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

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Citation
Meeuwsen, M. H. (2024, October 1). *TCRs as precision tools against B-cell and plasma cell malignancies*. Retrieved from https://hdl.handle.net/1887/4093497

Version:	Publisher's Version
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Note: To cite this publication please use the final published version (if applicable).

CHAPTER 2

A broad and systematic approach to identify B cell malignancy-targeting TCRs for multi-antigen-based T cell therapy

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Molecular therapy 2022
Vol. 30, Issue 2, Pages 564-578

ABSTRACT

CAR T-cell therapy has shown great promise for the treatment of B-cell malignancies. However, antigen-negative escape variants often cause disease relapse necessitating the development of multi-antigen-targeting approaches. We propose that a TCR-based strategy would increase the number of potential antigenic targets as peptides from both intracellular and extracellular proteins can be recognized. Here, we aimed to isolate a broad range of promising TCRs targeting multiple antigens for treatment of B-cell malignancies. As a first step, 28 target genes for B-cell malignancies were selected based on gene expression profiles. Twenty target peptides presented in either HLA-A*01:01, A*24:02, B*08:01 or B*35:01 were identified from the immunopeptidome of B-cell malignancies and used to form peptide-HLA-tetramers for T-cell isolation. Target-peptide specific CD8 T cells were isolated from HLA-mismatched healthy donors and subjected to a stringent stepwise selection procedure to ensure potency and eliminate cross-reactivity. In total, five T-cell clones specific for FCRL5 in HLA-A*01:01, VPREB3 in HLA-A*24:02 and BOB1 in HLA-B*35:01 recognized B-cell malignancies. For all three specificities, TCR gene transfer into CD8 T cells resulted in cytokine production and efficient killing of multiple B-cell malignancies. In conclusion, using this systematic approach we successfully identified three promising TCRs for T-cell therapy against B-cell malignancies.

INTRODUCTION

Adoptive T-cell therapy for the treatment of B-cell malignancies has shown great promise over the past decade. CD19-targeting chimeric antigen receptor (CAR) T-cell therapies have induced complete remission (CR) in 70-97% of patients with relapsed or refractory acute lymphoblastic leukemia (ALL).(1, 2) In aggressive relapsed or refractory diffuse large B-cell lymphoma (DLBCL) and transformed follicular lymphoma CR rates after CD19 CAR therapy are approximately 50%.(3, 4) In responding patients, antigen-loss escape variants are frequently observed, hampering long-term relapse-free survival.(5, 6) Multi-antigen-targeting T-cell therapy may reduce outgrowth of antigen-escape variants and enhance long-term remission rates of patients, as simultaneous loss of multiple antigens is more unlikely.(7, 8) However, in order to generate multi-antigen-targeting T-cell therapies, additional potent and safe T-cell therapies need to be developed.

Target antigens for immunotherapy of cancer should be expressed by the malignant cells while expression in essential healthy tissues must be absent.(9, 10) B-cells are considered a non-essential tissue, therefore antigens expressed by the healthy B-cell lineage could be safe targets for the treatment of B-cell malignancies. This was confirmed upon CD19 CAR T-cell therapy, which resulted in depletion of healthy B cells which was clinically managed by immunoglobulin administration.(11)

CAR T cells target epitopes of proteins located on the cell surface.(12) In contrast to CARs, T-cell receptors (TCRs) recognize peptides derived from proteins independent of cellular localization that are presented in HLA molecules on the cell surface.(13) Therefore, TCR based therapy theoretically allows targeting of all proteins, including those involved in essential intracellular pathways. So far, limited availability of safe and high-affinity TCRs has hampered clinical progress of TCR gene therapy. Furthermore, a large collection of high affinity TCRs targeting different peptide-HLA complexes would be required to allow TCR gene therapy for all patients.

T cells with high affinity TCRs for self-peptides presented in self-HLA alleles are deleted from the T-cell repertoire to prevent autoimmunity. Therefore, high avidity T cells targeting non-mutated peptides cannot be isolated from HLA-matched individuals.(14, 15) In contrast, high avidity T cells targeting non-mutated peptides have successfully been isolated from the T-cell repertoire of HLA-mismatched (allogeneic) healthy donors.(15-23) Previous efforts have mainly focused on identification of TCRs specific for peptides presented in HLA-A*02:01 and HLA-B*07:02.(15-19, 24) Here, to allow application of TCR gene therapy for individuals with other HLA genotypes, we aimed to identify TCR specific for peptides presented in HLA alleles A*01:01 (A1), A*24:02 (A24), B*08:01 (B8) and B*35:01 (B35). In this study, we performed a systematic approach for

