Isolation and analysis of T cell receptors against a drug-selected secondary mutation in BCR-ABL for adoptive T cell therapy

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1. Summary

Adoptive T cell therapy with T cell receptor (TCR) engineered T cells is a promising approach for cancer treatment. Target antigens are categorized into tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). TAAs are overexpressed on cancer cells and often shared between different cancers, however, targeting TAAs has the risk of inducing autoimmune responses. In contrast to TAAs, TSAs are expressed exclusively on cancer cells, but only some of them are shared between patients. Since drug-selected secondary mutations in chronic myeloid leukemia (CML) are both cancer-specific and shared between patients, they become attractive targets. CML is a myeloproliferative disease. Over 95% of CML cases are associated with Philadelphia (Ph) chromosome t(9;22). Such abnormal chromosome results in the BCR-ABL hybrid gene that encodes a constantly active tyrosine kinase. Currently, tyrosine kinase inhibitors (TKIs) are used as the first-line treatment, as they effectively avoid uncontrollable cell division by blocking the ATP binding site of the kinase. Nevertheless, drug resistance is often observed due to drug-selected secondary mutations that prevent the TKIs from binding to the BCR-ABL protein. As relapsed CML patients with compound mutations are resistant to most or all TKIs, TCR gene therapy could benefit these patients.

In this project, we aimed to discover neoepitopes from drug-selected secondary mutations in CML and to generate neoepitope-specific TCRs for clinical use. ABabDII mice expressing a human TCR repertoire and HLA-A2 molecules were immunized with two selected mutant peptides and analyzed. Although the peptide comprising mutation T315I did not elicit immune responses, two specific TCRs against the peptide comprising mutation E255V (ABL-E255V) were isolated from ABabDII mice. The T9141 TCR exhibiting a superior avidity was further analyzed for off-target toxicity. As no allo- and cross-reactivities were detected, T9141 was constructed to express minimal murinized human TCR constant region (T9141-mmc) as in the clinic. The expression and the functionality of T9141-mmc were comparable to T9141. Furthermore, ABL-E255V was demonstrated to be endogenously processed and presented by the ABL-minigene-E255V-transduced cancer cell lines. Taking a step forward, we introduced the point mutation E255V into Ph+ CML cell lines to simulate the antigen expression of blast cells in CML patients. Although no T cell responses against the genome-edited BV173 cells were found, detectable T cell responses against the genome-edited K562-A2 cells were observed, suggesting that the induced T cell responses varied with the BCR-ABL expression level in CML cell lines. Accordingly, the potency of T9141 could differ among CML patients.

Therefore, it is recommended to analyze the BCR-ABL expression level in corresponding CML patients, which should allow us to evaluate the efficacy of TCR gene therapy with T9141. Taken together, T9141 is optimal for TCR gene therapy; ABL-E255V could be a potential target.

2. Zusammenfassung

Die adoptive T-Zell-Therapie mit T-Zell-Rezeptor (TZR)-gesteuerten T-Zellen ist ein vielversprechender Ansatz für Krebsbehandlungen. Die Zielantigene werden in tumorassoziierte Antigene (TAAs) und tumorspezifische Antigene (TSAs) eingeteilt. TAAs sind auf Krebszellen überexprimiert und werden häufig von verschiedenen Krebsarten gemeinsam genutzt, doch die gezielte Bekämpfung von TAAs birgt das Risiko, Autoimmunreaktionen auszulösen. Im Gegensatz zu TAAs werden TSAs ausschließlich auf Krebszellen exprimiert, aber nur einige von ihnen werden von Patienten geteilt. Da medikamentös selektierte Sekundärmutationen bei der chronischen myeloischen Leukämie (CML) krebsspezifisch sind und von vielen Patienten geteilt werden, werden sie zu attraktiven Zielen. CML ist eine myeloproliferative Erkrankung. Über 95 % der CML-Fälle sind mit dem Philadelphia-Chromosom (Ph) t(9;22) assoziiert. Das abnorme Chromosom führt zum BCR-ABL-Hybridgen, das eine ständig aktive Tyrosinkinase kodiert. Derzeit werden Tyrosinkinase-Inhibitoren (TKI) als Erstlinienbehandlung eingesetzt, da sie eine unkontrollierte Zellteilung verhindern, indem sie die ATP-Bindungsstelle der Kinase wirksam blockieren. Dennoch wird häufig eine Resistenz gegen das Medikament beobachtet, die auf sekundäre Mutationen zurückzuführen ist, die die TKIs daran hindern, an die ATP-Bindungsstelle zu binden. Da rezidivierte CML-Patienten mit Compound-Mutationen gegen die meisten oder alle TKIs resistent sind, könnte eine TZR-Gentherapie diesen Patienten helfen.

In diesem Projekt zielten wir darauf ab, Neoepitope aus medikamentös selektierten Sekundärmutationen bei CML zu entdecken und neoepitopspezifische TZRs für den klinischen Einsatz zu generieren. ABabDII-Mäuse, die ein menschliches TZR-Repertoire und HLA-A2-Moleküle exprimieren, wurden mit zwei ausgewählten mutierten Peptiden immunisiert und analysiert. Obwohl das Peptid mit der Mutation T315I keine Immunantwort auslöste, wurden zwei spezifische TZRs gegen das Peptid mit der Mutation E255V (ABL-E255V) aus ABabDII-Mäusen isoliert. Der TZR T9141, der eine höhere Avidität aufwies, wurde weiter auf Off-Target-Toxizität untersucht. Da keine Allo- und Kreuz-Reaktivitäten festgestellt wurden, wurde T9141 so konstruiert, dass er die minimale murinisierte konstante Region des menschlichen TZR (T9141-mmc) wie im klinischen Einsatz exprimiert. Die Expression und die Funktionalität von T9141-mmc waren mit T9141 vergleichbar. Darüber hinaus wurde gezeigt, dass ABL-E255V endogen prozessiert und von den mit dem ABL-E255V-Minigen transduzierten Krebszelllinien präsentiert wird. Wir sind einen Schritt weiter gegangen und haben die

Punktmutation E255V in Ph+ Leukämiezelllinien eingeführt, um die Antigen-expression von Blastenzellen bei CML-Patienten zu simulieren. Obwohl keine T-Zell-Reaktionen gegen die Genom-editierte Leukämiezelllinie BV173 gefunden wurden, wurden nachweisbare T-Zell-Reaktionen gegen die Genom-editierte Leukämiezelllinie K562-A2 beobachtet. Dies deutet darauf hin, dass sich die induzierten T-Zell-Reaktionen je nach BCR-ABL-Expressionsniveau der CML-Zelllinien unterscheiden. Dementsprechend könnte die Wirksamkeit von T9141 auch bei CML-Patienten unterschiedlich sein. Die Analyse des Expressionsniveaus von BCR-ABL bei entsprechenden CML-Patienten sollte es uns daher ermöglichen, die Wirksamkeit der TZR-Gentherapie mit T9141 zu bewerten. Insgesamt ist T9141 optimal für die TZR-Gentherapie geeignet; ABL-E255V könnte ein potenzielles Zielantigen sein.

3. Introduction

3.1 Immune system and T cells

The immune system of vertebrates can be divided into innate and adaptive immunity. Innate immunity defends hosts by non-specific mechanisms, such as anatomic and chemical barriers and invariant pattern recognition receptors from innate immune cells (Broz et al., 2013; Brubaker et al., 2015). Adaptive immunity, on the other hand, responds to infection by a diverse antigen receptor repertoire, which recognizes structures specific to individual pathogens. In adaptive immunity, B cells and T cells are the two major types of lymphocytes derived from hematopoietic stem cells of the bone marrow (Cooper et al., 1965). B cells mature in the bone marrow and produce immunoglobulins against extracellular pathogens (Ehlich et al., 1993; Löffert et al., 1994), whereas T cells mature in the thymus and recognize antigens presented on infected cells or antigen-presenting cells (APCs) with T cell receptors (TCRs) (von Boehmer et al., 1988). T cells fall into two subgroups according to the expression of the co-receptors CD8 and CD4, which assist the recognition of T cells to antigens presented by major histocompatibility complex (MHC) class I and II, respectively (Gao et al., 2000; Vignali et al., 1994). CD8 T cells are known as cytotoxic T lymphocytes (CTLs), which kill their target cells by inducing apoptosis through cytotoxic proteins or Fas-Fas ligand interaction (Nagata and Golstein, 1995). In contrast to CD8 T cells, CD4 T cells differentiate into several subsets of effector T cells that promote the activation of different immune cells such as B cells and CD8 T cells (Shiku et al., 1975).

3.1.1 The development of T cells

T cell precursors originate in the bone marrow and migrate to the thymus for development. In the thymus, progenitor cells receive Notch signaling from thymic epithelial cells and differentiate into different T cell lineages (Radtke et al., 2010). The major population is α:β lineage, in which only cells expressing functional TCRs and both the CD8 and CD4 co-receptors undergo positive and negative selection in the thymus cortex (Shortman et al., 1990). During positive selection, the double-positive cells (CD4+CD8+) that recognize antigen in combination with self-MHC molecules receive a signal to survive and mature into CD8 or CD4 single-positive cells, whereas cells that cannot do so will enter apoptosis (Kisielow et al., 1988; Klein et al., 2014). During negative selection, T cells that respond strongly to self-peptide:self-MHC complexes (pMHC) undergo apoptosis (Klein et al., 2014; Palmer et al., 2003), which is

driven by bone marrow-derived antigen-presenting cells and AIRE (autoimmune regulator) expressing cells in the thymic medulla (Anderson et al., 2002). Currently, how the recognition of pMHC complexes by TCRs results in positive or negative selection is still unclear, but the selection process is thought to relate to the binding affinity between TCRs and pMHC complexes, known as the affinity hypothesis (Ashton-Rickardt et al., 1994). Low-affinity bindings allow the cells to survive and lead to positive selection, whereas high-affinity bindings induce cell apoptosis as negative selection (Ashton-Rickardt et al., 1994; Klein et al., 2014). These selections ensure that the mature T cells are both self-MHC-restricted and self-tolerant.

3.1.2 The T cell receptor

During T cell development, the α chain and the β chain undergo a series of TCR gene rearrangements (Oettinger, 1999). The TCRβ locus located on chromosome 7 contains 54 variable (V), 2 diversity (D), 13 joining (J), and 2 constant (C) genes, whereas the TCRα locus located on chromosome 14 contains 47 Vα genes, 57 Jα genes and one Cα gene (Arden et al., 1995). Throughout the process of generating functional TCRs, VDJ genes of TCRβ recombine to create unique TCRβ chains, while VJ genes of TCRα recombine to generate TCRα chains (Arden et al., 1995; Oettinger, 1999). In addition to the recombination, deletion of nucleotides by exonucleases and random addition of nucleotides by terminal deoxynucleotidyl transferase at the junction between different genes create highly variable complementarity determining regions 3 (CDR3) that contribute to the specificity of each TCR chain and mostly contact peptides presented by MHC molecules (Gauss et al., 1996; Komori et al., 1993). In contrast to CDR3, CDR1, and CDR2 are encoded within the germ line V genes. They make up peripheral regions of the antigen-binding site of TCRs and mainly contact the MHC molecules (Flutter et al., 2004; Garboczi et al., 1996; Garcia et al., 1996). To be a functional TCR pair expressed on the cell surface, TCRα and TCRβ chains form a disulfide-linked heterodimer and interact with the CD3 complex and the ζ chains. Each CD3 complex and ζ chain carry intracellular domains with immunoreceptor tyrosine-based activation motifs (ITAMs) and contribute to signal transmission upon TCR engagement (Call et al. 2002; Love et al., 2010). After a series of TCR generation processes, a highly diverse TCR repertoire in humans is estimated to comprise 25×10^6 T cell clones (Arstila et al., 1999).

3.1.3 Antigen processing and presentation

To trigger T cell responses, antigens need to be processed from native proteins and presented by MHC molecules. At the beginning of processing, proteins are ubiquitinated in the cytosol and degraded by an ATP-dependent multi-catalytic protease complex called 26S proteasome (Schubert et al., 2000), which is a cylindrical complex composed of one 20S catalytic core that associates with two 19S regulatory caps on either end as a 19S-20S-19S structure (Kloetzel, 2001; Orlowski, 1990; Rock et al., 1994). The 20S catalytic core consists of four stacked rings, each containing seven units. The two outer α rings are composed of distinct α units and are non-catalytic. The two inner β rings are composed of distinct β units, in which the proteolytic subunits β1, β2, and β5 form the catalytic chamber. Since the 20S catalytic core is responsible for degrading proteins, the 19S regulatory caps bind to ubiquitinated proteins, unfold them and pull them inside the catalytic chamber of 20S. Upon induction of interferon γ (IFNγ), the three units of the catalytic chamber, β1, β2, and β5, can be replaced by alternative subunits LMP2, MECL-1 and LMP7 (Gaczynska et al., 1993; Groettrup et al., 1996). The proteasome carrying the alternative subunits is called immunoproteasome. It exhibits altered enzymatic specificity by increasing cleavage of polypeptides following hydrophobic residues and decreasing cleavage after acidic residues (Boes et al., 1994; Driscoll et al., 1993; Kuckelkorn et al., 1995). The produced peptides have C-terminal residues that are preferred for binding to MHC I molecules and structures that are preferred for transporting by transporter associated with antigen processing (TAP) (Müller et al., 1994; Schumacher et al., 1994). Furthermore, IFNγ can induce the expression of the PA28 proteasome-activator complex, which binds to either or both ends of the 20S catalytic core in place of the 19S regulatory caps. The PA28 can enhance the production of antigenic peptides by increasing the efflux of peptides from the proteasome (Sijts et al., 2002).

Antigenic peptides degraded in the cytosol are transported into the endoplasmic reticulum (ER) by the ATP-dependent heterodimeric TAP (Androlewicz et al., 1993). Peptides are further trimmed from N-terminus to obtain fragments in length of 8-12 amino acids by ER aminopeptidase (ERAP) in humans, as well as ER aminopeptidase associated with antigen processing (ERAAP) in mice (Li et al., 2019; Jurtz et al., 2017). To be presented on the cell surface, peptides need to be loaded onto the MHC molecules to form peptide:MHC complexes. The newly synthesized MHC class I α chain that contains three domains (α1, α2, and α3) is first folded and bound to a β2-microglobulin (β2m) (Bjorkman et al., 1987). Because of variable human leukocyte antigen (HLA) alleles, the α chain encoded in the MHC locus is highly

polymorphic, whereas β2m is conservative and encoded on a different chromosome (Robinson et al., 2015). Without binding to a peptide, the partly folded MHC I α: β2m dimer is retained within ER by associating with the MHC I peptide-loading complex (PLC), which is composed of calreticulin, tapasin, and ERp57 (Farmery et al., 2000; Hughes and Cresswell, 1998; Peaper et al., 2008). Upon the binding of a peptide, the completely folded peptide:MHC complex is released and transported through the Golgi apparatus to the cell surface. Due to the MHC I polymorphism, the MHC I alleles have different preferences for particular peptide sequences (Apanius et al., 1997; Falk et al., 1991). Once CD8 T cells recognize antigenic peptides presented by the MHC I molecules, they induce apoptosis of target cells by secretion of perforin and granzymes (Grossman et al., 2004; Trapani et al., 1993), as well as Fas-Fas ligand cell surface interaction (Hanabuchi et al., 1994; Nagata and Golstein, 1995).

3.2 Tumor antigens

Antigens expressed on cancer cells can be categorized into two groups, tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs). TSAs are encoded by mutant genes that are expressed exclusively on tumor cells, whereas TAAs are encoded by non-mutant cellular genes that are expressed not only on tumor cells but also on normal tissues.

The specific immune responses against TSAs were first described in a study of early tumor transplantation in mice (Gross, 1943). They found that the mice recovered from methylcholanthrene (MCA) induced sarcoma could reject the same tumor upon renewed challenge. A later study further indicated the specificity of tumor antigens, as the mice which were inoculated with MCA-induced sarcoma cells, once grew to a tumor, which was excised, could reject the same tumor cells on challenge but not other tumors (Prehn and Main, 1957). Since TSAs are expressed exclusively on tumor cells and are able to induce specific immune responses, they become attractive targets for cancer therapy. Although TSAs can be very specific in each individual, high-throughput sequencing from tumor biopsies in combination with the predicted binding affinity to HLA-types of corresponding patients can give the information of potential antigen candidates (Engels et al., 2013; Kammertoens and Blankenstein, 2013; Sette et al., 1994). In addition, there are some TSAs highly shared between patients, such as antigens derived from mutated Ras and p53 that carry point mutations (Parada et al., 1982; Hollstein et al., 1991), from frameshift TGF-β R2 that is caused by a deletion within the gene (Linnebacher et al., 2001; Saeterdal et al., 2001), from fusion proteins BCR-ABL and

TEL-AML1 that are generated by chromosomal translocation (Pinilla-Ibarz et al., 2000; Yotnda et al., 1998 Jul) and from virus-related tumors (Heslop et al., 2010; Kenter et al., 2009).

In contrast to TSAs, TAAs are overexpressed on malignant cells but also found on normal cells. Although TAAs are encoded from cellular genes, the expression is sometimes relatively low in normal tissues or is restricted to certain cell lineages. As they are expressed in patients with the same or different types of cancers, TAAs could be used as universal targets. According to the location of expression, TAAs are subdivided into different groups. One group is the so-called cancer-testis antigens that are expressed in cells of the testis which is immune privileged and hidden from the immune system (Fijak and Meinhardt, 2006). Many TAAs from this group are discovered and widely studied, such as MAGE (van der Bruggen et al., 1991), BAGE (Boël et al., 1995), and GAGE (Van den Eynde et al., 1995), as well as NY-ESO-1 (Chen et al., 1997). Another group is so-called differentiation antigens that are expressed specifically in certain cell lineages, such as CD19 on B lymphocytes (Miller and Maus, 2015), and MART-1/Melan-A on melanocytes (Kawakami et al., 1994; Coulie et al., 1994). Proteins that are overexpressed in cancer cells in comparison with normal tissues are categorized into one group, such as p53 (Theobald et al., 1995), Her2 (Ioannides et al., 1993) and WT1 (Oka et al., 2000). They are often related to oncogenesis by increasing cell growth, proliferation, and survival. Finally, antigens found on embryonic or fetal tissues are categorized into the group called oncofetal antigens. The well-known antigens are carcinoembryonic antigen (CEA) (Tsang et al., 1995), α-fetoprotein (AFP) (Butterfield et al., 1999), and 5T4 (Griffiths et al., 2005). However, they are found also in other adult tissues, such as AFP, which is produced at a low level by the liver of normal adults (Ball et al., 1992; Christiansen et al., 2001), and CEA, which is expressed in the normal intestinal tissue. The risk of on-target toxicity has been shown by a clinical trial targeting CEA which resulted in a severe intestinal autoimmune response (Johnson et al., 2009; Parkhurst et al., 2011). Nevertheless, TAAs are still considered to be potential targets for immunotherapy, as they are shared in different tumor types.

3.3 Adoptive T cell therapy

3.3.1 Unmodified T cells

As many studies have shown that tumor antigens can elicit specific T cell responses (see 3.2), adoptive T cell therapy (ATT) can be a promising approach for cancer treatment. The potential of ATT was first seen in stem cell transplantation for treating chronic myeloid leukemia (CML),

where T cells within the allogeneic donor graft eliminated the residual leukemic cells (Odom et al., 1978). Later, a clinical study showed that the transfer of donor leukocytes to treat CML patients who relapsed after transplantation could achieve durable remissions (Kolb et al., 1995). Tumor-infiltrating lymphocytes (TILs) are also widely studied for ATT, as they are found to be TAAs- or TSAs-specific in tumors. In the cases of treating metastatic melanoma, TILs were expanded *ex vivo* unspecifically or specifically under the stimulation of autologous tumor cells and transferred back to patients, which accomplished complete regressions in some patients (Dudley et al., 2005; Rosenberg et al., 2017). However, TILs discovered are often targeting TAAs. Because of the negative selection in the thymus, those T cells recognizing self-antigens have generally low avidities, which could lead to inefficient immune responses. In addition, ATT with TILs is rarely extended to non-melanoma cancers, which could be due to potentially lower immunogenicity of the other types of cancer (Fousek and Ahmed, 2015; Rosenberg et al., 2008; van den Berg et al., 2020). Nevertheless, some successful cases targeting TSAs in different types of cancer have been reported, such as colorectal cancer (Rosenberg et al., 2017), breast cancer (Zacharakis et al., 2018), and cholangiocarcinoma (Tran et al., 2014).

