Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Identification of naturally presented HLA ligands on the surface of healthy and malignant hematopoetic cells and their therapeutic potential as targets for TCR-transgenic T cells

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades

eines Doktors der Naturwissenschaften

genehmigten Dissertation

Vorsitzender: Univ. -Prof. Dr. A. Skerra

Prüfer der Dissertation:
1. Univ.-Prof. Dr. I. Antes
2. Univ.-Prof. Dr. A. Krackhardt
3. Univ.-Prof. Dr. B. Küster

Die Dissertation wurde am 18.02.2014 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 03.12.2014 angenommen.

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Zusammenfassung

Für Patienten, die an einer rezidivierten Leukämie oder einer Leukämie, die nicht auf Standardtherapien anspricht, erkrankt sind, bleibt oftmals nur die allogene hämatopoetische Stammzelltransplantation. Es konnte gezeigt werden, dass der Therapieerfolg verbessert werden kann, wenn dem Patienten nach Transplantation Spenderlymphozyten-Infusionen gegeben werden. Die Zielstrukturen, die von diesen Zellen erkannt werden, sind jedoch größtenteils unbekannt. Eine optimierte Form dieser Therapie könnten T-Zell-Rezeptor (TZR)-transgene Spenderlymphozyten mit definierter Spezifität darstellen. Um geeignete Zielstrukturen für solche TZR zu identifizieren, wurden in dieser Arbeit sieben Leukämiezellen enthaltende Blut-Proben von Patienten mit myeloproliferativen Neoplasien mittels eines immunpeptidomischen Ansatzes analysiert. Hierbei wurden Peptide massenspektrometrisch sequenziert, die assoziiert mit HLA-Molekülen auf der Zelloberfläche präsentiert werden. Es konnten einige HLA-Liganden identifiziert werden, die von Genen abstammen, die restringiert im hämatopoetischen System exprimiert werden und auf verschiedenen HLA-Molekülen präsentiert werden. T Zellen, die spezifische Reaktivität gegen die ausgewählten HLA-Liganden zeigen, wurden im Rahmen der Einzel-HLA-Diskrepanz gesucht. Dazu wurde ein System etabliert, das die Generierung von Einzel-HLA-Diskrepanz dendritischen Zellen erlaubt, die das entsprechende HLA-Molekül exprimieren. Das System wurde mit sieben HLA Liganden getestet, die auf HLA-*B*07:02* präsentiert werden. Dazu wurde eine HLA-*B*07:02* kodierende *in vitro* transkribierte Ribonukleinsäure (RNS) in dendritische Zellen eines HLA-*B*07:02* negativen Blutspenders transfiziert und die Zellen mit den entsprechenden HLA-Liganden beladen. Nach Stimulation von naiven T-Zellen desselben Spenders konnten HLA Multimer positive Zellen in Antwort auf Stimulation mit drei von den sieben HLA-Liganden beobachtet werden. Einer dieser Liganden, MPO_5 stammt von MPO ab, einem Häm Protein, das ausschließlich in myeloischen Zellen exprimiert wird. Peptid spezifische Zellen wurden mit einem MPO_5 Multimer isoliert und mittels limitierender Verdünnung kloniert. Es konnte ein Klon und der entsprechende TZR isoliert werden, der im nachfolgenden 2.5D6 genannt wird. Der Klon zeigt Peptid-spezifische Reaktivität mit hoher funktioneller Avidität. TZR-transgene PBMC wurden detailliert charakterisiert und zeigten MPO spezifische Reaktivität und bislang keine MPO unabhängige Reaktivität. Desweiteren konnte keine toxische Aktivität der transgenen Zellen gegen gesunde hämatopoetische Stammzellen beobachtet werden. Die Zellen zeigten

weiterhin MPO spezifische Aktivität gegen Leukämiezelllinien und primäre Leukämie-Proben. Injektion von zentralen Gedächtnis T-Zellen, die mit dem TZR 2.5D6 transduziert wurden, führten im Vergleich zu den Kontrollgruppen zu einem verlängerten Überleben in Mäusen, die zuvor mit einer humanen Leukämiezelllinie inokuliert wurden.
Abschließend ist zu sagen, dass wir mit Hilfe eines immunpeptidomischen Ansatzes mehrere HLA-Liganden isolieren konnten, die von unterschiedlichen Genen stammen, die restringiert im hämatopoetischen System exprimiert werden. Nachdem eine Plattform zur Stimulation Peptid-spezifischer T-Zellen im Rahmen der Einzel-HLA-Diskrepanz etabliert war, konnte gegen ein HLA-*B*07:02* restringiertes Epitop von MPO ein spezifischer TZR isoliert werden. Dieser Rezeptor eignet sich zur Modifikation von Spenderlymphozyten-Infusionen, die im Rahmen einer Stammzelltransplantation gegeben werden. Das Vorgehen kann auf weitere HLA Liganden und HLA-Moleküle erweitert werden. Dies könnte Patienten mit rezidivierten oder Therapie-refraktären Leukämien eine verbesserte Behandlung ermöglichen.

Summary

The last option for patients with relapsed or therapy refractory leukemias is often the allogenic hematopoetic stem cell transplantation (HSCT). It has been shown, that T cells that are given in form of donor lymphocyte infusion (DLI) after transplantation can lead to a better outcome of the therapy. However, the target antigens that are recognized remain mostly unknown. An optimized form of this therapeutic approach could be represented by the administration of TCR-transgenic DLI with defined specificity. We applied an immunopeptidomic approach on seven leukemia samples of patients with MPN in order to seek for antigens that could serve as suitable targets for TCR-transgenic donor lymphocytes. We identified a number of ligands that are derived from different genes with expression restricted to the hematopoetic system and that are presented on different HLA molecules. In order to isolate T cells that are specific for selected HLA ligands in the single HLA-mismatched setting, a system was established to generate single HLA-mismatched DC expressing the respective HLA molecule. We tested the established system with seven HLA ligands that are presented on HLA-*B*07:02*. Therefore, DC from an HLA-*B*07:02*$^-$ blood donor were rendered HLA-*B*07:02* positive by electroporation and pulsed with the respective HLA ligands. Stimulation of naive T cells from the same donor with those cells resulted in expansion of HLA multimer positive cells in response to three of the seven HLA-*B*07:02* ligands. One of those ligands, MPO_5, is derived from MPO a heme protein expressed selectively in cells of the myeloid linage. Peptide specific T cells were isolated with an MPO_5 specific HLA multimer and cloned by limiting dilution. A clone and its TCR (named 2.5D6) could be isolated that shows peptide and antigen specific reactivity with high avidity. Detailed characterization of PBMC transgenic for the selected TCR showed no off target reactivity so far, lack of reactivity against healthy hematopoetic stem cells and MPO specific anti-leukemic reactivity *in vitro*. Furthermore, we could show increased overall survival in a murine model of human AML when animals were treated with TCR 2.5D6 transgenic T_{CM} compared to control groups.

Taken together, using the immunopeptidomic approach we could identify a number of HLA ligands derived from different genes with restricted expression to the hematopoetic system. After having established a platform for stimulation of peptide specific T cells in the single HLA-mismatched setting, we were able to isolate a highly specific TCR directed against an HLA-*B*07:02* restricted epitope derived from MPO. This TCR can be used as a tool to direct

the reactivity of DLI that are given after HSCT against leukemic cells. The approach can be extended to other HLA ligands and HLA molecules in order to provide better treatment options for patients with relapsed or therapy refractory leukemias.

Chapter 1

Introduction

1.1 Conventional therapeutic options for patients with myeloid neoplasms

Myeloid neoplasms are malignant diseases of the hematopoetic system and are characterized by uncontrolled proliferation of cells from the myeloid lineage. According to the world helath organization (WHO) guidelines, myeloid neoplasms are divided into MPN, myelodysplastic/myeloproliferative neoplasm (MDS/MPN) (not further described) and AML (Vardiman et al., 2009).

1.1.1 CML

The first type of leukemia that was described was CML a myeloid neoplasm that belongs to the group of MPN (Bennett, 1845; Craigie, 1845). The course of disease consists of three phases: chronic phase, characterized by an increase in myeloid precursors and granulocytes; accelerated phase, characterized by a rapid expansion of myeloid progenitor cells and blast crisis, that is similar to acute leukemia and leads to metastasis, organ failure and death (Faderl et al., 1999). The typical cytogenetic feature of the disease is the so called philadelphia (Ph) chromosome that was discovered in the 1960ies (Baikie et al., 1960; Nowell and Hungerford, 1960, 1961; Tough et al., 1961). The Ph chromosome is caused by a translocation of the long arm of chromosome 22 to the distal portion of the long arm of chromosome 9 [t(9;22)] (Rowley, 1973). It has been shown later, that this translocation leads to a transposition of the c-abl oncogene from its normal position (q34) on chromosome 9 to a BCR (q11) on chromosome 22 (Bartram et al., 1983; de Klein et al., 1982; Groffen et al., 1984). This results in translation of a novel mRNA that encodes for a constitutively active tyrosine kinase called $p210^{bcr\text{-}abl}$ (Konopka et al., 1984, 1985; Konopka and Witte, 1985). A remarkable success in therapy of CML could be achieved by targeting this tyrosine kinase with a small molecule called imatinib (Druker et al., 1996, 2001b,a). Binding of imatinib blocks the tyrosine kinase activity

and complete cytogenetic remission rates up to 87 % could be observed in the international randomized study of interferon and STI571 (IRIS) study (O'Brien et al., 2003; Druker et al., 2006). As a clear survival benefit with imatinib could be proofed by retrospective comparison to the previous standard therapy with interferon-α (IFN-α), imatinib is the standard therapy for CML nowadays. Despite the remarkable response rates to imatinib, leukemia cells can evade the therapy by mutations in the bcr-abl gene leading to imatinib resistance (Sawyers et al., 2002; Gorre et al., 2001). Therefore, second generation tyrosine kinase inhibitor (TKI)s were developed targeting mutated forms of the bcr-abl gene product, namely dasatinib and nilotinib (Weisberg et al., 2005; Shah et al., 2004; Golas et al., 2003). However, the genetic instability of leukemic stem cell (LSC) bears the risk of therapy resistant relapse caused by TKI mutations (Graham et al., 2002; Copland et al., 2006; Jorgensen et al., 2007) and the allogenic HSCT remains the only curative therapy for CML patients with TKI resistant disease (Silver et al., 1999).

1.1.2 BCR/ABL negative MPN

In case of the BCR/ABL negative MPNs polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) a mutation in the janus kinase 2 (JAK2) gene which leads to a valine to phenylalanine substitution at amino acid position 617 (V617F) has been described to be the prevalent genetic lesion. This mutation in the JH2 domain leads to disruption of the auto-inhibitory activity of JAK2 which is accompanied by a constitutive activity of the tyrosine kinase (TK) (Baxter et al., 2005; James et al., 2005). Standard therapies are aiming at reduction in platelet number to achieve an antithrombotic effect (e.g. low dose aspirin) in the case of PV and PMF (Landolfi et al., 2004; Cortelazzo et al., 1995; Harrison et al., 2005). High risk patients with PMF are considered for HSCT or investigational drug therapy (Tefferi and Vainchenker, 2011). Some of the investigational drug therapies for BCR/ABL negative MPNs are targeting the mutated janus kinase 1 (JAK1) and JAK2 gene products, resulting in a significant reduction in disease burden in PMF (Pardanani et al., 2011; Verstovsek et al., 2010). Although some clinical efficacy is observed, when PV or ET patients are treated with JAK1 and JAK2 inhibitors, the future role of those agents in standard therapy remains to be established (Verstovsek et al., 2009).

1.1.3 AML

AML is a clonal disorder of hematopoetic stem cells with a characteristic inhibition of differentiation of the affected cells (Fröhling et al., 2005). AML often involves multiple defects including fms-related tyrosine kinase 3 (FLT3) kinase internal tandem duplication (ITD) (Nakao et al., 1996), translocations targeting transcription factors or components of the transcriptional activation apparatus like core binding factor (CBF) (Miyoshi et al., 1993; Nucifora et al., 1993; Romana et al., 1995; Erickson et al., 1992; Kozu et al., 1993; Mitani et al., 1994;

Miyoshi et al., 1991; Liu et al., 1993), HOX family members (Borrow et al., 1996; Nakamura et al., 1996; Raza-Egilmez et al., 1998) and transcriptional coactivating proteins, such as CBP, p300 and TIF2 (Taki et al., 1997; Rowley et al., 1997; Giles et al., 1997; Carapeti et al., 1998; Liang et al., 1998). Depending on the response to conventional treatment as well as the cytogenetic and molecular genetic data, AMLs have been proposed to be divided into four groups. (1) Favorable, (2) Intermediate-I, (3) Intermediate-II and (4) Adverse (Döhner et al., 2010; Mrozek et al., 2012). Conventional treatment for AML starts with an induction phase with the aim to achieve a complete remission (CR) characterized by an undetectable level of leukemic cells. Therapy consists of cytarabine and an anthracycline, such as daunorubicin or idarubicin (Cheson et al., 2003; Fernandez et al., 2009). As it has been shown in the late 80ies, that almost all patients relapse without further treatment (Cassileth et al., 1988), the induction phase is followed by a consolidation phase today. This phase can either consist of a chemotherapy (Mayer et al., 1994; Bloomfield et al., 1998) or allogenic HSCT which leads to much lower relapse rates (Ferrara and Schiffer, 2013) and is explained in the next section.

1.2 Hematopoetic stem cell transplantation

As mentioned in the section before, HSCT is often the last option for patients with therapy resistant leukemia or leukemias with unfavorable cytogenetic conditions. The idea of HSCT for the treatment of leukemia is based on observations made by Lorenz et al. (1951) who could show survival of irradiated mice after infusion of healthy spleen or marrow cells whereas non transplanted animals died. However, due to limited knowledge about the HLA system, the first years of HSCT in humans were quite disappointing. Although 200 transplantations were conducted in the 1950ies and 1960ies none of them had been successful (Bortin, 1970). With the information obtained about HLA groups (Dausset, 1958; Van Rood et al., 1958) and the observations in studies of the dog leukocyte antigen (DLA) system that showed graft rejection or graft versus host disease (GvHD) in DLA mismatched settings (Epstein et al., 1968; Storb et al., 1968, 1970, 1971), trials of marrow grafting between HLA matched human siblings were conducted. Buckner et al. (1970) successfully transplanted a patient with CML after total body irradiation with the bone marrow of an HLA matched sibling. However, the patient died of cytomegalovirus (CMV) pneumonia, one of the problematic opportunistic infections occurring post transplantation (Sable and Donowitz, 1994; Maschke et al., 1999). Fefer et al. (1979) could show for the first time a graft versus leukemia (GvL) effect, represented by complete disappearance of the Ph chromosome in four CML patients treated with chemotherapy, irradiation and an identical twin marrow transplant. Control of CMV infections with ganciclovir (Schmidt et al., 1991; Goodrich et al., 1991), the use of hematopoetic stem cells from mobilized peripheral blood (Juttner et al., 1988; Gianni et al., 1989; Gluckman et al., 1989) and successful crossing of the HLA barrier that allows HLA-mismatched HSCT (Reis-

ner et al., 1983; Henslee-Downey et al., 1997; Aversa et al., 1998) has made HSCT a realistic therapeutic option for a broader patient population with leukemia nowadays. It has been furthermore shown by Kolb et al. (1990) that DLI transfused after marrow transplantation can increase the GvL effect mediated by the transplanted hematopoetic cells in CML patients. CML patients in chronic phase can achieve a complete cytogenetic response in 70-80 % and a potent GvL effect mediated by DLI could also be observed in patients with AML but to a lesser extend (15-30 %) (Kolb et al., 1995; Collins et al., 1997; Porter et al., 1999). The target structures recognized by DLI that are responsible for the beneficial GvL effect remain mainly unknown. Novel, leukemia specific epitopes, such as peptides derived from the TEL1 oncogene (TEL)-acute myeloid leukemia 1 protein (AML1) translocation or the BCR/ABL joining region are potential targets for DLI in AML and CML (Cai et al., 2012), respectively. However, as published by Popovic et al. (2011), the only proposed T cell epitope derived from the TEL-AML1 translocation is not naturally processed. Another group of potential targets in HLA-matched transplantation settings are minor histocompatibility antigens, that show restricted expression to the hematopoetic system (Kircher et al., 2002; Mutis et al., 1999; Heemskerk et al., 2003). The GvL effect observed in HLA-mismatched transplantation settings could be mediated by DLI that recognize targets with restricted expression to the hematopoetic system. The advent of technology offers the opportunity to genetic modification of DLI with TCR specific for defined target structures on the surface of leukemia cells to improve the GvL effect that is observed after transfusion of DLI.

1.3 Immunotherapies

Immunotherapies have been proven to be effective in different malignancies. There are several forms of immunotherapies including the inhibition of immune checkpoints, active immunization via vaccination approaches as well as passive immunization via antibodies or adoptive transfer of chimeric antigen receptor (CAR) or TCR transgenic T cells.

1.3.1 Immune checkpoint inhibitors

A very promising approach to support the endogenous anti-tumor reactivity in cancer patients is represented by the administration of so called immune checkpoint inhibitors. Those drugs target inhibitory receptors, like cytotoxic T lymphocyte antigen 4 (CTLA-4) (Krummel and Allison, 1996) and programmed cell death 1 (PD-1) (Vibhakar et al., 1997), on the surface of activated immune cells. The first immune checkpoint inhibitor that was approved by the food and drug administration (FDA) for the treatment of metastatic melanoma is ipilimumab that is binding to CTLA-4. It has been shown in animal models, that blockade of CTLA-4 leads to an increased antitumor function of $CD8^+$ T cells, an increased ratio of $CD8^+$ T cells to $Foxp3^+$ T regulatory cells and to an inhibition of the suppressive function of T regulatory

cells (Quezada et al., 2006). Hodi et al. (2010) could show a significant increase in median survival when melanoma patients were treated with ipilimumab compared to vaccination with a HLA-*A*02:01* restricted ligand derived from GP100 (10 vs. 6.4 month). There are only singular reports regarding the specificity of T cells that are involved in the beneficial effects of ipilimumab treatment. One study reports a potential positive correlation between T cell immunity against the cancer testis antigen NY-ESO1 and the response to ipilimumab treatment (Yuan et al., 2011). A case report published by van Rooij et al. (2013) describes a strongly increased T cell response against a mutated epitope derived from ataxia telangiectasia and Rad3-related protein (ATR) after ipilimumab therapy. A further promising target is PD-1, as blockade of PD-1 by the drug nivolumab lead to objective response in 28 % of the treated melanoma patients (Topalian et al., 2012). A combination of novilimumab and ipilimumab showed improved tumor regression compared to the experiences that were made with monotherapies (Wolchok et al., 2013).

1.3.2 Active and passive immunization

Anti-cancer immunity can either be induced by induction of an antigen specific immune response or by transfer of antigen specific immunity in form of antibodies or T cells.

1.3.2.1 Active immunization: Peptide and DC vaccination

One possible option to induce immunity against a selected target antigen is peptide vaccination. Walter et al. (2012) performed a phase 2 clinical trial in patients with renal cell carcinoma (RCC) by vaccination with *IMA901*, a vaccine consisting of 10 peptides identified by the immunopeptidomic approach. They could observe a correlation between the number of immune responses against vaccinated peptides and survival, speaking for efficacy of the approach. However, some patients could not benefit from the therapy as no or only a few immune responses against the vaccinated peptides could be induced. Another promising vaccination strategy is the application of DC, for example pulsed with a tumor specific antigen, in order to induce an anti-cancer immune response. The first cellular immunotherapy that showed effectiveness in a phase III clinical trial was the DC vaccine *Sipuleucel-T* as a treatment of asymptomatic metastatic hormone refractory prostate cancer (Small et al., 2006). Treatment with *Sipuleucel-T* resulted in a median survival of 25.9 month compared to 21.4 month in the placebo group in that study. The therapy is based on vaccination with autologous DC that have been pulsed *ex vivo* with a fusion protein consisting of prostatic acid phosphatase (PAP) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Small et al., 2000).

1.3.3 Passive immunization: Antibody therapy

Various antibodies that target tumor associated antigens are already used as therapeutic agents for the treatment of cancer patients. An antibody directed against the CD20 antigen that is expressed on more than 90 % of B cell lymphomas showed a response rate of 48 % in patients with relapsed low grade or follicular lymphoma (McLaughlin et al., 1998). Another example of an effective antibody is trastuzumab, a monoclonal antibody directed against human epidermal growth factor receptor 2 (HER2). Bang et al. (2010) showed a significantly increased median overall survival when patients with gastric or gastro-oesophageal junction cancer were treated with trastuzumab plus chemotherapy compared to chemotherapy alone. This antibody furthermore improved survival in $HER2^+$ breast cancer patients, when administered as an adjunvant treatment combined with an anthracycline-based regimen as well as a nonanthracycline regimen (Slamon et al., 2011).

1.3.4 Passive immunization: Adoptive T cell therapy

Adoptive T cell therapy is a form of passive immunization to help the immune system to fight cancer. The first experiences were made in patients with melanoma in the group of S. A. Rosenberg with autologous tumor infiltrating lymphocyte (TIL). They could show in several studies, that adoptive transfer of autologous, *in vitro* expanded TIL into lymphodepleted hosts can lead to objective cancer regression and increased survival in patients with melanoma (Rosenberg et al., 2011; Dudley et al., 2010, 2008). However, this approach is limited by the observation, that autologous TIL cannot be isolated in all patients (Rosenberg and Dudley, 2004) and that *ex vivo* culturing of TIL to numbers sufficient for adoptive transfer is time consuming and expensive (Dudley et al., 2003). It has been furthermore shown, that T cell clones derived from autologous TIL can have low avidity against tumor associated self antigens which results in limited efficiency of specific immune reactions mediated by those cells (Gervois et al., 1996). However, a clinical study published by Chapuis et al. (2013) demonstrated clinical efficacy of adoptively transferred high avidity T cell clones, recognizing an HLA-*A*02:01* restricted epitope of wilms tumor 1 (WT-1) in patients with acute lymphoblastic leukemia (ALL) after allogenic stem cell transplantation.
A possible source of T cells with high avidity for self antigens is the allogenic T cell repertoire. It has been shown in mouse experiments, that 1 to 7 % of the T cells of an individual animal show alloreactivity, meaning that those cells are able to recognize major histocompatibility complex (MHC) peptide complexes that were not encountered during thymic development (Lindahl and Wilson, 1977; Suchin et al., 2001). This recognition seems to be comparable to the conventional recognition of autologous HLA peptide complexes (Felix et al., 2007). This is also one basis of the GvL effect mediated by DLI that is observed in the context of allogenic stem cell transplantation (Kolb et al., 2004), as mentioned in section 1.2. The advantage of allogenic T cells compared to autologous T cells for tumor therapy is the missing negative

selection of T cells that show recognition of self antigens with high avidity. As many tumor antigens are self antigens, those allogenic T cells could be valuable tools for T cell mediated immunotherapy. In order to identify T cells that are specific for certain peptides in the allogenic system different approaches have been proposed. Sadovnikova et al. (1998); Gao et al. (2000); Schuster et al. (2007) used the transporter associated with antigen processing (TAP) deficient, HLA-*A*02:01* positive cell line T2 for stimulation of peptide specific T cells derived from HLA-*A*02:01* negative blood donors. This cell line is characterized by limited presentation of endogenous peptides on the cell surface. Wilde et al. (2009); Spranger et al. (2012) used a so called semi-allogenic stimulation approach for isolation of allorestricted, peptide specific T cells. They took DC from an HLA-*A*02:01* negative donor and rendered them positive for HLA-*A*02:01* by electroporation with HLA-*A*02:01* ivt-RNA. Those cells were then used for stimulation of T cells derived from the same donor. This resulted in expansion of peptide specific T cell clones that react with high avidity against the respective peptide.
Both, the autologous as well as the allogenic T cell repertoire have been used to isolate TCR specific for tumor associated antigens. Genetic modification of T cells (Heemskerk et al., 2003, 2004), PBMC (Clay et al., 1999; Schuster et al., 2007; Johnson et al., 2006) or hematopoetic progenitor cells (van Lent et al., 2007) led to successful transfer of the tumor specificity of the respective TCR. Morgan et al. (2006) were the first to translate TCR transgenic PBMC into the clinic. They could observe tumor regression in two out of 17 melanoma patients that were treated with TCR transgenic PBMC that recognize a melanoma antigen recognized by T cells 1 (MART1) derived HLA-*A*02:01* restricted peptide. Further attempts to translate TCR transgenic T cells into the clinic resulted in fatal outcomes due to unexpected adverse events (Linette et al., 2013; Morgan et al., 2013). This highlights the need for new, safe targets as well as TCR that are characterized extensively regarding their safety in *in vitro* studies and in suitable preclinical animal models.
A further possibility to generate efficient effector tools in form of specific T cells is the genetic modification with CAR. CAR are chimeric receptors that consist of an antigen specific binding domain (for example a single chain variable fragment (scFv) derived from an antibody variable chain) combined with a transmembranal activation domain (for example CD28 and CD137) that allows activation of transgenic T cells after antigen encounter via the CAR (Till et al., 2012; Hudecek et al., 2010). Clinical efficacy of such CAR-transgenic T cells are reported in several publications, manly for the B cell specific antigens CD19 and CD20 (Porter et al., 2011; Kalos et al., 2011; Till et al., 2012; Kochenderfer et al., 2012). Despite promising results of the present immunotherapeutic approaches a lot of patients can not benefit from such a therapy with CAR- or TCR-transgenic T cells caused, amongst others, by the lack of knowledge about suitable target structures.

1.3.4.1 Target identification: Direct immunology

There are two ways of direct identification of suitable targets for anti-cancer immunization therapies. The first method starts with tumor-reactive T cells followed by the identification of the recognized target structures. Advances in lymphocyte culture techniques allowed the generation of T cell lines from melanoma patients (Knuth et al., 1984) and isolation of tumor reactive T cell clones. Those clones were used to identify the recognized antigen and lead to identification of the first human tumor antigen melanoma-associated antigen 1 (MAGE-1) by an approach called direct immunology (van der Bruggen et al., 1991). This approach starts with stimulation of PBMC derived from a cancer patient with an autologous cancer cell line to obtain tumor reactive T cell clones. The HLA restriction element that is responsible for recognition by the T cell clone is identified and a copy desoxyribonucleic acid (cDNA) library is constructed from the autologous cancer cell line. cDNA pools are generated and transfected into target cells that bear the respective restriction element and cDNA pools are identified that lead to recognition of the cells by the tumor reactive T cell clone. Subcloning of the cDNA is carried out until the minimal sequence is identified and the recognized epitope is identified by the use of synthetic peptides that cover the minimal sequence (van der Bruggen et al., 1991; Wang, 2009). Due to the enormous time and cost effort and the fact, that tumor cell lines can not be generated from many tumor types an alternative approaches have been proposed (Celis et al., 1994; Maecker et al., 2001; Tanzarella et al., 1997).
The second possibility aims at the identification of antigens that are recognized by antibodies in sera of cancer patients. This method, called serological identification of antigens by recombinant expression cloning (SEREX) (Sahin et al., 1995), has been successfully used for identification of a number of tumor associated antigens (e.g. NY-ESO-1) potentially useful for T cell therapies (Chen et al., 1997; Krackhardt et al., 2002; Greiner et al., 2005). Immunogenic tumor antigens are identified by screening cancer derived cDNA expression libraries, that are expressed in *escherichia coli* (*E. coli*), for their recognition by immunoglobulin G (IgG) antibodies present in sera of cancer patients. After selection of immunogenic antigens, prediction algorithms can be used (e.g. SYFPEITHI, BIMAS) to predict potential HLA ligands. Those HLA ligands can then be used for *in vitro* T cell stimulation (Schuster et al., 2007) or peptide vaccinations (Jäger et al., 1998, 2000). However, *in silico* prediction of potential HLA ligands is one of the limitations of that approach as natural processing and presentation of predicted HLA ligands is not ensured.

1.3.5 Target identification: Immunopeptidomics

A straightforward method of target identification, that starts with identification of peptides that are presented on HLA molecules is the so called immunopeptidomic approach (Falk et al., 1991).

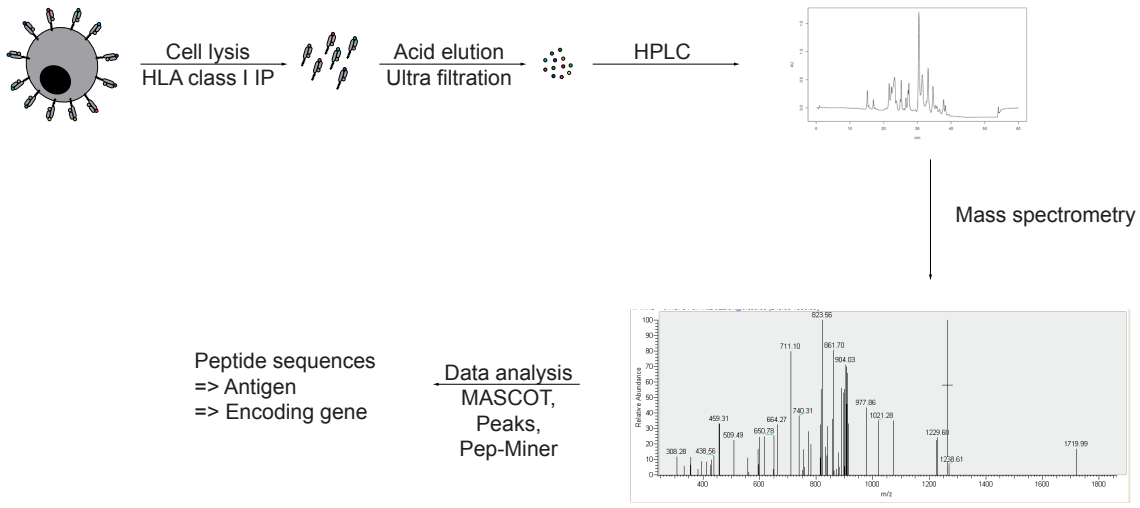

Figure 1.1: Schematic overview of the immunopeptidomic approach.
Cells are lyzed in detergent containing buffer and HLA molecules are precipitated with a specific antibody. HLA peptide complexes are disrupted by acid treatment and peptide solution is purified by ultrafiltration. The resulting peptide solution is fractionated by HPLC and fractions are submitted to mass spectrometry. Resulting mass spectra are interpreted manually or with different software tools to obtain peptide sequences and peptide coding genes.

HLA molecules (either class I or class II) are precipitated out of cell lysates by specific antibodies. Peptide HLA complexes are disrupted by acid treatment and a purified peptide solution free of larger molecules (HLA fragments, antibody molecules) is obtained by ultrafiltration through a 5 to 10 kDa molecular weight cutoff (MWCO) filter. The peptide solution is then subjected to reverse phase HPLC to reduce the sample complexity by separation of the peptides according to their hydrophobicity. The resulting fractions are then directly subjected to electrospray ionization (ESI) mass spectrometry where the masses of the intact peptides is determined followed by fragmentation and determination of the fragment masses. The obtained information is used to identify the sequences of the analyzed peptides by manual interpretation of the spectra (Depontieu et al., 2009; Stickel et al., 2009) or with different software tools (Bassani-Sternberg et al., 2010; Dutoit et al., 2012; Walter et al., 2012). Expression of the HLA ligand coding genes can then be analyzed by micro arrays (Stickel et al., 2009; Seliger et al., 2011) to select tumor specific or tumor associated HLA ligands. As Weinzierl et al. (2007) could show a distorted relation between mRNA expression and level of presentation of corresponding HLA ligands in some cases, methods have been established to compare the level of presentation of a certain HLA ligand on healthy cells and tumor cells (Lemmel et al., 2004). Some groups nowadays combine mRNA expression analysis and analysis of the presentation level of a certain HLA ligand to optimize selection of tumor associated HLA ligands (Walter et al., 2012; Dutoit et al., 2012). A schematic overview of the immunopeptidomic approach is depicted in figure 1.1.

1.4 Aim of the current study

Aggressive leukemias like AML and MPN that relapse or are resistant to standard therapies are difficult to treat and patients often die. T cells have been proven to be effective in the treatment of leukemias in form of DLI that are given in context of HSCT. An optimized therapy would include DLI or autologous T cells that bear TCR with defined tumor reactivity. The aim of the present study was to identify HLA ligands that could serve as targets for T cell mediated immunotherapies by using the immunopeptidomic approach. Primary material from patients with different hematologic malignancies should be analyzed to identify HLA ligands derived from different antigens and presented on different HLA molecules. A workflow consisting of *in silico* analysis should be established to estimate the suitability of the identified HLA ligands as targets for T cell mediated immunotherapies. Expression of selected candidates should be validated by qPCR and protein expression analysis.

To analyze the therapeutic potential of T cells derived from the allogenic repertoire that are specific for the selected HLA ligands, a system should be established that allows stimulation of naive T cells in a single HLA-mismatched setting. Therefore, selected HLA genes should be cloned together with eGFP to easily identify and isolated target cells (DC and different cell lines) rendered positive for the respective HLA molecule. Peptide specific T cells should be isolated with HLA multimers, cloned by limiting dilution and analyzed for their peptide specific cytotoxic reactivity.

