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**NOVEL T CELL-ENGAGING, BISPECIFIC ANTIBODIES  
FOR DEPLETION OF  
HUMAN IMMUNODEFICIENCY VIRUS-INFECTED CELLS**

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Dedicated to  
My Parents & My Wife

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# 1. INTRODUCTION

## 1.1. HUMAN IMMUNODEFICIENCY VIRUS-1 (HIV-1)

Of human pathogenic Retroviridae, two Orthoretrovirinae are known today: On the first hand the delta retroviruses with human T-lymphotropic viruses 1 and 2 (HTLV-1 and 2) and on the other hand the lentiviruses with human immunodeficiency viruses 1 and 2 (HIV-1 and 2). Whereas, the HTLV viruses can cause leukemia, the lentiviruses can cause the acquired immunodeficiency syndrome (AIDS).

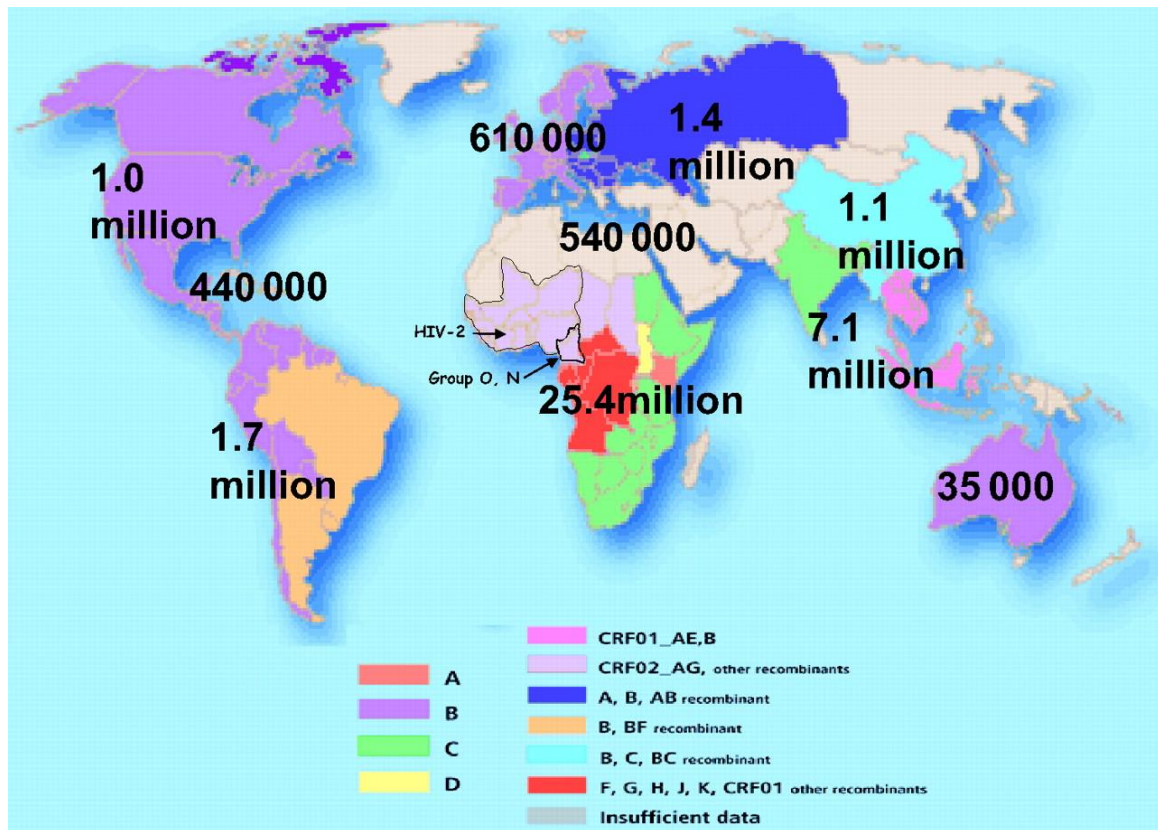
In 1981 the US center for disease control (CDC) reported that five young men previously in a healthy status have developed *Pneumocystis carinii* pneumonia of which two of them died [2]. Two years later, it became apparent that this report was the first scientific publication about HIV. Robert Gallo and Luc Montagnier independently from each other identified HIV to be the cause of AIDS in 1983 [3,4]. Sequencing studies showed that HIV viruses have developed from Simian Immunodeficiency Viruses (SIV) present in West-African monkeys [5]. Since the HIV-1 virus is highly homologous to the chimpanzee SIV (SIVcpz), found in *Pan troglodytes troglodytes*, it is thought to be derived from that virus. HIV-2 is also highly homologous to SIV found in sooty mangabeys (SIVsm) therefore being likely derived from that virus. Heeney et al. [6] published evidences for cross-specific transmissions of these viruses to humans via blood contact of humans to such infected animals. Further viral evolution within the human population led to an increased spread and finally to a worldwide pandemic.

According to the global AIDS report published by the United Nations in 2012 [7], about 35.3 million people were living with HIV. Recent efforts to curtail this pandemic showed positive results, visualized in a decreased number of new infections, a decrease in AIDS-related deaths and the increased number of people living with HIV. This trend is most probably based on prevention initiatives and the improved access to antiretroviral therapy for HIV patients.

HIV-1 is the prevalent HIV virus worldwide and is divided into four groups (Major, New, Outlying, P) with group P being newly identified in 2009 by Plantier et al. [8] from a Cameroonian woman whose HIV strain was closely related to gorilla SIV (SIVgor). The classification of viral isolates is based on sequence differences in their viral envelope protein (env). Inter-group sequence homologies range from 30-50%. Within the major group (group M) the env sequence differs ca. 20-30% [9]. Viruses of the M group are found in over 90% of the HIV infections, displaying the accountability of this group for the worldwide pandemic [8,10-12]. Because of their significant differences concerning their viral envelope protein sequences, the group M viruses have been further divided into nine different subtypes (A-D, F-H, J and K) plus circulating recombinant forms [13], which can be allocated to geographical regions (see Figure 1). The HIV-1 group M



subtypes not only differ genetically, but also consequently show differences in viral fitness and sensitivity to antiretroviral therapy.



**Figure 1: Map of the Global Distribution and Prevalence of HIV-1 Group M Subtypes and HIV-2 Distribution of 2006.** Modified from McCutchan et al. [14].

Given the fact that SIV is highly homologous to HIV-1, the infection with this virus shows comparable disease characteristics and disease progression, allowing *in vivo* studies of the disease for vaccine development and HIV treatments in non-human primate monkeys using a simian/human immunodeficiency virus; consisting of a HIV-1 virus with a SIV env protein (SHIV) [15].

### 1.1.1. HIV-1 GENOME & PROTEOME

Characteristic for all retroviruses, the genome of human immunodeficiency viruses consists of two copies of single stranded ca. 9.8 kb RNA molecules and is mainly coding for the env, gag and pol proteins. These unspliced RNA molecules are comparable to messenger RNA (mRNA) molecules, as they possess a 5' cap structure and a poly adenylated 3' tail. In total, the virus codes for 15 proteins, which are translated from nine

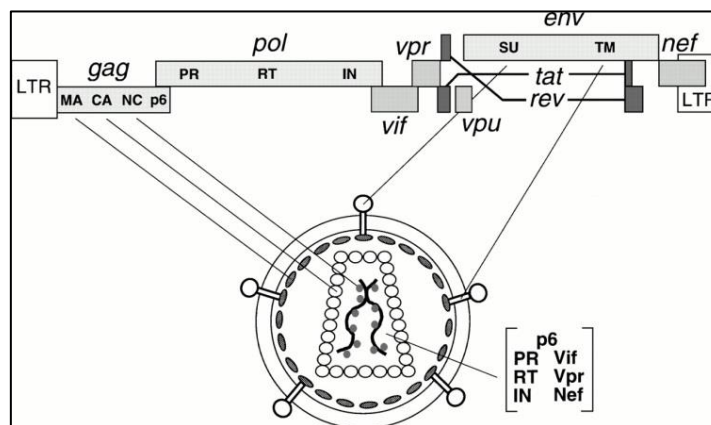
open reading frames and in part are post-translationally cleaved into individual proteins [16]. Differential splice variants of the RNA and ribosome shifting result in the individual viral proteins or the according precursor polyproteins, respectively.

The *env* open reading frame (ORF) codes for the HIV envelope protein (gp160) which is post-translationally cleaved into a surface protein (gp120) and a transmembrane protein (gp41). This protein binds to CD4 and a chemokine coreceptor present on the host cell and consequently initiates the viral entry into the cell by mediating the fusion process of the viral and host cell membrane.

The *pol* ORF codes for the protease, which cleaves the viral polyproteins into individual proteins, the reverse transcriptase, which generates the proviral DNA and the integrase, which integrates the proviral DNA into the host cell genome.

The *gag* ORF codes for four proteins (matrix, capsid, nucleocapsid and p6). The matrix protein (p17) lines the inner side of the viral envelope, the capsid protein (p24) forms the viral capsid within the viral envelope, the nucleocapsid protein (p7) forms the nucleocapsid harboring the RNA genome and the protein p6 directs the accessory *vpr* protein into the virion.

As described by Roy et al. [17] clustering and sequestration of HIV *env* molecules at the plasma membrane is controlled by the *gag* protein ensuring HIV *env* incorporation into viral particles. If HIV *env* is not bound to *gag*, HIV *env* can cause syncytium formation, so direct cell/cell contact via HIV *env* with CD4 of another cell with subsequent fusion of the cells leading to multinucleate enlarged cells.

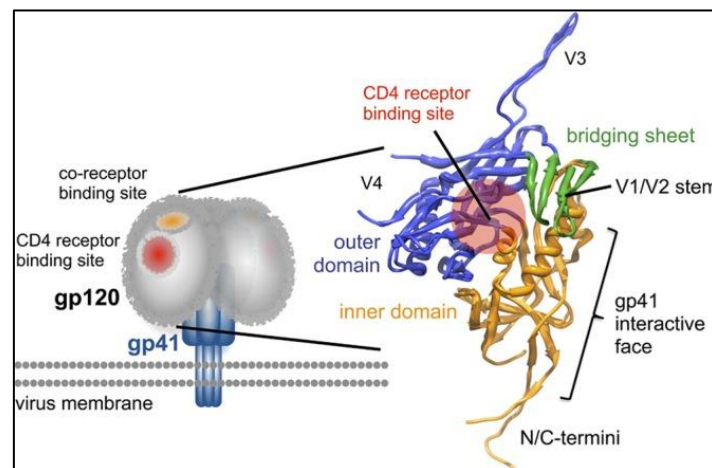


**Figure 2: HIV-1 Genome Structure with the Association of Open Reading Frames to Individual Protein Products and their Localization in the Virion.** The HIV genome consists of: Long terminal repeats (LTR) and open reading frames for *gag* (matrix, capsid, nucleocapsid, p6), *pol* (protease, reverse transcriptase, integrase), *vif*, *vpr*, *vpu* *env* (surface, transmembrane), *tat*, *rev*, *nef* proteins. Modified from Frankel et al. [16].

In addition, the HIV genome codes for accessory regulatory proteins like *vif* (involved in viral assembly/maturation), *vpu* (promoting degradation of gp160/CD4 complexes within the endoplasmic reticulum), *vpr* (transports nucleocapsid complexes to the host cell nucleus after viral entry and uncoating) and *nef* (downregulating CD4 in directing cell-surface CD4 to lysosomes). *Tat* and *rev*, are important regulatory proteins in HIV replication. The *Tat* protein not only increases the efficiency of the transcription of the viral genome, but can also boost the transcription rate of viral genes. *Rev* ensures the export of unspliced RNA to the cytoplasm ensuring the translation of later gene products and the export of full-length RNA for the virion assembly. Long terminal repeats (LTR) in the flanking regions of the genome enable the integrase to integrate the viral genome into the host genome.

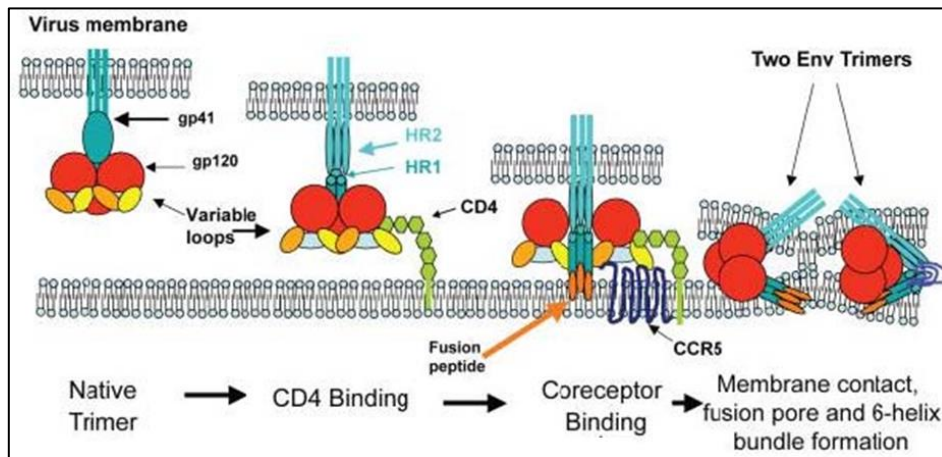
### 1.1.1.1. THE HIV ENVELOPE PROTEIN

The HIV *env* is the only known virus-specific protein that can be found on HIV virions or HIV infected cells. The *env* protein is translated as a precursor protein, which becomes post-translationally cleaved into two individual proteins named surface protein (gp120) and transmembrane protein (gp41) [18]. Prior to its processing the envelope protein is not functional as shown by McCune et al. [19]. Once the *env* protein is processed it gets exported to the cell membrane, where it forms homotrimeric complexes [20]. The extracellular part of one heterodimer is termed gp140.



**Figure 3: Structural Surface Model of the HIV Envelope Protein.** The HIV *env* protein forms homotrimeric complexes on the virus membrane (see left). One HIV *env* protein (on the right) consisting of a non-covalently bound heterodimer of gp120 (blue) and gp41 (yellow). Gp120 forms the outer domain with the exposed variable domains V1-V4, whereas the gp41 protein forms the inner domain of the HIV *env* protein. Credit: Guttman et al. [21].

The HIV env can be divided into five variable domains V1-V5 and six constant domains C1-C6. Whereas all variable domains and C1-C4 are located on the gp120 protein, the C5-C6 domains are located on the gp41 protein. The V1/V2 region forms part of the inner domain, followed by the bridging sheet, which consists of four antiparallel  $\beta$ -strands (two of the inner and two of the outer domain). According to the model of Roux et al. [22], the V1/V2 region shields the V3 loop. The V3 region itself is highly variable but fades into a rather conserved V3-loop harboring the binding site for the coreceptor and is target for neutralizing mAbs. After CD4 binding, the V3 loop with its coreceptor-binding site becomes exposed. The C3 region of gp120, located between V4 and V5, binds to the N-terminal CD4 domain which is immunoglobulin like and interacts with the CDR2 and CDR3 like regions within CD4 domain 1. The C4 region harbors the furin protease site where the precursor protein is cleaved into the individual gp120 and gp41 proteins. The C5 region plays a role in the membrane fusion for viral entry. The C6 region encodes for the transmembrane domain of gp41.



**Figure 4: Schematic Presentation of the Viral Entry Process of HIV.** Scheme of the HIV env dependent membrane fusion of the viral envelope and the host cell membrane. gp120 binds to CD4 that leads to subsequent conformational change of the variable domains 1-3 enabling the coreceptor-binding (CXCR4 or CCR5). The binding of the coreceptor mediates conformational rearrangements of the gp120 and the gp41 protein especially of the heptad regions 1-2 (HR1/2), resulting in the membrane fusion of the viral envelope and the host cell membrane. Modified from Redfield et al. [23].

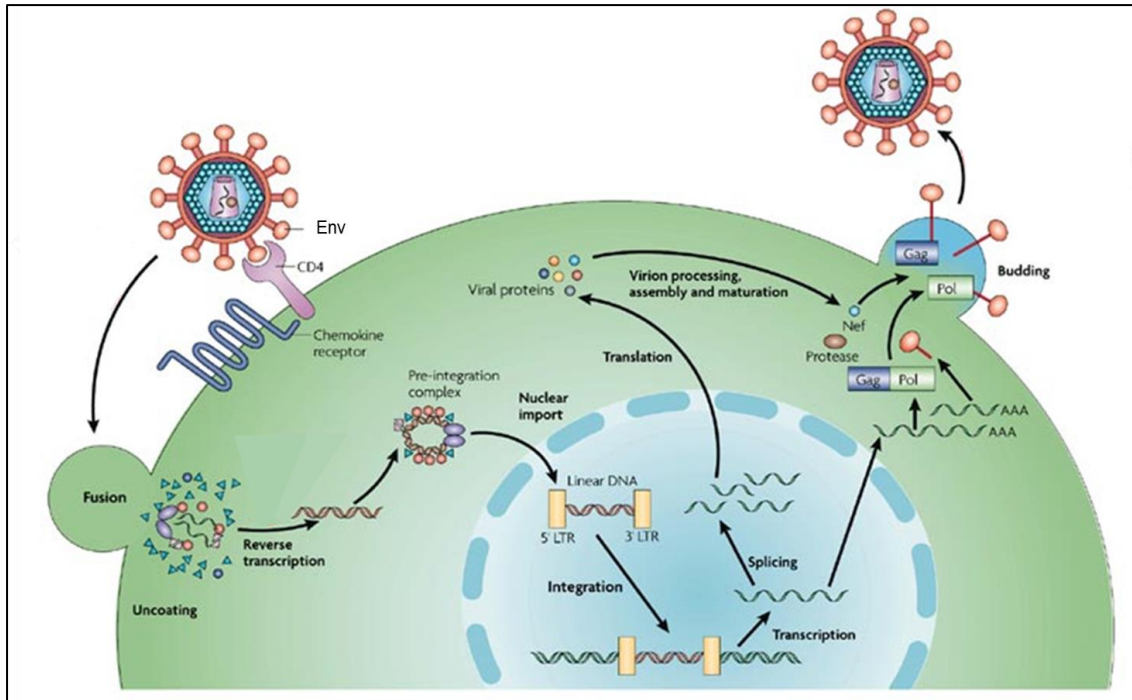
The role of the HIV env in the viral replication cycle is the binding of CD4 receptors (present on T cells, macrophages and monocytes) and chemokine coreceptors (CXCR4 or CCR5) depending on the viral strain's tropism [24,25]. Some viruses are dual-tropic and can bind to CXCR4 as well as CCR5 [26]. The glycoproteins gp120 and gp41 are non-covalently bound to each other and undergo conformational changes upon binding to CD4 [27-29]. In the native conformation of gp120, the coreceptor-binding site is hidden and only gets exposed by binding of gp120 to CD4.

After the initial binding of gp120 to CD4 and the subsequent conformational change enabling the binding to the coreceptor, gp41 changes its conformation and forms the six-helix-bundle formation, mediating the fusion of the viral and host cell membrane [30-32] (see Figure 4).

### *1.1.2. HIV-1 REPLICATION CYCLE*

After the fusion of the viral membrane with the host cell membrane, the viral capsid enters the cell, where the capsid proteins are released and the reverse transcription complex forms, which then reversely transcribes the RNA genome into the proviral double-stranded DNA provirus. After the generation of the provirus, the pre-integration complex is transported into the host cell nucleus. Because of its rather large size (20-30 nm), the import via the nuclear pores is not possible. Therefore, the import is dependent on mitotic action within the cell and especially on the disintegration of the nuclear membrane. This fact plays a major role for an efficient HIV infection, which requires proliferating cells. Within the nucleus, the proviral DNA is then integrated into the host cell genome, mediated by its long terminal repeats and the viral integrase.

Once the viral genome is integrated into the host cell genome, the ORFs can be transcribed by the RNA polymerase II. Due to the error rate of 1:1,000-1:10,000, variants within the viral proteins do commonly appear. Depending on the RNA transcript, these errors can be found temporarily in virions or in case of full length-genomic RNA molecules contribute to viral evolution. Viral RNA splice variants and full-length RNA molecules are exported to the cytoplasm where the viral (poly-) proteins are translated and if applicable subsequently cleaved into individual viral proteins. The viral proteins then assemble at the cell membrane, thereby generating virions, which upon contact with the genomic RNA molecules eventually bud from the cell membrane. The viral protease proteins become activated in the budding process and make the virions mature and infectious by cleaving the polyproteins into the individual proteins, enabling the infection of other target cells [33]. Accessory viral proteins take action on the host cell and on viral proteins at several points within the viral replication cycle, which are reviewed by Frankel et al. [16].



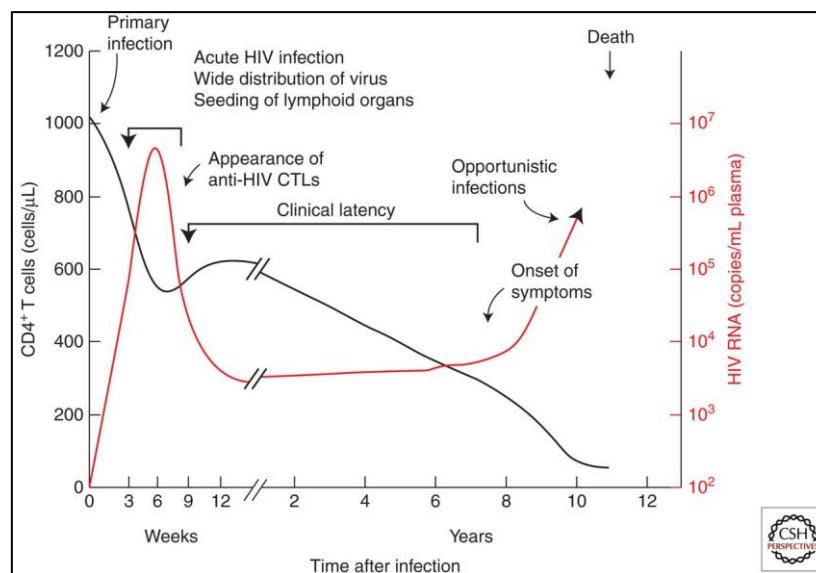
**Figure 5: Scheme of the HIV Replication Cycle.** Binding of HIV virion to CD4 and chemokine receptor, followed by membrane fusion and viral entry into the host cell. Then the viral capsid gets dissolved and the reverse transcription complex forms where the proviral DNA is generated and in form of the preintegration complex is imported into the cell nucleus. Inside the nucleus, the provirus is integrated into the host genome from where transcription of viral genes starts. Viral RNA transcripts get differentially spliced enabling the translation of all viral ORFs in the cytoplasm. Viral proteins gather at the plasma membrane, where they combine with precursor proteins like the gag-pol polyprotein. Together with viral full-length RNA molecules they form virions, which subsequently bud from the cell surface and get mature by proteolytic digestion of precursor proteins enabling the virions to infect new cells. Credit: Han et al. [34].

### 1.1.3. COURSE OF HIV INFECTION & DEVELOPMENT OF AIDS

The course of an HIV infection can be separated into three distinct periods. After transmission of the virus, the incubation phase of the virus usually takes up to four weeks during which the patient is symptom-free. The following primary infection with flu-like symptoms results in solid viral replication, which can be measured as a peak of viral RNA in the plasma, accompanied with a rapid loss of T cells and an immune response. With that peak in viral load, the patient gets systematically infected and HIV reservoirs establish in lymphoid organs. Once the immune response is established, the loss of T cells reaches a plateau phase and the viral RNA peak also drops to a plateau of about  $10^3$ - $10^4$  copies/ml plasma. After that primary infection, which usually takes up to three months, the cell titer and viral load stay stable for up to several years, depending on the patient's immune system and the viral strain. This phase is called clinical latency in which the virus is detectable, but controlled by the host's immune system. After that variable time

of clinical latency, symptoms arise resembling the ones of the primary infection with a characteristic loss of T cells and the rise of the viral titer in the plasma. This second onset of symptoms resembles the development of the acquired immune deficiency syndrome or AIDS, which is most likely caused by the exhaustion of the immune system, which was challenged by the virus over the years of clinical latency. In ca. 50% of HIV patients the coreceptor tropism of the virus changes from CCR5 to CXCR4 over the time of infection, which is thought to be associated with disease progression to AIDS [35].

This direct causality for the development of AIDS, however is still not exactly proofed today. In patients with AIDS, the immune system is no longer able to manage the viral infection efficiently, setting the stage for opportunistic infections, which also cannot be cleared by the immune system leading to fatal complications and ultimately to the patient's death. Generally, the periods of the infection stages represent an average timespan among patients, which can vary significantly between individuals (see Figure 6).



**Figure 6: Timeline of the HIV Infection and Development of AIDS.** Credit: Fauci and Desrosiers 1997 [36]. Primary infection with loss of CD4 T cells and peak in viral load, followed by a clinical latency phase with low constant viral load and slowly decreasing CD4 T cell level. After clinical latency, HIV viral load increases, CD4 T cell decrease further, symptoms arise and opportunistic infections cannot be managed leading to the death.

### 1.1.4. IMMUNE RESPONSE TO HIV INFECTION

Next to the viral fitness of the virus isolate, the immune response of the individual determines the course of the infection. For example, the antiviral response by cytotoxic T-lymphocytes (CTL) is directly linked to the dimension of viremia and survival of HIV patients [37]. There are two types of patients who can manage their HIV infection efficiently without antiretroviral therapy: Long-term nonprogressors, who keep a steady CD4<sup>+</sup> T cell count over five years of infection as well as elite controllers (EC), who manage to keep the viral load below detection limit without antiretroviral therapy. Simultaneously, HIV-specific CTL of regular HIV patients have been characterized and show signs of impaired effector functionality [38] and proneness to apoptosis [39]. Recently, Shai and colleagues revealed that a specific motif within the gp41 protein interacts with the TCR complex and inhibits T cell proliferation and IFN- $\gamma$  secretion *in vitro* and *in vivo* [40]. Larsson et al. reviewed that HIV facilitates the upregulation of immune-inhibiting proteins, subsequently suppressing T cell proliferation upon contact [41]. Therefore, an immunotherapy based on HIV specific BiTE antibody constructs might strengthen the immune response of regular HIV patients to that seen for ECs and circumvent virus-associated impacts on T cell function.

#### 1.1.4.1. Antibodies to the HIV Envelope Protein

As HIV env (gp160) consists of a non-covalently bound heterodimer of two glycoproteins (gp120/gp41), there are two possible target antigens to consider. Gp120 harbors the binding sites for CD4 and the coreceptor-binding site for CXCR4 or CCR5, which is accessible after a CD4-induced conformational change of gp120. Due to its exposed position (i.e., immunogenicity), the number of anti-gp120 specific mAbs exceeds that for isolated gp41 antibodies. Since gp120 is non-covalently attached to gp41, it can be shed and then detected as a soluble protein. Contrary to gp120, gp41 is anchored in the viral membrane. Gp41 alters its conformation upon the conformational change of gp120 after CD4 and the coreceptor-binding. The conformational change of gp41 eventually results in the fusion of the viral and cell membrane of the bound target cell. Gp41 in its native conformation is covered by gp120, however exposed to the immune system upon gp120 shedding. In close proximity to the plasma membrane the gp41 proteins show a conserved epitope called membrane proximal external region (MPER) to which broadly neutralizing antibodies have been identified.

Several antibodies have been identified, which either bind to the surface protein or to the transmembrane protein. In the following paragraphs anti-HIV envelope antibody classes (as illustrated in Figure 7) and exemplary antibodies are presented: Antibodies of these classes have been used in *in vivo* virus neutralization studies, for the generation of chimeric antigen receptors (CARs) or antibody-drug conjugates (ADCs).



### **Anti-HIV gp120 Antibodies**

#### ***Antibodies to V1/V2 domains***

The recently identified monoclonal antibody P9 targets the V1/V2 domains of gp120 [42] and is able to neutralize up to 77% of all major circulating HIV subtypes [43].

#### ***Antibodies to the CD4 Binding Site***

The monoclonal antibody B12 is broadly neutralizing (41% of major circulating HIV clades [44]) and well described in the scientific community. Its epitope shares the CD4 binding site and the V2 domain of gp120 [45,46].

A more recently discovered antibody is VRC01, whose epitope overlaps to 98% with the CD4 binding site on gp120 [47] and therefore is able to neutralize up to 91% of major circulating virus clades [44].

PGV04 also binds to the CD4 binding site, without enhancing the binding of mAb 17b, which binds specifically to the CD4-induced epitope. It is able to neutralize about 88% of the major HIV strains (n=162) tested [48].

The natural receptor of HIV, human CD4 can also be used to target gp120. This protein consists of four extracellular Ig-like domains, whereas the first two N-terminal domains can be functionally expressed and are able to bind gp120. Chen et al. [49] have engineered the N-terminal domain of human CD4 (domain 1) by the introduction of five point mutations in order to express the first domain solely and functionally. Experiments have shown that the first Ig-like domain of CD4 is the essential domain for the interaction with gp120 and that the omission of the second domain can lower side effects such as the interaction of CD4 with MHC class II complexes.

#### ***Antibodies to the CD4-Induced Binding Site***

The class of CD4-induced antibodies, bind to a hidden part of gp120, which becomes exposed upon the CD4-induced conformational change. This induced site on gp120 harbors the binding site for the coreceptor CXCR4 and/or CCR5. As an example, the antibody 17b binds to the CD4-induced epitope [50].

#### ***Anti-gp120 V3 Antibodies/Glycan-Specific Antibodies***

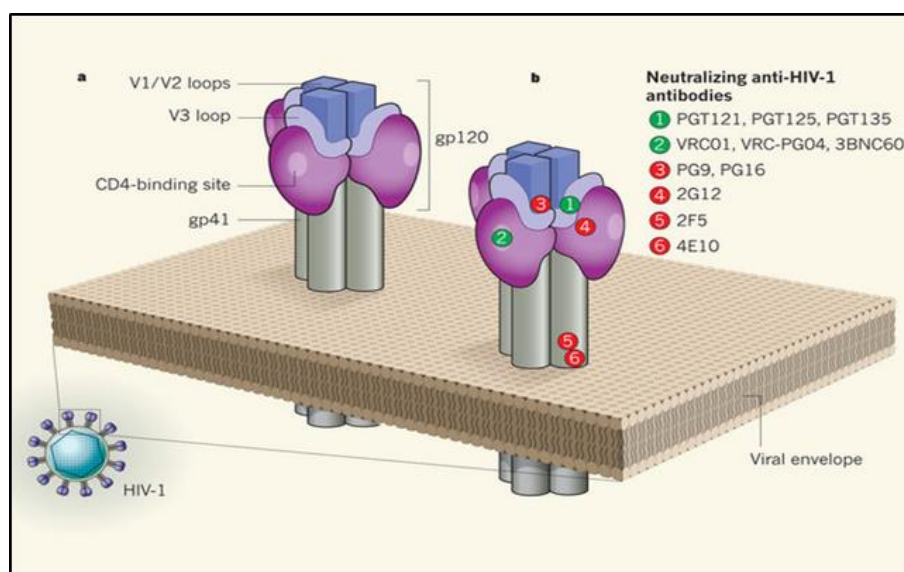
Antibodies against the V3 loop of gp120 interfere with the coreceptor-binding of the env resulting in the neutralization of the virus [51,52]. In 2011, Walker et al. identified a series of monoclonal antibodies (PGT 121-145) from elite-controllers, which interact with glycan-residues and do compete with 2G12 (see next paragraph), but also compete with

V3-specific antibodies [43,53] and can neutralize up to 78% of major circulating HIV isolates. Whereas most regular antibodies bind to amino acid epitopes, some are able to bind to the glycan shield of gp120 such as the mAb 2G12 [54]. 2G12 is reported to neutralize about 32% of all major circulating HIV strains [43].

### *Anti-HIV gp41 Antibodies*

#### *Antibodies to the Membrane proximal region (MPER)*

Broadly neutralizing antibodies specific for the transmembrane protein gp41 have been mapped to the MPER. As the name indicates this epitope is located directly at the plasma membrane [55]. Accessibility of this epitope is described to be CD4 dependent [56,57]. In addition, the conformational change within gp41 increases the accessibility of this epitope. Prominent antibodies defined as MPER specific are 2F5, 4E10 and 10E8 [58-60] (see Figure 7). 4E10 is reported to neutralize up to 96% of all major circulating HIV subtypes [43]. However, all identified anti-MPER antibodies show auto reactivity to phospholipids [61]. Therefore, this thesis focuses on gp120 specific antibodies (see Table 2).



**Figure 7: Model of the Trimeric Form of HIV env Heterodimers with Indicated Epitopes for Identified Broadly Neutralizing Antibodies.** Credit: Clapham et al. [62].

Due to the high number of isolated monoclonal antibodies to gp120, the described antibodies are only an excerpt of the available candidates and modifications thereof. The mentioned epitopes and candidates are the most prominent examples and should give a basic introduction into available gp120 antibodies (for more antibodies see [63,64]). Indicated neutralization breadths must not be interpreted as absolute numbers, since they depend on the virus panels tested in the respective underlying study and can therefore vary between publications.

## 1.2. HIV-1 THERAPY

To date, the infection with HIV is persistent. Despite many efforts in the past decades and some reported functional cures, the momentary standard therapy cannot cure the patients from HIV infection, but it can significantly improve the life of patients by extending the phase of clinical latency thereby shifting the HIV infection to an almost chronic disease. Next to post infection medication, pre-exposition prophylaxis and vaccination against HIV are active fields of research to curtail the pandemic.

### *1.2.1. COMBINED ANTIRETROVIRAL THERAPY*

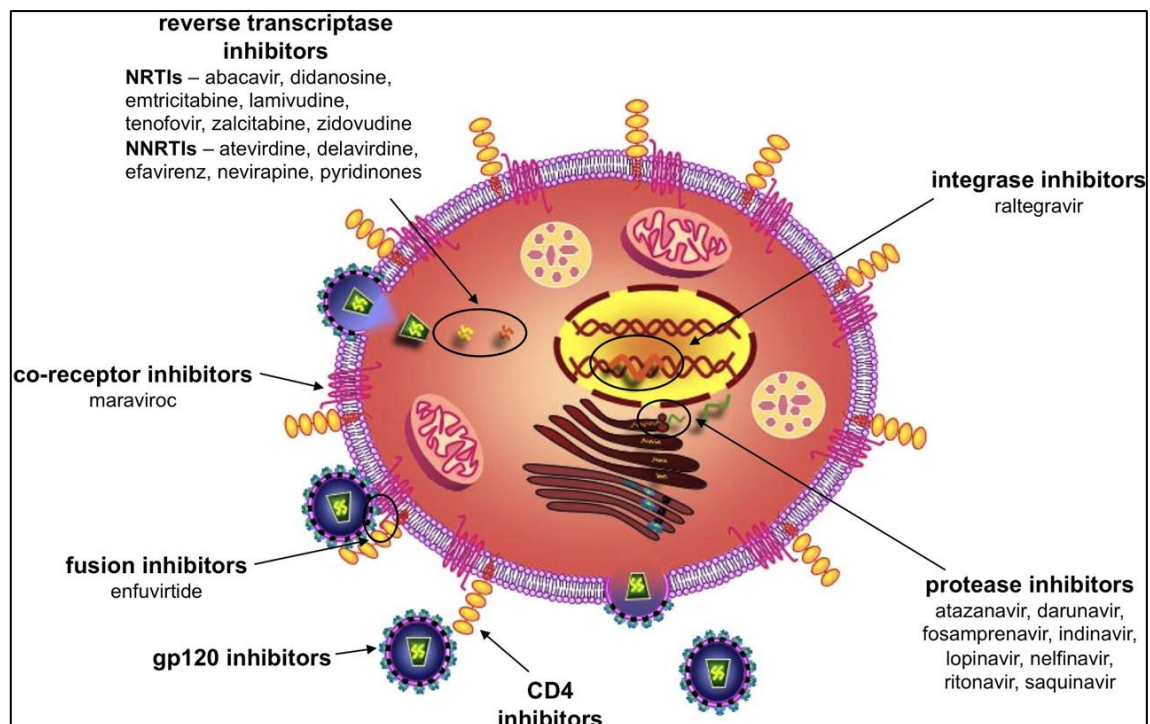
The current gold standard is a combined antiretroviral therapy (cART). In this combination, product classes used in the highly active anti-retroviral therapy (HAART) are combined to treat HIV infection. An exemplary overview of licensed active components of the different anti-retroviral drug classes, which interfere at different points within the viral replication cycle are shown in Figure 8.

As the name fusion inhibitors implicates, these molecules interfere with the fusion process of the virion with the host cell membrane (e.g., T-20; [65]). Likewise, coreceptor antagonists bind to coreceptor proteins (CXCR4/CCR5) and block the binding of the coreceptor to the HIV env protein thereby inhibiting subsequent membrane fusion. CD4 or HIV gp120 can also be blocked by specific inhibitors, which leads to blocking of the fusion.

Once the virus has entered the cell, reverse transcriptase (RT) inhibitors can block the activity of the RT and thereby prevent the generation of the provirus. Here, different inhibitors like nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs), or non-nucleoside reverse transcriptase inhibitors (NNRTIs) can block the elongation of proviral DNA polymerization.

Integrase inhibitors can block the integration of the viral genome (provirus) into the host genome.

After the integration of the HIV genome into the host cell genome, protease inhibitors can block the maturation of precursor or polyproteins into individual viral proteins, inhibiting the formation of mature and infectious virions. A combination of these antiretroviral therapeutic classes can efficiently block viral replication and lower the viral load below detection limits. This inhibition of viral replication at strict compliance allows the HIV patients to live an almost normal life and shifts the causes for death towards non-AIDS related malignancies [66].



**Figure 8: HIV Replication Cycle and Classes of Antiretroviral Drugs with Licensed Active Components.** Fusion inhibitors, gp120 inhibitors, CD4 inhibitors and co-receptor antagonists can block the viral entry into the cell. Once the virus has entered, the reverse transcriptase inhibitors can block the reverse transcription of the RNA genome. The integrase inhibitor can block the integration of the generated provirus into the host cell genome. After translation of the viral protein precursors, the protease inhibitors can block the maturation of the polyproteins into individual proteins and therefore hinder the maturation of virions. Credit to Pirrone et al. [1].

### 1.2.2. FUNCTIONAL CURES

Two functional cures of HIV have been reported to date. The first patient who was cured from HIV infection was a patient suffering not only from HIV infection, but also from relapsed acute myeloid leukemia (AML). Because of his leukemia, the patient received several rounds of chemotherapy and subsequent bone marrow transplantation. The donor of the engrafted bone marrow was homozygous for a rare deletion within the CCR5 gene (CCR5  $\Delta$ 32). This deletion of 32 amino acids, make these people immune to CCR5-tropic HIV strains, as CCR5 is no longer expressed on the cell surface [67]. Therefore, the engrafted bone marrow replaced his CD4<sup>+</sup> immune cells, depleting the original non-mutated CCR5<sup>+</sup> cells over time, so that this patient was eventually not only cured from leukemia, but also from his HIV infection since the virus was not able to establish HIV reservoirs [68,69]. This reported case, could mean a future therapy option for more patients. However, this outcome has to be shown repeatedly first to be established as a therapy and only becomes a possible therapy for those finding a matching CCR5  $\Delta$ 32 donor. Gene therapeutic approaches modifying CCR5 genes in autologous cells, would make this option applicable to more patients [70].

Another study from Henrich et al. [71] reported about the bone-marrow transplantation of two HIV-1 patients with a matching donor, homozygous for the wild-type CCR5 gene. Under ongoing cART, a post-transplantation decline of HIV RNA, cell-associated HIV DNA as well as anti-HIV antibody titers was observed. This observation suggests that the cART therapy is able to prevent the infection of the new donor cell population. This effect seems comparable to the post exposition prophylaxis, where potentially infected patients receive cART immediately after exposure to avoid HIV infection [72].

### 1.2.3. IMMUNOTHERAPEUTIC APPROACHES

Next to the classical approaches used in the current gold standard, immunotherapeutic approaches are developed to treat and potentially cure patients with HIV infection.

#### **Vaccination**

The engineering of HIV envelope proteins mimicking the *in vivo* structure of gp120, which are able to induce neutralizing antibody is the ultimate goal of vaccination research. However, the complex structure and conformational rearrangements of the env protein challenge the scientific community to provide a stable vaccine presenting the epitopes necessary for the induction of an efficient anti-HIV immune response [73].

### ***Histone Deacetylase Inhibitors (HDACI) – Reactivation of Viral Replication***

The reactivation of senescent T cells in order to reactivate viral replication in infected cells is tried by using inhibitors to histone deacetylases (HDACIs). The principle of the HDAC inhibition is to block the deacetylation of histones, thereby blocking the tight packaging of the genomic DNA by the histones. Since gene expression is regulated by the de-/acetylation of histones, the blocked deacetylation might help in restoring viral gene expression or block the latency of HIV provirus. The first HDACI, approved by the FDA, is licensed for the treatment of cutaneous T cell lymphoma. In the HIV context, the aim is to not only reactivate viral replication, but also to drive cells into a lytic cycle by causing overexpression of viral genes [74,75].

### ***TRE Recombinase - Excision of Viral DNA***

Another approach is the introduction of a lentivirus coding for the TRE Recombinase, which is an enzyme able to excise the HIV DNA provirus from infected cells. This approach would be able to cure infected cells by depleting the viral genetic information and has been shown to have an antiviral effect in a relevant mouse model [76].

### ***Antibody Neutralization Studies***

In experiments aiming to control HIV infection by neutralizing antibodies, engineered antibodies and combinations of anti-HIV antibodies have been applied to HIV positive cells *in vitro* [43] and have also been tested *in vivo* [77,78]. These results suggest that the sufficient administration of a mixture of anti-HIV antibodies can lower the viral load and number of HIV positive cells [79]. According to Barouch et al. the application of two broadly neutralizing antibodies to chronically infected rhesus macaques not only lowered the viral load below detection limits, but in some animals showed a prolonged antiviral effect after discontinuation of antibody exposure [80,81].

Soluble CD4 molecules (of domains 1 and 2) have also been used in clinical studies for neutralization of HIV virions, but failed due to the high sCD4 concentrations needed for efficient virus neutralization [82].

### ***Antibody Drug Conjugate (ADC)***

Recently Denton et al. [83] revealed that viral replication is still ongoing under ART. They were also able to show an additional antiviral effect in combination with ART, when applying an antibody drug conjugate directed to the HIV env to HIV infected BLT mice. Their result shows that even under ART HIV env<sup>+</sup> cells can be targeted by an antibody-based cytotoxic therapy targeting HIV env.

### ***Janusins - Bispecific Antibody Constructs***

In 1989, Traunecker et al. [84,85] tested the possibility of targeting gp120 positive cells with a bispecific antibody construct (termed Janusin) consisting of the first two N-terminal domains of human CD4 and an anti-human CD3 scFv-C $\kappa$  construct. In his publication, the bispecific antibody showed cytotoxic activity using T cell clones and HIV infected Jurkat cells *in vitro*. Using the Janusin antibody, Traunecker moreover observed target-independent lysis of uninfected Jurkat cells at Janusin concentrations 100 times higher than the concentration needed for the lysis of infected cells. In the publication, this effect was ascribed to the dimer content present in the Janusin preparation. No more related literature can be found by Traunecker et al. pursuing their antibody construct, suggesting the abandonment of their approach.

### ***Genetic Engineering of T Cells – Chimeric Antigen Receptor***

The gene therapeutic option with the introduction of chimeric antigen receptors (CAR) into autologous cytotoxic lymphocytes specific for the HIV env was shown to be functional. Masiero et al. [86] showed specific antiviral activity of CTLs equipped with CAR molecules *in vitro* by fusing an anti-gp120 (F105) scFv to the CD8 $\alpha$  hinge region and the T cell receptor  $\zeta$ .

