TCRs as precision tools against B-cell and plasma cell malignancies

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CHAPTER 6

Summary and general discussion

SUMMARY

CD19- and BCMA-targeting chimeric antigen receptor (CAR) T cells have shown significant anti-tumor effects in treating B-cell malignancies and multiple myeloma (MM) (1, 2). However, relapses post-therapy highlights ongoing challenges in T-cell therapy. Antigen escape is a common mechanism behind tumor escape, occurring when tumor cells with low or no surface antigen expression survive treatment(1, 3). To counter this, we advocate for multi-antigen-targeting approaches, simultaneously targeting several antigens. Adding more specificities to cellular products can decrease the chance of antigen escape(4).

To target multiple antigens, T-cell receptor (TCR) transduced T cells would be a promising component of multi-antigen-targeting therapy. TCRs have the intrinsic ability to recognize peptides from theoretically any antigen presented in HLA molecules on the cell surface, enabling them to target antigens inaccessible to CARs. Targeting B-cell lineage antigens consistently expressed in malignant cells is promising, as evidenced by the success of CAR T cells against CD19 and BCMA.

This thesis aimed to identify targets expressed in B-cell malignancies, that are not expressed in healthy cells except for the B-cell lineage. We searched for TCRs recognizing such targets effective against B-cell or plasma cell malignancies. Our approach involved identifying TCRs specific to peptides from B-cell antigens presented in various HLA alleles. These epitopes, being self-peptides, are not immunogenic in an HLA-matched setting. To overcome this, we isolated T-cell clones across HLA-barriers using donors lacking the target HLA-alleles. We used peptide-HLA (pHLA) multimers for isolation, followed by comprehensive potency and safety tests to select T cells with precise antigen recognition.

The TCRs of selected T-cell clones were sequenced, inserted into retroviral vectors, and introduced into third-party T cells. These T cells were then evaluated for their specificity and ability to target and eliminate B-cell malignancies expressing the antigen. For certain TCRs, we demonstrated anti-tumor effectiveness using *in vivo* xenograft models.

In **Chapter 2**, we performed a broad search to identify TCRs for therapy of B-/plasma cell malignancies. We started by selecting potential antigens for TCR-based therapy of B-/plasma cell malignancies. We utilized a gene expression dataset containing samples from acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and MM patients as well as healthy cells of various origins. We selected genes that were expressed in one of these malignancies while expression in healthy cells, with the exception of B cells, was negative. This approach yielded 28 proteins which could be promising

targets for therapy of B-/plasma cell malignancies. We identified peptides derived from these genes, presented in HLA on the surface of malignant B cells by peptide elution and mass spectrometry. Our selection of target HLA alleles included HLA-A*01:01, -A*24:01, -B*08:01, and -B*35:01, thereby focusing on commonly expressed alleles that have not been extensively studied in the past. Twenty target peptides derived from one of the selected genes and presented in one of the chosen HLA molecules were identified. To bypass self-tolerance, T-cell clones were isolated from healthy donors lacking the HLA alleles of interest. Employing pHLA-multimers, we isolated 5519 T-cell clones from 13 donors. High-throughput screenings yielded 23 T-cell clones specific for one of the target peptides, which demonstrated functional reactivity to naturally processed and presented antigen. We sequenced the TCRs of six T-cell clones which were sensitive enough to recognize and target B-cell malignancies naturally presenting the antigen. Following TCR-transfer, third-party T cells effectively recognized and lysed B-cell malignancies while antigen specificity was maintained. The selected TCRs target peptides derived from FCRL5 presented in HLA-A*01:01, VPREB-3 in HLA-A*24:02, and BOB1 in HLA-B*35:01. The potential therapeutic value of each TCR varies with the type of malignancy. For instance, the FCRL5-specific TCR showed promise for CLL therapy and possibly B-cell lymphomas. VPREB-3 is expressed in ALL and CLL, while expression is lower in CLL, VPREB-3 TCR T cells recognized CLL samples and might therefore be valuable for therapy of both. BOB1, expressed in ALL, CLL, and MM, was recognized by TCR T cells, making it a candidate for treating all three malignancies. Additionally, B-cell lymphoma cells were recognized.

