Exploiting HLA-alloreactivity in TCR gene therapy of B cell malignancies

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General Introduction and Aim of the Study

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Introduction

B Cell Development

B cells form an integral part of the host defense by guiding the immune system in the recognition and eradication of pathogens. B cells exert these functions through the expression of clonotypic immunoglobulins (Ig). Ig molecules can either be expressed as membrane-bound B cell receptors (BCR) or secreted as antibodies. An Ig molecule is composed of two heavy and two light chains. Within one Ig molecule, the two heavy and two light chains are identical. The heavy chains are linked to each other by disulfide bonds; each light chain is linked to one heavy chain by a disulfide bond. Both heavy and light chain contain a variable (V) and a constant (C) region. The C region provides primarily structural integrity and functional properties. Based on the C region of the heavy chain, Ig molecules can be distinguished into five classes – IgM, IgD, IgG, IgA and IgE. For the light chain two classes exists – lambda (λ) or kappa (κ). In contrast to the C region, the V region contains hypervariable domains that differ greatly in the peptide sequence between distinct Ig molecules. These hypervariable regions form the antigen-binding domain of the Ig molecule. The enormous variety in the V regions between Ig molecules produces a great diversity in the antibody repertoire allowing for the recognition of a vast and ever evolving array of pathogens. The C regions with their conserved sequences link the antigen specificity of Ig molecules to other components of the immune system.

The development, maturation, and differentiation of a B cell revolves around and is markedly influenced by the assembly of a functional BCR[1]. Pro-B cells, the earliest B-lineage cell, are derived from common lymphoid progenitor cells which are derived from hematopoietic stem cells residing in the bone marrow[2]. The bone marrow's cellular and cytokine microenvironment provide support for the developing pro-B cell through the pre-B cell stage into mature B cells. At each stage genetic rearrangement, V(D)J recombination, at the Ig gene locus occurs. Rearrangement starts with the heavy chain gene locus during the pre-B cell stage. Production of a functional IgM heavy chain is controlled by the expression of a pre-BCR. The rearranged heavy chain pairs with two invariant surrogate light chains called λ5 and VpreB that together form the pre-BCR. In addition, the invariant proteins Igα (CD79a) and Igβ (Cd79b) associate with the pre-BCR. As a heterodimer, CD79a/b are critically involved in BCR transport[3] and functionality by providing signaling capacity[4-8] to regulate processes such as allelic exclusion, proliferation, differentiation, anergy and apoptosis [9-11]. CD79a and CD79b will form an important part of the BCR expressed throughout the mature B cell stage. Transient expression of a functional pre-BCR leads to survival and proliferation of pro-B cells. Furthermore, B-lineage specific markers such as CD22 and CD19 are expressed for the first time. CD19 is crucial for setting intrinsic B cell signaling thresholds and modulates signaling driven by BCR-dependent and independent events[12-14]. CD19 is membrane-bound and exerts its function by interacting with various protein kinases[15]. In contrast, CD22 is only located in the cytosol at this stadium, but it will eventually be expressed extracellularly

at later developmental stages. Moreover, the membrane-embedded protein CD20 is weakly expressed in late pro-B cells and functions as a calcium channel modulating B cell responses[16]. The expression of CD20 will gradually increase with the maturation status of the B cell reaching its peak at the mature B cell stage. Development of pro-B cells continues by entering the pre-B cell stage and rearrangement of the light chain gene locus begins. If a functional light chain can be produced, it pairs with the rearranged IgM heavy chain and a functional BCR will be expressed on the cell surface; the pre-B cell enters the immature B cell stage. Immature B cells will express CD22 on the cell surface for the first time[17]. Failure to produce a functional heavy or light chain at any stage leads to apoptosis and elimination of the developing B cell.

Before immature B cells egress into the periphery as naïve B cells, the antigen specificity of the BCR is tested to exclude recognition of self-antigens. This process is known as central tolerance as it occurs in a central lymphoid organ, the bone marrow. Immature B cells with strong reactivity towards self-antigens are removed from the B cell repertoire by clonal deletion or arrestment of further development and entering a state of anergy. It is also possible for a self-reactive immature B cell to undergo additional receptor editing to produce a new BCR without specificity for self-antigens. Naïve B cells without strong reactivity for self-antigens leave the bone marrow and enter peripheral lymphoid organs such as the spleen and lymph nodes where the maturation and differentiation continues.