## 1.3. BISPECIFIC T CELL ENGAGER (BiTE<sup>1</sup>) TECHNOLOGY

In 1995, Mack et al. [87] have published their finding of a small bispecific antibody construct on a single polypeptide chain, which is able to mediate high tumor cell cytotoxicity. In contrast to bispecific antibodies, which have already been successfully tested before, this antibody construct against CD3 $\epsilon$  and epithelial 17-A1 antigen showed superior activity on relevant cell lines *in vitro* without the need for T cell co-stimulation. Since then, this format has been further developed, the original CD3 binding specificity has been de-immunized and meanwhile exchanged to a human anti-human CD3 scFv. BiTE antibody constructs of the different platform versions have been successfully used for several target antigens (see Figure 10). The principle of recruiting T cells by bispecific antibodies or antibody constructs is not a unique feature of the BiTE technology anymore, other antibody specificities and bispecific antibody formats have been identified that are able to recruit T cells [88]. However, the early development of the BiTE technology, the successful clinical proof of concept in patients and the gathered treatment experience with patients, who have benefited from this technology, mark the advantaged status of this technology.

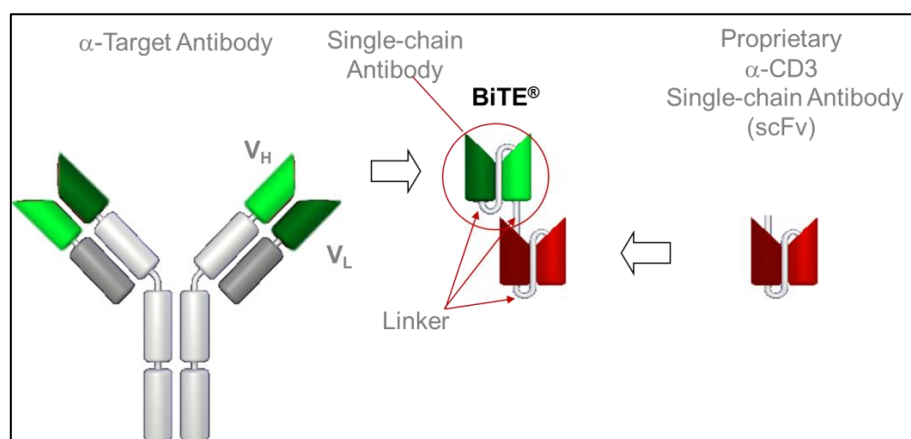
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<sup>1</sup> BiTE<sup>®</sup> is a registered trademark of AMGEN Inc.

### 1.3.1. DESIGN

The BiTE or bispecific T cell engager is a bispecific antibody construct, which consists of the variable domains (VH/VL) of two monoclonal antibody moieties in the scFv format on one single polypeptide chain.

The variable domains within each scFv are linked by a 15 AA glycine/serine linker ( $G_4S$ )<sub>3</sub>. While the N-terminal scFv is directed against the target of interest, the second scFv is linked via a  $G_4S$  linker to the first scFv and directed against a CD3 $\epsilon$  epitope.

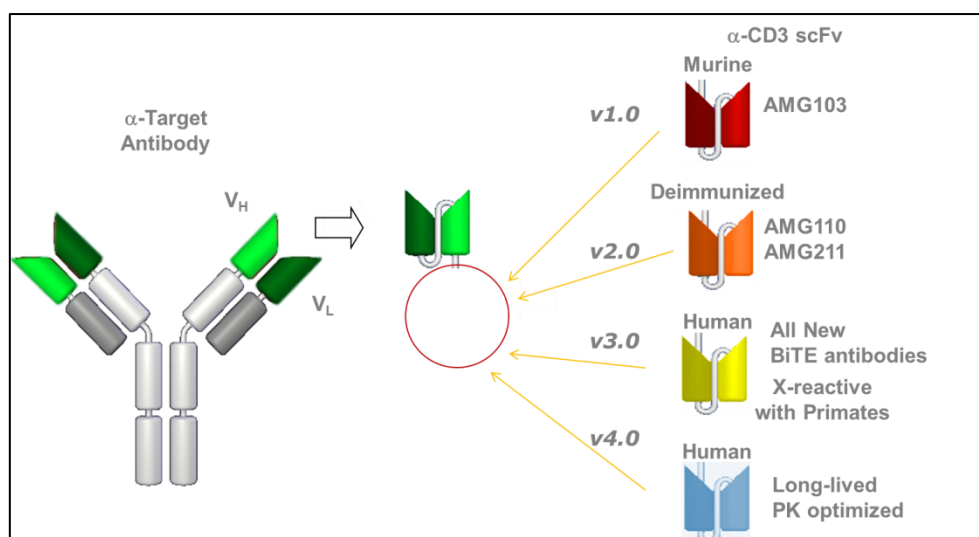


**Figure 9: Overview of the Construction and Design of BiTE Antibody Constructs.** Illustrated transfer of variable domains of an anti-target monoclonal antibody into the scFv format and its combination with a proprietary anti-CD3 scFv on a single polypeptide chain linked by an intra-domain ( $G_4S$ )<sub>3</sub> and an inter-domain  $G_4S$  linker peptide.

### 1.3.2. BiTE PLATFORM

The first version of the BiTE technology platform led to the first BiTE product candidate (MT103/AMG103), which has the most advanced development stage today. This BiTE targets CD19 and human CD3 $\epsilon$  via a murine scFv. The v2.0 BiTE platform comprised a de-immunized murine anti-human CD3 scFv and led to the BiTE antibody constructs AMG 110 (targeting human epithelial cell adhesion molecule (EpCAM) and AMG 211 (targeting carcinoembryonic antigen (CEA)). See Figure 10 for BiTE development candidates. All newer BiTE development programs use the third version of the BiTE platform comprising a human anti-human CD3 $\epsilon$  scFv, which is cross-reactive with primate CD3<sup>+</sup> cells, enabling the possibility of combined toxicity/efficacy *in vivo* studies in case of a cross-specific target-binding moiety. The v4.0 is the latest platform version offering prolonged half-life of BiTE antibody constructs *in vivo*.

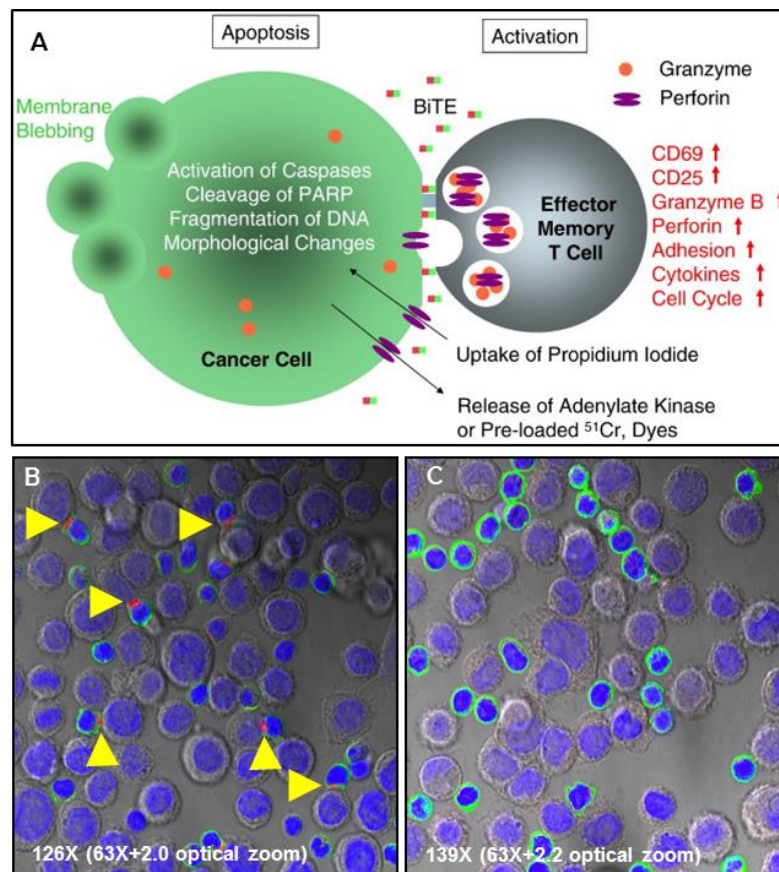




**Figure 10: Versions of the BiTE Platform Technology.** The proprietary anti-CD3 binding specificity in form of an anti-CD3 scFv was further developed since the funding of the BiTE technology. The first version (v1.0) consists of a murine anti-human CD3 scFv and is basis for AMG103, the second version comprised a de-immunized version of the anti-human CD3 scFv used for AMG110 & AMG211. With the third version the CD3 binding moiety changed to human anti-human CD3 scFv, which is fully cross-reactive with primate CD3. This version is used for the clinical candidate AMG212/BAY2010112. The latest version (v4.0) of the BiTE platform comprise PK-optimized BiTE antibody constructs, offering extended half-lives compared to earlier versions.

### 1.3.3. MODE OF ACTION

The human anti-CD3 $\epsilon$  specificity of the BiTE is able to bind CD3<sup>+</sup> effector cells and link them to target antigen positive cells via the anti-target specificity (e.g., tumor cells). CD3 proteins are associated with T cell receptors (TCRs) on effector cells become cross-linked with target antigens on target cells (e.g., cancer cells). Upon the linking of the effector cell with the target antigen positive cell by a critical number of BiTE antibody constructs leads to subsequent T cell activation (e.g. increase of CD25, CD69) and formation of a cytolytic synapse. Once the cytolytic synapse is established, perforins and granzymes are transferred into the target cell initiating the apoptosis of the target cell. This effect can be analyzed *in vitro* by the uptake of Propidium Iodide or release of <sup>51</sup>Cr labeled target cells.



**Figure 11: The Mode of Action of BiTE Antibody Constructs.** (A) Model of the BiTE mode of action: The cancer cell (green) is bound by BiTE antibody constructs, which also bind to a cytotoxic T cell via CD3 (gray). Upon sufficient cross-linking of TCR complexes, the T cell becomes activated and a cytolytic synapse is formed in which cytotoxic granula are transferred to the cancer cell. Perforin and granzyme B induce subsequent apoptosis of the cancer cell. Modified from Nagorsen et al. [89] (B) Picture was taken by a confocal laser-scanning microscope (CLSM) displaying cytolitic synapses formed by T cells (green circles) in the presence of BiTE antibody constructs. Several T cells can attack a single target cell. Moreover, one T cell can target more than one target cell at the same time as can be depicted from this photography. (C) T cells and target cells in the absence of BiTE. As can be seen no cytolitic synapses are formed. Modified from Ludmann et al. [90].

As shown by Hoffmann et al. T cells equipped with BiTE antibody constructs screen surrounding cells and can perform serial lyses of target positive cells upon cross-linking and subsequent TCR clustering by the BiTE antibody constructs [91].

BiTE-mediated T cell stimulation can be measured by standard activation markers and leads to T cell proliferation. For the BiTE-mediated redirected lysis of target cells, no co-stimulatory proteins are required [92]. Furthermore, the BiTE activity does not require MHC class I complexes or the presentation of peptide antigen [93]. The BiTE antibody constructs' recruiting potential is not limited to specific T cell clones or to TCR specificities. BiTE antibody constructs can recruit and use a diverse number of T cell subsets.

The main contribution for the redirected lysis comes from primed T cells of the CD8<sup>+</sup>/CD45RO<sup>+</sup>, CD8<sup>+</sup>/CD45RA<sup>+</sup> and CD4<sup>+</sup> T cell phenotype, whereas naïve T cells contribute very little to the BiTE-mediated redirected lysis [94].

#### 1.3.4. CLINICAL APPLICATIONS

To date, all BiTE development efforts in clinical studies target oncological diseases. The clinically most-advanced BiTE antibody is blinatumomab (AMG 103, formerly MT 103). This BiTE candidate targets human CD19<sup>+</sup> cells and is developed to treat B cell malignancies (i.e., acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL)) [95,96]. In NHL patients, blinatumomab showed regression of tumor cells in all treated patients (n=7) [97]. In a phase I/II study of pediatric patients, Blinatumomab showed antitumor activity in relapsed/refractory B cell precursor acute lymphoblastic leukemia (BCP-ALL) [98,99]. This antibody is currently analyzed in a pivotal study and expected to be marketed in the near future. The results so far, are showing high response rates of patients. Remarkably some patients, who did not benefit from the current standard of care (chemotherapy), showed responses to the BiTE treatments and enduring relapse-free survival [100,101].

MEDI-565/AMG211 is a tumor-targeting BiTE candidate targeting the carcinoembryonic antigen (CEA). This antigen was reported to be present on colorectal, breast and lung cancers. Among the identified CEA BiTE antibody constructs, a small group comprising MEDI-565/AMG211 revealed insensitivity towards soluble CEA proteins, strengthening the target specificity towards cell bound CEA [102]. This BiTE candidate is currently in a phase I clinical trial for the treatment of gastrointestinal adenocarcinoma.

In contrast to leukemia-related diseases, the BiTE candidate (AMG212/BAY2010112) is developed to treat patients suffering from prostate cancer, targeting the prostate-specific membrane antigen (PSMA). This BiTE antibody showed anti-tumor activity in human prostate xenograft mice and is currently tested in a phase I clinical trial [103].

AMG110 is under clinical investigation to determine its potential in targeting human EpCAM present on solid tumors. Its potential has been shown *in vitro* and *in vivo* in mediating redirected lysis of EpCAM positive primary human pancreatic cancer cells and cancer stem cells (CSC) [104,105].

## 1.4. HALF-LIFE EXTENSION OF THERAPEUTIC MOLECULES

Regular BiTE antibody constructs have a molecular weight of about 52 kDa. Due to this small size compared to other biologics, like recombinant IgG molecules, they experience a much shorter half-life *in vivo*. Generally speaking, most proteins smaller than 60 kDa are subject to renal filtration, limiting the exposure time of these molecules to a few hours [106]. This high turnover of small-sized therapeutics like BiTE antibody constructs allows to steer their exposure almost in an on and off mode with a time shift of a few hours. This characteristic offers a clear safety benefit in contrast to larger molecules like immunoglobulins. On the other hand, in order to maintain a constant BiTE plasma level, the short half-life of the BiTE requires continuous supply of BiTE antibody constructs by pumps or infusions and regular drug container exchange by professional staff, which consequently leads to higher immediate costs for the healthcare systems. Assuming that the therapy is compatible, handing patients a device that allows them to self-inject the therapeutics subcutaneously on a daily basis would decrease the costs and increase the comfort for patients. However, the prerequisite for that kind of therapy would be BiTE antibody constructs with an extended half-life to ensure constant effective exposure levels between less frequent applications.

Extended half-lives can be achieved in protecting molecules from proteolytic digestion and renal filtration. Acetylating the termini of molecules is one way to inhibit proteolytic degradation. However, there are several more ways of protecting proteins from degradation. Increasing the hydrodynamic volume or to increase the molecular weight of the protein to prolong the protein half-life are common methods. Another way is the fusion of a binding tag to long-lived proteins or to proteins involved in protein-recycling to the molecule of interest in order to increase its half-life [107].

### 1.4.1. INCREASING THE HYDRODYNAMIC VOLUME

#### 1.4.1.1. PEGylation

A common method to elongate the drug exposure *in vivo* is to conjugate the drug to polyethylene glycol (PEG). This method has been developed by FF. Davis [108,109] in 1977 and is commonly used for peptide or protein-based drugs. The elongated half-life is partly achieved due to higher stability concerning proteolytic digestion and an increase in the effective size of the PEGylated molecule, which protects it from renal filtration. Many PEGylated drug versions also experience higher efficacy and safety profiles [110].

### 1.4.1.2. PASylation

This technique was developed by Skerra et al. [111] and uses the same principle as the PEGylation of molecules with the difference that the PASylation is based on natural amino acids. PAS stands for proline, alanine and serine; repeats of these natural amino acids increase the effective size of the molecule of interest, offering similar protection from renal filtration as PEGylated proteins. The great advantage of this method is that the sequence information of the PAS-tag can be added to the ORF, which codes for the biomolecules of interest. This means that the biomolecule can already be expressed in its PASylated form, omitting the necessity of additional process steps after the purification of the biomolecules as required for the PEGylation. The amino acids used for the PASylation are natural amino acids, which can be degraded in contrast to PEG, which is not bio-degradable.

### *1.4.2. INCREASING THE MOLECULAR SIZE*

Fusing the molecule of interest to the Fc portion of an immunoglobulin G increases the effective size of the molecule again resulting in stabilization concerning proteolytic digestion and renal filtration. Next to the size-related effects, the wild-type Fc portion also harbors epitopes to other effector cells (e.g., NK cells) which has to be considered and offers recycling by the neonatal receptor (FcRn) (for details see 1.4.3.2). Other protein scaffolds (e.g., Protein A, lipocalins) have also been pursued and are reviewed here [112,113].

### *1.4.3. ADDITION OF BINDING TAGS TO ABUNDANT PROTEINS*

The increase in size by additive protein domains (Fc-fusion) or increase in hydrodynamic volume by molecules (PEG-, PASylation) offers a significant increase in size and the mentioned advantages concerning proteolytic digestion and renal filtration. An increase in size however interferes with the distribution of the protein and its ability to penetrate tissues as reported for proteins fused to different sized dextrans [114]. A possibility to circumvent this possible effect is the addition of small essential binding tags to the molecule enabling the binding to long-lived abundant proteins (e.g., human serum albumin) resulting in the protection of the molecule from degradation by benefiting from the FcRn recycling and minimized renal filtration.

### **1.4.3.1. Albumin Binding Peptides**

Adding an albumin binding peptide (ABP) like SA21 to the BiTE molecule, could increase the BiTE half-life as suggested by Dennis et al. [115] and others [116]. The half-life of human serum albumin (HSA) is about 19 days in human, thus offering a good basis to protect the ABP-tagged molecule from degradation/renal filtration [117-119]. Binding to HSA can also enhance the molecule's distribution [116]. Furthermore, HSA is also recycled by the neonatal receptor [107], which contributes to the extended half-life of HSA [120]. Additional engineering of the individual affinities to the target, CD3 and HSA could therefore lead to unaffected BiTE activity by simultaneously increased half-life.

### **1.4.3.2. Neonatal Receptor Binding Tags**

After unspecific endocytosis, Fc-fused proteins or Immunoglobulin G proteins can bind to the neonatal receptor (FcRn) at an acidic pH (pH 6.0) present in the endosomes. Binding to the FcRn allows these protein to be recycled and released at the cell membrane (pH 7.4), whereas all unbound protein is degraded in lysosomes. FcRn binding and subsequent exocytosis result in an prolonged exposure of these proteins in the organism [121]. Binding of the FcRn can also enable the crossing of cellular barriers (e.g., increased uptake from the gut lumen (transcytosis)). FcRn binding peptides have either been isolated from libraries [122,123] or have been isolated from Fc portions [124]. The usage of these FcRn binding tags allows the prolongation of the drug's half-life by keeping the molecular weight almost constant and minimalizes other Fc-portion related interactions like binding to NK cells.

## 1.5. AIMS OF THESIS

Next to the existing proof of concepts and successful application of the BiTE technology in treating oncological diseases, the aim of this thesis was to proof feasibility for BiTE antibody constructs to redirect lysis of HIV infected cells *in vitro* and *in vivo* using a relevant mouse model.

Therefore, antibodies to the HIV env protein should be identified and transferred into the BiTE context. Among these BiTE antibody constructs were scFv-based and CD4 protein domain-based BiTE antibody constructs. The characterization of a non-scFv-based target-binding moiety (consisting of the first two N-terminal CD4 domains 1+2) in the BiTE context should show the feasibility of non-scFv-based BiTE antibody constructs next to canonical scFv-based BiTE antibody constructs generated from well-known anti-HIV env mAbs B12 and VRC01. These BiTE antibody constructs were characterized for their intrinsic protein stability, affinity to HIV gp120 and human CD3 as well as for their potential to mediate redirected lysis *in vitro* using HIV gp140 transfected CHO cells and Jurkat T cells. The antiviral activity of the CD4(1+2) BiTE candidate should be demonstrated using HIV infected PBMCs and monocyte-derived macrophages (MDMs) *in vitro*. The antiviral activity should also be shown using a relevant mouse model representing the antiviral effect and potential of the BiTE technology *in vivo*.

With regard to the described target-independent cytotoxic activity of a bispecific CD4-based antibody construct (i.e., Janusin) when targeting Jurkat T cells [125], the applicability of the BiTE technology to redirect lysis to primary T cells should be determined evaluating potential limits in specificity and stability of BiTE monomers at high concentrations.

To enhance the half-life of the CD4(1+2) BiTE, the ideal position of an albumin binding peptide within the BiTE molecule should be identified by testing the affinity to HIV gp120, human serum albumin, human CD3 and the bioactivity of BiTE variants *in vitro*.

## 2. MATERIAL

### 2.1. ANTIGENS

#### 2.1.1. SOLUBLE PROTEINS

rgp120 HIV-1 <sub>IIIB</sub> (Euk)	Immunodiagnostics, Inc.
rgp130 SIV <sub>mac251</sub> (Euk)	Immunodiagnostics, Inc.
Human CD3 x human Fc fusion protein	Kindly provided by colleague Dr. Roman Kischel
Human Serum Albumin	Sigma Aldrich

#### 2.1.2. HIV ENV FUSION PROTEINS FOR RECOMBINANT EXPRESSION

HIV-1 <sub>HXB2</sub> gp140	P04578 (ENV_HV1H2); AA 33-684
HIV-1 <sub>SF162</sub> gp140	P19550 (ENV_HV1S1); AA 32-675
SIV <sub>mac251</sub> gp140	P08810 (ENV_SIVM2); AA 20-696
Human Epithelial Cell Adhesion Molecule (EpCAM)	P16422 (EPCAM_HUMAN); AA 266-314

Indicated viral antigen domains were fused to the indicated human EpCAM domains. Accession numbers refer to the protein knowledgebase - [www.uniprot.org](http://www.uniprot.org)

### 2.2. ANTIBODIES & CELL DYES

Anti-human CD3-PE, 1:200	BioLegend
Anti-human CD8-BV421, 1:100	BioLegend
Anti-humanCD14/anti-human CD56 MACS beads, see manual	Miltenyi Biotec
Anti-humanCD45-FITC, 1:200	BioLegend
Anti-humanCD4-PE-Cy7, 1:200	BioLegend
Goat anti-gp120 (ab21179), see manual	Abcam
Goat anti-mouse-APC, 1:200	Jackson Immunoresearch
Goat anti-mouse-PE, 1:200	Jackson Immunoresearch
Goat anti-mouse-POX, 1:5,000	Jackson Immunoresearch
Goat anti-rabbit POX, 1:5,000	Jackson Immunoresearch
Mouse anti-His mAb biotinylated, 1:5,000	Qiagen
Mouse anti-His mAb, 1:100	AbD Serotec
Mouse anti-His Fab-Alexa488	Kindly provided by colleague Patrick Hoffmann
Mouse anti-human MHC class II (L243), 1:20	Santa Cruz Biotechnologies



Mouse anti-humanCD28 (CD28.2), 1:1,000	Beckton Dickinson
Mouse anti-humanCD3 (OKT-3), 1:1,000	Orthoclone, Janssen-Cilag
Mouse anti- $\beta$ -Actin (C4), 1:200	Santa Cruz Biotechnologies
Propidium Iodide, 1:1,000	Sigma Aldrich
Rabbit anti-human EpCAM (E144), 1:2,500	Epitomics
Streptavidin-AP, 1:5,000	Promega
Vybrant Dio/DiD Cell Labeling Solution, 5 $\mu$ l/10 <sup>6</sup> cells	Life Technologies
Sytox Red dead cell stain, 1:1,000	Life Technologies

### 2.3. HIV-1 VIRUS STOCKS FOR *IN VITRO* INFECTION

<b>Viral Strain</b>	<b>Used for</b>
HIV <sub>NL4-3</sub> (CXCR4-tropic) C <sub>p24</sub> =85 ng/ml	<i>in vitro</i> p24 assay with PBMCs (see 4.7.2) <i>in vivo</i> experiment (see 4.7.3) BiTE-mediated infection (see 4.7.4)
HIV <sub>YU-2</sub> (CCR5-tropic) C <sub>p24</sub> =30 ng/ml	<i>in vitro</i> p24 assay with MDMs (see 4.7.2) BiTE-mediated infection (see 4.7.4)

### 2.4. BiTE ANTIBODY CONSTRUCTS

<b>BiTE Antibody Construct</b>	<b>Amino Acid Sequence</b>
CD4(1+2) $\alpha$ huCD3	KKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILG NQGSFLTkgpsklndradsrrslwdqgnfplliKNLkIE DSDTYICEVEDQKEEVQLLVFGLTANSdThLLQGQSLTL TLESPPGSSPSVQCRSPRGKNIQGGKTLsvSQLELQDSGT WTCTVLQNQKKVEFKIDIVVLAfQKASGGGGSEVQLVE SGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTA YLMNnlkTEDTAVYYCVRHGNFGNSYISYWAYWGQ GTLVTVSSGGGGSGGGGSQTvvTQEPSLTVSPG GTVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGT KFLAPGTPARFSGSLLGGKAALTLsgVQPEDEAEYYCVL WYSNRWVFGGGTKLTVLHHHHHH
CD4 (1+2) $\alpha$ murCD3	KKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILG NQGSFLTkgpsklndradsrrslwdqgnfplliKNLkIE DSDTYICEVEDQKEEVQLLVFGLTANSdThLLQGQSLTL TLESPPGSSPSVQCRSPRGKNIQGGKTLsvSQLELQDSGT WTCTVLQNQKKVEFKIDIVVLAfQKASGGGGSEVklVE SGGGLVKPGGSLKLSCAASGFTFSTFPMAWVRQSPAKR LEWVATLSPSGDSTYYRDSVKGRFTISRDNakNTLYLQ MSSLKSEDtATYYCTRVGFTTFYFDfWGQTTVTVSSG GGSGGGSGGGGSDIQMTQTPSFLSASVGDRVTINCK ASQNINKYLDWYQQKPDGTVKLLIYNINNLHSGVPSRFS GSGSGTDFSLTISNLEPEDVATYYCLQHRTGWTFGGGgTK LEIKHHHHHH

CD4 D1.1x $\alpha$ huCD3	<p>KKVVIGKKGDTVELTCTASQKKS IQFHWKNSNQIKILGN  QGSFLT KGPSKLNDRVDSRRSLWDQGNFPLIKNLKPED  SDTYICEVEDQKEEVQLLIVLGS GGGGSEVQLVESGGGLV  QPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLEWVA  RIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMNN  LKTEDTAVYYCVRHGNFGNSYISYWAYWGQGLVTVS  SGGGGSGGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTC  GSSTGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLAPGT  PARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNR  WVFGGGTKLTVLHHHHH</p>
CD4 D1.2x $\alpha$ huCD3	<p>KKVVYGGKGDVELTCTASQKKNIQFHWKNSNQIKILG  NQGSLFKGPSKLNDRADSRRLWDQGNFPLIKNLKPE  DSDTYICEVEDQKEEVQLVVVGS GGGGSEVQLVESGGG  LVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLEW  VARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQM  NNLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGLVTV  VSSGGGSGGGGSGGGGSQTVVTQEPSLTVSPGGTVTL  TCGSSTGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLAP  GTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSN  RWVFGGGTKLTVLHHHHH</p>
B12HLx $\alpha$ huCD3	<p>QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFVIHWV  RQAPGQRFWMGWINPYNGNKEFS AKFQDRVTFTADT  SANTAYMELRSLRSADTAVYYCARVGPYSWDDSPQDN  YYMDVWGKGTTVIVSSGGGGSGGGGSGGGGSEIVLTQS  PGTSLSPGERATFSCRSSH SIRSRRVAWYQHKGQAPR  LVIHGVS NRASGISDRFSGSGSGTDFTLTITRVEPEDFAL  YYCQVYGASSYTFGQGTKLERKSGGGGSEVQLVESGG  GLVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLE  WVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQ  MNNLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGLV  TVVSSGGGSGGGGSGGGGSQTVVTQEPSLTVSPGGTV  TLTCGSSTGAVTSGNYPNWVQQKPGQAPRGLIGGTKFL  APGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWY  SNRWVFGGGTKLTVLHHHHH</p>
B12LHx $\alpha$ huCD3	<p>EIVLTQSPGTLSPGERATFSCRSSH SIRSRRVAWYQHK  PGQAPRLVIHGVS NRASGISDRFSGSGSGTDFTLTITRVE  PEDFALYYCQVYGASSYTFGQGTKLERKGGGGSGGGGS  GGGGSQVQLVQSGAEVKKPGASVKVSCQASGYRFSNF  VIHWVRQAPGQRFWMGWINPYNGNKEFS AKFQDRVT  FTADTSANTAYMELRSLRSADTAVYYCARVGPYSWDD  SPQDNYYMDVWGKGTTVIVSSGGGGSEVQLVESGGGL  VQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLEWV  ARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMN  NLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGLVTV  SSGGGSGGGGSGGGGSQTVVTQEPSLTVSPGGTVTLT  CGSSTGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLAPG  TPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNR  WVFGGGTKLTVLHHHHH</p>

VRC01HLx $\alpha$ huCD3

QVQLVQSGGQMKKPGESMRISCRASGYEFIDSTLNWIRL  
APGKRPEWMGWLKPRGGAVNYARPLQGRVTMTRDVY  
SDTAFLELRSLTVDDTAVYFCTRGNADYNWDFEHWG  
RGTPVIVSSGGGGSGGGGSGGGGSEIVLTQSPGTLSPG  
ETAISCRTSQYGLAWYQQRPGQAPRLVIYSGSTRAAGI  
PDRFSGSRWGPDYNLTISNLESGDFGVYYCQQYEFFGQ  
GTKVQVDIKSGGGGSEVQLVESGGGLVQPGGSLKLSCA  
ASGFTFNKYAMNWVRQAPGKGLEWVARIRSKYNNYAT  
YYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYY  
CVRHGNFGNSYISYWAYWGQGTLLTVSSGGGGSGGGG  
SGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGAVTSGN  
YPNWVQQKPGQAPRGLIGGTKFLAPGTPARFSGSLLGG  
KAALTLSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTV  
LHHHHHH

VRC01LHx $\alpha$ huCD3

EIVLTQSPGTLSPGETAISCRTSQYGLAWYQQRPGQ  
APRLVIYSGSTRAAGIPDRFSGSRWGPDYNLTISNLESGD  
FGVYYCQQYEFFGQGTKVQVDIKGGGGSGGGGSGGGG  
SQVQLVQSGGQMKKPGESMRISCRASGYEFIDSTLNWIR  
LAPGKRPEWMGWLKPRGGAVNYARPLQGRVTMTRDV  
YSDTAFLELRSLTVDDTAVYFCTRGNADYNWDFEHW  
GRGTPVIVSSGGGGSEVQLVESGGGLVQPGGSLKLSCA  
ASGFTFNKYAMNWVRQAPGKGLEWVARIRSKYNNYAT  
YYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYY  
CVRHGNFGNSYISYWAYWGQGTLLTVSSGGGGSGGGG  
SGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGAVTSGN  
YPNWVQQKPGQAPRGLIGGTKFLAPGTPARFSGSLLGG  
KAALTLSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTV  
LHHHHHH

CD4L17bHLx $\alpha$ huCD3

KKVVLGKKGDTVELTCTASQKKSIFHWKNSNQIKILG  
NQSFLTKGPSKLNDRADSRRLWDQGNFPLIKNLKIE  
DSDTYICEVEDQKEEVQLLVFGLTANS DTHLLQGQSLTL  
TLESPPGSSPSVQCRSPRGKNIQGGKTLVSQLELQDSGT  
WTCTVLQNQKKVEFKIDIVVLAQKASGGGGSGGGGSG  
GGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESG  
AEVKKPGSSVKVSKASGDTFIRYSFTWVRQAPGQGLE  
WMGRIITILDVAHYAPHLQGRVTITADKSTSTVYLELRN  
LRSDDTAVYFCAGVYEGEADEGEYDNNGFLKHWGQGT  
LVTVSSGGGGSGGGGSGGGGSDIVMTQSPATLSVSPGE  
RATLSCRASESVSSDLAWYQKPGQAPRLLIYGASTRAT  
GVPARFSGSGSGAEFTLTISLQSEDFAVYYCQQYNNWP  
PRYTFGQGRLEIKSGGGGSEVQLVESGGGLVQPGGSLK  
LSCAASGFTFNKYAMNWVRQAPGKGLEWVARIRSKYN  
NYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDT  
AVYYCVRHGNFGNSYISYWAYWGQGTLLTVSSGGGG  
GGGGSGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGA  
VTSGNYPNWVQQKPGQAPRGLIGGTKFLAPGTPARFSG  
SLLGGKAALTLSGVQPEDEAEYYCVLWYSNRWVFGGG  
TKLTVLHHHHHH

Positive control BiTE $\alpha$ huCD19 $\times$ $\alpha$ huCD3 (45A10LH)	Kindly provided by colleague Evelyne Schaller
Neg. control BiTE Mec14LH $\times$ $\alpha$ huCD3	Kindly provided by colleague Dr. Ralf Lutterbuese
Irrelevant target $\times$ $\alpha$ huCD3 BiTE	Kindly provided by colleague Jochen Pendzialek

#### 2.4.1. ABP-TAGGED CD4(1+2) BiTE VARIANTS

SA21CD4(1+2) $\times$ $\alpha$ huCD3	RLIEDICLPRWGCLWEDDKKVVLGKKGDTVELTCTAS QKKSIFHWKNSNQIKILGNQGSFLTCKPSKLNDRADS RRSLWDQGNFPLIKNLKIEDSDTYICEVEDQKEEVQLL VFGLTANS DTHLLQGQSLTLTLESPPGSSPSVQCRSPRG KNIQGGKTLSVSQLELQDSGTWTCTVLQNQKKVEFKID IVVLA FQKASGGGGSEVQLVESGGGLVQPGGSLKLSA ASGFTFNKYAMNWVRQAPGKGLEWVARIRSKYNNYA TYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVY YCVRHGNFGNSYISYWAYWGQGT LVTVSSGGGGSGG GGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSSTGAVT SGNYPNWVQQKPGQAPRGLIGGTKFLAPGTPARFSGSL LGGKAALTL SGVQPEDEAEYYCVLWYSNRWVFGGGT KLTVLHHHHHH
SA21 G <sub>4</sub> S CD4(1+2) $\times$ $\alpha$ huCD3	RLIEDICLPRWGCLWEDDGGGGSKKVVLGKKGDTVEL TCTASQKKSIFHWKNSNQIKILGNQGSFLTCKPSKLN DRADSRRLWDQGNFPLIKNLKIEDSDTYICEVEDQKE EVQLLVFGLTANS DTHLLQGQSLTLTLESPPGSSPSVQC RSPRGKNIQGGKTLSVSQLELQDSGTWTCTVLQNQKK VEFKIDIVVLA FQKASGGGGSEVQLVESGGGLVQPGGS LKLSCAASGFTFNKYAMNWVRQAPGKGLEWVARIR KYNNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKT EDTAVYYCVRHGNFGNSYISYWAYWGQGT LVTVSSG GGSGGGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGS STGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLAPGTP ARFSGSL LGGKAALTL SGVQPEDEAEYYCVLWYSNRW VFGGGTKLTVLHHHHHH
SA21 (G <sub>4</sub> S) <sub>3</sub> CD4(1+2) $\times$ $\alpha$ huCD3	RLIEDICLPRWGCLWEDDGGGGSGGGGSGGGGSKKVV LGKKGDTVELTCTASQKKSIFHWKNSNQIKILGNQGS FLTCKPSKLNDRADSRRLWDQGNFPLIKNLKIEDSDT YICEVEDQKEEVQLLVFGLTANS DTHLLQGQSLTLTLE SPPGSSPSVQCRSPRGKNIQGGKTLSVSQLELQDSGTWT CTVLQNQKKVEFKIDIVVLA FQKASGGGGSEVQLVESG GGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGK LEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNSYISYWAYWGQ GTLVTVSSGGGGSGGGGSQTVVTQEPSLTVSPG GTVTLTCGSSTGAVTSGNYPNWVQQKPGQAPRGLIGG

	TKFLAPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYC VLWYSNRWVFGGGTKLTVLHHHHH
CD4(1+2)SA21 $\alpha$ huCD3	KKVVLGKKGDTVELTCTASQKKSIFHWKNSNQIKILG NQGSFLTKGPSKLNDRADSRRLWDQGNFPLIKNLKIE DSDTYICEVEDQKEEVQLLVFGLTANS DTHLLQGQSLT LTLESPPGSSPSVQCRSPRGKNIQGGKTLVSQLELQDS GTWTCTVLQNQKKVEFKIDIVVLA FQKARLIEDICLPR WGCLWEDDEVQLVESGGGLVQPGGSLKLSCAASGFTF NKYAMNWVRQAPGKGLEWVARIRSKYNNYATYYAD SVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRH GNFGNSYISYWAYWGQGLVTVSSGGGGSGGGGSGG GGSQTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYP NWWVQQKPGQAPRGLIGGTKFLAPGTPARFSGSLLGGK AALTLSGVQPEDEAEYYC VLWYSNRWVFGGGTKLTV LHHHHH
CD4(1+2) G <sub>4</sub> S SA21 G <sub>4</sub> S $\alpha$ huCD3	KKVVLGKKGDTVELTCTASQKKSIFHWKNSNQIKILG NQGSFLTKGPSKLNDRADSRRLWDQGNFPLIKNLKIE DSDTYICEVEDQKEEVQLLVFGLTANS DTHLLQGQSLT LTLESPPGSSPSVQCRSPRGKNIQGGKTLVSQLELQDS GTWTCTVLQNQKKVEFKIDIVVLA FQKAGGGGSR LIED ICLPRWGCLWEDDSGGGGSEVQLVESGGGLVQPGGSL KLSCAASGFTFNKYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTE DTAVYYCVRHGNFGNSYISYWAYWGQGLVTVSSGG GGSGGGGSGGGGSGTVVTQEPSLTVSPGGTVTLTCGS TGAVTSGNYPNWWVQQKPGQAPRGLIGGTKFLAPGTPA RFGSLLGGKAALTLSGVQPEDEAEYYC VLWYSNRWV FGGGTKLTVLHHHHH
CD4(1+2) (G <sub>4</sub> S) <sub>3</sub> SA21 (G <sub>4</sub> S) <sub>3</sub> $\alpha$ huCD3	KKVVLGKKGDTVELTCTASQKKSIFHWKNSNQIKILG NQGSFLTKGPSKLNDRADSRRLWDQGNFPLIKNLKIE DSDTYICEVEDQKEEVQLLVFGLTANS DTHLLQGQSLT LTLESPPGSSPSVQCRSPRGKNIQGGKTLVSQLELQDS GTWTCTVLQNQKKVEFKIDIVVLA FQKAGGGGSGGGG SGGGGSR LIEDICLPRWGCLWEDDGGGGSGGGGSGGG GSEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAMN WVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTI SRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYI SYWAYWGQGLVTVSSGGGGSGGGGSGGGGSGTVVT QEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWWVQQK PGQAPRGLIGGTKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYC VLWYSNRWVFGGGTKLTVLHHHHH
CD4(1+2) $\alpha$ huCD3SA21	KKVVLGKKGDTVELTCTASQKKSIFHWKNSNQIKILG NQGSFLTKGPSKLNDRADSRRLWDQGNFPLIKNLKIE DSDTYICEVEDQKEEVQLLVFGLTANS DTHLLQGQSLT LTLESPPGSSPSVQCRSPRGKNIQGGKTLVSQLELQDS GTWTCTVLQNQKKVEFKIDIVVLA FQKASGGGGSEVQ LVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQ

	<p>APGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDD  SKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISYW  AYWGQGTLLVTVSSGGGGSGGGGSGGGGSQTVVTQEP  SLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQQKPGQA  PRGLIGGTKFLAPGTPARFSGSLLGGKAALTLSGVQPED  EAEYYCVLWYSNRWVFGGGTKLTVLRLIEDICLPRWG  CLWEDDHHHHH</p>
CD4(1+2) $\alpha$ huCD3 G <sub>4</sub> S SA21	<p>KKVVLGKKGDTVELTCTASQKKSIFHWKNSNQIKILG  NQGSFLTKGPSKLNDRADSRRLWDQGNFPLIKNLKIE  DSDTYICEVEDQKEEVQLLVFGLTANS DTHLLQGQSLT  LTLESPPGSSPSVQCRSPRGKNIQGGKTLVSQLELQDS  GTWTCTVLQNQKKVEFKIDIVVLA FQKASGGGGSEVQ  LVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQ  APGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDD  SKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISYW  AYWGQGTLLVTVSSGGGGSGGGGSGGGGSQTVVTQEP  SLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQQKPGQA  PRGLIGGTKFLAPGTPARFSGSLLGGKAALTLSGVQPED  EAEYYCVLWYSNRWVFGGGTKLTVLGGGG SRLIEDIC  LPRWGCLWEDDHHHHH</p>
CD4(1+2) $\alpha$ huCD3 (G <sub>4</sub> S) <sub>3</sub> SA21	<p>KKVVLGKKGDTVELTCTASQKKSIFHWKNSNQIKILG  NQGSFLTKGPSKLNDRADSRRLWDQGNFPLIKNLKIE  DSDTYICEVEDQKEEVQLLVFGLTANS DTHLLQGQSLT  LTLESPPGSSPSVQCRSPRGKNIQGGKTLVSQLELQDS  GTWTCTVLQNQKKVEFKIDIVVLA FQKASGGGGSEVQ  LVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQ  APGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDD  SKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISYW  AYWGQGTLLVTVSSGGGGSGGGGSGGGGSQTVVTQEP  SLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQQKPGQA  PRGLIGGTKFLAPGTPARFSGSLLGGKAALTLSGVQPED  EAEYYCVLWYSNRWVFGGGTKLTVLGGGGSGGGGSG  GGG SRLIEDICLPRWGCLWEDDHHHHH</p>

## 2.5. BUFFER & SOLUTIONS

20X NuPAGE MES SDS Running buffer	Invitrogen
20X NuPAGE MES Transfer buffer	Invitrogen
4X NuPAGE LDS Sample buffer	Invitrogen
6X DNA Loading buffer	Fermentas
Acetate buffer pH 4.5	GE Healthcare
SEC Buffer	10 mM Citric Acid Monohydrate
	75 mM L-Lysine-Monohydrochloride
	Adjusted to pH 7.0
	Sterile filtrated

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FACS buffer	PBS 2% FCS
Erythrocyte lysis buffer	0.05% NaN <sub>3</sub> 150 mM NH <sub>4</sub> Cl 10 mM KHCO <sub>3</sub> 100 mM Na <sub>2</sub> EDTA Adjusted to pH 7.2 Sterile filtrated
HBS-EP buffer	GE Healthcare
IMAC buffer A	100 mM NaCl 20 mM Na <sub>2</sub> HPO <sub>4</sub> 5% (v/v) Glycerol Adjusted to pH 7.2 Sterile filtrated
IMAC buffer B	100 mM NaCl 20 mM Na <sub>2</sub> HPO <sub>4</sub> 500 mM Imidazole 5% (v/v) Glycerol Adjusted to pH 7.2 Sterile filtrated
SEC buffer	10mM Citrate 75mM Lysine pH 7.0 Sterile filtrated
BiTE buffer	10mM Citrate 75mM Lysine 4% (w/v) Trehalose pH 7.0 Sterile filtrated
Cell lysis buffer	PBS 1% (v/v) Triton X 100
MACS	PBS 0.5% (v/v) FCS
TAE buffer	2 mM EDTA 40 mM TRIS 20 mM Acetic Acid 2 mM EDTA Adjusted to pH 8.5
TfB I buffer	30 mM KAc 50 mM MnCl <sub>2</sub> 100 mM KCl 10 mM CaCl <sub>2</sub> 15% (w/v) Glycerol Sterile filtrated Stored at -20°C

TfB II buffer	10 mM MOPS (pH 7) 100 mM KCl 10 mM CaCl <sub>2</sub> 15% (w/v) Glycerol Sterile filtrated Stored at -20°C
PBS	Biochrom

## 2.6. CELL CULTURE MEDIA & ADDITIVES

293 fectin	Invitrogen
Carbenicillin	Sigma Aldrich
EDTA	Biochrom
Fetal calf serum un-/dialysed	Biochrom
Freestyle medium	Gibco
HEPES buffer	Biochrom
Human serum	PAA Laboratories
HyQ PS medium	PF CHO Liquid Soy Hy Clone 0.2% (v/v) Phenol red 100 U/ml Penicillin 100 µg/ml Streptomycin
Interleukin-2	Marien-Apotheke (Pharmacy)
LB agar	Carl Roth
LB medium	Carl Roth
Non-essential amino acids	Biochrom
Penicillin/Streptomycin	Biochrom
PF CHO Liquid Soy™ HyClone	Thermo Scientific
Phenolred	Sigma
Puromycin	Sigma
RPMI 1640 with L-Glutamine	Biochrom
RPMI <sup>+</sup> medium	RPMI 1640 with L-Glutamine 10% (v/v) FCS 100 U/ml Penicillin 100 µg/ml Streptomycin 1X NEAA 500 nM β-Mercaptoethanol 10 mM HEPES 1 mM Sodium Pyruvate
Sodium pyruvate	Biochrom
β-mercaptoethanol	Gibco

## 2.7. CHEMICALS

1-Step NBT/BCIP	Thermo Scientific
<sup>51</sup> Chromium	Hartmann Analytic
Acetic Acid	Carl Roth
Agarose	Sigma Aldrich



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Ampuwa H <sub>2</sub> O	Fresenius Kabi
Bovine Serum Albumin	Biowest
CaCl <sub>2</sub>	Carl Roth
Citric acid	Merck
Di-sodiumhydrogenphoshate-dihydrate	Merck
DMSO	Sigma Aldrich
DTT	Invitrogen
EDTA	Carl Roth
Eosin G	Carl Roth
Ethanol	Merck
Ethidium bromide solution 0.09%	AppliChem
Ficoll Separation medium	Biochrom
Glycerol	Merck
Glycine	Sigma Aldrich
Hydrochloric acid	Merck
Imidazole	Merck
InstantBlue Coomassie Stain	Expedeon
Kac	Carl Roth
KCl	Carl Roth
L-Lysine-Monohydrochloride	Merck
Milk powder	Fluka
MnCl <sub>2</sub>	Sigma Aldrich
MOPS	Carl Roth
Sodium Acetate	Merck
Sodium Chloride	Carl Roth
Sodium Hydroxide	Merck
Sulfuric Acid	Merck
SuperSignal West Femto Chemiluminescent Substrate	Thermo Scientific
TRIS	Carl Roth
Triton X 100	Sigma Aldrich
Tween 20	Carl Roth
Zinc Chloride	Merck
Empigen BB detergent	Sigma Aldrich
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS)	GE Healthcare

## 2.8. CONSUMABLES

Acrodisc Syringe Filter 0.2 µm	Pall Corp.
Acrylamide-gels NuPAGE 4-12% Bis-Tris	Invitrogen
Biacore CM5 Chip	GE Healthcare
Biomax MR Film	Kodak
Cell culture flasks	Greiner Bio-One, Nunc
Cell Culture Roller flasks 1 L	Corning
Cell strainer 70 µm, 100 µm	Beckton Dickinson
Centrifuge Tube 50 ml, 15 ml	Beckton Dickinson
Cryo tubes	Nunc
Developing Solution	Velopex
ELISA-Plates Maxisorb	Greiner Bio-One
Eppendorf Caps 1.5 ml, 2 ml	Eppendorf
Fixation Solution	Medivance
Fractogel EMD Chelate 40-90 µm	Merck
HiLoad 16/60 Superdex 200 prep grade	GE Healthcare
Hybond-P PVDF membrane	GE Healthcare
Leucosep tubes, 30 ml, sterile	Greiner Bio-One
MACS LS Columns	Miltenyi Biotec
Moxi Z Cassettes Type S	Orflo
Neubauer-Counting Chamber	Lo-Laboroptik
Omnican 0.3x12 mm 0.01-1 ml Syringes	Braun
PCR Softstrips 0.2 ml	Biozym Scientific
Petridishes, ø 8.5 cm	Greiner Bio-One
Pipet tips	Diamond
Polystyrol roundbottom tubes 15 ml	Becton Dickinson
PVDF membrane pore size 0.45 µm	Millipore
Scalpel, sterile	Ratiomed
Sponge for Westernblot	Invitrogen
Sterican100 0,05x16 mm 25 G x 5/8“ Needles	Braun
Sterile filter 0.2 µm	PALL
Tissue Culture Plates, 6-, 12-well	Becton Dickinson
Tissue Culture Plates, 96 well plate, V-, U-bottom	Greiner Bio-One
FACS 96 well plate, V-bottom	Hartenstein
VacuCap 90 PF Filter Unit	Pall Corp.
Vivasoin 6, 10,000 MWCO	Sartorius stedim
Vivaspin 500, 10,000 MWCO	Sartorius stedim
Rubber caps, type 2	GE Healthcare
Gilson vials, 0.8 ml	GE Healthcare

## 2.9. INSTRUMENTS

Aekta FPLC	Amersham Pharmacia Biotech
Agarose gel electrophoration system	Bio-Rad
Biacore T200/2000	GE Healthcare
Centrifuge Rotina 460RS	Hettich
CO <sub>2</sub> -Incubator Heracell 240	Kendro
ELISA reader	BioTek Instruments

FACS Accuri	Becton Dickinson
FACS Canto II	Becton Dickinson
FACS CyAn ADP Analyzer	Beckman Coulter
Fluorescence microscope	Nikon
Gel documentation station	Syngene
Genepulser II Electroporation System	BioRad
Horizontal shaker	Neolab
HP Scanjet G4050	Hewlett Packard
Light microscope Axiovert 25	Carl Zeiss
Magnet for cell isolation Dynal MPC-S	Life Technologies
Mini MACS magnet	Miltenyi Biotec
Moxi Z	Orflo Technologies
Nanodrop 2000	Thermo Scientific
Photometer	Eppendorf
Pipets	Abimed, Gilson
Power supply	Biorad, Novex
Pump P-50	Pharmacia Biotech
Safety cabinet Herasafe KSP	Thermo Scientific
Scales	Kern; Ohaus
SDS-PAGE gel chamber	Life Technologies
Tabletop centrifuge 5424	Eppendorf
Thermomixer/-block	Eppendorf
VP-DSC device	MicroCal LLC
Waterbath	Memmert
Wizard 300 gammacounter	Perkin Elmer
X-ray film developing machine (Extra-X)	Velopex

## 2.10. DNA & PROTEIN MARKER

GeneRuler DNA Ladder Mix	Fermentas
Novex Sharp Pre-Stained Protein Standard	Invitrogen

## 2.11. ENZYMES

Phosphatases	New England Biolabs
Restriction endonucleases	New England Biolabs
Trypsin	Biochrom



## 2.14. PLASMIDS

pEF DHFR

Plasmid vector encoding the dihydrofolate reductase allowing transfected CHO DHFR<sup>-</sup> cells to proliferate without exogenous nucleoside supply [127].

pEF Puro

Kindly provided by colleague Dr. Roman Kischel, who substituted the DHFR gene within the pEF DHFR plasmid with the puromycin N-acetyl-transferase (PAC) gene.