TCRs of T cell clones with peptide specific reactivity should be isolated, cloned and transferred into PBMC by retroviral transduction. Those PBMC should then further be used for detailed characterization of the respective TCR regarding functional avidity, antigen specificity, crossreactivity and leukemia reactivity. Furthermore, TCR with suitable recognition patterns should be tested for their leukemia reactivity *in vivo* after having established a suitable leukemia model in immunocompromised mice.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Technical equipment

Table 2.1: Technical equipment

Device	Company
Analytical balance 440-35N	Denver Instrument, Göttingen, Germany
Analytical balance SI-64	Denver Instrument, Göttingen, Germany
Axiovert 40C Mikroscope	Carl Zeiss Microscopy, Oberkochen, Germany
Centrifuge 5417R	Eppendorf AG, Hamburg, Germany
Centrifuge 5810R	Eppendorf AG, Hamburg, Germany
Centrifuge J2-HS	Beckman Coulter, Brea, USA
Electrophoresis Apparatus GPS 200/400	Pharmacia LKB Biotechnology, Uppsala, Sweden
Electrophoresis chamber Midi horizontal	G&P Kunststofftechnik, Kassel, Germany
FACS Canto	BD Biosciences, Franklin Lakes, USA
FACS Aria	BD Biosciences, Franklin Lakes, USA
Freezer (−80 °C)	BUCHNER Labortechnik. Pfaffenhofen, Germany
Freezer (−20 °C) profiline	Liebherr-International AG, Bulle, Switzerland
Gammacell 40	Atomic energy of Canda limited, Ottawa, Canda
Gene Pulser Xcell	Bio-Rad Laboratories, München, Germany
Incubator BBD 6220	Heraeus Holding, Hanau, Germany
Incubator Shaker	New Brunswick Scientific, Enfield, USA
LSRII	BD Biosciences, Franklin Lakes, USA
LTQ Orbitrap XL	Thermo Fisher Scientific, Waltham, USA

Table 2.1: Technical equipment *(continued)*

Device	Company
Laminar flow	BDK, Sonnenbühl-Genkingen, Germany
Laminar flow HERAsafe	Heraeus Holding, Hanau, Germany
LightCycler 480 System	Roche, Basel, Switzerland
MACS MultiStand	Miltenyi Biotec, Bergisch Gladbach, Germany
MACSmix Tube Rotator	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnet agitator	Janke & Kunkel, Staufen, Germany
Megafuge 1.0R	DJB Labcare Ltd, Buckinghamshire, England
MidiMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Mini Gel Casting Set	Cosmo Bio Co., LTD, Tokyo, Japan
Mini Gel Migration Through	Cosmo Bio Co., LTD, Tokyo, Japan
MiniMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Minishaker MS 2	IKA - Werke, Staufen, Germany
MoFlow™	Dako Germany, Hamburg, Germany
Multichannel pipets	Eppendorf AG, Hamburg, Germany
NanoDrop ND-1000	PEQLAB Biotechnologie, Erlangen, Germany
Neubauer counting chamber	Karl Hech, Sondheim/Röhn, Germany
Nucleofector	Lonza Group AG, Basel, Schweiz
Nunc-Immuno Wash 8	Nunc, Roskilde, Denmark
Pipetboy	INTEGRA Biosciences, Fernwald, Germany
Pipets	Eppendorf AG, Hamburg, Germany
Sunrise Photometer	Tecan, Groot-Bijgaarden, Belgium
TGradient Thermocycler	Biometra, Göttingen, Germany
Thermomixer comfort	Eppendorf AG, Hamburg, Germany
TopCount NXT	Perkin Elmer, Waltham, USA
UV-Transilluminator IP-CF01.SD	Vilber Lourmat Germany, Eberhardzell, Germany
Ultimate 3000	Dionex, Sunnyvale, USA
Vacufuge	Eppendorf AG, Hamburg, Germany
Vortexer	Bender & Hobe, Switzerland
XCell II™ Blot Module	Invitrogen, Carlsbad, USA
XCell SureLock™	Invitrogen, Carlsbad, USA
Mini-Cell	

2.1.2 Consumable supplies

Table 2.2: Consumable supplies

Consumable	Company
6-/12-/24-/96-well plates (tissue culture treated)	TPP Techno Plastic Products, Trasadingen, Switzerland
6-well plates (non treated)	BD Bioscience, Franklin Lakes, USA
24-well plates (non treated)	BD Bioscience, Franklin Lakes, USA
48-well plates (tissue culture treated)	BD Biosciences, Franklin Lakes, USA
Amicon Ultra-15 10 kDa	Millipore, Darmstadt, Germany
Aspiration pipet (2 ml	Sarstedt, Nümbrecht, Germany
Blunt end needles (16 G)	Stemcell Technologies, Vancouver, Canada
C_{18} spin tips	Protea Biosciences, Morgantown, USA
Cell culture flasks	Greiner Bio-One, Frickenhausen, Germany
Cell scraper (38 cm)	TPP Techno Plastic Products, Trasadingen, Switzerland
CryoPure tube (1.6 ml)	Sarstedt, Nümbrecht, Germany
EIA/RIA plates	Corning, New York, USA
Endotoxin-free plasmid DNA purification kit	Macherey-Nagel, Düren, Germany
Falcons (50/15 ml)	BD Biosciences, Franklin Lakes, USA
Filcon (30 µm)	Guenter Keul, Steinfurt, Germany
Filter tips (10 µl, 20 µl, 300 µl and 1000 µl)	Sarstedt, Nümbrecht, Germany
Gene Pulser Cuvette (0.4 cm)	Bio-Rad Laboratories, München, Germany
Gloves latex	Sempermed, Wien, Austria
Gloves nitrile	KCL, Eichenzell, Germany
Hamilton syringe (10/100 µl)	Hamilton, Bonaduz, Switzerland
Immuno Plates	Nunc, Roskilde, Denmark
Inoculating loops	VWR, Darmstadt, Germany
LUMA plates	Perkin Elmer, Waltham, USA
MACS Cell Separation Columns	Miltenyi Biotech, Bergisch Gladbach, Germany
Microtubes (1.2 ml)	Alpha Laboratories, Hampshire, UK
Mr. Frosty Freezing Container	Nunc, Roskilde, Denmark
Nunclon surface flasks	Nunc, Roskilde, Denmark

Table 2.2: Consumable supplies *(continued)*

Consumable	Company
($80\,cm^2$)	
Parafilm	Pechiney Plastic Packaging, Chicago, USA
Petri dishes	Greiner Bio-One, Frickenhausen, Germany
Petri dishes (35 mm)	BD Biosciences, Franklin Lakes, USA
Pierce Centrifuge Columns	Thermo Fisher Scientific, Waltham, USA
Pipet tips (10 µl, 200 µl and 1000 µl)	Sarstedt, N"umbrecht, Germany
Reaction tubes (0.5 ml)	Peqlab, Erlangen, Germany
Reaction tubes (1.5 ml and 2 ml)	Sarstedt, N"umbrecht, Germany
Round bottom FACS tubes	BD Biosciences, Franklin Lakes, USA
Round bottom tubes (5 ml)	BD Biosciences, Franklin Lakes, USA
Sealing foil	Alpha Laboratories, Hampshire, UK
Serological pipets	Sarstedt, N"umbrecht, Germany
(2 ml, 5 ml, 10 ml, 25 ml and 50 ml)	
Suq-Q (1 ml)	BD Biosciences, Franklin Lakes, USA
Syringe filter (0.22 µm)	TPP Techno Plastic Products, Trasadingen, Switzerland
Syringe filter (0.45 µm)	TPP Techno Plastic Products, Trasadingen, Switzerland
Syringes (1 ml) Luer-Lock	B.Braun, Melsungen, Germany
Syringes (5 ml, 10 ml and 50 ml)	BD Biosciences, Franklin Lakes, USA

2.1.3 Kits

Table 2.3: Kits

Kit	Company
AffinityScript Multiple	Agilent Technologies,
Temperature cDNA Synthesis Kit	Santa Clara, USA
BCA Protein Assay Reagent	Thermo Fisher Scientific, Waltham, USA
BD Cytofix/Cytoperm^{TM}	BD Biosciences, Franklin Lakes, USA
BD OptEIA^{TM}	BD Biosciences, Franklin Lakes, USA
IFN-γ ELISA kit	
Dynabeads Untouched^{TM}	Invitrogen, Carlsbad, USA
Human CD8$^+$ T cells	
Dynabeads Untouched^{TM}	Invitrogen, Carlsbad, USA
Human CD3$^+$ T cells	

Table 2.3: Kits *(continued)*

Kit	Company
Dynabeads Untouched^{TM} Human CD14^{+} Monocytes	Invitrogen, Carlsbad, USA
Dynabeads Untouched^{TM} Human CD19^{+} B cells	Invitrogen, Carlsbad, USA
Dynabeads Untouched^{TM} Human CD56^{+} NK cells	Invitrogen, Carlsbad, USA
HotstarTaq MasterMix Kit Plus	Quiagen, Hilden, Germany
KOD Polymerase	Merck, Darmstadt, Germany
Human CD45RO Micro Beads	Miltenyi Biotech, Bergisch Gladbach, Germany
Human CD57 MicroBeads	Miltenyi Biotech, Bergisch Gladbach, Germany
JETSTAR 2.0 Plasmid Purification Kit	Genomed, Löhne, Germany
KAPA SYBR FAST LightCycler 480	Peqlab, Peqlab, Erlangen, Germany
mMESSAGE mMACHINE T7	Invitrogen, Carlsbad, Germany
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel, Düren, Germany
Poly(A) Tailing Kit	Invitrogen, Carlsbad, Germany
RNeasy Mini Kit	Quiagen, Hilden, Germany
Wizard Genomic DNA Purification Kit	Promega, Madison, USA

2.1.4 Reagents and chemicals

Table 2.4: Reagents and chemicals

Reagent Chemical	Company
0.5 kb to 10 kb RNA Leiter	Invitrogen, Carlsbad, USA
100 bp DNA ladder	New England Biolabs, Ipswich, UK
1-Bromo-3-Chloro-Propan	Sigma Aldrich, Taufkirchen, Germany
7-AAD	Sigma Aldrich, Taufkirchen, Germany
AEC	Sigma Aldrich, Taufkirchen, Germany
Acetic acid	Merck, Darmstadt, Germany
Agarose	Roth, Karlsruhe, Germany
AIM-V	Invitrogen, Carlsbad, USA
Ampicillin	Sigma Aldrich, Taufkirchen, Germany

Table 2.4: Reagents and chemicals *(continued)*

Reagent Chemical	Company
Benzoase-endonuclease	Merck, Darmstadt, Germany
BSA	Sigma Aldrich, Taufkirchen, Germany
CHAPS	Sigma Aldrich, Taufkirchen, Germany
Chloroform-Isoamyl alcohol 24:1	Sigma Aldrich, Taufkirchen, Germany
CNBr sepharose	Sigma Aldrich, Taufkirchen, Germany
Complete Protease Inhibitor	Roche, Basel, Switzerland
Cr^{51}	Hartmann Analytic, Brauschweig, Germany
DEPC	Sigma Aldrich, Taufkirchen, Germany
DEPC H_2O	Invitrogen, Carlsbad, USA
DH5α	New England BioLabs, Ipswich, UK
DMEM	Invitrogen, Carlsbad, USA
DMF	Merck, Darmstadt, Germany
DMSO	Sigma Aldrich, Taufkirchen, Germany
D-PBS	Invitrogen, Carlsbad, Germany
DTT	Sigma Aldrich, Taufkirchen, Germany
Dynabeads Human T-Activator CD3/CD28 beads	Invitrogen, Carlsbad, USA
EMA	Invitrogen, Carlsbad, USA
Ethanol	Merck, Darmstadt, Germany
Ethidium Bromide	Roth, Karlsruhe, Germany
FCS	Invitrogen, Carlsbad, Germany
Ficoll	Biochrom, Berlin, Germany
Fibronectin (from bovine plasma)	Sigma Aldrich, Taufkirchen, Germany
Gentamycine	Biochrom, Berlin, Germany
GeneRuler 1kb DNA ladder	Peqlab, Erlangen, Germany
Glycin	Sigma Aldrich, Taufkirchen, Germany
Sulfuric acid	Roth, Karlsruhe, Germany
HBSS	Invitrogen, Carlsbad, Germany
Heparine	B.Braun, Melsungen, Germany
HEPES	Invitrogen, Carlsbad, Germany
HS	TU München, München, Germany
Isopropanol	Merck, Darmstadt, Germany
Gel Loading Dye (6 x)	Fermentas, St. Leon-Rot, Germany
LB Agar	Invitrogen, Carlsbad, Germany
LB Broth Base	Invitrogen, Carlsbad, Germany
NuPAGE LDS sample buffer (4x)	Invitrogen, Carlsbad, USA

Table 2.4: Reagents and chemicals *(continued)*

Reagent Chemical	Company
L-Glutamine	Invitrogen, Carlsbad, Germany
Milk powder	Sigma Aldrich, Taufkirchen, Germany
MPA	Sigma Aldrich, Taufkirchen, Germany
Na_2CO_3	Merck, Darmstadt, Germany
Na_2HCO_3	Merck, Darmstadt, Germany
Na_2HPO_4*H_2O_2	Merck, Darmstadt, Germany
NaCl	Merck, Darmstadt, Germany
NaN_3	Merck, Darmstadt, Germany
Non Essential Amino Acids	Invitrogen, Carlsbad, USA
NuPAGE LDS sample buffer	Invitrogen, Carlsbad, USA
NuPAGE MOPS running buffer	Invitrogen, Carlsbad, USA
NuPAGE Novex BisTris Gel (4-12 %)	Invitrogen, Carlsbad, USA
NuPAGE Transfer buffer	Invitrogen, Carlsbad, USA
OneShot TOP10	Invitrogen, Carlsbad, USA
PFA	Sigma Aldrich, Carlsbad, USA
PBS Dulbecco's	Biochrom, Berlin, Germany
Penicilline Streptomycin	Invitrogen, Carlsbad, USA
PFA	Sigma Aldrich, Taufkirchen, Germany
PGE_2	Sigma Aldrich, Taufkirchen, Germany
Propidiumiodide	Sigma Aldrich, Taufkirchen, Germany
Protaminsulfate	MP Biomedicals, Illkirch, France
P/S	PAA laboratories Pasching, Austria
Restriction enzymes	New England Biolabs, Ipswich, UK
RetroNectin	TaKaRa, Japan
RNase Out	Invitrogen, Carlsbad, USA
RPMI 1640	Invitrogen, Carlsbad, USA
Sheath fluid	Dako, Glostrup, Denmark
Sodium Pyruvate	Invitrogen, Carlsbad, USA
S.O.C. medium	Invitrogen, Carlsbad, USA
Streptavidin-HRP	Mabtech, Nacka Strand, Sweden
T4-Ligase	Fermentas, St. Leon-Rot, Germany
TAE Buffer	Invitrogen, Carlsbad, USA
TransIT-T293 transfection reagent	Mirus, Madison, USA
TRIzol Reagent	Invitrogen, Carlsbad, USA
Trypan blue	Invitrogen, Carlsbad, USA
Trypsine EDTA 0,5 %	PAA laboratories Pasching, Austria

Table 2.4: Reagents and chemicals *(continued)*

Reagent Chemical	Company
TFA	Sigma Aldrich, Taufkirchen, Germany
Tween 20	Sigma Aldrich, Taufkirchen, Germany
Western lightning ECL	Perkin Elmer, Waltham, USA

2.1.5 Cytokines and TLR ligands

Table 2.5: Cytokines and TLR ligands

Cytokine/TLR ligand	Company
CL075	InvivoGen, San Diego, USA
FLT3LG	PeproTech, London, UK
GM-CSF	PeproTech, London, UK
IFN-γ	PeproTech, London, UK
IL1-β	PeproTech, London, UK
IL-2	Chron Vaccines International, Marburg, Germany
IL-4	PeproTech, London, UK
IL-7	PeproTech, London, UK
IL-15	PeproTech, London, UK
IL-21	PeproTech, London, UK
SCF	PeproTech, London, UK
TNF-α	PeproTech, London, UK

2.1.6 Antibodies and HLA-multimers

Table 2.6: Antibodies used for flow cytometry

Antibody	Clone	Isotype	Conjugation	Company
α-hCD3	UCHT1	IgG1$_\kappa$	FITC, APC, AF700	BD Biosciences
α-hCD4	RPA-T4	IgG1$_\kappa$	FITC, PE, APC, V450	BD Biosciences
α-hCD8	HIT8a	mIgG1$_\kappa$	PE, APC, V450	BD Biosciences
α-hCD8	RPA-T8	mIgG1$_\kappa$	FITC	BD Biosciences

Table 2.6: Antibodies used for flow cytometry *(continued)*

Antibody	Clone	Isotype	Conjugation	Company
α-hCD14	M5E2	mIgG2$_a$	PE	BD Biosciences
α-hCD19	HIB19	mIgG1$_\kappa$	PE, APC	BD Biosciences
α-hCD33	WM53	mIgG1$_\kappa$	V450	BD Biosciences
α-hCD34	581	mIgG1$_\kappa$	PE	BD Biosciences
α-hCD45	J.33	mIgG1$_\kappa$	APC	Beckman Coulter
α-hCD45RA	HI100	mIgG1$_\kappa$	APC	BD Biosciences
α-hCD45RO	UCHL1	mIgG2$_a$	PE	BD Biosciences
α-hCD56	B159	mIgG1$_\kappa$	PE	BD Biosciences
α-hCD62L	DREG-56	mIgG1$_\kappa$	PE	BD Biosciences
α-hCD80	L307.4	mIgG1$_\kappa$	PE	BD Biosciences
α-hCD83	HB15e	mIgG1$_\kappa$	FITC	BD Biosciences
α-hCD86	2331 (FUN-1)	mIgG1$_\kappa$	FITC, PE	BD Biosciences
α-hMPO	1B10	mIgG1$_\kappa$	None	BD Biosciences
α-hMPO	5B8	mIgG1$_\kappa$	FITC	BD Biosciences
TCRm antibody	H57-597	AHIgG	FITC, PE, APC	Beckman Coulter
α-HLA ABC	w6/32	mIgG1$_\kappa$	FITC	BD Biosciences
Goat-α-mIgG			FITC, PE	Invitrogen
Isotype control	MOPC-21	mIgG1$_\kappa$	FITC, PE, APC, V450 AF700	BD Biosciences

Table 2.7: Antibodies used for western blot

Antibody	Clone	Company	Dilution
α-GAPDH	MAB374	Millipore	1:5000
α-HLA α-chain	EPR1394Y	Abcam	1:5000
α-hMPO	ERP4792	Abcam	1:1000
α-mIgG-HRP		Dianova	1:5000
α-rIgG-HRP		Dianova	1:4000

Table 2.8: HLA-*B*07:02*-multimers

HLA-multimer	Ligand sequence	Source
MPO_2	TPAQLNVL	AG Busch
MPO_5	NPRWDGERL	AG Busch
$MS4A3_1$	SPDYQKAKL	AG Busch
MYB_2	AQAPPTAQL	AG Busch
MYB_3	VPQPAAAAI	AG Busch
MYB_4	TPAIKRSIL	AG Busch
$pp65_{417\text{-}427}$	TPRVTGGGAM	AG Busch

2.1.7 Peptides

HLA-*B*07:02* ligands (kindly provided by S. Stevanovic) that were used for crossreactivity assays are listed in table E.1. All other peptides are listed in table 2.9.

Table 2.9: Peptides used in the present study

Peptide	Sequence	Company
$ELANE_1$	VQRIFENGY	IBA, Göttingen, Germany
EPX_1	NPRWNGDKL	GenScript, Hong Kong, China
$HMHA1_1$	RQLPEPLISF	IBA, Göttingen, Germany
$HMHA1_2$	RPRPTEATVSL	IBA, Göttingen, Germany
$ITGA2B_1$	PVADIFSSY	IBA, Göttingen, Germany
$ITGA2B_2$	AQAPVADIF	IBA, Göttingen, Germany
$LAT2_1$	SLREDQQSF	IBA, Göttingen, Germany
$LAT2_2$	YIDPIAMEY	IBA, Göttingen, Germany
LPO_1	NPQWDGEKL	GenScript, Hong Kong, China
MPO_1	EEAKQLVDKAY	IBA, Göttingen, Germany
MPO_2	TPAQLNVL	IBA, Göttingen, Germany
MPO_3	NQINALTSF	IBA, Göttingen, Germany
MPO_4	FVDASMVY	IBA, Göttingen, Germany
MPO_5	NPRWDGERL	IBA, Göttingen, Germany
$MPO_{5\text{-}1}$	APRWDGERL	GenScript, Hong Kong, China
$MPO_{5\text{-}2}$	NARWDGERL	GenScript, Hong Kong, China
$MPO_{5\text{-}3}$	NPAWDGERL	GenScript, Hong Kong, China
$MPO_{5\text{-}4}$	NPRADGERL	GenScript, Hong Kong, China
$MPO_{5\text{-}5}$	NPRWAGERL	GenScript, Hong Kong, China
$MPO_{5\text{-}6}$	NPRWDAERL	GenScript, Hong Kong, China
$MPO_{5\text{-}7}$	NPRWDGARL	GenScript, Hong Kong, China

Table 2.9: Peptides used in the present study *(continued)*

Peptide	Sequence	Company
MPO_{5-8}	NPRWDGEAL	GenScript, Hong Kong, China
MPO_{5-9}	NPRWDGERA	GenScript, Hong Kong, China
MPO_5 (mouse homologue)	NPRWNGEKL	GenScript, Hong Kong, China
$MS4A3_1$	SPDYQKAKL	IBA, Göttingen, Germany
$MS4A3_2$	GVFLGSLQY	IBA, Göttingen, Germany
MYB_1	SQPAVATSF	IBA, Göttingen, Germany
MYB_2	AQAPPTAQL	IBA, Göttingen, Germany
MYB_3	VPQPAAAAI	IBA, Göttingen, Germany
MYB_4	TPAIKRSIL	IBA, Göttingen, Germany
MYB_5	DESGIVAEF	IBA, Göttingen, Germany
$pp65_{417-426}$	TPRVTGGGAM	AG Busch

2.1.8 Primer and DNA vectors

All primers were purchased from Sigma Aldrich, Taufkirchen, Germany. Sequences are depicted in 5‘ → 3‘ direction. Primer used for TCR repertoire PCR are listed in tables C.1 and C.2.

Table 2.10: Primer used for cloning of HLA molecules and TCR

Primer	Sequence
HLA-A0101/0201/0301 NotI forward	TACAGGCGGCCGCCACCATGGCC GTCATGGCGCCCCGAACCCTC
HLA-B0702/0801/4201 NotI forward	TACAGGCGGCCGCCACCATGCTG GTCATGGCGCCCCGAACCGTCCTC
HLA-B1501 NotI forward	TACAGGCGGCCGCCACCATGCGGGTC ACGGCGCCCCGAACCGTCCTC
HLA-B1801 NotI forward	TACAGGCGGCCGCCACCATGCGGGTCA CGGCGCCCCGAACCCTCCTC
HLA-B4402 NotI forward	TACAGGCGGCCGCCACCATGCGGGTC ACGGCGCCCCGAACCCTCCTC
HLA-A0101/0201 SalI reverse	CCTGTCGACCACTTTACAA GCTGTGAGAGACACATCAGA
HLA-A0301 SalI reverse	AGGGTCGACCACTTTACAAG CTGTGAGGGACACATCAGA

Table 2.10: Primer used for cloning of HLA molecules and TCR *(continued)*

Primer	Sequence
HLA-B0702/1501/1801/4201/4402	AGGGTCGACAGCTGTGAGA
SalI reverse	GACACATCAGAGCCCTGGGC
TCR 2.5D6α NotI forward	TACAGGCGGCCGCCACCA
	TGCTCCTGGAGCTTATCCC
TCR 2.5D6β NotI forward	TACAGGCGGCCGCCACCAT
	GAGCCTCGGGCTCCTGTG
TCRα constant EcoRI reverse	TGGAATTCTCAGCTG
	GACCACAGCCGCAGC
TCRβ constant 1 EcoRI reverse	TGGAATTCTCAGAAA
	TCCTTTCTCTTGACC

Table 2.11: Primer used for preparation of expression vectors

Primer	Sequence
NotI20ntSalIP2A forward	TAGCGGCCGCCTACGTGGACTC
	CAAGCTGGGTCGACGGAAGCG
	GCGCCACGAACTTCTCTCTGTTA
eGFP EcoRI reverse	TAGAATTCTTACTTGTACAGCTCGTCCA
P2AdsRedExpress forward	TAGGAAGCGGCGCCACGAACTTCTCTCT
	GTTAAAGCAAGCAGGAGACGTGGAAGAA
	AACCCCGGTCCCATGGCCACAACCATGGATAG
dsRedExpress EcoRI reverse	TAGGAGAATTCCTACTGGAACAGGTGGTGGC

Table 2.12: Primer used for sequencing

Primer	Sequence
Primer	Sequence
MP71 forward	TGAAAATTAGCTCGACAAAG
MP71 reverse	GTAAATGATTGCCCCACCA
GFP reverse 1	AACTTCAGGGTCAGCTTGCC
dsRedExpress reverse 1	CTATCCATGGTTGTGGCCAT
T7	TAATACGACTCACTATAGGG
pcDNA3 reverse	GGCAACTAGAAGGCACAGTC
MPO forward 1	ATGTTCATGCAATGGGGCCA

Table 2.12: Primer used for sequencing *(continued)*

Primer	Sequence

Table 2.13: Primer used for cloning of MPO

Primer	Sequence
MPO NotI forward	TACAGGCGGCCGCCACCATGGGGGTTCCCTTCTTCTC
MPO EcoRI reverse	TAGTCGACGGAGGCTTCCCTCCAGGAAG

Table 2.14: Primer used for qPCR

Primer	Sequence
MPO qPCR forward 1	AATTGGCGAGGAAACTGATG
MPO qPCR reverse 1	GTGGTGATGCCTGTGTTGTC
ITGA2B qPCR forward 1	AGAATGAGACCAGGGTGGTG
ITGA2B qPCR reverse 1	TGTTCTGCTCCCTCTCACCT
MYB qPCR forward 1	GGCAGAAATCGCAAAGCTAC
MYB qPCR reverse 1	GCAGGGAGTTGAGCTGTAGG
ELANE qPCR forward 1	TGCTCAACGACATCGTGATT
ELANE qPCR reverse 1	TGCTCAACGACATCGTGATT
MS4A3 qPCR forward 1	TGCTGATTCTCACCTTGCTG
MS4A3 qPCR reverse 1	CGAGCAGGCTTATGTTCTCC
GAPDH qPCR forward 1	GAGTCAACGGATTTGGTCGT
GAPDH qPCR reverse 1	TTGATTTTGGAGGGATCTCG
HPRT1 qPCR forward 1	AAGCTTGCTGGTGAAAAGGAA
HPRT1 qPCR reverse 1	AAGCTTGCTGGTGAAAAGGAA
HMBS qPCR forward 1	AGGATGGGCAACTGTACCTG
HMBS qPCR reverse 1	TCGTGGAATGTTAACGAGCAG

Table 2.15: DNA vectors

Vector	Details	Source
pMP71G$_{PRE}$-eGFP	Retroviral vector coding for eGFP MPSV-LTR promotor	W. Uckert, Berlin
pcDNA3.1-MLV	Codes for group specific antigen	W. Uckert, Berlin

Table 2.15: DNA vectors *(continued)*

Vector	Details	Source
("gag-pol")	and polymerase genes	
pALF10A1-GALV	Codes for 10A1 virus envelope	W. Uckert, Berlin
("env")	gene of the murine Leukemia virus	
pcDNA3.1-	Suitable for *in vitro* transcription	Invitrogen,
	with a T7 polymerase	Carlsbad, USA
pMIberry	Codes for dsRedExpress 2	AG Duyster, München
pMK-RQ	TCR 2.5D6, murinized constant regions,	Invitrogen,
	codon optimized	Carlsbad, USA

2.1.9 Primary cells and cell lines

Table 2.16: Primary cells

Cells	Medium	Source
PBMC	cRPMI	Healthy blood donors,
		Patients (table: B)
DC	DC	Healthy blood donors
T cells	T cell	Healthy blood donors
HRGEC	ECM	ScienCell, Carlsbad, USA

Table 2.17: Cell lines

Cell line	Medium	Details	Source
T2	cRPMI	TAP deficient	ATCC, Manassas, USA
C1R	cRPMI	HLA class I deficient	S. Stevanovic, Tübingen
NB4	cRPMI	APL	CLS, Eppelheim, Germany
MV4-11	cRPMI	AML	CLS, Eppelheim, Germany
HL-60	cRPMI	APL	CLS, Eppelheim, Germany
ML-2	cRPMI	AML	The Cabri consortium
KG1a	cRPMI	AML	CLS, Eppelheim, Germany
K562	cRPMI	CML	ATCC, Manassas, USA
BJAB	cRPMI	Burkitt lymphoma	J. Mautner, München, Germany
Molt4	cRPMI	T-ALL	CLS, Eppelheim, Germany
NSO-IL15	cDMEM	mouse myeloma	S. Riddell, Seattle, USA

Table 2.17: Cell lines *(continued)*

Cell line	Medium	Details	Source
	(+ 2 µg/ml) MPA	hIL-15 producing	
SKOV-3	cDMEM	Ovarian carcinoma	ATCC, Manassas, USA
888-mel	cDMEM	Melanoma	NIH, Bethesda, USA
143TK$^-$	cDMEM	Osteosarcoma	R. Mocikat, München, Germany
SW-480	cDMEM	Colorectal carcinoma	ATCC, Manassas, USA
SKBR-3	cDMEM	Breast cancer	CLS, Eppelheim, Germany
293T	cDMEM	Human embryonic kidney cells	ATCC, Manassas, USA

All LCL were cultured in cRPMI-medium and are listed in table 2.18.

Table 2.18: LCL used for peptide independent and dependent HLA crossreactivity assays

LCL	IHW[1] number	HLA-*A**	HLA-*B**	HLA-*C**
LCL1	IHW09016	*02:04*, -:-	*51:01*, -:-	*15:02*, -:-
LCL2	IHW09005	*03:01*, -:-	*27:05*, -:-	*01:02*, -:-
LCL3	IHW09037	*29:02*, -:-	*40:02*, -:-	*02:02*, -:-
LCL4	IHW09064	*02:17*, -:-	*15:01*, -:-	*03:03*, -:-
LCL5	IHW09216	*02:09*, *03:01*	*35:01*, *38:01*	*04:01*, *12:03*
LCL6	IHW09021	*68:02*, *30:01*	*42:01*, -:-	*17:01*, -:-
LCL7	IHW09213	*02:08*, -:-	*50:01*, *08:01*	*07*, *06:02*
LCL8	IHW09079	*33:01*, -:-	*14:02*, -:-	*08:02*, -:-
LCL9	-	*02:01*, -:-	*07:02*, *15:01*	*03:04*, *12:03*
LCL10	IHW09043	*01:01*, -:-	*41:01*, -:-	*17:01*, -:-

2.1.10 Buffers and media

Table 2.19: Buffers

Buffer	Additives	Used for
ACK red cell lysis buffer	H_2O, 0.15 mol/l NH_4Cl 10 mmol/l $KHCO_3$ 0.1 mmol/l EDTA	Red cell lysis

[1] International HLA workshop

Table 2.19: Buffers *(continued)*

Buffer	Additives	Used for
Blocking reagent 1	PBS, 1 % milk powder	ELISA
Blocking reagent 2	PBS, 5 % milk powder	Western blot
CHAPS-lysis buffer A	PBS, 1.2 % CHAPS	Immunopeptidomics
	1x Complete (Roche)	
	2 mmol/l $NaVO_3$	
	2 mmol/l NaF	
	2 mmol/l PMSF	
CHAPS-lysis buffer B	50 % lysis buffer A,	Immunopeptidomics
	50 % PBS	
Coating buffer	0.1 mol/l $NaHCO_3$	ELISA
	0.03 mol/l Na_2CO_3	
	H_2O, pH 9.5	
Coupling buffer	H_2O, 0.5 mol/l NaCl	Immunopeptidomics
	0.1 mol/l $NaCO^3$	
ΔFCS	inactivated FCS	various
	(20 min at 58 °C)	
Fixation solution	PBS, 1 % PFA	Flow cytometry
ΔHS	inactivated HS	various
	(20 min at 58 °C)	
DEPC-H_2O	H_2O, 0.1 % DEPC	ivt-RNA production
FACS buffer	PBS, 1 % ΔFCS	Flow cytometry
Isolation buffer	PBS, 0.2 % ΔFCS	Cell purification
	2 mmol/l EDTA	(Dynabeads, MACS)
10x Isotonic ammonium chloride	H_2O, 1.55 mol/l NH_4Cl	Lysis of erythrocytes
	0.1 mol/l $NaHCO_3$	
Multimer staining buffer	PBS, 50 % ΔFCS,	Flow cytometry
	2 mmol/l EDTA	
RetroNectin solution	PBS, 12 µg/ml RetroNectin	Transduction
RIPA buffer	50 mmol/l Tris HCl, pH 7.5	Western blot
	150 mmol/l NaCl	
	1 % NP-40	
	0.25 % Na-deoxycholate	
	1 mmol/l PMSF	
	1x Complete (Roche)	
10x TAE buffer	0.4 mmol/l Tris-HCl, pH 7.8	DNA gel electrophoresis

Table 2.19: Buffers *(continued)*

Buffer	Additives	Used for
	10 mmol/l EDTA	
	0.2 mmol/l acetic acid	
	pH adjusted to 8.0	
10x TBE	DEPC H_2O	RNA gel electrophoresis
	1500 mmol/l NaCl	
	0.89 mol/l Tris HCl	
	0.89 mol/l boric acid	
	20 mmol/l EDTA	
TBS buffer	H_2O, 500 mmol/l Tris-HCl,	Western blot
	pH 7.4	
TBST	TBS buffer, 0.05 % Tween-20	Western blot
Washing buffer	PBS, 0.05 % Tween-20	ELISA

Table 2.20: Cell culture media

Medium	Composition
Freezing medium	90 % ΔFCS, 10 % DMSO
AIM-V (Invitrogen)	AIM-V
alpha-MEM	alpha-MEM (PAA), 2 % ΔFCS
DMEM	DMEM (Invitrogen, PAA)
cDMEM	DMEM (4.5 g/l Glucose), 10 % (v/v) ΔFCS
	2 mmol/l L-Glutamine, 10 mmol/l non essential AS
	1 mmol/l Sodium-Pyruvat, 100 IU ml P/S
RPMI	RPMI 1640 (Invitrogen, PAA)
cRPMI	RPMI 1640 with phenol red, 10 % (v/v) ΔFCS
	2 mmol/l L-Glutamine, 10 mmol/l non essential AS
	1 mmol/l Sodium-Pyruvat, 100 IU/ml P/S
DC	VLE RPMI, 1.5 % ΔHS
ECM	ECM medium (ScienCell), 5 % (v/v) ΔFCS
	5 ml ECGS, 5 ml P/S
MethoCult H4435 enriched	MethoCult H4435 enriched (Stem Cell Technologies)
OptiMEM (Invitrogen)	No additives
T cell	RPMI 1640 with phenol red, 5 % (v/v) ΔFCS
	5 % (v/v) ΔHS, 10 mmol/l HEPES,
	16.6 µg/ml Gentamycin, 2 mmol/l L-Glutamine,

Table 2.20: Cell culture media *(continued)*

Medium	Composition
	10 mmol/l non essential AS, 1 mmol/l Sodium-Pyruvat, 100 IU/ml P/S

2.1.11 Software

Table 2.21: Software and online tools

Software	Application	Company/website
FlowJo	Analysis of flow cytometric data	Tree Star, Ashland, USA
Clone Manager 7	Design of cloning experiments	Scientific Educational Software, Cary, USA
GraphPad Prism	Data analysis and presentation	GraphPad Software, San Diego, USA
IMGT	Analysis of TCR sequences	http://imgt.cines.fr
ImmunoSpot Software	ELISpot analysis	CTL-Europe, Bonn, Germany
LaTeX	Thesis preparation	`www.latex-project.org`
MASCOT	Mass spectrometry data analysis	`www.Matrixsciences.com`
Microsoft Office	Data analysis and presentation	Microsoft, Redmond, USA
mMass	Mass spectrometry data analysis	`www.mmass.org`
PEAKS	Mass spectrometry data analysis	Bioinformatics Solutions, Waterloo, Canada
Sequencer 4.5	Analysis of DNA data	Gene codes, Ann Arbout, USA
SYFPEITHI	HLA class I ligand binding predictions	`www.syfpeithi.de`

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 RNA isolation using TRIzol reagent

Values in parentheses indicated the number of cells and the volume of the different reagents when only limited material was available for RNA isolation.
For isolation of RNA out of different cell lines and primary cells, a pellet of up to 1×10^6 cells (approximately 0.1×10^6 cells) was resuspended in 1000 µl (200 µl) TRIzol reagent, vortexed and incubated for 5 min at room temperature (in the case of T cell clones 10 µg of Yeast-RNA was added additionally). 200 µl (40 µl) 1-Bromo-3-Chloro-Propan were added and after 10 min incubation at room temperature the solution was centrifuged for 15 min at 4 °C and 15 000 g. The aqueous, RNA containing phase was transferred into a new reaction tube containing 500 µl (100 µl) isopropanol. For precipitation of the RNA the solution was incubated for at least 1 h (up to 24 h) at −20 °C. After centrifugation for 20 min at 4 °C and 15 000 g the supernatant was removed and the pellet was washed using 1 ml (500 µl) ethanol. After centrifugation for 5 min at 4 °C and 15 000 g, supernatant was discarded and the remaining pellet was resuspended in 20 µl to 50 µl DEPC-H_2O after drying for about 5 min. RNA concentration was measured using a *NanoDrop ND-1000* device (PeqLab) and stored at −80 °C until further use.