3.3.2 Chimeric antigen receptor engineered T cells

The idea of chimeric antigen receptors (CARs) that combine antibody-like specificity and the effect of T cells was first proposed in the late 1980s (Gross et al., 1989; Kuwana et al., 1987). CARs typically consist of an extracellular domain that is responsible for antigen recognition and one or multiple intracellular signaling domains that mediate T cell activation. The extracellular domain is an antigen-binding single chain variable fragment (scFv) composed of the variable heavy and the variable light chain of an antibody linked by a peptide spacer (Mullaney et al., 2001). The intracellular signaling domain has developed into three generations. First generation CARs contain CD3ζ alone, second generation CARs contain one co-stimulation domain linked to CD3ζ, whereas third generation CARs contain multiple co-stimulation domains together with CD3ζ. Although the therapeutic effect of the first generation CARs has been shown in several studies (Darcy et al., 2000; Haynes et al., 2001; Pinthus et al., 2003; Pinthus et al., 2004), the clinical results were not promising due to short persistence and poor proliferation of CAR engineered T cells *in vivo* that were caused by insufficient single signaling (Kershaw et al., 2006; Lamers et al., 2006; Park et al., 2007). To enhance the signaling, second generation CARs were designed to comprise a co-stimulation domain such as 4-1BB or CD28. Many studies have shown that the incorporation of 4-1BB or CD28 in CARs

increased the potency of engineered T cells, as the survival and proliferation were significantly improved (Eshhar et al., 2001; Finney et al., 1998; Friedmann-Morvinski et al., 2005; Imai et al., 2004; Maher et al., 2002). Additionally, the studies comparing first and second generation CARs have also shown that the survival rate of mice increased when the mice were treated with second generation CARs (Carpenito et al., 2009; Milone et al., 2009). Following these studies, many successful clinical trials of second generation CARs have been reported, including chronic lymphocytic leukemia (Kalos et al., 2011; Porter et al., 2011) and multiple myeloma (Garfall et al., 2015; Ormhøj et al., 2017). The most well-known example is the application of autologous CD19 CAR T cells in acute lymphoblastic leukemia (Maude et al., 2014; Maude et al., 2018; Parket al., 2018) and diffuse large B-cell lymphoma (Neelapu et al., 2017; Schuster et al., 2017), that has been approved by Food and Drug Administration (FDA) and is now commercially available. Nowadays, CARs are studied intensively to extend to different types of cancers. As scFv of CARs can recognize cell surface proteins, CAR T cell-mediated responses are independent of antigen processing and the types of HLA-alleles. Moreover, CARs recognize also other molecules, such as glycoproteins and glycolipids (Ponterio et al., 2020). These advantages enable CARs to be powerful tools in the field of ATT.

3.3.3 T cell receptor engineered T cells

TCR, a heterodimer composed of one TCRα and one TCRβ chain, recognizes antigens presented by the MHC molecules. In contrast to CARs that recognize natively folded proteins on the cell surface, TCRs recognize antigens that are both intracellular and secreted. This feature broadens the range of potential targets for TCR gene therapy. To obtain TCR engineered T cells, antigen-specific TCRs are introduced into autologous T cells, in which dimerized TCRα and TCRβ chains interact with endogenous CD3 complex to form a functional TCR that redirects the T cell specificity. Antigen-specific TCRs can be isolated from different sources. One of the sources is TILs. As they are often clonally expanded with tumor antigen specificity in tumor tissues, they are commonly used for isolating tumor-specific TCRs (Lu et al., 2021; Tran et al., 2016; Zacharakis et al., 2018). The other source is autologous or allogeneic T cells. The strategy is stimulating peripheral blood T cells in an antigen-specific manner *in vitro*, which leads to clonal expansion of antigen-specific T cells. The typical approach to stimulate T cells is to use antigenic peptide pulsed or cDNA transfected antigen-presenting cells (APCs), usually dendritic cells (DCs) (Liao et al., 2004; Strobel et al., 2000; Tsai et al., 1997; Yee et al., 2002), or established artificial APCs transduced to express various HLA alleles stably (Turtle and

Riddell, 2010). Furthermore, T cell stimulation with cell-free systems has been established, in which beads and nanoparticles linked with HLAs and costimulatory components were used (Butler and Hirano, 2014; Kim et al., 2004; Neal et al., 2017).

To overcome the requirements of generating mature APCs and establishing optimal conditions for T cell stimulation with artificial APCs and a cell-free system, humanized transgenic mice serve as an alternative TCR source. As most human TAAs are not present in mice, the transgenic mice immunized with human TAAs enable the isolation of TAA-specific TCRs with high avidity, whereas isolated human TCRs against human TAAs mostly have low avidity. Several human TAA-specific TCRs that were isolated from HLA-A2 transgenic mice have been reported like p53 (Cohen et al., 2005; Kuball et al., 2005), CEA (Parkhurst et al., 2009), and gp100 (Johnson et al., 2009). However, human T cells expressing murine TCRs that were developed from murine TCR genes can induce unexpected immune responses against patients (Davis et al., 2010; Johnson et al., 2009; Parkhurst et al., 2011). To circumvent this problem, ABabDII and ABabDR4 transgenic mice are engineered to express human TCRs, as well as human MHC I molecules (HLA-A2) or human MHC II molecules (HLA-DR4), respectively (Li et al., 2010; Poncette et al., 2019). The studies using humanized transgenic mice for TCR isolation have shown that the human TAA-specific TCRs from transgenic mice exhibited better avidities in comparison with TCRs from humans, such as TCRs specific to MAGE-A1 and NY-ESO-1 (Obenaus et al., 2015; Poncette et al., 2019). Furthermore, humanized transgenic mice have been also used for isolating TSA and viral-antigen-specific TCRs, including Merkel cell polyomavirus-encoded T antigens and TEL-AML (Gavvovidis et al., 2018; Popovic et al., 2011). These studies demonstrated that the humanized transgenic mice with a diverse human TCR repertoire could serve as a source of TCRs. However, potential off-target toxicity of TCRs that are isolated from mice should be a concern. On average, 20% of the protein-coding regions of the human and mouse genomes are not identical (Waterston et al., 2002). Accordingly, TCRs developed in the murine thymus may not be tolerant to certain human self-peptides. Moreover, TCRs from the transgenic mice are restricted to specific human HLA alleles. These facts could result in potential cross-reactivity and allo-reactivity of TCRs, leading to unspecific immune responses. Therefore, TCRs isolated from mice should be characterized carefully before further application.

3.4 BCR-ABL in chronic myeloid leukemia

3.4.1 Clinical overview of chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a myeloproliferative disorder. The hallmark of this disease is a reciprocal chromosomal translocation between chromosome 9 and 22, which leads to the formation of the Philadelphia chromosome (Ph) that comprises the *BCR-ABL* fusion gene (Rowley, 1973; Kurzrock et al., 1988). The encoded BCR-ABL protein, a hyperactive tyrosine kinase, is involved in uncontrollable cell proliferation, inhibition of apoptosis, altered cell adhesion, and growth factor independence (Chereda and Melo, 2015; Daley et al., 1990; Ren, 2005). Since more than 95% of CML patients have the Ph chromosome (Quintás-Cardama and Cortes, 2006), karyotyping is usually performed for confirming the diagnosis. Additionally, polymerase chain reaction (PCR) is also often used for screening the *BCR-ABL* gene.

Although several symptoms of CML have been described, such as anaemia, splenomegaly, and exhaustion (Quintás-Cardama and Cortes, 2006), most patients are asymptomatic and diagnosed by unrelated medical checks. In general, the duration of the chronic phase (CP) takes three to five years without treatment, and CML progresses subsequently to the blast phase (BP) via an accelerated phase (AP), which is defined by the blast count in the peripheral blood as of 10-20% in AP and of more than 20% in BP (Kantarjian et al., 1987). Once the patients are in the BP, the survival rate decreases significantly due to infection, thrombosis, and anaemia, which are caused by a block of cell differentiation and the massive infiltration with immature blasts (Ilaria, 2005).

3.4.2 BCR-ABL and its oncogenic activities

The Ph chromosome formed by a t(9;22) (q34;q11) chromosomal translocation is mainly associated with CML but also found in some cases of acute lymphoid leukemia (ALL). The *BCR-ABL* fusion gene in the Ph chromosome is composed of the 5’ end of *breakpoint cluster region* (*BCR*) gene and the 3’ end of *Abelson* gene (ABL) (Figure 1A). Because of various breakpoints of BCR and ABL, BCR-ABL fusion proteins with different molecular weights, that are of 190, 210, and 230 kDa, are generated based on the location of breakpoints and the mRNA splicing (Melo, 1997; Score et al., 2010). Three breakpoint cluster regions within the *BCR* gene have been defined as minor (m-bcr), major (M-bcr), and micro (μ-bcr) (Chereda and Melo, 2015). The m-bcr locates between exon 1 (e1) and 2 (e2) and the μ-bcr locates between exon

19 (e19) and 20 (e20), whereas M-bcr locates between exon 13 and 15 that are also named b2 and b4, respectively. In the majority of CML cases, the fusion usually involves b2 and b3 of the *BCR* gene and exon 2 (a2) of the *ABL* gene, which encodes the fusion protein p210 with either b2a2 or b2a3 junction. In contrast to CML, the fusion protein p190 with e1a2 junction is usually found in ALL patients (Melo, 1997; Faderl et al., 1999; Goldman and Melo, 2003).

BCR-ABL p210 fusion protein contains different protein domains (Figure 1B), in which the dimerization domain (coiled-coil), the Rho/GEF domain, and the Ser/Thr kinase domain are from BCR, and the tyrosine kinase domain (SH), the proline-rich domain (PxxP), the DNA-binding domain and the actin-binding domain are from ABL (Chereda and Melo, 2015). A normal ABL kinase is regulated by autoinhibition in different ways. Under the unstimulated condition, the SH3 domain interacting with the SH2 domain causes a conformational change on the N-terminus of the kinase, which avoids the binding of the substrates (Barilá and Superti-Furga, 1998; Goga et al., 1993). The other mechanism is myristoylation on the N-terminus of ABL kinase. By binding of myristoyl group to the pocket on the catalytic domain, the conformation of ABL kinase is altered and thus is able to contribute to autoinhibition (Hantschel, 2012). This explains the property of the constitutive activity of BCR-ABL fusion protein, since it lacks the N-terminus of ABL kinase and loses the ability of autoinhibition.

The activity of the BCR-ABL fusion protein involves several intra-molecule interactions for downstream signaling (Figure 1C). At the BCR part of the fusion protein, the dimerized coiled-coil domain on the N-terminus leads to autophosphorylation at tyrosine residue 177 of BCR, which facilitates the formation of the GRB2/GAB2/SOS complex that activates the PI3K/AKT and Ras/MEK pathways. At the ABL part of the fusion protein, constitutive auto-phosphorylation of the tyrosine kinase domain (SH) activates the JAK/STAT pathway. The three pathways, PI3K/AKT, Ras/MEK, and JAK/STAT, are considered to be the first line of signaling (Goldman and Melo, 2003). In the PI3K/AKT pathway, PI3K activated by the GRB2/GAB2/SOS complex transduces a signal to the downstream effector AKT to regulate the activation of transcriptional factors, which uplifts the cell growth, cell survival, and the cell death inhibition (Adams et al., 2012; Sattler et al., 2002). Several studies have indicated that the PI3K/AKT pathway is essential in CML, as the inhibition of PI3K interferes with the transformation of hematopoietic cells mediated by BCR-ABL, leading to the death of CML cells (Skorski et al., 1997; Klejman et al., 2002). In the Ras/MEK pathway, Ras is also activated by the GRB2/GAB2/SOS complex. The activated Ras-GTPase triggers a series of signaling

through MEK to induce the transcription of certain cell growth factor genes (Steelman et al., 2011; Puil et al., 1994). Some studies have demonstrated that the inhibition of Ras/MEK signaling can prevent the development of CML induced by BCR-ABL in mice (Sattler et al., 2002; Baum and Ren, 2008). The other key pathway deregulated in CML is the JAK/STAT pathway, which is correlated with cell growth, cell proliferation, and cell survival (Lin et al., 2000). STAT being intracellular transcription factors are activated primarily by membrane-receptor associated JAK (Hennighausen et al., 2008). However, STAT5 in CML can be activated not only by phosphorylated JAK2 (Samanta et al., 2011), but also by autophosphorylated SH domain of BCR-ABL (Hantschel et al., 2012). Previous studies have shown that the constitutive activity of BCR-ABL directly increases the signaling of the JAK/STAT pathway (Chai et al., 1997; Warsch et al., 2013). Moreover, STAT5 is essential for CML development, as STAT5-knockout CML mice cannot develop a CML phenotype in the presence of BCR-ABL (Hoelbl et al., 2010; Walz et al., 2012).

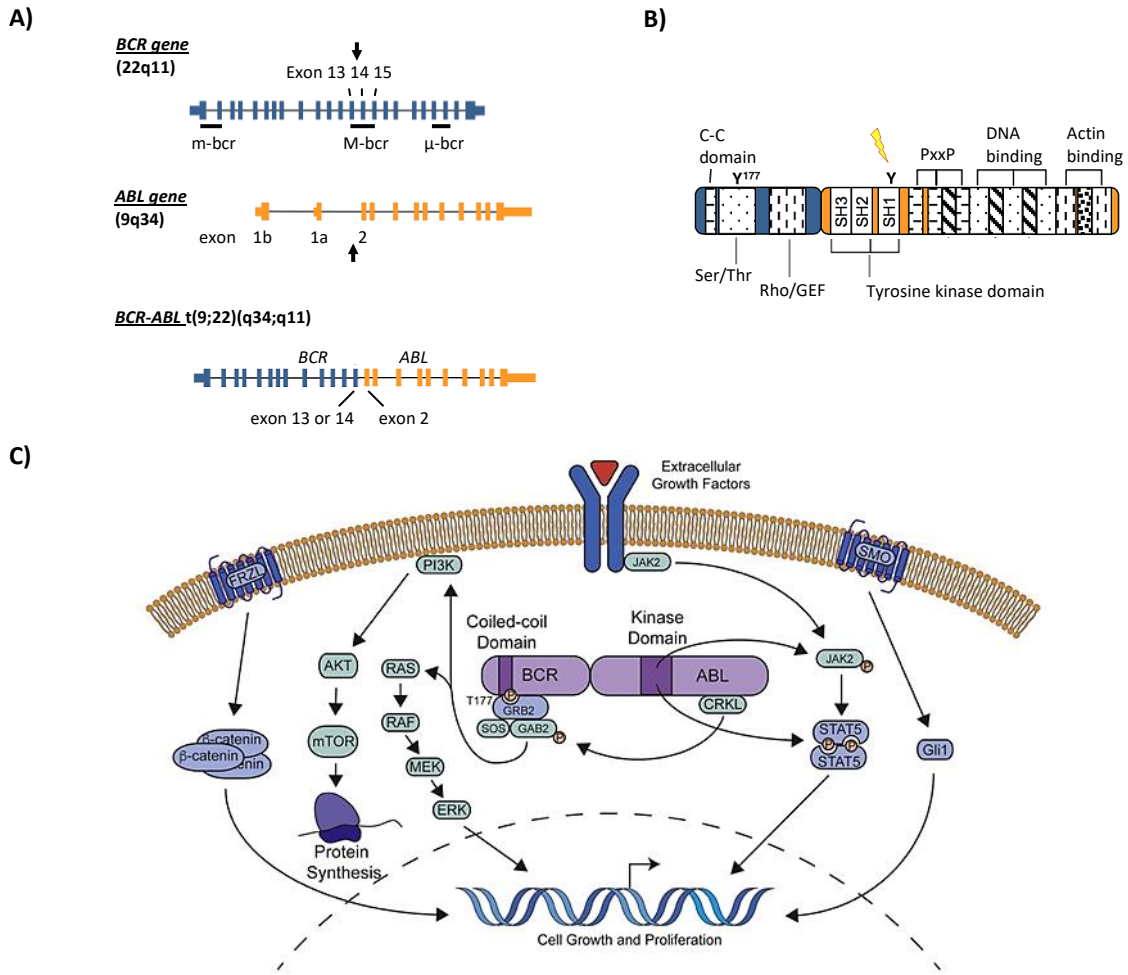


Figure 1. The gene, protein structure, and downstream signaling pathway of BCR-ABL. (A) The *BCR-ABL* fusion gene composed of the 5' end of the *BCR* gene and the 3' end of the *ABL* gene is a result of chromosomal

translocation t(9;22). The *BCR* gene contains three breakpoint cluster regions, minor (m-bcr, exon 1 -2), major (M-bcr, exon 13-15), and micro (μ-bcr, exon 19-20). In CML, the translocation usually involves M-bcr of the *BCR* gene and exon 2 of the *ABL gene* (Chereda and Melo, 2015). (B) The BCR-ABL fusion protein contains the dimerization domain (coiled-coil as C-C), the Ser/Thr kinase domain, and the Rho/GEF domain of BCR, as well as the tyrosine kinase domain (SH), the proline-rich domain (PxxP), the DNA-binding domain and the actin-binding domain of ABL (Chereda and Melo, 2015). (C) At the BCR part of the fusion protein, the dimerization of the C-C domain on N- terminus leads to autophosphorylation of tyrosine residue 177 in BCR, which facilitates the formation of the GRB2/GAB2/SOS complex that actives the PI3K/AKT and Ras/MEK pathways; at the ABL part, constitutive autophosphorylation of the tyrosine kinase domain (SH) activates the JAK/STAT pathway, where STAT5 is phosphorylated either by SH domain directly or by activated JAK2. The results of these downstream signaling pathways are cell growth and cell proliferation (Braun et al., 2020).

3.4.3 Secondary mutations in BCR-ABL selected by tyrosine kinase inhibitors (TKIs)

Allogeneic stem cell transplantation is a promising therapy for CML. Nevertheless, many patients are not suitable for this therapy due to a lack of a matched stem cell donor or advanced age (Goldman and Druker, 2001). Since BCR-ABL is required for the transformation of hematopoietic cells and is the key for the pathogenesis of CML, targeting BCR-ABL becomes favorable. Imatinib (also known as Glivec), formulated as methanesulfonate salt, has been developed as the first ATP-competitive inhibitor for BCR-ABL. The mechanism is that imatinib binds to the catalytic domain of ABL kinase to avoid ATP-dependent signaling, and thus, induces apoptosis of cells (Deininger et al., 1997; Druker et al., 1996). The treatment with imatinib is highly effective. More than 98% of CP-CML patients achieve a complete hematologic response, and 54% of patients display different levels of cytogenetic response (Drucker et al., 2001). Because of the high efficacy of imatinib, it is usually used as the first-line therapy in CML. Although imatinib brings remarkable success in treatment, approximately 25% of patients relapse due to the development of imatinib resistance (Marin et al., 2005). Several potential resistance mechanisms are described in previous studies, in which the occurrence of point mutations (also called secondary mutations) is found to be the most frequent case (Gorre and Sawyers, 2002; Gorre et al., 2001; Weisberg and Griffin, 2003). Currently, more than 80 different point mutations are reported to be predominately located in the tyrosine kinase domain of the BCR-ABL fusion protein and contribute to imatinib resistance (Baccarani et al., 2013).

Due to the emergence of the point mutations, second-generation TKIs have been developed including nilotinib and dasatinib. Nilotinib is a TKI functioning like imatinib but with a greater

potency (Weisberg et al., 2005; Golemovic et al., 2005). Clinical studies have demonstrated that nilotinib can target most imatinib-resistant point mutations like G250E, Q252H, F317L/C/V, etc. Nevertheless, nilotinib has only a limited effect on certain mutations, such as E255K/V and Y253F/H, and shows no impact on the most frequently occurring mutation T315I (Weisberg et al., 2005). The other second-generation TKI, dasatinib, is a multiple-tyrosine kinase inhibitor targeting BCR-ABL, Src kinase, etc. In comparison to imatinib and nilotinib which bind selectively to the inactivated form of ABL kinase, dasatinib binds to the activated conformation of ABL kinase by interacting with different amino acids (Schindler et al., 2000; Weisberg et al., 2006). Currently, dasatinib is recommended for treating CML patients who are imatinib-resistant. However, patients who relapse upon taking dasatinib are found frequently having V299L, F317L/V/I/C, as well as T315I mutation (Baccarani et al., 2013; Samimi et al., 2013). To target BCR-ABL with T315I mutation, a third generation TKI, ponatinib, was generated. It is a multi-tyrosine inhibitor like dasatinib, and it has great potency against BCR-ABL (Yang and Fu, 2015). Because of its special structure, ponatinib binds BCR-ABL by contacting several sites on the kinase, and thus, most point mutations including T315I are not able to prevent the binding from ponatinib (O'Hare et al., 2009). Presently, ponatinib is approved to treat CML patients and Ph+ ALL patients, who are resistant or intolerant to previous TKIs (Baccarani et al., 2013). Despite the efficacy of ponatinib, side effects with different severity levels are often observed, such as myelosuppression and cardiovascular events. Patients encountering side effects need to either reduce the dose or interrupt the therapy (Jain et al., 2015).