## 2.15. SERVICES

DNA sequencing

Sequiserve

Oligonucleotide synthesis

Metabion

Gene synthesis

GeneArt

HIV RNA RT-PCR

University Hospital Zurich, Switzerland

## 2.16. SOFTWARE & DATABASES

EndNoteX7

Thomson Reuters

FCS Express Research V4

De Novo Software

FlowJo Vx

TreeStar

Graph Pad Prism 6

Graph Pad Software

HP Scansoftware

Hewlett-Packard

Microsoft Office 2010

Microsoft

Unicorn 5.20

GE Healthcare

Vector NTI Advance 11

Invitrogen

NCBI blast protein/nucleotide

NCBI

UniProt

UniProt Consortium

SignalP 3.0

CBS, TU Denmark

## 3. METHODS

### 3.1. MOLECULAR BIOLOGICAL METHODS

#### 3.1.1. *AGAROSE GEL ELECTROPHORESIS*

The DNA samples were mixed with DNA loading dye and then applied to pockets within a homogeneous agarose gel. The agarose concentration of the gel was chosen according to the size of the DNA fragment. The gel was pre-stained with ethidium bromide to stain DNA molecules during electrophoresis. DNA molecules were separated according to their size by migration through the gel driven by their own negative charge at an applied constant voltage. When separation was sufficient, the run was stopped and DNA bands were documented using a UV light photo station and if applicable excised from the gel for further usage.

#### 3.1.2. *DESIGN OF EXPRESSION PLASMIDS*

For the generation of HIV antigen positive cell lines, the proteins of interest were identified using UniProt databases, designed theoretically using Vector NTI and obtained by gene synthesis from GeneArt. Upstream of the appropriate open reading frames, a kozak sequence and an immunoglobulin leader peptide was added to the antigen or BiTE coding sequence to achieve secretion of the BiTE antibody constructs into the cell culture medium or the localization of the target antigens at the plasma membrane respectively. All coding sequences were terminated by a stop codon. The complete ORF was enclosed by restriction sites for further cloning into expression vectors.

To generate antigen positive cells, the coding sequences of the according annotated ectodomain of HIV/SIV env (gp140) protein was fused to the coding sequence for the transmembrane and intracellular domain of the human epithelial cell adhesion molecule (EpCAM). Sequence-verified pEF DHFR or pEF Puro plasmid DNA clones were prepared and used to transfect CHO, HEK 293 or Jurkat cells respectively.

#### 3.1.3. *DIFFERENTIAL SCANNING CALORIMETRY*

The temperature stability of CD4(1+2) $\alpha$ huCD3 was analyzed by differential scanning calorimetry (DSC) to determine the intrinsic biophysical protein stability. The energy uptake of a sample containing CD4(1+2) BiTE (250  $\mu$ g/ml) was recorded from 20 to 90°C compared to a sample containing the BiTE formulation buffer. For recording of the respective melting curve, the overall sample temperature was increased stepwise and the energy uptake of the

sample and the formulation buffer reference was recorded for each temperature step. The difference in energy uptake  $C_p$  (kcal/mole/°C) of the sample minus the reference was plotted against the respective temperature to visualize the temperature at the peak of energy uptake.

#### 3.1.4. *DNA PREPARATION OF AGAROSE GELS*

For subsequent usage of DNA fragments, the bands of interest were excised under UV light using a sterile scalpel. DNA was purified from the excised gel piece using the QIAquick Gel Extraction Kit following the instructions of the manufacturer.

#### 3.1.5. *ELISA*

##### 3.1.5.1. *HIV P24 CAPSID ANTIGEN ELISA*

For analysis of viral replication, aliquots of the cell culture supernatant were collected at different days post infection. The sample volume of 50  $\mu$ l taken for the analysis was replenished at each time point. The capture ELISA for HIV p24 has been performed as described by Moore et al. [128]. Samples of cell culture supernatant, containing HIV virions, were diluted 1:5 in Empigen detergent and stored at -20°C until analysis. For the conduction a 96-well plate was coated with a polyclonal antibody to bind p24 antigen of sampled cell culture supernatant lysed by detergents. Bound p24 was detected with an AP-conjugated anti-p24 monoclonal antibody and a luminescent detection system. The absorbance values were plotted against the BiTE concentration to visualize antiviral effects in reference to the no BiTE control absorbance.

##### 3.1.5.2. *HIV GP120 ANTIGEN ELISA*

To determine the target affinity of the BiTE antibody constructs to gp120, 100  $\mu$ l/well of the recombinant gp120 (#1001, Immunodiagnosics) with a concentration of 3  $\mu$ g/ml were applied to a 96-well Maxisorp plate and incubated overnight at 4°C. On the next day, the plate was washed once with 250  $\mu$ l PBS-T and blocked with 250  $\mu$ l PBS +3% BSA at 37°C for 60 min. Subsequently, the blocking buffer was decanted and 100  $\mu$ l of the BiTE dilution series (diluted in PBS) was added to the plate and incubated at room temperature, 60 rpm for 1h. After a triple wash with PBS-T, 100  $\mu$ l of a mouse anti-histidine (1  $\mu$ g/ml) was added to the wells and incubated as before. Following a triple wash with PBS-T, 1  $\mu$ g/ml of a goat anti-mouse Fc POX conjugated antibody was added and incubated as before. After another washing step, 100  $\mu$ l of the OPD substrate were incubated for 5 to 10 minutes. The colorimetric reaction was stopped with 50  $\mu$ l of 3 M H<sub>2</sub>SO<sub>4</sub> upon sufficient colorimetric

signal. The absorption was measured at 492 nm using an ELISA plate reader. The absorbance with reference to the PBS control was plotted versus the according BiTE concentration to determine the curve's turning point representing the KD value of the BiTE.

### 3.1.6. *IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)*

After thawing of the BiTE containing cell culture supernatant, 5% of glycerol was added to the supernatant followed by sterile filtration using a 0.8/0.2  $\mu\text{m}$  Bottle Top Filter Unit. The IMAC was done with an AEKTA FPLC, where the BiTE supernatant was loaded onto a 10 ml Fractogel EMD chelate column preloaded with  $\text{ZnCl}_2$ . The column was pre-equilibrated with IMAC buffer A. In the presence of 10 mM Imidazole the BiTE supernatant was rinsed through the Fractogel column with a flow rate of 4 ml/min. Subsequently unbound proteins were removed by washing the column using IMAC buffer A with 10 mM Imidazole. The flow-through in combination with the washing volume was stored for later analysis. To elute, fractions of 10% and 100% IMAC buffer B elution steps (50 mM and 500 mM imidazole) of 5 column volumes each were collected and stored at 4°C. The BiTE fractions of the 100% IMAC buffer B elution step inducing a 280 nm peak were pooled, concentrated by spin columns and applied to size exclusion chromatography (SEC).

### 3.1.7. *INDIRECT IMMUNOFLUORESCENCE*

Transfected CHO cells were grown adherently in a 6-well plate. Before staining, the cells were washed in PBS/2% FCS and fixed using 3.75% paraformaldehyde for 20 min at room temperature. After a second washing step, the cells were lysed using PBS/2% saponin for 20 min at room temperature. After subsequent washing, the cells were stained using a rabbit anti-intracellular domain (IC) EpCAM antibody for 30 min at 4°C. Staining was visualized after washing using a FITC conjugated anti-rabbit antibody. Stained cells were analyzed using an inverse fluorescence microscope controlled by sole FITC-conjugated antibody staining (not shown).

### 3.1.8. *LIGATION*

Restriction digested DNA fragments were ligated by the use of the T4 DNA ligase. The insert fragment was applied to the reaction in a three molar excess to the vector fragment. Then the reaction was filled up to 10  $\mu\text{l}$  using T4 DNA ligase reaction buffer,  $\text{H}_2\text{O}$  dd. and 400 units of the T4 DNA ligase. The ligase reaction was incubated for 10 min at RT or 16°C overnight.



### 3.1.9. *PLASMID DNA (MINI-, MIDI-, MAXI-) PREPARATION*

Transformed *E. coli* bacteria were cultured overnight in lysogeny broth (LB) medium plus carbenicillin and harvested by centrifugation at 4,000 rpm, room temperature, 10 min depending on the volume of the bacterial cell culture. The cell culture supernatant was decanted and the plasmid DNA was isolated from the bacterial cells according to the instructions of the manufacturer.

### 3.1.10. *DNA RESTRICTION DIGEST*

In order to clone DNA fragments from one DNA molecule into another (e.g., change of vector plasmid), DNA restriction endonucleases were used which cut DNA at their specific restriction site within the DNA. These endonucleases were used in combination of up to three enzymes. Therefore, about one  $\mu\text{g}$  of plasmid DNA was combined with 10 units of the restriction endonuclease and restriction buffer suitable for the enzymes used in the digest. The total volume of the reaction was adjusted to the 10-fold volume of the enzyme volume in the reaction. The reaction was incubated at 37°C for about 60 min and subsequently stopped by the addition of DNA loading buffer. The resulting DNA fragments were separated by agarose gel electrophoresis and the fragment of interest was isolated by gel extraction for ligation reactions.

### 3.1.11. *SDS-PAGE GEL ELECTROPHORESIS & COOMASSIE STAINING*

To analyze the purification of a BiTE antibody or check transfected cells for antigen expression, the protein solutions were mixed with LDS sample buffer as well as 100 mM DTT and then heated up to 95°C for 3 min to denature the proteins. After a cool down step on ice, the protein solution was loaded onto a 4 – 12% Bis/Tris SDS gel and separated under a constant voltage of 200 volts for 45 minutes. Subsequently the gel was rinsed with water and either stained by a coomassie staining solution for 1 h at 60 rpm at room temperature followed by washing steps with H<sub>2</sub>O or used for western blot analysis.

### 3.1.12. *SIZE EXCLUSION CHROMATOGRAPHY (SEC)*

The BiTE eluate resulting from the IMAC was concentrated on a spin column pre-equilibrated with SEC buffer for a final volume of less than 3 ml. The concentrated BiTE solution was subsequently analyzed by SEC (flow rate: 1 ml/min) using the SEC buffer. Thereby the BiTE solution was divided by size, resulting in fractions of protein aggregate, BiTE multi-/dimers, BiTE monomer and if applicable BiTE degradation. Aliquots of these

protein samples were subjected to SDS-PAGE and Western Blot for analysis. Trehalose was added to the BiTE monomer fraction to a final concentration of 4% (w/v). After sterile filtration of the BiTE solution, the final protein concentration was calculated by multiplication of the absorbance at OD<sub>280 nm</sub> with the absorbance value according to Vector NTI with reference to the absorbance of the formulation buffer.

### 3.1.13. STATISTICAL ANALYSES

Statistical analyses were calculated using Graph Pad Prism 6.04. The statistics of the *in vitro* experiment in which infected PBMC and MDMs were incubated with BiTE antibody constructs were calculated by unpaired t-test with Welch's correction. In the BiTE-mediated infection experiments, samples without viral load were excluded from analysis. Here, statistics were also calculated using unpaired t-testing with Welch's correction. For analysis of the *in vivo* experiment one outlier in the BiTE treated cohort was excluded (determined by Grubb's test) and one outlier in the buffer control cohort was excluded (no viral load). Significances were determined for days 7 and 10 p.i. using two-way ANOVA with repeated measure followed by Holm-Sidak's multiple comparison test. Significant differences are indicated with p values. Sample values at day 16 p.i. are based on n=4, not included in analysis.

### 3.1.14. SURFACE-PLASMON RESONANCE (SPR) BASED TARGET AFFINITY

The BiTE antibody constructs were characterized concerning their HIV target affinity as well as their affinity to human CD3. For HIV, recombinant gp120 IIIB was used. For CD3 affinity measurements, a fusion protein was used that contains the first 27 amino acids of the human CD3 N-terminus fused to a human Fc portion. Long-lived CD4(1+2) BiTE antibody constructs (fused to an albumin binding peptide) have additionally been analyzed for their affinity to human serum albumin.

In detail, CM5 Sensor Chips were activated using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (5 µl/min). Subsequently, the CD3 construct was immobilized with 100 relative units (RU), the gp120 with roughly 900 RU or 800 RU of the human serum albumin diluted in acetate buffer pH 4.5. Inactivation was done as described by the manufacturer. Five aliquots of each BiTE candidate diluted in HBS-EP buffer were measured (50 nM – 25 nM – 12.5 nM – 6.25 nM – 3.125 nM).

**Table 1: Adjustments for the Determination of BiTE Affinities to HIV Gp120, Human CD3 and HSA by SPR.**

Antigen	Flow rate [ $\mu$ l/min]	Injection [sec]	Dissociation [sec]	Regeneration	Stabilization [sec]	Max. BiTE conc. [ $\mu$ g/ml]
gp120	30	240	480	2x 60 sec 4.5 M $MgCl_2$	60	2.5
huCD3	30	180	420	30 sec 0.1 M Gly 0.5 M NaCl pH 1.5	420	2.5
Human Serum Albumin	35	180	360	10 sec 0.1 M Gly 0.5 M NaCl pH 2.5	60	10

### 3.1.15. *TEMPERATURE STABILITY OF BiTE ANTIBODY CONSTRUCTS*

To assess the temperature stability of BiTE antibody constructs, aliquots (10  $\mu$ g/ml) were incubated at a temperature of 4, 37, 50, 60, 70, 80 or 90°C for 5 min, subsequently cleared for denatured proteins by centrifugation (4°C, 4,000 rpm, 10 min) and then used to stain HIV gp140<sup>+</sup> or CD3<sup>+</sup> cells. BiTE binding was determined via subsequent staining with mouse anti-histidine mAb and goat anti-Mouse mAb-APC and analyzed using FACS. The MFI values of the BiTE aliquots of each temperature were plotted with reference to the fluorescence signal of the detection antibodies in order to determine the temperature at the half-maximal signal intensity (TS<sub>50</sub>) of the BiTE.

### 3.1.16. *WESTERN BLOTTING*

Proteins separated by SDS PAGE were subsequently applied to a PVDF membrane, accompanied by sponge pillows and fixed in a Western Blot apparatus with NuPAGE transfer buffer. The applied constant voltage of 25 V for 60 min forced the negatively charged proteins (due to SDS denaturation) to transfer from the gel into the membrane located between the gel and the anode of the apparatus. After the transfer, the membrane was rinsed with water and incubated with 10% (w/v) milk emulsion for 60 min at 60 rpm. After the blocking step, the milk emulsion was decanted and the primary antibody (diluted in PBS-T) was incubated for 60 min at 60 rpm. After three washing steps with PBS-T of 5 min each at 60 rpm, the secondary antibody (diluted in PBS-T) was added to the membrane for 60 min at 60 rpm followed by another three washing steps.

The detection was done with 1-Step™ NBT/BCIP for AP-conjugated secondary antibodies or by Super Signal West Femto Chemiluminescent Substrate with subsequent x-ray film exposition and developing for HRP-conjugated secondary antibodies.

## 3.2. CELLULAR BIOLOGICAL METHODS

### 3.2.1. BACTERIAL CELL CULTURE

*E. coli* cell culturing was done in LB medium using Erlenmeyer flasks shaking with 200 rpm at 37°C rpm or on LB-agar petri dishes incubated at 37°C. Selection of transformed *E. coli* was achieved by carbenicillin (concentrated at 50 µg/ml for liquid cultures or 100 µg/ml for petri dish cultures). Aliquots of transformed *E. coli* single colony cultures were stored at 4°C for short-term storage or mixed 1:1 with 80% (v/v) glycerol and stored mid-term at -80°C.

### 3.2.2. BiTE EXPRESSION

Generally, a preparation of a nucleotide sequence verified plasmid DNA clone encoding the BiTE candidate was used for transfection. The BiTE antibody constructs were expressed stably either stationary or in roller flasks (37°C, 3 rpm, 7% CO<sub>2</sub>) using CHO cells transfected with pEF DHFR plasmids. Alternatively, BiTE antibody constructs were expressed transiently in HEK 293 F cells (37°C, 7% CO<sub>2</sub>, 130 rpm). Given the inserted immunoglobulin signal peptide leading sequence upstream of the BiTE coding sequence, the BiTE antibody constructs were secreted into the cell culture medium. The transfected cells were pelleted (4,000 rpm, 10 min) after the color shift of the pH indicator for CHO K1 cells or 72h after transfection for transient expressing HEK 293 cells. The supernatant was harvested and stored for protein purification at -20°C.

### 3.2.3. BiTE-MEDIATED HIV INFECTION ASSAY

To analyze potential BiTE-mediated infection, CD8<sup>+</sup> T cells have been isolated from PBMCs and incubated with 10 µg/ml of the BiTE antibody construct, an irrelevant target BiTE or no BiTE for 2 h in the incubator. Afterwards, the cells were washed before spinoculation (i.e., cells were incubated in virus containing cell culture supernatant and centrifuged (2300 rpm, room temperature, 2h). After the centrifugation step, the cells were washed with PBS and then incubated (37°C, 5% CO<sub>2</sub>, 95% rHu) with RPMI<sup>+</sup> medium for 7 days. After 7 days, the p24 level of the cell culture supernatant was determined by HIV p24 ELISA. In case of the CD4(1+2) BiTE the assay was repeated with prestimulated, isolated CD8<sup>+</sup> and CD4<sup>+</sup> T cells respectively.

### 3.2.4. *CYTOTOXICITY ASSAYS*

Characterization of BiTE antibody constructs concerning their cytotoxic activity was done by co-incubation of target antigen positive cells with effector cells (effector:target (E:T) ratio of 10:1) and BiTE dilutions for 18-72 h followed by subsequent analysis for the percentage of dead target cells.

Cytotoxicity assays were performed in RPMI<sup>+</sup> cell culture medium with 1:3 or 1:4 BiTE dilutions starting from 2 to 10 µg/ml in 200 µl per well. Target cells (i.e., transfected CHO or Jurkat cells), recombinantly expressing the target antigen, were pre-labeled using a fluorescent dye (Dio or DiD) according to the manual of the manufacturer. Duplicated samples of target ( $10^4$  cells/well) and effector cells ( $10^5$  cells/well) with the appropriate BiTE concentration were prepared on 96-well plates U-bottom plates.

The cytotoxicity assays were incubated for 18-24 h in case of prestimulated human CD8<sup>+</sup> effector T cells, 48 hours when using unstimulated CD14<sup>-</sup>, CD56<sup>-</sup> effector human PBMCs and 72 hours when using stimulated macaque T cells (41-19 LnPx) at 37°C, 95% relative humidity (rHu), 5% CO<sub>2</sub>. After the incubation, the wells were transferred to a 96-well v-bottom plate and pelleted (1,400 rpm, 4°C, 4 min) while the adherent CHO target cells were trypsinized before recombined with the suspension cells. The washed cells were then resuspended in PBS/2% FCS containing 1 µg/ml propidium iodide (PI) and analyzed by flow cytometry for PI-positive target cells.

Alternatively, cytotoxicity assays were performed using Chromium release assays. Therefore,  $10^6$  cells/ml target cells were pre-labeled with <sup>51</sup>Cr for 60 min at 37°C, 95% rHu, 5% CO<sub>2</sub>. Quadruplicates of labeled target cells ( $10^3$  cells/well) and prestimulated human CD8<sup>+</sup> effector T cells ( $10^4$  cells/well) in 200 µl/well were incubated with BiTE dilution series for 16-18 hours. For analysis, 50 µl of supernatant per well was analyzed for radioactivity using a gamma counter. The corresponding BiTE concentration at the half-maximum lysis of target cells (EC<sub>50</sub>) was determined using Graph Pad Prism software in plotting the percentage of PI positive labeled target cells or radioactive counts versus the corresponding BiTE concentration. Assays were controlled by BiTE independent lysis, spontaneous lysis and maximal lysis of target cells.

### 3.2.5. *DEPLETION OF CD14<sup>+</sup>, CD56<sup>+</sup> CELLS FOR THE USE OF UNSTIMULATED PBMCs*

Unstimulated PBMCs were depleted for CD14<sup>+</sup> (monocytes, macrophages) and CD56<sup>+</sup> (NK cells) cells before their application to cytotoxicity assays. Therefore, the isolated PBMCs were incubated with anti-CD14 and anti-CD56 MACS beads and separated from the unlabeled cells by magnetic separation. The isolation was done following the instructions of the manufacturer's manual.

### 3.2.6. CELL-BASED TARGET AFFINITY USING SCATCHARD-PLOT

A constant number of  $10^4$  antigen positive cells ( $CD3^+$  or  $gp140^+$  cells) was incubated with BiTE dilution series starting at 2.5  $\mu\text{g/ml}$  for 4 hours at  $4^\circ\text{C}$ . After the incubation, the cells were washed with PBS/2% FCS for excessive BiTE antibody constructs and incubated with 30  $\mu\text{g/ml}$  mouse anti-histidine Fab molecules conjugated to Alexa 488 for 30 min at  $4^\circ\text{C}$ . Followed by a washing step, cells were fixed for 20 min at room temperature using 3.75% paraformaldehyde before washed again and analyzed by FACS. The mean fluorescence intensity (MFI) values with reference to the secondary antibody control were plotted versus the according BiTE concentration to determine the curve's turning point representing the KD value of the BiTE candidate.

### 3.2.7. EUKARYOTIC CELL CULTURE

All cell lines and primary cells were incubated in cell incubators providing  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  and 95% rHu. Cell lines were passaged regularly (every 48-72 hours) before reaching a critical density. Primary cells and cell lines were grown in the following media:

<b>Cell Line/Primary Cells</b>	<b>Medium</b>
CHO DHFR <sup>-</sup>	HyQ PS medium + 1X Nucleosides
CHO pEF DHFR transfected	HyQ PS medium
HEK 293 F	Freestyle
HPB-ALL/Jurkat/Jurkat CCR5+/PBMC/41-19 LnPx/Raji	RPMI PLUS
Jurkat pEF Puro transfected	RPMI PLUS, 1 $\mu\text{g/ml}$ Puromycin

To store cell lines, aliquots of  $1 \times 10^6$  cells were pelleted, resuspended in 1 ml 90% FCS/10% DMSO and subsequently frozen at  $-80^\circ\text{C}$  using cryo tubes. For long-term storage, frozen cell aliquots were transferred to liquid nitrogen gas phase.

### 3.2.8. FACS ANALYSES

Flow cytometry or FACS is mainly used to visualize structures on cell surfaces respectively intracellular located molecules in native or fixed and lysed cells by fluorescent markers (e.g., fluorophor-conjugated antibodies). In this work, this technique was applied to visualize binding specificities of BiTE antibody constructs on different cell types as well as to analyze cytotoxicity assays. FACS analyses using BiTE antibody constructs was done with a standard concentration of 10  $\mu\text{g/ml}$ . Cells used for the staining were incubated in FACS buffer.

For FACS analyses using monoclonal antibodies, the recommended dilutions supplied with the antibodies were used. Secondary antibodies have been diluted as indicated in 2.2.

### 3.2.9. GENERATION OF COMPETENT BACTERIA & TRANSFORMATION

In order to generate transformation competent *E. coli*, a single colony of *E. coli XL-1 blue* was proliferated in 5 ml LB medium overnight (200 rpm, 37°C) and transferred into 100 ml LB medium (200 rpm, 37°C) and grown to a maximal  $OD_{600\text{ nm}} \leq 0.9$ . Subsequently, the bacteria were pelleted (3000 rpm, 10 min, 4°C) and resuspended in ice-cold 20 ml Tfb I buffer, pelleted and resuspended in 2 ml ice-cold Tfb II buffer, divided into 50 µl aliquots, shock-frozen in liquid N<sub>2</sub> and stored at -80°C.

### 3.2.10. INFECTION OF PBMCs IN VITRO

Infection of PBMCs for the *in vivo* experiment and *in vitro* assays was done by adding 25% (v/v) of sterile filtered cell culture supernatant containing virus ( $C_{p24} = 80$  ng/ml) to the total cell culture volume followed by a co-incubation overnight. The cells were triple washed the following day with PBS and subsequently incubated (37°C, 5% CO<sub>2</sub>, 95% rHu) in RPMI PLUS medium for *in vitro* analyses and in PBS for the *in vivo* experiment.

### 3.2.11. ISOLATION OF HUMAN PBMCs

Human PBMCs were either collected from blood of volunteering coworkers or provided by a collaborating blood bank. PBMCs were isolated by Ficoll centrifugation in Leucosep tubes (2200 rpm, 15 min, no brake). The interphase, enriched for human PBMCs, was retrieved, washed twice in PBS/2% FCS (1500 rpm, 6 min, room temperature). Possible impurities of erythrocytes were lysed by incubation of the PBMCs in erythrocyte-lysis buffer for 5 min at 4°C and subsequent washing of the cells in PBS/2% FCS.

### 3.2.12. ISOLATION OF PRIMARY B CELLS

To determine the cytotoxicity of the CD4(1+2) BiTE on primary B cells, human PBMCs were isolated and B cells were isolated by negative selection using a primary B cell isolation kit. The isolation was done according to the instructions provided in the manufacturer's manual.

### 3.2.13. *ISOLATION & DIFFERENTIATION OF CD14<sup>+</sup> CELLS*

To determine the cytotoxicity of the CD4(1+2) BiTE on monocyte-derived macrophages (MDMs), CD14<sup>+</sup> cells were isolated from human PBMCs by positive selection using anti-human CD14 beads. The isolation was done according to the instructions provided in the manufacturer's manual. Differentiation of monocytes into MDMs was achieved by incubation in RPMI medium supplemented with 2 mM L-glutamine, 5% FCS and 5% huAB serum at 37°C, 95% rHu, 5% CO<sub>2</sub> for 7 days.

### 3.2.14. *STIMULATION OF HUMAN PBMCs & ISOLATION OF CD8<sup>+</sup> T CELLS*

Ca. 4-6x10<sup>7</sup> PBMCs were resuspended in 100 ml RRMI<sup>+</sup> cell culture medium containing 20 U/ml IL-2 and plated onto a washed petri dish, which has previously been coated with 30 ml PBS, 30 µg anti-human CD3 (OKT-3) mAb and 30 µg anti-humanCD28 mAb for 60 min at 37°C. The PBMCs were incubated for 72 hours. Thereafter, the PBMCs were pelleted and incubated for further 24 h in fresh RPMI<sup>+</sup> cell culture medium containing 20 U/ml IL-2. CD8<sup>+</sup> T cells were purified following the instructions of the human CD8<sup>+</sup> T cell Isolation Kit.

### 3.2.15. *TRANSFECTIONS*

About 10<sup>6</sup> cells (e.g., CHO, Jurkat cells) were pelleted (1400 rpm, 4 min, rt), washed twice in sterile PBS and resuspended in 800 µl PBS. The washed cells were transferred into an ice-cooled electroporation cuvette, mixed with one µg of plasmid DNA and incubated on ice for 5 minutes. Electroporation was done in an electroporator using 260 Volts, 975 µF. Transfected cells were transferred into the appropriate medium and incubated for 24h (DHFR) or 48 h (Puromycin) without selection before selective pressure was applied by nucleoside removal (DHFR) or addition of 1 µg/ml Puromycin for Jurkat cells. After the second round of splitting, the cells were analyzed for the recombinant expression of the protein of interest.

## 3.3. *IN VIVO* METHODS

All *in vivo* work was planned and performed according to the Swiss animal protection laws, guidelines of the University of Zurich and Amgen internal standard operating procedures. In addition, the protocol was approved by the cantonal veterinary office of Zurich.



### 3.3.1. ANESTHESIA & RETROBULBAR BLOOD COLLECTION

The collection of blood samples for the analysis of blood cell status and viral load was done by retrobulbar venous plexus puncture. Therefore, the mice were put in an isoflurane chamber with a constant flow of 5% (v/v) isoflurane and 95% (v/v) O<sub>2</sub> until being in a comatose condition. Subsequently, the mice were put out of the chamber and the retrobulbar vein plexus was punctured using a glass capillary. After the blood collection and retraction of the capillary, the bleeding was stopped by applying adequate pressure onto the eye bulb using a sterile tissue. The vital signs were checked until the mice regained consciousness.

### 3.3.2. ANIMAL HANDLING

The animals used for this *in vivo* experiment were mouse females, 6-8 weeks of age. Animal handling was done in compliance with the Swiss animal protection laws, the guidelines of the University Hospital Zurich as well as the current Amgen internal guidelines. Upon delivery, the animals were health monitored and given an acclimatization time of 7 days. The single-ventilated cages were occupied by a maximal number of four mice. They were provided with nesting material, housing and autoclaved water and food pellets ad libitum.

### 3.3.3. INTRAPERITONEAL INJECTION

For the intraperitoneal injection of  $5 \times 10^6$  HIV infected human PBMCs in sterile PBS, the mice were anaesthetized and put on their back in a mouse restrainer allowing the punctuation of the abdomen. The vital signs were checked until the mice regained consciousness.

### 3.3.4. INTRAVENOUS INJECTION

For the daily application of the BiTE antibody or formulation buffer, the mice were put into a fixation restrainer allowing the manipulation of the tail. To display the tail veins for easier access, the tail was incubated shortly in warm water to dilate the blood vessels. The intravenous injection was performed using a 1 ml syringe with a short 26 G needle.

## 4. RESULTS

### 4.1. THE HIV ENV PROTEIN – THE ANTIGEN ON HIV INFECTED CELLS

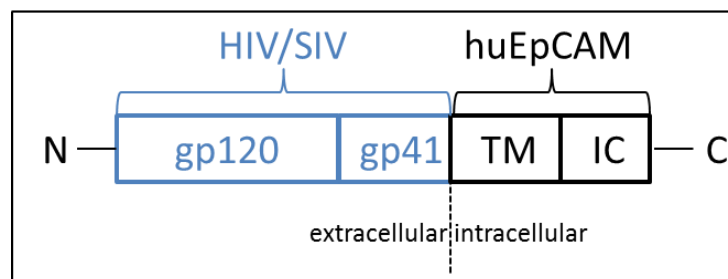
Extracellular accessibility of the targeted antigen on the plasma membrane of target cells is key for the BiTE technology. In general, the number of proteins encoded by HIV and therefore specific for HIV infected cells is very limited, offering only a small number of targetable antigens. To my knowledge, there is no HIV-specific upregulation of human proteins on the cell's surface, which would be specific for HIV infected cells. Non HIV-related proteins, which by accident can become part of the HIV virion membrane during the budding process from the cell surface, can be found on virions [129-131]. However, it seems that there is no further virus-specific protein present on the viral membrane besides the HIV envelope protein. As typical for all RNA retroviruses the HIV virions form and bud directly from the plasma membrane of the infected cell. Therefore, the HIV env can be detected on the surface of the infected cell prior to the budding process, making the infected cell sensitive towards HIV env targeting therapies.

Perelson et al. estimated that about  $10.3 \times 10^9$  virions bud in an infected individual on a daily basis [132]. Jouvenet et al. showed that most HIV virus-like particles bud within 6-8 minutes [133]. In average, 5 to 15 spikes of the HIV envelope proteins are present on a virion [134-136]. This described continuous formation of virions and exposure of antigens on the cell's membrane should be sufficient to make an infected cell targetable for BiTE antibody constructs.

### 4.2. GENERATION & CHARACTERIZATION OF HIV ENV<sup>+</sup> CELL LINES

In order to characterize the BiTE antibody constructs, cell lines were generated, which stably express HIV env. Since BiTE antibody constructs are able to bind to their target in addition to human CD3, Chinese hamster ovary (CHO) cells (negative for CD3) were chosen in the first place for recombinant antigen expression instead of human T cells as natural host cells. Furthermore, the extracellular part of the selected HIV env (gp140) was fused to the transmembrane and intracellular domain of the human epithelial cell adhesion molecule (EpCAM). Therefore, plasmids coding for full-length gp120 and the extracellular domain of gp41 were fused to the transmembrane and intracellular domain of human EpCAM and transfected into CHO cells. This anchoring of the target antigen by human EpCAM was successfully applied to different antigens before and provided an additional possibility of antigen detection via EpCAM-specific antibodies.

In case of HIV env, where two non-covalently bound proteins (gp120 and gp41) form a heterodimer, it was possible to detect the gp120 and gp41-EpCAM proteins individually thereby proofing post-translational processing of the precursor HIV env.



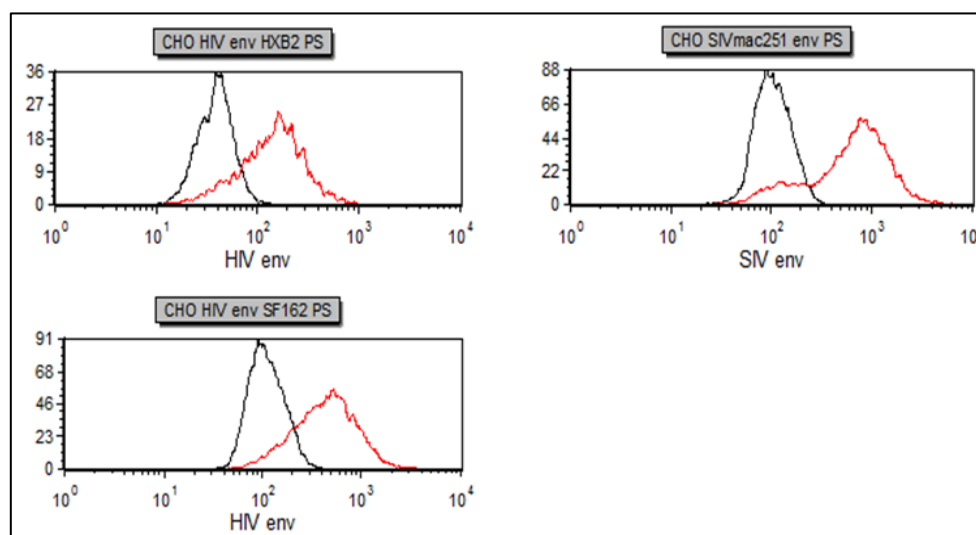
**Figure 12: Scheme of the HIV/SIV env Fusion Protein Used for Recombinant Expression in CHO and Jurkat Cell Lines.** The glycoprotein (gp120) is non-covalently bound to gp41, which is anchored in the plasma membrane via the transmembrane domain (TM) of human EpCAM. The transmembrane domain is followed by the intracellular domain (IC) of human EpCAM, which is used as an intracellular tag permitting the proof of correct intracellular localization and processing of the fusion protein.

With regard to the differing coreceptor usage of the existing HIV strains [137], two different CHO cell lines were generated expressing HIV envelope proteins with different coreceptor tropism in order to characterize the BiTE antibody constructs. The coverage of CXCR4 and CCR5 tropism of gp120 proteins allowed an approximate determination of the impact on BiTE affinity & activity caused by sequence variation within gp140. Next to the ectodomain of gp160 (gp140) of the HIV-1<sub>HXB2</sub> (Group M, subtype B) that uses CXCR4 as a coreceptor for viral entry, the CCR5-tropic clinical isolate SF162 (HIV group M, unknown subtype) was used for recombinant target expression. As human CD4 is known to bind to SIV envelope proteins as well [138], the ectodomain of the CCR5-tropic SIV<sub>mac251</sub> env was also fused to human EpCAM and transfected into CHO cells. Next to expression in CHO cells, the fusion protein of the HIV<sub>HXB2</sub> strain was transfected into Jurkat cells to allow characterization of the BiTE antibody constructs in a human T cell setting.

As can be seen in Figure 13, the transfection of CHO cells with the plasmid DNA constructs as described above, resulted in sufficient antigen expression detected by an intracellular anti-EpCAM staining using flow cytometry.

Western blotting of the transfected CHO cell lysates was done using an anti-gp120 as well as an anti-huEpCAM antibody, controlled by a cell lysate of untransfected CHO cells. The staining via the EpCAM-specific mAb (Figure 14) resulted in high background. The protein bands, at ca. 50 kDa and 100 kDa seem to be unspecific, since they are also present in the untransfected CHO lysate sample. Most likely, they appeared due to antibody cross-reactivity to the EpCAM protein of *Cricetulus griseus* that was not described for the mAb. However, the staining proofed the correct processing of target

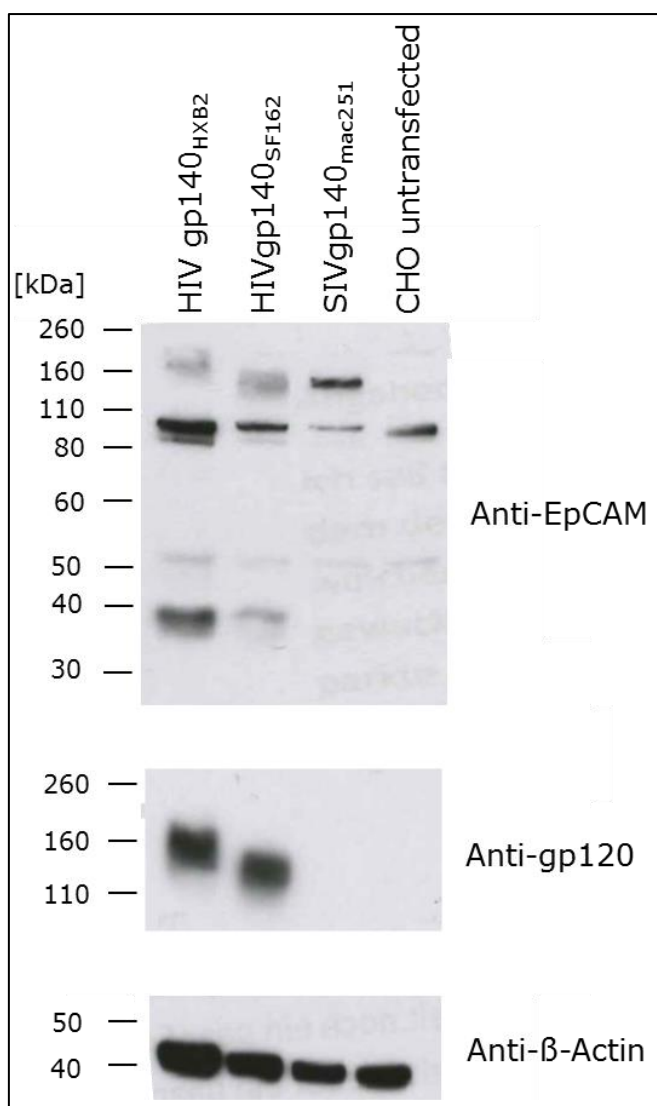
antigens for both HIV gp140 transfected CHO cells, as can be seen from the gp41-EpCAM band at 40 kDa. Not all HIV gp140-EpCAM proteins were processed as can be seen from the bands at around 160 kDa. Concluding from this staining the post-translational processing of the gp140 fusion precursor protein seems to be effective in HIV gp140 transfected CHO cells.



