeway *et al.*, 2005]. The key substrates for CD45 in T and B cells are the SFK members Lck and Lyn, respectively [Saunders and Johnson, 2010]. By decreasing the threshold of antigen receptor signalling in T and B cells, CD45 makes these cells more sensitive to stimulation by a specific antigen. CD45 also regulates antigen-triggered Fc-receptor signalling in mast cells and Toll-like receptor signalling in dendritic cells. Moreover, CD45 may have an impact on immune cell adhesion and migration as well as cytokine production. Thus, it seems to be involved in the regulation of adaptive and innate immune responses.

CD45 is one of the most abundant leucocyte cell surface glycoproteins, comprising up to 10 % of all cell surface proteins in lymphocytes [Thomas, 1989]. About 200.000

CD45 molecules are expressed per cell on average [van der Jagt *et al*., 1992]. CD45 is also expressed on most haematological malignancies, including 85 – 90 % of acute leukaemias, while not being found on non-haematopoietic tissues [Matthews *et al.*, 1991]. Furthermore, CD45 is not internalised or shed upon binding of an anti-CD45 antibody [Press *et al.*, 1994]. Due to this feature, its abundance, and the fact that it is the most specific marker of haematopoietic lineage, CD45 appears as an attractive target for developing immunotherapy strategies for the treatment of haematological malignancies. New therapeutical approaches are especially required since acute myeloid leukaemia (AML), for example, still kills 60 – 70 % of those affected despite improvements in induction chemotherapy and haematopoietic stem cell transplantation [Lin *et al.*, 2006]. In fact, researchers have already focused on targeting CD45 for radioimmunotherapy. A number of radiolabelled CD45-specific antibodies were developed for conditioning prior to allogeneic transplantation in AML and myelodysplastic syndrome (MDS) and have been tested in clinical studies [Orozco *et al.*, 2012], revealing the potency of CD45 as target of blood cancer. For example, an ^{131}I-labelled anti-CD45 antibody (clone BC8) was used as radioimmunotherapy in advanced acute leukaemia and MDS [Matthews *et al*., 1999]. This CD45-targeted radiotherapy could be safely combined with a reduced-intensity conditioning regimen leading to encouraging overall survival for older, high-risk patients with AML or MDS [Pagel *et al.*, 2009]. In order to enhance the radiation at the malignant CD45-expressing sites and to diminish it in normal tissues, a pretargeted radioimmunotherapy in the form of a recombinant anti-CD45 tetravalent single-chain antibody-streptavidin fusion protein (scFv4SA) was developed [Lin *et al.*, 2006]. This antibody can hence be bound via streptavidin to radiolabelled DOTA-biotin for the treatment of leukaemia. In a human xenograft model, using an α-emitting radionuclide, this approach demonstrated highly specific tumour targeting and minimal toxicity to normal tissue [Pagel *et al.*, 2011]. The same scFv, derived from a murine anti-CD45 antibody (clone BC8) that recognises all human CD45 isoforms, was used in the present study.

2.3 Therapeutic Antibodies in Cancer Therapy

Antibodies are part of the adaptive immune system. These proteins are produced by B cells in a vast range of antigen specificity. Their main function is to specifically bind pathogens and to recruit immune cells or molecules to destroy the pathogen. The fact that antibodies bind to their antigen with a high affinity and selectivity makes them a promising tool for targeted therapy. Monoclonal antibodies, deriving from one B cell, have long been eyed as a potential new class of therapeutics targeting cancer and other diseases [Booy *et al.,* 2006]. Monoclonal antibodies have become an established and clinically well tolerated therapeutic option for a number of diseases [Reichert, 2011; Nelson *et al.*, 2010]. In cancer therapy, most of them target tumour-associated

antigens. Their anti-tumour effects derive from the ability to elicit effector mechanisms of the immune system mediated by the Fc region, such as antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CMC), and phagocytosis. However, antibodies are only able to recruit effector cells bearing the respective Fc receptors, such as macrophages, granulocytes, monocytes, and NK cells. In contrast, the most potent effector cells, namely the cytotoxic T lymphocytes, lack Fc receptors and thus cannot be recruited and activated. Another limitation of monoclonal antibodies is that they can only target a single antigen. Due to the often multifactorial nature of cancer, dual-targeting approaches which target two molecules with one antibody, a so-called bispecific antibody, ought to be therapeutically more effective [Kontermann, 2012]. In addition, bispecific antibodies are able to redirect an immune response to the tumour site, circumventing the (sometimes disadvantageous) Fc-mediated or MHC-restricted activation of effector cells [Müller and Kontermann, 2007].

2.3.1 The Concept of Bispecific Antibodies

The principle of bispecific molecules for immunotherapy is based on the selective retargeting of effector cells to tumour cells. Bispecific antibodies carry one specificity for a trigger molecule on an effector cell and another one for a tumour antigen on the target cell. Hence, they are able to recruit an effector mechanism to a target. The effector cells can be recruited by different trigger molecules: for example, CD3 on T cells, FcγRIII (CD16) on NK cells, FcγRI (CD64) or FcαRI (CD89) on monocytes and granulocytes [Peipp and Valerius, 2002; van Spriel *et al.*, 2000].

2.3.2 Recombinant Bispecific Antibody Formats

Recombinant bispecific antibodies offer several advantages over conventionally produced antibodies. Initially, bispecific antibodies were generated by chemical cross-linking of two different monoclonal antibodies or by fusion of two hybridoma clones. Since the first generation of therapeutic antibodies were mainly of mouse origin, their repeated application resulted in a rapid elimination by the immune system induced by the production of neutralising antibodies against the mouse bispecific antibodies (HAMA, human anti-mouse antibodies) [Klee, 2010]. The next antibodies were constructed as chimeric antibodies, with human constant antibody domains (~ 70 %) and only antigen-binding domains being of mouse origin, leading to a greatly reduced immunogenicity [Morrison *et al.*, 1984; Galizia *et al.*, 2007]. Further improved properties were achieved with humanised antibodies. In those, only the complementarity-determining regions (CDR) that determine the antigen specificity of an antibody were retained of mouse origin [Jones *et al.*, 1986; Riechmann *et al.*, 1988; Hudis, 2007]. The generation of entirely human antibodies was enabled by the phage display technology [Hoogenboom and Chames, 2000] or by using transgenic mice [Lonberg, 2005].

Although humanised or human antibodies are less immunogenic and can therefore act longer due to a prolonged half-life in humans, a generation of human anti-human antibodies (HAHA) was found [Getts *et al.*, 2010]. The HAHA response, in turn, can be further reduced by 'deimmunisation', *i.e.* by the removal of the responsible T-cell epitopes [Hellendoorn *et al.,* 2004; Jones *et al.*, 2004]. The large-scale production of human or humanised antibodies is performed by using prokaryotic, yeast, or mammalian expression systems.

In order to reduce immunogenicity and to avoid Fc-mediated side effects such as cytokine-release syndrome, recombinant bispecific antibodies, which lack the Fc-region and retain only the antigen-binding variable domains of an antibody, can be achieved by proteolytic cleavage or genetic engineering. Various formats of such antibodies have been developed in recent years. The most widely used formats, such as tandem scFv (taFv), diabody (Db), and single-chain diabody (scDb), are based on two single-chain variable fragments (scFv) specific for different antigens (Fig. 2.5 A – C). A scFv fragment is one of the smallest antigen-binding molecules. It consists of the variable domains of the heavy (V_H) and the light chain (V_L) of an antibody joined by a flexible linker of 15 – 20 residues, providing the suitable folding into a functional scFv fragment [Huston *et al.,* 1988; Bird *et al.*, 1988]. Bearing only one antigen-binding site, scFv fragments are monovalent and monospecific. The reduction of the linker length to about 5 – 10 amino acids sterically inhibits the assembly of the variable domains of one chain to a functional unit and therefore forces the two V_H-V_L chains to homodimerise crosswise, resulting in a diabody with two antigen-binding sites [Holliger *et al.,* 1993]. Thus, diabodies are bivalent molecules, either monospecific or bispecific. Bispecific diabodies express two polypeptide chains for the V_H-V_L configuration within the same cell, V_HA-V_LB and V_HB-V_LA. In the further developed single-chain diabody (scDb) format, the two diabody-forming polypeptide chains are joined by an additional middle linker of 15 – 20 residues. A scDb assembles due to the different linker lengths into a bispecific and monomeric molecule, for example in the V_HA-V_LB-linker-V_HB-V_LA orientation [Brüsselbach *et al.,* 1999]. Another possibility is the tandem scFv (taFv) achieved by the linkage of two scFv fragments, for example into a V_HA-V_LA-linker-V_HB-V_LB configuration [Mallender and Voss, 1994; Mack *et al.*, 1995]. Lastly, several other genetically engineered recombinant antibodies which lack any constant domains were established (Fig. 2.5 D), as reviewed by Müller and Kontermann [2011] and Kontermann [2012].

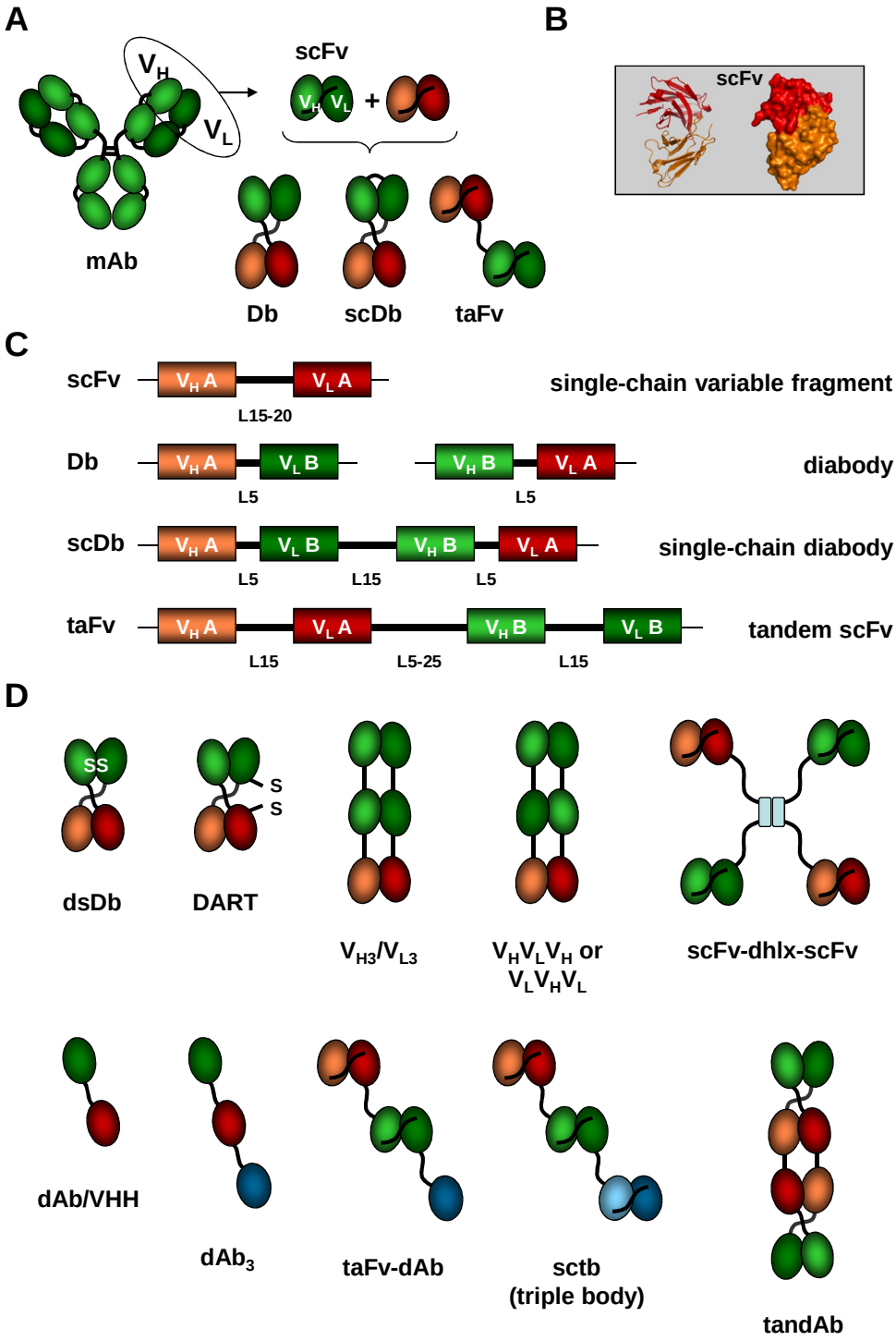


Fig. 2.5: **Recombinant bispecific antibody formats. A)** Structures of the recombinant bispecific antibody formats: diabody (Db), single-chain diabody (scDb), and tandem scFv (taFv) derived from single-chain variable fragments (scFv). All formats are based on the variable domains of the heavy chain (V_H) and the light chain (V_L) of an antibody joined by linkers of different lengths. **B)** Ribbon and surface structure of scFv. **C)** Domain and linker arrangements in scFv, Db, scDb, and taFv molecules. Linker lengths are indicated with the number of amino acids. Adapted from Müller and Kontermann [2011]. **D)** Further examples of recombinant bispecific antibody formats which lack constant domains. *mAb*, monoclonal antibody. Adapted from Kontermann [2011a].

The construction of all these formats through genetic means allows further manipulation to add novel functions such as the fusion to toxins, costimulatory molecules, or polyethylene glycol (PEG) to improve pharmacokinetics [Chapman, 2002; Kontermann, 2011b]. All these antibody fragments may be expressed in soluble form in the periplasm of *E. coli* or using a mammalian expression system. As the taFv fragments were often found to form insoluble aggregates in bacteria, mammalian expression systems are nowadays used with preference [Kontermann, 2005]. Although mammalian expression often provides higher yields, better purity, and essential posttranslational modification, it is considerably more expensive and time-consuming than prokaryotic expression.

The small size of antibody fragments, such as the above-mentioned (scFv ~ 25 kDa; Db, scDb, and taFv ~ 50 – 60 kDa in comparison to IgG ~ 150 kDa), allows for good penetration into the tumour tissue in therapeutical applications, but also gives rise to a rapid elimination from the circulation via renal clearance [Kipriyanov *et al.*, 1999; Tryggvason and Wartiovaara, 2005]. In addition, the recycling processes mediated by the neonatal Fc receptor, which prolong the half-life of IgGs, have no effect, as these small fragments lack the Fc region. On account of their short serum half-lives, small fragments may not circulate long enough to induce therapeutic effects. Currently, different approaches are pursued in order to improve pharmacokinetics: i) N-glycosylation, ii) the fusion of PEG, iii) the fusion to Fc or an IgG-binding domain, iv) the fusion to human serum albumin, and v) the fusion of an albumin-binding domain from streptococcal protein G [Kontermann, 2011b; Müller *et al.,* 2007; Stork *et al.,* 2007; Stork *et al.,* 2008].

2.4 Bispecific Antibodies for Targeting Immune Effector Cells

As mentioned above, bispecific antibodies are able to recruit effector cells of the immune system towards the tumour, which provides a promising approach to circumvent immune evasion mechanisms of the tumour [Dunn *et al.*, 2004; Garcia-Lora *et al.*, 2003]. Cytotoxic T cells have been chosen as potential tools in most retargeting approaches in cellular immunotherapy, as they rank among the most potent effector cells of the immune system. The following section describes the molecular mechanisms of T-cell activation in general and subsequently outlines a range of bispecific antibodies targeting tumour-associated antigens, which were developed for this purpose in recent years.

2.4.1 Activation of Naive T Cells

In the course of an adaptive immune response, naive T cells differentiate upon activation into armed effector T cells such as cytotoxic T cells. An efficient T-cell activation requires two independent signals delivered by the same antigen-presenting

cell (APC), namely a dendritic cell, macrophage, or B cell. The primary signal is received through the T-cell receptor complex. The second, or costimulatory, signal is provided by the interaction between costimulatory molecules, such as CD28 on the T cell and B7 molecules on the APC. Antigen binding to the T-cell receptor in the absence of costimulation may induce programmed cell death (apoptosis) as well as anergy, a state of unresponsiveness to subsequent antigen presentation [Chappert and Schwartz, 2010]. In contrast, the costimulatory signal alone has no effect on the T cell (Fig. 2.6). The expression of costimulatory molecules on APCs is generally induced by a range of microbial components that are recognised by receptors of the innate immune system to prevent immune responses to self antigens.

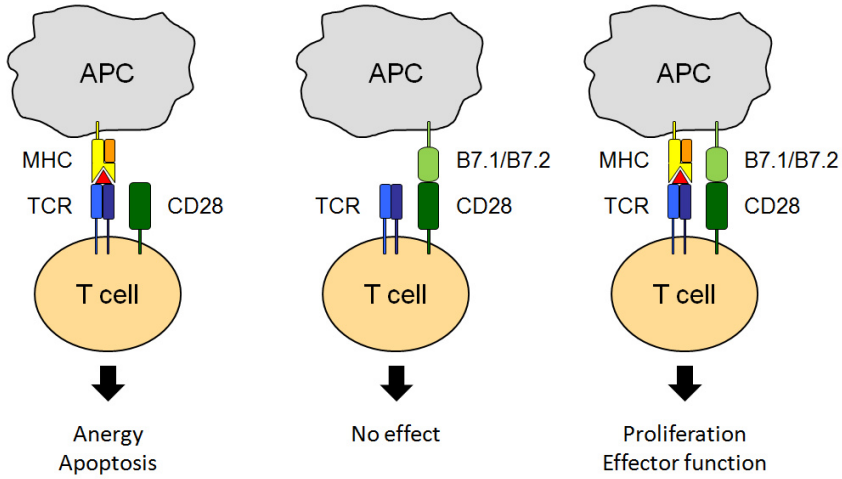


Fig. 2.6: T-cell fate under different conditions of TCR engagement. Simultaneous recognition of a specific major histocompatibility complex (MHC)-peptide complex by the T-cell receptor (TCR) and of B7.1 (CD80) or B7.2 (CD86) by the costimulatory receptor CD28 results in T-cell activation, cytokine production, proliferation, and differentiation. In the absence of CD28 ligation, T cells undergo apoptosis or become anergic, whereas the costimulatory signal alone has no apparent effect. APC, antigen-presenting cell. Adapted from Alegre *et al.* [2001].

Clonal expansion and differentiation into armed effector cells upon T-cell activation is mainly driven by the cytokine interleukin-2 (IL-2), which is produced by the activated T cell itself. IL-2 induces T-cell proliferation and serves as a survival factor for these cells. Interestingly, for the synthesis and secretion of IL-2, the costimulatory signal is necessary. Armed effector cytotoxic T cells release lytic granules upon recognition of antigen on target cells. These granules contain the cytotoxic proteins perforin, granzymes, and granulysin. Perforin forms pores into the membrane of the target cell through which the granzymes can enter and induce apoptosis. Furthermore, cytotoxic T cells perform their killing function by activating apoptosis through the CD95 (Fas) ligand they express [Janeway *et al.*, 2005].

2.4.2 Primary Signal for T-Cell Activation

The primary signal, which is antigen-specific, is triggered through the T-cell receptor engaging with an MHC-peptide complex, and its coreceptor, CD4 or CD8. In tumour defence, cytotoxic T cells bearing the coreceptor CD8 play an important role. TCRs recognise peptides presented by MHC molecules on an APC following protein processing. TCRs are heterodimers which are composed of two membrane-spanning chains, α and β, and only responsible for antigen recognition. The juxtaposition of both chains forms the antigen-binding site, thus accounting for the peptide-binding specificity of the TCR. The TCR is effective only in conjunction with the individual's own MHC molecules. This dual recognition is called MHC restriction and was first discovered by Zinkernagel and Doherty in 1974. In contrast, the actual signalling as such is transmitted by the invariant accessory chains, namely the intracellular ζ-chains (homodimer) and the CD3 complex comprising a heterodimer of an ε-chain associated with either a δ-chain or a γ-chain (Fig. 2.7). These chains have ITAMs enabling them to signal upon antigen ligation. ITAMs are phosphorylated by activated tyrosine kinases of the Src-family (Lck and Fyn), followed by the activation of ZAP-70 (ζ-chain associated protein), a further tyrosine kinase propagating the signal onward. Ultimately, three important signalling pathways are initiated, culminating in the activation of the transcription factors NFκB, NFAT, and AP-1 in the nucleus. The activation of new gene transcription finally results in the differentiation, proliferation, and effector functions of T cells [Janeway *et al.*, 2005].

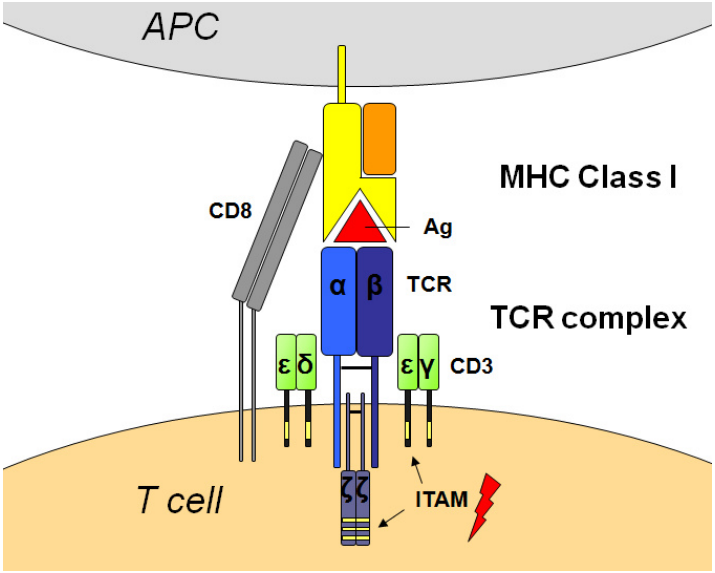


Fig. 2.7: Primary T-cell activation through the T-cell receptor (TCR). An antigen (Ag) bound to an MHC class I molecule on an antigen-presenting cell (APC) is recognised by the TCR complex of a T cell bearing the coreceptor CD8. Signalling is transmitted by the invariant accessory chains, the CD3 complex and ζ-chains, bearing immunoreceptor tyrosine-based activation motifs (ITAMs).

Antibodies targeting the CD3 ε-chain, the constant molecule of the TCR complex, circumvent both MHC restriction, *i.e.* antigen recognition by the TCR only in conjunction with self-MHC molecules, and the clonotypic antigen specificity of T cells. They are able to activate T cells in an MHC- and costimulation-independent manner by cross-linking CD3. However, they induce an even stronger, polyclonal T-cell response in the presence of appropriate costimulation. Examples of such antibodies are TR66 [Lanzavecchia and Scheidegger, 1987; Traunecker *et al.,* 1991] and UCHT-1 [Beverley and Callard, 1981]. The CD3-specific moiety of all bi- and trispecific constructs that are used for T-cell activation in the present study derives from diL2K [Brischwein *et al.*, 2006], a deimmunised variant of the human CD3ε-specific mouse mAb L2K [Dreier *et al.*, 2002], which, in turn, is identical with TR66. Several bispecific antibodies have been developed for effector cell retargeting directed against CD3 and a tumour antigen (Table 2.1, p. 22). These are outlined in the following.

2.4.3 Bispecific Antibodies for the Retargeting of T Cells

A broad range of recombinant bispecific antibodies for T-cell activation, targeting tumour-associated antigens and CD3, have been developed and tested for anti-tumour activity *in vitro* and *in vivo* over the last years (Table 2.1, p. 22). A few of them are currently being tested in clinical trials [Müller and Kontermann, 2007; Kontermann, 2011a]. For the construction of such bispecific antibodies, several different targets were chosen that are overexpressed by tumour cells, such as CD19, CD20, EpCAM (epithelial cell adhesion molecule), EGFR (epidermal growth factor receptor), HER2 (human epidermal growth factor receptor 2), and CEA (carcinoembryonic antigen).

Interestingly, CD3-specific antibodies of the BiTE (bispecific T-cell engager) format are able to induce T-cell activation in an MHC- and costimulation-independent manner. Several of such bispecific antibodies deriving from the monoclonal antibody TR66 were developed. BiTEs are tandem scFv molecules. Their second specificity was designed against different tumour antigens, including CD19 [Löffler *et al.*, 2000; Dreier *et al.*, 2002; 2003], EpCAM [Schlereth *et al.*, 2005; Brischwein *et al.*, 2006], tyrosine kinase EphA2 [Hammond *et al.*, 2007], CEA [Lutterbuese *et al.*, 2009], EGFR [Lutterbuese *et al.*, 2010], or the large melanoma-associated proteoglycan MCSP [Bluemel *et al.*, 2010]. *In vitro* studies revealed a high efficiency in tumour cell lysis at low concentrations and low effector-to-target cell ratios [Baeuerle *et al.*, 2009; Hammond *et al.*, 2007; Hoffmann *et al.*, 2005]. The BiTE directed against the B-cell antigen CD19 was characterised in great detail. It was described to inhibit the tumour growth very efficaciously in mice bearing human B cell lymphoma xenografts [Dreier *et al.,* 2003] and to induce T-cell activation and B-cell depletion in chimpanzees [Schlereth *et al.*, 2006]. This BiTE, called blinatumomab or MT103, is currently being investigated in clinical trials. In a phase I dose-escalating study, partial or, in some cases, even

complete tumour regression was observed at very low doses in patients with non-Hodgkin's lymphoma (NHL) [Bargou *et al.*, 2008]. In addition, clinical phase I and II trials are ongoing for the treatment of acute lymphoblastic leukaemia (ALL). For a completed phase II study, promising results have been reported [Topp *et al.*, 2011]; another one is underway. A second BiTE antibody named MT110, targeting the pan-carcinoma antigen EpCAM, was shown to eradicate established subcutaneous tumours in immunodeficient mice with human tumour xenografts [Schlereth *et al.*, 2005; Brischwein *et al.*, 2006]. MT110 is currently being tested in a dose-escalating phase I study for the treatment of solid tumours, including gastrointestinal, lung, breast, ovarian, and prostate cancer. Both BiTEs were the first (recombinant) bispecific antibodies of the second generation to enter the clinic. The outstanding potency and costimulation independence of BiTEs is assumed to be accounted for by a highly effective T-cell activation, which is achieved by inducing the formation of cytolytic synapses with high frequency [Wolf *et al.*, 2005; Offner *et al.,* 2006] and by supporting serial target cell lysis [Hoffmann *et al.*, 2005]. On the other hand, dosage variation appears to be the only way to regulate the application of BiTEs, which may lead to unwanted and uncontrolled T-cell activation owing to their high and MHC-independent reactivity.

Alternatively to anti-CD3 antibodies, superagonistic anti-CD28 bispecific antibodies were developed that induce effective tumour cell-restricted T-cell activation without concurrent primary signal through the TCR complex [Grosse-Hovest *et al.*, 2003; 2005; Otz *et al.*, 2009]. One of them, named rM28, is specific for a melanoma-associated proteoglycan and was tested in clinical phase I/II trials for local treatment of metastatic melanoma (ClinicalTrials.gov; identifier NCT00204594); the results are not available yet. However, the usage of superagonists bears a potentially high risk to generate a systemic T-cell activation and an excessive immune response by circumventing physiological control mechanisms as was revealed for another superagonistic but monoclonal anti-CD28 antibody (TGN1412) that caused unforeseen disastrous side effects in a phase I clinical trial [Beyersdorf *et al.,* 2006; Suntharalingam *et al.*, 2006]. Another anti-CD28 antibody was used for the construction of a trispecific CEA × CD3 × CD28 antibody by fusing the V_H domain of a CD28-specific antibody to a bispecific CEA × CD3 tandem scFv molecule [Wang *et al.*, 2004]. This antibody fusion protein, providing both signals required for T-cell activation in one molecule, was able to activate and recruit T cells to tumour cells *in vitro* and thus to induce tumour-specific cell lysis.

Table 2.1: Recombinant bispecific antibodies for cancer therapy targeting CD3 on T cells. Adapted from Müller and Kontermann [2007; 2011].

Target CD3 × …	Type	Efficacy	Potential indication	Reference
BCL-1	Db	*in vitro*	B-cell tumours	[Holliger *et al.*, 1996]
CD19	taFv (BiTE)	*in vitro* / *in vivo*	B-cell tumours	[Dreier *et al.*, 2003; Bargou *et al.*, 2008; Topp *et al.*, 2011]
CD19	Db	*in vitro* / *in vivo*	B-cell tumours	[Kipriyanov *et al.*, 1998; 2002; Cochlovius *et al.*, 2000a]
CD19	TandAb	*in vitro* / *in vivo*	B-cell tumours	[Kipriyanov *et al.*, 1999; Cochlovius *et al.*, 2000b; Reusch *et al.*, 2004; Le Gall *et al.*, 2004]
CD20	Db	*in vitro* / *in vivo*	B-cell tumours	[Xiong *et al.*, 2002; Liu *et al.*, 2010]
CD105	scDb	*in vitro*	Solid tumours	[Korn *et al.*, 2004]
CEA	Db	*in vitro* / *in vivo*	Various carcinomas	[Holliger *et al.*, 1999, Blanco *et al.*, 2003, Compte *et al.*, 2007; 2009; 2010]
CEA	scDb	*in vitro*	Various carcinomas	[Müller *et al.*, 2007]
EGFR	Db	*in vitro* / *in vivo*	Various carcinomas	[Hayashi *et al.*, 2004]
EpCAM	taFv (BiTE)	*in vitro* / *in vivo*	Various carcinomas	[Schlereth *et al.*, 2005; Brischwein *et al.*, 2006]
EpCAM	Db	*in vitro*	Various carcinomas	[Helfrich *et al.*, 1998]
EpCAM	taFv	*in vitro* / *in vivo*	Various carcinomas	[Ren-Heidenreich *et al.*, 2004]
EphA2	taFv (BiTE)	*in vitro* / *in vivo*	Various carcinomas, metastatic melanoma	[Hammond *et al.*, 2007]
FAP	taFv (BiTE)	*in vitro*	Various carcinomas	[Wüest *et al.*, 2001]
HER2	Db	*in vitro*	Ovarian and breast cancer	[Zhu *et al.*, 1996]
HER2	taFv (BiTE)	*in vitro*	Ovarian and breast cancer	[Maletz *et al.*, 2001]
Lewis Y	taFv (BiTE)	*in vitro*	Various carcinomas	[Maletz *et al.*, 2001]
MUC1	Db	*in vitro*	Various carcinomas	[Takemura *et al.*, 2000]
Pgp	Db	*in vitro* / *in vivo*	Drug-resistant cancers	[Gao et al., 2004; Guo *et al.*, 2008]
Pgp	dsDb	*in vitro* / *in vivo*	Drug-resistant cancers	[Liu *et al.*, 2009]
PSMA	Db	*in vitro* / *in vivo*	Prostate cancer	[Bühler *et al.*, 2008; 2009]
Wue-1	taFv (BiTE)	*in vitro*	Multiple myeloma	[Hönemann *et al.*, 2004]

BCL-1, surface idiotype of the mouse lymphoma line BCL-1; *BiTE*, bispecific T-cell engager; *CEA*, carcinoembryonic antigen; *Db*, diabody; *dsDb*, disulfide-stabilised diabody; *EGF*R, epithelial growth factor receptor; *EpCAM*, epithelial cell adhesion molecule; *EphA2*, ephrin A2 receptor; *FAP*, fibroblast activation protein; *HER2*, human epidermal growth factor receptor 2; *MUC1*, mucin-1; *Pgp*, P-glycoprotein; *PSMA*, prostate-specific membrane antigen; *scDb*, single-chain diabody; *taFv*, tandem scFv; *TandAb*, tandem single-chain diabody; *Wue-1*, an antigen on human plasma cells.

2.5 Dual Targeting with Bispecific Antibodies

In the past years, various dual targeting bispecific antibodies were developed and were recently reviewed in great detail by Kontermann [2012]. Most of them target pathological factors such as receptors, ligands, or a combination of both. This appears beneficial, since complex diseases are often multifactorial. Besides cancer, they were developed for the treatment of inflammatory and infectious diseases and may act in an activating or inhibiting manner on the target molecule.

An example for dual-targeting bispecific antibodies of small format is a bs-scFv (conforms taFv) directed against ErbB2 and ErbB3 (HER2 and HER3), which was shown to inhibit the growth of tumour cells *in vitro* [Robinson *et al.*, 2008]. In addition, a bispecific immunotoxin called DT2219 was generated by fusing a truncated diphtheria toxin (DT) N-terminally to a taFv targeting CD22 and CD19, both found on B cells [Vallera *et al.*, 2005; 2009]. In proliferation assays, this immunotoxin demonstrated a greater anti-tumour activity against double-positive tumour cells than its monospecific counterparts DT2222 and DT1919. Furthermore, in a mouse xenograft model of B-cell malignancy, DT2219 effectively inhibited the growth of established primary tumours, and it also prolonged survival in a model of systemic leukaemia-like malignancy. However, the functionality of this agent is not dependent on the simultaneous presence of both antigens. Thus, it may also eliminate single-positive cells. Nevertheless, the applicability of such an immunotoxin was suggested to lie in the treatment of all B-cell malignancies. This is why a clinical phase I trial for the treatment of B-cell leukaemia and lymphoma was initiated for an optimised version of this agent, called DT2219ARL.

Other bispecific antibodies use dual targeting for retargeting effector molecules or effector cells. Trispecific antibodies, called single-chain Fv triple bodies (sctb), which were created simply by extending the taFv format by a third scFv appear to be an advantageous approach [Kellner *et al.*, 2008; Kügler *et al.*, 2010; Schubert *et al.*, 2011; 2012]. Triple bodies were developed for dual retargeting of effector cells. One of those triple bodies targets two antigens associated with AML (CD33 and CD123) and the low affinity FcγRIII (CD16), a trigger molecule on NK cells, neutrophils, and macrophages. The centrally located scFv against CD16 comprises a disulfide bond for more stabilisation. This trispecific CD123 × CD16 × CD33 triple body was shown to bind to both tumour antigens at the same time and to mediate ADCC of AML cells *in vitro* with high potency [Kügler *et al.*, 2010]. The same group generated also another triple body of CD33 × CD16 × CD19 specificity [Schubert *et al.*, 2011]. Here, in addition to the CD16-specific scFv, the C-terminal scFv against the B-cell antigen CD19 also contained a stabilising disulfide bond. This triple body mediated strong redirected lysis of tumour cells expressing both tumour antigens, CD33 and CD19. In addition, it

revealed a higher binding avidity to double-positive tumour cells than its respective bispecific counterparts (CD33 × CD16 and CD19 × CD16), which served as controls and are capable of monovalent targeting only. For a third triple body of HLA-DR × CD16 × CD19 specificity, lysis of double-positive cells was shown to occur with preference over single-positive cells [Schubert *et al.*, 2012]. However, dual targeting with such a trispecific one-molecule strategy seems not to be able to strictly discriminate between double-positive tumour cells and single-positive healthy cells. Nevertheless, dual retargeting of effector cells appears to be a promising tool to increase efficacy and selectivity.

In conclusion, such dual-targeting strategies require the existence and knowledge of two appropriate and specific (tumour) targets. In contrast, up to now no studies have been reported on a combinatorial dual-targeting approach, which exploits two conventional antigens in combination for specific tumour targeting and whose functionality strictly depends on the presence of and binding to both antigens at the same time.

2.6 Thesis Aims and the Concept of a Dual Antigen-Restricted Trispecific Antibody

In recent years preclinical and clinical studies revealed the great potential of recombinant bispecific antibodies for the retargeting of T cells as the most potent effector cells in cancer immunotherapy. BiTE antibodies emerged as the most successful recombinant bispecific molecules and are well characterised. In order to live up to the success of the BiTE antibody technology, one aim of the present study was the generation and functional characterisation of a bispecific tandem scFv (taFv) antibody against AChRγ and CD3 for the treatment of aggressive rhabdomyosarcoma (Fig. 2.8 on the next page). Through the simultaneous binding of this antibody to both antigens, T cells are supposed to be activated, thus leading to tumour-specific cell lysis.

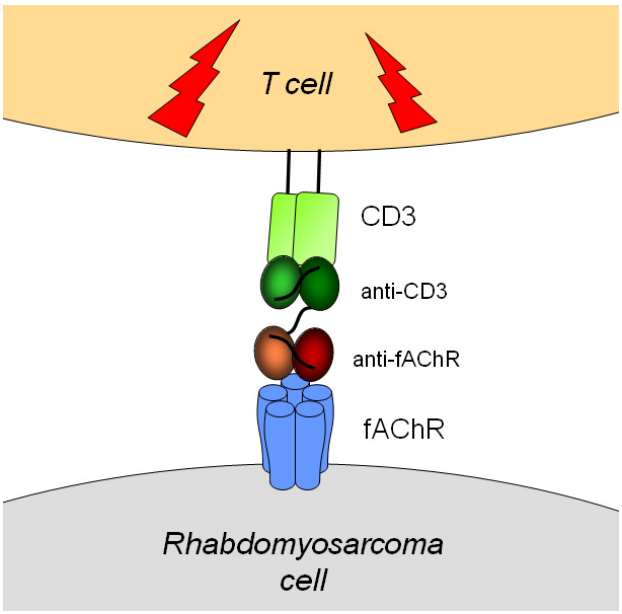


Fig. 2.8: Bispecific Antibody against Rhabdomyosarcoma. One molecule strategy for T-cell activation and the retargeting of T cells to tumour cells (here rhabdomyosarcoma) provided by a bispecific T-cell engager (BiTE) molecule directed against CD3 and fetal acetylcholine receptor (fAChR).

As the second and major aim of this study, a similar but dual antigen-restricted trispecific concept was to be realised for the treatment of blood cancer in the context of haematopoietic stem cell transplantation (HSCT). For an HLA-mismatched setting where the patient is HLA-A2-positive, but the donor is not, it was aimed to exploit the HLA disparity for targeted immunotherapy by using a complementing trispecific antibody. This trispecific antibody should target CD3 and a combination of two other antigens, HLA-A2 and CD45. It should further be formed upon binding to the latter two antigens, which were chosen, because HLA-A2 is one of the most common HLA alleles, and because CD45 is a highly specific haematopoietic marker. This trispecific HLA-A2 × CD3 × CD45 antibody should assemble from two parts in such a way that the third specificity against CD3 is only created upon the prior binding of the two other antigens at the same cell. A high tumour specificity should be reached by this antibody due to the combination of two non-tumour-specific antigens, HLA-A2 and CD45, their coexpression on the recipient's malignant haematopoietic cells, and the single expression of CD45 on the graft and HLA-A2 on the recipient's non-haematopoietic cells, respectively. Thus, single positive cells should remain unaffected (Fig. 2.9).

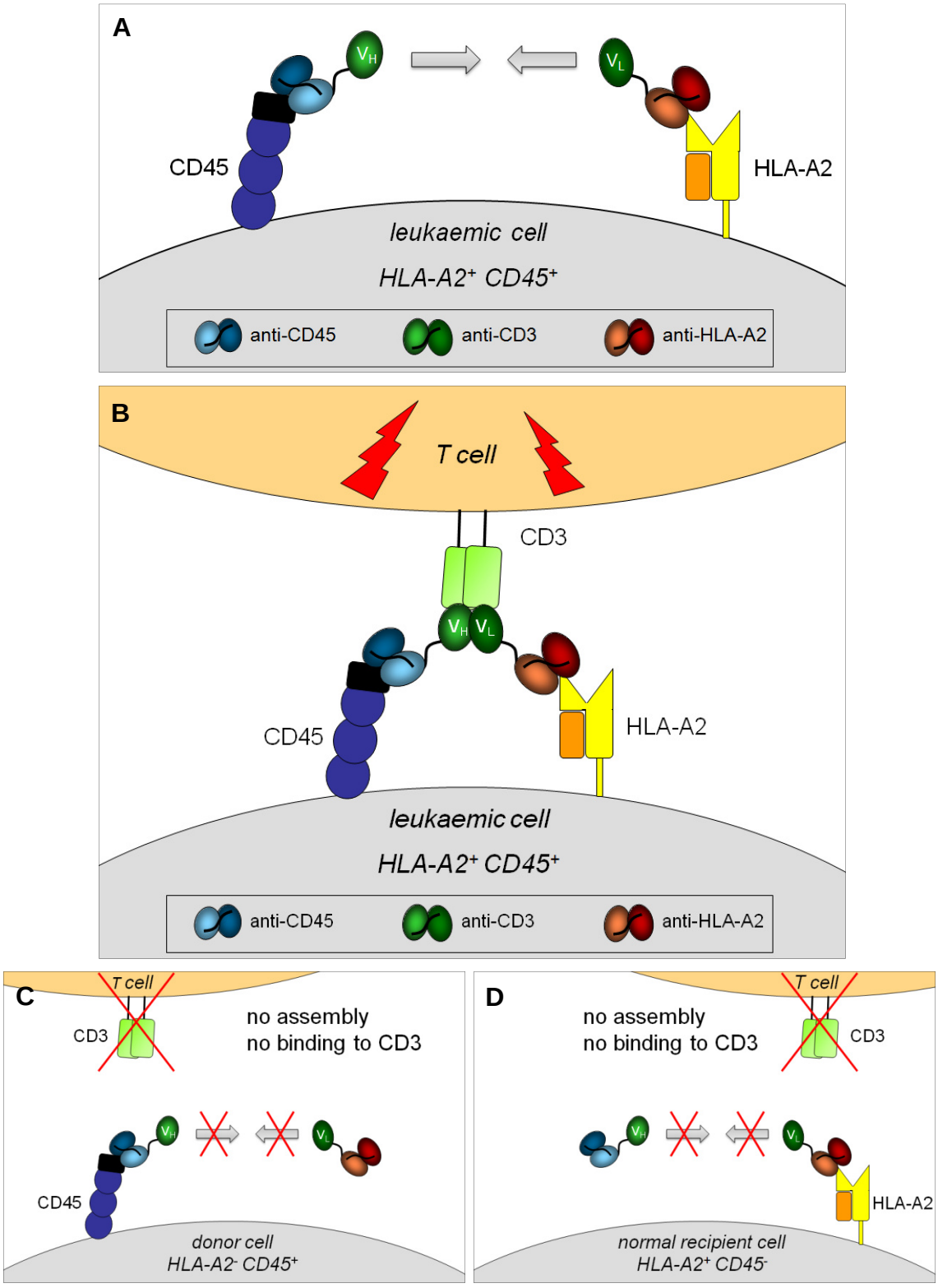


Fig. 2.9: **Concept of a dual antigen-restricted trispecific antibody against leukaemia. A)** Two parts of a trispecific antibody bind to a double positive leukaemic cell (HLA-A2$^+$ CD45$^+$) and assemble. **B)** The complemented trispecific antibody now can activate T cells via CD3. **C) + D)** The presence of a single antigen does not suffice for binding to CD3 and for T cell activation so that single positive cells remain unaffected.

3 Materials and Methods

The descriptions of method details in this section are partly adapted from my diploma thesis at the University of Stuttgart [2008].

