Suitability of the TEL-AML1 Chromosomal Translocation for Targeting by Adoptive T Cell Therapy of Leukemia: An Investigation in a Novel Humanized Mouse Model

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1 Summary

Adoptive therapy with T cell receptor (TCR)-engineered T cells is a promising approach in cancer treatment. While usage of T cells specific for tumor-associated antigens (TAAs) can lead to serious side effects due to autoimmunity, targeting true tumor-specific mutations, such as the products of translocations in leukemias, should reduce such risk. A potentially ideal target might be the chimeric protein TEL-AML1, which results from the chromosomal translocation 12;21 and represents the most common fusion gene in childhood B cell precursor acute lymphoblastic leukemia (BCP-ALL). Within the fusion region of TEL-AML1, a single epitope has been described by reverse immunology as immunogenic in HLA-A*0201 restriction settings. Engineering T cells with a TCR, which would specifically recognize the epitope, should enable design of a widely applicable adoptive cell therapy protocol.

As a potential source of TCRs specific for the TEL-AML1 epitope we have used a novel mouse model, termed AB*ab*DII, expressing a human TCR-αβ repertoire and human major histocompatibility complex (MHC) class I. In order to show that AB*ab*DII mice present a valid model for investigation of human HLA-A*0201-restricted T cell epitopes, we tested if they could mount specific functional responses against some previously characterized epitopes derived from human tumor antigens. Specific responses could indeed be induced, arguing for existence of a functionally diverse human TCR repertoire in these mice. Additionally, we showed that effector T cells differentiating in the mice were functional, *bona fide* cytotoxic T lymphocytes (CTLs). Finally, we confirmed that AB*ab*DII mice, as shown previously for other murine models, were capable of correctly processing human HLA-A*0201-restricted epitopes. Therefore, we propose AB*ab*DII mice as a suitable model for testing immunogenicity of human HLA-A*0201-restricted epitopes.

The described TEL-AML1 peptide, however, could not prime specific CTLs in AB*ab*DII mice, the most probable reason being its unexpectedly low HLA-A*0201-binding affinity. This problem could be overcome by modifying an anchor-residue. The modification rendered the peptide immunogenic and the induced effectors retained the specificity for the native TEL-AML1 peptide. Surprisingly, however, we found that although specific functional $CD8^+$ T cell responses against the peptide could be evoked, the described epitope was in fact not endogenously processed. Analyses done with a potent antigen presenting cell line, as well as with purified human proteasomes, support the conclusion that this peptide cannot be proposed as a potential target in immunotherapy of ALL in HLA-A*0201-restricted fashion.

Further investigation showed that the TEL-AML1 fusion comprises no other HLA-A*0201-restricted epitopes, hence cannot be recognized as foreign by HLA-A*0201-restricted CTLs. Alternative target molecules for immunotherapy of BCP-ALL, possibly some of the newly characterized ALL-specific mutations should be considered. Based on our findings presented here, we propose that inducing specific CTLs might not be the limiting step in determining antigenic potential of such tumor-specific mutations. Testing whether a putative epitope would be naturally processed and presented for efficient recognition by specific effectors should, therefore, be performed with a relatively high specificity threshold set, to enable identification of genuine targets suitable for therapy.

2 Zusammenfassung

Adoptive Therapie mit T-Zellrezeptor (TCR, *engl*. T cell receptor)-gentechnisch ausgestatteten T-Zellen ist eine vielversprechende Krebsbehandlungsmethode. Die Verwendung von T-Zellen, die spezifisch für Tumor-assoziierte Antigene sind, kann jedoch zu ernsthaften Nebenwirkungen durch Autoimmunreaktion führen. Hingegen sollte ein Ansatz, bei dem man auf Tumor-spezifische Antigene, wie beispielsweise Produkte von Chromosomentranslokationen in Leukämien, abzielt, dieses Risiko reduzieren. Ein potentiell ideales Zielantigen könnte das chimäre Protein TEL-AML1 sein, das durch die Chromosomentranslokation 12;21 entsteht und das häufigste Fusionsgen in pädiatrischer Precursor-B-Zell-akuter-lymphoblastischer-Leukämie (BCP-ALL, *engl*. B cell precursor acute lymphoblastic leukemia) ist. Mittels “reverser Immunologie” ist innerhalb der TEL-AML1-Fusionsregion ein einziges Epitop beschrieben worden. Die Erkennung dieses Epitops war HLA-A*0201-restringiert. Gentechnische Ausstattung von T-Zellen mit einem TCR, der dieses Epitop spezifisch erkennt, sollte ein breit anwendbares Protokoll für adoptive Zelltherapie ermöglichen.

Als potentielle Quelle eines solchen TEL-AML1-Epitop-spezifischen TCR haben wir ein neues Mausmodell (AB*ab*DII genannt) verwendet, das ein humanes TCR-αβ-Repertoire und humane Haupthistokompatibilitätskomplex-Klasse-I (MHC class I, *engl*. major histocompatibility complex) Moleküle ausprägt. Um zu zeigen, dass AB*ab*DII Mäuse ein geeignetes Modellsystem für Untersuchungen humaner HLA-A*0201-restringierter T-Zell-Epitope sind, haben wir analysiert, ob spezifische funktionelle Reaktionen gegen bekannte humane Tumorantigen-abgeleitete Epitope in den Mäusen ausgelöst werden können. Spezifische Reaktionen gegen diese Antigene konnten tatsächlich induziert werden, was dafür spricht, dass AB*ab*DII Mäuse ein funktionell diverses Repertoire ausprägen. Außerdem konnten wir zeigen, dass Effektor-T-Zellen, die in den Mäusen differenzieren, funktionelle, bona fide zytotoxische T-Lymphozyten (CTLs, *engl*. cytotoxic T lymphocytes) sind. Schließlich haben wir bestätigt, dass AB*ab*DII Mäuse fähig sind, humane HLA-A*0201-restringierte Epitope korrekt zu prozessieren. Deshalb schlagen wir vor, dass AB*ab*DII Mäuse ein geeignetes Modell darstellen, die Immunogenität von humanen HLA-A*0201-restringierten Epitopen zu untersuchen.

Das beschriebene TEL-AML1 Epitop konnte jedoch keine spezifischen CTLs in AB*ab*DII Mäusen auslösen, vermutlich wegen der unerwartet niedrigen HLA-A*0201-Bindungsaffinität. Die Einführung einer Anker-Aminosäure erhöhte die Immunogenität des Peptids. Die induzierten Effektor-T-Zellen behielten die Spezifität für das native Peptid. Überraschenderweise, obwohl spezifische funktionelle $CD8^+$ T-Zell-Antworten gegen das Peptid induziert werden konnten, haben wir gefunden, dass das beschriebene Epitop nicht endogen prozessiert wird. Analysen, die sowohl mit effektiven Antigen-präsentierenden Zellen als auch mit gereinigten humanen Proteasomen durchgeführt wurden, befürworteten die Schlußfolgerung, dass dieses Peptid als potentielles HLA-A*0201-restringiertes Zielantigen für Immuntherapie nicht vorgeschlagen werden kann.

Weitere Untersuchungen haben gezeigt, dass das TEL-AML1 Fusionsprotein keine anderen HLA-A*0201-restringierten Epitope enthält, daher wird diese Mutation nicht als Fremdantigen von HLA-A*0201-restringierten CTLs erkannt. Alternative Ziel-Moleküle für eine BCP-ALL-Immuntherapie,

eventuell solche kürzlich charakterisierter ALL-spezifischer Mutationen, könnten in Betracht gezogen werden. Aufgrund der hier präsentierten Ergebnisse schlagen wir vor, dass das Auslösen spezifischer CTLs kein limitierender Faktor bei der Immunogenitätsbestimmung solcher Tumor-spezifischer Mutationen sein sollte. Analysen, die zeigen sollten, ob das mutmaßliche Epitop endogen prozessiert und präsentiert wird, um effizient von spezifischen Effektoren erkannt zu werden, sollten deshalb mit einer hohen Spezifitätsschwelle durchgeführt werden. Dies sollte ermöglichen, geeignete Zielantigene für adoptive T-Zelltherapie zu identifizieren.

3 Introduction

3.1 Tumors can be recognized by T lymphocytes

Neoplasm, or tumor, literally means “new growth”. Tumor cells have the ability to replicate autonomously; however, based on the manner in which they grow, one can make a distinction between benign and malignant tumors. A benign tumor is a growth that remains confined to its original location. A malignant tumor, referred to as *cancer*, is *invasive* - it infiltrates and thereby destroys adjacent normal tissue and *metastatic* - it spreads to distant organ sites through the circulatory system (Cooper 1995). There is substantial evidence that cancers are result of multiple sequential mutations, most of them being acquired (somatic mutations), but some being germline encoded (Schreiber 2003). While acquiring multiple mutations, a tumor progresses to its fully malignant phenotype.

Immunity is a reaction to foreign substances, including microbes, but also macromolecules, such as proteins and polysaccharides (Abbas and Lichtman 2003). Being mutated, i.e. foreign, cancerous cells should be recognized by immune system. Indeed, it has been established that immune system has the ability to recognize and destroy cancer cells. Moreover, among the plethora of immune mediators, T lymphocytes have been shown to be the main cells which mediate anti-tumor immunity. The existence of tumor-antigens and their recognition by cellular adaptive immunity was established in a series of tumor-transplantation studies with chemically induced tumors in the 1950s and 1960s. Study by Prehn and Main has shown that methylcholantrene (MCA)-induced sarcomas were antigenically distinct from the normal tissue of the host (Prehn and Main 1957). By transplanting the tumors in inbred mice, they succeeded in inducing tumor-specific immunity, referred to as isologous immunity (designating animals or tissues of supposedly identical genetic constitution). Moreover, they were able to show that this isologous tumor immunity was not a consequence of possible residual heterogeneity within the inbred mouse strains used. Namely, immunity against transplanted MCA-induced cancers could be induced only by implants of the tumor tissue, and not by the normal tissue derived from the very same animal from which the tumor originated. Conversely, tumor implants could not immunize against skin grafts. Their groundbreaking study showed that the tumor rejection-antigens were specific for the tumor tissue. One has to note, however, that the normal tissues used for attempted immunization against tumor implants consisted of spleen, liver, heart, lymph node and skeletal muscle; therefore, one still could not formally exclude the existence of residual heterozygosity, which could have had a stronger impact, if some other tissues had been used. Namely, different tissues may respond differently to weak differences in histocompatibility (Winn et al. 1958). Nevertheless, the formal exclusion of residual heterozygosis as a possible cause of tumor rejection in such transplantation experiments in inbred mice came from the study by Klein et al. (Klein et al. 1960). They showed that resistance against MCA-induced mouse sarcomas could be induced in the primary autochtonous host, after operative removal of the tumor followed by serial injection of irradiated tumor cells, and finally followed by challenge with viable tumor cells. Also, they could show

that lymph node cells, but not serum (even in the presence of complement), of an immunized isologous host were capable of neutralizing the sarcoma cells *in vitro*. These findings were further substantiated by Old et al. (Old 1962), who showed that intrastrain immunity to MCA-induced tumor could be transferred by injecting lymph node, spleen, or peritoneal cells mixed with tumor cells from the immune mice. Immune mice-derived serum could not confer immunity. Furthermore, this cellularly-transferred immunity was highly specific, since there was no cross-protection between different MCA-induced tumors from mice of the same inbred strain. Altogether, it became clear from these studies that the protection was mediated by adaptive immunity – it was specific; furthermore, it was mediated by cellular arm of adaptive immunity – T cells.

Further studies confirmed involvement of T cells in anti-tumor response in other tumor models. It was shown that UV-induced tumors completely regressed when transferred to normal syngeneic recipients, but mice immunosuppressed by thymectomy supported the growth of these tumors (Kripke 1974). Furthermore, the $CD8^+$ T cell subset seems to play a substantial role, since UV-induced regressor tumors grow progressively in the majority of $CD8^+$ T cell-depleted mice, whereas mice depleted of $CD4^+$ cells remain capable of rejecting the tumors (Ward et al. 1990). However, since cancer is not a single disease, it is most probable that different subsets of T cells play prominent roles in different types of cancer.

3.2 Autochthonous (primary) antigenic tumors are usually not spontaneously rejected

The studies described above established that tumors were perceived as antigenically foreign by the immune system. However, as emphasized by Klein et al. in their study, an important question remained: what was the explanation for the paradoxical finding that antigenically foreign cell clones could develop into a tumor in an animal and were not automatically eliminated by the immune response (Klein et al. 1960)? The study on UV-induced tumors cited above (Kripke 1974) left the same question open: how such highly antigenic tumors became established and grew in the primary host? Clearly, transplantable tumor models cannot answer this question, but sporadic (spontaneous) cancer models should be able to. An insightful model is a mouse model of sporadic cancer (LoxP-Tag) with a defined tumor-specific rejection antigen - SV40 T antigen (Willimsky and Blankenstein 2005). It enables one to analyze spontaneous immune response against tumors developing from single cells, and shows that a primary immunogenic tumor is unable to induce or sustain a protective immune response, but instead induces tolerance. Two postulated ways of tolerance induction in the LoxP-Tag model (Blankenstein 2007) are the following: (i) a large necrotic tumor releases high amount of antigen, however, under non-stimulatory conditions anergic $CD8^+$ T cells are induced, (ii) a small latent tumor releases persistently a small amount of antigen by apoptotic cells, and under such tolerogenic conditions non-functional T cell are induced or even deleted. Besides these two mechanisms, other possible scenarios can also explain outgrowth of primary immunogenic tumors (Blankenstein 2007), such as *sneaking through* – whereby a small tumor does not release enough antigen to be cross-presented by dendritic cells (DCs) leading to T cell ignorance, or *concomitant*

immunity – in order to release a sufficient amount of antigen, tumors have to grow to a size too large to be rejected even in the presence of functional endogenous T cells.
The bottom line is that, although the immune system has the ability to recognize tumors, this ability, due to any of the reasons described above, seems not to suffice for a primary tumor to be rejected. Therefore, one needs to manipulate the immune system in order to achieve autochtonous tumor eradication.

3.3 Adoptive T cell therapy

Immunotherapy of cancer through adoptive transfer of T cells might be a promising treatment approach (Schumacher and Restifo 2009), since it should allow overcoming inefficiencies of immune response to cancer described in the previous section – it should allow manipulating anti-cancer cellular immune response in a desirable way to achieve cancer rejection. This approach consists of obtaining a small number of tumor antigen-specific T cells, which can be manipulated *in vitro*: they can be tested for tumor recognition, expanded, activated free from endogenous inhibitory factors to obtain desired anti-tumor effector functions and transferred to cancer patients. Finally, it is also possible to manipulate the host prior to cell transfer to provide optimal environment for the transferred cells to exert their anti-cancer functions (Rosenberg et al. 2008).
While naturally occurring autologous tumor reactive cells can be grown from (some) patients with melanoma, for a wide variety of other cancers it would be desirable to artificially endow T cells with tumor antigen specificity, which is feasible by genetic engineering (Schumacher 2002). T lymphocytes recognize tumor antigens by the heterodimeric T cell receptor (TCR) expressed on their surface, which is composed of the TCR alpha and beta chains (Krogsgaard and Davis 2005). It has been shown that the transfer of TCR alpha and beta chain genes was necessary and sufficient to endow recipient T cells with the specificity of the donor cell (Dembic et al. 1986). TCR gene therapy, as a mode of adoptive cell therapy employing T cells engineered with a TCR of desired specificity, should therefore enable targeting virtually any tumor antigen by transferred T cells. Importantly, the choice of tumor antigen to be targeted by T cells might well be decisive for the success of adoptive cell therapy (Offringa 2009). If the tumor antigen recognized by the transferred cells is not expressed exclusively on tumor cells, but also on healthy tissues, an unwanted immune attack on that healthy tissue can occur.
The landmark study performed more than two decades ago (Kolb et al. 1990) showed the success and potential of adoptive immunotherapy approach: donor leukocytes could treat relapsed chronic myelogenous leukemia (CML). The target antigens recognized on host leukemic cells by the infused HLA-identical donor lymphocytes were minor histocompatibility antigens, not potential leukemia-specific antigens. Complete remission up to 91 weeks was achieved, with percentage of Philadelphia chromosome (the translocation characterizing CML) positive cells dropping to zero. However, graft-versus-host disease also developed (which could be treated by immunosuppression). Autologous tumor infiltrating lymphocytes (TILs) were also used as a source of T cells for adoptive transfer in

melanoma patients (Dudley et al. 2002). This led to objective clinical response in some patients, accompanied by autoimmune attack manifested as vitiligo and uveitis, since most of the infused T cells were specific for melanoma differentiation antigens Melan-A/MART-1 and gp100, which were also expressed on healthy melanocytes. Similarly, adoptive transfer of autologous peripheral lymphocytes genetically engineered to express TCRs specific for Melan-A/MART-1 and gp100 in melanoma patients led to a partial clinical response, accompanied by pathologies in the skin, retina and inner ear (Johnson et al. 2009). Targeting ERBB2, which is overexpressed in a variety of tumors, through chimeric antigen receptor (CAR)-transduced lymphocytes in a colon cancer patient resulted in a serious adverse event, due to recognition of low levels of ERBB2 on normal lung cells (Morgan et al. 2010). These studies point to the great potential of adoptively transferred T cells for destroying cancer cells, but they also reveal their equally great potential for inflicting damage to healthy tissues, if tissue-distribution pattern of their cognate antigens is not strictly tumor-specific.

3.4 Tumor antigens

Antigens recognized on tumor cells can be encoded by normal non-mutant cellular genes which are expressed by certain cancer cells, but also by at least one subset of normal adult cells. Therefore, such antigens are not strictly tumor specific and are designated as tumor-associated antigens (TAAs) (Schreiber 2003). An example is a tumor antigen MAGE-A1, which was initially reported to be encoded by a non-mutated cellular gene expressed only in melanoma and some other cancer types (van der Bruggen et al. 1991), but later found expressed in the testis (Takahashi et al. 1995). As such, it is a representative of *cancer-testis antigens,* or *oncospermatogonal antigens.* Nevertheless, its importance has been substantial, as the first human tumor antigen epitope identified was a nonapeptide derived exactly from MAGE-A1 (Traversari et al. 1992). MAGE-A1 belongs to a family of related genes, including MAGE-A10. MAGE-A10 is expressed in a variety of carcinomas, such as lung, bladder, head and neck and esophageal carcinomas as well as in melanomas; as is the case for MAGE-A1, it is also expressed in testis, and additionally in placenta (De Plaen et al. 1994; Huang et al. 1999). *Differentiation antigens* are another class of TAAs expressed on tumor cells, but also during at least some stage of differentiation on nonmalignant cells of the cell lineage from which the tumor developed. An example is Melan-A/MART-1, which is expressed on melanomas and normal melanocytes (Coulie et al. 1994; Kawakami et al. 1994a), or gp100 with the same pattern of expression (Kawakami et al. 1994b). *Oncofetal and carcinoembrionic antigens* are expressed on embryonic or fetal tissue, and aberrantly expressed on tumor cells as well; however, they are also present in low levels in nonmalignant, nonfetal adult tissues. Alpha-fetoprotein is an oncofetal antigen (Vollmer et al. 1999) deregulated in the majority of human hepatocellular carcinomas (HCCs) and produced normally by fetal liver, but also present in low levels in serum throughout life.

There exist, however, antigens encoded by mutant cellular genes expressed exclusively on cancer cells, designated as tumor-specific antigens (TSAs). An example is a cyclin-dependent kinase 4 (CDK4) mutant formed by a single nucleotide transition which translates into an amino acid

replacement, creating a novel HLA-A*0201-restricted epitope (Wolfel et al. 1995). Such epitopes would provide an exquisite target for T cells in the settings of adoptive cell transfer. This particular mutation has been identified in one additional melanoma patient tested. True tumor-specific mutations shared among a larger number of patients would enable design of widely applicable adoptive T cell therapy protocols.

3.5 Antigenic epitopes recognized by αβ T cell receptors are peptides

T cells recognize their antigens presented in the context of polymorphic major histocompatibility complex (MHC)-encoded molecules, i.e. they are MHC-restricted (Zinkernagel and Doherty 1974). Antigenic epitopes recognized by $CD8^+$ T cells are peptides bound to MHC class I molecules, in humans termed HLA molecules, shortened from human leukocyte antigen (Klein and Sato 2000). This was initially suggested when the crystal structure of an HLA class I allele, HLA-A2, was determined (Bjorkman et al. 1987a; Bjorkman et al. 1987b). The dimensions of the antigen binding site suggested that HLA molecule bound a peptide. This was consistent with the previous findings (Shimonkevitz et al. 1983) from an MHC class II-restricted model system, which showed that foreign antigens were not recognized as native three-dimensional structures by T cells, but were degraded intracellularly into short peptides, which was both necessary and sufficient condition for antigen presentation. Not long afterwards, naturally processed peptides presented on the cell surface by MHC class I molecules derived from minor histocompatibility antigens (Rotzschke et al. 1990b) and from viral antigens (Rotzschke et al. 1990a) were identified. By sequencing self-eluted peptides from MHC molecules, allele specific motifs were revealed (Falk et al. 1991). For example, ligands for HLA-A*0201 allele (designating a variant of serologically defined HLA-A2 molecule, which can be precisely determined by molecular typing) were found to be predominantly nonapeptides, but also decapeptides (Rammensee et al. 1995), both with Leu or Met at position 2 and Val or Leu at the carboxyl terminus (position 9 or 10), which appeared to be anchor residues. Crystal structure of a TCR-peptide/MHC complex, as first determined by Garcia et al. in 1996, precisely showed antigenic peptide bound to α1 and α2 domains of the MHC class I molecule (which is a heterodimer containing the polymorphic alpha chain comprising domains α1, α2 and α3 and the nonpolymorphic β_2-microglobulin (β_2m) chain), interacting with the antigen recognizing domain of the αβ TCR (consisting of variable portions of the two chains) (Garcia et al. 1996). Although the αβ TCR fully determines the specificity of a T cell for its antigen, by binding to the antigenic peptide presented in the α1/α2 peptide binding cleft of the MHC class I molecule, expression of CD8 molecules facilitates the interaction with its target cell (Dembic et al. 1986; Dembic et al. 1987). Indeed, while αβ TCR interacts with α1 and α2 domains of the MHC class I molecule, it has been shown that the CD8 co-receptor simultaneously binds to its α3 domain (Salter et al. 1990).

3.6 Processing of MHC class I-restricted peptide epitopes

Since αβ T cell receptor recognizes only short peptides, tumor antigen proteins have to be degraded into short peptide fragments and presented at the cell surface by MHC class I molecules to $CD8^+$ T cells. The key component in the cellular class I antigen processing pathway is the proteasome, an ATP-dependent, multisubunit protease (Kloetzel 2001). This was inferred from the fact that application of proteasome-specific inhibitors impaired assembly of MHC class I molecules and antigen presentation (Rock et al. 1994). The peptide aldehydes identified in this study inhibited proteasome-mediated degradation in intact cells, thus enabling analysis of the role of the proteasome in biological processes; prior to their findings, extralysosmal degradation of most cellular proteins could be prevented only by unselective inhibitors - metabolic poisons which depleted cells of ATP, and which therefore could not aid in studying physiological functions of proteasomal degradation pathway. The proteolytic active sites of the proteasome are found within the 20S core complex, while the 19S regulator contains ATPase subunits and non-ATPase substrate-binding subunits; the 20S and two 19S units together form the 26S proteasome (Kloetzel 2001). Most proteasome substrates are conjugated with a multi-ubiquitin chain, which is recognized by the proteasome and targets the proteins for degradation (Young et al. 1998). However, ubiquitylation is not an absolute prerequisite for class I antigen processing, as shown on the example of ovalbumin – epitopes derived from this protein could be efficiently presented even when ubiquitylation was prevented by methylating all the lysine residues in the protein (Carbone et al. 1989). Soon afterwards, it was shown that 26S proteasome indeed catalyzes a ubiquitin-independent proteolysis as well, as shown on the example of ornithine decarboxylase, the most rapidly turned over mammalian enzyme (Murakami et al. 1992). In any event, the peptides resulting from proteasomal degradation are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) complex (Elliott 1997). Peptide binding to MHC class I heterodimers stabilizes these molecules, and the resulting peptide/MHC complexes are transported to the cell surface through the Golgi apparatus, for presentation to $CD8^+$ T cells (Pamer and Cresswell 1998).

In addition to proteasomal processing of peptide epitopes, which is necessary for generation of carboxyl (C) termini of epitopes, cellular aminopeptidases can generate amino (N) termini, by trimming N-terminally extended peptide precursors that contain the correct C terminus of the given epitope (Mo et al. 1999).

3.7 Identification of T cell epitopes derived from tumor antigens

T cell epitopes derived from tumor antigens can be identified either by classical, direct approach starting from a pre-detected T cell response, or alternatively starting from a candidate tumor antigen sequence (Stevanovic 2002).

The classical immunological approach for identification of tumor antigen derived T cell epitopes is exemplified by identification of MAGE-A1 derived epitope (van der Bruggen et al. 1991; Traversari et

al. 1992). A cosmid library was prepared with cDNA from a melanoma cell line, and by transfecting smaller and smaller pools of cosmids into antigen-negative test cell line, the antigen which sensitized this cell line against the pre-existing CTL clone recognizing the original melanoma cell line was identified. Another direct immunological approach has been employed to identify an epitope derived from gp100 (Cox et al. 1994). Peptides naturally present on a melanoma cell line were eluted and tandem mass spectrometry was used to identify the exact peptide which reconstituted the epitope on the T2 cell line, when tested with the pre-existing melanoma-specific CTL lines.

An alternative approach (Stevanovic and Rammensee 1994) termed “reverse immunology” is based on defined HLA-allele-specific peptide motifs (Falk et al. 1991), which are used to predict an epitope out of a candidate tumor antigen sequence. The following steps are then performed (Stevanovic 2002; Viatte et al. 2006): the predicted peptide is synthesized and used to induce specific T cells, either *in vivo* by immunizing HLA-transgenic mice, or *in vitro* by stimulation of precursor T cells derived from healthy blood donors or human patients. The final proof that the identified HLA-binding and immunogenic peptide is a true tumor-derived epitope, which is naturally processed and presented on the surface of tumor cells for recognition by T cells, is obtained when the specific T cells induced are able to recognize tumor cells endogenously expressing the antigen.