**Figure 13: Intracellular FACS Analysis of HIV/SIV env Transfected CHO Cells.** Cells were fixed, lysed and stained using an antibody directed against the intracellular domain of huEpCAM (red) binding to the intracellular part of the recombinant target fusion proteins. Secondary antibody staining was performed as a control (black). Transfected CHO cells showed sufficient HIV env expression. The SIV env transfected CHO cells showed a fraction of non-expressing cells, but SIV env expression in the majority of cells.

The SIV fusion protein is expressed in CHO cells, however no processed gp41-EpCAM protein can be detected, indicating that the SIV gp140 fusion protein cannot be processed by the CHO furin protease. The correct localization and extracellular accessibility was tested via indirect immunofluorescence and extracellular FACS staining using the generated BiTE antibody constructs presented in Figure 20.

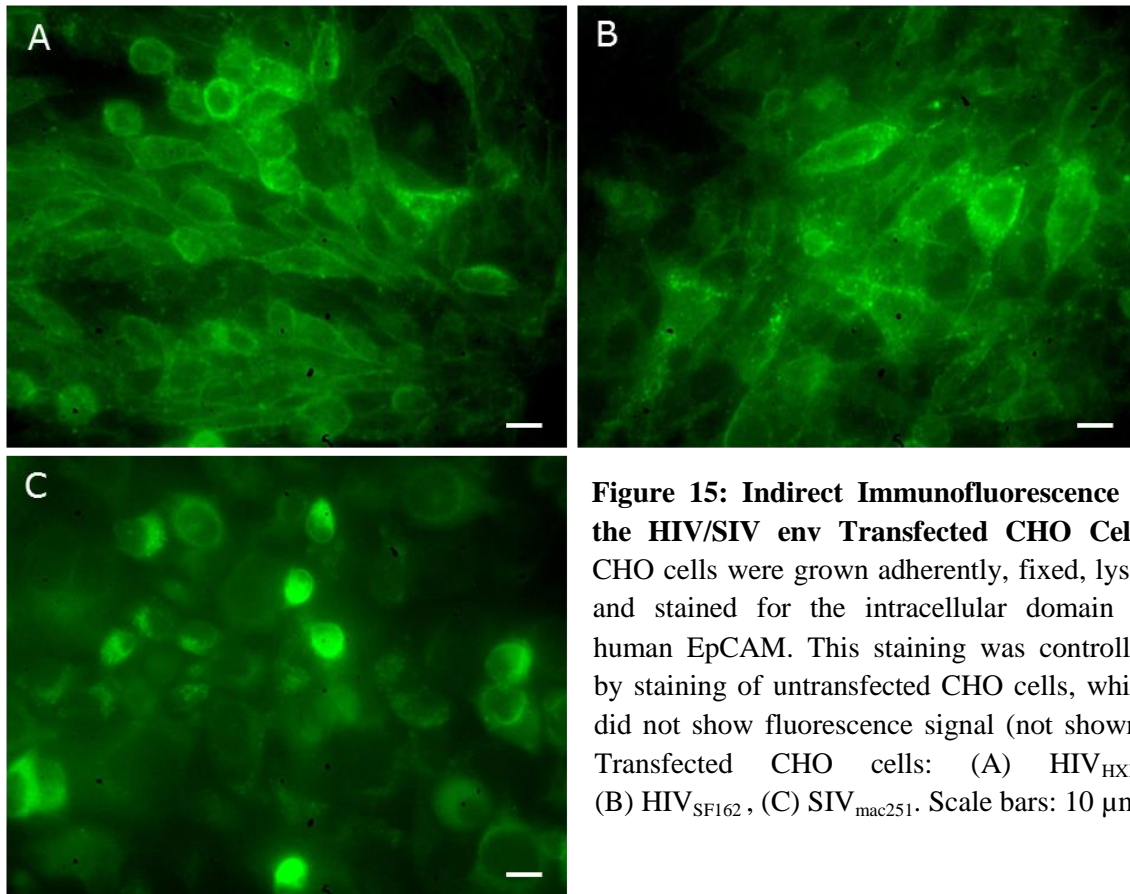
The anti-gp120 staining showed a positive signal for the HIV env transfected CHO cells. The HIV<sub>HXB2</sub> and HIV<sub>SF162</sub> gp140 transfected CHO cells showed one broad band at ca. 120-160 kDa, consisting most probably of processed (gp120) as well as unprocessed gp120-gp41EpCAM (ca. 160 kDa). The anti-gp120 antibody was not cross-specific to the SIV envelope protein. Therefore, the SIV transfected CHO lysate was not stained by this mAb.



**Figure 14: Western Blotting of Cell Lysates of the HIV/SIV env Transfected CHO Cells.** CHO cell lysates were applied to SDS-PAGE, blotted and stained via an anti-gp120 antibody, anti-huEpCAM or  $\beta$ -Actin-specific antibody, detected by HRP-conjugated secondary antibody and visualized by x-ray film exposure to chemiluminescent substrate incubation.

The transfected CHO cells were additionally analyzed by indirect immunofluorescence to proof the correct localization of the target antigen at the plasma membrane. This was done for all generated CHO cell lines and showed correct localization of the HIV fusion proteins at the plasma membrane. For the SIV fusion protein, the staining revealed protein expression at the plasma membrane (Figure 15), but also showed staining in the endoplasmatic reticulum and cytosol confirming the result of western blotting that the processing of the SIV fusion protein in CHO cells seems to be impaired.

Besides the intracellular detection and analysis of the recombinant envelope proteins, the expression of target antigens tested in Figure 20 showed positive target expression by FACS analysis using the generated BiTE antibody constructs.



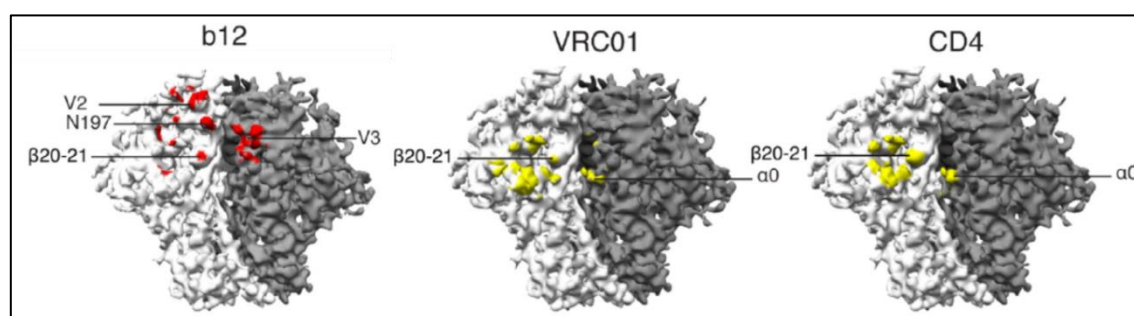
**Figure 15: Indirect Immunofluorescence of the HIV/SIV env Transfected CHO Cells.** CHO cells were grown adherently, fixed, lysed and stained for the intracellular domain of human EpCAM. This staining was controlled by staining of untransfected CHO cells, which did not show fluorescence signal (not shown). Transfected CHO cells: (A) HIV<sub>HXB2</sub>, (B) HIV<sub>SF162</sub>, (C) SIV<sub>mac251</sub>. Scale bars: 10  $\mu$ m.

#### 4.3. SELECTION OF BINDING MOIETIES FOR ANTI-HIV BiTE ANTIBODY CONSTRUCTS

Two human monoclonal anti-gp120 antibodies were converted into BiTE antibody constructs. On one hand, the mAb B12, first described by Burton et al. [45] was used as a model candidate. This neutralizing antibody has a nM affinity to gp120, is broadly neutralizing across different HIV strains and well characterized by the scientific community. The B12 epitope partially overlaps with the conserved CD4 binding site on gp120. On the other hand, the VRC01 antibody, a more recently discovered neutralizing monoclonal antibody was chosen to be expressed as a BiTE. The epitope of this mAb overlaps with the CD4 binding site to 98%, resulting in a comparable specificity as human CD4 [47,139].

Generally, the HIV epitopes of monoclonal antibodies are prone to viral escape due to the high rate of sequence alteration during viral replication. Therefore, the first two N-terminal domains (1+2) of CD4 (the natural receptor of HIV-1), were also fused to the anti-huCD3 scFv, resulting in a new, non-scFv target-binding BiTE format: CD4(1+2) $\alpha$ huCD3.

Viral evolution by mutations within the HIV envelope protein, making the virus unable to bind CD4 are unlikely, because CD4 is the major receptor for HIV entry into the cell. Binding to CD4 is necessary for the virion in order to change the conformation of the env protein so that it is able to bind to the coreceptor (CXCR4/CCR5) and ultimately enter the target cell. Rare exclusions however do exist. Some HIV strains have developed that are CD4 insensitive. In these cases, the HIV env proteins are in a “chronically” engaged conformation enabling them to bind directly to the coreceptor. That is why even CD4, as the major receptor of HIV, is only able to bind to 92% of existing HIV strains.



**Figure 16: HIV gp120 Trimer Model and Epitopes of mAbs Selected for BiTE Generation.** The structure models show two adjacent gp120 monomers (light/dark grey). The amino acids representing the respective epitopes are color-coded and their position within the gp120 molecule is indicated. The B12 epitope only shares a part of the CD4 binding site on HIV gp120, whereas the VRC01 epitope is almost congruent to the entire interacting amino acids, which represent the CD4 binding site. Modified from Lyumkis et al. [140].

Furthermore, two engineered constructs (CD4 D1.1/D1.2) designed by Chen et al. [49], were transferred into BiTE antibody constructs, comprising a mutated version of the first domain of human CD4, which is essential for binding to gp120 [141]. According to Chen et al. this isolation and engineering of the essential CD4 domain increases the target affinity and decreases the affinity to human MHC II complexes. The sole expression of the wild-type CD4 domain 1 is reported to be not functional, because of the clustering of hydrophobic amino acids that are usually buried in the interface to the second CD4 domain.

#### 4.4. DESIGN OF HIV BiTE ANTIBODY CONSTRUCTS

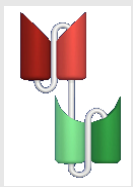
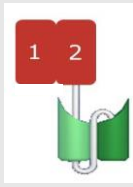
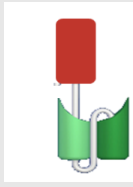
Generation of the B12 and VRC01 BiTE antibody constructs was done using their variable domains in heavy-light chain or light-heavy chain orientation respectively. Their variable domains were linked by a 15 amino acid linker of glycines and serines (G<sub>4</sub>S)<sub>3</sub>.

The resulting targeting scFv antibodies were linked to the human anti-CD3 scFv by a G<sub>4</sub>S linker, resulting in VH-(G<sub>4</sub>S)<sub>3</sub>-VL-G<sub>4</sub>S- $\alpha$ CD3 scFv or VL-(G<sub>4</sub>S)<sub>3</sub>-VH-G<sub>4</sub>S- $\alpha$ CD3 scFv. The anti-CD3 scFv was kept constant in a heavy light chain orientation (target-binding moiety-G<sub>4</sub>S-VH <sub>$\alpha$ CD3</sub>-(G<sub>4</sub>S)<sub>3</sub>-VL <sub>$\alpha$ CD3</sub>) as indicated in

Table 2.

The CD4(1+2) BiTE antibody constructs were generated by fusing the engineered CD4 domain 1 in case of D1.1/D1.2 candidates or the first two domains in case of CD4(1+2) of wild type CD4 (AA 1-182) to the anti-CD3 scFv separated by a G<sub>4</sub>S linker. This resulted in the theoretical sequence: (CD4 D1.1/1.2 or CD4(1+2))-G<sub>4</sub>S-VH <sub>$\alpha$ CD3</sub>-(G<sub>4</sub>S)<sub>3</sub>-VL <sub>$\alpha$ CD3</sub>. In order to be able to check the effect of soluble CD4(1+2) on infected cells as well as HIV env<sup>+</sup> expression on transfected T cells, the first two CD4 domains CD4(1+2) were also fused to an anti-murine CD3 scFv, which is not cross-reactive to human CD3.

**Table 2: Overview of the Generated Anti-HIV BiTE Antibody Constructs.** The candidates are split into scFv-based candidates and CD4-based candidates. The anti-HIV mAbs B12 and VRC01 have been transferred into the scFv format and linked to the proprietary anti-CD3 scFv. In case of the CD4-based BiTE antibody constructs, either the first two N-terminal domains of human CD4 or two candidates of an engineered human CD4 domain1 have been transferred into the BiTE context.

Structure	Candidates	Characteristics
	B12 HL / LH x $\alpha$ huCD3	<ul style="list-style-type: none"> <li>MAb, partially overlapping with CD4 epitope</li> <li>Broad HIV strain specificity, well characterized</li> </ul>
	VRC01 HL / LH x $\alpha$ huCD3	<ul style="list-style-type: none"> <li>MAb; 98 % overlap with CD4 epitope</li> <li>Broad HIV strain specificity</li> </ul>
	CD4 (D1+2) x $\alpha$ huCD3	<ul style="list-style-type: none"> <li>CD4 domains 1+2 fusion</li> <li>Broad specificity</li> </ul>
	CD4 D1.1 / D1.2 x $\alpha$ huCD3	<ul style="list-style-type: none"> <li>Two CD4 domain 1 candidates</li> <li>Increased HIV gp120 affinity</li> <li>Lowered affinity to MHC class II complexes</li> </ul>



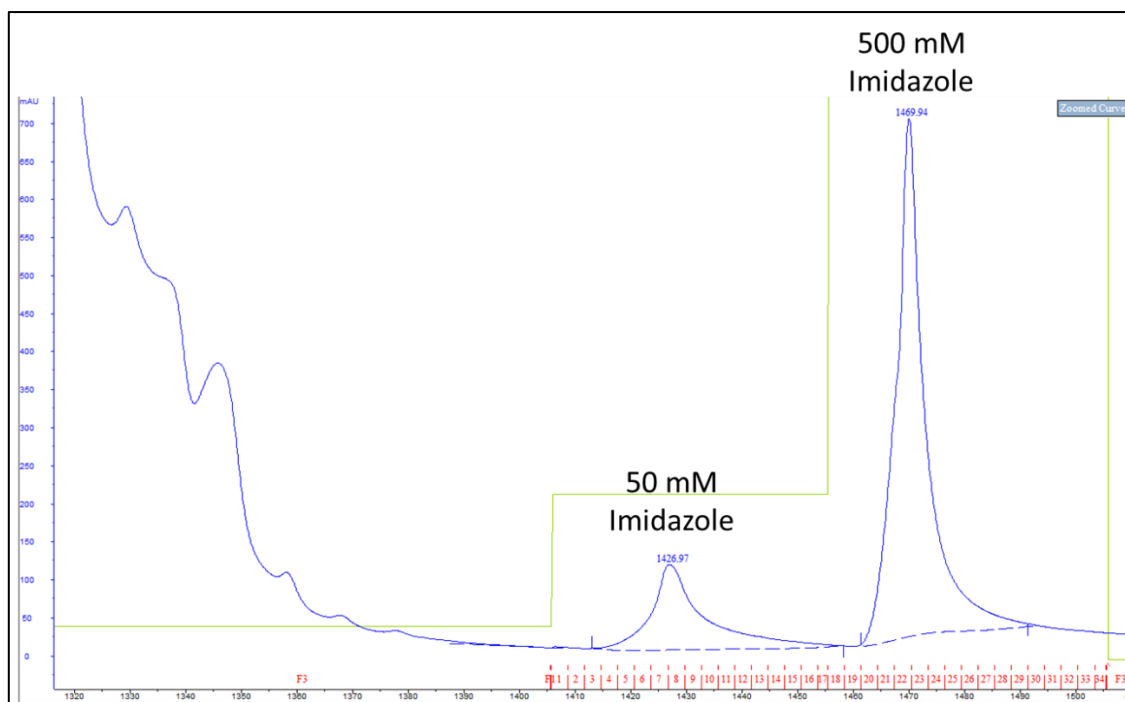
#### 4.5.FUNCTIONAL CHARACTERIZATION OF HIV BiTE ANTIBODY CONSTRUCTS

The BiTE antibody constructs were produced stably in CHO or transiently in HEK 293 cells and purified by immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). The purification process of the BiTE antibody constructs was analyzed by SDS-PAGE, subsequent coomassie staining and western blotting. The BiTE monomer fractions were pooled and split in aliquots before shock-frozen in liquid nitrogen and storage at -80°C. Next to protein stability determined by heat denaturation, the functional activity of both binding moieties was checked qualitatively by FACS analysis and quantitatively by surface plasmon resonance (SPR), ELISA and/or Scatchard Plot. The bioactivity of the candidates was determined visually by light microscopy-based assays or quantitatively by <sup>51</sup>Cr release or FACS-based cytotoxicity assays.

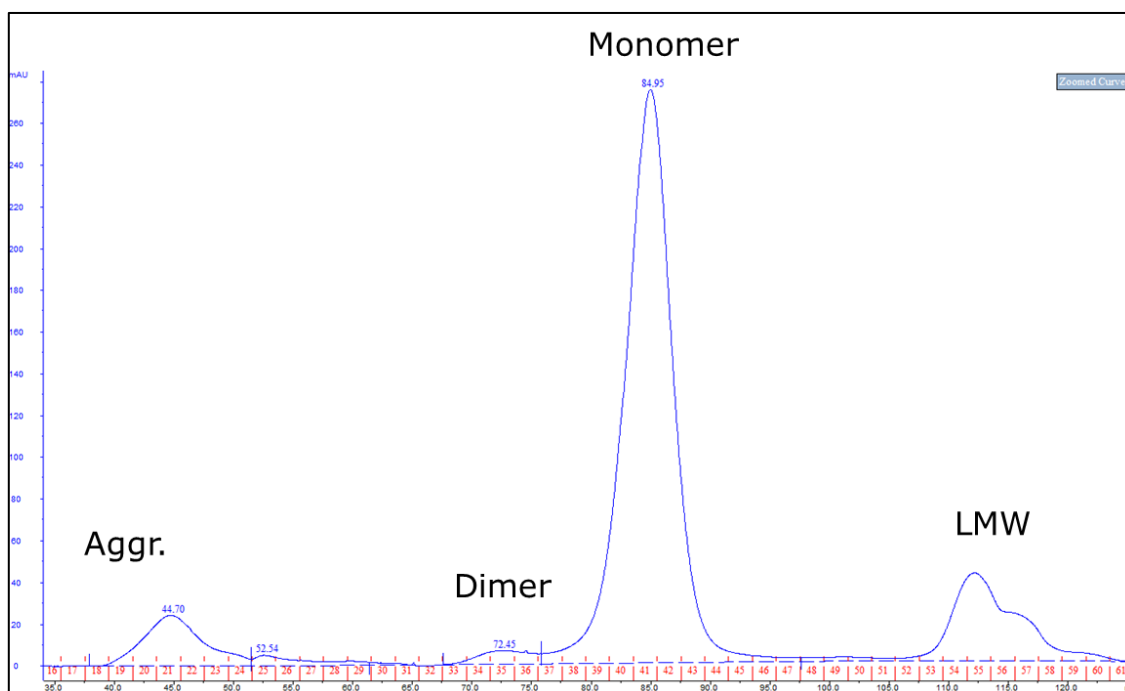
##### 4.5.1. PURIFICATION OF BiTE PROTEINS

BiTE antibody constructs were purified via their c-terminal hexa-histidine-tag on an IMAC column. Using a two-step elution process of different imidazole concentrations, unspecific proteins bound to the IMAC column were eluted at a low imidazole concentration (50 mM). The BiTE antibody constructs were eluted in a second elution step at a higher concentration of imidazole (500 mM). Fractions of the elution volume were stored at 4°C. Fractions containing the second elution peak of the IMAC were concentrated in spin columns and adjacently applied to size exclusion chromatography (SEC).

High molecular weight protein aggregates containing non-BiTE protein are typically observed in SEC and eluted first, potentially followed by a multimer and/or dimer peak of the BiTE antibody. The main peak at around 80 ml elution volume contained the BiTE monomer fraction (ca. 50 kDa). Occasionally, depending on the BiTE candidate, degradation can be observed in form of a peak following the BiTE monomer peak. The last peak in a typical chromatogram shows low molecular weight (LMW) proteins or salts (see Figure 18).

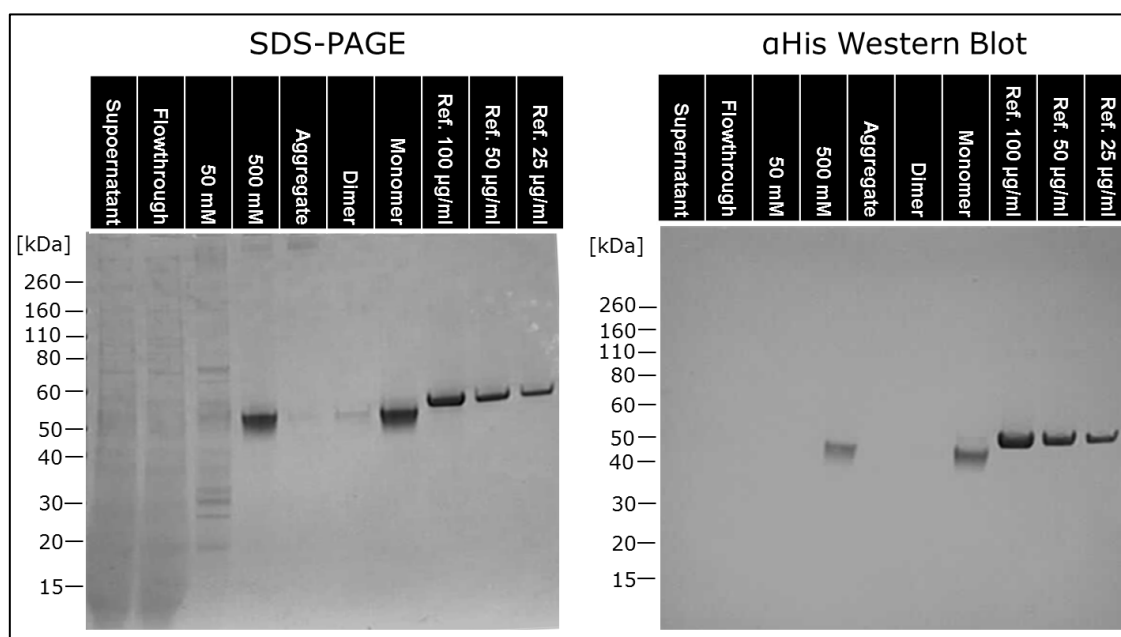


**Figure 17: Immobilized Metal Affinity Chromatography of B12HLx̄huCD3.** The 50 mM imidazole peak resembles the elution of non-BiTE proteins derived from the cell culture supernatant. The 500 mM imidazole peak mainly resembles BiTE as can be seen from the analytical samples. Legend: Imidazole concentration (green), OD<sub>280</sub> (blue) and fraction numbers (red).



**Figure 18: Size Exclusion Chromatography of B12HLx̄huCD3.** The first peak resembles high molecular weight mostly non-BiTE protein aggregates (Aggr.), followed by BiTE dimer and the BiTE monomers. Low molecular weight (LMW) resembles small proteins, polypeptides and salt divided from the BiTE antibody constructs. Legend: OD<sub>280</sub> (blue), fraction numbers (red).

Analytical samples of the purification processes were stored. These samples were analyzed by SDS-PAGE under denaturing and reduced conditions and by their His-tag in Western Blot. As shown in Figure 19, which is representative for the purification of the generated BiTE antibody constructs, the cell culture supernatant and flow-through show a variety of protein bands, resembling the cell culture supernatant. Overexpressed protein bands of the BiTE antibody are not always clearly visible, most probably due to low concentration. The 50 mM imidazole elution peak shows the elution of unspecific proteins, whereas the 500 mM elution sample shows one specific band at the assumed size of ca. 47-54 kDa depending on the BiTE. In the SEC analysis, the aggregate sample as well as the dimer sample display a weak band at the size of the BiTE antibody. The band of the monomer sample at ca. 50 kDa shows a prominent band at the correct size and no other bands assuring the purity of the BiTE monomer purification. A two-step detection system for the His-tag of the BiTE antibody constructs by western blotting resulted in staining of the 100% elution peak of the IMAC, the monomer fraction of the SEC and the reference BiTE antibody constructs at the expected size. The CD4(1+2) BiTE was additionally analyzed for its endotoxin level to confirm its usability for the *in vivo* experiment.

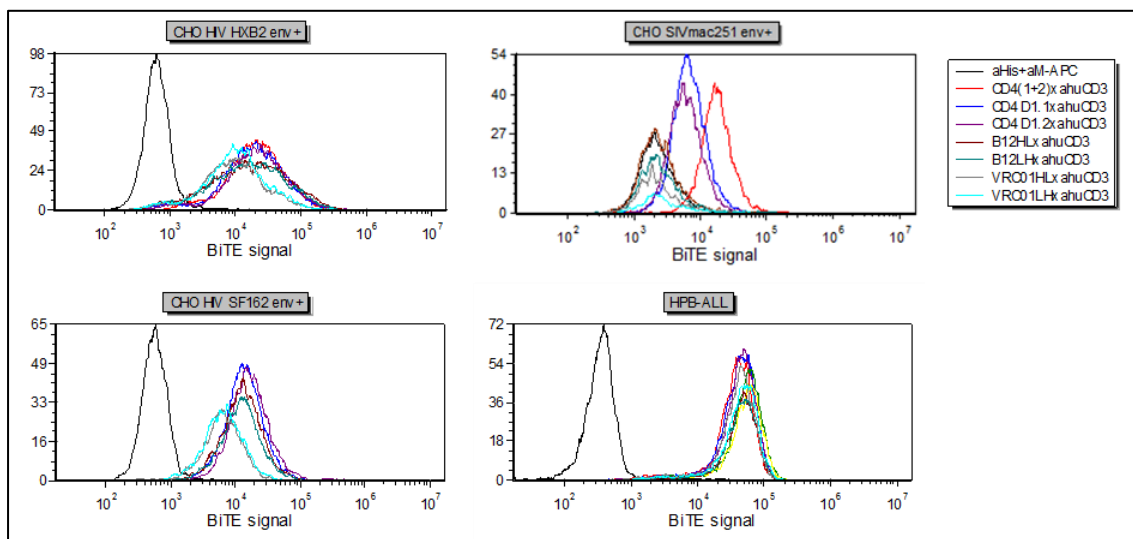


**Figure 19: Analysis of BiTE Purification of B12HLxahuCD3.** Samples of the IMAC purification: Cell culture supernatant, flow through of the IMAC column, 50 mM elution and 500 mM imidazole elution. Samples of the SEC purification: Protein aggregates, BiTE dimer and BiTE monomer. Reference samples of purified BiTE monomer at 100 µg/ml, 50 µg/ml and 25 µg/ml. SDS-PAGE was performed under denaturing, reduced conditions. Western blotting was performed using an anti-His mAb directed against the His-tag of the BiTE antibody constructs.

#### 4.5.2. FACS BINDING TO HIV ENV<sup>+</sup> CHO CELLS AND T CELLS

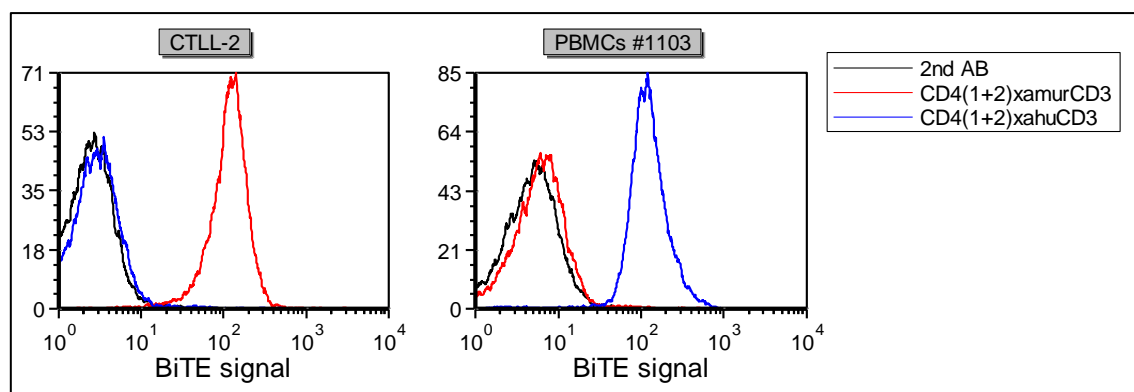
After purification of the BiTE antibody constructs, the purified monomer fraction was tested for its functionality in binding to HIV/SIV env<sup>+</sup> and human/maaque CD3<sup>+</sup> cells respectively using flow cytometry (Figure 20). All generated BiTE antibody constructs showed binding to HIV env transfected CHO cells. VRC01 BiTE antibody constructs showed a lower signal as compared to the CD4-based, and B12-based BiTE antibody constructs. Binding to human CD3<sup>+</sup> cells revealed the functionality of the anti-CD3 $\epsilon$  scFv. No signal graduation was observed between the BiTE antibody constructs, indicating that the target-binding protein does not affect the functionality of the constant CD3 binding scFv within the BiTE.

In contrast to the scFv-based HIV BiTE antibody constructs, the CD4-based BiTE antibody constructs were able to bind to the SIV env<sup>+</sup> CHO cells. Here, the CD4(1+2) BiTE showed an increased signal compared to the CD4 domain 1 candidates (CD4 D1.1/CD4 D1.2).



**Figure 20: Functional Proof of Generated BiTE Antibody Constructs by Binding to HIV/SIV env Transfected CHO and Human CD3<sup>+</sup> Cells.** Purified monomeric BiTE antibody constructs at 10  $\mu$ g/ml (color-coded, see legend) were tested by FACS analysis using CHO cells positive for binding to the according HIV or SIV env and CD3<sup>+</sup> on HPB-ALL cells.

The generated CD4(1+2) control BiTE containing the CD4 domains 1 and 2 fused to a murine CD3 scFv was additionally tested on a murine T cell line (CTLL-2) proofing its murine specificity. This property enabled the analysis of the target-binding moiety on human T cells independently of human CD3 binding. FACS binding analysis confirmed the specificity of the anti-murine CD3 scFv to murine CD3. Binding to human CD3 was not observed nor cytotoxic activity (not shown) when testing this BiTE with human PBMCs *in vitro*.

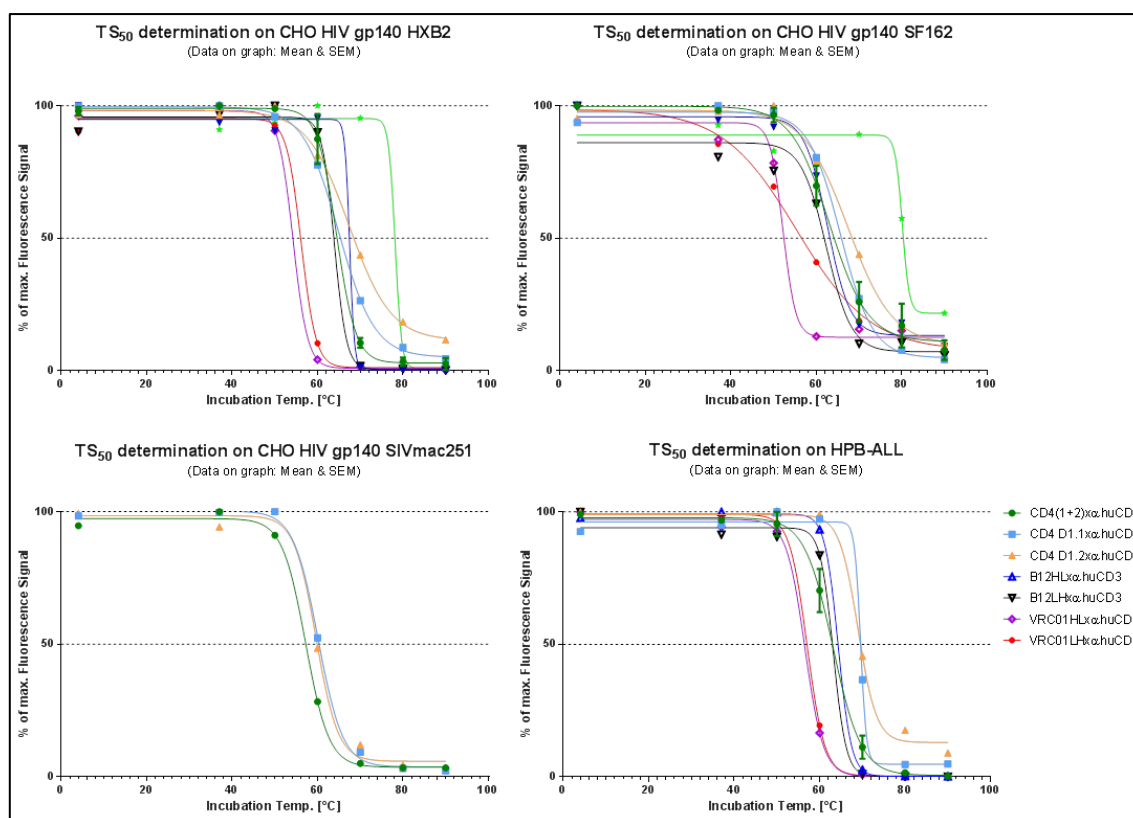


**Figure 21: FACS Analysis of Murine CD3 Specificity of CD4(1+2)xantimurCD3 Control BiTE.** CD4(1+2) BiTE and CD4(1+2) control BiTE was incubated with CTLL-2 (murine T cells) and human PBMCs and applied to FACS analysis to proof the specificity of both BiTE antibody constructs.

#### 4.5.3. TEMPERATURE STABILITY OF HIV BiTE ANTIBODY CONSTRUCTS

In order to characterize the BiTE antibody constructs for their temperature stability, aliquots of the BiTE antibody constructs were incubated for five minutes at temperatures ranging from 4 to 90°C. The supernatants of the BiTE aliquots were subsequently incubated on ice and analyzed for their binding ability to HIV env<sup>+</sup> and CD3<sup>+</sup> cells via FACS analysis. Though the BiTE antibody constructs become denatured during the incubation at high temperatures, later refolding processes after the heat denaturation step that interfere with the FACS assay cannot be excluded.

To scrutinize the stability of the different BiTE antibody constructs, their TS<sub>50</sub> value (temperature at the half-maximum FACS signal of the BiTE candidate) was determined.



**Figure 22: Temperature Stability of BiTE Antibody Constructs Using FACS Analysis.** BiTE antibody constructs were incubated at temperatures ranging from 4 to 90°C and subsequently analyzed for their binding to target or CD3 positive cells. The temperature at the half-maximum fluorescence signal ( $TS_{50}$ ) was determined.

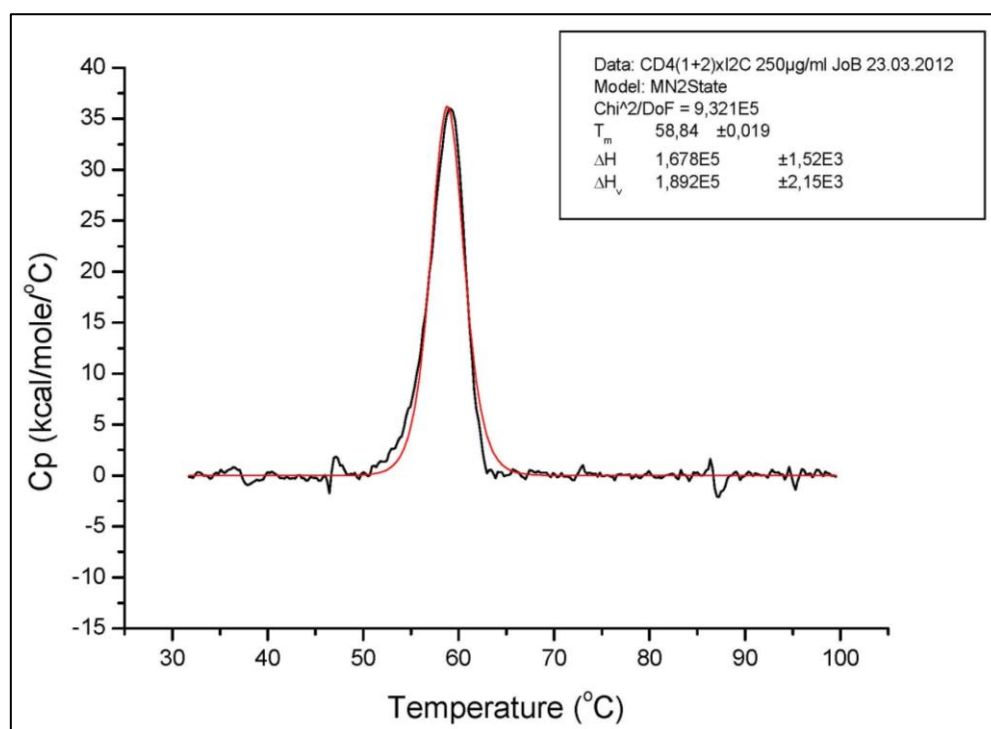
As can be seen from Table 3, CD4 and B12 BiTE antibody constructs showed a temperature stability around 65°C when tested on HIV  $env^+$  CHO cells and CD3<sup>+</sup> cells.

**Table 3:  $TS_{50}$  Overview of BiTE Antibody Constructs Tested on CHO HIV  $env^+$  and CD3<sup>+</sup> Cells**

$TS_{50}$ [° C]	CHO <sub>HXB2</sub>	CHO <sub>SF162</sub>	CHO SIV <sub>mac251</sub>	HPB-ALL
CD4(1+2)xα,huCD3	64.48	63.07	57.20	62.48
CD4 D1.1xα,huCD3	64.94	65.67	60.13	69.57
CD4 D1.2xα,huCD3	67.37	67.43	59.67	68.95
B12HLxα,huCD3	67.51	62.60	NA	64.32
B12LHxα,huCD3	63.92	62.36		63.17
VRC01HLxα,huCD3	54.25	52.18	NA	56.54
VRC01LHxα,huCD3	56.17	55.37		57.05
CD4(1+2)xα,murCD3	78.16	80.11	ND	NA

The temperature stability on HIV env<sup>+</sup> of VRC01 BiTE antibody constructs was around 55°C, thus lower than for B12 or CD4-based BiTE antibody constructs. This decreased temperature stability also affects the constant CD3 scFv of the VRC01 BiTE showing a TS<sub>50</sub> in the same range (ca. 55°C) on CD3<sup>+</sup> cells instead of the TS<sub>50</sub> of ca. 65°C as seen for the other BiTE antibody constructs. The CD4 control BiTE CD4(1+2) $\alpha$ murCD3 showed the highest temperature stability of almost 80°C, only tested on HIV env<sup>+</sup> cells. CD4-based BiTE antibody constructs showed a TS<sub>50</sub> at ca. 60°C on SIV env<sup>+</sup> CHO cells.

The stability of CD4(1+2) $\alpha$ huCD3 was further analyzed using differential scanning calorimetry (DSC) with reference to the BiTE formulation buffer. According to the DSC graph, the melting process of the CD4(1+2) BiTE starts at ca. 50°C and peaks at 58.84°C, revealing method-related differences in the determination of temperature stability (FACS TS<sub>50</sub> of 64.5°C compared to 58.8°C in DSC).



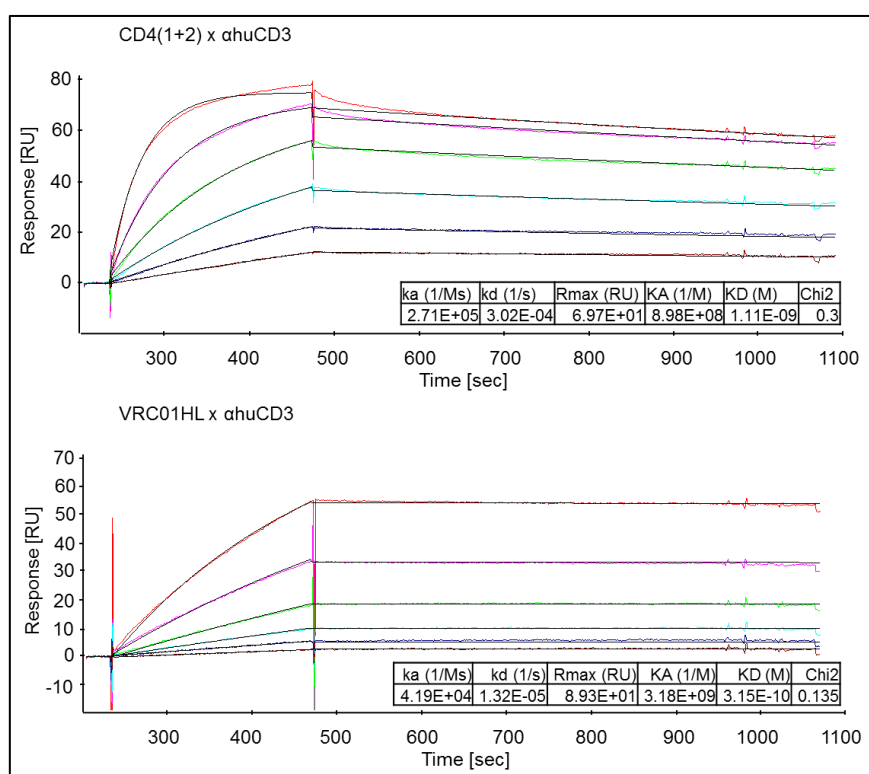
**Figure 23: Differential Scanning Calorimetry of CD4(1+2) $\alpha$ huCD3.** The BiTE candidate was adjusted to a concentration of 250 µg/ml and applied to DSC showing a homogeneous peak at 58.8°C with a Cp of ca. 36 kcal/mole/°C. BiTE formulation buffer was used as a reference.

#### 4.5.4. DETERMINATION OF BiTE AFFINITY TO HIV / SIV ENV PROTEINS

For characterization of the BiTE antibody constructs, the affinities of both binding moieties of each BiTE were examined using soluble antigens in SPR and ELISA or cell-anchored antigens of transfected cells in FACS analyses using Scatchard Plot analyses.

#### 4.5.4.1. Determination of BiTE Target Affinity Using SPR

The target affinity of the CD4(1+2) and VRC01HL BiTE measured by surface plasmon resonance (SRP) is shown in Figure 24. The association and dissociation rates of the CD4(1+2) BiTE shown are representative for the B12 HL/LH and the CD4 domain 1 candidates. The association rate of CD4-based and B12 candidates was about  $2.7 \times 10^5$  1/Ms compared to a lower association rate of  $4.2 \times 10^4$  1/Ms for the VRC01 constructs. However, the dissociation rate of VRC01 constructs was lower compared to CD4 or B12 constructs, leading to a KD of 0.3 nM for VRC01 candidates versus ca. 1 nM for CD4 and B12 constructs respectively (see Table 4 for details).