3.1 Materials

3.1.1 Instruments

Balances	Basic BA 2100 [Sartorius, Göttingen, DE]
Blotter	PerfectBlue™ Semi-Dry Electroblotter SEDEC M [Peqlab, Erlangen, DE]
Centrifuges	Eppendorf 5417C [Eppendorf, Hamburg, DE], J2-HS with rotors JA14 and JA20 [Beckman Coulter, Krefeld, DE], Heraeus Megafuge 1.OR (cell culture), Varifuge 3.OR (cell culture), and Pico 17 Microcentrifuge [Thermo Scientific, Langenselbold, DE]
CO_2 Incubator	Steri-Cycle with Class 100 HEPA [Thermo Scientific, Langenselbold, DE]
Electrophoresis Systems	Mini-PROTEAN® Tetra cell [Bio-Rad, Munich, DE], PerfectBlue™ Gel System Mini S [Peqlab, Erlangen, DE], Electrophoresis Power Supply E834 [Consort, Turnhout, BE], Electrophoresis Power Supply EPS 301 [GE Healthcare, Freiburg, DE]
ELISA Plate Reader	Lucy 2 [Anthos Labtec, Krefeld, DE]
Flow Cytometry System	BD FACSCalibur [BD BioScience, Heidelberg, DE]
Gel Imager	Gel iX Imager Windows Version with UV-Transilluminator [INTAS Science Imaging Instruments, Göttingen, DE]
Heating Block	Labtherm [Gebr. Liebisch, Bielefeld, DE]
Infrared Imaging System	Odyssey® 3.0 with Software [Li-Cor Biosciences, Bad Homburg, DE]
Laminar Flow Cabinets	Heraeus HB 2448 [Thermo Scientific, Langenselbold, DE], BDK [BDK Luft- und Reinraumtechnik, Sonnenbühl-Genkingen, DE]
Magnetic Stirrer	MR 2002 [Heidolph Instruments, Schwabach, DE]
Microscope	Digital Inverted Fluorescence Microscope, Evos® fl, Cell Imaging System [Advanced Microscopy Group, Bothell, US]
PCR Cycler	Primus 96 Plus Thermal Cycler [MWG Biotech, Ebersberg, DE]
Shaking Incubators	Stuart Orbital Incubators SI50 and SI500 [Stuart Scientific, Redhill, UK]
Spectrophotometer	GeneQuant *pro* RNA / DNA Calculator [Pharmacia Biotech, Uppsala, SE]
Vortex Mixer	7-2020 [neoLab, Heidelberg, DE]
X-ray film processor	Cawomat 2000 IR [Cawo, Schrobenhausen, DE]

3.1.2 Special Implements

Centrifuge Bottles	Nalgene® wide-mouth with sealing caps, 250 ml, style 3141, # Z353736 [Sigma-Aldrich, Steinheim, DE]
CD14 MicroBeads	Anti-human CD14 conjugated magnetic beads [Miltenyi Biotec, Bergisch Gladbach, DE]
Chromatography Columns	Poly-Prep # 731-1550 with Cap # 731-1555 [Bio-Rad, Munich, DE]
Dialysis Membrane	Visking MWCO 14,000; 34 mm, # 1784.1 [Roth, Karlsruhe, DE]
ELISA Microplates	MICROLON® 600, 96 well [Greiner Bio-One, Frickenhausen, DE]
Filter Paper	FN 100, 0.35 mm, 195g/m^2 [Munktell & Filtrak, Bärenstein, DE]
Folded Filter	Rotilabo®, Type 600P [Roth, Karlsruhe, DE]
IMAC Affinity Matrix	Ni-NTA-Agarose, 25 ml, # 30210 [Qiagen, Hilden, DE]
Nitrocellulose Membrane	Whatman™ Protran™ BA83, 0.2 µm pore size [GE Healthcare, Freiburg, DE]
Sterile Filter	Acrodisc® Syringe Filters, 0.2 µm [Pall Life Sciences, Dreieich, DE]
Tissue Culture Flasks/Dishes	CellStar® [Greiner Bio-One, Frickenhausen, DE]
UV-Cuvettes	Semi-micro cuvette, # 67.742 [Sarstedt, Nümbrecht, DE], Micro, center height 15 mm, 70 µl, # 7592 20 [Brand, Wertheim, DE]
X-Ray Films	Amersham Hyperfilms™ ECL [GE Healthcare, Freiburg, DE]

3.1.3 Chemicals, Solutions, and Media

Chemicals

Chemicals were purchased with the highest purity grade available from AppliChem [Darmstadt, DE], Roche [Mannheim, DE], Roth [Karlsruhe, DE], and Sigma-Aldrich [Steinheim, DE] unless otherwise indicated.

Buffers and Solutions

Blocking Buffer	1 % BSA, 0.05 % NaN_3 in sterile PBS
Blotting Buffer 1 ×	20 % methanol, 192 mM glycine, 25 mM Tris base, pH 8.3
Bradford Solution 5 ×	Bio-Rad Protein Assay [Bio-Rad, Krefeld, DE]
Destaining Solution	45 % methanol, 10 % glacial acetic acid in H_2O
DNA Loading Dye 6 ×	10 mM Tris-HCl (pH 7.6), 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 60 % glycerol and 60 mM EDTA, supplied with GeneRuler™ DNA Ladder Mix [Fermentas, St. Leon-Rot, DE]
ECL substrate solution	4 ml of solution A (0.1 M Tris-HCl, 1.4 mM luminal, pH 8.6) + 400 µl solution B (6.7 mM *p*-coumaric acid in DMSO) + 1.2 µl 30 % H_2O_2)
FastAP™ Buffer 10 ×	supplied with FastAP™ Thermosensitive Alkaline Phosphatase
FastDigest® (Green) Buffer 10 ×	supplied with FastDigest® restriction enzymes
$MgCl_2$	25 mM, supplied with *Taq* DNA Polymerase
$(NH_4)_2SO_4$ *Taq* Buffer 10 ×	supplied with *Taq* DNA Polymerase [Fermentas, St. Leon-Rot, DE]

PBA	2 % FCS, 0.02 % NaN_3 in sterile PBS
PBS 1 ×	140.1 mM NaCl, 18.8 mM Na_2HPO_4, 2.3 mM NaH_2PO_4 • H_2O or PBS powder without Ca^{2+}, Mg^{2+} (instamed Dulbecco) for dialysis, # L 182-50 [Biochrom, Berlin, DE]
Periplasmic Preparation Buffer	30 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 20 % (w/v) sucrose in H_2O
Reagent Diluent	0.1 % BSA, 0.05 % Tween 20, 20 mM Tris base, 150 mM NaCl in H_2O, pH 7.2 – 7.4, sterile filtrated
SDS-PAGE Loading Buffer 5 ×	25 % β-mercaptoethanol (only for reducing loading buffer), 25 % glycerol, 10 % (w/v) SDS, 0.05 % bromophenol blue, 313 mM Tris-HCl pH 6.8
SDS-PAGE Running Buffer 10 ×	1.92 M glycine, 250 mM Tris base, 1 % (w/v) SDS, pH 8.3
Sodium Phosphate Buffer 5 ×	250 mM sodium phosphate (36.2 g Na_2HPO_4 • 2 H_2O + 6.4 g NaH_2PO_4 • 2 H_2O), 1.25 M NaCl, pH 7.5, ad 1 l H_2O
Staining Solution	0.25 % Coomassie Blue R 250 in destaining solution
TAE Buffer 50 ×	2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA, pH 8.3, ad 1 l H_2O
TMB Substrate Solution	100 µg/ml TMB in 66,7 % DMSO, 0.006 % H_2O_2, 100 mM sodium acetate buffer pH 6.0

Bacterial Culture

Ampicillin	Stock solution 100 mg/ml in H_2O, # K029.1 [Roth, Karlsruhe, DE]
Kanamycin	Stock solution 100 mg/ml in H_2O K 4000 [Sigma-Aldrich, Steinheim, DE]
IPTG	Isopropyl β-D-thiogalactopyranoside, stock solution 1 M in H_2O, # CN08.2 [Roth, Karlsruhe, DE]
$LB_{amp/kan,\ glc}$ Agar Plates	LB medium, 1.5 % agar autoclaved, 100 µg/ml ampicillin/kanamycin, 1 % glucose added after cooling-down
LB Medium 1 ×	LB medium (Lennox), 20 g/l in H_2O (including 21 % tryptone, 0.5 % yeast extract, 0.5 % NaCl), # X964.2 [Roth, Karlsruhe, DE]
TY Medium 2 ×	2 × YT medium, 31 g/l in H_2O (including 1.6 % tryptone, 1 % yeast extract, 0.5 % NaCl, # X966.2 [Roth, Karlsruhe, DE]

Cell Culture

Fetal Calf Serum (FCS)	Fetal Bovine Serum, Standard Quality, EU approved, A15-101 [PAA Laboratories, Cölbe, DE], heat inactivated at 56°C for 30 min
Ficoll	Lymphocyte Separation Medium LSM 1077, based on Ficoll 400 and sodium diatrizoate [PAA Laboratories, Cölbe, DE]
Lipofectamine™ 2000	Transfection reagent, # 11668-027 [Gibco® Invitrogen, Karlsruhe, DE]
Opti-MEM® I 1 ×	Reduced Serum Medium, liquid, # 31958-054 [Gibco® Invitrogen, Karlsruhe, DE]
Penicillin-Streptomycin-Glutamine (Pen-Strep) 100 ×	liquid, 10^4 U/ml of penicillin, 10 mg/ml of streptomycin, # P11-010 [PAA Laboratories, Cölbe, DE]
PBS (Dulbecco's) 1 ×	Without Ca and Mg, # H15-002 [PAA Laboratories, Cölbe, DE]
Puromycin	Selective reagent, liquid, 10 mg/ml, diluted to 1 mg/ml in sterile H_2O [InvivoGen, San Diego, US]
RPMI 1640 Medium 1 ×	liquid, with L-glutamine, # E15-840 [PAA Laboratories, Cölbe, DE]

DMEM Medium 1 ×	liquid, high glucose (4.5 g/l), with L-glutamine, # E15-810 [PAA Laboratories, Cölbe, DE]
Trypan Blue	0.4 % trypan blue, liquid and sterile-filtered, T 8154 [Sigma-Aldrich, Steinheim, DE]
Trypsin-EDTA 10 ×	liquid, 0.5 % Trypsin, 0.2 % EDTA, # L11-003 [PAA Laboratories, Cölbe, DE] diluted to 1 × in sterile PBS

3.1.4 Antibodies and their Working Dilution

anti-AChRγ unconjugated, mouse IgG1, clone C9 [MCA1330, AbD Serotec, Düsseldorf, DE]	1 : 1	Flow cytometry
anti-FLAG® tag FITC conjugate, mouse IgG1, clone M2 [F 4049, Sigma-Aldrich, Steinheim, DE]	1 : 100	Flow cytometry
anti-His_6 tag unconjugated, mouse IgG1, clone H-3 [sc-8036, Santa Cruz Biotechnology, Heidelberg, DE]	1 : 1,000	Western blot
anti-His_6 tag FITC conjugate, mouse IgG1κ, clone 13/45/31-2 [DIA 920, Dianova, Hamburg, DE]	1 : 50 – 1 : 200	Flow cytometry
anti-His_6 tag HRP conjugate, mouse IgG1, clone H-3 [sc-8036 HRP, Santa Cruz Biotechnology, Heidelberg, DE]	1 : 1,000	Western blot
anti-human CD3ε unconjugated, mouse IgG1κ, clone UCHT-1 [# 21620030, ImmunoTools, Friesoythe, DE]	5 µg/ml	Flow cytometry
anti-human CD3ε FITC conjugate, mouse IgG1κ, clone UCHT-1 [# 21620033, ImmunoTools, Friesoythe, DE]	1 : 20	Flow cytometry
anti-human CD45 FITC conjugate, mouse IgG1, clone 2D-1 [sc-1187, Santa Cruz Biotechnology, Heidelberg, DE]	1 : 44	Flow cytometry
anti-human HLA-A2 PE conjugate, mouse IgG2bκ, clone BB7.2 [# 558570, BD Pharmingen™, BD Biosciences, Heidelberg, DE]	1 : 10	Flow cytometry
anti-mouse IgG (H+L) IRDye® 800CW conjugate, goat polyclonal [# 926-32210, Li-Cor, Bad Homburg, DE]	1 : 15,000	Western blot
anti-mouse IgG (whole molecule) PE conjugate, goat polyclonal [P 9670, Sigma-Aldrich, Steinheim, DE]	1 : 100	Flow cytometry
mouse IgG1κ unconjugated, isotype control, clone MOPC-21 [M 5284, Sigma-Aldrich, Steinheim, DE]	1 : 1 or 5 µg/ml	Flow cytometry
mouse IgG1 FITC conjugate, isotype control, clone MOPC-21 [# 555748, BD Pharmingen™, BD Biosciences, Heidelberg, DE]	1 : 20 or 1 : 44	Flow cytometry
mouse IgG2bκ PE conjugate, isotype control, clone 27-35 [# 555743, BD Pharmingen™, BD Biosciences, Heidelberg, DE]	1 : 10	Flow cytometry

3.1.5 Antigens, Enzymes, Kits, and Markers

Antigens, Nucleotides, Cytokines, Lectins, and Fluorescent Dyes

BSA	Bovine Serum Albumin, A 3059, (A 7030, A 4503) [Sigma-Aldrich, Steinheim, DE]
dNTP Mix	2 mM each deoxynucleotide, molecular biology grade, # R0241 [Fermentas, St. Leon-Rot, DE]
IL-2	Recombinant Human Interleukin-2, # 11340023 [ImmunoTools, Friesoythe, DE]
PHA-L	Phytohemagglutinin-L, # 11 249 738 001 [Roche Diagnostics, Mannheim, DE]
7-AAD	7-AAD (7-aminoactinomycin D) Viability Staining Solution, # 420404 [BIOZOL, Eching, DE]
CFSE (CFDA SE)	see Kits

Enzymes

Alkaline Phosphatase	1 U/µl, FastAP™ Thermosensitive Alkaline Phosphatase, # EF0654 [Fermentas, St. Leon-Rot, DE]
Lysozyme	Muramidase; N-acetylmuramide glycanohydrolase from hen egg white, 10 g, # 10 837 059 001 [Roche Diagnostics, Mannheim, DE]
Restriction Enzymes	*Pvu*I (10 U/µl) and FastDigest® enzymes *Age*I, *Bam*HI, *Eco*RI, *Hind*III, *Nco*I, *Not*I, *Pst*I, *Sac*I, *Sfi*I, *Spe*I (1 FDU/µl) [Fermentas, St. Leon-Rot, DE]
T4 DNA Ligase	5 U/µl, # EL0014 [Fermentas, St. Leon-Rot, DE]
Taq DNA Polymerase	1 U/µl, recombinant, # EP 0403 [Fermentas, St. Leon-Rot, DE]

Kits

GeneJET™ Plasmid Miniprep Kit	[Fermentas, St. Leon-Rot, DE]
Human IL-2 DuoSet ELISA Development Kit, DY202	[R&D Systems, Wiesbaden-Nordenstadt, DE]
PureYield™ Plasmid Midiprep System	[Promega, Mannheim, DE]
REDTaq® ReadyMix™ PCR Reaction Mix (0.06 U/µl)	[Sigma-Aldrich, Steinheim, DE]
Venor® *GeM* Mycoplasma Detection Kit	[Minerva Biolabs, Berlin, DE]
Vybrant® CFDA SE Cell Tracer Kit	[Invitrogen, Karlsruhe, DE]
Wizard® SV Gel and PCR Clean-Up System	[Promega, Mannheim, DE]

Markers

GeneRuler™ DNA Ladder Mix, # SM0333	[Fermentas, St. Leon-Rot, DE]
PageRuler™ Prestained Protein Ladder Plus, # SM1811	[Fermentas, St. Leon-Rot, DE]

3.1.6 Oligonucleotides

Primers for Cloning

Numbered primers were purchased from Metabion [Martinsried, DE].

1_*Sfi*I-*Nco*I-mut-His-back (32 bp)

```
...  SfiI
5′-AG CCG G CC ATG GCA AGC AGC CAT CAC CAT CAT-3′
          NcoI                 His6 tag
```

3_scFvCD45-stop-*Eco*RI-for (40 bp)

```
5′-CCG GAA TTC TTA TTA GCT CAC GGT CAC GCT GGT GCC CTG G-3′
       EcoRI  stop                scFvCD45
```

6_LMB4 (18 bp)

```
5′-GCA AGG CGA TTA AGT TGG-3′
```

8_V_HHLA-A2-*Bam*HI-back (44 bp)

```
5'-TAT GGA TCC GGT GGA GGA GGT TCA CAG GTG CAG CTG GTG CAG TC-3'
       BamHI                        VHHLA-A2
```

10_V_LCD3-*Nco*I-back (34 bp)

```
5'-CAT GCC ATG GCC GAC ATT GTA CTG ACC CAG TCT C-3'
          NcoI              VLCD3
```

12_V_HCD45-*Nco*I-*Spe*I-back (42 bp)

```
5'-CAT ACC ATG GCC ACT AGT ACT GGT CAG GTG CAG CTG GTG GAA-3'
          NcoI      SpeI              VHCD45
```

13_V_HCD45-*Age*I-for (83 bp)

```
5'-ATA ACC GGT ACC TCC TCC TGA ACC ACC TCC TCC TGA ACC TCC TCC ACC…
          AgeI

…GCT CAC GGT CAC GCT GGT GCC CTG GCC CCA ATA A-3'
                VHCD45
```

15_V_LCD45-*Not*I-for (48 bp)

```
5'-A TAG TTG TGC GGC CGC TTT AAT TTC CAG TTT GGT GCC GCT GCC AAA GG-3'
                NotI                VLCD45
```

16_V_HCD3-*Hind*III-back (31 bp)

```
5'-CCT AAG CTT GAC GTC CAA CTG GTG CAG TCA G-3'
          HindIII           VHCD3
```

17_V_LCD3-*Sfi*I-for (48 bp)

```
5'-T TAT GGC CGG CTG GGC CGA TTT GAT CTC CAC CTT GGT CCC GCC ACC GA-3'
             SfiI                VLCD3
```

18_V_LAChR-*Bam*HI-back (43 bp)

```
5'-TAT GGA TCC GGT GGA GGT TCA GTG ATG ACC CAG TCT CCA TCC T-3'
       BamHI                     VLAChR
```

19_V_HAChR-*Not*I-*Sac*I-for (20 bp)

```
5'-GCT CAG CCT AAT CAT CTA GC-3'
          multi stop
```

20_V_HCD3-*Not*I-back (47 bp)

```
5'-TA TTA TTA GCG GCC GCA GGT GGT TCA GAC GTC CAA CTG GTG CAG TCA-3'
          NotI                VHCD3
```

21_V_LCD3-stop-*Sac*I-for (44 bp)

5'-GGC GAG CTC TTA TTA TTT GAT CTC CAC CTT GGT CCC GCC ACC GA-3'
*Sac*I stop V_LCD3

23_V_HCD3-*Sac*I-back (33 bp)

5'-TT GAG CTC GGT GAC GTC CAA CTG GTG CAG TCA G-3'
*Sac*I V_HCD3

24_V_LAChR-*Sfi*I-back (40 bp)

5'-TAA TAG GCC CAG CCG GCC ATG GCC GTG ATG ACC CAG TCT C-3'
*Sfi*I V_LAChR

25_V_HCD3-*Bam*HI-for (37 bp)

5'-T ATG GAT CC T GAG GAG ACG GTG ACC GTG GTG CCT TGG-3'
*Bam*HI V_HCD3

26_V_LHLA-A2-*Sac*I-for (37 bp)

5'-CTT GAG CTC TCC GCC ACC ACG TTT GAT ATC CAC TTT G-3'
*Sac*I V_LHLA-A2

27_LMB3 (18 bp)

5′-CAG GAA ACA GCT ATG ACC-3′

Primers for Screening or Sequencing

Primers were available in-house or at GATC [Konstanz, DE].

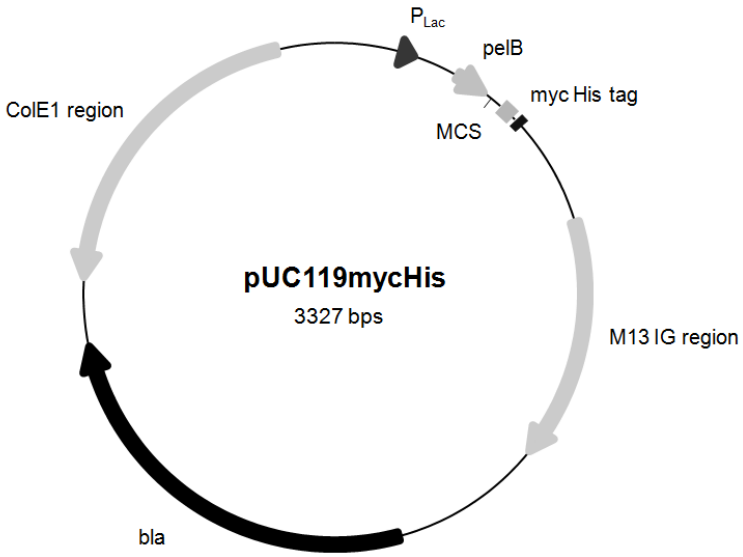
pBM1.1:	T-7	(forward, 20 bp)	5′–TAA TAC GAC TCA CTA TAG GG–3′
	pET-24a	(reverse, 23 bp)	5'-GGG TTA TGC TAG TTA TTG CTC AG-3'
	alternative:		
	19_V_HAChR-*Not*I-*Sac*I-for	(reverse, 20 bp)	sequence as stated above
pIRES:	CMV-F	(forward, 21 bp)	5′–CGC AAA TGG GCG GTA GGC GTG–3′
	pIRES-RP	(reverse, 19 bp)	5'-TAT AGA CAA ACG CAC ACC G-3'
pUC119:	27_LMB3	(forward, 18 bp)	5′–CAG GAA ACA GCT ATG ACC–3′
	6_LMB4	(reverse, 18 bp)	5′–GCA AGG CGA TTA AGT TGG–3′
	alternative:		
	LMB2	(reverse, 18 bp)	5′–TGT AAA ACG ACG GCC AGT–3′
	1_*Sfi*I-*Nco*I-mut-His-back	(forward, 32 bp)	sequence as stated above
	3_scFvCD45-stop-*Eco*RI-for	(reverse, 40 bp)	sequence as stated above
	8_V_HHLA-A2-*Bam*HI-back	(forward, 44 bp)	sequence as stated above
	16_V_HCD3-*Hind*III-back	(forward, 31 bp)	sequence as stated above

3.1.7 Vectors

pUC119mycHis: Vector for Periplasmic Protein Expression in *E. coli* TG1

The vector backbone used, pUC119mycHis (derived from the pUC119 plasmid), comprises a *pelB* leader sequence for periplasmic secretion, a myc tag and a hexahistidine tag for purification. Furthermore, this vector contains the *lacI* repressor gene, the *lacZ* promoter, the intergenic region of M13 bacteriophage (M13 IG), the ColE1 origin of replication, and a multiple cloning site (MCS). It also expresses the *bla* gene conferring ampicillin resistance (β-lactamase) needed for prokaryotic selection (Fig. 3.1) [Low *et al.*, 1996].

A



B

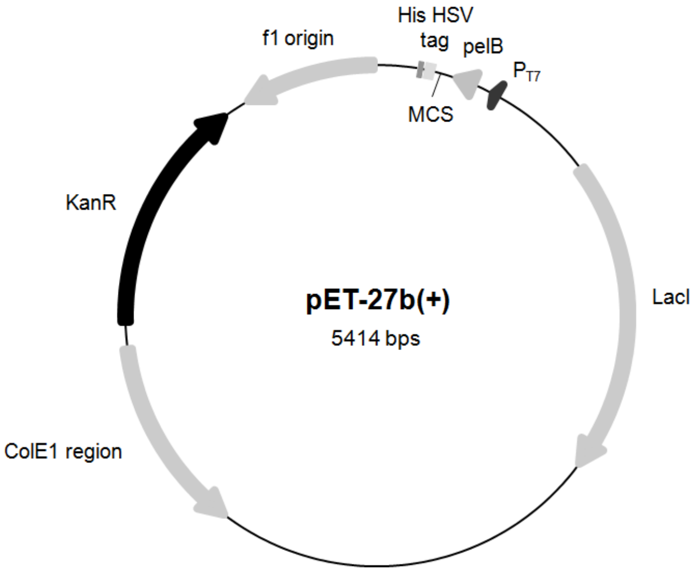
```
                                                  HindIII                 RBS
...........caggaaacagctatgaccatgattacgccaagcttgcatgcaaattctatttcaaggagacagtcata
                  LMB3
                                                                        SfiI
atg aaa tac cta ttg cct acg gca gcc gct gga ttg tta tta ctc gcg gcc cag ccg gcc
 M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L   A   A   Q   P   A
 pelB leader sequence

                PstI    SalI    XhoI                        NotI
atg gcc cag gtg cag ctg cag gtc gac ctc gag atc aaa cgg gcg gcc gca gaa caa aaa
 M   A   Q   V   Q   L   Q   V   D   L   E   I   K   R   A   A   A   E   Q   K
                                                                    myc tag…

ctc atc tca gaa gag gat ctg aat ggg gcc gca cat cac cat cat cac cat taa taa
 L   I   S   E   E   D   L   N   G   A   A   H   H   H   H   H   H   *   *
…myc tag                                    His tag

EcoRI
gaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatcgccttgc....
          LMB2                                                  LMB4
```

Fig. 3.1: **A) Schematic representation of the expression vector pUC119mycHis.** P_{Lac}, *lacZ* promoter; *pelB*, leader sequence; MCS, multiple cloning site; myc and His_6 tag; M13 IG, intergenic region; *bla*, encoding β-lactamase (ampicillin resistance gene); ColE1, origin of replication. **B) Extract of pUC119mycHis sequence.** The positions of primers LMB2, LMB3, and LMB4, ribosomal binding site (RBS), *pelB* leader sequence, MCS, myc and His_6 tag are indicated.

pET-BM1.1: Vector for Periplasmic Protein Expression in *E. coli* BL21 (DE3)

The other vector backbone used, pBM1.1, was cloned from the pET-27b(+) plasmid from Novagen by Matthey *et al.* [1999]. The pET-27b(+) plasmid comprises a *pelB* leader sequence for periplasmic secretion, a HSV tag and a hexahistidine tag for purification. Furthermore, this vector contains the T-7 promoter, the *lacI* coding sequence, the ColE1 and f1 origin of replication, and a multiple cloning site (MCS). It also expresses a gene conferring kanamycin resistance needed for prokaryotic selection (Fig. 3.2).

A



B

```
                                         ............taatacgactcactataggg
                                                              T7
                    XbaI                              RBS
gaattgtgagcggataacaattcccctctagaaataattttgtttaactttaagaaggagatatacat

atg aaa tac ctg ctg ccg acc gct gct gct ggt ctg ctg ctc ctc gct gcc cag ccg gcg
 M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L   A   A   Q   P   A
 pelB leader sequence
                                                EcoRI
     NcoI                          BamHI              SacI    SalI   HindIII NotI…
atg gcc atg gat atc gga att aat tcg gat ccg aat tcg agc tcc gtc gac aag ctt gcg
 M   A   M   D   I   G   I   N   S   D   P   N   S   S   S   V   D   K   L   A

…NotI   XhoI
gcc gca ctc gag atc aaa cgg gct agc cag cca gaa ctc gcc ccg gaa gac ccc gag gat
 A   A   L   E   I   K   R   A   S   Q   P   E   L   A   P   E   D   P   E   D
                                 HSV tag

gtc gag cac cac cac cac cac cac tga gatccggctgctaacaaagcccgaaaggaagctgagttggctg
 V   E   H   H   H   H   H   H   *
         His tag

        CelII
ctgccaccgctgagcaataactagcataaccc....
                 pET-24a
```

Fig. 3.2: **A) Schematic representation of the expression vector pET-27b(+).** P_{T7}, T7 promoter; *pelB*, leader sequence; MCS, multiple cloning site; HSV and His_6 tag; KanR, encoding kanamycin resistance gene; ColE1 and f1, origin of replication; LacI, *lacI* repressor. **B) Extract of pET-27b(+) sequence.** The positions of primers T7 and pET-24a, ribosomal binding site (RBS), *pelB* leader sequence, MCS, HSV and His_6 tag are indicated.

pIRESpuro2 HLA-A2: Vector for Eukaryotic Protein Expression in HEK-293T Cells

The cDNA of HLA-A2 based on genomic DNA, thus including promoter and introns of the human HLA-A0201, was kindly provided by Dr. Grosse-Hovest, Department of Immunology, University of Tübingen, in the vector pIRESpuro2 [Clontech, Heidelberg, DE]. This vector comprises the human cytomegalovirus (CMV) immediate early enhancer and promoter, a synthetic intron (IVS), an internal ribosome entry site (IRES), a gene coffering puromycin resistance needed for eukaryotic selection, and the bovine growth hormone (BGH) poly-A signal. Additionally, ColE1, *bla*, and an MCS are present (Fig. 3.3).

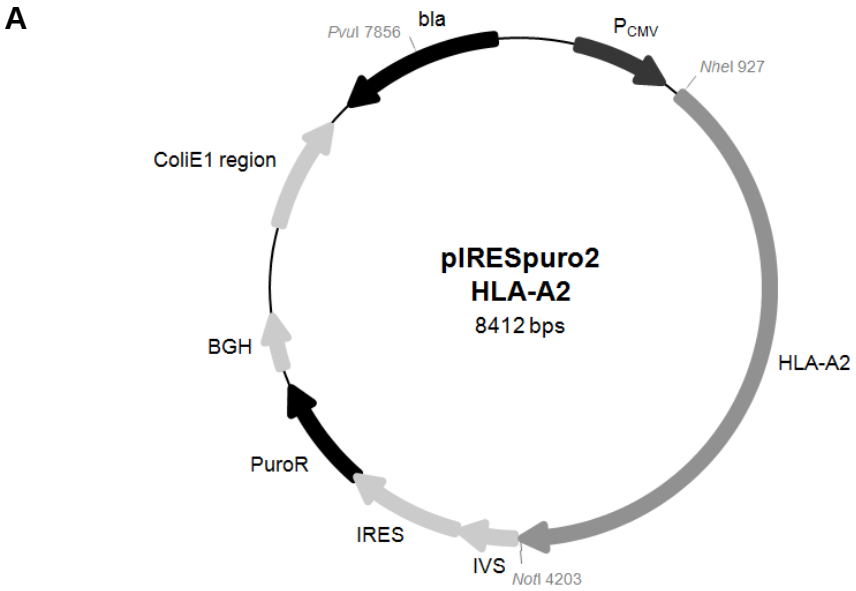


Fig. 3.3: A) Schematic representation of the expression vector pIRESpuro2 HLA-A2. P_{CMV}, human cytomegalovirus immediate early promoter; IVS, synthetic intron; IRES, internal ribosome entry site; BGH, bovine growth hormone poly-A signal; PuroR, encoding puromycin resistance gene; ColE1, origin of replication; *bla*, encoding β-lactamase (ampicillin resistance gene).

3.1.8 *E. coli* Strains

BL21 Star™ (DE3): F- *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm rne131*(DE3)

[Invitrogen by Life Technologies GmbH, Darmstadt, DE]

NEB 5-alpha: *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

[New England Biolabs GmbH, Frankfurt, DE]

TG1: Δ(*lac-pro*), *sup*E, *thi, hsd*D5 [F' *tra*D36, *pro*AB, *lacI*^{q}, *lacZ*ΔM15]

[DSMZ, Braunschweig, DE]

3.1.9 Mammalian Cell Lines

Table 3.1: Mammalian cell lines and corresponding culture conditions.

Cell Line	Cell Type	Medium
A-204	human rhabdomyosarcoma; $fAChR^-$	RPMI 1640 + 10 % FCS
AX-OH-1	human alveolar rhabdomyosarcoma; $fAChR^+$	RPMI 1640 + 10 % FCS
HEK-293T	highly transfectable derivative of the human embryonal kidney cell line HEK-293 carrying a plasmid containing the temperature sensitive mutant of SV-40 large T antigen; $HLA\text{-}A2^-$	DMEM + 10 % FCS
HEK-293T A2+	HEK-293T cells stably transfected with the human MHC class I molecule HLA-A201	DMEM + 10 % FCS
Jurkat	human T-cell leukaemia (acute lymphoblastic) cells; $CD3^+$, $CD45^+$, $HLA\text{-}A2^-$	RPMI 1640 + 10 % FCS
KMS-12-BM	human multiple myeloma (B cell); $CD45^-$, $HLA\text{-}A2^-$	RPMI 1640 + 10 % FCS
L-363	human plasma cell leukaemia related to multiple myeloma (B cell); $CD45^-$, $HLA\text{-}A2^+$	RPMI 1640 + 10 % FCS
MCF-7	human breast adenocarcinoma; $HLA\text{-}A2^{++}$	RPMI 1640 + 10 % FCS
PBMC	peripheral blood mononuclear cells of human origin, isolated from blood donor buffy coat by Ficoll density gradient centrifugation; largely monocyte-depleted; $CD3^+$, $CD45^+$, $HLA\text{-}A2^{+/-}$	RPMI 1640 + 10 % FCS + Pen-Strep
Raji	human Burkitt's lymphoma (B cell); $CD45^+$, $HLA\text{-}A2^-$	RPMI 1640 + 10 % FCS
RD	human embryonal rhabdomyosarcoma; $fAChR^+$	DMEM + 10 % FCS
TE-671	human embryonal rhabdomyosarcoma; $fAChR^+$, a derivative of the RD cell line	DMEM + 10 % FCS
U266	human multiple myeloma (B cell); $CD45^+$, $HLA\text{-}A2^+$	RPMI 1640 + 10 % FCS

Cells were cultured at 37°C in a humidified (60 % rel. humidity) incubator with a 5 % CO_2 atmosphere and were tested for the presence of mycoplasma by using the Venor® *GeM* Mycoplasma Detection Kit from Minerva Biolabs according to the manufacturer's instructions.

3.2 Strategies for Cloning of Recombinant Antibody Constructs

3.2.1 Construction of Recombinant Bispecific AChRγ × CD3 taFvs

The cDNA of the human scFv against AChRγ with an N-terminal His_{10} cluster was kindly provided by Prof. S. Gattenlöhner, Institute of Pathology, University of Würzburg. This scFv originates from a human Fab-fragment (Fab35) that was isolated from a thymic combinatorial cDNA library from a myasthenia gravis patient with high levels of AChRγ-specific autoantibodies [Matthews *et al.*, 2002]. The conversion of Fab35 into the scFv format is detailed in Gattenlöhner *et al.* [2010] [see Section 8.1].

The deimmunised mouse scFv fragment diL2K [Brischwein *et al.*, 2006] was chosen as the CD3-specific antibody fragment. It derives from the human CD3ε-specific mouse mAb L2K [Dreier *et al.*, 2002], which is identical with the mAb TR66 [Lanzavecchia and Scheidegger, 1987, Traunecker *et al.*, 1991]. The cDNA sequence of the scFv diL2K was obtained from a patent specification accessible online [Kinch *et al.*, 2007] and was commercially synthesised [GenScript, Piscataway, US] after the addition of a C-terminal $FlagHis_6$ tag and several desired restriction sites.

scFvAChR had been available in the prokaryotic expression vector pBM1.1 [Matthey *et al.*, 1999] with a flexible $(G_4S)_2$-G-$(GS)_2$-linker between V_L and V_H, which allows the suitable folding into a functional scFv.

scFvCD3 was cloned from the provided cloning vector pUC57 from GenScript into the prokaryotic expression vector pUC119 scFvHLA-A2 [see Section 8.5] via *Nco*I and *Eco*RI, generating pUC119 scFvCD3 [see Section 8.2]. Here, V_H and V_L were joined by a flexible GEGTST-$(GSG)_3$-GAD-linker.

For the cloning of His-taFvCD3-AChR, the coding sequence of scFvCD3 was amplified by PCR with the primers *16_V_HCD3-HindIII-back* and *17_V_LCD3-SfiI-for*, with plasmid pUC57 scFvCD3 as a template to introduce a *Hind*III restriction site at the N-terminus and an SAQPA-linker/*Sfi*I restriction site at the C-terminus of scFvCD3. Then scFvCD3 was inserted N-terminal to scFvAChR into pBM1.1 scFvAChR by digestion with *Hind*III and *Sfi*I, resulting in pBM1.1 His-taFvCD3-AChR.

For the construction of taFvCD3-AChR-FlagHis, the cDNA of scFvAChR was amplified from pBM1.1 scFvAChR by using the primers *18_V_LAChR-BamHI-back* and *19_V_HAChR-NotI-SacI-for* to introduce a *Bam*HI/GSG_3S-encoding linker sequence at the N-terminus of scFvAChR. scFvAChR was then digested with *Bam*HI/*Not*I. scFvCD3 was cut from pUC57 scFvCD3 by *Sfi*I/*Bam*HI. Both inserts, scFvCD3 (*Sfi*I/*Bam*HI) and scFvAChR (*Bam*HI/*Not*I), were introduced into pUC119 V_HCD3-scFvCD45$(V_L$-$V_H)$ [for cloning, see Section 3.2.2] via *Sfi*I/*Not*I, generating pUC119 taFvCD3-AChR-FlagHis.

Amplification of the scFvCD3 coding sequence from pUC57 scFvCD3 with the primers *20_V_HCD3-NotI-back* and *21_V_LCD3-stop-SacI-for* introduced *Not*I/A_3G_2S-linker at the N-terminus and stop/*Sac*I at the C-terminus. Hence, scFvCD3 could be inserted C-terminally to scFvAChR into pBM1.1 scFvAChR via *Not*I/*Sac*I, resulting in pBM1.1 His-taFvAChRCD3.

For the construction of taFvAChR-CD3-FlagHis, the cDNA of scFvCD3 was amplified from pUC57 scFvCD3 by using the primers *23_V_HCD3-SacI-back* and *06_LMB4* to introduce *Sac*I N-terminal to scFvCD3. In addition, the cDNA of scFvAChR was amplified from pBM1.1 scFvAChR by using the primers *24_V_LAChR-SfiI-back* and *19_V_HAChR-NotI-SacI-for* to introduce *Sfi*I at the N-terminus of scFvAChR. Then scFvAChR and scFvCD3 were digested with *Sfi*I/*Sac*I and *Sac*I/*Not*I, respectively. Both inserts, scFvAChR (*Sfi*I/*Sac*I) and scFvCD3 (*Sac*I/*Not*I), were introduced into pUC119 V_HCD3-scFvCD45$_{(V_L\text{-}V_H)}$ via *Sfi*I/*Not*I, generating pUC119 taFvAChR-CD3-FlagHis with an A_3ELG-linker between the two scFvs.

3.2.2 Construction of a Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibody

The cDNA of the HLA-A2-specific scFv (clone 3PF12) with a C-terminal mycHis$_6$ tag was kindly provided by Dr. Nicholas A. Watkins, Department of Haematology, University of Cambridge. scFvHLA-A2 was isolated from an alloimmunised patient and selected by V gene phage display technology [Watkins *et al.*, 2000] [see Section 8.5].

The selected CD45-specific scFv is derived from human CD45-specific mouse mAb BC8 (hybridoma), which is reactive with all CD45 isoforms [Lin *et al.*, 2006]. The cDNA sequence of scFvCD45 was obtained from the given publication and commercially synthesised [GenScript, Piscataway, US] in the V_L-V_H orientation after the addition of a His$_6$Flag tag and a TCS at the N-terminus, GFP11 at the C-terminus, as well as several desired restriction sites.

scFvHLA-A2 was available in the prokaryotic expression vector pUC119mycHis with a flexible $(G_4S)_3$-linker between V_H and V_L, which allows the suitable folding into a functional scFv.

The situation with scFvCD45 was different, as the cDNA of scFvCD45 was achieved from a sequence of scFvCD45$_{(V_L\text{-}V_H)}$-GFP11, which had already been acquired by purchase. First, the cDNA of scFvCD45$_{(V_L\text{-}V_H)}$-GFP11 was amplified by PCR from the provided cloning vector pUC57 scFvCD45$_{(V_L\text{-}V_H)}$-GFP11 by using the primers *01_SfiI-NcoI-mut-His-back* and *27_LMB3*. On this occasion, the last amino acid in the *pelB* leader in the incorrectly ordered sequence was amended. Then scFvCD45$_{(V_L\text{-}V_H)}$-GFP11 was introduced into pUC119 scFvHLA-A2 via *Nco*I/*Eco*RI, generating pUC119 scFvCD45$_{(V_L\text{-}V_H)}$-GFP11 with the corrected *pelB* leader sequence. PCR amplification of

the scFvCD45(V_L-V_H) coding sequence from pUC119 scFvCD45(V_L-V_H)-GFP11 with the primers *01_SfiI-NcoI-mut-His-back* and *03_scFvCD45-stop-EcoRI-for* introduced stop/*Eco*RI C-terminal to scFvCD45(V_L-V_H). Thus, scFvCD45(V_L-V_H) could be inserted into the prokaryotic expression vector pUC119 scFvHLA-A2 via *Nco*I/*Eco*RI, resulting in pUC119 scFvCD45(V_L-V_H). Here, V_L and V_H were joined by a flexible KIS-$(G_4S)_3$-S-linker.

Due to suboptimal features of scFvCD45(V_L-V_H), which emerged in flow cytometry [see Fig. 4.17 in Section 4.4.4], scFvCD45(V_H-V_L) was additionally constructed. For this purpose, the cDNA of V_HCD45 and V_LCD45 was amplified from pUC119 scFvCD45(V_L-V_H) by using the primer pair *12_V_HCD45-NcoI-SpeI-back/13_V_HCD45-AgeI-for* and *27_LMB3/15_V_LCD45-NotI-for*, respectively. In doing so, *Nco*I/*Spe*I was introduced N-terminal to V_HCD45, $(G_4S)_2$-G_3TG-linker/*Age*I C-terminal to V_HCD45, *Age*I N-terminal to V_LCD45, and *Not*I C-terminal to V_LCD45. Then V_HCD45 and V_LCD45 were digested with *Nco*I/*Age*I and *Age*I/*Not*I, respectively. Both inserts, V_HCD45 (*Nco*I/*Age*I) and V_LCD45 (*Age*I/*Not*I), were introduced into pUC119 V_HCD3-scFvCD45(V_L-V_H) [for cloning, see below in this section] via *Nco*I/*Not*I, generating pUC119 scFvCD45(V_H-V_L) with a $(G_4S)_2$-G_3TG-linker between V_H and V_L.

For the construction of V_LCD3-scFvHLA-A2, the cDNA of V_LCD3 was amplified from pUC57 scFvCD3 by using the primers *10_V_LCD3-NcoI-back* and *06_LMB4*, thus introducing *Nco*I at the N-terminus of V_LCD3. The cDNA of scFvHLA-A2 was amplified from pUC119 scFvHLA-A2 by using the primers *08_V_HHLA-A2-BamHI-back* and *06_LMB4*, thus introducing *Bam*HI/GS-(G_4S)-linker at the N-terminus of scFvHLA-A2. Then V_LCD3 and scFvHLA-A2 were digested with *Nco*I/*Bam*HI and *Bam*HI/*Not*I, respectively. Both inserts, V_LCD3 (*Nco*I/*Bam*HI) and scFvHLA-A2 (*Bam*HI/*Not*I), were introduced into pUC119 scFvHLA-A2 via *Nco*I/*Not*I, generating pUC119 V_LCD3-scFvHLA-A2 with a GS-(G_4S)-linker between V_LCD3 and scFvHLA-A2.

For the cloning of V_HCD3-scFvCD45(V_L-V_H), the cDNA of V_HCD3 was cut from pUC57 scFvCD3 by *Nco*I/*Age*I and inserted into pUC119 scFvCD45(V_L-V_H)-GFP11 via *Nco*I/*Age*I, generating pUC119 V_HCD3-scFvCD45(V_L-V_H)-GFP11 as an intermediate product. Then the coding sequence of a $FlagHis_6$ tag was cloned from pUC57 scFvCD3 into pUC119 V_HCD3-scFvCD45(V_L-V_H)-GFP11 via *Not*I/*Eco*RI, resulting in pUC119 V_HCD3-scFvCD45(V_L-V_H) with a GEGTSTG-linker between V_HCD3 and scFvCD45(V_L-V_H).

pUC119 V_HCD3-scFvCD45(V_H-V_L) with the same GEGTSTG-linker between V_HCD3 and scFvCD45(V_H-V_L) was obtained by cloning the cDNA of *pelB*-V_HCD3 from pUC119 V_HCD3-scFvCD45(V_L-V_H)-GFP11 into pUC119 scFvCD45(V_H-V_L) via *Hind*III/*Spe*I.

For the construction of V_HCD3-scFvHLA-A2-mycHis, the cDNA of V_HCD3 was amplified from pUC119 scFvCD3 by using the primers *27_LMB3* and *25_V_HCD3-BamHI-for*, thus

introducing *Bam*HI at the C-terminus of V_HCD3. Then V_HCD3 was inserted into pUC119 V_LCD3-scFvHLA-A2 via *Nco*I/*Bam*HI, generating pUC119 V_HCD3-scFvHLA-A2-mycHis with a GS-(G_4S)-linker between V_HCD3 and scFvHLA-A2.

Thereof V_HCD3-scFvHLA-A2 was subsequently cloned into pUC119 scFvCD45$_{(V_H\text{-}V_L)}$ via *Nco*I/*Not*I, resulting in pUC119 V_HCD3-scFvHLA-A2-FlagHis.

3.2.3 Construction of Bispecific HLA-A2 × CD3 taFvs

For cloning taFvCD3-HLA-A2-mycHis, the cDNA of scFvCD3 was cut from pUC119 taFvCD3AChR-FlagHis by *Nco*I/*Bam*HI. Then scFvCD3 was inserted into pUC119 V_LCD3-scFvHLA-A2 via *Nco*I/*Bam*HI, generating pUC119 taFvCD3-HLA-A2-mycHis with a GS-(G_4S)-linker between scFvCD3 and scFvHLA-A2.

Thereof taFvCD3-HLA-A2 was then cloned into pUC119 V_HCD3-scFvCD45$_{(V_L\text{-}V_H)}$ via *Sfi*I/*Not*I, resulting in pUC119 taFvCD3-HLA-A2-FlagHis.

For construction of taFvHLA-A2-CD3-mycHis, the cDNA of scFvHLA-A2 was amplified from pUC119 scFvHLA-A2 by using the primers *27_LMB3* and *26_V_LHLA-A2-SacI-for*, thus introducing G_3EL-linker/*Sac*I at the C-terminus of scFvHLA-A2. Then scFvHLA-A2 was introduced into pUC119 taFvAChR-CD3-FlagHis [see Section 8.7] via *Sfi*I/*Sac*I, generating pUC119 taFvHLA-A2-CD3-mycHis with a G_3ELG-linker between the two scFvs.

The subsequent cloning of taFvHLA-A2-CD3 into pUC119 scFvHLA-A2 via *Nco*I/*Not*I resulted in pUC119 taFvCD3-HLA-A2-mycHis.