Currently, diagnosed CML patients are treated with suitable TKIs according to the sequencing results, and are suggested to change to different TKIs while showing resistance. A consequence of sequential TKI therapy is, however, found to be a clinical problem, since many patients end up acquiring more than one mutation, potentially conferring resistance to multiple TKIs (Shah et al., 2007). A previous study using ultra-deep sequencing has demonstrated that 50% of Ph+ CML patients receiving sequential TKI treatment carried compound mutations, in which 76% were double mutations, 21% were triple mutations and 3% were quadruple mutations (Soverini et al., 2013). Although ponatinib, a third generation TKI, is capable of targeting BCR-ABL that harbors certain mutations, the emergence of compound mutations becomes a challenge. For example, preclinical studies showed decreasing efficacy of ponatinib against certain double mutations (O'Hare et al., 2009), and an *in vitro* study demonstrated that compound mutations confer varying resistance to all available TKIs including ponatinib (Zabriskie et al., 2014).

Since TKI-resistant patients carrying compound mutations lack an available targeted therapy, developing new therapeutic strategies is necessary and urgent.

3.5 Aim of this thesis

As CML patients carrying compound mutations are resistant to most or all available TKIs (Zabriskie et al., 2014), TCR gene therapy targeting cancer-specific secondary mutations provides a highly specific treatment for these patients. In this thesis, we aimed to discover HLA-A2 restricted neoepitopes from secondary mutated BCR-ABL and to generate target-specific TCRs from transgenic ABabDII mice for clinical application.

3.5.1 Secondary mutated BCR-ABL as targets

HLA-A2 restricted binding affinity of the frequently occurring secondary mutations from BCR-ABL were evaluated with two prediction programs, the Immune Epitope Database (IEDB) with stabilized matrix method (SMM) (Peters et al., 2005) and the NetMHC 3.4 (Nielsen et al., 2003; Lundegaard et al., 2008). As the formation of stable pMHC complexes was essential for T cell recognition, peptides having affinity score (IC_{50}) lower than 100 nmol/L were presumed to be good binders and selected as candidates. Importantly, the parental peptide sequences of chosen targets were confirmed to be identical between human and mice, which could preclude potential cross-recognition of transgenic mice derived TCRs against wild type peptides. Once the candidate peptides had the ability to induce T cell responses, the processing of these peptides in cells was analyzed. In the present thesis, different human cancer cell lines naturally expressing HLA-A2 were transduced with the minigene encoding approximately 300 amino acids surrounding the target mutation. Moreover, we performed CRISPR-Cas9 system to introduce the point mutation into HLA-A2 expressing Ph+ leukemia cell lines, which could simulate the antigen expression of leukemic cells in CML patients. The results of a series of tests enabled us to evaluate the potential of candidate peptides in TCR gene therapy.

3.5.2 Generation of antigen-specific TCRs for TCR gene therapy

To generate antigen-specific TCRs, we immunized ABabDII mice (Li et al., 2010) with candidate peptides derived from secondary mutated BCR-ABL. Specific T cells of responsive mice were isolated for TCR identification. To test the potency of determined TCRs for clinical use, the specificity of the TCRs against candidate peptides was tested. As TCR avidity toward

pMHC complexes had a great impact on the result of therapy (Kammertoens and Blankenstein, 2013), TCR avidity was examined through functionality assay. To avoid potential mispairing with autologous TCRs, the TCR with optimal avidity was constructed with the human constant regions containing nine amino acids from counterparts in mice and a cystine-bridge referred as minimally murinized constant regions (mmc) (Sommermeyer and Uckert, 2010; Cohen et al. 2007). The expression and the functionality of the TCR-mmc were analyzed. Furthermore, off-target toxicity of the identified TCR was also tested to prevent unexpected immune responses in clinical applications.

4. Materials and methods

4.1 Materials

4.1.1 Mice

The transgenic ABabDII mice contain the entire human TCR α and β gene loci and a chimeric molecule *HLA-A2-H2-D^b* linked to human β2-microglobulin (*B2m*). These mice are deficient for mouse TCRαβ (*tcra* and *tcrb*), mouse *B2m*, and *H2-Db* genes. All animal experiments were approved by the German national institute LaGeSo (Landesamt für Gesundheit und Soziales) and performed in accordance with German animal welfare guidelines. All mice were bred and housed in the animal facility of Max-Delbrück-Center Berlin-Buch.

4.1.2 Cells

All cells were maintained at 37°C in 95% humidified air containing 5% CO_2. All medium were supplemented with 10% (v/v) heat-inactivated fetal calf serum (PAN Biotech) unless otherwise indicated.

Name	Description	Medium	Source
HEK-GALV	Human embryonic kidney cells expressing stably GALV-env and MLV-gag/pol	DMEM (Gibco)	**
T2	Human lymphoma cells defective in TAP	RPMI (Gibco)	ATCC
BV173	Human chronic myeloid leukemia cells	RPMI, 20% FCS	DSMZ
K562-A2	Human chronic myeloid leukemia cells (transduced with HLA-A2)	RPMI, 20% FCS	*
LB373.Mel	Human melanoma cells	HMC medium: DMEM, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, and 1× nonessential amino acids (all Gibco)	**
SK.Mel 37	Human melanoma cells	HMC medium	**
624.Mel 38	Human melanoma cells	HMC medium	**
MZ2.Mel 43	Human melanoma cells	HMC medium	**

LCLs	Lymphoblastoid cell lines	RPMI, 10% FCS, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, and 1× nonessential amino acids	**

* Research group of Prof. Uckert in Max-Delbrück-Center Berlin-Buch.

** Research group of Prof. Blankenstein in Max-Delbrück-Center Berlin-Buch.

4.1.3 Peptides

All peptides were purchased from GenScript as lyophilized and HPLC-purified products with >95% purity. Peptides were dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C. Peptides used in the alanine scan and the analysis of peptides with the TCR T9141-recognition motif are listed in appendix 1 and appendix 2, respectively.

Name	Gene	Sequence
ABL-E255V	ABL	KLGGGQYG**V** (position 247-255)
ABL-E255WT	ABL	KLGGGQYGE
ABL-T315I	ABL	YII**I**EFMTYGN (position 312-322)
ABL-T315WT	ABL	YIITEFMTYGN
MAGE-A1$_{278}$	MAGE-A1	KVLEYVIKV

4.1.4 Oligonucleotides

All oligonucleotides were purchased as lyophilized products and dissolved in ddH_2O for usage.

Oligonucleotides for SMARTer™ RACE cDNA amplification:

Primers	Sequence (5' to 3')	Source
hTRAC-R	CGGCCACTTTCAGGAGGAGGATTCGGAAC	Eurofins
hTRBC-R	CCGTAGAACTGGACTTGACAGCGGAAGTGG	Eurofins

Oligonucleotides for cloning of P2A-linked TCR chains (T9141-mmc):

Primers	Sequence (5' to 3')	Source
TRBV2_Not1-F	ATTGCGGCCGCCATGGATACCTGGCTCGTATGC	Eurofins
hTRBCco-R	GGGGGGGAACACGTTCTTCAGGTCCTC	Eurofins
hTRBCco-F	GAGGACCTGAAGAACGTGTTCCCCCCCG	Eurofins
P2Aco-R	GGGGCCAGGGTTCTCTTCCACGTCGCC	Eurofins
P2Aco-TRAV38-2-F	GAGAACCCTGGCCCCATGGCCTGCCCTGG	Eurofins

hTRAVco-start-R	CTGTCCCGCAGCTGGTACACGGCGGGGTC	Eurofins
hTRAVco-F	GACCCCGCCGTGTACCAGCTGCGGGACAG	Eurofins
hTRACco-end-EcoRI-R	CCCGAATTCATCAGCTGCTCCACAGCCGC	Eurofins

Oligonucleotides for cloning of ABL-minigene with E255V mutation:

Primers	**Sequence (5' to 3')**	**Source**
ABL-NotI-F	AAAGCGGCCGCCACCATGAAGCTGGTGGGCTGCAAAT	Eurofins
E255V-R	CCTCGTACAC<u>C**A**C</u>CCCGTACT	Eurofins
E255V-F	AGTACGGG<u>G**T**G</u>GTGTACGAGG	Eurofins
ABL-SalI-RR	CGCGTCGACCTATGACGAGATCTGAGTGGCCAT	Eurofins

Oligonucleotides for sequencing of genes:

Primers	**Sequence (5' to 3')**	**Source**
pMP71-F	CTGTCTGACTGTGTTTCTGTATTTGTCTG	Eurofins
pMP71-R	CCCCACCATTTTGTTTTATTAACATTT	Eurofins
IRES-R	GTGCGTCTAAGTTACGGGAAGG	Eurofins
BCR_exon 9-F	AGCTGCTGAAGGACAGCTTCATGGTG	Eurofins
ABL_a7-R	TGGAGAACTTGTTGTAGGCCAGGCTC	Eurofins
BCR_exon 9_inner-F	TGCTTCTCTGCACCAAGCTC	Eurofins
ABL_a7_inner-R	GCCAAAATCAGCTACCTTCACC	Eurofins
ABL-isoform a_F	ACCGAGCTGGGAGAGGGGTTC	Eurofins
ABL-isoform b_F	GGAACTCCTGCTTGCAAGTGTCAAC	Eurofins
ABL-CRISPR-outside-F	TGGCACCCACTGCATTGTTGCTTTC	Eurofins
ABL-CRISPR-outside-R	TGCAGAATTGGAACCCACTGGGGATG	Eurofins

Single strand oligo DNA for homology-directed repair (HDR):

Oligos	**Sequence (5' to 3')**	**Source**
Temp_ABL_ exon 4-130 (ssODN 3)	TGGAACGCACGGACATCACCATGAAGCACAAGCTGG GCGGGGGCCAGTACGGG<u>G**T**G</u>GTGTA**T**GAGGGCGTGT GGAAGAAATACAG**T**CTGACGGTGGCCGTGAAGACCT TGAAGGTAGGCTGGGACTG	Eurofins
Temp_ABL_ exon4-60r (ssODN 6)	CCCGGCAGTCCCAGCCTACCTTCAAGGTCTTCACGGC CACCGTCAGGCTGTATTTCTTCCACACGCC**T**TC**A**TA**G** AC<u>C**A**C</u>CCCGTACTGGCCCCCGCCCAGCTTGTGCTTCA TGGTGATGT	Eurofins

CRISPR RNA (crRNA) used in CRISPR-Cas9 engineering:

gRNA	Sequence (5' to 3')	Source
ABL_exon 4-130 (crRNA 3)	AGGTCTTCACGGCCACCGTCAGG	Integrated DNA Technologies
ABL_exon 4-60r (crRNA 6)	CAGTACGGGGAGGTGTACGAGGG	Integrated DNA Technologies

Antibodies and dextramer:

Specificity	Conjugate	Clone	Isotype	Host	Source
Mouse CD3ε	FITC	145-2C11	IgG	Armenian Hamster	Biolegend
Mouse CD8a	APC	53-6.7	IgG2a,κ	Rat	Biolegend
Mouse CD4	purified	RM4-5	IgG2a,κ	Rat	Biolegend
Mouse IFNγ	PE	XMG1.2	IgG1,κ	Rat	Biolegend
Mouse TCRβ chain	FITC, PE	H57-597	IgG2, λ1	Armenian Hamster	Biolegend
Human CD3	purified FITC	OKT3 HIT3α	IgG2a,κ IgG2a,κ	Mouse Mouse	Biolegend
Human CD8	APC, BV421, PE	SK1	IgG1,κ	Mouse	Biolegend
Human CD28	purified	CD28.2	IgG1,κ	Mouse	Biolegend
Human TCR Vβ22 (Human TRBV2)	PE	IMMU 546	IgG1	Mouse	Beckman Coulter
Human HLA-A2	APC	BB7.2	IgG2b,κ	Mouse	Biolegend
HLA-A2-KLGGGQYGV (dextramer)	APC, PE	-	-	-	Immudex

4.2 Methods

4.2.1 Immunization of ABabDII mice

For priming, ABabDII mice were injected subcutaneously with 100 nmol of peptide in a 200 µl 1:1 solution of incomplete Freund's adjuvant (Sigma-Aldrich) and PBS supplemented with 50 µg CpG. Successive boosts were performed with at least four weeks interval.

4.2.2 Detection of antigen specific CD8 T cell responses

CD8 T cell responses from immunized ABabDII mice were analyzed seven days after each boost. Blood samples or splenocytes were lysed by 1 or 3 ml ammonium-chloride-potassium lysing buffer (ACK buffer: 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 2 min followed by two washes with T cell medium (RPMI supplemented with 10% FCS, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 1× nonessential amino acids, and 1 mM HEPES). Cells were resuspended and plated in a tissue culture treated 96-well round bottom plate. For re-stimulation, cells were cultured with 10^{-6} M ABL-E255V or ABL-T315I peptide overnight. ABL-E255WT or ABL-T315WT was used as negative control and anti-mouse CD3/CD28 dynabeads (Invitrogen) as positive control. After 2 hrs incubation at 37°C, GolgiPlug (BD Cytofix/Cytoperm solution kit, BD biosciences) was added to all samples. After 12 hrs, intracellular cytokine staining was performed following the manufacturer's instruction. Cells were analyzed by flow cytometry.

4.2.3 *In vitro* expansion of antigen specific CD8 T cells from mice

Single cell suspensions were prepared from spleens and inguinal lymph nodes of responder ABabDII mice at day 10 to 12 after the last boost. After lysis of erythrocytes, cells were resuspended in T cell medium at a final concentration of 4×10^6 cells/ml. Subsequently, 20 U/ml IL2 and 10^{-9} M of peptides were added to the single cell suspension and 10 µl/ml anti-mouse CD4 (1 mg/ml) was added to block CD4 cells. Finally, the cell suspension was distributed in 24-well plates with 1.5 ml/well and maintained at 37°C in 95% humidified air containing 5% CO_2 for 10 to 12 days.

4.2.4 Mouse IFNγ secretion assay

To isolate antigen specific CD8 T cells, Mouse IFNγ Secretion Assay (Miltenyi) was carried out as follows. Cells were stimulated with 10^{-6} M peptides or anti-mouse CD3/28 dynabeads (Invitrogen) as positive control for 3 hrs. After three times washes with MACS buffer (PBS with 0.5% bovine serum albumin and 2 mM EDTA), cells were labelled by IFNγ Catch Reagent for 5 min and diluted by up to 50 ml warm T cell medium. Following incubation on a rotator at 37°C for 45 min, cells were washed twice and stained by IFNγ Detection Antibody and anti-mouse CD8 antibody. Prior to cell sorting by flow cytometry, 7-AAD (Biolegend) or SYTOX™

Blue (Thermo Fisher) was added to exclude dead cells. Labelled cells were sorted directly into RTL Plus Lysis Buffer (RNeasy Plus Micro Kit, Qiagen).

4.2.5 Isolation of TCRs

Total RNA from sorted cells was extracted using RNeasy Plus Micro kit (Qiagen) according to the manufacturer's instructions. To reverse transcript mRNA into 5'-rapid amplification of cDNA ends (RACE) ready cDNA, the reaction is performed with SMARTer™ RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. Subsequent TCR-specific amplification was carried out in a 50 µl reaction with 5 µl cDNA, 200 µM dNTP, 1x Phusion HF buffer, 1 U Phusion High-Fidelity DNA polymerase (Thermo Scientific), 1x universal primer A mix (SMARTer™ RACE cDNA amplification kit, Clontech) and 100 nM of either *hTRAC* (5'-CGG CCA CTT TCA GGA GGA GGA TTC GGA AC-3') or *hTRBC* (5'-CCG TAG AAC TGG ACT TGA CAG CGG AAG TGG-3') primers. Initial denaturation was performed at 98 °C for 2 min followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at 68 °C for 20 sec, and elongation at 72 °C for 45 sec. Final elongation was performed at 72 °C for 10 min. The amplicons were separated on a 1% agarose gel and extracted using a Zymoclean™ Gel DNA recovery kit (Zymo research). Purified DNA was cloned into TOPO vector using a Zero Blunt TOPO PCR Cloning Kit (Life Technologies) and used to transform competent *Escherichia coli*. Plasmids from individual clones were isolated and sequenced using T3 primer at Eurofins Genomics.

4.2.6 Generation of transgenes

The dominant TCR α and β chains per mouse were matched. All TCR transgene cassettes were codon-optimized for human expression and synthesized by GeneArt/Life Technologies. Pairing TCR α and β chains were linked with the porcine teschovirus-1 2A peptide (P2A) as described before (Leisegang et al., 2008). The human TCR constant regions were either completely replaced by the murine constant regions (Cohen et al., 2006), or modified by exchanging nine amino acids with their counterparts in the murine constant regions, as well as an additional cysteine bridge (TCR-mmc) (Cohen et al., 2007; Kuball et al., 2007; Sommermeyer and Uckert, 2010). The transgenes were cloned into pMP71-PRE (Schambach et al., 2000) using NotI and EcoRI restriction sites.

ABL-minigene-E255V cassette was constructed of a DNA fragment of size 1045 bp (position 19-1063 in open reading frame, ORF) comprising the E255V point mutation. The ABL-minigene-E255V was cloned out from the CML cell line BV173, and the E255V point mutation was introduced by replacing adenine with thymine in position 764 in ORF by using overlap extension PCR. For the cloning *STPG4* gene, a complete ORF of *STPG4* was synthesized by GeneArt/Life Technologies. Transgenes were cloned into pMP71 vectors, linked to a fluorescent reporter gene via an internal ribosomal entry site (IRES) by using NotI and SalI restriction sites. The transduced cancer cells were sorted based on GFP or mCherry expression.

4.2.7 Retroviral transduction

Transduction was carried out as described (Engels et al., 2003). Packaging 293-GALV cells (HEK-293 cells expressing stably GALV-env and MLV-gag/pol) were grown to approximately 70% confluence and transfected with 3 μg of plasmid carrying transgene in the presence of 10 μl Lipofectamine2000 (Life Technologies). Retrovirus containing supernatant was harvested 48 and 72 hours after transfection.

To transfer TCR gene, human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors by ficoll gradient centrifugation. A total of 1×10^6 freshly isolated or frozen hPBMCs per well were stimulated with anti-CD3 (OKT3) and anti-CD28 (CD28.2: BD Pharmingen)-coated 24-well plates in the presence of 300 U/ml recombinant human interleukin 2 (hIL-2, Peprotech). Transductions were done 48 and 72 hrs after stimulation by the addition of retrovirus containing supernatant and 4 μg/ml protamine sulfate followed by spinoculation for 90 min at 800 *g* and 32 °C (1st transduction) or preloading of virus onto retronectin (Takara)-coated plates and spinoculation for 30 min at 800 *g* and 32 °C (2nd transduction). Transduced T cells were kept in the presence of 300 U/ml hIL-2 for a total of 2 weeks followed by at least 2 days of culture in the presence of 30 U/ml hIL-2 before they were used for experiments.

To transfer ABL-minigene-E255V in cancer cell lines, a total of 1×10^5 cells were grown to approximately 60% confluence and transduced with retrovirus containing supernatant as described above. After transduction, cancer cells were cultured for 10 days for expansion and sorted by flow cytometry according to GFP or mCherry expression to gain a high purity of transduced cells.

4.2.8 Genome editing with CRISPR-Cas9 system

The CRISPR RNA (crRNA) targeting the *ABL* gene was designed using the CRISPOR program (Concordet and Haeussler, 2018). To prepare the guide RNA (gRNA) at a final concentration of 50 µM, an equal molar of crRNA and tracrRNA were annealed by heating at 95°C for 5 min. After cooling to RT, the formed gRNA and Alt-R HiFi Cas9 V3 protein (61 µM) were mixed to a final concentration of 5 µM and 4 µM, respectively. The mixture was incubated at RT for 20 min to form the ribonucleoprotein (RNP). During the incubation time, a total of 3×10^5 BV173 or K562-A2 cells were resuspended in 20 µl supplemented Nucleofector solution R (Lonza Bioscience). To prepare nucleofection, the cell suspension was mixed with 5 µl RNP, 1 µl of 100 µM single strand oligo DNA (ssODN), and 1 µl of 100 µM electroporation enhancer. The mixture was electroporated by using the DN-100 program of the 4D-Nucleofector device (Lonza Bioscience). The electroporated cells were immediately transferred to pre-warmed conditioned medium and cultured at 37°C in 95% humidified air containing 5% CO_2. All CRISPR-Cas9 components used here were purchased from Integrated DNA Technologies (IDT).

4.2.9 Co-culture experiments

In addition to the allo-reactivity assay using 2×10^4 effector cells and 2×10^4 target cells per well, all co-culture experiments were performed with 1×10^4 effector cells and 1×10^4 target cells per well in 96-well round bottom plates and cultured at 37°C in 95% humidified air containing 5% CO_2 for 24 hrs. Effector cells cultured with target cells without peptides or effector cells cultured alone served as negative controls, and effector cells cultured with 50 ng/ml phorbol-12-myristate 13-acetate (PMA) and 5 µg/ml ionomycin were used as positive control. Human IFNγ secretion in the supernatant was measured by ELISA (BD OptEIA) following the manufacturer's instruction.