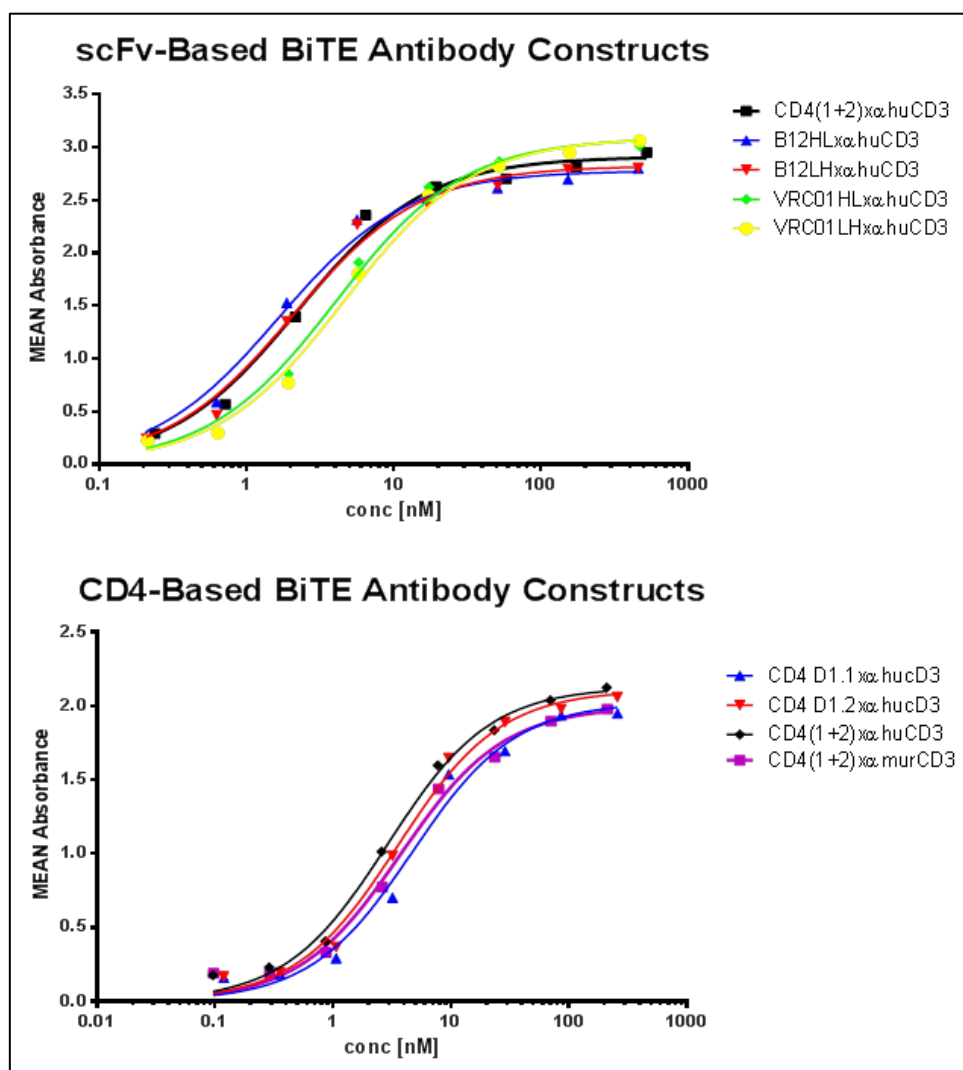


**Figure 24: gp120 Surface Plasmon Resonance Data Representing the Affinity Kinetics of CD4(1+2)ahucd3 and VRC01HLahucd3 to Immobilized HIV<sub>IIIB</sub> gp120 Recombinant Protein.** The kinetic of the CD4(1+2) BiTE candidate is representative for the B12 and CD4 domain 1 BiTE antibody constructs.



#### 4.5.4.2. Determination of BiTE Affinity Using ELISA

In addition to SRP, the affinity of the BiTE antibody constructs to HIV and SIV was examined by enzyme linked immunosorbent assay (ELISA). In this format, the recombinant solubilized env of HIV<sub>IIIB</sub> or SIV<sub>mac251</sub> was coated on a 96-well plate, incubated with BiTE dilution series and detected via the hexa-histidine-tag of the BiTE antibody constructs. Since the detection was done using bivalent immunoglobulins (IgG) this method is less precise than the monovalent Biacore and Scatchard Plot FACS-based measurements.



**Figure 25: HIV gp120 Affinity of scFv-Based and CD4-Based BiTE Antibody Constructs Analyzed by ELISA.** Soluble HIV<sub>IIIB</sub> gp120 was coated on a Maxisorp plate. BiTE antibody constructs were added and detected via a peroxidase-conjugated antibody directed against the BiTE antibody constructs' histidine-tag. CD4 domain 1 BiTE antibody constructs showed no increased affinity compared to the CD4(1+2) BiTE. The VRC01 BiTE antibody constructs showed a decreased affinity compared to B12 and CD4-based BiTE antibody constructs. For more details, see Table 4.

Figure 25 shows the absorption measured at the corresponding BiTE concentrations. The amount of BiTE at the half-maximal absorbance, shows the affinity constant  $K_D$  [nM] for each BiTE. CD4(1+2) and B12 BiTE antibody constructs showed a gp120 affinity of ca. 2 nM, whereas VRC01 candidates presented a slightly lower  $K_D$  of ca. 4 nM.

CD4(1+2) and CD4 D1.2 BiTE antibody constructs displayed target affinities at ca. 2.7 nM (see lower plot). The control BiTE CD4(1+2)x  $\alpha$ murCD3 and CD4 D1.1 candidate showed a  $K_D$  of ca. 3.6 nM.

#### 4.5.4.3. Determination of Cell-Based Affinity Using CHO Env<sup>+</sup> Cells

Using the CHO cell lines stably transfected with the ectodomain of the HIV env of the HIV<sub>HXB2</sub> strain, HIV<sub>SF162</sub> isolate or SIV<sub>mac251</sub>, the cell-based affinity of the BiTE antibody constructs was determined.

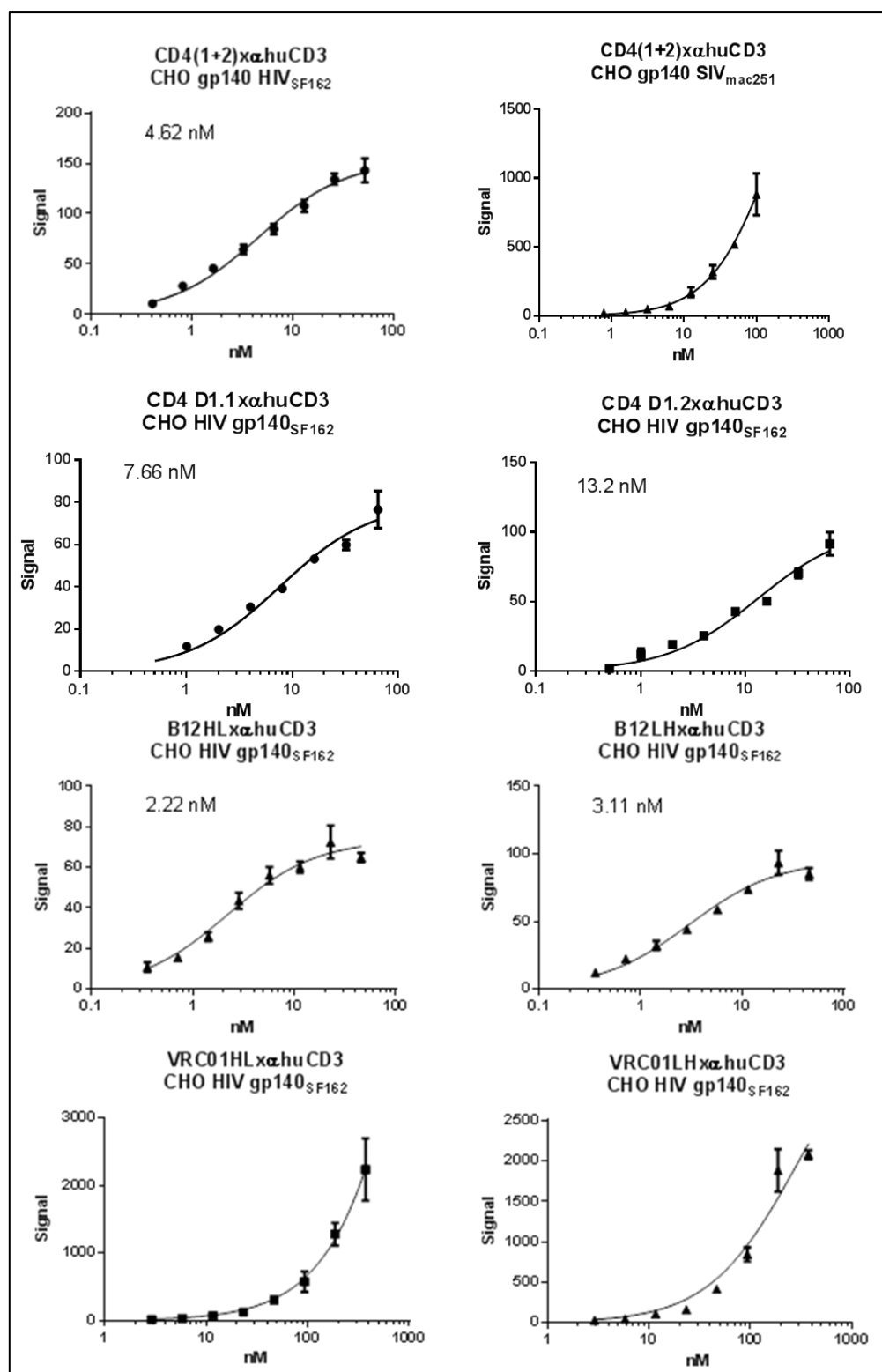
The cell-based HIV env affinity was determined by four-hour incubation of BiTE dilution series with target positive cells, subsequent washing, staining with a monovalent detection system using fluorophor-labeled anti-histidine-Fab fragments, further washing, fixation and subsequent FACS analysis.

The cell-based affinity of the CD4(1+2) BiTE to HIV env of the SF162 isolate was about 4.62 nM, whereas the B12 candidates showed a slightly better cell-based target affinity with ca. 2 nM.

CD4 domain 1 BiTE antibody constructs (D1.1/D1.2) showed lower affinities (7 and 11 nM respectively) in cell-based measurements than the CD4(1+2) candidate.

The VRC01 candidates did not reach the saturation plateau in order to determine a precise  $K_D$  value.

The affinity of CD4-based BiTE antibody constructs to SIV env could not be examined using SIV transfected CHO cells (see Figure 25 bottom), because the FACS signal did not reach the saturation plateau (most likely because of insufficient BiTE concentration). However, their target affinity could be analyzed using soluble SIV gp120 antigen in the ELISA format.



**Figure 26: Scatchard Plot Analyses of Anti-HIV BiTE Antibody Constructs for the Determination of HIV/SIV gp120 Affinity on Transfected CHO Cells.** BiTE dilution series were incubated with target positive cells for four hours at 4°C on a shaker and subsequently stained using a monovalent detection system and analyzed by FACS. For candidates reaching the saturation plateau, the KD values are indicated. The affinity of VRC01 on HIV gp120 or CD4-based BiTE antibody constructs on SIV gp120 could not be determined using transfected CHO cells, because their FACS signal did not reach the saturation plateau. Mean values are displayed, error bars indicate SEM.

**Table 4: Overview of HIV gp120 Affinity Measurements for BiTE Antibody Constructs.**

Method	SPR		ELISA		Scatchard		ELISA
Antigen	HIV <sub>IIIB</sub>				HIV <sub>HXB2</sub>	HIV <sub>SF162</sub>	SIV <sub>mac251</sub>
Affinity	ka [1/Ms]	kd [1/s]			KD [nM]		
CD4(1+2)x ahuCD3	2.93E+05	2.08E-04	1	2.5	5.79	10.49	94.32
B12HLx ahuCD3	5.26E+05	1.23E-03	2	1.8	3.50	8.76	NA
B12LHx ahuCD3	4.64E+05	1.03E-03	2.3	2.3	5.57	11.48	
VRC01HLx ahuCD3	4.19E+04	1.32E-05	0.3	4.3	ND	ND	
VRC01LHx ahuCD3	3.72E+04	3.41E-07	0.3	4.9	ND	ND	

Overall, the determined affinities to gp120 ranged in the single digit to low nM range for the generated BiTE antibody constructs (except for VRC01 BiTE antibody constructs) and matched the published affinities for the parental mAbs or CD4 molecules respectively.

VRC01 BiTE antibody constructs showed a HIV env affinity of ca. 300 pM in SPR, but ca. 4 nM in the ELISA assay. Their cell-based concentration could not be determined since their concentration was insufficient, so that their FACS signal did not reach the saturation plateau.

The SIV gp120 affinity determined for CD4(1+2) is about 50- to 100-fold (depending on the method) lower than the affinity which was examined for the HIV gp120 proteins.

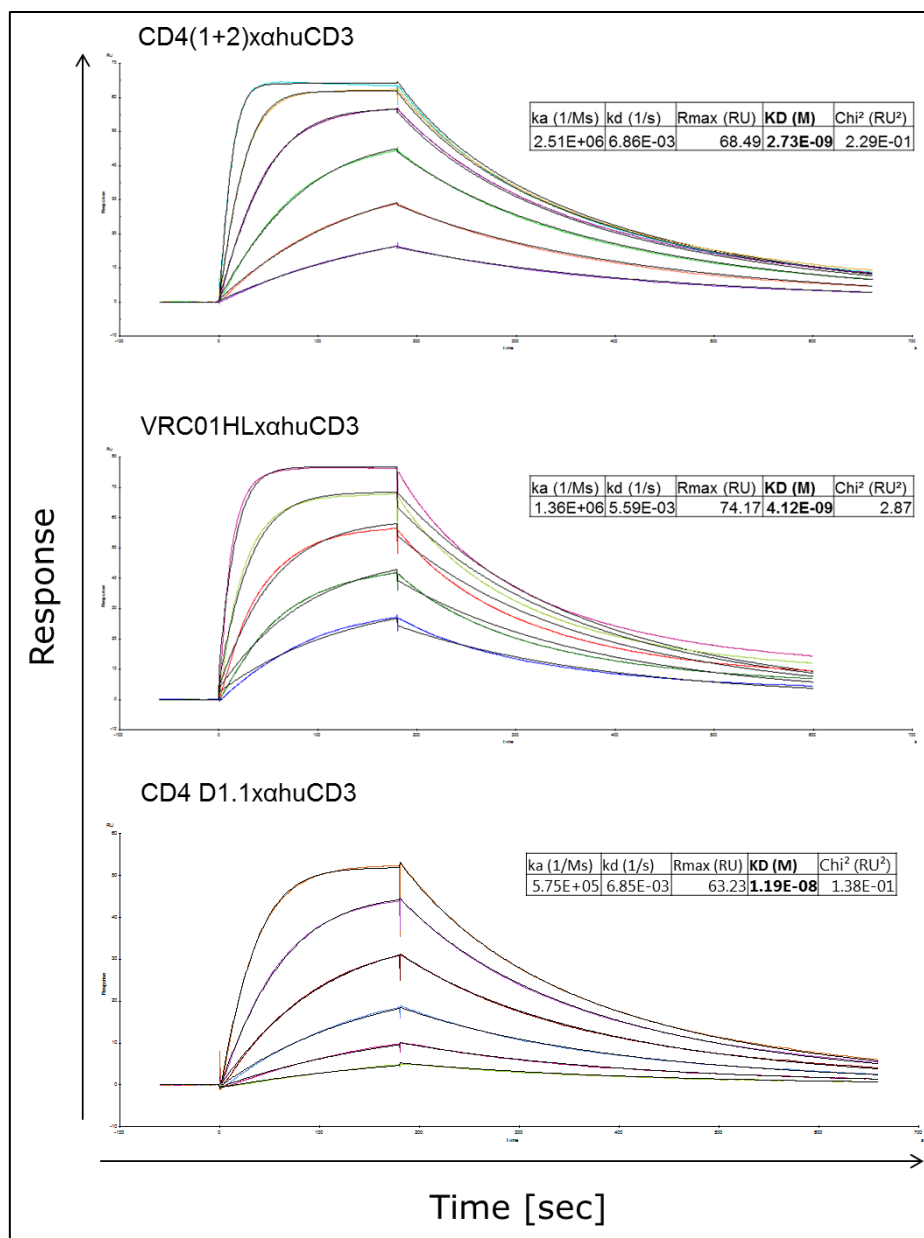
Generally, the measured cell-based affinities were slightly lower than the results using the soluble gp120 antigens, most probably due to an altered conformation of the antigen in context of the plasma membrane and potentially influenced by the EpCAM transmembrane domain.

#### 4.5.5. DETERMINATION OF BiTE AFFINITY TO HUMAN CD3

The CD3 $\epsilon$  binding moiety of the BiTE antibody constructs was also analyzed using Biacore and Scatchard Plot analysis.

#### 4.5.5.1. BiTE Affinity to Human CD3 Using SPR

Affinity analyses of the BiTE antibody constructs to human CD3 were performed using a fusion protein of the extracellular domain of CD3 fused to human Fc or binding analyses on human CD3<sup>+</sup> cells. The three Biacore plots shown here represent the affinity kinetics of the BiTE antibody constructs to human CD3. For an overview of the determined values, refer to Table 5.

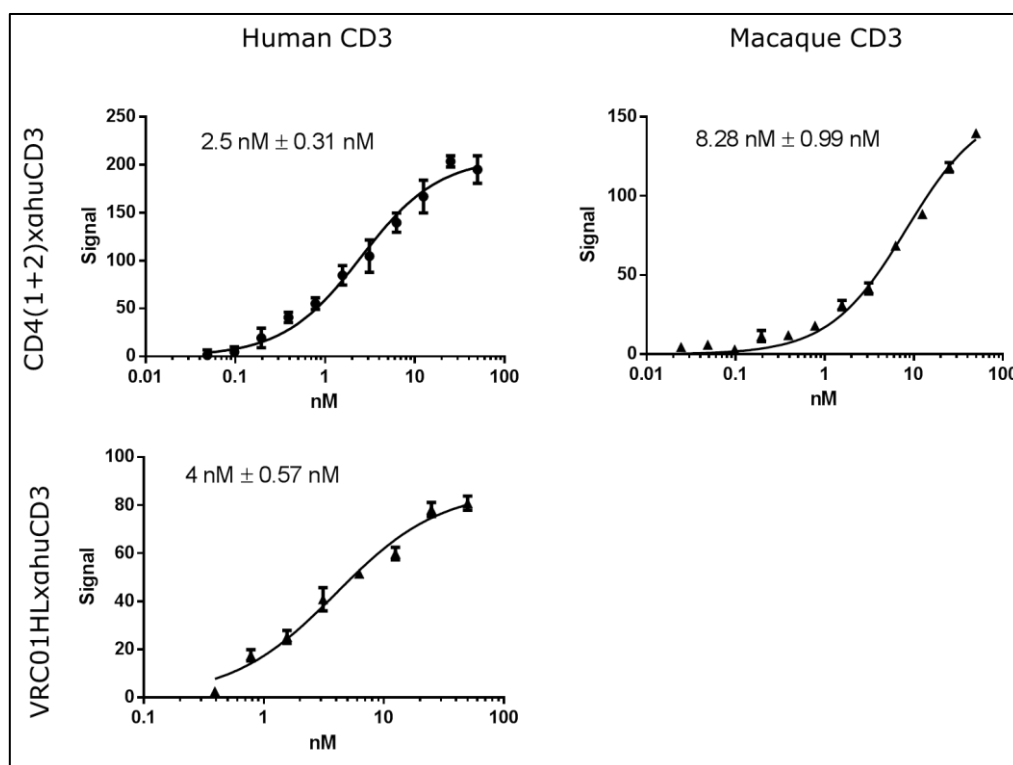


**Figure 27: Excerpt of Human CD3 Affinity Measurement of the CD4(1+2), VRC01 and CD4 D1 BiTE Antibody Constructs Using SPR.** Soluble human CD3 peptide was coated onto a CM5 chip. BiTE antibody constructs were added and affinity was determined. CD4 domain 1.1 candidate showed a decreased CD3 affinity compared to CD4(1+2) and VRC01 BiTE antibody constructs, which are representative for B12 and CD4 domain 1.2 BiTE antibody constructs.

As expected for the constant anti-CD3 $\epsilon$  scFv of the BiTE antibody constructs, the Biacore analysis showed only slight differences in human CD3 affinity for most candidates. The KD values ranged between 1.5 and 4.1 nM except for CD4 D1.1x $\alpha$ huCD3, which showed a human CD3 affinity of 11.9 nM (see Figure 27).

#### 4.5.5.2. Cell-Based BiTE Affinity to Human and Macaque CD3

Next to affinity measurements with recombinant soluble CD3 fusion proteins, the cell-based CD3 affinity of BiTE antibody constructs was examined by a monovalent detection method using Scatchard Plot analysis, see 3.2.6 for details. Human CD3 $^+$  cells (PBMCs & HPB-ALL) or macaque CD3 $^+$  cells (41-19 LnPx) were used to determine the CD3 affinity of the BiTE antibody constructs.



**Figure 28: Determination of Cell-Based CD3 Affinity Using Human or Macaque CD3 $^+$  Cells.** BiTE dilution series were incubated with human or macaque CD3 $^+$  positive cells for 4 hours at 4 $^{\circ}$ C and subsequently stained using a monovalent detection system and analyzed by FACS. Mean values are displayed, error bars indicate SEM.

The affinity determined by Scatchard Plot analysis ranged between 1-4 nM for all BiTE antibody constructs. The lower affinity of CD4 D1.1xahuCD3 determined by SPR was not confirmed using human CD3<sup>+</sup> cells. The KD values of CD4-based BiTE antibody constructs to macaque CD3<sup>+</sup> were slightly lower than for human CD3 ranging at ca. 4-8 nM.

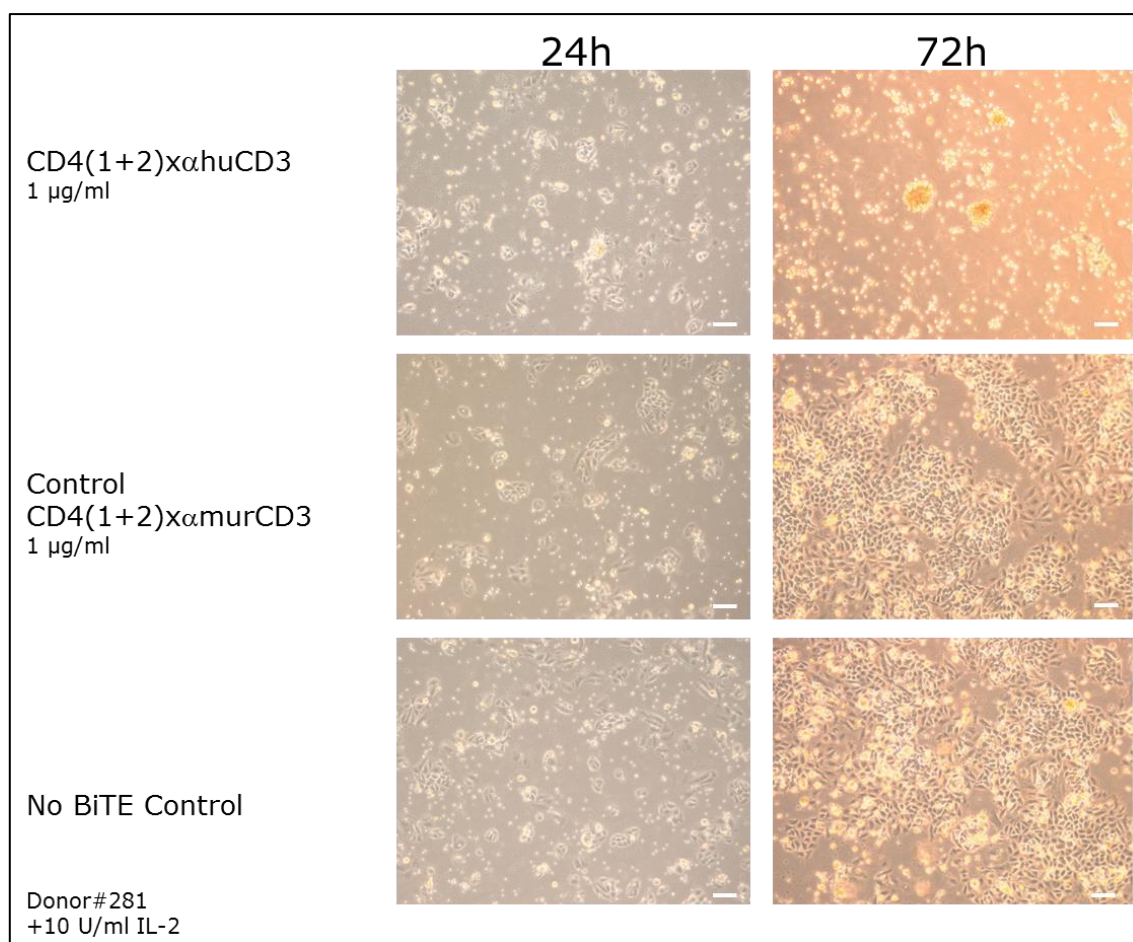
**Table 5: Summary of Human and Macaque CD3 Affinity of the BiTE Antibody Constructs.**

Method		SPR		Scatchard		
Antigen		huCD3			maCD3	
Affinity		ka [1/Ms]	kd [1/s]	KD [nM]		
BiTE Antibody Constructs	CD4(1+2)xahuCD3	2.82E+06	6.33E-03	2.3	2.42	8.28
	CD4 D1.1xahuCD3	5.75E+05	6.85E-03	11.9	2.76	8.21
	CD4 D1.2xahuCD3	2.32E+06	5.93E-03	2.6	0.97	3.9
	B12 HLxahuCD3	3.15E+06	5.61E-03	1.8	1.61	ND
	B12 LHxahuCD3	4.42E+06	6.65E-03	1.5	1.49	
	VRC01HLxahuCD3	1.36E+06	5.59E-03	4.1	3.74	
	VRC01LHxahuCD3	1.43E+06	4.10E-03	2.9	5.8	

#### 4.5.6. QUALITATIVE DETERMINATION OF CYTOTOXIC ACTIVITY ON HIV ENV<sup>+</sup> CHO CELLS

The cytotoxic activity of the BiTE antibody constructs was determined visually in cell culture and quantitatively by <sup>51</sup>Cr release or FACS-based cytotoxicity assays. Unstimulated human PBMCs or isolated primary CD8<sup>+</sup> T cells, prestimulated with anti-CD3/anti-CD28/rIL-2, served as effector cells in cytotoxicity assays. These effector cells were incubated with HIV env<sup>+</sup> CHO cells and a dilution series of a BiTE.

The visual cytotoxicity assay (Figure 29) shows adherent HIV env<sup>+</sup> CHO cells grown on a 6-well plate co-incubated with prestimulated primary CD8<sup>+</sup> T cells 24h after setup of the assay. Two days later no more adherent CHO cells can be detected in the CD4(1+2) BiTE containing well. However, T cell aggregates can be detected pointing to T cell stimulation. In the control wells however (CD4(1+2)xamurCD3 or no BiTE), the CHO cells proliferated in the presence of stimulated CD8<sup>+</sup> T cells demonstrating the BiTE dependent lysis by cytotoxic T cells as seen with CD4(1+2)xahuCD3.



**Figure 29: Qualitative Determination of Cytotoxic Activity of the CD4(1+2) BiTE.** Anti-CD3/anti-CD28/IL-2 prestimulated human primary CD8<sup>+</sup> T cells were co-incubated for 72h with adherent HIV env<sup>+</sup> CHO cells (Effector:target ratio of 10:1) in presence of IL-2. Scale bars: 50 μm.

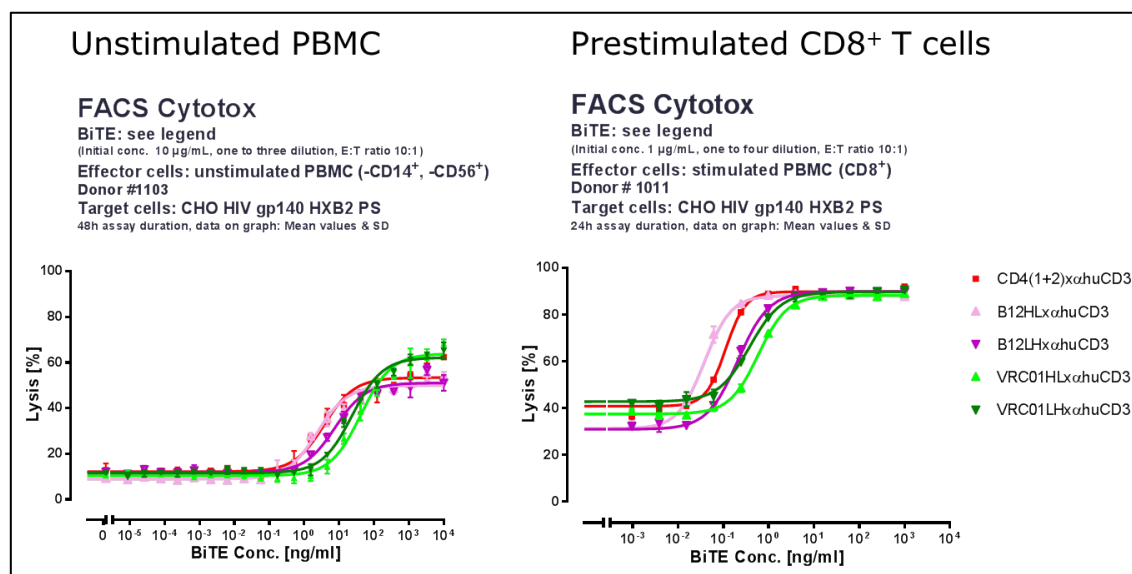
#### 4.5.7. QUANTITATIVE DETERMINATION OF BIOACTIVITY IN VITRO

In order to quantify the cytotoxic activity and rank the BiTE antibody constructs for their activity, <sup>51</sup>Cr-release or FACS-based cytotoxicity assays were performed using prestimulated and native PBMCs as effector cells.

In short, a BiTE dilution series adjusted for the BiTE concentration of the candidate was prepared in 96-well plate. Afterwards, HIV/SIV env transfected CHO cells labeled with a fluorescent dye were mixed with a 10-fold excess of effector cells and added to the pre setup BiTE dilution series. After co-incubation of 18-48 hours, the cells were incubated with propidium iodide (PI). The percentage of dead target cells was analyzed (see Figure 30) by gating for labeled and PI-positive cells.



Since the molecular weight of BiTE antibody constructs is not identical, their  $EC_{50}$  values were determined based on their protein concentration as well as their molarity. See Table 6 for molarity-based  $EC_{50}$  values.



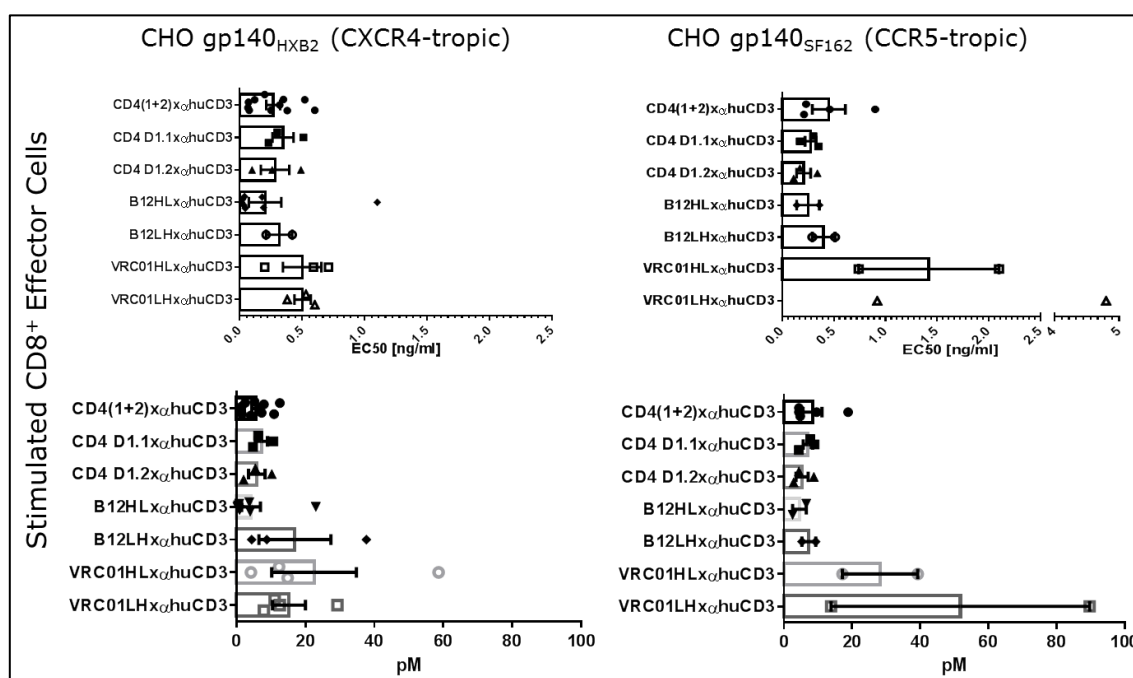
**Figure 30: Exemplary FACS-Based Cytotoxicity Assays Using HIV  $env^+$  CHO Target Cells and Either Unstimulated Native PBMCs or Prestimulated  $CD8^+$  Purified Primary T Cells.** After co-incubation of the labeled target cells with the effector cells and BiTE dilution series, the total cell population was stained using propidium iodide and the percentage of PI positive labeled target cells at the according BiTE concentration was determined. For inter-BiTE comparison, the corresponding BiTE concentration at the half-maximum lysis ( $EC_{50}$ ) was used. Mean values are displayed, error bars indicate SEM.

#### 4.5.7.1. Bioactivity Using Stimulated $CD8^+$ Effector Cells

To compare the BiTE antibody constructs under ideal conditions, prestimulated primary human  $CD8^+$  T cells were used as effector cells and co-incubated with HIV  $env^+$  CHO cells at an effector to target ratio of 10:1 (E:T: 10:1) in presence of BiTE dilution series. The activity of the BiTE antibody constructs was ranked using their  $EC_{50}$  values with regard to their concentration and molarity. Variability of  $EC_{50}$  values results from donor variation.

For the HIV<sub>HXB2</sub>  $env$  transfected CHO cells, the BiTE antibody constructs showed comparable results regarding their cytotoxic activity. B12HL and CD4(1+2) showed the best cytotoxic activity in this panel ( $EC_{50}$ : 4.2-5.6 pM) with B12HLxαhuCD3 showing the best  $EC_{50}$  mean. VRC01 BiTE antibody constructs showed slightly higher  $EC_{50}$  values ( $EC_{50}$ : 15.2-22.5 pM). This trend of inferior cytotoxic activity of VRC01-based BiTE antibody constructs became clearer when targeting HIV<sub>SF162</sub>  $env$  transfected CHO cells ( $EC_{50}$ : 28.2-51.8 pM). The residual BiTE antibody constructs (B12 and CD4-based) showed similar ranges of  $EC_{50}$  values targeting HIV<sub>SF162</sub>  $env$  transfected CHO cells

( $EC_{50}$ : 4.5-8.4 pM), with CD4 D1.2 $\alpha$ huCD3 showing the best  $EC_{50}$  mean. For detailed  $EC_{50}$  values, see Table 6.

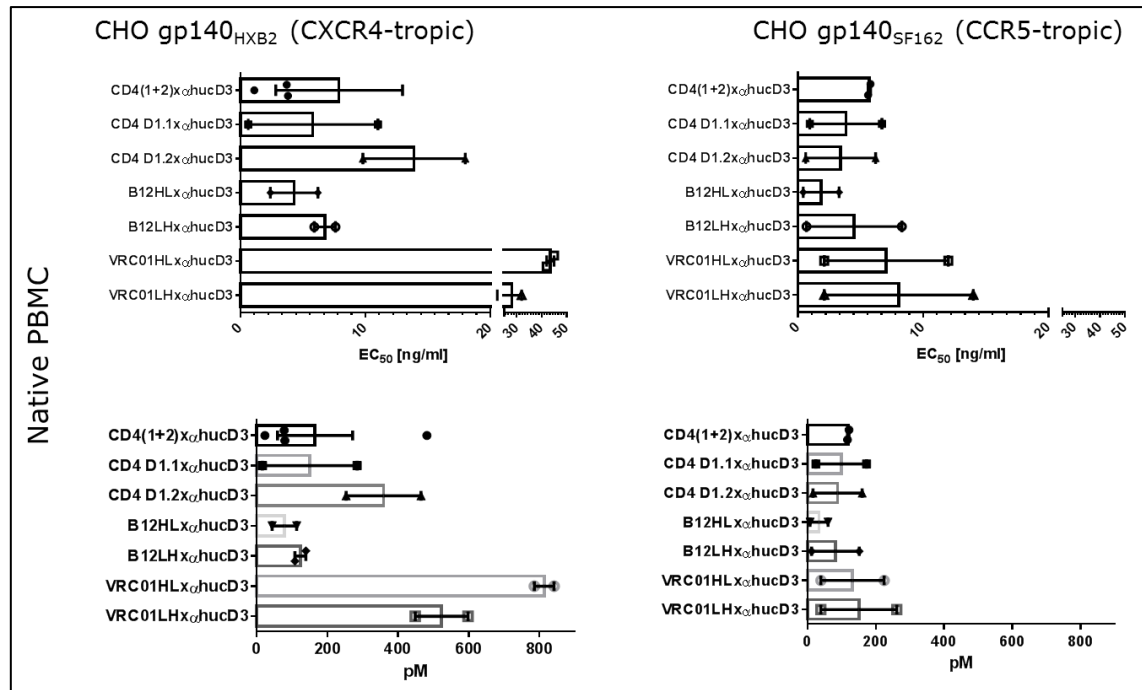


**Figure 31:  $EC_{50}$  Ranking of BiTE Antibody Constructs Using Stimulated Human Primary  $CD8^+$  T Cells and HIV gp140 Transfected CHO Cells.** Data points represent single  $EC_{50}$  values; columns represent mean values. Upper panel shows the associated BiTE concentration in ng/ml; the lower panel represents the values in pM. The cytotoxicity assays were performed at least with two independent donors. B12HL BiTE and CD4-based BiTE antibody constructs show the best  $EC_{50}$  values for both HIV env transfected CHO cell lines. CD4 domain 1 candidates showed no advantage. Mean values are displayed, error bars indicate SEM.

#### 4.5.7.2. Bioactivity Using Native PBMC Effector Cells

Next to the determination of cytotoxic activity under ideal circumstances, the potential of BiTE antibody constructs to engage native human primary T cells to perform redirected lysis of HIV env<sup>+</sup> CHO cells was analyzed using the same assay format.

Targeting HIV<sub>HXB2</sub> env transfected CHO cells with unstimulated PBMCs led to  $EC_{50}$  values of 78-359 pM for CD4-based and B12 candidates. The VRC01 BiTE antibody constructs showed higher  $EC_{50}$  values at 523-813 pM. For the HIV<sub>SF162</sub> env, the overall  $EC_{50}$  range was tighter ranging from 34-150 pM, with B12HL $\alpha$ huCD3 showing the best bioactivity using native human PBMCs. For  $EC_{50}$  values of all candidates, see Table 6.

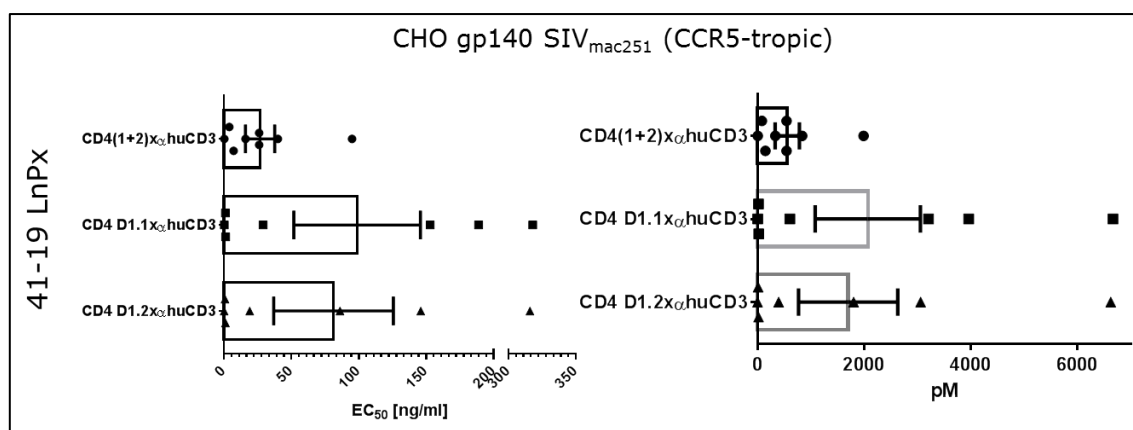


**Figure 32: EC<sub>50</sub> Ranking of BiTE Antibody Constructs Using Unstimulated Human Primary PBMCs.** Data points represent single EC<sub>50</sub> values; columns represent mean values and error bars indicate SEM. Upper panel shows the associated BiTE concentration in ng/ml; the lower panel shows the pM values. The cytotoxicity assays were performed at least twice.

#### 4.5.8. CD4-BASED BiTE BIOACTIVITY USING MACAQUE T CELLS

The current version of the BiTE technology platform offers a pan-specific human anti-CD3 antibody that can also bind and engage macaque T cells. Since the human CD4 protein is cross specific for the env of HIV and SIV, the CD4-based BiTE antibody constructs are fully cross-reactive to SIV and therefore ideal candidates for combined toxicity/efficacy *in vivo* studies in non-human primates.

To show the cross-reactive bioactivity of CD4-based BiTE antibody constructs, a prestimulated macaque T cell line was used in cytotoxicity assays and co-incubated with BiTE dilution series and SIV<sub>mac251</sub> env transfected CHO cells. In these assays, the CD4-based BiTE antibody constructs showed cross-reactive bioactivity using stimulated macaque T cells and SIV gp140<sup>+</sup> CHO cells *in vitro*. The CD4(1+2) BiTE showed the best EC<sub>50</sub> value at 561 pM, followed by CD4 D1.2αhuCD3 (1.7 nM) and CD4 D1.1αhuCD3 (2.01 nM).



**Figure 33: EC<sub>50</sub> Ranking of BiTE Antibody Constructs Using Prestimulated Macaque Effector T cell line and SIV env<sup>+</sup> CHO Target Cells.** Data points represent single EC<sub>50</sub> values; columns represent mean values, error bars indicate SEM. Left panel shows the associated BiTE concentration in ng/ml; the right panel represents the values in pM. The highly variable EC<sub>50</sub> values of the 41-19 LnPx cells might be due to the IL-2 stimulation of the cell culture.

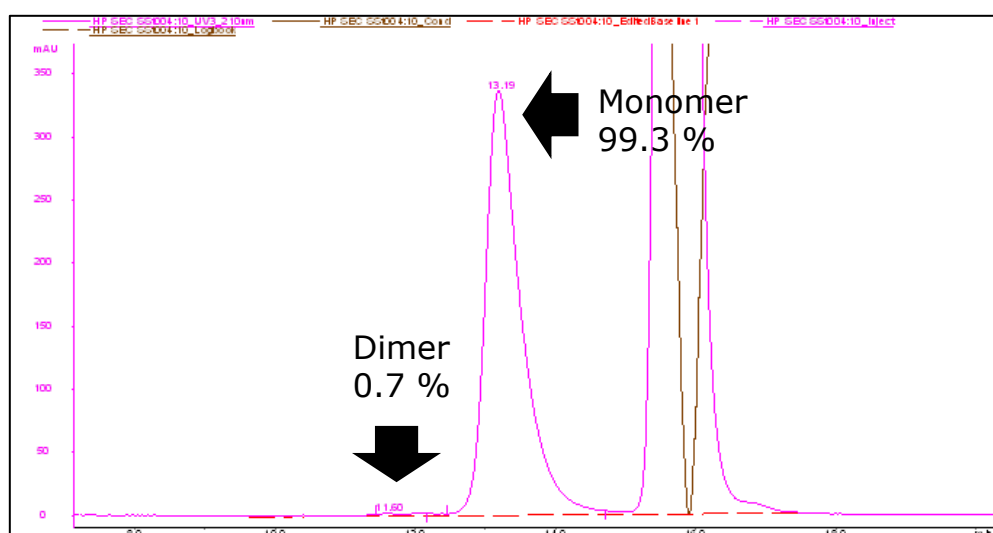
**Table 6: Overview of Cytotoxic Activity (EC<sub>50</sub> mean) of HIV BiTE *in vitro* Using Isolated Stimulated Human CD8<sup>+</sup> T Cells, Native Human PBMCs or Stimulated 41-19 LnPx Macaque T Cell Line Effector Populations and HIV/SIV env Transfected CHO Target Cells.**

EC <sub>50</sub> [pM]	Stim. CD8 <sup>+</sup> T Cells			Native PBMCs		Macaque T Cells	
	HIV		SIV	HIV		SIV	
	HXB2	SF162	mac251	HXB2	SF162	mac251	
CHO env <sup>+</sup>							
CD4(1+2)xαhuCD3	5.6	8.4	33.3	165.5	119.4	559.3	561
CD4 D1.1xαhuCD3	7.3	7.1	139.7	150.3	99.1	2,288.8	2,069
CD4 D1.2xαhuCD3	5.9	5.3	47.4	359.3	88.1	2,054.9	1,702
B12HLxαhuCD3	4.3	4.6		78.6	34.0		
B12LHxαhuCD3	17	7.3		123.6	82.2		
VRC01HLxαhuCD3	22.5	28.3		813.9	131.9		
VRC01LHxαhuCD3	15.2	51.8		523.7	150.6		

#### 4.5.9. BIOACTIVITY TARGETING HIV ENV<sup>+</sup> JURKAT T CELLS

To determine the cytotoxic activity of the CD4(1+2) BiTE in the T cell context, cells of the Jurkat T cell line were transfected with the HIV<sub>HXB2</sub> env-EpCAM construct.