The spleen and lymph nodes are highly structured organs where the adaptive immune response to pathogens is initiated. Each compartment is characterized by the interaction between different types of lymphocytes. Naïve B cells entering the lymph nodes and spleen will pass through the paracortical area and the periarteriolar lymphoid sheet, respectively. In these areas antigen-presenting dendritic cells and helper T (T_H) cells interact with and can activate naïve B cells that have encountered their cognate antigen. Activated B cells migrate further into the primary and secondary lymphoid follicles that contain germinal centers. In germinal centers, activated B cells undergo immunoglobulin somatic hypermutation, further altering the V regions of the Ig molecule to increase its affinity for the cognate antigen[18]. This process is paired with the massive proliferation of activated B cells displacing many resting B cells to the neighboring mantle zone. In B cells that have successfully altered their Ig molecules, class-switch recombination of the C region of the heavy chain permanently determines the class of the Ig molecule. CD22 is highly expressed on B cells during the germinal center reaction acting predominately as an inhibitory coreceptor and playing an important role in BCR signaling threshold. Via its extracellular domain CD22 binds to sialylated carbohydrates, while its intracellular domain contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) capable of activating phosphatases which in turn can dampen positive components of the B cell receptor signaling cascade[19]. Fully matured B cells egress from the germinal centers either as plasma cells or memory B cells. Memory B cells circulate in the periphery and drive secondary immune responses to the same pathogen. As fully matured B cells, memory B cells continue to express typical B-lineage markers such as CD19, CD20, CD22 and CD79a/b. In contrast, plasma cells homing to the bone marrow or spleen,

where they will secrete high affinity antibodies providing life-long immunity, cease to express the aforementioned B cell markers.
In the spleen, the follicles are surrounded by marginal zones harboring marginal zone B cells. These are mature non-circulating B cells that play an important role to combat blood-borne pathogens.

B Cell Malignancies

The process of V(D)J recombination to produce functional Ig molecules involves double-stranded DNA breaks[20]. DNA breaks are then resolved by non-homologous end-joining, a process that carries the risk of aberrant chromosomal translocation. Similarly, somatic hypermutation and class-switch recombination necessitate the introduction of DNA breaks and, therefore, also carry the chance of faulty DNA repair and translocations[21-23]. Chromosomal translocation can result in disruptive expression of genes that regulate survival and proliferation. Therefore, B cell development can give rise to uncontrollably dividing cells, and indeed B cell malignancies characteristic for all developmental stages have been described[24, 25].
The earliest B-lineage cells such as pro- and pre-B cell can give rise to acute lymphoblastic leukemia (ALL)[26]. ALL is characterized by highly proliferating cells occupying the blood and bone marrow, eventually disrupting and inhibiting normal hematopoiesis[27]. Similar to their normal counterparts, the V regions of the Ig gene locus have not been altered yet and leukemia's stemming from pre-B cells can express the pre-BCR complex. As early progenitors in the B cell lineage, ALL cells often express CD34 also found on hematopoietic stem cells. Furthermore, ALL blast can be positively identified if they express any two of the earliest B-lineage markers CD19, CD22 or CD79a while other markers commonly found on early B cell progenitors are lacking[28]. Other differentiation markers such as CD20 of more developed B cells can be expressed depending on the maturation status of the diseased cells.
The most prevalent leukemia, chronic lymphocytic leukemia (CLL), arises from uncontrollably dividing activated or memory B cells in the blood[29]. Malignant cells of CLL origin are characterized by the expression of CD79a and CD79b, and CD19. Often, cells also express CD20 and CD22 albeit to a weaker extent[30]. The course of disease can vary greatly between patients with some surviving for many years without therapy, whereas in other cases CLL rapidly progresses despite aggressive therapy[31]. CLL can be categorized into cells expressing unmutated or mutated BCRs, while CLLs expressing unmutated BCRs are more aggressive[32].
Besides leukemias, aberrantly proliferating B cells can give rise to lymphomas, i.e. neoplasms residing in the lymphoid tissues. Lymphomas can be categorized based on the cell of origin[33, 34]. Naïve, pre-germinal center B cells residing in the mantle zone of secondary follicles can give rise to mantle cell lymphoma (MCL) and account for 7-8 % of all lymphomas[35, 36]. Similar to their healthy counterpart, MCL cells express CD19 and CD20. MCLs most commonly express BCRs in which the V regions have not been altered[36]. Patients suffering from mantle cell lymphoma remain difficult to treat with survival rates among the first 3-5 years not

exceeding 50 % and cure rates of less than 10 %[30].

Germinal center B cells can give rise to follicular lymphoma (FL) and Burkitt's lymphoma (BL). Follicular lymphoma is the most frequently encountered lymphoma and accounts for approximately 25 % of all B cell neoplasm. Cells of follicular lymphomas can be characterized by the expression of CD19 and CD20 among other cell markers. Ig molecules of malignant clones are often mutated and somatic hypermutation can be ongoing[37]. Nonetheless, FL cells seem to require a structurally integer and functional BCR for survival since a selective bias is observed that appears to prevent the introduction of deleterious mutation in the Ig molecules[38, 39]. Patients of indolent FL can survive many years without treatment, however, resistance to common therapy or transformation to a more aggressive form can quickly worsen the prognosis. This observation is mirrored in survival rates exceeding 70 % at 10 years, while the cure rate remains low, under 20 %[30]. In contrast, Burkitt's lymphoma is a rarely encountered B cell malignancy representing less than 1 % of all cases. Malignant cells can be immunophenotypically characterized by the expression of CD19, CD20, and CD22. Although a disease of the lymph node, malignant cells can spread to the abdomen, ovaries, kidneys and other sites[40]. BLs remain among the lymphomas with the highest cure rate of about 80 %[37].