2.2.1.2 RT PCR

For synthesis of cDNA by RT PCR, the *AffinityScript Multiple Temperature Reverse Transcriptase* was used according to the manufacturer's instructions with slight modifications. Up to 1 µg of RNA was mixed with 1 µl of a oligo(dT) primer solution ($\widehat{=}$500 ng) and DEPC-H_2O was added to a final volume of 14.5 µl. After incubation for 5 min at 65 °C the sample was cooled to room temperature for 20 min. Afterwards, 2 µl of the AffinityScript buffer, 2 µl dNTPs (Fermentas, 10 mmol/l each), 0.5 µl RNase Block and 1 µl of the AffinityScript polymerase were added. The mixture was incubated for 1 h at 47.5 °C followed by incubation for 15 min at 70 °C in a *TGradient Thermocycler* (Biometra). cDNA was stored at 20 °C until further use.

2.2.1.3 PCR

For amplification of selected genes out of different cell lines, RNA was isolated and cDNA was synthesized (sections 2.2.1.1 and 2.2.1.2). The resulting cDNA was used as a template in a PCR reaction with the respective primer pairs (tables 2.10, 2.11 and 2.13). The PCR reaction was done using the *KOD Hot Start Polymerase* kit (Merck).

Table 2.22: Reaction mixture for PCR with the KOD polymerase

Volume	Reagent
10x KOD buffer	10 µl
dNTP Mix (2 mmol/l)	10 µl
$MgSO_4$ (25 mmol/l)	6 µl
Primer forward (15 µmol/l)	3 µl
Primer reverse (15 µmol/l)	3 µl
cDNA	2 µl
KOD	2 µl
H_2O	*ad* 100 µl

The reaction mixture was separated into two 0.5 ml reaction tubes and incubated in a *TGradient Thermocycler*.

Table 2.23: PCR program with KOD polymerase

Temperature	Time	
95 °C	2 min	
95 °C	30 s	
55 °C	30 s	30 cycles
70 °C	1 min	
70 °C	10 min	
4 °C	∞	

2.2.1.4 TCR α and β repertoire PCR

After isolation of RNA out of T cell clones and reverse transcription into cDNA (section 2.2.1.1 and 2.2.1.2) the TCR Vα and Vβ repertoire was analyzed using different Vα (table C.1) and Vβ (table C.2) primers binding in different variable TCR regions. For the PCR the *HotStar MasterMix Plus* (Qiagen) was used. The composition of the reaction mixtures is listed in table 2.24 for the α and in table 2.25 for the β repertoire. The reaction mixtures were incubated in a *TGradient Thermocycler* with the program listed in table 2.26.

Table 2.24: Reaction mixture for the TCR Vα PCR using *HotStar MasterMix Plus*

Reagent	Volume
HotStar MasterMix	12.5 µl
P-5'αST (5 µmol/l)	1.5 µl

Table 2.24: Reaction mixture for the TCR Vα PCR using *HotStar MasterMix Plus* *(continued)*

Reagent	Volume
P-3'αST (5 µmol/l)	1.5 µl
3'-Cα (5 µmol/l)	2 µl
cDNA	0.55 µl
Coral load	2.5 µl
DEPC-H_2O (5 µmol/l)	1.4 µl
Vαx (5 µmol/l)	3 µl

Table 2.25: Reaction mixture for the TCR Vβ PCR using *HotStar MasterMix Plus*

Reagent	Volume
HotStar MasterMix	12.5 µl
P-5'βST (5 µmol/l)	2.5 µl
P-3'βST (5 µmol/l)	2.5 µl
3'-CβII (5 µmol/l)	2.5 µl
cDNA	0.77 µl
Coral load	2.5 µl
DEPCH_2O (1.73 µmol/l)	1.4 µl
Vβx (5 µmol/l)	3 µl

Table 2.26: PCR program with *HotStar MasterMix Plus*

Temperature	Time	
95 °C	5 min	
94 °C	1 min	
54 °C	1 min	35 cycles
72 °C	1 min	
72 °C	10 min	
4 °C	∞	

Family specific gel bands were exsized from the gel, DNA was purified (section 2.2.1.8) and sent for sequencing with the respective Vα or Vβ primer to Eurofins MWG.

2.2.1.5 *In silico* modification of TCR sequences

It has been shown, that transgenic expression of TCR can be improved by different modifications of the native TCR sequences. *In silico* modification of TCR sequences implemented (1) generation of a bicistronic construct starting with the *beta*-chain, followed by a porcine teschovirus-1 peptide P2A (P2A) element and the *alpha*-chain (Leisegang et al., 2008). (2) Replacement of the human constant chains by mouse constant chains for improved pairing of the exogenous *alpha*- and *beta*-chain (Cohen et al., 2006). (3) Insertion of an additional disulfide bond for improvement of TCR expression (Cohen et al., 2007). Modified sequences were submitted to GeneArt for codon optimization for improved transgene expression (Scholten et al., 2006) and synthesis of the TCR construct. The modified, bicistronic TCR construct was then further cloned into the retroviral vector pMP71 for further experiments

2.2.1.6 qPCR using SYBR Green I

For relative quantification of the expression of different genes on the mRNA level, qPCR was performed on a *LightCycler 480* system (Roche) using the *KAPA SYBR FAST LightCycler 480* master mix (PeqLab) after RNA purification and cDNA synthesis (sections 2.2.1.1 and 2.2.1.2). Cp values of target genes were normalized against the geometric mean of Cp values of the housekeeping genes GAPDH, HMBS and HPRT1. Relative quantitative expression in comparison to PBMC was calculated using the ΔΔCt method as described previously (Pfaffl, 2001). Sequence identity of the amplified product was analyzed by gel electrophoresis (section 2.2.1.7), gel extraction (section 2.2.1.8) and DNA sequencing in selected cases. Primers used for qPCR are listed in table 2.14, reaction mixture and cycling program are listed in tables 2.27 and 2.28.

Table 2.27: qPCR reaction mix

Reagent	Volume
SYBR FAST	10 µl
Primer forward (15 µmol/l)	0.26 µl
Primer reverse (15 µmol/l)	0.26 µl
cDNA	2 µl
DEPC-H_2O	18 µl

Table 2.28: *LightCycler 480* program

Temperature	Time
95 °C	3 min
95 °C	3 s

Table 2.28: ***LightCycler 480*** **program** ***(continued)***

Temperature	Time	
65 °C	1 min	40 cycles
72 °C	1 s	
95 °C	1 s	
65 °C	1 s	Melting
97 °C	1 s	curve
45 °C	10 s	

Melting curve analysis was performed to identify samples with more than one product (for example: primer dimers).

2.2.1.7 Agarose DNA electrophoresis

For analysis of DNA fragments gel electrophoresis was done using 1-1.7 % agarose gels. Therefore, the respective amount of agarose (w/v) was cooked in 1x TAE buffer and ethidium bromide was added to a final concentration of 0.8 µg/ml. Samples were mixed with the respective amount of 6x DNA loading dye except in the cases where Coral load has been added during PCR preparation. DNA fragments were separated for 20 min at 130 V in a *Electrophoresis chamber Midi horizontal* (G & P Kunststofftechnik).
For analysis of RNA and ivt-RNA 1.5 % agarose gels were used. The buffer system used was 1x TBE instead of 1x TAE. For destruction of secondary RNA structures, RNA as well as the RNA ladder was incubated with 50 % formamide (v/v) for 10 min at 65 °C. After cooling on ice for 2 min, the respective amount of 6x RNA loading dye was added and samples were loaded. Electrophoresis was conducted in a *Mini Gel Migration Through* system (Cosmo Bio) for 35 min and 100 V. 0.5x TBE buffer was used.
Results were visualized and documented using a *BioDoc Analyze digital* system (BioMetra).

2.2.1.8 Gel extraction

For purification of DNA from agarose gels the *NucleoSpin Gel and PCR Clean-up Kit* (Macherey-Nagel) was used according to the manufacturer's instructions. Elution of DNA was done using 30 µl DEPC-H_2O. Concentration was determined on a *NanoDrop ND-1000* device.

2.2.1.9 Restriction digest

For further cloning into a DNA vector, the purified PCR product (sections 2.2.1.3 and 2.2.1.8) as well as 15 µl of the respective DNA vector was digested for 2 h at 37 °C. Reaction was

terminated by incubation for 15 min at 65 °C. Composition of the reaction mixture is listed in table 2.29.

Table 2.29: Reaction mix for restriction digests

Reagent	Volume/amount
DNA vector/	15 µg
PCR product	30 µl
10x buffer	10 µl
Enzyme 1	2 µl
Enzyme 2	2 µl
DEPC-H_2O	ad 100 µl

Digested DNA vectors were separated by gel electrophoresis (section 2.2.1.7), DNA bands of the respective size were excized and DNA was purified (section 2.2.1.8). Digested PCR products were purified without prior gel electrophoresis. Concentration was determined on a *NanoDrop ND-1000* device.

2.2.1.10 Ligation

For ligation of digested PCR products into the respective DNA vector, ligation was done at molare ratios (DNA vector : PCR fragment) of 1:5 and 1:3. The amount of PCR fragment was calculated using the following formula:

$$m\ (PCR\ fragment\ (\text{ng})) = 5\ x\ (size\ PCR\ fragment\ (\text{bp}))\ x\ \frac{\text{m (DNA vector (ng))}}{\text{size (DNA vector (bp))}}$$

Reaction mixture was pipetted as depicted in table 2.30 and incubated for 16 h at 16 °C.

Table 2.30: Pipetting scheme for ligation reactions

Reagent	Volume/amount
DNA vector	100 ng
PCR fragment	x ng
10x T4 ligase buffer	1 µl
T4 ligase	1 µl
DEPC-H_2O	ad 10 µl

2.2.1.11 Transformation of chemically competent bacteria

Chemically competent *E. coli* bacteria were thawed on ice for 10 min. After addition of 2 µl ligation mixture (section 2.2.1.10) to 25 µl cell suspension, the mixture was incubated on ice for 30 min. A heat shock was performed at 42 °C for 30 s and 250 µl S.O.C medium were added after incubation on ice for 2 min. Cells were shaked for 1 h at 300 rpm in a *Thermomixer comfort* (Eppendorf), different dilutions were plated on prewarmed LB-agar plates containing 100 µg/ml Ampicillin and incubated over night at 37 °C.
In the case of retransformation of DNA vectors, 100 ng DNA was mixed with 25 µl thawed cell suspension, incubated for 2 min on ice. After the heat shock, cells were incubated on ice for 2 min, shaked for 10 min at 37 °C and plated as described above.

2.2.1.12 Plasmid preparation

To analyze transformation of bacteria with the respective DNA vector, single colonies (section 2.2.1.11) were transferred into 5 ml LB-medium (+ 100 µg/ml Ampicillin) and incubated over night at 37 °C and 265 rpm in a *Incubator Shaker* (New Brunswick Scientific). Plasmids were preparated out of 1.5 ml over night culture using the *JetStar 2.0 Plasmid Purification Kit* (Genomed) according to the manufacturer's instructions.
For purification of DNA vectors for cloning or transfection (section 2.2.4.1) plasmids were retransformed as described in section 2.2.1.11. A 3 ml starter culture (+ 100 µg/ml Ampicillin) was inoculated with a single colony and incubated for 6 h. Afterwards, 250 µl were used for inoculation of a 250 ml maxi culture. Plasmids were purified after over night incubation at 37 °C and 265 rpm using the *JetStar 2.0 Plasmid Purification Kit* according to the manufacturer's instructions. I case of DNA plasmids coding for TCRs the *Endotoxin-free plasmid DNA purification kit* (Macherey-Nagel) was used. DNA concentration was determined using a *NanoDrop ND-100*.

2.2.1.13 Analytical digest

After plasmid preparation (section 2.2.1.12) an analytical digest was performed to seek for clones with expected digestion patterns. The composition of the reaction mixture is listed in table 2.31.

Table 2.31: Pipetting scheme for analytical digests

Reagent	Volume
DNA vector	1 µl
10x buffer	1 µl
Enzyme 1	0.5 µl
Enzyme 2	0.5 µl

Table 2.31: Pipetting scheme for analytical digests *(continued)*

Reagent	Volume
DEPC-H_2O	ad 10 µl

Plasmid with the correct digestion pattern were sent for sequencing to Eurofins MWG with the respective primer (table 2.12).

2.2.1.14 Generation of vectors for simultaneous expression of HLA or target genes and fluorescent dyes

As specific flow cytometry antibodies are only available for a few HLA molecules and some target antigens show intracellular expression, we generated bicistronic constructs coding for the respective HLA molecule or the target and eGFP or dsRed separated by a P2A element. This allowed us to follow gene transfer efficiency by flow cytometry as well as cell sorting without the need for specific antibodies. We modified the vector pMP71-eGFP to contain a *NotI* restriction site followed by a nonsense part of 20 nucleotide, a *SalI* restriction site, a P2A element and an eGFP. Additionally, a vector containing a *NotI* restriction site followed by a nonsense part of 20 nucleotides, a *SalI* restriction site, P2A and a dsRed was generated. Therefore, a PCR (section 2.2.1.3) was done to add the P2A element to the dsRed gene using the primer pair P2AdsRedExpress forward and dsRedExpress EcoRI reverse. The generated product was then further modified by PCR (section 2.2.1.3) using the primer pair NotI20ntSalIP2A forward and dsRedExpressEcoRI reverse and cloned into the vector pMP71. Both vectors allowed us to easily clone the genes of interest via the *NotI* and *SalI* restriction sites to obtain bicistronic constructs. For generation of vectors suitable for production of ivt-RNA, the bicistronic cassette was excised by digestion with *NotI* and *EcoRI* and cloned into the vector pcDNA3.1-.

2.2.1.15 *In vitro* transcription and polyadenylation

For synthesis of ivt-RNA pcDNA3.1 vectors containing the respective gene were linearized using the restriction enzyme HindIII (section 2.2.1.9). The success of digestion was analyzed by gel electrophoresis (section 2.2.1.7) and DNA was purified by addition of 10 µl sodium acetate (3 mol/l), 5 µl EDTA (0.5 mol/l) and 200 µl ethanol (100 %). DNA was precipitated over night at −20 °C. After centrifugation for 15 min at 15 000 g supernatant was discarded and pellet was air dried for 10 min. DNA was resuspended in 15 µl DEPC-H_2O and concentration was determined on a *NanoDrop ND-1000*.
In vitro transcription was done using the *mMessage mMachine T7 Kit* (Invitrogen) followed by polyadenylation with the *Poly(A) tailing Kit* (Invitrogen) according to the manufacturer's

instructions. After polyadenylation, RNA was purified using the *RNeasy Mini Kit* (Qiagen) according to the manufacturer's instructions. Elution was done two times using 30 µl DEPC-H_2O in each elution step. 0.5 µl of the reaction mixture before polyadenylation as well as 1.5 µg polyadenylated RNA was analyzed by gel electrophoresis as described in section 2.2.1.7. Concentration was determined on a *NanoDrop ND-1000* device and ivt-RNA was stored at −80 °C until further use.

2.2.2 Cell culture methods

All cell culture methods were performed under sterile conditions and work with retroviruses, epstein-barr virus $(EBV)^+$ cell lines or human blood samples was done in accordance with S2 guidelines. Unless otherwise stated, all centrifugation steps were done at 500 g for 5 min at room temperature.

2.2.2.1 Freezing and thawing of cells

Cells were pelleted prior freezing , resuspended in 1 ml freezing medium (table 2.20), transferred in a cryo vial and kept in a −80 °C freezer for one day before transfer into liquid nitrogen for long term storage.
For thawing of cells, the cryo vial was warmed shortly in warm water and cells transferred into 10 ml medium. After centrifugation supernatant was discarded, cells were resuspended in the appropriate medium (tables 2.20, 2.16 and 2.17) and transferred into a suitable culture vessel.
In the case of clumping cells (some leukapheresis products, cells from MPN patients), benzoase-endonuclease was added to 5 ml medium to a final concentration of 25 IU/ml before thawing of the cells.

2.2.2.2 Counting of cells

Cells were counted using a *Neubauer* counting chamber. For dead cell exclusion, cell suspension was diluted in trypan blue solution. Blue cells were excluded during counting and concentration of cells was calculated using the following formula:

$$c\ (Cells) = \frac{\text{Number of counted cells x dilution-factor}}{4}\ x\ 10^4$$

2.2.2.3 Culture of cell lines

Suspension and adherent cell lines were cultured in the respective medium (tables 2.20 and 2.17) and split every three to four days. For splitting of adherent cell lines (except T293 cells), medium was removed and cells were washed with PBS. Cells were incubated for 2 min to 5 min at 37 °C after addition of trypsine. Loosening of cells was checked under a microscope and

medium was added to stop trypsine reaction. Cell suspension was transferred into a 50 ml tube, pelleted and resuspended in the appropriate amount of culture medium.

2.2.3 Purification of primary cells

2.2.3.1 PBMC isolation from heparinized whole blood

PBMC were isolated from heparinized whole blood by Ficoll density gradient centrifugation. Therefore, blood was diluted 1:1 (or in the case of blood from leukemia patients up to 1:10) and carefully layered on 15 ml ficoll in a 50 ml tube. After centrifugation for 20 min without break at 880 g and room temperature buffy coats were carefully harvested, transferred into a new 50 ml tube and washed twice in RPMI. After resuspension in appropriate buffer or medium, cells were counted and used for downstream applications.
Cell pellets for western blot analysis (section 2.2.8.2) and immunopeptidomic analysis (section 2.2.8.1) were prepared by centrifugation for 10 min at 1500 g and room temperature. After quickfreezing in liquid nitrogen, pellets were stored at −80 °C until further use.

2.2.3.2 Isolation of $CD8^+CD45RO^-CD57^-$ naive T cells

For stimulation of naive T cells with naturally presented HLA ligands, $CD8^+CD45RO^-CD57^-$ cells were negatively selected out of PBMC (section 2.2.3.1). Therefore, $CD8^+$ T cells were isolated using the *Dynabeads Untouched Human CD8 T Cells Kit* (Invitrogen). Antigen experienced $CD8^+$ T cells were depleted using *Human CD45RO* and *CD57 MicroBeads* (Miltenyi Biotec) according to the manufacturer's instructions. The success of isolation was checked by flow cytometry (section 2.2.6).

2.2.3.3 Purification $CD8^+CD62L^+CD45RA^-$ T_{CM}

For the *In vivo* experiment 2, TCR transgenic $CD8^+CD62L^+CD45RA^-$ T_{CM} were injected into BRG mice to analyze *in vivo* tumor reactivity of the TCR 2.5D6. Therefore, 2×10^8 PBMC (section 2.2.3.1) were resuspended in 200 µl isolation buffer. For depletion of $CD4^+$ and $CD45RA^+$ cells, 20 µl of a hαCD4-FITC and a hαCD45RA-APC antibody was added. After 15 min incubation at 4 °C, cells were washed by addition of 10 ml isolation buffer and pelleted. Cells were resuspended in 960 µl isolation buffer and 320 µl *Anti-FITC* and *Anti-APC MicroBeads* (Miltenyi Biotec) was added. Cells were rotated for 15 min at 4 °C and washed by addition of 10 ml isolation buffer. After pelleting, cells were resuspended in 800 µl isolation buffer and loaded onto a *LD* column (Miltenyi Biotec) according to the manufacturer's instructions. The $CD4^-CD45RA^-$ fraction was resuspended in 100 µl isolation buffer and cells were stained with 10 µl of an hαCD62L-PE antibody for 15 min at 4 °C and washed. For selection of $CD62L^+$ cells, the pellet was resuspended in 160 µl isolation buffer and 40 µl *Anti-PE MicroBeads* were added. After rotation for 15 min at 4 °C and a washing step, the cell

suspension was loaded onto a *MS* column (Miltenyi Biotec) according to the manufacturer's instructions. Phenotype and purity of the cells was analyzed by flow cytometry (section 2.2.6).

2.2.3.4 Differentiation and maturation of three day DC

Monocytes were enriched out of 1×10^8 to 1.3×10^8 PBMC (section 2.2.3.1) by plastic adherence in Nunclon surface cell culture flasks (80 cm^2) in 15 ml DC medium. After incubation for 1.5 h at 37 °C, non adherent cells were removed by washing three times with prewarmed RPMI medium. 15 ml fresh DC medium was added containing IL-4 (20 ng/ml) and GM-CSF (100 ng/ml). 48 h later the maturation cocktail was added (table 2.32) and cells were incubated at 37 °C.

Table 2.32: DC maturation cocktail

Cytokine/TLR ligand	End concentration
TNF-α	10 ng/ml
IL1-β	10 ng/ml
IFN-γ	5000 IU/ml
PGE_2	250 ng/ml
CL075	1 µg/ml
IL-4	20 ng/ml
GM-CSF	100 ng/ml

24 h later cells were harvested by flushing with PBS + 0.5 % ΔHS. Cells were pelleted, resuspended and washed two times with PBS before electroporation with ivt-RNA (section 2.2.5.1). Phenotype of DC was analyzed by flow cytometry (section 2.2.6) using antibodies binding to CD80, CD83, CD86, CD14 and CD209.

2.2.3.5 Purification of granulocytes out of EDTA treated whole blood by differential centrifugation

For purification of granulocytes, blood was taken from healthy donor with 9 ml *Monovettes* (Sarstedt), containing 1.6 mg EDTA per ml blood. Erythrocytes were lyzed by incubating 4 ml blood with 16 ml ice cold 1x isotonic ammonium chloride solution for 15 min on ice. After centrifugation at 160 g for 10 min at room temperature, supernatant was discarded and cells were resuspended in 10 ml HBSS. Cells were centrifuged at 55 g for 10 min at room temperature, supernatant was discarded and cells were resuspended in 5 ml HBSS. After a final centrifugation for 5 min at 55 g and room temperature, supernatant was discarded and cells were kept on ice after resuspension in 2 ml HBSS until further use.

2.2.4 Gene transfer techniques

2.2.4.1 Production of virus particles

For generation of retroviral particles T293 cells were used as a producer cell line. Therefore, 0.2×10^6 to 0.3×10^6 T293 cells were seeded in 3 ml cDMEM medium per well of a tissue culture treated 6-well plate 24 h prior transfection. On the next day, 9 µl *TransIT-293 Transfection reagent* (Mirus) were mixed with 200 µl DMEM and incubated for 20 min at room temperature. 1 µg of the DNA vectors pcDNA3.1-MLV ("gag-pol"), pALF10A1-GALV ("env") and pMP71 coding for the gene of interest was added, mixed by pipetting and incubated for 30 min at room temperature. After changing medium, the transfection mixture was added dropwise to the cells under gentle agitation. After incubated for 48 h at 37 °C supernatant was harvested and cleared by centrifugation at 500 g for 5 min followed by filtering through a 0.45 µm syringe filter. In cases of pMP71 DNA vectors coding for eGFP or dsRedExpress2, transfection efficiency in T293 cells was analyzed by flow cytometry (section 2.2.6).

2.2.4.2 Transduction of activated PBMC

Transduction of activated PBMC was done in RetroNectin coated non treated 24-well plates. Therefore, 400 µl of a RetroNectin solution (end concentration: 12.5 mg/ml) was added to each well and incubated for 24 h. After removal of the RetroNectin solution, wells were blocked with 500 µl PBS + 2 % BSA for 30 min at room temperature. Wells were washed with 2 ml PBS + 2.5 % (v/v) HEPES and stored at 4 °C after addition of 1 ml PBS + 2.5 % (v/v) HEPES.
PBMC (section 2.2.3.1) were seeded at 1×10^6cells per ml in a 12-well plate (final volume 2 ml) in T cell medium and activated in the presence of IL-2 (50 IU/ml and OKT3 (30 ng/ml). Two days later, cells were counted (section 2.2.2.2) and resuspended at a concentration of 1×10^6cells per ml in T cell medium. 1 ml of activated PBMC was pipetted into a RetroNectin coated well of a 24-well plate. After addition of 1 ml virus supernatant (section 2.2.4.1), protamine sulfate (4 µg/ml, IL-2 (100 IU/ml) and 1 % HEPES the plate was centrifuged for 90 min at 939 g and 32 °C. Cells were incubated over night at 37 °C and washed on the next day prior two a second round of transduction. Cells were transferred into tissue culture treated plates or cell culture flasks on the next day in T cell medium containing IL-7 and IL-15 at a final concentration of 5 ng/ml. Three two four days later, transduction efficiency was analyzed by flow cytometry (section 2.2.6) and functionality was assessed as described in section 2.2.7.

2.2.4.3 Transduction of T_{CM}

T_{CM} were purified as described in section 2.2.3.3. Transduction was done as described in section 2.2.4.2 with modified activation of the cells. Therefore, T_{CM} were resuspended in T cell medium at a concentration of 1×10^6 per ml and IL-2 was added to a final concentration of 50 IU/ml. *Dynabeads Human T-Activator CD3/CD28* (Invitrogen) were prepared according to the manufacturer's instruction and added to a T_{CM}:bead ratio of 1:1. After two days incubation at 37 °C cells were transduced as described in the previous section. T_{CM} were purified as described in section 2.2.3.3. Transduction was done as described in section 2.2.4.2 with modified activation of the cells. Therefore, T_{CM} were resuspended in T cell medium at a concentration of 1×10^6 per ml and IL-2 was added to a final concentration of 50 IU/ml. *Dynabeads Human T-Activator CD3/CD28* (Invitrogen) were prepared according to the manufacturer's instruction and added to a T_{CM}:bead ratio of 1:1. After two days incubation at 37 °C cells were transduced as described in the previous section.

2.2.4.4 Transduction of cell lines

Cell lines were transduced in tissue culture treated 24-well plates. Therefore, 0.3×10^6 were seeded per well in 1 ml of the respective medium and 1 ml retroviral supernatant was added. After addition of protamine sulfate (4 µg/ml) the plate was centrifuged for 90 min at 939 g and 32 °C. Cells were incubated for 24 h at 37 °C, washed and cultured further in a T25 flask. Transduction efficiency was analyzed by flow cytometry (section 2.2.6).

2.2.5 Expansion and isolation of antigen specific T cell clones

For expansion of peptide specific T cells recognizing naturally presented HLA ligands (section 2.2.8.1) a single HLA-mismatched stimulation approach was used.

2.2.5.1 Electroporation of DC with ivt-RNA

For generation of single HLA-mismatched DC, 2×10^6 to 6×10^6 three day DC (section 2.2.3.4) were resuspended in 350 µl OptiMEM and transferred into a 4 mm cuvette (Bio-Rad) and incubated on ice for 3 min. 20 µg to 40 µg ivt-RNA (section 2.2.1.15, coding for the respective HLA molecule) was added and the cell suspension was mixed with a pipet tip. Electroporation was done using a *Gene Pulser Xcell* system (Bio-Rad) with an exponential protocol at 300 V, 300 µF. Cells were transferred into 2 ml prewarmed DC medium in a non tissue culture treated 6-well plate and incubated over night at 37 °C. Electroporation efficiency was analyzed by determination of the eGFP expression by flow cytometry (section 2.2.6).

2.2.5.2 Peptide specific T cell expansion

0.1×10^6 single HLA-mismatched DC (section 2.2.5.1) were resuspended in AIM-V medium and pulsed with 0.1 µmol/l of the respective HLA ligand for 2 h at 37 °C. After washing with 10 ml RPMI, cells were resuspended in 500 µl T cell medium and seeded into one well of a 24-well plate. 1×10^6 $CD8^+$ naive T cells (section 2.2.3.2) were added in 500 µl T cell medium (effector:target ratio = 10:1) and cells were incubated for 72 h at 37 °C after addition of IL-21 (30 ng/ml). IL-7 and IL-15 were added every two to three days at a final concentration of 5 ng/ml and splitted when necessary. Cells were restimulated on day 10 with C1R cells, transduced with the respective HLA molecule and pulsed with 0.1 µmol/l of the respective peptide. Expanding cells were analyzed on day 20 using HLA multimers and sorted on a *MoFlo* cell sorter.
A schematic overview of the process is shown in figure 2.1.

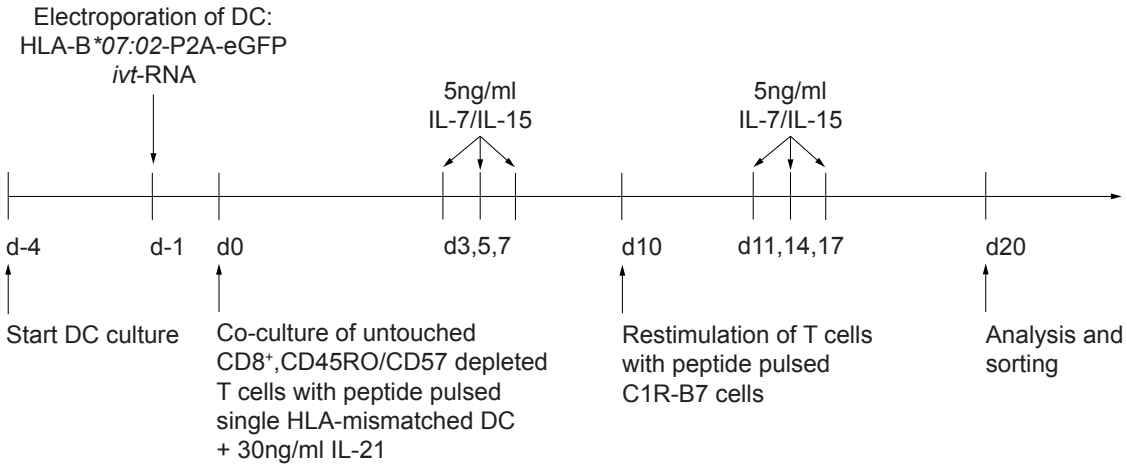

Figure 2.1: Single HLA-mismatched T cell stimulation.
Schematic overview of the single HLA-mismatched stimulation of naive T cells for the expansion of T cells specific for naturally presented HLA-*B*07:02* ligands.