3.8 HLA-A*0201 transgenic mice as models for characterization of HLA-A*0201-restricted T cell responses

Mouse models transgenic for HLA molecules have a great potential as tools for identification and characterization of human HLA-restricted epitopes, potentially tumor epitopes as well. Several mouse models transgenic for the HLA-A*0201 allele have been developed so far, which is found in large proportion of the human population, precise percentage depending on the particular ethnic group considered (Fernandez-Vina et al. 1992). However, HLA-A*0201-restricted responses were not always successfully induced in previously generated human class I transgenic mouse models. Mice generated by Epstein et al. (Epstein et al. 1989) could not mount influenza virus specific CTLs, although in humans the HLA-A*0201 antigen is an efficient restriction element for this virus. A possible explanation might have been the poor interaction between the mouse CD8 and the human HLA molecules. Indeed, $CD8^+$ T cell responses to the dominant human HLA-A*0201-restricted epitope derived from the influenza virus matrix protein were successfully induced in another model, A2.1/K^b mouse (Vitiello et al. 1991), which utilized a chimeric form of HLA-A*0201 molecule generated by Sherman and colleagues, consisting of α1 and α2 domains of HLA-A2 and α3, transmembrane and cytoplasmic regions of K^b (Irwin et al. 1989). Murine CD8 molecule interacted more efficiently with the class I molecule that contained the species-matched α3 domain, which was the most probable reason for successful induction of immune responses. $CD8^+$ T cells in A2.1/K^b mouse model are educated on human HLA-A*0201, as well as on mouse MHC class I. A further improvement came several years later when HHD mouse model was generated (Pascolo et al. 1997). This model utilizes another class I chimeric construct consisting of human β2m fused to HLA-A*0201 α1 and α2 domains, and to α3,

transmembrane and cytoplasmic regions of murine H-2D^b allele. Since β2m and mouse H-2D^b have been knocked out, mouse TCRs in this model are forced to use exclusively human HLA-A*0201 during their education and selection in thymus. However, although CD8$^+$ HLA-A*0201-restricted T cell responses against different human epitopes have been successfully induced in these previous models, probes used to detect specific responses in all of these models were mouse TCRs. A similar mouse model system which would utilize human TCRs might provide novel insights into T cell responses of even higher therapeutic relevance. Additionally, such human TCRs could potentially be isolated for therapeutic purposes, since they would not be immunogenic in humans.

3.9 AB*ab*DII mice as a model for characterization of HLA-A*0201-restricted T cell responses

AB*ab*DII mouse is a model generated in our group, engineered to express human TCRs restricted to HLA-A*0201 (Li et al. 2010). TCR-α and TCR-β genes comprise the V (variable), D (diversity; present only in the case of the β chain), J (joining) and C (constant) gene segments homologous to immunoglobulin (Ig) coding segments. They are separated in the germ line cells and most of the somatic cells, but in T cells the V, (D) and J gene segments are somatically recombined at the genomic level during T cell differentiation (Chien et al. 1984; Hedrick et al. 1984a; Hedrick et al. 1984b; Yanagi et al. 1984). This somatic recombination produces enormous clonal diversity among the developing T cells. AB*ab*DII mice are transgenic for the complete human TCR-α and TCR-β gene loci, which have been introduced via yeast artificial chromosomes and embryonic stem cell fusion. They are also deficient for murine TCR-α and TCR-β, since they have been crossed to Tcra-/- and Tcrb-/- mice, in which the corresponding murine constant gene segment loci have been knocked out. Additionally, they are transgenic for the above described HHD molecule (modified HLA-A*0201), and deficient for murine H-2D^b and β2m, since they have been crossed to HHD mice (alternative designation: HHDII mice) described above (Pascolo et al. 1997). The resulting mice are designated AB*ab*DII (capital letters designating knocked-in human TCR loci, lower letters designating deficient murine TCR loci, and DII pertaining to HHDII). Since CD8$^+$ T cells in AB*ab*DII mice express human TCRs educated exclusively on human class I molecule HLA-A*0201, these mice should represent an exquisite tool for testing the immunogenicity of potential HLA-A*0201-restricted tumor antigen epitopes and for isolation of therapeutic TCRs. However, the potential usefulness of this mouse model in characterizing class I restricted T cell responses against human antigens crucially depends on two factors: (i) on the ability of the mouse antigen processing and presentation machinery to generate ‘correct’ human epitopes, i.e. those which would be generated by the human antigen processing and presentation machinery and (ii) on the presence of a functional and diverse TCR repertoire, which will enable T cells to respond to these epitopes.

Regarding the first issue of whether the mouse processing and presentation machinery is capable of generating human antigen derived epitopes, there is little doubt left by the studies conducted so far that the answer is positive when HLA-A*0201-restricted epitopes are concerned. It was shown already in the study by Gomard et al. that murine cells expressing HLA-A*0201 and infected by human

influenza A virus were specifically lysed by HLA-A*0201-restricted human antiviral T cells (Gomard et al. 1986). This issue has also been addressed in all of the previously generated HLA transgenic mouse models. The original study in which the HHD mice were characterized reported that the mice were able to correctly process the influenza A matrix peptide which is the immunodominant HLA-A*0201-restricted epitope in humans (Pascolo et al. 1997). Later, it has also been shown that HHD mice were capable of correctly processing multiple hepatitis B virus (HBV) surface antigen derived epitopes which were described in the human infection (Loirat et al. 2000). DNA immunization of HHD mice with a melanoma polyepitope construct resulted in specific responses against human melanoma epitopes (Firat et al. 1999). An HLA-A*0201 transgenic mouse model was successfully used to predict endogenously processed HCV structural protein derived epitopes, which were recognized by human CTLs from HCV-infected patients (Shirai et al. 1995). A2.1/K^b mice could develop a CTL response towards the same influenza matrix protein epitope which was dominant in the equivalent human response (Vitiello et al. 1991). Furthermore, A2.1/K^b mice processed the same epitopes derived from human TAA gp100 as human antigen presenting cells (Yang et al. 2000). A2.1/K^b mice were successfully used for identification of major epitopes derived from Mycobacterium tuberculosis Ag85B that are recognized in humans (Geluk et al. 2000). $CD8^+$ T cells specific for vaccinia virus (VACV) derived peptides induced in A2.1/K^b mice recognized VACV-infected human cells - HLA-A*0201/K^b Jurkat cells (Pasquetto et al. 2005). Successful generation of human HLA-A*0201-restricted epitopes in mice should not be surprising, in view of the fact that the major differences between mouse and human processing machineries concern TAP specificities. Human TAP can translocate peptides with both hydrophobic and basic C termini, whereas mouse TAP prefers peptides with hydrophobic termini (Momburg et al. 1994). Therefore, this difference should be of no relevance in the case of HLA-A*0201 transgenic mouse models, since HLA-A*0201 ligands have hydrophobic C termini (Falk et al. 1991). Of note, even HLA-A11/K^b transgenic mice generated an A11/K^b-restricted CTL response following immunization with influenza virus A/PR/8/34, suggesting that they could, at least to some extent, process and present HLA-A*1101-restricted epitopes which have basic C termini. Indeed, the epitope identified in this study had a lysine at the C terminus (Alexander et al. 1997). Also, a minigene construct encoding nine human HBV or HIV CTL epitopes, six of them HLA-A*0201-restricted and three of them HLA-A*1101-restricted, induced CTL responses in both HLA-A2.1/K^b and HLA-A11/K^b transgenic mice (Ishioka et al. 1999).

In view of the described findings, we did not think it would be of the utmost importance to address the question of antigen processing pathway in AB*ab*DII mice as a separate issue, during the initial characterization of $CD8^+$ T cell responses in this new model. However, we did confirm that antigen processing and presentation pathways in mouse and human indeed are conserved, on the example of one human TAA in AB*ab*DII mice and additionally, on another human TAA using antigen presenting cells (APCs) which we generated from murine NIH fibroblasts, as will be described later in the text.

On the other hand, the issue regarding the presence of a functional and diverse TCR repertoire in AB*ab*DII mouse model has to be addressed since TCR-α and TCR-β gene loci determine the 'germline' TCR repertoire (which is therefore human in these mice), but they are not the sole factors which determine the TCR repertoire found in the periphery, i.e. outside the thymus where T cells

develop. After random assembly of V(D)J gene segments to form 'germline' TCRs (although they, strictly speaking, also include nucleotide additions and deletions not encoded in the germline), T cell repertoire is further shaped in the thymus by the processes of positive and negative selection (Bevan 1997). A subset of developing T cells reacting weakly to self-MHC/self-peptide complexes survives in the process of positive selection, while thymocytes that react too strongly with self-MHC/self-peptide complexes are subjected to death by negative selection. T cell clonotypes which survive should therefore be capable of efficiently recognizing self-MHC/foreign peptide complexes, while not being able to strongly react against self-MHC/self-peptide complexes. Since in AB*ab*DII mice, human TCRs expressed on $CD8^+$ T cells are selected on complexes composed of a single human class I molecule (the modified HLA-A*0201) and murine self-peptides, it is necessary to test whether this would have a significant functional impact on peripheral TCR repertoire. This issue regarding the presence of a functionally diverse TCR repertoire, which would enable T cells to respond to human antigen derived epitopes, we have addressed by asking the following question: could AB*ab*DII mice mount specific functional $CD8^+$ T cell responses against some of the known, previously described HLA-A*0201-restricted epitopes derived from human TAAs?

3.10 The TEL-AML1 translocation as a potential target in adoptive T cell therapy

As stressed earlier, adoptive T cell therapy, and particularly TCR gene therapy, might present a powerful mode of cancer treatment, if a TCR used would recognize a target antigen expressed exclusively on cancer cells. Such strictly specific tumor antigens might be generated as results of chromosomal translocations observed in human cancer. Either a gene segment from TCR or Ig loci is erroneously recombined with a proto-oncogene, thereby activating it, or the breaks occur within a gene on each chromosome involved, creating a fusion gene encoding a chimeric protein (Rabbitts 1994). Although one might think that translocations involving TCR or Ig loci would be prevalent since, as described earlier, these loci undergo normal, physiological somatic gene recombination during the generation of their antigen receptors (Chien et al. 1984), generation of tumor-specific fusion proteins is in fact more commonly observed (Rabbitts 1994). When such a translocation produces an in-frame fusion gene, this novel open reading frame (ORF) will code for a novel protein. The new amino acid sequence at the fusion site provides an opportunity for a novel epitope to be formed exclusively in a cancer cell, which might then be targeted by T cells, avoiding the potential risk of autoimmunity.

The TEL-AML1 (alternative designations: ETV6-AML1, ETV6-RUNX1) fusion protein is a result of the karyotypically cryptic t(12;21)(p13;q22) chromosomal rearrangement. The translocation has been cloned by two groups in 1995 (Golub et al. 1995; Romana et al. 1995a). From these and the studies conducted very soon afterwards (Romana et al. 1995b; Shurtleff et al. 1995) it became clear that this translocation was present exclusively in the leukemic cells of children diagnosed with B cell precursor acute lymphoblastic leukemia (BCP-ALL), also known as common ALL (cALL). Consistent with early pre-B stage of B cell maturation, these leukemic blasts were immunophenotyped as $CD10^+$, $CD19^+$, $CD22^+$, cytoplasmic IgM^+, surface Ig^- (Golub et al. 1995). None of the patients with acute myeloid

leukemia (AML) had this particular translocation. The exclusive detection of this mutation in the cases of childhood BCP-ALL immediately implicated it in the pathogenesis of this disease. Moreover, the two fusion partners, TEL and AML1, had been initially discovered as fused to other genes in chronic myelomonocytic or acute myeloid leukemias, respectively (Miyoshi et al. 1991; Golub et al. 1994). Both of the molecules were found to be transcription factors and seemed to have transforming potential when their function was deregulated/changed by fusion to other genes. Importantly, the precise sequence of the novel fusion protein was identified. Golub et al. employed ribonuclease protection assays to determine the exact translocation breakpoint – the nucleotide (nt) 1033 in the TEL transcripts. It was fused to nt 58 of the AML1 coding sequence (Golub et al. 1995). The resulting fusion sequence is shown in Fig. 1. This novel sequence obviously presents a potential source of putative novel fusion epitopes. Moreover, exactly the same nucleotide fusion sequence was found in the vast majority of patients investigated in all of the studies cited above. Namely, translocation breakpoints are preferentially clustered within the intron following the exon 5 of TEL and the intron preceding the exon 2 of AML1, so that subsequent mRNA splicing leads to generation of the identical transcripts (as shown in Fig. 1) and to translation of the identical fusion protein (Wiemels et al. 2000). This was highly significant in a sense that not only a potential mutated, i.e. specific, tumor antigen was discovered, but it was also shared among different patients. Indeed, the TEL-AML1 translocation was found to be the most common genetic lesion in pediatric ALL, present in 22% of patients (Shurtleff et al. 1995). This finding made it extremely attractive as a potential target in immune therapy protocols.

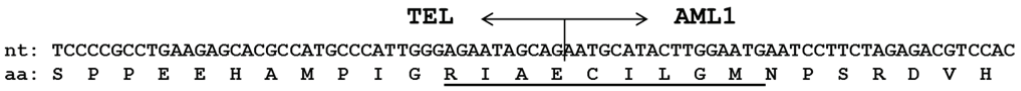


Figure 1. Sequence of the TEL-AML1 fusion region. The nucleotide (nt) and amino acid (aa) sequence spanning the TEL-AML1 fusion region are shown. The breakpoint occurs after the nt 1033 of TEL, which is fused to the nt 58 of AML1 in the in-frame fusion transcript coding for the chimeric protein (Golub et al. 1995). The sequence of the single previously described T cell epitope (Yotnda et al. 1998) derived from the TEL-AML1 fusion protein is underlined.

Besides being a specific and a shared tumor mutation, the third factor which makes the TEL-AML1 protein a promising target for TCR gene therapy is its role in the leukemogenesis process. As stated above, its exclusive appearance in childhood BCP-ALL correlated it with the pathogenesis of the disease, but numerous later studies directly confirmed its involvement in the transformation process. Studies of concordant cALL in twins, as well as retrospective analysis of Guthrie cards (neonatal blood spots) of non-twinned children with cALL showed that the TEL-AML1 fusion occured already in

utero (Greaves et al. 2003). This implied that the initiating, clinically covert TEL-AML1$^+$ preleukemic clone had to acquire additional genetic changes (Mullighan et al. 2007) to convert to frank leukemia after a relatively long latent period. However, it has been shown that TEL-AML1 may, as a single mutation, be sufficient to generate a population of preleukemic stem cells, which may act both as initiating and cancer-propagating cell in BCP-ALL (Hong et al. 2008). Another model proposed TEL-AML1 as the initiating lesion present already in hematopoietic stem cells (HSCs) before commitment to the lymphoid lineage, showing that TEL-AML1 expanded and sustained altered HSCs that were available for subsequent genetic insults (Schindler et al. 2009). In that model, TEL-AML1 was indeed shown to act as a cooperating oncogene with an additional, second hit (achieved experimentally by chemical mutagenesis). Being the crucial, initiating mutation in BCP-ALL, TEL-AML1 is not expected to be lost from leukemic cells easily, even under a strong selective pressure which would be imposed in the settings of adoptive T cell therapy targeting this mutation. Appearance of antigen-loss variants poses an inherent hurdle for cancer immunotherapy, and indeed for any kind of cancer therapy, for the following reason: if the targeted mutation is not essential for a malignant cell, the cell might, being genetically unstable, lose the expression of the mutation. Therefore it becomes obvious that TEL-AML1, being essential for the leukemic cell, is a potentially very suitable target.

Finally, apart from being an extremely promising potential target for adoptive T cell cancer therapy, an exact T cell epitope derived from the TEL-AML1 fusion protein has been identified (Figure 1) (Yotnda et al. 1998). This single TEL-AML1 derived epitope was HLA-A*0201-restricted. The authors used reverse immunology approach. First, a peptide derived from the known TEL-AML1 fusion sequence which bound well to HLA-A*0201 was identified. This peptide was subsequently used to induce CTL lines from a healthy donor and a patient, which recognized both patient's autologous leukemic cells and the leukemic cell line REH. Therefore, we set out to isolate a TCR recognizing this identified TEL-AML1 epitope. As a potential source of such a TCR we have used AB*ab*DII mice, which express HLA-A*0201-restricted human TCRs. Additionally, since the epitope in question was the only TEL-AML1 derived epitope known so far, we intended to characterize some new putative HLA-A*0201-restricted epitopes which might span the TEL-AML1 fusion region.

4 Aims

Prompted by the above discussed findings, we aimed at answering the following questions:

i. Do AB*ab*DII mice express a TCR repertoire that is functionally diverse enough to enable recognition of human epitopes?
ii. Can a specific functional response against the described TEL-AML1 epitope be induced in AB*ab*DII mice?
iii. Can TCRs recognizing this TEL-AML1 epitope be employed in TCR gene therapy of leukemia?
iv. Does the TEL-AML1 chimeric protein comprise some novel, yet unidentified, HLA-A*0201-restricted epitopes suitable for targeting by adoptive T cell therapy?

5 Materials and Methods

5.1 Peptides

The peptides used had purity of >95% and were purchased either from GenScript (Piscataway, NJ) or JPT (Berlin, Germany). Nomenclature refers to the position of the first amino acid of a peptide in its corresponding protein, as well as to the position of anchor-modification in the epitope. Anchor modifications in epitopes are underlined, as well as exact epitopes within the longer 30-mer peptides. The peptides were: MAGE-A10 derived epitope GLYDGMEHL (MAGE-A10$_{254}$), α-fetoprotein derived FMNKFIYEI (α-fetoprotein$_{158}$), anchor-modified peptides derived from gp100 YLEPGPVTV (gp100$_{280\text{-}9V}$, designated gp100) and SSGTLISRALVVTHTYLEPGPVTVQVVLQA (gp100$_{265\text{-}9V}$) , and from Melan-A/MART-1 ELAGIGILTV (Melan-A/MART-1$_{26\text{-}2L}$, designated Melan-A/MART-1). Also, the following TEL-AML1 derived peptides were used: TEL-AML1 RIAECILGM (TEL-AML1$_{334}$, designated TEL-AML1), anchor-modified TEL-AML1 RIAECILGV (TEL-AML1$_{334\text{-}9V}$, designated TEL-AML1-9V), anchor modified TEL-AML1 RLAECILGM (TEL-AML1$_{334\text{-}2L}$, designated TEL-AML1-2L), TEL-AML1$_{331}$ PIGRIAECIL, anchor-modified TEL-AML1$_{331\text{-}2L}$ PLGRIAECIL, long TEL-AML1 MVSVSPPEEHAMPIGRIAECILGMNPSRDV (TEL-AML1$_{319}$), long anchor-modified TEL-AML1-9V MVSVSPPEEHAMPIGRIAECILGVNPSRDV (TEL-AML1$_{319\text{-}9V}$) and long anchor-modified TEL-AML1$_{316\text{-}2L}$ NHIMVSVSPPEEHAMPLGRIAECILGMNPS. The long 30-mer peptides were deduced from the natural sequence of each protein, as previously described (Zwaveling et al. 2002). Unspecific peptides used for *in vitro* restimulation in control experiments were either Melan-A/MART-1 or NY-BR-1$_{960}$ SLSKILDTV (designated NY-BR-1). Peptides were stored at -20 °C, as 10^{-2} M stock solutions, diluted either in PBS or DMSO (Sigma-Aldrich, St. Louis, MO) according to their hydropathy indices calculated as described by Kyte and Doolittle (Kyte and Doolittle 1982).

5.2 Antibodies

The following antibodies were used: CD8a FITC (clone 53-6.7), CD3e APC (clone 145-2C11), purified CD16/CD32 (clone 2.4G2), CD3e Pacific Blue® (clone 500A2), CD8a PE Cy7 (clone 53-6.7), CD107a PE (clone 1D4B) (all BD Biosciences, Heidelberg, Germany), IFNγ PE (clone AN18.17.24; Miltenyi Biotec, Bergisch Gladbach, Germany), HLA-A2 Alexa Flour® 647 (clone BB7.2; AbD Serotec, Düsseldorf, Germany) and CD8 APC-eFluor® 780 (clone 53-6.7; eBioscience, San Diego, CA).

5.3 Cells

NIH/3T3 fibroblasts (CRL-1658™; American Type Culture Collection (ATCC), Manassas, VA) were cultured in DMEM with 10% FBS (PAN Biotech, Aidenbach, Germany) and 100 IU/ml Penicillin-Streptomycin. NIH/3T3 cells transduced to express HHD, HHD and TEL-AML1 minigene or HHD and Melan-A/MART-1 (designated NIH-HHD, NIH-HHD-TEL-AML1 and NIH-HHD-Melan-A/MART-1) were cultured in the same way as NIH/3T3 cells. Plat-E cells were cultured as described (Morita et al. 2000) in DMEM with 10% FBS and selection drugs blasticidin (10 µg/ml) and puromycin (1 µg/ml), but with addition of 10 µg/ml gentamicin and 50 µM β-mercaptoethanol. T2 cells (CRL-1992™; ATCC) were cultured in RPMI with 10% heat-inactivated FBS (PAN Biotech), 10 mM HEPES and 1% Penicillin-Streptomycin. Splenocytes and LN cells were cultured in RPMI with 10% heat-inactivated FBS (PAN Biotech), 1 mM sodium pyruvate, 100 µM MEM non-essential amino-acids, 50 µM β-mercaptoethanol and 10 µg/ml gentamicin. Dissociation of adherent cell lines (NIH/3T3 and its derivatives, as well as Plat-E) was performed with Trypsin/EDTA 0.05/0.02% (PAN-Biotech, Aidenbach, Germany). All cell culture reagents were purchased from Invitrogen (Darmstadt, Germany), unless otherwise stated.

5.4 Mice

AB*ab*DII mice are transgenic for entire human TCR-α and TCR-β gene loci, as well as for HHD molecule (Pascolo et al. 1997), and deficient for the murine TCR-α and -β chains, as well as for murine β2m and H2-D^b genes. The generation of AB*ab*DII mice has been described in detail (Li et al. 2010). Mice were genotyped by PCR using DNA isolated from tail biopsy tissue by alkaline lysis in the following manner: the tissue was incubated in 0.5 ml 0.05 M NaOH at 95 °C for 1 hour, and the lysis was stopped by adding 50 µl of 1 M Tris-Cl pH 8.0 with 10 mM EDTA (Sigma-Aldrich). Each PCR reaction was performed in a total volume of 10 µl, with 1 µl lysed DNA, 0.1 µl DNA Polymerase (FastStart Taq DNA Polymerase, Roche, Mannheim, Germany; only in the case of β2m specific reactions InnuTaq HOT-A DNA Polymerase, AnalytikJena, Jena, Germany, was used), 0.2 mM dNTPs (PCR Grade Nucleotide Mix, Roche) and 0.5 µM primers (forward + reverse), in the corresponding manufacturer's buffer. PCR genotyping primers are listed in Table 1. PCR thermal cycling conditions were the following: 1. Initial denaturation: 95 °C, 5 min; 2. Denaturation 95 °C, 30 s; 3. Annealing 64 °C, 40s; 4. Extension 72 °C, 1min 10 s; 5. Steps 2-4 performed 35 times; 6. Final extension 72 °C, 10 min. The mice used in the study were 2 to 6-month-old and were housed at the Max-Delbrück-Center animal facility. All animal experiments were approved by the Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit, Berlin, Germany.

Table 1

Primers	Forward primer sequence	Reverse primer sequence	Size (bp)
TRAV10	ATGGCAAAAACCAAGTGGAG	TTTGCTTTGTGTCTGCATCC	221
TRBV19	CACATTAGGCCAGGAGAAGC	CCTGCTTAGTGGCTGAGTGG	691
A2-1	HLA-A2 134 CTACAGAGCCTAGCAGGGTGTCCTTGGCAG	HLA-A2 385 CTCTGAGTTTCTGTGTGAGTCCAGGACATCTCC	303
A2-2	HLA-A2 964 CATTGAGACAGAGCGCTTGGCACAGAAGCAG	B2M 1332 GGATGACGTGAGTAAACCTGAATCTTTGGAGTACGC	435
Db WT	SMO 130 ATTGGGAGCGGGAAACACAG	SMO 131 TCCGACCCCAAGTCACAG	340
Db KO	Neo 771 TCGCCTTCTTGACGAGTTCT	Db-Intrn5 GCAGAGGCATGTGAATTTGA	878
B2m WT	SMO 126 GTCAGATATGTCCTTCAGCAAG	SMO 124 GATGCTGATCACATGTCTCG	657
B2m KO	Neo-mittle2 CTTGCCGAATATCATGGTGGAAAA T	SMO 124 GATGCTGATCACATGTCTCG	900
Tcra WT	oIMR0733 ACTGTGCTGGACATGAAAGC	oIMR0734 CCATAGATTTGAGCCAGGAGG	160
Tcra KO	KOneo3A TACCGGTGGATGTGGAATGT	oIMR0733 ACTGTGCTGGACATGAAAGC	164
Tcrb WT	TRBko5´-II TGAGCCATCAAAAGCAGAGA	TRBko3´-II GAAGTGGTTGCGAGGATTGT	219
Tcrb KO	KOneo3A TACCGGTGGATGTGGAATGT	TRB-3B TTCTAGACCCCCACCTAGAGC	248

Table 1. AB*ab*DII mouse genotyping primers. Primers used for detection of the transgenes - human TCR-α (TRAV10), TCR-β (TRBV19), HHD (A2-1 and A2-2), as well as for confirming the gene-deficiencies in murine TCR-α (Tcra), TCR-β (Tcrb), H2-D^b (Db) and β2m (B2m) loci are shown.

5.5 Construction of retroviral vectors for expression of the TEL-AML1 minigene and Melan-A/MART-1

The nucleotide sequence comprising the TEL-AML1 fusion region (TEL-AML1 minigene): atggtctctgtctccccgcctgaagagcacgccatgcccattgggagaatagcaGaatgcatacttggaatgaatccttctagagacgtc (upper case – the fusion nucleotide, underlined – the sequence corresponding to the TEL-AML1 peptide), coding for 30 amino acids of the fusion protein, was cloned as an open reading frame (ORF) comprising enhanced green fluorescent protein (EGFP) and a yeast ubiquitin (Ub) moiety into the retroviral vector pMP71 (Engels et al. 2003). This was done in the following way: the TEL-AML1 minigene was first subcloned into the lentiviral vector pRRL.CMV.GFP.sin18 (Garcia Casado et al. 2008), which was based on Ub/protein/reference (UPR) technique (Levy et al. 1996) and contained EGFP and the yeast Ub moiety in an ORF with tumor antigen NY-ESO-1, while NY-ESO-1 was excised. More precisely, a DNA fragment comprising the sequence of TEL-AML1 and additional 185 bp corresponding to the Ub sequence (placed upstream of the TEL-AML1): cggtaaaaccaatgcattggaagttgaatcttccgataccatcgacaacgttaagtcgaaaattcaagacaaggaaggtatccctccagatca acaaagattgatctttgccggtaggcagctagaaggcggtagaacgctgtctgattacaacattcagaaggagtccaccttacatcttgtgctaa ggctccgcggtggcatggtctctgtctccccgcctgaagagcacgccatgcccattgggagaatagcagaatgcatacttggaatgaatccttc tagagacgtctaaaggatccgcg was digested with BstXI and BamHI restriction enzymes (Fermentas, St. Leon-Rot, Germany) out of the purchased carrying vector pMA (Geneart, Regensburg, Germany). The restriction was performed in a 30 µl volume reaction, with approximately 2.5 µg DNA. Lentiviral vector pRRL.CMV.GFP.sin18 was similarly digested with the same enzymes and dephosphorylated with 1U calf intestine alkaline phosphatase (Roche). Restriction fragments were separated by agarose gel electrophoresis and purified using DNA purification kit (Easy Pure, Biozym, Hessisch Oldendorf, Germany). The TEL-AML1 containing fragment and linearized pRRL.CMV.GFP.sin18 vector were ligated in the ratio of 3:1 (molecules) with T4 DNA Ligase (Rapid DNA Ligation Kit, Roche), according to the manufacturer's instruction. The resulting construct was transformed into XL10-Gold Ultracompetent bacteria for amplification, according to manufacturer's instruction (Stratagene, Agilent Technologies, Waldbronn, Germany). Plasmid DNA was isolated by using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), and afterwards in larger quantities with QIAGEN Plasmid Maxi Kit. The resulting construct contained EGFP-Ub-TEL-AML1 in a single ORF and was designated pRRL.CMV.GFP.sin18-TEL-AML1. The sequence was verified by restriction analysis with BstXI and BamHI, and by sequence analysis with sequencing primer seqEGFPdown_fwd (Table 2). Next, the ORF EGFP-Ub-TEL-AML1 (1131 bp in length) was cloned into pMP71 in the following way: the whole ORF was amplified from pRRL.CMV.GFP.sin18-TEL-AML1 plasmid (0.5 µg) with 0.5 µl Phusion® Hot Start High-Fidelity Polymerase (Finnzymes, Espoo, Finland) and 0.5 µM primers designed to introduce NotI and EcoRI restriction sites (forward primer fwd_EGFP-up and reverse primer rv_TEL-AML1-down; Table 2) in a 50 µl PCR reaction, in the supplemented manufacturer's buffer (Finnzymes), with 0.2 mM DNTPs (PCR Grade Nucleotide Mix, Roche). The PCR temperature cycles were designed as follows: 1. Initial denaturation: 98 °C, 30 s; 2. Denaturation 98 °C, 10 s; 3. Annealing 58 °C, 10s; 4. Extension 72 °C, 20s; 5. Steps 2-4 performed 35 times; 6. Final extension 72

°C, 5 min. The amplified DNA was precipitated by ethanol and subsequently digested with NotI and EcoRI (New England Biolabs, Frankfurt, Germany) in a 30 µl digestion reaction. pMP71 plasmid (2.5 µg) was similarly digested with the same restriction enzymes (whereby EGFP present in the vector was excised), dephosphorylated, and the linearized pMP71 vector and EGFP-Ub-TEL-AML1 fragment were ligated, amplified and isolated as described above for pRRL.CMV.GFP.sin18-TEL-AML1. The sequence of the resulting construct designated pMP71-TEL-AML1 was verified by restriction analysis with SpeI (New England Biolabs), and by sequence analysis with the sequencing primers: forward primer seqLEADfwd and reverse primer seqPRErev (Table 2).