In **Chapter 3**, we identified TCRs recognizing peptides from the joining chain (Jchain) that can be used to treat MM. In Chapter 2, the Jchain was already identified as one of the target proteins for therapy of B-/plasma cell malignancies. In healthy plasma cells the Jchain functions as a linker molecule between monomers of IgA and IgM when secreted in multimeric forms. Expression in MM cells is irrespective of the immunoglobulin isotype that is produced and the Jchain is highly expressed in most MM samples. The Jchain could therefore be a broadly applicable target antigen for MM. The Jchain is a secreted protein and can thus not be targeted with CAR T cells, instead TCR T cells specific for Jchain derived peptides in HLA can be used to target *JCHAIN* expressing cells. Our goal in this chapter was to identify TCRs that could recognize Jchain peptides presented in various HLA-A alleles. Target peptides derived from the Jchain presented in HLA were identified from the HLA class-I peptidome of MM cells. pHLA-multimer technology as used in Chapter 2, was applied to isolate Jchain specific T-cell clones. This resulted in the discovery of four T-cell clones that effectively recognized Jchain peptides presented in HLA-A*01:01, -A*03:01, -A*11:01, or -A*24:02. These Jchain-specific T cells showed promising safety profiles. Upon equipping T cells with the Jchain-TCRs, we observed that they successfully lysed MM tumor cells from various patients but only when the *JCHAIN*

was expressed. Additionally, Jchain-TCR T cells drastically reduced tumor burdens in a pre-clinical *in vivo* model of established MM. Thus, these TCRs hold substantial potential for further development as MM therapies. Given their favorable HLA restrictions, these four TCRs collectively have the potential to treat approximately 60% of individuals with *JCHAIN*-positive MM.

In **Chapter 4**, we focused further on identification of TCRs applicable for MM therapy. MM cells are the malignant counterpart of plasma cells and therefore often secrete high amounts of immunoglobulins of a specific isotype. Most MM cells are of IgG isotype and 22% is of IgA isotype, but IgA expression is associated with an unfavorable prognosis. We therefore aimed to target these immunoglobulins, specifically peptides from the constant domains of IgG and IgA heavy chains, identified from the HLA-I peptidome of B-cell malignancies. Following the approaches as in Chapter 2 and Chapter 3, we aimed to identify T cells specific to these immunoglobulins. Extensive efforts led to the discovery of T-cell clones specific for the LMI peptide from the IgG heavy chain constant domain presented in HLA-A*02:01 or the SPK or HPR peptides from the IgA constant domain presented in HLA-B*07:02. These clones effectively recognized and lysed MM cell lines expressing the right isotype. Specificity and functionality was maintained upon TCR sequencing and transfer to third party CD8 T cells. Immunoglobulin-TCR T cells did not recognize healthy cells of hematopoietic origin other than B cells. These T cells only lysed isotype-positive B cells while sparing others, suggesting a potential clinical advantage by partially preserving B-cell immunity, especially when IgA-TCR T cells are applied. Furthermore, dendritic cells co-culture with high concentrations of IgG immunoglobulins were recognized by IgG-TCR T cells in a dose-dependent manner, most likely as of result of IgG uptake, cross-presentation, and subsequent presentation of the respective IgG peptide in HLA-A*02:01 to TCR T cells. Immunoglobulin-specific TCR-transgenic T cells specifically lysed patient-derived MM cells when the respective immunoglobulin and targeted HLA allele were expressed. Furthermore, IgA-TCR T cells effectively reduced tumor burdens in MM bearing mice.

Chapter 5 delves into an interesting aspect of the HLA class-I ligandome: the presence of unconventionally long peptides. In the method applied in Chapters 2-4, as well as by others working on T-cell epitope identification or prediction, the general assumption has been applied that HLA class-I presents short peptides of 8-11 amino acids in length. However, the HLA class-I ligandome contains a substantial fraction of longer peptides which are often dismissed as contaminants from HLA class-II. In **Chapter 5** an unconventionally long peptide was explored for its potential origin from HLA class-I. We examined a 20-amino acid (20-mer) FLP peptide from the IL27 receptor alpha, identified in HLA-A*02:01 expressing cells and investigated its binding to HLA-A*02:01. Successful refolding of FLP:HLA-A*02:01 complexes demonstrated that the FLP peptide

could indeed bind to HLA-A*02:01. Crystal structure analysis revealed that the first 11 amino acids bound HLA in a conventional linear manner, while the remaining portion formed an extended 'peptide tail'. We isolated T cells recognizing the FLP peptide, as in previous chapters, to understand how such a long peptide could be recognized. Our findings indicated that while some T cells were activated by the core peptide alone, others required the C-terminal tail, losing recognition if it was trimmed or altered. This chapter highlights the diverse way peptides can bind to HLA and shows that T cells can recognize peptides bound to HLA in unconventional manners.