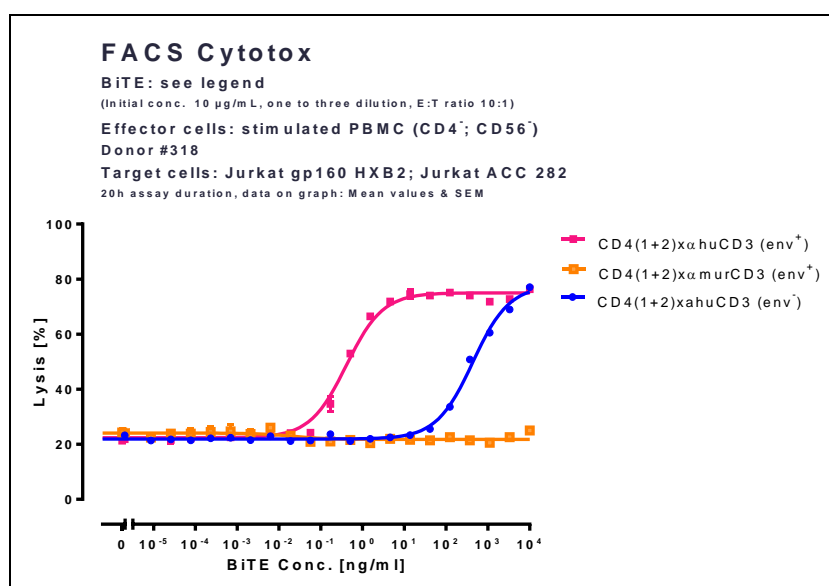
As had already been shown by Traunecker et al. [84], bispecific antibody constructs (called Janusins) are able to redirect lysis of cytotoxic T-lymphocyte clones to infected T-lymphocytes *in vitro*. In addition, he also observed bispecific antibody-dependent lysis of uninfected T cells when applying more than 100 ng/ml Janusins to the assay. The window between lysis of uninfected T cells and lysis of infected T cells was about factor 100. Because the Janusins were solely purified by affinity chromatography, this phenomenon was ascribed to Janusin dimers (estimated 5%) present within the applied Janusin fraction.



**Figure 34: HP-SEC Data of CD4(1+2) BiTE Monomer.** An aliquot of CD4(1+2) $\alpha$ huCD3 was directly applied to HP-SEC analysis after thawing from -80°C storage to determine possible dimer content.

Because of the claim that dimers of the bispecific antibody could cause the target-independent lysis of T cells, an aliquot of the purified CD4(1+2) BiTE batch was analyzed by HP-SEC showing a dimer content of 0.7% (Figure 34).

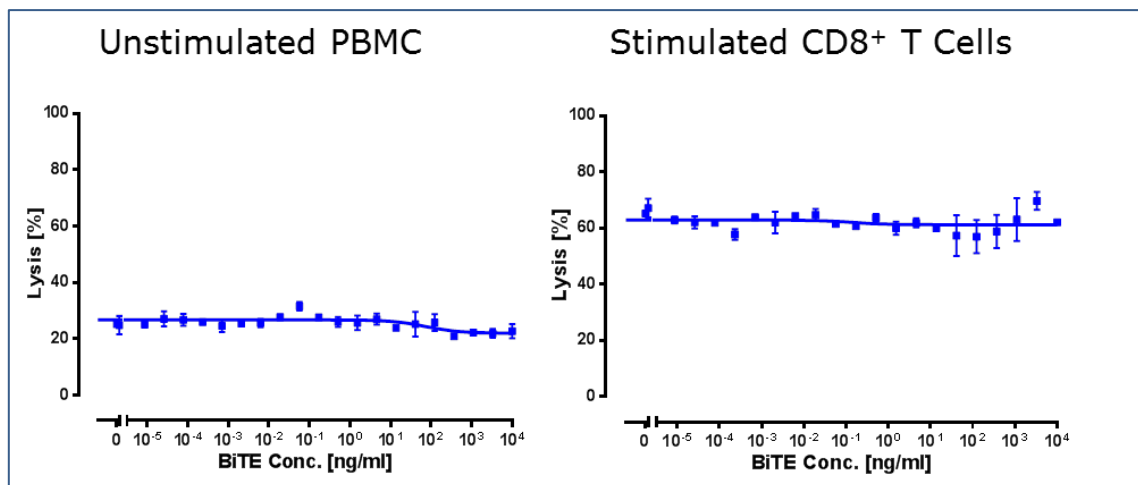
However, this phenomenon of target-independent lysis persisted when applying BiTE monomer fractions isolated by SEC to a comparable assay format. The CD4(1+2) BiTE showed lysis of HIV  $env^+$  Jurkat cells starting at double digit pg/ml. However at higher concentrations (double digit ng/ml) the CD4(1+2) BiTE also showed cytotoxic activity on HIV  $env^-$  Jurkat cells (factor 1,000 difference). This phenomenon was already described for Janusins by Traunecker et al. [84], who described a factor 100 difference between specific and target-independent lyses. Although, the phenomenon persisted, the window between HIV<sub>HXB2</sub> gp140 transfected Jurkat cells and untransfected Jurkat cells by BiTE antibody constructs increased to factor 1,000 (Figure 35) and may also have been caused by residual BiTE dimer in the purified BiTE monomeric fraction.



**Figure 35: Cytotoxic Activity of CD4(1+2) BiTE on HIV  $env^+$  and HIV  $env^-$  Jurkat T Cells.** Prestimulated human CD8<sup>+</sup> T cells were co-incubated with labeled (un-) transfected Jurkat T cells (E:T 10:1) in the presence of BiTE dilutions of CD4(1+2) BiTE antibody constructs. After 24 hours of co-incubation of the cells, propidium iodide was added and the percentage of PI positive labeled target cells at the according BiTE concentration was determined. Mean values are displayed, error bars indicate SEM.

4.5.10. *BIOACTIVITY OF CD4(1+2) BiTE ON PRIMARY T CELLS*

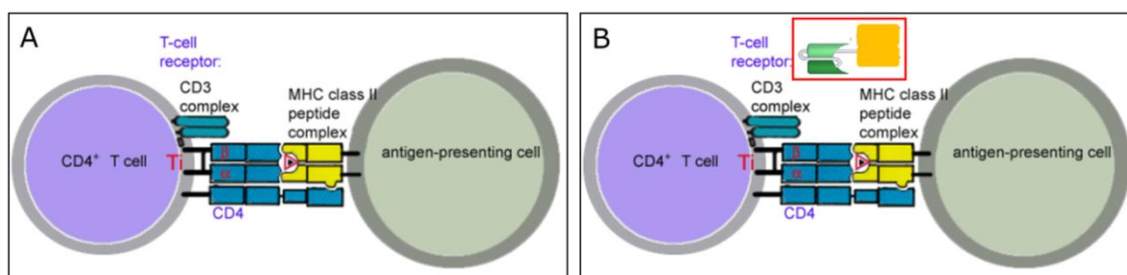
The CD4-based Janusin as well as the CD4(1+2) BiTE showed cytotoxic activity on Jurkat T cells *in vitro*. To determine whether this effect plays a role using primary T cells, the cytotoxic activity of CD8<sup>+</sup> T cells on autologous non-infected CD4<sup>+</sup> T cells was examined in an un-stimulated and pre-stimulated setting. In contrast to the cytotoxic activity of the CD4(1+2) BiTE on Jurkat T cells, no significant BiTE-dependent cytotoxic activity was measurable when co-incubating primary untransfected and HIV-negative CD4<sup>+</sup> T cells with autologous CD8<sup>+</sup> T cells. The BiTE independent lysis of CD4<sup>+</sup> T cells was increased compared to the unstimulated setting. A donor-dependent small increase in dead CD4<sup>+</sup> T cells at high BiTE concentrations can be observed when using prestimulated cells. In parallel to this assay, the stimulated CD8<sup>+</sup> effector T cells have been applied to a cytotoxicity assay incubated with on target-positive cells and BiTE where they showed comparable cytotoxic activity to previous assays and proofed cytotoxic potential (data not shown).



**Figure 36: CD4(1+2) BiTE Cytotoxic Activity Using Primary CD4<sup>+</sup> T Cells as Target Cells.** Left: Isolated, unstimulated CD8<sup>+</sup> T cells were co-incubated with autologous unstimulated CD4<sup>+</sup> T cells for 72h. Right: Isolated CD8<sup>+</sup> and CD4<sup>+</sup> T cells were prestimulated with CD3/CD28/IL-2 and co-incubated with CD4(1+2) BiTE for 24h. After co-incubation of the cells, propidium iodide was added and the percentage of PI positive labeled target cells at the according BiTE concentration was determined. CD8<sup>+</sup> T cells were checked for their cytotoxic activity on target positive cells (data not shown). Mean values are displayed, error bars indicate SEM.

#### 4.5.11. *MHC II<sup>+</sup> / CD4-BASED BiTE INTERACTION*

In the physiological setting, the native human CD4 protein accompanies the binding of the T cell receptor complex to MHC class II complexes [142]. The binding of the CD4(1+2) $\times$ huCD3 BiTE could replace this physiological interaction by binding to MHC class II complexes via its CD4 domains and to the T cell receptor associated CD3 protein via its anti-huCD3 scFv (see Figure 37). Therefore, the CD4-based BiTE antibody constructs could cause lysis of MHC class II<sup>+</sup> cells. To analyze whether this theoretical concern proves to be relevant, binding as well as cytotoxic activity of CD4(1+2) $\times$ huCD3 on B cells was analyzed *in vitro*.

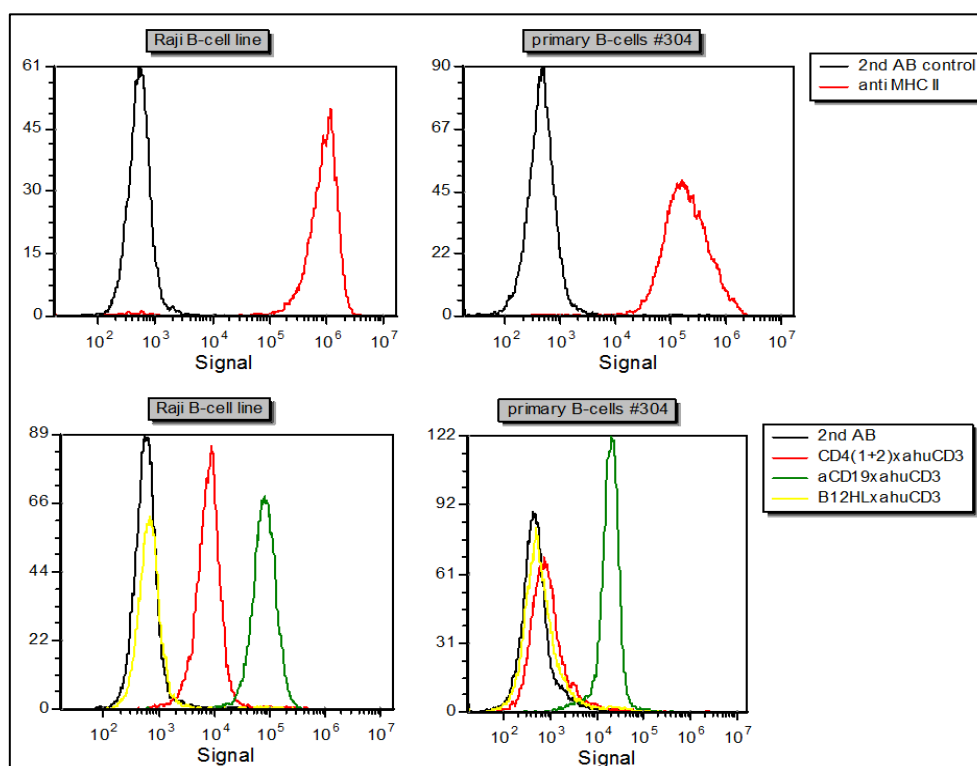


**Figure 37: T Cell Stimulation by B Cells via Binding of MHC II Complex to the T Cell Receptor.** (A) Physiological setting: T cell receptor binds to peptide loaded MHC II complex. This binding is accompanied by CD4 binding to the MHC II complex and CD40 Ligand of the T cell binding to CD40 on the B cell. (B) Potential BiTE setting: CD4(1+2) BiTE (red box) binds to CD3 (accompanying the T cell receptor complex) and binds to the MHC II complex via CD4(1+2). Thereby the T cell is activated, potentially resulting in the lysis of the B cell. Modified from [143].



#### 4.5.11.1. Binding of CD4(1+2) BiTE to MHC II<sup>+</sup> Cells

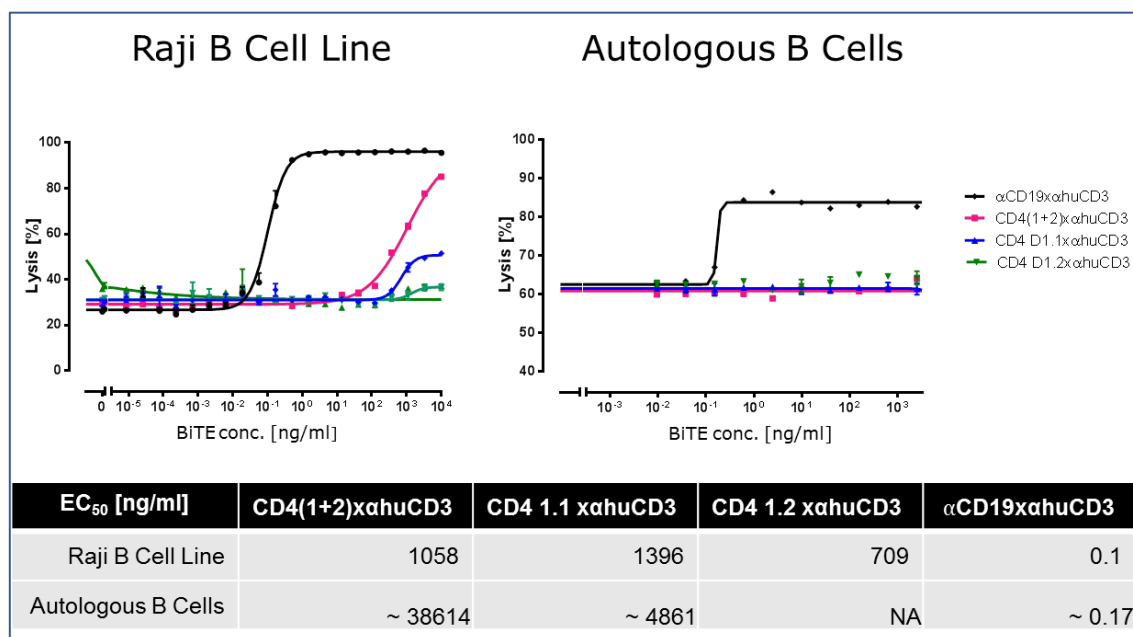
According to the FACS analysis (Figure 38, lower panel), the CD4(1+2) BiTE binds to cells of the Raji B cell line. However, the CD4(1+2) BiTE bound only weakly to primary B cells. This trend is confirmed by the observation that the signal for MHC II complexes on Raji B cells is significantly higher than on primary B cells (see Figure 38, upper panel). Thus, Raji cells seem to have more MHC II complexes on their cell surface, thereby offering more epitopes for the CD4(1+2) BiTE than primary B cells, which eventually leads to a stronger signal for Raji B cell binding of the CD4(1+2) BiTE. The anti-CD19 BiTE and the B12HL BiTE served as positive or negative controls respectively.



**Figure 38: Analysis of B cell Binding of CD4(1+2) BiTE and MHC II Expression Analyzed Using Raji B Cell Line and Primary B Cells.** Raji B cell line cells and primary B cells have been analyzed with FACS for their MHC II expression by staining with an anti-MHC II mAb (upper panel). Indicated BiTE antibody constructs were incubated with Raji B cells or primary B cells (lower panel). Binding was analyzed via FACS using a secondary antibody directed against the histidine-tag of the BiTE antibody constructs.

#### 4.5.11.2. CD4-Based BiTE Activity Targeting MHC II<sup>+</sup> Cells

To determine whether the binding of the CD4(1+2) BiTE antibody constructs results in redirected lysis of B cells, the cytotoxic activity of the CD4-based BiTE was analyzed for Raji B cells as well as primary B cells in combination of prestimulated CD8<sup>+</sup> T cells (autologous to the primary B cells).




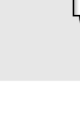
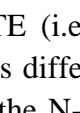


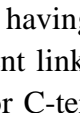
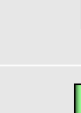

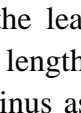

**Figure 39: Cytotoxic Activity of CD4-Based BiTE Antibody Constructs on MHC II<sup>+</sup> Cells:** Prestimulated CD8<sup>+</sup> T cells were used as effector cells to test cytotoxic activity on MHC class II<sup>+</sup> cells (Left: Raji B cell line, Right: autologous B cells), E:T ratio 10:1. An anti-CD19 BiTE served as a positive control (black), CD4(1+2) BiTE (pink). CD4 domain 1 BiTE 1.1 (blue), 1.2 (green). Mean values are displayed, error bars indicate SEM.

The left plot in Figure 39 illustrates that there is cytotoxic activity of the CD4(1+2) BiTE at high concentrations when targeting Raji B cells (EC<sub>50</sub> of 1058 ng/ml). This cytotoxic activity was also observed for the CD4 domain 1 BiTE antibody constructs. Those CD4 domain 1 BiTE antibody constructs showed decreased activity as can be depicted from the lower amplitude at higher BiTE concentrations, which is consistent with the lowered affinity of CD4 domain 1 constructs for MHC class II complexes as reported by Chen et al. [49]. When targeting primary autologous B cells however, there was no cytotoxic activity detected for CD4-based BiTE antibody constructs (right plot). The effector function of the T cells was unbiased as the positive control (anti-CD19 BiTE) showed comparable activity (EC<sub>50</sub> of 170 pg/ml) compared to 100 pg/ml in case of the Raji B cells. When targeting autologous B cells using prestimulated isolated CD8<sup>+</sup> T cells, the BiTE independent lysis increased to 60% compared to 30% lysis of Raji B cells.

#### 4.6. HALF-LIFE EXTENDED CD4(1+2) BiTE ANTIBODY CONSTRUCTS

To increase the convenience for the patient, BiTE antibody constructs were generated that have been extended for an albumin-binding peptide (ABP). The long-term goal is to achieve sufficient BiTE exposure with daily subcutaneous BiTE applications while not being dependent on continuous BiTE infusion as is currently the standard. This means an increase in comfort for the patient and lower immediate costs.

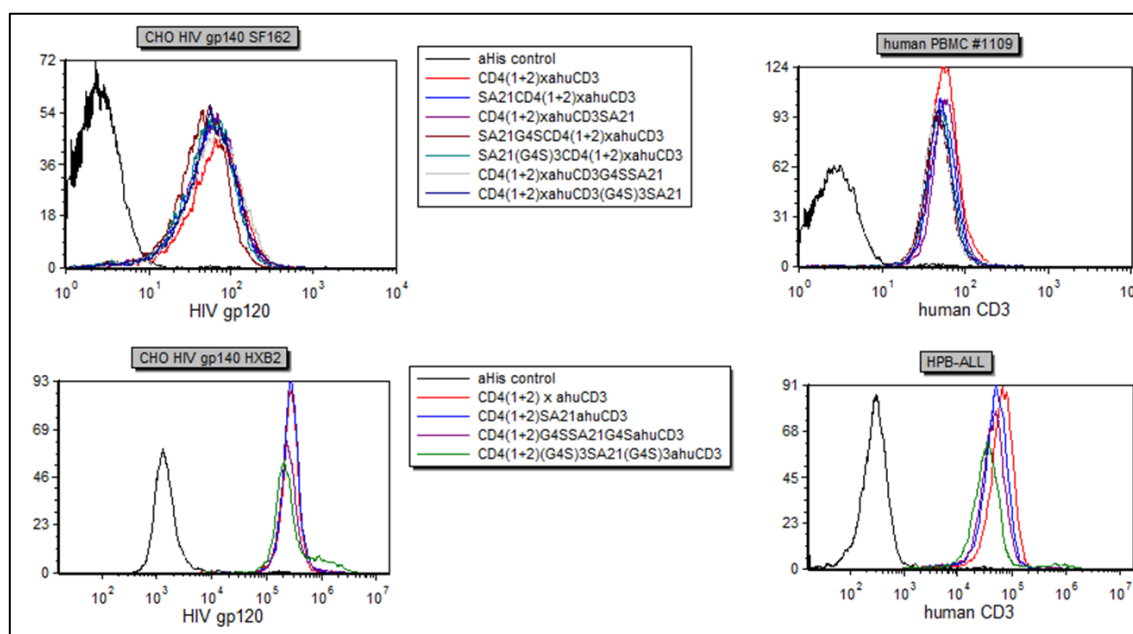
**Table 7: Overview of Generated SA21-Tagged CD4(1+2) BiTE Antibody Constructs.** The SA21-tag was fused directly to the N-terminus of CD4(1+2), positioned between CD4 and the anti-huCD3 scFv or fused to the C-terminus of the anti-huCD3 scFv. In addition to the direct fusion of the SA21-tag,  $G_4S$  or a  $(G_4S)_3$  linker were tested. For the internally-tagged candidates, the linkers surrounded the SA21-tag.

Position	Fusion	$(G_4S)$ Linker	$(G_4S)_3$ Linker
N-Terminus			
C-Terminus			
Internal			
			

An albumin-binding peptide described by Dennis et al. [115] named SA21, was tested in combination with the parental BiTE CD4(1+2) $\times$ ahuCD3. To examine the ideal position of the ABP within the BiTE (i.e., having the least impact on the cytotoxic activity), different positions as well as different linker lengths were tested *in vitro*. In general, the ABP-tag was positioned at the N- or C-terminus as well as between both binding arms. Different linker lengths were tested to fuse the ABP-tag to the BiTE (see Table 7).

#### 4.6.1. FACS BINDING TO HIV ENV<sup>+</sup> CELLS AND CD3<sup>+</sup> CELLS

As a first indicator for functionality, the purified BiTE antibody constructs were applied to FACS for analysis of binding to HIV env<sup>+</sup> and CD3<sup>+</sup> cells. All tested candidates were functional with respect to HIV env and human CD3 binding (see Figure 40). All BiTE antibody constructs were adjusted to 10 µg/ml for FACS analysis. However, slight signal differences were observed, which suggest differences in binding kinetics of the SA21-tagged BiTE antibody constructs. N-terminally-tagged BiTE antibody constructs show slightly decreased signals in gp120 binding and internally tagged BiTE variants in human CD3 binding. For a more exact determination of binding kinetics, the HIV env, human CD3 and HSA affinities were determined by SPR (see 4.6.2).



**Figure 40: FACS Binding Analysis of SA21-Tagged CD4(1+2) BiTE Antibody Constructs for HIV Env and CD3 Binding.** Indicated BiTE antibody constructs were incubated with HIV env HXB2 or SF162 transfected CHO cells for gp120 binding. For human CD3 binding PBMCs or HPB-ALL T cells were stained respectively. FACS binding was analyzed via a secondary antibody directed against histidine-tag of the BiTE antibody constructs (used as negative control staining).

#### 4.6.2. DETERMINATION OF HIV GP120, HUMAN CD3 AND HUMAN ALBUMIN AFFINITY

To determine the impact of the albumin binding peptide on its neighboring domains, the affinities to gp120 and human CD3 were compared for the terminally tagged BiTE antibody constructs.

Concerning the target affinity to gp120, the C-terminally SA21-tagged BiTE variants showed superior affinity over the N-terminally tagged BiTE antibody constructs independently of the linker length (see Table 8). The best target affinity was detected for CD4(1+2) $\alpha$ huCD3SA21 with 2.9 nM compared to 0.9 nM for the parental CD4(1+2) BiTE.

This trend was also true for the affinity to human CD3. The C-terminally tagged SA21 BiTE variants with direct SA21 fusion or (G<sub>4</sub>S)<sub>3</sub> linker showed a single digit nM affinity to CD3 (4.3 and 7.6 nM), whereas the G<sub>4</sub>S linked SA21 variant showed an affinity of 32 nM to human CD3. N-terminally tagged variants showed constant KD values between 12-14 nM to human CD3.

The CD4(1+2) BiTE with the C-terminally direct SA21 fusion showed the best HSA affinity (141 nM), followed by the N-terminal direct fusion (226 nM). The addition of a five or 15 AA linker did not lead to an increase in HSA affinity of the SA21-tag for N- or C-terminally tagged BiTE antibody constructs. The internally tagged variants showed a linker length dependent decrease in HSA affinity. The direct fusion of the SA21-tag between the CD4(1+2) domains and anti-CD3 scFv led to the best KD for internally SA21-tagged CD4(1+2) BiTE antibody constructs of 465 nM for HSA.

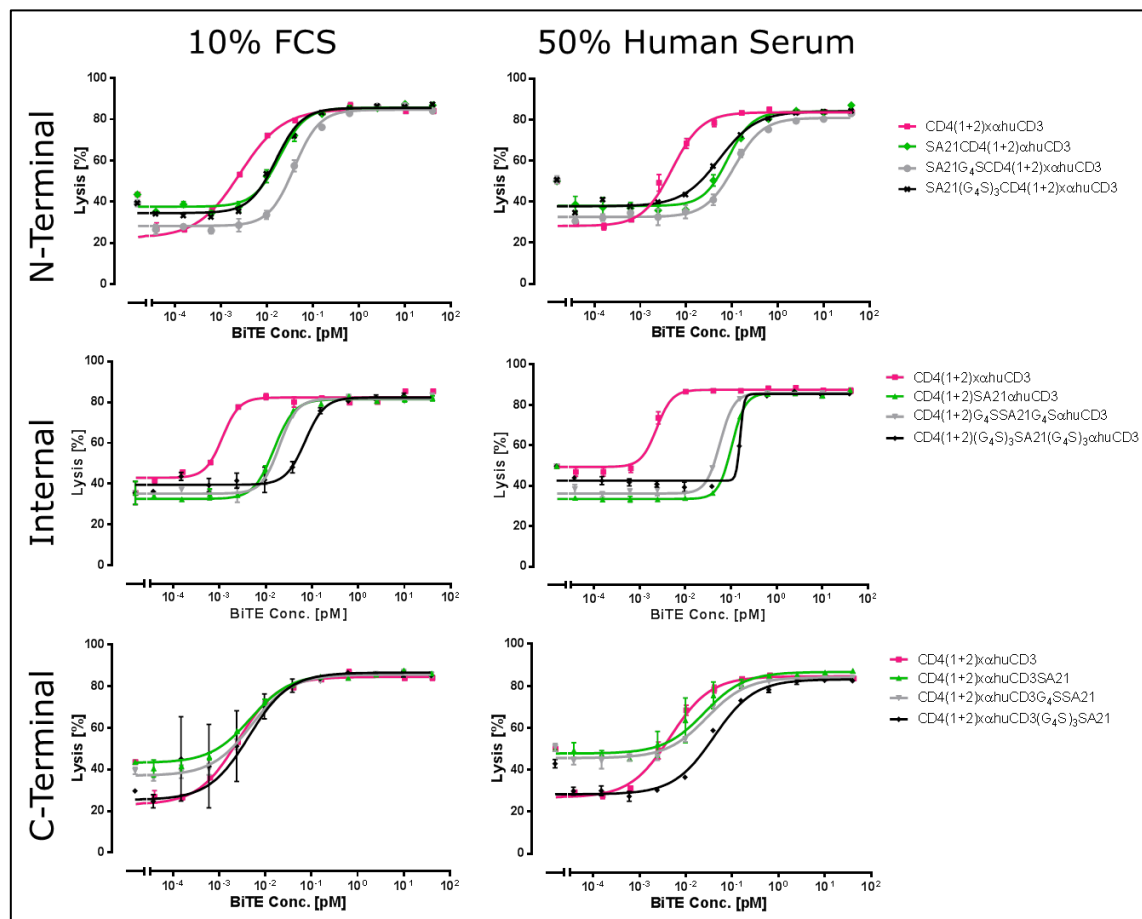
As can be depicted from Table 8 the CD4(1+2) $\alpha$ huCD3SA21 BiTE showed for all three binding partners (HIV env, huCD3 and HSA) the best overall KD. Whereas different SA21-tagged BiTE antibody constructs share the second best affinity values depending on the antigen.

**Table 8: SPR Affinity Overview of HIV env, Human CD3 and HSA for SA21-Tagged CD4(1+2) BiTE Antibody Constructs.** Soluble antigens were coated on a CM5 chip and the affinity kinetics were determined with reference to the BiTE signal in an uncoated flow cell. Affinity measurement for internally SA21-tagged CD4(1+2) BiTE antibody constructs did not show interpretable data on HIV gp120. HIV gp120 and human CD3 binding was proofed by FACS analysis and cytotoxicity assays.

SPR Affinity		HIV gp120			huCD3			HSA		
		Ka [1/Ms]	Kd [1/s]	KD [M]	Ka [1/Ms]	Kd [1/s]	KD [M]	Ka [1/Ms]	Kd [1/s]	KD [M]
N-Terminal	SA21-Tagged CD4(1+2) BiTE									
	Fusion	6.45E+04	2.98E-04	<b>4.61E-09</b>	4.34E+05	5.34E-03	<b>1.23E-08</b>	3.63E+04	8.22E-03	<b>2.26E-07</b>
	G <sub>4</sub> S	6.69E+03	1.76E-04	<b>2.63E-08</b>	5.50E+05	3.68E-03	<b>1.47E-08</b>	3.26E+04	1.75E-05	<b>5.36E-07</b>
	(G <sub>4</sub> S) <sub>3</sub>	3.07E+04	2.05E-04	<b>6.70E-09</b>	4.34E+05	5.34E-03	<b>1.23E-08</b>	3.90E+04	1.02E-02	<b>2.60E-07</b>
Internal	Fusion	1.17E+05	3.26E-04	<b>2.79E-09</b>	1.73E+05	3.30E-03	<b>1.91E-08</b>	8.26E+03	3.84E-03	<b>4.65E-07</b>
	G <sub>4</sub> S				4.31E+04	7.23E-03	<b>1.68E-07</b>	3.78E+04	2.92E-02	<b>7.73E-07</b>
	(G <sub>4</sub> S) <sub>3</sub>				2.24E+05	2.87E-03	<b>1.28E-08</b>	1.36E+03	6.15E-03	<b>4.52E-06</b>
C-Terminal	Fusion	1.07E+05	2.81E-04	<b>2.63E-09</b>	1.03E+06	4.48E-03	<b>4.33E-09</b>	2.19E+05	3.08E-02	<b>1.41E-07</b>
	G <sub>4</sub> S	4.24E+04	2.53E-04	<b>5.95E-09</b>	1.81E+05	5.76E-03	<b>3.18E-08</b>	4.54E+03	1.09E-02	<b>2.41E-06</b>
	(G <sub>4</sub> S) <sub>3</sub>	7.55E+04	2.79E-04	<b>3.70E-09</b>	7.16E+05	5.43E-03	<b>7.59E-09</b>	1.18E+05	3.53E-02	<b>2.99E-07</b>

#### 4.6.3. BIOACTIVITY ON HIV ENV TRANSFECTED CELLS

Besides the affinity determination for the half-life extended BiTE antibody constructs, they were also tested for their cytotoxic activity *in vitro* to discriminate for their bioactivity under low and high abundance of HSA. The SA21 characterization by Dennis et al. reported low affinity to bovine serum albumin compared to high affinity for human serum albumin. The testing of the BiTE antibody constructs without the influence of serum albumin was not possible, due to its necessity for human PBMC cell culturing. Therefore, the bioactivity was analyzed under the influence of 10% (v/v) fetal calf serum (FCS) versus 50% (v/v) human serum. The 10% FCS setting should offer a good approximation to the unbiased bioactivity of the BiTE antibody constructs, whereas the testing with 50% human serum should give rise to their bioactivity in the presence of naturally high levels of HSA.













**Figure 41: Determination of *in vitro* Cytotoxic Activity of SA21-tagged BiTE Antibody Constructs with 10% FCS or 50% Human Serum.** Prestimulated human CD8<sup>+</sup> T cells were co-incubated with labeled HIV env transfected CHO cells (E:T 10:1) in the presence of BiTE dilutions and the presence of 50% human serum or 10% FCS. After 24 h of co-incubation, PI was added to the assay and the percentage of PI positive labeled target cells at the according BiTE concentration was determined. Mean values are displayed, error bars indicate SEM.

As can be seen in Figure 41, the C-terminally tagged BiTE antibody constructs showed almost no decrease in activity compared to the parental molecule when assayed using 10% FCS. Comparably low abundance of bovine serum albumin to SA21 (10% FCS), revealed the ideal SA21-tag position within the BiTE independently of HSA. As can be seen from Table 9, the C-terminally tagged BiTE antibody constructs showed the highest bioactivity with the lead of the direct fusion (factor 2 decrease compared to the parental CD4(1+2) BiTE), followed by the (G<sub>4</sub>S)<sub>3</sub> linked variant (factor 3 decrease) and the G<sub>4</sub>S linked CD4(1+2) BiTE candidate (factor 4 decrease).

The best N-terminally tagged candidate is the candidate with the (G<sub>4</sub>S)<sub>3</sub> linked SA21 (factor 7 decrease), followed by the directly linked SA21 candidate (factor 8) and the G<sub>4</sub>S linked SA21 candidate with a factor 17 decrease.

The internally tagged BiTE candidate without linkers showed a better bioactivity (factor 14) as the N-terminally tagged candidate with the directly fused SA21 (factor 17). The G<sub>4</sub>S and G<sub>4</sub>S<sub>3</sub> linked candidates show the lowest bioactivity in the presence of 10% FCS (factor 18 and 67 respectively). The internally tagged BiTE antibody constructs showed a linker-length dependent impact on the cytotoxic activity in the presence of 10% FCS.






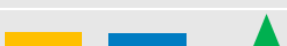




**Table 9: Bioactivity Ranking of SA21-Tagged CD4(1+2) BiTE Antibody Constructs with 10% FCS.** For the ranking of the BiTE antibody constructs, the EC<sub>50</sub> value of the parental CD4(1+2) BiTE was taken as a basis and the factor of decreased activity was noted for each SA21-tagged BiTE.

Rank	SA21-tagged CD4(1+2) BiTE Antibody Constructs	50% Serum	10% FCS
1		1	1
2		8	2
3		23	3
4		13	4
5		11	7
6		17	8
7		46	14
8		23	17
9		26	18
10		73	67



In presence of 50% human serum, the ranking of the BiTE antibody constructs according, showed that the CD4(1+2) $\times$ ahuCD3SA21 maintained the best cytotoxic activity with a 8-fold decreased activity compared to the parental CD4(1+2) BiTE. Thereafter, N- and C-terminally linked variants interchange in the ranking positions without displaying a clear trend. The internally tagged BiTE antibody constructs showed the biggest negative impact in cytotoxic activity (up to factor 73).

**Table 10: Bioactivity Ranking of SA21-Tagged CD4(1+2) BiTE Antibody Constructs with 50% Human Serum.** For the ranking of the BiTE antibody constructs, the EC<sub>50</sub> value of the parental CD4(1+2) BiTE was taken as a basis and the factor of decreased activity noted for each SA21-tagged BiTE.

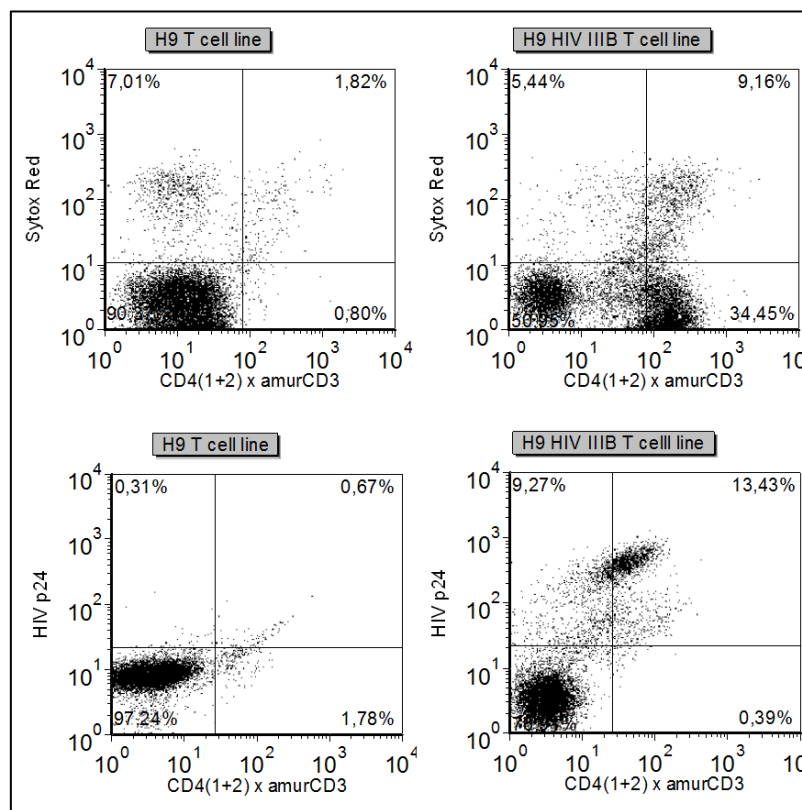
Rank	SA21-tagged CD4(1+2) BiTE Antibody Constructs	50% Serum	10% FCS
1		CD4(1+2) $\times$ ahuCD3	1
2		CD4(1+2) $\times$ ahuCD3SA21	8
3		SA21(G <sub>4</sub> S) <sub>3</sub> CD4(1+2) $\times$ ahuCD3	11
4		CD4(1+2) $\times$ ahuCD3(G <sub>4</sub> S)SA21	13
5		SA21CD4(1+2) $\times$ ahuCD3	17
6		CD4(1+2) $\times$ ahuCD3(G <sub>4</sub> S) <sub>3</sub> SA21	23
6		SA21(G <sub>4</sub> S)CD4(1+2) $\times$ ahuCD3	23
7		CD4(1+2)(G <sub>4</sub> S)SA21(G <sub>4</sub> S) $\times$ ahuCD3	26
8		CD4(1+2)SA21ahuCD3	46
9		CD4(1+2)(G <sub>4</sub> S) <sub>3</sub> SA21(G <sub>4</sub> S) <sub>3</sub> ahuCD3	73

#### 4.7. PROOF OF CONCEPT FOR CD4(1+2) BiTE USING HIV INFECTED CELLS

The CD4(1+2) BiTE was chosen for further investigation due to its high potential in redirecting T cell lysis, because of its intrinsic broad recognition of different HIV strains and cross-reactivity to SIV env. Proof of concept was performed by showing its antiviral activity using HIV infected primary T cells and MDMs *in vitro* and *in vivo* by using NSG mice engrafted with HIV infected human PBMCs.

#### 4.7.1. FACS BINDING OF CD4(1+2) BiTE TO HIV INFECTED CELLS

As a first experiment, the ability of the CD4(1+2) BiTE target-binding domain to bind to HIV infected cells was tested using the CD4(1+2) $\times$ amurCD3 BiTE antibody to stain a chronically HIV infected T cell line. The cells were stained for intracellular HIV p24 protein, cell viability and BiTE signal with reference to the uninfected cell line counterpart (see Figure 42).

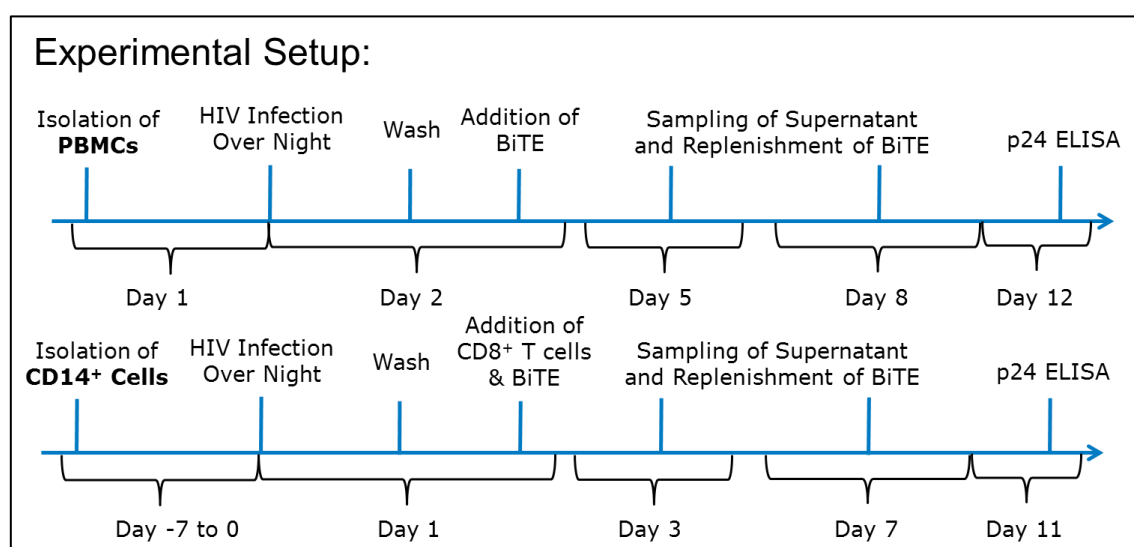


**Figure 42: FACS Staining of HIV<sup>+</sup> H9 IIB T cells Versus HIV<sup>-</sup> H9 T Cells Using the CD4(1+2) $\times$ amurCD3 Control BiTE.** HIV<sup>+</sup> (H9 HIV<sub>IIB</sub>) and HIV<sup>-</sup> (H9) T cells were stained to determine the correlation of the BiTE signal with vital and HIV p24<sup>+</sup> cells. Staining with CD4(1+2) $\times$ amurCD3 and Sytox Red dead cell stain (upper panel) or with CD4(1+2) $\times$ amurCD3 and intracellular HIV p24 (lower panel).

The upper panel of Figure 42 shows the uninfected H9 T cell line versus the chronically HIV<sub>IIB</sub> infected H9 cell line stained with the CD4(1+2) $\times$ amurCD3 control BiTE. As a control for living cells, the cells were co-stained with Sytox Red to confirm the target positivity on vital cells. The BiTE stained mainly live infected cells, however a simultaneous increase in dead and BiTE positive cells can be observed. A BiTE co-staining with an intracellular staining of the HIV p24 core protein of these two cell lines, showed positive correlation, also confirming the specificity of the BiTE for HIV infected cells (Figure 42, lower panel).

#### 4.7.2. ANTIVIRAL ACTIVITY OF CD4(1+2) BiTE IN VITRO

To test the antiviral potential of the CD4(1+2) BiTE in treating a primary HIV infection, PBMCs and primary MDMs were infected overnight using virus containing cell culture supernatant. After a washing step, a BiTE dilution series was added to HIV<sub>NL4-3</sub> (CXCR4-tropic) infected PBMCs as well as to HIV<sub>YU-2</sub> (CCR5-tropic) infected MDMs supplemented with autologous CD3/CD28/IL-2 prestimulated CD8<sup>+</sup> T cells. To determine the antiviral impact of the BiTE, the level of viral core protein (p24) was analyzed at day 5, 8 and 10 post infection. The higher the measured p24 level, the higher the number of vital cells that replicate virus, so the lower the antiviral effect.