The nucleotide sequence of Melan-A/MART-1 (comprising the anchor-modified decamer epitope - underlined):

atgccaagagaagatgctcacttcatctatggttaccccaagaaggggcatggccactcttacaccacggctgaagagctcgctgggatcgg catcctgacagtgatcctgggagtcttactgctcatcggctgttggtattgtagaagacgaaatggatacagagccttgatggataaaagtcttca tgttggcactcaatgtgccttaacaagaagatgcccacaagaagggtttgatcatcgggacagcaaagtgtctcttcaagagaaaaactgtga acctgtggttcccaatgctccacctgcttatgagaaactctctgcagaacagtcaccaccaccttattcaccttaa was cloned into the pMP71 in a similar way: an ORF (1395 bp) consisting of EGFP, Ub and Melan-A/MART-1 was PCR-amplified (Phusion® Hot Start High-Fidelity Polymerase, Finnzymes) from the lentiviral vector pRRL.CMV.GFP.sin18 (Chapatte et al. 2006) with primers designed to introduce NotI and EcoRI restriction sites (forward primer fwd_EGFP-up and reverse primer rv_Melan-A-down; Table 2) in the 50 µl PCR reaction, as described above, with slightly changed temperature cycling conditions: 1. Initial denaturation: 98 °C, 30 s; 2. Denaturation 98 °C, 10 s; 3. Annealing 58 °C, 10s; 4. Extension 72 °C, 30s; 5. Steps 2-4 performed 35 times; 6. Final extension 72 °C, 5 min. The extension time is prolonged in order to enable complete synthesis of the longer 1395 bp EGFP-Ub-Melan-A/MART-1 segment (in comparison to 1131 bp of EGFP-Ub-TEL-AML1). The ORF and pMP71 vector were subsequently NotI/EcoRI digested, ligated and amplified as described above for the TEL-AML1 fragment. The sequence of the resulting construct designated pMP71-Melan-A/MART-1 was verified in the same way by restriction analysis with SpeI, and by sequence analysis with the sequencing primers: forward - seqLEADfwd and reverse – seqPRErev.

Sequences of the primers used for cloning and sequencing are listed in Table 2. All primers were synthesized by Eurofins MWG Operon, Martinsried, Germany.

Table 2

Name	Sequence	Used for
fwd_EGFP-up	ATGCGGCCGCC ACCATGGTGAG CAAGGGCG	cloning of ORF EGFP-Ub-TEL-AML1 from pRRL.CMV.GFP.sin18-TEL-AML1 into pMP71-TEL-AML1; cloning of ORF EGFP-Ub-Melan-A/MART-1 from pRRL.CMV.GFP.sin18 into pMP71-Melan-A/MART-1
rv_TEL-AML1-down	ATGAATTCTTAG ACGTCTCTAGAA GGAT	cloning of ORF EGFP-Ub-TEL-AML1 from pRRL.CMV.GFP.sin18-TEL-AML1 into pMP71-TEL-AML1
rv_Melan-A-down	ATGAATTCTTAA GGTGAATAAGGT GGTGGT	cloning of ORF EGFP-Ub-Melan-A/MART-1 from pRRL.CMV.GFP.sin18 into pMP71-Melan-A/MART-1
seqEGFPdown_fwd	CGATCACATGGT CCTGCTGG	sequencing of pRRL.CMV.GFP.sin18 and pRRL.CMV.GFP.sin18-TEL-AML1
seqCMVPROMdown_fwd	AACAACTCCGCC CCATTGAC	sequencing of pRRL.CMV.GFP.sin18
seqLEADfwd	CAGCATCGTTCT GTGTTGTCT	sequencing of pMP71-TEL-AML1 and pMP71-Melan-A/MART-1
seqPRErev	CATTTAAATGTA TACCCAAATCAA	sequencing of pMP71-TEL-AML1 and pMP71-Melan-A/MART-1

Table 2. Cloning and sequencing primers. Primers used for construction of plasmids pRRL.CMV.GFP.sin18-TEL-AML1, pMP71-TEL-AML1 and pMP71-Melan-A/MART-1, as well as for sequencing of these and the original pRRL.CMV.GFP.sin18 plasmids are shown.

5.6 Generation of NIH-HHD, NIH-HHD-TEL-AML1 and NIH-HHD-Melan-A/MART-1 cell lines

NIH/3T3 cells were retrovirally transduced to express HHD, HHD and TEL-AML1 minigene or HHD and Melan-A/MART-1.

5.6.1 Transfection of packaging cells

To produce retrovirus-containing supernatant, ecotropic Plat-E packaging cells were transfected either with pMP71-HHD, pMP71-TEL-AML1 or pMP71-Melan-A/MART-1 in the following way: the cells were plated at 1 x 10^6 pro well (in 6-well plates) in 2 ml of the medium without the selection drugs. On the next day, 3 µg DNA was added, dissolved in 500 µl Opti-MEM medium (Invitrogen) with the addition of 10 µl Lipofectamin 2000 (Invitrogen). 6 hours later, after the transfection was completed, the medium was exchanged for fresh NIH/3T3 cell medium. Two days later, the viral supernatant was collected and filtered through 0.45 µm filters. Cells transfected with pMP71-TEL-AML1 or pMP71-Melan-A/MART-1 could be visualized (due to EGFP expression) by fluorescence microscopy, using *Olympus FSX100.*

5.6.2 Retroviral transduction and cell sorting

NIH/3T3 fibroblasts were plated at 5 x 10^4 cells per ml in 24-well plates one day before the collection of the viral supernatant and on the following day spinoculated (32° C, 800 g, 90 min) with the viral supernatant, 1 ml in total, containing retroviral MP71-HHD, MP71-HHD + MP71-TEL-AML1 or MP71-HHD + MP71-Melan-A/MART-1 particles, with the addition of 4 µg/ml polybrene (Sigma-Aldrich). The spinoculation procedure was repeated on the following day. The resulting cells were designated NIH-HHD, NIH-HHD-TEL-AML1 and NIH-HHD-Melan-A/MART-1. Expression of TEL-AML1 and Melan-A/MART-1 was measured by determining the level of EGFP expression by flow cytometry, and expression of HHD was measured after staining with HLA-A2 specific antibody (approximately 0.5 x 10^6 cells were stained with 2 µl of antibody in 50 µl PBS) also by flow cytometry. After expanding the cells for approximately two weeks, they were stained for HHD using anti-HLA-A2 antibody and sorted based on HHD and EGFP expression to more than 97% purity on BD FACSAria II. Cells were sorted in PBS supplemented with 1% FCS, 1.75 µg/ml Fungizone (Invitrogen) and 3x Antibiotic/Antimycotic (Invitrogen).

5.7 Peptide immunization

Mice were immunized s.c. in the tail base either with 100 nmol (~100 µg) of the nonamer peptides, 50 nmol (~50 µg) of the highly immunogenic decamer peptide Melan-A/MART-1, or 40 nmol (125 – 130 µg) of the long peptides, diluted in 100 µl PBS with ≤10% v/v DMSO, mixed with 50 µg CpG 1826 oligodeoxynucleotides (CpG ODN) (TIB MOLBIOL, Berlin, Germany) and emulsified in 100 µl of incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, St. Louis, MO). The total volume injected was 200 µl pro mouse. Some of the mice were subsequently boosted once or twice with the same peptide emulsions as used for the initial immunization in two-three weeks intervals. Pooled splenocytes and inguinal LN cells were analyzed from each mouse separately 10-14 days after the immunization/boosting. Unspecific peptides used for *in vitro* restimulation in control experiments were either Melan-A/MART-1 or NY-BR-1_{960} SLSKILDTV (designated NY-BR-1).

5.8 In vitro peptide stimulation and intracellular cytokine staining

Analysis of T cell function by measuring effector cytokine production was performed in the following way: spleen and inguinal LNs were isolated from immunized AB*ab*DII mice, prepared as single cell suspensions in the splenocyte medium and cultured from each mouse separately. Erythrocytes were lysed with ACK lysing buffer (Lonza, Walkersville, MD). Lysis was performed with 2 ml of the lysing buffer per one spleen and pair of isolated LNs, for 2 min at room temperature, before it was stopped with 30 ml medium. Pooled splenocytes and inguinal LN cells were washed two times and counted using Neubauer hemocytometer (dead cells were excluded by trypan blue staining). 1 x 10^6 cells / 200

µl / well were incubated overnight in 96-well plates either with 1 µM nonamer peptide (specific or irrelevant peptide) or 25 µl CD3/CD28 Dynabeads® (Invitrogen) (referred to as anti-CD3/CD28 antibodies in the further text). 1 µl/ml GolgiPlug™ (containing brefeldin A; BD Biosciences) was added one hour after beginning of the stimulation. On the following day, cells were fixed using Cytofix/Cytoperm Plus Kit (BD Biosciences) and stained for CD8, CD3 and IFN-γ. Precisely, purified CD16/CD32 antibody (0.25 µg per 1 x 10^6 cells) was first applied for 10 min at 4 °C to achieve Fc receptor blocking, the cells were then stained with CD8a FITC (0.5 µg per 1 x 10^6 cells in 50 µl PBS) for 15 min at 4 °C, washed with PBS two times and then permeabilized with 100 µl/well of the Fixation/Permeabilization solution for 20 min at 4 °C. Afterwards, they were washed twice with BD Perm/Wash™ buffer and stained intracellularly with CD3e APC (0.1 µg per 1 x 10^6 cells) and IFN-γ PE (1 µl per 1 x 10^6 cells) diluted in 50 µl BD Perm/Wash™ buffer for 30 min at 4 °C. After two final washes with BD Perm/Wash™ buffer, cells were resuspended in 200 µl PBS and analyzed by flow cytometry.

5.9 CD107a mobilization assay

Induction of effector CTLs capable of releasing cytolytic granules was measured in the CD107a mobilization assay as described (Betts et al. 2003; McElroy et al. 2007), with minor modifications. Spleens and inguinal LNs were isolated from immunized AB*ab*DII mice, prepared as single cell suspensions and cultured from each mouse separately. Erythrocytes were lysed, pooled splenocytes and inguinal LN cells were washed and counted, as described above (section 5.8). 1 x 10^6 cells / 200 µl / well were incubated for 6 hours in 96-well plates either with 1 µM nonamer peptide (specific or irrelevant peptide) or 25 µl CD3/CD28 Dynabeads® (Invitrogen). PE-conjugated anti-CD107a antibody (BD Biosciences) was present during the whole period of stimulation, at the final concentration 5 µl/ml (1 µg per 1 x 10^6 cells). 1 µl/ml GolgiStop™ (containing monensin; BD Biosciences) was added one hour after the beginning of stimulation. Cells were subsequently washed, incubated with purified CD16/CD32 antibody (0.25 µg per 1 x 10^6 cells) for 10 min at 4 °C, and then stained with CD8a FITC (0.5 µg per 1 x 10^6 cells) and CD3e APC (0.2 µg per 1 x 10^6 cells)) for 15 min at 4 °C in 50 µl PBS. The cells were subsequently washed with PBS, resuspended in 200 µl PBS and analyzed by flow cytometry.

5.10 Coculture experiments

For peptide loading experiments, NIH-HHD cells were trypsinized, washed and incubated in serum-free medium with 10^{-5} M peptide at room temperature for 4h. Afterwards, they were washed 3 times, resuspended in the splenocyte medium and 2 x 10^5 cells were cultured overnight with 1 x 10^6 pooled splenocytes and LN cells in 200 µl total volume in 96-well plates. NIH-HHD-TEL-AML1 and NIH-HHD-

Melan-A/MART-1 cells, as well as NIH-HHD not loaded with peptides were plated with splenocytes and LN cells in the same fashion. GolgiPlug™ was added one hour after plating the cells, and T cell function was analyzed on the next day by intracellular cytokine staining as described for peptide stimulation experiments, but with different combination of colors: either CD8 APC-eFluor® 780, CD3e Pacific Blue® and IFNγ PE, or CD8a PE Cy7, CD3e APC and IFNγ PE. These modified color combinations were used in order to prevent spectral overlapping with EGFP-transduced NIH/3T3 fibroblasts. The following amounts of antibodies were used: 0.05 µg of CD8 APC-eFluor® / 50 µl / 1 x 10^6 cells, 0.1 µg of CD3e Pacific Blue® / 50 µ / 1 x 10^6 cells and 0.025 µg of CD8a PE Cy7 / 50 µl / 1 x 10^6 cells.

5.11 Magnetic sorting of $CD8^+$ T cells

Spleen and inguinal LNs were isolated from immunized AB*ab*DII mice and prepared as single cell suspension in PBS. $CD8^+$ T cells were then negatively magnetically sorted using $CD8^+$ T Cell Isolation Kit and autoMACS Separator (Miltenyi Biotec). More precisely, pooled splenocytes and LNs were resuspended in autoMACS Running Buffer and labeled with the biotin-antibody cocktail, containing antibodies against CD4 (L3T4), CD11b (Mac-1), CD45R (B220), DX5 and Ter-119. Next, anti-biotin monoclonal antibodies conjugated to magnetic MicroBeads were added, and after incubation at 4 °C, the cells were washed and non-$CD8^+$ T cells were depleted in autoMACS Separator, according to the manufacturer's instruction.

5.12 T2 cell assay

HLA-A*0201 stabilization assay was done using the T2 cell line as described (Casati et al. 2003), with minor modifications. T2 cells are HLA-A*0201 positive and transporter associated with antigen processing (TAP) negative human cells expressing unstable HLA-A*0201 molecules, which can be stabilized at the cell surface by providing exogenous HLA-A*0201 binding peptide. The cells (1 x 10^6 / ml) were incubated overnight at 28 °C in medium containing 1 x 10^{-5} M peptide TEL-AML1, TEL-AML1-2L, TEL-AML1-9V, TEL-$AML1_{331}$, TEL-$AML1_{331\text{-}2L}$ or Melan-A/MART-1, or left uncoated. On the next day, the cells were transferred to 37 °C, an aliquot (0.5 ml) was taken at the time point zero, the cells were then washed with the medium without FBS to remove free peptides and aliquots were taken during a period of six hours. The aliquots were stained for HLA-A*0201 (2 µl of antibody per 0.5 x 10^6 cells in 50 µl PBS), washed with PBS and resuspended in 200 µl PBS for determination of MFI by flow cytometry. Fluorescence index (FI) was calculated at the time point zero according to formula FI = MFI [T2 cells with peptide] / MFI [T2 cells without peptide] – 1.

5.13 Flow cytometry analysis

Multiparametric flow cytometry analysis was performed on BD FACScalibur or FACScanto II. Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

5.14 Proteasome purification, reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry (MS)[1]

Proteasomes were isolated following standard procedures (Kuckelkorn et al. 2002) from LCL (lymphoblastoid cell lines), which are human B lymphocytes immortalized with EBV mostly expressing active immunoproteasomes. The purity of 20S proteasome preparation was verified by SDS-PAGE, using 12.5% polyacrylamide gel stained with Coomassie dye.

In vitro digestion of TEL-AML1-319 peptide and a control peptide listeriolysin O (designated LLO-291; sequence AYISSVAYGRQVYLKLSTNSHSTKVKA) was performed as described (Kuckelkorn et al. 2002). 1 µg of proteasome was incubated with 10 µg of peptide in a final volume of 100 µl of 20 mM Hepes, pH 7.8, 2 mM magnesium acetate, 2 mM dithiothreitol for the indicated times at 37 °C. Digests were stopped with 0.1 volume of trifluoroacetic acid. All digests were repeated at least three times.

Peptide analysis and quantification were done as follows: samples were analyzed by reverse-phase HPLC, using an HP1100 system (Hewlett-Packard, Palo Alto, CA) equipped with an RPC C2/C18 SC 2.1/10 column (GE Healthcare, München, Germany). Analysis was performed online with an LCQ ion trap MS equipped with an electrospray ion source (ThermoQuest, Bremen, Germany) (Sun et al. 2002). Ion counts of each reaction were normalized to the 9GPS standard peptide, which was added in equal amounts prior to analysis (Kuckelkorn et al. 2002).

[1] Experiments performed using the methods described in this section are the result of cooperation with Prof. Peter Michael Kloetzel, Institute of Biochemistry, Medical Faculty, Charité, Berlin, Germany.

6 Results

6.1 CD8$^+$ T cell responses specific for diverse human tumor antigens can be induced in AB*ab*DII mice

In order to test if AB*ab*DII mice express functional and diverse TCR repertoire, we asked whether they could mount functional responses against three known human TAAs: alpha-fetoprotein, MAGE-A10 and gp100. In the cases of all three TAAs, HLA-A*0201-restricted epitopes derived from these antigens had been identified. Importantly, their sequences differ from the corresponding sequences found in the homologous murine proteins (Fig. 2).

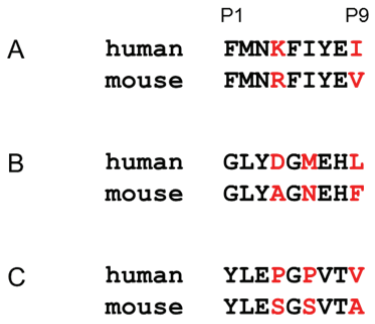


Figure 2. Sequences of the human TAA-derived peptide epitopes compared to the corresponding sequences found in the homologous murine proteins. Sequences of (A) α-fetoprotein$_{158}$, (B) MAGE-A10$_{254}$ and (C) gp100$_{280\text{-}9V}$ epitopes are shown. The nomenclature refers to the amino acid positions of the epitopes within the corresponding human TAA proteins. Differing amino acids are shown in red. Positions 1 (P1) to 3 and 9 (P9) are primary and secondary anchor residues for binding to HLA-A*0201; positions 4 to 8 are directly involved in TCR recognition (Parkhurst et al. 1996). Note that the human α-fetoprotein$_{158}$ has a single residue primarily recognized by TCR which is different from the mouse residue (position 4); furthermore, the discordant amino acid is a conservative substitution. The gp100$_{280\text{-}9V}$ peptide is anchor-modified at the position P9 (as explained in the further text, section 6.1.3); the native human gp100 sequence has an alanine residue at P9.

6.1.1 AB*ab*DII mice mount CD8$^+$ T cell responses specific for α-fetoprotein

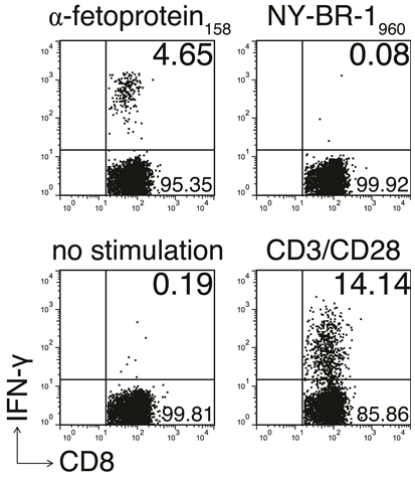
It has been previously shown that the nonamer α-fetoprotein$_{158}$ epitope could be used to induce human T cells specific for HLA-A*0201$^+$/ α-fetoprotein$^+$ tumors cells (Butterfield et al. 2001). AB*ab*DII mice were therefore immunized with α-fetoprotein$_{158}$ peptide emulsified in IFA and mixed with CpG

ODN. The combination of IFA and CpG ODN has been described as very effective in inducing CTLs against a human TAA, Melan-A/MART-1 (Miconnet et al. 2002). Immune responses were tested ten days after the immunization or boosting (some of the mice were additionally boosted with the same mixture of peptides in adjuvants). Splenocytes and draining LNs were isolated and restimulated *in vitro* overnight with 10^{-6} M α-fetoprotein$_{158}$ peptide. Functionality of $CD8^+$ T cells was detected by intracellular IFN-γ staining of the stimulated cells, accompanied with staining for CD3 and CD8, to enable distinguishing between different cellular populations and determining whether IFN-γ was secreted exclusively by $CD8^+$ T cells. Figure 3A shows flow cytometry analysis profiles for one representative out of four analyzed mice. A distinct population of $CD8^+$ T cells was detected (4.65% IFN-γ^+ cells out of $CD3^+CD8^+$ lymphocytes), which responded to restimulation with the specific peptide, while not responding to restimulation with an irrelevant peptide derived from NY-BR-1, which served as a specificity control (0.08% IFN-γ^+ cells). $CD8^+$ T cells also responded to polyclonal stimulation with anti-CD3/CD28 antibodies and did not produce significant amount of IFN-γ when cultured without stimulation (Figure 3A). Percentages of functionally responding $CD8^+$ T cells in the individual mice analyzed, immunized with α-fetoprotein$_{158}$ are shown in Fig. 3B. Specific $CD8^+$ T cells were induced in all of the analyzed mice, although the background response without stimulation and upon stimulation with the unspecific peptide was slightly higher in the mouse No.4, compared to the other analyzed mice.

6.1.2 AB*ab*DII mice mount $CD8^+$ T cell responses specific for MAGE-A10

Analysis of the responses towards MAGE-A10$_{254}$ epitope, previously identified as a target of autologous tumor-reactive cells from a melanoma patient (Huang et al. 1999), is shown in Figure 4. AB*ab*DII mice were immunized with the MAGE-A10$_{254}$ peptide in adjuvants as described above for α-fetoprotein$_{158}$. Immune responses were tested ten days after the immunization or boosting, after pooled splenocytes and LNs were restimulated *in vitro* with 10^{-6} M MAGE-A10$_{254}$ peptide, unspecific peptide or anti-CD3/CD28 antibodies, or left unstimulated. Firuge 4A shows flow cytometry analysis profiles for one representative out of four analyzed mice, after intracellular IFN-γ staining of the cells. The response was weaker than the response to α-fetoprotein$_{158}$, however, as in the case of α-fetoprotein$_{158}$, a distinct population of $CD8^+$ T cells was detected, which responded specifically to the MAGE-A10$_{254}$ peptide, without nonspecific IFN-γ secretion. Figure 4B shows percentages of functionally responding $CD8^+$ T cells in the individually analyzed mice, immunized with MAGE-A10$_{254}$. Although lower percentages of responding cells were detected, specific $CD8^+$ T cells responses were induced in all of the analyzed mice.

A



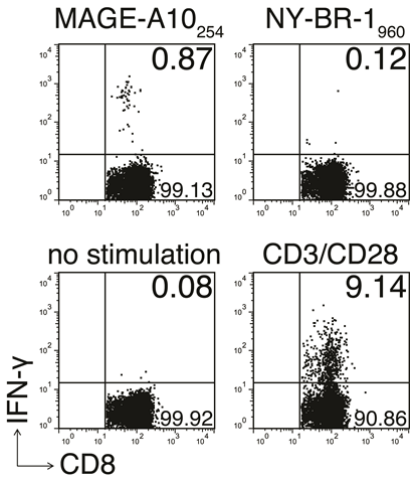
B

Mouse No.	α-fetoprotein$_{158}$	unspecific peptide	no stimulation	CD3/CD28
1	2.48[a]	0.59	0.23	7.51
2	0.9	0.15	0.11	5.16
3	4.65	0.08	0.19	14.14
4	2.83	1.2	1.04	9.35
	2.71±1.54[b]	0.5±0.51	0.4±0.43	9.04±3.81

[a] Percentage of IFN-γ$^+$ cells, out of CD3$^+$CD8$^+$ lymphocytes
[b] Average (arithmetic mean) ± SD

Figure 3. AB*ab*DII mice mount specific CD8$^+$ T cell responses against α-fetoprotein$_{158}$. (A) Specific CD8$^+$ T cell response against α-fetoprotein$_{158}$ in one representative out of four analyzed mice. The mouse was immunized s.c. with α-fetoprotein$_{158}$ peptide with 50 µg CpG ODN and 100 µl IFA and subsequently boosted with the same peptide-containing emulsion. Splenocytes and draining LN cells were isolated 10 days after the boosting, pooled and restimulated *in vitro* overnight as indicated. The cells were stained for CD8, as well as for CD3 and IFN-γ intracellularly, and analyzed by flow cytometry. Events shown are gated on CD3$^+$CD8$^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants (IFN-γ$^+$ cells residing in the upper right). (B) CD8$^+$ T cell responses against α-fetoprotein$_{158}$ in individually analyzed AB*ab*DII mice, immunized as described in (A). Mice No.1 and No.2 were immunized once and analyzed after 10 days without boosting; as shown, they also responded specifically. Numbers in the table designate percentages of IFN-γ$^+$ cells, out of CD3$^+$CD8$^+$ lymphocytes, determined by flow cytometry as described in (A), and the column headings indicate mode of stimulation. NY-BR-1$_{960}$ was used for unspecific stimulation, and in the case of the mouse No.1 Melan-A/MART-1 peptide was used.