GENERAL DISCUSSION

Combining CAR and TCR T cells to improve outcomes of cellular therapy

Most B-cell malignancies, unlike tumors with high mutational burdens, often lack neo-antigens that can be targeted by therapies designed to exploit mutational diversity(5). The absence of neo-antigens in B-cell malignancies limits the efficacy of treatments that rely on high mutational burdens for targeting, such as tumor infiltrating lymphocyte (TIL) therapy or immune checkpoint blockade to boost neo-antigen specific T cells. Instead, these malignancies can be treated by targeting B-cell lineage antigens, which are expressed throughout various stages of B-cell development and for which expression is often maintained after malignant transformation(6). Initially, rituximab, an antibody therapy that targets CD20 expressed on B cells, demonstrated efficacy for treatment of lymphoma, chronic lymphocytic leukemia (CLL) and other B-cell mediated diseases such as rheumatoid arthritis(7). Depletion of healthy B cells as side effect of treatment increased the risk of infections but this side effect could be overcome by treatment with immunoglobulin replacement therapy(8).

In addition to antibody mediated therapy for B-cell associated diseases, T-cell-based therapies have emerged more recently. T-cell therapies are currently based on the introduction of a chimeric antigen receptor (CAR) that targets a B-cell lineage antigen on the surface of malignant B cells like CD19 and BCMA for treatment of B-cell malignancies and multiple myeloma (MM), respectively. Despite achieving high initial response rates many patients relapse after CAR T-cell therapy(9, 10). Some patients relapse with antigen positive malignancy, often attributed to suboptimal performance of CAR T cells(11). Additionally, outgrowth of antigen low or negative tumor cells can contribute tumor escape(3). These relapses highlight the need to keep advancing cellular therapy and demonstrate that new targets should be included as part of combination therapy to mitigate the risk of antigen negative relapse. CAR T cells recognize proteins on the surface of target cells. This characteristic combined with the requirement for B-cell restricted expression limits identification of suitable CAR targets. T-cell receptor (TCR)

transgenic T cells could complement CAR T cells in one crucial aspect. TCRs possess the capability to recognize peptides derived from any protein, irrespective of its cellular localization. Therefore, TCRs can be used to target any antigen of choice if the criteria regarding a safe expression profile are met. This ability opens many avenues to explore new target proteins for treatment of B-cell malignancies. Because CARs and TCRs have a different recognition mode as well as a different composition/design, we believe that combination of TCR and CAR T cells is expected to enhance the efficacy of T-cell therapy as each modality brings unique advantages while balancing the limitations of the other and thereby improving overall outcomes following T-cell therapy. In recent years the CAR T-cell field has rapidly developed, and many clinical trials have been performed that provided new insights in the advantages and disadvantages of CAR T cells. An advantage of CAR T cells is that specific surface proteins independent of HLA are targeted, making them broadly applicable across patients independent of HLA type. CARs are artificial receptors that combine an antigen binding domain, a signaling domain and one or more costimulatory domains in a modular manner, because of this modular design CARs can easily be adapted and improved by changing an individual module. Throughout different generations of CARs, the effector function was initially enhanced by incorporation of a co-stimulatory domain and later by incorporation of alternative co-stimulatory domains(12). Additionally, CARs contain a CD3zeta signaling domain which makes CARs independent of endogenous T-cell signaling and therefore also independent of the CD8 or CD4 co-receptor. Therefore, CARs function in both cytotoxic CD8 T cells as well as CD4 helper T cells. CD4 T-cell help has been shown to be important for the effectiveness of CD19 CAR T cells. Preclinical studies have shown that CAR T cells in a defined CD8:CD4 ratio outperformed CAR T cells that were derived from CD4 or CD8 T cells alone, indicating some degree of synergy between receptor engineered CD4 and CD8 T cells(13). CD4 T cells not only ‘help’ other cells through specific cytokine signals or by providing costimulatory ligands but can also directly exert effector functions and anti-tumor activity(14). Furthermore, some patients demonstrated decade long persistence of CAR T cells after administration(15). Interestingly, those CAR T cells were CD4 T cells, suggesting that CD4 T cells may play a role in establishing long-term memory of engineered T cells.