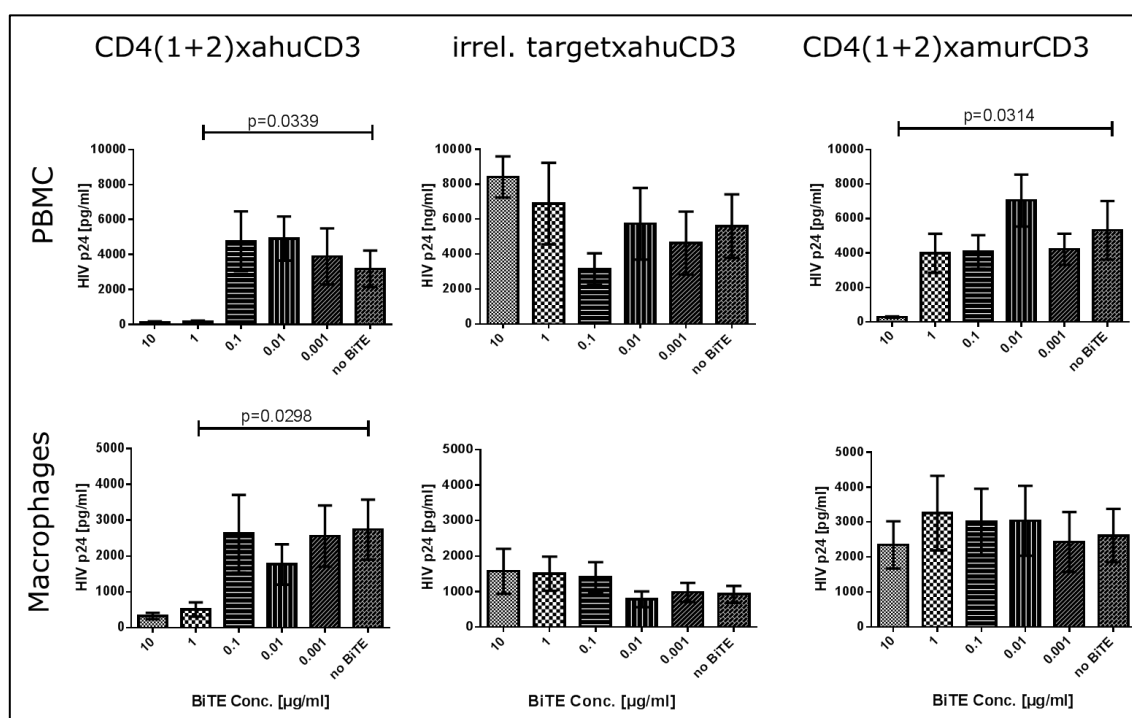


**Figure 43: Scheme for the Timeline of the *in vitro* Experiment with HIV Infected PBMCs or MDMs & CD8<sup>+</sup> T cells.** PBMCs were isolated and infected with HIV overnight. In case of MDMs, CD14<sup>+</sup> cells were isolated and differentiated for 7 days to generate MDMs. Then, the MDMs were infected overnight, washed and incubated with autologous stimulated CD8<sup>+</sup> T cells and BiTE dilutions. Supernatant was sampled and replenished at indicated days and aliquots were used for HIV p24 ELISA.

Since the BiTE activity on HIV infected cells was unknown; samples of the cell culture supernatant were taken at different days post infection. The p24 raw data of the different BiTE concentrations at different time points was analyzed in order to compare the p24 protein level and the antiviral activity over time. Obtained sample volumes were replenished with the according BiTE dilution in cell culture medium. The statistical significance of the HIV p24 values for PBMC and MDM/CD8<sup>+</sup> T cell setups increased over time and is displayed in Figure 44 for day 12 (PBMCs) and day 11 (MDMs) post infection.

For infected PBMCs, the CD4(1+2) BiTE showed significant antiviral activity at concentrations higher than 100 ng/ml compared to the no BiTE control. At the highest concentration of 10  $\mu\text{g/ml}$ , the CD4(1+2) control BiTE containing the anti-murine CD3 scFv, showed significant antiviral activity. For the irrelevant target-binding BiTE, no antiviral effect was observed in both settings. In contrast, high doses of the irrelevant BiTE showed an increase in p24 levels in both settings.

The control BiTE CD4(1+2) $\times$ amurCD3 showed no inhibition of viral replication when incubated with the infected MDMs plus autologous CD8<sup>+</sup> T cells. Likewise to the PBMC setting the CD4(1+2) $\times$ ahuCD3 BiTE showed statistically significant antiviral activity at concentrations (i.e., >100 ng/ml) on infected MDMs. The irrelevant target control BiTE showed slightly increased HIV p24 levels at concentrations of 100 ng/ml and higher concentrations.

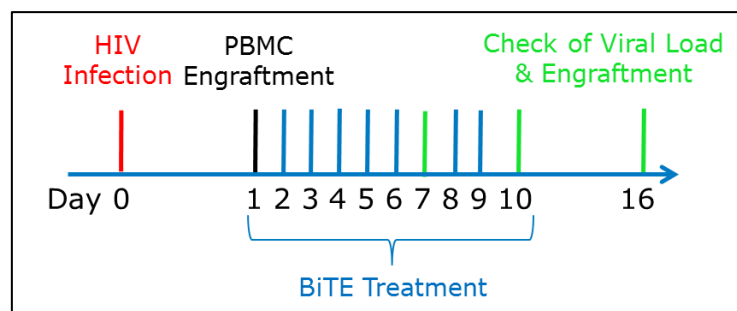


**Figure 44: Determination of Antiviral Activity of the CD4(1+2) BiTE and Controls on Primary Infected Cells *in vitro*.** HIV p24 level (marker for viral replication) was determined for CD4(1+2) $\times$ ahuCD3, irrelevant target $\times$ ahuCD3 and CD4(1+2) $\times$ amurCD3 control BiTE dilution series applied to infected PBMCs (upper panel) or infected MDMs plus autologous CD8<sup>+</sup> T cells (lower panel) at day 12 post infection. The assays were performed using three independent donors. Significances were determined using unpaired t-tests with Welch's correction. Mean values are indicated, error bars indicate SEM. Statistical significance at  $p < 0.05$ .

#### 4.7.3. ANTIVIRAL ACTIVITY OF CD4(1+2) BiTE IN VIVO

A first *in vivo* experiment designed to treat an established latent HIV infection using a humanized mouse model [144] failed due to inhomogeneity of human cell engraftment and viral load within the cohorts. Despite the equal application of CD34<sup>+</sup> human cells, derived from fresh cord blood, the engraftment level of human cells within the mice differed significantly. Thus, HIV RNA levels as well as human cell engraftment levels within the cohorts did not result in interpretable data (data not shown).

In the interest of time, the *in vivo* approach was adapted to an acute HIV infection model using NSG mice engrafted with HIV infected human PBMCs. The experiment started with the infection of human PBMCs with CXCR4-tropic HIV<sub>NL4-3</sub>. These infected PBMCs were injected into the mice one day post infection (p.i.). With the day of engraftment, the mice (n=8) were treated daily with 50 µg of the CD4(1+2) BiTE for 10 consecutive days. The BiTE treated cohort was controlled by a cohort (n=8) that was treated with BiTE formulation buffer. On day 7, 10 and 16 p.i., blood was collected from the mice to check their human cell status and HIV RNA level.

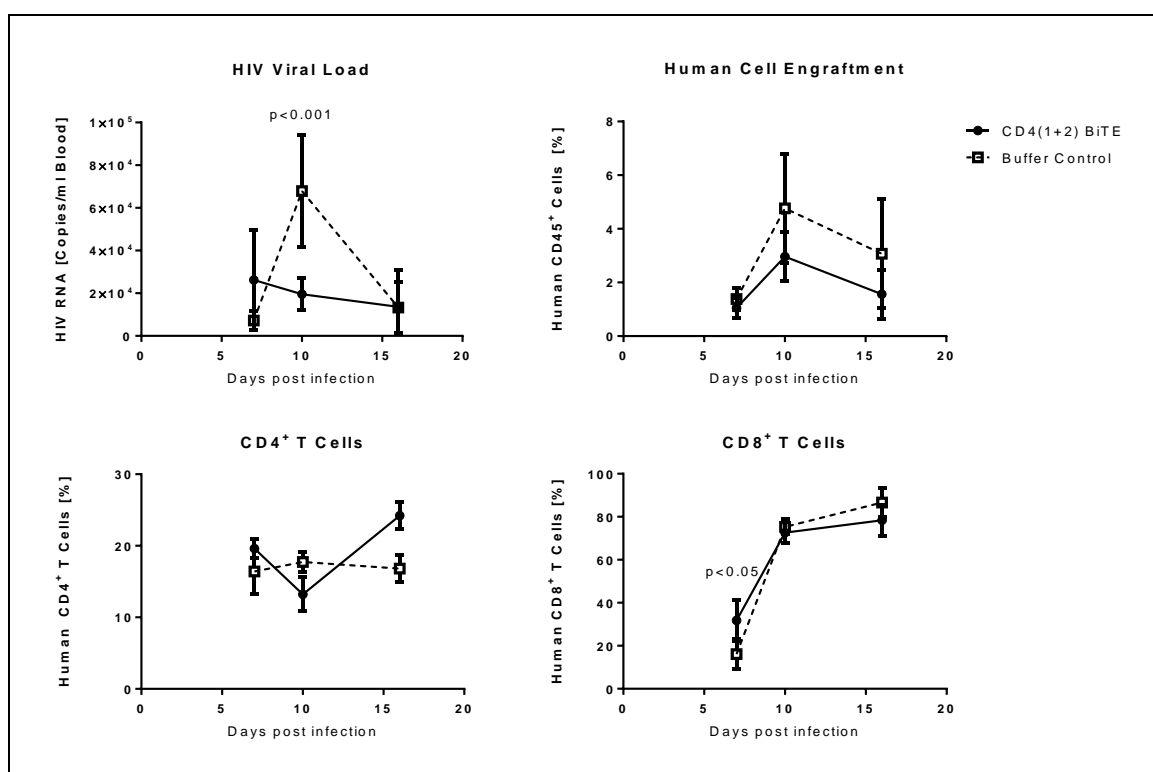


**Figure 45: Design of the *in vivo* Experiment.** Human PBMCs were infected *in vitro*, washed, split equally and injected i.p. into the mice. Daily BiTE treatment of 50 µg i.v. was started on the day of engraftment and continued for 10 consecutive days. The mice were analyzed for human cell status and HIV RNA level on day seven, 10 and day 16 post infection.

The bleeding of the mice on day 7 post infection (p.i.) showed an insignificantly increased viral load in the BiTE treated cohort compared to the buffer control cohort. Whereas the individual values were closely related within the control cohort, the BiTE treated cohort showed three mice with 40,000-70,000 copies/ml blood and four mice with comparable levels to the buffer control (ca. 10,000 copies/ml blood). Three days later (day 10 p.i.), the viral load of the BiTE treated cohort was significantly lower than for the buffer control cohort.

HIV RNA copy numbers for the BiTE treated group ranged between 10,000-30,000 copies/ml blood and 50,000-100,000 copies/ml blood in the buffer control cohort. Six days after the end of treatment (day 16 p.i.), the viral loads in both cohorts dropped to the same level of ca. 10,000 copies/ml blood.

The overall engraftment level of human PBMCs was monitored using human CD45 as a human cell marker. The CD45<sup>+</sup> cell levels of both cohorts seven days p.i. were comparable at 1.5%. On day 10 and day 16 p.i. the human cell level in the buffer control group was insignificantly increased compared to the BiTE treated cohort, but followed the same trend.



**Figure 46: Viral Load and Analysis of Human PBMC Engraftment of HIV<sup>+</sup> PBMCs in NSG Mice.** Mice serum samples were used for the determination of viral load by RT-PCR. Human PBMC engraftment level (huCD45<sup>+</sup> cells) and human CD4<sup>+</sup>/CD8<sup>+</sup> T cell levels were determined by FACS analysis. Mice were treated with CD4(1+2) BiTE or buffer for 10 consecutive days. Blood samples were taken at day 7, 10 and 16 p.i. Mean values are plotted, error bars indicate SD. Statistical significance at p < 0.05.

Of the human CD45<sup>+</sup>/human CD3<sup>+</sup> cells, the CD4<sup>+</sup> HIV target cells were stained to analyze a potential impact on their abundance by the infection and if applicable by the BiTE treatment. The BiTE treated cohort showed a significant decrease in CD4<sup>+</sup> T cell numbers between day seven and day ten p.i. (in average from 20% to 12% of CD45<sup>+</sup>/CD3<sup>+</sup> cells), whereas the control cohort showed steady CD4<sup>+</sup> T cell numbers at ca. 15%.

After the end of treatment at day 16 p.i., the CD4<sup>+</sup> T cell levels in the BiTE treated group increased hardly insignificant to ca. 22%, whereas the buffer control group showed steady CD4<sup>+</sup> T cell levels at ca. 15%.

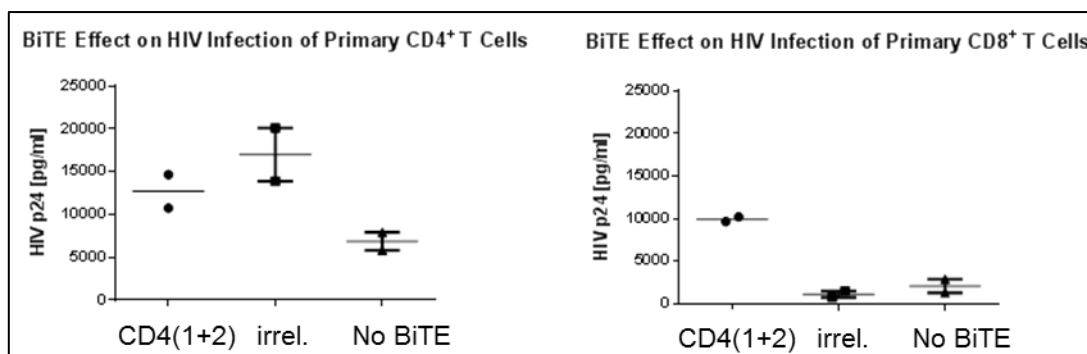
The BiTE treated cohort showed a significant increased human CD8<sup>+</sup> T cell level at day seven p.i. compared to the buffer control cohort (30% compared to 15% of human CD45<sup>+</sup>/CD3<sup>+</sup>/CD8<sup>+</sup> cells). Both cohorts showed a comparable increase in CD8<sup>+</sup> T cell levels between day 7 and day 10 p.i. (ca. 70%) and a further increase between day 10 and day 16 p.i. to ca. 80%.

#### 4.7.4. HIV BiTE-MEDIATED INFECTION OF T CELLS

The fact that a CD4<sup>+</sup>/CD3<sup>+</sup> T cell, can bind to the CD4(1+2) BiTE and thereby becomes positive for CD4 domains essential for binding to HIV, raised the concern that T cells bound to BiTE antibody constructs could experience higher infectivity because of their increased CD4 positivity. For a first test whether the binding of the CD4(1+2) BiTE results in a BiTE-mediated infection of non-target CD8<sup>+</sup> T cells or increased infection of CD4<sup>+</sup> T cells, primary CD4<sup>+</sup> and CD8<sup>+</sup> T cells were isolated and incubated with 10 µg/ml of the CD4(1+2) BiTE for two hours. Followed by a washing step, the cells were infected with HIV. Subsequently, the cells were washed and incubated for seven days. Thereafter, the p24 level of the isolated T cell subpopulations incubated with CD4(1+2) BiTE were determined with reference to a negative control (BiTE consisting of an irrelevant target-binding moiety and the anti-huCD3 scFv) and a no BiTE control.

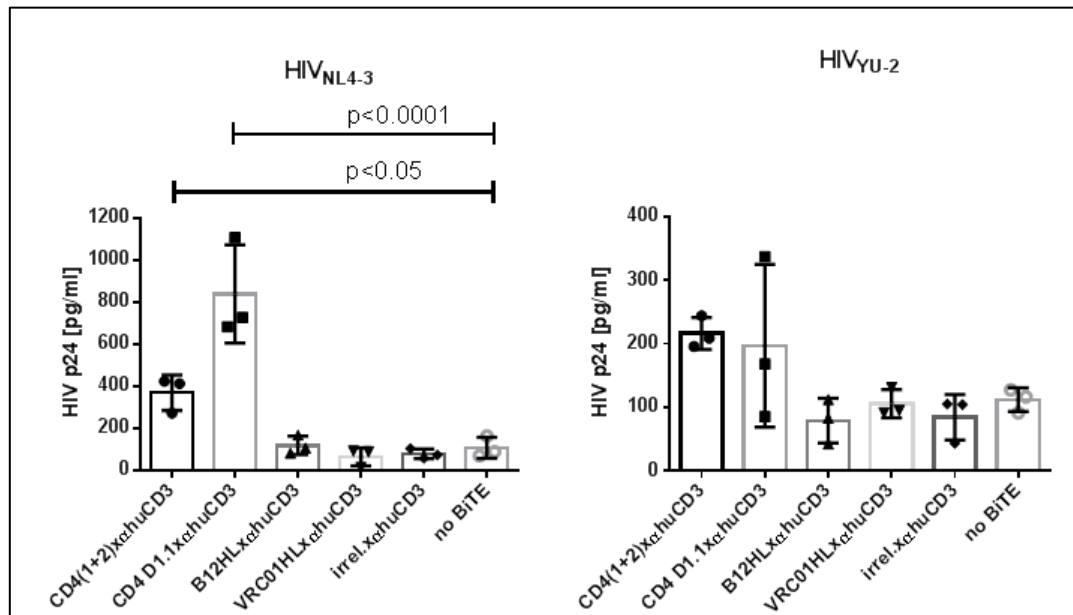
Isolated CD4<sup>+</sup>/CD8<sup>-</sup> T cells showed a slight increase when pre-incubated with BiTE antibody constructs in relation to the no BiTE control. This increase of p24 level was independent of the target specificity of the BiTE.

Isolated CD8<sup>+</sup>/CD4<sup>-</sup> T cells, which usually are not a target for HIV, showed a specific increase in HIV p24 level when pre-incubated with CD4(1+2) BiTE. Cells pre-incubated with the control BiTE and the no BiTE control showed comparably lower levels of HIV p24.



**Figure 47: CD4(1+2) BiTE-mediated Infection of T Cells.** Primary isolated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were incubated with CD4(1+2) BiTE, a negative control BiTE or no BiTE for 2h, washed subsequently and then infected with HIV<sub>NL4-3</sub> via spinoculation. Thereafter, the cells were washed and incubated for 7 days. The HIV p24 level was determined by ELISA using cell culture supernatant. The assay was performed using two independent donors. Mean values are displayed, error bars indicate SD.

The experiment was repeated by incubating unstimulated CD8<sup>+</sup>/CD4<sup>-</sup> T cells with 10 µg/ml of all generated BiTE antibody constructs to see whether this effect is also present when using a gp120 neutralizing binding moiety like B12 or VRC01 in the BiTE format.



**Figure 48: BiTE-Mediated Infection of CD8<sup>+</sup>/CD4<sup>-</sup> T Cells.** Primary isolated and unstimulated CD8<sup>+</sup> T cells were incubated with BiTE antibody constructs (at 10 µg/ml) for 2 hours, washed subsequently and then spinoculated with HIV<sub>NL4-3</sub> (CXCR4-tropic) or HIV<sub>YU-2</sub> (CCR5-tropic) for 2h, washed subsequently and incubated for 7 days. The HIV p24 level was determined by ELISA using cell culture supernatant. The assay was performed using three independent donors. Mean per donor (dots) and mean per BiTE (column) are displayed, error bars indicate SEM. Statistical significance at p<0.05.



Here, the CD4(1+2) BiTE as well as the CD4 D1.1 BiTE showed higher levels of HIV p24 compared to B12, VRC01-based BiTE antibody constructs, the irrelevant BiTE and the no BiTE control. The CD4 domain 1 BiTE showed an even higher level of HIV p24 compared to the CD4(1+2) BiTE when infected with HIV<sub>NL4-3</sub>. The canonical BiTE antibody constructs based on the neutralizing binding moieties B12 or VRC01 did not show this elevation in HIV p24 level. As expected, CCR5 expression in unstimulated human CD8<sup>+</sup> T cells was lower compared to CXCR4 (measured by FACS, data not shown), resulting in lower HIV p24 levels. However, the HIV<sub>YU-2</sub> infected CD8<sup>+</sup> T cells showed the same trend for CD4-based BiTE antibody constructs as measured for the HIV<sub>NL4-3</sub> infected cells.

#### 4.8. GENERATION OF A DUAL-TARGETING BiTE: CD4L17BXAHUCD3

As seen from previous experiments, the neutralization of HIV virions by CD4-based BiTE antibody constructs is insufficient, most likely because the coreceptor-binding site of HIV gp120 is not blocked by CD4. Cells decorated with CD4(1+2) BiTE can bind to HIV env present on a virion, which then seems to induce the engagement of the HIV env proteins, which eventually can lead to viral entry upon binding of the engaged HIV gp120 to the coreceptor that is also present on the cell.

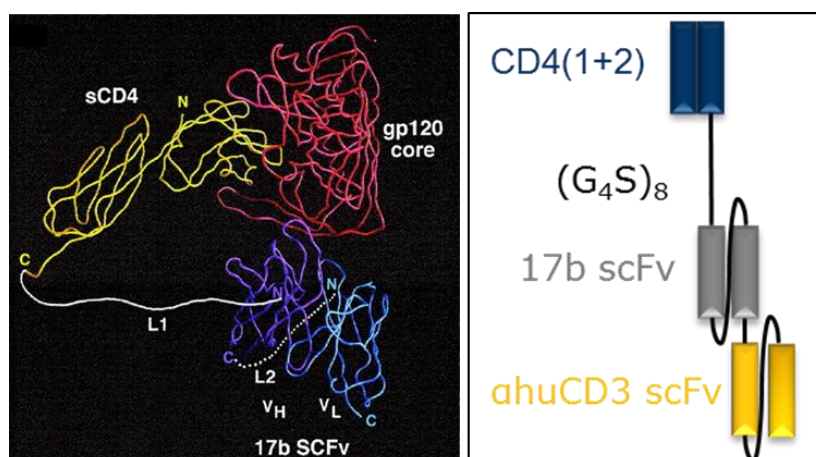
In contrast to CD4-based BiTE antibody constructs, neutralizing HIV antibodies like B12 or VRC01 may also be used to achieve complete neutralization as reported previously [145] and seen in the BiTE-mediated infection experiments. Those antibodies neutralize gp120 by binding and locking the gp120 protein in the unengaged, native conformation. So, these mAbs are able to block CD4 binding and subsequent engagement of HIV env, because of their overlapping epitope with the CD4 binding site.

To further use the broad specificity of CD4 for HIV virus strains, a CD4(1+2) BiTE was designed that is theoretically able to fully neutralize the HIV env protein. Therefore, the generation of a dual-targeting BiTE was tested. The idea was to add a scFv to the existing CD4(1+2) BiTE, preventing the coreceptor from binding to gp120 thereby inhibiting subsequent viral entry. This combination of the natural receptor CD4 and the blockade of coreceptor-binding by a scFv should offer a good breadth regarding circulating HIV strains. Similarly to single-targeting antibody-based BiTE antibody constructs, the breadth of this scFv portion is limited with regard to viral strains that are sensitive to the anti coreceptor-binding site scFv.

The well-known monoclonal antibody 17b was used for the addition to the CD4(1+2) BiTE, because its structure showed blocking of coreceptor-binding site on HIV<sub>HXB2</sub>, because of its partially overlapping epitope with the coreceptor-binding site on HIV gp120 [141]. This CD4L17b BiTE should be able to bind to the CD4 binding site, induce the conformational change of gp120 thereby exposing the CD4-induced epitope which then can be bound by the scFv 17b blocking subsequent coreceptor-binding. This

neutralization property might prevent the virion from subsequently entering the T cell. Salzwedel et al. have shown that the infection of cells can be enhanced by soluble CD4, but inhibited by further adding the mAb 17b to the cells [146].

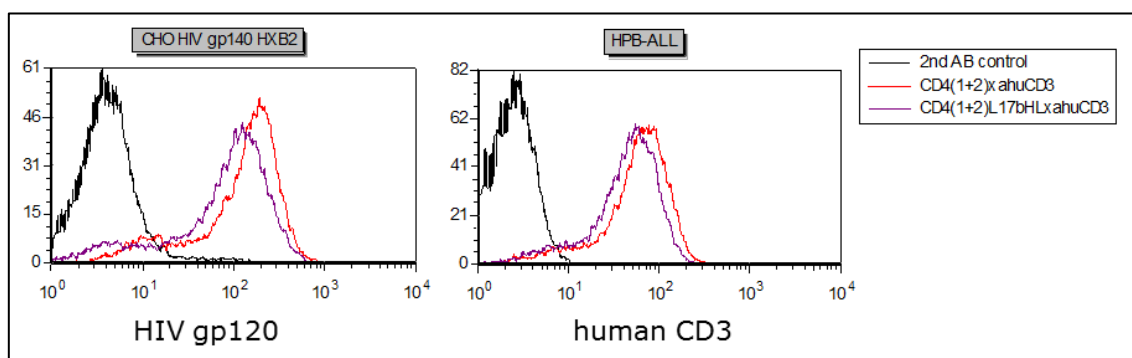
Lagenaur et al. presented the CD4L17b dual-targeting molecule first and analyzed the additive effect in neutralization assays. They determined the best linker length between CD4(1+2) and 17b in order to allow simultaneous binding of both epitopes [147,148]. The described optimal linker length of  $(G_4S)_8$  was adopted for the construction of this dual-targeting BiTE molecule.



**Figure 49: Model of sCD4-17b and Structural Scheme of the Dual-Targeting HIV BiTE CD4L17bHLx $\alpha$ huCD3.** The scFv of the anti-coreceptor-binding site mAb 17b was added between the CD4 domains 1+2 and the anti-huCD3 scFv. A 40 AA  $(G_4S)_8$  linker was introduced between the CD4 domains and the 17b scFv according to Lagenaur et al. [147]. Credit for CD4L17b/gp120 core model [148].

#### 4.8.1. FUNCTIONAL PROOF OF THE CD4L17bHLxAHuCD3 BiTE

The first characterizing step of the dual-targeting candidate was to test its binding ability to HIV  $env^+$  or human  $CD3^+$  cells respectively. The FACS binding showed functionality of the CD4L17b BiTE for both arms of the BiTE. In relation to the parental CD4(1+2) BiTE, the dual-targeting BiTE CD4L17bx $\alpha$ huCD3 showed a small reduction in signal on gp120 and human CD3 binding when incubated at the same concentration.

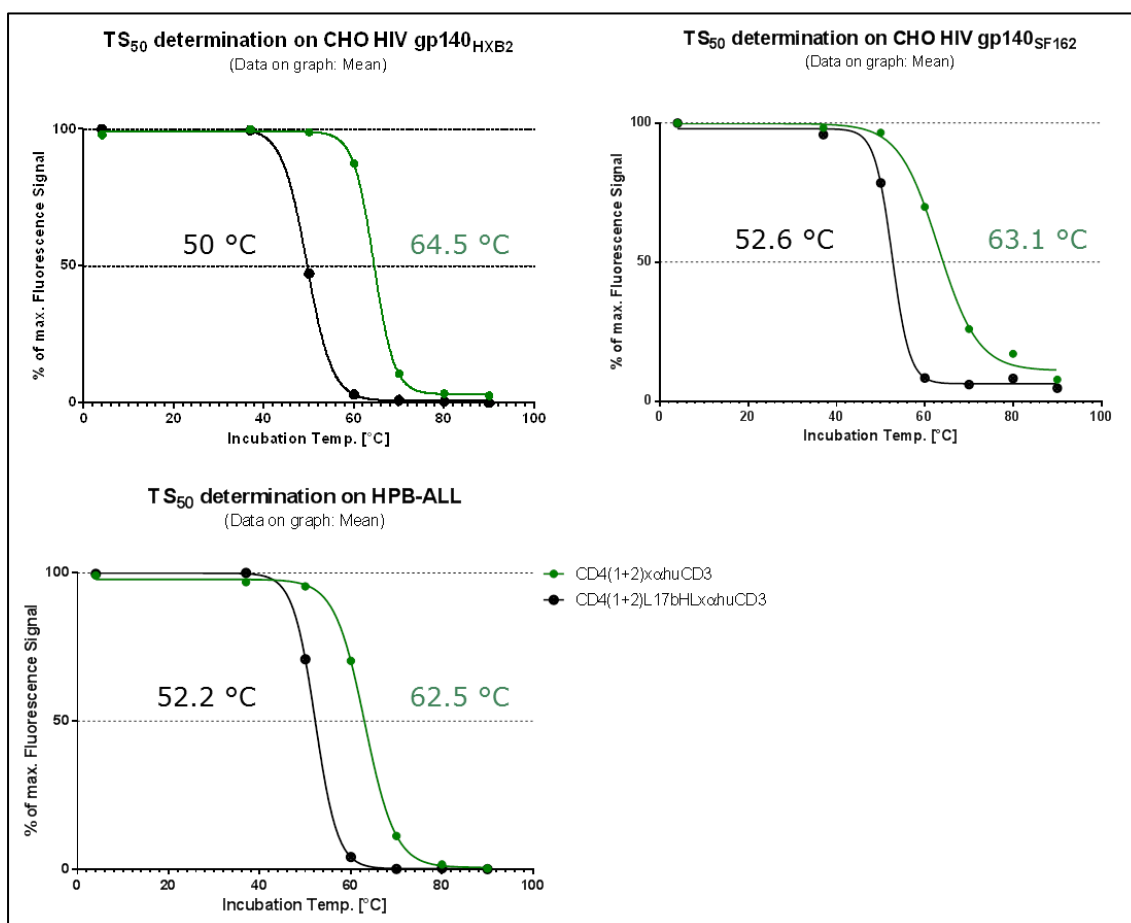


**Figure 50: FACS Analysis of the Dual-Targeting BiTE With HIV  $env^+$  CHO and Human  $CD3^+$  Cells.** Indicated BiTE antibody constructs were incubated with HIV  $env$  transfected CHO cells and huCD3 positive HPB-ALL cells and analyzed by FACS using a secondary anti-histidine mAb. The dual targeting BiTE showed slightly decreased binding to HIV  $env^+$  or human  $CD3^+$  cells at normalized concentrations.

#### 4.8.2. TEMPERATURE STABILITY OF THE CD4L17BHLXAHUCD3 BiTE

To determine the influence of the second targeting domain on the BiTE the temperature stability of this dual-targeting BiTE was tested with reference to the parental CD4(1+2) BiTE.

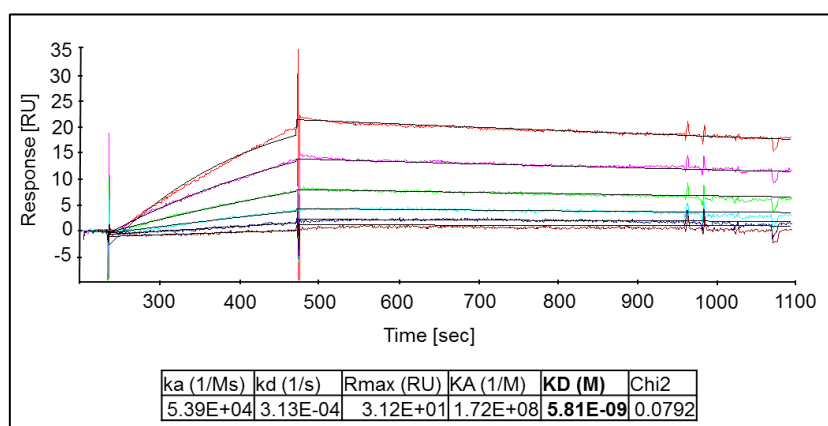
The dual-targeting BiTE showed decreased stability in comparison with the parental CD4(1+2) BiTE when analyzing for its binding to HIV  $env^+$  or human  $CD3^+$  cells. The signal obtained from the FACS analysis showed a decreased  $TS_{50}$  value of 50-52.6°C compared to 62.5-64.5°C for the regular CD4(1+2) BiTE.



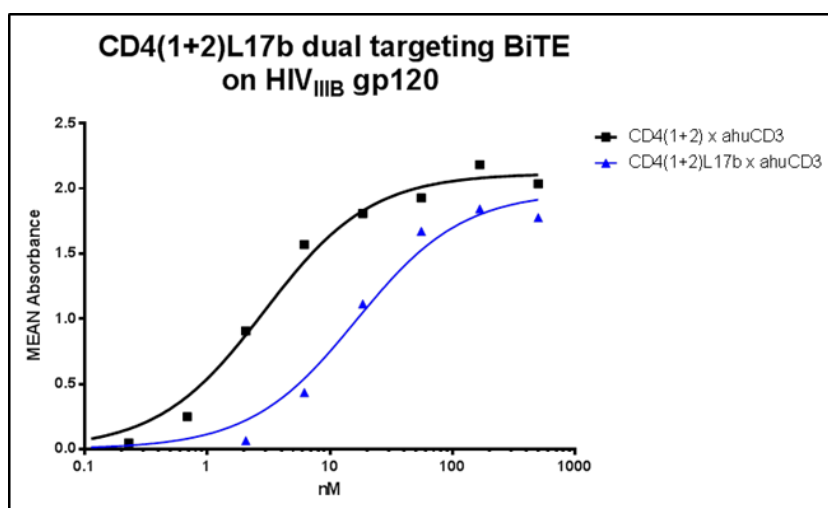
**Figure 51: Temperature Stability Analysis of the Dual-Targeting BiTE on HIV  $env^+$  CHO and Human  $CD3^+$  Cells.** BiTE antibody constructs were incubated at indicated temperatures before they have been used to stain antigen positive cells. The temperature equaling the half-maximal FACS signal gives rise to the indicated  $TS_{50}$  values.

#### 4.8.3. HIV Gp120 AFFINITY OF THE CD4L17bHLxahUCD3 BiTE

The effect of the second target-binding domain on the overall target affinity of CD4(1+2)L17b to HIV env was tested by SPR and ELISA analysis. The Biacore data showed a decrease in target affinity of factor 6 (KD 5.8 nM) for the dual-targeting BiTE compared to the single-targeting BiTE (KD 1 nM). The determination of the target affinity by ELISA showed a KD of 16.4 nM in comparison to 2.9 nM for the CD4(1+2) BiTE (ca. 5.6-fold reduction). The SPR value comparison of the two BiTE antibody constructs showed a decrease in the association rate for the dual-targeting BiTE, whereas the dissociation rate showed roughly constant values for both candidates.



**Figure 52: SPR HIV gp120 Affinity of CD4L17bHLxahUCD3.** Soluble gp120<sub>IIIB</sub> was coated on a CM5 chip. BiTE affinity kinetics were calculated with reference to an uncoated flow chamber.



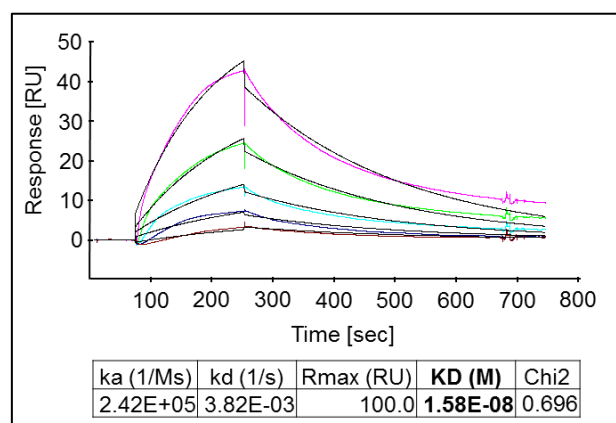
**Figure 53: HIV gp120 Affinity of Dual-Targeting CD4(1+2) BiTE Versus Single-Targeting CD4(1+2) BiTE Using ELISA.** Soluble gp120<sub>IIIB</sub> was coated on a Maxisorp plate. BiTE antibody constructs were added and analyzed by a peroxidase-conjugated antibody specific for the histidine-tag of the BiTE.

**Table 11: Overview of the CD4L17bxahuCD3 Affinity to HIV<sub>III</sub>B gp120.**

Method	SPR			ELISA
Antigen	HIV <sub>III</sub> B gp120			
Affinity	ka [1/Ms]	kd [1/s]	KD [nM]	
CD4(1+2)xahuCD3	2.93E+05	2.08E-04	1	2.9
CD4(1+2)L17bHLxahuCD3	5.39E+04	3.13E-04	5.81	16.4

#### 4.8.4. HUMAN CD3 AFFINITY OF THE CD4L17BHLXAHUCD3 BiTE

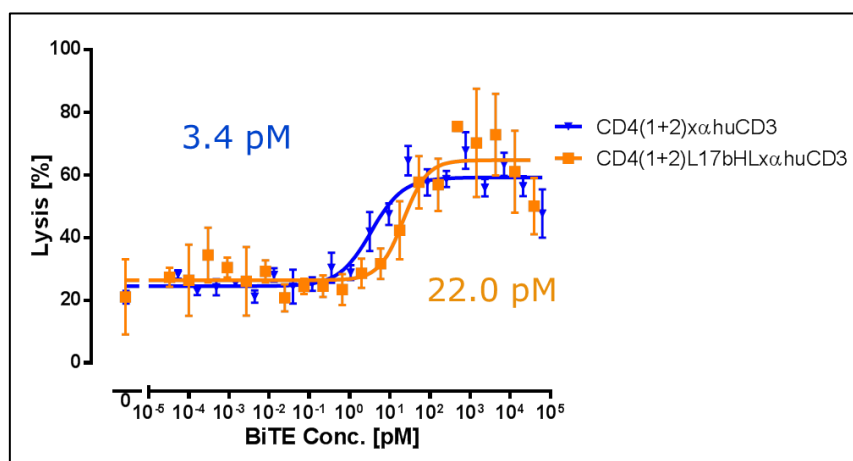
The affinity of the dual-targeting BiTE was also tested for its affinity to human CD3. However, this affinity revealed unusual kinetics that could not be fitted exactly using the standard fit models. The net result of the best possible fit showed a seven-fold decrease in human CD3 affinity (KD of 15.8 nM) compared to the parental CD4(1+2) BiTE (KD of 2.3 nM).



**Figure 54: SPR Analysis of CD4L17bxahuCD3 to Human CD3.** Soluble human CD3 (Fc-fusion of the extracellular CD3 domain) was coated on a CM5 chip. BiTE affinity kinetics could not be calculated exactly, because of the volatile association rate of the dual-targeting BiTE.

#### 4.8.5. CYTOTOXIC ACTIVITY OF THE CD4L17bHLxAHUCD3 BiTE

The ability of the dual-targeting BiTE to redirect lysis of primary prestimulated human CD8<sup>+</sup> T cells to HIV env<sup>+</sup> CHO cells was tested *in vitro*. Next to binding and affinity analyses, the cytotoxicity assay confirmed the lower performance of this dual-targeting BiTE when compared to the CD4(1+2) BiTE. The impact on the EC<sub>50</sub> value ranged at factor 7 in molar activity (22 pM compared to 3.4 pM activity of the CD4(1+2) BiTE).

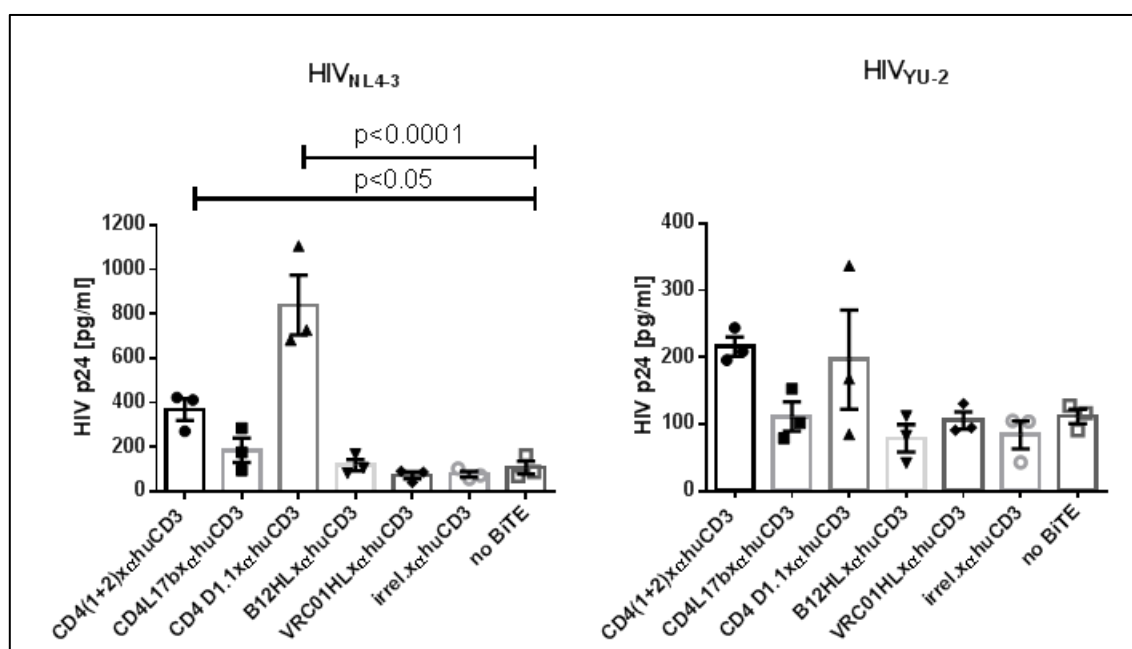


**Figure 55:** <sup>51</sup>Cr Release Cytotoxicity Assay Comparing the Bioactivity of the CD4(1+2) and CD4(1+2)L17b BiTE Antibody Constructs. HIV env<sup>+</sup> CHO cells were labeled with <sup>51</sup>Cr before co-incubation with prestimulated human CD8 T cells and BiTE dilution series. After co-incubation of 16 h, the <sup>51</sup>Cr amount in the cell culture supernatant was determined. With reference to a maximal lysis of target cells, the percentage of dead target cells was calculated. Mean values are displayed, error bars indicate SEM.

#### 4.8.6. CD4L17B BiTE-MEDIATED INFECTION OF CD8<sup>+</sup> T CELLS

To examine whether the mAb 17b directed to the CD4-induced coreceptor-binding site and integrated into the CD4(1+2) BiTE is able to mediate full neutralization of HIV env, the CD4L17b BiTE was tested in parallel to the canonical BiTE antibody constructs for its potential to mediate HIV infection of primary CD4<sup>+</sup>/CD8<sup>+</sup> T cells.

As can be seen from Figure 56, the CD4L17b dual-targeting BiTE showed a reduction in HIV p24 level compared to the mono-targeting CD4-based BiTE antibody constructs with both viruses. In case of HIV<sub>NL4-3</sub>, the HIV p24 level of the CD8<sup>+</sup> T cells pre-incubated with CD4L17b BiTE was not significantly different from those of the neutralizing scFv-based BiTE antibody constructs and the no BiTE control. In contrast, the CD4-based BiTE antibody constructs mediated significantly higher HIV p24 levels when using HIV<sub>NL4-3</sub> (as already presented in 4.7.4).



**Figure 56: CD4(1+2) Dual-Targeting BiTE-Mediated Infection of CD8<sup>+</sup>/CD4<sup>+</sup> T Cells.** Primary isolated, unstimulated CD8<sup>+</sup> T cells were incubated with indicated BiTE antibody constructs (at 10 μg/ml) for 2 hours, washed and then spinoculated with HIV<sub>NL4-3</sub> (CXCR4-tropic) or HIV<sub>YU-2</sub> (CCR5-tropic), washed and incubated for 7 days. The HIV p24 level was determined by ELISA using a fraction of the cell culture supernatant. The assay was performed using three independent donors. Mean per donor (dots) and mean per BiTE (column) are displayed, error bars indicate SEM. Statistical significance at p<0.05.