A



B

Mouse No.	MAGE-A10$_{254}$	unspecific peptide	no stimulation	CD3/CD28
1	1.33[a]	0.15	0.28	8.09
2	2.59	0.21	0.34	11.26
3	0.87	0.12	0.08	9.14
4	0.85	0.24	0.28	7.56
	1.41±0.82[b]	0.18±0.05	0.24±0.11	9.01±1.64

[a] Percentage of IFN-γ^+ cells, out of $CD3^+CD8^+$ lymphocytes
[b] Average (arithmetic mean) ± SD

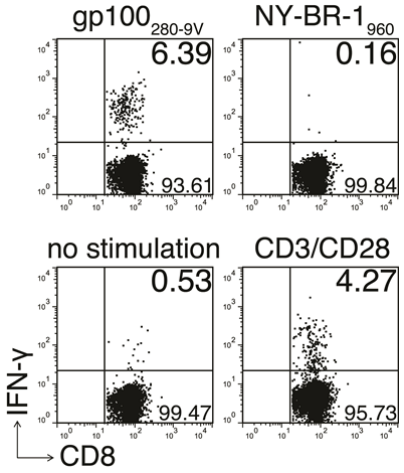
Figure 4. AB*ab*DII mice mount specific $CD8^+$ T cell responses against MAGE-A10$_{254}$. (A) Specific $CD8^+$ T cell response against MAGE-A10$_{254}$ in one representative out of four analyzed mice. The mouse was immunized with MAGE-A10$_{254}$ peptide in adjuvants, boosted, and splenocytes and LNs were isolated 10 days later, as described in Fig.3. Pooled splenocytes and LNs were *in vitro* restimulated overnight as indicated, stained and analyzed as described in Fig. 3. Events shown are gated on $CD3^+CD8^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. (B) $CD8^+$ T cell responses against MAGE-A10$_{254}$ in individually analyzed AB*ab*DII mice, immunized as described in (A). Mice No.1 and No.2 were immunized once and analyzed after 10 days without boosting; as shown, they also responded specifically. Numbers in the table designate percentages of IFN-γ^+ cells, out of $CD3^+CD8^+$ lymphocytes, determined as described in (A), and the column headings indicate mode of stimulation. The same peptides as in Fig. 3 were used for unspecific stimulation (NY-BR-1$_{960}$ / Melan-A/MART-1 in the case of the mouse No.1).

6.1.3 AB*ab*DII mice mount $CD8^+$ T cell responses specific for gp100

Numerous peptide epitopes encoded within gp100 protein have been identified; however, in the case of the epitope $gp100_{280}$, it was shown that it was specifically recognized by HLA-A*0201-restricted CTL lines isolated out of five melanoma patients (Cox et al. 1994). Moreover, an anchor-modified peptide $gp100_{280\text{-}9V}$, comprising valine (V) at the position 9 instead of the original alanine has been designed and it was shown, that it could very efficiently induce melanoma-reactive CTLs out of peripheral blood mononuclear cells (PBMCs) from patients (Parkhurst et al. 1996). This modification did not change the specificity of the peptide, since it did not involve any of the residues primarily recognized by TCR, but it did enhance binding of the peptide to MHC. Therefore, we used the same $gp100_{280\text{-}9V}$ epitope which induced response in patients' cells to immunize AB*ab*DII mice. Analysis of the responses in AB*ab*DII mice towards $gp100_{280\text{-}9V}$ epitope is shown in Figure 5. Mice were immunized with $gp100_{280\text{-}9V}$ peptide in adjuvants as decribed above for $\alpha\text{-fetoprotein}_{158}$. Immune responses were tested ten to fourteen days after the immunization or boosting. Splenocytes and LNs were restimulated *in vitro* with 10^{-6} M $gp100_{280\text{-}9V}$ peptide, unspecific peptide or anti-CD3/CD28 antibodies, or cultured unstimulated. Figure 5A shows flow cytometry analysis profiles for one representative out of thirteen analyzed mice, after intracellular IFN-γ staining of the cells; a specific $CD8^+$ T cell response was successfully induced. Figure 5B shows percentages of functionally responding $CD8^+$ T cells in the thirteen individually analyzed mice, immunized with $gp100_{280\text{-}9V}$. Specific responses against gp100 could be reproducibly induced. Background levels of IFN-γ production, seen in some of the mice, were similar in magnitude both in the case of stimulation with unspecific peptide and without any stimulation. Also, the level of this unspecific IFN-γ production stayed the same, when different additional peptides were used for unspecific stimulation (data not shown). However, in all of the mice a considerable fraction of the $CD8^+$ T cells produced IFN-γ upon *in vitro* restimulation with the specific peptide, but not with the unspecific peptide, the specific IFN-γ release being at least three times higher than the unspecific release in more than 50% of the mice.

The results described here showed that AB*ab*DII mice could mount functional and specific $CD8^+$ T cell responses against HLA-A*0201-restricted epitopes derived from TAAs, known to be immunogenic in humans. They further encouraged us to use AB*ab*DII mice as a tool to induce specific $CD8^+$ T cell response against the TEL-AML1 fusion epitope.

A



B

Mouse No.	$gp100_{280\text{-}9V}$	unspecific peptide	no stimulation	CD3/CD28
1	4.69[a]	1.87	1.58	10.95
2	6.75	0.77	0.44	5.83
3	4.1	0.66	0.6	3.98
4	5.17	3.19	2.58	6.35
5	6.39	0.16	0.53	4.27
6	3.57	0.93	0.98	6.82
7	6.52	2.16	2.58	6.12
8	21.32	3.39	3.59	9.85
9	3.08	1.6	1.58	4.03
10	1.33	0.32	0.28	2.93
11	4.02	1.82	1.68	6.7
12	4.29	1.06	1.53	3.27
13	4.63	1.26	1.03	4.78
	5.83±4.88[b]	1.48±1.0	1.46±0.98	5.84±2.41

[a] Percentage of IFN-γ^+ cells, out of $CD3^+CD8^+$ lymphocytes
[b] Average (arithmetic mean) ± SD

Figure 5. AB*ab*DII mice mount specific $CD8^+$ T cell responses against $gp100_{280\text{-}9V}$. Mice were immunized with $gp100_{280\text{-}9V}$ peptide in adjuvants, and splenocytes and draining LNs were isolated 10-14 days after the immunization/boosting. Pooled splenocytes and LNs were *in vitro* restimulated overnight as indicated. The cells were stained and analyzed as described in Fig. 3. (A) One representative out of thirteen analyzed mice is shown. Events shown are gated on $CD3^+CD8^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. (B) $CD8^+$ T cell responses against $gp100_{280\text{-}9V}$ in individually analyzed AB*ab*DII mice. Mouse No.1 was analyzed after a single immunization, mice No.2 to No.6 after a single boost, and mice No.7 to No.13 were boosted twice. Numbers in the table designate percentages of IFN-γ^+ cells, out of $CD3^+CD8^+$ lymphocytes, determined as described in (A), and the column headings indicate mode of stimulation. Peptides used for unspecific stimulation were the same as in Fig. 3 ($NY\text{-}BR\text{-}1_{960}$ / Melan-A/MART-1).

6.2 The native TEL-AML1 epitope is not immunogenic in AB*ab*DII mice

In order to induce TEL-AML1-specific $CD8^+$ T cell responses, AB*ab*DII mice were immunized with the exact nonamer peptide fusion epitope RIAECILGM (TEL-AML1) with CpG ODN in IFA, as described in the previous section for the three TAAs tested. Pooled splenocytes and LNs were subsequently restimulated *in vitro* either with TEL-AML1 peptide, with an irrelevant peptide, with anti-CD3/CD28 antibodies of left unstimulated, and functionality of $CD8^+$ T cells was detected by intracellular IFN-γ staining. As shown in Figure 6, no TEL-AML1-specific $CD8^+$ T cell response could be detected in any of the five analyzed mice, although the mice were boosted two times. However, the $CD8^+$ T cells were functional, since they responded well to nonspecific polyclonal stimulation through CD3/CD28: 19.41% of IFN-γ producing $CD8^+$ T cells were detected (mean ± SD, 9.26 ± 5.74%).

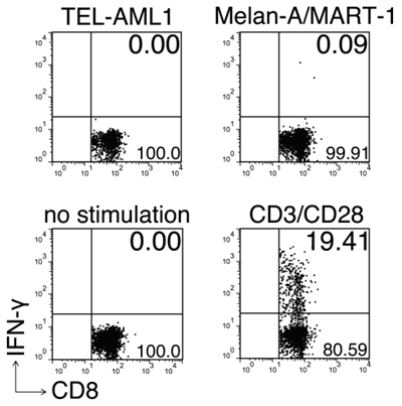


Figure 6. The native TEL-AML1 peptide cannot induce specific $CD8^+$ T cells in AB*ab*DII mice. Mice were immunized s.c. with the TEL-AML1 peptide as described in Fig. 3. They were boosted twice, and splenocytes and draining LNs were isolated 10-14 days later. Pooled splenocytes and LNs were *in vitro* restimulated as indicated. The cells were stained and analyzed as described in Fig 3. Events shown are gated on $CD3^+CD8^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. One representative out of five analyzed mice is shown.

6.3 An anchor-modification is needed to render the described TEL-AML1 epitope immunogenic

Since the described TEL-AML1 epitope could not induce specific $CD8^+$ T cell responses in AB*ab*DII mice, we tried to modify the primary anchor residues in the peptide, at positions 2 and 9 (Falk et al. 1991). As mentioned above, this strategy has been first described as successful in inducing a response against a tumor antigen in the case of the gp100 epitope (Parkhurst et al. 1996). We designed two peptides, where we either introduced a valine instead of the original methionine at the position 9 (RIAECILGV), or a leucine instead of the original isoleucine at the position 2 (RLAECILGM); the peptides were designated TEL-AML1-9V or TEL-AML1-2L, respectively.

Figure 7 shows the result of the immunization with TEL-AML1-9V with addition of CpG ODN and IFA, performed as described above for the native peptide TEL-AML1. The modification rendered the peptide immunogenic, so that 2.99% of $CD8^+$ T cells responded specifically to *in vitro* restimulation

with TEL-AML1-9V (mean ± SD, 2.64 ± 0.44%), while not responding to restimulation with an irrelevant peptide Melan-A/MART-1 (0.16% of IFN-γ producing $CD8^+$ T cells; mean ± SD 0.18 ± 0.06%). However, although the primary anchor positions in HLA class I epitopes should preferentially bind to HLA molecules, without affecting interaction with TCR, it was shown that modifying anchor residues can in some cases abolish the specificity of the induced effector cells for the native peptide (Parkhurst et al. 1996). Therefore, we next tested whether the modification introduced into the TEL-AML1-9V peptide affected the specificity of the peptide-TCR interaction. After immunizing AB*ab*DII mice with TEL-AML1-9V, the cells were restimulated *in vitro* both with the modified and the native peptide, as well as with the appropriate controls – unspecific Melan-A/MART-1, anti-CD3/CD28 antibodies, or left unstimulated. Figure 8 shows that the specificity of the anchor-modified peptide TEL-AML1-9V was retained. The $CD8^+$ T cells induced *in vivo* by the anchor-modified peptide specifically recognized the native peptide upon *in vitro* restimulation. Restimulation with the anchor-modified TEL-AML1-9V induced 1.95% IFN-γ producing $CD8^+$ T cells (mean ± SD, 2.78 ± 0.78%) and restimulation with the native one induced 1.34% IFN-γ producing $CD8^+$ T cells (mean ± SD, 1.62 ± 0.25%), while restimulation by irrelevant Melan-A/MART-1 did not induce almost any IFN-γ production (0.08%; mean ± SD; 0.16 ± 0.07%).

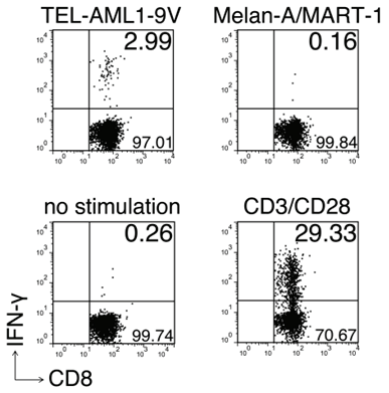


Figure 7. Anchor modification at position 9 renders the TEL-AML1 peptide immunogenic. AB*ab*DII mice were immunized with the anchor modified TEL-AML1-9V as described for the native TEL-AML1 in Fig. 6. Splenocytes and LN cells were isolated, restimulated as indicated and analyzed as described in Fig. 6. Events shown are gated on $CD3^+CD8^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. One representative out of three analyzed mice is shown.

The alternatively modified peptide at the position 2, TEL-AML1-2L, was also tested for its immunogenicity. In a preliminary experiment, an AB*ab*DII mouse was immunized with TEL-AML1-2L and 2.77% of $CD8^+$ T specifically responding to *in vitro* peptide restimulation were detected (Fig. 9). Obviously, the anchor modification at the position 2 also rendered the peptide immunogenic in AB*ab*DII mice; however, as it was not necessary to use this additional peptide in further experiments, its specificity for the native TEL-AML1 peptide was not further tested.

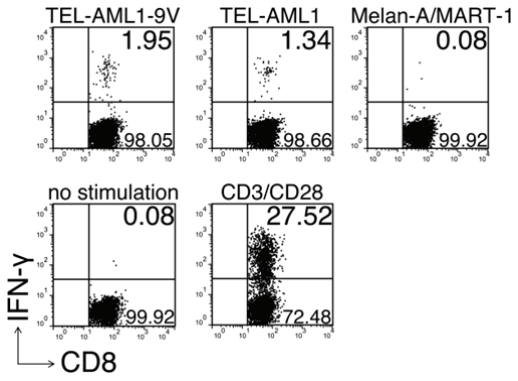


Figure 8. Anchor-modified TEL-AML1-9V induced CD8$^+$ T cells retain the specificity for the native TEL-AML1 peptide. AB*ab*DII mice were immunized with the anchor-modified TEL-AML1-9V, and pooled spleen and LNs were restimulated with the anchor-modified, the native, the irrelevant peptide, anti-CD3/CD28 antibodies, or left unstimulated and analyzed as described in Fig. 6. Events shown are gated on CD3$^+$CD8$^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. One representative out of three analyzed mice is shown.

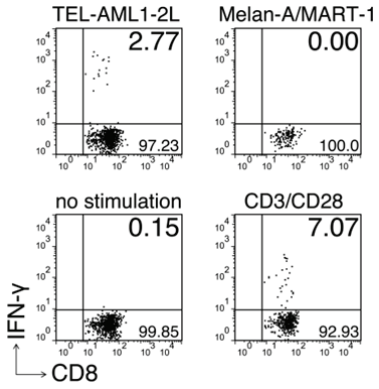


Figure 9. Anchor modification at position 2 renders the TEL-AML1 peptide immunogenic. An AB*ab*DII mouse was immunized with TEL-AML1-2L, boosted once, and splenocytes and LN cells were restimulated as indicated and analyzed as described in Fig. 3.

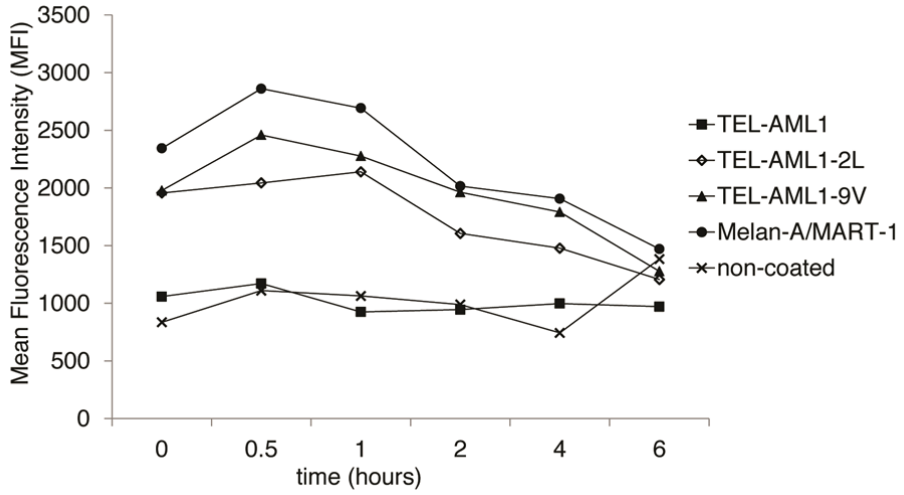
6.4 The native peptide TEL-AML1 is a poor binder to HLA-A*0201 molecules

Although the native TEL-AML1 peptide was reported as immunogenic, we could not detect this in our system, unless appropriate anchor-modifications were introduced. In an attempt to resolve why the native peptide did not evoke any specific CD8$^+$ T cell response, we tested its binding to HLA-A*0201$^+$, by performing a kinetic binding assay using the HLA-A*0201$^+$, TAP-deficient T2 cell line. We compared how well the native TEL-AML1 peptide would stabilize HLA-A*0201 molecules at T2 cell surface, compared with the immunogenic TEL-AML1-9V and TEL-AML1-2L peptides we designed. As a positive control for binding to HLA-A*0201, we coated the cells with the known human epitope Melan-A/MART1, and in order to determine the basal level of HLA-A*0201 expression at the T2 cell surface, we also left a portion of cells uncoated. The cells were incubated with peptides overnight at 28 °C, since lower temperatures were shown to stabilize HLA-A*0201 molecules on T2 cells (Ljunggren et al. 1990), and on the next day the cells were transferred to 37 °C and washed to

remove the unbound peptides. Aliquots were then taken during 6 hours to determine how long HLA-A*0201 molecules would stay at the cell surface, aided only by the bound peptide. Surprisingly, as shown in Fig. 10A, the native peptide bound poorly to HLA-A*0201, as opposed to good binding of the both anchor-modified TEL-AML1 peptides, which induced good and relatively stable expression of HLA-A*0201 molecules at the cell surface. The positive control Melan-A/MART-1 also bound very well to HLA-A*0201. Fluorescence indices (FI) calculated at the time point zero for each of the peptides are shown in Fig. 10B.

In the previous section we showed that $CD8^+$ T cells specific for the native TEL-AML1 peptide could be induced by modifying the anchor residue (Fig. 8), which shows that TCRs specific for TEL-AML1 were present in the AB*ab*DII repertoire. Therefore, the poor binding of the native peptide provided the most probable explanation for the absence of its immunogenicity in AB*ab*DII mice.

A



B

	FI(0) = MFI[T2 with peptide] / MFI[T2 w/o peptide] - 1
TEL-AML1	0.27
TEL-AML1-2L	1.34
TEL-AML1-9V	1.37
Melan-A/MART-1	1.81

Figure 10. The native TEL-AML1 peptide binds only weakly to HLA-A*0201. T2 cell assay: cells were either coated with TEL-AML1, TEL-AML1-2L, TEL-AML1-9V or Melan-A/MART-1 at the final concentration 10^{-5} M, or left uncoated. On the next day, they were stained for HLA-A*0201 and analyzed by flow cytometry. (A) The graph shows changes in MFI during 6 hours. (B) Fluorescence index (FI) for each of the peptides was calculated at the time point zero according to the formula shown. One out of two experiments with similar results is shown.

6.5 Anchor-modification in the TEL-AML1 peptide is needed to induce effector CTLs capable of degranulation

The unexpected absence of immunogenicity of the native TEL-AML1, as opposed to the anchor-modified peptide, prompted us to address this issue by an additional functional assay. We tested whether the peptides could induce cytotoxic T lymphocyte (CTL) effectors, by measuring increase of CD107a (LAMP-1) on the $CD8^+$ T cell surface as a result of degranulation, which directly correlated with their ability to kill (Betts et al. 2003). Immunization with the native TEL-AML1 peptide (Fig. 11A) could not induce specific CTLs in any of the five mice tested. Upon TEL-AML1 peptide restimulation, surface CD107a was detected only in 1.98% of $CD8^+$ T cells (mean ± SD, 2.36 ± 0.79%), which was

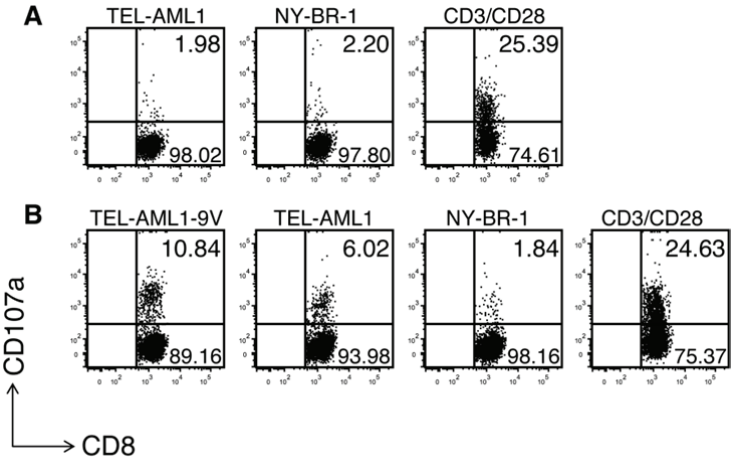


Figure 11. Anchor-modification in the TEL-AML1 peptide is needed to induce effector CTLs capable of degranulation. AB*ab*DII mice were immunized s.c. with (A) the native peptide TEL-AML1 or (B) the anchor-modified peptide TEL-AML1-9V, mixed with CpG ODN, PBS and IFA. They were boosted, and splenocytes and draining LNs were isolated 10 days later. The cells were *in vitro* restimulated for 6 hours with 10^{-6} M specific peptides (TEL-AML1 or TEL-AML1-9V), with an irrelevant peptide (NY-BR-1), or with anti-CD3/CD28 antibodies, as indicated. Pre-staining for CD107a was performed by including anti-CD107a antibodies during the whole period of stimulation, with the addition of monensin. The cells were subsequently stained for CD3 and CD8 and analyzed by flow cytometry. Events shown are gated on $CD3^+CD8^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. One representative out of five (A) and one out of six (B) mice is shown, from two independent experiments with similar results.

at the background level, as measured upon restimulation with an irrelevant peptide NY-BR-1 (2.2%; mean ± SD, 2.07 ± 0.14%), or without stimulation (data not shown). However, specific $CD8^+$ T cells induced by immunization with the anchor-modified TEL-AML1-9V were *bona fide* CTLs capable of degranulation, as 10.84% of $CD8^+$ T cells specifically responded to restimulation with TEL-AML1-9V

peptide (Fig. 11B). Specific responses were seen in all six mice tested, ranging from 2.57% to 10.84% (mean ± SD, 5.27 ± 3.13%). As further depicted in Fig. 11B, CTLs induced *in vivo* by the anchor-modified peptide specifically reacted by degranulation upon *in vitro* restimulation with the native TEL-AML1 peptide; specific reactions against the native peptide were seen in five out of six mice, although apparently to a lower extent than against the anchor-modified peptide (ranging from 2.6% to 6.02%; mean ± SD, 3.72 ± 1.27%). After restimulation with the unspecific NY-BR-1, only background levels of CD107a expressing CD8+T cells were detected (ranging from 1.29% to 2.65%; mean ± SD, 1.88 ± 0.45%). Altogether, functionality of specific $CD8^+$ T cell effectors, as detected based on IFN-γ production (Figures 6-8) correlated with their cytotoxic activity (Fig. 11), confirming that the native TEL-AML1 was not immunogenic according to any of the criteria applied.

Importantly, besides confirming our finding regarding the absence of immunogenicity of the described native TEL-AML1 peptide, the results presented in this section show that $CD8^+$ T cells developing in AB*ab*DII mice are capable of differentiating into *bona fide* effector killer cells.

6.6 Extended peptides comprising either the native or the modified TEL-AML1 cannot induce $CD8^+$ T cells

In an attempt to enhance the immune response to the TEL-AML1 peptide, we immunized the mice with extended 30-mer peptides comprising the exact epitope in adjuvants, both CpG ODN and IFA. Immunization with long peptides complemented with CpG ODN has been proven as superior compared to immunization with exact epitopes, since longer peptides could be taken up and processed only by professional APCs, which were able to present them in the context of MHC class I molecules (Zwaveling et al. 2002). Another study shows that addition of IFA, which serves as a peptide-depot, but also as an inducer of a local inflammatory response, leads to predominant local presentation of long peptides in the inflamed draining lymph nodes, leading to efficient CTL priming (Bijker et al. 2007). Therefore, we complemented long peptides with both CpG ODN and IFA, hoping that both of the effects would take place and lead to induction of effector $CD8^+$ T cells. When applying this immunization strategy, we were interested to see whether enhanced immunogenicity of long peptides could compensate for the very low HLA-A*0201 binding affinity of the native TEL-AML1 nonamer. For the immunizations we used 30-mer peptides comprising either the native TEL-AML1 9-mer peptide (designated $TEL\text{-}AML1_{319}$, with the sequence: MVSVSPPEEHAMPIGRIAECILGMNPSRDV) or comprising the anchor modified TEL-AML1-9V 9-mer peptide (designated $TEL\text{-}AML1_{319\text{-}9V}$, with the sequence: MVSVSPPEEHAMPIGRIAECILGVNPSRDV). The flanking residues surrounding the exact nonamer peptides (underlined) were deduced from the natural sequence of TEL-AML1 fusion protein. In order to control whether the immunization strategy would be efficient in AB*ab*DII mice, we also used a 30-mer peptide $gp100_{265\text{-}9V}$ comprising the $gp100_{280\text{-}9V}$ epitope, for which we previously showed (Fig. 5) that it could induce specific $CD8^+$ T cell responses, when applied as the exact nonamer peptide.

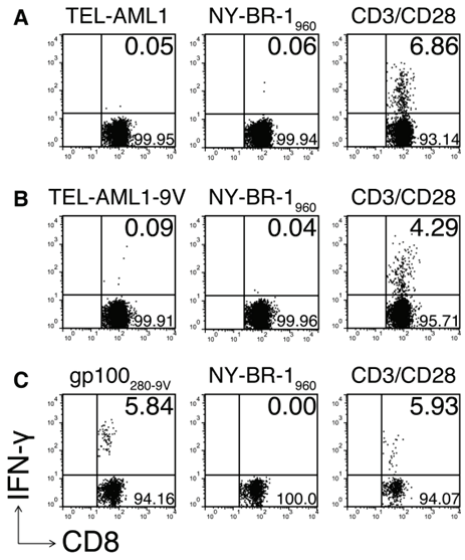


Figure 12. Elongated peptides comprising the TEL-AML1 epitope cannot evoke CD8$^+$ T cell responses. AB*ab*DII mice were immunized s.c. in the tail base with the following 30-mer peptides: (A) TEL-AML1$_{319}$, which comprises the native TEL-AML1 sequence, (B) TEL-AML1$_{319\text{-}9V}$, comprising the anchor-modified TEL-AML1-9V sequence, or (C) gp100$_{265\text{-}9V}$, comprising the anchor-modified gp100 epitope, mixed with 50 µg CpG ODN and 100 µl IFA. In the cases of the TEL-AML1 native and anchor-modified peptide, some of the mice were additionally boosted after two weeks with the corresponding peptides. Spleen and draining LNs were isolated 10 days after the immunization, or boosting. The cells were *in vitro* restimulated overnight either with 10^{-6} M corresponding nonamer peptide (TEL-AML1, TEL-AML1-9V or gp1000$_{280\text{-}9V}$), an irrelevant peptide (NY-BR-1$_{960}$) or with anti-CD3/CD28 antibodies. The cells were stained for CD8, CD3 and IFN-γ, and analyzed. Events shown are gated on CD3$^+$CD8$^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. In the case of gp100 one representative out of three analyzed mice is shown, and in the cases of the TEL-AML1 anchor-modified, as well as the native peptide, one representative out of six analyzed mice is shown.