3.3 Cloning of Recombinant Antibody Constructs

3.3.1 Polymerase Chain Reaction (PCR)

PCR enables selective amplification of specific regions of DNA. The following PCR master mix was prepared to amplify the chosen DNA fragments from corresponding vectors:

Volume	Compound (concentration)	Final conc.
10 µl	DNA template (1 ng/µl)	10 ng
5 µl	$(NH_4)_2SO_4$ buffer 10 ×	1 ×
4 µl	$MgCl_2$ (25 mM)	2 mM
1 µl	forward primer (10 pmol/µl)	0.2 µM
1 µl	reverse primer (10 pmol/µl)	0.2 µM
1.25 µl	dNTP set (4 × 2 mM)	4 × 50 µM
1.25 µl	*Taq* DNA-Polymerase (1 U/µl)	
ad 50 µl	H_2O	

For amplification of DNA fragments, the same PCR programme was used, but with adapted annealing temperature:

3 min 94°C	initial denaturation	
30 sec 94°C	denaturation	30 ×
30 sec 51 – 62°C	annealing	
1 min 72°C	extension	
5 min 72°C	final extension	

Agarose gel electrophoresis was performed with a 1 % agarose gel in order to confirm that the amplification succeeded and to verify the size of the amplified DNA fragments. Following separation, PCR fragments were extracted from the gel by using the Wizard® SV Gel and PCR Clean-Up System from Promega (see Section 3.3.3) according to the manufacturer's instructions.

3.3.2 Restriction Digestion

For restriction digestion, 2 (– 10) µg of vector DNA, 1 (– 5) µg of insert DNA, or the entire amount of PCR product DNA extracted from an agarose gel (see Section 3.3.1) were digested in a total volume of 20 (– 50) µl. In each digestion sample, 0.5 – 3 µl of the respective restriction enzyme were used in the corresponding buffer. Digestion occurred for 5 – 30 min under appropriate temperature conditions according to the manufacturer's instructions.

Finally, 1 µl of alkaline phosphatase was added to digested vector DNA and incubated for at least 10 min at 37°C. This enzyme enhances the efficiency of the following ligation by dephosphorylation of the 5' end and thus preventing the DNA from self-ligation.

3.3.3 Agarose Gel Electrophoresis and DNA Gel Extraction

Horizontal agarose gel electrophoresis was performed in order to analyse PCR products, to estimate the size of DNA molecules following restriction enzyme digestion, and to purify digested DNA. For this purpose, DNA samples were mixed with DNA loading dye and separated on a 0.8 – 1.5 % agarose gel containing 0.05 µg/ml ethidium bromide in TBE buffer. For size estimation, 3 or 5 µl of GeneRuler™ DNA Ladder Mix from Fermentas were loaded on each gel as well. After a gel was run for 1 h at 100 V, relevant DNA bands were excised under ultraviolet light, and the DNA fragments were extracted from the gel by using the Wizard® SV Gel and PCR Clean-Up System from Promega according to the manufacturer's instructions. Finally, DNA was eluted from the column into 35 µl H_2O.

3.3.4 Ligation

For covalent binding of a linearised, dephosphorylated vector and an insert, T4 DNA ligase was used. Ligation was performed with 2.5 U of T4 DNA ligase in ligation buffer in a total volume of 20 µl, containing vector and insert at a ratio of 1 : 3 up to 1 : 16.5. For ligations of two fragments into one vector, ratios of 1 : 3 : 3 up to 1 : 10 : 10 ($V : I_1 : I_2$) were prepared. As a negative control solely the vector was used. After ligation occurred for at least 1 h at room temperature (RT), 10 µl of each ligation sample were transformed into chemically competent cells (see Section 3.3.6).

3.3.5 Preparation of Chemically Competent Cells

Making bacterial cells chemically competent enables them to take up plasmid DNA, which is needed for the expression of recombinant proteins. In this case, cells of the *E. coli* strain TG1 and BL21 Star™ (DE3) were made chemically competent using $CaCl_2$ as follows:

100 ml of LB medium, including 1 % glucose, were inoculated with 1 ml of an overnight culture of bacterial cells and grown to exponential phase (OD_{600} 0.4 – 0.8) at 37°C. Then the bacterial suspension was chilled on ice for 15 min, and cells were harvested by centrifugation for 10 min at 2,000 × g and 4°C. Following gentle resuspension in 2 × 30 ml of 100 mM ice cold $CaCl_2$, bacterial cells were centrifuged as stated above. Finally, in order to be suitable for storage, chemically competent cells were gently resuspended in 2 × 2.5 ml of 50 mM ice cold $CaCl_2$ containing 20 % glycerol and then frozen in aliquots at -80°C.

In order to determine the transformation efficiency of the chemically competent cells, 100 µl of cells were transformed with 10 ng of pUC119 scFvHLA-A2 plasmid DNA, 20 pg of pUC119 control plasmid DNA, or H_2O as a negative control (3.3.6). The transformation efficiency is defined as the number of transformed cells generated by 1 µg of plasmid DNA in a transformation reaction (indicated as 'colony-forming units' per µg). The resulting transformation efficiency usually amounted to $10^5 – 10^6$ cfu/µg.

3.3.6 Transformation of Chemically Competent Cells

For transformation, 100 µl of chemically competent cells were thawed on ice and added to 10 µl of ligated DNA, then mixed up gently and incubated on ice for 15 min. Next, cells were subjected to a heat shock at 42°C for exactly 1.5 min in a water bath and subsequently cooled down on ice for 1 min. Since the plasmids used in this study carry ampicillin- or kanamycin-resistance genes, successfully transformed cells could be selected by ampicillin or kanamycin, respectively. Thus, 1 ml of LB medium was added and the cells were incubated for 45 min at 37°C with shaking to allow the expression of the resistance protein. Cells were then centrifuged for 1 min at

17,900 × g. Finally, after the supernatant was discarded, cells were resuspended in the backflow, plated on $LB_{amp/glc}$ or $LB_{kan/glc}$ agar plates and incubated overnight at 37°C.

3.3.7 Screening of Positive Transformants by PCR

In order to screen positively transformed cells, the REDTaq® ReadyMix™ PCR Reaction Mix from Sigma-Aldrich was used. Single colonies grown overnight were picked and analysed by PCR to check for incorporation of the insert. Simultaneously, the same colonies were streaked on a master plate and incubated overnight at 37°C. A PCR sample contained 10 µl of the REDTaq® ReadyMix™, including 0.6 U of *Taq* DNA polymerase, 1 µM of each primer, and H_2O from the kit in a total volume of 20 µl. In order to achieve an unambiguous screening result, appropriate primers were chosen to amplify the DNA fragment inserted into the vector. The following PCR programme was used:

5 min 94°C	initial denaturation	
1 min 94°C	denaturation	30 ×
1 min 50°C	annealing	
2 min 72°C	extension	
5 min 72°C	final extension	

Finally, PCR fragments were separated by agarose gel electrophoresis, and positive clones were identified by the size of the bands. One of the positive clones was then used for plasmid DNA isolation.

3.3.8 Plasmid DNA Isolation (Mini and Midi Preparation)

For the isolation of plasmid DNA, overnight cultures were prepared in LB medium containing 1 % glucose and 100 µg/ml ampicillin or kanamycin. 4 ml of overnight culture were prepared for mini preparation, and 100 ml for midi preparation. Each overnight culture was inoculated with a single positively screened clone from the master plate. The next day, a glycerol stock (26 % glycerol) was prepared from an aliquot of the bacterial suspension for long-term storage of the clone, and the remaining cells were harvested by centrifugation for 2 min at 7,000 × g (mini) or 15 min at 6,000 × g (midi) and 4°C. The plasmid DNA isolation was performed by using the GeneJET™ Plasmid Miniprep Kit from Fermentas or the PureYield™ Plasmid Midiprep System from Promega following the manufacturers' instructions. The eluted DNA was finally stored at –20°C. The DNA concentration was measured photometrically at OD_{260} using the spectrophotometer GeneQuant *pro* from Pharmacia.

In order to confirm the identity of the isolated plasmid DNA, a control digestion with appropriate restriction enzymes and / or sequence analysis was performed.

3.3.9 Sequence Analysis

For sequence analysis, plasmid DNA at 28 – 87 ng/µl and the appropriate primers at 10 pmol/µl were sent to GATC Biotech [Konstanz, DE] in H_2O in a total volume of 30 µl. Sequence alignment was performed using the Blast algorithm [Tatusova and Madden 1999], and the sequence was analysed by aid of Clone Manager 7 [Scientific & Educational Software, Cary, US].

3.4 Expression and Purification of Recombinant Antibodies

3.4.1 Periplasmic Protein Expression

All antibody fragments were expressed in the periplasm of the *E. coli* strains TG1 or BL21 Star™ (DE3), using the prokaryotic expression vector pUC119 and pBM1.1, respectively. For each antibody fragment, two litres of 2 × TY medium, including 0.1 % glucose and 100 µg/ml ampicillin or kanamycin, were inoculated with 20 ml of an overnight culture of transformed bacterial cells and grown to exponential phase (OD_{600} 0.8 – 0.9) at 37°C. Since the antibody fragments are under control of the lactose promoter, protein expression was induced by addition of 1 mM IPTG, followed by incubation at RT with shaking for additional 3 h. Cells were harvested by centrifugation for 15 min at 1,700 × g and 4°C and resuspended in 100 ml periplasmic preparation buffer. Cell lysis was performed by adding 50 µg/ml freshly dissolved lysozyme [Roche Diagnostics] and incubating for 25 min on ice. Following, 10 mM $MgSO_4$ were added to stabilise spheroblasts, and cells were centrifuged for 10 min at 6,000 × g and 4°C. Finally, the obtained supernatant, containing the periplasmic protein, was dialysed against PBS overnight at 4°C and was then centrifuged again for 15 min as stated above. Afterwards, recombinant proteins were purified by Ni-NTA-IMAC as described in the following section.

3.4.2 Immobilised-Metal Affinity Chromatography (IMAC)

For purification of recombinant proteins owning a His_6 tag or a His_{10} cluster, an IMAC was performed by means of immobilised nickel-nitrilotriacetic acid (NTA) agarose beads [Qiagen]. First, a column of 1 ml Ni-NTA agarose needed to be equilibrated with approximately 10 ml of sterile 1 × sodium phosphate buffer with 20 mM imidazole. Then, crude protein, dialysed from periplasmic expression, was gradually applied to the column. After washing the column with about 20 ml of an appropriate IMAC wash buffer (1 × sodium phosphate buffer with 30 – 35 mM imidazole) until no more protein was detectable in the flow, bound protein was eluted from the column in 500 µl fractions with 1 × sodium phosphate buffer including 200 mM imidazole. All solutions were kept ice-cold throughout the entire purification.

All collected wash and elution fractions were tested for presence of protein by a qualitative Bradford assay by adding 10 µl of each sample to 90 µl of 1 × Bradford solution. Verification of the purification process was performed by an SDS-PAGE analysis. For this purpose, eluted fractions were run in parallel with crude protein, flow, and wash fraction under reducing conditions. Finally, positive fractions determined by the colorimetric reaction were pooled into peak and minor fractions and dialysed against PBS overnight at 4°C. For usage in stimulation assays, 0.01 % sodium azide was added to purified proteins as an alternative to lossy sterile filtration, and their concentration was determined. In addition, after protein quantification, (0.5 –) 2 µg of all purified proteins were also analysed by Western blotting under reducing conditions.

3.4.3 Determination of Protein Concentration

Protein concentration was determined by photometric measurement at OD_{280} [GeneQuant *pro*], using the calculated molecular weight and ε value of each protein.

3.5 Biochemical Characterisation

3.5.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to characterise the purified proteins with regard to molecular weight, purity, and potential degradation, an SDS-PAGE was performed. For this purpose, SDS gels with 12 % of polyacrylamide were prepared as follows:

	12 % Resolving Gel		**5 % Stacking Gel**	
	2 gels	**4 gels**	**2 gels**	**4 gels**
H_2O	3.3 ml	6.6 ml	2.1 ml	5.5 ml
30 % polyacrylamide	4 ml	8 ml	500 µl	1.3 ml
1.0 M Tris-HCl pH 6.8	–	–	380 µl	1 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	5 ml	–	–
10 % SDS	100 µl	200 µl	30 µl	80 µl
10 % APS	100 µl	200 µl	30 µl	80 µl
TEMED	4 µl	8 µl	3 µl	8 µl

Each sample was mixed up with reducing SDS-PAGE loading buffer, heated for 5 – 10 min at 95°C and loaded on the gel. A prestained protein standard was used to estimate protein size. Then, gels were run for approximately 1.5 h at 150 V (stacking gel 80 V) and stained for 1 h with shaking, using staining solution containing Coomassie Blue R 250. The gels were first destained with destaining solution until single separated protein bands were discernible and then additionally destained with H_2O overnight. The next day, a digital image was taken by a conventional scanner or the infrared imaging scanner Odyssey®.

3.5.2 Western Blot Analysis

Purified recombinant proteins were further verified by Western blotting in order to confirm their identity. To this end, they were separated on 12 % SDS-PAGE gels and transferred onto nitrocellulose membranes by semi-dry blotting (Fig. 3.4) for 45 min (1 gel) – 90 min (4 gels) at 47 mA per gel up to 10 V. Further proceeding differed depending on the detection system.

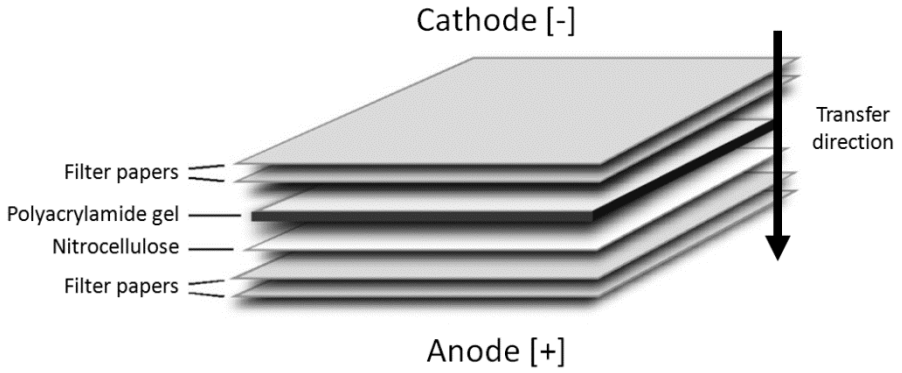


Fig. 3.4: Western blot set-up.

Fluorescent Imaging System

After the blocking of remaining binding sites on the membrane with 5 % skimmed milk in PBS for 1 h at RT with shaking, the membrane was washed 3 × with PBS containing 0.05 % Tween 20 and once with PBS alone for 5 min each. Next, bound proteins (His-tagged antibody constructs) were sequentially detected by incubation with unconjugated mouse anti-His_6 tag IgG_1 antibody 1 : 1,000 diluted in PBS, 0.02 % NaN_3 overnight at 4°C. After renewed washing (4 × with PBS containing 0.05 % Tween 20), bound antibody was detected by polyclonal goat anti-mouse IgG antibody conjugated with IRDye® 800CW, 1 : 15,000 diluted in blocking solution containing 0.1 % Tween 20, for 1 h at RT protected from light with shaking. Finally, the blot was washed again (last wash with PBS) and scanned at 800 nm by using the infrared imaging system Odyssey®.

ECL (Enhanced Chemiluminescence) System

Here, remaining binding sites were blocked with 5 % skimmed milk in PBS containing 0.1 % Tween 20, and the membrane was washed 3 × with PBS containing 0.05 % Tween 20. For protein detection, the membrane was incubated with an HRP-conjugated mouse anti-His_6 tag IgG_1 antibody 1 : 1,000 diluted in blocking solution (see above) overnight at 4°C. After renewed washing (last washing with PBS devoid of Tween 20), bound antibody was developed by the ECL system. For this purpose, the membrane was incubated with the ECL substrate solution for 1 – 2 min and was

subsequently exposed to an X-ray film for a few seconds. The film was then automatically developed in an X-ray film processor.

3.6 Cell Culture Techniques

3.6.1 Cell Cultivation

Mammalian cells were cultivated in T_{75} tissue culture flasks in 20 ml of the appropriate culture medium (see Section 3.1.9) in a humidified (60 % rel. humidity) incubator at 37°C with 5 % CO_2 atmosphere. Cells were split every 2 – 3 days. Adherent cells first needed to be detached with 1 × trypsin-EDTA. Cells were counted using the vital stain trypan blue. For storage, cells of 60 – 80 % confluence were harvested by centrifugation for 4 min at 1200 × g, resuspended in FCS with 10 % DMSO, aliquoted in cryovials, and gradually frozen to a temperature of -80°C in a cryobox containing isopropanol. Cells were thawed quickly at 37°C in a water bath and cautiously added to 5 ml medium. In order to remove DMSO, cells were centrifuged again, resuspended in fresh medium and transferred into a tissue culture flask.

3.6.2 Stable Transfection

Since only a relatively low amount of the DNA that is introduced into the cell during the transfection process is inserted into the nuclear genome, the foreign DNA is usually lost rather quickly in the absence of selection pressure. In order to enable stable expression of HLA-A2 on HEK-293T cells, a gene conferring puromycin resistance was transfected in the same plasmid allowing stable transfectants to proliferate under puromycin selection pressure, while other cells die. In order to facilitate stable transfection, the HLA-A2-encoding plasmid DNA was linearised overnight with the restriction enzyme *Pvu*I.

For stable transfection, HEK-293T cells (tested negative for HLA-A2 and CD45) were seeded in a 6-well plate at a density of 10^6 cells per well in 2 ml of culture medium and incubated overnight at 37°C and 5 % CO_2. Then, 166 µl Opti-MEM® I medium were incubated for 5 min with 6.7 µl of the transfection reagent Lipofectamine™ 2000. In parallel, additional 166 µl Opti-MEM® I medium were mixed with 2.66 µg of linearised plasmid DNA to be transfected. Both solutions were combined, gently mixed, and incubated for 20 min at RT. Meanwhile, the FCS containing medium on the cells was replaced by 1.33 ml Opti-MEM® I medium. Then, the DNA-Lipofectamine™ 2000 complexes were cautiously added to the cells and incubated overnight. Medium was exchanged by Opti-MEM® I medium containing Pen-Strep after at least 6 h to remove remaining Lipofectamine™ 2000. 24 h after transfection, the cells were transferred into a ø 10 cm tissue culture plate in 8 ml RPMI 1640 medium containing 15 % FCS. After another 24 h, 3 µg/ml puromycin were added for selection of stable transfectants. This

selection medium was changed every 3 – 4 days until those cells that had the DNA inserted into their genome (resistant colonies) proliferated. This occurred approximately after 5 weeks. Finally, the selective reagent was decreased to 1 µg/ml, and the HLA-A2 expression was tested by flow cytometry (see Section 3.7.1).

Stocks of stably transfected cells cultured at each puromycin concentration were stored at -80°C.

3.6.3 Preparation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC, comprising lymphocytes and monocytes, had been isolated from the buffy coat (leukapheresis) of a healthy human donor by density-gradient centrifugation using the Ficoll-based lymphocyte separation solution LSM 1077 (PAA, Cölbe, DE) according to the manufacturer's instructions. Furthermore, monocytes were depleted by magnetic-activated cell sorting using anti-CD14 conjugated MicroBeads (Miltenyi Biotec, Bergisch Gladbach, DE) according to the manufacturer's instructions. Remaining PBMC, essentially including lymphocytes, were available in-house and were tested in flow cytometry for the expression of HLA-A2. Finally, they were frozen in aliquots for usage in T-cell activation assays, PBMC stimulation (see Section 3.7.2) and cytotoxicity assays (see Section 3.7.4). PBMC from a handful of different donors were used, but the HLA-A2-negative PBMC that were used for PBMC stimulation assays derived from one and the same donor.

3.7 Functional Studies

3.7.1 Flow Cytometry

Binding studies with all antibody constructs and antigen surface expressions were tested by flow cytometry.

In order to prove the surface expression of AChRγ, 5×10^5 rhabdomyosarcoma cells were incubated with a mouse anti-AChRγ antibody or an appropriate isotype control at 4°C for 2 h. After threefold washing with 150 µl of PBA, cells were incubated with PE-conjugated goat anti-mouse antibody at RT for 30 min and then washed again twice. For gating, of each cell type one sample of unstained cells was additionally prepared. Finally, cells were resuspended in 500 µl of PBA, transferred into FACS tubes, and analysed by flow cytometry using BD FACSCalibur. Received data were analysed with the aid of WinMDI 2.8 software.

The surface expression of the molecules HLA-A2, CD3, and CD45 was examined by incubating 5×10^5 cells with directly FITC- or PE-labelled antibodies against those surface molecules or the appropriate isotype control at RT for 30 min. The following washing and analysis was performed as described above.

In order to provide evidence that the original scFvCD3 is indeed specific for CD3ε, a blocking assay was performed. 5×10^5 Jurkat cells were preincubated with 10 µg/ml of anti-human CD3ε or an isotype control in 50 µl of PBA at RT for 30 min, followed by the addition of 2 µg/ml of scFvCD3 in 50 µl of PBA and further incubation at 4°C for 2 h. The resulting final concentrations were 5 µg/ml IgG and 1 µg/ml scFvCD3, respectively. After threefold washing as stated above, cells were incubated with FITC-conjugated anti-His_6 tag antibody at RT for 30 min, followed by washing and analysis as described above.

The epitope binding specificity of taFvCD3-HLA-A2-FlagHis was assayed on HLA-A2-expressing HEK-293T $A2^+$ cells in a similar blocking assay. 5×10^5 cells were preincubated with 4 µM of scFvHLA-A2-mycHis, followed by the addition of 40 nM of taFvCD3-HLA-A2-FlagHis, resulting in final concentrations of 2 µM and 20 nM, respectively. After washing, bound taFvCD3-HLA-A2-FlagHis was detected by FITC-conjugated anti-Flag antibody, again followed by washing and analysis as described above.

Specific binding of the single antibody constructs to different antigen-presenting cells was also tested by flow cytometry. For this purpose, 5×10^5 cells were incubated with 10 µg/ml of antibody construct in 100 µl of PBA per well on a 96-well V-shaped plate at 4°C for 2 h. After threefold washing as stated above, cells were incubated with FITC-conjugated anti-His_6 tag antibody at RT for 30 min. Subsequent procedure was conducted as described above.

Both the antibodies used and their appropriate dilutions are listed in Section 3.1.4.

3.7.2 PBMC Stimulation Assay

The stimulatory activity of recombinant antibodies was tested in a cell-based stimulation assay. Therein, T-cell activation mediated by bi- or trispecific antibody constructs *in vitro* was assessed by quantifying the induced interleukin-2 (IL-2) release.

Stimulatory Activity of Bispecific AChRγ × CD3 taFv Fragments

Adherent antigen-expressing tumour target cells (RD and HEK-293T $A2^+$) were seeded in a flat-bottomed 96-well cell culture plate at a density of 10^5 cells per well in 100 µl of culture medium and incubated overnight at 37°C and 5 % CO_2 to allow the cells to adhere. The next day, after removing the supernatant from the cells, (titrated) stimulatory antibodies (taFv or scFv as control) were added in 100 µl medium per well and preincubated for 1 h at 37°C and 5 % CO_2 to ensure binding to AChRγ. Unstimulated monocyte-depleted PBMC (HLA-$A2^+$) (see Section 3.6.3) had been thawed the day before and cultured in a ø 10 cm tissue culture plate overnight, allowing the adherence of potentially remaining monocytes to the plate. Those PBMC that remained in solution were harvested and then added to the target cells at a density of

2×10^5 per well in 100 µl medium and incubated for 24 h at 37°C and 5 % CO_2. Finally, plates were centrifuged for 4 min at 1,200 × g to harvest 150 µl cell-free supernatants for IL-2 quantification in ELISA (see Section 3.7.3).

Stimulatory Activity of Bispecific HLA-A2 × CD3 taFv Fragments

Adherent antigen-expressing tumour target cells (HEK-293T $A2^+$ and RD) were stimulated with bispecific HLA-A2 × CD3 taFv fragments in the presence or absence of unstimulated HLA-A2-positive PBMC as described above.

Suspension antigen-expressing tumour target cells (U266) were seeded in a U-shaped 96-well cell culture plate at a density of 10^5 cells per well in 50 µl of culture medium and incubated at 37°C and 5 % CO_2 until antibody dilutions were prepared. Then titrated stimulatory taFv fragments were added in 100 µl medium per well and preincubated for 1 h at 37°C and 5 % CO_2 to ensure binding to HLA-A2. Unstimulated monocyte-depleted HLA-A2-negative PBMC (see Section 3.6.3) were freshly thawed and then added to the target cells at a density of 2×10^5 per well in 50 µl medium and incubated for 24 h at 37°C and 5 % CO_2. Finally, cell-free supernatants were achieved for IL-2 quantification in ELISA as described above.

Stimulatory Activity of Two-Part HLA-A2 × CD3 × HLA-A2 Trispecific Antibody

Here, the assay was performed with adherent target cells (HEK-293T $A2^+$), basically according to the description for bispecific AChRγ × CD3 taFv fragments above, but with the following exception. First, the antibody constructs for complementation (V_LCD3-scFvHLA-A2 and V_HCD3-scFvHLA-A2) were mixed (55 µl of each). Then the antibody combination was added in 100 µl medium per well to the target cells. The assay was further performed as stated above.

Stimulatory Activity of Two-Part HLA-A2 × CD3 × CD45 Trispecific Antibody

All antigen-expressing tumour target cells (adherent and suspension) were seeded in a U-shaped 96-well cell culture plate at a density of 10^5 cells per well in 50 µl of culture medium and incubated at 37°C and 5 % CO_2 until antibody dilutions were prepared. The antibody constructs for complementation (V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45) were first mixed (55 µl of each) and subsequently titrated if applicable. In the following, the (titrated) antibody combination was added in 100 µl medium per well to the target cells (U266, Raji, KMS-12-BM, L-363, and MCF-7). Then 50 µl of unstimulated monocyte-depleted HLA-A2-negative PBMC were added to the target cells as stated above. Thus, all compounds amounted to a final volume of 200 µl as in the other experiments above. After 24 h of incubation at 37°C and 5 % CO_2, finally, cell-free supernatants were harvested for IL-2 ELISA.

In order to prove the dual-antigen dependence of the trispecific antibody, the following blocking experiment was performed. HLA-$A2^+$ $CD45^+$ target cells (U266) were first

incubated with 80 µl of scFvHLA-A2 or scFvCD45(V_H-V_L) in 100 × molar excess (2.7 µM final concentration) at RT for 30 min, followed by the addition of 10 µl of V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45(V_H-V_L) (27 nM final concentration), respectively. Apart from that, samples were treated as stated above.

All experiments were performed *in duplicates* and repeated twice.

3.7.3 IL-2 Sandwich ELISA (Enzyme-Linked Immunosorbent Assay)

As an indicator for the stimulatory activity, T-cell activation induced by bi- or trispecific antibodies was measured in terms of the IL-2 release. Upon PBMC stimulation, the concentration of secreted IL-2 in the supernatant was quantified in an IL-2 sandwich ELISA by using the Human IL-2 DuoSet ELISA development kit from R&D Systems.

First, a 96-well ELISA plate was coated with 400 ng / 100 µl per well of mouse anti-human IL-2 antibody overnight at RT, followed by the saturation of nonspecific binding sites with blocking buffer for at least 1 h at RT. In the meantime, serial 1 : 2 dilutions of an IL-2 standard were prepared *in duplicate* in reagent diluent, starting with a maximum IL-2 concentration of 1,000 pg/ml. Then, supernatants containing IL-2 were appropriately diluted in RPMI 1640 medium containing 10 % FCS and Pen-Strep. Both diluted supernatants and standards were transferred into the ELISA plate and incubated for 2 h at RT. Subsequently, IL-2 was detected by incubation with 25 ng / 100 µl per well of biotinylated goat anti-human IL-2 antibody for 2 h at RT. Finally, 100 µl of HRP-conjugated streptavidin, 1 : 200 diluted in reagent diluent, was added per well and incubated for 20 min at RT while protected from light. HRP catalyses a colorimetric reaction by using TMB as a substrate. Thus, 100 µl of freshly prepared TMB substrate solution was added per well and incubated until sufficient blue colour appeared. After stopping the reaction with 50 µl of 1 M H_2SO_4, the absorbance was measured in an ELISA plate reader at 450 nm. In order to achieve a background signal, at least 2 wells on each plate were incubated with either reagent diluent or medium only and the detecting antibody plus TMB. Between each incubation step, the plate was washed three times with PBS containing 0.05 % Tween 20 and once with PBS only.

A seven point standard curve was created by plotting the absorbance signals of each standard sample against the IL-2 concentration. Thus, the amount of IL-2 of each supernatant could be determined by interpolation of the standard curve fitted with the nonlinear regression equation for one phase exponential association using the software GraphPad Prism®.

3.7.4 7-AAD/CFSE Cell-Mediated Cytotoxicity Assay

Cell-mediated cytotoxicity represents the cytolysis of target cells by effector cells such as T cells or NK cells. Here, cell-mediated cytotoxicity has been assessed using

a fluorescent assay, which consequently is free of radioactivity. In this method, the target cells are labelled with the green fluorescent CFSE (CFDA SE, 5-(6)-carboxyfluorescein diacetate, succinimidyl ester) and a red fluorescent compound, namely 7-AAD (7-aminoactinomycin D). The highly cell permeable CFDA SE passively diffuses into cells. It is colourless and nonfluorescent until intracellular esterases remove its acetate groups and convert the molecule to the highly fluorescent carboxyfluorescein succinimidyl ester (CFSE). CFSE is retained within cells for a long time. It is inherited by daughter cells and therefore diluted with time. The fluorescent intercalator 7-AAD penetrates only dead or damaged cells and undergoes a spectral shift upon association with double stranded DNA. The combination of both allows the identification of four different cell populations by multicolour flow cytometry: double positive dead target cells ($CFSE^+$ 7-AAD^+), live target cells ($CFSE^+$ 7-AAD^-), and live ($CFSE^-$ 7-AAD^-) or dead effector cells ($CFSE^-$ 7-AAD^+).

In order to examine whether the bi- and trispecific antibodies are able to mediate cytotoxicity by inducing the formation of a lytic synapse, the redirected lysis was measured in a cytotoxicity assay using 7-AAD and CFSE.

Cytotoxicity of Bispecific AChRγ × CD3 taFv

For this purpose, pelleted target cells were incubated with 100 µl of 1.5 µM CFSE in PBS per 2×10^6 cells for 10 min at RT while protected from light. In the following, cells were washed with 2 ml of FCS and allowed to stand for 1 min at RT for saturation of nonspecific binding sites. The cells were then washed twice with the appropriate medium containing 1 % FCS. Subsequently, 2.5×10^5 CFSE-labelled target cells were incubated with 27 nM of antibody constructs in the presence or absence of 2.5×10^6 unstimulated PBMC (E : T ratio of 10 : 1) in 1 ml medium containing 10 % FCS per well on a 48-well plate at 37°C for 24 h. As control for spontaneous cell death, CFSE-labelled target cells were incubated either alone or with PBMC only, *i.e.* in the absence of any antibody constructs. Maximum cell death was determined by incubating target cells with 0.1 % Triton X-100 only. After 0 h, 4 h, and 24 h, respectively, 250 µl of cell suspension was obtained and stained with 5 µl of 7-AAD per 10^6 cells for 10 min at RT while protected from light. Finally, cells were analysed by multicolour flow cytometry using BD FACSCalibur (BD BioScience, Heidelberg, DE). Since PBMC alone also led to some target cell death, the specific cell death was calculated as follows:

$$\frac{\%\,\text{‘Ab+PBMC’} - \%\,\text{‘PBMC’}}{(100 - \%\,\text{‘PBMC’})} \times 100\,\%$$

Ab: antibody construct

Cytotoxicity of Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibody

The cytotoxicity assay with U266 as target cells was performed as described above with the following exceptions. 5 ml of FCS were used for washing and blocking. 5×10^5

CFSE-labelled target cells were incubated with 27 nM of antibody constructs in the presence or absence of unstimulated HLA-A2-negative PBMC in 2 ml per well on a 24-well plate for 72 h. After 0 h, 24 h, 48 h, and 72 h, respectively, 330 µl of cell suspension was obtained and stained with 7-AAD. Finally, cells were analysed by multicolour flow cytometry and the specific cell death was calculated as described above. Cell death was determined after 24 h and 48 h only, since no effect was visible after 0 h and too low cell counts were measured after 72 h.

For a second experiment, 2.5×10^5 CFSE-labelled target cells were incubated with titrated antibody constructs in the presence or absence of unstimulated HLA-A2-negative PBMC in 1 ml per well on a 48-well plate for 24 h. After 0 h and 24 h, respectively, 250 µl of cell suspension was obtained and stained with 7-AAD. Data from multicolour flow cytometry were normalised to the lowest value using GraphPad Prism® (24 h data only).

4 Results

In the following, the generation and functional characterisation of antibody constructs of two concepts will be described: first, bispecific antibodies of the taFv format against CD3 and AChRγ for the treatment of aggressive rhabdomyosarcoma, and second, construct pairs for complementation of a trispecific HLA-A2 × CD3 × CD45 antibody for the treatment of leukaemia and other haematological malignancies in the context of haematopoietic stem cell transplantation.

4.1 Bispecific AChRγ × CD3 Antibodies of taFv Format

4.1.1 Construction of Recombinant Bispecific AChRγ × CD3 taFvs

In order to live up to the success of the BiTE antibody technology, one aim of the present study was to generate an effector-cell retargeting bispecific antibody against AChRγ and CD3. For this purpose, the taFv format was chosen, the same that was used for the BiTE antibodies [Baeuerle *et al.*, 2009]. This format is achieved by the linkage of two different scFv fragments in tandem. Thus, scFv fragments against AChRγ and CD3 were required as a basis for the construction of the taFv constructs but also as positive or negative controls depending on the experimental assay. The antibody fragment specific for the γ subunit of the fAChR used in the present study for the generation of the bispecific AChRγ × CD3 taFvs originates from “scFv35” (analogous to Fab35) and is referred to as scFvAChR in the following. The cDNA of the human scFvAChR was kindly provided by Prof. S. Gattenlöhner. For targeting T cells, the human CD3ε-specific mouse scFv diL2K (deimmunised L2K) was used (see also details in Section 3.2.1).

Both scFvCD3 and scFvAChR were equipped with a 15 – 18 aa long flexible linker between the variable domains, which allows the suitable folding into a functional scFv (Fig. 4.1 A). For detection and purification, scFvAChR includes an N-terminal His_{10} cluster, and scFvCD3 includes a C-terminal $FlagHis_6$ tag.

Four taFv variants were created in order to identify a construct showing favourable expression, binding properties, and activity in functional assays. The four variants evaluated differ in the orientation of the two scFvs, in the location of the tags for detection and purification, and in the expression vector used (Fig. 4.1 B). They contain either a $FlagHis_6$ tag at the C-terminus in the pUC119 vector or a His_{10} cluster and an ECS at the N-terminus in the pBM1.1 vector. All four variants target T cells via CD3, on the one hand, and AChRγ-expressing tumour cells, on the other hand, so they represent bispecific molecules.

All four taFv constructs were equipped with short middle linkers (5 – 6 aa), which allow folding of the two scFv fragments into a functional taFv. They include either an N-terminal His_{10} cluster or a C-terminal $FlagHis_6$ tag for detection and purification, as do scFvAChR and scFvCD3, respectively. All sequences are provided in the appendix.

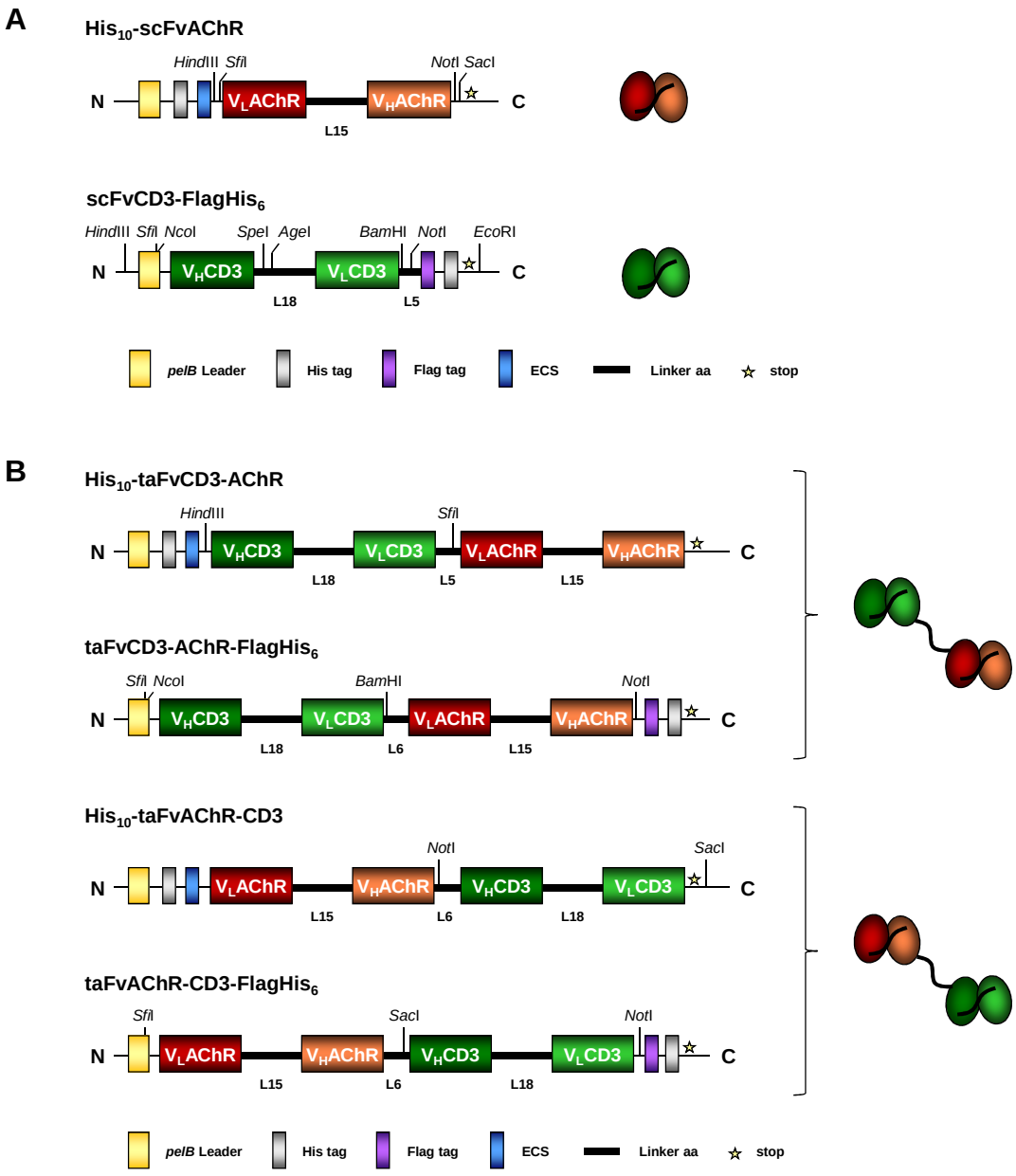


Fig. 4.1: Schematic illustration of the structure of the scFv and the bispecific taFv constructs targeting AChRγ and CD3. A) scFv fragments specific for AChRγ and CD3, respectively. The variable domains of the heavy (V_H) and light chain (V_L) of antibodies specific for AChRγ or CD3ε were joined by a flexible linker into the scFv format. **B)** Bispecific taFv constructs targeting AChRγ and CD3 evaluated in this study. Two scFv fragments, scFvAChR and scFvCD3, were joined by a 5 – 6 aa linker into the taFv format. The figure shows also the variable antibody domains, *pelB* leader sequences, important restriction sites, linkers and their lengths, His and Flag tags, ECS, and stop codons.

4.1.2 Expression of Bispecific AChRγ × CD3 taFvs

The two scFvs, used as controls, and the four bispecific AChRγ × CD3 taFvs were periplasmically expressed in *E. coli* TG1 or BL21(DE3) using the following prokaryotic expression vectors: pUC119mycHis for scFvCD3, taFvCD3-AChR-FlagHis, and taFvAChR-CD3-FlagHis, and pBM1.1 for scFvAChR, His-taFvCD3-AChR, and His-taFvAChRCD3. Subsequently, all proteins were purified by IMAC (see Sections 3.4.1 and 3.4.2). In order to verify the purification process and to determine the degree of purity, protein samples of all fractions were subjected to SDS-PAGE, followed by Coomassie Blue staining for visualisation of proteins. For each recombinant protein, a major band appeared on the gel in fractions containing the eluates. As an example, the purifications of the scFvs and taFvCD3-AChR-FlagHis are depicted in Fig. 4.2 A - C on the next page. Aliquots of all purified proteins were separated by SDS-PAGE under reducing conditions and analysed by Western blot (Fig. 4.2 D, p. 58) as described in Section 3.5.2. This revealed proteins migrating with apparent molecular weights that reasonably correspond to the calculated molecular weights deduced from the amino acid sequence of the constructs (Table 4.1).

Table 4.1: Overview of molecular weights (MW) and yields of the recombinant antibody constructs. The most important constructs of this study are highlighted in bold.

Construct	Apparent MW SDS-PAGE [kDa]	Predicted MW Sequence [kDa]	Yield [mg/l]	*E. coli* strain
His-**scFvAChR**	31	31	0.5	BL21(DE3)
scFvCD3-FlagHis	35	28	1.0	TG1
His-taFvAChR-CD3	71	57	0.2	BL21(DE3)
His-taFvCD3-AChR	68	57	0.2	BL21(DE3)
taFvCD3-AChR-FlagHis	63	55	0.2	TG1
taFvAChR-CD3-FlagHis	63	55	0.1	BL21(DE3)

The Western blot analysis revealed a single protein band for scFvAChR, scFvCD3, and taFvAChR-CD3-FlagHis, whereas for His-taFvAChR-CD3, His-taFvCD3-AChR, and taFvCD3-AChR-FlagHis minor bands appeared at a lower molecular weight in addition to a predominant band. These minor bands presumably derived from some His-containing cleavage products, corresponding to one, two, or three residual variable domains. By periplasmic expression, the highest yields were obtained for scFvs with 0.5 – 1.0 mg/l, as listed in Table 4.1. The yields obtained for taFvs amounted to only 0.1 – 0.2 mg/l.

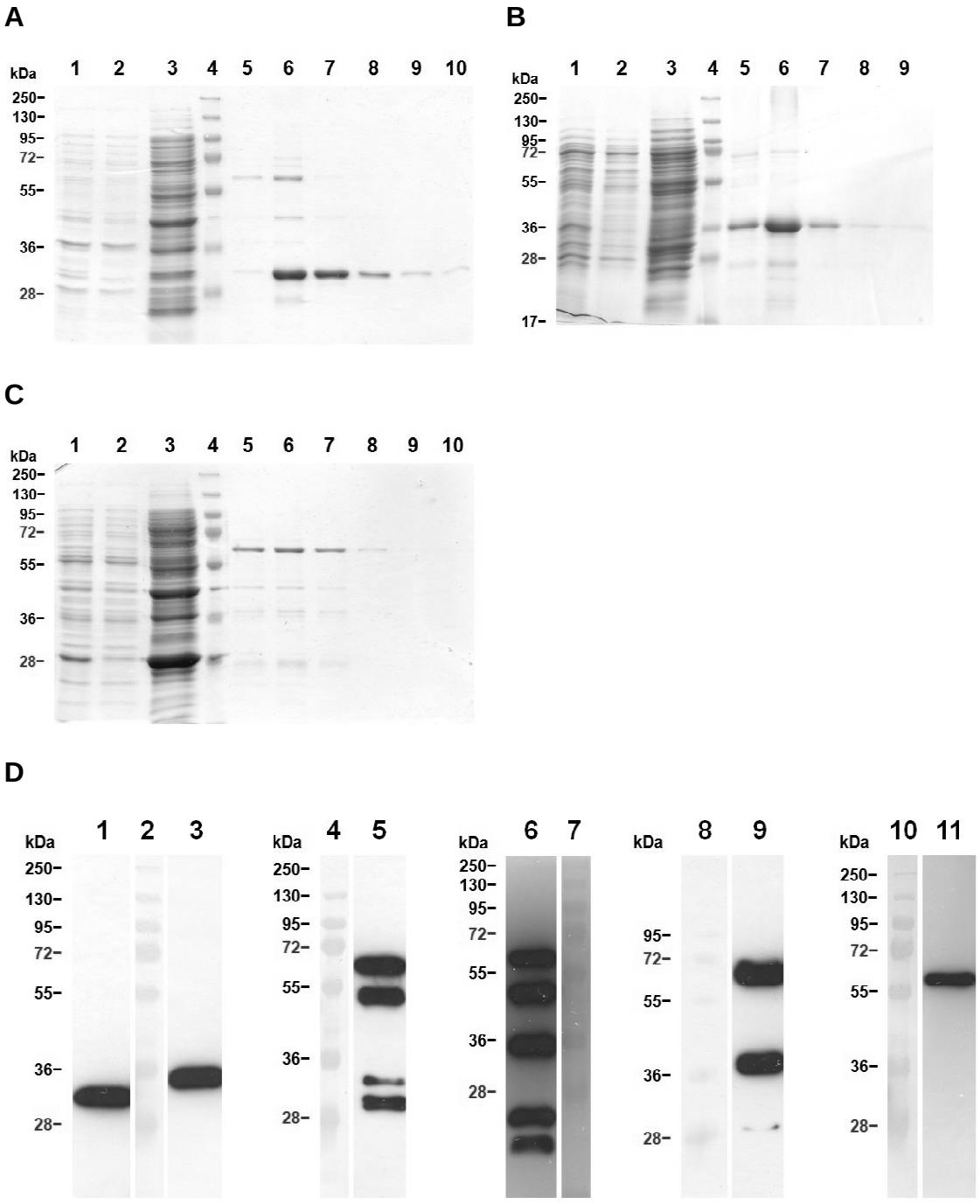


Fig. 4.2: SDS-PAGE and Western blot analysis of purified recombinant antibody constructs. Purification process of **A)** scFvAChR, **B)** scFvCD3, and **C)** taFvCD3-AChR-FlagHis. The following protein fractions were separated by 12 % SDS-PAGE under reducing conditions and visualised by Coomassie Blue staining: *lane 1*, crude extract; *2*, flow-through; *3*, wash; *4*, protein marker; *5 – 10*, eluates 1 – 6. **D)** 1 µg (scFv) or 2 µg (taFv) of purified protein per lane were separated by 12 % SDS-PAGE under reducing conditions. Proteins were detected by HRP-conjugated mouse anti-His_6 tag IgG_1 antibody. *Lane 1*, scFvAChR; *2*, *4*, *7*, *8*, *10*, protein marker; *3*, scFvCD3; *5*, His-taFvAChR-CD3; *6*, His-taFvCD3-AChR; *9*, taFvCD3-AChR-FlagHis; *11*, taFvAChR-CD3-FlagHis.