2.2.5.3 Cloning of T cells by limiting dilution

Peptide specific T cells were cloned by limiting dilution after sorting with HLA multimers (section 2.2.5.2). Therefore, PBMC pooled from three different caucasian healthy blood donors were γ-irradiated with 30 Gy and used as feeder cells. 0.5 to 1 sorted T cells were coincubated with 5×10^4 irradiated feeder cells per well of a 96-well plate in 200 µl T cell medium containing IL-2 (50 IU/ml), IL-7 and IL-15 (5 ng/ml each). IL-2 was added two times a week in 10 µl T cell medium to a final concentration of 50 IU/ml. Growing clones were analyzed for their functionality between day 10 and 14 (section 2.2.7) and restimulated on day 14 with feeder cells as described above.

2.2.6 Flow cytometry

Cell pellets were obtained by centrifugation for 5 min at 500 g and 4 °C. Flow cytometry was done in all cases on a *LSRII* or a *FACS Canto II* device (both: BD). Sorting of cells was performed either on a *FACS Aria* (BD) or a *MoFlo* high performance cell sorter (Dako).

2.2.6.1 Staining of surface molecules

For analysis of molecules expressed on the surface of cells, 0.1×10^6 to 1×10^6 cells were pelleted, washed once in FACS buffer (table 2.19) and F_c receptors were blocked by resuspension in 100 µl ΔHS and incubation on ice for 10 min. After washing, 1.5 µl of each antibody (table 2.6) was added in a total volume of 50 µl FACS buffer and incubated in the dark for 20 min on ice. For live dead cell discrimination, 7-AAD was added to a final concentration of 5 ng/µl during incubation. After a final washing step, cells were resuspended in 200 µl FACS buffer prior analysis. In some cases cells were fixed in 200 µl fixation solution (table 2.19).

2.2.6.2 Staining of intracellular MPO

For staining of MPO, cell pellets were resuspended in 50 µl ΔHS+4 µg/ml EMA solution for blocking of F_c receptors and staining of dead cells. Samples were incubated 10 min on ice in the dark followed by incubation for 10 min on ice under strong light for covalent linkage of EMA to DNA in dead cells. After washing in FACS buffer, surface molecules were stained as described above, 7-AAD was not used. For permeabilization and fixation of cells the *Cytofix/Cytoperm* reagent kit (BD) was used according to the manufacturer's instructions. Cells were resuspended in 200 µl FACS buffer after the last step and analyzed.

2.2.6.3 Staining of T cells with HLA multimers

For analysis and sorting of peptide specific T cells after stimulation in the single HLA-mismatched setting, cells were stained with the respective HLA multimers (table 2.8). Therefore, F_c receptors were blocked after washing of cells by resuspension in 100 µl ΔHS and incubation on ice for 10 min. 1.5 µl of the respective HLA multimer was diluted in 50 µl multimer staining buffer (table 2.19) and centrifuged for 5 min at 12 000 g and 4 °C. Cells were resuspended after a washing step in 50 µl of the carefully harvested supernatant containing the HLA multimer. For staining of surface molecules and dead cells, 1.5 µl of the respective antibodies were added as well as 7-AAD to a final concentration of 5 ng/µl. After incubation on ice for 15 min cells were washed and resuspended in 200 µl FACS buffer prior analysis. In cases of sorting, propidium iodide (PI) was added instead of 7-AAD to a final concentration of 1 µg/µl directly before sorting.

2.2.7 Functional characterization of T cells

2.2.7.1 Coincubation assays

Unless otherwise stated, TCR transgenic PBMC or T_{CM} as well as T cell clones were stimulated at an effector:target ratio of 2:1 using 2×10^4 effector cells and 1×10^4 target cells per well of a 96-well plate in 200 µl T cell medium. When T2 cells were used as target cells, cells were pulsed over night with 1 µmol/l of the respective peptide in cRPMI medium. Cells were used for stimulation after washing and resuspension in T cell medium. All other target cells were pulsed with the respective peptide by directly adding the peptide to a final concentration of 1 µmol/l during stimulation. All adherent cell lines, myeloid cell lines and primary patient samples were pretreated with 500 IU/ml IFN-γ over night before used for stimulation assays. Samples of patients with MPN were additionally cultured for 6 days in cRPMI medium containing SCF and FLT3LG (100 ng/ml each). Every reaction condition was plated in triplicates. After incubation for 20 h to 24 h at 37 °C, supernatants were harvested and stored at −20 °C until further use.

2.2.7.2 IFN-γ ELISA

The amount of IFN-γ produced by stimulated T cells (section 2.2.7.1) was measured in the supernatant using the *Human IFN-γ ELISA Set* (BD) with a slightly modified protocol. ELISA plates were coated with 50 µl/well of a 1:250 dilution of capture antibody in coating buffer (table 2.19). After over night incubation at 4 °C, plates were washed three times in washing buffer (table 2.19). Unspecific binding sites were blocked for 1 h with 200 µl blocking buffer (table 2.19) at room temperature. After three washing steps serial dilutions of a IFN-γ standard (1000 ng/ml to 31.25 ng/ml in T cell medium, 50 µl/well) were plated in duplicates and samples were plated in triplicates (50 µl/well). After incubation for 1 h to 2 h at room temperature, plates were washed five times with washing buffer and 50 µl/well of detection solution (biotinylated detection antibody, and Streptavidin-HRP conjugate, diluted in PBS+1 % milk powder) was added. Plates were washed seven times after incubation for 1 h. After the last washing step, 100 µl/well of substrate solution (*TMB Substrate Reagent Set*, BD) was added. Reaction was stopped after 10 min to 15 min, when the standard was visible from strong to light blue by addition of 50 µl H_2SO_4 (1 mol/l). Extinction was measured at 450 nm on a *Sunrise Photometer* (Tecan).

2.2.7.3 FACS based cytotoxic assay

Cytotoxic activity of TCR transgenic effector cells was assessed by flow cytometry after coincubation with $eGFP^+$ target cells (section 2.2.7.1) after 20 h to 24 h (unless otherwise stated). Therefore, cells from triplicates were harvested, pooled and washed in FACS buffer (table 2.19). Cells were resuspended in 50 µl FACS buffer and 7-AAD was added to a final con-

centration of 5 ng/µl. After incubation on ice for 15 min, cells were washed in FACS buffer and analyzed on a flow cytometer. Cytotoxic activity was determined by analysis of the percentage of eGFP expressing, 7-AAD$^-$ target cells.

2.2.7.4 Chromium release assay

T cell clones were tested for their cytotoxic activity in a standard ^{51}Cr release assay. Therefore, 1×10^6 to 2×10^6 target cells were resuspended in 100 µl ΔFCSand labeled with 0.185 MBq ^{51}Cr for 90 min at 37 °C. 5×10^4 T cells were seeded in 50 µl cRPMI per well of a 96-well plate and 2×10^3 cells were added in 50 µl cRPMI after two washing steps. After incubation for 4 h at 37 °C, 50 µl supernatant was transferred on a *Luma* plate (Perkin Elmer). After drying over night, radioactivity was measured on a *TopCount NXT* (Perkin Elmer). Cytotoxic activity was determined as follows:

$$\%\ (Lysis) = \frac{\text{x - spontaneous lysis}}{\text{maximal lysis-spontaneous lysis}}$$

2.2.7.5 CFU assay

To assess the cytotoxic activity of TCR transgenic effector cells against hematopoetic progenitor cells CFU assays were done using *MethoCult H4435* (Stem Cell Technologies) according to the manufacturer's instructions. PBMC samples containing a total of five hundred CD34$^+$ (as assessed by flow cytometry, section 2.2.6) cells derived from leukemia patients or from a granulocyte-colony stimulating factor (G-CSF) mobilized healthy donor were coincubated with either TCR transgenic or untransduced PBMC. Incubation was performed in 250 µl alpha MEM medium supplemented with 2 % ΔFCS at different effector to target ratios for 15 min or 1 h at 37 °C. Subsequently, cell suspensions were mixed with 2.5 ml *MethoCult H4435* and plated in duplicates of 1.1 ml in 35 mm petri dishes resulting in 200 CD34$^+$ cells per plate. After 12 d to 14 d of incubation at 37 °C, plates were analyzed for the number of colonies under a light microscope. In some experiments, percent killing was calculated as follows:

$$100 - \left(\frac{\text{Number of colonies (E:T = x:1)}}{\text{Number of colonies (E:T = 0:1)}} * 100 \right)$$

2.2.8 Biochemical methods

2.2.8.1 Immunopeptidomics

For analysis of HLA class I ligands, HLA class I molecules were first immunoprecipitated using the pan-HLA class I specific antibody w6/32. Therefore, 10 ml *Pierce Centrifugation Columns* (Pierce) were pretreated by washing two times with 10 % acetic acid. CNBr sepharose (60 mg per 1×10^9 cells) was activated in 10 ml 1 mmol/l ice cold HCl in pretreated columns by rotation for 1 h at room temperature. Column was packed by removing HCl by gravity flow

followed by a centrifugation step for 4 min at 60 g without break. Column was washed once with 10 ml coupling buffer (table 2.19). For coupling of the antibody to the sepharose, antibody (1.5 mg per 1×10^9 cells) was added to the resin in 10 ml coupling buffer (table 2.19) and incubation was done by over night rotation at 4 °C.

The coupling solution was removed on the next day by gravity flow and centrifugation for 1 min at 60 g without break. 10 ml 200 mmol/l glycine was added for blocking and the column was rotated at room temperature for 1 h. Resin was dryed by gravity flow and centrifugation as described above. Column was washed two times with cold PBS and resin was stored at 4 °C in PBS.

Quickfrozen cell pellets (section 2.2.3.1) were resuspended in lysis buffer A (table 2.19) on the next day (5 ml per 1×10^9 cells). After addition of lysis buffer B (5 ml per 1×10^9 cells) the suspension was vortexed, incubated on ice for 1 h and vortexed every 10 min. Suspension was sonificated for complete cell disruption and shearing of DNA for three 20 s cycles at 50 % power. Lysate was kept on ice and sonification was paused for 20 s between the cycles to remain a low sample temperature. Afterwards, lysis buffer B was added (5 ml per 1×10^9 cells) and lysate was cleared by centrifugation at 20 000 g for 1 h at 4 °C followed by filtration through a 0.22 µm syringe filter.

For precipitation of HLA class I molecules the lysate was loaded two times over the column. Column was centrifuged and washed with two column volumes PBS + 0.9 % CHAPS, 4 column volumes PBS and 4 column volumes H_2O. Plastic material used for all further steps was pretreated by washing two times with 10 % acetic acid (for removal of plastic components) and two times with 0.2 mmol/l glycine. A *Hamilton* syringe was used for pipeting in all steps. For elution of HLA molecules from the antibody and disruption of the peptides, 500 µl 10 % acetic acid was added to the resin and column was shaked for 10 min at room temperature. Fraction was collected by centrifugation for at 800 g for 1 min. Elution was repeated five times, fractions were pooled and incubated for 10 min at 70 °C for complete disruption of HLA peptide complexes. Elution pool was loaded onto a pretreated 10 kDa *Amicon Ultra-15* (Millipore) for separating peptides from larger molecules. The filter was centrifuged at maximum speed and 4 °C until all liquid had passed through. The peptide solution was desalted and concentrated on *C_{18} SpinTips* (Protea) according to the manufacturer's instructions. Elution reagent was evaporated on a *Vacufuge* (Eppendorf) and samples were stored at −80 °C until mass spectrometry analysis.

Peptide sequencing by mass spectrometry was done in the Core facility proteomics of the Helmholtz Zentrum München, Neuherberg, Germany. In brief, peptides were analyzed by nano-HPLC (Ultimate 3000, Dionex) coupled to a linear quadruple ion trap-Orbitrap (LTQ Orbitrap XL) mass spectrometer (Thermo Fisher) equipped with a nano-ESI source, allowing for fragemntation of peptides with charges +1 to +3.

MS/MS data were analyzed using three different data analysis tools, namely MASCOT (Ma-

trix Science), Peaks (Bioinformatics Solutions Inc.) (Zhang et al., 2012) and Pep-Miner (IBM Haifa Research Lab) (Beer et al., 2004). Database searches were performed using the human part of the NCBI and UniProt database and the Human Short Peptide Variation Database (`http://srs.bioinformatics.nl/hspv/search.php`). Error tolerance was set to 10ppm for parent ions and 0.5 Da for fragment ions. Peptides with a length of 8-11 AS, a MASCOT score $\geq$ 30, a Peaks -logP10 $\geq$ 15 or a Pep-Miner score $\geq$ 70 were analyzed for described anchor residues (`www.syfpeithi.de`) for the respective HLA type of the patient samples. To screen for genes with suitable expression patterns for T cell targeting, the expression of peptide coding genes was determined by database and literature research (`http://genome.ucsc.edu/cgi-bin/hgBlat`, `www.biogps.org`, `www.ncbi.nlm.nih.gov/pubmed`). For sequence validation of selected candidate ligands, synthetic counterparts (IBA) were analyzed by mass spectrometry. After normalization, spectra were matched using mMass (Niedermeyer and Strohalm, 2012).

2.2.8.2 Western Blot

For expression analysis on the protein level, cell pellets were resuspended in an appropriate amount of RIPA buffer (table 2.19), incubated on ice for 40 min and vortexed every 10 min. Protein concentration was determined with the *BCA Protein Assay Reagent* (Pierce) according to the manufacturer's instructions. 50 µg were diluted with an appropriated amount *NuPAGE LDS sample buffer (4x)* (Invitrogen) and DTT was added to a final concentration of 0.1 mol/l. After denaturation for 5 min at 95 °C, samples were loaded onto a *NuPAGE Novex 4-12 % Bis-Tris Gel* (Invitrogen) and electrophoresis was done at a constant voltage of 200 V for 1 h. Proteins were blotted on a nitrocellulose membrane using a *XCell IT Blot Module* for 1 h at a constant voltage of 30 V according to the manufacturer's instructions. Success of blotting was checked by Ponceau staining and membrane was blocked for 1 h in blocking solution (table 2.19) at room temperature afterwards. The membrane was incubated at 4 °C over night with a dilution of the primary antibody (table 2.7). After washing of the membrane by shaking in washing buffer (table 2.19) for 5 min three times, a dilution of the secondary antibody was added for 1 h. Membrane was washed three times as described above and 1 ml of a substrate (*Western Lightning Chemiluminescence Reagent* Perkin Elmer) was added for 1 min. Chemiluminescence was visualized on chemiluminescence films by exposure to the membrane for 10 s, 1 min and 5 min.

2.2.9 BRG mouse model

For *in vivo* experiments with TCR transgenic T cells in a xenogenic mouse model of human AML immunocompromised BRG mice (C.Cg-Rag2^{tm1Fwa} Il2rg^{tm1Sug}/JicTac) (Taconic) were used. Mice were maintained at the *Zentrum für präklinische Forschung, TU München* according to the institutional guidelines and approval by local authorities. Mice were sub-

lethally irradiated with 3.5 Gy on day 0 and 5×10^6 or 1×10^6 NB4 cells transduced with HLA-*B*07:02*-P2A-eGFP (NB4-B7 cells) cells were injected intravenously (i.v.) on the next day. One or three days later, mice received either 200 µl PBS, 1×10^6 untransduced or 1×10^6 TCR transgenic cells (sections 2.2.4.3 and 2.2.4.2). To provide a constant systemic supply of human IL-15, 1.5×10^7 NSO-IL15 cells were injected intraperitoneally twice per week in 200 µl PBS. Cells were irradiated with 80 Gy prior injection to omit *in vivo* cell growth. Health status of mice was monitored daily by an independent, experienced research assistant in the animal facility. Mice that suffered from health problems indicated by weight loss (>10 %), tumor manifestation (>1 cm), rough hair coat, changes in breathing rate or reduced mobility were sacrificed. Tumor load and T cell engraftment was analyzed by flow cytometry (section 2.2.6) after organ collection and preparation of single cell suspensions. Erythrocytes were lyzed by resuspension of cell pellets in 1 ml ACK lysis buffer (table 2.19) and incubation at room temperature for 2 min. After washing in FACS buffer cells were stained with the respective antibodies and analyzed on a flow cytometer (section 2.2.6).

Chapter 3

Results

3.1 Immunopeptidomic identification of naturally presented HLA ligands on the surface of malignant hematopoetic cells

To define suitable epitopes for T cell mediated immunotherapies for the treatment of hematopoetic malignancies, the immunopeptidome of seven patients with MPN was analyzed together with Sebastian Schober during his medical doctor (MD) thesis. We identified, by mass spectrometry, 4386 unique peptides with adequate peptide length and proper anchor residues to serve as potential ligands of diverse HLA class I molecules. These peptides were evaluated as potential candidates for T cell mediated immunotherapies by literature and expression database research. We did not find peptides with mutated sequences or peptides derived from cancer testis antigens. However, 17 candidate peptides derived from seven different genes with restricted expression to the hematopoetic system could be identified (table 3.1). Expression on the mRNA level was confirmed by qPCR (Expression data MD-thesis Sebastian Schober, expression data MPO: figure 3.1, not done for linker for activation of T-cells family member 2 (LAT2) and histocompatibility (minor) HA-1 (HMHA1)). Sequence identity of selected peptides (except for LAT2 and HMHA1 derived peptides) was confirmed by matching the spectra of the eluted peptides to the spectra of the synthetic counterparts. As shown in figure 3.2 and figure A.1, all spectra showed - despite some background peaks - overlapping fragmentation patterns.

Table 3.1: Selected HLA-ligands

Name	Sequence	Gene	HLA-Restriction	Phenotype frequency[1]
$ELANE_1$	VQRIFENGY	ELANE	*B*15:01*	15.3%
$HMHA1_1$	RQLPEPLISF	HMHA1	*B*15:01*	15.3%
$HMHA1_2$	RPRPTEATVSL	HMHA1	*B*07:02*	24.5%

Table 3.1: Selected HLA-ligands *(continued)*

Name	Sequence	Gene	HLA-Restriction	Phenotype frequency[1]
$LAT2_1$	SLREDQQSF	LAT2	*A*03:01*	28.6%
$LAT2_2$	YIDPIAMEY	LAT2	*A*01:01*	27.7%
$ITGA2B_1$	PVADIFSSY	ITGA2B	*A*03:01*	28.6%
$ITGA2B_2$	AQAPVADIF	ITGA2B	*B*15:01*	15.3%
MPO_1	EEAKQLVDKAY	MPO	*B*44:02*	23.9%
MPO_2	TPAQLNVL	MPO	*B*07:02*	24.5%
MPO_3	NQINALTSF	MPO	*B*15:01*	15.3%
MPO_4	FVDASMVY	MPO	*A*01:01*	27.7%
MPO_5	NPRWDGERL	MPO	*B*07:02*	24.5%
$MS4A3_1$	SPDYQKAKL	MS4A3	*B*07:02*	24.5%
$MS4A3_2$	GVFLGSLQY	MS4A3	*A*03:01*	28.6%
MYB_1	SQPAVATSF	MYB	*B*15:01*	15.3%
MYB_2	AQAPPTAQL	MYB	*B*07:02*	24.5%
MYB_3	VPQPAAAAI	MYB	*B*07:02*	24.5%
MYB_4	TPAIKRSIL	MYB	*B*07:02*	24.5%
MYB_5	DESGIVAEF	MYB	*B*18:01*	9.0%

3.1.1 Detailed characterization of MPO as a target for T cell mediated immunotherapies for the treatment of myeloid leukemias

MPO was selected as a particularly interesting target antigen due to its restricted expression and function in myeloid cells (Su et al., 2004; Klebanoff et al., 2013). We determined expression of MPO on the mRNA level (figure 3.1 **(A)**) by qPCR and observed highly increased expression in comparison to PBMC in most samples of patients with MPN and AML as well as in fetal liver and bone marrow. No increased expression could be observed in differentiated myeloid cells as $CD14^+$ monocytes and granulocytes. Interestingly, $CD14^+$ monocytes and granulocytes show expression of MPO on the protein level (figure 3.9). MPO immunohistochemistry was done in cooperation with Dr. Slotta-Huspenina from the pathology department. As shown in figure 3.1 **(B)**, MPO was only detected in the bone marrow and in single myeloid cells in all other organs. As shown in table 3.1, five HLA ligands derived from MPO could be identified that are presented on four different HLA-molecules. Spectra of eluted MPO peptides and the synthetic counterparts are shown in figure 3.2 and show, despite some background peaks overlapping patterns.

[1] http://www.allelefrequencies.net/,(Brown et al., 2000)

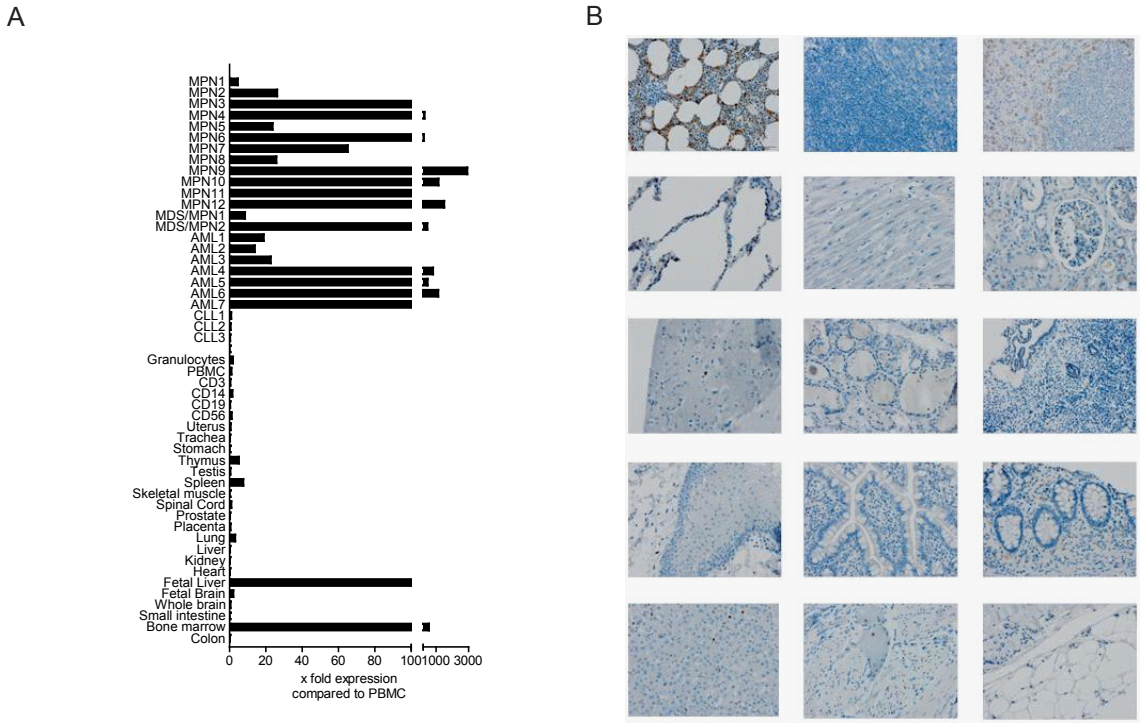

Figure 3.1: Expression of MPO on the mRNA and protein level.
The expression of MPO on the mRNA level was determined by qPCR in a panel of non-transformed and malignant human tissues **(A)**. Ct values were normalized against the geometric mean of three housekeeping genes (GAPDH, HMBS and HPRT1) using the ddCT method. Relative expression is shown in comparison to PBMC. MPO immunohistochemistry in diverse healthy tissue samples is shown in **(B)** (from left to right and top to bottom: bone marrow, lymph node, spleen, lung, heart, kidney, brain, thyroid gland, endometrium, esophagus, intestinal mucosa, colon mucosa, liver, vessels, soft tissue)

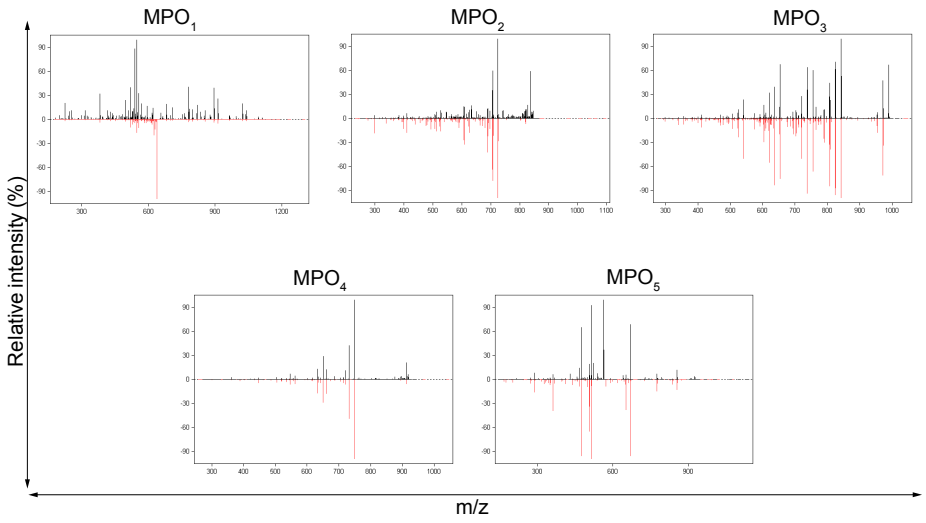

Figure 3.2: Sequence validation of identified MPO-derived HLA ligands.
MPO HLA ligands identified by the immunopeptidomic approach were synthesized, sequenced by mass spectrometry and matched to the spectra of eluted peptides after normalization with the mMass tool for sequence validation. Spectra of synthetic peptides are shown in red (lower) and spectra of eluted peptides in black (upper).

3.2 Establishment of a system to stimulate and characterize peptide specific T cells

As we were able to identify a number of candidate peptides presented on different HLA class I molecules a system for the stimulation and functional characterization of peptide specific T cells was established together with Martina Rami during her master thesis. For priming of naive T cells we decided to use HLA-ivt-RNA electroporated, single HLA-mismatched DC. Therefore, ivt-RNA coding for the respective HLA molecules (table 3.1) and eGFP, separated by a P2A element were transferred into DC by electroporation. As an example DC of an HLA-*B*07:02* negative blood donor are shown after transfer of HLA-*B*07:02*-ivt-RNA in figure 3.3 **(A)**. In total, 58.8 % express eGFP and 42.3 % show also expression of HLA-*B7*.
To generate cell lines for restimulation and functional characterization of T cells, different cell lines were transduced with retroviral supernatants, coding for the respective HLA molecules (table 3.1) and eGFP. As an example, transduction of the HLA class I negative cell line K562 with HLA-*B*07:02* is shown in figure 3.3 **(B)**. All cells that express eGFP show surface expression of HLA class I. For details refer to Martina Rami's master thesis.

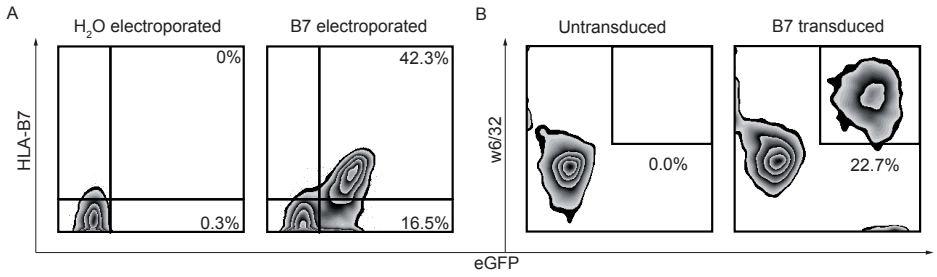

Figure 3.3: Transfer of HLA-*B*07:02*-P2A-eGFP into DC and K562 cells.
(A) Mature DC of an HLA-*B*07:02* negative donor were electroporated with HLA-*B*07:02*-P2A-eGFP ivt-RNA. 16 hours later expression of eGFP and HLA-*B*07:02* was analyzed by flow cytometry with an HLA-*B7* specific antibody. As a control, DC electroporated with H_2O were analyzed in the same way. **(B)** K562 cells were transduced with a retroviral supernatant coding for HLA-*B*07:02*. Seven days later cells were stained with the HLA class I specific antibody w6/32 followed by staining with an anti-mouse IgG-PE antibody. Percentage of eGFP and HLA class I expressing cells was analyzed by flow cytometry. As a control, untransduced K562 cells were analyzed in the same way.

3.3 Expansion of T cells specific for HLA-*B*07:02* restricted candidate peptides

After having established the electroporation of DC with HLA-coding ivt-RNA we decided to start the expansion of T cells with specificity against peptides restricted to HLA-*B*07:02*. With a phenotype frequency of 23.9%, HLA-*B*07:02* represents the third most abundant HLA-*B* phenotype in the european caucasian population (Brown et al., 2000). We stimulated $CD8^+$

naive T cells of a HLA-*B*07:02* negative donor with peptide pulsed autologous DC in the single HLA-mismatched system for expansion of peptide specific T cells. As shown in figure 3.4, stimulation of T cells with MYB_3, MPO_2 and MPO_5 lead to well-defined populations of $CD8^+$, HLA-multimer positive cells compared to the background produced by the respective HLA-multimer when T cells stimulated with an irrelevant peptide were stained. No defined populations could be observed when T cells were stimulated with MYB_2, MYB_4 and $MS4A3_1$.

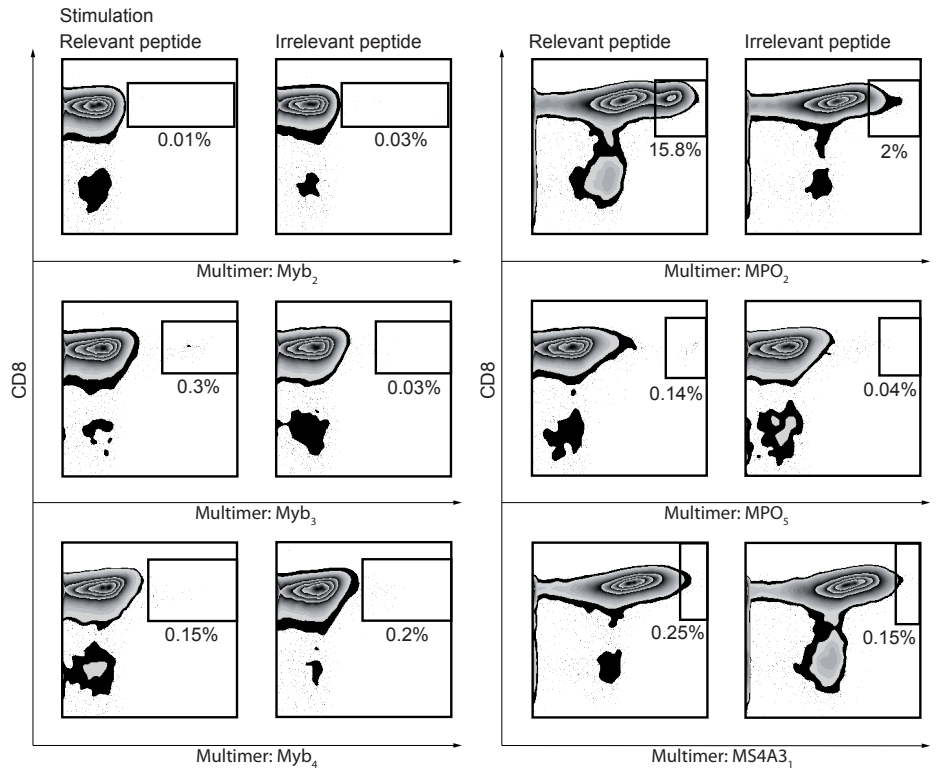

Figure 3.4: Expansion of T cells specific for HLA-*B*07:02* restricted peptide ligands. $CD8^+$ naive T cells of a HLA-*B*07:02* negative donor were stimulated with semi-allogenic DC pulsed with 0.1 µmol/l of the respective peptide. Cells were restimulated with peptide pulsed C1R-B7 cells on day 10. On day 20, expansion of peptide specific T cells was analyzed by staining of each condition with the respective HLA-multimer and an CD8 specific antibody followed by analysis on a flow cytometer. As a control for unspecificity of the respective HLA-multimer, a condition that was stimulated with an irrelevant peptide was analyzed in parallel.

3.3.1 Isolation of a MPO_5 specific TCR

HLA-multimer positive cells were sorted on a MoFlow high performance cell sorter and further cloned by limiting dilution. After 10 - 14 days, clones were analyzed for their reactivity against T2 cells transduced with HLA-*B*07:02*-P2A-eGFP (T2-B7 cells) pulsed with either an irrelevant peptide or the relevant peptide. As manufacturing of the MPO_2-multimer was not finished at the time of sorting, data is only shown for MPO_5 and MYB_3. Despite a

theoretical number of 2400 clones that were seeded in 96-well plates, only 7 clones showed proliferation and could be tested for cytotoxic activity. As shown in figure 3.5 all five clones that were sorted with the MPO_5 HLA-multimer lyzed T2-B7 cells specifically, when pulsed with the MPO_5 peptide whereas non of the two clones that were sorted with the MYB_3 HLA-multimer showed reactivity.

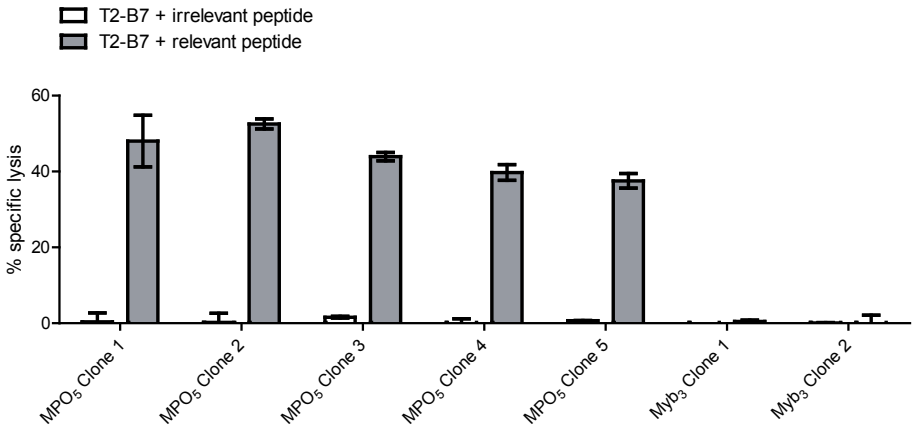

Figure 3.5: Cytotoxic activity of MPO_5 and MYB_3 HLA-multimer sorted T cell clones. MPO_5 and MYB_2 HLA-multimer positive cells were sorted on a MowFlow high performance cell sorter and cloned by limiting dilution. Resulting clones were analyzed for their peptide specificity in a chromium release assay. T2-B7 cells were used as targets, either pulsed with an irrelevant or the relevant peptide.