While the design of CARs brings many benefits, CAR T cells also have some disadvantages which we previously described in the introduction of this thesis. In short: Depending on the CAR design the artificial signaling mechanism of CAR T cells can induce tonic signaling, which can lead to antigen-independent proliferation, differentiation, and upregulation of co-inhibitory molecules(16). Upon antigen-dependent activation, CAR T cells are sensitive to overactivation, which may compromise their efficacy especially in settings with high tumor burdens or in solid tumors. Another limitation of CAR T cells is the risk of antigen escape which can occur for various reasons: heterogeneous

expression levels, genetic loss of target antigen, trogocytosis or target protein cleavage which has been described in the case of BCMA(17).

We believe that TCR T cells can be used to overcome disadvantages associated with CAR T cells because TCR T cells have distinct advantages. TCRs are the natural receptors of T cells that do not contain costimulatory or signaling domains, additionally TCRs are exclusively activated upon cognate antigen encounter. TCR T cells might therefore be less sensitive to overstimulation compared to CAR T cells, increasing their ability to deal with relatively high tumor burdens and potentially leading to increased persistence *in vivo*(18). The degree of trogocytosis that occurs between a T cell and target antigen is dependent on the receptor affinity for its ligand(19). TCRs generally have lower affinity for their ligand than CARs(20), which likely underlies the limited trogocytosis by TCR T cells. Furthermore, TCR T cells have the theoretical capability to target peptides derived from any protein presented by HLA molecules, thereby expanding their targetable protein repertoire to include secreted proteins or those essential for tumor cell survival, such as certain transcription factors. Targeting antigens essential for survival makes it unlikely that tumor cells evade TCR T cells through complete loss of these vital proteins. Although TCR T cells offer several advantages some limitations should also be considered. For instance, the HLA-restricted recognition of TCR T cells restricts the applicability of TCR T cells to patients expressing the targeted HLA restriction element. In addition, HLA class-I-restricted TCRs often depend on presence of the CD8 co-receptor for optimal functionality, limiting their applicability in CD4 T cells(21). This observation is confirmed in **Chapters 2, 3 and 4** where it is demonstrated that all HLA class-I restricted TCRs are dependent on the CD8 co-receptor for optimal functionality. CD8-dependent TCRs are not functional in CD4 T cells, and CD4 T-cell help can thus not be provided by introducing the TCR alone into CD4 T cells. In a setting of combination therapy with CAR T cells, this might not be a problem as the TCR could be inserted in CD8 T cells only, while the CAR could be introduced in both CD8 and CD4 T cells. Consequently, CAR-CD4 T cells could provide help to the TCR-engineered CD8 T cells. An alternative approach is to functionalize TCR-CD4 T cells by co-introducing the CD8 molecule alongside the TCR in CD4 cells to generate functional TCR-engineered CD4 T cells(22).

While antigen escape can compromise efficacy of CAR T-cell therapy, antigen escape after TCR T-cell therapy can also occur, partially through alternative mechanisms. After TCR T-cell therapy, tumor cells can escape that do not or no longer present the targeted epitope or HLA molecule(23). Epitope negativity can occur when the protein from which the epitope is derived is not expressed or expression is lost, when problems in the peptide processing and presentation machinery hamper presentation of the targeted epitope, or when a mutation in the targeted epitope arises resulting from the genetic instability of the malignancy. In cancer, mutations in the peptide processing and presentation

machinery can lead to HLA negative tumor cells that could escape TCR-mediated recognition(24). Alternatively, full genetic loss of one of the HLA loci can occur as a result of immunological pressure. This is sometimes observed after haploidentical allogeneic stem cell transplantation for treatment of leukemia and the same might following TCR T cell therapy(25). Overall tumor cells can escape TCR-mediated recognition via multiple mechanisms, some of which may not overlap with potential escape mechanisms following CAR T cell therapy.

In summary, CAR and TCR T cells have distinct mechanisms of antigen recognition as well as various functional differences. In CAR and TCR combination therapy, one subset might be able to cope better with low tumor burdens while the other subset might cope better with high tumor burdens. Additionally, both CD4 and CD8 tumor-targeting T cells can be part of a combination product. Furthermore, the incorporation of multi-antigen-targeting through different mechanisms should ensure that tumor cells escaping recognition by one T-cell population can still be targeted by the other. Consequently, CAR and TCR therapies are likely to complement each other very well, improving the overall outcome of T-cell therapy. In this combined approach multiple TCRs and CARs could be incorporated. By including multiple receptors, the likelihood of antigen escape is further reduced. When selecting multiple TCRs, an optimal combination should target different antigens as well as different HLA alleles, thereby reducing the chance of HLA-based escape or escape through target protein loss.