The results of immunizations with long peptides are shown in Figure 12. We found, as shown in Fig. 12A, that none of the six mice immunized with the extended peptide TEL-AML1$_{319}$ comprising the native TEL-AML1 peptide could mount specific CD8$^+$ T cell response (0.05% IFN-γ producing CD8$^+$ T cells; mean ± SD, 0.09 ± 0.06%). The cells responded to polyclonal stimulation through CD3/CD28 (6.86% IFN-γ producing CD8$^+$ T cells; mean ± SD, 7.07 ± 2.82%). Such a result should not be surprising, considering the very low HLA-A*0201 binding affinity of TEL-AML1. Surprisingly however, immunization by the 30-mer peptide TEL-AML1$_{319\text{-}9V}$ comprising the immunogenic anchor-modified TEL-AML1-9V nonamer could not induce a CD8$^+$ T cell response either, as a single immunization or complemented by boosting (Fig. 12B). Only 0.09% IFN-γ producing CD8$^+$ T cells were detected (mean ± SD, 0.17 ± 0.18%). CD8$^+$ T cells were functional, since polyclonal stimulation induced 4.29% IFN-γ producing CD8$^+$ T cells (mean ± SD, 5.82 ± 3.49%). The immunization strategy per se was effective, since functional CD8$^+$ T cells specific for the gp100$_{280\text{-}9V}$ epitope were successfully induced

with the corresponding extended peptide $gp100_{265\text{-}9V}$. Figure 12C shows that 5.84% of $CD8^+$ T cells specific for $gp100_{280\text{-}9V}$ were detected (mean ± SD, 3.56 ± 2.07%), while they did not respond to unspecific restimulation with NY-BR-1 (0.0% IFN-γ producing $CD8^+$ T cells; mean ± SD, 0.07 ± 0.07%). The cells also responded well to polyclonal stimulation (5.93% IFN-γ producing $CD8^+$ T cells; mean ± SD, 9.32 ± 4.99%).

Since the anchor-modified nonamer peptide TEL-AML1-9V is immunogenic in AB*ab*DII mice (Figures 7, 8 and 11B), the absence of any specific response to the extended anchor-modified peptide TEL-$AML1_{319\text{-}9V}$ could be best explained by the absence of endogenous processing of the described epitope. However, although our control peptide $gp100_{265\text{-}9V}$ showed that the immunization method worked (Fig. 12C), we could not formally exclude that additional factors apart from peptide processing, such as different *in vivo* peptide degradation kinetics, might have accounted for the response towards gp100 and the absence of such a response towards TEL-AML1. Therefore, we next designed an experimental strategy which should enable us to test endogenous processing of the fusion TEL-AML1 region, without influence of other factors affecting success of immunization.

It should be noted that the results presented here, showing correct processing of the human epitope $gp100_{280\text{-}9V}$ out of the $gp100_{265\text{-}9V}$ long peptide in AB*ab*DII mice *in vivo*, confirm the previous findings in multiple murine models regarding the capability of the murine antigen processing machinery of generating human HLA-A*0201-restricted epitopes.

6.7 The TEL-AML1 peptide is not endogenously processed

In order to directly test endogenous processing of the described peptide out of the TEL-AML1 fusion region, without the impact of other factors which might influence outcome of immunization, we intended to perform a following experiment: we would immunize AB*ab*DII mice with the exact anchor-modified 9-mer peptide TEL-AML1-9V, since we showed, that it induced $CD8^+$ T cells specific for the described TEL-AML1 epitope (Fig. 8 and 11B), and we would coculture induced $CD8^+$ T cells with appropriate antigen presenting cells (APCs) which would endogenously express the TEL-AML1 fusion region. For that purpose, we needed to generate suitable APCs. More precisely, we needed a cell line which would express the TEL-AML1 fusion region and the appropriate restriction element, HHD, to enable presentation of the peptide epitope to $CD8^+$ T cell effectors. As a positive control, we needed an equivalent HHD-expressing cell line, which would express a known human tumor antigen, and process and present a known epitope derived from that antigen to $CD8^+$ T cell efectors, which would in parallel be induced in AB*ab*DII mice. We chose to use Melan-A/MART-1 as a control tumor antigen since the immunodominant HLA-A*0201-restricted epitope in humans was known (Kawakami et al. 1994c; Romero et al. 1997). Furthermore, an anchor-modified peptide (designated Melan-A/MART-1 in the present text) has been identified, which more efficiently induced CTLs reactive to the native epitope (Valmori et al. 1998) and which we therefore intended to use for our control immunizations. Finally, we would need an equivalent cell line which would express only HHD molecules, to serve as a specificity control in coculturing experiments. The generation of constructs for expression of the TEL-

AML1 fusion region and Melan-A/MART-1, as well as the generation of antigen presenting cell lines is described next.

6.7.1 Generation of constructs for expression of the TEL-AML1 fusion region and Melan-A/MART-1

The TEL-AML1 fusion region and Melan-A/MART-1 were cloned into the retroviral vector pMP71 (Engels et al. 2003), which should enable stable and high-level expression of the transgenes. The restriction element HHD was expressed from pMP71 as well (designated pMP71-HHD; the plasmid was a gift from Matthias Leisegang), so we intended to introduce both HHD, as well as the test antigen (TEL-AML1 minigene) or the control tumor antigen (Melan-A/MART-1) into cells by means of retroviral transduction. Melan-A/MART-1 was cloned as a complete protein consisting of 118 amino acids, since the immunodominant HLA-A*0201-restricted epitope was known, as described above. TEL-AML1, on the contrary, has been cloned as a minigene 90 nucleotides long, comprising only the fusion region. This was done to enable direct testing of the specific $CD8^+$ T cell response against the described fusion epitope, and to avoid potential dominant responses against any putative epitopes that could be present in either the TEL or the AML1 portion of the fusion protein, which is 797 amino acids long.

Furthermore, we utilized the Ub/protein/reference (UPR) technique (Levy et al. 1996) for the expression of the two transgenes, TEL-AML1 minigene and Melan-A/MART-1 (Fig. 13). In UPR-based constructs a reporter gene, a Ub moiety and a protein of interest constitute a single ORF. EGFP is used as a reporter gene. Such a tripartite fusion is cleaved, cotranslationally or nearly so, by Ub-specific proteases, producing equimolar amounts of the protein of interest (TEL-AML1 or Melan-A/MART-1) and the reference protein (EGFP) bearing a C terminal Ub moiety (76 amino acids long). This is enabled by usage of a modified Ub moiety, in which lysine at position 48 had been replaced by arginine; this precludes the possibility of C terminal Ub moiety in EGFP-Ub acting as a ubiquitylation/degradation signal (Lys-48 of Ub is one of the major sites of Ub-Ub bond formation within a multi-Ub chain, whereas an Arg residue cannot be ubiquitylated). Therefore, the reference protein (EGFP) cannot be rapidly degraded, and its expression level (measured by flow cytometry) enables determination of the expression level of the protein of interest.

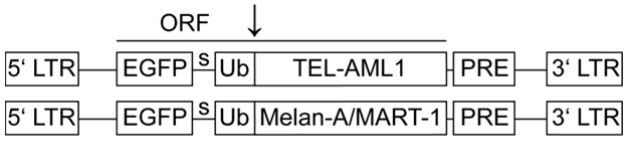


Figure 13. Schematic representation of the generated UPR-based MP71 expression constructs. EGFP, Ub and either TEL-AML1 minigene or Melan-A/MART-1 constitute a single ORF within the MP71 construct. Site of cleavage by Ub-specific proteases is indicated by arrow. LTR - long terminal repeat derived from murine myeloproliferative sarcoma virus, PRE - woodchuck hepatitis virus posttranscriptional regulatory element, s – spacer peptide.

As shown in Fig. 14, the TEL-AML1 minigene was first subcloned into a UPR-based lentiviral vector pRRL.CMV.GFP.sin18 (Garcia Casado et al. 2008). First, the TEL-AML1 coding fragment was BamHI/BstXI digested (Fig. 14A); pRRL.CMV.GFP.sin18 vector was digested in the same manner, in order to excise NY-ESO-1 containing fragment (Fig. 14A). After ligation of the appropriate fragments, the resulting pRRL.CMV.GFP.sin18-TEL-AML1 was tested by restriction analysis (Fig. 14B), and by sequencing. The complete EGFP-Ub-TEL-AML1 ORF was subsequently cloned into pMP71, as shown in Fig. 15. First, the whole ORF was PCR-amplified from the pRRL.CMV.GFP.sin18-TEL-AML1 plasmid to introduce NotI/EcoRI restriction sites, it was subsequently NotI/EcoRI digested (Fig. 15A), and ligated into linearized pMP71 (previously digested with the same enzymes to excise the existing EGFP; Fig 15A). The resulting construct designated pMP71-TEL-AML1 was verified by restriction analysis (Fig 15B), and by sequencing. Plasmid map of the generated pMP71-TEL-AML1 is shown in Fig. 17. Cloning of the Melan-A/MART-1 antigen is shown in Fig. 16. The ORF already containing Melan-A/MART-1 fused to EGFP and Ub was PCR-amplified from the lentiviral vector pRRL.CMV.GFP.sin18 (Chapatte et al. 2006) to introduce NotI/EcoRI restriction sites, it was subsequently NotI/EcoRI digested (Fig. 16A), and ligated into pMP71 linearized in the same way as described above (Fig 16A). The resulting construct pMP71-Melan-A/MART-1 was verified by restriction analysis (Fig 16B), and by sequencing. Plasmid map of the generated pMP71-Melan-A/MART-1 is shown in Fig. 18.

6.7.2 Generation of antigen presenting cell lines NIH-HHD, NIH-HHD-TEL-AML1 and NIH-HHD-Melan-A/MART-1

We have chosen NIH/3T3 cell line as a basis for generation of APCs, since they were shown to be capable of processing and presenting the same viral and tumor HLA-A*0201-restricted epitopes, which were immunogenic in humans (Papanicolaou et al. 2003; Dupont et al. 2005). In order to produce retroviral particles for transduction of NIH/3T3 cells, we transfected the ecotropic packaging cells Plat-E either with pMP71-HHD, pMP71-TEL-AML1 or pMP71-Melan-A/MART-1. Plat-E cells transfected with EGFP-coding pMP71-TEL-AML1 or pMP71-Melan-A/MART-1 were readily visualized by fluorescence microscopy (Fig. 19). NIH/3T3 cells were subsequently transduced with filtered viral supernatant containing retroviral MP71-HHD, MP71-HHD + MP71-TEL-AML1 or MP71-HHD + MP71-Melan-A/MART-1 particles, and after staining with anti-HLA-A2 antibody, transduction efficacies were measured by flow cytometry (Fig. 20A). The generated cell lines NIH-HHD, NIH-HHD-TEL-AML1 and NIH-HHD-Melan-A/MART-1 were subsequently sorted by FACS to more than 97% purity, based on EGFP and HHD expression (Fig. 20B). The UPR-based constructs enabled direct comparison of the expression levels of TEL-AML1 and Melan-A/MART-1 in the generated NIH-HHD-TEL-AML1 and NIH-HHD-Melan-A/MART-1 cell lines, based on comparison of EGFP expression levels in the two cell lines. As shown in Fig. 20C, the expression levels of the test antigen TEL-AML1 and the control antigen Melan-A/MART-1 were virtually the same. The levels of HHD expression were also very similar in the three generated cell lines (Fig. 20C right).

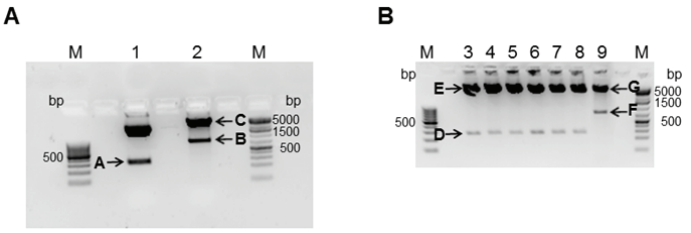


Figure 14. Subcloning of the TEL-AML1 minigene into pRRL.CMV.GFP.sin18. (A) Preparative agarose gel showing BamHI/BstXI restriction DNA fragments of the carrying vector pMA containing the TEL-AML1 minigene sequence (lane 1) and of the original pRRL.CMV.GFP.sin18-NY-ESO-1 plasmid (lane 2). Fragments are labeled with capital letters as follows: A: TEL-AML1-comprising fragment, B: NY-ESO-1-comprising fragment, C: linearized pRRL.CMV.GFP.sin18. (B) Restriction analysis of the resulting pRRL.CMV.GFP.sin18-TEL-AML1 construct (lanes 3-8) and of the original pRRL.CMV.GFP.sin18-NY-ESO-1 plasmid (lane 9) with BamH/BstXI, after amplification in bacteria and plasmid DNA preparation. The labeled fragments are: D: TEL-AML1-comprising fragment, E and G: linearized pRRL.CMV.GFP.sin18 vectors, F: NY-ESO-1-comprising fragment. M - size marker, bp - base pairs.

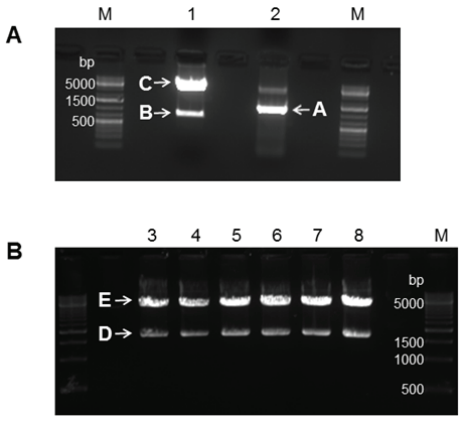


Figure 15. Cloning of the EGFP-Ub-TEL-AML1 ORF into pMP71. (A) Preparative agarose gel showing DNA fragments resulting from NotI/EcoRI restriction of the pMP71 plasmid (lane 1) and of the PCR amplified EGFP-Ub-TEL-AML1 fragment from the plasmid pRRL.CMV.GFP.sin18-TEL-AML1 (lane 2). The labeled fragments are: A: PCR amplified EGFP-Ub-TEL-AML1, B: EGFP, C: linearized pMP71. (B) Restriction analysis of the resulting pMP71-TEL-AML1 construct with SpeI, after amplification in bacteria and plasmid DNA preparation (lanes 3-8). The labeled fragments are: D: EGFP-Ub-TEL-AML1-comprising fragment, E: linearized pMP71. M - size marker, bp - base pairs.

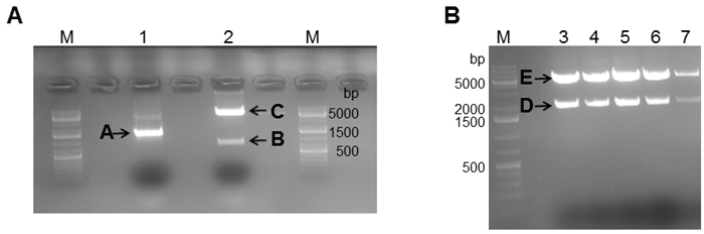


Figure 16. Cloning of the EGFP-Ub-Melan-A/MART-1 ORF into pMP71. (A) Preparative agarose gel showing DNA fragments resulting from NotI/EcoRI restriction of the pMP71 plasmid (lane 2) and of the PCR amplified EGFP-Ub-Melan-A/MART-1 fragment from the plasmid pRRL.CMV.GFP.sin18 (lane 1). The labeled fragments are: A: PCR amplified EGFP-Ub-Melan-A/MART-1, B: EGFP, C: linearized pMP71. (B) Restriction analysis of the resulting pMP71-Melan-A/MART-1 construct with SpeI, after amplification in bacteria and plasmid DNA preparation (lanes 3-7). The labeled fragments are: D: EGFP-Ub-Melan-A/MART-1-comprising fragment, E: linearized pMP71. M - size marker, bp - base pairs.

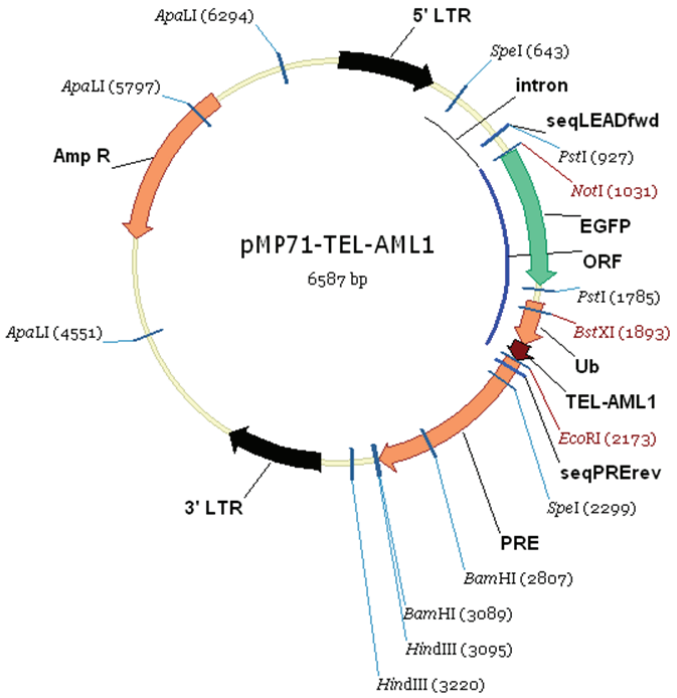


Figure 17. Restriction map of the pMP71-TEL-AML1 plasmid. LTR – long terminal repeat derived from murine myeloproliferative sarcoma virus, PRE - woodchuck hepatitis virus posttranscriptional regulatory element, Amp R - ampicillin resistance gene. Note the ORF (in blue) comprising EGFP, Ub and TEL-AML1 minigene. Positions of the sequencing primers (seqLEADfwd and seqPRErev; see Table 2) are indicated.

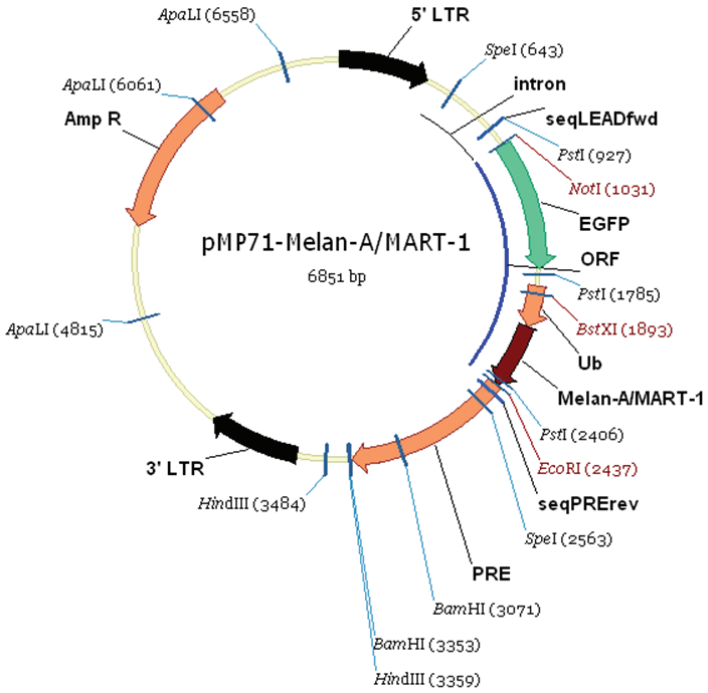


Figure 18. Restriction map of the pMP71-Melan-A/MART-1 plasmid. Labeling is the same as in Fig. 16.

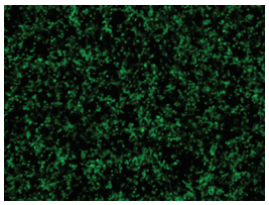


Figure 19. Plat-E cells transfected with pMP71-TEL-AML1. The cells were visualized by fluorescence microscopy (*Olympus FSX100*) due to EGFP expression.

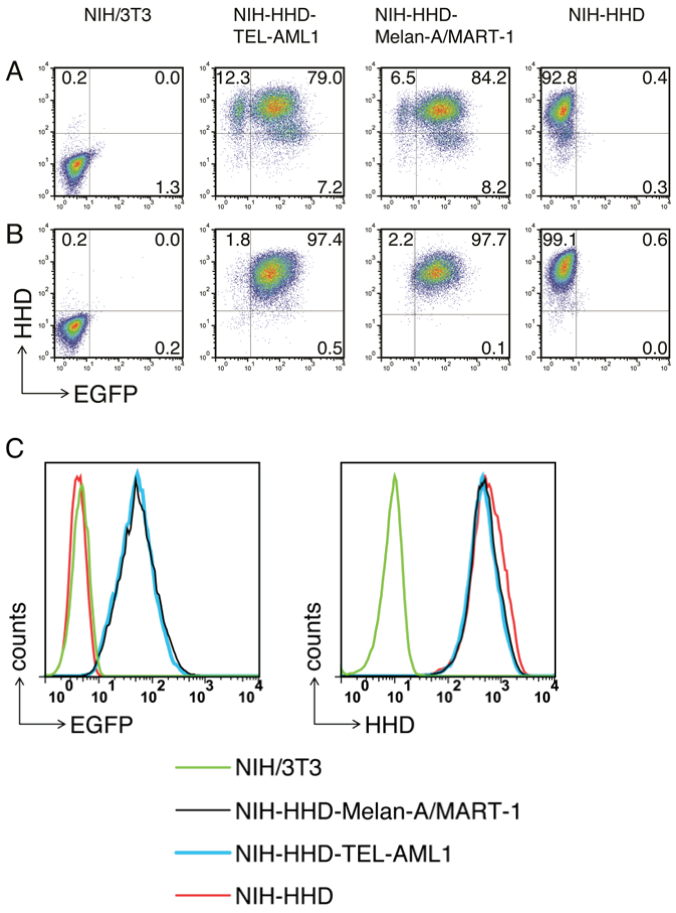


Figure 20. Generated APCs express comparable and high levels of TEL-AML1 minigene and Melan-A/MART-1 and of the restriction element HHD. NIH-HHD, NIH-HHD-TEL-AML1, NIH-HHD-Melan-A/MART-1 and nontransduced NIH/3T3 cells were stained with HLA-A2 specific antibody and expression of HHD and EGFP was analyzed by flow cytometry. Events shown are gated on live cells; numbers indicate percentages of cells in the respective quadrants. (A) Cell lines after retroviral transduction. (B) Cell lines after fluorescence-activated cell sorting (FACS), based on EGFP and HHD expression (after staining with anti-HLA-A2 antibody). (C) Comparison of the expression levels of TEL-AML1 and Melan-A/MART-1, measured by EGFP expression (left), and of HHD, measured after staining with HLA-A2 specific antibody (right) in the sorted cell lines.

6.7.3 TEL-AML1 cannot be recognized by specific effectors when endogenously expressed in APCs

In order to test whether the generated NIH-HHD cells can efficiently function as APCs, we loaded the cells with the exact peptide epitopes TEL-AML1-9V or Melan-A/MART-1 and cocultured them with pooled spleen and LN cells from AB*ab*DII mice, previously immunized with the corresponding peptide epitopes with CpG ODN and IFA. As shown in Fig. 21, after overnight coculture, $CD8^+$ T cells specifically responded to the corresponding peptide used for immunization in both cases. More precisely, 1.8% of $CD8^+$ T cells from the TEL-AML1-9V immunized mouse specifically responded to the peptide presented by NIH-HHD cells (mean ± SD, 1.87 ± 0.73%), while not recognizing NIH-HHD cells without exogenously loaded peptide (0.0% of IFN-γ producing $CD8^+$ T cells; mean ± SD, 0.1 ± 0.14%) (Fig. 21A). Cells from mice immunized with the highly immunogenic Melan-A/MART-1 peptide responded strongly to the peptide exogenously loaded onto NIH-HHD cells. Fig. 21B shows that 18.71% IFN-γ^+ cells among $CD8^+$ T cells were detected (mean ± SD, 24.85 ± 5.66%). The response was specific, since stimulation with the NIH-HHD cells without exogenously loaded peptide gave only 3.97% IFN-γ^+ $CD8^+$ T cells (mean ± SD, 6.81 ± 2.48%). The slight background seen in the case of Melan-A/MART-1, as suggested by a preliminary experiment shown in Fig. 22, was likely due to retention of this high-affinity binding peptide on APCs present in spleen, since it was abolished when the $CD8^+$ cells were additionally magnetically negatively sorted.

However, although specific $CD8^+$ T cells were induced in TEL-AML1-9V immunized mice, they were not able to recognize NIH-HHD-TEL-AML1 cells, which endogenously expressed TEL-AML1. Figure 21A shows that no IFN-γ^+ $CD8^+$ T cells could be detected upon coculturing with NIH-HHD-TEL-AML1 cells (0.1% IFN-γ^+ $CD8^+$ T cells; mean ± SD, 0.15 ± 0.05%). On the contrary, $CD8^+$ T cells from Melan-A/MART-1 immunized mice responded very well to stimulation with the cells endogenously expressing Melan-A/MART-1. Figure 21B shows that 24.39% IFN-γ^+ $CD8^+$T cells were detected upon coculturing with NIH-HHD-Melan-A/MART-1 cells (mean ± SD, 32.56 ± 7.47%). The response was even slightly stronger than the response to NIH-HHD cells exogenously loaded with the peptide. This argued that the NIH-HHD cells not only efficiently presented peptides to T cells, but also endogenously processed and presented the UPR-derived polypeptides even more efficiently than they presented the exogenous peptides. Therefore, if the TEL-AML1 fusion region would have been processed, we would have been able to detect it, most probably as a stronger response in comparison to the one seen with the exogenously added peptide.

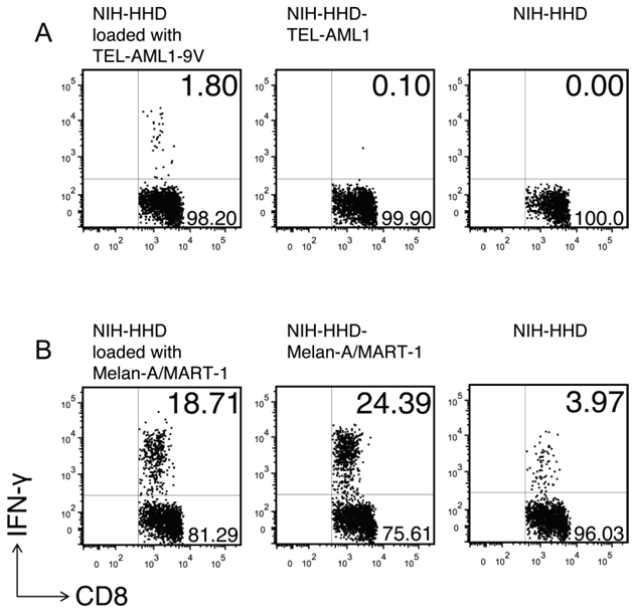


Figure 21. The TEL-AML1 peptide is not naturally processed. AB*ab*DII mice were immunized either with (A) TEL-AML1-9V or (B) Melan-A/MART-1 peptide as described in Figure 3 and boosted after 14 days. Ten days after the boost spleen and LNs were isolated, pooled and cocultured overnight either with the NIH-HHD cells loaded with the corresponding peptide (TEL-AML1-9V or Melan-A/MART-1), or with NIH-HHD endogenously expressing TEL-AML1 minigene (NIH-HHD-TEL-AML1) or Melan-A/MART-1 (NIH-HHD-Melan-A/MART-1). Additionally, they were cultured with NIH-HHD cells only. On the next day, the cells were stained for CD8, as well as for CD3 and IFN-γ intracellularly, and analyzed by flow cytometry. Events shown are gated on $CD3^+CD8^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. In each case one representative out of three analyzed mice is shown.