simultaneous identification of clinically relevant TCRs targeting multiple epitopes. We started by identifying genes with expression restricted to B-cell malignancies and the healthy B-cell lineage. Target peptides derived from these genes presented in target HLA alleles HLA-A1, A24, B8 or B35 were selected from the immunopeptidome of B-cell malignancies. To allow identification of high avidity T cells targeting these epitopes, T cells were isolated from the allogeneic (allo) T-cell repertoire using HLA-mismatched healthy donor PBMCs. Multiple efficacy and safety screenings resulted in the selection of clinically relevant TCRs recognizing antigens in HLA-A1, HLA-A24 and HLA-B35 presented on B-cell malignancies. Finally, upon TCR gene transfer, selected TCRs induced specific lysis of different B-cell malignancies including a multiple myeloma (MM) cell line and patient-derived ALL, chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL) and hairy cell leukemia (HCL).

RESULTS

Target gene selection for treatment of B-cell malignancies

To ensure targeting of malignant B cells while limiting the risk of toxicity, data obtained from an in-house-generated microarray database were used to identify genes expressed in B-cell malignancies with expression restricted to the B-cell lineage. This database was previously generated and validated for selection of target antigens valuable for immunotherapy of hematological malignancies with lineage restricted expression. (25) Gene expression was measured by probe fluorescence represented as mean fluorescence intensity (MFI), with a lower detection limit of MFI=50. Potent T-cell recognition occurs when target antigens are highly expressed in B-cell malignancies, and therefore, a threshold of gene expression MFI≥250 in primary ALL, CLL or MM was set for each probe (**Figure S1**). To prevent recognition of healthy tissues (excluding B cells), a threshold was set at MFI=100 to define very low expression and all genes expressed MFI>100 in healthy tissues were excluded. In total, 28 genes highly expressed in primary B-cell malignancies with very low or no expression in healthy tissues were selected as target genes (**Figure 1**). Of these genes, 14 were highly expressed (MFI≥250) in either ALL, CLL or MM, while 13 genes were expressed in two types and the BOB1 encoding gene *POU2AF1* was expressed in all three types of primary B-cell malignancies.

Identification of candidate epitopes from the immunopeptidome of malignant B-cells

To identify target peptides for TCR based recognition of B-cell malignancy antigens, the HLA-presented peptide repertoire of B-cell malignancies was determined. Patient material was obtained from nine ALL patients, two CLL patients, one hairy cell leukemia (HCL) and one follicular lymphoma patient expressing HLA-A1, A24, B8 and/or B35 (**Table S1**).