Diffuse large B cell lymphoma (DLBCL) forms a heterogeneous disease with clinical outcomes varying greatly between patients[41]. Diseased cells arise from germinal center B cells, however, a subclass of DLBCL resembles post-germinal center B cells. Malignant cells express typical B cell markers such as CD19, CD20, and CD22. Ig genes can be highly mutated and ongoing SHM can be observed in malignant clones[42]. DLBCL accounts for about 32 % of all lymphomas and conventional chemotherapy cures about 40 % of patients[30].

Hodgkin lymphomas (HL) represent about 10 % of all lymphoid malignancies and are most prevalent in young adults. HL stems from germinal center B cells in which the V regions of Ig genes have been mutated[43]. Although derived from mature B cells, diseased cells of Hodgkin Lymphoma have mainly lost their B cell phenotype and therefore often lack the expression of CD19, CD20 and CD22[44, 45]. Current therapies achieve cure rates upward of 80 % in patients suffering from HL[30].

Finally, multiple myeloma is a neoplasm of the antibody secreting plasma cells. As such, they do not express the typical B cell markers CD19, CD20, and CD22. While CD79a is expressed by healthy and diseased plasma cells, CD79b is not[46]. Malignant plasma cells can occupy bone marrow but also various extramedullary sites where normal hematopoiesis is destroyed and bone destruction is observed. Furthermore, secretion of high amounts of paraprotein, an abnormal antibody, can cause kidney failure[47]. Multiple myeloma accounts for 13 % of hematological cancers.

Immunotherapy of B Cell Malignancies

Chemotherapy and radiation therapy remain the standard treatment modality for B cell malignancies. Both treatment options aim at arresting cell division or inflicting genomic

damage leading to apoptosis and cell death. Malignant cells are especially impacted due to their fast cell cycles and lack of properly functioning DNA repair mechanism. Although systemic administration of chemotherapy and radiation therapy also causes severe damage to healthy tissue, intact DNA repair mechanisms and slower cell growth reduce the toxicity inflicted to healthy cells compared to cancerous cells. Besides chemo- and radiotherapy, patients can be subjected to targeted therapies better tailored to exploit weaknesses in malignant cells. For instance, small molecules can be used to inhibited important components of the BCR signaling cascade since BCR signaling is essential for B cell lymphomagenesis[48]. Additionally, genes that have gained activating mutations and drive tumorigenesis can be the focus of targeted therapy[49]. Furthermore, disrupting cellular function such as protein degradation by proteasome inhibition or ubiquitination pathways have emerged as viable strategies in the treatment of lymphoproliferative disorders[50, 51].

However, considerable advancement in the therapy of B cell malignancies has been achieved by amending conventional regimens with immunotherapeutic interventions. One such immunotherapeutic approach can involve the administration of monoclonal antibodies (mAbs) targeting cell surface-expressed antigens such as CD20 and CD22[52-55]. Monoclonal antibodies bind with high antigen specificity to target cells and mark them for destruction by various mechanisms. mAbs can induce direct apoptosis of the target cells, however, eradication by activating the immune system is a more common mechanism of action. Cells labelled with mAbs can either be lysed by complement-dependent cytotoxicity (CDC)[56] or antibody-induced cellular cytoxicity (ADCC)[57]. CDC is mediated by a cascade of proteolytic enzymes present in the blood serum. Ultimately, activation of the complement system leads to the formation of the membrane-attack complex making the membrane porous resulting in cell lysis and death. In contrast ADCC, is mediated by immune cells such as NK cells but also macrophages, neutrophils, and eosinophils. NK cells ligate via their Fc-receptors to the C regions of mAbs that are bound to their cognate antigen. Upon ligation, NK cells become activated and release cytokines such as IFN-γ but also granzymes such as proteases and perforin that induce lysis of targeted cells. Besides activation of the immune system, mAbs can also be conjugated to drugs or radioactive particles[58, 59]. Antibody-drug conjugates allow for the delivery of a cytotoxic dose to cancerous cells with higher precision than possible with systemic administration of the active drug. Nonetheless, refractory or relapsed disease have been reported after treatment with mAbs. Mechanism of resistance can vary from downregulation of the target-antigen[60-62], internalization of the antigen-antibody complex before activation of the immune system can take place[63, 64], and upregulation of inhibitors of the complement cascade[65-67]. Furthermore, the effectiveness of mAbs can be hampered by a weakened or suppressed patient's immune system due to additional chemo- or radiotherapy. More recently, chimeric antigen receptors (CARs) used to modify the antigen specificity of T cells have had a great impact in the treatment of hematological malignancies[68-73]. CARs are extracellular transmembrane designer molecules that can install desired antigen specificities onto T cells[74, 75]. The antigen-binding moiety is derived from the antigen-binding domain of antibodies. The antigen-binding moiety is linked via a stem structure and an