4.1.3 Binding Studies with Bispecific AChRγ × CD3 taFvs by Flow Cytometry

Expression of CD3 on the Surface of PBMC and Jurkat Cells

In order to prove the functionality of the CD3-specific scFv domain of the bispecific taFv fragments, binding to PBMC and Jurkat cells was analysed. First, the anticipated expression of CD3 on these cells was verified with an anti-CD3 mAb by flow cytometry. For this purpose, human PBMC were isolated from the buffy coat of a healthy human donor by Ficoll-based density gradient centrifugation and monocytes were mostly depleted (see Section 3.6.3), so that the further used PBMC essentially consisted of lymphocytes. In the following, both PBMC and Jurkat cells were incubated with a FITC-conjugated antibody specific for CD3ε or the corresponding isotype control and analysed by flow cytometry (Fig. 4.3).

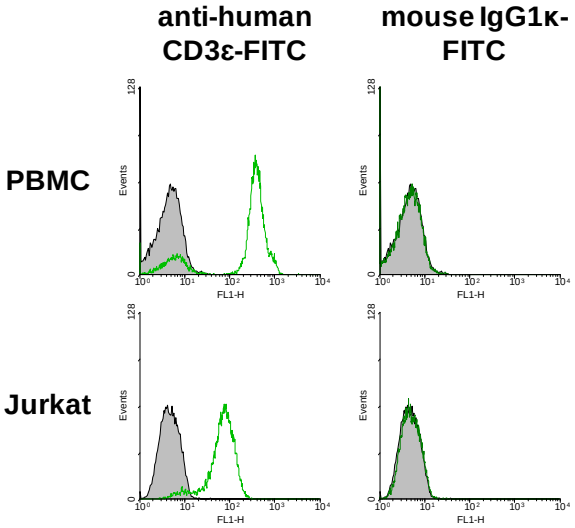


Fig. 4.3: Expression of CD3ε on PBMC and Jurkat cells, analysed by flow cytometry. Detection was performed by incubating cells with FITC-conjugated antibody specific for CD3ε (clone UCHT-1) or mouse IgG1κ-FITC as an isotype control. Grey filled, unstained cells; green line, antibody against CD3ε molecule or isotype control.

As expected, both the PBMC and the human T-cell leukaemia cell line Jurkat revealed high amounts of CD3 expression, on PMBC more than on the Jurkat cell line.

Analysis of Specific Binding of scFvCD3 by Flow Cytometry

In order to prove that the original scFvCD3, which was used for the construction of the bispecific AChRγ × CD3 taFv fragments, is indeed specific for CD3ε, a blocking assay was performed, as described in Section 3.7.1. Therein, CD3-expressing Jurkat cells were preincubated with a mouse mAb directed against human CD3ε (clone UCHT-1) before the addition of scFvCD3. As shown in Fig. 4.4, the binding of scFvCD3 to CD3ε was thereby entirely blocked. On the contrary, binding of scFvCD3 occurred in the presence of an isotype control instead. Consequently, scFvCD3 clearly recognises the

same or at least an overlapping epitope on CD3ε as the commercially available antibody of proven specificity for CD3ε.

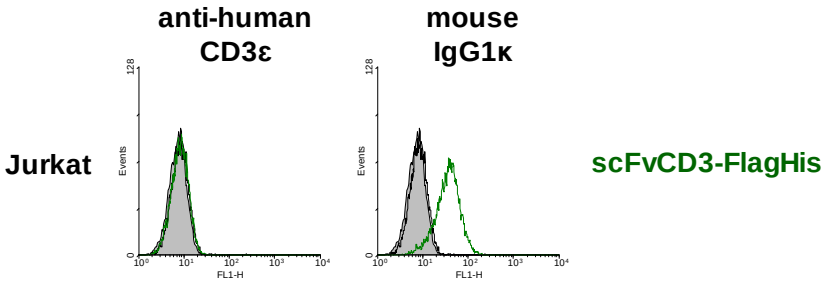


Fig. 4.4: Binding specificity of scFvCD3 to CD3 expressed on Jurkat cells, analysed by flow cytometry. Blocking of binding of 1 µg/ml of scFvCD3 to CD3-expressing Jurkat cells by preceding incubation with 10 µg/ml anti-human CD3ε antibody (clone UCHT-1) or mouse IgG1κ as an isotype control (resulting in 5 µg/ml final concentration each). Binding was detected by FITC-conjugated anti-His_6 tag antibody. Grey filled, unstained cells; black line, secondary antibody alone; coloured line, scFvCD3.

Expression of AChRγ on the Surface of Rhabdomyosarcoma Cell Lines

Initially, the rhabdomyosarcoma cell lines TE-671, RD, and AX-OH-1 were tested for AChRγ expression by flow cytometry; namely by incubating these cell lines with a mouse anti-AChRγ antibody and by subsequently detecting the binding with a PE-conjugated goat anti-mouse antibody (Fig. 4.5).

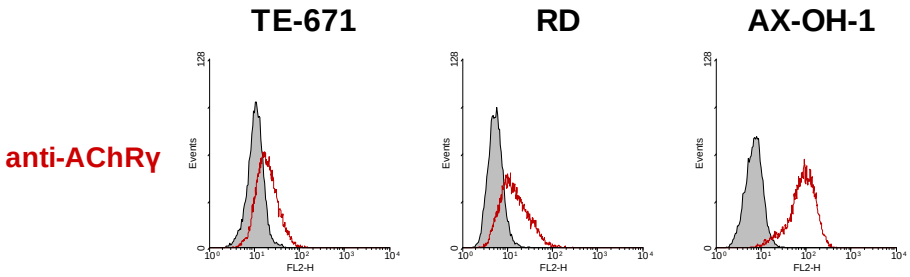


Fig. 4.5: Expression of AChRγ on the rhabdomyosarcoma cell lines TE-671, RD, and AX-OH-1, analysed by flow cytometry. Cells were incubated with a mouse anti-AChRγ antibody. Binding to AChRγ was detected by PE-conjugated goat anti-mouse IgG antibody. Grey filled, secondary antibody alone; red line, detected mAb against AChRγ.

As expected, all three rhabdomyosarcoma cell lines expressed AChRγ, but to a variable extent. AX-OH-1 cells showed the highest expression, whereas TE-671 revealed the lowest expression.

Binding Studies with scFvAChR by Flow Cytometry

Specific binding of the supposedly AChRγ-specific scFv was tested by flow cytometry. For this purpose, the three AChRγ-expressing rhabdomyosarcoma cell lines described above were each incubated with scFvAChR, followed by incubation with a FITC-conjugated anti-His_6 tag antibody (Fig. 4.6 A, p. 62). scFvAChR exhibited the strongest

binding to TE-671, followed by AX-OH-1 and, lastly, RD cells. Unexpectedly, the results did not correlate well with the expression determined with the anti-AChRγ mAb (see Fig. 4.5). Here, AX-OH-1 cells showed the highest expression, whereas TE-671 revealed the lowest expression. Since it remained unclear which cells are most suitable for further experiments, all three cell lines were used for binding studies by flow cytometry.

Binding Studies with Bispecific AChRγ × CD3 taFvs by Flow Cytometry

The binding abilities of the bispecific AChRγ × CD3 taFv fragments to antigen-expressing cells were also tested by flow cytometry. The taFv fragments were tested for binding to CD3 by using PBMC and Jurkat cells. Additionally, binding to AChRγ was tested on TE-671, RD, and AX-OH-1 cells. For reasons of clarity, only the results for Jurkat and TE-671 cells are shown (Fig. 4.6 B, p. 62).

Three of the four taFv fragments revealed specific binding to CD3-expressing cells as well as AChRγ-expressing TE-671 cells. Surprisingly, for one of them, namely His-taFvAChR-CD3, no significant binding at all was detected to any of the tested cell lines. This finding occurred despite repeating the experiment on TE-671 and AX-OH-1 cells. A possible reason is assumed to be the high level of protein degradation indicated in Western blot analysis (see Fig. 4.2 D). His-taFvCD3-AChR revealed moderate binding properties to both CD3-expressing cells and AChRγ on TE-671. taFvAChR-CD3-FlagHis showed the best binding to TE-671, which was even slightly better than that of scFvAChR, and a strong binding to CD3. taFvCD3-AChR-FlagHis showed best binding to CD3, which was even a little (PBMC, not shown) or significantly (Jurkat) better than scFvCD3, and moderate binding to TE-671. scFvAChR did not show any binding to (AChRγ-negative) PBMC (not shown). The results for AX-OH-1 complied well with those for TE-671, but with an overall lower binding for all constructs (not shown). However, surprisingly, poor or even non-significant binding was observed to RD cells (not shown), although they have previously displayed an even higher AChRγ expression than TE-671 (see Fig. 4.5 and discussion in Section 5.1). Despite showing the highest AChRγ expression, as detected by the commercial anti-AChRγ antibody in this study (see Fig. 4.5), AX-OH-1 cells were rather difficult to cultivate and therefore not suitable for functional assays. On that account, and since the AChRγ expression detected by using the commercial antibody was higher on RD than on TE-671 cells, RD cells were chosen for preliminary stimulation and cytotoxicity assays.

In sum, taFvAChR-CD3-FlagHis and taFvCD3-AChR-FlagHis proved themselves able to strongly bind both CD3-expressing cells and AChRγ-expressing cells.

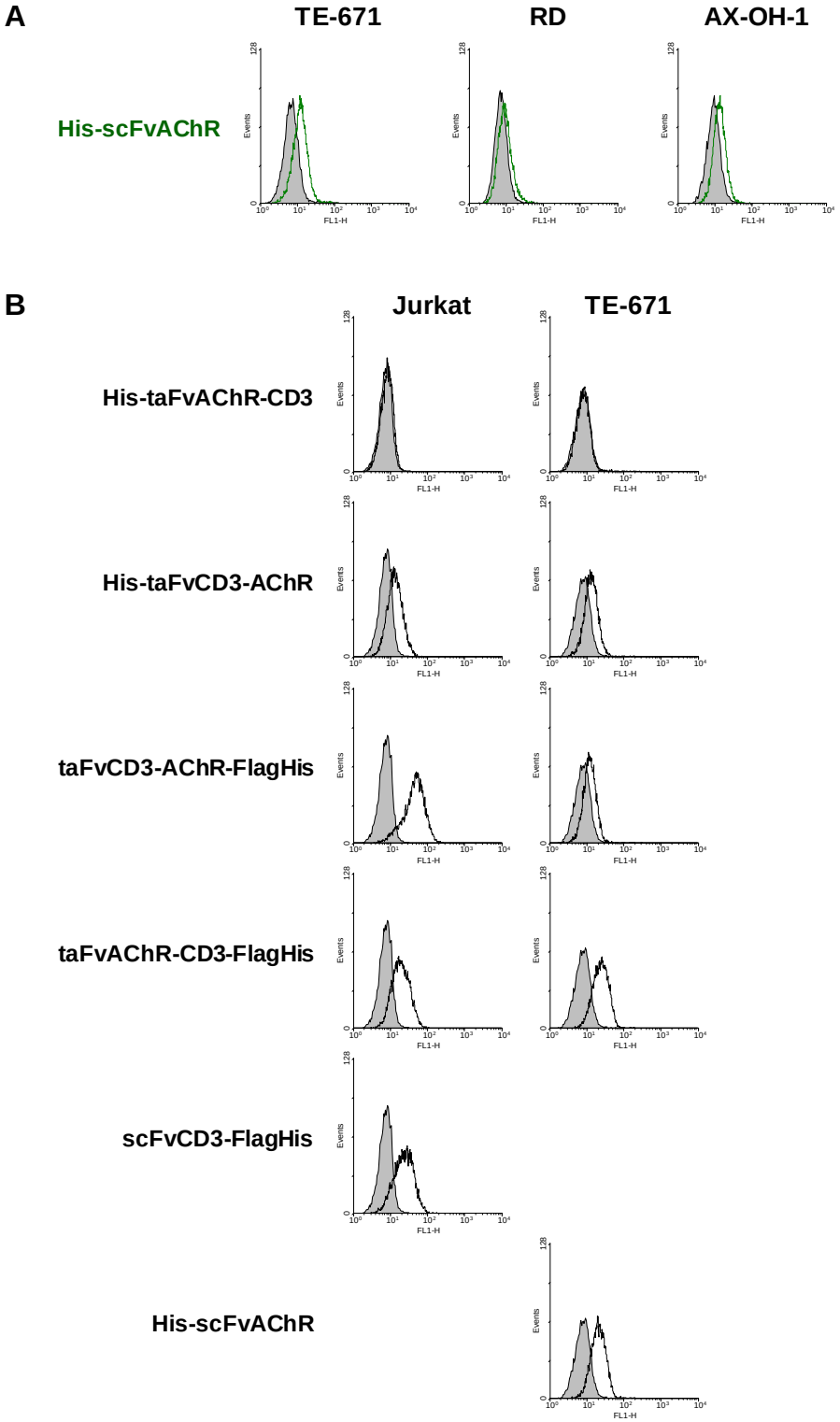


Fig. 4.6: Binding of scFvAChR to AChRγ and of bispecific AChRγ × CD3 taFv fragments to CD3 and AChRγ, analysed by flow cytometry. A) Rhabdomyosarcoma cell lines TE-671, RD, AX-OH-1 (all AChRγ$^+$) were incubated with 10 µg/ml of His-scFvAChR. **B)** Binding of 10 µg/ml of each bispecific taFv fragment to Jurkat (CD3$^+$) or TE-671 (AChRγ$^+$) cells. Binding was detected by FITC-conjugated anti-His_6 tag antibody. Grey filled, secondary antibody alone; green line, His-scFvAChR; black line, antibody fragment.

4.2 Stimulatory Activity of Bispecific AChRγ × CD3 taFvs

Since CD3-specific antibodies of the BiTE (bispecific T-cell engager) format [Baeuerle *et al.*, 2009] are able to induce T-cell activation in a costimulation-independent manner, their ability to activate T cells can easily be evaluated by using *in vitro* functional assays. As the recombinant bispecific taFv fragments generated in this study target the T-cell receptor complex molecule CD3 and the tumour antigen AChRγ, they should be able to activate T cells in the same way when simultaneously bound to the tumour target antigen. Thus, T cells are activated in a target cell-dependent manner. In order to assess the potential of the generated recombinant bispecific AChRγ × CD3 taFv fragments to activate T cells *in vitro*, a cell-based stimulation assay was performed as described in Section 3.7.2. Therein, PBMC were stimulated by bispecific antibodies that were previously bound to target cells. Representing an early T-cell activation marker, the T-cell growth factor IL-2 is secreted upon the activation of T cells. In this way, T-cell activation was then measured after 24 h in terms of the induced IL-2 release.

For the reasons stated in Section 4.1.3, p. 61, RD cells were chosen as AChRγ-expressing target cells to examine the stimulatory potential of bispecific AChRγ × CD3 taFv fragments. For this purpose, the four bispecific constructs were titrated from 0.3 – 243 nM on RD cells, followed by the addition of unstimulated PBMC at an E : T ratio of 2 : 1 (Fig. 4.7 A). As a control, background signals of IL-2 were achieved by incubating PBMC with target cells only, *i.e.* in the absence of antibody constructs.

Three of four constructs exhibited a stimulatory effect on PBMC in a concentration-dependent manner. The most effective stimulation was achieved with taFvCD3-AChR-FlagHis at 243 nM, resulting in a maximum IL-2 concentration of about 250 pg/ml and indicating an incipient saturation of AChRγ-binding sites on the target cells. At the same concentration, the resulting IL-2 release amounted to 56 pg/ml for His-taFvCD3-AChR, 21 pg/ml for taFvAChR-CD3-FlagHis, and 3 pg/ml for His-taFvAChR-CD3, which corresponds to approximately 23 %, 9 %, and 1 % of the IL-2 release achieved by stimulation with taFvCD3-AChR-FlagHis. The background signal was at 4 pg/ml (2 % of taFvCD3-AChR-FlagHis). In contrast, all four constructs had a far lower stimulatory effect on PBMC when AChRγ-negative target cells (HEK-293T $A2^+$) were used instead (Fig. 4.7 B). Here, the background signal was at 11 pg/ml. For three of four constructs, the IL-2 signal was up to twice as high as the background (below 23 pg/ml IL-2), indicating some unspecific binding to HEK-293T $A2^+$ cells. As expected, scFvCD3 and scFvAChR, used as negative controls, had no stimulatory effect on PBMC. Since soluble scFvCD3 is not able to crosslink CD3, T-cell activation is not induced. In conclusion, three of the four constructs do mediate T-cell activation, with taFvCD3-AChR-FlagHis being the most potent one by far. T-cell stimulation seems to

be impaired when mediated by constructs with scFvAChR or a His_{10} cluster at the N-terminus.

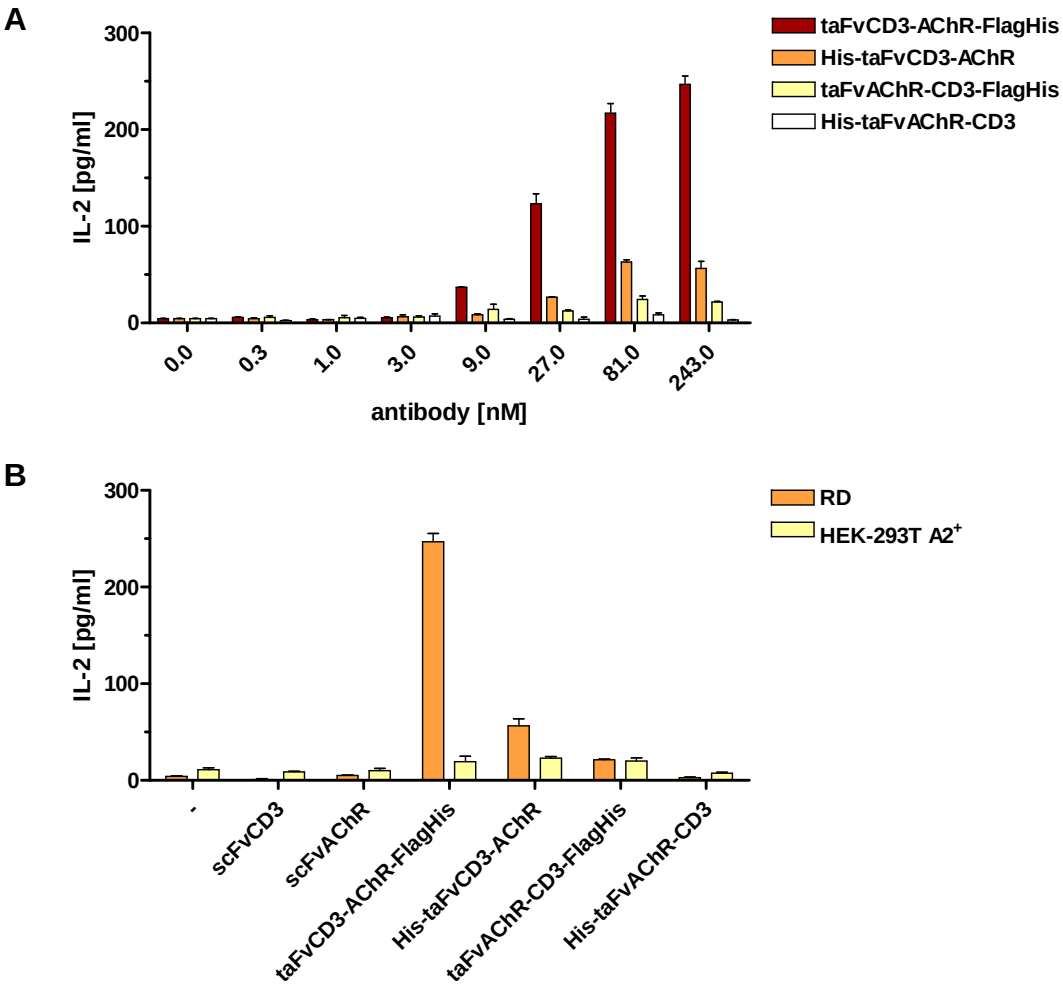


Fig. 4.7: **T-cell activation mediated by bispecific AChRγ × CD3 taFv fragments. A)** AChRγ-expressing target cells (RD) were incubated with titrated antibody constructs (0.3 – 243 nM) *in duplicate*. **B)** RD and HEK-293T $A2^+$ ($AChRγ^-$) cells, respectively, were incubated with 243 nM of the indicated antibody constructs *in duplicate*. In the following, unstimulated PBMC were added (E : T ratio of 2 : 1) and IL-2 release was measured in ELISA after 24 h. These results are representative of three independent experiments.

4.3 Cytotoxicity of a Bispecific AChRγ × CD3 taFv

Having shown that taFvCD3-AChR-FlagHis activates T cells in terms of the IL-2 release induced, it was then examined whether it also induces the formation of a lytic synapse. For this purpose, the redirected lysis by cytotoxic T cells was measured *in vitro* in a flow cytometry-based cytotoxicity assay. Basically, PBMC were stimulated by bispecific antibodies that were previously bound to CFSE-labelled target cells. The fluorescent intercalator 7-AAD mainly penetrates cells without intact cell membranes, thus representing a marker for apoptosis. So after 0 h, 4 h, and 24 h, apoptosis was

then detected by staining with 7-AAD. Since dead target cells should be double positive ($CFSE^+$ $7\text{-}AAD^+$), they can easily be identified by multicolour flow cytometry, *i.e.* distinguished from live target cells ($CFSE^+$ $7\text{-}AAD^-$), and live ($CFSE^-$ $7\text{-}AAD^-$) or dead PBMC ($CFSE^-$ $7\text{-}AAD^+$).

In order to examine the mediated cytotoxicity, CFSE-labelled AChRγ-expressing target cells (RD) were preincubated with 27 nM taFvCD3-AChR-FlagHis in the presence or absence of unstimulated PBMC at an E : T ratio of 10 : 1 (Fig. 4.8 A). As negative control, the AChRγ-negative rhabdomyosarcoma cell line A-204 [Gattenlöhner *et al.*, 2006] was additionally tested in parallel (Fig. 4.8 B). As control for spontaneous cell death, CFSE-labelled target cells were incubated either alone or with PBMC only, *i.e.* in the absence of taFv. Maximum cell death was determined by incubating target cells with 0.1 % Triton X-100 only. Each data set was normalised to the lowest value.

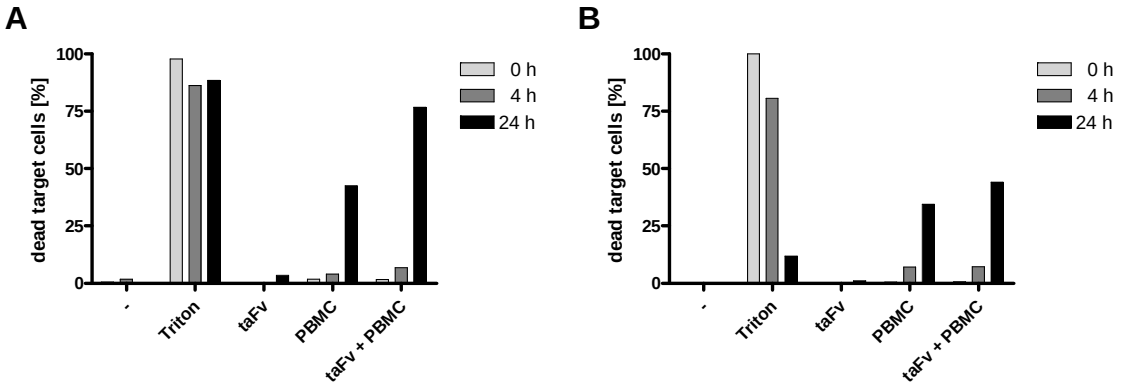


Fig. 4.8: Cytotoxicity mediated by taFvCD3-AChR-FlagHis. CFSE-labelled **A)** RD ($AChR\gamma^+$) cells or **B)** A-204 ($AChR\gamma^-$) cells were incubated with either 27 nM taFvCD3-AChR-FlagHis, unstimulated PBMC (E : T ratio of 10 : 1), or both. Samples were taken after 0 h, 4 h, and 24 h and stained with 7-AAD. Dead target cells ($CFSE^+$ $7\text{-}AAD^+$) were identified by multicolour flow cytometry.

Specific cytotoxic effects did not occur until after 24 h. After this time, taFvCD3-AChR-FlagHis had no effect on the RD cells in the absence of PBMC, whereas a considerable cytotoxicity was observed in conjunction with PBMC (black bars). Since PBMC alone also led to some target cell death, the specific cell death was calculated as described in Section 3.7.4. For taFvCD3-AChR-FlagHis at 27 nM, the percentage of cytotoxic activity amounted to 60 % on RD cells versus 15 % on A-204 cells, indicating some unspecific binding to A-204. So the cytotoxic effect was much lower when the AChRγ-negative A-204 served as target cells. The spontaneous cell death amounted to 1.2 %, 13.6 %, and 29.9 % of RD cells and 0.2 %, 3.2 %, and 2.6 % of A-204 cells after 0 h, 4 h, and 24 h, respectively. In a similar second experiment without A-204 cells and the taFv alone sample, a comparable cytotoxic activity was achieved for taFvCD3-AChR-FlagHis on RD cells, indicating that the findings were reproducible (data not shown). However, these data are preliminary as they were performed only twice, so

that further experiments are required for verification. Thus, it was shown that at nanomolar concentration, one of the four bispecific AChRγ × CD3 taFv fragments, namely taFvCD3-AChR-FlagHis, is not only able to specifically activate T cells to release IL-2, but also to induce cytotoxicity to AChRγ-expressing tumour cells.

4.4 Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibody

After the successful generation of a functional bispecific AChRγ × CD3 antibody, the major aim of this study was to extend this approach to a dual antigen-restricted trispecific concept for targeted immunotherapy. More precisely, this study aimed to figure out, if it is possible to assemble a functional trispecific HLA-A2 × CD3 × CD45 antibody from two parts in such a way that the third specificity against CD3 is only created upon the prior binding of the two other antigens at the same cell. This would allow approaches which require a dual-targeting strategy, such as the treatment of leukaemia and other haematological malignancies in the context of haematopoietic stem cell transplantation (HSCT).

Patients with, for example, leukaemia or multiple myeloma often require HSCT (with preceding radiation or chemotherapy). Unfortunately, for one out of five patients, no matching donor can be found, because an HLA-identical donor is very rare. The probability of finding an HLA-compatible donor (with a 10/10 match in HLA-A, -B, -C, -DRB1, and -DQB1) varies between one in 20,000 and one in several million [DKMS]. If no matching donor can be identified, grafts with one mismatch are increasingly employed. However, initial therapy, including HSCT, sometimes fails to eliminate all cancer cells, leading to a subsequent relapse of the original malignancy. Many recipients of donor transplants relapse: among those receiving HLA-identical sibling transplants about 40 – 45 % relapse and among those receiving unrelated donor transplants about 35 % relapse. Thus, relapse remains the most frequent cause of treatment failure and mortality [Barrett and Battiwalla, 2010].

For an HLA-mismatched setting where the patient is HLA-A2-positive, but the donor is not, it was aimed to exploit the HLA disparity for targeted immunotherapy by using a complementing trispecific antibody. Besides CD3, this trispecific antibody should target HLA-A2, one of the most common HLA alleles, and CD45, a highly specific haematopoietic marker, at the same time. More precisely, this combinatorial approach was to be realised by the development of two self-assembling single-chain antibody constructs: a scFv specific for HLA-A2, and a scFv against CD45, each linked to the V_L and the V_H domain of a CD3ε-specific antibody, respectively. After the binding of each scFv to its respective target, HLA-A2 and CD45, coexpressed on the same cell, the unpaired variable domains should assemble to a functional scFv against CD3ε. This assembly should exclusively occur on the recipient's malignant haematopoietic cells,

causing the complementation of the trispecific antibody, then able to activate T cells via CD3, and finally leading to T cell-mediated tumour-specific cell destruction. In the present dual-targeting approach both antigens, HLA-A2 and CD45, are tumour-specific only when combined, *i.e.* not by themselves. In this way, residual blood cancer cells of host origin could be specifically recognised and finally eliminated shortly after HSCT, thus preventing a relapse of disease. Furthermore, the preceding radiation or chemotherapy could be substantially reduced or – ideally – even omitted.

Moreover, this study aimed to figure out if the affinity of V_HCD3 and V_LCD3 to each other would be sufficient for a self-assembly of both single-chain antibody constructs or if this approach would require further affinity enhancement through an incorporation of heterodimerising peptides, such as jun-fos leucine zippers [de Kruif and Logtenberg, 1996; Kostelny *et al.*, 1992; Pack and Plückthun, 1992; O'Shea *et al.*, 1989; Arndt *et al.*, 2001]. However, the affinity must not be too high to avoid binding to single-positive target cells.

4.4.1 Construction of a Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibody

The constructs for complementation were achieved by the linkage of a complete scFv specific for HLA-A2 or CD45 to the V_L and V_H domain of a CD3-specific antibody, respectively. These constructs are referred to as V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45 in the following. For a first proof of concept, antibody constructs which consist of a complete scFv of the same specificity, namely HLA-A2, and a split anti-CD3 moiety were additionally constructed (V_LCD3-scFvHLA-A2 and V_HCD3-scFvHLA-A2). Furthermore, scFv fragments against CD3, HLA-A2, and CD45 were required as basis for the construction of the above mentioned constructs and as positive or negative control, depending on the experimental assay. For the use as positive control in functional assays, bispecific HLA-A2 × CD3 taFv constructs were additionally created.

The human HLA-A2-specific antibody fragment used in this study, 3PF12, was isolated from an alloimmunised patient by phage display with a nanomolar affinity for its target HLA-A2 and does not cross-react with HLA-A2-negative platelets [Watkins *et al.*, 2000]. The trispecific HLA-A2 × CD3 × (CD45 / HLA-A2) and bispecific HLA-A2 × CD3 antibody constructs used in the present study for effector-cell retargeting originate from 3PF12. In the following, it is referred to as scFvHLA-A2. The cDNA of scFvHLA-A2 (clone 3PF12) was kindly provided by Dr. Nicholas A. Watkins.

The human CD45-specific scFv that was used in the present study for the construction of the trispecific HLA-A2 × CD3 × CD45 antibody derived from a mouse scFv (clone BC8) that recognises all human CD45 isoforms [Lin *et al.*, 2006]. It was commercially synthesised according to the cDNA sequence specified by Lin *et al.* [2006] and is

referred to as scFvCD45 in the following. More details regarding the encoding sequences, their origin, and the cloning of scFvCD45 are given in Section 3.2.2.

Both scFvHLA-A2 and scFvCD45 (in V_L-V_H and V_H-V_L orientation) were equipped with a 15 – 19 aa long flexible linker between the variable domains, which allows the suitable folding into a functional scFv (Fig. 4.9).

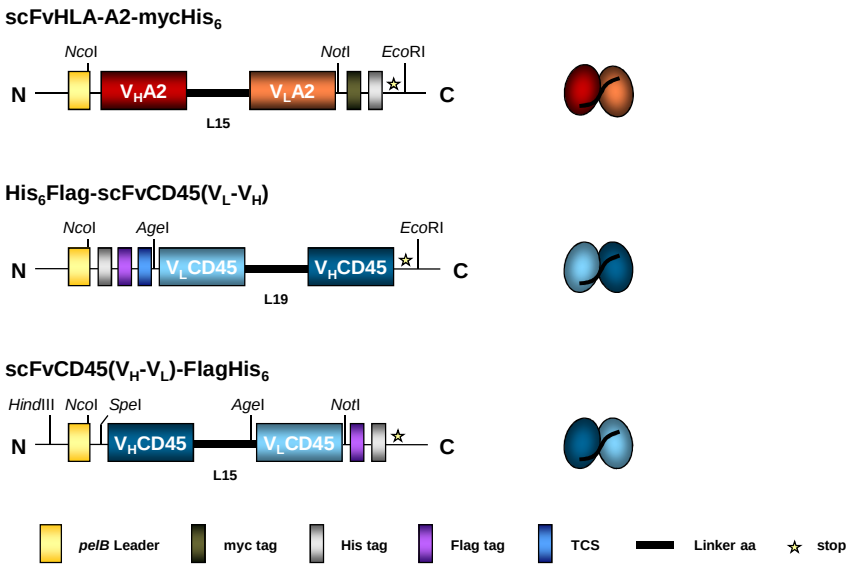


Fig. 4.9: Schematic representation of the structure of the scFv fragments specific for HLA-A2 and CD45, respectively. The variable domains of the heavy (V_H) and light chain (V_L) of antibodies specific for HLA-A2 or CD45 were joined by a flexible linker into the scFv format. Furthermore, *pelB* leader sequences, important restriction sites, linkers and their lengths, myc, His, and Flag tags, TCS, and stop codons are indicated.

For the assembling V_H and V_L domains, the same CD3ε-specific scFv served as a template, as set out above in Section 4.1.1. With scFvCD3, scFvHLA-A2, scFvCD45(V_L-V_H), and scFvCD45(V_H-V_L) as a basis, five constructs were developed for the trispecific concept. V_LCD3-scFvHLA-A2, V_HCD3-scFvCD45(V_L-V_H), and V_HCD3-scFvCD45(V_H-V_L) were developed for complementation on cells expressing both HLA-A2 and CD45. For a first proof of concept on HLA-A2-expressing cells (*i.e.* for assembly with V_LCD3-scFvHLA-A2), V_HCD3-scFvHLA-A2-mycHis and V_HCD3-scFvHLA-A2-FlagHis were additionally constructed (Fig. 4.10).

All five created constructs for complementation were equipped with short linkers (7 aa) between V_H or V_L and scFv to provide flexibility and prevent an incorrect folding of the constructs. They include either a myc or a Flag tag in addition to a His_6 tag at the C-terminus for detection and purification, as do scFvHLA-A2 and scFvCD45(V_H-V_L), whereas scFvCD45(V_L-V_H) contains a His_6Flag tag at the N-terminus.

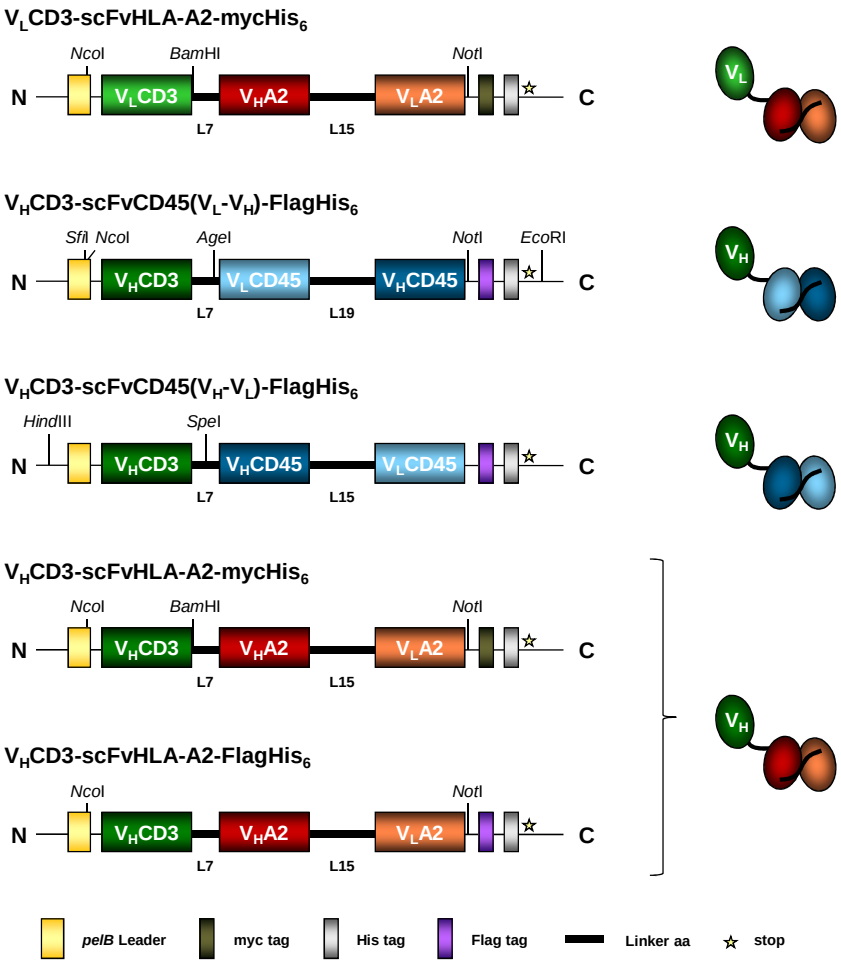


Fig. 4.10: Schematic illustration of the structure of the constructs for complementation on cells expressing either both HLA-A2 and CD45 or HLA-A2 only. The variable domains of the heavy (V_H) and light chain (V_L) of antibodies specific for HLA-A2, CD45, or CD3ε were joined by flexible linkers into the respective $V_{H/L}$CD3-scFv format. The figure shows also *pelB* leader sequences, important restriction sites, linkers and their lengths, myc, His and Flag tags, and stop codons.

The accurate cloning strategies of all constructs are detailed in Section 3.2 and all sequences are included in the appendix.

4.4.2 Construction of Recombinant Bispecific HLA-A2 × CD3 taFvs

For the use as a positive control, a bispecific taFv antibody against HLA-A2 and CD3 was created. More precisely, four variants were created in order to be able to identify the construct showing the best features (Fig. 4.11). The four variants differ in the orientation of the two scFvs and the location of tags for detection and purification. All four variants target T cells via CD3 on the one hand and HLA-A2-expressing cells on the other hand, thus representing bispecific molecules.

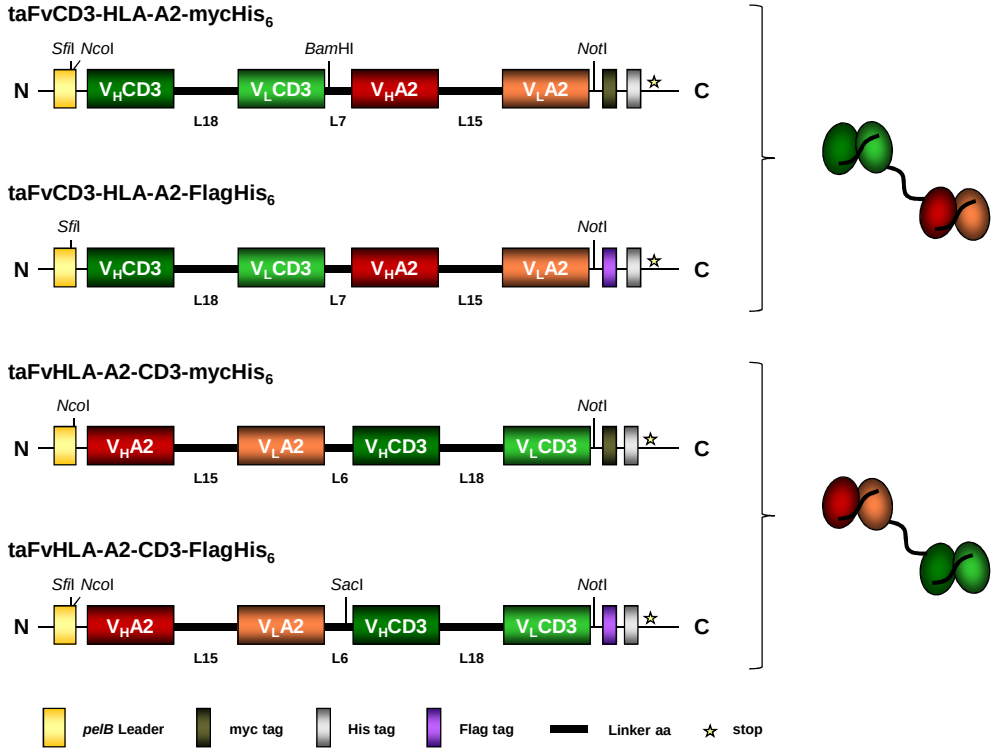


Fig. 4.11: Schematic representation of the structure of bispecific taFv constructs targeting HLA-A2 and CD3. Two scFv fragments, scFvHLA-A2 and scFvCD3, were joined by a 6 – 7 aa linker into the taFv format. Furthermore, *pelB* leader sequences, important restriction sites, linkers and their lengths, myc, His, and Flag tags, and stop codons are indicated.

All four created taFv constructs were equipped with short middle linkers (6 – 7 aa) to provide a suitable folding of the two scFv fragments into a functional taFv and include either a myc or a Flag tag in addition to a His_6 tag at the C-terminus for detection and purification.

4.4.3 Expression of Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibodies

The five constructs for complementation were periplasmically expressed in *E. coli* TG1 by using the prokaryotic expression vector pUC119mycHis. The four scFvs and the four bispecific HLA-A2 × CD3 taFvs were produced in the same way. Subsequently, all proteins were purified by IMAC (see Sections 3.4.1 and 3.4.2).

The purification process was verified and the degree of purity was determined by SDS-PAGE followed by Coomassie Blue staining to visualise all proteins. A major band appeared on the gel for each recombinant protein in the eluate fractions. As an example, the purifications of scFvHLA-A2, scFvCD45(V_H-V_L), V_LCD3-scFvHLA-A2, V_HCD3-scFvCD45(V_H-V_L), and taFvCD3-HLA-A2-mycHis are depicted in Fig. 4.12. The purification of scFvCD3 is shown above in Fig. 4.2, p. 58.