For functionality assay, effector cells were cultured with T2 cells loaded with peptides in a range of 10^{-6} to 10^{-12} M. For alanine scan, effector cells were cultured with T2 cells loaded with either 10^{-5} or 10^{-8} M peptides (ABL-E255V-1A to 9A). Each peptide contained one amino acid replaced by alanine at one position of the sequence of ABL-E255V (Appendix 1). For human HLA-A2 self-peptide library scan, T2 cells were loaded with peptides at 10^{-5} M and cultured with effector cells. To titrate the peptides carrying the TCR T9141-recognition motif, T2 cells were loaded with peptides in a range of 10^{-5} to 10^{-9} M and cultured with effector cells. For

peptide processing assay, effector cells were cultured with the ABL-minigene E255V or the *STPG4*-transduced cancer cell lines. For allo-reactivity assay, effector cells were cultured with a panel of Epstein-Barr virus-transformed B lymphoblastoid cell lines (B-LCLs) expressing an abundance of HLA alleles (Appendix 3).

4.2.10 Quantitative PCR (q-PCR)

Total RNA was extracted from 1×10^6 cells using Quick-RNA™ miniprep kit (Zymo Research) according to the manufacturer's instruction. To synthesize cDNA, 100 ng total RNA were reverse transcribed using ProtoScript® II First Strand cDNA synthesis kit (BioLabs). The genes selected for Taqman® gene expression assay (ThermoFisher) were BCR-ABL (b2a2, Hs03024541_ft) and ABL (exon 3-4, Hs00245445_m1). ABL (exon 8-9, Hs01104728_m1) was used as an endogenous control. The qPCR was performed using QuantStudio3 Real-Time PCR system (ThermoFisher) and analyzed according to the comparative $2^{-\Delta\Delta Ct}$ Method.

4.2.11 *In silico* analysis of peptides

Peptide binding affinity to HLA-A2 molecules was predicted by NetMHC 3.4 (Nielsen et al., 2003; Lundegaard et al., 2008) and IEBD-SMM (Peters et al., 2005). The isolated TCRs were analyzed by IMGT tool - IMGT/V-QUEST (Brochet et al., 2008; Giudicelli et al., 2011). The screening of peptides carrying the identified TCR recognition motif in human peptidome was done by ScanProsite (de Castro et al., 2006).

4.2.12 Flow cytometry

Cells were analyzed by BD FACSCanto™ II (BD Biosciences) or MacsQuant (Miltenyi Biotech). Cell sorting was performed with BD FACSAria™ II or Fusion (BD Biosciences). FACS data analysis was performed using FlowJo (TreeStar).

5. Results

5.1 ABL-E255V but not ABL-T315I induced a specific immune response in ABabDII mice

Based on predicted binding affinities of frequently occurring secondary mutations, we selected two candidate peptides, ABL-E255V (KLGGGQYG**V**) and ABL-T315I (YII**I**EFMTYG), for short peptide immunization (Table 1). It was confirmed that the parental sequences of the two candidate peptides were identical between human and mice, avoiding potential cross-reactivity of isolated TCRs to wild type peptides (Figure 2A). ABabDII mice were boosted with four weeks intervals. The response of each immunized mouse was tested seven days after each boost by intracellular cytokine staining following *ex vivo* peptide re-stimulation of blood. As a result, specific CD8 T cell responses were observed in several ABL-E255V-immunized mice (Figure 2B), whereas no response was found in ABL-T315I-immunized mice (Data not shown).

5.2 ABL-E255V-specific TCRs were isolated from ABabDII mice

After ABL-E255V-specific CD8 T cell responses were detected, splenocytes from immune responding ABabDII mice were cultured *in vitro* with 10^{-9} M ABL-E255V peptide for 7 to 10 days to expand specific CD8 T cells. Prior to flow cytometry sorting, ABL-E255V-specific CD8 T cells were stimulated with 10^{-6} M ABL-E255V peptide for 3 hours and labelled by using mouse IFNγ secretion kit (Figure 2C). To identify TCRs, RT-PCR was carried out with RNA extracted from sorted CD8 T cells. Obtained cDNA was used for TCRα and β chain-specific 5'RACE PCR. After sequencing, dominant TCRα and β chains were matched and linked by P2A element for equimolar expression (Figure 3A). Moreover, human TCR constant regions were substituted with murine constant regions, enabling preferential pairing and avoiding mispairing with endogenous TCRs (Cohen et al., 2006). All constructed TCR gene cassettes were cloned into the retroviral vector pMP71 for re-expression. Finally, two TCRs were identified from two individual ABabDII mice (Table 2).

Table 1. Predicted binding affinity of secondary mutated BCR-ABL toward HLA-A2

Gene	Mutation	Sequence of peptide	NetMHC 3.4 IC_{50} (nM)		IEDB (SMM) IC_{50} (nM)	
			MUTANT	WT	Mutant	WT
ABL	E255V	KLGGGQYG**V**	11	17489	25	7206
ABL	T315I	YII**I**EFMTYG	1070	789	103	118

Table 2. V-J segments and CDR3 regions of functional TCRs

TCR	V-CDR3-J	Clone frequency
T9141	TRAV38-2 - CAYRSPQGGSEKLVF - TRAJ57	100%
	TRBV2 - CASSEWPPSSYNEQFF - TRBJ2-1	83%
T8922	TRAV39*01-CAVDGDDKIIF-TRAJ30*01	46%
	TRBV7-8*01-CASSFGPVYEQYF-TRBJ2-7*01	67%

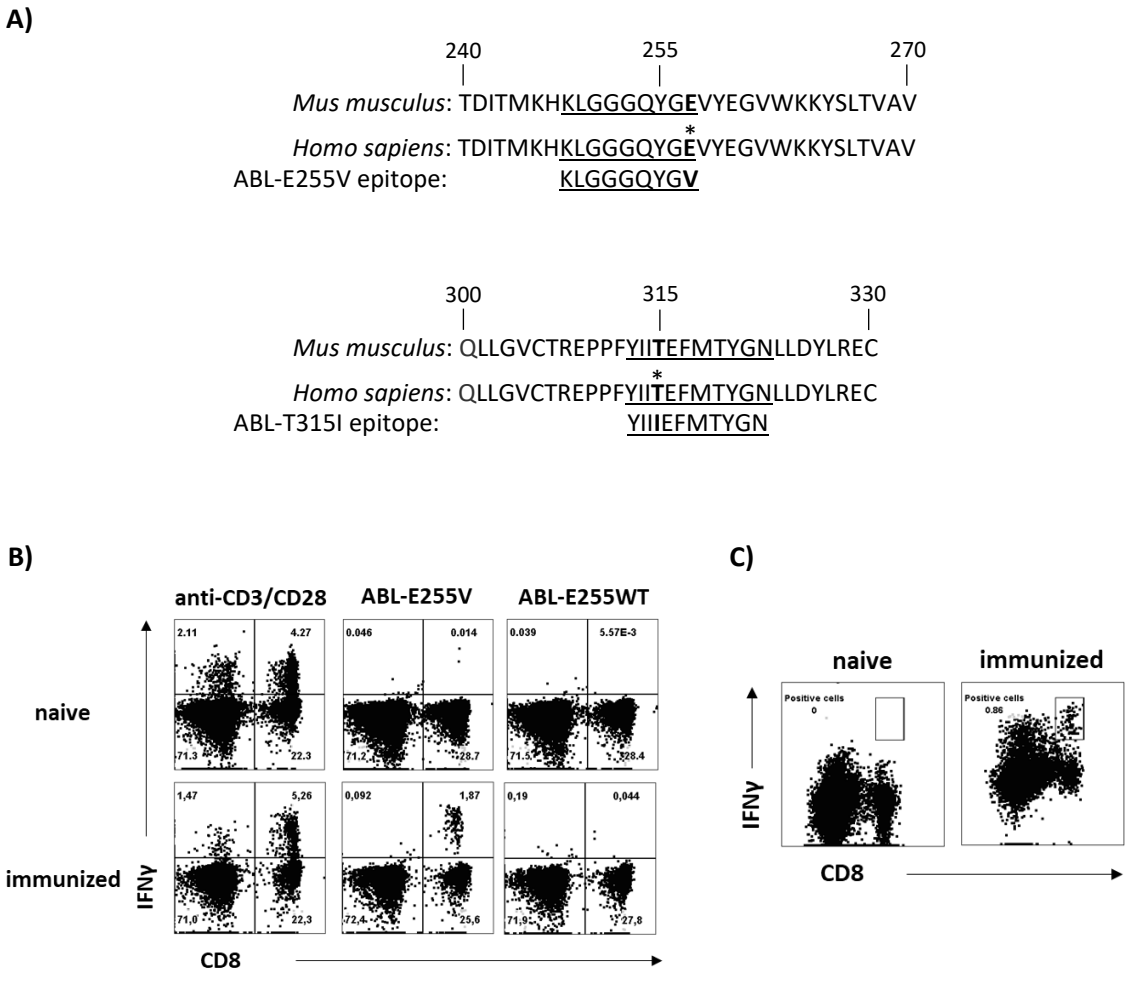


Figure 2. ABL-E255V but not ABL-T315I induced specific CD8 T cell responses in AB*ab*DII mice. (A) The sequence of ABL-E255V and ABL-T315I, and the parental sequence in human and mouse homolog. Epitopes are underlined. (B) Representative dot plots showing intracellular IFNγ staining as indicator of mouse CD8 T cell responses after peptide stimulation *in vitro* from unimmunized or ABL-E255V peptide immunized ABabDII mouse. Plots were gated on CD3 cells. (C) Representative dot plots showing clonal CD8 T cell expansion detected by IFNγ secretion assay following ABL-E255V peptide stimulation *in vitro* for 10 days. Plots were gated on CD3 cells.

5.3 ABL-E255V-specific TCRs exhibited different functional avidities

Human T cells were retrovirally transduced to re-express the paired TCRs (Figure 3B). To verify the specificity of isolated TCRs, TCR-transduced human T cells were co-cultured with T2 cells loaded with 10^{-6} M ABL-E255V or ABL-E255WT peptide. All isolated TCRs recognized ABL-E255V but not ABL-E255WT (Figure 3C). To further investigate the functionality of each ABL-E255V-specific TCR, we co-cultured TCR-transduced human T cells with T2 cells loaded with ABL-E255V in a range of 10^{-6} to 10^{-12} M. The two tested TCRs exhibited different functional avidities. T9141 responded to ABL-E255V up to 10^{-11} M and had a measured EC_{50} of 10^{-10} M, while T8922 responded up to 10^{-10} M and had a measured EC_{50} of 10^{-9} M (Figure 3D and E). Since T9141 exhibited a superior avidity, we cloned T9141 with codon-optimized human constant regions containing nine amino acids from the counterparts of the murine constant regions and an additional cysteine bridge (T9141-mmc) to minimize potential immunogenicity, as a version for clinical use. The expression level of TCRs was determined by dextramer, showing that the expression of T9141-mmc was slightly better than that of T9141 (Appendix 4A and B). In addition, the functionality assay showed that T9141-mmc achieved similar IFNγ production in response to different ABL-E255V peptide concentrations to T9141 (Appendix 4C and D), suggesting that the replaced mmc-constant region did not influence the TCR expression and the functionality. Taken together, the two isolated TCRs recognized ABL-E255V specifically and exhibited different avidities; T9141-mmc exhibited similar expression and functionality to T9141.

5.4 ABL-E255V was endogenously processed and presented in the ABL-minigene-E255V transduced cancer cell lines

Epitopes employed as targets for TCR gene therapy should be capable of eliciting a specific immune response and being endogenously processed and presented by cancer cells. Since a specific immune response induced by ABL-E255V was observed, we transduced three human HLA-A2+ melanoma cell lines, 624.Mel 38, SK.Mel 37 and LB373.Mel, and one human HLA-A2+ CML cell line, BV173, to test the processing of ABL-E255V epitope. As a negative control, the HLA-A2 negative human melanoma cell line, MZ2.Mel 43, was also transduced. The construct used for transduction was ABL-minigene-E255V, a fragment in the length of ca. 1 kb derived from *ABL* gene exon 1 to exon 6 with the point mutation E255V in exon 4 and linked to a GFP reporter gene by internal ribosome entry site (IRES) (Figure 4A). Expression of ABL-minigene-E255V was determined according to GFP by flow cytometry (Figure 4B).

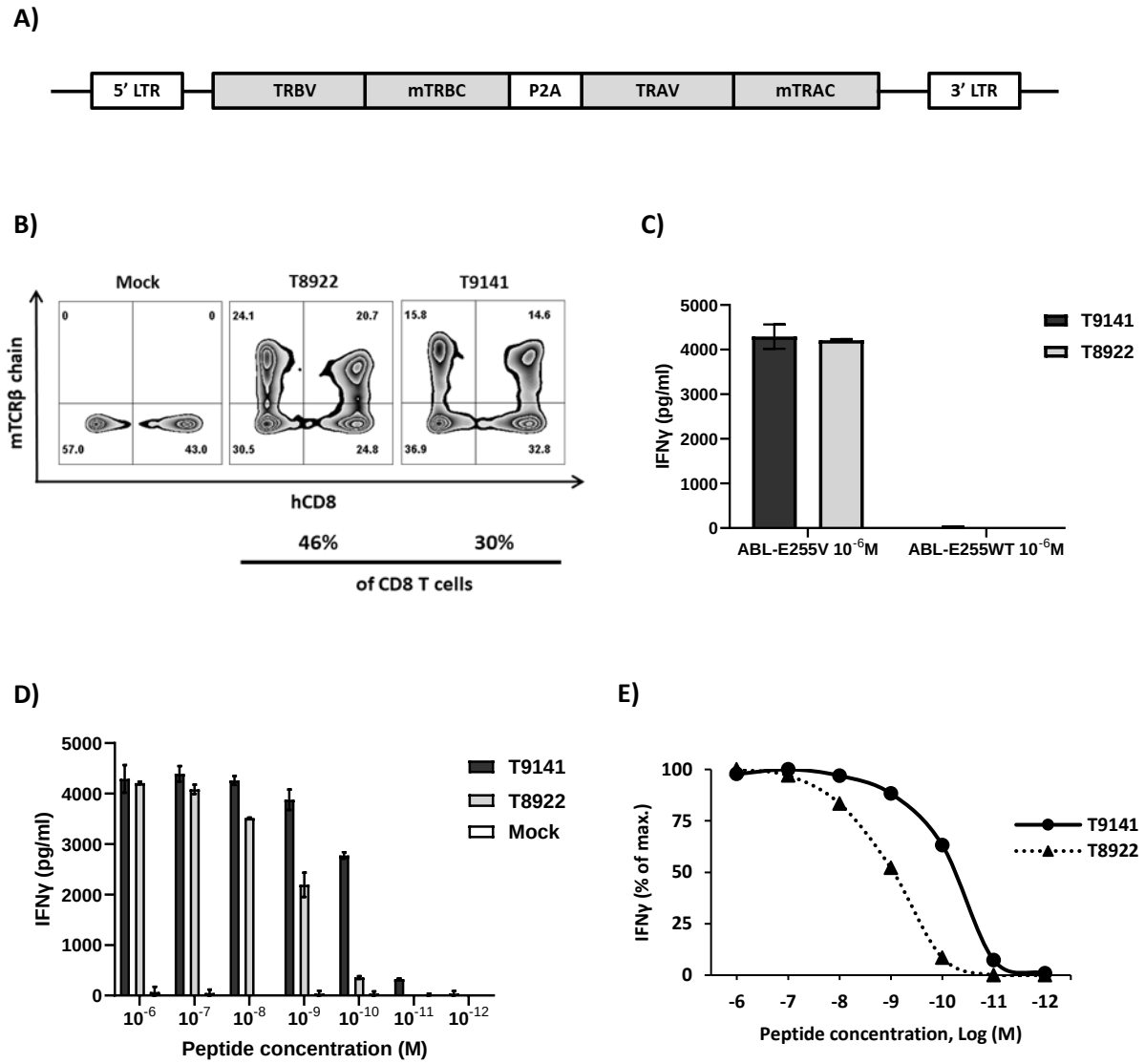


Figure 3. Specificity and functional avidity of ABL-E255V-specific TCRs derived from ABabDII mice. (A) Schematic of constructed TCR α and β chain linked by P2A cleavage element and flanked by 5' and 3' long terminal repeats (LTR) in pMP71 vector. Each TCR chain contains the murine constant region instead of the human constant region. (B) Representative plots showing CD8 and murine TCR β constant region (mTCRβ) staining as indicators of TCR-transduced human T cells. Numbers represent percentage of cells in the respective quadrant. (C) IFNγ production of T9141- or T8922-transduced human T cells after co-culturing with T2 cells loaded with ABL-E255V or ABL-E255WT peptide at 10^{-6} M. Mean values of duplicate cultures with standard deviations (SD) are shown. The results are representative of three independent experiments using PBMCs of different donors. (D and E) IFNγ production of T9141- or T8922-transduced human T cells after co-culturing with T2 cells loaded with ABL-E255V at 10^{-6} to 10^{-12} M. (D) Data shows absolute IFNγ production. Mean values of duplicate cultures with SD are shown. The results are representative of three independent experiments using PBMCs of different donors. (E) Responses of transduced human T cells were normalized to maximum IFNγ release.

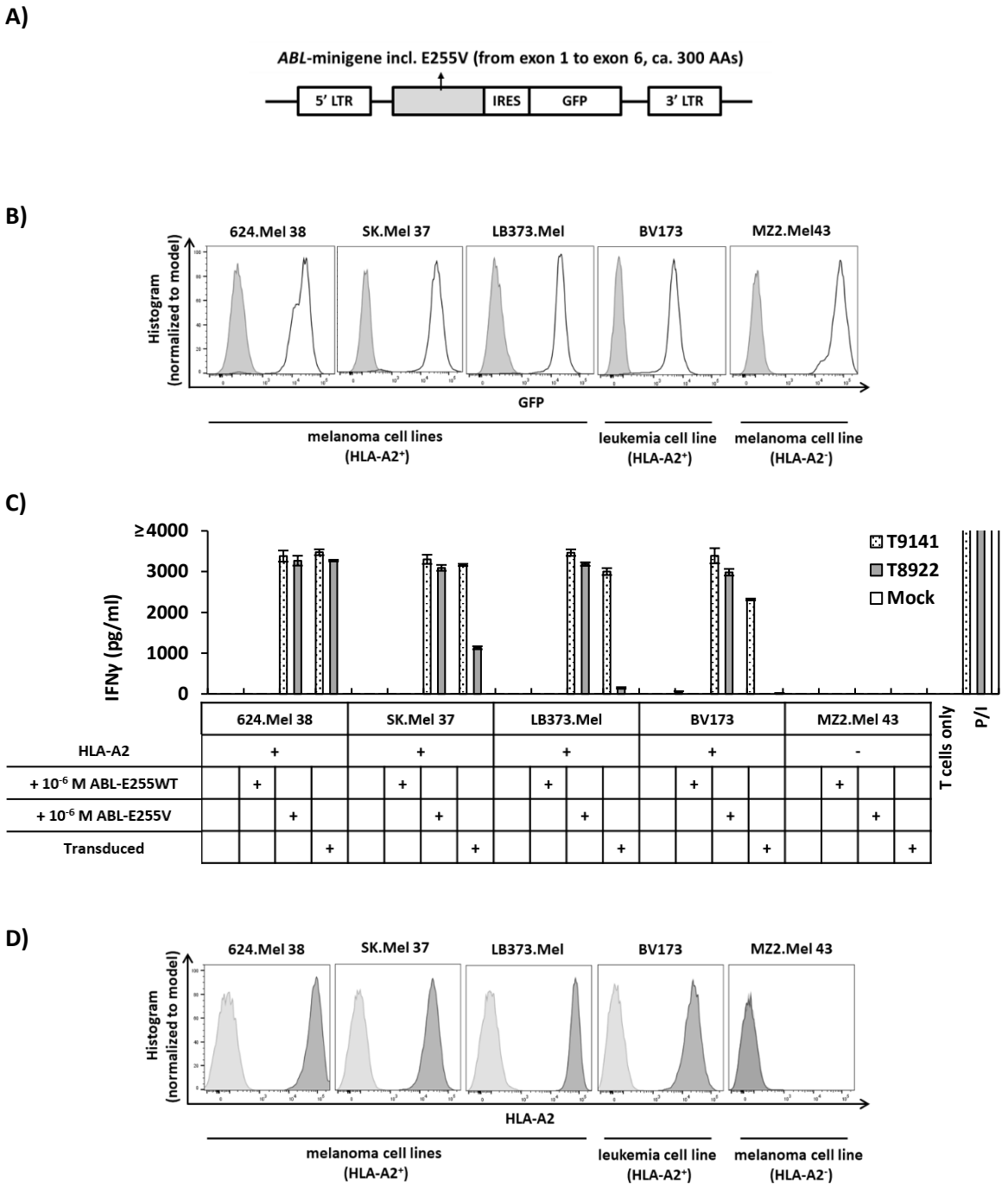


Figure 4. ABL-E255V epitope was endogenously processed. (A) Schematic of ABL-minigene-E255V linked to a GFP reporter gene by IRES and flanked by 5' and 3' LTR in pMP71 vector. (B) Expression of ABL-minigene-E255V in transduced 624.Mel 38 (HLA-A2$^+$), SK.Mel 37 (HLA-A2$^+$), LB373.Mel (HLA-A2$^+$), BV173 (HLA-A2$^+$) and MZ2.Mel 43 (HLA-A2$^-$) cell lines. Expression was determined by GFP. (C) IFNγ production of T9141- or T8922-transduced human T cells after co-culturing with ABL-minigene-E255V-transduced or untransduced cancer cell lines, as well as cancer cells loaded with 10^{-6} M ABL-E255V or ABL-E255WT. Unspecific T cell stimulation with PMA/ionomycin (P/I) was used as a positive control. Mean values of duplicate cultures with SD are shown. The results are representative of three independent experiments using PBMCs of different donors. (D) Expression of HLA-A2 of tested cancer cell lines. Expression was determined by HLA-A2 staining.