membrane-spanning region to intracellular signaling molecules that cause activation of T cells upon binding of the CAR to its respective antigen. The intracellular signaling domains are often derived from the same molecule that are important for T cell activation upon TCR engagement such as CD28 and the CD3ζ-chain. CARs bind to their antigen directly which induces T cell activation[76]. However, the CAR-targeted antigen must be cell surface-expressed to be available for binding to the CAR. While the former greatly increases the applicability of CAR-engineered T cells irrespective of the human leukocyte antigen (HLA) genotype of a patient, the latter restricts the antigen target-pool available for CARs as promising intracellular antigens are not accessible to CARs. Nonetheless, CAR-engineered T cells have been successfully applied in the treatment of ALL, CLL and lymphomas by targeting the B cell-specific antigen CD19. Especially in the treatment of ALL, complete remission rates of 50-70% have been achieved[69-72, 77-79].

Besides CAR-based immunotherapies, the cytotoxic effector function of T cells is also exploited in a novel approach utilizing bispecific T cell engager (BiTe) molecules[80]. BiTes are designer molecules composed of two different antigen-binding domains derived from monoclonal antibodies linked by a polypeptide chain. One antigen-binding moiety is directed against a cell-surface antigen expressed on the malignant cell population, while the other antigen-binding domain is specific for the CD3ε-chain expressed on T cells. As a result, T cells are effectively crosslinked to the targeted cell population and T cell activation through the engagement of the CD3ε-chain occurs. Following crosslinking, activated T cells secrete perforins and granzymes leading to apoptosis of the targeted cell[81]. Similar to CARs, BiTes can be applied irrespective of the HLA genotype of a patient. However, the targeted antigen on the malignant cell population must be cell surface expressed.

B cell-expressed differentiation antigens such as CD19 and CD20 have been successfully targeted with CAR-engineered T cells[68-73, 82] and mAbs[52-55]. Complete remissions have been reported in the treatment of CD19- and CD20-expressing ALL and lymphomas. In both treatment strategies, depletion of the healthy B cell compartment was observed[53, 71, 73, 83, 84]. Ablation of healthy B cells correlated with the continued persistence of circulating CAR-modified T cells in patients. Nonetheless, resulting hypogammaglobulinema due the lack of an existing B cell compartment is well manageable and can be treated with immunoglobulin transfusions[73, 85, 86].

T Cells

T cells form a subpopulation of lymphocytes and are distinguishable from other white blood cells by the expression of a clonotypic T cell receptor (TCR). The TCR is a transmembrane-spanning heterodimer composed of an α and β chain (TCRα and TCRβ, respectively) that are linked by a disulfide bond. Embedded in the cell membrane, the TCR is associated with the CD3 protein complex which provides intracellular signaling capacity. Both, the TCRα and TCRβ chain, can be segmented into a variable and constant region. Similar to the BCR, the V regions of both chains form the antigen-binding domain whereas the C regions provides

structural integrity. During T cell development the rearrangement of these chains and the introduction of random mutations in the complementary determining regions (CDRs) within the V regions make up the vast diversity of the TCR repertoire. On the cell surface, the TCR is associated with the CD3 protein complex. Three different co-receptor chains called CD3γ, CD3δ, CD3ε form the CD3 protein complex in association with the CD3ζ chain. Together, the TCR and CD3 protein complex form the TCR complex that induces T cell activation upon antigen encounter.

T cell development starts with the migration of T cell progenitors that are derived from hematopoietic stem cells residing in the bone marrow. T cell progenitors home to the thymus where V(D)J recombination of the TCRα and TCRβ chain occur to form a functional TCR. TCR-expressing T cells are subjected to positive and negative selection[87, 88]. T cells expressing TCRs that bind weakly to HLA molecules presenting self-peptides receive important survival signals ensuring the further development of the T cell. Furthermore, this step of positive selection of T cells ensures that T cells egressing from the thymus express TCRs capable of interacting with HLA molecules presenting peptides derived from pathogens. In contrast, T cells that interact strongly via their TCRs with self-peptides bound to HLA molecules are deleted from the T cell repertoire. Negative selection of self-reactive T cells establishes immunological tolerance and limits the occurrence of autoimmune diseases. T cells having successfully navigated thymic development egress either as $CD4^+$ or $CD8^+$ T cells into the periphery.