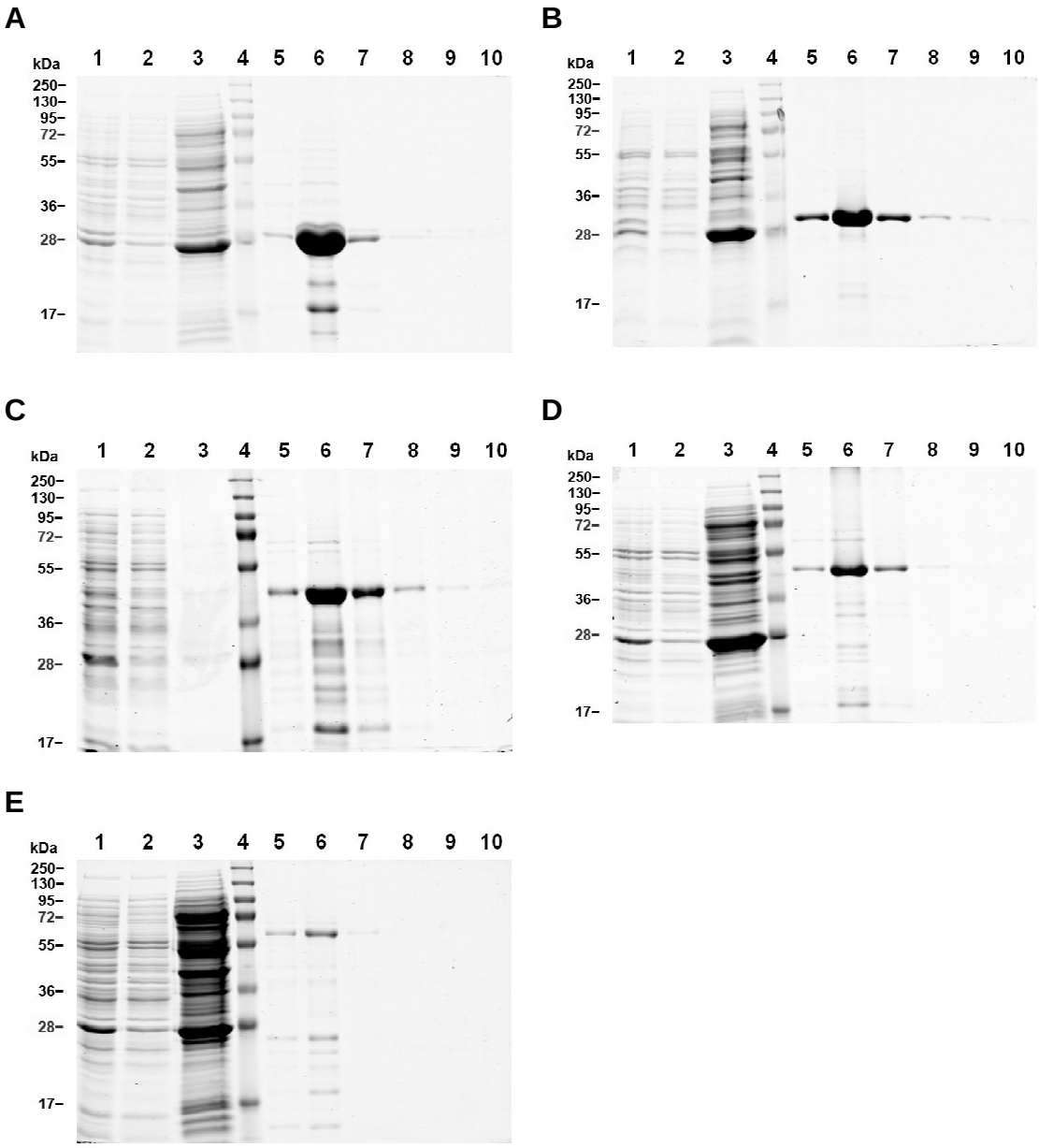


Fig. 4.12: SDS-PAGE analysis of the purification process of the recombinant antibody fragments. Purification of **A)** scFvHLA-A2, **B)** scFvCD45(V_H-V_L), **C)** V_LCD3-scFvHLA-A2, **D)** V_HCD3-scFvCD45(V_H-V_L), and **E)** taFvCD3-HLA-A2-mycHis. The following protein fractions were separated by 12 % SDS-PAGE under reducing conditions and visualised by Coomassie Blue staining: *lane 1*, crude extract; *2*, flow-through; *3*, wash; *4*, protein marker; *5 – 10*, eluates 1 – 6.

In order to identify the purified antibody constructs and to characterise them with regard to molecular weight and potential degradation, 2 µg of each purified construct – with the exception of scFvCD45(V_L-V_H) (0.5 µg) and taFvCD3-A2-FlagHis (1 µg) – were separated by SDS-PAGE under reducing conditions and analysed by Western blot (Fig. 4.13). The identity of the recombinant proteins was confirmed by Western blot analysis as described in Section 3.5.2.

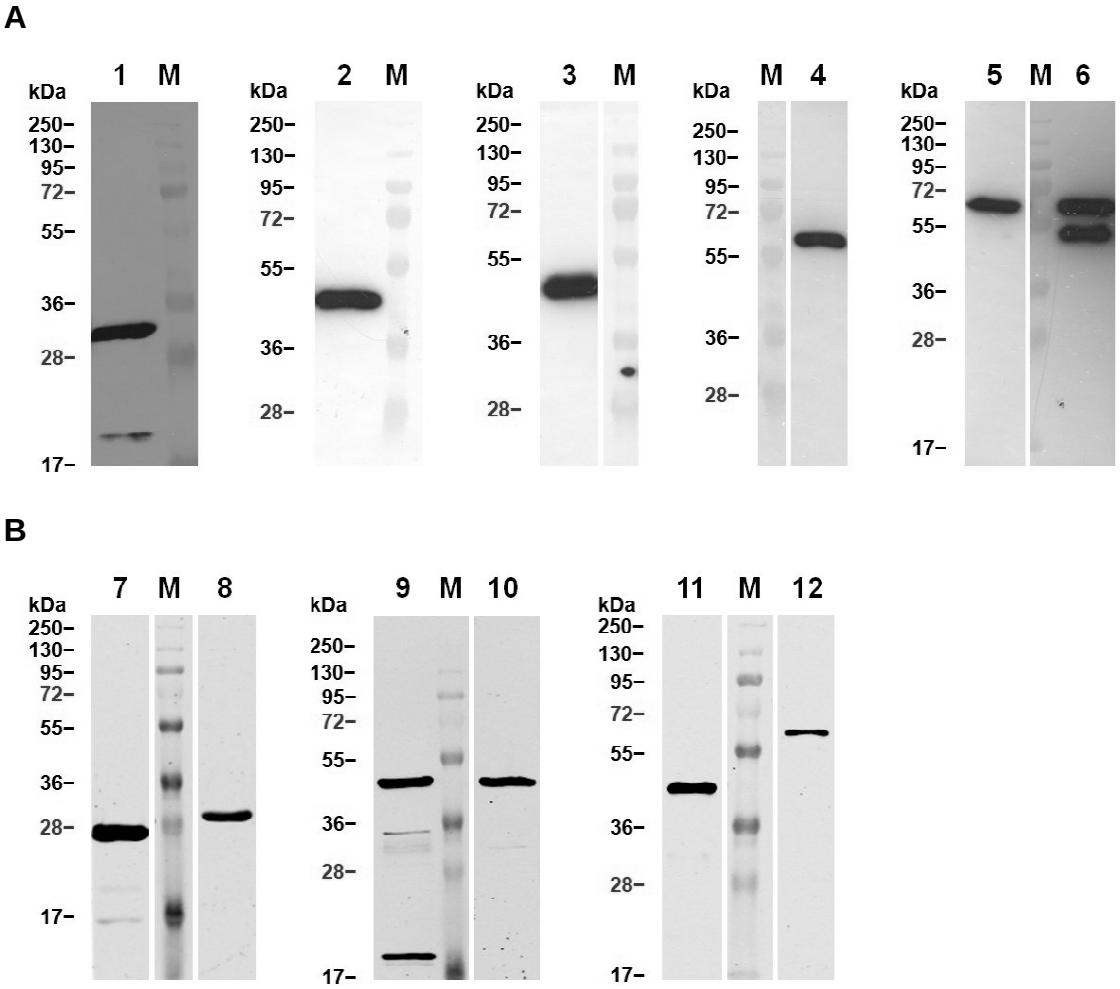


Fig. 4.13: Western blot analysis of purified recombinant antibody constructs. 2 µg of purified protein per lane were separated by 12 % SDS-PAGE under reducing conditions (0.5 µg in case of scFvCD45(V_L-V_H) and 1 µg of taFvCD3-A2-FlagHis). For Western blot analysis, proteins were detected **A)** by HRP-conjugated mouse anti-His_6 tag IgG_1 antibody or **B)** by mouse anti-His_6 tag IgG_1 antibody and IRDye 800 conjugated polyclonal goat anti-mouse IgG antibody. *Lane 1*, scFvCD45(V_L-V_H); *M*, protein marker; *2*, V_HCD3-scFvHLA-A2-FlagHis; *3*, V_HCD3-scFvHLA-A2-mycHis; *4*, taFvCD3-HLA-A2-FlagHis; *5*, taFvHLA-A2-CD3-FlagHis; *6*, taFvHLA-A2-CD3-mycHis; *7*, scFvHLA-A2; *8*, scFvCD45(V_H-V_L); *9*, V_HCD3-scFvCD45(V_L-V_H); *10*, V_HCD3-scFvCD45(V_H-V_L); *11*, V_LCD3-scFvHLA-A2; *12*, taFvCD3-HLA-A2-mycHis.

SDS-PAGE under reducing conditions and subsequent Western blot analysis revealed proteins migrating with apparent molecular weights that reasonably correspond to the calculated molecular weights deduced from the amino acid sequence of the constructs (Table 4.2).

Western blot analysis revealed a major protein band for each antibody construct. For scFvCD45(V_L-V_H), scFvHLA-A2, V_HCD3-scFvCD45(V_L-V_H), V_HCD3-scFvCD45(V_H-V_L), V_LCD3-scFvHLA-A2, and taFvHLA-A2-CD3-mycHis, additional minor bands appeared at a lower molecular weight. These minor bands presumably derived from some His-

containing cleavage products, corresponding to one, two or three residual variable domains. With the exception of taFvHLA-A2-CD3-mycHis, these bands are negligible.

Table 4.2: Overview of molecular weights (MW) and yields of the recombinant antibody constructs. The most important constructs of this study are highlighted in bold.

Construct	Apparent MW SDS-PAGE [kDa]	Predicted MW Sequence [kDa]	Yield [mg/l]
scFvCD3-FlagHis	35*	28	1.0
scFvHLA-A2-mycHis	27	29	1.5
HisFlag-scFvCD45(V_L-V_H)	30	30	0.1
scFvCD45(V_H-V_L)-FlagHis	31	29	1.0
V_LCD3-scFvHLA-A2-mycHis	47	41	0.8
V_HCD3-scFvCD45(V_L-V_H)-FlagHis	49	43	0.3
V_HCD3-scFvCD45(V_H-V_L)-FlagHis	49	42	0.4
V_HCD3-scFvHLA-A2-mycHis	50	42	0.2
V_HCD3-scFvHLA-A2-FlagHis	48	42	0.2
taFvCD3-HLA-A2-mycHis	64	55	0.2
taFvCD3-HLA-A2-FlagHis	64	55	0.2
taFvHLA-A2-CD3-mycHis	67	55	0.2
taFvHLA-A2-CD3-FlagHis	67	55	0.3

* data from Western blot analysis shown above in Fig. 4.2 D in Section 4.1.2

The highest yields from periplasmic expression in *E. coli* TG1 were obtained for scFvs with 1.0 – 1.5 mg/l, as listed in Table 4.2. The yield of scFvCD45(V_L-V_H) was exceptionally low with 0.1 mg/l. The yield obtained for V_LCD3-scFvHLA-A2 amounted to 0.8 mg/l, whereas the yields of V_H-scFvs (0.2 – 0.4 mg/l) were similar to those of taFvs (0.2 – 0.3 mg/l).

4.4.4 Binding Studies with the Self-Assembling Constructs by Flow Cytometry

Expression of CD3 and CD45 on Jurkat and HLA-A2 on HEK-293T $A2^+$ cells

In order to prove the binding properties of the five constructs for complementation, their binding to Jurkat cells ($CD3^+$ $CD45^+$) and to HEK-293T cells stably transfected with HLA-A2 (HEK-293T $A2^+$) was analysed. First, the anticipated expression of CD3, CD45, and HLA-A2 on these cells was verified by flow cytometry. For this purpose, Jurkat and HEK-293T $A2^+$ cells were incubated with a PE-conjugated antibody specific for HLA-A2, FITC-conjugated antibody specific for CD3ε or CD45, or the corresponding isotype control, mouse IgG2b-PE or mouse IgG1κ-FITC. Subsequently, the respective antigen expression was analysed by flow cytometry (Fig. 4.14). The CD3-expression on HEK-293T $A2^+$ was not tested, as these cells should be CD3-negative, because of their non-haematopoietic origin.

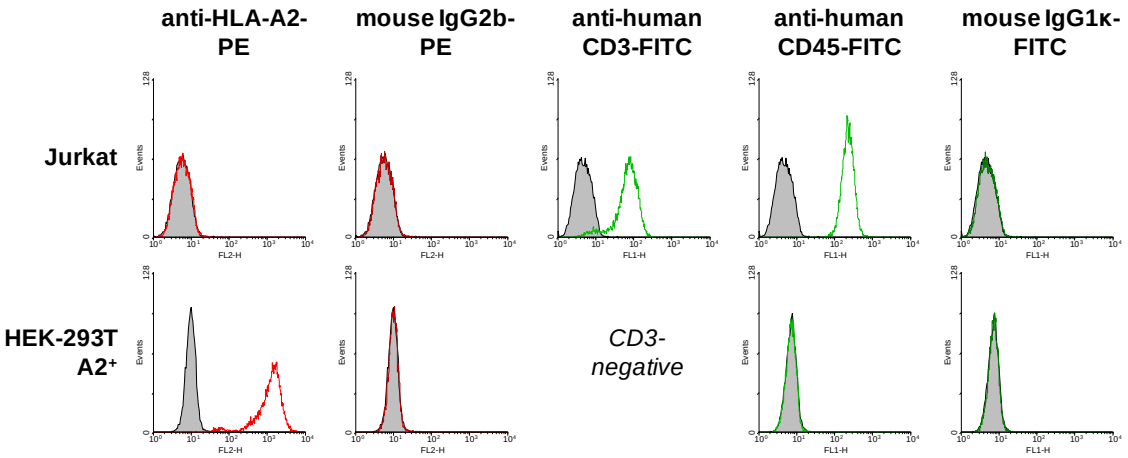


Fig. 4.14: Expression of HLA-A2, CD3, and CD45 on Jurkat cells and HEK-293T A2$^+$ cells, analysed by flow cytometry. Detection was performed by incubating cells with anti-HLA-A2-PE, anti-CD3-FITC, anti-CD45-FITC antibody, or the corresponding isotype control, mouse IgG2b-PE or mouse IgG1-FITC. Grey filled, unstained cells; coloured line, antibody against a cell-surface molecule or isotype control.

As expected, the human T-cell leukaemia cell line Jurkat revealed high amounts of CD3 and CD45, and HEK-293T A2$^+$ cells expressed high amounts of HLA-A2, but no CD45. More importantly, Jurkat cells appeared to be HLA-A2-negative. Thus, both cell lines proved to be appropriate for binding studies with the constructs for complementation.

Binding Studies with Bispecific HLA-A2 × CD3 taFvs by Flow Cytometry

The binding ability of the bispecific HLA-A2 × CD3 taFv fragments to antigen-expressing cells was tested by flow cytometry. The taFv fragments were tested for binding to HEK-293T A2$^+$ cells and to Jurkat cells expressing high levels of CD3 (Fig. 4.15).

All four taFv fragments revealed a strong binding to HEK-293T A2$^+$ cells, as did their parental monospecific equivalent scFvHLA-A2. They also bound to CD3-expressing Jurkat cells, but to a variable extent. taFvHLA-A2-CD3-FlagHis revealed good binding to CD3, but it was exceeded by taFvCD3-HLA-A2-mycHis and taFvHLA-A2-CD3-mycHis. taFvCD3-HLA-A2-FlagHis showed the best binding to CD3, which was even better than that shown by the monospecific equivalent scFvCD3. scFvHLA-A2, having been used as a negative control on Jurkat cells, did not show any binding to CD3. Likewise, scFvCD3 did not bind to HEK-293T A2$^+$ cells.

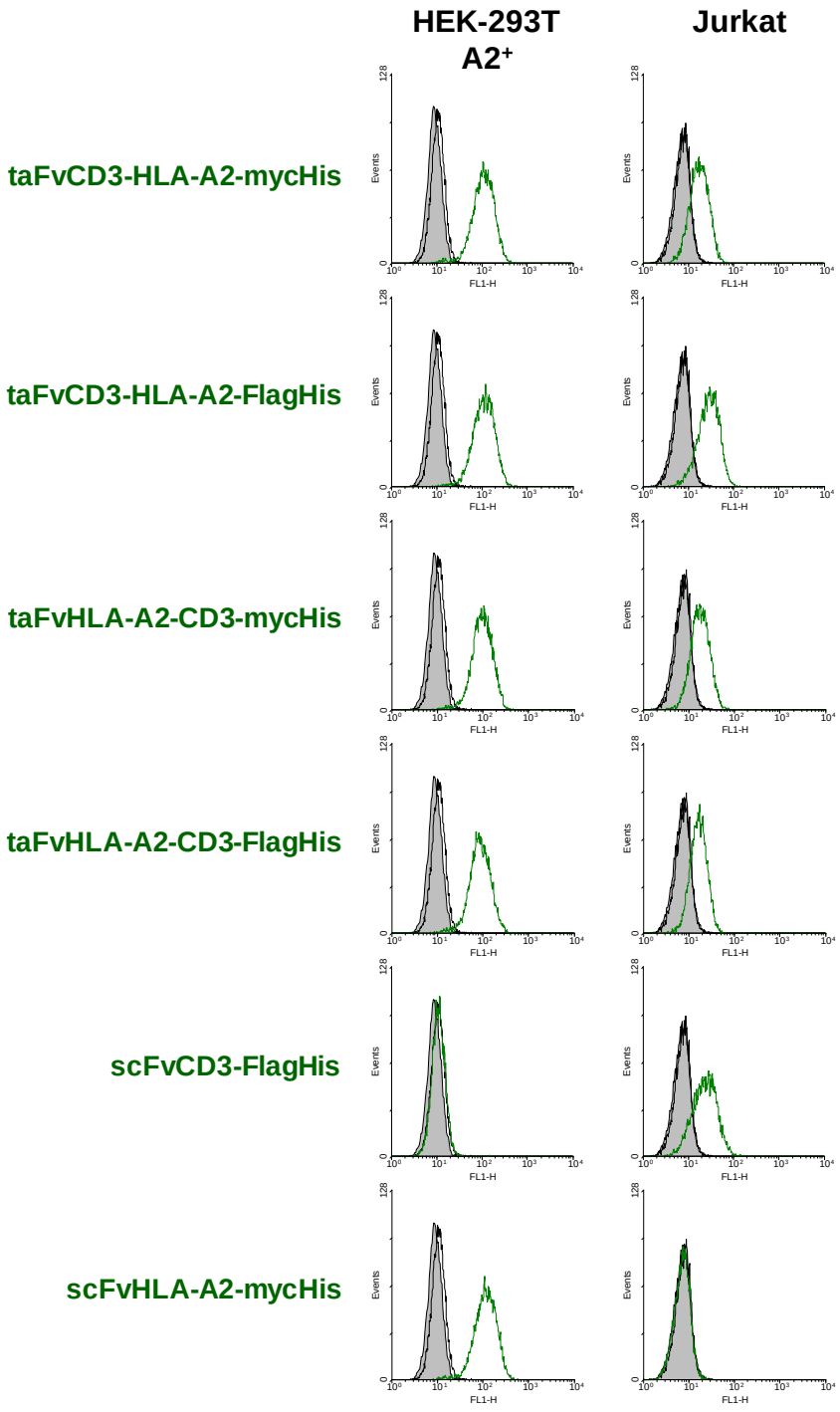


Fig. 4.15: Binding of bispecific HLA-A2 × CD3 taFv fragments to HLA-A2 and CD3, analysed by flow cytometry. Binding of 10 µg/ml of each bispecific taFv fragment to HEK-293T A2+ and Jurkat ($CD3^+$) cells. Binding was detected by FITC-conjugated anti-His_6 tag antibody. Grey filled, unstained cells; black line, secondary antibody alone; green line, antibody construct.

In order to rule out unspecific binding of the bispecific HLA-A2 × CD3 taFvs to HEK-293T $A2^+$ cells, a blocking assay was performed as described in section 3.7.1. Therein, HEK-293T $A2^+$ cells were preincubated with scFvHLA-A2 before the addition of taFvCD3-HLA-A2-FlagHis as an example for the bispecific taFvs. Thus, the binding of taFvCD3-HLA-A2-FlagHis was entirely blocked. On the contrary, complete binding occurred in the absence of scFvHLA-A2 (Fig. 4.16). Consequently, the observed binding of taFvCD3-HLA-A2-FlagHis is clearly accomplished via HLA-A2 through its HLA-A2-specific antibody moiety.

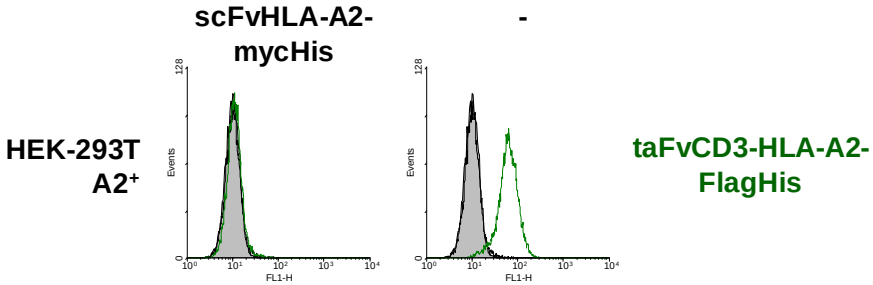


Fig. 4.16: Binding specificity of taFvCD3-HLA-A2-mycHis to HLA-A2, analysed by flow cytometry. Binding of 20 nM taFvCD3-HLA-A2-FlagHis to HEK-293T $A2^+$ cells in the presence or absence of preincubated scFvHLA-A2-mycHis at 4 µM (2 µM final concentration). Binding was detected by FITC-conjugated anti-Flag tag antibody. Grey filled, unstained cells; black line, secondary antibody alone; green line, taFvCD3-HLA-A2-FlagHis.

In sum, all four taFv fragments proved themselves able to strongly bind to both HEK-293T $A2^+$ and CD3-expressing Jurkat cells according to their specificities. taFvCD3-HLA-A2, with either FlagHis or mycHis tag, represents the best candidate for use as a positive control in subsequent functional assays with the constructs for complementation.

Binding Studies with the Constructs for Complementation by Flow Cytometry

The binding ability of the individual constructs for complementation to antigen-expressing cells was tested by flow cytometry. The individual constructs were tested for binding to HEK-293T $A2^+$ cells and Jurkat cells (Fig. 4.17).

All five constructs for complementation individually bound to cells expressing the respective antigen according to the specificity of the complete scFv molecule they harbour. The three HLA-A2-specific ones, V_LCD3-scFvHLA-A2, V_HCD3-scFvHLA-A2-mycHis, and V_HCD3-scFvHLA-A2-FlagHis, revealed a binding to HEK-293T $A2^+$ cells that was as strong as that of scFvHLA-A2. As expected, no binding of the HLA-A2-specific constructs (including scFvHLA-A2) to Jurkat cells was observed, indicating that neither the V_LCD3 domain nor the V_HCD3 domain interacts with CD3 on Jurkat cells. The complete scFvCD3 construct, on the other hand, strongly bound to Jurkat, but not to HEK-293T $A2^+$ cells.

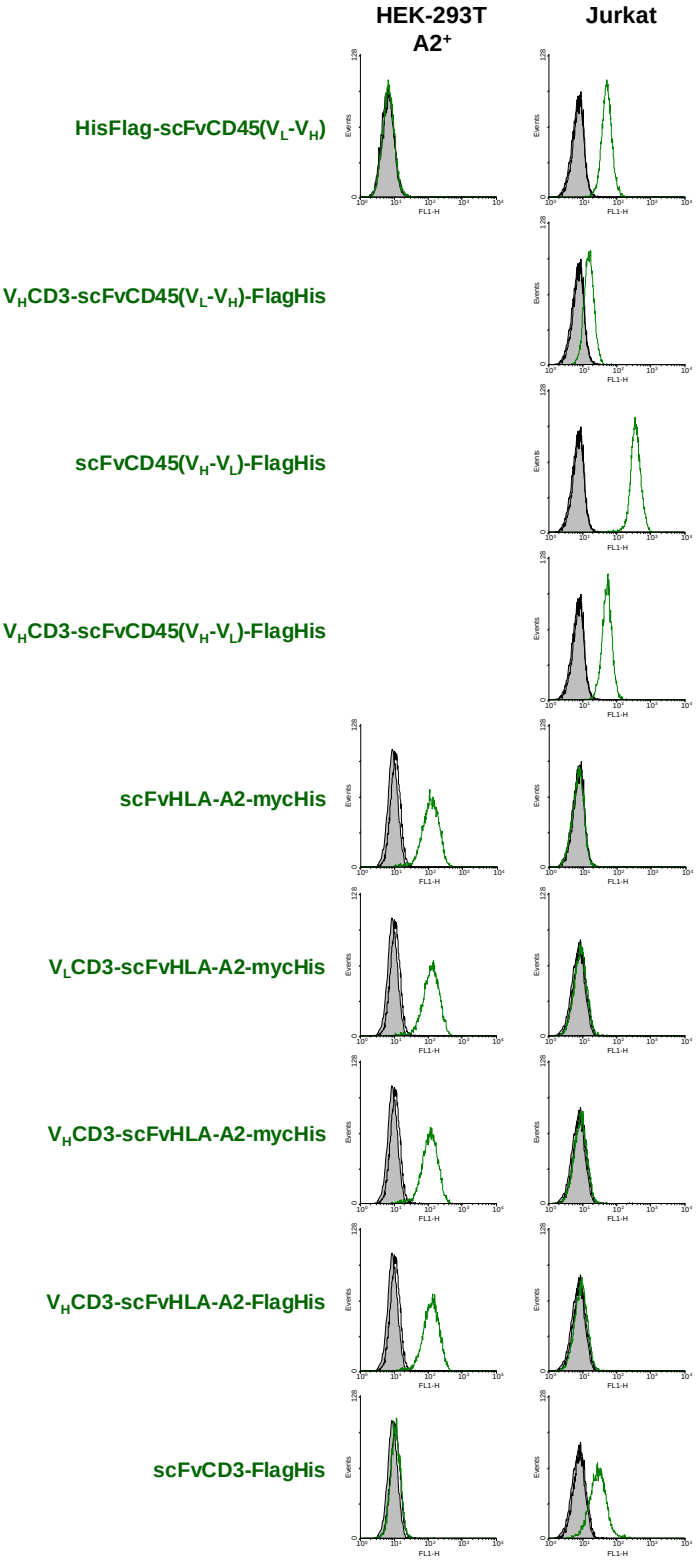


Fig. 4.17: Binding of the antibody constructs for complementation to HLA-A2, CD3, or CD45, analysed by flow cytometry. Binding of 10 µg/ml of each antibody construct to HEK-293T $A2^+$ cells or to Jurkat cells ($CD3^+$ $CD45^+$). Binding was detected by a FITC-conjugated anti-His_6 tag antibody. Grey filled, unstained cells; black line, secondary antibody alone; green line, antibody construct.

The CD45-specific constructs bound to Jurkat cells to a variable extent. scFvCD45(V_H-V_L) showed the best binding to Jurkat cells, which was in fact better than that of scFvCD45(V_L-V_H). V_HCD3-scFvCD45(V_H-V_L) and V_HCD3-scFvCD45(V_L-V_H) revealed a slightly less strong binding than their respective scFv equivalent. As expected, no binding of scFvCD45(V_L-V_H) to HEK-293T $A2^+$ cells was detected.

4.5 Complementation of Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibody

As a first proof of the occurrence of the self-assembly of V_LCD3-scFv and V_HCD3-scFv on double-positive cells, the same *in vitro* functional assay that was described for the bispecific AChRγ × CD3 taFv fragments in Sections 4.2 and 3.7.2 was performed. This assay was used to investigate the potential of the generated two-part trispecific HLA-A2 × CD3 × CD45 antibodies and the bispecific HLA-A2 × CD3 taFv fragments to activate T cells *in vitro*.

4.5.1 Expression of HLA-A2 and CD45 on Target Cells for PBMC Stimulation Assay

In order to prove the stimulatory activity of the two-part trispecific HLA-A2 × CD3 × CD45 antibody variants, in addition to HLA-A2-negative PBMC, different target cells were required that express both HLA-A2 and CD45, either HLA-A2 or CD45, or neither of them. Since additional transfections of HEK-293T and HEK-293T $A2^+$ cells with CD45 repeatedly led to a very low CD45-expression (not shown), other target cell lines expressing the respective antigens were searched. Therefore, the expression of HLA-A2 and CD45 was examined on different cell lines by flow cytometry. For this purpose, in addition to PBMC, also MCF-7, Raji, and ten different human multiple myeloma cell lines were incubated with a PE-conjugated antibody specific for HLA-A2, FITC-conjugated antibody specific for CD45, or the corresponding isotype control, mouse IgG2b-PE or mouse IgG1κ-FITC (Fig. 4.18).

Of the tested human multiple myeloma cell lines solely U266 revealed high expressions of both HLA-A2 and CD45. L-363 expressed high amounts of HLA-A2, but no detectable CD45, whereas the human Burkitt's lymphoma cell line Raji expressed CD45, but no detectable HLA-A2. On KMS-12-BM cells neither HLA-A2 nor CD45 was detected. The human breast cancer cell line MCF-7 appeared to be HLA-A2-positive. The PBMC were HLA-A2-negative. CD45-expression on MCF-7 and PBMC was not tested, as MCF-7 cells should be CD45-negative, because of their non-haematopoietic origin, whereas the PBMC are definitely CD45-positive due to their haematopoietic origin. Other PBMC were tested CD45-positive and were well bound by the CD45-specific scFvs (data not shown).

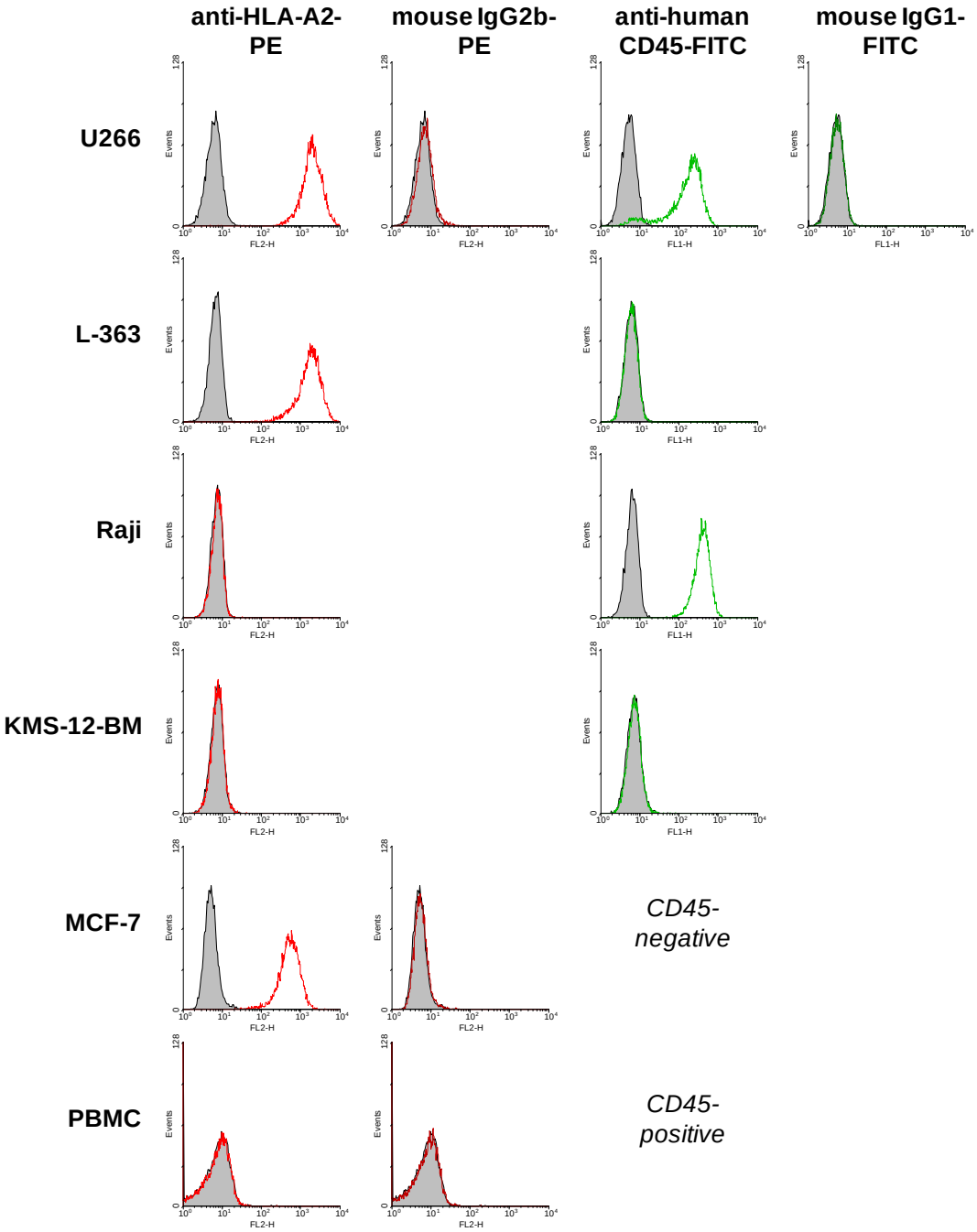


Fig. 4.18: Expression of HLA-A2 and CD45 on target cells and PBMC for stimulation assays, analysed by flow cytometry. Detection was performed by incubating cells with anti-HLA-A2-PE, anti-CD45-FITC antibody, or the corresponding isotype control, mouse IgG2b-PE or mouse IgG1-FITC. Grey filled, unstained cells; coloured line, antibody against a cell-surface molecule or isotype control.

4.5.2 Stimulatory Activity of Bispecific HLA-A2 × CD3 taFvs

The stimulatory potential of bispecific HLA-A2 × CD3 taFv fragments was examined on HLA-A2-expressing target cells, namely HEK-293T $A2^+$ (see Fig. 4.14, p. 74) or U266 (see Fig. 4.18 above). For this purpose, the four bispecific constructs were titrated from

0.3 – 243 nM on U266 cells, followed by the addition of unstimulated HLA-A2-negative PBMC at an E : T ratio of 2 : 1 (see Fig. 4.18 above). As a control, background signals of IL-2 were achieved by incubating PBMC with target cells only, *i.e.* in the absence of any antibody constructs (Fig. 4.19 A).

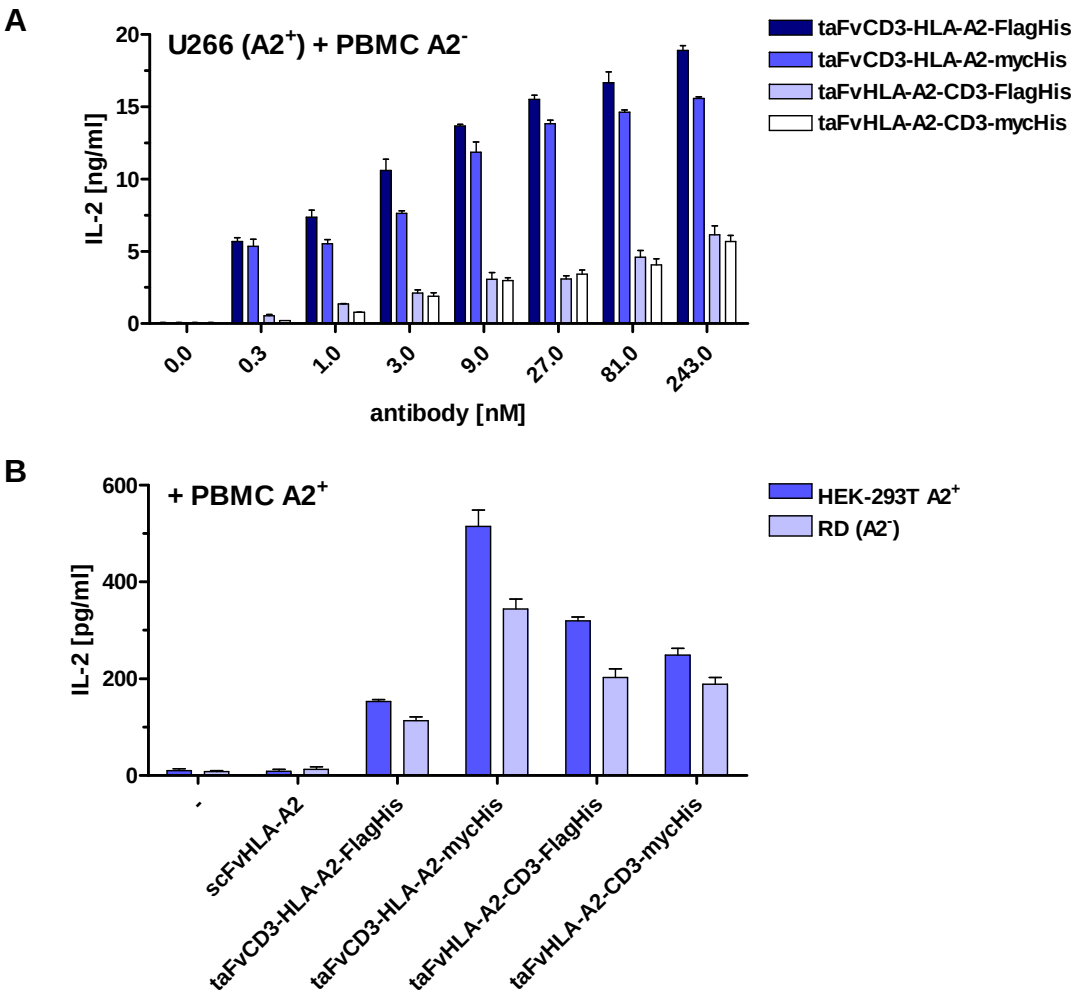


Fig. 4.19: T-cell activation mediated by bispecific HLA-A2 × CD3 taFv fragments (positive control). **A)** HLA-A2-expressing target cells (U266) were incubated with titrated antibody constructs (0.3 – 243 nM) *in duplicate*, followed by addition of unstimulated HLA-A2-negative PBMC (E : T ratio of 2 : 1). **B)** HEK-293T $A2^+$ and RD ($HLA\text{-}A2^-$) cells were incubated with 27 nM of the indicated antibody constructs *in triplicate*, respectively, prior to an addition of unstimulated HLA-A2-positive PBMC (E : T ratio of 2 : 1). IL-2 secretion was measured in ELISA after 24 h. The results of **A)** are representative of three independent experiments. Please note that U266 cells express much higher HLA-A2 levels than HEK-293T cells explaining the superior IL-2 inducibility in the assays with U266 cells.

All four taFv fragments exhibited a stimulatory effect on PBMC in a concentration-dependent manner. The most effective stimulation was achieved with taFvCD3-HLA-A2-FlagHis at 243 nM, resulting in a maximum IL-2 concentration of about 19 ng/ml. At the same concentration, the resulting IL-2 release amounted to 16 ng/ml for taFvCD3-HLA-A2-mycHis and 6 ng/ml for taFvHLA-A2-CD3-FlagHis as well as taFvHLA-A2-

CD3-mycHis. These IL-2 amounts correspond to approximately 82 % and 32 % of the IL-2 release achieved by the stimulation with taFvCD3-HLA-A2-FlagHis. An incipient saturation of HLA-A2-binding sites on the target cells is indicated for taFvCD3-HLA-A2-mycHis. Interestingly, in a preliminary experiment, all four taFvs had also a stimulatory effect when HLA-A2-negative target cells (RD) and HLA-A2-positive PBMC (PBMC $A2^+$) were used instead (Fig. 4.19 B). Here, the IL-2 release was a little lower but comparable to that reached with HEK-293T $A2^+$ used in parallel with PBMC $A2^+$. This indicates a mutual stimulation of the PBMC $A2^+$ regardless of the target cells RD (see discussion, Section 5.2). On average, a 30 % loss in IL-2 release could be observed. As expected, scFvHLA-A2, used as negative control, had no stimulatory effect on PBMC. In conclusion, all four constructs mediate T-cell activation with the two taFvCD3-HLA-A2 variants being most potent.

4.5.3 Stimulatory Activity of HLA-A2 × CD3 × HLA-A2 Specific Antibodies

In order to facilitate the self-assembly of V_LCD3 and V_HCD3, as a first proof of concept, the self-assembly of V_LCD3-scFvHLA-A2 and V_HCD3-scFvHLA-A2 was tested on HLA-A2-expressing cells. Here, both the V_LCD3 and V_HCD3 domain was linked to the same complete scFv specific for HLA-A2. Thus, differences in epitope localisation, size, or expression level of the two potentially targeted antigens should not have any impact on complementation. For the examination of the stimulatory activity, HEK-293T $A2^+$ cells were preincubated with 27 nM V_LCD3-scFvHLA-A2-mycHis, V_HCD3-scFvHLA-A2-mycHis, and V_HCD3-scFvHLA-A2-FlagHis, either alone or in the combination V_LCD3-scFvHLA-A2 + V_HCD3-scFvHLA-A2. Subsequently, unstimulated HLA-A2-negative PBMC were added at an E : T ratio of 2 : 1. The bispecific taFvCD3-HLA-A2-mycHis served as positive control. Subsequent measurement of IL-2 release after 24 h revealed the following IL-2 concentrations (Fig. 4.20).

Stimulation with 27 nM taFvCD3-HLA-A2-mycHis led to an IL-2 secretion of 262 pg/ml. In comparison to that, the combination of V_LCD3-scFvHLA-A2-mycHis with V_HCD3-scFvHLA-A2-mycHis (Combn A + B) amounted to 55 pg/ml and that of V_LCD3-scFvHLA-A2-mycHis with V_HCD3-scFvHLA-A2-FlagHis (Combn A + C) to 71 pg/ml. In both cases, each $V_{H/L}$CD3-scFv was used at 27 nM concentration, which corresponds to the same amount of the assembled CD3-specific moiety in taFv. In contrast, as expected, these constructs for complementation had no stimulatory effect on PBMC when applied individually. Neither had scFvCD3 nor scFvHLA-A2, which were used as negative controls. Regarding the stimulation with taFvCD3-HLA-A2, the IL-2 release achieved by stimulation with the combinations resulted in 21 % (A + B) and 27 % (A + C), respectively. These values are approximately 10 fold over the background achieved by incubating PBMC with target cells only (6 pg/ml). Evidently,

complementation of a functional scFvCD3 occurred in both cases. In conclusion, both combinations mediated an activation of T cells, with A + C tending to be slightly more potent than A + B. In addition, as expected, these constructs for complementation had no stimulatory effect when HLA-A2-negative target cells (RD) were used instead (data not shown).

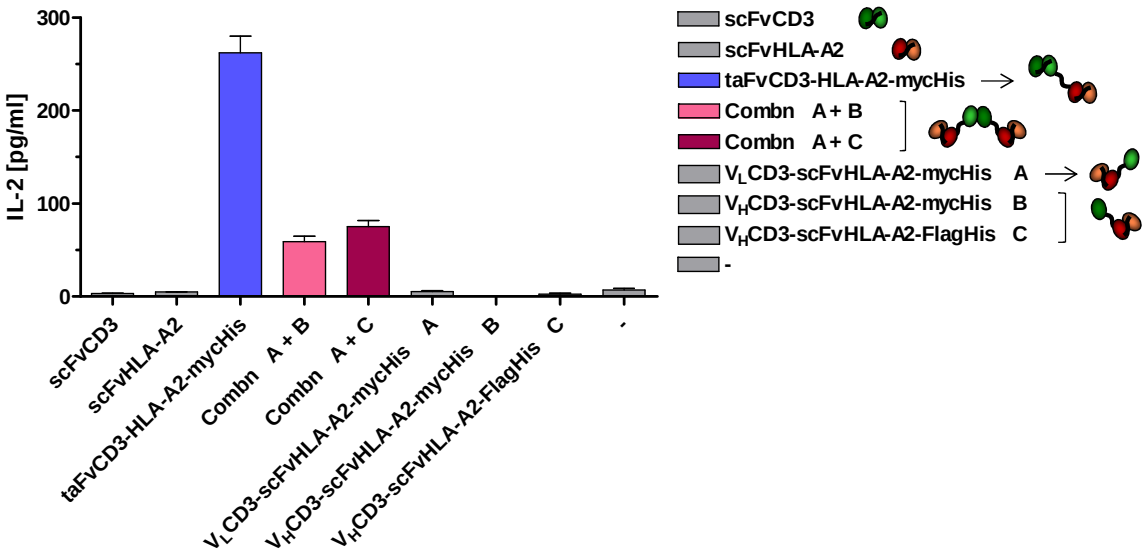


Fig. 4.20: Complementation and stimulatory activity of two-part trispecific HLA-A2 × CD3 × HLA-A2 constructs on HLA-A2$^+$ cells. HLA-A2-expressing target cells (HEK-293T A2$^+$) were incubated with 27 nM antibody constructs *in duplicate*, followed by addition of unstimulated HLA-A2-negative PBMC (E : T ratio of 2 : 1). IL-2 release was measured in ELISA after 24 h.

4.5.4 Stimulatory Activity of Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibodies

In a second step, the strategy of the self-assembling trispecific antibody was transferred to a system that indeed depends on two different antigens, namely HLA-A2 and CD45. The stimulatory properties of the two variants of trispecific HLA-A2 × CD3 × CD45 antibody were examined on U266 cells that express high levels of HLA-A2 as well as CD45 (see Fig. 4.18, p. 79). For this purpose, V_LCD3-scFvHLA-A2, V_HCD3-scFvCD45$_{(V_L\text{-}V_H)}$, and V_HCD3-scFvCD45$_{(V_H\text{-}V_L)}$, either alone or in the combination V_LCD3-scFvHLA-A2 + V_HCD3-scFvCD45, were titrated from 0.04 – 243 nM on U266 cells. Subsequently, unstimulated HLA-A2-negative PBMC were added (E : T ratio of 2 : 1) and the IL-2 release was measured after 24 h (Fig. 4.21).