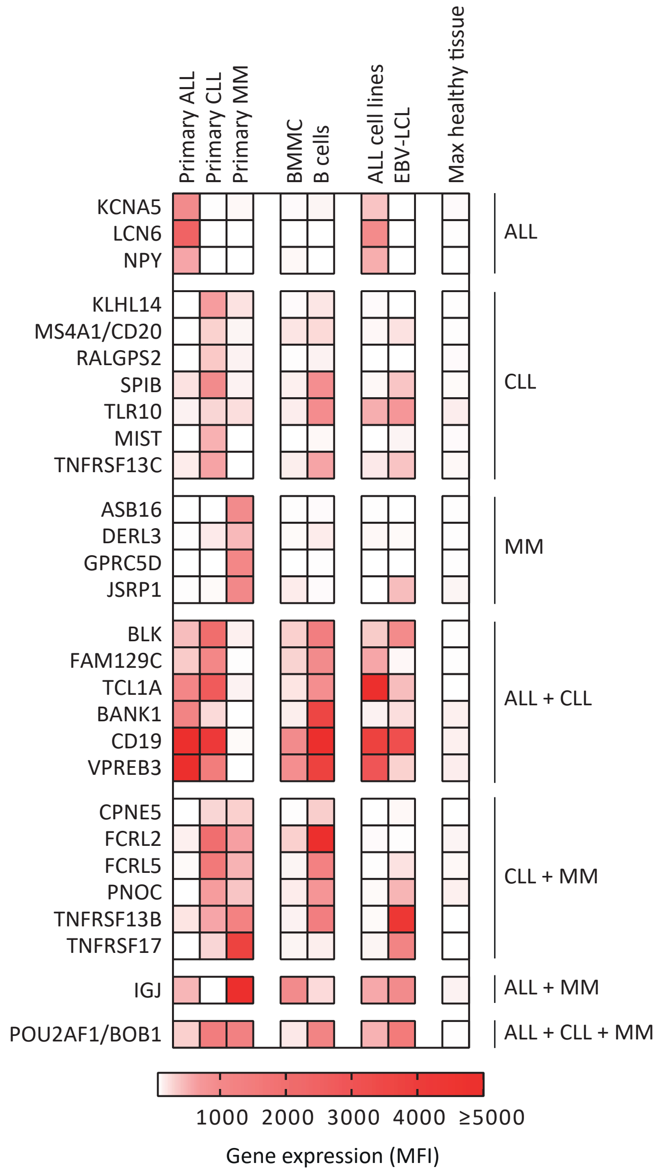

Figure 1. Gene expression of target genes selected for treatment of B-cell malignancies. Gene expression was retrieved from an Illumina HT12.0 microarray dataset.(25) Per gene the average mean fluorescence intensity (MFI) of samples is shown for different types of patient derived (primary) B-cell malignancies, BMMCs, B cells ($CD19^{pos}$), ALL cell lines and EBV-LCLs. The final column represents the highest gene expression as measured in any healthy tissue other than B cells, PBMCs and BMMCs as included in the microarray dataset and shown in supplementary figure 1. Genes are clustered according to the type of B-cell malignancy in which they are expressed (MFI≥250). Abbreviations: MFI, mean fluorescence intensity; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; BMMC, bone marrow mononuclear cells; EBV-LCL; Epstein-Barr virus-transformed lymphoblastoid cell lines.

Since materials from MM patients lacked sufficient cell numbers for peptide elution, the MM cell line UM9 was included instead. Cells from each malignant cell population were lysed, and peptides were eluted before subsequent separation by HPLC and mass spectrometry analysis. Per sample, between 539 and 82,504 unique peptides, with an Ion Score (IS) ≥20, were identified (**Table S1**). The Ion Score indicates the confidence of correctly matching the observed mass spectrum of a peptide to the reference database spectrum. Therefore, a cutoff of Ion score ≥20 was used to ensure a high chance of correct peptide identification. Eluted peptides were matched to HLA alleles by combining predicted HLA binding (netMHC3.4) with the HLA typing of the material from which the peptides originated. Twenty peptides were identified to be derived from one of the target genes and presented by target HLA alleles HLA-A1, A24, B8 or B35 (**Table S2, Table 1**). Peptide sequences of these peptides were validated by comparing mass spectra of eluted peptides to spectra of synthetically generated peptides (**Figure S2**). After peptide verification, target HLA binding was confirmed by stable peptide-HLA (pHLA)-monomer refolding (data not shown). From these monomers PE-labeled pHLA-tetramers were generated and used for T-cell isolation.

Table 1. Target gene derived peptides presented in HLA-A1, A24, B8 or B35 by B-cell malignancies

Protein	HLA	Assigned peptide nr	Sequence	Affinity (nM)[a]	SB/WB[a]
RALGPS2	A1	p242	LTDSEKGNSY	24	SB
FCRL5	A1	p243	LTEGHSGNYY	15	SB
FCRL5	A1	p263	TTENSGNYY	9	SB
MIST	A1	p248	ESEYADTHY	102	WB
TLR10	A1	p265	YLDHNSFDY	8	SB
IGJ	A1	p268	YTAVVPLVY	7	SB
BLK	A24	p246	AYIERMNSI	75	WB
VPREB3	A24	p269	YYCSVGYGF	37	SB
RALGPS2	A24	p258	QYIEELQKF	78	WB
TLR10	B8	p247	ELFKRTIQL	54	WB
TLR10	B8	p256	LPHLKTLIL	26	SB
IGLL1	B8	p251	HGLLRPTAA	588	<WB[b]
KLHL14	B8	p239	DMNTKRAIHTL	396	WB
FCRL2	B8	p252	IVKIKVQEL	252	WB
FAM129C	B8	p255	LPALRAQTL	32	SB
FAM129C	B8	p271	YLRLLDAL	726	<WB[b]
RALGPS2	B8	p261	TLKIRAEVL	43	SB
TNFRSF13B	B35	p259	SADQVALVY	11	SB
BOB1	B35	p233	APAPTAVVL	196	WB
BOB1	B35	p236	LPHQPLATY	6	SB

[a] According to NetMHC 3.4. [b] HLA-B8 most likely origin of peptides, highest predicted binding.

Isolation and selection of T-cell clones with on-target functional specificity

To isolate high avidity T cells recognizing target peptides, pHLA-tetramers were incubated with PBMCs from healthy donors negative for the target HLA alleles (**Table S3**). pHLA-tetramer bound cells were enriched and pHLA-tetramer^{pos} CD8^{pos} T cells were single-cell sorted and clonally expanded. In total 12,336 (Range; 192-2,640) T cells were sorted from 13 healthy donors (**Table 2**). On average 59% (Mean; range 14-83%) of the T-cell clones expanded. Expanded T-cell clones were initially screened for on-target functional specificity. The HLA-negative myeloid leukemia cell line K562 was transduced (Td) with target HLA alleles and mixed into two pools containing HLA-A1 and HLA-A24 or HLA-B8 and HLA-B35. All target peptides were mixed and loaded onto K562 before incubation with the T-cell clones (**Table 1**). As measured by cytokine production, unloaded K562 recognition was frequently observed revealing off-target recognition and these clones were discarded to prevent off-target toxicity (**Table 2**). Additionally, 34-98% of expanded T-cell clones were not reactive and were discarded due to lack of target peptide recognition. On-target recognition was defined when only peptide mix loaded K562 cells were recognized. From these 13 donors, 23 T-cell clones recognized only HLA-A1/A24^{pos} K562 cells loaded with the target peptide mix (**Table 2**). In addition, 23 T-cell clones only recognized target peptide loaded HLA-B8/B35^{pos} K562 cells (**Table 2**). In summary, 46 T-cell clones demonstrated specific, on-target recognition and were selected for further screening.