For the activation of naïve $CD8^+$ T cells various simultaneous signals are required. First, the TCR should bind to its cognate peptide bound to a HLA class I molecule. The interaction between TCR and peptide-HLA (pHLA) complex is further stabilized by the CD8 coreceptor that also engages the HLA molecule. Furthermore, essential costimulation is provided by the interaction between CD28 on T cells and CD80 and CD86 on antigen presenting cells (APC). Proper activation and upregulation of costimulatory molecules on APCs is promoted by $CD4^+$ T_H cells. Furthermore, $CD4^+$ T_H cells secrete cytokines that provide additional stimuli to $CD8^+$ T cells and drive their proliferation. Once activated, $CD8^+$ T cells will also produce cytokines such as IFN-γ, GM-CSF and TNF-α, but also actively engage antigen-presenting target cells. Secretion of perforin and granzymes by $CD8^+$ T cells will lead to the lysis of the target cells[89, 90]. $CD8^+$ T cells can also induce apoptosis by interacting with FAS molecules on target cells. Antigen encounter also results in the massive proliferation of antigen-specific effector T cells. Upon clearance of target cells, this effector T cell pool contracts and the formation of a T cell memory compartment provides immunological memory that guides a quicker and more efficient secondary immune response upon reencounter of the antigen[91-93].

The degree of interaction between T cells and target cells is markedly influenced by the expression of costimulatory and adhesion molecules on both cells as well as the binding strength of the TCR to its cognate pHLA molecule. The strength of interaction between TCR and pHLA molecule is termed affinity, whereas the sum of all interaction between T cell and target cell determines the avidity. Naïve T cells rely on the interaction not only of the TCR with its cognate pHLA complex to reach their activation threshold, but also on the simultaneous signaling of the costimulatory molecules expressed on APCs[94, 95]. Once activated, naïve T cells

express chemokine receptors allowing them to migrate to infected tissue and perform their effector functions. In contrast, antigen-experienced memory and effector T cells require less to none costimulation. The binding of the TCR to its antigen often suffices for the activation of memory and effector T cells resulting in the lysis of target cells. The activation of antigen-experienced T cells is therefore, solely dependent on the affinity of the TCR.

Antigen Recognition by T Cells

The antigenic structure recognized by T cells via their T cell receptor (TCR) is composed of the cell surface expressed major histocompatibility complex (MHC) molecules presenting short polypeptide sequences. In humans, MHC molecules are called human leukocyte antigen (HLA). HLA molecules can be divided into two classes. Both classes are transmembrane molecules. HLA class I molecules are composed of a β_2 microglobulin and heavy α-chain. Due to the closed peptide-binding groove, HLA class I molecules are limited to the presentation of 8-15 amino acid-long peptides[96, 97]. In contrast, HLA class II molecules are heterodimers composed of an α-chain and β-chain that together form the peptide-binding groove. The structural difference of HLA class II molecules results in an open binding grove than can accommodate 12-25 amino acid-long peptides[96]. HLA class I molecules present peptides derived from endogenous proteins to $CD8^+$ T cells, whereas MHC class II molecules sample the extracellular space and present these peptides to $CD4^+$ T cells. The presentation of peptides of HLA class I starts with the ubiquitination of proteins marking them for degradation by proteasomes[98, 99]. Proteasomes release peptide fragments that may be further shortened by peptidases. Small peptides are shuttled from the cytosol to the lumen of the endoplasmatic reticulum (ER) by the transporter associated with antigen processing (TAP)[100]. In the ER lumen, HLA class I molecules are loaded with these peptides. HLA class I molecules display a preference as to the amino acids at a certain positions within the peptide. These positions are called anchor positions and interact closely with residues in the peptide-binding pocket of the HLA molecule. Anchor residues matching the preference of a HLA molecule lead to the formation of a stable peptide-HLA complex that is transported to the cell surface for presentation to $CD8^+$ T cells. The HLA-presented peptidome is limited to self-HLA molecules and samples the cellular proteome[101]. Since HLA class I molecule sample the endogenous proteome of a cell, HLA class I molecules constantly communicate the healthy or diseased status of the cell. Therefore, HLA class I molecules can be found on all nucleated cells. In contrast, HLA class II molecules are loaded with peptides derived from proteins of the extracellular space that are acquired by endocytosis. The expression of HLA class II molecules is mainly restricted to cells of hematopoietic origin such as B cells, monocytes, dendritic cells and macrophages. However, inflammation can induce the expression of HLA class II also on nonhematopoietic cells[102, 103].

HLA class I and II molecules represent a diverse class of molecules that are structurally highly similar but can differ nonetheless in regions that interact with the antigen-binding domain of TCRs.