TCRs targeting immunoglobulins

Targeting immunoglobulin heavy or light chains via TCR-mediated therapy might be a promising strategy for immunotherapy of multiple myeloma (MM). MM cells are the malignant counter part of plasma cells and are therefore characterized by high production and secretion of monoclonal immunoglobulins. In MM, 54% of cases produce IgG immunoglobulins and 22% produced IgA, but IgA production correlates with poorer prognosis(26). Other immunoglobulin subtypes are rare, and 20% of the MM cases MM secrete immunoglobulin light chains only(27). As B cells develop into plasma cells BCR surface expression is lost. Consequently, malignant plasma cells do not express surface BCR preventing targeting by CAR T cells. Instead, TCR mediated targeting of constant domains of immunoglobulin heavy or light chains forms an attractive approach for TCRs with widespread applicability for therapy of MM. In **Chapter 4** of this thesis, TCRs specific for peptides from IgG and IgA immunoglobulin heavy chain constant domains, presented by HLA-A*02:01 and HLA-B*07:02, respectively, were identified. MM cells isolated from patient bone marrow samples expressed IgA or IgG immunoglobulin at levels 100-1000-fold higher compared to housekeeping genes. Effective lysis of MM cells by immunoglobulin-TCR T cells combined with lack of recognition of healthy cells other than B cells, underscores the potential of immunoglobulin-specific TCR T cells as

a therapeutic strategy for MM.

Immunoglobulin-specific TCR T cells offer a theoretical benefit over CD19 or BCMA CAR therapy, as they only deplete the peripheral blood B cells expressing the targeted isotype, while leaving B cells of another isotype intact. Especially in the context of IgA-specific TCR T cells for therapy of MM or other IgA+ malignancies, preserving IgG+ B/plasma cells holds significant advantages. IgG serves as the primary antibody isotype responsible for systemic protection against pathogens. By sparing IgG+ B/plasma cells, patients would retain this crucial aspect of B-cell immunity. Consequently, this preservation could potentially alleviate the need for patients to receive soluble immunoglobulins after T-cell therapy and thereby improve health and reduce the need of additional therapeutic interventions, leading to a better quality of life.

Previously, immunoglobulin kappa light chain specific CAR T cells have been developed to treat surface BCR positive B-cell malignancies(28). However, efficacy of IgK CAR T cells has fallen behind that of CD19 CAR T cells, hampering their further application. The functionality of IgK CAR T cells was inhibited by immunoglobulin proteins present in human serum(29, 30). Additionally, CARs covered by circulating antigen lose their ability to respond to antigen positive tumor cells(17). Given these functional differences, we hypothesized that TCR T cells might be influenced differently when the target protein is present in serum. TCR T cells recognizing peptides from proteins present in circulation might indirectly recognize cells that naturally don't express the targeted protein. In **Chapter 4**, we proposed that cells proficient in endocytosis, such as antigen presenting cells (APCs), might take-up full protein and sequentially process and cross-present peptides from these proteins in HLA class-I on their cell surface. We investigated the effect of co-culturing dendritic cells (DCs) as well as various adherent cells of different tissue origins with varying concentrations of soluble IgG immunoglobulins. DCs co-cultured with varying concentrations of soluble IgG immunoglobulins stimulated IgG-TCR T cells in a dose dependent manner, while adherent cells co-cultured with high concentrations of immunoglobulins did not generally induce T-cell activation. Only limited recognition of fibroblasts from one of two donors was observed after co-culture with the highest dose of immunoglobulins. These data suggest that uptake of immunoglobulins followed by cross-presentation and recognition by IgG-TCR T cells is dose dependent and mostly restricted to cells with high endocytic capacity and high T-cell stimulatory capacity such as DCs. We hypothesize that immunoglobulin-TCR T cells *in vivo* will be boosted by cross-presenting APCs, stimulating the anti-tumor response of immunoglobulin-TCR T cells. This phenomenon is likely restricted to the initial period after start of therapy when immunoglobulin serum concentrations are high. When immunoglobulin-specific TCR T cells are effective and MM tumor burdens reduce, serum immunoglobulin concentrations will reduce simultaneously abrogating cross-

presentation of immunoglobulin peptides. While it hasn't been investigated in this study it is reasonable that as a consequence of T-cell recognition DCs cross-presenting these immunoglobulin epitopes are killed. In natural immune responses, CTLs lyse antigen expressing DCs as part of the negative feedback loop after initiation of an immune response(31). While cross-presentation by APCs is expected to benefit TCR T cells, cross-presentation by other cell types might result in undesired on-target off-tumor toxicity. Our data showed that cross-presentation of immunoglobulin peptides by adherent cells is very limited, but it might still occur under specific circumstances. It remains difficult to predict how observations made in an *in vitro* co-culture assay translate to an *in vivo* setting. If cross-presentation of immunoglobulin peptides occurs more broadly than anticipated and leads to unexpected toxicity it should be considered that recognition of cross-presented immunoglobulin peptides was dose-dependent and consequently reducing the concentrations of serum immunoglobulin will reduce cross-presentation by bystander cells and subsequent consequences.