Defining T-cell specificity and confirming recognition of endogenously processed target antigen

To determine the peptide specificity of each selected T-cell clone, target peptides were assorted into combinatorial mixes revealing unique recognition patterns for all specificities. Mixes were loaded onto HLA-A1/A24^{pos} or HLA-B8/B35^{pos} K562 cells accordingly and incubated with each T-cell clone. For example, T-cell clones 1F3.4 and 6B10.12, recognized two peptide mixes loaded on HLA-A1/A24^{pos} K562 cells (**Figure 2A**). This recognition pattern revealed specificity for peptide 243 (p243) derived from FCRL5 presented in the context of HLA-A1 (**Table 1**). Following this approach, all 46 T-cell clones were tested revealing 11 different peptide specificities (**Table 2**). Since recognition of endogenously processed and presented antigen correlates with T-cell avidity and recognition of malignant cells (26), T-cell clones were also screened for recognition of K562 cells Td with target genes and target HLA alleles. T-cell clones demonstrating no or weak recognition of endogenously processed and presented antigen were discarded. In Figure 2, 23 T-cell clones, recognizing eight different B-cell specific peptides, that exhibited strong endogenous recognition are shown. These 23 T-cell clones were selected for further investigation of clinical relevance.

Table 2. Identification of target peptide specific T-cell clones from tetramer^{pos} CD8^{pos} sorted T cells using healthy donor PBMCs

			Reactivity of T-cell clones [d]				Peptide specificity of T-cell clones [e]										
Donor [a]	T cells sorted (n) [b]	Clones exp. (n) [c]	Not reactive (n)	Off-target (n)	HLA-A1/A24 (n)	HLA-B8/B35 (n)	p236 POU2AF1 B35	p243 FCRL5 A1	p247 TLR10 B8	p248 MIST A1	p252 FCRL2 B8	p256 TLR10 B8	p259 TNFRSF13B B35	p261 RALGPS2 B8	p263 FCRL5 A1	p265 TLR10 A1	p269 VPREB3 A24
3	288	239	233	6	0	0											
4	192	156	101	54	1	0		1x									
5	192	129	44	83	0	2							2x				
6	528	433	235	180	6	12	6x		1x	2x	3x		1x	1x		3x	1x
7	288	207	181	26	0	0											
8	384	311	289	22	0	0											
12	864	639	320	314	5	0		1x		2x					2x		
13	1728	1002	891	108	2	1						1x					2x
14	1296	384	330	53	0	1						1x					
15	864	449	399	49	0	1								1x			
16	1920	1018	907	105	3	3				1x			2x	1x			2x
17	2640	360	351	9	0	0											
18	1152	192	143	40	6	3				2x	2x	1x				2x	2x
Total	12336	5519	4424	1049	23	23	6x	2x	1x	7x	5x	3x	5x	3x	2x	5x	7x

[a] Arbitrary donor numbers assigned to healthy donors from which PBMCs were obtained, in accordance with Table S3. [b] Number of pHLA-tetramer^{pos} CD8^{pos} T cells single-cell sorted from each donor. [c] Number of single-cell sorted T cells that expanded and were tested for peptide specificity. [d] Expanded T-cell clones were stimulated with a 1:1 mixture of HLA-A1 and HLA-A24 transduced K562 cells or HLA-B8 and B35 transduced K562 cells, unloaded or loaded with a mixture of all target peptides (100nM). Reactivity was assessed after overnight co-culture based on IFN-γ ELISA, for T-cell clones not producing IFN-γ, GM-CSF was used instead. Recognition was categorized in; not reactive, off-target recognition, HLA-A1/A24 restricted peptide specific recognition (HLA-A1/A24) and HLA-B8/B35 restricted peptide specific recognition (HLA-B8/B35). [e] The number of peptide specific T-cell clones per donor is indicated, specificity was determined as demonstrated in Figure 2. No T-cell clones were identified for peptides not included in the table.