For identification of the TCRs recognizing the MPO_5 peptide, α- and β-chains were analyzed by PCR. Figure 3.6 shows the TCR repertoire of a MPO_5 specific T cell clone. The products that were amplified additionally to the internal controls (α1 (400base pairs (bp)), α1.1 (400bp), β13.1 (250bp) and β13.2 (250bp and 600bp) were excised from the gel, DNA was purified and sent for sequencing. Sequence analysis revealed a functional TCR-α-chain with the same sequence for α1 and α1.1, no functional β-chain for β13.1 and a functional TCR-β-chain for both β13.2 products (data is not shown for the other MPO_5 specific T cell clones because all clones showed the same α and β chain). The TCR and T cell clone is further called 2.5D6.

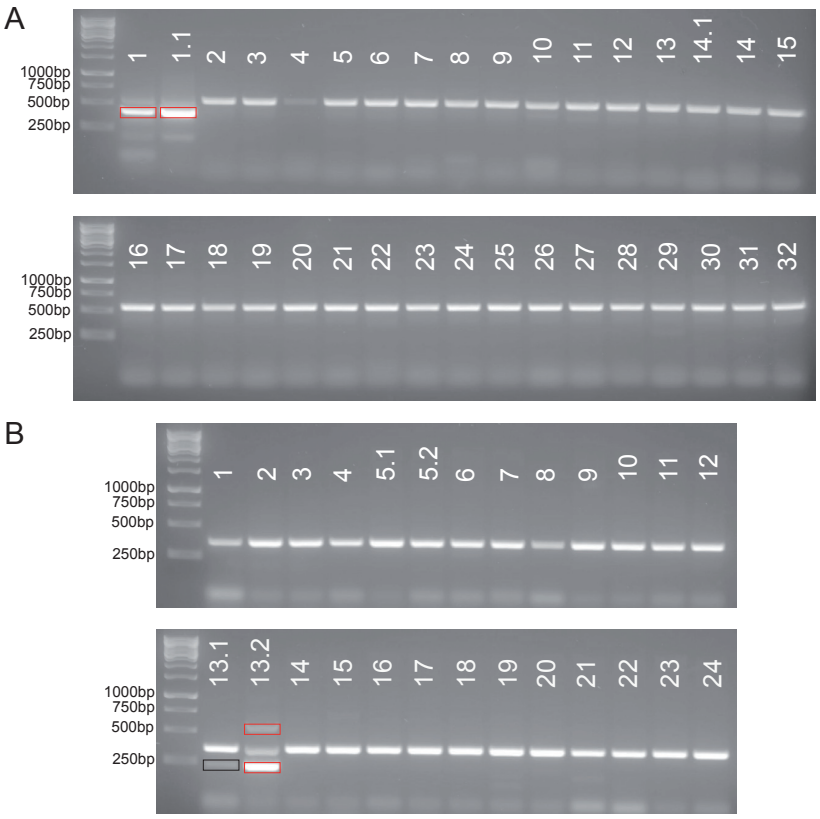

Figure 3.6: TCR repertoire of the MPO_5 specific TCR 2.5D6.
RNA of a MPO_5 specific T cell clone was isolated and cDNA was synthesized. Variable α **(A)** and β **(B)** chains were identified by PCR using a set of specific primers. As an internal control, a part of the TCR constant region was amplified with expected products at 550bp **(A)** and 350bp **(B)**, respectively.

After cloning of the complete α- and β-chain into the retroviral vector pMP71 the full sequence of the TCR could be identified (D.1). The sequences of the complementarity determining region 3 (CDR3)-regions and the VDJ segments were identified by database searches (IMGT) (table 3.2).

Table 3.2: α- and β-chains of the TCR 2.5D6

	IMGT [1]					
Chain	V	D	J	Arden [2]	Gel	CDR3-region
α	TRAV8-3*02		TRAJ17*01	8S3	1/1.1	AGRAAGNKLT
β	TRBV6-2*01	TRBD2*01	TRBJ1-5*01	13S2a	13.2	ASSYSSGQPQH

[1] (Folch and Lefranc, 2000)
[2] (Arden et al., 1995)

3.3.1.1 Transfer of the TCR 2.5D6 into activated human PBMC

A prerequisite for T cell mediated immunotherapy with TCR-transgenic T cells is the transfer of a defined TCR and its reactivity into T cells. For detailed characterization of the TCR 2.5D6, murinized and codon optimized α- and β-chains were retrovirally transferred into activated PBMC (section 2.2.3.1). The success of transduction was analyzed by flow cytometry and is shown in figure 3.7 **(A)**. Expression of the TCR could be observed in $CD8^+$ and $CD4^+$ T cells, although with reduced mean fluorescence intensity (MFI) in the $CD4^+$ population, indicating a limited CD8 dependence of the TCR. The percentage of cells that bind the TCRm antibody, which is directed against the murinized region of the TCR is somewhat higher than the percentage of cells that bind both, the antibody and the MPO_5-multimer (40.1 % vs. 20.1 %). The percentage of cells that only bind the TCRm antibody is higher in $CD4^+$ cells compared to $CD8^+$ cells (34.8 % vs. 11.4 %). Cytotoxic activity of the TCR 2.5D6 transduced PBMC in comparison to the T cell clone 2.5D6 is shown in figure 3.7 **(B)**, untransduced PBMC were used as a negative control. The flow cytometry based assay shows a reduction of eGFP expressing T2-B7 cells in the lifegate, when pulsed with the MPO_5 peptide compared to T2-B7 cells pulsed with an irrelevant peptide. This reduction could only be observed, when target cells were incubated with the T cell clone (0.4 % vs. 57 % eGFP expressing cells) or TCR transgenic PBMC (0.3 % vs. 39.8 % eGFP expressing cells), but not when incubated with untransduced PBMC (46.4 % vs. 34.2 % eGFP expressing cells). Production of IFN-γ could only be observed when the T cell clone 2.5D6 or TCR 2.5D6 transduced PBMC were incubated with T2-B7 cells, pulsed with the MPO_5 peptide.

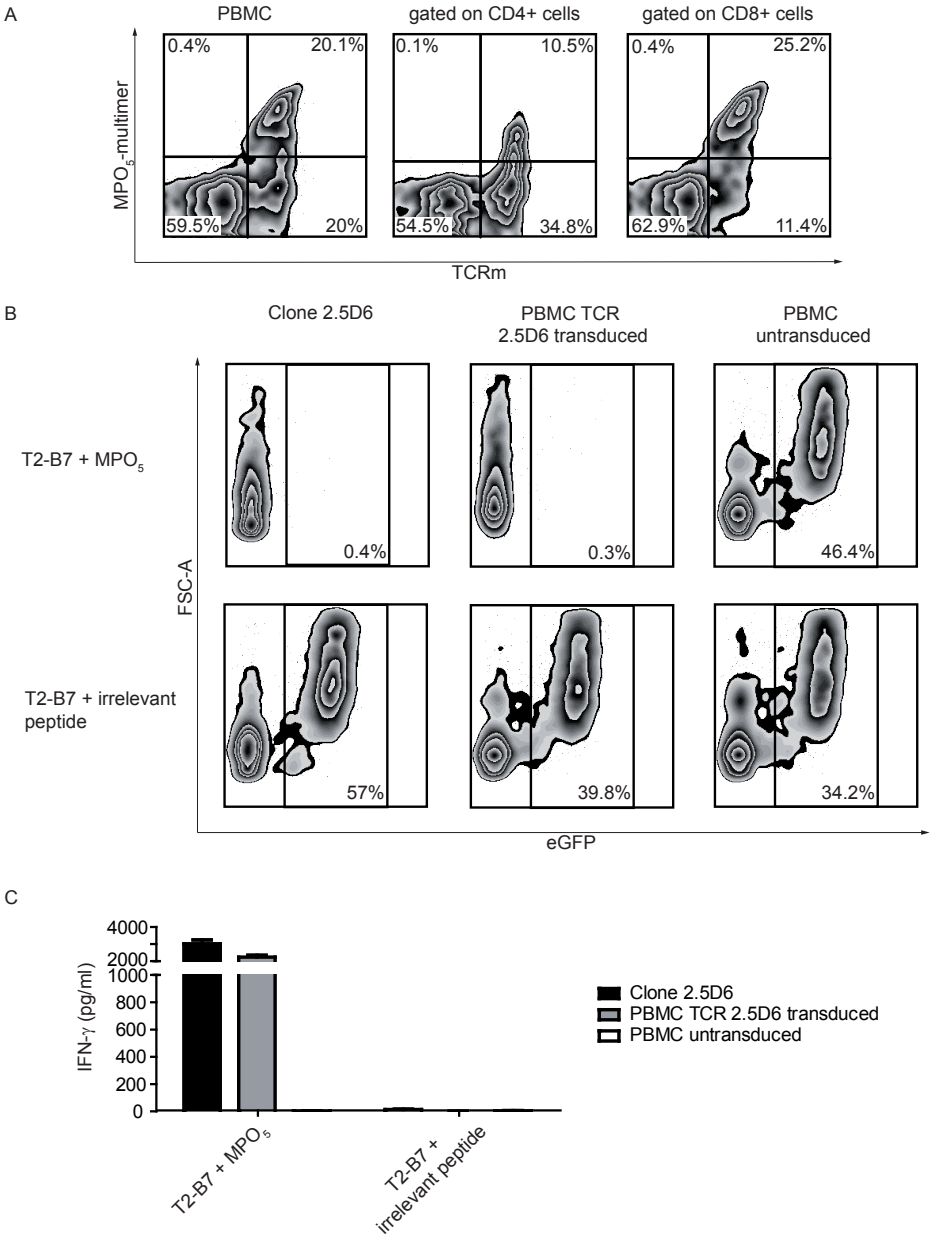

Figure 3.7: Transduction of the TCR 2.5D6 into activated PBMC.
The codon optimized and murinized TCR 2.5D6 was transduced into activated human PBMC. **(A)** The expression of the TCR in $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry using the MPO_5-multimer and the TCRm antibody. Functionality of transduced cells was analyzed after coincubation with T2-B7 cells either pulsed with an irrelevant peptide or the MPO_5 peptide. Cytotoxic activity was measured 20 hours later by flow cytometry (according to eGFP positive cells in the lifegate) **(B)** and production of IFN-γ was measured in the supernatant by ELISA **(C)**. The clone 2.5D6 was used as a positive control, untransduced PBMC as a negative control.

3.4 Functional characterization of the TCR 2.5D6

The following section deals with the functional characterization of the TCR 2.5D6 transduced PBMC described in 3.3.1.1. Untransduced PBMC were always used as a control.

3.4.1 Functional avidity and antigen specificity

As a first step the functional avidity, a biological measure that describes how well a T cell responds *in vitro* to a defined concentration of a ligand, of the TCR was analyzed. As shown in figure 3.8 **(A)**, we could observe a half maximal production of IFN-γ at a concentration of 150 pmol/l of the MPO_5 peptide when TCR 2.5D6 transduced PBMC were used. This was about tenfold higher than the functional avidity observed with the T cell clone (1 nmol/l, figure 3.8 **(B)**). No reactivity could be observed when T2-B7 cells were pulsed with 20 µmol/l of an irrelevant peptide or when untransduced PBMC were used as effector cells (data not shown). We next analyzed the MPO specific reactivity of TCR 2.5D6 transduced PBMC. Therefore, HLA-*B*07:02* transduced cell lines of B cell origin that are not recognized by TCR 2.5D6 transduced PBMC were transduced with a construct coding for MPO-P2A-dsRed-Express2 (dsRed). As shown in figure 3.8 **(B)**, IFN-γ production could only be observed by TCR transduced PBMC, when incubated with C1R cells transduced with HLA-*B*07:02*-P2A-eGFP and MPO-P2A-dsRed (C1R-B7/MPO cells) as well as BJAB cells transduced with HLA-*B*07:02*-P2A-eGFP and MPO-P2A-dsRed (BJAB-B7/MPO cells). Additional pulsing of C1R-B7/MPO cells and BJAB-B7/MPO cells with the MPO_5 peptide lead to an increase in IFN-γ production and served as a positive control. Untransduced PBMC showed no reactivity at all.

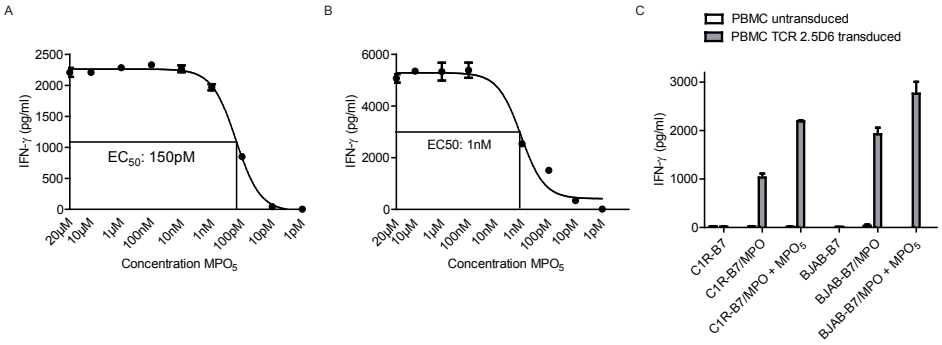

Figure 3.8: Functional avidity and antigen specificity of the TCR 2.5D6.
For determination of the functional avidity of the TCR 2.5D6 either TCR 2.5D6 transduced PBMC **(A)** or the T cell clone 2.5D6 **(B)** were coincubated for 20 hours with T2-B7 cells that were pulsed with graded amounts of MPO_5 peptide. The antigen specificity of TCR transduced PBMC was analyzed by transduction of the MPO negative C1R-B7 cells and BJAB-B7 cells with a construct coding for MPO and dsRed separated by a P2A element. As a positive control C1R-B7/MPO cells and BJAB-B7/MPO cells were pulsed with 1 µmol/l of the MPO_5 peptide, reactivity of untransduced PBMC was analyzed as a negative control. Supernatants were collected and IFN-γ-production was determined by ELISA.

3.4.2 Reactivity against HLA-*B*07:02* positive primary PBMC subsets and granulocytes

We next investigated potential reactivity of TCR 2.5D6 transduced PBMC against different PBMC subsets and granulocytes from a healthy, HLA-*B*07:02* positive blood donor. As shown in figure 3.9 **(A)**, no reactivity against negatively isolated PBMC subsets, namely $CD3^+$ T cells, $CD56^+$ NK-cells, $CD19^+$ B cells and $CD14^+$ monocytes could be observed. Furthermore, TCR2.5D6 transgenic PBMC showed no reactivity against granulocytes isolated by differential centrifugation. Pulsing of the target cells with the MPO_5 peptide lead to IFN-γ production by TCR transgenic PBMC, whereas no reactivity of untransduced PBMC could be observed at all. Expression analysis of MPO on the protein level revealed expression only in $CD14^+$ monocytes and granulocytes (figure 3.9 **(B)**). This is in strong contrast to the qPCR data, where neither $CD14^+$ monocytes nor granulocytes show increased expression compared to the other PBMC subsets (figure 3.1).

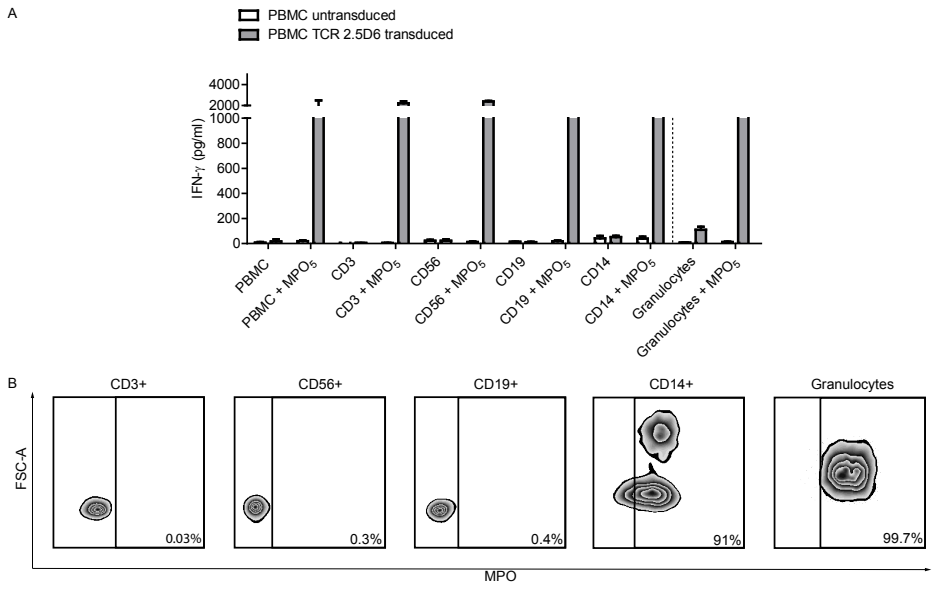

Figure 3.9: Reactivity of the TCR 2.5D6 against different HLA-*B*07:02* positive hematopoetic cells.
(A) PBMC of a HLA-*B*07:02* positive healthy donor were isolated by ficoll gradient centrifugation and CD3 positive T cells, CD56 positive NK cells, CD19 positive B cells and CD14 positive monocytes were negatively isolated. Granulocytes were purified from whole blood using differential centrifugation. After 20 hours coincubation with either untransduced or TCR 2.5D6 transduced PBMC supernatants were collected and IFN-γ was measured by ELISA. As a positive control all target cells were pulsed with 1 µmol/l of the MPO_5 peptide. **(B)** Expression analysis of MPO on the protein level in the different hematopoetic cells was analyzed by FACS. Therefore, purified PBMC subsets were gated according to the expression of the respective CD molecule and granulocytes were identified according to size and granularity in the forward and side scatter. MPO-expression was visualized by intracellular staining with an MPO specific antibody.

3.4.3 Cytotoxic activity against healthy colony forming hematopoetic stem cells

The reactivity of TCR 2.5D6 transduced PBMC against healthy, $CD34^+$ hematopoetic stem cells of a healthy HLA-*B*07:02* positive blood donor was additionally investigated in CFU assays. As shown in figure 3.10 **(A)**, TCR 2.5D6 transduced PBMC showed no increased cytotoxicity against G-CSF mobilized hematopoetic stem cells in bulk PBMC compared to untransduced PBMC. Data is shown for a representative CFU assay where effector cells were incubated with target cells for 1 hour before plating in methylcellulose. Similar results were obtained when cells were incubated for 15 minutes or 24 hours, respectively (data not shown). In contrast, when target cells were pulsed with 1 µmol/l of the MPO_5 peptide, a strong reduction in CFU could be observed when cells were incubated with TCR 2.5D6 transduced PBMC (3.10 **(B)**). This effect was not observed, when target cells were incubated with untransduced PBMC.

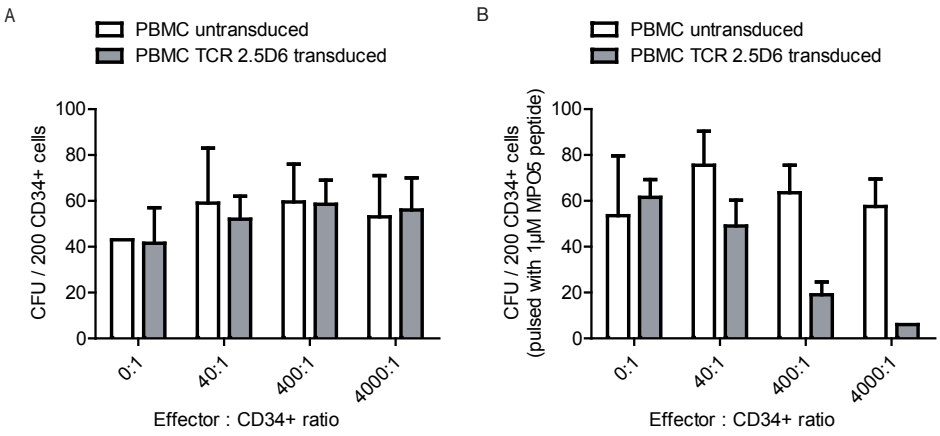

Figure 3.10: Hematopoetic stem cell cytotoxicity of the TCR 2.5D6.
Reactivity of TCR 2.5D6 transduced PBMC against HLA-*B*07:02* positive, $CD34^+$ hematopoetic stem cells was assessed in CFU assays. Five hundred $CD34^+$ cells within PBMC were incubated at different effector to target ratios with untransduced or TCR 2.5D6 transduced PBMC for 1 hour **(A)**. As positive control, target cells were pulsed with 1 µmol/l of the MPO_5 peptide **(B)**. Cells were plated in duplicates in methylcellulose medium. Colonies were counted 14 days later under a light microscope, the mean of duplicates is shown.

3.4.4 Peptide independent and MPO_5 specific crossreactivity against different HLA molecules

To analyze the reactivity of the TCR 2.5D6 against allo HLA molecules as well as the potential of the MPO_5 peptide to bind to other HLA molecules than HLA-*B*07:02*, different LCLs were analyzed for their recognition by TCR 2.5D6 transduced PBMC. HLA-types of the LCLs are listed in table 2.18. As shown in figure 3.11 **(A)**, despite some background IFN-γ production against LCL3, LCL7 and LCL10 no peptide independent allo HLA reac-

tivity could be observed. When the LCLs were pulsed with 1 µmol/l of the MPO_5 peptide, TCR 2.5D6 transduced PBMC produced IFN-γ in response to LCL6 and LCL9. LCL9 is HLA-*B*07:02* positive and served as a positive control, LCL6 is HLA-*B*07:02* negative and recognition was unexpected. *In silico* analysis of binding motifs for HLA molecules expressed by LCL6 revealed a potential binding of MPO_5 to HLA-*B*42:01*. To proof presentation of the MPO_5 peptide on HLA-*B*42:01*, C1R cells were transduced with a construct coding for HLA-*B*42:01* and a construct coding for MPO. As shown in figure 3.11 **(B)**, C1R cells were only recognized, when transduced with both, the restriction element and MPO. Pulsing with 1 µmol/l of the MPO_5 peptide served as a positive control and lead to an increase in IFN-γ production by TCR 2.5D6 transduced PBMC. Untransduced PBMC served as negative controls and, despite some background reactivity against LCL3, LCL7 and LCL10 no reactivity could be observed at all.

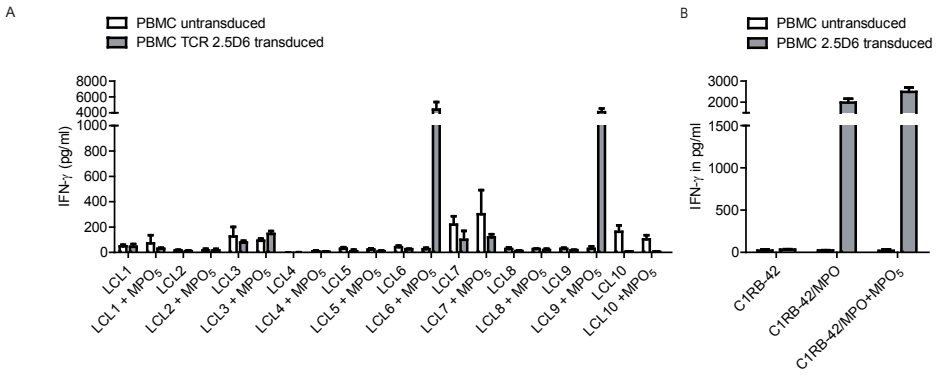

Figure 3.11: MPO_5 independent and dependent reactivity against different LCLs. **(A)** Different LCLs (HLA-type: 2.18) were coincubated with either untransduced or TCR 2.5D6 transduced PBMC. For analysis of peptide dependent crossreactivity LCL were pulsed with 1 µmol/l of the MPO_5 peptide. **(B)** Untransduced or TCR 2.5D6 transduced PBMC were stimulated with C1R-B42 cells. Target cells were rendered MPO positive by MPO gene transfer (C1R-B42/MPO cells) and additional peptide pulsing. Supernatants were collected after 20 hours and IFN-γ was measured by ELISA.

3.4.5 Reactivity against cell lines of non hematopoetic origin

The crossreactivity of TCR 2.5D6 transduced PBMC was further analyzed by coincubation with different cell lines of non hematopoetic origin. The cell lines SKOV-3 (ovarian carcinoma), 888-mel (melanoma), 143TK$^-$ (osteosarcoma), SW-480 (colorectal adenocarcinoma) and SKBR-3 (breast cancer) transduced with a construct coding for HLA-*B*07:02* as well as primary HLA-*B*07:02* positive HRGEC were investigated for their recognition by TCR 2.5D6 transduced PBMC. As shown in figure 3.12, non of the cell lines was recognized by TCR 2.5D6 transduced PBMC unless targets were pulsed with 1 µmol/l MPO_5 peptide. Analysis of MPO expression on the protein level by western blot revealed absent MPO protein in all cell lines except in HL-60 cells that served as a western blot positive control (figure 3.12).

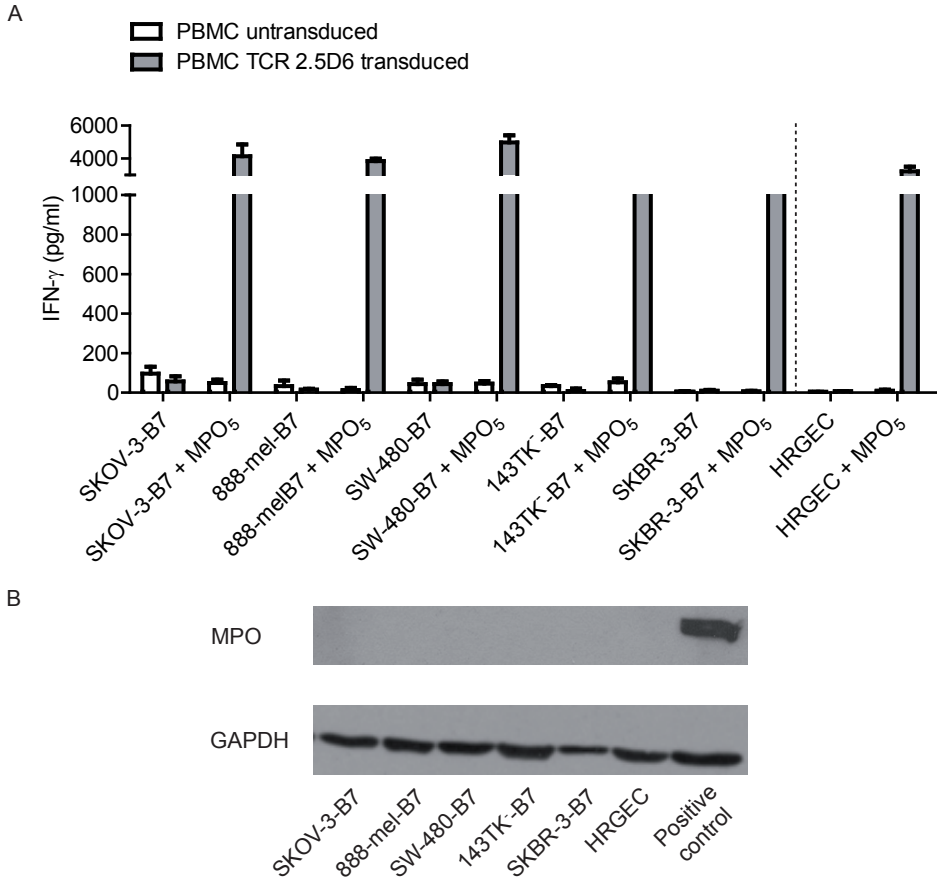

Figure 3.12: Analysis of crossreactivity against MPO negative cell lines of non hematopoetic origin.
(A) HLA-*B*07:02* transduced non myeloid cell lines SKOV-3, 888mel, SW480, 143TK$^-$, SKBR-3 (transduction rate 25-50% without sorting and cloning) as well as primary HRGEC (HLA-*B*07:02* positive) were coincubated for 20 hours with untransduced as well as TCR 2.5D6 transduced PBMC. Supernatants were harvested and IFN-γ was measured by ELISA. **(B)** Expression of MPO on the protein level was analyzed by western blot. As a positive control lysate of the MPO positive leukemia cell line HL-60 was loaded. As a loading control the expression of GAPDH was analyzed.

3.4.6 Alanine scan of the MPO_5 peptide

To assess the peptide specificity of the TCR 2.5D6, a set of synthetic peptides was pulsed on T2-B7 cells or LCL with endogenous HLA-*B*07:02* expression with alanine (A) substitutions at every amino acid position of the MPO_5 peptide. As shown in figure 3.13 **(A)** and **(B)**, pulsing of peptides with an exchange of proline (P) at position 2, arginine (R) at position 3, tryptophane (W) at position 4 and aspartic acid (D) at position 5 onto both target cells led to a strong decrease in IFN-γ production by TCR 2.5D6 transduced PBMC in comparison to the wild type peptide. Interestingly, pulsing of LCL with variants containing an exchange of arginine (R) at position 8 and leucine (L) at position 9 additionally resulted in a strong decrease in IFN-γ production (figure 3.13 **(B)**). This results in the recognition pattern

XPRWDXXRL, when LCL are used as target cells. *In silico* analysis with the ScanProsite tool (`www.http://prosite.expasy.org/scanprosite`) revealed MPO being the only human protein with this specific pattern.

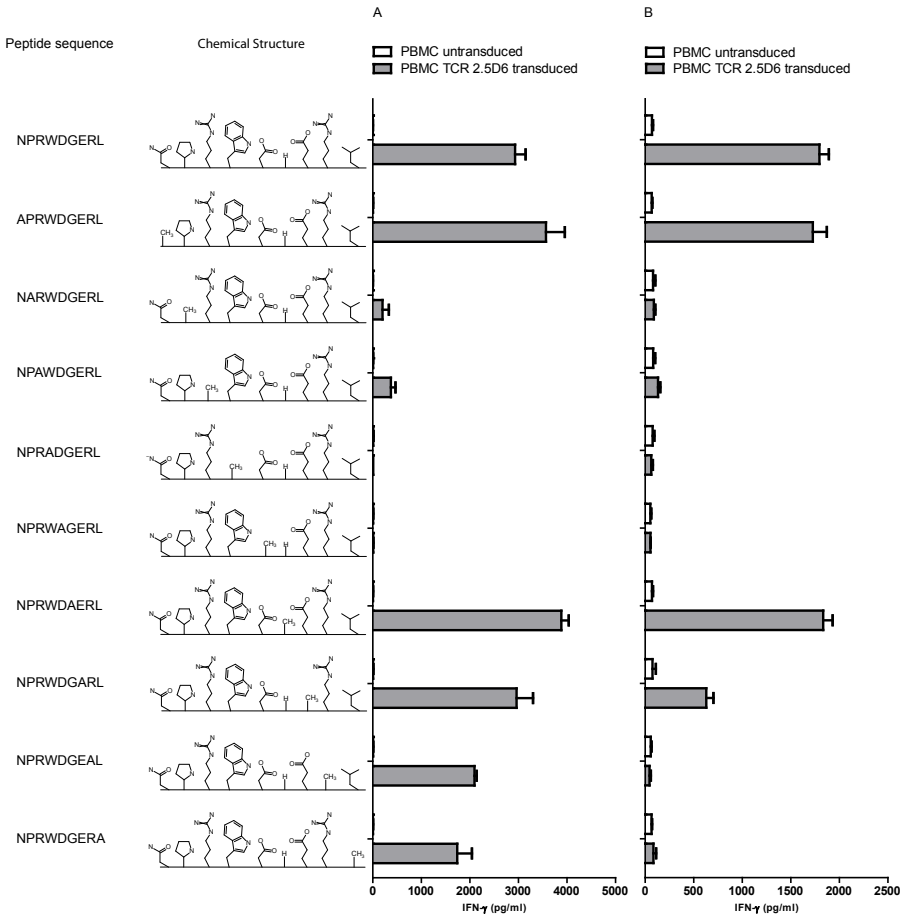

Figure 3.13: Recognition of MPO_5 peptides containing substitutions of alanine by TCR 2.5D6 transduced PBMC.
Peptides with single alanine substitutions at position 1-9 were pulsed on T2-B7 cells **(A)** or HLA-*B*07:02*$^+$ LCL at a concentration of 1 µmol/l. After 20 hours coincubation with either untransduced or TCR 2.5D6 transduced PBMC supernatants were collected and IFN-γ was measured by ELISA. As a positive control target cells pulsed with the MPO_5 peptide were used.

In case of T2-B7 cells, exchange of asparagine (N) at position 1 and glycine (G) at position 6 lead to a slight increase, exchange of glutamic acid (E) at position 7, arginine (R) at position 8 and leucine (L) at position 9 to a slight decrease in IFN-γ production (figure 3.13 **(A)**). When HLA-*B*07:02*$^+$ LCL were used as target cells, exchange of asparagine (N) at position 1 and glycine (G) at position 6 did not lead to a change in IFN-γ production, whereas exchange of glutamic acid (E) at position 7 lead to a moderate decrease. No reactivity of untransduced PBMC could be observed at all.