MM is typically characterized by the secretion of monoclonal immunoglobulin(27). However, 20% of MM cases secrete immunoglobin light chains only, and some MM are classified as non-secreters(27). When considering TCR therapy targeting immunoglobulins, it becomes essential to establish whether these malignancies completely lack production of immunoglobulin heavy chains or if secretion is hampered while production continues. If secretion is impaired but immunoglobulins are still produced, peptides derived from these proteins can still be presented in HLA molecules, enabling TCR T cells to recognize these malignancies. On the other hand, if light-chain-only or non-secreting MM completely lack heavy chain production, these malignancies cannot be treated with immunoglobulin heavy chain-targeting TCR T-cell therapy.

In addition to MM, TCRs targeting IgA and IgG could be explored in the context of other B-cell mediated diseases such as autoimmune disorders. Recently, CD19-targeting CAR T cells have shown promise in treating patients with refractory systemic lupus erythematosus and other B-cell-mediated autoimmune diseases such as anti-synthetase syndrome and myasthenia gravis(32, 33). However, CD19 CAR T cells deplete all B cells irrespective of their Ig subtype. Most autoimmune disorders are thought to be associated with B cells of an IgG subtype. The subtype-specificity of IgG-targeting TCRs could therefore provide an interesting conceptual advantage by sparing healthy B cells of non-IgG subtypes such as IgA, which plays a crucial role in mucosal immunology. Additionally, IgA-targeting TCR T cells could be an interesting approach to treat patients suffering from IgA nephropathy, as it would leave IgG producing B cells intact.

Verifying epitope presentation prior to therapy

As T-cell therapy is an extremely costly treatment it will be very important to select

and treat patients for which a good response to therapy is expected. For TCR T cells to recognize malignant cells the target proteins need to be expressed, the target HLA needs to be expressed and epitopes derived from the target protein need to be processed and presented in HLA. In this thesis, expression of the target gene in the malignant population of interest was measured by qPCR. QPCR determines the average expression level of the target gene on a bulk level. In this thesis as well as in other studies we observed that in most cases, target gene expression combined with expression of the respective HLA allele as determined by genotyping can be used to predict lysis by TCR T cells. However, exceptions might exist in which HLA and gene expression do not precisely predict lysis by TCR T cells. In **Chapter 3**, an example was observed: Jchain HLA-A*24:02 specific TCR T cells did not lyse MM materials MM.J3 and MM.J4 despite high *JCHAIN* expression of 14- and 39-fold compared to housekeeping genes, respectively. An HLA-A*24:02 restricted control T-cell clone did lyse these MM materials, indicating that HLA-A*24:02 was indeed expressed. In contrast, Jchain positive, HLA-A*24:02 positive MM sample MM.J2 was lysed. These data suggest that despite expression of the target gene, the Jchain A24 epitope is not always presented by HLA-A*24:02 positive MM cells. Rather than a general lack of processing and presentation of this target peptide it seems that the targeted peptide is absent in HLA molecules from some cell populations.

Cells of the hematopoietic lineage as well as MM cell can express immunoproteasomes, which preferentially cleaves peptides with hydrophobic c-terminal amino acid(34). The Jchain A24 epitope contains such an c-terminal amino-acid and predictions suggest that the Jchain A24 epitope relies on processing and presentation by the immunoproteasome(35). Activity of the immunoproteasome varies between MM tumors (36), potentially influencing processing and presentation of the Jchain A24 epitope. To test this, immunoproteasome activity in MM samples should be determined and correlated to recognition by Jchain A24 TCR T cells. The discrepancy between gene expression and TCR T-cell recognition observed in this case underscores the inadequacy of solely relying on gene expression as a diagnostic tool to predict recognition by TCR T cells.