Recognition of B-cell malignancies identifies T-cell clones which are candidates for TCR-sequencing

To identify candidates for TCR sequencing, the 23 selected T-cell clones were screened for recognition of B-cell malignancies. T-cell clones were tested for cytokine production upon stimulation with patient derived CLL material, ALL cell lines or MM cell lines. Per specificity the appropriate cell types were selected based on gene expression profiles (**Figure 1**). Target gene expression in materials used was determined by qPCR (**Figure S3**). Despite recognition of endogenously processed antigen in target gene Td k562 cells, MIST HLA-A1, FCRL2 HLA-B8, RALGPS2 HLA-B8 and TNFRSF13B HLA-B35 restricted T-cell clones failed to produce cytokine upon co-culture with malignant B-cells (data not shown). FCRL5 HLA-A1 specific T-cell clones 1F3.4 and 7E8.12, recognizing p243 and p263 respectively, were co-cultured with *FCRL5* expressing patient derived CLL samples.

T-cell clone 6B10.12 did not expand and could therefore not be included. Recognition of HLA-A1^{pos} CLL samples was observed for clone 1F3.4 (**Figure 3A**), but not for clone 7E8.12 (data not shown). Allo-HLA T-cell clones were included as positive controls for stimulatory capacity and HLA expression of the target cells. Furthermore, 3 out of 6 VPREB3 HLA-A24 restricted T-cell clones (6A2.18, 6E2.18 and 10G3.16) efficiently recognized all *VPREB3* expressing HLA-A24^{pos} ALL cell lines, whereas HLA-A24^{neg} ALL cell lines were not recognized (**Figure 3B**). In addition, BOB1 HLA-B35 restricted T-cell clone 1C5.6, demonstrated efficient recognition of all *POU2AF1* (BOB1) expressing HLA-B35^{pos} ALL and MM cell lines tested (**Figure 3C**). BOB1 HLA-B35 restricted T-cell clone 4H5.6 did not recognize these B-cell malignancies (data not shown). Of note, malignant cell recognition by the VPREB3 HLA-A24 and BOB1 HLA-B35 T-cell clones correlated with a high avidity as measured by peptide titration (**Figure S4**). To summarize, 5 out of 22 tested T-cell clones recognized B-cell malignancies and were therefore promising candidates for TCR gene therapy. These T-cell clones were specific for FCRL5 in HLA-A1, VPREB3 in HLA-A24 and BOB1 in HLA-B35.

Safety screenings reveal high on-target specificity of selected T-cell clones

To study the specificity of the 5 selected T-cell clones, safety profiles were investigated. Cross-reactivity with other HLA-alleles was assessed using an Epstein-Barr virus transformed lymphoblastoid cell line (EBV-LCL) panel expressing all HLA-I alleles with an allele frequency >1% in the Caucasian population (**Figure 4, Table S4**).(27) Additionally, cross-reactivity with other peptides presented in target HLA alleles was determined by stimulating with HLA-A1 and B8 or HLA-A24 and B35 Td cell lines of non-B-cell origins (**Figure 4**). The respective allo HLA-A1, A24 and B35 T-cell clones recognized the HLA transduced cell lines (**Figure S5**), demonstrating that sufficient HLA was expressed to allow T-cell recognition. T-cell clones 1F3.4 (FCRL5 HLA-A1), 6A2.18 (VPREB3 HLA-A24), 10G3.16 (VPREB3 HLA-A24) and 1C5.6 (BOB1 HLA-B35) did not demonstrate cross-reactivity with any of the target cells included in the screenings (**Figure 4A-C**). However, T-cell clone 6E2.18 (VPREB3 HLA-A24) produced IFN-γ when stimulated with EBV-LCLs from donor URN (EBV-LCL URN) and upon stimulation with HLA-A24/B35 Td CASKI cells (**Figure 4B**). This was a result of cross-reactivity with two possible peptide-HLA complexes. Recognition of EBV-LCL URN resulted from cross-reactivity with a peptide presented in HLA-B*08:01 or B*50:01, since these HLA alleles were expressed by EBV-LCL URN but not by other EBV-LCLs in the panel. Recognition of HLA-A24/B35 Td CASKI cells was caused by cross-reactivity with a peptide in HLA-A24 or B35, since untransduced CASKI cells were not recognized (data not shown). In summary, most T-cell clones targeting FCRL5 in HLA-A1, VPREB3 in HLA-A24 or BOB1 in HLA-B35 revealed promising safety profiles for clinical application in TCR gene therapy of B-cell malignancies.