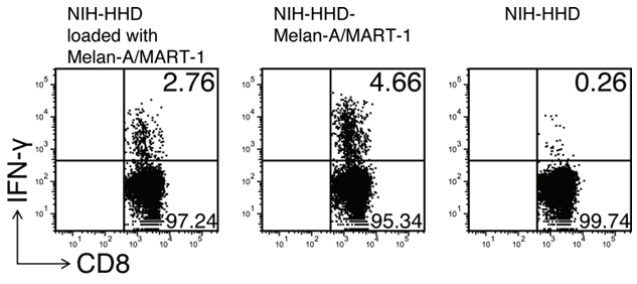


Figure 22. A small amount of the high-affinity Melan-A/MART-1 peptide might be retained on spleen-derived APCs during short-term culture. $CD8^+$ T cells from a mouse immunized with Melan/MART-1 peptide (as described in Fig. 21) were magnetically negatively sorted, cocultured overnight with NIH-HHD-Melan/MART-1, NIH-HHD cells loaded with the Melan-A/MART-1 peptide, or with NIH-HHD only, and further analyzed as described in Fig. 21.

6.8 The TEL-AML1 peptide is not processed by human proteasomes *in vitro*

As discussed in detail in the introductory section, there is substantial evidence suggesting a large functional redundancy between murine and human antigen processing machineries in general, and particularly with regard to HLA-A*0201-restricted epitopes. Moreover, our results confirm that the murine antigen processing machinery correctly generates the same tumor-derived epitopes, which are processed in humans - both in AB*ab*DII mice ($gp100_{280\text{-}9V}$ epitope, Fig. 12C) and in murine NIH/3T3 fibroblasts (Melan-A/MART-1 epitope, Figures 21B, 22 and 25). However, we wanted to confirm that the TEL-AML1 epitope could not be processed by human proteasomes. To that end, the 30 amino acid long peptide spanning the TEL-AML1 fusion region ($TEL\text{-}AML1_{319}$) was digested by purified lymphoblastoid cell lines (LCL) 20S proteasomes. Peptide fragments were detected by reverse-phase liquid chromatography coupled online with tandem mass spectrometry (LC-MS/MS) (Fig. 23A). The described epitope was not identified. Moreover, longer fragments with the appropriate C-terminus, which could serve as potential precursors for amino-terminal trimming by aminopeptidases present in the cytosol and ER were not identified either. Instead, kinetic analysis identified a large number of destructive cleavages within the epitope, which formed relatively early during the digestion and destroyed the epitope. Control digestion of a polypeptide derived from bacterial listeriolysin O (LLO-291) resulted in the efficient generation of both the MHC class I epitope LLO-296-304, as well as its N-terminally elongated precursor (Fig. 23B), showing that the cleavage within the epitope in that case did not affect the overall predominance of the epitope generation.

Figure 23. In vitro digestion of synthetic peptides with human proteasomes. (A) Digestion of the peptide TEL-AML1-319. Predominant cleavage products and cleavage sites are shown. Synthetic peptides were incubated until 50% of the peptide substrate was turned over by purified LCL (lymphoblastoid cell lines) 20S proteasomes, which mainly expresses immunoproteasomes. Peptide fragments are detected by LC-MS/MS (reversed phase liquid chromatography coupled online with tandem mass spectrometry) using a normal triple method. Arrows of various thickness are proportional to the relative cleavage intensity. Note the large number of destructive cleavages within the epitope (in red) and the complete absence of a potentially functional N-terminal cleavage. (B) Generation of the LLO-296-304 $CD8^+$ T cell epitope by digestion of synthetic polypeptide LLO-291 derived from bacterial listeriolysin O. The experiment was performed as described in (A). The dominant relevant cleavage products and the MHC class I ligand Val_{296} - Leu_{304} (in red) as well as the N-terminally elongated epitope precursor peptide thereof (in green) are shown. Arrows indicate major and minor cleavage sites. Note the strong cleavage behind the C-terminal Leu_{304} residue, resulting in the predominant generation of the epitope. Cleavage within the epitope does not affect the overall predominance of Val_{296} - Leu_{304} peptide generation. Numbers designate amino acid residue positions in the corresponding proteins (TEL-AML1 and listeriolysin O). *(The data presented in this figure are the result of cooperation with Prof. Peter Michael Kloetzel, Institute of Biochemistry, Medical Faculty, Charité, Berlin, Germany).*

6.9 The TEL-AML1 fusion region is not immunogenic in HLA-A*0201-restriction settings

The experimental results described in the previous sections revealed that the only described T cell epitope derived from the TEL-AML1 translocation was not naturally processed. Therefore, having previously made potent APCs for presentation of HLA-A*0201-restricted epitopes to effector $CD8^+$ T cells induced in AB*ab*DII mice, we intended to use the model system we established to investigate whether some unidentified TEL-AML1 derived epitopes existed.

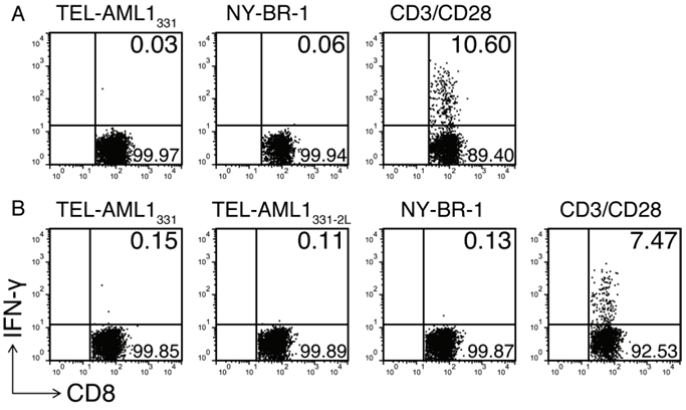


Figure 24. Elongated peptides comprising the putative TEL-AML1$_{331}$ epitope cannot evoke $CD8^+$ T cell responses. AB*ab*DII mice were immunized s.c. in the tail base with the following 30-mer peptides: (A) TEL-AML1$_{319}$, comprising the TEL-AML1$_{331}$ sequence, or (B) TEL-AML1$_{316\text{-}2L}$, comprising the anchor-modified TEL-AML1$_{331\text{-}2L}$ sequence and boosted, as described in Fig. 12. In the case of the anchor modified TEL-AML1$_{316\text{-}2L}$, some of the mice were analyzed already after single immunization. Spleen and draining LNs were isolated 10 days after the immunization/boosting. The cells were *in vitro* restimulated overnight either with nonamer peptides TEL-AML1$_{331}$/TEL-AML1$_{331\text{-}2L}$, the irrelevant peptide (NY-BR-1$_{960}$) or with anti-CD3/CD28 antibodies. The cells were stained and analyzed by flow cytometry. Events shown are gated on $CD3^+CD8^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. In the case of TEL-AML1$_{319}$ one representative out of three analyzed mice is shown, and in the case of the anchor modified TEL-AML1$_{316\text{-}2L}$, one representative out of fourteen analyzed mice is shown.

From the digestion with human proteasomes (Fig. 23) it was evident that there was a single peptide of the appropriate length (10 amino acids) for binding to HLA-A*0201, which spanned the fusion region, starting at the position 331 in the fusion protein, which was produced by human proteasomes *in vitro*. This peptide, with the sequence PIGRIAECIL, was designated TEL-AML1$_{331}$. We tested whether this peptide might be processed *in vivo*. AB*ab*DII mice were immunized with the 30-mer peptide TEL-AML1$_{319}$ (MVSVSPPEEHAMPIGRIAECILGMNPSRDV), which comprised the putative epitope TEL-AML1$_{331}$ (underlined). Another group of mice was immunized with 30-mer peptide TEL-AML1$_{316\text{-}2L}$ (NHIMVSVSPPEEHAMPLGRIAECILGMNPS), which contained an anchor-modification (I → L) at the

position 2 in TEL-AML1$_{331}$, in order to increase immunogenicity of the putative epitope. However, as shown in Figure 24, none of the three mice immunized with TEL-AML1$_{319}$, and also none of the fourteen mice immunized with TEL-AML1$_{316\text{-}2L}$ showed any specific CD8$^+$ T cell response upon *in vitro* restimulation with the nonamer peptides TEL-AML1$_{331}$ or the anchor-modified TEL-AML1$_{331\text{-}2L}$. T cells did respond well to polyclonal stimulation (10.6% of IFN-γ producing CD8$^+$ T cells; mean ± SD, 6.75 ± 3.91% in the case of TEL-AML1$_{319}$ immunized mice; 7.47% of IFN-γ producing CD8$^+$ T cells; mean ± SD, 7.92 ± 2.99% in the case of TEL-AML1$_{316\text{-}2L}$ immunized mice).

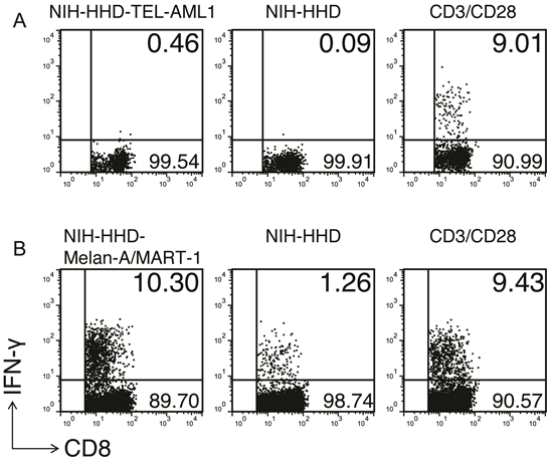


Figure 25. CD8$^+$ T cells from mice immunized with the long peptide TEL-AML1$_{319}$ do not recognize NIH-HHD-TEL-AML1 cells. AB*ab*DII mice were immunized either with (A) TEL-AML1$_{319}$ or (B) Melan-A/MART-1, as described in the previous figure, and boosted. Ten days after the boost spleen and LNs were isolated, pooled and cocultured overnight with NIH-HHD-TEL-AML1 / NIH-HHD-Melan-A/MART-1 cells, NIH-HHD cells, or stimulated with anti-CD3/CD28 antibodies. On the next day, the cells were stained and analyzed. Events shown are gated on CD3$^+$CD8$^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. In each case one representative out of three analyzed mice is shown.

Moreover, as shown in Fig. 25A, when we immunized mice with the 30-mer peptide TEL-AML1$_{319}$ which spans the fusion region and cocultured pooled splenocytes and LN cells with NIH-HHD-TEL-AML1 cells, no specific CD8$^+$ T cell response could be detected, although stimulation through CD3/CD28 induced 9.01% responding cells (mean ± SD, 6.39 ± 2.47%). On the contrary, mice immunized in parallel with Melan-A/MART-1 specifically recognized NIH-HHD-Melan-A/MART-1 cells (Fig. 25B). Upon coculturing with NIH-HHD-Melan-A/MART-1, 10.3% CD8$^+$ T cells responded specifically (mean ± SD, 8.55 ± 2.69%), while only 1.26% CD8$^+$ T cells responded to control NIH-HHD cells (mean ± SD, 1.83 ± 0.94%). These Melan-A/MART-1 specific cells responded to polyclonal stimulation (9.43% CD8$^+$ T cells; mean ± SD, 10.18 ± 3.64%) to the same extent as the cells from mice immunized with TEL-AML1$_{319}$. This experiment shows that no HLA-A*0201-restricted CD8$^+$ T cell response specific for the TEL-AML1 fusion region can be induced in AB*ab*DII mice, when

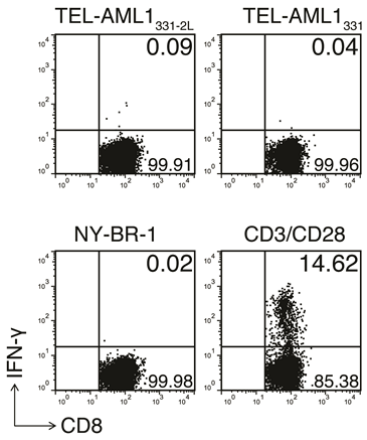
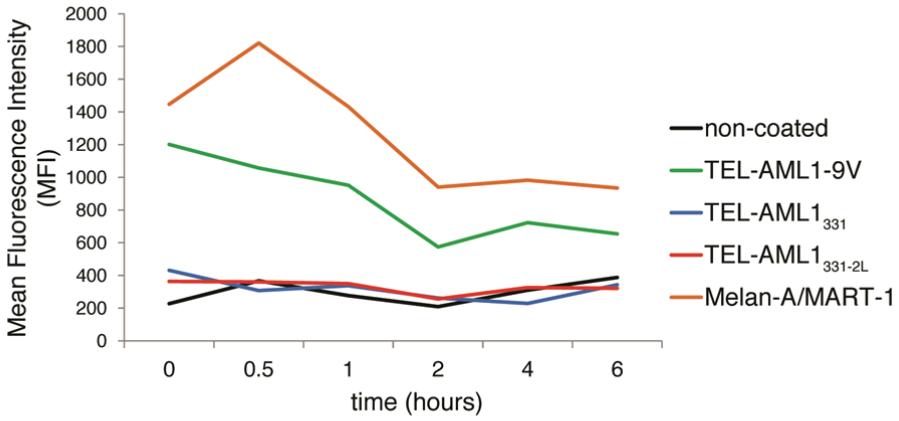


Figure 26. The anchor modified TEL-AML1$_{331\text{-}2L}$ peptide is not immunogenic in AB*ab*DII mice. Mice were immunized with the TEL-AML1$_{331\text{-}2L}$ peptide as described in Fig. 3. Some of the mice were additionally boosted. Splenocytes and draining LNs were isolated 10 days later, restimulated overnight with 10^{-6} M TEL-AML1$_{331\text{-}2L}$, TEL-AML1$_{331}$, the irrelevant NY-BR-1$_{960}$, with anti-CD3/CD28 antibodies, or left unstimulated, stained and analyzed. Events shown are gated on $CD3^+CD8^+$ lymphocytes; numbers indicate percentages of cells in the respective quadrants. One representative out of four analyzed mice is shown.

endogenous processing is required. This shows that the complete fusion region is inefficiently processed, and therefore cannot serve as a source of potential HLA-A*0201-restricted epitopes, as could be predicted by *in vitro* human proteasome analysis. However, the notable exception was the peptide TEL-AML1$_{331}$; in the case of this peptide, it seemed that the results obtained *in vivo* and *in vitro* were discordant.

To resolve why the putative epitope TEL-AML1$_{331}$ could not evoke specific response in the long peptide-immunization experiments, although it was generated by human proteasome *in vitro*, we immunized AB*ab*DII mice with the exact TEL-AML1$_{331\text{-}2L}$ 10-mer peptide (with the anchor modification at position 2, in order to make the peptide more immunogenic if possible). Figure 26 shows that no specific $CD8^+$ T cells were induced. Two possible explanations might exist: either the peptide TEL-AML1$_{331}$ is such a weak binder to HLA-A*0201 that even the anchor modification introduced could not render it immunogenic, or T cells which might recognize this peptide were not present in AB*ab*DII TCR repertoire. To resolve this, we performed the T2 cell binding assay with TEL-AML1$_{331}$ and TEL-AML1$_{331\text{-}2L}$ peptides. As a positive control we used both Melan-A/MART-1 and the previously designed anchor-modified TEL-AML1-9V peptide. Figure 27 shows that the peptide TEL-AML1$_{331}$ did not efficiently bind HLA-A*0201; furthermore, the binding affinity could not be improved by modifying the anchor residue. We conclude that the peptide TEL-AML1$_{331}$, identified as processed out of the TEL-AML1 fusion region by human proteasomes *in vitro*, does not have an appropriate structure to serve as an HLA-A*0201 ligand, which is the reason for the absence of its immunogenicity. Altogether, the results presented here show that the TEL-AML1 fusion region does not comprise any HLA-A*0201-restricted epitopes.

A



B

	FI (0) = MFI [T2 with peptide] / MFI [T2 w/o peptide] - 1
TEL1-AML1$_{331}$	0.89
TEL-AML1$_{331\text{-}2L}$	0.59
TEL1-AML1-9V	4.27
Melan-A/MART-1	5.35

Figure 27. Neither the native TEL-AML1$_{331}$ nor the anchor modified TEL-AML1$_{331\text{-}2L}$ peptide can efficiently bind to HLA-A*0201. T2 cell assay: cells were coated with TEL-AML1$_{331}$, TEL-AML$_{331\text{-}2L}$, TEL-AML1-9V or Melan-A/MART-1 and the assay was performed as described in Fig. 10. (A) The graph shows changes in MFI during 6 hours. (B) Fluorescence indices (FI) of the peptides used are shown.

7 Discussion

7.1 AB*ab*DII mice express a functionally diverse TCR repertoire

AB*ab*DII mice might present an exquisite experimental tool for investigating human HLA-A*0201-restricted epitopes, provided they would express a functionally diverse peripheral TCR repertoire. We have addressed this issue by asking whether AB*ab*DII mice could mount specific functional $CD8^+$ T cell responses against some of the known, previously described HLA-A*0201-restricted epitopes derived from human TAAs.

We have chosen three human TAAs: α-fetoprotein, MAGE-A10 and gp100. Indeed, specific functional responses against all of them were induced in AB*ab*DII mice. To induce specific $CD8^+$ T cell responses we used an assay which included immunization with exact peptide epitopes (with the addition of adjuvants CpG ODN and IFA), followed by *in vitro* overnight peptide restimulation. One has to note that we were able to induce specific responses against all of the antigens tested by using this protocol, without the need for prolonged *in vitro* peptide restimulation. This speaks in favor of proper functionality of $CD8^+$ T cells developing in AB*ab*DII mice. For example, in one of the previous HLA transgenic mouse models, HLA-B*0702 mice, prolonged *in vitro* peptide stimulation over 6 days was needed for induction of effector cells (Alexander et al. 2003).

Since MHC class II locus in AB*ab*DII mice is murine, it was not possible to use cognate human HLA class II-restricted epitopes to aid induction of HLA-A*0201-restricted responses against the human TAAs tested. Therefore we used an immunization strategy which includes administration of CpG ODN, shown to be useful in induction of anti-Melan-A/MART-1 responses, when the exact class I-restricted epitope without any cognate helper epitopes was used (Miconnet et al. 2002). CpG ODN induce maturation of DCs, which should then be able to activate CTL precursors via signaling through CD40 on DCs in $CD4^+$ helper T cell-independent manner (Bennett et al. 1998; Ridge et al. 1998; Schoenberger et al. 1998). Indeed, this strategy proved to be useful in our system as well. Besides CpG ODN which served as immunostimulant, another adjuvant included in this immunization protocol, IFA, an oil-in-water emulsion, served primarily as a depot for peptides (Reed et al. 2009). Further, this strategy of immunization with exact peptide epitopes in adjuvants seemed to be effective even after a single immunization, independent of additional boosting: two booster immunizations improved response only in a single mouse immunized with $gp100_{280\text{-}9V}$ (Fig. 5B), but this had to be attributed to individual variation. This was in concordance with the results from the study by Zwaveling et al, who similarly found that prime-boost regimen of vaccination with minimal CTL epitopes showed $CD8^+$ T cell responses of similar strength as those seen after one vaccination (Zwaveling et al. 2002).

Successful induction of specific responses against all of the tested TAA-derived epitopes might seem unexpected, since AB*ab*DII mice exhibit a decrease in total $CD3^+$ cell number in the spleen, compared to wild type mice (Li et al. 2010). However, total $CD3^+$ cell number in the spleen is only slightly decreased in comparison to HHD mice, but AB*ab*DII mice have higher percentage of $CD8^+$ T cells in the blood in comparison to HHD mice. HHD mice have, nevertheless, successfully been used for

induction of specific responses against human TAA-derived HLA-A*0201-restricted epitopes (Firat et al. 1999). Further, the responses in AB*ab*DII mice could be reproducibly induced, although magnitude of the responses to all three TAAs varied in individual mice analyzed. This variability in the magnitude of response might be due to the fact that AB*ab*DII mice are outbred, since multiple strains had to be crossed in order to generate the mice. For example, 129Sv, BALB/c and C57BL/6 strains were used for generation of AB mice (Li et al. 2010); additionally SJL strain was involved in generation of HHD mice (Pascolo et al. 1997). But although this might lead to variability in individual responses in AB*ab*DII mice, it might be advantageous for a population as a whole, making AB*ab*DII mice responsive to higher number of different antigens. For example, presence of various MHC class II locus linked alleles might exert influence on generation of $CD8^+$ T cell responses. Another possible explanation for variations seen among responses in individual mice might be the relatively low expression level of HHD restriction element (Pascolo et al. 1997).

Therefore, although human TCRs expressed on $CD8^+$ T cells in AB*ab*DII mice are educated on a single human class I allele (HLA-A*0201) forming complexes with murine self-peptides, they enable specific functional responses to different human TAA derived epitopes. This might not be unexpected, if one considers how the repertoire of T lymphocytes is shaped in the thymus by the processes of positive and negative selection. Positive selection of antigen-specific, class I MHC-restricted $CD8^+$ T cells in the thymus requires the specific interaction of the αβ TCR with the restricting class I MHC molecule (Kisielow et al. 1988b; Sha et al. 1988). This process selects a TCR repertoire which is skewed towards recognition of antigen in the context of self-MHC molecules. Negative selection, on the other hand, eliminates developing thymocytes which react to self-MHC, ensuring tolerance to self-MHC (Kappler et al. 1987; Kisielow et al. 1988a). In both processes, developing T cells are selected based on interaction of TCRs with MHC/self-peptide complexes. But the outcome depends on the affinity of the interaction: a high affinity MHC/peptide complex deletes the interacting TCR, while a low affinity MHC/peptide complex positively selects the TCR, as measured directly by Biacore (Alam et al. 1996). Selected TCRs, which recognized a self-MHC/self-peptide complex with a low affinity in thymus, should hopefully recognize a self-MHC/foreign peptide with high affinity at the periphery. TCRs which recognized a self-MHC/self-peptide complex with high affinity should be deleted, which should prevent autoimmunity. Therefore, since the used human TAA derived epitopes had different sequences from the corresponding murine sequences, it might not be surprising that the TCR repertoire selected in AB*ab*DII mice was competent for efficient and specific recognition of these human epitopes. These human peptides represent foreign antigens for the mouse. However, human TAA derived epitopes used possess only one to two amino acid differences in the region of the peptide preferentially recognized by TCR (since position 9 represents a primary MHC-binding anchor residue), as compared to the murine sequences. Especially in the case of α-fetoprotein$_{158}$ this similarity is striking, since the only differing residue primarily recognized by TCR is a conservative substitution K → R. Yet, mice were not tolerant to human α-fetoprotein$_{158}$ and specific responses were successfully induced. Furthermore, one can propose that precisely because of such similarity of human TAA derived epitopes to murine proteins, the TCR repertoire selected in AB*ab*DII is especially suited for specifically recognizing these human epitopes. Namely, as first suggested in the study by

Nikolić-Zugić and Bevan (Nikolic-Zugic and Bevan 1990), self-peptides are critically involved in positively selecting TCR repertoire. The authors analyzed the ability of variant H-2K^b molecules to positively select T cells that could respond to H-2K^b with ovalbumin. The ability of different H-2K^b variants to select this response in thymus correlated with their ability to present the ovalbumin peptide, which indicated that a self-peptide mimic of the foreign peptide could be involved in positive selection. A study by Hogquist et al. soon afterwards directly identified peptides with the ability to induce positive selection, by using fetal organ thymic culture, an *in vitro* method for studying positive selection (Hogquist et al. 1994). All of the peptides able to induce positive selection indeed represented variants of the antigenic peptide (having from 1-4 amino acids substitutions) and were identified as TCR antagonists in their study. Unrelated peptides were very inefficient in inducing positive selection. Therefore, it is conceivable that in AB*ab*DII mice, the murine peptides, being similar to human TAAs, might even aid in selecting TCR repertoire specific for the human TAAs. However, at present this question remains unresolved.

Importantly, another human TAA, Melan-A/MART-1, used as a positive control in the experiments shown in Figures 21, 22 and 25, also induced specific functional responses in AB*ab*DII mice. Its amino acid sequence (E<u>L</u>AGIGIL<u>T</u>V) differs from the murine corresponding sequence (E<u>A</u>AGIGIL<u>I</u>V) only in the MHC anchor residue at position 2 (A → L), which is a modified residue described previously (Valmori et al. 1998), and in the residue at the position 8 which serves primarily for recognition by TCR (I → T). However, one should point out that specific functional CD8$^+$ T cell responses against the described TEL-AML1 peptide, which is a completely foreign peptide both in humans and mice, were successfully induced in AB*ab*DII mice as well (but only provided a short nonamer peptide is used, since the peptide was in fact not naturally processed, as revealed by the present study and as would be discussed in detail in the further text). Altogether, five out of five HLA-A*0201-restricted, previously described human epitopes used in this study induced specific functional CD8$^+$ T cell responses in AB*ab*DII mice, pointing to the existence of a functionally diverse human TCR repertoire.

Our results showing the functional overlap between TCR repertoires in humans and AB*ab*DII mice are not unexpected in view of the previous findings in different mouse models transgenic for human HLA class I molecules. In the first study describing A2.1/K^b mice, it was reported that the mice were a valid model for investigating human epitopes, since priming *in vivo* with PR8 influenza virus led to the generation of influenza-specific A2.1/K^b-restricted CTLs, and a portion of the response was directed toward the dominant human HLA-A*0201-restricted influenza epitope (Vitiello et al. 1991). Already from this early study, it seemed that even in xenogeneic combinations (whereby murine TCRs interacted with human MHC class I), determinant selection by MHC was the major factor in dictating the antigen-specific TCR repertoire. Indeed, a subsequent study confirmed that a good correlation existed between the CTL repertoire of A2.1/K^b mice and HLA-A*0201-positive individuals, using different viral epitopes derived from HBV, HCV and HPV16 (Wentworth et al. 1996). A number of additional studies confirmed that a large functional redundancy existed between murine TCR repertoires educated on human HLA class I molecules and human TCR repertoire, when responses against epitopes derived from human viral and non-viral pathogens were investigated. HLA-A11/K^b

transgenic mice were shown to have a functional CTL repertoire, able to respond to similar HLA-A*1101-restricted epitopes derived from HIV, EBV and HBV, which were also recognized in humans (Alexander et al. 1997). Eleven peptides from HCV proteins, with HLA-A*0201-binding motifs, were tested for induction of CTLs in HLA-A*0201 transgenic mice and HCV-infected patients and virtually identical repertoire was revealed – the same four out of eleven peptides were strongly recognized by both human and murine CTLs (Shirai et al. 1995). Recognition of similar epitopes derived from HBV, HIV, Mycobacterium tuberculosis Ag85B, influenza A and *Plasmodium falciparum* by murine CTLs in HLA-A*0201, A2.1/K^b, HLA-A11/K^b, HHD and HLA-B*0702 transgenic murine models, as compared to recognition by human CTLs was described in numerous studies (Pascolo et al. 1997; Ishioka et al. 1999; Geluk et al. 2000; Loirat et al. 2000; Alexander et al. 2003). Therefore, since in all those models xenogeneic interactions between murine TCRs and human class I permitted development of broad TCR repertoires functionally similar to human, it should be expected that the TCR repertoire in AB*ab*DII mice would be at least as functional and diverse as in those previous murine models, since human TCRs in AB*ab*DII mice were educated on human HLA class I.