Cancer Therapy by TCR Gene Transfer

As mentioned earlier, T cells engineered to express CARs targeting cell surface-expressed B cell-specific antigens have revolutionized the field of immunotherapy for B cell malignancies. The adoptive cell transfer (ACT) of unmodified T cells in the treatment of hematological malignancies has, however, been applied for decades in the form of donor lymphocyte infusions (DLI) in the context of allogeneic hematopoietic stem cell transplantation (alloHSCT)[104-106]. Donor-derived T cells contained in the graft can mediate a potent graft-versus-leukemia (GvL) effect resulting in the life-long suppression of leukemic cells[107, 108]. The benefit of curative treatment is, however, offset by the occurrence of life-threatening graft-versus-host disease (GvHD). The risk of developing fatal GvHD may outweigh the chance for curative potential, especially in older, more fragile patients. Therefore, combinations of chemo- and radiotherapy with monoclonal antibodies remain the standard treatment for most lymphomas and chronic leukemias.

Investigations into the GvL effect and GvHD have demonstrated that donor-derived T cells can recognize genetic disparities between patient and donor cells[109-112]. Minor histocompatibility antigens (MiHAs) are one class of genetic disparities and arise from nonsynonymous single nucleotide polymorphism (SNPs) altering the amino acid sequence of transcribed and translated DNA sequences resulting in immunogenic T cell epitopes[113-118]. More recently, patients suffering from melanoma have benefited from the isolation of tumor infiltrating lymphocytes (TILs) from surgically removed tumor masses and tumor-draining lymph nodes[119-123]. It has been demonstrated that tumor-reactive T cell populations contained in TILs can mediate tumor regression by targeting neo-antigens, i.e. peptides containing amino acid changes arising from mutations in the genome of cancerous cells[124-127]. Neo-antigens and MiHAs are interesting targets for the specific eradication of tumors; tumor-restricted expression confines toxicity to cancerous tissue while the lack of central tolerance towards these highly immunogenic peptides has not deleted high avidity T cells. However, neo-antigens are often person-specific as they arise sporadically in DNA due to exposure to mutagenic agents, therefore, limiting their potential to be exploited in a greater patient group. Similarly, unfavorable distributions of MiHAs among a given population can present a serious obstacle for the widespread applicability of an identified MiHA.

Treatment of B cell malignancies with CAR-engineered T cells and monoclonal antibodies has demonstrated the permissiveness of eradicating the healthy B cell compartment in the course of treatment[53, 71, 73, 83, 84]. By targeting differentiation antigens, i.e. a class of proteins whose expression is limited to cancerous cells and healthy tissue, the entire B cell compartment including diseased and healthy B cells is targeted. Therefore, T cells expressing TCRs specific for differentiation antigens could likewise mediated B cell eradication and provide curative treatment. Since the antigen specificity of T cells is solely determined by the expressed TCR, transfer of the TCR encoding genes will install antigen specificity onto the recipient cells. Indeed, the feasibility of TCR gene transfer has been shown by various studies in vitro and in vivo[128-139]. TCR genes packaged into retroviral and lentiviral expression vectors allow for the stable integration and long-term expression of TCRs in recipient T cells. Patients suffering

from melanoma were successfully treated with T cells engineered to expressed TCRs specific for melanoma-expressed antigen MART1[135, 140], gp100[140] or NY-ESO-1[141, 142]. Several studies have demonstrated the clinical efficacy of TCR gene transfer, however, the broad applicability of TCR gene transfer is limited by the breadth of well characterized TCRs available for the treatment of various hematological and solid tumors. Greater coverage of different cancer types and patient groups with varying HLA genotypes can be achieved by an ever increasing off-the-shelf TCR library targeting increasing numbers of peptides presented in various HLA alleles.

Suitable Antigens for TCR Gene Transfer

The success of monoclonal antibodies such as rituximab and ofatumumab is likely influenced by their great applicability to various B cell malignancies originating from the early to mature B cell stage since the antigenic target CD20 is highly expressed on these subsets. Moreover, the relative ease of administration has greatly facilitated the widespread use of mAbs. These factors have certainly sparked efforts to identify additional markers such as CD22 which are now also under investigation as antigenic targets[59, 143-145]. Similarly, CD19 is sufficiently expressed from the pro-B cell stage to mature B cells, greatly contributing to the success of CD19-targeting CAR T cells in the treatment of ALL and lymphomas. Although heavily exploited in CAR- and mAbs-based approaches, cell surface-expressed B cell-specific antigens may still form attractive targets for TCR-based immunotherapeutic interventions. As mentioned earlier, refractory or relapsed disease has been described after administration of mAbs due to insufficient or downregulated cell surface-expression of the targeted antigen. B cell malignancies expressing only very low amounts of an antigen may still be efficiently targeted by T cells since only very low quantities of agonistic pHLA complex are required for TCR triggering and T cell activation[146-148]. In contrast, CAR and antibody-based approaches necessitate greater abundance of the antigenic target[149].