In addition to investigating gene or protein expression, it is essential to establish HLA expression as well as peptide presentation before initiating therapy. Ideally, peptide:HLA complexes should be detected in a fast and direct manner, for example by staining the peptide:HLA complex of interest. Target peptide:HLA complexes on malignant cells can be detected by staining with a soluble variant of the therapeutic TCR. However, sensitivity currently only allows detection of artificially overexpressed peptide:HLA complexes and further optimization would be needed to achieve sufficient sensitivity to detect natural levels of peptide:HLA complexes on cells(37). Rather than direct detection of the target peptide:HLA complex, functional analysis of TCR T-cell recognition might be a more

feasible way to assess targetability of malignancies. Potential TCR T-cell recognition of a patient sample could be swiftly assessed by using TCR transgenic T-cell reporter cell lines like the Jurkat 76-based triple parameter reporter(38). These cells allow detection of TCR triggering by flowcytometry within one day of co-culture with target cells of interest. Confirming TCR T-cell recognition of malignancies prior to therapy will be critical, especially for certain epitopes. Incorporating such diagnostics into clinical trials could be valuable regardless of the targeted epitope, as the generated data could ultimately be used for outcome predictions or to refine therapies in the future.

Expand coverage of TCRs for therapy of MM

As the field of TCR therapy advances, identifying new TCRs with alternative HLA restrictions and specificities becomes crucial to extend the patient population eligible for TCR therapy. Maximizing accessibility will require identification of TCRs targeting less frequently expressed HLA alleles. Among the various HLA types, HLA-A alleles exhibit the lowest degree of polymorphism, positioning these alleles as the most interesting restriction alleles for TCRs to efficiently achieve broad coverage within the population(39). Alternatively, HLA-C shares a similar degree of polymorphism. However, identification of HLA-C restricted TCR comes with some challenges, which became apparent when we tried to identify HLA-C restricted Jchain specific TCRs (data not shown). T cells expressing certain KIR molecules bound HLA-C pHLA-multimers via the KIR receptor instead of their TCR. Interference by KIRs could be overcome by counterstaining and exclusion of KIR+ T cells(40).

Attempts to identify TCR targeting less common HLA alleles currently face considerable obstacles, including limited research funding, publication bias, a lack of data on rarer HLA alleles, and the challenge of recruiting patients for clinical trials. Scientific data on rare HLA alleles is limited for example in peptidome databases, which mostly collected data from materials expressing common HLA alleles. As a result, peptide binding motifs for rare alleles are weakly defined, hampering identification of peptides presented by rare alleles, which is a fundamental starting point of the TCR identification pipeline. Specific attempts are needed to establish peptide binding motifs for rare HLA alleles. Approaches may involve introduction of single HLA molecules into HLA class-I negative cells followed by peptidome analysis. Alternatively, HLA positive malignant cell lines could be used, for which endogenous HLA knockout followed by introduction of a single HLA allele should be performed prior to peptidome analysis.

Furthermore, the identification of new target peptide-HLA combinations is central to extending the repertoire of available TCRs, a process that could be improved by targeted mass spectrometry. Targeted mass spectrometry involves spiking the peptide of interest, labeled with a stable epitope, into the biological sample, followed by parallel reaction

monitoring (PRM) mass spectrometry(41). Targeted mass spectrometry is a refined approach with increased sensitivity and selectivity over regular methods.

Moreover, the limited availability of patient material for pre-clinical testing underscores the necessity for comprehensive biobanking and the open exchange of diagnostic samples among institutes. This collaborative spirit is vital for the pre-clinical evaluation of therapies targeting rare HLA types.