It is also evident that the majority of the studies cited above, which investigated functional overlap of murine TCR repertoires in HLA class I transgenic mice and the human TCR repertoire, employed known human viral epitopes as probes. Specific CTL responses against viral epitopes were successfully induced both in A2.1/K^b mice, which expressed murine MHC class I alleles, as well as in HHD mice, which did not express murine MHC class I. Good performance of both of the systems might seem surprising, since transgenic mice which still express murine MHC class I show a bias in favor of H-2-restricted CTL responses (Firat et al. 1999). However, viral CTL epitopes are known to be among the highest affinity MHC binding peptides (Celis et al. 1995). Studies of CTL induction in HLA-A*0201/K^b and HLA-B*0702 transgenic mice showed that for high affinity binding peptides 'holes in the repertoire' were more difficult to find (Sette et al. 1994; Alexander et al. 2003). This could partially explain relatively good outcome of testing TCR repertoire in different transgenic models by viral epitopes. But, in a study where CTL induction in transgenic mice against epitopes derived from human TAAs was evaluated, most of the peptides were found to be immunogenic in HHD mice, whereas only a minority of them induced responses in mice transgenic for HLA-A*0201/K^b, which still expressed murine H-2 (Firat et al. 1999). From these studies, it seems that induction of specific $CD8^+$ T cell responses towards human TAAs should present a more stringent way of testing functional diversity of a transgenic TCR repertoire, compared to induction of anti-viral responses, which were in general successfully induced in the different transgenic models developed previously. This is the reason we used human TAA-derived epitopes for functional testing of the TCR repertoire in AB*ab*DII mice.

It is important to stress that these previous studies in HLA transgenic mice (Vitiello et al. 1991; Sette et al. 1994; Alexander et al. 2003) provided evidence in favor of the determinant selection hypothesis, which stated that the genetic control of T cell responses mainly was the consequence of the capacity of certain antigenic determinants to bind specific MHC alleles. An alternative view, proposing that the tolerance mechanisms, such as deletion or functional inactivation of autoreactive T cells, would lead to the absence of certain TCR specificities, creating 'holes in the T cell repertoire', was not

substantiated to a greater extent. Namely, in these studies (Sette et al. 1994; Alexander et al. 2003), panels of peptides were synthesized based on HLA-allele-specific peptide motifs, tested for HLA binding *in vitro*, and then tested for immunogenicity in transgenic mice. Indeed, the great majority of high affinity HLA binding peptides proved to be immunogenic. Interestingly, in our study, among the six peptides which we directly tested for HLA-A*0201 binding capacity (TEL-AML1, TEL-AML1-9V, TEL-AML1-2L, TEL-AML1$_{331}$, TEL-AML1$_{331\text{-}2L}$ and Melan-A/MART-1), only three that turned out to be very poor HLA-A*0201 binders (TEL-AML1, TEL-AML1$_{331}$ and TEL-AML1$_{331\text{-}2L}$) were incapable of *in vivo* CTL priming in AB*ab*DII mice. The good binders (TEL-AML1-9V, TEL-AML1-2L and Melan-A/MART-1) could prime CTLs. These results seem to be in concordance with the above studies, implying that the negative selection process involving murine self-peptides in the thymus apparently does not result in any 'holes' in the functional human TCR repertoire in AB*ab*DII mice, and that the presence of the HHD restriction element should indeed enable development of a diverse repertoire.

Finally, although AB*ab*DII mice carry a small deletion present in the human TCR-β locus (comprising Vβ5.1 and Vβ6.1 segments), the above considerations imply that, although having impact on the 'germline' TCR repertoire, this should not have any significant impact on the functional peripheral TCR repertoire; however, at the moment it is too early for any firm conclusions.

Successful induction of specific CD8$^+$ T cell responses towards human TAAs in AB*ab*DII mice might also be important for reasons other than testing competence of their TCR repertoire. These TAAs are expressed, as discussed in the introduction (section 3.4), in different types of cancers, but also in a limited number of peripheral healthy tissues. This would imply that T cell responses to these antigens should be subject to post-thymic peripheral tolerance mechanisms (Stockinger 1999). However, medullary thymic epithelial cells (mTECs) have been shown to promiscuously express a diverse range of tissue-specific antigens (Derbinski et al. 2001); T cell clonotypes specific for these tissue-specific antigens are therefore subject to central tolerance mechanisms as well. In other words, deletion of TCRs recognizing these peripheral self-antigens with high affinity is likely to occur due to negative selection in the thymus. Among the antigens reported to be expressed in the thymus in the original study were, importantly, both α-fetoprotein and gp100 (Derbinski et al. 2001). However, increasing number of tissue-specific antigens expressed in the thymus has been reported since, including other TAAs (Cloosen et al. 2007). In the settings of TCR gene therapy of cancer, which would target some of these TAAs (provided one would be able to control potential autoimmune attack on self-tissues), it might be crucial to use high-affinity TCRs for successful eradication of cancer cells (Schumacher 2002). Such high-affinity TCRs might be difficult to isolate by *in vitro* priming of human cells, since they might be already deleted, due to their promiscuous expression in the thymus. An approach which enables circumventing this hurdle is to prime human CD8$^+$ T cells *in vitro* using DCs expressing an allogeneic MHC; such allo-restricted CTLs can express TCRs of higher affinity compared to self-restricted CTLs, since they have never been exposed to negative selection in the thymus (Wilde et al. 2009). *In vivo* induction of immune responses in AB*ab*DII mice, on the other hand, also provides an opportunity for isolation of high-affinity TCRs out of a non-tolerant repertoire. Namely, since most of the human TAAs have sequences different from the homologous murine proteins, high-affinity HLA-A*0201-restricted human TCRs specific for the human TAA-derived epitopes should not undergo

negative selection in the thymus in AB*ab*DII mice. Importantly, we have shown that even in the case of α-fetoprotein$_{158}$ epitope, with a very high similarity to the corresponding mouse sequence, T cells were not tolerant to the human epitope and specific CD8$^+$ T cells were successfully induced. One might expect that the CD8$^+$ T cells specifically reacting to human TAAs, which we detected in the present study, might express high-affinity TCRs, and might therefore be used in TCR gene therapy. This might be particularly attractive in the case of MAGE-A10 antigen, since its expression in testis (and in placenta) (De Plaen et al. 1994) should not lead to autoimmune attack by transferred T cells in patients, as these tissues might not express MHC class I antigens. However, when considering potential application of CD8$^+$ T cell responses specific for MAGE-A10 we detected, expression of MAGE-A10 would have to be thoroughly investigated, in order to make sure that it is not expressed in additional healthy tissues. Important for the success of a therapy targeting MAGE-A10 would further be to investigate if this antigen is homogeneously expressed in individual tumors. Response against alpha-fetoprotein which we detected could also be potentially employed in therapy, provided detailed tests can be made to ensure that low levels of alpha-fetoprotein in serum, which can be detected in humans independently of HCC, are not produced by some vital cells in the body. Importantly, clinical application of these TCRs should be feasible, since they would not be immunogenic in humans. Nevertheless, potential exploitation for TCR therapy of the responses specific for human TAAs, detected here, as well as potential higher affinity of the responding TCRs in comparison to those which might be isolated from humans, is still hypothetical. The immediate importance of the successful induction of specific functional CD8$^+$ T cell responses against the previously described human TAA-derived epitopes used in this study is that this points to the existence of a functionally diverse TCR repertoire in AB*ab*DII mice.

Finally, along with the expression of a functionally diverse TCR repertoire, we were able to show that CD8$^+$ T cells developing in AB*ab*DII mice were capable of properly differentiating into effector CTLs. This conclusion is based on (i) their efficient production of the effector cytokine IFN-γ after a short overnight *ex vivo* restimulation and (ii) their capability of degranulation, which is the crucial property of *bona fide* effector killer cells. Further, we confirmed that AB*ab*DII mice, as shown previously for other murine models, were capable of correctly processing human HLA-A*0201-restricted epitopes. We therefore propose AB*ab*DII mice as a valid tool for investigation of human HLA-A*0201-restricted epitopes.

7.2 The only described T cell epitope derived from the TEL-AML1 translocation is not naturally processed

7.2.1 T cell responses specific for the TEL-AML1 peptide can be induced in AB*ab*DII mice

Having established that the peptide immunization strategy allowed successful induction of responses towards different human TAAs in AB*ab*DII mice, we applied the strategy to induce specific CD8$^+$ T cell response against the described TEL-AML1 epitope. We immunized AB*ab*DII mice with the exact 9-

mer TEL-AML1 peptide in adjuvants; however, no TEL-AML1-specific $CD8^+$ T cell response could be detected even after two additional boosts. In order to try to render the peptide immunogenic, we applied the strategy of modifying MHC anchor residues in this peptide (Parkhurst et al. 1996). The peptide modified at the position 9, TEL-AML1-9V, induced specific responses in ABabDII mice. Notably, another peptide we designed, with a modification at the position 2, TEL-AML1-2L, induced a comparable response in a preliminary experiment. The effector $CD8^+$ T cells primed *in vivo* with the anchor-modified variant TEL-AML1-9V retained the specificity for the native TEL-AML1, as they specifically responded to *in vitro* restimulation with the native peptide by IFN-γ secretion and by degranulation, although this cytolytic response was apparently lower. Somewhat lower cytotoxic response triggered by the native peptide might not be surprising, taking into account its very poor HLA-A*0201 binding affinity.

Although the native TEL-AML1 peptide was described as immunogenic in the study by Yotnda et al. (Yotnda et al. 1998), we could not detect this in our system, unless an anchor-residue was modified. We wouldn't have expected such an outcome of immunization of AB*ab*DII mice, since Yotnda and colleagues reported that the synthetic (native) TEL-AML1 peptide was successfully used for primary CTL induction from human PBMCs. Another study reported that *in vitro* induction of human primary CTL responses from PBMCs might have lower sensitivity then murine *in vivo* assay, whereby CTLs were induced by peptide immunization of HLA-A*1101/K^b mice followed by *in vitro* peptide restimulation, the result having been obtained with two known dominant human epitopes derived from HIV (Alexander et al. 1997). In that study, epitopes HIV pol 325 and HIV pol 507 failed to generate CTL responses in the human primary *in vitro* system but scored positive in the HLA-A*1101/K^b transgenic mice, although their human primary *in vitro* system was successfully used for induction of responses to other epitopes tested in parallel. Therefore, we would have expected that our AB*ab*DII mouse *in vivo* assay should enable induction of TEL-AML1 specific $CD8^+$ T cells, especially since we have successfully established the method on the other human TAAs tested. The reason more to be puzzled by the absence of immunogenicity of the native TEL-AML1 in AB*ab*DII mice was the fact that TCRs recognizing the native peptide were clearly present in the AB*ab*DII TCR repertoire, as $CD8^+$ T cells specific for the native TEL-AML1 could be induced by immunizing the mice with the anchor-modified TEL-AML1-9V.

In an attempt to resolve why the native peptide did not evoke any specific $CD8^+$ T cell response, we tested its binding to HLA-A*0201^+ in a kinetic binding assay using the T2 cell line. Surprisingly, the native peptide bound very poorly to HLA-A*0201, as opposed to good binding of the two anchor-modified TEL-AML1 peptides and Melan-A/MART-1. In the study by Yotnda et al. a moderate dissociation rate of TEL-AML1/HLA-A*0201 complexes was reported, with the half-life of complexes of 3 hours, measured also in the kinetic binding assay using T2 cells. However, since the data were not shown, we could not comment on possible causes of this discrepancy. Nevertheless, taken into account that binding of the native peptide is very weak, it might seem surprising that the $CD8^+$ T cells primed with the anchor-modified peptide could afterwards recognize the native one at all. On the other hand, it has been reported that for generating effector $CD8^+$ T cells by using heteroclitic peptides, the rate limiting step was the priming step and once the effector cells were generated, the binding affinity

of the native peptide to MHC class I was not limiting in the capacity of these cells to perform effector functions (Zirlik et al. 2006). The same study reported increased killing of targets expressing native peptides with FI equaling zero. FI for the native TEL-AML1 peptide equals 0.27 (Fig. 10B), which might explain its specific recognition by anchor-modified peptide primed $CD8^+$ T cells. In any event, since TCRs specific for the native TEL-AML1 clearly were present in the AB*ab*DII repertoire, poor binding of the native peptide provided the most probable explanation for the absence of its immunogenicity in AB*ab*DII mice.

7.2.2 TEL-AML1 is not processed in any of the settings: AB*ab*DII mice, potent APCs or by purified human proteasomes

Our attempt to enhance immunogenicity of the native TEL-AML1 peptide, by utilizing another immunization strategy with extended 30-mer peptides comprising the exact epitope in adjuvants, revealed the lack of processing of this peptide in AB*ab*DII mice *in vivo*. Namely, since the anchor-modified 9-mer TEL-AML1-9V is immunogenic in AB*ab*DII mice, the absence of specific response to the long anchor-modified peptide TEL-$AML1_{319\text{-}9V}$ can be best explained by absence of endogenous processing of the described epitope. There are two distinct antigen processing pathways for extracellular and intracellular antigens, controlled by two separate MHC classes and leading to stimulation of either $CD4^+$ or $CD8^+$ T cells, whereby extracellular antigens are processed in the endosomal compartment and end up bound to MHC class II for presentation to $CD4^+$ T cells, while intracellular antigens are processed in the cytosol mainly by proteasome, ending up bound to MHC class I to stimulate $CD8^+$ T cells. However, it is well established that alternative antigen presentation pathways do exist as well (Reimann and Kaufmann 1997). Professional APCs, such as macrophages and DCs, are capable of internalizing extracellular antigens and cross-presenting them to class I-restricted $CD8^+$ T cells. This cross-presentation mechanism into the class I pathway is relevant for the comparison of immunization strategies with short and long peptides. While short, exact epitope peptides, may bind to MHC class I molecules on all nucleated cells (both professional APCs, as well as all other cells), long peptides need to be taken up and processed by professional APCs, since their size excludes direct binding to MHC class I; the professional APCs are then able to cross-present these exogenous long peptides in the context of MHC class I molecules. This indeed takes place in the presence of DC-activating agents such as CpG ODN, leading to more efficient induction of $CD8^+$ T cells by long compared to short peptides (Zwaveling et al. 2002). Importantly, it has been shown that this cross-presentation of exogenous antigens internalized into phagosomes of macrophages was resistant to chloroquine, which inhibits acidic hydrolysis in the lysosomal compartment, meaning that this pathway was separate from endosomal class II presentation pathway (Kovacsovics-Bankowski and Rock 1995). The same study showed that this cross-presentation was blocked by inhibitors of the proteasome, by a mutation in the TAP transporter, and by Brefeldin A (which inhibited exocytosis of proteins from the ER and Golgi complex and prevented peptide/MHC class I complexes from reaching the cell surface). This shows that internalized extracellular antigens do enter the cytosol from the phagosome and that they are indeed processed by the proteasome in the same manner as the endogenously synthesized antigens, and also presented through TAP, ER and Golgi complex in the

same way as endogenous antigens. Importantly, this shows that the same class I epitopes should be processed and presented through both the classical class I pathway and the alternative cross-presenting pathway. Indeed, the correct $gp100_{280\text{-}9V}$ epitope was processed and presented after immunization with the long peptide, since $gp100_{280\text{-}9V}$-specific $CD8^+$ T cells were successfully induced. This confirms that the exogenous long peptides were internalized, processed and presented in the context of MHC class I by professional APCs (presumably induced by CpG ODN) in AB*ab*DII mice. Therefore, the absence of specific response to the long anchor-modified peptide TEL-$AML1_{319\text{-}9V}$ allows us to conclude that the described TEL-AML1 epitope is in fact not endogenously processed.

Importantly, it is most unlikely that putative species-specific differences in antigen processing in mice and humans could have accounted for the observed results since, on the example of human TAA gp100, we show that antigen processing and presenting machinery in ABabDII mice is capable of correctly generating a human HLA-A*0201-restricted epitope. Moreover, we thus confirm the findings of multiple studies (Gomard et al. 1986; Vitiello et al. 1991; Shirai et al. 1995; Pascolo et al. 1997; Firat et al. 1999; Ishioka et al. 1999; Geluk et al. 2000; Loirat et al. 2000; Yang et al. 2000; Pasquetto et al. 2005), showing that the murine antigen processing and presenting machinery correctly generates human HLA-A*0201-restricted epitopes. However, although the control peptide $gp100_{265\text{-}9V}$ clearly induced specific response, we could not formally exclude that some additional factors apart from peptide processing might have accounted for the response towards gp100 and the absence of such a response towards TEL-AML1. For example, *in vivo* peptide degradation kinetics of the two peptides could be so drastically different, that we could not detect any response towards TEL-AML1 on day 10 after immunization, although the response towards gp100 was clearly visible at the same time after immunization. One has to point out that this is highly unlikely, since previous studies using diverse long peptides for immunization reported that response was consistently visible at day 10, and was even detectable at day 30 (Zwaveling et al. 2002; Bijker et al. 2007). Nevertheless, to formally exclude this possibility, we next designed an experimental strategy which directly addressed the issue of endogenous processing of TEL-AML1, without influence of other factors affecting success of immunization.

This approach indeed confirmed the lack of endogenous processing of TEL-AML1. Effector $CD8^+$ T cells were induced in AB*ab*DII mice by immunization with the immunogenic, anchor-modified nonamer peptide and the effectors were tested for the recognition of NIH-HHD-TEL-AML1 cells, endogenously expressing the TEL-AML1 fusion region. Although exogenously loaded peptides onto NIH-HHD cells were efficiently recognized, there was no recognition of the endogenously expressed TEL-AML1. The generated control cell line efficiently processed and presented another human TAA Melan-A/MART-1. Indeed, the generated cells were potent APCs, as judged by the finding that the response to the endogenously expressed control antigen Melan-A/MART-1 was repeatedly slightly stronger than the response to NIH-HHD cells exogenously loaded with the Melan-A/MART-1 peptide. Hence, the NIH/3T3-based cell lines, besides functioning as efficient APCs for presentation of exogenous peptides, were capable of even more efficiently processing and presenting the UPR-based polypeptides. Therefore, if the TEL-AML1 fusion region would be endogenously processed, we would have certainly been able to detect it in our system, taken into account that we do detect the response

towards the exogenously loaded peptide. Importantly, our model system enabled precise quantitative comparison of the expression levels of the control Melan-A/MART-1 and the test TEL-AML1 antigen in the APCs. The levels were virtually identical, hence could not account for the observed results.

Although both the proteasome, as well as aminopeptidases, are involved in the generation of MHC class I restricted ligands, efficient recognition of NIH-HHD-Melan-A/MART-1 cells implies that proteasomal degradation might be preferentially involved in the generation of exact epitopes out of UPR-based polypeptides in our system. Namely, the control antigen Melan-A/MART-1 was not chosen solely based on the fact that the immunodominant HLA-A*0201-restricted epitope in humans was known (Kawakami et al. 1994c; Romero et al. 1997), but also since the intracellular degradation pathway leading to the generation of this immunodominant epitope has been extensively characterized in previous studies. Proteasome-dependent degradation was found to be an essential proteolytic pathway for the presentation of this epitope, since the recognition by specific CTLs of NA8-MEL melanoma cells transiently transfected with Melan-A/MART-1 encoding plasmids (carrying the same anchor-modification as used here) was dramatically reduced by addition of the proteasome inhibitor lactacystin (Rimoldi et al. 2001). Importantly, using the UPR technique and comparing recognition of NA8-MEL cells transfected with constructs expressing either the exact Melan-A/MART-1 epitope, a C-terminally extended precursor or an N-terminally extended precursor, it was found that the final antigenic Melan-A/MART-1 peptides directly produced by the proteasome are preferentially selected for presentation by HLA-A*0201 molecules (Chapatte et al. 2004). Their study indicated that postproteasomal processing of N-terminally extended Melan-A/MART-1 peptides by aminopeptidases might not significantly contribute to the pool of antigenic peptides loaded and presented by HLA-A*0201 molecules. Therefore, proteasomal processing of both the C terminus (as is the case for all MHC class I ligands), but also of the N terminus, seems to be needed for efficient presentation of the Melan-A/MART-1 epitope. Since Melan-A/MART-1 epitope is very efficienty endogenously processed and presented in our system, this implies that proteasomal degradation indeed is highly involved in processing and presentation of UPR-based constructs in the APCs we generated here. Therefore, besides clearly showing that the described TEL-AML1 epitope is not endogenously processed, the results of the experiments using NIH/3T3-based cell lines point towards inefficient processing by the proteasome as the main reason for the absence of recognition of the endogenously expressed TEL-AML1.

Efficient recognition of the Melan-A/MART-1 epitope processed and presented by NIH-HHD-Melan-A/MART-1, derived from murine NIH/3T3 fibroblasts, additionally confirms that the murine antigen processing and presenting machinery can correctly generate human HLA-A*0201-restricted epitopes. This is in concordance with the previous studies, which used APCs derived from the same NIH/3T3 murine cell line to process and present human antigens. Papanicolaou et al. successfully used those cells to stimulate CMV-specific human CTLs (Papanicolaou et al. 2003). Interestingly, besides showing that NIH/3T3-derived APCs correctly processed the investigated CMV_{495} epitope, they found that the cell line expressing the epitope within the context of the natural CMV protein sequence was even more efficient in generating CTLs, compared to the cells expressing a preprocessed exact nonamer CMV_{495} epitope. This is also in agreement with our own results, where Melan-A/MART-1

epitope endogenously processed out of the flanking natural amino acid sequences is very potent in stimulating the responding $CD8^+$ T cells. Dupont et al. similarly showed that NIH/3T3-derived APCs correctly processed and presented epitopes derived from a human tumor epitope, human telomerase reverse transcriptase (hTERT) (Dupont et al. 2005). There is another interesting aspect of using NIH/3T3 fibroblasts for derivation of APCs for presentation of human antigens. High efficiency of NIH/3T3-derived artificial APCs in inducing human CTLs was actually first shown in the study by Latouche et al, where the authors introduced costimulatory molecule B7.1 (CD80) and adhesion molecules ICAM-1 (CD54) and LFA-3 (CD58), normally expressed at high levels on DCs (which are able to induce naïve T lymphocytes), in addition to the restriction element HLA-A*0201 (Latouche and Sadelain 2000). The APCs we generated here, efficiently stimulated effector $CD8^+$ T cells without any need for introduction of costimulatory/adhesion molecules. The most probable reason might be that, since naïve $CD8^+$ T cells are primed *in vivo* in our experimental system, high-level presentation of antigen by APCs in the context of the appropriate restriction element (HHD) is sufficient for detection of the recall *in vitro* response, without additional costimulation.

As stated above, the results of the experiments using NIH/3T3-derived APCs not only showed that the TEL-AML1 epitope was not endogenously processed, but also pointed towards absence of its processing by the proteasome. To formally prove that the proposed epitope was in fact not processed by the proteasome, the long peptide spanning the TEL-AML1 fusion region was digested by purified 20S proteasomes. Furthermore, to confirm that the TEL-AML1 epitope could not be processed by human proteasomes, the proteasomes purified from LCL, which are human B lymphocytes immortalized with EBV, have been used. LC-MS/MS analysis of the recovered fragments indeed showed that human proteasomes generated multiple cleavages within the fusion TEL-AM1 region, leaving neither the proposed epitope, nor any potential N-terminally extended precursor intact.

The reductionist approach applied in our study, starting with the investigation of processing of the TEL-AML1 peptide in AB*ab*DII mice *in vivo*, followed by revealing the lack of processing in the generated antigen presenting cell line and concluded by investigation of proteasomal digestion *in vitro*, allowed us to draw a formal conclusion that the TEL-AML1 peptide was not processed by the proteasome. As the destruction of the TEL-AML1 by human proteasomes *in vitro* confirmed our findings obtained *in vivo* in mice and in intact cells, it is important to note that numerous previous studies similarly showed that generation of MHC class I ligands from polypeptide substrates by 20S proteasomes *in vitro* closely reflected their generation in intact cells, both qualitatively and quantitatively. This has been shown, among others, for epitopes derived from murine CMV (Eggers et al. 1995), HBV core antigen (Sijts et al. 2000a), adenoviral early 1B protein (Sijts et al. 2000b) and lymphocytic choriomeningitis virus (LCMV) (Schwarz et al. 2000). Such concordance might actually seem surprising. Namely, one has to keep in mind that the 20S proteasome constitutes merely the proteolytic core of the larger 26S complex found in vivo, which comprises the 19S regulator as well (Kloetzel 2001). However, as the main function of the 19S regulator complexes *in vivo* is in ATP-dependent opening of the central gate leading to the 20S core catalytic chamber, as well as the reverse-chaperone-like unfolding of protein substrates (Kloetzel 2004), it becomes understandable how the isolated 20S core complex correctly digests the polypeptide substrates without the aid of the

regulators. Namely, substrates for *in vitro* digestion are polypeptides which are considerably shorter than whole protein substrates and which cannot form intricate secondary or higher structures, thereby abolishing the need for help in unfolding and channeling them into the catalytic chamber. By the same token, although substrates for proteasomal degradation are classically conjugated to Ub prior to destruction, the substrate-linked multi-Ub chain which binds to the 19S regulator probably serves to decrease the rate of dissociation of a substrate-proteasome complex, thereby aiding in unfolding of a relevant region of the substrate and increasing the probability of substrate degradation (Varshavsky 1997). Therefore, it also becomes understandable how polypeptide substrates are correctly processed by the proteasome *in vitro* without the need for prior ubiquitylation. Of note, as discussed in the introduction (section 3.6), numerous studies also showed that ubiquitylation is not an absolute prerequisite for class I antigen processing (Carbone et al. 1989; Murakami et al. 1992). Additionally, although Michalek et al. proposed, based on investigations in cells exhibiting a temperature-sensitive defect in Ub-conjugation, that Ub-dependent proteolytic pathway was necessary for generation of class I epitopes (Michalek et al. 1993), a later study using the same cell line revealed some residual Ub-conjugating activity on non-permissive temperatures (Cox et al. 1995). Further, by using an additional temperature sensitive mutant cell line, the authors showed that cells in fact might not need an intact Ub-conjugating system for processing of endogenous antigens (Cox et al. 1995). These findings are in agreement with our results, which show concordance between *in vivo* and *in vitro* processing of MHC class I epitopes.

Concordant results revealing lack of processing of TEL-AML1 both in murine model systems (AB*ab*DII mice and murine NIH/3T3-derived APCs) and in a human model (LCL-derived proteasomes) were to be expected in view of the study showing that the ability of proteasomes to generate potentially immunocompetent peptides evolved well before the vertebrate immune system (Niedermann et al. 1997). This study presented a remarkably high degree of evolutionary conservation of the proteasomal cleavage patterns. Several major human or mouse self-epitopes, originally eluted from MHC class I molecules, were found to be the predominant products generated after digesting of their respective source polypeptides by invertebrate 20S proteasomes, such as insects- and yeast-derived proteasomes. It was concluded that the proteasomes may have influenced the evolution of the polymorphic MHC class I system in vertebrates, and not vice versa.