In order to broaden the exploitable antigen target pool and make immunotherapeutic approaches accessible to additional B cell malignancies, novel antigens should be identified. On the one hand, a likely strategy for the identification of potential targets could be based on the functional role of the protein. For instance, the BCR associated heterodimer CD79a/b is critically involved in BCR transport[3] and functionality by providing signaling capacity[4-8] to regulate processes such as allelic exclusion, proliferation, differentiation, anergy and apoptosis [9-11]. On the other hand, an approach focusing solely on the identification of genes based on a B cell restricted expression profile can result in the identification of potential antigens that have not been considered earlier or whose function is not fully understood. Moreover, HLA molecules constantly sample the entire endogenous proteome of cells, therefore, both extracellular and intracellular antigens can be presented in the context of HLA molecules. Since the antigenic structure recognized by a TCR are cell surface-expressed pHLA complexes, the potential antigen pool available to TCR-based approaches encompasses both extracellular and intracellular proteins.

Identification of HLA-presented Peptides

Following the selection of a candidate antigen, HLA-presented antigenic peptides derived from the candidate antigen must be identified. TCRs targeting monomorphic peptides, i.e. peptides showing no amino acid sequence variability between individuals, will ensure that a single TCR will suffice to treat a wide patient group suffering from tumors that express the antigen from which the monomorphic peptide is derived. Public computer-generated algorithms can be used to predict T cell epitopes from a given protein's amino acid sequence based on potential proteosomal cleavage sites and presence of amino acid residues within short peptide sequences that form anchor residues in the binding groove of HLA class I molecules[150-152]. The feasibility of this approach has been demonstrated by the identification of several antigenic peptides inducing $CD8^+$ T cell responses. However, the efficiency of this approach can be undermined by the inclusion of biologically irrelevant peptides, i.e. peptides that are efficient HLA-ligands but are not naturally processed and presented by antigen-expressing cells[153, 154]. A study from our group has demonstrated that T cells specific for such peptides can be isolated but failed to recognize antigen-expressing cells due to lack of efficient antigen processing and peptide presentation[153]. The inclusion of data gained from studies investigating the HLA-presented ligandome of various cell subsets and tumor tissues can significantly increase the probability of identifying naturally HLA-presented peptides[155, 156]. Using acid elution, HLA-bound peptides are separated from their HLA molecules and fractionated by high performance liquid chromatography (HPLC)[97, 157-160]. Tandem mass spectrometry generates fragmentation patterns of peptides contained in these fractions. Peptides are then identified by matching of the tandem mass spectra of the eluted peptides with a data base containing theoretical spectra of possible peptides contained in the human proteome. Scores indicating the goodness of fit between spectra can guide and further increase the probability of selecting good candidate peptides. Furthermore, matching tandem mass spectra obtained from the eluted and synthesized peptide of same amino acid sequence serve as an important quality control and can verify correct identification.

Identification and Isolation of Peptide-specific T Cells

Several approaches have been described to induce T cell responses towards one or several candidate peptides. Peptide-MHC (pMHC) tetramers composed of the candidate peptide bound to the HLA molecule can be used to investigate the presence of antigen-specific T cells in a given cell population[161]. Fluorochrome-labelled pMHC tetramers can be used to visualize pMHC tetramer-binding T cells in blood or tissue samples. In addition, pMHC tetramers-binding T cells can be isolated from cell populations by magnetic activated cell sorting (MACS) or fluorescent activated cell sorting (FACS). In the former, magnetic beads binding to pMHC tetramers will retain pMHC tetramer-binding cells if placed in a magnetic field and thus separate them from pMHC $tetramer^{neg}$ cells. Similar, FACS can be used to identified pMHC tetramer-binding cells based on to the fluorescent signal emitted from the fluorochrome. Our group and others have used these methods to monitor the existence of

antigen-specific T cells in patients as well as to isolate antigen-specific T cell lines and T cell clones[153, 155, 156, 162-164].

In a different approach, T cells can be repeatedly stimulated with irradiated antigen-presenting cells that have been exogenously loaded with the candidate peptides, transfected with mRNA coding for the candidate peptides[165, 166], or are coated with peptide-HLA complexes containing the candidate peptides[167].

Using pMHC tetramers to directly isolate antigen-specific T cells or stimulating T cell populations with antigen-loaded antigen-presenting cells to favor the expansion of antigen-reactive T cells still requires careful assessment of the generated T cells[165-167]. Although T cells express TCRs that are highly peptide-specific, reactivity towards peptides bound in the same or different HLA molecule than the targeted peptide are not uncommon[168-171]. Therefore, extensive screening of T cells expressing promising TCRs should be conducted. Such screening should include the stimulation with a broad and diverse panel of cell populations that do not express the targeted antigen but are positive for the HLA allele presenting the antigenic peptide. Observed reactivity to antigen-negative stimulator cells is a likely indication for cross-reactivity with other peptides increasing the chance for toxicity towards undesired cell populations. Furthermore, crossreactivity with other HLA molecules can be assessed by measuring T cell reactivity towards a stimulator panel consisting of cells expressing common and rare HLA alleles. Reactivity to cells lacking the expression of the targeted HLA allele probably indicates HLA crossreactivity.