The processing and presentation of ABL-E255V epitope were tested by co-culturing TCR-transduced human T cells with ABL-minigene-E255V-transduced cancer cell lines. As a result, T9141-transduced T cells responded to all HLA-A2+/ABL-minigene-E255V+ cancer cell lines (Figure 4C), indicating that ABL-E255V could be endogenously processed and presented on the cell surface. The responses of T9141-transduced T cells to the three transduced melanoma cell lines were comparable with that to 10^{-6} M ABL-E255V-loaded cancer cells, whereas a lower response to transduced CML cell line, BV173, was detected. These results reflected that a lower level of ABL-E255V was presented by transduced BV173 that was detected to express one-log lower ABL-minigene-E255V in comparison with other transduced cancer cells, thereby inducing a weaker T cell response (Figure 4B and C). Despite a lower avidity of T8922, T8922-transduced T cells exhibited a strong response to transduced 624.Mel 38 and a significant level of IFNγ release to transduced SK.Mel 37. However, a weak and no recognition of T8922 to transduced LB373.Mel and BV173 pointed out again that T8922 was less sensitive when confronting ABL-E255V below a certain level. To note, the three transduced cancer cell lines, 624.Mel 38, SK.Mel 37 and LB.373.Mel, expressed similar levels of the ABL-minigene-E255V (Figure 4B), but elicited different responses of T8922-transduced T cells (Figure 4C). These data indicated that unequal amounts of ABL-E255V were presented by these transduced cancer cell lines, a result of different peptide processing efficiency in each cell line. Although HLA-A2 expression level on the cell surface could affect the magnitude of T cell response as well, it was not a concern here as each cancer cell line expressed HLA-A2 at a similar level (Figure 4D). Taken together, even though each cancer cell line showed different processing efficiency and different ABL-E255V expression level, ABL-E255V was endogenously processed and presented for TCR recognition.

5.5 Characterization of T9141 TCR

T9141 TCR with a superior functional avidity is selected as a potential candidate for clinical trials. As human HLAs are highly diverse, TCRs derived from HLA-mismatched donors could potentially cause off-target toxicity. In addition, approximately 20% of the protein-coding regions of the human and mouse genomes are not identical (Waterston et al., 2002). T9141 may not be tolerant of certain human self-peptides. To avoid such possibilities, T9141 was analyzed for allo-reactivity and cross-reactivity.

5.5.1 T9141 TCR exhibited no allo-reactivity

As ABabDII mice lack human MHC molecules other than HLA-A2, potential HLA allo-reactivity was investigated. We tested T9141-transduced T cells with a panel of Epstein-Barr virus-transformed B lymphoblastoid cell lines (B-LCLs) expressing diverse HLA molecules (Appendix 3). Although a minor background was observed from mock T cells, no allo-reactivity was detected from T9141-transduced T cells (Figure 5A).

5.5.2 T9141 TCR exhibited no cross-reactivity

5.5.2.1 No recognition was detected in human HLA-A2 self-peptide library scan

The murine thymus does not present the whole human peptidome, thus, the TCR repertoire in ABabDII mice can potentially cross-recognize certain human peptides. To investigate such cross-reactivity, we analyzed T9141-transduced T cells against T2 cells loaded with 114 known HLA-A2 restricted human self-peptide (Morgan et al., 2013) at a concentration of 10^{-5} M. No cross-reactivity was detected (Figure 5B).

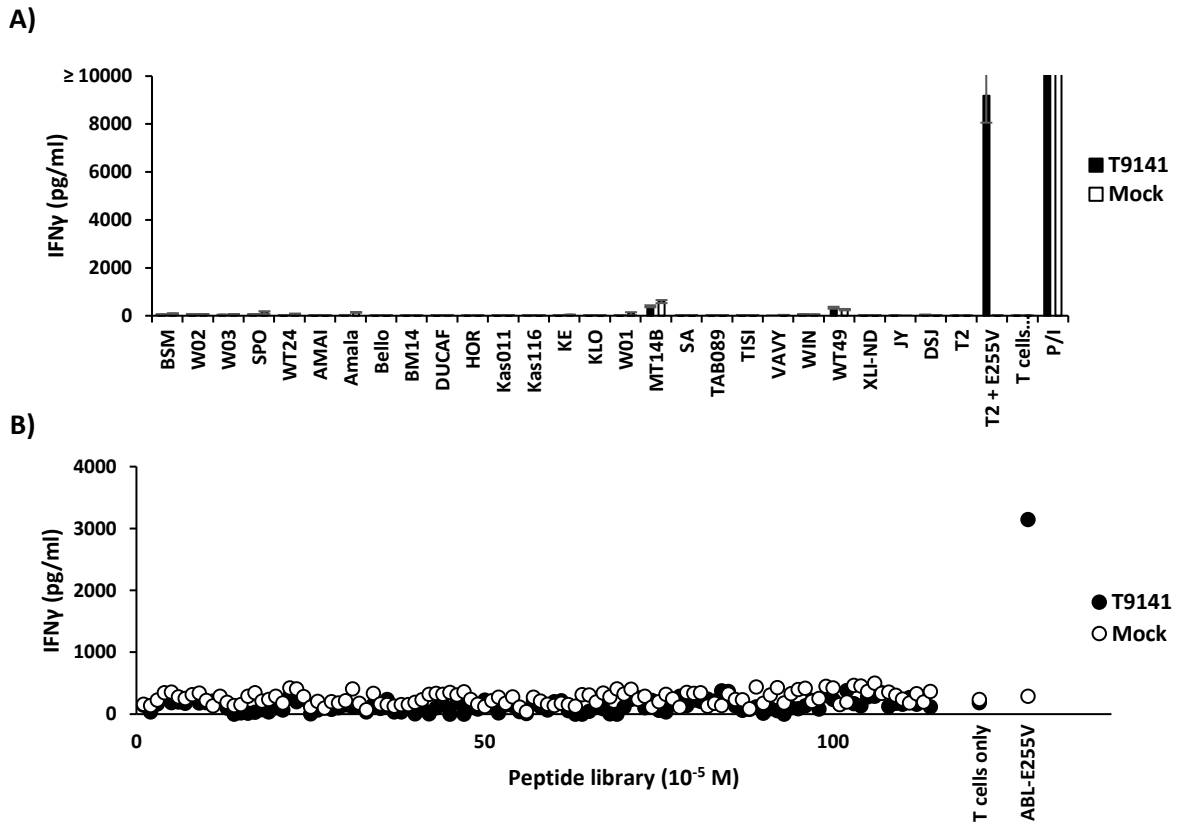


Figure 5. The ABabDII-derived TCR T9141 exhibited no allo-reactivity and no cross-reactivity to human self-peptides. (A) IFNγ production of T9141-transduced human T cells after co-culturing with a panel of B-LCLs expressing variable HLA allotypes (Appendix 3). Unspecific T cell stimulation with P/I was used as a positive

control. Mean values of duplicate cultures with SD are shown. The results are representative of two independent experiments using PBMCs of different donors. (B) IFNγ production of T9141-transduced human T cells after co-culturing with T2 cells loaded with 114 different HLA-A2-restricted human self-peptides or ABL-E255V at 10^{-5} M. T2 cells without peptide loading (W/O) was used as a negative control. The results are representative of two donors.

5.5.2.2 The STPG4 peptide containing the T9141-recognition motif was recognized only at high concentrations and was not endogenously processed

Certain human self-peptides carrying similar sequences to ABL-E255V could potentially induce cross-recognition. To identify the amino acids within ABL-E255V responsible for TCR recognition, the so-called TCR recognition motif, we employed alanine scan by replacing each amino acid of ABL-E255V with alanine (Appendix 1). Position 2, 5, 6, and 7 (x-L-x-x-G-Q-Y-x-x) of ABL-E255V were determined as essential for T9141 TCR recognition, when a threshold of 20% of the maximal IFNγ production was set as a minimal T cell response (Figure 6A). Following the analysis of the database ScanProsite (de Castro et al., 2006), we found 36 peptides within the human proteome carrying the same motif, that were not present in mice (Appendix 2). From the 36 peptides, eight peptides inducing responses of T9141-transduced T cells were identified as potential candidates for cross-reactivity, when a threshold was set at 250 pg/ml of IFNγ release (Figure 6B). The peptides eliciting T cell responses below the threshold were neglected, since they could elicit only very weak T cell responses at 10^{-5} M, a peptide concentration well above physiological levels. We further tested the T cell responses to the eight peptides with T9141-recognition motif by down-titrated peptide concentrations, in which the peptide derived from STPG4 protein (sperm-tail PG-rich repeat containing 4) was recognized by T9141-transduced T cells at a peptide concentration down to 10^{-8} M, when the other seven peptides were recognized at 10^{-5} M to 10^{-6} M (Figure 6C). As the seven peptides elicited T cell responses only at high peptide concentrations, they were unlikely to induce cross-reactivity of T9141 under physiological levels. Therefore, we focused on the role of the STPG4 peptide in the cross-reactivity of T9141. To testify, T9141-transduced T cells were co-cultured with full-length *STPG4*-transduced cancer cell lines, the same cancer cell lines used in section 5.4 (Figure 7A and B). No recognition was detected. Importantly, T9141-transduced T cells responded only to 624.Mel38 cells that were loaded externally with STPG4 at 10^{-4} M (Figure 7C), indicating that STPG4 peptide could not compete HLA-A2 with other endogenous peptides for presentation at physiological levels. Taken together, no cross-reactivity of T9141 was detected.

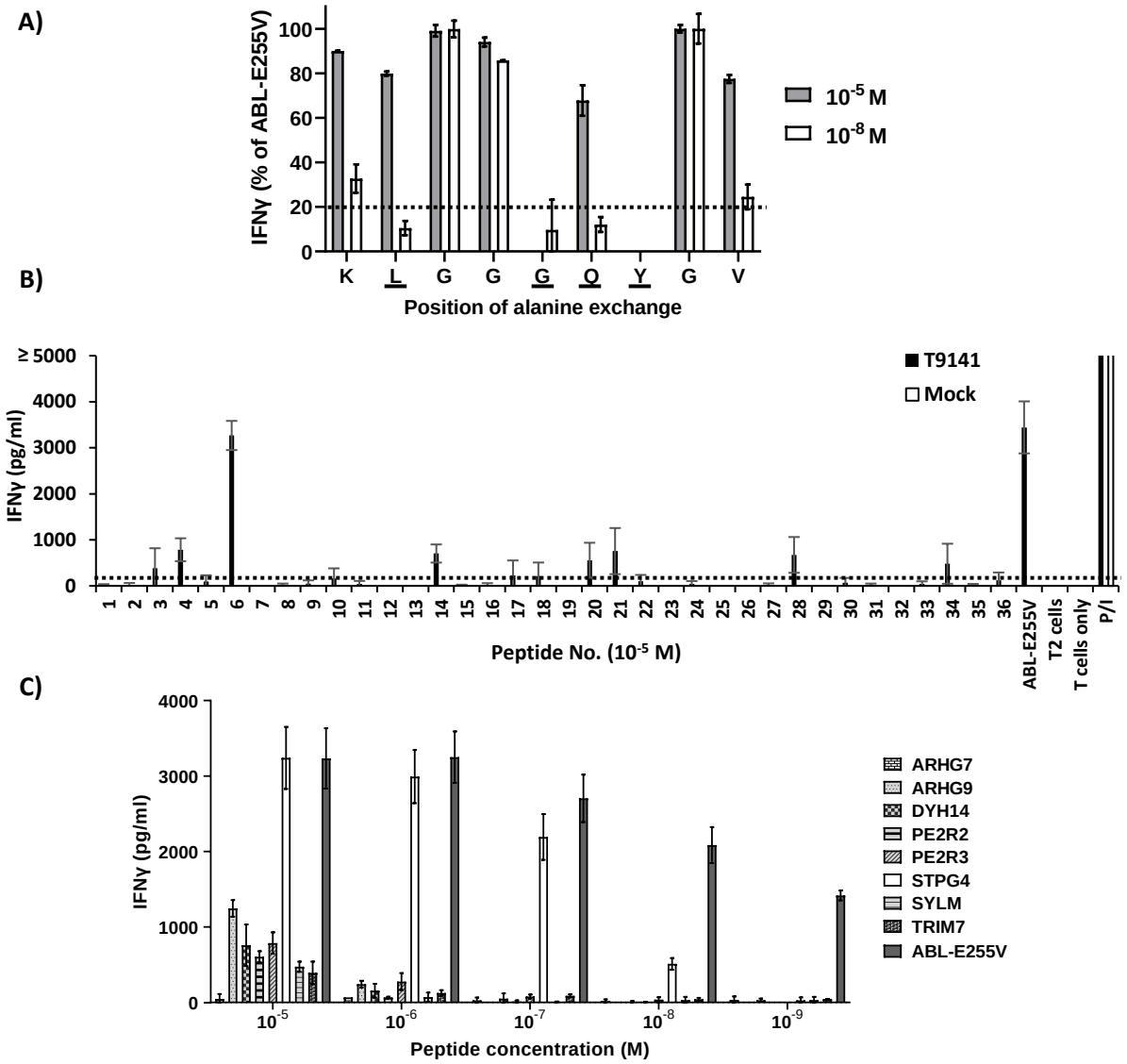


Figure 6. Part of the peptides containing the T9141-recognition motif was recognized by T9141 TCR but only at high peptide concentrations. (A) IFNγ production of T9141-transduced human T cells after co-culturing with T2 cells loaded with nine different peptides at either 10^{-5} or 10^{-8} M. Each peptide contains one alanine substitution sequentially from ABL-E255V (Appendix 1). Data were normalized to IFNγ production in response to ABL-E255V peptide. The dash line indicates a threshold of 20% of IFNγ production. Underlined amino acids indicate the T9141 recognition motif. Mean values of duplicate cultures with SD are shown. The results are representative of three independent experiments using PBMCs of different donors. (B) IFNγ production of T9141-transduced human PBMCs after co-culturing with T2 cells loaded with 36 peptides containing the T9141 recognition motif (x-L-x-x-G-Q-Y-x-x) at 10^{-5} M. T cell stimulation with ABL-E255V and P/I were used as a parameter and a positive control, respectively. The 36 peptides carrying the T9141 recognition motif are listed in appendix 2. The dash line indicates a threshold of 250 pg/ml of IFNγ production. The diagram shows mean values of two independent experiments using PBMCs of two donors with SD. (C) IFNγ production of T9141-transduced human PBMCs after co-culturing with T2 cells loaded with eight different peptides with down-titrated peptide concentrations. The diagram shows mean values of two independent experiments with SD.

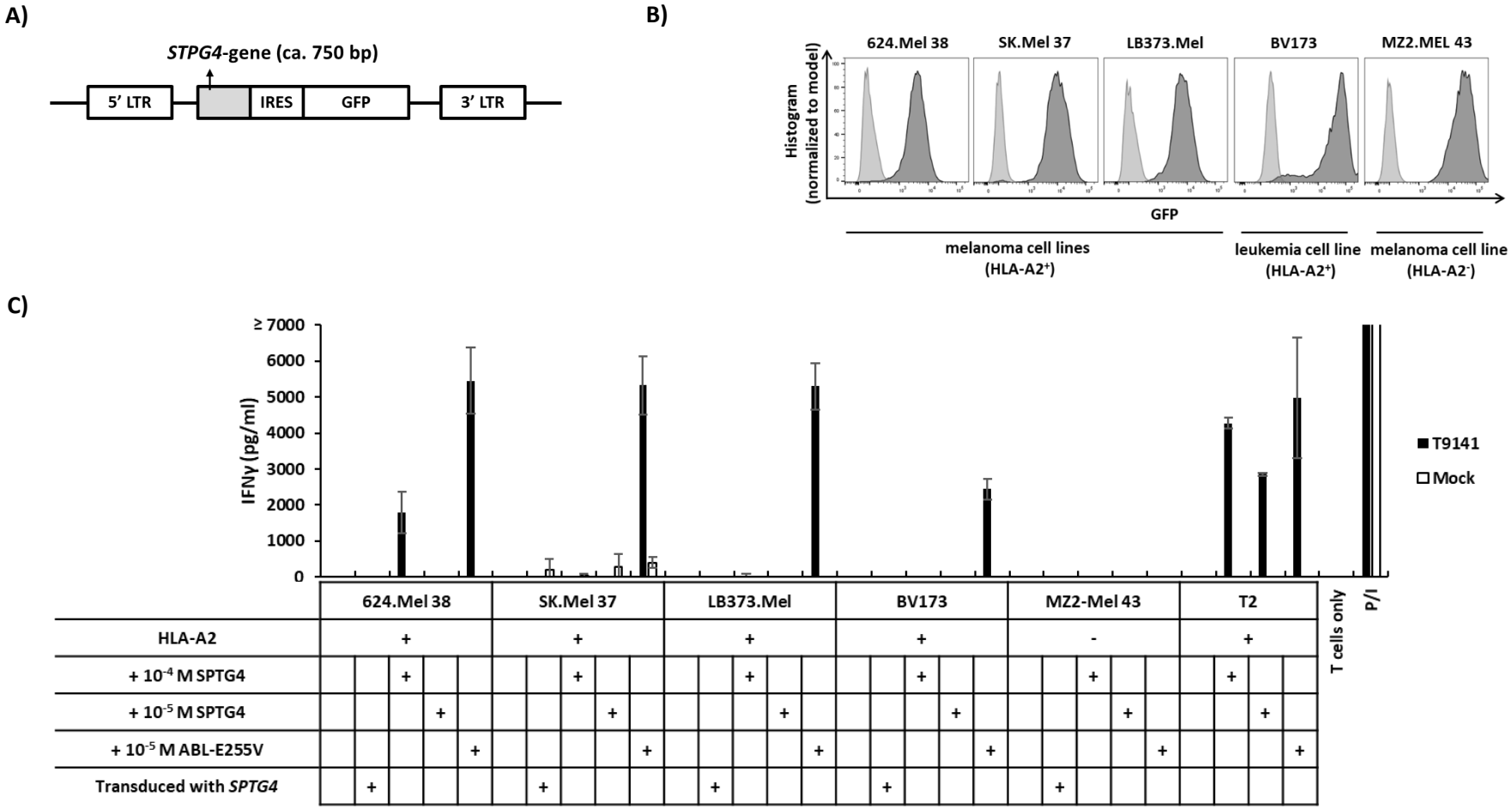


Figure 7. STPG4 peptide was not endogenously processed. (A) Schematic of the *STPG4* gene linked to a GFP reporter gene by IRES and flanked with 5' and 3' LTR in pMP71 vector. (B) Expression of STPG4 in transduced 624.Mel 38 (HLA-A2$^+$), SK.Mel 37 (HLA-A2$^+$), LB373.Mel (HLA-A2$^+$), BV173 (HLA-A2$^+$) and MZ2.Mel43 (HLA-A2$^-$). Expression was determined by GFP. (C) IFNγ production of T9141-transduced T cells after co-culturing with STPG4-transduced, untransduced cancer cell lines and cells loaded with STPG4 or ABL-E255V peptide at indicated concentrations. Unspecific T cell stimulation with P/I was used as a positive control. Mean values of duplicate cultures with SD are shown. The results are representative of three independent experiments using PBMCs of different donors.