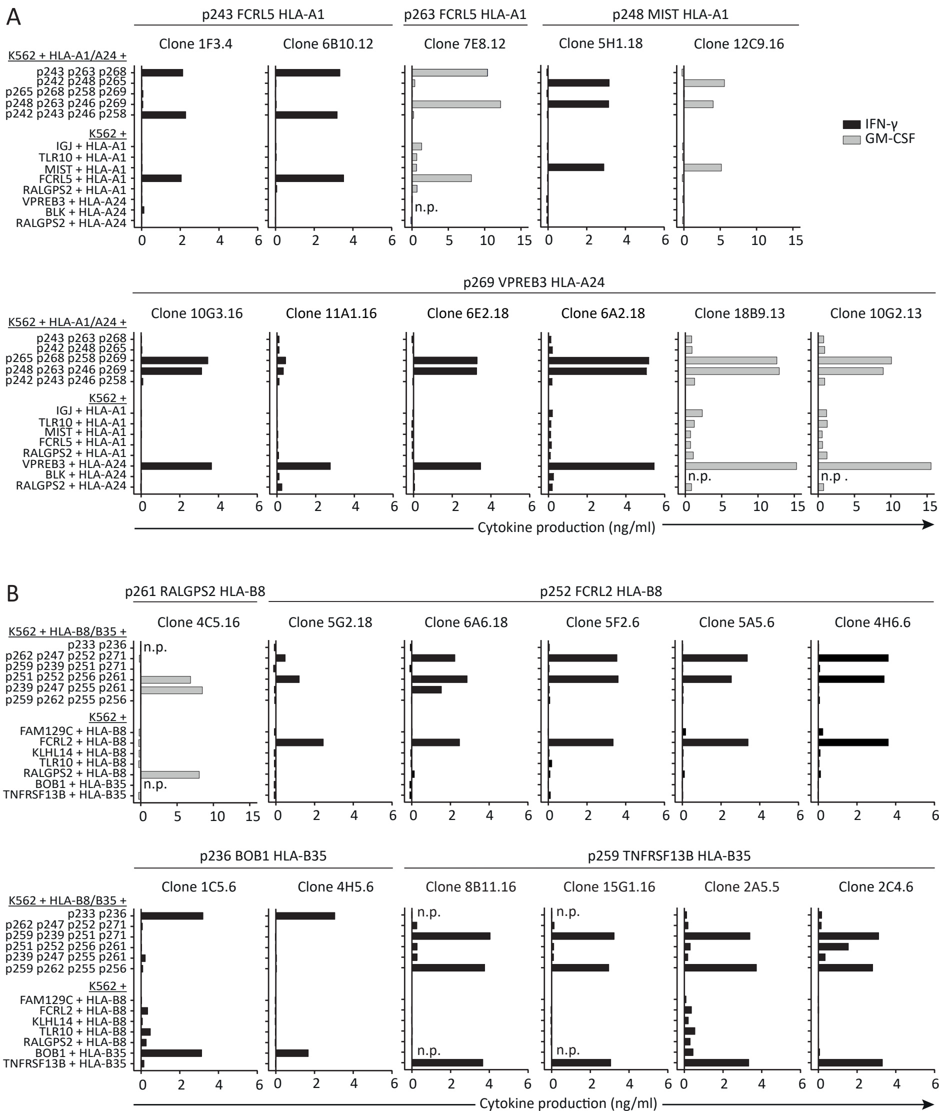

Figure 2. Peptide specificity and target gene recognition by selected T-cell clones. T-cell clones were stimulated with the appropriate HLA-expressing K562 cells in an effector:target ratio of 1:6 loaded with combinatorial combinations of target peptides to determine peptide specificity (upper part) and K562 cells Td with target gene and HLA (bottom part) to determine recognition of endogenously processed and presented peptide. IFN-γ (in black) or GM-CSF (in grey) production by T-cell clones categorized as high endogenous recognition. GM-CSF production is shown for clones that do not produce IFN-γ. T-cell clones isolated from different donors were tested in separate experiments. **A)** T-cell clones recognizing HLA-A1 or A24 presented target peptides. **B)** T-cell clones recognizing HLA-B8 or B35 presented target peptides, peptide 233 and 236 were not part of combinatorial mixes. For clone 1C5.6 and 4H5.6 specificity for p236 was determined by pHLA-tetramer stain (data not shown). Abbreviations: n.p., not performed