3.4.7 Recognition of MPO_5 homologue peptides

A very close model for the investigation of the safety of the TCR 2.5D6 is represented by a humanized mouse that is transgenic for HLA-*B*07:02* which will be available at Taconic soon. A prerequisite for using this model is the recognition of the mouse homologue peptide of MPO_5. Although peptides differ at positions 5 and 8, similar production of IFN-γ by TCR 2.5D6 transduced PBMC could be observed in response to T2-B7 cells pulsed with the mouse homologue peptide compared to cells pulsed with the MPO_5 peptide (3.14 **(A)**). However, when the mouse homologue peptide was pulsed onto HLA-*B*07:02*$^+$ LCL, no reactivity could be observed (figure 3.14 **(B)**). If the mouse homologue peptide is processed and presented on the surface of MPO-expressing cells that are derived from the HLA-*B*07:02* transgenic mouse is currently under investigation.

To predict potential off target reactivities in the clinical setting by binding of the TCR to homologue peptides of MPO_5, a blast search in the NCBI non-redundant protein sequence database was done. The top two homologous peptides, derived from lactoperoxidase (LPO) and eosinophil peroxidase (EPX) were synthesized and pulsed on T2-B7 cells. As shown in figure 3.14 **(A)**, IFN-γ production by TCR 2.5D6 transduced PBMC could be observed in response to both peptides, although reduced compared to the MPO_5 peptide. As observed with the mouse homologue peptide of MPO_5 no reactivity could be observed when the peptides were pulsed onto HLA-*B*07:02*[5] LCL (3.14 **(B)**).

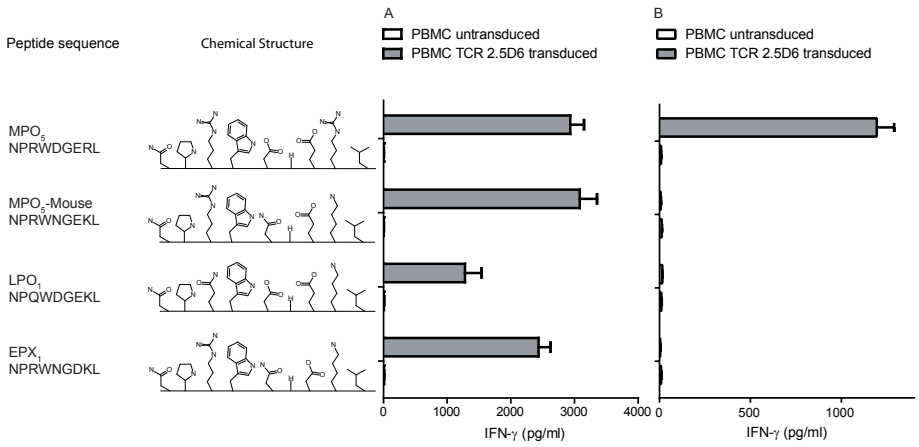

Figure 3.14: Crossreactivity of TCR 2.5D6 transduced PBMC against MPO_5 homologue peptides.
Homologue peptides of MPO_5 were searched in the NCBI non-redundant protein sequence database. Selected peptides and the murine MPO_5-counterpart were synthesized and loaded onto T2-B7 cells (**(A)**) or HLA-*B*07:02*$^+$ LCL (**(B)**) at a concentration of 1 µmol/l. Target cells were coincubated with either untransduced or TCR 2.5D6 transduced PBMC and IFN-γ was measured in the supernatant after 20 hours. As a positive control target cells pulsed with the MPO_5 peptide were used.

3.4.8 Reactivity against a panel of HLA-*B*07:02* ligands

To analyze crossreactivity against a panel of 58 HLA-*B*07:02* ligands (kind gift of S. Stevanovic), reactivity of TCR 2.5D6 transduced PBMC against T2-B7 cells pulsed with peptide pools was analyzed. As shown in figure 3.15, no reactivity against the peptide pools could be observed neither by untransduced nor TCR 2.5D6 transduced PBMC could be observed. However, IFN-γ was produced exclusively by TCR 2.5D6 transduced PBMC in response to T2-B7 cells pulsed with the MPO_5 peptide. A detailed list of the peptide pools is shown in table E.1.

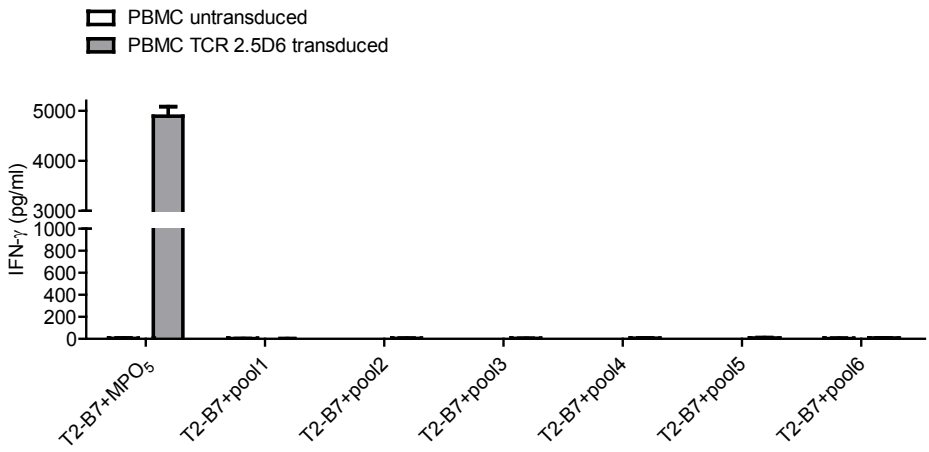

Figure 3.15: Reactivity of TCR transduced PBMC against naturally presented HLA-*B*07:02* ligands.
58 HLA-*B*07:02* ligands (kind gift of S. Stevanovic) were divided into eight peptide pools and were pulsed on T2-B7 cells at a concentration of 1 µmol/l for each peptide (table E.1). As a positive control, T2-B7 cells pulsed with 1 µmol/l of the MPO_5 peptide were used. PBMC either untransduced or transduced with the TCR 2.5D6 were coincubated with target cells for 20 hours. Supernatants were harvested and IFN-γ was measured by ELISA.

3.5 *In vitro* tumor reactivity of the TCR 2.5D6

After having analyzed the functionality and crossreactivity of the TCR 2.5D6 we sought to analyze the tumor reactivity *in vitro*.

3.5.1 Leukemia cell lines

We first started with analysis of the reactivity of TCR 2.5D6 transduced PBMC against different HLA-*B*07:02* transduced cell lines. HL-60 cells transduced with HLA-*B*07:02*-P2A-eGFP (HL-60-B7 cells) (APL), NB4-B7 cells (APL) and ML-2-B7 cells (AML French-American-British (FAB) M4) were recognized by TCR 2.5D6 transduced PBMC also when not pulsed with the MPO_5 peptide. In contrast, the cell lines Molt4-B7 (T-ALL), KG1a-B7

(AML), K562-B7 (CML) and MV4-11-B7 (AML FAB M5) were only recognized, when pulsed with the defined peptide (figure 3.16 **(A)**). Analysis of the MPO expression on the protein level by western blot revealed expression of MPO only in HL-60-B7, NB4-B7 and ML-2-B7 cells (figure 3.16 **(B)**). No reactivity of untransduced PBMC in response to the cell lines could be observed.

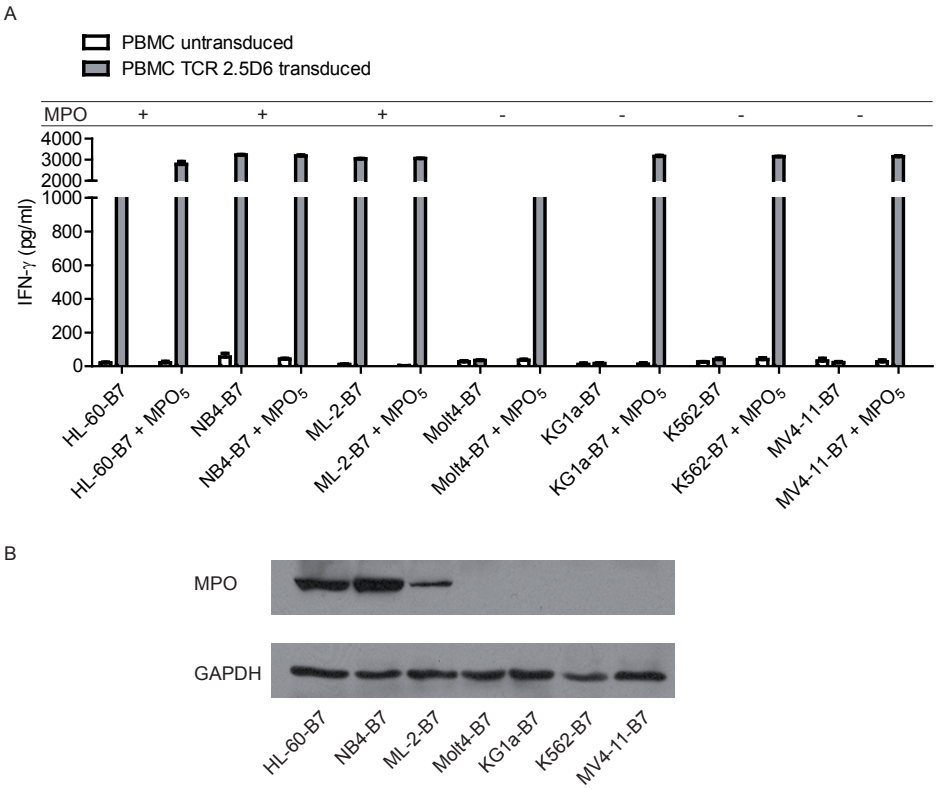

Figure 3.16: Reactivity of the TCR 2.5D6 against leukemic cell lines.
(A) Different myeloid and lymphoblastic leukemia cell lines were transduced with a construct coding for HLA-*B*07:02* and eGFP separated by a P2A element and coincubated with either untransduced or TCR 2.5D6 transduced PBMC for 20 hours. As a control, target cells were pulsed with 1 µmol/l of the MPO_5 peptide. IFN-γ was measured in the supernatant by ELISA. **(B)** Expression of MPO on the protein level in the leukemic cell lines was analyzed by western blot. The expression of GAPDH was analyzed as a loading control.

3.5.2 Primary leukemias

We next analyzed IFN-γ production of TCR 2.5D6 transduced PBMC in response to different primary samples of patients with MPN and AML (patient characteristics: table B) (figure 3.17 **(A)**, expression data **(B)**). Reactivity could only be observed, when 2.5D6 transduced PBMC were incubated with the HLA-*B*07:02* and MPO positive samples MPN1, MPN2, MPN5, MPN6 and AML6. The MPO positive but HLA-*B*07:02* negative sample MPN3 and the MPO negative, HLA-*B*07:04* positive sample AML2 were not recognized. As pulsing

of AML2 with the MPO_5 peptide resulted in recognition of the sample by TCR transgenic PBMC, MPO_5 potentially binds also to the HLA-*B7* subtype *B*07:04*.

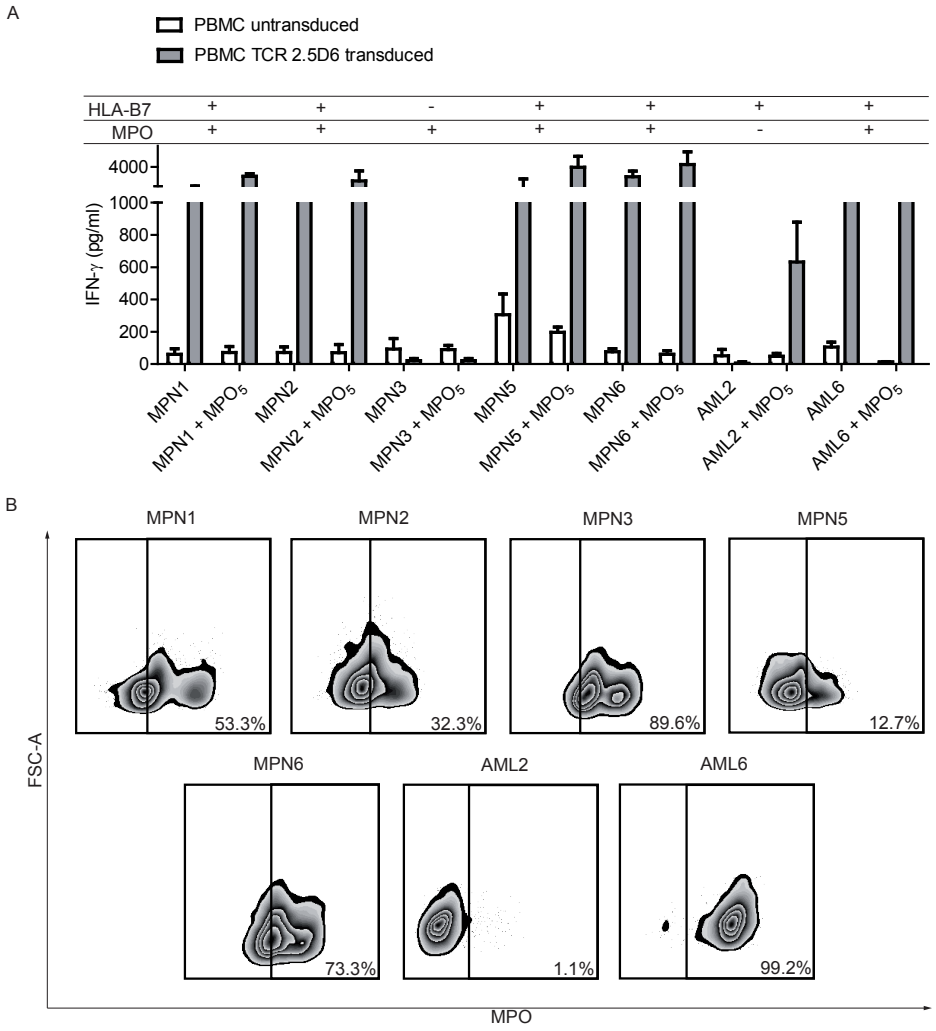

Figure 3.17: Reactivity of the TCR 2.5D6 against primary leukemia samples.
(A) Primary samples of patients with MPN were cultured for six days in the presence of 100 ng/ml SCF and FLT3LG. MPN and AML samples were pretreated with 500 IU/ml IFN-γ over night. MPN1, MPN2, MPN5, MPN6 and AML6 express HLA-*B*07:02*, MPN3 does not express HLA-*B*07:02* and AML2 does express HLA-*B*07:04* (B). As a control, cells were pulsed with the MPO_5 peptide at a concentration of 1 µmol/l. **(B)** Expression of MPO on the protein level was analyzed by intracellular staining with an MPO specific antibody followed by flow cytometry analysis.

3.5.3 Cytotoxic reactivity against leukemic progenitor cells

As eradication of leukemic progenitor cells is the essential aim of an effective leukemia therapy, we next tested the cytotoxic reactivity of TCR 2.5D6 transduced PBMC against leukemic precursor cells in CFU assays. As shown in figure 3.18 **(A)** we could observe cytotoxic activity of TCR 2.5D6 transduced PBMC against leukemic progenitor cells of the sample

MPN2 (HLA-*B*07:02* and MPO positive). This is represented by a reduction in CFU by 72 %, 22 % and 15 % at an effector to target ratio of 4000, 400 and 40 PBMC, respectively to one $CD34^+$ cell of the patient sample. Pulsing of the MPN2 derived target cells with the MPO_5 peptide resulted in a reduction in CFU by 90.3 %, 77.6 % and 50 % (figure 3.18 **(B)**). No impact of untransduced PBMC on the colony forming capacity could be observed in both cases. As a proof of specificity we also performed CFU assays with cells of the patient MPN3 (MPO positive, HLA-*B*07:02* negative). As shown in figure 3.18 **(C)**, no specific cytotoxic reactivity of TCR 2.5D6 transduced PBMC could be observed.

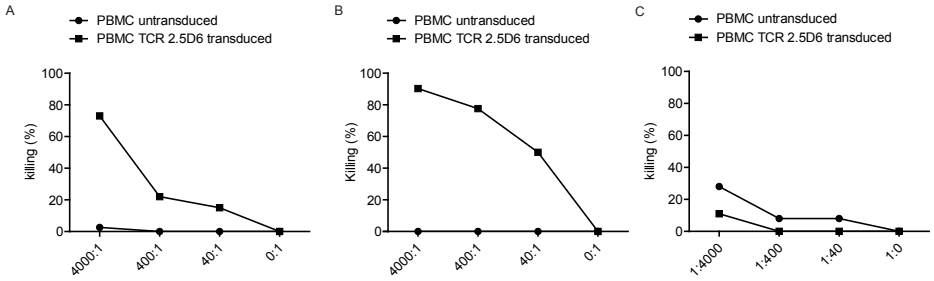

Figure 3.18: Leukemic progenitor cell cytotoxicity of TCR 2.5D6.
Reactivity of TCR 2.5D6 transduced PBMC against leukemic progenitor cells was assessed in CFU assays. Therefore, five hundred $CD34^+$ cells within PBMC were incubated at different effector to target ratios with untransduced or TCR 2.5D6 transduced PBMC for 1 hour and cells were plated thereafter in duplicates in methylcellulose medium. Colonies were counted 14 days later under a light microscope. **(A)** CFU assay with cells of the MPO and HLA-*B*07:02* positive patient MPN2. **(B)** As positive control, cells of the patient MPN2 were pulsed with 1 µmol/l of the MPO_5 peptide. **(C)** As a negative control cells of the MPO positive and HLA-*B*07:02* negative patient MPN3 were used. Untransduced PBMC served as negative control in all experiments. Percent killing was calculated as follows: $100 - \left(\frac{\text{Number of colonies (E:T = x:1)}}{\text{Number of colonies (E:T = 0:1)}} * 100 \right)$

3.6 *In vivo* tumor reactivity of the TCR 2.5D6

Having proven tumor reactivity of the TCR 2.5D6 *in vitro* we next sought to investigate tumor reactivity in a xenogenic mouse model with eGFP expressing NB4-B7 cells, as they are recognized by TCR 2.5D6 transduced PBMC *in vitro* (figure 3.16).

3.6.1 *In vivo* tumor reactivity experiment 1

Figure 3.19 **(A)** shows the typical phenotype of a mouse that had to be sacrificed when suffering from health problems as described in section 2.2.9. Massive infiltration of human $CD45^+$, $eGFP^+$ tumor cells could be observed in all mice by flow cytometry (data not shown). We could not observe a significant prolonged survival when mice were treated with TCR 2.5D6 transduced PBMC compared to treatment with untransduced PBMC or no treatment at all (figure 3.19 **(B)**, median survival: 31 vs. 28 vs. 27 days). However, as shown in figure

3.19 **(C)**, infiltration of human CD45$^+$, eGFP$^+$ cells into the bone marrow was significantly decreased in mice that received TCR 2.5D6 transduced PBMC ($0.0840\% \pm 0.1781$) compared to mice that received untransduced PBMC ($2.042\% \pm 1.654$) or mice that received no PBMC at all ($5.972\% \pm 5.337$). No significant difference in infiltration into the spleen and lung could be observed.

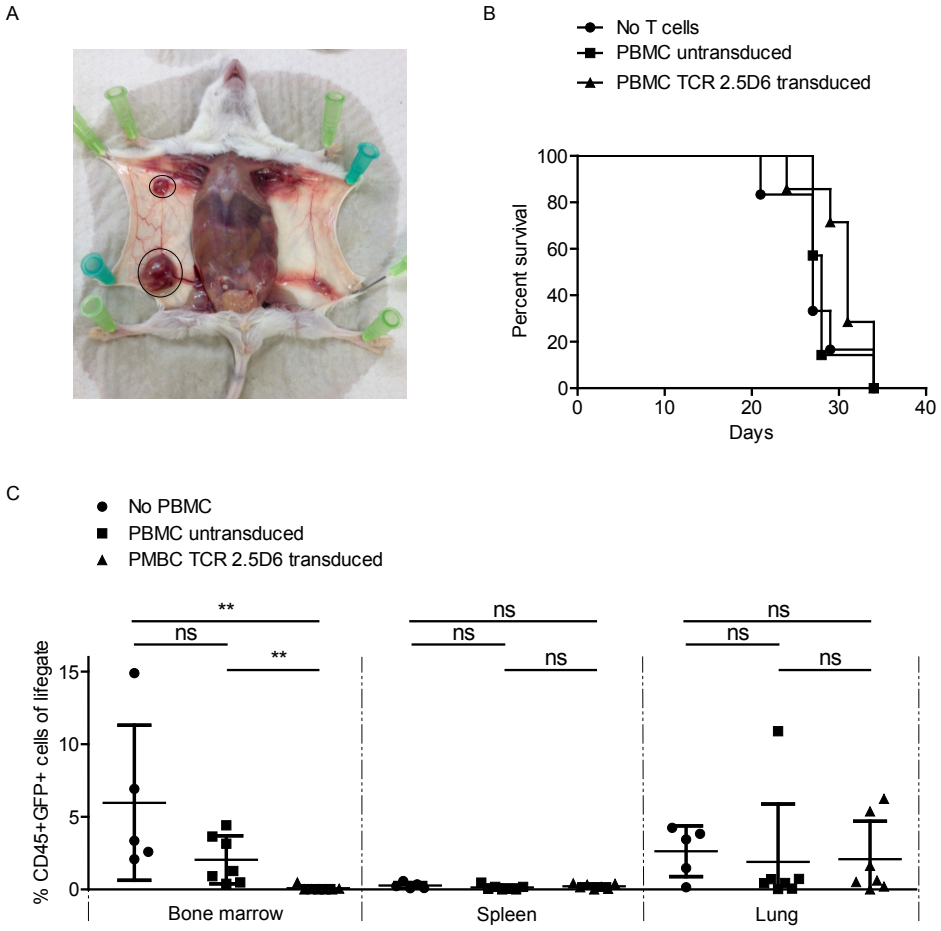

Figure 3.19: *In vivo* experiment 1: Survival and tumor cell infiltration.
20 BRG mice were irradiated with 3.5 Gy and inoculated i.v. with 5×10^6 NB4-B7 tumor cells on the next day. Three days later, six mice received no PBMC, seven mice received 1×10^7 untransduced PBMC and seven mice received 1×10^7 TCR 2.5D6 transduced PBMC i.v.. Mice were sacrificed between day 27 and 34 when suffering from health problems. **(A)** shows the typical phenotype of a sacrificed mouse with massive infiltration of the lymphnodes with human CD45$^+$, eGFP$^+$ NB4-B7 cells. **(B)** Kaplan-Meier curves are shown for all three groups. Statistical analysis was done between animals receiving no PBMC, untransduced PBMC and TCR 2.5D6 transduced PBMC using the Mantel-Cox test. **(C)** Organ infiltration of human CD45$^+$, eGFP$^+$ NB4-B7 cells was analyzed in single cell suspensions obtained from resected organs by flow cytometry. Percentage of CD45$^+$, eGFP$^+$ cells in the lifegate is shown. Statistic analysis between all groups was done using Mann-Whitney test ($^{**}P \leq 0.0025$)

3.6.2 *In vivo* tumor reactivity experiment 2

As we observed reduced leukemic bone marrow infiltration after treatment with TCR-transgenic T cells, we decided to repeat the *in vivo* experiment with optimized parameters. We reduced the number of leukemia cells down to 1×10^6 per mouse, injected the T cells 24 hours after tumor cell inoculation and used T_{CM} instead of whole PBMC for transduction with the TCR 2.5D6. Phenotype of T_{CM} and transduction efficiency of the transduced cells is shown in figure 3.20. Only a slight difference in the percentage of $CD4^-$, $CD8^+$ T_{CM} could be observed between untransduced and TCR 2.5D6 transduced T_{CM} (79.6 % vs. 84.4 %). Also the percentage of $CD62L^+$, $CD45RA^-$ cells was similar in the untransduced and the TCR 2.5D6 transduced condition (89.5 % vs. 81.1 %) (figure 3.20 **(A)**). No $CD8^+$, MPO_5-multimer positive cells could be observed in the untransduced condition, whereas the percentage of $CD8^+$ TCRm antibody binding cells was 2.1 %. In contrast, the percentage of $CD8^+$ cells that bound to the MPO_5-multimer was 45.8 % in the TCR 2.5D6 transduced condition and the percentage of $CD8^+$ cells that bound the TCRm antibody was 56.1 %. 10.3 % of the TCR 2.5D6 transduced $CD8^+$ T_{CM} only bound the TCRm antibody, not the MPO_5-multimer (figure 3.20 **(B)**).

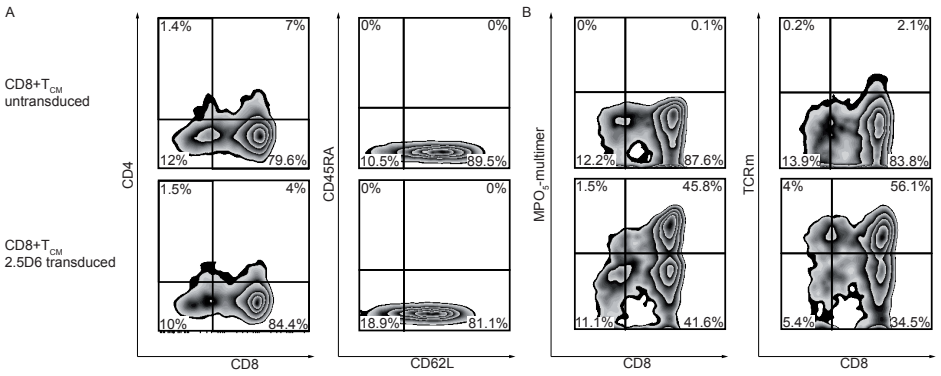

Figure 3.20: *In vivo* experiment 2: Phenotype and transduction efficiency of TCR 2.5D6 transduced T_{CM}.
For mouse experiments, $CD8^+CD62L^+CD45RA^-$ T_{CM} were isolated on day 0 by magnetic bead cell depletion and selection. Cells were activated for two days in the presence of CD3/CD28 beads and IL-2 and transduced with TCR 2.5D6 virus supernatant or left untreated. Untransduced and transduced cells were analyzed on day 10 after transduction for expression of the surface markers CD4, CD8, CD45RA and CD62L **(A)**. Expression of the transgenic T cell receptor chains was analyzed by staining with the MPO_5-multimer and the TCRm antibody **(B)**.

Functionality of T_{CM} was tested before *in vivo* application and results are shown in figure 3.21. In comparison to the untransduced control, reduction of eGFP expressing cells in the lifegate was only observed, when TCR 2.5D6 T_{CM} cells were incubated with T2-B7 cells pulsed with the MPO_5 peptide or with NB4-B7 cells. Pulsing of NB4-B7 cell lead to a slightly increased cytotoxic activity of the TCR transgenic T_{CM} cells (figure 3.21 **(A)**). Similar results were seen regarding the production of IFN-γ, as TCR 2.5D6 transduced T_{CM} produced IFN-

γ only in response to T2-B7 cells pulsed with the MPO_5 peptide, NB4-B7 cells as well as NB4-B7 cells pulsed with the MPO_5 peptide (figure 3.21 **(B)**).

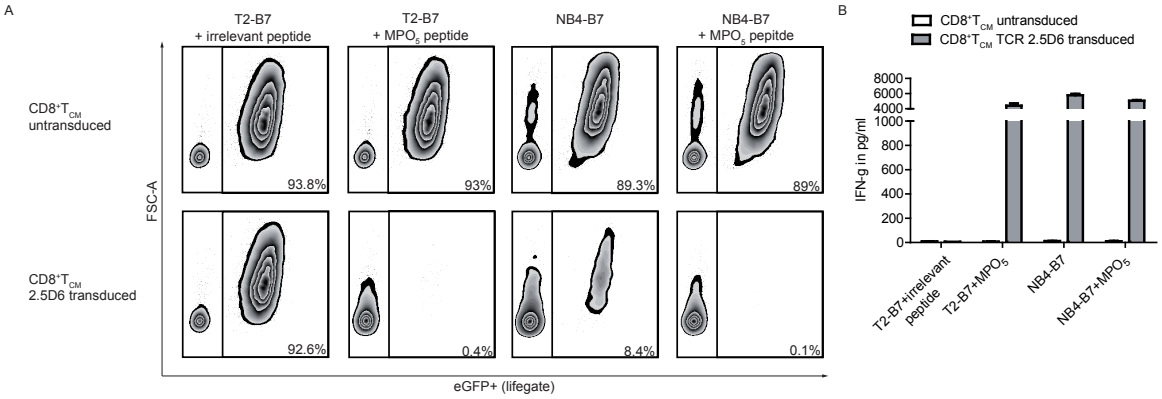

Figure 3.21: *In vivo* experiment 2: Functionality of TCR 2.5D6 transduced T_{CM}. Functionality of the untransduced or the TCR 2.5D6 transduced $CD8^+T_{CM}$ (figure 3.20) was assessed by coincubation with T2-B7 cells pulsed with an irrelevant peptide or the MPO_5 peptide or NB4-B7 leukemia cells unpulsed or pulsed with the MPO_5 peptide at an effector to target ratio of 2:1. Supernatants were harvested after 20 hours for IFN-γ measurement by ELISA **(A)** and cytotoxic activity was assessed after 52 hours by determination of eGFP expressing target cells in the lifegate by flow cytometry **(B)**.

Mice were sacrificed between day 30 and 51 after the beginning of the experiment, except for one long term surviving mouse in the group that was treated with TCR 2.5D6 transduced T_{CM}. This mouse was sacrificed at the endpoint of the experiment at day 112 and showed no signs of tumor cell infiltration in any organs. As shown in figure 3.22 **(A)**, we could observe a significant prolonged survival in the group of mice that received TCR 2.5D6 transduced T_{CM} compared to the group that received untransduced T_{CM} (median survival: 44 days vs. 34 days, $P = 0.00048$) and the group that received no T_{CM} (median survival: 44 days vs. 37 days, $P = 0.0115$). No significant difference was observed between the group that received no T_{CM} and the group that received untransduced T_{CM} (37 days vs. 34 days, $P = 0.2229$). Analysis of organ infiltration with human $CD45^+$, $eGFP^+$NB4-B7 cells, revealed again a significantly reduced infiltration of tumor cells into the bone marrow in the group that was treated with TCR 2.5D6 transduced T_{CM} compared to the group that received untransduced T_{CM} as well as no T_{CM} at all ($P = 0.0247$ and $P = 0.0087$). No significant difference in tumor infiltration could be observed in the spleen or the lung.

We also analyzed the number of eGFP expressing human $CD45^+$ NB4-B7 cells in tumors of all mice and could observe nearly 100 % eGFP expressing $CD45^+$ cells in all mice that received no or untransduced T_{CM} (example in figure 3.23 **(A)**). In contrast, in four of five mice that received TCR 2.5D6 transduced T_{CM} and developed delayed tumors we observed loss of eGFP expression in the $CD45^+$ population. We therefore additionally analyzed the HLA-*B7* expression in one mouse. As shown in figure 3.23 **(B)**, loss of eGFP expression

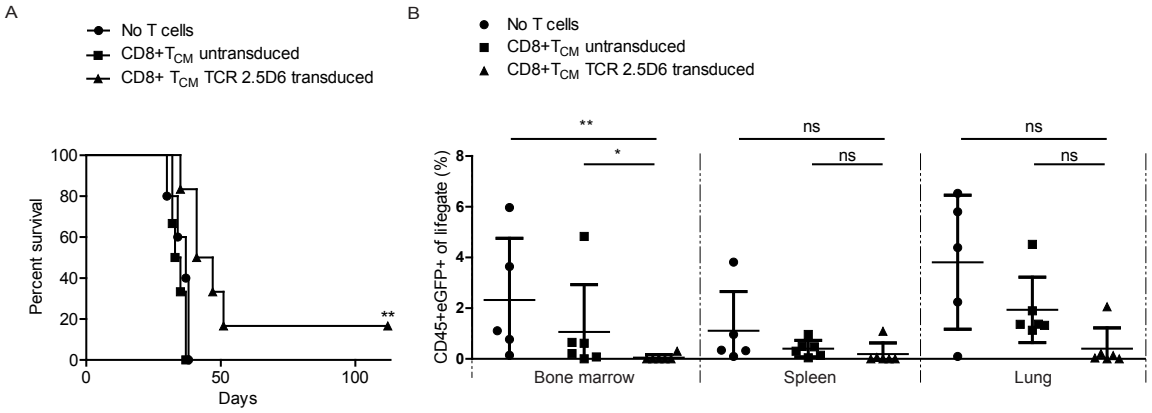

Figure 3.22: *In vivo* experiment 2: Survival and tumor cell infiltration in different organs. BRG mice were irradiated with 3.5 Gy and inoculated 24 hours later with 1×10^6 eGFP$^+$ NB4-B7 cells. One day later, mice received either 200 µl PBS (n=5), 1×10^7 untransduced T_{CM} (n=6) or TCR 2.5D6 transduced T_{CM} (n=6) i.v. (figure 3.20: Phenotype and transduction efficiency, figure 3.21: Functionality). Continuous support of human IL-15 was provided by biweekly i.p. injection of 1.5×10^6 IL-15 producing γ-irradiated NSO cells. Mice were sacrificed when suffering from health problems as described in section 2.2.9 and survival is shown for all three groups in **(A)**. Statistical analysis was done between animals receiving T_{CM} transduced with TCR 2.5D6 and untransduced T_{CM} as well as no T_{CM} using the Mantel-Cox test ($^{**}P = 0.0048$ and $P = 0.0115$, respectively). Analysis of the infiltration with human CD45$^+$, eGFP$^+$ in the bone marrow, the spleen and in the lung was analyzed by flow cytometry in single cell suspensions after organ removal **(B)**. Data of the groups were compared using Mann-Whithney test (** $P = 0.0087$,* $P = 0.0247$)

correlated with a loss in HLA-*B7* expression. However, expression of the targeted antigen MPO was preserved.