For both combinations where functional scFvCD3 complementation is possible [V_LCD3-scFvHLA-A2 + V_HCD3-scFvCD45$_{(V_L\text{-}V_H)}$ (Combn A + D) and V_LCD3-scFvHLA-A2 + V_HCD3-scFvCD45$_{(V_H\text{-}V_L)}$ (Combn A + E)], a stimulatory effect on PBMC could be observed in a concentration-dependent manner. The most effective stimulation was achieved at 243 nM, resulting in a maximum IL-2 concentration of about 13 ng/ml,

without reaching saturation. Individually applied V_HCD3-scFvCD45(V_L-V_H) and V_HCD3-scFvCD45(V_H-V_L) had no stimulatory effect on PBMC. Only V_LCD3-scFvHLA-A2 showed a marginal effect, leading to an IL-2 release of 712 pg/ml at 243 nM concentration. This corresponds to only approximately 5 % of the IL-2 release achieved by the stimulation with the assembled trispecific antibody at this concentration, but clearly exceeding the background, reached without any antibody (101 pg/ml), by a factor of seven. In conclusion, both combinations mediated the activation of T cells, indicating that it is possible to make the assembly of a functional scFvCD3 molecule dependent on the availability of two distinct cell surface antigens.

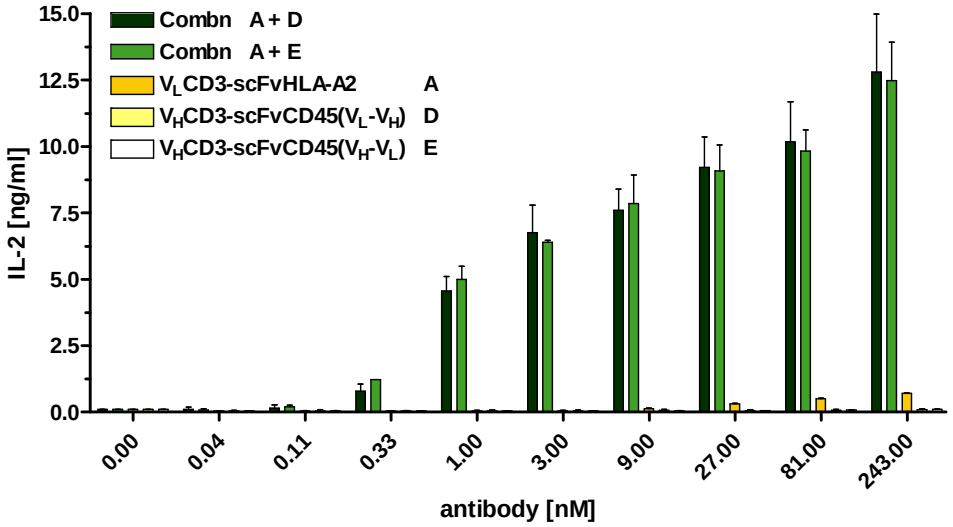


Fig. 4.21: Complementation and stimulatory activity of two-part trispecific HLA-A2 × CD3 × CD45 constructs on HLA-A2$^+$ CD45$^+$ cells. Target cells expressing both HLA-A2 and CD45 (U266) were incubated with titrated antibody constructs (0.04 – 243 nM) *in duplicate.* In the following, unstimulated HLA-A2-negative PBMC (E : T ratio of 2 : 1) were added and IL-2 secretion was measured in ELISA after 24 h. Results are representative of four independent experiments.

For better illustration and to compare the two-part trispecific antibody with the bispecific positive control, a similar experiment was performed (Fig. 4.22). Here, simultaneously with taFvCD3-HLA-A2-mycHis, the V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45(V_H-V_L) constructs, either alone or in combination, were titrated from 0.3 – 243 nM on U266 cells, followed by the addition of unstimulated HLA-A2-negative PBMC (E : T ratio of 2 : 1) and measurement of IL-2 release after 24 h.

At lower concentrations (0.3 – 3 nM), a slightly higher stimulatory effect was observed for the two-part trispecific antibody. Here, on average, stimulation with the two-part trispecific antibody led to an IL-2 release that was 12 % higher than stimulation with the bispecific positive control. In contrast, at higher concentrations (9 – 243 nM), the bispecific positive control was considerably more potent. Here, stimulation with the two-part trispecific antibody averaged 65 % of the IL-2 release achieved by the bispecific

positive control. In conclusion, the two-part trispecific HLA-A2 × CD3 × CD45 construct (Combn A + E) mediated activation of T cells tendentially less well than taFvCD3-HLA-A2-mycHis used as bispecific positive control.

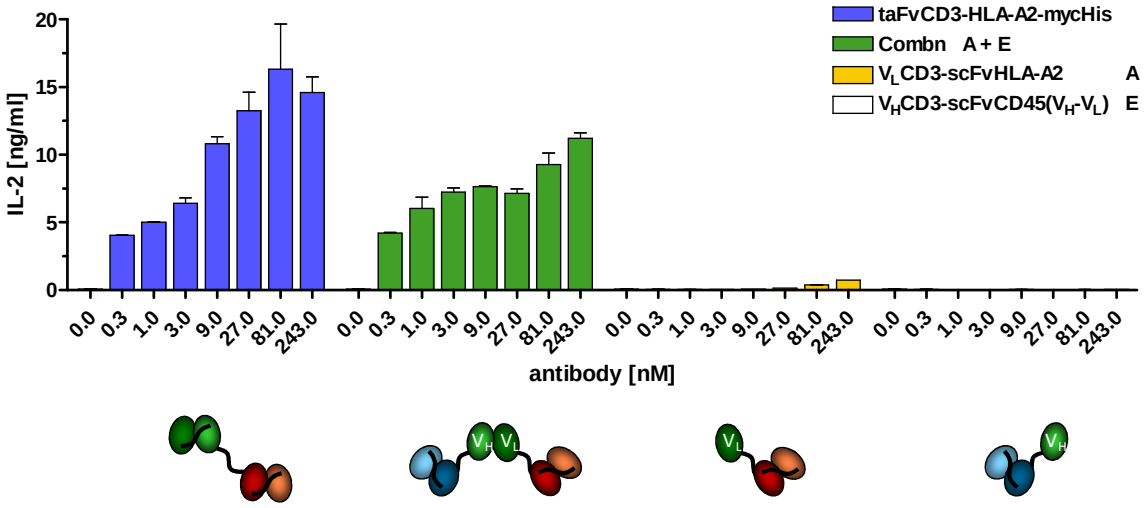


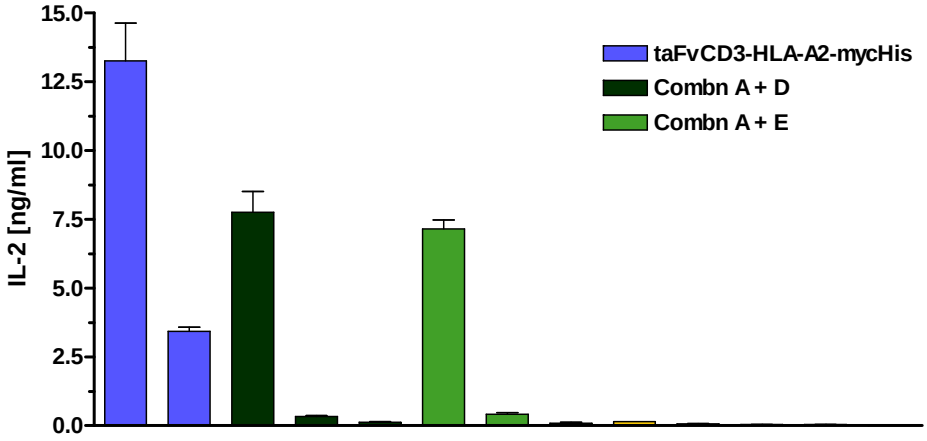
Fig. 4.22: Stimulatory activity of two-part trispecific HLA-A2 × CD3 × CD45 constructs compared to bispecific HLA-A2 × CD3 taFv on HLA-A2$^+$ CD45$^+$ cells. Target cells expressing both HLA-A2 and CD45 (U266) were incubated with titrated antibody constructs (0.3 – 243 nM) *in duplicate*, followed by addition of unstimulated HLA-A2-negative PBMC (E : T ratio of 2 : 1). IL-2 release was measured in ELISA after 24 h.

4.5.5 Dual-Antigen Restriction of Trispecific HLA-A2 × CD3 × CD45 Antibody

Under the described experimental conditions, it has to be ruled out that a spontaneous and premature self-assembly of V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45$_{(V_H\text{-}V_L)}$ occurs in solution without contact to at least one antigen, HLA-A2 or CD45. According to this, the interaction of either V_LCD3-scFvHLA-A2 with HLA-A2 or V_HCD3-scFvCD45 with CD45 on U266 cells was blocked by prior incubation with HLA-A2- and CD45-specific scFv, respectively (Fig. 4.23).

The stimulatory activity of both trispecific antibody pairs at 27 nM was blocked by preincubation with 2.7 µM scFv. The induced IL-2 secretion was reduced to 4.4 % by blocking with scFvHLA-A2 for the combination A + D and to 5.9 % for the combination A + E. In comparison, the stimulatory activity of the bispecific taFvCD3-HLA-A2-mycHis was only reduced to 26 % and not, as it was expected, to the same extent as the trispecific antibodies. The IL-2 release induced by the combinations was reduced to 1.7 % (Combn A + D) and 1.4 % (Combn A + E), respectively, by blocking with scFvCD45$_{(V_H\text{-}V_L)}$ to the level achieved by each single construct for complementation on its own (last block of bars). In summary, the stimulatory activity of both trispecific antibodies was confirmed to be dual antigen-restricted, since the affinity of V_HCD3-scFv

and V_LCD3-scFv to each other is definitely insufficient for complementation in the absence of at least one antigen, HLA-A2 or CD45.



taFvCD3-HLA-A2	+	+											
V_LCD3-scFvHLA-A2			+	+	+	+	+	+	+				
V_HCD3-scFvCD45(V_L-V_H)			+	+	+					+			
V_HCD3-scFvCD45(V_H-V_L)						+	+	+			+		
100× scFvHLA-A2		+		+			+					+	
100× scFvCD45					+			+					+

Fig. 4.23: Dual antigen-restricted complementation of two-part trispecific HLA-A2 × CD3 × CD45 constructs on HLA-A2$^+$ CD45$^+$ cells. Target cells expressing both HLA-A2 and CD45 (U266) were preincubated with or without 5.4 µM scFvHLA-A2 or scFvCD45(V_H-V_L) (each 2.7 µM final concentration), followed by an incubation with 27 nM antibody constructs *in duplicate*. Unstimulated HLA-A2-negative PBMC were added (E : T ratio of 2 : 1) and IL-2 release was measured in ELISA after 24 h. These results are representative of two independent experiments. *Combn A + D*, V_LCD3-scFvHLA-A2 + V_HCD3-scFvCD45(V_L-V_H); *Combn A + E*, V_LCD3-scFvHLA-A2 + V_HCD3-scFvCD45(V_H-V_L).

In order to prove the dual-antigen restriction of the trispecific construct in another way, the stimulatory activity of the two variants of trispecific HLA-A2 × CD3 × CD45 antibody was examined on target cells expressing high levels of only one antigen at a time, HLA-A2 or CD45, or neither of them (see Fig. 4.18, p. 79). Here, the respective target cells were preincubated with the trispecific combinations A + D and A + E, as described above for Fig. 4.21 in Section 4.5.4, but at 27 nM of A, D, and E each. In the following, unstimulated HLA-A2-negative PBMC were added (E : T ratio of 2 : 1) and IL-2 release was measured in ELISA after 24 h. The following IL-2 concentrations resulted with target cells expressing high levels of CD45, but no detectable HLA-A2 (Raji, Fig. 4.24 A) or neither of these antigens (KMS-12-BM, Fig. 4.24 B). Here, the T-cell stimulant PHA-L served as positive control.

Stimulation with 1 µg/ml PHA-L led to an IL-2 secretion of 34 ng/ml on Raji cells and of 33 ng/ml on KMS-12-BM cells. As expected, both variants of the trispecific HLA-A2 × CD3 × CD45 antibody had no stimulatory effect on PBMC. Stimulation with both trispecific combinations resulted in IL-2 concentrations in the order of the

background signals which were achieved by incubating PBMC with target cells only (last bar in each diagram), approximately 500 pg/ml on Raji and 80 pg/ml on KMS-12-BM cells. In conclusion, both variants of the trispecific HLA-A2 × CD3 × CD45 antibody were not able to active T cells in the presence of only CD45 or in the absence of both HLA-A2 and CD45. Consequently, it is ensured that the self-assembly of V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45 only occurs when both antigens, HLA-A2 and CD45, are present.

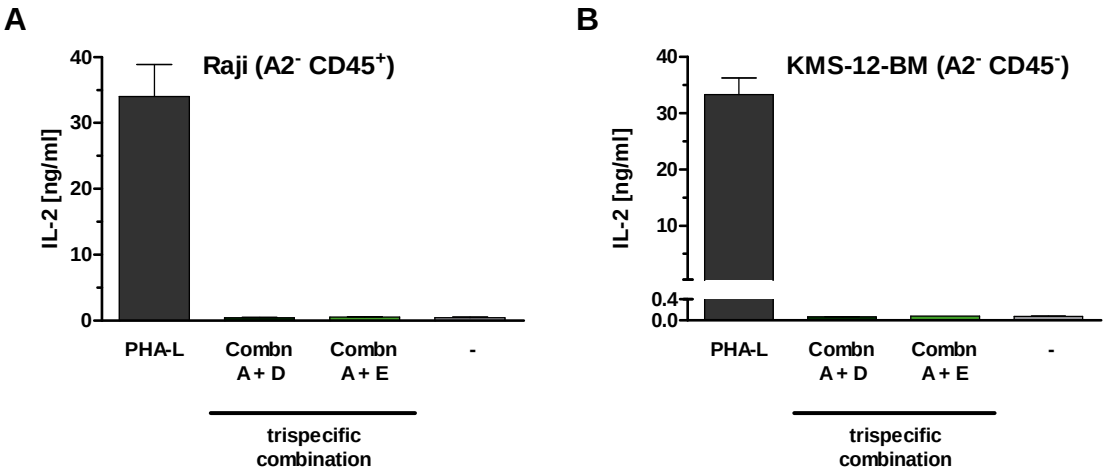


Fig. 4.24: No complementation of two-part trispecific HLA-A2 × CD3 × CD45 constructs on HLA-A2$^-$ CD45$^+$ and HLA-A2$^-$ CD45$^-$ cells. Target cells expressing **A)** no HLA-A2 but CD45 (Raji) or **B)** neither HLA-A2 nor CD45 (KMS-12-BM) were incubated with 1 µg/ml PHA-L as positive control or 27 nM antibody constructs *in duplicate*, respectively. Unstimulated HLA-A2-negative PBMC (E : T ratio of 2 : 1) were added and IL-2 secretion was measured in ELISA after 24 h. Results are representative of **A)** four and **B)** three independent experiments. *A*, V_LCD3-scFvHLA-A2; *D*, V_HCD3-scFvCD45(V_L-V_H); *E*, V_HCD3-scFvCD45(V_H-V_L).

Furthermore, target cells expressing HLA-A2, but no detectable CD45 (L-363 and MCF-7), were preincubated with 27 nM taFvCD3-HLA-A2-mycHis as a bispecific positive control and the trispecific combinations, A + D and A + E (Fig. 4.25). MCF-7 cells were additionally preincubated with the constructs for complementation, A, D, and E, on their own. Apart from that, the assay was further performed as above. It should be noted that the MCF-7 cells are known to be homozygous for HLA-A2 [Christensen *et al.*, 2009].

As expected, due to its ability to crosslink between HLA-A2 on target cells and CD3 on T cells, the bispecific taFvCD3-HLA-A2-mycHis had a stimulatory effect on PBMC, leading to an IL-2 secretion of 10 ng/ml at 27 nM on both L-363 and MCF-7 cells. Unfortunately, the combination of V_LCD3-scFvHLA-A2 with V_HCD3-scFvCD45 also led to a stimulation of PBMC. The stimulation with the trispecific combination led to an IL-2 release of approximately 7 ng/ml on L-363 and 5 ng/ml on MCF-7 cells. It is worthy of note that each construct for complementation on its own had no stimulatory effect on PBMC, as shown by way of example for MCF-7 cells (Fig. 4.25 B). In conclusion, for

unknown reasons, both variants of the trispecific HLA-A2 × CD3 × CD45 antibody unspecifically mediated the activation of T cells, reaching 68 % and 48 % of the stimulatory activity of the bispecific positive control on L-363 and MCF-7 cells, respectively.

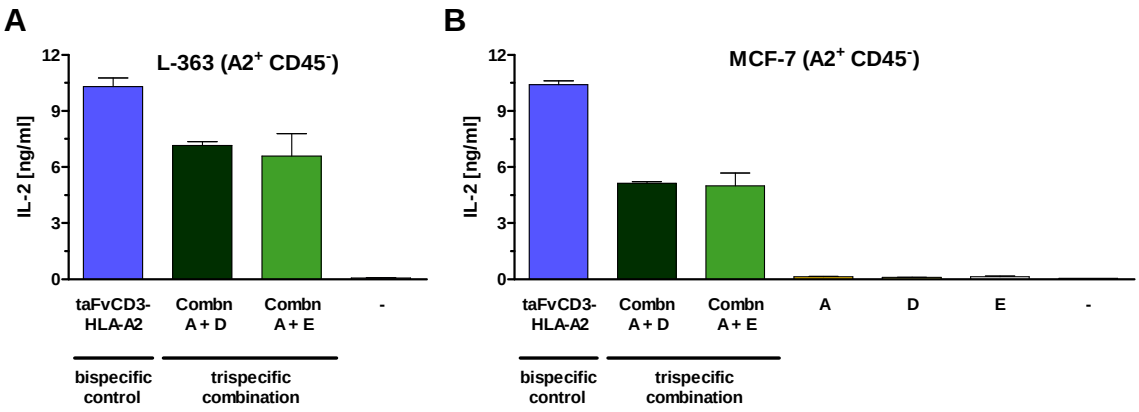


Fig. 4.25: Unspecific activity of two-part trispecific HLA-A2 × CD3 × CD45 constructs on HLA-A2$^+$ CD45$^-$ cells. Target cells expressing HLA-A2, but no CD45, **A)** L-363 and **B)** MCF-7, were incubated with 27 nM antibody constructs *in duplicate*, followed by addition of unstimulated HLA-A2-negative PBMC (E : T ratio of 2 : 1). IL-2 release was measured in ELISA after 24 h. Results are representative of **A)** three and **B)** two independent experiments. *A*, V_LCD3-scFvHLA-A2; *D*, V_HCD3-scFvCD45(V_L-V_H); *E*, V_HCD3-scFvCD45(V_H-V_L).

4.6 Cytotoxicity of Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibody

So far, it was shown in this study that the complementation of the two-part trispecific HLA-A2 × CD3 × CD45 constructs is functional, dual antigen-restricted, and able to activate T cells by inducing IL-2 release in a costimulation-independent manner. For further evaluation, the trispecific constructs were investigated for the ability to recruit T cells for lysis of target cells expressing both HLA-A2 and CD45. In order to measure tumour cell lysis by cytotoxic T cells *in vitro,* the same flow cytometry-based cytotoxicity assay was performed as described for the bispecific AChRγ × CD3 taFv fragments in Section 4.3 (see method details in Section 3.7.4).

CFSE-labelled target cells expressing both HLA-A2 and CD45 (U266) were preincubated with 27 nM V_LCD3-scFvHLA-A2, V_HCD3-scFvCD45(V_L-V_H), and V_HCD3-scFvCD45(V_H-V_L), either alone or in the combination V_LCD3-scFvHLA-A2 + V_HCD3-scFvCD45 in the presence or absence of unstimulated PBMC at an E : T ratio of 10 : 1 (Fig. 4.26). The bispecific taFvCD3-HLA-A2-mycHis served as positive control. Cell death was determined after 24 h and 48 h only, since after 0 h no effect was visible (as expected) and after 72 h too low cell counts were measured. As control for spontaneous cell death, CFSE-labelled target cells were incubated either alone or with

PBMC only, *i.e.* in the absence of antibody constructs. Maximum cell death was achieved by incubating target cells with 0.1 % Triton X-100.

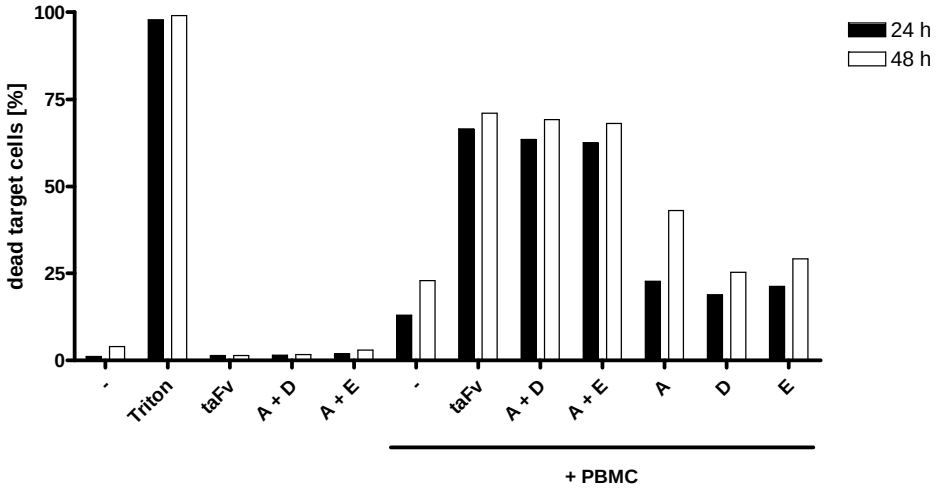


Fig. 4.26: Cytotoxicity mediated by trispecific HLA-A2 × CD3 × CD45 constructs. CFSE-labelled target cells expressing both HLA-A2 and CD45 (U266) were incubated with 27 nM antibody constructs in the presence or absence of unstimulated HLA-A2-negative PBMC (E : T ratio of 10 : 1). After 24 h and 48 h, samples were taken and stained with 7-AAD. Dead target cells ($CFSE^+$ $7\text{-}AAD^+$) were identified by multicolour flow cytometry. *taFv*, taFvCD3-HLA-A2-mycHis; *A*, $V_LCD3\text{-}scFvHLA\text{-}A2$; *D*, $V_HCD3\text{-}scFvCD45(V_L\text{-}V_H)$; *E*, $V_HCD3\text{-}scFvCD45(V_H\text{-}V_L)$.

As expected, in the absence of PBMC, neither of the antibody constructs had a cytotoxic effect on U266 – not even after 48 h. In contrast, in conjunction with PBMC, a considerable cytotoxic effect was observed when U266 were stimulated with the bispecific taFv or the trispecific antibody variants. Due to some target cell death induced by PBMC alone, the specific cell death was calculated as described in Section 3.7.4. For the bispecific taFv and the trispecific combinations at 27 nM, the percentage of cytotoxic activity on U266 amounted to approximately 59 % (57 – 61) and 60 % (59 – 62) after 24 h and 48 h, respectively. On the other hand, for individually applied $V_LCD3\text{-}scFvHLA\text{-}A2$, $V_HCD3\text{-}scFvCD45(V_L\text{-}V_H)$, and $V_HCD3\text{-}scFvCD45(V_H\text{-}V_L)$, the percentage of cytotoxic activity only reached approximately 9 % (7 – 11) after 24 h. After 48 h, only $V_LCD3\text{-}scFvHLA\text{-}A2$ had a higher cytotoxic effect than expected, leading to a specific cell death of 26 % versus 3 % for $V_HCD3\text{-}scFvCD45(V_L\text{-}V_H)$ and 8 % for $V_HCD3\text{-}scFvCD45(V_H\text{-}V_L)$. For both trispecific variants, the cytotoxic activity on U266 was comparable to that of the bispecific taFvCD3-HLA-A2-mycHis. One should note that these data are preliminary, as they were performed only once, so that further experiments are required for detailed and quantitative verification.

For a more precise evaluation of the cytotoxic activity of $V_LCD3\text{-}scFvHLA\text{-}A2$ in combination with $V_HCD3\text{-}scFvCD45(V_H\text{-}V_L)$ (Combn A + E), both antibody constructs were titrated from 0.04 – 27 nM on CFSE-labelled U266 cells in the presence or absence of unstimulated PBMC at an E : T ratio of 10 : 1. Subsequently, the

percentage of dead U266 cells ($CFSE^+$ $7\text{-}AAD^+$) was determined after 24 h by multicolour flow cytometry (Fig. 4.27). As control for spontaneous cell death, CFSE-labelled U266 were incubated with PBMC only. As negative control, individually applied V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45(V_H-V_L) were additionally tested at 27 nM in parallel. Data were normalised to the lowest value.

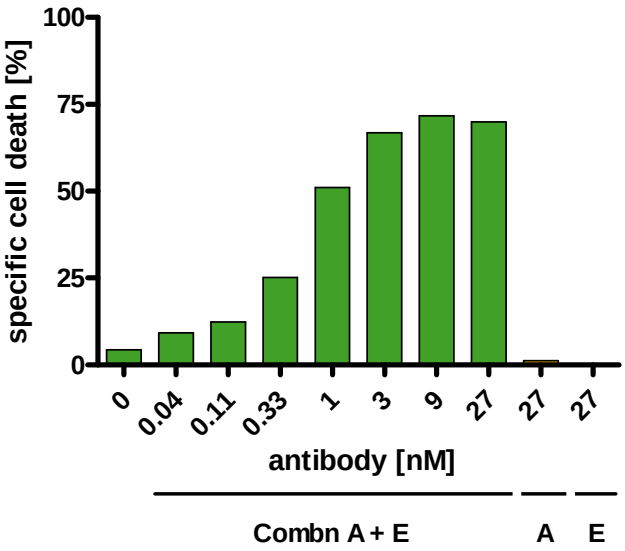


Fig. 4.27: Cytotoxicity mediated by two-part trispecific HLA-A2 × CD3 × CD45 construct. CFSE-labelled target cells expressing HLA-A2 and CD45 (U266) were incubated with titrated antibody constructs (0.04 – 27 nM) in the presence of unstimulated HLA-A2-negative PBMC (E : T ratio of 10 : 1). After 24 h, samples were taken and stained with 7-AAD. Dead target cells ($CFSE^+$ $7\text{-}AAD^+$) were identified by multicolour flow cytometry. *A*, V_LCD3-scFvHLA-A2; *E*, V_HCD3-scFvCD45(V_H-V_L).

The complemented trispecific antibody exhibited a cytotoxic effect on U266 in a concentration-dependent manner. The most effective redirected cell lysis of U266 was achieved at 9 nM, resulting in a maximum specific cell death of 72 %, and was not further increased by a higher concentration of antibody constructs. The determined EC_{50} value was 0.6 nM. As expected, the individual constructs for complementation did not reveal any cytotoxic activity on U266 cells at the highest tested concentration of 27 nM. The spontaneous cell death amounted to 12.9 % and 35.7 % after 0 h and 24 h, respectively. These data were confirmed in a second experiment.

Finally, it was shown that, at nanomolar concentration, the trispecific antibody assembled from V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45(V_H-V_L) is not only able to specifically activate T cells to release IL-2, but also to induce cytotoxicity to tumour cells expressing both antigens, HLA-A2 and CD45.

5 Discussion

In order to overcome the side effects caused by the current conventional cancer treatments, new targeted therapies based on recombinant antibodies are gaining ground. This work introduces two such targeted therapies, one of which being of combinatorial and highly innovative nature.

5.1 Bispecific AChRγ × CD3 Antibodies of taFv Format

For the treatment of the aggressive rhabdomyosarcoma, which predominantly children suffer from and which is treatment-resistant after the occurrence of metastasis or relapse, a bispecific AChRγ × CD3 antibody of taFv format was developed by analogy with the BiTE antibody format [Baeuerle *et al.*, 2009].

The AChRγ expression on the three rhabdomyosarcoma cell lines available for the present study was determined by means of incubation of a commercial anti-AChRγ antibody (clone C9) (see Fig. 4.5, p. 60). Unfortunately, binding of the scFvAChR used in this study did not correlate well with the AChRγ expression data obtained with the commercial antibody. This was unexpected, because a scFvAChR-related monovalent Fab was found to bind specifically to AChRγ by Matthews *et al.* [2002], as it efficiently blocked binding of two bivalent mAb specific for fetal AChR, one of which being C9. Also Gattenlöhner *et al.* [2010] described the same scFvAChR but fused to exotoxin A (scFv35-ETA), binding specifically and equally to RD and TE-671 cells in flow cytometry. In the present study, scFvAChR exhibited the strongest binding to TE-671, although these cells revealed the lowest expression, whereas AX-OH-1 cells with the highest expression were only moderately bound by scFvAChR. Possible reasons for this discrepancy, for instance, might be the unknown exact antigen specificity of scFvAChR, differential AChR isoform expression on these cells, or the trypsinisation of TE-671 and RD cells. For detachment, the very sensitive AX-OH-1 cells were treated with Accutase instead of trypsin, which is less damaging to cells. The mild treatment might have led to the highest apparent AChRγ expression. In contrast, as proteolysis of AChR with trypsin was reported by Neumann *et al.* [1986], a cleavage of AChR has to be taken into account on TE-671 and RD cells, especially because these cell lines are highly adherent and were needed to be treated with trypsin for several minutes to detach. Unfortunately, using a cell scraper did not lead to evaluable results in flow cytometry due to highly unspecific binding of the detecting anti-His-FITC antibody despite thorough washing (not shown). Thus, binding studies with scFvAChR and the bispecific AChRγ × CD3 taFv fragments need to be repeated under optimised

conditions, e.g. by dissociation with Accutase instead, or by using a different cell line expressing fetal AChR.

Despite the confirmed high affinity of the Fab, from which scFvAChR is derived, the γ subunit specificity of scFvAChR can only be assumed [Matthews *et al.*, 2002], because the fetal AChR is composed of five subunits ($\alpha_2\beta\gamma\delta$) [Unwin, 2000]. The two acetylcholine binding sites are located at the α/δ and either the α/γ or α/ε interfaces [Unwin, 2005]. The majority of mAb specific for AChR derived from patients with myasthenia gravis (autoimmune neuromuscular disease) are thought to target the main immunogenic region on the α subunit [Tzartos *et al.*, 1998], whereas antibodies against the fetal AChR seem to bind a site at the α/γ interface [Riemersma *et al.*, 1996] (see Fig. 2.1, p. 7). The α/γ interface is also bound by acetylcholine, so that it was suggested that antibodies binding this site inhibit the function of fetal AChR. It is very likely that scFvAChR belongs to this antibody group, as it derives from the same patient described. Thus, scFvAChR supposedly binds the γ subunit, but in combination with the α subunit. The expression of the α subunit on the three rhabdomyosarcoma cell lines used in the present study have not been tested, but it might be informative whether the expression differs. In order to figure out to which subunit exactly scFvAChR is binding, single subunits or a combination of α and γ of AChR could be expressed on cells and tested for specific binding by scFvAChR. However, it is questionable whether the AChR subunits are able to be individually expressed.

However, two of the four constructed taFvs revealed good binding to CD3-expressing cells as well as AChRγ-expressing TE-671 cells, with taFvCD3-AChR-FlagHis binding better to CD3 and taFvAChR-CD3-FlagHis binding better to AChRγ (see Fig. 4.6, p. 62). Obviously, the location of the particular scFv on the polypeptide chain is decisive for its binding properties. The N-terminal location seems to be less hindering for the respective antigen-binding site and, thus, more advantageous. The taFv fragments with an N-terminal His_{10} cluster seem to be prone to degradation and to a reduced binding activity, although His-taFvCD3-AChR revealed moderate binding properties to both CD3 and AChRγ.

Despite the fact that the results from the binding studies in flow cytometry still have room for improvement, one of the four taFvs, namely taFvCD3-AChR-FlagHis, showed some stimulatory and cytotoxic activity on AChRγ-expressing cells (see Figs. 4.7 and 4.8, pp. 64 + 65). taFvCD3-AChR-FlagHis was able to mediate T-cell activation in a concentration-dependent manner at nanomolar concentrations of 81 nM, as measured by IL-2 release, and revealed 60 % cytotoxic activity at 27 nM to AChRγ-expressing rhabdomyosarcoma versus 15 % to AChRγ-negative rhabdomyosarcoma. This might be advantageous since, for therapeutic application, an efficacy at low concentrations is wanted, which minimises side effects, production costs, and above

all, the amount of protein required to reach the tumour. Unfortunately, the relatively high spontaneous cell death of RD cells (30 % after 24 h versus 3 % for A-204 cells) reflects a suboptimal condition of the RD cells. This might be due to a too short period of time after thawing, again trypsinisation of the RD cells, or simply a suboptimal condition of the thawed batch of cells. Since these data are preliminary and only in parts fully convincing, further experiments are required for verification with cells that have not been treated with trypsin and display a higher AChRγ expression. They will certainly lead to a higher stimulatory and cytotoxic activity, confirming the potential of this bispecific antibody. Likewise, using purified T cells instead of PBMC for both stimulatory and cytotoxic assays would presumably reveal a higher activity for this bispecific antibody.

A future aim for this approach is to reach results that are at least similar to those reported by Gattenlöhner *et al.* [2010] for an anti-fAChR immunotoxin (scFv35-ETA), which contains the same scFvAChR. This immunotoxin displayed a specific cytotoxic effect on fAChR-positive rhabdomyosarcoma cell lines in a dose-dependent manner and led to a delay in tumour development in a murine transplantation model. However, the application of the anti-fAChR immunotoxin was insufficient for a real cure of the tumour in mice, as tumours occurred shortly after the end of treatment. However, these results showed some activity *in vitro* and *in vivo*, thus, demonstrating fAChR to be a prominent target and the bispecific AChRγ × CD3 antibody to be a promising candidate for future preclinical studies.

5.2 Two-Part Trispecific HLA-A2 × CD3 × CD45 Antibody

Leukaemia and other blood cancers are mostly treated with chemotherapy or even radiation therapy. In certain cases, haematopoietic stem cell transplantation is performed. Sometimes initial therapy fails to eliminate all cancer cells, leading to a recurrence of the cancer and a subsequent relapse of disease. This study presents the development of a two-part trispecific HLA-A2 × CD3 × CD45 antibody for the treatment of leukaemia and other haematological malignancies in the context of haematopoietic stem cell transplantation. A future application is suggested for a treatment after haematopoietic stem cell transplantation in order to prevent disease recurrence.

Dual-specific antibodies have already been reported. Recently Kontermann [2012] summarised a plethora of “Dual targeting strategies with bispecific antibodies”. In some cases, heterodimeric antibodies bearing two different antigen-binding sites were developed. These are IgG-like bispecific antibodies which aim to take advantage of the IgG-related long half-life and effector functions. Bispecific antibodies of smaller recombinant formats have also been developed. These strategies have shown that two

tumour-specific antigens are helpful for tumour targeting by increasing the binding avidity. Most molecules of these two types have a prefolded or fixed shape in common. Moreover, several techniques have been developed to achieve multivalent antibodies [Deyev and Lebedenko, 2008]. Some of them consist of two or more modules, which have to assemble first before application and are thus produced to act as an overall entity as well. Alternatively, in another approach, a small trispecific CEA × CD3 × CD28 antibody construct was developed [Wang *et al.,* 2004]. This trispecific antibody was shown to induce tumour-specific cell lysis following an efficient T-cell activation. However, the results of this study also demonstrated the risk of a target-independent T-cell activation and, thus, the risk of inducing severe side effects due to superagonistic properties of such a trispecific one-molecule strategy. Another trispecific antibody, namely a single-chain Fv triple body (sctb), was developed, which extended a taFv, like the one used for BiTE antibodies, by a third scFv [Kügler *et al.*, 2010; Schubert *et al.*, 2011]. For this trispecific CD123 × CD16 × CD33 triple body, which targets two antigens associated with acute myeloid leukaemia (CD33 and CD123) and the low affinity FcγRIII (CD16), dual retargeting of NK cells to myeloid leukaemia was demonstrated [Kügler *et al.*, 2010]. Another triple body of CD33 × CD16 × CD19 specificity, which was generated by the same group [Schubert *et al.*, 2011], mediated strong redirected lysis of tumour cells expressing both tumour antigens, CD33 and CD19 (B-cell antigen). Although showing high efficacy and selectivity due to an increased functional affinity for the tumour, the presence of both antigens is not an obligatory prerequisite for tumour targeting through these triple bodies. Theoretically, only one tumour antigen should be sufficient for tumour targeting. Furthermore, such approaches require even more, namely two, tumour-specific antigens. On this point, our strategy mainly differs from those mentioned above in ways that will be explained in the following.

One of the major limitations of using highly cytotoxic approaches such as BiTE antibodies [Baeuerle *et al.*, 2009] is the lack of tumour-specific antigens. On that account, the unique idea to condition the cytotoxic activity on the coincident presence of two antigens to enlarge the specificity for tumour tissue appears to be a promising tool for targeted therapy. Incidentally, the two antigens do not need to be tumour specific in itself as do HLA-A2 and CD45 in the present study, in which a complementing trispecific antibody for such a combinatorial approach was developed.

An assembly of an effector molecule-targeting moiety by two antibody molecules has not yet been reported. The success of such an assembly approach might depend, to a great extent, on the particular antibody for assembly, on the spatial arrangement of all three antigen-binding sites, and on the distribution of target molecules on the cell surface. For that reason, the well-studied anti-CD3 antibody from the BiTE technology

was selected, and target antigens were chosen that are expressed in large amounts on the cell, namely HLA-A2 and CD45 [Matthews *et al.*, 1991]. Additionally, differences in epitope localisation regarding the distance to the target cell membrane and the size of the two targeted antigens might have an impact on the complementation of the trispecific construct, as these parameters were described to determine the potency of T cell-mediated lysis by BiTE antibodies by Bluemel *et al.* [2010]. More precisely, it was postulated that the formation of a lytic synapse may be more efficient the closer the membranes of T cell and target cell can adhere to each other. The longitudinal dimension of HLA-A2 (HLA class I molecule) is about 7 nm [Bjorkman *et al.*, 1987, Barclay *et al.*, 1997]. The epitope recognized by the scFvHLA-A2 is assumed to be formed by the amino acid residues 142 and 145 (TTKH) located on the α2 domain of the heavy chain of the HLA class I molecule close to the peptide binding cleft [Watkins *et al.*, 2000]. Since the parental monoclonal antibody of scFvCD45, BC8, is reactive with all CD45 isoforms, only the C-terminal part of the extracellular domain of CD45, which is identical in all isoforms, comes into question as the recognised epitope. This conserved part comprises a cysteine-rich domain and three fibronectin type III domains with an overall dimension of 28 nm [McCall *et al.*, 1992]. The length of CD3ε is approximately 4 nm [Sun *et al.*, 2001]. For comparison, the TCR-peptide-MHC complex spans approximately 14 nm [Garboczi *et al.*, 1996; Garcia *et al.*, 1998]. Thus, the size difference between the two antigens, HLA-A2 and CD45, might evoke difficulties to reach an optimal cell-to-cell distance for T-cell activation due to steric orientation. In order to provide enough flexibility to enable functionality of all three antigen-binding sites of the trispecific construct, linkers of 7 aa length were inserted between the variable domains of anti-CD3 and the respective scFv fragments. Furthermore, V_HCD3 and V_LCD3 were N-terminally fused to the scFvs to avoid both restrictions caused by and affecting the C-terminal tags.

First of all, this study shows that a self-assembly of V_H and V_L (in this case of an anti-CD3 antibody) is feasible, when brought into close proximity of each other on the surface of a single cell. Secondly, such a complemented Fv is functional. The constructed two-part trispecific antibodies were shown to be able to activate T cells and to induce cytotoxic T cell responses in a concentration-dependent manner at nanomolar concentrations of 3 – 243 nM (Fig. 4.21, p. 83) and 9 nM (Fig. 4.27, p. 89), respectively. Again, this can be regarded as favourable since, for therapeutic application, an efficacy at low concentrations is preferable in order to minimise side effects, production costs, and, above all, the amount of protein needed to reach the tumour. Nevertheless, an efficacy at lower concentrations is probably sufficient for targeting blood cancer (e.g. leukaemia), since blood cancer is assumed to be more accessible for such targeting approaches than solid tumours, which require higher concentrations to be targeted effectively [Müller and Kontermann, 2010]. Over and

above this, the fact that blood cells express high amounts of costimulatory molecules might likewise be beneficial.

In the first proof of concept experiment, the stimulatory activity of the two-part trispecific HLA-A2 × CD3 × HLA-A2 construct turned out to be diminished compared to the bispecific taFv (see Fig. 4.20, p. 82). However, it was still considerable in spite of the initial expectations that an insertion of heterodimerising peptides, such as jun-fos leucine zippers [de Kruif and Logtenberg, 1996; Kostelny *et al.*, 1992; Pack and Plückthun, 1992; O'Shea *et al.*, 1989; Arndt *et al.*, 2001], would be required to improve the affinity of V_H and V_L for each other. The diminished stimulatory activity might be understood as a result of the lesser amount of HLA-A2 antigen that was available for binding due to competition between V_LCD3-scFvHLA-A2 and V_HCD3-scFvHLA-A2. Such competitive effects should not occur when complementation relies on two different antigens. In the comparative experiment with the actually intended trispecific HLA-A2 × CD3 × CD45 antibody, the bispecific control antibody again tended to be more potent (see Fig. 4.22, p. 84). BiTE antibodies have consistently been reported to mediate redirected tumour cell lysis at half-maximal effective concentrations in the pM to nM range [Baeuerle *et al.*, 2009; Hoffmann *et al.*, 2005; Bluemel *et al.*, 2010; Lutterbuese *et al.*, 2009], whereas the present trispecific concept indicates an efficacy in the nM range (see Figs. 4.20, 4.21, and 4.27, pp. 82 + 83, 89). In the comparative experiment mentioned above, it has to be considered that both the bispecific and the two-part combinatorial trispecific construct are of different designs and, thus, probably subjected to different binding kinetics. As a consequence, the reduced activity of the trispecific construct might simply be explained by the fact that its two halves first have to meet, and V_H and V_L of the anti-CD3 moiety need to assemble before being able to bind and stimulate T cells. It was assumed that the affinity of V_HCD3 and V_LCD3 is low, since the stability of the noncovalently-associated V_H and V_L in fact varies from antibody to antibody, but an isolated Fv fragment usually tends to dissociate rapidly due to the weak interface between V_H and V_L. For Fv fragments, dissociation constants have been reported ranging from 10^{-5} M to 10^{-8} M [Skerra and Plückthun, 1988; Glockshuber *et al.*, 1990; Jäger and Plückthun, 1999; Mallender *et al.*, 1996; Polymenis and Stollar, 1995]. Within the bispecific control antibody, however, V_HCD3 and V_LCD3 are already available in a prefolded form (scFvCD3). Hence, the reduced activity of the trispecific antibodies is reasonable.

At the same time, for the new approach presented here, a low affinity of V_H and V_L for each other might be beneficial, as high affinity involves the risk of premature complementation and, thus, of redirecting cytotoxic T cells to cells expressing only one of the two target antigens. Especially an attack on cells expressing only HLA-A2 would be devastating, as almost all nucleated cells in the body express HLA class I molecules. Driven by a much higher affinity of V_LCD3 and V_HCD3 for each other,

a premature self-assembly of V_LCD3-scFvHLA-A2 and V_HCD3-scFvCD45 would already occur in solution without contact to at least one antigen, HLA-A2 or CD45. Both molecules would then probably combine into a trispecific antibody in solution, thus being able to activate T cells in dependence of the presence of only one antigen. Therefore, a low to medium affinity of V_LCD3 and V_HCD3 is requested, *i.e.* an affinity high enough to enable self-assembly, but low enough to preclude premature complementation. Incidentally, even in the case of a soluble trispecific antibody, T-cell activation would probably not work in a target cell-independent fashion, since soluble scFvCD3 is not able to crosslink CD3 on T cells (see Figs. 4.7 and 4.20, pp. 64 + 82). Likewise, in the absence of target cells, neither the bispecific control antibody nor the constructs for complementation had a stimulatory effect on T cells (data not shown).