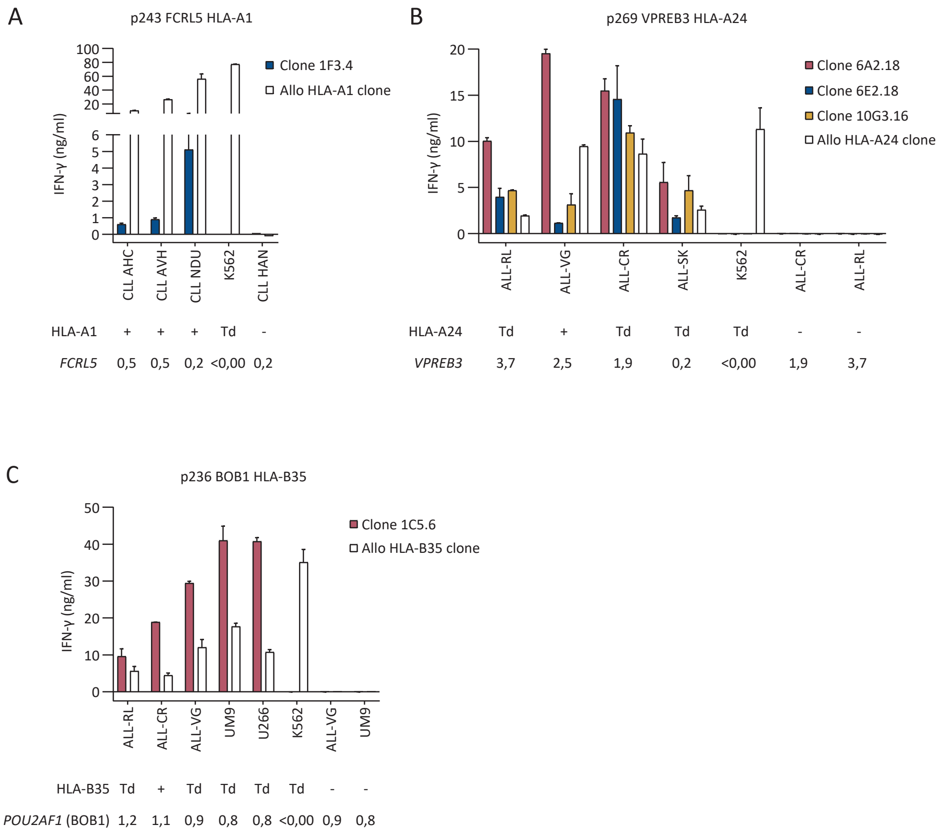

Figure 3. Recognition of malignant B cells by selected T-cell clones. IFN-γ production by T-cell clones after overnight stimulation with various target cells. Values and error bars represent mean and standard deviations of technical duplicates. Target cells were positive (+), negative (-) or transduced (Td) with target HLA. Target gene expression was measured by qPCR and is depicted below targets as expression relative to housekeeping genes (HKG set to 1). Allo HLA T-cell clones were included as positive controls for HLA expression. **A)** FCRL5 HLA-A1 specific T-cell clone stimulated with patient derived chronic lymphocytic leukemia (CLL) samples, in an effector:target (E:T) ratio of 1:20 to correct for cell size and negative control K562 cells in E:T ratio 1:6. T-cell clone 6B10.12 could not be tested due to lack of expansion. **B)** VPREB3 HLA-A24 clones stimulated with different acute lymphoblastic leukemia (ALL) cell lines and K562 cells in E:T ratio 1:6. **C)** BOB1 HLA-B35 clone stimulated with different ALL cell lines, multiple myeloma cell lines UM9 and U266 and K562 cells in E:T ratio 1:6.