5.6 Recognition of T9141-transduced T cells to CML cell lines harboring E255V

In section 5.4, we demonstrated that the neoepitope ABL-E255V was endogenously processed and presented by transduced cancer cell lines. As the LTR of the retroviral vector leads to overexpression of ABL-minigene-E255V, the recognition of T9141 against CML cells acquired the E255V mutation in the *BCR-ABL* gene should be tested. To simulate the natural scenario, we performed CRISPR-Cas9 system to introduce the point mutation E255V into the CML cell lines that carried the fusion gene *BCR-ABL* and expressed HLA-A2 molecules. Accordingly, the ABL-E255V epitope expressed by CRISPR-edited CML cells was solely derived from BCR-ABL fusion protein. After editing by CRISPR-Cas9, cells modified through homology directed repair would carry the point mutation valine (G**T**G) instead of glutamic acid (G**A**G). Besides the E255V mutation, additional silence mutations were introduced to change the PAM sequence and to disable one of the digestion sites of the HPY8I restriction enzyme for screening (Figure 8). To identify cell clones harboring the E255V mutation, two rounds of identification were carried out. In the first round, a specified DNA fragment was amplified from genomic DNA, which allowed us to produce PCR products from a great number of clones simultaneously and facilitated the processes of screening by avoiding tortuous procedures like RNA extraction and cDNA synthesis. Following digestion of the PCR products, clones showing an extended DNA fragment (ca. 1.2 kb) due to the absence of one of the two HPY8I restriction sites were chosen for the second round of identification. The two CML cell lines used here carried a Ph chromosome comprising the *BCR-ABL* gene, a result of translocation between one chromosome 9 and one chromosome 22, but also a normal chromosome 9 comprising the *ABL* gene. Therefore, the modified *ABL* sequence might be located in the *BCR-ABL* gene, the wild type *ABL* gene, or both. To verify that the E255V mutation was in the *BCR-ABL* gene, we sequenced the PCR products that were amplified from cDNA of CRISPR-edited clones by the 5' primer targeting the *BCR* gene and the 3' primer targeting the *ABL* gene. Once selected clones were confirmed to carry the E255V point mutation, the CRISPR-edited clones were co-cultured with T9141-transduced T cells to test the T cell response.

5.6.1 No responses to BV173 cells harboring E255V were detected

After digestion screening and sequencing, three CRISPR-edited BV173 clones were confirmed to carry the E255V mutation in the *BCR-ABL* gene (Figure 9A and B). We co-cultured the three CRISPR-edited BV173 clones with T9141-transduced T cells. However, no specific T cell responses were detected (Figure 10A). To exclude the possibility that the processing machinery

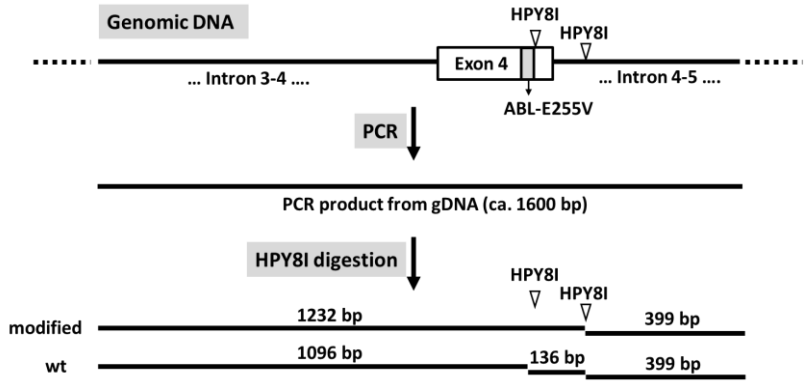
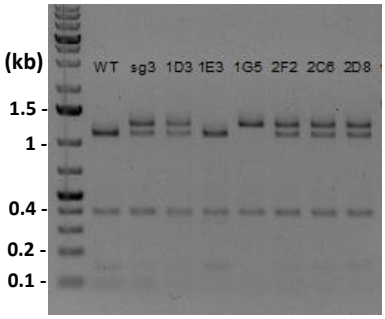


Figure 8. The screening strategy for identifying CRISPR-edited cell clones. Schematic of screening strategy for identifying CRISPR-edited cell clones. PCR product was amplified from genomic DNA of each cell clone. Following digestion of HPY8I restriction enzyme, DNA fragment with a length of ca. 1.2 kb served as an indicator of cells carrying modified sequences.

of BV173 cells was altered after a series of manipulation and long-term culture, the CRISPR-edited BV173 clones were transduced with ABL-minigene-E255V (Figure 10B). The results of co-culture demonstrated that the CRISPR-edited BV173 clones were functional to process and present ABL-E255V to T cells (Figure 10A). However, these clones induced lower IFNγ production on average in comparison with the parental BV173, suggesting that the processing machinery of the CRISPR-edited BV173 clones became less efficient, thereby expressing less ABL-E255V. BCR-ABL is often highly expressed in CML leukemic cells, as it is essential for neoplastic transformation and survival of CML leukemic cells (Barnes et al., 2005 Oct; Issaad et al., 2000; Modi et al., 2007). We quantified the expression of the inherited *BCR-ABL* gene and the transduced ABL-minigene-E255V by q-PCR, and determined that the expression of ABL-minigene-E255V was 20-fold higher than that of *BCR-ABL* gene (Figure 10C). The data suggested that the inability of CRISPR-edited BV173 clones to induce responses in T9141-transduced T cells was due to insufficient ABL-E255V processed from a low level of BCR-ABL expression. Of note, the ability to present antigens was similar between the parental BV173 and the three CRISPR-edited clones, as all types of BV173 externally loaded with 10^{-6} M ABL-E255V induced comparable T cell responses. In summary, BV173 harboring the E255V mutation did not induce responses of T9141-transduced T cells due to the low level of BCR-ABL expression despite functional processing machinery and presentation of peptides.

A)



B)

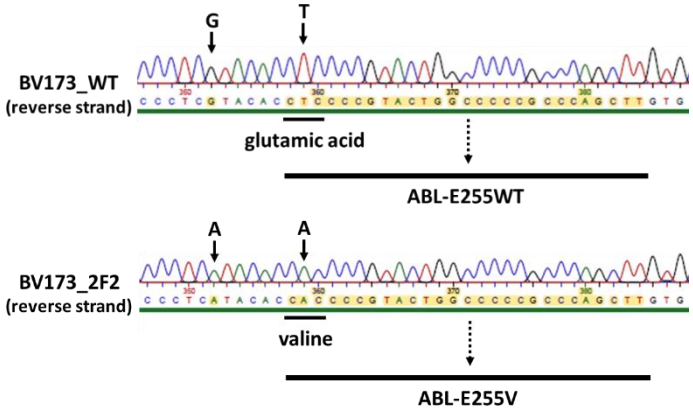


Figure 9. BV173 cells harboring the E255V mutation in the *BCR-ABL* gene were identified following CRISPR-editing. (A) Digestion of PCR products amplified from genomic DNA of CRISPR-edited BV173 clones by the HPY8I restriction enzyme. Clones carrying wild type sequences showed three DNA fragments in the length of 1 kb, 0.4 kb, and 0.13 kb, indicating that the specified sequences were not modified by CRISPR-Cas9; clones carrying the modified *ABL* sequence on either the *BCR-ABL* gene or the wild type *ABL* gene showed fragments of four lengths (1.2 kb, 1 kb, 0.4 kb and 0.13 kb), in which the fragments in the length of 1 kb, 0.4 kb and 0.13 kb were from the unmodified gene, and the fragments in the length of 1.2 kb and 0.4 kb were from the modified gene; clones carrying the modified *ABL* sequences on both of the *BCR-ABL* gene and the wild type *ABL* gene showed two fragments in the length of 1.2 kb and 0.4 kb. To note, the fragment in the length of 0.4 kb was present in both modified and unmodified sequences. WT represents wild type; sg3 represents CRISPR-edited bulk BV173 cells using crRNA 3 and ssODN 3. (B) Sequencing of a specified region on the *BCR-ABL* gene that was amplified from cDNA. Results of reverse strand are shown. The upper result is the sequencing of unmodified BV173 cells (BV173_WT); the lower result is the sequencing of the CRISPR-edited clone, BV173_2F2, which is representative of three CRISPR-edited clones.

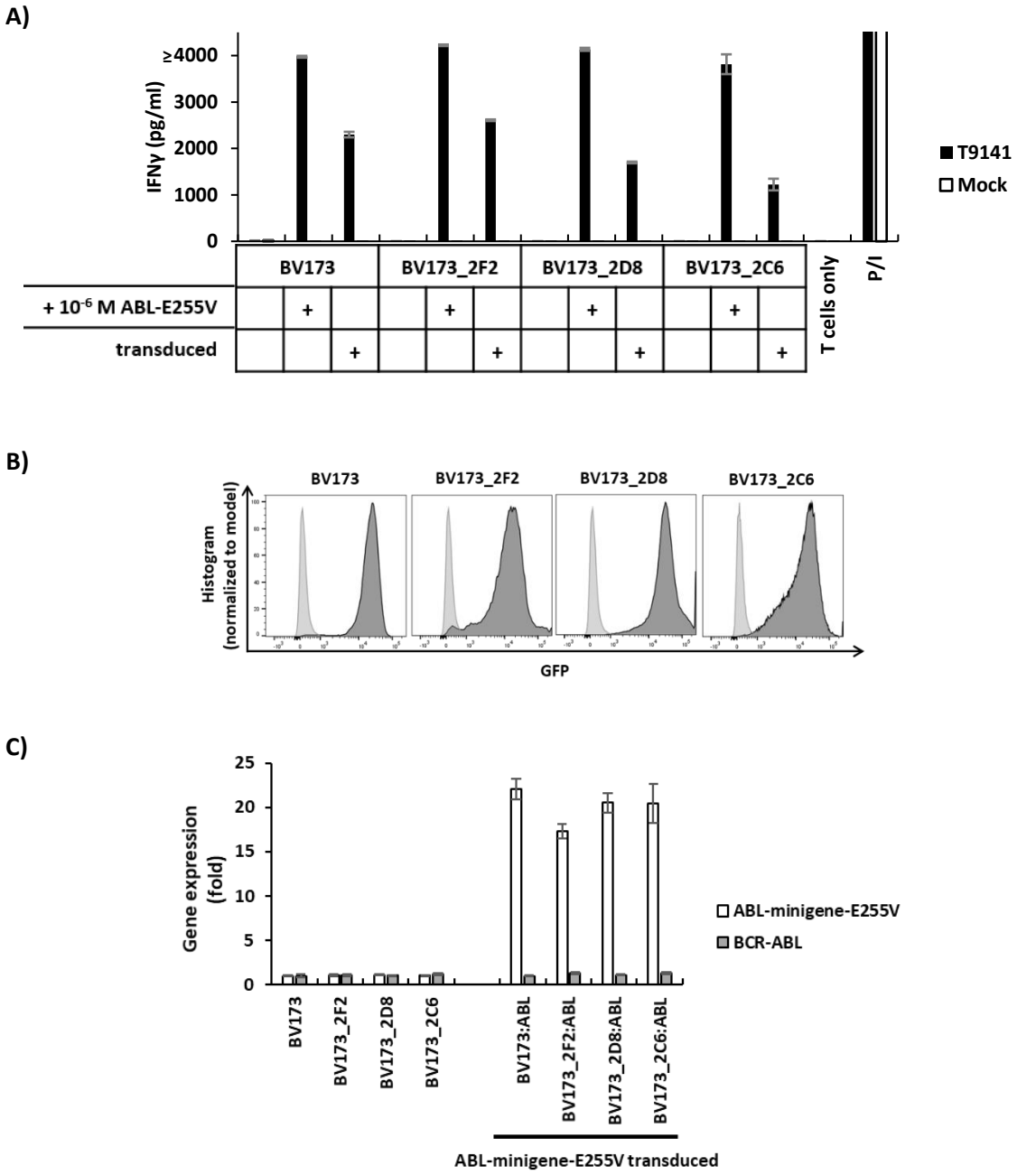


Figure 10. The CRISPR-edited BV173 clones did not induce specific responses of T9141-transduced T cells despite functional processing machinery and presentation of peptides. (A) IFNγ production of T9141-transduced human T cells after co-culturing with untransduced and ABL-minigene-E255V-transduced CRISPR-edited BV173 clones, and cells loaded with 10^{-6} M ABL-E255V. Unspecific T cell stimulation with P/I was used as a positive control. Mean values of duplicate cultures with SD are shown. The results are representative of two independent experiments using PBMCs of different donors. (B) Expression of ABL-minigene-E255V in transduced BV173 cells and three CRISPR-edited BV173 clones. Expression was determined by GFP. (C) Gene expression of the *BCR-ABL* gene and ABL-minigene-E255V in parental BV173 cells and three CRISPR-edited BV173 clones that were either untransduced or ABL-minigene-E255V-transduced. The value of gene expression is presented as relative quantification (RQ) in fold calculated by the method $2^{-(\Delta\Delta Ct)} \pm \mathrm{RQ_{max}}$ and $\mathrm{RQ_{min}}$.

5.6.2 Responses to K562-A2 cells harboring E255V were detected

As each CML cell line expresses a different level of BCR-ABL (Clapper et al., 2020; Koch et al., 2008; Pereira et al., 2017; Sharma et al., 2015; Tamai et al., 2018) and possesses different processing efficiency, it is necessary to include CML leukemic cells from different patients for co-culture experiment. Due to a lack of primary CML leukemic cells and other CML cell lines that were both E255V+ and HLA-A2+, we used a CML cell line K562-A2 that was transduced to express HLA-A2 for CRISPR-Cas9-editing. Following digestion screening and sequencing, we identified one clone harboring E255V in the *BCR-ABL* gene (Figure 11A and B).

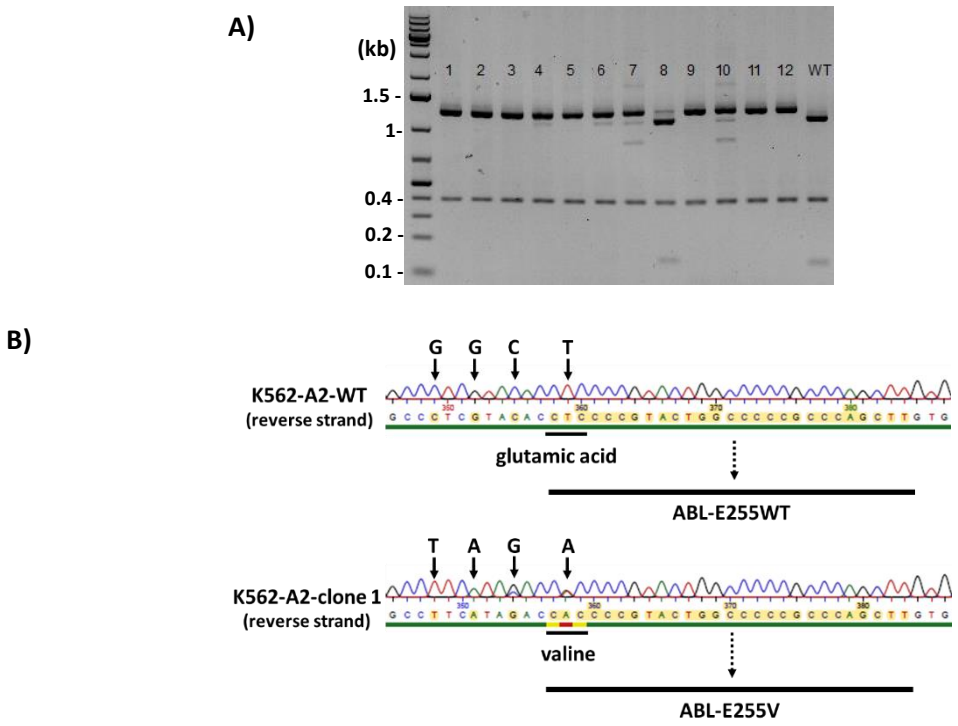


Figure 11. K562-A2 cells harboring the E255V mutation in the *BCR-ABL* gene were identified following CRISPR-editing. (A) Digestion of PCR products amplified from genomic DNA of CRISPR-edited K562-A2 clones by the HPY8I restriction enzyme. Clones carrying wild type sequences showed three DNA fragments in the length of 1 kb, 0.4 kb, and 0.13 kb, indicating that the specified sequences were not modified by CRISPR-Cas9; clones carrying the modified *ABL* sequence on either the *BCR-ABL* gene or the wild type *ABL* gene showed fragments of four lengths (1.2 kb, 1 kb, 0.4 kb, and 0.13 kb), in which the fragments in the length of 1 kb, 0.4 kb, and 0.13 kb were from the unmodified gene, and the fragments in the length of 1.2 kb and 0.4 kb were from the modified gene; clones carrying the modified *ABL* sequences on both of the *BCR-ABL* gene and the wild type *ABL* gene showed two fragments in the length 1.2 kb and 0.4 kb. To note, the fragment in the length of 0.4 kb was present in both modified and unmodified sequences. (B) Sequencing of a specified region on the *BCR-ABL* gene that was amplified from cDNA. Results of reverse strand are shown. The upper result is the sequencing of unmodified K562-A2 cells (K562-A2_WT); the lower result is the sequencing of the CRISPR-edited K562-A2_clone 1. K562-A2 cells were CRISPR-Cas9-edited by using crRNA 6 and ssODN 6.

After co-culturing with T9141-transduced T cells, the CRISPR-edited K562-A2 clone 1 induced a specific, albeit weak T cell response (Figure 12A). To compare the capability of processing and presenting peptides, parental K562-A2 cells and the CRISPR-edited K562-A2 clone 1 were transduced with ABL-minigene-E255V and used for co-culture (Figure 12A and B). As a result, T cell responses induced by the transduced CRISPR-edited K562-A2 clone were comparable to transduced parental K562-A2 cells, indicating that the processing efficiency of the CRISPR-edited clone was not altered after manipulations and long-term culture. Moreover, the T cell responses induced by transduced cells were even slightly higher than that by K562-A2 loaded with 10^{-6} M ABL-E255V, indicating that a high level of ABL-E255V was efficiently processed from translated ABL-minigene-E255V. The detected T cell responses were not influenced by the HLA-A2 density of K562-A2 cells. Although HLA-A2 was retrovirally transduced in K562 cells, the expression level of HLA-A2 was not overexpressed but was comparable to other cancer cell lines expressing HLA-A2 naturally (Figure 4D and 12C). Taken together, K562-A2 cells with an efficient processing machinery and a higher level of BCR-ABL expression induced specific responses of T9141-transduced T cells.

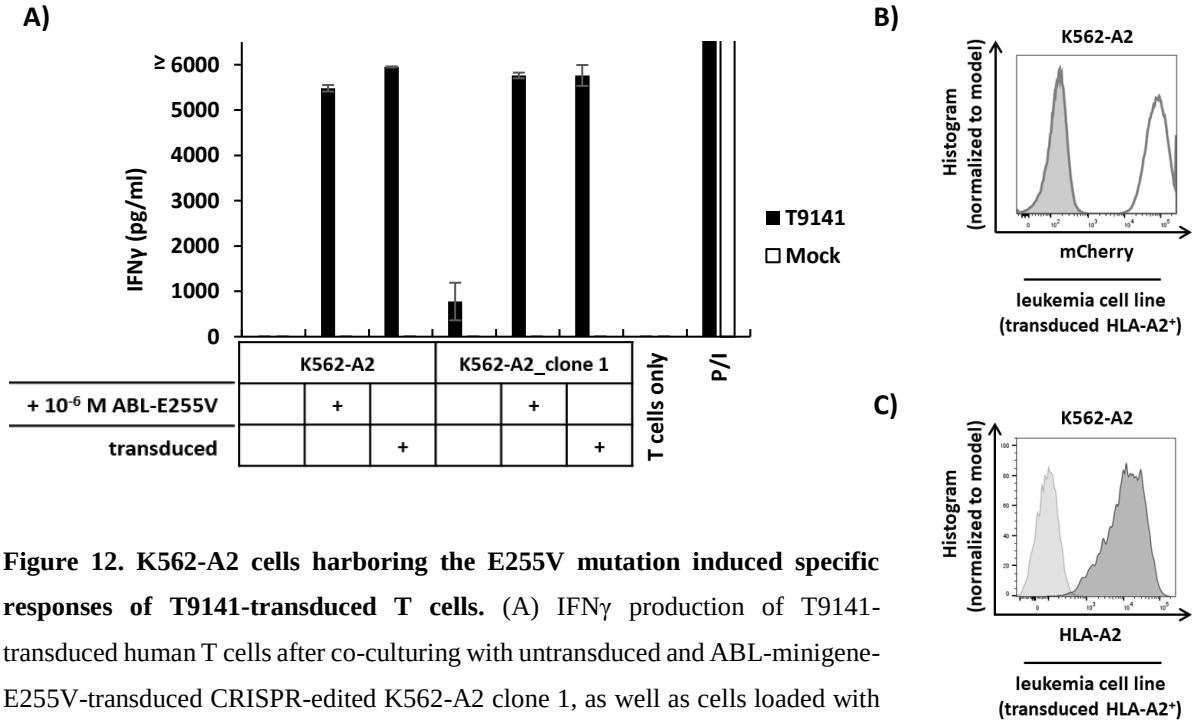


Figure 12. K562-A2 cells harboring the E255V mutation induced specific responses of T9141-transduced T cells. (A) IFNγ production of T9141-transduced human T cells after co-culturing with untransduced and ABL-minigene-E255V-transduced CRISPR-edited K562-A2 clone 1, as well as cells loaded with ABL-E255V at 10^{-6} M. Unspecific T cell stimulation with P/I was used as a positive control. Mean values of duplicate cultures with SD are shown. The results are representative of two donors. (B) Expression of ABL-minigene-E255V in transduced K562-A2. Expression was determined by mCherry. (C) Expression of HLA-A2 in transduced K562 (designated as K562-A2). Expression was determined by HLA-A2 staining.