## 5. DISCUSSION

### 5.1. THE HIV ENVELOPE PROTEIN AS TARGET ANTIGEN

The HIV gp160 or env protein is the only known extracellularly accessible HIV-specific antigen on infected cells. Its posttranslational cleavage into gp120 and gp41, offers two possible target proteins for antibodies or BiTE antibody constructs respectively. HIV gp120 as the surface protein of the HIV envelope heterodimer, offers several conserved epitopes like the CD4 binding site or the CD4-induced coreceptor-binding site. Many broadly neutralizing antibodies have been identified from patients, which bind to those conserved epitopes. The respective antibodies have been characterized and mostly crystallized in complex with the gp120 core protein. Next to its location on the cell membrane, gp120 can be found associated to virions or because of its non-covalent bond to the transmembrane protein gp41 also as a soluble protein. Because soluble gp120 proteins and budded virions possibly bind to BiTE antibody constructs or to T cells engaged with BiTE antibody constructs, the effective BiTE concentration in patients probably needs to be higher than for exclusively membrane-standing target antigens as can be found in oncological diseases. As demonstrated in this thesis, targeting of HIV infected cells via gp120 is feasible *in vitro* and *in vivo* to treat HIV infection. Moreover, Denton et al. showed an additional antiviral effect of an anti-gp120 antibody drug conjugate (ADC) in mice treated with ART, suggesting that targeting gp120 *in vivo* even under ART is feasible in order to lyse HIV infected cells [83].

The transmembrane protein gp41, the second protein of the env heterodimer is covered by gp120 and as the name indicates, is anchored in the cell or viral membrane respectively. With its membrane proximal position the gp41 protein would be well-suited to serve as antigen for BiTE antibody constructs as Bluemel et al. showed that the shorter the distance of the epitope to the target cell membrane, the better is the cytotoxic activity of the respective BiTE [149]. However, the two prominent human anti-gp41 monoclonal antibodies 2F5 and 4E10 do show auto-reactivity. Both bind to phospholipids via their hydrophobic CDR3 domain and therefore usually are depleted during the maturation process of B cells [150]. Additionally, recent data from Chakrabarti et al. indicates that the accessibility of their epitope, the conserved membrane proximal external region (MPER), is CD4 dependent [151]. This means that the 2F5 or 4E10 specificities themselves do not seem to be able to bind to gp41 on infected cells prior to CD4 engagement. This hypothesis was confirmed by the observation that the 4E10 mAb only binds to the MPER epitope upon spontaneous or induced MPER exposure [57]. In 2012, Huang et al. [60] described a new gp41 specific antibody (10E8) with improved neutralization activity and without auto-reactivity as confirmed by Kwong et al. [152]. In addition, this mAb was reported to show improved binding in membrane-surface context in comparison to mAbs 2F5 and 4E10. The described characteristics of 10E8 might sound advantageous for the generation of an anti-gp41 BiTE candidate. However, Chen et al. have shown that 10E8 also binds to phospholipids and propose this auto-reactivity to be a general property of anti-MPER antibodies [153], because of their characteristic hydrophobic CDR3 domains.

Based on this data, available anti-gp41 antibody specificities do not seem to be applicable for the generation of BiTE antibody constructs. Therefore, this thesis focused on gp120 as target for HIV BiTE antibody constructs.

## 5.2. THE NATURAL HIV RECEPTOR CD4 AS A TARGETING MODULE

For the binding of CD4 to gp120, only the first N-terminal immunoglobulin-like domain seem to be essential as shown by Diskin et al. [154]. Omitting the following second extracellular domain of CD4 might lower possible side effects. However, according to Chen et al. [49] the sole expression of the wild-type N-terminal CD4 domain 1 failed, because of the hydrophobic amino acids within this domain, which usually form a hydrophobic interface between CD4 domain 1 and 2. These hydrophobic amino acids negatively affect the functional structure of domain 1, if expressed solely. Chen et al. introduced five point mutations within the first CD4 domain enabling its functional expression independently of the second CD4 domain. On the upside, this engineering effort of CD4 should increase the affinity to gp120, which however was not seen for the generated CD4 domain 1 BiTE antibody constructs. On the downside, the introduction of any (point-) mutation into wild type CD4 alters the natural protein and makes it potentially vulnerable to viral escape. Furthermore, a CD4 domain 1 BiTE candidate has a lower molecular weight (ca. 39 kDa instead of 48 kDa for the CD4(1+2) BiTE), which might also be disadvantageous considering its potential half-life *in vivo*. With the omission of the second CD4 domain, the affinity of CD4 domain 1 BiTE antibody constructs to MHC class II complexes should also be lowered. Since CD4(1+2) is known to interact with MHC class II complexes, a CD4(1+2) BiTE possibly mediates the binding of any T cell via CD3 to a MHC class II<sup>+</sup> cell thereby initiating its lysis [155]. However, the affinity of wild-type CD4 to MHC class II complexes is reported to be very low (ca. 200  $\mu$ M to 2 mM) [156], which might offer a therapeutic window for a BiTE therapy below that concentration.

Own experiments showed cytotoxic activity of the CD4-based BiTE antibody constructs at concentrations higher than one  $\mu$ g/ml (21 nM) only for a B cell line. Here, CD4 domain 1 BiTE antibody constructs showed lower cytotoxic activity than the CD4(1+2) BiTE. However, when targeting primary B cells, no BiTE-dependent cytotoxic activity was detected for the CD4(1+2) BiTE as well as for CD4 domain 1 BiTE antibody constructs up to 10  $\mu$ g/ml. Therefore, CD4 domain 1 BiTE antibody constructs showed no advantage compared to the CD4(1+2) BiTE when targeting primary B cells. As the FACS experiment revealed, the MHC class II expression of Raji cells was higher than for primary B cells, which may well explain their higher sensitivity towards the CD4(1+2) BiTE redirected lysis. MHC class II complexes have not been shown to be the interacting protein on Raji or primary B cells of CD4(1+2) in this FACS experiment. However, available literature and signal correlation of MHC class II and CD4(1+2) BiTE signal suggest that MHC class II complexes are the interacting partner of the CD4(1+2) BiTE (see Figure 38) [157]. The increased BiTE independent lysis rate of autologous B cells in the cytotoxicity assay using prestimulated CD8<sup>+</sup> T cells, might be caused by binding of the MHC class II complexes of B cells to

matching T cell receptors of prestimulated CD8<sup>+</sup> T cells resulting in lysis of these B cells (see Figure 39).

In general, using the first two domains of CD4 for binding of gp120 offers the best chance to avoid viral escape since CD4 is the natural receptor of HIV. Furthermore, CD4-based BiTE antibody constructs offer cross-reactivity with SIV gp120 and macaque CD3 enabling combinational *in vivo* studies allowing to determine toxicity and efficacy in non-human primates.

On the downside however CD4(1+2) is known to induce shedding of gp120 upon binding [158,159], which might theoretically be disadvantageous when targeting HIV infected cells. MPER-specific antibodies 2F5 and 4E10 have also been shown to induce gp120 shedding as well as the bnAb B12, which has previously been reported otherwise [160]. However, this fact does not seem to play an essential role since the *in vitro* and *in vivo* experiments revealed antiviral activity of the CD4(1+2) and B12 BiTE. The shedding of gp120 causes neutralization of HIV virions, which might be advantageous for the inhibition of viral spread. However, it could be more beneficial to use a gp120 binding moiety for BiTE generation, which does not induce gp120 shedding, like 2G12 [160] because this candidate might mediate more stable binding of effector cells to HIV infected cells. This however would be accompanied by a limited breadth in specificity as 2G12 neutralizes only ca. 32% of relevant HIV viruses [43].

However, the main handicap of the CD4-based BiTE antibody constructs resulted from the BiTE-mediated infection assays. The concern was that the CD4(1+2) BiTE possibly mimics CD4<sup>+</sup> T cells by binding via its anti-CD3ε scFv to CD4<sup>-</sup>/CD8<sup>+</sup>/CD3<sup>+</sup> cells or increase infectivity of CD4<sup>+</sup>/CD8<sup>-</sup>/CD3<sup>+</sup> cells by increasing the number of CD4(1+2) domains present on the cell surface. This concern was confirmed *in vitro* using prestimulated CD8<sup>+</sup> T cells. These cells were incubated with different BiTE antibody constructs prior to their infection *in vitro* and showed that the CD4(1+2) BiTE facilitates subsequent HIV infection. In this preliminary experiment, the incubation with CD4(1+2) BiTE before infection caused an increased HIV p24 level for CD8<sup>+</sup> T cells. The incubation of CD8<sup>+</sup> T cells with an irrelevant BiTE did not lead to an increased HIV p24 level as well as the control without BiTE. For primary CD4<sup>+</sup> T cells, the samples, which have been pre-exposed to the CD4(1+2) BiTE and an irrelevant BiTE showed comparably elevated HIV p24 levels in contrast to the no BiTE control. The repetition of this preliminary experiment in which all generated BiTE antibody constructs were analyzed for their potential in mediating infection of unstimulated CD4<sup>-</sup>/CD8<sup>+</sup> T cells, confirmed the trend for the mediated infection by CD4-based BiTE antibody constructs. In contrast, BiTE antibody constructs based on neutralizing mAbs B12 or VRC01 showed no significant increase of HIV p24. The HIV<sub>NL4-3</sub> p24 level for CD8<sup>+</sup> T cells incubated with the CD4 D1.1 BiTE was even higher than the one seen for the CD4(1+2) BiTE. This is most likely due to a higher number of CD4 D1.1 BiTE molecules in the assay, since the BiTE antibody constructs were adjusted to 10 µg/ml, independently of their molecular weight. In case of HIV<sub>YU-2</sub>, the low CCR5-coreceptor expression of the CD8<sup>+</sup> T cells most probably did not lead to significantly elevated HIV p24 levels of the CD4-based BiTE antibody constructs, further suggesting that the coreceptor is necessary for viral entry.

As already mentioned, CD4-based BiTE antibody constructs do not fully neutralize the HIV env protein. Since subpopulations of CD8<sup>+</sup> T cells express the HIV coreceptors CXCR4 or CCR5, the HIV env molecules are most likely engaged by the CD4 domains of the CD4-based BiTE antibody constructs and subsequently interact with the coreceptors present on the cell, leading to the fusion of the virion with the host cell membrane.

Although it is known that CD3 is recycled by T cells [161], this mechanism does not seem to play a major role in mediating the infection of CD8<sup>+</sup> T cells, since the B12 or VRC01 BiTE antibody constructs did not show increased HIV p24 values compared to the irrelevant or no BiTE control in the BiTE-mediated infection assays. Furthermore, even if the HIV virion would be co-internalized with the BiTE and CD3 it remains questionable if the virion becomes degraded or the viral membrane is able to fuse with the vesicle membrane in order to infect the cell.

### 5.3. COMPLETE NEUTRALIZATION OF HIV ENV

#### 5.3.1. NEUTRALIZING MONOCLONAL ANTIBODY MOIETIES

Due to the results discussed in the previous subchapter, the full neutralization of gp120 seems to be crucial for an anti-HIV BiTE. Since the neutralization of gp120 by CD4 is not complete and the epitopes of broadly neutralizing antibodies (bnAbs) like B12 or VRC01 fully neutralize many HIV env proteins, the application of those BiTE antibody constructs comprising neutralizing mAb specificities would have a double benefit: Neutralization of circulating virions and the mediation of redirected lysis of HIV infected cells. Therefore, the question arises: Would not the usage of a HIV neutralizing BiTE be more advantageous compared to the CD4-based BiTE, which does not neutralize the coreceptor-binding site on gp120?

In general: Yes, a complete neutralization of HIV env (i.e., the CD4 binding site and the coreceptor-binding site) would be beneficial. However, sequence alteration of the HIV genome is common during viral replication and can lead to the failure of a neutralizing antibody-based treatment [162-165]. Therefore, antibody-based BiTE antibody constructs can potentially be overrun by viral escape. However, the combined antiretroviral therapy, as of today, results in a limited number of virus clones circulating in the plasma as described by Bailey et al. [166]. Therefore, the combination of cART and BiTE treatment potentially limits HIV sequence variation and might ensure antiviral efficacy of a scFv-based BiTE. Since the effect of the cART regimen in limiting the number of HIV clones is strictly dependent on the compliance of the patient, the bnAb-based BiTE antibody constructs could still experience a limited breadth in viral specificity. Even the VRC01 epitope, which almost completely matches with the CD4 binding site (98%, [47]) making this mAb comparable in breadth to CD4 still has a higher probability of experiencing viral escape than the natural receptor itself [145,167].

### 5.3.2. CD4-BASED DUAL-TARGETING BiTE ANTIBODY CONSTRUCTS

To achieve full neutralization of HIV env using CD4 as a target-binding moiety would require the additional blocking of the coreceptor-binding site on gp120. One could combine a CD4-based BiTE molecule with individual coreceptor inhibitors against CXCR4 or CCR5 as part of an ART (e.g., CCR5-inhibitor Maraviroc [168,169]). Thereby, HIV infected cells could be targeted with the broadest specific target-binding moiety CD4, ensuring low potential for viral escape. In addition, BiTE-mediated infection of T cells could potentially be abolished in inhibiting the coreceptor-binding of CD4(1+2) BiTE-engaged HIV env molecules.

In contrast to the combinatorial approach of coreceptor antagonists, a second target-binding moiety within the CD4(1+2) BiTE molecule could be introduced which blocks the coreceptor-binding site. The generated dual-targeting BiTE CD4L17b $\alpha$ huCD3, which consists of the CD4 domains 1+2 and the scFv of mAb 17b, which binds to the CD4-induced epitope on HIV gp120, could have the further advantage of being able to target cells infected with HIV strains, which are non-sensitive to CD4, but sensitive to mAb 17b. HIV sequence variations leading to the insensitivity to human CD4 are rare (ca. 8%) [47]. However, strains with such mutations in the env protein exist. In those cases, the env is in a “chronically” engaged conformation and allows direct binding of the coreceptor CXCR4 and/or CCR5 for viral entry [170-175], which could be bound by a dual-targeting BiTE construct like CD4L17b $\alpha$ huCD3.

However, the first results of the *in vitro* characterization of this dual-targeting BiTE revealed a decreased temperature stability compared to the parental CD4(1+2) BiTE ( $\Delta T_{S_{50}} = 10^{\circ}\text{C}$ ) and a decreased overall gp120 affinity of 6 nM compared to 1 nM for the CD4(1+2) BiTE. This observation could be related to Zhang et al. [176], who reported that upon 17b binding the gp120 affinity to CD4 is decreased, presumably to allow conformational rearrangements of gp120. Moreover, the human CD3 affinity seemed to be impacted by the dual-targeting BiTE design leading to a KD of ca. 15 nM compared to 2.3 nM for the CD4(1+2) BiTE in SPR. The cytotoxic activity when targeting HIV env<sup>+</sup> CHO cells showed a seven-fold decrease in molar activity (1.7 ng/ml; 22 pM) compared to the parental BiTE (0.16 ng/ml; 3.3 pM), which could be well explained by the reduced affinities.

However, the dual-targeting BiTE showed a benefit when compared with the parental CD4(1+2) BiTE when analyzed for its potential in mediating HIV infection of CD8<sup>+</sup> T cells. Because of the remaining functional characterization of the scFv 17b within the BiTE, the benefit cannot be surely claimed to result from the blocking of the coreceptor-binding site. Although it is unlikely, it cannot be excluded that the effect was caused by the inferior affinity of the dual-targeting BiTE construct to HIV gp120 or by steric hindrance of the membrane fusion by a non-functional 17b scFv at this point. The data generated with unstimulated CD8<sup>+</sup> T cells, which were infected with HIV<sub>NL4-3</sub> suggests that the dual-targeting CD4(1+2)-based BiTE can reduce BiTE-mediated infection of T cells. In case of HIV<sub>YU-2</sub> with generally lower HIV p24 levels, the dual-targeting BiTE also showed a reduction in HIV p24 level. Most probably due to the low CCR5 coreceptor expression on CD8<sup>+</sup> T cells, the CD4-based canonical BiTE antibody constructs did not lead to significantly elevated HIV p24 levels.

Thus, the application of a dual targeting CD4(1+2) BiTE that also inhibits coreceptor-binding of HIV gp120 potentially increased the safety of the CD4(1+2) BiTE, but also reduced its bioactivity *in vitro*.

As stated, the originally published dual-targeting construct CD4(1+2)L17b [147] was transferred into the BiTE context without further functional proof of the individual target-binding domains. Therefore, these results are preliminary and need to be further evaluated to conclude on the functionality of the 17b specificity and the simultaneous binding capability of CD4(1+2) and 17b in order to evaluate this dual-targeting BiTE approach. A reverse order in the binding moieties on the single polypeptide chain might have positive effects as seen by Quinlan et al. for CD4 and CCR5-mimicking peptides expressed on a single polypeptide chain [177]. Nevertheless, the gathered data so far suggests an impact on the BiTE stability and the CD3 affinity of the proprietary anti-CD3 $\epsilon$  scFv caused by the addition of the (G<sub>4</sub>S)<sub>8</sub> linker and/or the second target-binding domain (17b) itself. If an optimized version of this dual-targeting BiTE candidate does not show improved binding and cytotoxic activity, these impacts seem to be indispensable for an improved specificity and an enhanced neutralization of gp120 proteins by a dual-targeting CD4-based BiTE.

Next to the addition of an antibody moiety specific for the coreceptor-binding site, a CD4-based BiTE could also be linked to a gp41 neutralizing molecule like the oligopeptide T-20 [65]. Due to the potential auto-reactivity of MPER antibodies (2F5, 4E10 or 10E8), the addition of a gp41-binding peptide (like T-20) might be advantageous. This dual-targeting approach would fully neutralize the HIV env protein in binding to gp120 and gp41, thereby blocking membrane fusion.

Both target-binding approaches with scFv-based or CD4-based candidates will have their own limits. HIV gp120 neutralizing scFv-based BiTE antibody constructs usually experience a limit in breadth regarding the divergence of HIV viral env sequences. In case of the CD4(1+2) BiTE, it will be crucial to abolish BiTE-mediated infection of (CD8<sup>+</sup>) T cells. In case of the dual-targeting CD4(1+2) BiTE, the second targeting domain to the coreceptor-binding site or gp41 will most probably also be limited in breadth, which limits the complete gp120 neutralization capability of the CD4-based BiTE. However, the potential of dual-targeting might offer avidity effects in binding to HIV env. In addition, the individual binding moieties might complement each other in their specificity that might contribute to an increased breadth. Complete neutralization of gp120 molecules could potentially also be achieved by co-administration of individual coreceptor inhibitors along with the CD4(1+2) BiTE as part of a cART, which has to be shown *in vitro* first.

In a clinical setting, the BiTE treatment is likely to be performed in combination with a standard antiretroviral therapy, which should interfere with the viral spread and viral entry of HIV. The first choice for a BiTE seems to be a neutralizing scFv-based BiTE like B12 or VRC01, as they showed comparable HIV p24 levels as the irrelevant BiTE and no BiTE controls in the BiTE-mediated infection assay. The addition of a third binding domain in a BiTE antibody might decrease the bioactivity of the BiTE, as the preliminary results of CD4L17b $\alpha$ huCD3 suggested a factor 7 decrease in EC<sub>50</sub> when targeting recombinantly gp120 expressing CHO cells.

#### 5.4. PROOF OF CONCEPT FOR THE CD4(1+2) BiTE *IN VITRO*

Regardless of CD4(1+2) BiTE-mediated infection of T cells, the presented *in vitro* assays with HIV infected cells showed its significant antiviral activity. At concentrations higher than 100 ng/ml, the CD4(1+2) BiTE showed almost complete inhibition of viral replication when added to infected PBMCs and MDMs supplemented with autologous CD8<sup>+</sup> T cells.

Interestingly, the CD4(1+2) control BiTE specific for murine CD3 showed also antiviral activity at 10 µg/ml when incubated with HIV infected PBMCs. This antiviral effect of the CD4(1+2)αmurCD3 BiTE might be mediated by the binding of the CD4(1+2) domains to gp120 proteins and subsequent interference with the spread of infection (e.g. by mediating shedding of gp120 [178]). However, this potential neutralization effect of the CD4 control BiTE was not observed in the MDM setting. Here, the p24 level of the CD4(1+2)αmurCD3 sample showed the same HIV p24 range as the no BiTE control. The reason for this effect is unclear. In contrast to the HIV<sub>NL4-3</sub> CXCR4-tropic HIV strain used for the PBMCs, the HIV<sub>YU-2</sub> CCR5-tropic strain used to infect the MDMs might be less sensitive to soluble CD4(1+2) [179,180]. Furthermore, the exact budding process of HIV virions in macrophages is discussed controversially. Koppensteiner et al. described that virions bud into intracellular virus-containing compartments that are only temporarily connected to the plasma membrane [181]. However, Welsch et al. report that HIV virions bud mainly at the plasma membrane of primary macrophages [182]. Depending on the exact budding mechanism, the virions might not be accessible for the CD4(1+2) control BiTE antibody constructs. Furthermore, Sullivan et al. described enhanced infection when incubating COS-1 cells with HIV<sub>YU-2</sub> and sub-neutralizing concentrations of soluble CD4 [178]. This might have contributed to the increased HIV p24 values of the CD4(1+2) control BiTE compared to the lower HIV p24 values seen for the irrelevant target-binding BiTE candidate when incubated with infected MDMs.

Nevertheless, the CD4(1+2)αhuCD3 BiTE has shown its antiviral potential at concentrations above 100 ng/ml on infected PBMCs and infected MDMs supplied with stimulated CD8<sup>+</sup> T cells.

The administration of an irrelevant target-binding BiTE showed no antiviral effect in both settings even at high doses. This observation suggests the specificity of the antiviral activity of the CD4(1+2) BiTE in this context. Bearing in mind that Jurkat T cells were lysed target-independently at high CD4(1+2) BiTE concentrations *in vitro* (see Figure 35), high concentrations of the irrelevant target-binding BiTE could also have led to target-independent lysis of T cells when incubated with infected PBMCs. In such a case, the HIV p24 level would have remained low, because of less vital virus-replicating cells. Therefore, it remains questionable if this cell line phenomenon matters when targeting primary T cells. In addition, *in vitro* analysis of isolated autologous CD4<sup>+</sup> versus CD8<sup>+</sup> T cells revealed no obvious BiTE-dependent cytotoxic activity independently of prior T cell activation (see Figure 36), despite of their regular cytotoxic activity with BiTE on target-positive CHO cells (data not shown).

### 5.5. PROOF OF CONCEPT FOR THE CD4(1+2) BiTE *IN VIVO*

Unfortunately, the initially conducted *in vivo* experiment failed, in which the CD4(1+2) BiTE was tested in a humanized mouse model resembling a chronic HIV infection by using human CD34<sup>+</sup> stem cell engrafted and HIV infected mice [144]. The high variance in viral load and human cell engraftment within the cohorts, made it impossible to interpret the gathered results. In the second experiment, in which HIV infected human PBMCs were injected into NSG mice resembling an acute HIV infection, showed comparable viral loads and comparable human cell engraftments within the cohorts.

The determination of viral load at day seven post infection (p.i.) showed an insignificantly increased viral load in the BiTE treated cohort compared to the buffer control cohort. This unexpected result might be caused by the insignificantly elevated number of CD4<sup>+</sup> T cells determined in the BiTE treated group compared to the CD4<sup>+</sup> T cell number of the buffer control cohort. This higher number of CD4<sup>+</sup> T cells might be due to the presence of BiTE antibody constructs stimulating T cell proliferation in the presence of target as described by Kufer et al. [183]. The significantly higher level of CD8<sup>+</sup> T cells in the BiTE treated group at day 7 p.i., supports this theory. Since HIV replication is enhanced in activated T cells [184], this might have led to the higher viral load on day seven p.i. compared to the control animals. Besides T cell stimulation by the CD4(1+2) BiTE, the BiTE-mediated infection, which was provoked *in vitro* at a high BiTE concentration, potentially also played a role in the increased viral load on day seven p.i. As can be seen three days later, the viral load in the control cohort was significantly higher than for the BiTE treated cohort. The declining trend of the CD4<sup>+</sup>/CD3<sup>+</sup> subpopulation in combination with the significantly lower viral load at day 10 in the BiTE treated cohort, suggests the lysis of HIV infected CD4<sup>+</sup> T cells by the CD4(1+2) BiTE. In comparison, the buffer control cohort showed almost steady CD4<sup>+</sup> T cell levels over the whole duration. *In vitro*, high concentrations of the CD4(1+2) BiTE-mediated infection of CD8<sup>+</sup> T cells. *In vivo*, no decline of CD8<sup>+</sup> T cells was observed, being aware that a decline of CD8<sup>+</sup> T cells caused by the lysis of HIV-infected CD8<sup>+</sup> T cells, could have been balanced by proliferation of CD8<sup>+</sup> T cells. Interestingly, six days after the end of treatment the CD4<sup>+</sup>/CD3<sup>+</sup> cell level recovered to the level seen at day seven p.i. in the BiTE treated group, whereas the viral load dropped further likewise to the buffer cohort.

In a future experiment, the application of a neutralizing scFv-based BiTE candidate, which did not show mediated-infection of CD8<sup>+</sup> T cells *in vitro* could have shown an increased antiviral effect compared to the CD4(1+2) BiTE *in vivo*. Likewise, higher dosing and prolonged application of a BiTE could emphasize the antiviral activity and putative curative potential of BiTE antibody constructs *in vivo*. As a further control, the application of an irrelevant target-binding BiTE comprising the  $\alpha$ huCD3 scFv needs to proof the specific antiviral effect of an anti-HIV BiTE candidate in a parallel cohort. A chronic HIV infection model in human CD34<sup>+</sup> engrafted mice would be ideally suited to also show the BiTE efficacy in chronic disease, ideally in combination with cART.



If one wants to pursue the CD4-based BiTE, the CD4(1+2) BiTE could theoretically be tested in combination with a coreceptor inhibitor as part of a cART. A CD4-based dual-targeting candidate that fully neutralizes the HIV env could also be an alternative option.

In contrast to the first experiment using the CD34<sup>+</sup> mouse model, the pooling of CD34<sup>+</sup> cell donors seems to be a reasonable approach to balance engraftment levels and achieve comparable viral loads in the humanized mice. Moreover, the BiTE approach relies primarily on primed differentiated cytotoxic T-lymphocytes [94]. *Ex vivo* experiments of murine T cells revealed that the effector function of naïve T cells is limited and therefore these T cells cannot show the full potential of the BiTE technology (colleague Dr. Markus Muenz, personal communication). Thus, mice engrafted with human CD34<sup>+</sup> cells probably need to be pre-challenged before the actual experiment to prime the immune system. The establishment of an HIV infection in mice for the time span of two to three months, could be sufficient to prime the immune system, but should be confirmed in a pilot study before the actual experiment.

When the BiTE technology shows efficacy in combination with ART, it might be possible to avoid increased viral replication and subsequent viral spread by activated CD3<sup>+</sup> effector cells by simultaneously eradicating HIV infected cells.

## 5.6. BIOACTIVITY OF ABP-TAGGED CD4(1+2) BiTE ANTIBODY CONSTRUCTS *IN VITRO*

First, the bioactivity of ABP-tagged CD4(1+2) BiTE antibody constructs was tried to be determined without the influence of a relevant serum albumin in order to measure the intrinsic BiTE properties and their influence on the bioactivity. According to Dennis et al. [115] the hydrophobic SA21 ABP-tag has a high affinity to HSA and a low affinity to BSA. However, the presence of FCS was essential for the activity of the effector T cells *in vitro*. Therefore, the candidates could only be tested with 10% FCS for their nearly unbiased bioactivity.

When ranking the SA21-tagged BiTE antibody constructs according to their nearly unbiased bioactivity with 10% FCS, there was a clear trend that the C-terminally tagged BiTE antibody constructs showed the best bioactivity of all tested variants. The decrease in activity between the C-terminally tagged BiTE antibody constructs was very small as can be seen from the two to four-fold decline in activity compared to the parental CD4(1+2) BiTE. This means that the overall activity decrease independently of the linker insertion was very low for C-terminally tagged CD4(1+2) BiTE antibody constructs. Therefore, this data suggested that the C-terminal position to be ideally suited with respect to the nearly unbiased bioactivity in presence of 10% FCS.

Next to the almost unbiased bioactivity of the ABP-tagged BiTE antibody constructs, the BiTE antibody constructs were ranked according to their bioactivity in the presence of 50% human serum. In general, the physiological abundance of HSA in the serum is very high (34-54 g/l; [107]). The *in vitro* characterization of ABP-tagged CD4(1+2) BiTE antibody

constructs in presence of 50% human serum also displayed the best bioactivity (8-fold decrease) for the CD4(1+2) BiTE with the SA21-tag directly fused to its C-terminus.

In addition, the C-terminal position of the SA21-tag also theoretically seems to be favorable, since its location at the C-terminus is on the opposite side of the anti-CD3 scFv binding interface and distant from the target-binding interface. Therefore, the impact that can be caused at the C-terminal position theoretically seems to be the lowest possible within a BiTE. Locating the ABP-tag at the N-terminus or between the anti-target and anti-CD3 domain theoretically interferes with the first or second domain respectively.

The introduction of linkers between the SA21-tag and the C-terminus of the BiTE led to a linker-length dependent decrease in bioactivity. Similarly, the addition of a linker peptide did not increase the HSA affinity of the SA21-tag as can be seen from the SPR results of the BiTE antibody constructs. The insertion of a linker could provide more flexibility for the arrangement of the individual domains within the whole molecule, which might have negative impacts on the functionality of the SA21-tag, the anti-CD3 scFv or the CD4(1+2) domains (e.g., steric hindrance of the scFv and the ABP-tag leading to decreased bioactivity of the BiTE).

The BiTE antibody constructs with the SA21-tag located between the CD4(1+2) domains and the anti-CD3 scFv showed the highest decrease in bioactivity when tested in 50% human serum. The introduction of the G<sub>4</sub>S linker at both ends of the SA21-tag led to the best bioactivity of all internally tagged BiTE antibody constructs, displaying a 26-fold reduction in bioactivity compared to the parental BiTE.

For the N-terminal SA21-tag position, the introduction of a (G<sub>4</sub>S)<sub>3</sub> linker led to a higher bioactivity of the BiTE, despite no influence on the gp120 affinity compared to the directly fused N-terminal SA21-tagged BiTE. This suggests that the allowance of more flexibility between the CD4(1+2) and SA21-tag, can also positively affect the BiTE bioactivity. Since it is the N-terminal CD4 domain that is essential for gp120 binding, the respective epitope is located at the N-terminus of CD4 (AA 25-64). Therefore, the introduction of a linker could also be beneficial for targeting gp120 by shifting the hydrophobic SA21-tag aside from the interaction of CD4(1+2) with gp120.

Concerning the HSA affinity, the CD4(1+2) BiTE with the SA21 peptide directly fused to the N-terminus showed with 168 nM the best affinity of all candidates to HSA, followed by the candidate with the SA21-tag directly fused to the C-terminus (201 nM).

Of all ABP-tagged CD4(1+2) BiTE antibody constructs tested, irrespectively of the ABP-tag position and the linker length, the CD4(1+2) $\alpha$ huCD3SA21 candidate showed the best overall bioactivity *in vitro* in the presence of 50% human serum. Its average affinity to gp120 was 2.6 nM and 4.3 nM to human CD3 and therefore comparable to the affinity seen for the parental CD4(1+2) $\alpha$ huCD3. If this candidate with the best *in vitro* bioactivity also reveals a maximal prolonged half-life and best bioactivity *in vivo* has to be determined.

The presented results are based on the CD4(1+2) BiTE and do not necessarily apply to other canonical BiTE antibody constructs, since parallel observations using different (scFv-based) BiTE antibody constructs have shown advantages in N-terminal positioning of the SA21-tag for some BiTE antibody constructs (colleague Dr. Holger Dulat, personal communication).

### 5.7. TARGET CELL SPECIFICITY OF BiTE ANTIBODY CONSTRUCTS AT HIGH CONCENTRATIONS

As already tested by Traunecker et al. [84] T cells are able to kill cells of the same kind when using CD4-based bispecific antibody constructs. Due to the results of the *in vitro* characterization in this thesis, an effective BiTE concentration appears to begin in the high ng/ml to low µg/ml concentration for antiviral efficacy when using infected cells. Therefore, the dosing of BiTE antibody constructs might need to be higher as seen for the treatment of oncological diseases. As argued by Traunecker et al., the target-independent cytotoxic activity on uninfected Jurkat T cells at high concentrations of bispecific antibodies seems to be dimer-related. These Jurkat cell line related findings did not seem to be relevant when targeting primary T cells with the CD4(1+2) BiTE (as shown in Figure 36). Furthermore, when incubating HIV-infected PBMCs with an irrelevant target-binding BiTE *in vitro*, no unspecific antiviral effect was observed (see 4.7.2).

However, the general prevention of BiTE dimer formation will play a major role in the clinical application of BiTE antibody constructs in cases where high concentrations are needed *in vivo* or are needed in terms of storing the BiTE monomers after protein purification. Therefore, the stabilization of BiTE monomers will be a major step towards higher homogeneity at high BiTE concentrations and lower the risk of dimer-related side effects. The formation of BiTE dimers can happen at various points within the BiTE production and purification. Dimers can form during protein synthesis within the cells. In the course of the protein purification, these dimers need to be depleted to the highest possible degree by purification processes as already performed using SEC, ion exchange, etc. After purification of the BiTE monomers, the storage buffer and storage conditions do have a big impact on the BiTE monomer stability. Repetitive freeze and thaw processes, temperature or pH shifts can provoke the formation of dimers or BiTE aggregates, which can be balanced or exacerbated depending on the buffer conditions.

Denaturation and dimer formation of scFvs can also theoretically happen *in vitro* or *in vivo* by thermal fluctuation during close proximity of BiTE antibody constructs. Most variable domains have an intrinsic interdomain affinity between VH and VL of  $10^{-6}$  to  $10^{-8}$  M [185-187], which stabilizes the scFv monomer. Thus, a low interdomain affinity could be a basis for dimer formation. In order to achieve a stable BiTE monomer, which is more likely to be inert to dimer formation, there are two options:

1. Perpetuation of current BiTE format with screening for increased interdomain affinity scFv variants
  
2. Change of BiTE format:
  - a. Transfer of scFv into single chain Fab (scFab) molecules. This potentially offers higher stability, because of the fusion of constant domains to the variable domains and further stabilization by a disulfide bond between the constant domains [188]. The increased size of scFab BiTE antibody constructs might in addition lead to a prolonged half-life *in vivo*. However, the increased size also could interfere with tissue penetration and bioactivity.
  - b. The change to non-IgG scaffolds (e.g., anticalins [189]) could also minimize oligomerization, because of their provision of a stable protein backbone.
  - c. Usage of single domain binding proteins for targeting and/or CD3 binding (as evaluated with CD4 domain 1 candidates for target-binding or by the use of camel-derived single domain antibodies). On the downside, single binding domain antibodies in a BiTE context lead to smaller molecules and most likely provide a shorter half-life *in vivo*. On the upside single domain antibodies have shown less sensitivity in terms of oligomerization [190].

Since the current format seems to be stable at efficient doses when targeting primary cells and format changes might negatively affect the BiTE antibody constructs' potential, the development of BiTE antibody constructs in the current BiTE format should not be withheld. However, the testing and development of improvements of the current format or format changes addressing the potential to minimize undesirable side effects should be pursued in parallel to evolve the BiTE technology.

## 5.8. BiTE TREATMENT IN CHRONIC HIV INFECTION

The chronic infection status with HIV is multifaceted; there are reservoirs of latently HIV infected cells that show no viral replication. However, there is active viral replication of infected cells, which results in a base level of viral load throughout the course of the disease. The cART lowers the viral load in most cases below detection limit, but is not able to fully stop viral replication. Interestingly, although the virus is replicating, sequence alteration was not observed in patients under cART [166]. Besides the actively virus replicating cells, which could be targeted by BiTE antibody constructs, the latently infected cells, probably do not express viral proteins (e.g. env) in sufficient amounts. However, those cells could become points of active viral replication upon cell activation. In the presence of target (e.g. env), the binding of the BiTE antibody constructs to CD3 on T cells can activate the T cell as seen in the *in vitro* characterization when using unstimulated PBMCs for redirected lysis of HIV env<sup>+</sup> CHO cells. This activation might lead to active viral replication and subsequently to the redirected lysis of the infected cells. Infected macrophages, as they are CD3<sup>+</sup>, could also become activated as bystanders through cytokine release from activated T cells. As a result, the BiTE treatment could theoretically provoke active viral replication and subsequent lysis of infected cells without an additional activating substance. If the cell activation by BiTE is insufficient, the application of histone deacetylases inhibitors (HDACIs) might help to reactivate viral gene expression [191-193]. It was shown that HIV replication can be restored from latently infected patients under ART using HDACIs [194]. HDACIs are currently licensed for the treatment of oncological diseases.

The addition of cART based on coreceptor inhibitors, fusion inhibitors and protease inhibitors potentially minimizes or at best blocks viral spread during BiTE treatment, which otherwise might be caused by BiTE-dependent cell activation. The mentioned coreceptor-/fusion- and protease inhibitors seem to be ideally suited as co-medication for a BiTE treatment, as they do not interfere with viral replication in previously infected cells. Infected cells treated with these drugs would still be targetable via their HIV env expression. However, the cART would interfere with the spread of virus in blocking the maturation of virions and in blocking the viral entry of HIV virions into uninfected cells. The addition of non-competing neutralizing antibodies to HIV env that reduce the viral load and viral spread without competing with the HIV env epitope of the BiTE antibody constructs would also be a possible combination.

As shown recently [83], the targeting of gp120 on HIV infected cells is possible in a humanized mouse model in combination with ART by an antibody-based approach. This data suggests that the BiTE treatment has the potential to offer an additive antiviral effect when combined with ART and might enable the eradication of HIV infected cells.

## 6. SUMMARY

In 2012, over 35 million people were infected with HIV worldwide. The current standard of care is the combined antiretroviral therapy (cART). It can provide patients with an almost non-restricted life in lowering their viral load below detection limit, thereby enabling average life expectancy. On the downside, the available therapy is not curative. To cure HIV patients, all infected cells need to be eradicated. This could be accomplished using the bispecific T cell engaging (BiTE) technology, as BiTE antibody constructs have shown sustained depletion of cancer cells in patients.

Many broadly neutralizing antibodies to the HIV envelope protein (env), which is present on infected cells, have been identified. However, all suffer from limited breadth concerning the diverse HIV env sequences of circulating viral isolates and are therefore prone to viral escape. BiTE antibody constructs built from scFv of well-known monoclonal antibodies like B12 and VRC01 were compared to a new BiTE format using the natural HIV receptor CD4. By targeting HIV env with the essential domains of CD4, that BiTE has the broadest possible specificity and lowest risk of viral escape. Affinity, stability and cytotoxic activity measurements with recombinant HIV env showed no significant differences between scFv-based and CD4-based BiTE antibody constructs *in vitro* showing its successful application in the BiTE context.

The *in vitro* proof of concept for the CD4 domain 1+2 BiTE was shown by its significant antiviral activity when adding the BiTE to infected PBMCs as well as to infected macrophages supplemented with autologous CD8<sup>+</sup> effector cells. In an acute HIV infection mouse model, the CD4(1+2) BiTE lowered the viral load significantly compared to the buffer control cohort. Likewise, the number of CD4<sup>+</sup> T cells dropped during the BiTE treatment in contrast to the control displaying the cytotoxic activity of the CD4(1+2) BiTE *in vivo*.

Due to their small size, BiTE antibody constructs experience a short half-life *in vivo*. This offers a safety benefit that comes with decreased patient convenience, because of the required continuous supply of BiTE antibody constructs. Therefore, the CD4(1+2) BiTE molecule was extended for an albumin binding peptide (ABP) at diverse positions within the molecule and characterized *in vitro*. Overall, the direct C-terminal fusion of the ABP-tag to the CD4(1+2) BiTE antibody showed the best bioactivity of all tested variants *in vitro* in the presence of human serum.

In summary, antiviral activity of the BiTE technology using HIV infected cells was shown *in vitro* and in an acute infection model *in vivo*. CD4 as a non scFv-based target-binding protein was successfully transferred into the BiTE format and showed antiviral activity. The CD4-based BiTE antibody constructs, although efficacious *in vivo*, mediated the HIV infection of CD8<sup>+</sup> T cells *in vitro* that usually are no host cells of HIV. Interestingly, this effect was not observed for scFv-based BiTE antibody constructs based on antibodies B12 or VRC01.

In the future, it has to be shown if the BiTE treatment is also effective in a chronic HIV infection setting and ideally mediates a curative effect.

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## 8. ABBREVIATION INDEX

<b>Abbreviation</b>	<b>Explanation</b>
(v/v)	Volume per volume
°C	Degree Celsius
µg	Microgram
µl	Micro liter
41-19 LnPx	Macaque T cell line
<sup>51</sup> Cr	Radioactive chromium isotope
AA	Amino acid
ADC	Antibody drug conjugate
AIDS	Acquired immunodeficiency syndrome
AML	Acute myeloid leukemia
ART	Antiretroviral therapy
BiTE	Bispecific T cell engager BiTE <sup>®</sup> is a registered trademark of AMGEN Inc.
bnAb	Broadly neutralizing antibody
cART	Combined antiretroviral therapy
CCR5 Δ32	CCR5 gene mutation, deletion of a 32 aa fragment
CD	Cluster of differentiation
CD4 D1.1/D1.2	Engineered CD4 domain 1 constructs
CD4(1+2)	CD4 domain 1 and 2
CDC	US center for disease control
CHO	Chinese hamster ovarian carcinoma (cell line)
CO <sub>2</sub>	Carbon dioxide
CTL	Cytotoxic T-lymphocytes
DHFR	Dihydrofolate reductase
DSC	Differential Scanning Calorimetry
<i>E. coli</i>	<i>Escherichia coli</i>
E:T	Effector:target ratio
EC	Elite controllers
EC <sub>50</sub>	Corresponding BiTE concentration for half-maximal lysis of target cells
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
env	Envelope protein
EpCAM	Epithelial cell adhesion molecule
FACS	Fluorescent activated cell sorting or flow cytometry
FCS	Fetal calf serum
G <sub>4</sub> S/(G <sub>4</sub> S) <sub>3</sub>	Linking peptide of 4x glycine plus 1x serine or three repeats thereof
Gly	Glycine
gp120	HIV glycoprotein 120 or surface protein
gp140	Extracellular part of the HIV envelope protein
gp41	HIV glycoprotein 41 or transmembrane protein
HAART	Highly active antiretroviral therapy
HDACI	Histone deacetylase inhibitors
HEK	Human embryonic kidney cell line
HIV	Human immunodeficiency virus



<b>Abbreviation</b>	<b>Explanation</b>
HL/LH	Heavy→light / light→heavy chain orientation of variable domains
HPB-ALL	T cell line, derived from an acute lymphoblastic leukemia patient
HSA	Human serum albumin
HTLV	Human T-lymphotropic virus
huCD3	Human CD3
IMAC	Immobilized metal affinity chromatography
Jurkat	Human T cell line
kcal	Kilo calories
KD	Dissociation constant
L	Liter
LB	Lysogeny broth
LMW	Low molecular weight
LTR	Long terminal repeats
M	Molar
MDMs	Monocyte-derived macrophages
MHC	Major histo-compatibility complex
min	Minute(s)
ml	Milliliter
mM	Millimolar
MPER	membrane proximal external region (epitope on gp41)
murCD3	Murine CD3
ng	Nanogram
NK cell	Natural killer cell
nM	Nanomolar
NRTIs/NtRTIs/NNRTIs	Nucleoside, nucleotide or non-nucleoside RT inhibitors
NSG	NOD-SCID gamma
O <sub>2</sub>	Oxygen
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pg	Picogram
pM	Picomolar
PSMA	Prostate-specific membrane antigen
PVDF	Polyvinylidene fluoride
rHu	Relative humidity
Rpm	Rounds per minute
RT	Reverse transcriptase
scFv	Single chain fragment variable
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SEM	Standard error mean
SHIV	Chimeric simian/human immunodeficiency virus
SIV	Simian immunodeficiency virus
SPR	Surface plasmon resonance
TS <sub>50</sub>	Corresponding temperature at half-maximal FACS signal
VH/VL	Variable domain of the heavy/light chain of an immunoglobulin