All resected organs were also analyzed for infiltration with human T cells. A gating strategy for analysis of infiltrating T cells in the spleen of a mouse that received TCR 2.5D6 transduced T_{CM} is shown in figure 3.24 **(A)**. Comparison of the number of CD45$^+$eGFP$^-$CD3$^+$CD8$^+$ infiltrating T cells in the bone marrow, tumor, lung and spleen between the groups of mice that received untransduced or TCR 2.5D6 transduced T_{CM} revealed no significant difference (figure 3.24 **(B)**). In contrast, analysis of infiltrating human CD45$^+$eGFP$^-$CD3$^+$CD8$^+$TCRm$^+$ cells in the group that was treated with TCR 2.5D6 transduced T_{CM} revealed significant higher infiltration in tumor ($P = 0.0117$) and spleen ($P = 0.0022$) as compared to the background of the TCRm antibody staining in organs of mice that received untransduced T_{CM}.

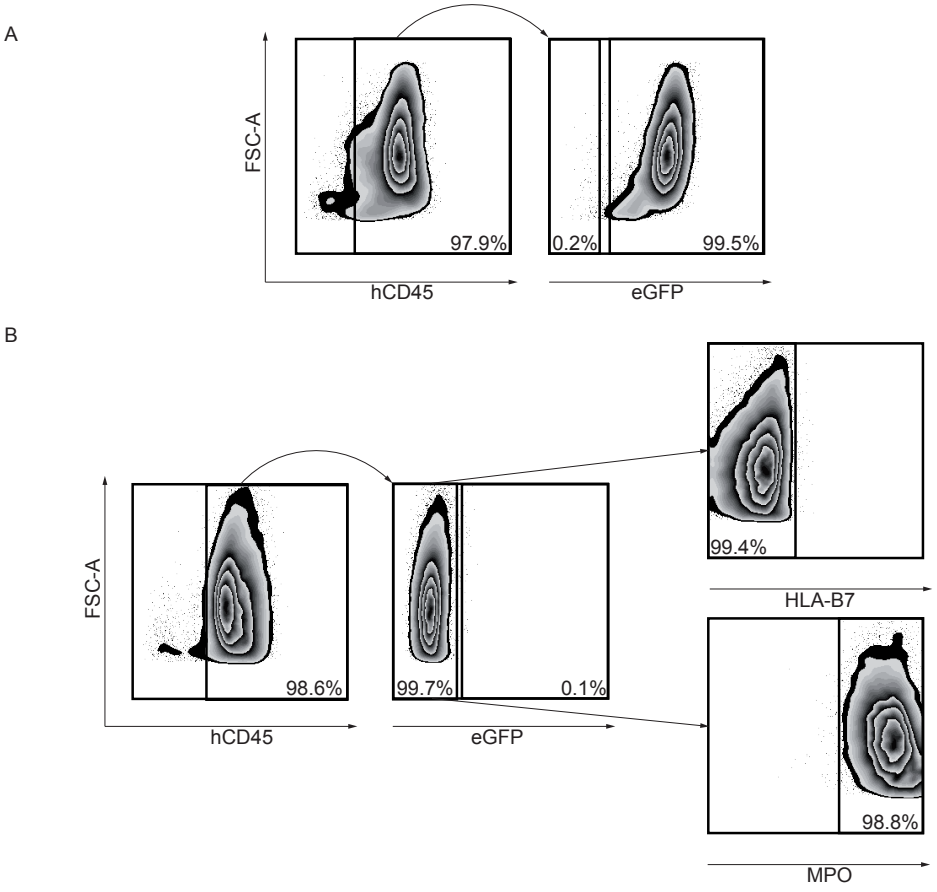

Figure 3.23: *In vivo* experiment 2: Loss of eGFP expression in tumors of mice treated with TCR 2.5D6 transduced T_{CM}.
After obtaining single cell suspensions of tumors resected from diseased mice after sacrification, infiltration of eGFP expressing human $CD45^+$ cells was analyzed by flow cytometry. **(A)** Analysis of a tumor of a mouse that received no T_{CM}. **(B)** The tumor of a mouse that was treated with TCR 2.5D6 transduced T_{CM} was additionally analyzed by staining with an HLA-*B7* and an MPO specific antibody.

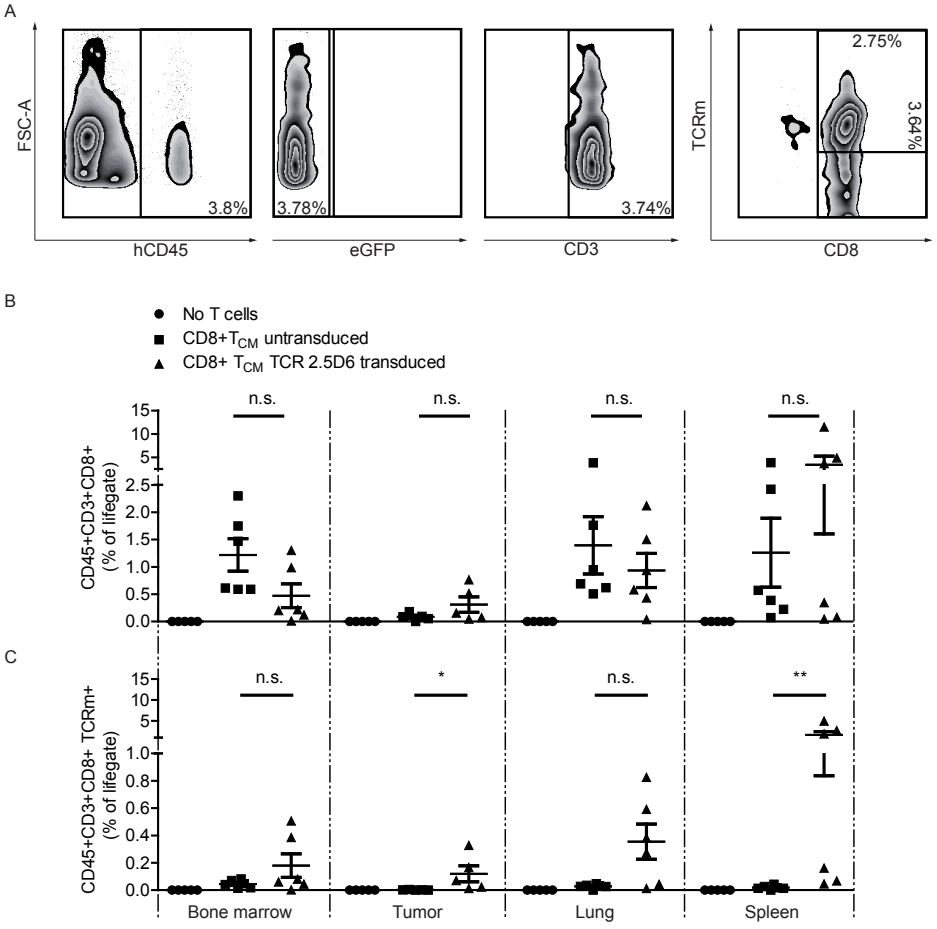

Figure 3.24: *In vivo* experiment 2: T cell infiltration.
Single cell suspensions of organs were analyzed for their percentage of $CD45^+eGFP^-CD3^+CD8^+$ and $CD45^+eGFP^-CD3^+CD8^+TCRm^+$ T cells. A gating strategy is shown in **(A)**, results for $CD45^+eGFP^-CD3^+CD8^+$ cells are shown in **(B)** and for $CD45^+eGFP^-CD3^+CD8^+TCRm^+$ cells in **(C)**

Chapter 4

Discussion

4.1 Immunopeptidomic identification of potential targets for T cell mediated immunotherapy in leukemia

Adoptive transfer of TCR transgenic T cells has been proven to be effective in several clinical settings. Targeting melanoma-associated differentiation antigens such as GP100 and MART1 led to objective cancer regressions in up to 30 %. However, some patients had severe side effects affecting normal melanocytes, whereas others did not respond to therapy (Morgan et al., 2006; Johnson et al., 2009). Targeting a broader expressed antigen like NY-ESO-1 showed clinical efficiency also in patients with sarcoma, highlighting that this approach is not restricted to melanoma. Those findings highlight the power of T cell mediated immunotherapies. However, as not all cancer patients respond to therapies that target antigens like GP100, new target structures need to be identified. Furthermore, as severe side effects occurred in some cases (Morgan et al., 2013; Linette et al., 2013), preclinical toxicity tests need to be developed to improve the safety of immunotherapies. With regard to leukemia therapy, it has been shown in the early 90'ies that DLI that were given in the context of hematopoetic stem cell transplantation can induce a curative GvL effect (Kolb et al., 1990; Kolb, 2008). However, target structures recognized by donor lymphocytes remain mostly unknown and expanding knowledge is of great value. WT-1 may be a candidate target antigen in leukemia patients for TCR transgenic T cells as *in vivo* anti-leukemic reactivity has been observed in a mouse model of human leukemia (Xue et al., 2005). Furthermore, targeting an HLA-*A*02:01* restricted epitope derived from WT-1 with autologous T cell clones showed some clinical activity post transplantation without any signs of GvHD (Chapuis et al., 2013). Another potential target is supposed to be an HLA-*A*02:01* restricted epitope derived from neutrophil elastase and proteinase 3 called PR1. T cells transgenic for PR1-specific constructs are currently under investigation as potential therapeutic tools for the treatment of CML and AML (Yong et al., 2007; Sergeeva et al., 2011). Another potential group of targets are peptides carrying cancer

specific mutations. It has been shown in this regard in some patients with melanoma that were treated with autologous TILs that T cells can recognize peptides derived from mutated genes (Robbins et al., 2013). However, these mutations and their HLA restricted presentation are highly patient specific, limiting the number of patients that could benefit from a TCR recognizing such antigens.

4.1.1 Target selection

In order to define potential targets for TCR transgenic T cells presented on the surface of primary leukemia cells, we investigated the immunopeptidome of seven patients with MPN. We could not identify any mutated peptides. This is probably caused by technical hurdles to identify patient specific mutations that are not enclosed in the databases used for analysis of mass spectrometry data. We tried to circumvent the use of databases by using the *de novo* sequencing software PEAKS. However, due to incomplete fragmentation of peptides during mass spectrometry, resulting in incomplete fragmentation spectra, the use of this software did not result in satisfactory results regarding mutated peptides. We furthermore looked for leukemia specific antigens like WT-1 but did not find any ligands of such antigens. This could be caused by insufficient sensitivity of the mass spectrometry device resulting in detection of only the most abundant HLA ligands. What we could identify were 17 HLA ligands derived from seven genes that show, according to database searches, restricted expression to the hematopoetic system. One ligand is derived from ELANE, a serine proteinase that is secreted by neutrophils and macrophages during inflammation (Belaaouaj et al., 2000). Two ligands are derived from HMHA1 and are presented on two different HLA molecules. HMHA1 codes for the first described minor histocompatibility antigen (mHag) HA-1 which has been proposed to be a suitable target in leukemia, due to high expression in the hematopoetic system but low or absent expression in non hematopoetic cells (Wilke et al., 2003). LAT2 has been selected as a potential target due to restricted expression in the hematopoetic system (Su et al., 2004). Nevertheless, the expression of LAT2 is not fully understood so far. Expression seems to be downregulated in AML1/ETO positive AML but upregulated in cases of AML with myelomonocytic features (Duque-Afonso et al., 2011). Two ligands encoded by ITGA2B were selected as potential targets, as expression of ITGA2B is described to be restricted to hematopoetic cells like hematopoetic stem cell (HSC)s, megacaryocytes and platelets (Su et al., 2004; Dumon et al., 2012). MPO was selected as a possible target antigen and is discussed in detail in section 4.1.2. Two peptides derived from MS4A3 were selected as potential candidates for TCR transgenic T cells, as MS4A3 also known as HTm4 is an important regulator of hematopoetic differentiation (Donato et al., 2002). Last but not least MYB was selected as a potential antigen and five HLA ligands presented on three different HLA molecules could be identified by the immunopeptidomic approach. MYB seems to play an important role in leukemogenesis (Waldron et al., 2012; Lidonnici et al., 2008) and accord-

ing to database searches expression is mainly restricted to the hematopoetic system (Su et al., 2004). Nevertheless, MYB is described to play an important role for progenitor cell homeostasis in colonic crypts which bears the risk of immune reactions against colon tissue mediated by MYB-specific TCR transgenic T cells. However, induction of immunity against MYB by application of a DNA vaccine in a mouse model of colon cancer led to anti-tumor immunity while no autoimmune pathology could be observed in MYB expressing tissues (Williams et al., 2008). If the same effect can be achieved by therapy with high avidity TCRs targeting MYB derived peptides is unknown and needs to be investigated in the future. qPCR results confirmed the expression patterns described in the literature and in databases (not done (ND) for LAT2 and HMHA1 so far).
In conclusion, the immunopeptidomic analysis of samples from patients with MPN led to the identification of peptides derived from seven genes with expression restricted to the hematopoetic system. Detailed characterization as their potential and safety as targets for TCR transgenic T cells for the treatment of leukemia needs further investigation. MPO was selected as target in the current study and is discussed in the next section.

4.1.2 Characterization of MPO as a target for T cell mediated immunotherapy in myeloid leukemia

MPO was chosen as a potential target because of its restricted expression to myeloid precursors, mature neutrophils, monocytes and macrophages. It plays an important role in the phagocyte oxygen-dependent intracellular microbicidal system (Prokopowicz et al., 2012) and provides a highly suitable candidate target for the treatment of patients with myeloid leukemias. Five HLA ligands that are derived from MPO could be identified by the immunopeptidomic approach and are presented on four different HLA molecules. We could verify all five HLA ligands by matching the mass spectra with spectra of the synthetic counterparts. To confirm restricted expression described in the literature and in databases we performed qPCR, immunohistochemistry (IHC) and flow cytometry and could indeed confirm the restricted expression in myeloid cells. Furthermore, most of the PBMC samples derived from patients with myeloid leukemias showed increased expression compared to the reference PBMC derived from healthy donors. Flow cytometric analysis revealed high MPO expression on the protein level in monocytes and granulocytes and we therefore expected increased expression compared to PBMC also on the mRNA level. Surprisingly this was not the case and has already been described in the literature (Fouret et al., 1989; Tobler et al., 1988). They found out that MPO is highly expressed on the mRNA level in myeloid precursor cells and expression declines during differentiation. Detection of MPO on the protein level in differentiated monocytes and granulocytes is caused by the high stability of the protein.
MPO thereby represents a highly attractive target for the treatment of myeloid leukemias with TCR transgenic T cells especially in the haploidentical or single HLA-mismatched trans-

plantation setting. Donor derived myeloid precursor cells are spared in those settings, when TCRs are used that target MPO ligands presented on mismatched HLA molecules.

4.2 Establishment of a system for stimulation of peptide specific T cells

All antigens that were selected for targeting by TCR transgenic T cells are derived from self antigens. We did not expect to identify TCR with high avidity in the autologous TCR repertoire due to negative selection of self reactive TCR with high avidity in the thymus. A potential source of TCR recognizing HLA ligands derived from self antigens with high avidity is the allogenic TCR repertoire (de Witte et al., 2006; Wilde et al., 2009; Spranger et al., 2012). We therefore decided to search for peptide specific T cells in the allogenic repertoire. However, the portion of peptide specific T cells that are stimulated during incubation with completely allogenic DC is expected to be rather low caused by up to six HLA mismatches between T cell and DC donor. Wilde et al. (2009) and Spranger et al. (2012) reported a system to optimize the isolation of T cells specific for HLA-*A*02:01* ligands out of the single HLA-mismatched repertoire. They stimulated T cells from an HLA-*A*02:01* negative donor with DC from the same donor, that have been transfected with ivt-RNA coding for HLA-*A*02:01*. We decided to use this single HLA-mismatched stimulation approach but had to optimize it further, as the selected HLA ligands are not presented on HLA-*A*02:01* but on different HLA molecules. We generated ivt-RNA coding for the respective HLA molecules and eGFP as a marker to follow transfection efficiency into DC. As a specific antibody is available for HLA-*B*07:02* we could confirm surface expression of HLA-*B7* in eGFP expressing DC after transfection. Other target cell lines could be successfully modified in a similar way, by retroviral transduction with a construct coding for the respective HLA molecules and eGFP.

4.3 Expansion of specific T cells by stimulation with HLA-*B*07:02* ligands

In order to find TCR with high avidity for the six identified HLA-*B*07:02* ligands we stimulated T cells of a HLA-*B*07:02*$^+$ donor in a single HLA-mismatched approach. Stimulation resulted in defined HLA multimer positive populations only in response to three of six peptides. As the HLA multimers have not been tested before and positive controls are not available, lack of functionality regarding the HLA multimers can not be excluded. Another explanation could be the fact that some T cells do not bind to HLA multimers even if they show peptide specific reactivity (Rubio-Godoy et al., 2001; Lyons et al., 2006). We analyzed in this regard IFN-γ production in those T cell lines in response to target cells pulsed with an irrelevant or the relevant peptide. We could observe high numbers of IFN-γ producing cells but with no

difference between the two target cell conditions. It is possible that peptide specific T cells are present at a low frequency but are missed due to the high background reactivity against the target cells itself. Functional sorting of the reactive cells followed by cloning and analysis of the T cell clones could be a possibility to isolate peptide specific T cells that are present at low frequencies. Another reason could simply be the fact that T cells reactive against the respective HLA-*B*07:02* ligand are not present in the naive repertoire of the specific blood donor. Therefore, single HLA-mismatched stimulation experiments need to be repeated with cells from different HLA-B*07:02 negative blood donors to elucidate if the ligands represent T cell epitopes.
Nevertheless, we were able to isolate a T cell clone and its receptor (further called 2.5D6) that specifically recognizes the HLA-*B*07:02* restricted MPO epitope MPO_5. The detailed characterization is discussed in the following section (4.4)

4.4 Detailed characterization of the TCR 2.5D6

4.4.1 Functional characterization and antigen specificity

A prerequisite for generation of T cell therapeutics with defined specificity is the genetic modification of T cells with defined TCR chains. Newly introduced TCR chains can pair with endogenous TCR chains upon gene transfer, leading to formation of TCR with undefined specificities. It has been described that this can lead to lethal autoimmune reactions after TCR gene transfer in mice (Bendle et al., 2010). This has so far not been observed in human studies with TCR transgenic T cells potentially caused by the long culture period in human studies prior to adoptive T cell transfer, resulting in diminished T cell function *in vivo* (Bendle et al., 2010). Nevertheless, *in vitro* studies with human TCRs revealed toxic neoreactivities caused by mispairing of exogenous and endogenous TCR chains (van Loenen et al., 2010). Several possibilities exist to reduce mispairing. The most obvious approach consists of excision of the endogenous TCR chains with specific zink finger nucleases followed by introduction of the desired TCR as described previously (Provasi et al., 2012). Nevertheless, as this would involve numerous steps of viral gene transfer and cell sorting such an approach will be very cost intensive when done under good manufacturing practise (GMP) conditions. Another option to reduce mispairing is the murinization of the constant regions, which has been described to improve expression and functionality of the exogenous TCR (Cohen et al., 2006). As this transfers murine genetic material into the human system this could potentially result in rejection of the transferred T cells *in vivo*. However, rejection of TCR derived from the murine system has for example not been observed in a study conducted by Abate-Daga et al. (2013).
For improved expression and reduced mispairing with endogenous TCR chains, we performed *in silico* codon optimization, murinization of the constant regions, addition of an additional

cystein bridge and cloning of the TCR chains as a bicistronic construct separated by a P2A element as described previously (Cohen et al., 2006; Kuball et al., 2007; Scholten et al., 2006). We could successfully transfer the peptide specific reactivity of the T cell clone 2.5D6 into activated PBMC by retroviral gene transfer. Furthermore, expression of the TCR was also observed in $CD4^+$ T cells which has been associated with a high functional avidity (Ray et al., 2010; Kuball et al., 2005; Morris et al., 2005). However, although different modifications have been introduced in the TCR, the fact that a higher percentage of cells bound to the TCRm antibody compared to the percentage of cells that bound the specific HLA multimer could still argue for a certain degree of mispairing. Another explanation for this discrepancy could be explained by low cell surface expression of the exogenous TCR chains on some cells, not enabling binding of the multimer.

We further analyzed the functional avidity of the TCR 2.5D6. The functional avidity is a biological measure that describes how strong a T cell responses *in vitro* to a given concentration of a ligand, in our case the MPO_5 peptide in complex with HLA-*B*07:02*. The functional avidity is, in addition to the expression of the TCR and co-receptors as well as the distribution of signaling molecules (Viola and Lanzavecchia, 1996; Valitutti et al., 1996), depending on the affinity of the specific TCR for the peptide/HLA complex (Schamel et al., 2005; Cawthon et al., 2001). The TCR affinity describes the physical strength of the binding between a single TCR molecule and its cognate peptide/HLA complex. The affinity can be measured by determination of the dissociation constant (K_D) which is influenced by the on- as well as the off-rate of the TCR on the respective peptide/HLA complex. A lower K_D, which speaks for a higher affinity, has been associated with a better T cell response in several reports (Holler et al., 2001; Tian et al., 2007). Analysis of the functional avidity of TCR 2.5D6 transgenic PBMC led to a nearly tenfold increased half maximal effective concentration (EC_{50}) value compared to the T cell clone. This could be explained by exhaustion of the T cell clone at the time of analysis due to culture over several weeks and repeated stimulation with allogenic feeder cells. TCR transgenic PBMC showed a rather high avidity with an EC_{50} of 150 pmol/l compared to a report of the EC_{50} values for seven allogenic TCR with specificity for an HLA-*A*02:01* restricted epitope derived from tyrosinase (mean EC_{50} = 2.5 nmol/l (Wilde et al., 2009)). However, difficulties in comparing EC_{50} values from different TCR tested in different laboratories are expected due to different assays and conditions. We further sought to proof antigen specificity of the TCR 2.5D6. Antigen specificity of a TCR can either be analyzed by knockdown of the respective antigen (Amir et al., 2011; Spranger et al., 2012) in antigen positive target cells or by transfer of the antigen into antigen negative target cells. We could demonstrate antigen specificity of the TCR 2.5D6 by transfer of MPO into two cell lines of B cell origin that naturally do not express the antigen.

4.4.2 On target toxicity

On target toxicity refers in the context of TCR transgenic T cells to recognition of healthy cells or tissues, expressing the targeted antigen. Experiments to reveal if a certain antigen with restricted expression to the hematopoetic system can safely be targeted by TCR transgenic T cells in context of an HLA-matched or HLA-mismatched transplantation setting is crucial. Targeting such an antigen in the HLA-matched setting comes along with eradication of all cells of the donor and recipient expressing the antigen. In contrast, when an epitope of such an antigen is targeted in the mismatched setting and the recognized epitope is presented on an HLA molecule which is only present on patient cells, cells of the patient that express the respective antigen are attacked exclusively. To analyze on target toxicity of the TCR 2.5D6 we analyzed recognition of different subsets of PBMC from an HLA-*B*07:02* positive blood donor. We expected reactivity against monocytes and granulocytes as they express MPO on the protein level. However, we did not observe reactivity against HLA-*B*07:02* positive monocytes and only very low reactivity against granulocytes. This could be explained by the fact, that MPO has a low protein turnover (discussed in section 4.1.2 and newly synthesized proteins seem to be the mature source for substrates used by the TAP (Reits et al., 2000) leading to low MPO peptide presentation in mature myeloid cells. We therefore expect recognition of myeloid progenitor cells, that show high MPO expression on the mRNA level, by TCR 2.5D6 transduced T cells, but were so far not able to analyze this *in vitro*. Those experiments will be done in the future. A prerequisite for targeting MPO in an HLA matched setting is the lack of reactivity against healthy hematopoetic stem cells. Otherwise, hematopoetic stem cells of the donor would be destroyed after infusion of TCR 2.5D6 transduced T cells leading to engraftment failure. We have not observed any reactivity against healthy $CD34^+$ stem cells, as shown by CFU assays. This is consistent with previous observations that report low or absent expression of MPO in healthy $CD34^+$ hematopoetic stem cells (Majeti et al., 2009; Kim et al., 2010). The lack of cytotoxicity against hematopoetic stem cells tempted us to consider this TCR for treatment in the matched transplantation setting or even apart from HSC. However, implementation of a suicide mechanism (Griffioen et al., 2009; Ciceri et al., 2007) to deplete TCR transgenic T cells after treatment is going to be important to allow regeneration of healthy myeloid progenitor cells in the bone marrow in the autologous setting. Additionally this safety mechanism might be of great value also in the HLA-mismatched transplantation setting to limit unexpected on and off target toxicity.

4.4.3 Off target toxicity

Determination of the off target toxicity, which refers to recognition of different antigens than the desired by TCR transgenic T cells, is a very important step before transfer into the clinic. Recent efforts to target melanoma-associated antigen 3 (MAGE-A3) with T cells transgenic for affinity enhanced TCR constructs were compromised by fatal clinical outcomes. In a clin-

ical study published by Morgan et al. (2013) patients died because of unexpected neurological toxicity caused by expression of proteins derived from the melanoma-associated antigen (MAGE) family in the brain, that show similarities to the MAGE-A3 protein. In another study, where patients were treated with a TCR construct specific for a MAGE-A3 derived HLA-*A*01:01* ligand, Linette et al. (2013) observed cytotoxic reactivity against contracting cardiac myocytes. Recognition of those cells was not expected, as they do not express MAGE-A3. In order to seek for the reason for off target toxicity, the authors performed alanine scan experiments with the MAGE-A3 peptide to identify the AS residues that are responsible for recognition by the TCR. They used the ScanProsite tool to identify peptides that contain residues critical for recognition by the TCR and found a Titin derived peptide to be responsible for off target toxicity (Cameron et al., 2013). Regarding specificity of the TCR 2.5D6, lacking crossreactivity against cells of the hematopoetic system that do not express MPO on the mRNA level is discussed in the previous section. We furthermore analyzed diverse non-hematopoetic cell lines that do not express MPO for their recognition by TCR 2.5D6 transduced PBMC and could not observe any off target toxicity so far. In order to determine the AS residues in the MPO_5 peptide that are responsible for recognition by TCR 2.5D6 transduced T cells, we also performed Alanine scan experiments. Those experiments revealed position 2, 3, 4 and 5 of the MPO_5 peptide as essential for recognition by the TCR 2.5D6 when T2-B7 cells were used as target cells. In contrast, when LCL with endogenous HLA-*B*07:02* expression were used as target cells, amino acids at position 2, 3, 4, 5, 8 and 9 revealed to be essential for recognition. It is already described in the literature, that T2 cells have more peptide-receptive HLA molecules available, caused by a defect in the TAP, compared to cells with intact antigen processing and peptide presentation (Luft et al., 2001). Therefore, the differences in the recognition patterns could be caused by the fact, that alanine exchanges at position 8 and 9 result in peptides that bind to HLA-*B*07:02* with low affinity. We hypothesize, that those low affinity peptides can bind to empty HLA-*B*07:02* molecules in T2-B7 cells, whereas the affinity to HLA-*B*07:02* is to low to compete with endogenous HLA-*B*07:02* peptides in LCL. We additionally searched for MPO_5 homologous peptides and identified LPO_1 and EPX_1. LPO_1 has a polar, uncharged glutamine at position 3 compared to a positively charged arginine in the MPO_5 peptide. In the case of the EPX_1 peptide, a negatively charged aspartic acid is exchanged to a polar, uncharged asparagine on position 5. Based on the results of the alanine scan experiments recognition would have not been expected. However, the TCR 2.5D6 demonstrated reactivity against both homologous peptides, when pulsed on T2-B7 cells. This could be explained by a publication of Stone and Kranz (2013), reporting that the structural similarity of a given peptide can play a major role for crossreactivity of a TCR, not only the sequence similarity. Similar to the results of the alanine scan experiment, we could observe differences between T2-B7 cells and HLA-*B*07:02* expressing LCL, as LCL were not recognized, when pulsed with the LPO_1 or the EPX_1 pep-

tide. This could again be caused by a low affinity of those peptides to HLA-*B*07:02*. As the LCL represent a cell line with natural HLA-*B*07:02* expression, we are convinced that this reflects a more suitable model to study the recognition pattern of a TCR. Taken together, new tools need to be developed, as alanine scans alone are not sufficient to cover the full range of possible crossreactivities. One limitation of the alanine scan experiment is the potential loss of binding to a certain HLA molecule (HLA-*B*07:02* in the case of the MPO_5 peptide) in alanine variants. This could be overcome by using a panel of LCL expressing common HLA alleles as target cells. The same panel of LCL could further be transfected with a DNA vector library coding for triple repeats of selected peptides followed by proteasomal cleavage sites as described by Engels et al. (2013). This would represent a more physiological and cheaper approach compared to pulsing cells with synthetic peptides. However, the feasibility of such an approach in a large scale needs to be investigated in the future. Another possibility to investigate crossreactivities of a given TCR are mouse models that are transgenic for HLA. A suitable mouse model to test HLA-*B*07:02* restricted on and off target effects of the TCR 2.5D6 is represented by a transgenic mouse that expresses HLA-*B*07:02* (Rohrlich et al., 2003). However, limitations of such a model are the partial genome homology between mice and humans as well as differences in antigen processing and presentation (Street et al., 2002; Kotturi et al., 2009). Such a model would also only shed light on crossreactivity against one HLA molecule, but T cells have been described to have a certain degree of polyspecificity (Felix et al., 2007). We therefore could not exclude recognition of a peptide in complex with an other HLA molecule than HLA-*B*07:02*. Furthermore, transgenic mouse models are only available for a limited number of HLA molecules.

4.4.4 Leukemia reactivity of the TCR 2.5D6 *in vitro* and *in vivo*

The most important prerequisite for suitability of a TCR for anti leukemic immunotherapy is tumor reactivity. We analyzed reactivity of TCR 2.5 transgenic PBMC *in vitro* and observed reactivity against leukemia cell lines that endogenously express MPO. We furthermore could observe MPO and HLA-*B7* specific reactivity against primary samples derived from patients with MPN as well as AML. As it has been proposed that leukemias as CML (Holyoake et al., 1999, 2001) and AML (Bonnet and Dick, 1997) are hierarchically organized diseases, emerging from uncontrolled proliferation of leukemic stem cells, an effective therapy should also target those cells. As the phenotype of such leukemia initiating cells is being discussed controversially (Sarry et al., 2011; Eppert et al., 2011), we performed CFU assays with unselected PBMC of a patient with MPN. Indeed we could observe MPO and HLA-*B*07:02* specific reactivity of the TCR 2.5D6 against leukemic precursor cells resulting in a reduction in colony forming cells. This is consistent with reports that describe expression of MPO in leukemia stem cells of MPO high expressing leukemias. Stem cells were defined in those reports as $CD34^+CD38^-$ cells (Kim et al., 2010; Gal et al., 2006) and $CD34^+CD38^-CD123^+$ or $CD34^+CD38^-CD90^-$

(Majeti et al., 2009).

Encouraged by the *in vitro* results, we next sought to analyze tumor reactivity of the TCR 2.5D6 *in vivo*. We therefore chose a model of human APL represented by NB4-B7 cells as this cell line was recognized by the TCR *in vitro*. This cell line is published to induce leukemia in a non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model (McCormack et al., 2007). We decided to use the immunocompromised BRG mouse strain as those mice lack functional T cells, B cells and NK cells (Goldman et al., 1998; Mazurier et al., 1999). The same is true for NOD.Cg-Prkdc^{scid} Il2r^{gtm1Wjl}/SzJ (NSG) mice, however, as reported by Ali et al. (2012) they seem to be more susceptible to graft versus host effects caused by transferred human PBMC. Indeed, we did not observe any xenoreactivity in the BRG mice after transfer of human PBMC or T_{CM}. In the first *in vivo* experiment, where 5×10^6 NB4-B7 cells haven been injected, we did not see a significant increase in survival in the group that was treated with TCR 2.5D6 transduced PBMC. This could be caused by the fact, that we injected a rather high number of tumor cells compared to other leukemia models (for example Spranger et al. (2012): 1×10^6 THP-1 cells per mouse). It has been shown furthermore, that T_{CM} show better *in vivo* engraftment compared to t effector cell (T_E) (Wang et al., 2011; Berger et al., 2008). As we did not select for a defined effector subtype in the first *in vivo* experiment, the number of T_{CM} injected can be assumed to be very low (Wang et al. (2012): 0.4 % in PBMC). It is therefore possible, that the transferred T cells did not survive long enough to efficiently destroy the tumor cells. Reduction of the number of injected tumor cells, reduction in the time span between tumor cell and T cell injection and injection of T_{CM} in the second *in vivo* experiment led to a significant prolonged survival in the group of mice that received TCR transgenic T_{CM}. This model reflects a state of minimal residual disease (MRD) and proofs, that a therapy with TCR 2.5D6 transgenic T cells would be suitable to reduce the risk of relapse after chemotherapy or HSCT. Experiments that show reduction in leukemia burden in a mouse model of a progressed leukemia are ongoing. We could observe human T cells in mice that received either untransduced or TCR transduced T_{CM} up to day 112, although at very low frequencies. We could furthermore observe significant reduction in the percentage of tumor cells in the bone marrow, the preferential site of leukemic cell infiltration (Woiterski et al., 2013), as shown in both experiments. This is, in addition to the prolonged survival in the second experiment a hint for *in vivo* anti leukemic reactivity of the TCR 2.5D6 in both experiments. Although survival was prolonged in the group that was treated with TCR 2.5D6 transduced T_{CM}, a majority of mice developed delayed progression of myeloid tumors and had to be euthanized. Four of the five mice with tumor progression developed tumors that showed human CD45 expression but no expression of eGFP, as it was expected after injection of eGFP$^+$ NB4-B7 cells. This loss did take place although the leukemia cells had been sorted for eGFP and cloned by limiting dilution prior injection, minimizing the risk that untransduced NB4 cells have been transferred. As NB4-B7 cells

are transgenic for a bicistronic construct coding for HLA-*B*07:02* and eGFP, loss of HLA-*B*07:02* was also expected and could be shown by flow cytometry. This indicates genetic or epigenetic instability of the transgene under selection pressure as described previously (Frank et al., 2004). This was not observed in any mouse of the other groups and our model simulates therefore a realistic scenario, as loss of HLA molecules in leukemic cells has been described previously to be responsible for relapse in patients after haploidentical stem cell transplantation (Vago et al., 2009). This observation highlights the need for simultaneous targeting of peptides that are presented on different HLA molecules. Loss of HLA expression is one possible escape scenario of cancer cells by immunoediting. Another possibility for cancer cells to evade immune pressure is loss of the targeted antigen (DuPage et al., 2012), what we could not observe as MPO expression was preserved. However, a more natural model of human primary leukemia would represent a situation that is closer to the human system. We tried to establish a primary AML that is HLA-*B*07:02* and MPO positive, but unfortunately we were not able yet to collect a patient sample that shows *in vivo* engraftment in the BRG mouse model. This will be further pursued in the future.