However, this trispecific approach was shown to be dual antigen-restricted and to activate T cells in a target-cell dependent fashion. As shown in Fig. 4.24 (p. 86), HLA-A2-negative T cells were not activated by the trispecific HLA-A2 × CD3 × CD45 antibody, when the target cells expressed neither HLA-A2 nor CD45 or CD45 only. Thus, a spontaneous premature complementation can definitely be excluded. Unfortunately, the trispecific antibody activated T cells when using HLA-A2-positive target cells (see Fig. 4.25, p. 87), reaching 48 % (MCF-7) to 68 % (L-363) of the stimulatory activity of the bispecific positive control. Three possible reasons that might explain this fact have to be taken into account. First, in case of L-363, some residual CD45 expression is conceivable, even though it was undetectable in flow cytometry (see Fig. 4.18, p. 79 and Colette *et al.*, 2007). During maturation, this multiple myeloma cell line might have undergone an incomplete loss of CD45 [Kumar *et al.*, 2005]. Secondly, in case of the breast cancer cell line MCF-7, some unspecific binding of the anti-CD45 moiety has to be considered. It cannot be ruled out that MCF-7 cells express molecules related to CD45, which is also known as PTPRC, protein tyrosine phosphatase, receptor type, C. For example, MCF-7 cells have been reported to express PTPγ, another member of the protein tyrosine phosphatase family [Liu *et al.*, 2004]. As the CD45-specific scFv used in this study is derived from an anti-CD45 antibody, which was described to be reactive with all CD45 isoforms [Lin *et al.*, 2006], its specificity might be directed against an epitope also expressed on other protein tyrosine phosphatases, including fibronectin type III domains. This problem might be solved by using different HLA-A2-expressing cells or by exchanging the anti-CD45 moiety with a more specific anti-CD45 antibody. However, the results from Fig. 4.25 (p. 87) suggest a higher specificity and selectivity of scFvHLA-A2 to HLA-A2 than of scFvCD45 to CD45. The third possible reason might be that CD45 is provided by a T cell that is different to the one targeted via CD3 and, thus, serves as a second target cell besides the intended HLA-A2-positive target cell. In such a case, the assembled trispecific antibody would have to stretch between CD45 on a T cell and HLA-A2 on the

intended target cell, while binding CD3 on a different T cell. Then, HLA-A2-positive target cells and HLA-A2-negative T cells alone would be sufficient for stimulation. Although sterically unfavourable, such a constellation remains to be verified. Nevertheless, the stimulatory activity of the trispecific antibodies was reaffirmed to be dual antigen-restricted (Fig. 4.23, p. 85). Here it was shown that, if just one of both target antigens is not available for binding, the binding affinity of V_HCD3-scFvCD45 and V_LCD3-scFvHLA-A2 for each other is not high enough for complementation of the trispecific antibody, thus leading to an almost completely prevented T-cell activation. Hence, the presence of both antigens is imperative for T-cell activation by the two-part trispecific HLA-A2 × CD3 × CD45 antibody. Only in case the third possibility proves true, the blocking experiment with scFvCD45 should be verified as well, since CD45 might also be blocked on T cells. Furthermore, the trispecific antibody mediated cytotoxicity of HLA-A2- and CD45-expressing cells with a maximum specific cell death of 72 % at 9 nM and with a half-maximal effective concentration of 0.6 nM (see Fig. 4.27, p. 89). Thus, despite the much larger magnitude of CD45 than HLA-A2, the trispecific concept succeeded. The formation of a lytic synapse or cross-linkage of T cells was obviously not impaired by the size of CD45. It bears mentioning that these results were obtained with unstimulated PBMC.

Despite the promising results, this two-part trispecific concept also implicates some minor obstacles related to the future therapeutic application. For one thing, the immunogenicity of the trispecific antibody might lead to HAMA responses due to the anti-CD45 moiety of murine origin. Thus, adverse events or a decrease in the effectiveness of the treatment may be induced [Moldenhauer, 2011]. It is worth considering to circumvent such HAMA responses by humanisation [Jones *et al.*, 1986; Riechmann *et al.*, 1988; Hudis, 2007], deimmunisation [Hellendoorn *et al.,* 2004; Jones *et al.*, 2004], or, more simply, by exchanging this antibody moiety.

For another thing, the uncombined V_H and V_L expose highly hydrophobic interfaces and have the propensity to aggregate when expressed individually, V_H more so than V_L. This might also lead to aggregation of the whole antibody construct including V_H and V_L. In fact, some aggregation was observed for more or less all constructs generated in this study, especially after thawing. Aggregates were visibly reduced after storage at 4°C and always discarded by centrifugation before functional assays were performed. Before therapeutic application, the aggregate formation of the components of the trispecific antibody described here should be studied in more detail, since aggregates may lead to reduced bioactivity and increase immunogenicity. Several methods have been developed in order to i) isolate non-aggregating V_H domains from synthetic libraries [Jespers *et al.*, 2004; Christ *et al.*, 2007], ii) reduce the hydrophobicity of the former light chain interface in the course of the development of single domain antibodies [Davies and Riechmann, 1994; 1996], and iii) prevent

aggregation [Barthelemy *et al.*, 2008; Dudgeon *et al.*, 2012]. However, an application of these methods on the approach presented here would require a balancing act, since, in the end, changing residues in the interface would also affect the assembly of V_H and V_L. Another, more encouraging strategy was developed by Schaefer and Plückthun [2012], according to which negatively charged residues attached to the N-terminus of human IgG molecules confirmed a beneficial effect on aggregation resistance. These residues, which remained from the used *Pichia* secretion system, are hypothesised to increase the net negative charge of the antibody, while not affecting the antigen binding.

Despite the free hydrophobic interfaces being prone to aggregation, this two-part trispecific antibody effectively activates T cells. However, for unknown reasons, some slight stimulatory and cytotoxic activity was also observed for V_LCD3-scFvHLA-A2 at the highest tested concentration (see Figs. 4.21, 4.22, and 4.26, pp. 83 + 84, 88), even though no binding was detected to Jurkat cells ($CD3^+$ HLA-$A2^-$) by flow cytometry (see Fig. 4.17, p. 77). Indeed, one can argue that the stimulation and cytotoxic assays are more sensitive than the binding studies in flow cytometry. Although the effect seems negligible, it remains to be determined whether it is utterly out of the question that V_LCD3-scFvHLA-A2 shows some activity, if only minor, due to specific antigen binding by itself. Even though uncombined V_L often have reduced affinity and solubility, they can retain noticeable antigen-binding specificity [Colby *et al.*, 2004; van den Beucken *et al.*, 2001; Mei *et al.*, 1991; Edelman *et al.*, 1963; Roholt *et al.*, 1964; Sun *et al.*, 1994]. A more precise answer to that question for the present concept might be provided by binding studies with the bioluminescent protein *Gaussia* luciferase (GLuc) [Tannous *et al.*, 2005] in fusion with a dimeric single-chain CD3 molecule following the example set by Law *et al.* [2002], who constructed a single-chain CD3δε-Ig fusion protein. Unfortunately, preliminary GLuc (ELISA) assays with CD3δε-GLuc from mammalian expression did not prove to be suitable for binding by scFvCD3, although it was bound well by an anti-CD3ε antibody (clone UCHT-1) (data not shown). Since most anti-CD3 antibodies recognise an immunodominant conformational epitope on CD3ε, which is only expressed when CD3ε is bound either to CD3δ or to CD3γ [Salmerón *et al.*, 1991], CD3γε-GLuc and CD3γε were additionally constructed, but have not yet been tested. However, it remains possible that scFvCD3 has a different affinity for CD3δε than for CD3γε heterodimer or that the conformational epitope even depends on the intact TCR complex, like it was assumed for the anti-CD3ε antibody OKT3 [Kjer-Nielsen *et al.*, 2004]. Moreover, scFvCD3 derives from the anti-CD3ε antibody TR66 [Lanzavecchia and Scheidegger, 1987; Traunecker *et al.*, 1991], which does not recognise isolated CD3ε, whereas UCHT-1 does [Tunnacliffe *et al.*, 1989]. Thus, it remains unclear, whether CD3γε-GLuc or CD3γε would be more suitable for binding by scFvCD3 than CD3δε-GLuc was.

Remarkably, the bispecific HLA-A2 × CD3 taFv fragments also had a stimulatory effect on HLA-A2-positive PBMC, regardless of whether HLA-A2-positive or -negative target cells were being used (see Fig. 4.19 B, p. 80). Thus, HLA-A2-positive T cells ($HLA\text{-}A2^+$ $CD45^+$ $CD3^+$) seem to kill each other in the manner of fratricide. Such a scenario is also conceivable for the trispecific HLA-A2 × CD3 × CD45 antibody. It might be worth considering to benefit from this approach by treating the recipient (e.g. leukaemia patient) directly prior to the haematopoietic stem cell transplantation, since graft rejection may occur due to some remaining T cells of host origin. In this way, a complete elimination of malignant cells as well as a prevention of graft rejection could be realised at the same time.

Another limiting factor of the present approach is the small size of the self-assembling constructs. With around 40 kDa in size they might be cleared rapidly from the blood circulation due to the renal filter with a cut-off of 60 kDa. For instance, the small antibody molecules scFv (~ 25 kDa) and scDb (~ 55 kDa, like taFv) exhibit terminal half-lives of only 1 – 4 h and 5 – 6 h, while albumin (~ 67 kDa) and IgG (~ 150 kDa) have a long half-life of 19 days and 3 – 4 weeks in humans, respectively [Müller and Kontermann, 2010]. A range of different approaches have therefore been developed to extend the circulation time of these molecules to facilitate application and to improve efficacy [Kontermann, 2009; 2011b]. At large, they intend either to enlarge the molecular weight by increasing the hydrodynamic radius, for example through PEGylation, or to use recycling processes mediated by the neonatal Fc receptor (FcRn). There are a few possibilities to achieve the latter strategy: i) fusion of the therapeutic molecule to Fc or to human serum albumin, or ii) binding of the therapeutic molecule to human serum albumin or IgG. However, before making any effort on half-life extension, the present approach should first prove successful *in vivo*. Studies with mice are ongoing and will show if the promising *in vitro* findings can be confirmed.

5.3 Conclusions and Future Directions

In conclusion, both the bispecific AChRγ × CD3 antibody against rhabdomyosarcoma and the trispecific HLA-A2 × CD3 × CD45 antibody against blood cancer seem to be promising approaches for the recruitment of T cells. The dual antigen-restricted trispecific antibody approach in particular achieved astonishing results. Thus, providing T-cell activation, V_LCD3-scFvHLA-A2 in conjunction with V_HCD3-scFvCD45 are worth being tested in further assays, such as cytotoxicity assays, proliferation assays, and subsequent *in vivo* animal studies to confirm these findings and to evaluate their whole therapeutic potential for targeted cancer immunotherapy. Such assays and functional studies in mice are in progress.

6 References

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7 Abbreviations

µ	micro (10^{-6})
7-AAD	7-aminoactinomycin D, red fluorescent dye
aa	amino acid
AChRγ, fAChR	acetylcholine receptor gamma, fetal AChR
APC	antigen-presenting cell
APS	ammonium peroxodisulfate
B7.1/B7.2 (CD80/CD86)	costimulatory molecules
BE	Belgium
BiTE	bispecific T-cell engager
bp	base pairs
BSA	bovine serum albumin
CD	cluster of differentiation
CD3	part of T-cell receptor complex implicated in T-cell activation
CEA	carcinoembryonic antigen, tumour antigen
CFSE, CFDA SE	5-(6)-carboxyfluorescein diacetate, succinimidyl ester, green fluorescent dye
CO_2	carbon dioxide
Db	diabody
DE	Germany
DMSO	dimethyl sulfoxide
DNA or cDNA	deoxyribonucleic acid, coding DNA
dNTP	deoxyribonucleoside triphosphate
DT	diphtheria toxin
EC_{50}	half maximal effective concentration
ECL	enhanced chemiluminescence
ECS	enterokinase cleavage site (DYKDDDDK)
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
E : T	effector cell to target cell ratio
ETA	*Pseudomonas* exotoxin A
Fab	fragment antigen binding, part of an antibody, cleaved with enzyme papain
FACS	fluorescence activated cell sorter
Fc	Fragment crystallisable, constant part of an antibody
FCS	fetal calf serum, FBS
Fig	Figure
FITC	fluorescein-5-isothiocyanate, green fluorescent dye
Flag	polypeptide protein tag (DYKDDDDK)
g	standard gravity of earth, 9.80665 m/s^2
GFP11	green fluorescent protein, the smaller GFP fragment of the split GFP assay
GLuc	*Gaussia* luciferase
h	hours
Her2/Her3	human epidermal growth factor receptor 2/3
$His_{6/10}$	polyhistidine-tag or cluster (HHHHHH or HHHHHHHHHH)
H_2SO_4	sulphuric acid
HAHA	human anti-human antibody

HAMA	human anti-mouse antibody
HRP	horseradish peroxidise, enzyme used for TMB/H_2O_2 colour reaction in ELISA
Ig	immunoglobulin
IL-2	interleukin-2, T-cell growth factor
IMAC	Immobilised-metal affinity chromatography
ITAM	immunoreceptor tyrosine-based activation motif
kDa	kilo Dalton = 10^3 Dalton, unified atomic mass unit, 1 g/mol
m	milli (10^{-3}) or meter
M	molar mass
mAb	monoclonal antibody
mol	amount of chemical substance
MCS	multiple cloning site
MHC	major histocompatibility complex
MW	molecular weight
MWCO	molecular weight cut-off
n	nano (10^{-9})
OD_{600}	optical density at 600 nm
p	pico (10^{-12})
PAGE	polyacrylamide gel electrophoresis
pBM1.1	prokaryotic expression vector
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin, red fluorescent dye
PEG	polyethylene glycol
Pen/Strep	penicillin/streptomycin
PHA-L	phytohemagglutinin L, T-cell stimulant
pIRESpuro2	eukaryotic expression vector
pUC119	prokaryotic expression vector
RT	room temperature
scDb	single-chain diabody, bispecific antibody format
scFv	single-chain variable-fragment, comprises a monovalent binding site for the antigen
SDS	sodium dodecyl sulphate
SE	Sweden
TAA	tumour-associated antigen
taFv	tandem single-chain variable fragment (scFv), bispecific antibody format
TCS	thrombin cleavage site (LVRGS)
TCR	T-cell receptor
TEMED	N,N,N,N-Tetramethylethylendiamin
TMB	3,3',5,5'-tetramethylbenzidine, colour substrate in ELISA
Tris	tris(hydroxymethyl)aminomethane
Tween	polyoxyethylene sorbitan monolaurate
U	unit
UK	United Kingdom
US	United States
V	Volt
V_H, V_L	immunoglobulin variable region (heavy or light chain)
w/v	weight/volume

8 Sequences

8.1 His-scFvAChR

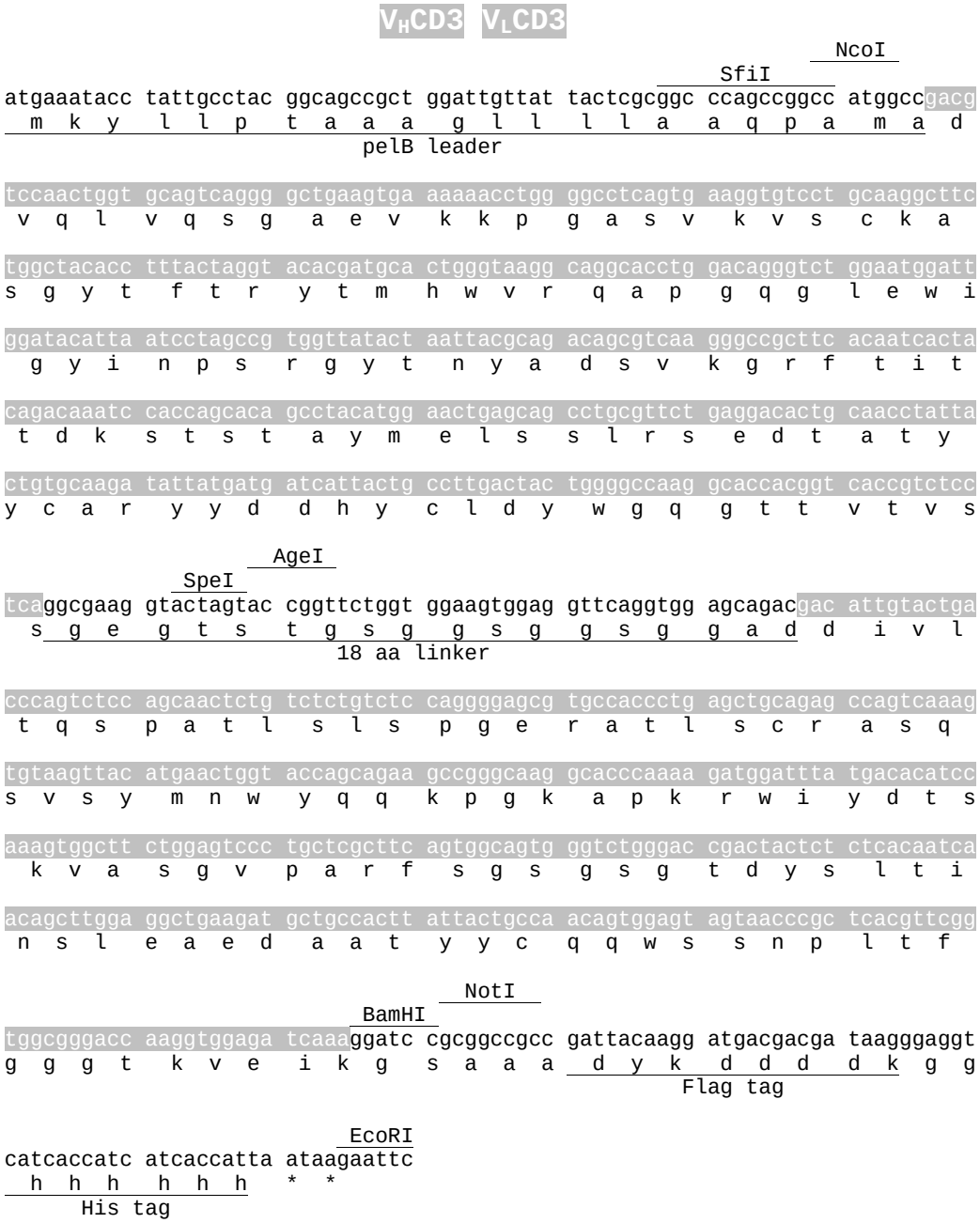
(in pBM1.1)

V_LAChR V_HAChR

```
  1  atgaaatacc tgctgccgac cgctgctgct ggtctgctgc tcctcgctgc ccagccggcg atggccatgg
       m  k  y   l  l  p   t  a  a  a   g  l  l   l  l  a   a  q  p  a   m  a  m
                                 pelB leader

                                                                          HindIII…
 71  gccatcatca tcatcatcat catcatcatc acagcagcgg ccatatcgac gacgacgaca agcatatgaa
      g  h  h   h  h  h  h   h  h  h   h  s  s   g  h  i  d   d  d  d   k  h  m
                  His cluster                              ECS

      ____  ____SfiI_____
141  gcttatggcc cagccggccg tgatgaccca gtctccatcc tccctgtctg catctgtagg agacagagtc
     k  l  m  a   q  p  a   v  m  t   q  s  p  s   s  l  s   a  s  v   g  d  r  v

211  accatcgctt gccgggcaag tcagaccatt agcaactatt taaattggta tcagcagaaa ccagggaaag
       t  i  a   c  r  a   s  q  t  i   s  n  y   l  n  w   y  q  q  k   p  g  k

281  cccctaagct cctgatctat ggtgcatcca gtttgcaaag tggggtccca tcaaggttca gtggcagtgg
      a  p  k   l  l  i  y   g  a  s   s  l  q   s  g  v  p   s  r  f   s  g  s

351  atctgggaca gatttcactc tcaccatcag cagtctgcaa cctgaagatt ttgcaactta ctactgtcaa
     g  s  g  t   d  f  t   l  t  i   s  s  l  q   p  e  d   f  a  t   y  y  c  q

421  cagagttaca gtacccctcc gacgtacact tttggccagg ggaccaagct ggagatcaaa ggtggcggtg
       q  s  y   s  t  p   p  t  y  t   f  g  q   g  t  k   l  e  i  k  g  g  g

491  gctcgggcgg tggtgggtcg ggtggcagcg gatcatcggg gggcgacttg gtccagccgg gggggtccct
     g  s  g   g  g  g  s   g  g  s   g  s  s   g  g  d  l   v  q  p   g  g  s
          …(G4S)2-G2S-GS-linker (15 aa)

561  gagagtctcc tgtgtagcct ctggatttac atttaggacc tatgtgatga actgggtccg ccaggctcca
     l  r  v  s   c  v  a   s  g  f   t  f  r  t   y  v  m   n  w  v   r  q  a  p

631  ggaaaggggc tggagtgggt ggcccacata agtccagagg gaactgaaga atactatgcg gaccctgtga
       g  k  g   l  e  w   v  a  h  i   s  p  e   g  t  e   e  y  y  a   d  p  v

701  agggccgatt taccgtctcc agagacaacg cgaagaattc agtatttctg caaatgaata gtctgagagg
      k  g  r   f  t  v  s   r  d  n   a  k  n   s  v  f  l   q  m  n   s  l  r

771  cgaggacacg gctgtgtatt attgcgcgag agtccgacgc tatggtccct ctacgctcag tccgttcacc
     g  e  d  t   a  v  y   y  c  a   r  v  r  r   y  g  p   s  t  l   s  p  f  t

                                                                        NotI…
841  tggaaggaca atcactacgc catggacgtc tggggccaag ggacaacggt caccgtctct ccagcggccg
       w  k  d   n  h  y   a  m  d  v   w  g  q   g  t  t   v  t  v  s   p  a  a

      _ SacI
911  cagagctcta gatgattagc tagatgatta g
      a  e  l   *           *
                  *           *
                      *           *
                  stop in 3 frames
```

8.2 scFvCD3-FlagHis

(in pUC119)



8.3 scFvCD45(V_H-V_L)-FlagHis

(in pUC119)

V_HCD45 V_LCD45

```
                                                                                                  NcoI    SpeI…
  1 atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccacta
      m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  t
                                  pelB leader

 71 gtactggtca ggtgcagctg gtggaaagcg gtggcggact ggtgcagccg ggcggcagcc tgaaactgag
      s  t  g  q  v  q  l  v  e  s  g  g  g  l  v  q  p  g  g  s  l  k  l

141 ctgtgccgcc agcggttttg attttagccg ttattggatg agctgggtgc gtcaggcgcc gggcaaaggc
      s  c  a  a  s  g  f  d  f  s  r  y  w  m  s  w  v  r  q  a  p  g  k  g

211 ctggaatgga ttggcgaaat taacccgacc agcagcacca ttaactttac cccgagcctg aaagataaag
      l  e  w  i  g  e  i  n  p  t  s  s  t  i  n  f  t  p  s  l  k  d  k

281 tgtttattag ccgtgataac gcgaaaaaca ccctgtatct gcagatgagc aaagtgcgta gcgaagatac
      v  f  i  s  r  d  n  a  k  n  t  l  y  l  q  m  s  k  v  r  s  e  d

351 cgcgctgtat tattgcgcgc gtggcaacta ttatcgttat ggcgatgcga tggattattg gggccagggc
      t  a  l  y  y  c  a  r  g  n  y  y  r  y  g  d  a  m  d  y  w  g  q  g

                                                                                    AgeI
421 accagcgtga ccgtgagcgg tggaggaggt tcaggaggag gtggttcagg aggaggtacc ggtgatattg
      t  s  v  t  v  s  g  g  g  g  s  g  g  g  g  s  g  g  g  t  g  d  i
                        (G4S)2-G3-TG-linker (15 aa)

491 ttctgaccca gagcccggcg agcctggcgg ttagcctggg tcagcgtgcc accattagct gccgtgcgag
      v  l  t  q  s  p  a  s  l  a  v  s  l  g  q  r  a  t  i  s  c  r  a

561 caaaagcgtg agcaccagcg gctatagcta tctgcattgg tatcagcaga aaccgggcca gcctccaaaa
      s  k  s  v  s  t  s  g  y  s  y  l  h  w  y  q  q  k  p  g  q  p  p  k

631 ctgctgattt atctggccag caacctggaa agcggtgtgc cggcccgttt tagcggcagc ggcagcggta
      l  l  i  y  l  a  s  n  l  e  s  g  v  p  a  r  f  s  g  s  g  s  g

701 ccgattttac cctgaacatt catccggtgg aagaagaaga tgcggcgacc tattattgcc agcatagccg
      t  d  f  t  l  n  i  h  p  v  e  e  e  d  a  a  t  y  y  c  q  h  s

                                                                NotI
771 tgaactgccg tttacctttg gcagcggcac caaactggaa attaaagcgg ccgccgatta caaggatgac
      r  e  l  p  f  t  f  g  s  g  t  k  l  e  i  k  a  a  a  d  y  k  d  d
                                                                          Flag tag…

841 gacgataagg gaggtcatca ccatcatcac cattaataa
      d  d  k  g  g  h  h  h  h  h  h  *  *
Flag tag          His tag
```

8.4 HisFlag-scFvCD45(V_L-V_H)

(in pUC119)

V_LCD45 V_HCD45

```
                                                                                                NcoI
  1 atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggcaagca
      m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  s
                                  pelB leader

 71 gccatcacca tcatcaccat agcgattaca aggatgacga cgataagagc ggcctggtgc cgcgcggcag
      s  h  h  h  h  h  h  s  d  y  k  d  d  d  d  k  s  g  l  v  p  r  g_
            His tag                    Flag tag                          TCS…

       AgeI
141 caccggtgat attgttctga cccagagccc ggcgagcctg gcggttagcc tgggtcagcg tgccaccatt
    s  t  g  d  i  v  l  t  q  s  p  a  s  l  a  v  s  l  g  q  r  a  t  i

211 agctgccgtg cgagcaaaag cgtgagcacc agcggctata gctatctgca ttggtatcag cagaaaccgg
      s  c  r  a  s  k  s  v  s  t  s  g  y  s  y  l  h  w  y  q  q  k  p

281 gccagcctcc aaaactgctg atttatctgg ccagcaacct ggaaagcggt gtgccggccc gttttagcgg
      g  q  p  p  k  l  l  i  y  l  a  s  n  l  e  s  g  v  p  a  r  f  s

351 cagcggcagc ggtaccgatt ttaccctgaa cattcatccg gtggaagaag aagatgcggc gacctattat
    g  s  g  s  g  t  d  f  t  l  n  i  h  p  v  e  e  e  d  a  a  t  y  y

421 tgccagcata gccgtgaact gccgtttacc tttggcagcg gcaccaaact ggaaattaaa aagatctctg
      c  q  h  s  r  e  l  p  f  t  f  g  s  g  t  k  l  e  i  k  k  i  s

491 gtggcggcgg ctcgggtggt ggtgggtcgg gcggcggcgg ctcgagccag gtgcagctgg tggaaagcgg
    g  g  g  g  s  g  g  g  g  s  g  g  g  g  s  s  q  v  q  l  v  e  s
        …KIS-(G4S)3-S-linker (19 aa)

561 tggcggactg gtgcagccgg gcggcagcct gaaactgagc tgtgccgcca gcggttttga ttttagccgt
    g  g  g  l  v  q  p  g  g  s  l  k  l  s  c  a  a  s  g  f  d  f  s  r

631 tattggatga gctgggtgcg tcaggcgccg ggcaaaggcc tggaatggat tggcgaaatt aacccgacca
      y  w  m  s  w  v  r  q  a  p  g  k  g  l  e  w  i  g  e  i  n  p  t

701 gcagcaccat taactttacc ccgagcctga aagataaagt gtttattagc cgtgataacg cgaaaaacac
      s  s  t  i  n  f  t  p  s  l  k  d  k  v  f  i  s  r  d  n  a  k  n

771 cctgtatctg cagatgagca aagtgcgtag cgaagatacc gcgctgtatt attgcgcgcg tggcaactat
    t  l  y  l  q  m  s  k  v  r  s  e  d  t  a  l  y  y  c  a  r  g  n  y

                                                                              EcoRI
841 tatcgttatg gcgatgcgat ggattattgg ggccagggca ccagcgtgac cgtgagctaa taagaattc
      y  r  y  g  d  a  m  d  y  w  g  q  g  t  s  v  t  v  s  *  *
```

8.5 scFvHLA-A2-mycHis

(in pUC119)

V_HHLA-A2 V_LHLA-A2

```
                                                                                                  NcoI
  1 atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggcccagg
      m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  q
                                  pelB leader

 71 tgcagctggt gcagtctggg ggaggcgtgg tccagcctgg ggggtccctg agagtctcct gtgcagcgtc
      v  q  l  v  q  s  g  g  g  v  v  q  p  g  g  s  l  r  v  s  c  a  a

141 tggggtcacc ctcagtgatt atggcatgca ttgggtccgc caggctccag gcaaggggct ggagtggatg
      s  g  v  t  l  s  d  y  g  m  h  w  v  r  q  a  p  g  k  g  l  e  w  m

211 gcttttatac ggaatgatgg aagtgataaa tattatgcag actccgtgaa gggccgattc accatctcca
      a  f  i  r  n  d  g  s  d  k  y  y  a  d  s  v  k  g  r  f  t  i  s

281 gagacaactc caagaaaaca gtgtctctgc aaatgagcag tctcagagct gaagacacgg ctgtgtatta
      r  d  n  s  k  k  t  v  s  l  q  m  s  s  l  r  a  e  d  t  a  v  y

351 ctgtgcgaaa aatggcgaat ctgggccttt ggactactgg tacttcgatc tctggggccg tggcaccctg
      y  c  a  k  n  g  e  s  g  p  l  d  y  w  y  f  d  l  w  g  r  g  t  l

421 gtcaccgtgt cgagtggtgg aggcggttca ggcggaggtg gctctggcgg tggcggatcg gatgttgtga
      v  t  v  s  s  g  g  g  g  s  g  g  g  g  s  g  g  g  g  s  d  v  v
                                  (G4S)3-linker (15 aa)

491 tgactcagtc tccatcctcc ctgtctgcat ctgtaggaga cagagtcacc atcacttgcc aggcgagtca
      m  t  q  s  p  s  s  l  s  a  s  v  g  d  r  v  t  i  t  c  q  a  s

561 ggacattagc aactatttaa attggtatca gcagaaacca gggaaagccc ctaagctcct gatctacgat
      q  d  i  s  n  y  l  n  w  y  q  q  k  p  g  k  a  p  k  l  l  i  y  d

631 gcatccaatt tggaaacagg ggtcccatca aggttcagtg gaagtggatc tgggacagat tttactttca
      a  s  n  l  e  t  g  v  p  s  r  f  s  g  s  g  s  g  t  d  f  t  f

701 ccatcagcag cctgcagcct gaggattttg caacttatta ctgccaacaa tatagtagtt ttccgctcac
      t  i  s  s  l  q  p  e  d  f  a  t  y  y  c  q  q  y  s  s  f  p  l

                                                 NotI
771 tttcggcgga gggaccaaag tggatatcaa acgtgcggcc gcagaacaaa aactcatctc agaagaggat
      t  f  g  g  g  t  k  v  d  i  k  r  a  a  a  e  q  k  l  i  s  e  e  d
                                                                        myc tag…

                                                       EcoRI
841 ctgaatgggg ccgcacatca ccatcatcac cattaataag aattc
      l  n  g  a  a  h  h  h  h  h  h  *  *
    myc tag              His tag
```

8.6 His-taFvAChR-CD3

(in pBM1.1)

V_LAChR **V_HAChR** **V_HCD3** **V_LCD3**

```
   1 atgaaatacc tgctgccgac cgctgctgct ggtctgctgc tcctcgctgc ccagccggcg atggccatgg
       m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  m
                                  pelB leader

  71 gccatcatca tcatcatcat catcatcatc acagcagcgg ccatatcgac gacgacgaca agcatatgaa
       g  h  h  h  h  h  h  h  h  h  h  s  s  g  h  i  d  d  d  d  k  h  m
                   His cluster                                 ECS

 141 gcttatggcc cagccggccg tgatgaccca gtctccatcc tccctgtctg catctgtagg agacagagtc
       k  l  m  a  q  p  a  v  m  t  q  s  p  s  s  l  s  a  s  v  g  d  r  v

 211 accatcgctt gccgggcaag tcagaccatt agcaactatt taaattggta tcagcagaaa ccagggaaag
         t  i  a  c  r  a  s  q  t  i  s  n  y  l  n  w  y  q  q  k  p  g  k

 281 cccctaagct cctgatctat ggtgcatcca gtttgcaaag tggggtccca tcaaggttca gtggcagtgg
         a  p  k  l  l  i  y  g  a  s  s  l  q  s  g  v  p  s  r  f  s  g  s

 351 atctgggaca gatttcactc tcaccatcag cagtctgcaa cctgaagatt ttgcaactta ctactgtcaa
       g  s  g  t  d  f  t  l  t  i  s  s  l  q  p  e  d  f  a  t  y  y  c  q

 421 cagagttaca gtacccctcc gacgtacact tttggccagg ggaccaagct ggagatcaaa ggtggcggtg
         q  s  y  s  t  p  p  t  y  t  f  g  q  g  t  k  l  e  i  k  g  g  g

 491 gctcgggcgg tggtgggtcg ggtggcagcg gatcatcggg gggcgacttg gtccagccgg gggggtccct
       g  s  g  g  g  g  s  g  g  s  g  s  s  g  g  d  l  v  q  p  g  g  s
         …(G4S)2-G2S-GS-linker (15 aa)

 561 gagagtctcc tgtgtagcct ctggatttac atttaggacc tatgtgatga actgggtccg ccaggctcca
       l  r  v  s  c  v  a  s  g  f  t  f  r  t  y  v  m  n  w  v  r  q  a  p

 631 ggaaaggggc tggagtgggt ggcccacata agtccagagg gaactgaaga atactatgcg gaccctgtga
         g  k  g  l  e  w  v  a  h  i  s  p  e  g  t  e  e  y  y  a  d  p  v

 701 agggccgatt taccgtctcc agagacaacg cgaagaattc agtatttctg caaatgaata gtctgagagg
         k  g  r  f  t  v  s  r  d  n  a  k  n  s  v  f  l  q  m  n  s  l  r

 771 cgaggacacg gctgtgtatt attgcgcgag agtccgacgc tatggtccct ctacgctcag tccgttcacc
       g  e  d  t  a  v  y  y  c  a  r  v  r  r  y  g  p  s  t  l  s  p  f  t

                                                                      NotI…
 841 tggaaggaca atcactacgc catggacgtc tggggccaag ggacaacggt caccgtctct ccagcggccg
         w  k  d  n  h  y  a  m  d  v  w  g  q  g  t  t  v  t  v  s  p  a  a

 911 caggtggttc agacgtccaa ctggtgcagt caggggctga agtgaaaaaa cctggggcct cagtgaaggt
       a  g  g  s  d  v  q  l  v  q  s  g  a  e  v  k  k  p  g  a  s  v  k
       …6 aa linker

 981 gtcctgcaag gcttctggct acacctttac taggtacacg atgcactggg taaggcaggc acctggacag
       v  s  c  k  a  s  g  y  t  f  t  r  y  t  m  h  w  v  r  q  a  p  g  q

1051 ggtctggaat ggattggata cattaatcct agccgtggtt atactaatta cgcagacagc gtcaagggcc
         g  l  e  w  i  g  y  i  n  p  s  r  g  y  t  n  y  a  d  s  v  k  g

1121 gcttcacaat cactacagac aaatccacca gcacagccta catggaactg agcagcctgc gttctgagga
       r  f  t  i  t  t  d  k  s  t  s  t  a  y  m  e  l  s  s  l  r  s  e

1191 cactgcaacc tattactgtg caagatatta tgatgatcat tactgccttg actactgggg ccaaggcacc
       d  t  a  t  y  y  c  a  r  y  y  d  d  h  y  c  l  d  y  w  g  q  g  t
```

```
1261  acggtcaccg tctcctcagg cgaaggtact agtaccggtt ctggtggaag tggaggttca ggtggagcag
        t  v  t  v  s  s  g  e  g  t  s  t  g  s  g  g  s  g  g  s  g  g  a
                                                                18 aa linker…

1331  acgacattgt actgacccag tctccagcaa ctctgtctct gtctccaggg gagcgtgcca ccctgagctg
       d  d  i  v  l  t  q  s  p  a  t  l  s  l  s  p  g  e  r  a  t  l  s

1401  cagagccagt caaagtgtaa gttacatgaa ctggtaccag cagaagccgg gcaaggcacc caaaagatgg
      c  r  a  s  q  s  v  s  y  m  n  w  y  q  q  k  p  g  k  a  p  k  r  w

1471  atttatgaca catccaaagt ggcttctgga gtccctgctc gcttcagtgg cagtgggtct gggaccgact
        i  y  d  t  s  k  v  a  s  g  v  p  a  r  f  s  g  s  g  s  g  t  d

1541  actctctcac aatcaacagc ttggaggctg aagatgctgc cacttattac tgccaacagt ggagtagtaa
      y  s  l  t  i  n  s  l  e  a  e  d  a  a  t  y  y  c  q  q  w  s  s

                                                                 SacI
1611  cccgctcacg ttcggtggcg ggaccaaggt ggagatcaaa taataagagc tc
      n  p  l  t  f  g  g  g  t  k  v  e  i  k  *  *
```

8.7 taFvAChR-CD3-FlagHis

(in pUC119)

V_LAChR **V_HAChR** **V_HCD3** **V_LCD3**

```
                                                                 SfiI
   1  atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccgtga
        m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  v
                                    pelB leader

  71  tgacccagtc tccatcctcc ctgtctgcat ctgtaggaga cagagtcacc atcgcttgcc gggcaagtca
       m  t  q  s  p  s  s  l  s  a  s  v  g  d  r  v  t  i  a  c  r  a  s

 141  gaccattagc aactatttaa attggtatca gcagaaacca gggaaagccc ctaagctcct gatctatggt
      q  t  i  s  n  y  l  n  w  y  q  q  k  p  g  k  a  p  k  l  l  i  y  g

 211  gcatccagtt tgcaaagtgg ggtcccatca aggttcagtg gcagtggatc tgggacagat ttcactctca
        a  s  s  l  q  s  g  v  p  s  r  f  s  g  s  g  s  g  t  d  f  t  l

 281  ccatcagcag tctgcaacct gaagattttg caacttacta ctgtcaacag agttacagta cccctccgac
       t  i  s  s  l  q  p  e  d  f  a  t  y  y  c  q  q  s  y  s  t  p  p

 351  gtacactttt ggccagggga ccaagctgga gatcaaaggt ggcggtggct cgggcggtgg tgggtcgggt
      t  y  t  f  g  q  g  t  k  l  e  i  k  g  g  g  g  s  g  g  g  g  s  g
                                              (G4S)2-G2S-GS-linker (15 aa)…

 421  ggcagcggat catcgggggg cgacttggtc cagccggggg ggtccctgag agtctcctgt gtagcctctg
        g  s  g  s  s  g  g  d  l  v  q  p  g  g  s  l  r  v  s  c  v  a  s

 491  gatttacatt taggacctat gtgatgaact gggtccgcca ggctccagga aaggggctgg agtgggtggc
       g  f  t  f  r  t  y  v  m  n  w  v  r  q  a  p  g  k  g  l  e  w  v

 561  ccacataagt ccagagggaa ctgaagaata ctatgcggac cctgtgaagg gccgatttac cgtctccaga
      a  h  i  s  p  e  g  t  e  e  y  y  a  d  p  v  k  g  r  f  t  v  s  r

 631  gacaacgcga agaattcagt atttctgcaa atgaatagtc tgagaggcga ggacacggct gtgtattatt
        d  n  a  k  n  s  v  f  l  q  m  n  s  l  r  g  e  d  t  a  v  y  y

 701  gcgcgagagt ccgacgctat ggtccctcta cgctcagtcc gttcacctgg aaggacaatc actacgccat
       c  a  r  v  r  r  y  g  p  s  t  l  s  p  f  t  w  k  d  n  h  y  a
```

```
                                                                  SacI
771  ggacgtctgg ggccaaggga caacggtcac cgtctctcca gcggccgcag agctcggtga cgtccaactg
     m  d  v  w  g  q  g  t  t  v  t  v  s  p  a  a  a  e  l  g  d  v  q  l
                                                6 aa linker

841  gtgcagtcag gggctgaagt gaaaaaacct ggggcctcag tgaaggtgtc ctgcaaggct tctggctaca
       v  q  s  g  a  e  v  k  k  p  g  a  s  v  k  v  s  c  k  a  s  g  y

911  cctttactag gtacacgatg cactgggtaa ggcaggcacc tggacagggt ctggaatgga ttggatacat
       t  f  t  r  y  t  m  h  w  v  r  q  a  p  g  q  g  l  e  w  i  g  y

981  taatcctagc cgtggttata ctaattacgc agacagcgtc aagggccgct tcacaatcac tacagacaaa
     i  n  p  s  r  g  y  t  n  y  a  d  s  v  k  g  r  f  t  i  t  t  d  k

1051 tccaccagca cagcctacat ggaactgagc agcctgcgtt ctgaggacac tgcaacctat tactgtgcaa
       s  t  s  t  a  y  m  e  l  s  s  l  r  s  e  d  t  a  t  y  y  c  a

1121 gatattatga tgatcattac tgccttgact actggggcca aggcaccacg gtcaccgtct cctcaggcga
       r  y  y  d  d  h  y  c  l  d  y  w  g  q  g  t  t  v  t  v  s  s  g

1191 aggtactagt accggttctg gtggaagtgg aggttcaggt ggagcagacg acattgtact gacccagtct
     e  g  t  s  t  g  s  g  g  s  g  g  s  g  g  s  g  g  a  d  d  i  v  l  t  q  s
           …18 aa linker

1261 ccagcaactc tgtctctgtc tccaggggag cgtgccaccc tgagctgcag agccagtcaa agtgtaagtt
       p  a  t  l  s  l  s  p  g  e  r  a  t  l  s  c  r  a  s  q  s  v  s

1331 acatgaactg gtaccagcag aagccgggca aggcacccaa aagatggatt tatgacacat ccaaagtggc
       y  m  n  w  y  q  q  k  p  g  k  a  p  k  r  w  i  y  d  t  s  k  v

1401 ttctggagtc cctgctcgct tcagtggcag tgggtctggg accgactact ctctcacaat caacagcttg
     a  s  g  v  p  a  r  f  s  g  s  g  s  g  t  d  y  s  l  t  i  n  s  l

1471 gaggctgaag atgctgccac ttattactgc caacagtgga gtagtaaccc gctcacgttc ggtggcggga
       e  a  e  d  a  a  t  y  y  c  q  q  w  s  s  n  p  l  t  f  g  g  g

                                  NotI
1541 ccaaggtgga gatcaaagga tccgcggccg ccgattacaa ggatgacgac gataagggag gtcatcacca
       t  k  v  e  i  k  g  s  a  a  a  d  y  k  d  d  d  d  k  g  g  h  h
                                                Flag tag

1611 tcatcaccat taataa
     h  h  h  h  *  *
     …His tag
```

8.8 His-taFvCD3-AChR

(in pBM1.1)

V_HCD3 **V_LCD3** **V_LAChR** **V_HAChR**

```
1    atgaaatacc tgctgccgac cgctgctgct ggtctgctgc tcctcgctgc ccagccggcg atggccatgg
       m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  m
                                  pelB leader

                                                                        HindIII…
71   gccatcatca tcatcatcat catcatcatc acagcagcgg ccatatcgac gacgacgaca agcatatgaa
       g  h  h  h  h  h  h  h  h  h  h  s  s  g  h  i  d  d  d  d  k  h  m
                    His cluster                                 ECS

141  gcttgacgtc caactggtgc agtcaggggc tgaagtgaaa aaacctgggg cctcagtgaa ggtgtcctgc
     k  l  d  v  q  l  v  q  s  g  a  e  v  k  k  p  g  a  s  v  k  v  s  c
```

```
211  aaggcttctg gctacacctt tactaggtac acgatgcact gggtaaggca ggcacctgga cagggtctgg
       k  a  s  g  y  t  f  t  r  y  t  m  h  w  v  r  q  a  p  g  q  g  l

281  aatggattgg atacattaat cctagccgtg gttatactaa ttacgcagac agcgtcaagg gccgcttcac
       e  w  i  g  y  i  n  p  s  r  g  y  t  n  y  a  d  s  v  k  g  r  f

351  aatcactaca gacaaatcca ccagcacagc ctacatggaa ctgagcagcc tgcgttctga ggacactgca
     t  i  t  t  d  k  s  t  s  t  a  y  m  e  l  s  s  l  r  s  e  d  t  a

421  acctattact gtgcaagata ttatgatgat cattactgcc ttgactactg gggccaaggc accacggtca
       t  y  y  c  a  r  y  y  d  d  h  y  c  l  d  y  w  g  q  g  t  t  v

491  ccgtctcctc aggcgaaggt actagtaccg gttctggtgg aagtggaggt tcaggtggag cagacgacat
     t  v  s  s  g  e  g  t  s  t  g  s  g  g  s  g  g  s  g  g  a  d  d
                                  18 aa linker

561  tgtactgacc cagtctccag caactctgtc tctgtctcca ggggagcgtg ccaccctgag ctgcagagcc
     i  v  l  t  q  s  p  a  t  l  s  l  s  p  g  e  r  a  t  l  s  c  r  a

631  agtcaaagtg taagttacat gaactggtac cagcagaagc cgggcaaggc acccaaaaga tggatttatg
       s  q  s  v  s  y  m  n  w  y  q  q  k  p  g  k  a  p  k  r  w  i  y

701  acacatccaa agtggcttct ggagtccctg ctcgcttcag tggcagtggg tctgggaccg actactctct
     d  t  s  k  v  a  s  g  v  p  a  r  f  s  g  s  g  s  g  t  d  y  s

771  cacaatcaac agcttggagg ctgaagatgc tgccacttat tactgccaac agtggagtag taacccgctc
     l  t  i  n  s  l  e  a  e  d  a  a  t  y  y  c  q  q  w  s  s  n  p  l

                                                  SfiI
841  acgttcggtg gcgggaccaa ggtggagatc aaatcggccc agccggccgt gatgacccag tctccatcct
       t  f  g  g  g  t  k  v  e  i  k  s  a  q  p  a  v  m  t  q  s  p  s
                                      5 aa linker

911  ccctgtctgc atctgtagga gacagagtca ccatcgcttg ccgggcaagt cagaccatta gcaactattt
     s  l  s  a  s  v  g  d  r  v  t  i  a  c  r  a  s  q  t  i  s  n  y

981  aaattggtat cagcagaaac cagggaaagc ccctaagctc ctgatctatg gtgcatccag tttgcaaagt
     l  n  w  y  q  q  k  p  g  k  a  p  k  l  l  i  y  g  a  s  s  l  q  s

1051 ggggtcccat caaggttcag tggcagtgga tctgggacag atttcactct caccatcagc agtctgcaac
       g  v  p  s  r  f  s  g  s  g  s  g  t  d  f  t  l  t  i  s  s  l  q

1121 ctgaagattt tgcaacttac tactgtcaac agagttacag tacccctccg acgtacactt ttggccaggg
       p  e  d  f  a  t  y  y  c  q  q  s  y  s  t  p  p  t  y  t  f  g  q

1191 gaccaagctg gagatcaaag gtggcggtgg ctcgggcggt ggtgggtcgg gtggcagcgg atcatcgggg
     g  t  k  l  e  i  k  g  g  g  g  s  g  g  g  g  s  g  g  s  g  g  s  s  g
                          (G4S)2-G2S-GS-linker (15 aa)

1261 ggcgacttgg tccagccggg ggggtccctg agagtctcct gtgtagcctc tggatttaca tttaggacct
       g  d  l  v  q  p  g  g  s  l  r  v  s  c  v  a  s  g  f  t  f  r  t

1331 atgtgatgaa ctgggtccgc caggctccag gaaaggggct ggagtgggtg gcccacataa gtccagaggg
       y  v  m  n  w  v  r  q  a  p  g  k  g  l  e  w  v  a  h  i  s  p  e

1401 aactgaagaa tactatgcgg accctgtgaa gggccgattt accgtctcca gagacaacgc gaagaattca
     g  t  e  e  y  y  a  d  p  v  k  g  r  f  t  v  s  r  d  n  a  k  n  s

1471 gtatttctgc aaatgaatag tctgagaggc gaggacacgg ctgtgtatta ttgcgcgaga gtccgacgct
       v  f  l  q  m  n  s  l  r  g  e  d  t  a  v  y  y  c  a  r  v  r  r

1541 atggtccctc tacgctcagt ccgttcacct ggaaggacaa tcactacgcc atggacgtct ggggccaagg
       y  g  p  s  t  l  s  p  f  t  w  k  d  n  h  y  a  m  d  v  w  g  q

1611 gacaacggtc accgtctctc cagcggccgc agagctctag atgattagct agatgattag
     g  t  t  v  t  v  s  p  a  a  a  e  l  *              *
                                                *           *
                                                    *           *
                                                stop in 3 frames
```