Exploiting the Immunogenicity of Allogeneic HLA Molecules to Targeted Monomorphic Self-peptides

The class of differentiation antigens is composed of a subset of human proteins and can thus be described as self-antigens. Therefore, monomorphic peptides derived from these antigens present self-peptides with existing immunological tolerance; negative selection during thymic development deletes T cells expressing high affinity TCRs for self-peptides to prevent autoimmune reactivity. Therefore, it is unlikely that T cells will express high affinity TCRs specific for these self-peptides. However, since tolerance is only induced towards self-peptides presented in the context of self-HLA, self-peptides presented in allogeneic HLA could be highly immunogenic due to the immunogenicity of allogeneic HLA. Indeed, our lab and others have demonstrated that high avidity T cells can be raised towards self-antigens in an HLA-mismatched setting[167, 172-175]. Furthermore, T cells recognizing self-peptides presented in allogeneic HLA can do so with avidities greatly exceeding that of self-HLA restricted T cells[172, 173]. Moreover, our lab demonstrated that T cells recognizing self-peptides presented in allogeneic HLA do so in an HLA-restricted, peptide-specific manner making them ideal tools to overcome self-tolerance to self-peptides[168, 172].

Aim of the Study

Immunotherapy of cancer using adoptive T cell transfer of TCR-engineered T cells has demonstrated clinical efficacy in many studies. However, the widespread application is hampered by a lack of a diverse TCR library in order to target different malignancies. Furthermore, emergence of antigen-loss tumor-escape variants after the administration of adoptive T cell transfer urges the need to identify additional targetable proteins. The research described in this thesis develops a high throughput methodology for the identification of TCRs specific for monomorphic self-peptides derived from B cell differentiation antigens.

In **chapter 2,** we analyzed the potential targeting of to the B cell-specific antigen CD20 by TCR gene transfer strategy. CD20 has been successfully targeted using mAb such as rituximab and ofatumumab. However, refractory disease has emerged after the administration of CD20-targeting mAbs due to downregulation of CD20. Although downregulated or cell surface expression is decreased in malignant cell samples, we speculate that a high affinity CD20-targeting TCR will still efficiently recognize CD20-expressing cells independent of cellular location of CD20. We describe a general high throughput strategy using peptide-MHC tetramers for the identification of TCRs targeting monomorphic self-peptides in allogeneic HLA. Successes and pitfalls in the selection procedure of promising candidate T cell clones are discussed. Finally, we demonstrate how high affinity CD20-targeting TCRs may be a valuable addition to current immunotherapeutic approaches aimed to treat B cell malignancies such as ALL, CLL and MCL.

Chapter 3 focuses on the B cell-expressed antigen CD22 that is currently evaluated as a target for CAR- and mAbs-based immunotherapies. We hypothesize that a CD22-targeting TCR could be a valuable addition for the treatment of B cell malignancies in cases where CD22 cell surface expression is downregulated. A CD22-derived HLA-B*07:02-presented peptide is identified from the HLA ligandome of B lymphocytes. Using the same pMHC tetramer-based methodology, a high affinity TCR is isolated from a T cell clone of a HLA-B*07:02-negative individual. We assess the clinical relevance of this TCR by targeting acute lymphoblastic leukemia. However, we also indicate an important caveat of CD22 that may limit its attractiveness as a target for cellular based immunotherapies.

In **chapter 4** we aim to broaden the applicability of TCR-based immunotherapies by exploring CD79b as a potential antigen. The BCR associated protein CD79b was selected due to its high expression on healthy B cells and malignant ALL and CLL cells. In addition, CD79b expression has been implicated in poor prognosis and disease progression. Again, we demonstrate the usefulness of our platform starting from the identification of HLA-presented peptides to the successful isolation of CD79b-specific high affinity TCRs. Based on the reactivity profiles of our CD79b-specific T cell clones we highlight critical parameters that need to be considered when selecting candidate targets.

In **chapter 5**, we identify a novel target antigen exploitable for the treatment of B cell malignancies including multiple myeloma by incorporating mRNA expression data into our pipeline. A TCR against this antigen is isolated from the HLA-mismatched T cell allorepertoire.

The clinical relevance of this TCR is assessed by using a stimulator panel composed of several B cell malignancies and healthy cell subsets. We corroborate our results by employing an in vivo xenograft mouse model of established myeloma. This study highlights the importance of identifying additional target antigens to broaden the applicability of immunotherapy.
In the **General Discussion**, we outline the next steps in antigen selection, further optimization of the T cell isolation protocol as well as risk factors inherit with TCR-based therapies.

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