6. Discussion

In the present thesis, ABL-E255V, one of the frequently occurring mutations in BCR-ABL of relapsed CML patients after TKI treatment, was identified to be an immunogenic neoepitope. Although the expression level of ABL-E255V in CML patients requires further investigation, our results have demonstrated that ABL-E255V was endogenously processed and presented on the cell surface. Moreover, we isolated two ABL-E255V-specific TCRs from immunized ABabDII mice. The TCR T9141 with a superior avidity in contrast to T8922 was further analyzed. As no off-target toxicity was detected, T9141 is determined as a suitable TCR for clinical usage, which can benefit CML patients carrying compound mutations.

6.1 Immune response against secondary mutated BCR-ABL in ABabDII mice

6.1.1 ABabDII mice as a valid tool for TCR gene therapy

In previous studies, it has been proven that ABabDII mice are functional, as they can give specific CD8 T cell responses to a panel of human TAAs, including AFP, gp100, MAGE-A1, MAGE-A10, NY-ESO-1 and STEAP (Li et al., 2010; Obenaus et al., 2015). Specific immune responses against virus-derived tumor antigens and TSAs were also shown in ABabDII mice, such as Merkel cell polyomavirus (MCV)-encoded T antigens (Tag) (Gavvovidis et al., 2018), mutated KRAS (Willimsky et al., 2021), and personal neoepitopes from two individual patients (Grunert et al., 2022). Moreover, ABabDII mice have been demonstrated that they exhibited similar TCR usage to human, as one study showed that CD8 T cell clones against Melan-A-derived peptide (ELA) from ABabDII mice preferentially used TRAV12-2 gene with limited TRVB genes, which were similar to ELA-specific CD8 T cell clones isolated from humans (Li et al., 2010). Since ABabDII mice expressing a diverse human TCR repertoire have been reported as a valid tool for isolating TCRs against HLA-A2 restricted antigens, we employed ABabDII mice in this project to test the immunogenicity of ABL-E255V and ABL-T315I and to generate specific TCRs against the target antigens.

6.1.2 Selection of potential neoepitopes from drug-selected secondary mutations in BCR-ABL

Immunotherapy has been focused on the BCR-ABL fusion protein, as BCR-ABL is exclusively expressed on leukemic cells and junction peptides could serve as shared neoepitopes. BCR-

ABL was first described as a neoepitope in the early 1990s (Chen et al., 1992). Later, several studies reported that epitopes derived from BCR-ABL junctions could bind to human HLAs and induce cytotoxic T cell responses (Clark et al., 2001; Yotnda et al., 1998 May). However, it is debatable whether junction peptides can be processed and presented on HLA molecules, as the results are not consistent in studies (Berke et al., 2000; Bocchia et al., 1995; Buzyn et al., 1997; Cullis et al., 1994). Additionally, one comprehensive study has reported that no epitopes derived from BCR-ABL junctions were found in HLA ligandome (Bilich et al., 2019), which suggested that junction peptides might not be endogenously processed. Due to poor evidence that junction peptides could be immunogenic neoepitopes, many studies started to investigate the drug-selected secondary mutations of BCR-ABL in CML, as they were also expressed exclusively on leukemic cells and shared across patients. Currently, over 100 mutations have been identified in more than 50 hotspot positions (Balabanov et al., 2014), in which two third of the mutations occurred in T315, Y253, E255, M315, G250, F359, and H396 (Tadesse et al., 2021). Although second and third generation TKIs have been employed to treat CML patients carrying secondary mutated BCR-ABL, the appearance of compound mutations conferred different levels of resistance to TKIs (Zabriskie et al., 2014). Hence, those frequently occurring mutations become attractive targets for ATT. One example was an HLA-A3 restricted neoepitope derived from BCR-ABL E255K, which has been reported to be capable of eliciting specific CD8 T cell responses in PBMCs of CML patients and was endogenously processed (Cai et al., 2012). In the present thesis, we put frequently occurring secondary mutations in the prediction algorithms, the IEDB and the NetMHC 3.4, to acquire the binding affinity toward HLA-A2 *in silico*. Based on the predicted binding score, we choose two peptide candidates ABL-E255V and ABL-T315I for investigation.

6.1.2 Immunization with ABL-E255V

Glutamic acid (E) in position 255 of the ABL domain replaced by lysine (K) or valine (V) was often detected in relapsed CML patients (Apperley, 2007). According to the analysis *in silico*, ABL-E255V (KLGGGQYG**V**) was predicted as a good binder toward HLA-A2, as it had estimated IC_{50} of 11 nM in NetMHC 3.4 and of 25 nM in IEDB. These results reflected the preferential binding motif of HLA-A2, which favored peptides in the length of nine amino acids with leucine (L) in position 2 and valine in position 9. These two positions are known as anchor residues that provide the main contact with HLA-A2 molecules (Parker et al., 1992; Binkowski et al., 2012). Conversely, the parental peptide ABL-E255WT (KLGGGQYG**E**) carrying

glutamic acid in position 9 had predicted IC_{50} of 17489 nM in NetMHC 3.4 and of 7206 nM in IEDB. The predicted results were in accordance with the previous studies that the disliked amino acids at the anchor positions could disrupt the binding of peptides to HLA-A2 molecules (Drijfhout et al., 1995; Binkowski et al., 2012). As ABL-E255V carried good binding affinity that contributes to the formation of stable pMHC complexes, ABL-E255V has successfully induced specific immune responses in ABabDII mice following two to three boosts. Of note, the sequence of ABL-E255WT in human is identical to the sequence in mice. This should preclude the probability that the ABabDII-derived ABL-E255V-specific TCRs cross-recognized wild type peptides, as T cells expressing TCRs that recognized self-antigens were eliminated in the thymus during negative selection. Additionally, the poor binding affinity of ABL-E255WT also declined the potential of cross-recognition.

6.1.3 Immunization with ABL-T315I

T315I is the most common mutation in relapsed CML patients (Apperley, 2007; Balabanov et al., 2014). As a gatekeeper mutation, the substitution of threonine (T) in position 315 to isoleucine (I) contributes to the resistance to most of TKIs by disabling the formation of a hydrogen bond in position 315 with TKIs (An et al., 2010). As T315I has been the most frequently occurring mutation, we analyzed the binding affinity of peptides comprising the T315I mutation. Although ABL-T315I (YII**I**EFMTYG) was predicted with an IC_{50} of 1070 nM in NetMHC 3.4, it had a predicted IC_{50} of 103 nM in IEDB. Before immunization, we confirmed that the parental sequence (YII**T**EFMTYG) in mice was identical to the sequence in human to avoid potential cross-recognition. However, no immune responses were detected in ABabDII mice following several boosts. We assumed that ABL-T315I might bind poorly to HLA-A2 molecules, as ABL-T315I did not contain the most preferential amino acids at the two anchor positions. The moderate binding affinity predicted by IEDB might be due to the isoleucine in position 2, which was the third preferential amino acid in that anchor position (Drijfhout et al., 1995; Binkowski et al., 2012). Due to a lack of additional information, we could not conclude how the binding between T315I and HLA-A2 is. Nevertheless, ABL-T315I was not an immunogenic peptide according to our results.

6.2 Isolated ABL-E255V-specific TCRs

6.2.1 Functionality of ABL-E255V-specific TCRs

Following boost immunizations with the ABL-E255V peptide, specific CD8 T cell responses were detected in several ABabDII mice. Two specific TCRs isolated from each individual mouse could recognize ABL-E255V specifically but not ABL-E255WT. To determine their functional avidities, functionality assay with T2 cells was performed. As a result, T9141 and T8922 had a half maximal IFNγ production (EC_{50}) at 10^{-10} M and 10^{-9} M, respectively. The differences in functionality were expected, as the two TCRs were unique to each other. T9141 consisted of TRAV38-2 and TRBV2, whereas T8922 consisted of TRAV39 and TRBV7-8. Importantly, the CDR3 from each TCR shared no similarity. Previous studies have indicated that CDR1 and CDR2 were responsible for contacting MHC I, while hypervariable CDR3 predominately interacted with the peptide (Davis and Bjorkman, 1988; Krogsgaard and Davis, 2005). The CDR3 region of TCRα and β chain defines not only the TCR specificity but also contributes to the unique binding pattern of each TCR (Danska et al., 1990). Thus, we could anticipate that the two TCRs varied not only in functionality but also in their recognition pattern. As our aim in this project was to generate TCRs for clinical use, T9141 that exhibited a superior avidity was chosen and analyzed for off-target toxicity.

6.2.2 Allo-reactivity

As ABabDII mice contain only one human HLA allele, TCRs that are trained in the murine thymus could exhibit allo-reactivity toward other human HLA alleles. Of note, the risk of allo-reactivity is not restricted to murine-derived TCRs, but also to the ones derived from human. Because human HLAs are highly variable, TCRs derived from HLA-mismatched donors could potentially cause the same problem. Several studies have tested the allo-reactivity by co-culturing TCR-transduced T cells with different B lymphoblastoid cell lines (B-LCLs) expressing diverse human HLA alleles (Kunert et al., 2017; Luo et al., 2020; Obenaus et al., 2015; Sanderson et al., 2019). The efficacy of this method has been pointed out in one study, which identified that an HLA-A*02:01 restricted MAGE-A4-specific TCR allo-reacted to HLA-A*02:05, suggesting that the treatment with this TCR should exclude patients expressing HLA-A*02:05 (Sanderson et al., 2019). In our study, T9141 did not exhibit allo-reactivity.

6.2.3 Cross-reactivity to human self-peptides

To apply therapeutic TCRs in clinic, it is important to investigate potential off-target toxicity beforehand. Previous studies have shown severe consequences due to unspecific cross-reactivity, such as the clinical trial using the affinity-matured MAGE-A3-specific TCR (TCR a3a). In that study, TCR a3a cross-reacted to a Titin-derived peptide that was expressed by cardiomyocytes, resulting in the death of two patients (Cameron et al., 2013; Linette et al., 2013). This case points out the importance of investigating cross-reactivity toward non-target peptides. To examine the potential cross-reactivity, alanine scan, that replaces amino acid with alanine in each position of cognate peptide, is often employed to identify the amino acids responsible for TCR recognition, the so-called TCR recognition motif. Other peptides carrying the same motif in human peptidome are searched by web tools like BLAST and ScanProsite; the potential cross-reactivity toward them is then investigated (Cai et al., 2020; Chandran et al., 2022; Obenaus et al., 2015).

In this thesis, we identified the TCR recognition motif of T9141 by alanine scan and found 36 human peptides that carried the same motif and were not present in mice. Although part of the 36 peptides was predicted as poor binders toward HLA-A2, T cell responses to all peptides were tested for the safety issue. Peptides that could only induce T cell responses under high concentration were neglected, as peptides with such poor binding affinities could not form stable pMHC complexes for T cell recognition. To concern the potential risk, the STPG4-derived peptide that could be recognized at 10^{-8} M on T2 cells was further tested. The results indicated that the STPG4-derived peptide was a poor binder and was not endogenously processed. Besides peptides carrying the same TCR recognition motif, peptides that are dissimilar to the cognate peptide should be concerned for cross-reactivity as well (Bijen et al., 2018). Hence, we employed a peptide library containing 114 known HLA-A2 restricted human self-peptides for cross-reactivity assay regardless of the TCR recognition motif (Morgan et al., 2013; Obenaus et al., 2015). As no T cell responses were detected, cross-reactivity mediated by T9141 was unlikely to happen while confronting peptides with the same motif, as well as peptides with no similarity.

6.3 Expression of ABL-E255V

6.3.1 ABL-minigene-E255V-transduced cancer cell lines

Being a target for TCR gene therapy, the epitope should not only be capable of inducing immune responses, but should also be endogenously processed and presented by cancer cells. Thus, we transduced different cancer cell lines with ABL-minigene-E255V, a fragment in the length of ca. 1 kb derived from *ABL* gene exon 1 to exon 6 with the E255V mutation in exon 4. According to the results of co-culture, ABL-E255V was endogenously processed and presented. Although the response of T9141-transduced T cells against the CML cell line BV173 was lower, the responses against the other transduced cancer cell lines were comparable to the responses against the cancer cells loaded with 10^{-6} M ABL-E255V peptide. In contrast to T9141, T cells expressing T8922 with a suboptimal avidity exhibited different levels of response to the transduced cancer cell lines, in which only one transduced cancer cell line induced a T cell response comparable to cancer cells loaded with 10^{-6} M ABL-E255V, while the other three transduced cancer cell lines induced relatively low to no responses. According to the result of functionality assay, the functional avidity of TCRs decreased dramatically while confronting a peptide concentration lower than the dose inducing a half-maximal T cell response (EC_{50}). For the same reason, T8922 might have lost its functionality against the transduced cancer cell lines, while the ABL-E255V expression level of the transduced cancer cells was lower than the EC_{50} of T8922. Moreover, although three out of four transduced cancer cell lines exhibited similar expression levels of ABL-minigene-E255V according to the MFI of the GFP linked to it, the results from T8922 suggested that each cancer cell line possessed different peptide processing efficiency, leading to distinct ABL-E255V expression levels on the cell surface. Of note, the expression level of ABL-minigene-E255V in BV173 cells was lower than the other transduced cancer cell lines. Since the transduced BV173 cells induced a lower response of T9141-transduced T cells and no response of T8922-transduced T cells, we assumed that the processing efficiency of BV173 was not able to compensate for the lower expression of the transduced minigene, resulting in a low level of ABL-E255V expression on the cell surface.

Furthermore, the expression of HLA-A2 should be assessed, because the expression level of HLA-A2 molecules directly devotes to the density of pMHC complexes, and the T cell activation depends on the binding of TCR to pMHC complexes (González et al., 2005). Here, we analyzed the expression of HLA-A2 in each cancer cell line and found no significant

differences. This result suggested that the HLA-A2 expression was not the reason for different magnitudes of T cell response against the transduced cancer cell lines.

6.3.2 BV173 cells harboring E255V

Our previous experiment demonstrated that ABL-E255V was endogenously processed and presented by the ABL-minigene-E255V-transduced cancer cells. However, overexpression of the minigene controlled by LTR should be a concern, because such expression level did not reflect the physiological situation. As there were no available primary CML cells and no reported CML cell lines being both HLA-A2+ and E255V+, we employed the CRISPR-Cas9 system to introduce valine in position 255 of the BCR-ABL protein by replacing adenine (GAG) to thymine (GTG) in the specified sequence of BV173, a CML cell line carrying the Ph chromosome and expressing naturally HLA-A2. The established CRISPR-edited BV173 clones should express ABL-E255V derived only from BCR-ABL fusion protein. Nevertheless, the CRISPR-edited BV173 clones did not elicit responses of T9141-transduced T cells in our experiment.

Since establishing CRISPR-edited BV173 clones took months, we investigated whether the peptide processing efficiency of these clones was altered after a long-term culture. We transduced the CRIPSR-edited BV173 clones with ABL-minigene-E255V and tested their capability of inducing responses of T9141-transduced T cells. Following co-culture, all transduced CRISPR-edited BV173 clones elicited specific responses of T9141-transduced T cells, however, in a lower level in contrast to the ABL-minigene-E255V-transduced parental BV173 cells that were maintained for only two to three weeks in culture. This result indicated that the transduced CRISPR-edited BV173 clones following a long-term culture presented a decreased level of ABL-E255V, thereby inducing lower T cell responses. Furthermore, we quantified the expression of ABL-minigene-E255V in all transduced BV173 by q-PCR and found that the expression level of the transduced CRISPR-edited BV173 clones was similar to that of transduced parental BV173 cells. This result demonstrated that the lower ABL-E255V expression in the transduced CRISPR-edited BV173 clones was not due to different levels of ABL-minigene-E255V expression but less efficient peptide processing machinery after a series of manipulation and long-term culture. Accordingly, the CRISPR-edited BV173 clones might process and express ABL-E255V from BCR-ABL fusion protein at a level that was lower than it should be.

Moreover, the result of q-PCR showed that the expression of ABL-minigene-E255V enhanced by LTR was 20-fold higher than that of the *BCR-ABL* gene in both transduced parental and CRISPR-edited BV173 cells. Despite a such high level of ABL-minigene-E255V expression in transduced BV173 cells, the results of ABL-E255V processing assay showed that transduced BV173 cells were able to induce a relatively lower T cell response in comparison with other transduced cancer cell lines, as well as the 10^{-6} M peptide loaded BV173 cells (see 6.3.1). Therefore, BCR-ABL protein that was expressed at a much lower level in contrast to ABL-minigene-E255V in BV173 cells might be processed to present ABL-E255V in a level that was too low to induce specific T cell responses. In the functionality assay, we observed that T9141 had a measured EC_{50} of 10^{-8} M when co-cultured with peptide-loaded BV173, whereas an EC_{50} of 10^{-10} M was determined when co-cultured with peptide-loaded T2 cells (data not shown). This result again pointed out that ABL-E255V needed to be processed and presented in a sufficient amount to compete with other endogenous peptides for HLA-A2 molecules, thereby activating T cells. However, it was not achieved by the CRISPR-edited BV173 clones in our experiments.

As previous studies have shown that a high level of BCR-ABL expression was required for neoplastic transformation (Barnes et al., 2005 Oct; Issaad et al., 2000; Modi et al., 2007), one could assume that high levels of peptides derived from such overexpressed BCR-ABL would be presented on the cell surface. Surprisingly, the CRISPR-edited BV173 clones were not recognized by T9141 in our experiment. Other than the inefficient peptide processing machinery of BV173 cells, the BCR-ABL expression should be considered, as the expression level correlates directly to the amount of ABL-E255V presented. Several studies have observed that the BCR-ABL expression level differed significantly in different CML cell lines (Clapper et al., 2020; Koch et al., 2008; Pereira et al., 2017; Sharma et al., 2015; Tamai et al., 2018). In the study by Koch et al., BCR-ABL expression determined by western blotting was different among four different CML cell lines, in which the BCR-ABL expression level of K562 was the highest and that of BV173 was the lowest (Koch et al., 2008). This result has been consistent with the study of Alagiozian-Angelova et al., who showed that the BCR-ABL fusion transcript of K562 cells was stronger than that of BV173 cells (Alagiozian-Angelova et al., 2007). Accordingly, BV173 with relatively low BCR-ABL expression might not be the most optimal CML cell line for test, as a low level of BCR-ABL could lead to insufficient ABL-E255V expression for T cell activation. In this part of project, we chose BV173 due to a lack of primary CML cells and CML cell lines that were both HLA-A2+ and E255V+. In addition, detailed

information on HLA alleles from available CML cell lines is mostly lacking. Although BV173 edited to carry E255V could not induce T cell responses, the results highlighted the effect of BCR-ABL expression level in T cell activation.

6.3.3 K562-A2 cells harboring E255V

To investigate the impact of BCR-ABL expression level on the potency of T9141, we chose another CML cell line, K562, for our experiments. Although K562 did not express naturally HLA-A2, it expressed a higher level of BCR-ABL in comparison with other CML cell lines (Clapper et al., 2020; Koch et al., 2008; Pereira et al., 2017; Sharma et al., 2015). Here, K562 cells transduced to express HLA-A2 were used (K562-A2). The HLA-A2 expression level was verified to be similar to other cancer cell lines expressing HLA-A2 naturally, which allowed us to exclude the biases in T cell responses caused by different amount of HLA-A2 on the cell surface. To test whether K562-A2 harboring the E255V mutation could induce specific T cell responses, T9141-transduced T cells were co-cultured with a CRISPR-edited K562-A2 clone. As a result, transduced T cells from two healthy donors showed low but specific T cell responses, indicating that ABL-E255V was processed and expressed in a sufficient amount for T cell activation. The capability of K562-A2 cells to elicit T cell responses was not only due to a higher level of BCR-ABL expression but also to an efficient peptide processing machinery. The processing efficiency could be observed in the co-culture experiment with ABL-minigene-E255V-transduced K562-A2 cells, where T cell responses induced by transduced K562-A2 cells were slightly higher than that by 10^{-6} M peptide loaded K562-A2 cells. In comparison, T cell responses of transduced BV173 were obviously lower. One might argue that the ABL-minigene-E255V expression of transduced K562-A2 was one-log higher than that of transduced BV173, however, inefficient processing machinery could still lead to a low level of ABL-E255V. The case has been shown by the transduced LB373.Mel cell line which expressed a high level of ABL-minigene-E255V but could not present sufficient ABL-E255V after processing to elicit responses of T8922-transduced T cells (see 6.3.1). These data supported the assumption that K562-A2 cells harboring E255V could process and present ABL-E255V, but the co-culture experiment with CRISPR-edited K562-A2 cells should be repeated, since it has been done once with one clone due to time limitation. Summarizing the current data, although T cell responses induced by the CRISPR-edited K562-A2 cells were low, it was demonstrated that K562-A2 cells with a high level of BCR-ABL expression and efficient processing

machinery were capable of presenting sufficient ABL-E255V derived from BCR-ABL for T cell activation.