Since multiple evidence presented here, both in murine and human model systems, *in vivo* as well as *in vitro*, concordantly reveal lack of natural processing of the previously described TEL-AML1 epitope, we conclude that this peptide cannot be proposed as a potential target in immunotherapy of leukemia in HLA-A*0201-restricted fashion.

Our results are in contrast with those from the study by Yotnda et al. (Yotnda et al. 1998). In their study, the TEL-AML1 peptide was used to prime CTLs, which were reported to specifically recognize autologous leukemic cells and the REH cell line, a human ALL cell line expressing the TEL-AML1 protein. However, most of the cytotoxicity assays in the study were performed with T cell lines, not with isolated T cell clones. Since T cell specificity is inherently cross-reactive (Mason 1998; Wilson et al. 2004), a polyclonal population can be expected to respond non-specifically to some extent. In the aforementioned study, cytotoxic activities of the T cell line and the isolated CTL clones were low, with

high levels of background killing, making it difficult to judge whether the reactivity was indeed specific for the TEL-AML1 fusion peptide. Moreover, comparable (and low) levels of killing of both the HLA-A*0201-transfected and the HLA-A*0201-negative REH line pose a similar question regarding HLA-A*0201 restriction.

Regarding our conclusion that the TEL-AML1 peptide is not a suitable target for adoptive cell therapy due to absence of its endogenous processing, it is important to stress that the proteasome consists of different subunits and their abundance within the cell determines predominance of certain proteasomal forms, such as standard and immunoproteasome (Kloetzel 2001). The 20S proteolytic core is composed of multiple α and β subunits and three β subunits (two copies of each) - β1, β2 and β5 - harbor catalytically active sites. Upon IFN-γ induction, the catalytically active subunits are exchanged for inducible LMP2/β1i, MECL1/β2i and LMP7/β5i subunits, to form immunoproteasome. Besides in IFN-γ-stimulated cells, immunoproteasomes are expressed constitutively in some cells, mainly in the lymphoid tissues such as thymus, spleen and lymph nodes (Sijts and Kloetzel 2011). In line with the high evolutionary conservation of the proteasome (Niedermann et al. 1997), another study which performed direct comparison of peptide fragments generated by standard proteasomes isolated from mouse C4 fibroblasts and human carcinoma HeLa cells, as well as such comparison involving immunoproteasomes isolated from the same murine and human cells after IFN-γ treatment, revealed no species-specific proteasomal cleavage properties (Sijts et al. 2000a). Immunoproteasomes compared to standard proteasomes, however, do exhibit altered cleavage site preferences, and therefore generate certain epitopes with very different efficiencies (Sijts and Kloetzel 2011). Nevertheless, direct mass spectrometric (MS) analyses of digested polypeptides comprising such epitopes revealed large, yet solely quantitative differences in the epitopes (or epitope precursors) generated by standard, as compared to immunoproteasomes. For instance, although CTLs specific for a human TAA MAGE-A3-derived epitope could efficiently recognize MAGE-A3 expressing cell lines only after IFN-γ treatment or transfection with immunosubunits, direct MS analyses of generated fragments after digestion with either standard or immunoproteasomes revealed that the antigenic peptide was present in both digests, but it was much more abundant after immunoproteasomal digestion (Schultz et al. 2002). Further, processing of an adenoviral early 1B (E1B) protein-derived epitope could be considered as immunoproteasome-dependent, since target cells were lysed above background levels only after induction of immunosubunits. However, the effector CTLs secreted TNF-α after recognition of both the targets expressing standard, as well as immunoproteasomes, although in very different amounts. Direct biochemical MS analysis further confirmed that both types of proteasomes generated the epitope, although with strikingly different efficiencies and kinetics (Sijts et al. 2000b). Interestingly, the same study reported complete independence of immunosubunits of another adenoviral E1A-derived epitope. Immunoproteasomes were shown to be essential for clearance of *Lysteria monocytogenes* in nonlymphoid tissues, thus characterizing the listeriolysin O derived epitope LLO_{296} responsible for immune recognition in this model as immunoproteasome-dependent; however, digestion analysis again revealed that immunoproteasomes strongly, but only quantitatively influenced the processing of the epitope (Strehl et al. 2006). Similarly, digestion of an epitope derived from LCMV nucleoprotein, characterized as

immunoproteasome-dependent by immunological assays, revealed only quantitative differences in epitope and precursor fragments generated by two types of proteasomes (Schwarz et al. 2000). Therefore, it seems that both standard and immunoproteasomes are able to generate even those epitopes, which could be immunologically characterized as either standard- or immunoproteasome-dependent. Human LCL-derived proteasomes used for digestion analysis in this study are predominantly immunoproteasomes. As we do not know the precise composition of proteasomes which might be expressed in leukemic blasts *in vivo*, we cannot completely exclude that in these cells the TEL-AML1 peptide might be generated. However, in view of the discussed studies (Schwarz et al. 2000; Sijts et al. 2000b; Schultz et al. 2002; Strehl et al. 2006), and taking into account multiple destructive cleavages within the proposed TEL-AML1 epitope detected, that should be unlikely. Furthermore, mere avoidance of the complete destruction might not necessarily allow this peptide to act as a tumor rejection antigen. One might hypothesize that the TEL-AML1 peptide would have to be generated very efficiently, to allow appearance of a sufficient number of surface TEL-AML1/MHC class I complexes, in order to sustain an effective T cell response leading to complete eradication of leukemia-propagating cells. Potential requirement for a high level of processing seems justified, as TEL-AML1 exerts indeed a very poor ability of stabilizing MHC class I heterodimeric complexes and high peptide amounts might be therefore needed to override dissociation of the unstable complexes that would form. By the same token, although it cannot be excluded that leukemic blasts *in vivo* might acquire a proteasomal processing defect, which would allow the TEL-AML1 peptide to appear at the cell surface in MHC class I peptide binding cleft at very low levels, relying on such a putative deficiency in achieving complete therapeutic effect might be questionable. Hence, we conclude that the previously described TEL-AML1 epitope cannot be proposed as a suitable target for adoptive T cell therapy of leukemia.

7.3 Implications for identification of epitopes suitable for targeting by adoptively transferred T cells

Besides specific implications for applicability of the previously described TEL-AML1 epitope in the adoptive cell therapy of childhood BCP-ALL, there are generic implications of our results for defining epitopes suitable for targeting by T cells in the settings of adoptive cell therapy. Namely, our results point to the step in the reverse immunology approach, in which endogenous processing of putative epitopes is tested, as important for the specificity of this approach. In other words, by performing rigorous examinations in this step of the reverse immunological method, the specificity of the method could be increased, thus reducing the chance of detecting a false positive epitope. The first successful identification of a TAA-derived human epitope by reverse immunology was described almost two decades ago, when an HLA-A1-restricted epitope derived from MAGE-A3 was discovered (Celis et al. 1994). The study clearly showed feasibility of the approach and its applicability to any MHC class I-restricted CTL system where the sequence of the (potential) antigen and the MHC anchor peptide motifs were known. It is important to notice that the choice of the starting candidate antigen protein, MAGE-A3, in that study was based on the previous finding that another member of

this gene family, MAGE-A1, coded for an HLA-A1-restricted epitope (van der Bruggen et al. 1991; Traversari et al. 1992). As the specific CTL responses directed against the MAGE-A1 epitope had been previously documented, this was in the study by Celis et al. (correctly) taken as an indication that immunogenic peptides might be encoded by other gene family members. One should indeed think that such peptides from other gene family members might represent genuine epitopes, likely to be successfully processed and presented out of the homologous protein sequences. And indeed, Celis et al. reported that the MAGE-A3-derived epitope that was identified in their study turned out to be a homolog of the previously described MAGE-A1 CTL epitope, a nonamer with only three amino acid differences. On the contrary, when a candidate tumor antigen is chosen based solely on nonimmunological methods, e.g. based on differential analysis of tumor and healthy tissues, such as proteomics or microarray analysis, mutated tumor proteins thus identified provide a rich source of potential peptide epitope sequences, yet their immunogenicity has to be rigorously tested. Why would testing of endogenous processing be critical for proving immunogenicity of a potential epitope and the specificity of reverse immunological method? A probable reason might lie in a relatively high sensitivity, but insufficient specificity of the first steps in the reverse immunology approach for epitope identification. Namely, as discussed above regarding the issue of TCR repertoire selection (section 7.1), previous studies presented evidence in favor of the determinant selection hypothesis, which stated that the ability to bind to a certain MHC molecule was the main factor impacting on a peptide's immunogenicity (Vitiello et al. 1991; Sette et al. 1994; Alexander et al. 2003). This implies that, if a peptide can efficiently bind to a certain MHC allele, there should exist a TCR capable of recognizing it within any TCR repertoire, since other processes, such as negative selection in the thymus, should not significantly impact on the functional repertoire, i.e. there should be no 'holes in the T cell repertoire'. Our data presented in this study seem to be in agreement with the determinant selection hypothesis, since AB*ab*DII mice mount specific responses against diverse HLA-A*0201-restricted human epitopes, and since among the tested peptides, only those which virtually do not bind to HLA-A*0201 are not capable of priming T cells. Another important consideration would be that, as Mason argues in his study (Mason 1998), a very high degree of T cell cross-reactivity exists, whereby an individual T cell clonotype might productively react with up to 10^6 different MHC-associated minimal peptide epitopes (although with differing affinities). He proposes that such a high level of cross-reactivity should be an inherent feature of the TCR, which enables that on average one T cell in a few thousand can respond to a foreign peptide epitope. Importantly, the mathematical model Mason develops is indeed in agreement with experimental data obtained from different model systems, such as estimates of cross-reactivity based on combinatorial peptide libraries or based on APC function. Coming back to a typical reverse immunology procedure, peptide candidates which have been initially chosen from a candidate protein antigen sequence based on the presence of HLA binding peptide motifs and then verified empirically in HLA binding assays, are next tested for immunogenicity either *in vivo* in HLA transgenic mice or *in vitro* by stimulating potential precursor T cells derived from human blood donors. Based on the determinant selection hypothesis, the peptides detected in the previous steps as efficient binders to a particular HLA molecule should be almost certainly recognized by T cells, independently of the TCR repertoire used as a probe. Furthermore, taking into account the high

degree of TCR cross-reactivity, a peptide candidate might be recognized, even if it does not represent the specific ligand for a given T cell clonotype which is actually naturally presented *in vivo*. It follows that, if a given peptide candidate binds to an HLA molecule with a certain high affinity, it will probably successfully pass all these early selection steps in the reverse immunology procedure, although it might not necessarily be genuine peptide epitope processed and presented *in vivo*. Obviously, the method is highly sensitive, as a potential epitope should be rarely missed. However, the following steps, which test the recognition of targets endogenously expressing the candidate antigen by induced T cells, must therefore be performed rigorously, i.e. with a relatively high specificity threshold set, in order to filtrate out some false positive epitopes which might have been detected in the preceding steps. These considerations make it understandable how a peptide, such as the described TEL-AML1 epitope, might induce CTLs, and yet might not represent a true, naturally processed and presented T cell epitope. Thorough testing of endogenous processing is therefore needed, in order to increase the specificity of the highly sensitive reverse immunology approach, which should lead to identification of efficiently processed and presented peptides suited as targets for therapy.

7.4 The TEL-AML1 fusion protein is not a suitable target for HLA-A*0201-restricted T cells

As our results have shown that the only previously described CTL epitope derived from the TEL-AML1 translocation was not naturally processed, we used the model system established here to investigate whether some unidentified HLA-A*0201-restricted TEL-AML1 derived epitopes existed. We initially tested if the decamer peptide TEL-AML1$_{331}$ spanning the TEL-AML1 fusion region and found to be generated by human proteasomes *in vitro*, might be processed *in vivo*. Immunization of AB*ab*DII mice with the long TEL-AML1$_{319}$ peptide did not show induction of a specific response towards TEL-AML1$_{331}$; moreover, introducing an amino-acid exchange at the anchor-residue position of the putative epitope, which should increase its MHC class I binding, could not evoke a response either. Furthermore, when we employed the generated NIH-HHD-TEL-AML1 cell line to present the TEL-AML1 fusion region to CD8$^+$ T cell effectors isolated from mice immunized with the long TEL-AML1$_{319}$ peptide, there were no specific responses detectable. The inability to detect processing of any potential HLA-A*0201-restricted epitope spanning the TEL-AML1 fusion region in AB*ab*DII mice and NIH-HHD-TEL-AML1 cells seemed to be in agreement with the proteasome digestion analysis. Namely, the digestion analysis did not reveal generation of any peptide of suitable length for an HLA-A*0201-restricted epitope, however with the notable exception of the peptide TEL-AML1$_{331.}$ This apparent discrepancy between *in vivo* and *in vitro* processing data for the peptide TEL-AML1$_{331}$ would not be expected, based on the previous studies showing concordance between *in vivo* and *in vitro* processing assays (Eggers et al. 1995; Schwarz et al. 2000; Sijts et al. 2000a; Sijts et al. 2000b). Similarly, the data presented in this study concordantly revealed the lack of processing of the described TEL-AML1 peptide, confirming agreement between the *in vivo* and *in vitro* analyses. However, we further discovered that the TEL-AML1$_{331}$ cannot induce any specific response in AB*ab*DII mice even when applied as an exact short peptide, with or without the anchor-modification.

The most probable reason for the absence of its immunogenicity was its poor ability of stabilizing HLA-A*0201 complexes, which could not be improved by introducing an anchor-modification. These analyses showed that there was in fact no disagreement between the *in vivo* and *in vitro* processing results obtained, and that they all point towards the conclusion that the complete TEL-AML1 fusion region is not immunogenic in HLA-A*0201-restricted settings. Importantly, although based on the results of proteasome digestion *in vitro* we could not formally exclude that some of the generated longer fragments spanning the fusion region might serve as precursors for additional N-terminal trimming *in vivo*, the complete absence of recognition of the TEL-AML1 fusion-expressing APCs by $CD8^+$ T cell effectors isolated from AB*ab*DII mice renders this possibility highly unlikely. It has to be concluded that no HLA-A*0201-restricted epitopes spanning the TEL-AML1 fusion region exist, hence the mutation cannot be recognized as foreign by HLA-A*0201-restricted CTLs.

However, a cautionary note has to be made. One needs to make a distinction between our conclusions regarding the lack of processing of the previously described TEL-AML1 epitope, and the lack of immunogenicity of the complete TEL-AML1 fusion region in HLA-A*0201-restriction settings. The evidence for the lack of processing of the described TEL-AML1 peptide (section 7.2) is based on usage of specific CTL effectors as probes, which could be efficiently induced in AB*ab*DII mice, and as such can be considered as conclusive. On the other hand, when interpreting the results showing the absence of any processed HLA-A*0201-restricted epitope in the TEL-AML1 fusion region, it cannot be formally excluded that our conclusion might be dependent on the read-out system used. In other words, we cannot formally exclude that in another model system it might be possible to induce CTL effectors specific for some putative novel epitopes derived from this fusion protein. For instance, immunization of some of the other HLA-A*0201 transgenic mice, harboring different TCR repertoires, might enable induction of specific CTLs, which we do not detect in our model. This is however not very likely, since transgenic mice lacking murine MHC class I (as our AB*ab*DII model) performed better than those still expressing murine MHC class I, which showed a bias in favor of H-2-restricted CTL responses when tested for CTL induction against human TAAs (Firat et al. 1999). Alternatively, human *in vitro* priming systems could be used, such as CTL induction from autologous PBMCs by peptide pulsed DCs (Brossart et al. 1999). We cannot formally exclude that such human TCR repertoire would contain T cell clonotypes capable of recognizing some novel TEL-AML1 fusion epitopes. However, as our investigation of TCR repertoire in AB*ab*DII mice provided evidence for a functionally diverse repertoire, enabling the mice to respond to multiple human HLA-A*0201-restricted epitopes, it must be regarded as very unlikely that using other model systems would lead to detection of some novel TEL-AML1 epitopes.

Already in the early study by Shurtleff et al. it was reported that the TEL-AML1 translocation characterized the largest genetically defined group in childhood ALL and that it might be the initiating molecular lesion in the disease. The study further reported that TEL-AML1 expression identified a uniform patient subgroup characterized by an age between 1 and 10 years, with B precursor, nonhyperdiploid leukemic blast phenotype and a favorable prognosis, i.e. relatively prolonged event-free survival (Shurtleff et al. 1995). Some later risk- and response-based classifications of BCP-ALL confirmed the value of TEL-AML1 fusion as a good prognostic factor, suggesting underlying biologic

features that mediated treatment outcome (Schultz et al. 2007). Interestingly, in the study by Yotnda et al, where the TEL-AML1 peptide was originally described as a valid HLA-A*0201-restricted epitope, the favorable prognosis for patients with TEL-AML1$^+$ leukemic blasts was also pointed out, as one of the incentives to investigate whether it might represent a specific CTL target (Yotnda et al. 1998). This is reminiscent of immunological surveillance as proposed by Burnet, stating that the essential function of the cellular adaptive immunity is in preventing the emergence of malignant mutant cells (Burnet 1964; Burnet 1970), whereby specific endogenous T cells might be able to recognize and control spontaneous outgrowth of TEL-AML1$^+$ leukemic blasts *in vivo* in patients. However, as described in detail in the introduction (sections 3.1 and 3.2), whereas T cells do have the ability to efficiently recognize specific antigens on transplanted tumors, they cannot control the outgrowth of primary (autochthonous) tumors, although these might be equally immunogenic. Indeed, all direct evidence argues that the cancer immunosurveillance does not play a significant role in preventing primary tumor outgrowth, except in the case of virus-induced tumors, which can be explained by the absence of evolutionary pressure in the settings of spontaneous non-virally induced cancer as a disease of age (Blankenstein 2007). Moreover, even if one takes into account that cALL occurs in childhood, putative cancer immunosurveillance is very unlikely to explain better prognosis for patients with TEL-AML1$^+$ leukemic blasts, since our study shows that this mutation harbors no epitopes which could be processed and presented to HLA-A*0201-restricted CD8$^+$ T cells, which is an HLA allele occurring very frequently in humans. Therefore, we propose that the relatively longer relapse-free survival in TEL-AML1$^+$ cALL patients, observed in multiple studies, is not an immune-mediated phenomenon, but instead represents a result of altered cycling properties of TEL-AML1$^+$ preleukemic cells. Namely, Schindler et al. have shown that TEL-AML1 has the potential to induce premalignant HSCs which enter an altered steady state. They are more quiescent, i.e. cycle more slowly, than the wild-type HSCs, but they persist with a relatively small population size for prolonged time periods and are available for further genetic insults (Schindler et al. 2009). The study by Hong et al. presents slightly discordant results regarding the stage of cell differentiation at which the TEL-AML1 fusion first arises generating a preleukemic cell (Hong et al. 2008). They identify a population of cells already committed to the B lymphoid lineage (characterized as CD34$^+$CD38$^{-/low}$CD19$^+$), as the earliest TEL-AML1 expressing, preleukemic stem cells. Nevertheless, they also conclude that such preleukemic cell population can persist for a very long time period before progressing to overt leukemia. Finally, a very recent study (van Delft et al. 2011) seems to provide evidence for the following course of disease in some of the patients presenting with TEL-AML1$^+$ cALL: TEL-AML1$^+$ preleukemic clones, probably as a consequence of slow growth or quiescence, might be relatively resistant to ablation by chemotherapy, would persist during and after maintenance chemotherapy and occasionally give rise to another *de novo* cALL masquerading as a conventional though ‘late’ off-treatment relapse. The authors identified a subgroup of patients whose leukemic blasts, analyzed at presentation and at the time of (late) relapse, had only the TEL-AML1 fusion in common, but all the other, multiple mutations they identified were different between presentation and relapse. This indeed suggested that the preleukemic TEL-AML1$^+$ clone persisted from presentation throughout chemotherapy until the ‘very late relapse’ or, as the authors designated it, *de novo* cALL. Therefore, multiple evidences seem to be in agreement with

our favored explanation that the TEL-AML1 expression can indeed alter the cycling properties of the target preleukemic population leading to lengthy remissions, without any need for implicating immunological processes in the observed favorable prognosis for patients.

The findings presented in this study imply that, although the TEL-AML1 fusion protein is a true leukemia-specific mutation, it does not represent a suitable target for adoptive cell therapy in HLA-A*0201-restriction settings. We do not know whether the TEL-AML1 fusion might harbor some epitopes restricted by other HLA alleles. However, as HLA-A*0201 represents one of the most common alleles in the human population, alternative target molecules for immunotherapy of BCP-ALL, which would provide new HLA-A*0201-restricted epitopes should be considered. Genome-wide analysis of genetic alterations in ALL, using high-resolution Affymetrix single nucleotide polymorphism (SNP) arrays, led to the discovery of copy number changes in multiple genes encoding principal regulators of B lymphocyte development and differentiation (Mullighan et al. 2007). As the TEL-AML1 translocation seems to possess only a weak transforming activity and acts as the ‘first hit’ requiring the action of additional cooperating oncogenes (Greaves et al. 2003; Hong et al. 2008; Schindler et al. 2009), it is likely that some of these mutations affecting the B lineage pathway could represent the necessary additional transforming factors. Hence, they might represent potentially suitable immunotherapy targets, as the possibility of outgrowth of antigen-loss cancer cell variants would in that case be diminished. The study by Mullighan et al. reported recurring deletions in, among others, PAX5, EBF1 (early B-cell factor) and IKZF1 (IKAROS). A deleted sequence, of course, cannot serve as a source of novel epitopes. However, the high-resolution analysis of copy number changes enabled the detection of several deletions within the PAX5 gene, which spanned only a subset of PAX5 exons resulting in the expression of in-frame spliced transcripts and, as confirmed by Western blot analysis, in the expression of truncated PAX5 proteins of the predicted sizes. If some of these truncating PAX5 mutations would turn out to be shared among different patients, they might be considered as potential target candidates for development of widely applicable protocols for adoptive cell therapy of cALL. However, the crucial question would be whether such mutated protein would be antigenic. Based on the findings presented in this study, we propose that inducing specific CTLs might not be the limiting step in determining antigenic potential of such mutation. Testing whether a putative epitope would be naturally processed and presented at the cell surface for efficient recognition by the specific effectors should therefore be rigorous. This will aid in exploitation of growing collection of tumor-specific mutations in future successful adoptive cell therapy trials, which will target true tumor-specific antigens.

8 Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	antigen presenting cell
ATP	adenosine-5'-triphosphate
BCP-ALL	B cell precursor acute lymphoblastic leukemia
C	carboxyl
cALL	common acute lymphoblastic leukemia
CML	chronic myelogenous leukemia
CpG ODN	CpG oligodeoxynucleotides
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EBV	Epstein-Barr virus
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FI	fluorescence index
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
HSC	hematopoietic stem cell
IFA	incomplete Freund’s adjuvant
Ig	immunoglobulin
LC	liquid chromatography
LCL	lymphoblastoid cell lines
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
MCA	methylcholantrene
MHC	major histocompatibility complex
MS	mass spectrometry
mTEC	medullary thymic epithelial cell
N	amino
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SV40	Simian virus 40
TAA	tumor-associated antigen

TAP	transporter associated with antigen presentation
TCR	T cell receptor
TILs	tumor infiltrating lymphocytes
TSA	tumor-specific antigen
Ub	ubiquitin
UPR	ubiquitin/protein/reference
VACV	vaccinia virus

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11 Publications

Popović, J., L.P. Li, P. M. Kloetzel, M. Leisegang, W. Uckert and T. Blankenstein (2011). "The only proposed T cell epitope derived from the TEL-AML1 translocation is not naturally processed" Blood doi: 10.1182/blood-2010-12-325035.

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12 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

13 Addendum

13.1 Sequence of the ORF comprising EGFP, Ub and TEL-AML1 minigene in the pMP71-TEL-AML1 plasmid

The following nucleotide sequence of the ORF EGFP-Ub-TEL-AML1 was determined by sequencing the pMP71-TEL-AML1 plasmid using the primers seqLEADfwd and seqPRErev (listed in Table 2):

5'-1041-

atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgt
ccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccc
tcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaa
ggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtg
aaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgt
ctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccg
accactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaa
gaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtccg
gactcagatctcgaaattctgcagtcgacggtacctacccatacgatgtcccagattacgccgctttccttgcgggtagtgggtcgaccatgcag
attttcgtcaagactttgaccggtaaaaccataacattggaagttgaatcttccgataccatcgacaacgttaagtcgaaaattcaagacaagga
aggtatccctccagatcaacaaagattgatctttgccggtaggcagctagaaggcggtagaacgctgtctgattacaacattcagaaggagtc
caccttacatcttgtgctaaggctccgcggtggcatggtctctgtctccccgcctgaagagcacgccatgcccattgggagaatagcagaatgc
atacttggaatgaatccttctagagacgtctaa -2171-3'.

The numbers designate nucleotide positions in the pMP71-TEL-AML1 plasmid, as labeled on the restriction map shown in Figure 17.

Sequencing was performed in Eurofins MWG Operon, Martinsried, Germany.

13.2 Sequence of the ORF comprising EGFP, Ub and Melan-A/MART-1 in the pMP71-Melan-A/MART-1 plasmid

The following nucleotide sequence of the ORF EGFP-Ub-Melan-A/MART-1 was determined by sequencing the pMP71-Melan-A/MART-1 plasmid using the primers seqLEADfwd and seqPRErev (listed in Table 2):

5'-1041-

atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgt
ccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccc
tcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaa
ggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtg
aaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgt

ctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccg
accactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaa
gaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtccg
gactcagatctcgaaattctgcagtcgacggtacctacccatacgatgtcccagattacgccgctttccttgcgggtagtgggtcgaccatgcag
attttcgtcaagactttgaccggtaaaaccataacattggaagttgaatcttccgataccatcgacaacgttaagtcgaaaattcaagacaagga
aggtatccctccagatcaacaaagattgatctttgccggtaggcagctagaaggcggtagaacgctgtctgattacaacattcagaaggagtc
caccttacatcttgtgctaaggctccgcggtggcatgccaagagaagatgctcacttcatctatggttaccccaagaaggggcatggccactctt
acaccacggctgaagagctcgctgggatcggcatcctgacagtgatcctgggagtcttactgctcatcggctgttggtattgtagaagacgaaa
tggatacagagccttgatggataaaagtcttcatgttggcactcaatgtgccttaacaagaagatgcccacaagaagggtttgatcatcgggac
agcaaagtgtctcttcaagagaaaaactgtgaacctgtggttcccaatgctccacctgcttatgagaaactctctgcagaacagtcaccaccac
cttattcaccttaa -2435-3’.

The numbers designate nucleotide positions in the pMP71-Melan-A/MART-1 plasmid, as labeled on the restriction map shown in Figure 18.

Sequencing was performed in Eurofins MWG Operon.

14 Statement (Eidesstatliche Erklärung)

Hiermit bestätige ich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Außerdem versichere ich, dass die Arbeit in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt worden ist.

Berlin, den 9.06.2011 Jelena Popović