8.9 taFvCD3-AChR-FlagHis

(in pUC119)

V_HCD3 V_LCD3 V_LAChR V_HAChR

NcoI
SfiI
1 atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccgacg
m k y l l p t a a a g l l l l a a q p a m a d
pelB leader

71 tccaactggt gcagtcaggg gctgaagtga aaaaacctgg ggcctcagtg aaggtgtcct gcaaggcttc
v q l v q s g a e v k k p g a s v k v s c k a

141 tggctacacc tttactaggt acacgatgca ctgggtaagg caggcacctg gacagggtct ggaatggatt
s g y t f t r y t m h w v r q a p g q g l e w i

211 ggatacatta atcctagccg tggttatact aattacgcag acagcgtcaa gggccgcttc acaatcacta
g y i n p s r g y t n y a d s v k g r f t i t

281 cagacaaatc caccagcaca gcctacatgg aactgagcag cctgcgttct gaggacactg caacctatta
t d k s t s t a y m e l s s l r s e d t a t y

351 ctgtgcaaga tattatgatg atcattactg ccttgactac tggggccaag gcaccacggt caccgtctcc
y c a r y y d d h y c l d y w g q g t t v t v s

421 tcaggcgaag gtactagtac cggttctggt ggaagtggag gttcaggtgg agcagacgac attgtactga
s g e g t s t g s g g s g g s g g a d d i v l
18 aa linker

491 cccagtctcc agcaactctg tctctgtctc caggggagcg tgccaccctg agctgcagag ccagtcaaag
t q s p a t l s l s p g e r a t l s c r a s q

561 tgtaagttac atgaactggt accagcagaa gccgggcaag gcacccaaaa gatggattta tgacacatcc
s v s y m n w y q q k p g k a p k r w i y d t s

631 aaagtggctt ctggagtccc tgctcgcttc agtggcagtg ggtctgggac cgactactct ctcacaatca
k v a s g v p a r f s g s g s g t d y s l t i

701 acagcttgga ggctgaagat gctgccactt attactgcca acagtggagt agtaacccgc tcacgttcgg
n s l e a e d a a t y y c q q w s s n p l t f

BamHI
771 tggcgggacc aaggtggaga tcaaaggatc cggtggaggt tcagtgatga cccagtctcc atcctccctg
g g g t k v e i k g s g g g s v m t q s p s s l
6 aa linker

841 tctgcatctg taggagacag agtcaccatc gcttgccggg caagtcagac cattagcaac tatttaaatt
s a s v g d r v t i a c r a s q t i s n y l n

911 ggtatcagca gaaaccaggg aaagccccta agctcctgat ctatggtgca tccagtttgc aaagtggggt
w y q q k p g k a p k l l i y g a s s l q s g

981 cccatcaagg ttcagtggca gtggatctgg gacagatttc actctcacca tcagcagtct gcaacctgaa
v p s r f s g s g s g t d f t l t i s s l q p e

1051 gattttgcaa cttactactg tcaacagagt tacagtaccc ctccgacgta cacttttggc caggggacca
d f a t y y c q q s y s t p p t y t f g q g t

1121 agctggagat caaaggtggc ggtggctcgg gcggtggtgg gtcgggtggc agcggatcat cgggggggcga
k l e i k g g g g s g g g g s g g g s g s s g g
$(G_4S)_2$-G_2S-GS-linker (15 aa)

1191 cttggtccag ccgggggggt ccctgagagt ctcctgtgta gcctctggat ttacatttag gacctatgtg
d l v q p g g g s l r v s c v a s g f t f r t y v

1261 atgaactggg tccgccaggc tccaggaaag gggctggagt gggtggccca cataagtcca gagggaactg
m n w v r q a p g k g l e w v a h i s p e g t

```
1331  aagaatacta tgcggaccct gtgaagggcc gatttaccgt ctccagagac aacgcgaaga attcagtatt
       e  e  y   y  a  d  p   v  k  g   r  f  t   v  s  r  d   n  a  k   n  s  v

1401  tctgcaaatg aatagtctga gaggcgagga cacggctgtg tattattgcg cgagagtccg acgctatggt
      f  l  q  m   n  s  l   r  g  e   d  t  a   v  y  y  c   a  r  v   r  r  y  g

1471  ccctctacgc tcagtccgtt cacctggaag gacaatcact acgccatgga cgtctggggc caagggacaa
        p  s  t   l  s  p   f  t  w  k   d  n  h   y  a  m   d  v  w  g   q  g  t

                          NotI
1541  cggtcaccgt ctctccagcg gccgccgatt acaaggatga cgacgataag ggaggtcatc accatcatca
       t  v  t   v  s  p  a   a  a  d   y  k  d   d  d  d  k   g  g  h   h  h  h
                                      Flag tag                    His tag…

1611  ccattaataa
      h  h  *  *
```

8.10 taFvCD3-HLA-A2-FlagHis / -mycHis

(in pUC119)

V_HCD3 **V_LCD3** **V_HHLA-A2** **V_LHLA-A2**

```
                                                                    NcoI
                                                          SfiI
  1  atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccgacg
       m  k  y   l  l  p   t  a  a  a   g  l  l   l  l  a   a  q  p  a   m  a  d
                                  pelB leader

 71  tccaactggt gcagtcaggg gctgaagtga aaaaacctgg ggcctcagtg aaggtgtcct gcaaggcttc
       v  q  l   v  q  s  g   a  e  v   k  k  p   g  a  s  v   k  v  s   c  k  a

141  tggctacacc tttactaggt acacgatgca ctgggtaagg caggcacctg gacagggtct ggaatggatt
      s  g  y   t  f  t  r   y  t  m   h  w  v   r  q  a  p   g  q  g   l  e  w  i

211  ggatacatta atcctagccg tggttatact aattacgcag acagcgtcaa gggccgcttc acaatcacta
       g  y  i   n  p  s  r   g  y  t   n  y  a   d  s  v  k   g  r  f   t  i  t

281  cagacaaatc caccagcaca gcctacatgg aactgagcag cctgcgttct gaggacactg caacctatta
       t  d  k   s  t  s  t   a  y  m   e  l  s   s  l  r  s   e  d  t   a  t  y

351  ctgtgcaaga tattatgatg atcattactg ccttgactac tggggccaag gcaccacggt caccgtctcc
      y  c  a   r  y  y  d   d  h  y   c  l  d   y  w  g  q   g  t  t   v  t  v  s

421  tcaggcgaag gtactagtac cggttctggt ggaagtggag gttcaggtgg agcagacgac attgtactga
       s  g  e   g  t  s  t   g  s  g   g  s  g   g  s  g  g   a  d  d   i  v  l
                                  18 aa linker

491  cccagtctcc agcaactctg tctctgtctc caggggagcg tgccaccctg agctgcagag ccagtcaaag
       t  q  s   p  a  t  l   s  l  s   p  g  e   r  a  t  l   s  c  r   a  s  q

561  tgtaagttac atgaactggt accagcagaa gccgggcaag gcacccaaaa gatggattta tgacacatcc
      s  v  s   y  m  n  w   y  q  q   k  p  g   k  a  p  k   r  w  i   y  d  t  s

631  aaagtggctt ctggagtccc tgctcgcttc agtggcagtg ggtctgggac cgactactct ctcacaatca
       k  v  a   s  g  v  p   a  r  f   s  g  s   g  s  g  t   d  y  s   l  t  i

701  acagcttgga ggctgaagat gctgccactt attactgcca acagtggagt agtaacccgc tcacgttcgg
       n  s  l   e  a  e  d   a  a  t   y  y  c   q  q  w  s   s  n  p   l  t  f

                                  BamHI
771  tggcgggacc aaggtggaga tcaaaggatc cggtggagga ggttcacagg tgcagctggt gcagtctggg
       g  g  g   t  k  v  e   i  k  g   s  g  g   g  g  s  q   v  q  l   v  q  s  g
                                  7 aa linker
```

```
 841  ggaggcgtgg tccagcctgg ggggtccctg agagtctcct gtgcagcgtc tggggtcacc ctcagtgatt
        g  g  v  v  q  p  g  g  s  l  r  v  s  c  a  a  s  g  v  t  l  s  d

 911  atggcatgca ttgggtccgc caggctccag gcaaggggct ggagtggatg gcttttatac ggaatgatgg
        y  g  m  h  w  v  r  q  a  p  g  k  g  l  e  w  m  a  f  i  r  n  d

 981  aagtgataaa tattatgcag actccgtgaa gggccgattc accatctcca gagacaactc caagaaaaca
       g  s  d  k  y  y  a  d  s  v  k  g  r  f  t  i  s  r  d  n  s  k  k  t

1051  gtgtctctgc aaatgagcag tctcagagct gaagacacgg ctgtgtatta ctgtgcgaaa aatggcgaat
        v  s  l  q  m  s  s  l  r  a  e  d  t  a  v  y  y  c  a  k  n  g  e

1121  ctgggccttt ggactactgg tacttcgatc tctggggccg tggcaccctg gtcaccgtgt cgagtggtgg
        s  g  p  l  d  y  w  y  f  d  l  w  g  r  g  t  l  v  t  v  s  s  g

1191  aggcggttca ggcggaggtg gctctggcgg tggcggatcg gatgttgtga tgactcagtc tccatcctcc
      g  g  g  s  g  g  g  g  s  g  g  g  g  s  d  v  v  m  t  q  s  p  s  s
          …(G4S)3-linker (15 aa)

1261  ctgtctgcat ctgtaggaga cagagtcacc atcacttgcc aggcgagtca ggacattagc aactatttaa
        l  s  a  s  v  g  d  r  v  t  i  t  c  q  a  s  q  d  i  s  n  y  l

1331  attggtatca gcagaaacca gggaaagccc ctaagctcct gatctacgat gcatccaatt tggaaacagg
       n  w  y  q  q  k  p  g  k  a  p  k  l  l  i  y  d  a  s  n  l  e  t

1401  ggtcccatca aggttcagtg gaagtggatc tgggacagat tttactttca ccatcagcag cctgcagcct
      g  v  p  s  r  f  s  g  s  g  s  g  t  d  f  t  f  t  i  s  s  l  q  p

1471  gaggattttg caacttatta ctgccaacaa tatagtagtt ttccgctcac tttcggcgga gggaccaaag
        e  d  f  a  t  y  y  c  q  q  y  s  s  f  p  l  t  f  g  g  g  t  k

                        NotI
1541  tggatatcaa acgtgcggcc gccgattaca aggatgacga cgataaggga ggtcatcacc atcatcacca
       v  d  i  k  r  a  a  a  d  y  k  d  d  d  d  k  g  g  h  h  h  h  h  h
                                  Flag tag                    His tag…

1611  ttaataa
      h  *  *

 or

...

                        NotI
1541  tggatatcaa acgtgcggcc gcagaacaaa aactcatctc agaagaggat ctgaatgggg ccgcacatca
       v  d  i  k  r  a  a  a  e  q  k  l  i  s  e  e  d  l  n  g  a  a  h
                                     myc tag

1611  ccatcatcac cattaataa
      h  h  h  h  h  *  *
      …His tag
```

8.11 taFvHLA-A2-CD3-FlagHis / -mycHis

(in pUC119)

V$_H$HLA-A2 V$_L$HLA-A2 V$_H$CD3 V$_L$CD3

```
                                                                             NcoI
                                                                   SfiI
   1  atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggcccagg
        m  k  y   l  l  p   t  a  a  a   g  l  l   l  l  a   a  q  p  a   m  a  q
                                  pelB leader

  71  tgcagctggt gcagtctggg ggaggcgtgg tccagcctgg ggggtccctg agagtctcct gtgcagcgtc
       v  q  l   v  q  s  g   g  g  v   v  q  p   g  g  s  l   r  v  s   c  a  a

 141  tggggtcacc ctcagtgatt atggcatgca ttgggtccgc caggctccag gcaaggggct ggagtggatg
      s  g  v  t   l  s  d   y  g  m   h  w  v  r   q  a  p   g  k  g   l  e  w  m

 211  gcttttatac ggaatgatgg aagtgataaa tattatgcag actccgtgaa gggccgattc accatctcca
        a  f  i   r  n  d   g  s  d  k   y  y  a   d  s  v   k  g  r  f   t  i  s

 281  gagacaactc caagaaaaca gtgtctctgc aaatgagcag tctcagagct gaagacacgg ctgtgtatta
       r  d  n   s  k  k  t   v  s  l   q  m  s   s  l  r  a   e  d  t   a  v  y

 351  ctgtgcgaaa aatggcgaat ctgggccttt ggactactgg tacttcgatc tctggggccg tggcaccctg
      y  c  a  k   n  g  e   s  g  p   l  d  y  w   y  f  d   l  w  g   r  g  t  l

 421  gtcaccgtgt cgagtggtgg aggcggttca ggcggaggtg gctctggcgg tggcggatcg gatgttgtga
        v  t  v   s  s  g   g  g  g  s   g  g  g   g  s  g   g  g  g  s   d  v  v
                              (G4S)3-linker (15 aa)

 491  tgactcagtc tccatcctcc ctgtctgcat ctgtaggaga cagagtcacc atcacttgcc aggcgagtca
       m  t  q   s  p  s  s   l  s  a   s  v  g   d  r  v  t   i  t  c   q  a  s

 561  ggacattagc aactatttaa attggtatca gcagaaacca gggaaagccc ctaagctcct gatctacgat
      q  d  i  s   n  y  l   n  w  y   q  q  k  p   g  k  a   p  k  l   l  i  y  d

 631  gcatccaatt tggaaacagg ggtcccatca aggttcagtg gaagtggatc tgggacagat tttactttca
        a  s  n   l  e  t   g  v  p  s   r  f  s   g  s  g   s  g  t  d   f  t  f

 701  ccatcagcag cctgcagcct gaggattttg caacttatta ctgccaacaa tatagtagtt ttccgctcac
       t  i  s   s  l  q  p   e  d  f   a  t  y   y  c  q  q   y  s  s   f  p  l

                                                          SacI
 771  tttcggcgga gggaccaaag tggatatcaa acgtggtggc ggagagctcg gtgacgtcca actggtgcag
      t  f  g  g   g  t  k   v  d  i   k  r  g  g   g  e  l   g  d  v   q  l  v  q
                                                   6 aa linker

 841  tcaggggctg aagtgaaaaa acctggggcc tcagtgaagg tgtcctgcaa ggcttctggc tacaccttta
        s  g  a   e  v  k   k  p  g  a   s  v  k   v  s  c   k  a  s  g   y  t  f

 911  ctaggtacac gatgcactgg gtaaggcagg cacctggaca gggtctggaa tggattggat acattaatcc
       t  r  y   t  m  h  w   v  r  q   a  p  g   q  g  l  e   w  i  g   y  i  n

 981  tagccgtggt tatactaatt acgcagacag cgtcaagggc cgcttcacaa tcactacaga caaatccacc
      p  s  r  g   y  t  n   y  a  d   s  v  k  g   r  f  t   i  t  t   d  k  s  t

1051  agcacagcct acatggaact gagcagcctg cgttctgagg acactgcaac ctattactgt gcaagatatt
        s  t  a   y  m  e   l  s  s  l   r  s  e   d  t  a   t  y  y  c   a  r  y

1121  atgatgatca ttactgcctt gactactggg gccaaggcac cacggtcacc gtctcctcag gcgaaggtac
       y  d  d   h  y  c  l   d  y  w   g  q  g   t  t  v  t   v  s  s   g  e  g


1191  tagtaccggt tctggtggaa gtggaggttc aggtggagca gacgacattg tactgaccca gtctccagca
      t  s  t  g   s  g  g   s  g  g   s  g  g  a   d  d  i   v  l  t   q  s  p  a
              …18 aa linker
```

```
1261  actctgtctc tgtctccagg ggagcgtgcc accctgagct gcagagccag tcaaagtgta agttacatga
        t  l  s  l  s  p  g  e  r  a  t  l  s  c  r  a  s  q  s  v  s  y  m

1331  actggtacca gcagaagccg ggcaaggcac ccaaaagatg gatttatgac acatccaaag tggcttctgg
       n  w  y  q  q  k  p  g  k  a  p  k  r  w  i  y  d  t  s  k  v  a  s

1401  agtccctgct cgcttcagtg gcagtgggtc tgggaccgac tactctctca caatcaacag cttggaggct
      g  v  p  a  r  f  s  g  s  g  s  g  t  d  y  s  l  t  i  n  s  l  e  a

1471  gaagatgctg ccacttatta ctgccaacag tggagtagta acccgctcac gttcggtggc gggaccaagg
        e  d  a  a  t  y  y  c  q  q  w  s  s  n  p  l  t  f  g  g  g  t  k

                          NotI
1541  tggagatcaa aggatccgcg gccgccgatt acaaggatga cgacgataag ggaggtcatc accatcatca
       v  e  i  k  g  s  a  a  a  d  y  k  d  d  d  d  k  g  g  h  h  h  h
                                   Flag tag                    His tag…

1611  ccattaataa
      h  h  *  *
```

or

...

```
                          NotI
1541  tggagatcaa aggatccgcg gccgcagaac aaaaactcat ctcagaagag gatctgaatg gggccgcaca
       v  e  i  k  g  s  a  a  a  e  q  k  l  i  s  e  e  d  l  n  g  a  a__
                                      myc tag

1611  tcaccatcat caccattaat aa
      h  h  h  h  h  h  *  *
      …His tag
```

8.12 V_HCD3-scFvCD45(V_H-V_L)-FlagHis

(in pUC119)

V_HCD3 V_HCD45 V_LCD45

```
   1  atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccgacg
        m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  d
                                  pelB leader

  71  tccaactggt gcagtcaggg gctgaagtga aaaaacctgg ggcctcagtg aaggtgtcct gcaaggcttc
        v  q  l  v  q  s  g  a  e  v  k  k  p  g  a  s  v  k  v  s  c  k  a

 141  tggctacacc tttactaggt acacgatgca ctgggtaagg caggcacctg gacagggtct ggaatggatt
        s  g  y  t  f  t  r  y  t  m  h  w  v  r  q  a  p  g  q  g  l  e  w  i

 211  ggatacatta atcctagccg tggttatact aattacgcag acagcgtcaa gggccgcttc acaatcacta
          g  y  i  n  p  s  r  g  y  t  n  y  a  d  s  v  k  g  r  f  t  i  t

 281  cagacaaatc caccagcaca gcctacatgg aactgagcag cctgcgttct gaggacactg caacctatta
        t  d  k  s  t  s  t  a  y  m  e  l  s  s  l  r  s  e  d  t  a  t  y

 351  ctgtgcaaga tattatgatg atcattactg ccttgactac tggggccaag gcaccacggt caccgtctcc
        y  c  a  r  y  y  d  d  h  y  c  l  d  y  w  g  q  g  t  t  v  t  v  s

                    SpeI
 421  tcaggcgaag gtactagtac tggtcaggtg cagctggtgg aaagcggtgg cggactggtg cagccgggcg
          s  g  e  g  t  s  t  g  q  v  q  l  v  e  s  g  g  g  l  v  q  p  g
                7 aa linker

 491  gcagcctgaa actgagctgt gccgccagcg gttttgattt tagccgttat tggatgagct gggtgcgtca
        g  s  l  k  l  s  c  a  a  s  g  f  d  f  s  r  y  w  m  s  w  v  r

 561  ggcgccgggc aaaggcctgg aatggattgg cgaaattaac ccgaccagca gcaccattaa ctttaccccg
        q  a  p  g  k  g  l  e  w  i  g  e  i  n  p  t  s  s  t  i  n  f  t  p

 631  agcctgaaag ataaagtgtt tattagccgt gataacgcga aaaacaccct gtatctgcag atgagcaaag
          s  l  k  d  k  v  f  i  s  r  d  n  a  k  n  t  l  y  l  q  m  s  k

 701  tgcgtagcga agataccgcg ctgtattatt gcgcgcgtgg caactattat cgttatggcg atgcgatgga
        v  r  s  e  d  t  a  l  y  y  c  a  r  g  n  y  y  r  y  g  d  a  m

 771  ttattggggc cagggcacca gcgtgaccgt gagcggtgga ggaggttcag gaggaggtgg ttcaggagga
        d  y  w  g  q  g  t  s  v  t  v  s  g  g  g  g  s  g  g  g  g  s  g  g
                                              (G4S)2-G3-TG-linker (15 aa)…

 841  ggtaccggtg atattgttct gacccagagc ccggcgagcc tggcggttag cctgggtcag cgtgccacca
          g  t  g  d  i  v  l  t  q  s  p  a  s  l  a  v  s  l  g  q  r  a  t

 911  ttagctgccg tgcgagcaaa agcgtgagca ccagcggcta tagctatctg cattggtatc agcagaaacc
        i  s  c  r  a  s  k  s  v  s  t  s  g  y  s  y  l  h  w  y  q  q  k

 981  gggccagcct ccaaaactgc tgatttatct ggccagcaac ctggaaagcg gtgtgccggc ccgttttagc
        p  g  q  p  p  k  l  l  i  y  l  a  s  n  l  e  s  g  v  p  a  r  f  s

1051  ggcagcggca gcggtaccga ttttaccctg aacattcatc cggtggaaga agaagatgcg gcgacctatt
          g  s  g  s  g  t  d  f  t  l  n  i  h  p  v  e  e  e  d  a  a  t  y

1121  attgccagca tagccgtgaa ctgccgttta cctttggcag cggcaccaaa ctggaaatta aagcggccgc
        y  c  q  h  s  r  e  l  p  f  t  f  g  s  g  t  k  l  e  i  k  a  a

1191  cgattacaag gatgacgacg ataagggagg tcatcaccat catcaccatt aataa
        a  d  y  k  d  d  d  d  k  g  g  h  h  h  h  h  h  *  *
                Flag tag                 His tag
```

8.13 V_HCD3-scFvCD45(V_L-V_H)-FlagHis

(in pUC119)

V_HCD3 V_LCD45 V_HCD45

```
                                                                                NcoI
                                                                  SfiI
   1 atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccgacg
       m  k  y   l  l  p   t  a  a  a   g  l  l   l  l  a   a  q  p  a   m  a  d
                                      pelB leader

  71 tccaactggt gcagtcaggg gctgaagtga aaaaacctgg ggcctcagtg aaggtgtcct gcaaggcttc
      v  q  l   v  q  s  g   a  e  v   k  k  p   g  a  s  v   k  v  s   c  k  a

 141 tggctacacc tttactaggt acacgatgca ctgggtaagg caggcacctg gacaggggtct ggaatggatt
     s  g  y  t   f  t  r   y  t  m   h  w  v  r   q  a  p   g  q  g   l  e  w  i

 211 ggatacatta atcctagccg tggttatact aattacgcag acagcgtcaa gggccgcttc acaatcacta
       g  y  i   n  p  s   r  g  y  t   n  y  a   d  s  v   k  g  r  f   t  i  t

 281 cagacaaatc caccagcaca gcctacatgg aactgagcag cctgcgttct gaggacactg caacctatta
      t  d  k   s  t  s  t   a  y  m   e  l  s   s  l  r  s   e  d  t   a  t  y

 351 ctgtgcaaga tattatgatg atcattactg ccttgactac tggggccaag gcaccacggt caccgtctcc
     y  c  a  r   y  y  d   d  h  y   c  l  d  y   w  g  q   g  t  t   v  t  v  s

                                  AgeI
 421 tcaggcgaag gtactagtac cggtgatatt gttctgaccc agagcccggc gagcctggcg gttagcctgg
       s  g  e   g  t  s   t  g  d  i   v  l  t   q  s  p   a  s  l  a   v  s  l
                 7 aa linker

 491 gtcagcgtgc caccattagc tgccgtgcga gcaaaagcgt gagcaccagc ggctatagct atctgcattg
      g  q  r   a  t  i  s   c  r  a   s  k  s   v  s  t  s   g  y  s   y  l  h

 561 gtatcagcag aaaccgggcc agcctccaaa actgctgatt tatctggcca gcaacctgga aagcggtgtg
     w  y  q  q   k  p  g   q  p  p   k  l  l  i   y  l  a   s  n  l   e  s  g  v

 631 ccggcccgtt ttagcggcag cggcagcggt accgatttta ccctgaacat tcatccggtg gaagaagaag
       p  a  r   f  s  g   s  g  s  g   t  d  f   t  l  n   i  h  p  v   e  e  e

 701 atgcggcgac ctattattgc cagcatagcc gtgaactgcc gtttaccttt ggcagcggca ccaaactgga
      d  a  a   t  y  y  c   q  h  s   r  e  l   p  f  t  f   g  s  g   t  k  l

 771 aattaaaaag atctctggtg gcggcggctc gggtggtggt gggtcgggcg gcggcggctc gagccaggtg
     e  i  k  k   i  s  g   g  g  g   s  g  g  g   g  s  g   g  g  g   s  s  q  v
                              KIS-(G4S)3-S-linker (19 aa)

 841 cagctggtgg aaagcggtgg cggactggtg cagccgggcg gcagcctgaa actgagctgt gccgccagcg
       q  l  v   e  s  g   g  g  l  v   q  p  g   g  s  l   k  l  s  c   a  a  s

 911 gttttgattt tagccgttat tggatgagct gggtgcgtca ggcgccgggc aaaggcctgg aatggattgg
      g  f  d   f  s  r  y   w  m  s   w  v  r   q  a  p  g   k  g  l   e  w  i

 981 cgaaattaac ccgaccagca gcaccattaa ctttaccccg agcctgaaag ataaagtgtt tattagccgt
     g  e  i  n   p  t  s   s  t  i   n  f  t  p   s  l  k   d  k  v   f  i  s  r

1051 gataacgcga aaaacaccct gtatctgcag atgagcaaag tgcgtagcga agataccgcg ctgtattatt
       d  n  a   k  n  t   l  y  l  q   m  s  k   v  r  s   e  d  t  a   l  y  y

1121 gcgcgcgtgg caactattat cgttatggcg atgcgatgga ttattggggc cagggcacca gcgtgaccgt
      c  a  r   g  n  y  y   r  y  g   d  a  m   d  y  w  g   q  g  t   s  v  t

         NotI                                                              EcoRI…
1191 gagcgcggcc gccgattaca aggatgacga cgataaggga ggtcatcacc atcatcacca ttaataagaa
     v  s  a  a   a  d  y   k  d  d   d  d  k  g   g  h  h   h  h  h   h  *  *
                     Flag tag                           His tag

1261 ttc
```

8.14 V_HCD3-scFvHLA-A2-FlagHis

(in pUC119)

V_HCD3 **V_HHLA-A2** **V_LHLA-A2**

```
                                                                                                  NcoI
   1  atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccgacg
        m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  d
                                    pelB leader

  71  tccaactggt gcagtcaggg gctgaagtga aaaaacctgg ggcctcagtg aaggtgtcct gcaaggcttc
        v  q  l  v  q  s  g  a  e  v  k  k  p  g  a  s  v  k  v  s  c  k  a

 141  tggctacacc tttactaggt acacgatgca ctgggtaagg caggcacctg gacagggtct ggaatggatt
        s  g  y  t  f  t  r  y  t  m  h  w  v  r  q  a  p  g  q  g  l  e  w  i

 211  ggatacatta atcctagccg tggttatact aattacgcag acagcgtcaa gggccgcttc acaatcacta
          g  y  i  n  p  s  r  g  y  t  n  y  a  d  s  v  k  g  r  f  t  i  t

 281  cagacaaatc caccagcaca gcctacatgg aactgagcag cctgcgttct gaggacactg caacctatta
        t  d  k  s  t  s  t  a  y  m  e  l  s  s  l  r  s  e  d  t  a  t  y

 351  ctgtgcaaga tattatgatg atcattactg ccttgactac tggggccaag gcaccacggt caccgtctcc
        y  c  a  r  y  y  d  d  h  y  c  l  d  y  w  g  q  g  t  t  v  t  v  s

           BamHI
 421  tcaggatccg gtggaggagg ttcacaggtg cagctggtgc agtctggggg aggcgtggtc cagcctgggg
          s  g  s  g  g  g  g  s  q  v  q  l  v  q  s  g  g  g  v  v  q  p  g
                7 aa linker

 491  ggtccctgag agtctcctgt gcagcgtctg gggtcaccct cagtgattat ggcatgcatt gggtccgcca
        g  s  l  r  v  s  c  a  a  s  g  v  t  l  s  d  y  g  m  h  w  v  r

 561  ggctccaggc aaggggctgg agtggatggc ttttatacgg aatgatggaa gtgataaata ttatgcagac
        q  a  p  g  k  g  l  e  w  m  a  f  i  r  n  d  g  s  d  k  y  y  a  d

 631  tccgtgaagg gccgattcac catctccaga gacaactcca agaaaacagt gtctctgcaa atgagcagtc
          s  v  k  g  r  f  t  i  s  r  d  n  s  k  k  t  v  s  l  q  m  s  s

 701  tcagagctga agacacggct gtgtattact gtgcgaaaaa tggcgaatct gggcctttgg actactggta
        l  r  a  e  d  t  a  v  y  y  c  a  k  n  g  e  s  g  p  l  d  y  w

 771  cttcgatctc tggggccgtg gcaccctggt caccgtgtcg agtggtggag gcggttcagg cggaggtggc
        y  f  d  l  w  g  r  g  t  l  v  t  v  s  s  g  g  g  g  s  g  g  g  g
                                                    (G4S)3-linker (15 aa)…

 841  tctggcggtg gcggatcgga tgttgtgatg actcagtctc catcctccct gtctgcatct gtaggagaca
          s  g  g  g  g  s  d  v  v  m  t  q  s  p  s  s  l  s  a  s  v  g  d

 911  gagtcaccat cacttgccag gcgagtcagg acattagcaa ctatttaaat tggtatcagc agaaaccagg
        r  v  t  i  t  c  q  a  s  q  d  i  s  n  y  l  n  w  y  q  q  k  p

 981  gaaagcccct aagctcctga tctacgatgc atccaatttg gaaacagggg tcccatcaag gttcagtgga
        g  k  a  p  k  l  l  i  y  d  a  s  n  l  e  t  g  v  p  s  r  f  s  g

1051  agtggatctg ggacagattt tactttcacc atcagcagcc tgcagcctga ggattttgca acttattact
          s  g  s  g  t  d  f  t  f  t  i  s  s  l  q  p  e  d  f  a  t  y  y

                                                                                        NotI
1121  gccaacaata tagtagtttt ccgctcactt tcggcggagg gaccaaagtg gatatcaaac gtgcggccgc
        c  q  q  y  s  s  f  p  l  t  f  g  g  g  t  k  v  d  i  k  r  a  a

1191  cgattacaag gatgacgacg ataagggagg tcatcaccat catcaccatt aataa
        a  d  y  k  d  d  d  d  k  g  g  h  h  h  h  h  h  *  *
                Flag tag                    His tag
```

8.15 V_HCD3-scFvHLA-A2-mycHis

(in pUC119)

V_HCD3 **V_HHLA-A2** **V_LHLA-A2**

```
                                                                                                  NcoI
   1 atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccgacg
       m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  d
                                  pelB leader

  71 tccaactggt gcagtcaggg gctgaagtga aaaaacctgg ggcctcagtg aaggtgtcct gcaaggcttc
       v  q  l  v  q  s  g  a  e  v  k  k  p  g  a  s  v  k  v  s  c  k  a

 141 tggctacacc tttactaggt acacgatgca ctgggtaagg caggcacctg gacagggtct ggaatggatt
       s  g  y  t  f  t  r  y  t  m  h  w  v  r  q  a  p  g  q  g  l  e  w  i

 211 ggatacatta atcctagccg tggttatact aattacgcag acagcgtcaa gggccgcttc acaatcacta
         g  y  i  n  p  s  r  g  y  t  n  y  a  d  s  v  k  g  r  f  t  i  t

 281 cagacaaatc caccagcaca gcctacatgg aactgagcag cctgcgttct gaggacactg caacctatta
       t  d  k  s  t  s  t  a  y  m  e  l  s  s  l  r  s  e  d  t  a  t  y

 351 ctgtgcaaga tattatgatg atcattactg ccttgactac tggggccaag gcaccacggt caccgtctcc
       y  c  a  r  y  y  d  d  h  y  c  l  d  y  w  g  q  g  t  t  v  t  v  s

                BamHI
 421 tcaggatccg gtggaggagg ttcacaggtg cagctggtgc agtctggggg aggcgtggtc cagcctgggg
         s  g  s  g  g  g  g  s  q  v  q  l  v  q  s  g  g  g  v  v  q  p  g
               7 aa linker

 491 ggtccctgag agtctcctgt gcagcgtctg gggtcaccct cagtgattat ggcatgcatt gggtccgcca
       g  s  l  r  v  s  c  a  a  s  g  v  t  l  s  d  y  g  m  h  w  v  r

 561 ggctccaggc aaggggctgg agtggatggc ttttatacgg aatgatggaa gtgataaata ttatgcagac
       q  a  p  g  k  g  l  e  w  m  a  f  i  r  n  d  g  s  d  k  y  y  a  d

 631 tccgtgaagg gccgattcac catctccaga gacaactcca agaaaacagt gtctctgcaa atgagcagtc
         s  v  k  g  r  f  t  i  s  r  d  n  s  k  k  t  v  s  l  q  m  s  s

 701 tcagagctga agacacggct gtgtattact gtgcgaaaaa tggcgaatct gggcctttgg actactggta
       l  r  a  e  d  t  a  v  y  y  c  a  k  n  g  e  s  g  p  l  d  y  w

 771 cttcgatctc tggggccgtg gcaccctggt caccgtgtcg agtggtggag gcggttcagg cggaggtggc
       y  f  d  l  w  g  r  g  t  l  v  t  v  s  s  g  g  g  g  s  g  g  g  g
                                                  (G4S)3-linker (15 aa)…

 841 tctggcggtg gcggatcgga tgttgtgatg actcagtctc catcctccct gtctgcatct gtaggagaca
         s  g  g  g  g  s  d  v  v  m  t  q  s  p  s  s  l  s  a  s  v  g  d

 911 gagtcaccat cacttgccag gcgagtcagg acattagcaa ctatttaaat tggtatcagc agaaaccagg
       r  v  t  i  t  c  q  a  s  q  d  i  s  n  y  l  n  w  y  q  q  k  p

 981 gaaagcccct aagctcctga tctacgatgc atccaatttg gaaacagggg tcccatcaag gttcagtgga
       g  k  a  p  k  l  l  i  y  d  a  s  n  l  e  t  g  v  p  s  r  f  s  g

1051 agtggatctg ggacagattt actttcacc atcagcagcc tgcagcctga ggattttgca acttattact
       s  g  s  g  t  d  f  t  f  t  i  s  s  l  q  p  e  d  f  a  t  y  y

                                                                                        NotI
1121 gccaacaata tagtagtttt ccgctcactt tcggcggagg gaccaaagtg gatatcaaac gtgcggccgc
       c  q  q  y  s  s  f  p  l  t  f  g  g  g  t  k  v  d  i  k  r  a  a

1191 agaacaaaaa ctcatctcag aagaggatct gaatggggcc gcacatcacc atcatcacca ttaataa
       a  e  q  k  l  i  s  e  e  d  l  n  g  a  a  h  h  h  h  h  h  *  *
                     myc tag                          His tag
```

8.16 V_LCD3-scFvHLA-A2-mycHis

(in pUC119)

V_LCD3 **V_HHLA-A2** **V_LHLA-A2**

```
                                                                                                  NcoI
   1 atgaaatacc tattgcctac ggcagccgct ggattgttat tactcgcggc ccagccggcc atggccgaca
       m  k  y  l  l  p  t  a  a  a  g  l  l  l  l  a  a  q  p  a  m  a  d
     pelB leader

  71 ttgtactgac ccagtctcca gcaactctgt ctctgtctcc aggggagcgt gccaccctga gctgcagagc
       i  v  l  t  q  s  p  a  t  l  s  l  s  p  g  e  r  a  t  l  s  c  r

 141 cagtcaaagt gtaagttaca tgaactggta ccagcagaag ccgggcaagg cacccaaaag atggatttat
       a  s  q  s  v  s  y  m  n  w  y  q  q  k  p  g  k  a  p  k  r  w  i  y

 211 gacacatcca aagtggcttc tggagtccct gctcgcttca gtggcagtgg gtctgggacc gactactctc
       d  t  s  k  v  a  s  g  v  p  a  r  f  s  g  s  g  s  g  t  d  y  s

 281 tcacaatcaa cagcttggag gctgaagatg ctgccactta ttactgccaa cagtggagta gtaacccgct
       l  t  i  n  s  l  e  a  e  d  a  a  t  y  y  c  q  q  w  s  s  n  p

                                                BamHI
 351 cacgttcggt ggcgggacca aggtggagat caaaggatcc ggtggaggag gttcacaggt gcagctggtg
       l  t  f  g  g  g  t  k  v  e  i  k  g  s  g  g  g  g  s  q  v  q  l  v
                                                7 aa linker

 421 cagtctgggg gaggcgtggt ccagcctggg gggtccctga gagtctcctg tgcagcgtct ggggtcaccc
       q  s  g  g  g  v  v  q  p  g  g  s  l  r  v  s  c  a  a  s  g  v  t

 491 tcagtgatta tggcatgcat tgggtccgcc aggctccagg caaggggctg gagtggatgg cttttatacg
       l  s  d  y  g  m  h  w  v  r  q  a  p  g  k  g  l  e  w  m  a  f  i

 561 gaatgatgga agtgataaat attatgcaga ctccgtgaag ggccgattca ccatctccag agacaactcc
       r  n  d  g  s  d  k  y  y  a  d  s  v  k  g  r  f  t  i  s  r  d  n  s

 631 aagaaaacag tgtctctgca aatgagcagt ctcagagctg aagacacggc tgtgtattac tgtgcgaaaa
       k  k  t  v  s  l  q  m  s  s  l  r  a  e  d  t  a  v  y  y  c  a  k

 701 atggcgaatc tgggcctttg gactactggt acttcgatct ctggggccgt ggcaccctgg tcaccgtgtc
       n  g  e  s  g  p  l  d  y  w  y  f  d  l  w  g  r  g  t  l  v  t  v

 771 gagtggtgga ggcggttcag gcggaggtgg ctctggcggt ggcggatcgg atgttgtgat gactcagtct
       s  s  g  g  g  g  s  g  g  g  g  s  g  g  g  g  s  d  v  v  m  t  q  s
                  (G4S)3-linker (15 aa)

 841 ccatcctccc tgtctgcatc tgtaggagac agagtcacca tcacttgcca ggcgagtcag gacattagca
       p  s  s  l  s  a  s  v  g  d  r  v  t  i  t  c  q  a  s  q  d  i  s

 911 actatttaaa ttggtatcag cagaaaccag ggaaagcccc taagctcctg atctacgatg catccaattt
       n  y  l  n  w  y  q  q  k  p  g  k  a  p  k  l  l  i  y  d  a  s  n

 981 ggaaacaggg gtcccatcaa ggttcagtgg aagtggatct gggacagatt ttactttcac catcagcagc
       l  e  t  g  v  p  s  r  f  s  g  s  g  s  g  t  d  f  t  f  t  i  s  s

1051 ctgcagcctg aggattttgc aacttattac tgccaacaat atagtagttt tccgctcact ttcggcggag
       l  q  p  e  d  f  a  t  y  y  c  q  q  y  s  s  f  p  l  t  f  g  g

                                          NotI
1121 ggaccaaagt ggatatcaaa cgtgcggccg cagaacaaaa actcatctca gaagaggatc tgaatggggc
       g  t  k  v  d  i  k  r  a  a  a  e  q  k  l  i  s  e  e  d  l  n  g
                                                   myc tag

1191 cgcacatcac catcatcacc attaataa
     a  a  h  h  h  h  h  h  *  *
           His tag
```

9 Published Abstracts

9.1 Oral Presentation

Parts of this study were presented as an oral presentation at the following conference:

Banaszek, A., Stuhler, G. (2012). Dual Antigen-Restricted Complementation of a Trispecific Antibody Construct for Targeted Immunotherapy of Blood Cancer. *8th Fabisch-Symposium for Cancer Research and Molecular Cell Biology, 3rd Targeted Tumor Therapies*

(Berlin, 21st – 23rd March 2012)

9.2 Poster Presentation

Banaszek, A., Kontermann, R. E., Müller, D. (2008). Generation and Characterization of Costimulatory B7-Antibody Fusion Proteins for Targeted Cancer Immunotherapy. *CIMT, Cancer Immunotherapy, 6th Annual Meeting*

(Mainz, 15th -16th May 2008; based on Diploma thesis)

9.3 Other

Müller, D., Stork, R., Campigna, E., Robert, B., Diebolder, P., Zettlitz, K., Frey, K., Banaszek, A., Rothdiener, M., Kontermann, R. (2009). Bispecific and Bifunctional Antibody Molecules for Targeted Cancer Immunotherapy. *4th Fabisch-Symposium for Cancer Research and Molecular Cell Biology, 2nd Targeted Tumor Therapies*

(Berlin, 1st – 3rd April 2009; taken from Diploma thesis)

10 Publications

Parts of the present study were submitted for publication as part of the following research article (revisions in process for Nature Medicine):

Banaszek, A.*, Bumm, T. G. P.*, Wölfl, M., Vornberger, N., Schwinn, S., Rasche, L., Hönemann, D., Bargou, R. C., Einsele, H., Riethmüller, G., Stuhler, G. A bimolecular T-cell engaging antibody construct for combinatorial cancer immune therapy.

11 Supplementary Notes

The **experimental work** for this dissertation was performed from 1st December 2008 to 31st May 2012 under the direction of Dr. med. Gernot Stuhler at the Department of Internal Medicine II, but principally at the laboratories of Prof. Dr. rer. nat. Harald Wajant at the Division of Molecular Internal Medicine, Julius-Maximilians-Universität Würzburg.

The data and results of this thesis regarding the trispecific antibody were substantially used as a proof of concept in the following **patent specification**: *Dual antigen-induced bipartite functional complementation.* Julius-Maximilians-Universität Würzburg. Patent No. WO 2013/104804.

Furthermore, the development of the trispecific antibody concept presented by this study helped to win the **m^4 Award 2013**: *"Neuartige bi-molekulare T-Zell aktivierende Antikörper für die zielgerichtete kombinatorische Tumor-Immuntherapie (bi-ATAK)"*.

12 Curriculum Vitae

For reasons of data protection the CV has been removed.

Der Lebenslauf wurde aus Datenschutzgründen entfernt.

For reasons of data protection the CV has been removed.

Der Lebenslauf wurde aus Datenschutzgründen entfernt.