4.5 Conclusion and outlook

In conclusion, using the immunopeptidomic approach, we could identify a number of highly attractive target structures for the treatment of patients with leukemia by immunotherapy with TCR transgenic T cells. Those target structures are derived from different genes that show expression restricted to the hematopoetic system. We established a system that allows single HLA-mismatched stimulation of T cells with HLA ligands presented on several different HLA molecules. Using this system, we could isolate a TCR recognizing an HLA-*B*07:02* restricted epitope from MPO, a gene that is restrictedly expressed in myeloid cells. The TCR shows MPO specific anti-leukemic reactivity *in vivo* and *in vitro*, but lacks reactivity against mature myeloid cells and, more importantly, against $CD34^+$ hematopoetic stem cells and cell lines derived from MPO^- tissues. Furthermore, alanine scan experiments with HLA-*B*07:02*$^+$ LCL revealed a MPO unique recognition pattern of the TCR 2.5D6. This TCR represents a highly attractive tool for the treatment of patients with myeloid leukemias after hematopoetic stem cell transplantation. A potential clinical protocol for the treatment of patients with myeloid leukemia in context of an allogenic HSCT with TCR 2.5D6 transgenic DLI is shown in figure 4.1.

Further homologous peptides need to be characterized according to recognition by the defined TCR to estimate safety of the TCR before transfer into the clinic. Detailed characterization of side effects observed in a transgenic HLA-*B*07:02* mouse model will further shed light on the safety of the MPO specific TCR. Finally, to provide efficient treatment options with TCR transgenic T cells to a broader part of leukemia patients, isolation and characterization of

TCRs directed against other identified HLA ligands are ongoing.

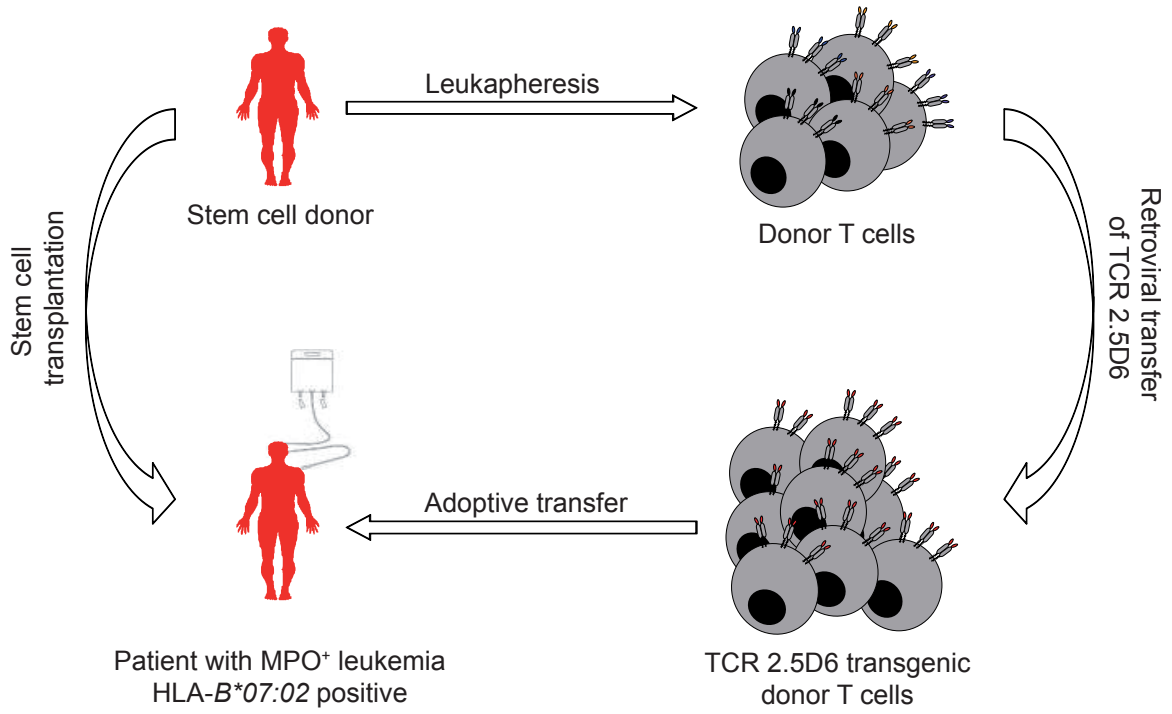

Figure 4.1: Potential clinical application of the TCR 2.5D6.
An HLA-*B*07:02*$^+$ patient with an MPO$^+$ leukemia is transplanted with hematopoetic stem cells of an HLA matched or mismatched donor. Donor lymphocytes are harvested and transduced with the TCR 2.5D6 and TCR transgenic cells are adoptively transferred to the leukemia patient.

Appendix A

Sequence verification of naturally presented HLA ligands

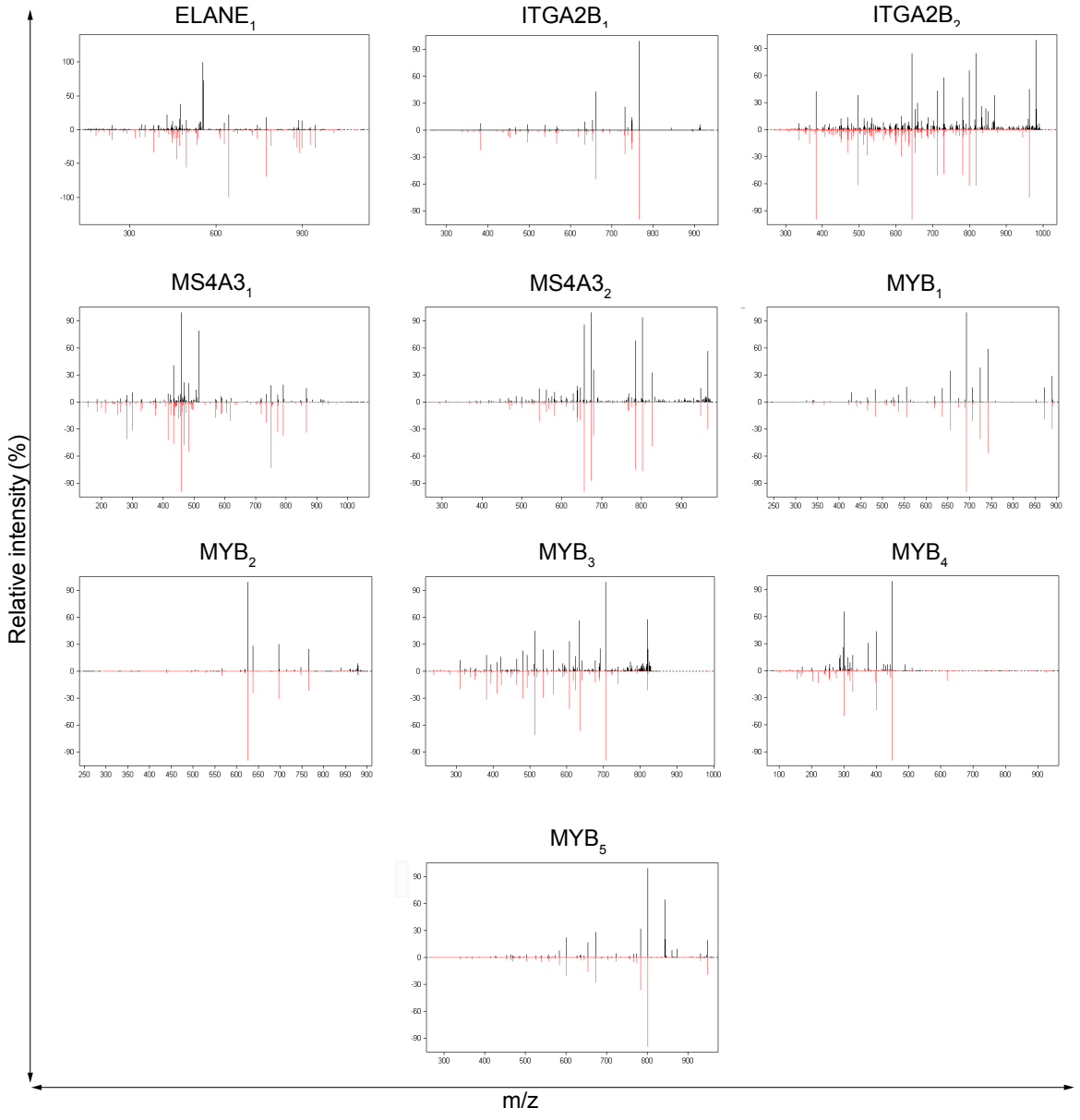

Figure A.1: Sequence verification of naturally presented HLA ligands.
HLA ligands identified by the immunopeptidomic approach were synthesized, sequenced by mass spectrometry and matched to the spectra of eluted peptides after normalization with the mMass tool for sequence verification. Spectra of synthetic peptides are shown in red (lower) and spectra of eluted peptides in black (upper).

Appendix B

Patient characteristics

Table B.1: Patient characteristics

Patient	Age	Sex	Diagnosis	Previous therapy prior blood venipuncture	Stage/Type	HLA typing
MPN1	74	M	Atypical CML	Hydrocyurea	Blast crisis	*A*26:01*, *A*68:01* *B*07:02*, *B*58:01*
MPN2	42	M	CML	None	Blast crisis	*A*03:01*, *A*25:01* *B*07:02*, *B*15:01*
MPN3	54	F	CML	None	Chronic phase	*A*01:01*, *A*24:02* *B*08:01*, *B*18:01*

Table B.1: Patient characteristics *(continued)*

Patient	Age	Sex	Diagnosis	Previous therapy prior blood venipuncture	Stage/Type	HLA typing
MPN4	78	M	CML	Cytarabin	Blast crisis	*A*01:01*, *A*-:-* *B*08:01* *B*-:-*
MPN5	68	M	CML	Imatinib, Nilotinib, Cytarabin, Etoposid	Blast crisis	*A*01:01*, *A*33:01* *B*07:02*, *B*14:02*
MPN6	71	M	PV	Hydroxurea	Proliferative phase	*A*24:02*, *A*32:01* *B*07:02*, *B*44:02*
MPN7	68	F	CML	None	Chronic phase	*A*01:01*, *A*03:01* *B*55:01*, *A*57:03*
MPN8	75	M	CML	Litalir etc.	Blast crisis	ND
MPN9	80	M	CML	None	Chronic phase	ND
MPN10	61	M	CML	None	Chronic phase	ND

Table B.1: Patient characteristics *(continued)*

Patient	Age	Sex	Diagnosis	Previous therapy prior blood venipuncture	Stage/Type	HLA typing
MPN11	61	M	CML	Imatinib, Dasatinib, Ponatinib, Hydroxurea	Blast crisis	*A*02:01*, *A*03:01* *B*07:02*, *B*15:01*
MPN12	30	M	CML	None	Accelerated phase	*A*02:01*, *A*24:02* *B*15:01*, *B*-:-*
MDS/MPN1	76	M	CMML	Hydroxyurea		ND
MDS/MPN2	93	M	CMML	Hydroxurea		ND
AML1	37	F	AML	None	M5a	*A*02:01*, *A*32:01* *B*14.02*, *B*27:05*
AML2	33	M	AML	None	M5	*A*01:01*,*A*03:01* *B*07:02*,*B*57:01*
AML3	56	M	AML	Cytarabin, Idarubicin, Etoposid, Mitoxantron, allo HSCT	M2	*A*01:01*,*A*02:01* *B*44:02*,*B*44:03*

Table B.1: Patient characteristics *(continued)*

Patient	Age	Sex	Diagnosis	Previous therapy prior blood venipuncture	Stage/Type	HLA typing
AML4	44	F	AML	None	M2	*A*01:01*, *A*66:01* *B*08:01*,*B*35:02*
AML5	56	M	AML	None	M4Eo	ND
AML6	21	F	AML	None	M4Eo	*A*01:01*,*A*33:03* *B*07:02*,*B*58:01*
CLL1	77	M	B-CLL	Fludarabine, Cyclophosphamide	Binet C	ND
CLL2	69	F	B-CLL	None	Binet A	ND
CLL3	77	F	B-CLL	Chlorambucil	Binet C	ND

Appendix C

Primer for TCR repertoire PCR

Table C.1: TCR α variable gene segment family specific primer

Primer	Sequence
P-5'αST	CTG TGC TAG ACA TGA GGT CT
P-3'αST	CTT GCC TCT GCC GTG AAT GT
3'-Cα	GGT GAA TAG GCA GAC AGA CTT GTC ACT GGA
Vα1	AGA GCC CAG TCT GTG ASC CAG (S=C/G)
Vα1.1	AGA GCC CAG TCR GTG ACC CAG (R=A/G)
Vα2	GTT TGG AGC CAA CRG AAG GAG
Vα3	GGT GAA CAG TCA ACA GGG AGA
Vα4	TGA TGC TAA GAC CAC MCA GC
Vα5	GGC CCT GAA CAT TCA GGA
Vα6	GGT CAC AGC TTC ACT GTG GCT A
Vα7	ATG TTT CCA TGA AGA TGG GAG
Vα8	TGT GGC TGC AGG TGG ACT
Vα9	ATC TCA GTG CTT GTG ATA ATA
Vα10	ACC CAG CTG CTG GAG CAG AGC CCT
Vα11	AGA AAG CAA GGA CCA AGT GTT
Vα12	CAG AAG GTA ACT CAA GCG CAG ACT
Vα13	GAG CCA ATT CCA CGC TGC G
Vα14.1	CAG TCC CAG CCA GAG ATG TC
Vα14	CAG TCT CAA CCA GAG ATG TC
Vα15	GAT GTG GAG CAG AGT CTT TTC
Vα16	TCA GCG GAA GAT CAG GTC AAC
Vα17	GCT TAT GAG AAC ACT GCG T
Vα18	GCA GCT TCC CTT CCA GCA AT
Vα19	AGA ACC TGA CTG CCC AGG AA

Table C.1: TCR α variable gene segment family specific primer *(continued)*

Primer	Sequence
Vα20	CAT CTC CAT GGA CTC ATA TGA
Vα21	GTG ACT ATA CTA ACA GCA TGT
Vα22	TAC ACA GCC ACA GGA TAC CCT TCC
Vα23	TGA CAC AGA TTC CTG CAG CTC
Vα24	GAA CTG CAC TCT TCA ATG C
Vα25	ATC AGA GTC CTC AAT CTA TGT TTA
Vα26	AGA GGG AAA GAA TCT CAC CAT AA
Vα27	ACC CTC TGT TCC TGA GCA TG
Vα28	CAA AGC CCT CTA TCT CTG GTT
Vα29	AGG GGA AGA TGC TGT CAC CA
Vα30	GAG GGA GAG AGT AGC AGT
Vα31	TCG GAG GGA GCA TCT GTG ACT A
Vα32	CAA ATT CCT CAG TAC CAG CA

Table C.2: TCR β variable gene segment family specific primer

Primer	Sequence
P-5'βST	AAG CAG AGA TCT CCC ACA C
P-3'βST	GAG GTG AAG CCA CAG TCT G
P-3'CβII	GAT GGC TCA AAC ACA GCG ACC TC
Vβ1	GCA CAA CAG TTC CCT GAC TTG GCA C
Vβ2	TCA TCA ACC ATG CAA GCC TGA CCT
Vβ3	GTC TCT AGA GAG AAG AAG GAG CGC
Vβ4	ACA TAT GAG AGT GGA TTT GTC ATT
Vβ5.1	ATA CTT CAG TGA GAC ACA GAG AAA C
Vβ5.2	TTC CCT AAC TAT AGC TCT GAG CTG (Vβ5.2 + Vβ5.2T 1:1 MIX)
Vβ6.1	GCC CAG AGT TTC TGA CTT ACT TC
Vβ6.2	ACT CTG ASG ATC CAG CGC ACA (S=C/G)
Vβ6.3	ACT CTG AAG ATC CAG CGC ACA
Vβ7	CCT GAA TGC CCC AAC AGC TCT C
Vβ8	ATT TAC TTT AAC AAC AAC GTT CCG
Vβ8.3	GCT TAC TTC CGC AAC CGG GCT CCT
Vβ9	CCT AAA TCT CCA GAC AAA GCT
Vβ10	CTC CAA AAA CTC ATC CTG TAC CTT
Vβ11	TCA ACA GTC TCC AGA ATA AGG ACG

Table C.2: TCR β variable gene segment family specific primer *(continued)*

Primer	Sequence
Vβ12	AAA GGA GAA GTC TCA GAT
Vβ13.1	CAA GGA GAA GTC CCC AAT
Vβ13.2	GGT GAG GGT ACA ACT GCC
Vβ14	GTC TCT CGA AAA GAG AAG AGG AAT
Vβ15	AGT GTC TCT CGA CAG GCA CAG GCT
Vβ16	AAA GAG TCT AAA CAG GAT GAG TCC
Vβ17	CAG ATA GTA AAT GAC TTT CAG
Vβ18	GAT GAG TCA GGA ATG CCA AAG GAA
Vβ19	CAA TGC CCC AAG AAC GCA CCC TGC
Vβ20	AGC TCT GAG GTG CCC CAG AAT CTC
Vβ21	AAA GGA GTA GAC TCC ACT CTC
Vβ22	CAT CTC TAA TCA CTT ATA CT
Vβ23	GCA GGG TCC AGG TCA GGA CCC CCA
Vβ24	ATC CAG GAG GCC GAA CAC TTC T

Appendix D

Complete sequence of the TCR 2.5D6

D.1 α chain

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        M  L  L  E  L  I  P  L  L  G  I  H  F  V  L  R  T  A
alpha   ATGCTGCTGGAACTGATCCCCCTGCTGGGAATCCACTTCGTGCTGAGGACAGCCA    55

        R  A  Q  S  V  T  Q  P  D  I  H  I  T  V  S  E  G  A  S
alpha   GAGCCCAGTCTGTGACCCAGCCCGACATCCACATCACCGTGTCTGAGGGCGCCAG   110

          L  E  L  R  C  N  Y  S  Y  G  A  T  P  Y  L  F  W  Y
alpha   CCTGGAACTGCGGTGCAACTACTCTTACGGCGCCACCCCCTACCTGTTTTGGTAC   165

        V  Q  S  P  G  Q  G  L  Q  L  L  L  K  Y  F  S  G  D
alpha   GTGCAGAGCCCCGGACAGGGACTGCAGCTGCTGCTGAAGTACTTTAGCGGCGACA   220

        T  L  V  Q  G  I  K  G  F  E  A  E  F  K  R  S  Q  S  S
alpha   CCCTGGTGCAGGGCATCAAGGGATTCGAGGCCGAGTTCAAGCGGAGCCAGAGCAG   275

          F  N  L  R  K  P  S  V  H  W  S  D  A  A  E  Y  F  C
alpha   CTTCAACCTGCGGAAGCCCTCCGTGCATTGGAGCGACGCCGCCGAGTATTTCTGT   330

        A  G  R  A  A  G  N  K  L  T  F  G  G  G  T  R  V  L
alpha   GCCGGCAGAGCAGCCGGCAACAAGCTGACATTTGGCGGCGGAACCAGGGTGCTCG   385

        V  K  P  N  I  Q  N  P  E  P  A  V  Y  Q  L  K  D  P  R
alpha   TGAAGCCCAACATCCAGAACCCCGAGCCCGCCGTGTACCAGCTGAAGGACCCTAG   440

          S  Q  D  S  T  L  C  L  F  T  D  F  D  S  Q  I  N  V
alpha   AAGCCAGGACAGCACCCTGTGCCTGTTCACCGACTTCGACTCCCAGATCAACGTG   495

        P  K  T  M  E  S  G  T  F  I  T  D  K  T  V  L  D  M
alpha   CCCAAGACCATGGAAAGCGGCACCTTCATCACCGACAAGACCGTGCTGGACATGA   550
```

```
        K  A  M  D  S  K  S  N  G  A  I  A  W  S  N  Q  T  S  F
alpha   AGGCCATGGACAGCAAGAGCAACGGCGCCATTGCCTGGTCCAATCAGACCAGCTT   605

          T  C  Q  D  I  F  K  E  T  N  A  C  Y  P  S  S  D  V
alpha   CACATGCCAGGACATCTTCAAAGAGACAAACGCCTGCTACCCCAGCAGCGACGTG   660

           P  C  D  A  T  L  T  E  K  S  F  E  T  D  M  N  L  N
alpha   CCCTGTGATGCCACCCTGACCGAGAAGTCCTTCGAGACAGACATGAATCTGAACT   715

        F  Q  N  L  S  V  M  G  L  R  I  L  L  L  K  V  A  G  F
alpha   TCCAGAACCTGAGCGTGATGGGCCTGAGAATCCTGCTGCTGAAAGTGGCCGGCTT   770

          N  L  L  M  T  L  R  L  W  S  S  .
alpha   CAATCTGCTGATGACCCTGAGACTGTGGTCCAGCTGA   807
```

D.2 β chain

```
         M  S  L  G  L  L  C  C  G  V  F  S  L  L  W  A  G  P
beta    ATGTCTCTGGGACTGCTGTGTTGCGGCGTGTTCAGCCTGCTGTGGGCCGGACCTG    55

        V  N  A  G  V  T  Q  T  P  K  F  R  V  L  K  T  G  Q  S
beta    TGAATGCCGGCGTGACCCAGACCCCCAAGTTCCGGGTGCTGAAAACCGGCCAGAG   110

          M  T  L  L  C  A  Q  D  M  N  H  E  Y  M  Y  W  Y  R
beta    CATGACCCTGCTGTGCGCCCAGGACATGAACCACGAGTACATGTATTGGTACAGA   165

         Q  D  P  G  M  G  L  R  L  I  H  Y  S  V  G  E  G  T
beta    CAGGACCCCGGCATGGGCCTGCGGCTGATCCACTATTCTGTGGGCGAGGGCACCA   220

        T  A  K  G  E  V  P  D  G  Y  N  V  S  R  L  K  K  Q  N
beta    CCGCCAAGGGCGAAGTGCCTGATGGCTACAACGTGTCCCGGCTGAAGAAGCAGAA   275

          F  L  L  G  L  E  S  A  A  P  S  Q  T  S  V  Y  F  C
beta    CTTCCTGCTGGGCCTGGAAAGCGCCGCTCCTAGCCAGACCAGCGTGTACTTCTGC   330

         A  S  S  Y  S  S  G  Q  P  Q  H  F  G  D  G  T  R  L
beta    GCCAGCAGCTACAGCAGCGGCCAGCCTCAGCACTTTGGCGACGGCACCAGACTGA   385

        S  I  L  E  D  L  R  N  V  T  P  P  K  V  S  L  F  E  P
beta    GCATCCTGGAAGATCTGCGGAACGTGACCCCCCCCCAAGGTGTCCCTGTTCGAGCC   440

          S  K  A  E  I  A  N  K  Q  K  A  T  L  V  C  L  A  R
beta    TAGCAAGGCCGAGATCGCCAACAAGCAGAAAGCCACCCTCGTGTGCCTGGCCAGA   495

         G  F  F  P  D  H  V  E  L  S  W  W  V  N  G  K  E  V
beta    GGCTTCTTCCCCGACCACGTGGAACTGTCTTGGTGGGTCAACGGCAAAGAGGTGC   550

        H  S  G  V  S  T  D  P  Q  A  Y  K  E  S  N  Y  S  Y  C
beta    ACAGCGGCGTGTCCACCGATCCCCAGGCCTACAAAGAGAGCAACTACAGCTACTG   605
```

```
        L  S  S  R  L  R  V  C  A  T  F  W  H  N  P  R  N  H
beta  CCTGTCCAGCAGACTGAGAGTGTGCGCCACCTTCTGGCACAACCCCCGGAACCAC   660

       F  R  C  Q  V  Q  F  H  G  L  S  E  E  D  K  W  P  E
beta  TTCAGATGCCAGGTGCAGTTTCACGGCCTGAGCGAAGAGGACAAGTGGCCCGAGG   715

      G  S  P  K  P  V  T  Q  N  I  S  A  E  A  W  G  R  A  D
beta  GCAGCCCTAAGCCCGTGACACAGAATATCAGCGCCGAAGCCTGGGGCAGAGCCGA   770

        C  G  I  T  S  A  S  Y  H  Q  G  V  L  S  A  T  I  L
beta  CTGTGGAATCACCAGCGCCAGCTACCATCAGGGCGTGCTGAGCGCCACCATCCTG   825

       Y  E  I  L  L  G  K  A  T  L  Y  A  V  L  V  S  G  L
beta  TACGAGATTCTGCTGGGCAAGGCCACCCTGTACGCCGTGCTGGTGTCTGGCCTGG   880

      V  L  M  A  M  V  K  K  K  N  S  .
beta  TGCTGATGGCCATGGTCAAGAAGAAGAACAGCTAA   915
```

Appendix E

HLA-*B*07:02* ligands details

Table E.1: Details of the panel of HLA-*B*07:02* ligands (kind gift of S. Stevanovic)

Pool	Sequence	Gene
1	EPRPVFAV	?
	FPESLMVGLAV	?
	IARNLTQQL	ADFP
	RPELVRPAL	AKR1C3
	GPALGRSFL	CD70
	APRGPHGGAASGL	CTG1B
	KPGKFVTTL	DCAM
	FPNIPGKSL	DEGS1
	IPQIRNPSL	DPYD
	NPRTQTHATL	DPYD
2	RPQGGSRPEFV	EAD_EBV
	RPQGGSRPEFVKL	EAD_EBV
	RPQGGSRPEF	EAD_EBV
	RPPIFIRRL	EBNA3_EBV
	WPMGYRTAT	EBNA6_EBV
	KPNANRIAL	LGALS3
	LPHAPGVQM	HDAC1
	APAPIHNQF	HERP1
	RPTLWAAAL	IBP3
	APRAPRVPR	IE63_EBV
3	VPTRGSLEL	IMP3
	RPRAATVVA	KAP1
	TPKEKAQAL	KI67
	VPLIQSRI	KLK2

Table E.1: Details of the panel of HLA-*B*07:02* ligands *(continued)*

Pool	Sequence	Gene
	RPPPIGAEV	MCL1
	HPEYNRPLL	KLK4
	FPKGFSVEL	LRC42
	RPKSSLPPVL	MYLK
	SPQQVDFRSVL	MYLK
	SPIKVTLATL	NPM
4	RPILTIITL	P53
	LPDAQRLY	P96258
	APRGKSGAAL	PARP1
	QPMEPNPRVPL	PERM
	LPNGTRVPM	PIM1
	RPHERNGFTVL	pp65_HCMVA
	TPRVTGGGAM	pp65_HCMVA
	RPHERNGFTVL	pp65_HCMVA
	IPQRLVNVVL	PRTN3
	QPVPHGTQCL	PRTN3
5	SPSSILSTL	PPTG1
	KPNANRIAL	Q6FGLO
	KPSKDGVTV	Q6FHD2
	GPRTAALGLL	RCN2
	GPRYSTQRGV	RET3
	GPHYSTQRGV	RET3
	TPHQTFVRL	RPN2
	IPRAALLPLL	HTRA1
	SPYQNIKIL	SPSY
	YPDRIMNTF	TBB2A
6	APYSRPKQL	THOC4
	HPTSVISGY	TCPA
	LPDDKVTAL	TMPS3
	RPRALPGHL	VHL
	RPMSLRSTII	VO01
	LPNVGKSTLF	OLA1
	QPAKTSSVSL	ZCCHV
	EPAKTSSVSL	ZCCHV

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Danksagung

Mein erster Dank gilt Prof. Dr. med. Angela Krackhardt für die Bereitstellung des Themas, die ausgezeichnete Betreuung und dafür, dass sie trotz doppelter Belastung aufgrund ihrer klinischen Verpflichtungen für ausgiebige Diskussionen und Ratschläge immer Zeit gefunden hat.

Einen großen Dank möchte ich Frau Prof. Dr. Iris Antes für die Übernahme des Erstgutachtens meiner Doktorarbeit aussprechen!

Bei Herrn Prof. Dr. Arne Skerra möchte ich mich recht herzlich für die Übernahme des Prüfungsvorsitzes bedanken.

Ein großer Dank geht an alle Mitglieder der Arbeitsgruppe Krackhardt für die schöne Zeit und die Unterstützung! Besonders möchte ich mich bei Martina Rami, Sebastian Schober, Sabine Mall und Stephanie Rämisch bedanken, die maßgeblich am Erfolg des Projekts mitgewirkt haben. Eva Bräunlein danke ich für Diskussionen und Blutspenden! Den ehemaligen Mitgliedern der AG Krackhardt Luise Weigand, Xiaoling Liang und Elfriede Eppinger danke ich für das Einlernen und eine schöne Zeit im Labor.

Herrn Prof. Dr. med. Christian Peschel danke ich dafür, dass es mir möglich war die Laborräume der III.Medizinischen Klinik am Klinikum rechts der Isar für meine Doktorarbeit zu nutzen.

Der letzte und größte Dank gebührt natürlich Kathrin, die mich während der Zeit mit ihrer Liebe und Zuneigung immer unterstützt hat!

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die der Fakultät für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:
Identification of naturally presented HLA ligands on the surface of healthy and malignant hematopoetic cells and their therapeutic potential as targets for TCR-transgenic T cells
im Department für Biowissenschaftliche Grundlagen der Technischen Universität München unter der Anleitung und Betreuung durch Univ. Prof. Dr. sc. nat. Iris Antes ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 5 angegebenen Hilfsmittel benutzt habe.
(x) Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.
( ) Die vollständige Dissertation wurde in .....veröffentlicht.
Die Fakultät für .....
hat der Vorveröffentlichung zugestimmt.
(x) Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.
( ) Ich habe bereits am ..... bei der Fakultät für ..... der Hochschule ..... unter Vorlage einer Dissertation mit dem Thema ..... die Zulassung zur Promotion beantragt mit dem Ergebnis: ..... Die Promotionsordnung der Technischen Universität München ist mir bekannt.

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Publikationen

Klar R., Schober S., Rami M., Mall S., Merl J., Hauck S.M., Ueffing M., Admon A., Slotta-Huspenina J, Stevanovic S., Oostendorp R.A.J., Busch D.H., Peschel C and Krackhardt A.M. **Therapeutic targeting of naturally presented myeloperoxidase-derived HLA peptide ligands in myeloid leukemia cells by TCR-transgenic T cells.** *Leukemia.* 2014 Dez;28(12):2355-66

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Kongressbeiträge

ITOC 2014, München (poster): **Targeting naturally presented, leukemia-derived HLA ligands with TCR-transgenic T cells for the treatment of therapy refractory leukemias.** (**Klar R.**, Schober S., Rami M., Mall S., Merl J., Slotta-Huspenina J., Oostendorp R., Stevanovic S., Busch D.H., Peschel C., Krackhardt A.M.)

CIMT 2013, Mainz (poster): **Immunopeptidomic identification of naturally presented myeloperoxidase epitopes presented on primary myeloid leukemia cells and their potential as targets for T cell receptor gene modified donor lymphocyte infusions.** (**Klar R.**, Schober S., Rami M., Rämisch S., Merl J., Hauck S., Ueffing M., Admon A., Barnea E., Kremmer E., Oostendorp R., Stevanovic S., Busch D.H., Peschel C., Krackhardt A.M.)

EBMT 2013, London (poster): **Immunoproteomic identification of naturally presented epitopes presented on primary myeloid leukemia cells and their potential as targets for T cell receptor gene modified donor lymphocyte infusions.** (**Klar R.**, Schober S., Rami M., Sarioglu H., Hauck S., Ueffing M., Admon A., Barnea E., Kremmer E., Oostendorp R., Stevanovic S., Busch D.H., Peschel C., Krackhardt A.M.)

DGHO 2011, Basel (poster): **Immunoproteomic identification of epitopes presented on healthy and malignant hematopoetic cells and their potential as targets for T cell mediated novel immunotherapies in B cell neoplasms.** (**Klar R.**, Sarioglu H., Hauck S., Ueffing M., Admon A., Stevanovic S., Kremmer E., Busch D.H., Peschel C., Krackhardt A.M.)

CIMT 2011, Mainz (oral presentation): **"Immunoproteomic identification of epitopes presented on healthy and malignant hematopoetic cells and their potential as targets for T cell mediated immunotherapy in patients with leukemia".** (**Klar R.**, Sarioglu H., Hauck S., Ueffing M., Eppinger E., Admon A., Ringshausen I., Stevanovic S., Kremmer E., Busch D.H., Peschel C., Krackhardt A.M.)