In short, our data suggest that the ability of CML cells with the E255V mutation to induce T cell responses should depend on their peptide processing efficiency and the amount of expressed BCR-ABL. As the expression of BCR-ABL in each CML cell line varies greatly (Clapper et al., 2020; Koch et al., 2008; Pereira et al., 2017; Sharma et al., 2015; Tamai et al., 2018), the two CML cell lines here may not adequately represent the broad CML population. Therefore, it is recommended to include more CML cell lines for investigation, which should reflect the efficacy of T9141-transduced T cells in different CML patients, as different levels of BCR-ABL expression have been observed also in CML patients (Barnes et al., 2005 Oct; Donato et al., 2004; Guo et al., 1994; Guo et al., 1996). Nevertheless, potential overexpression of HLA-A2 should be a concern if the used CML cell lines are transduced to express HLA-A2.

6.4 TCR gene therapy for CML

Several immunotherapies are under development for CML treatment, in which TCR gene therapy is being intensively studied since the expression of BCR-ABL is restricted to CML leukemic cells. Nevertheless, to date, no available TCR gene therapies for CML have been reported. Some neoepitopes derived from BCR-ABL junctions were suggested to be immunogenic, but no specific TCRs were investigated in these studies, and also, no follow-up studies confirmed these results (Chen et al., 1992; Clark et al., 2001; Yotnda et al., 1998 May). The study analyzing HLA-ligandome in CML even indicated that no junction peptides were found on MHC I or II molecules (Bilich et al., 2019). Besides BCR-ABL junctions, secondary mutated BCR-ABL can serve as ideal targets as well. Cai et al. have shown that an HLA-A3-restricted neoepitope comprising the point mutation E255K could induce specific cytotoxic T cell responses and were endogenously processed and presented. However, no TCRs were identified from established E255K-specific T cell clones. Also, the E255K-specific T cell clones that could recognize minigene-transduced target cells were not tested with primary CML cells. Thus, it remains unclear whether there are E255K-specific TCRs available and whether the E255K-epitope is truly presented on CML cells.

In the present thesis, we generated ABL-E255V-specific TCRs. T9141 TCR with superior functional avidity was tested for off-target toxicity. As no allo-reactivity and cross-reactivity were detected, T9141 was verified as a suitable therapeutic TCR for CML treatment. To be

targeted, CML cells should express ABL-E255V endogenously. Here, we demonstrated that ABL-E255V was endogenously processed and presented by testing T cell responses to three minigene-transduced human melanoma cell lines and two minigene-transduced human CML cell lines. Moreover, we tested whether T9141-transduced T cells recognize the CRISPR-edited CML cell lines that were engineered to have the point mutation E255V. These cell lines expressed ABL-E255V solely derived from endogenous BCR-ABL protein. Nevertheless, no T cell response or low levels of T cell responses were detected in CRISPR-edited BV173 and K562-A2 cells, respectively. We could not rule out the possibility that the avidity of T9141 was not sufficient to recognize very low levels of ABL-E255V endogenously expressed in CML cells. Although TCR-maturation *in vitro* could enhance the avidity, such TCRs could lose their specificity and increase the risk of cross-recognition (Jaigirdar et al., 2016; Linette et al., 2013; Morgan et al., 2013). In addition, the avidity of T9141 was only slightly weaker than T1367, a MAGE-A1-specific TCR exhibiting a great sensitivity of recognition (Appendix 5) (Obenaus et al., 2015), suggesting that the sensibility of T9141 should be sufficient. T cell activation is dependent on the binding of TCRs to pMHC complexes, which in turn is influenced by the expression level of epitopes on the cell surface (González et al., 2005). Thus, weak T cell responses to CRISPR-edited CML cells should be a result of low levels of ABL-E255V expression. However, the results of the two CML cell lines could not represent the extended population of CML patients, as divergent BCR-ABL expression levels have been observed in different CML cell lines and CML patient samples. Our results have also shown that different peptide processing efficiency of each cancer cell line contributed to different levels of ABL-E255V expression. Importantly, upregulation of BCR-ABL in CML leukemic cells was observed in accelerated and blast phase, as well as under drug treatment (see 6.4.1 and 6.4.2), which might enable strong responses mediated by T9141-transduced T cells. Hence, the efficacy of T9141 should be tested with CML cells from different origins to give a broader view in CML treatment.

6.4.1 Expression of BCR-ABL in different phases of CML

BCR-ABL is known to be essential for CML initiation and progression, since it has been demonstrated that BCR-ABL alone could initiate CML-like myeloproliferative neoplasm in BCR-ABL transduction-transplantation models (Daley et al., 1990; Gishizky et al., 1993; Zhang and Ren, 1998) and transgenic CML models (Honda et al., 1998; Inokuchi et al., 1993). CML is starting with chronic phase (CP), which is characterized by increasing numbers of

myeloid precursors and mature cells and lasts approximately three to five years without treatment (Faderl et al., 1999; Skorski, 2012). Evidence suggests that progression from CP to accelerated phase (AP) and blast phase (BP) is a time-dependent process involving two factors, BCR-ABL-dependent pathways and BCR-ABL-independent DNA damage caused by inefficient and unfaithful DNA repair (Radich, 2011; Skorski, 2011; Yin et al., 2019). Along with the progression, the two factors facilitate the development from CML-CP cells to CML-AP cells and eventually CML-BP cells. However, the significantly elevated BCR-ABL expression in CML-AP and -BP cells are believed to be responsible for the disease progression, as cell survival, growth, and proliferation pathways, such as RAS/STAT/PI3K, are enhanced by elevated BCR-ABL level, leading to the aggressive phenotype of CML-BP cells (Calabretta and Perrotti, 2004; Chereda and Melo, 2015; Lee et al., 2021; Osman and Deininger, 2021). Patients that enter the CML-BP are highly resistant to TKIs (Branford et al., 2003; Johansson et al., 2002; Zheng et al., 2006). Since BCR-ABL expression has been reported to increase significantly in CML-AP and -BP cells (Barnes et al., 2005 Sep; Gaiger et al., 1995), it might promote specific T cell responses mediated by T9141.

6.4.2 Expression of BCR-ABL under TKIs treatment

TKIs are highly effective, however, 25% of patients relapse due to drug resistance (Marin et al., 2005). The common mechanism of acquired drug resistance is BCR-ABL with secondary mutations that prevent BCR-ABL kinase protein from binding to TKIs. Nevertheless, drug resistance caused by BCR-ABL amplification rather than secondary mutations was also observed in relapsed patients and resistant CML cell lines (Gorre et al., 2001; Karimiani et al., 2014; le Coutre et al., 2000). The BCR-ABL amplification is a result of multiple copies of the *BCR-ABL* gene that appears after drug treatment (Gorre et al., 2001). Resistant CML cells with elevated BCR-ABL expression exhibit active BCR-ABL downstream signaling involving MAPK, PI3K, SRC, and JAK/STAT (Braun et al., 2020) and no induction of apoptosis despite the presence of TKIs (le Coutre et al., 2000). Interestingly, increased BCR-ABL expression was also found in BCR-ABL mutant CML cells of patients, indicating that the expression of BCR-ABL harboring secondary mutation was elevated upon drug exposure (Branford et al., 2003). Thus, relapsed patients, who are under drug treatment, might have elevated BCR-ABL expression which could enable effective T cell treatment mediated by T9141.

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8. Appendix

Appendix 1. Sequences of peptides used in the alanine scan for identifying the T9141 TCR recognition motif.

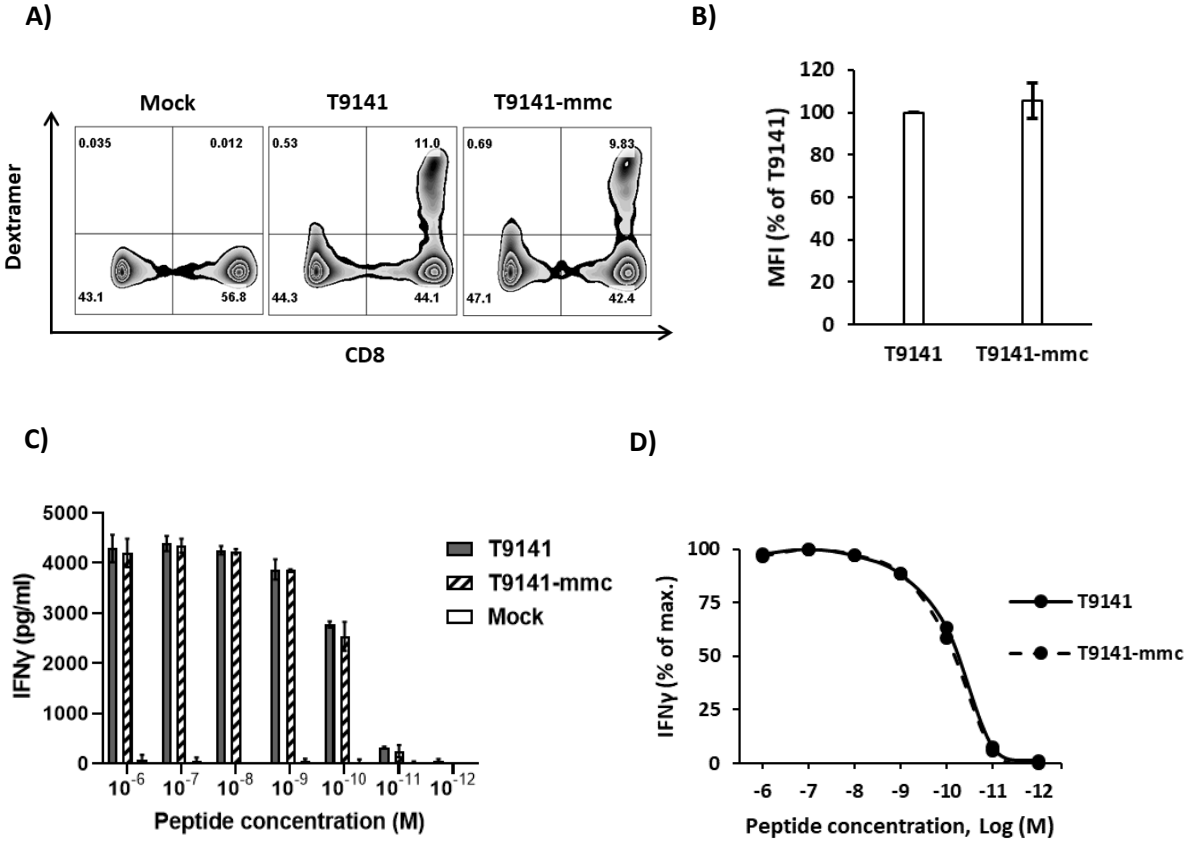
Peptide	Sequence								
ABL-E255V	K	L	G	G	G	Q	Y	G	V
ABL-E255V-1A	**A**	L	G	G	G	Q	Y	G	V
ABL-E255V-2A	K	**A**	G	G	G	Q	Y	G	V
ABL-E255V-3A	K	L	**A**	G	G	Q	Y	G	V
ABL-E255V-4A	K	L	G	**A**	G	Q	Y	G	V
ABL-E255V-5A	K	L	G	G	**A**	Q	Y	G	V
ABL-E255V-6A	K	L	G	G	G	**A**	Y	G	V
ABL-E255V-7A	K	L	G	G	G	Q	**A**	G	V
ABL-E255V-8A	K	L	G	G	G	Q	Y	**A**	V
ABL-E255V-9A	K	L	G	G	G	Q	Y	G	**A**

Appendix 2. Peptides carrying the T9141 recognition motif within the human peptidome. Peptides included in the table were not present in the murine peptidome. Predicted binding affinity of each peptide was analyzed by NetMHC 3.4. Peptides induced responses of T9141-transduced T cells at 10^{-5} M were bold and marked with gray background.

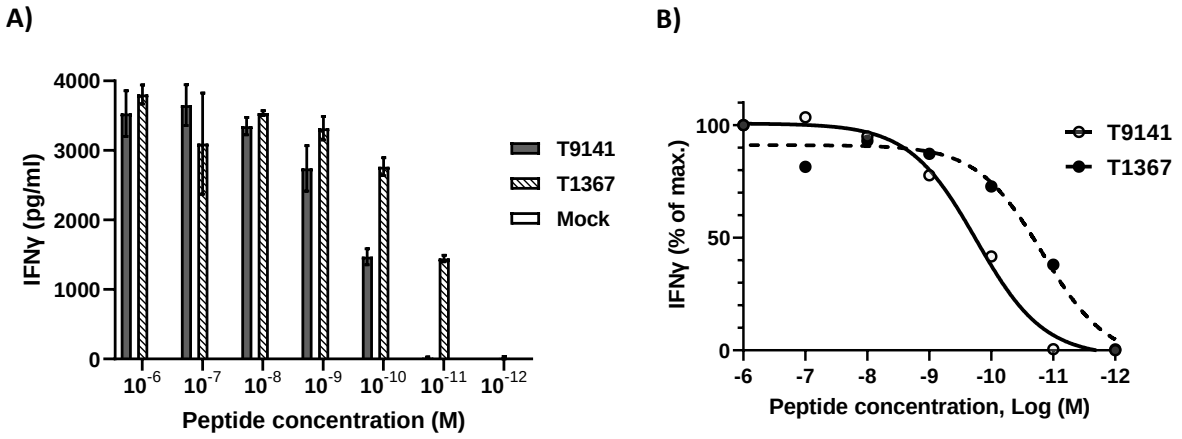
Peptide No.	Gene	Sequence with motif x-L-x-x-G-Q-Y-x-x	IC_{50} (nM)
1	ADIPL	QLQAGQYAS	7625
2	AGRF3	YLPQGQYLR	8587
3	**ARHG7**	**KLFQGQYRS**	**763**
4	**ARHG9**	**PLNHGQYLV**	**675**
5	BAZ2B	KLSSGQYPN	7237
6	**CB061/STPG4**	**QLSPGQYNV**	**42**
7	CD69	ALSVGQYNC	5880
8	CF132	DLRPGQYGQ	29026
9	CGAT1	QLRNGQYQA	6772
10	CP8B1	KLDFGQYAK	9424
11	CR018	GLPPGQYAT	1316
12	CSPG4	ALKNGQYWV	87
13	DAPLE	PLKPGQYVK	29108
14	**DYH14**	**GLPHGQYSV**	**19**
15	ERFE	NLTSGQYRA	639
16	ITB2	KLIYGQYCE	13182
17	LRP2	ILERGQYCK	23820
18	NU133	LLSLGQYLW	14094
19	OBSCN	TLREGQYVE	24626
20	**PE2R2**	**LLDYGQYVQ**	**9128**
21	**PE2R3**	**VLGVGQYTV**	**25**
22	PGBD1	LLERGQYPY	17117
23	PHF19	KLTEGQYVL	14
24	RRP5	KLKVGQYLN	24098
25	SARAF	FLSDGQYSP	389
26	SETD9	PLAVGQYVN	29358
27	SNX20	CLRAGQYPR	21596
28	**SYLM**	**RLPSGQYLQ**	**8970**
29	TBC31	ALTKGQYPV	9
30	TDRD3	QLHQGQYRS	16604
31	TENS3	KLSLGQYDN	20255
32	TM2D1	DLKVGQYIC	27915
33	TRI69	KLNLGQYKG	13467
34	**TRIM7**	**QLNGGQYWA**	**356**
35	ZAN	QLKNGQYGC	19404
36	ZMIZ2	YLQGGQYAP	2122

Appendix 3. HLA allotypes expressed by the different lymphoblastoid B cell lines (B-LCLs). Part of B-LCLs are homozygous in all MHC-I loci. Allele designations follow the 2004 HLA nomenclature report.

B-LCL	HLA-A*		HLA-B*		HLA-Cw*	
AMAI	68:02		53:01		04:01	
AMALA	02:17:01		15:01:01:01		03:03	
Bello	02:02	11:01	41:01	52:01	12:02	17:01
BM14	03:01		07:02		07:02	
BSM	02:01:01		15:010101		03:04:01	
DUCAF	30:02		18:01		05:01	
HOR	33:03:01		44:03:01		14:03	
JY	02:01		07:02:01		07:02:01:01	
KAS011	01:0101		37:01		06:02	
KAS116	24:02:01:01		51:01		12:03	
KE	02:01	29:02	44:03	44:05	02:02	16:01
KLO	02:08	01:01:01:01	08:01:01	50:01:01	07:01:01:01	06:02:01:02
SA	24:02:01:01		07:02:01		07:02	
SPO	02:01		44:02		05:01	
TAB089	02:07		46:01		01:02	
TISI	24:02:01:01		35:08		04:01	
VAVY	01:01		08:01		07:01	
W-01	03:01	24:02	15:01	35:01	03:03	04:01
W-02	02:01	26:01	38:01	44:02	38:01	44:02
W-03	02:01	23:01	15:01	58:01	03:04	07:01
WIN	01:01		57:01:01		06:02	
WT24	02:0101		27:0502		02:0202	
WT49	02:05:01		58:0101		07:18	
XLI-ND	02:10	30:01	13:02	40:06:01:01	06:02	08:01



Appendix 4. T9141 carrying minimal murinized human constant region (T9141-mmc) exhibited comparable expression and functionality to T9141. (A) Representative plots showing CD8 and dextramer staining as indicators of TCR-transduced human CD8 T cells. Numbers represent percentage of cells in the respective quadrant. (B) Mean fluorescence intensity (MFI) of dextramer labeled TCR-transduced T cells. The diagram represents means of two donors with SD. (C) IFNγ production of T9141-mmc and T9141-transduced human T cells after co-culturing with T2 cells loaded with ABL-E255V at 10^{-6} M to 10^{-12} M. Mean values of duplicate cultures with SD are shown. The results are representative of two independent experiments using PBMCs of different donors. (D) Responses of transduced human PBMCs were normalized to maximum IFNγ release.



Appendix 5. T9141 TCR exhibited one-log lower functional avidity compared to MAGE-A1-specific T1367 TCR. (A) IFNγ production of T9141- and T1367-transduced human T cells after co-culturing with T2 cells loaded with ABL-E255V or MAGE-A1 peptide at 10^{-6} M to 10^{-12} M. Mean values of duplicate cultures with SD are shown. The results are representative of three independent experiments using PBMCs of different donors. (B) Responses of transduced human PBMCs were normalized to maximum IFNγ release.

9. Abbreviations

ABL	Abelson gene
AFP	α-fetoprotein
ALL	acute lymphoid leukemia
AP	accelerated phase
APC	antigen-presenting cell
ATT	adoptive T cell therapy
B2m	β2-microglobulin
BCR	breakpoint cluster region gene
B-LCL	B lymphoblastoid cell line
BP	blast phase
CAR	chimeric antigen receptors
CDR	complementarity determining region
CEA	carcinoembryonic antigen
CML	chronic myeloid leukemia
CP	chronic phase
CRISPR	clustered regularly interspaced short palindromic repeats
CTL	cytotoxic T lymphocyte
ER	endoplasmic reticulum
ERAP	ER aminopeptidase
ERAAP	ER aminopeptidase associated with antigen processing
FCS	fetal calf serum
HLA	human leukocyte antigen
HDR	homology-directed repair
IEDB	Immune Epitope Database
IFN	interferon
IRES	internal ribosomal entry site
ITAM	immunoreceptor tyrosine-based activation motifs
LTR	long terminal repeat
MCA	methylcholanthrene
MHC	major histocompatibility complex
ORF	open reading frame
P2A	porcine teschovirus-1 2A peptide
PBMCs	peripheral blood mononuclear cells
Ph	Philadelphia chromosome
PLC	peptide-loading complex
PMA	Phorbol-12-myristate 13-acetate
pMHC	peptide-MHC complex
q-PCR	Quantitative PCR
RNP	ribonucleoprotein
SD	standard deviation
SMM	stabilized matrix method
TAA	tumor-associated antigen
TAP	transporter associated with antigen processing
TCR	T cell receptor
TIL	Tumor-infiltrating lymphocytes
TKI	tyrosine kinase inhibitor
TSA	tumor-specific antigen

10. Publications

Welters C, Lammoglia Cobo MF, Stein CA, **Hsu MT**, Ben Hamza A, Penter L, Chen X, Buccitelli C, Popp O, Mertins P, Dietze K, Bullinger L, Moosmann A, Blanc E, Beule D, Gerbitz A, Strobel J, Hackstein H, Rahn HP, Dornmair K, Blankenstein T, Hansmann L. Immune phenotypes and target antigens of clonally expanded bone marrow T cells in treatment-naïve multiple myeloma. Cancer Immunol Res. 2022 Sep 19:CIR-22-0434.

Hsu MT, Willimsky G, Hansmann L, Blankenstein T. T cell receptors against a drug-selected secondary mutation in BCR-ABL. (in progress)

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