Combination therapy with TCRs

Effective viral clearance after infection is achieved through oligo or polyclonal T-cell responses directed towards multiple antigens and multiple HLA restriction alleles(42). Similarly, effectiveness of allogeneic stem cell transplantation (allo-SCT) for hematologic malignancies has been attributed to polyclonal T-cell responses(43). In transgenic T-cell therapy, single-antigen-targeting regimens are associated with antigen escape and disease relapse. To improve efficacy, it would be ideal to include multiple receptors with different characteristics in a transgenic T-cell product to mimic the polyclonal immune responses underlying effective viral defense or cure after allo-SCT for malignancies(44). For multi-targeting TCR therapy of B-/plasma cell malignancies different types of target antigens are explored, one type of targets are tumor-associated antigens (TAAs). TAAs are proteins that are expressed by tumors of various origins while expression in healthy adult tissues is absent or restricted to immune privileged sites. TAAs like MAGE family members, PRAME and NY-ESO-1 are expressed in some MM cases. TAAs, despite heterogeneous expression, might be appealing targets as they often appear in advanced disease stages and expression inversely correlated with survival (19, 45). Many TAA-specific TCRs have already been identified but these TCRs typically target commonly expressed HLA alleles and therefore the target HLA diversity of such TCRs is limited(46). The efficacy of an HLA-A*02:01 restricted TCR targeting NY-ESO-1 has been investigated for therapy of MM. NY-ESO-1 is expressed in one-third of the MM cases (45). 70% of the patients with NY-ESO-1+ tumor had a (near) complete response after treatment with autologous stem cell transplantation followed by NY-ESO-1 TCR T-cell infusion (23). Specific depletion of NY-ESO-1+ tumor cells demonstrated on-target activity of NY-ESO-1 TCR T cells and simultaneously revealed that antigen escape variants can arise when targeting heterogenous TAAs (23, 45). To achieve full tumor clearance with TCR T cells, especially when targeting heterogeneous antigens like TAAs, combination therapy should be employed. However, for many patients TAA-targeting TCRs are not available, either because TAAs are not expressed in the patient's malignant cells or due to the limited HLA diversity of currently available TAA-targeting TCRs. Therefore, many patients would benefit from an additional set of TCRs with different HLA restriction and targeting different types of antigens, such as the B-cell antigen targeting TCRs identified in this thesis.

Throughout this thesis, multiple TCRs targeting proteins expressed in MM cells are described, namely TCRs specific for peptides from BOB1 in HLA-B*35:01, Jchain in HLA-A*01:01, Jchain in HLA-A*03:01, Jchain in HLA-A*11:01, Jchain in HLA-A*24:02, IgG in HLA-A*02:01, and IgA in HLA-B*07:02 TCR. Previous work has identified a BOB1-targeting TCR recognizing a peptide presented in HLA-B*07:02(47). Collectively, these TCRs target 3 different target proteins: BOB1, the Jchain, and immunoglobulin heavy chains. Each of these target proteins presents a promising candidate for TCR T-cell therapy because of favorable characteristics. BOB1, encoded by the *POU2AF1* gene, is a transcriptional coactivator and BOB1 is expressed in at normal levels in MM. Expression of BOB1 has been shown to be essential for the survival of MM cells by us and others(unpublished data)(48). MM cells with knockout of the BOB1 protein do not survive, indicating that it is unlikely that MM cells escape BOB1-TCR therapy by loss of the target protein. All patient-derived MM samples tested to date express the BOB1 protein. While we did not analyze single cell-based gene expression profiles, the necessity of BOB1 suggests homogenous expression within MM tumors. The Jchain represents a different type of antigen, which is expressed in approximately 80% of MM samples independent of the produced immunoglobulin isotypes(49, 50). Currently, there is no evidence for an indispensable role for the Jchain in MM cells. A benefit of targeting the Jchain is the enormous amount of Jchain protein produced in MM. As established in **Chapter 3** Jchain gene expression exceeds expression of housekeeping genes by many-fold. Immunoglobulin heavy chains share characteristics with the Jchain. An essential role for immunoglobulins in MM seems unlikely, but the enormous levels of immunoglobulins produced by MM cells enables potent recognition by immunoglobulin-specific TCR T cells. Our data has demonstrated that both Jchain- and immunoglobulin-targeting TCR T cells can effectively eliminate MM cells from patient BM samples in short term co-cultures. For highly expressed target proteins, the likelihood of identifying TCRs with sufficient avidity to recognize malignant cells increases(51), which probably contributed to the identification of TCRs targeting these proteins in this thesis. BOB1, Jchain and immunoglobulin TCRs identified in this thesis have various HLA restrictions. In a combination regimen, our TCRs can be used to target different proteins, each with their own characteristics, across different HLA restriction alleles.

To explore the hypothetical MM patient coverage that could be treated with one or more of these TCRs, several factors need to be considered, including gene expression frequencies of target antigens and HLA allele frequencies in the population. The expression frequencies of the target proteins are in MM: 100% for BOB1, 80% for Jchain, 52% for IgG and IgA is expressed in 21% of cases. Calculations including gene expression and HLA alleles frequencies of the identified TCRs reveal that TCRs identified in this thesis can be used to treat approximately 67% of patients with MM. For 28% of the patients more than one TCR would be available. Together these identified TCRs can

significantly contribute to the availability of TCRs for patients with MM as well as the potential option to generate multi-targeting TCR products for therapy of patients with MM complementing TAA-targeting TCRs such as the NY-ESO-1 TCR.

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