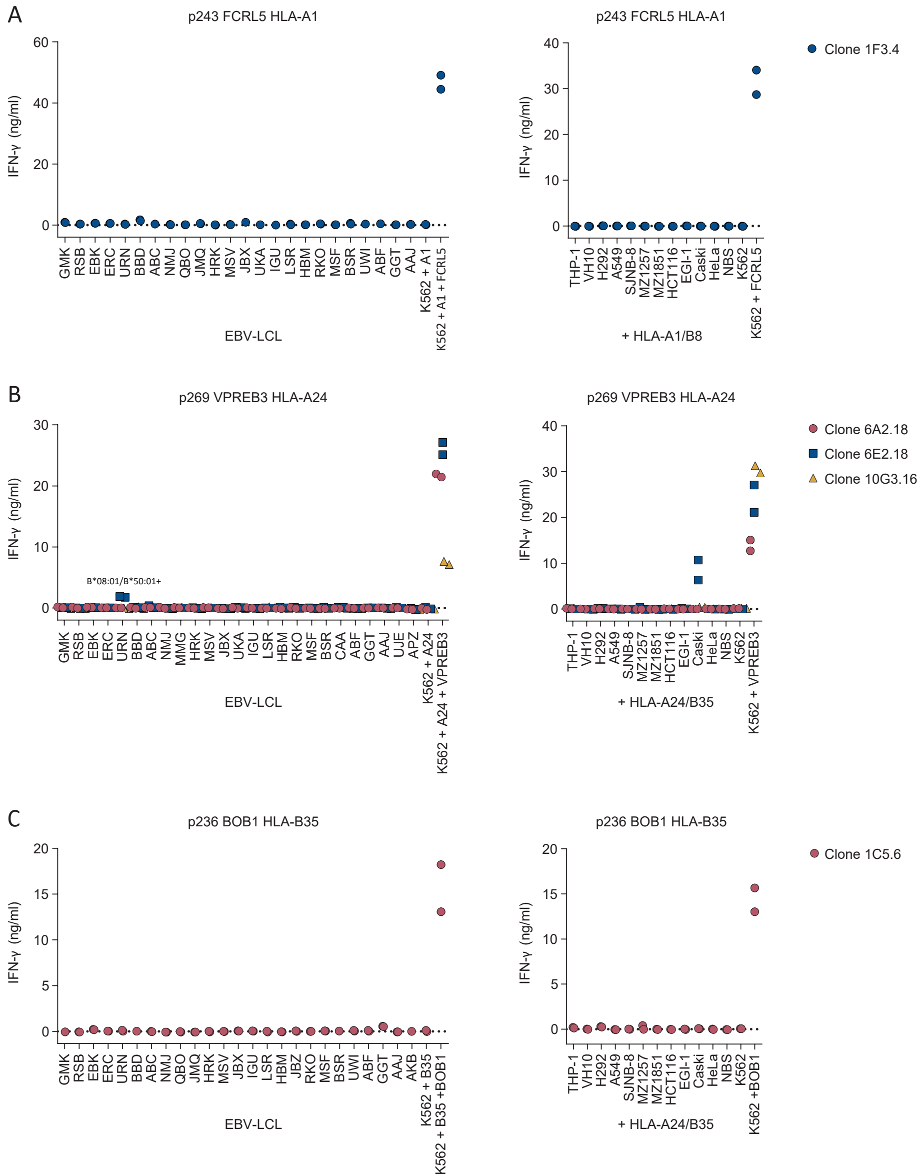

Figure 4. Safety screenings for selected T-cell clones. IFN-γ production by T cells measured by ELISA after overnight co-culture, technical duplicates are depicted. T-cell clones stimulated with an Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) panel not expressing target HLA alleles (left panels). Target gene and HLA transduced K562 cells were included as positive control for T-cell function. T cells were stimulated in effector:target (E:T) ratio 1:6. T-cell clones stimulated with NBS and VH10 fibroblast cell lines, Hela and Caski cervix carcinoma cell lines, EGI-1 bile duct carcinoma cell lines, HCT116 colon carcinoma cell line, MZ1851 and MZ1257 renal cell carcinoma cell lines, SJNB-8 neuroblastoma cell line, A549 and H292 lung carcinoma cell lines transduced with HLA-A1 and HLA-B8 (+HLA-A1/B8) or HLA-A24 and HLA-B35 (+HLA-A24/B35) (right panels). Lack of *FCRL5*, *VPREB3* and *POU2AF1* (BOB1) expression in these cell lines was confirmed by qPCR (data not shown). **A)** Safety screenings of FCRL5 HLA-A1 specific T-cell clones. **B)** Safety screenings of VPREB3 HLA-A24 T-cell clones. **C)** Safety screenings of the BOB1 HLA-B35 T-cell clone.

TCR gene transfer to CD8 T cells reveals promising candidates for TCR gene therapy of B-cell malignancies

To study the potential for clinical application in TCR gene therapy, TCRs of the five selected T-cell clones were sequenced and retrovirally transferred to healthy donor CD8 T cells. Additionally, the TCR of T-cell clone 6B10.12 (FCRL5 HLA-A1), which was not screened for recognition of B-cell malignancies due to lack of *in vitro* expansion, was included. All six identified TCRs, including the TCRs from T-cell clones 6A2.18 and 6E2.18 which were isolated from the same donor, had unique TCR sequences (data not shown). Moreover, all TCRs were functional upon transfer into CD8 T cells as demonstrated by pHLA-tetramer binding. The intensity of the staining, however, was weaker in TCR Td CD8 T cells than in the parental T-cell clones (**Figure 5A**). To determine clinical relevance of identified TCRs, cytokine production by TCR Td T cells after co-culture with primary B-cell malignancies of multiple origins was assessed. HCL, MCL, ALL and CLL patient derived samples were included depending on availability of material expressing target HLA in the LUMC biobank. TMD8 and U266 were used as representatives for DLBCL and MM respectively, as primary malignant cells were not available for these diseases.

2

The results depicted in Figure 5B demonstrate that FCRL5 HLA-A1 specific TCR 6B10.12 Td T cells efficiently recognized *FCRL5* expressing HLA-A1^{pos} patient derived HCL, MCL and CLL samples, as well as HLA-A1 Td DLBCL cell line TMD8. Reactivity by TCR 6B10.12 Td T cells was more efficient than by TCR 1F3.4 Td T cells (**Figure 5B**), which corresponded with the difference observed in tetramer binding (**Figure 5A**).

VPREB3 HLA-A24 specific TCR 6A2.18 Td T cells consistently outperformed TCRs 6E2.18 and 10G3.16 for recognition of HLA-A24^{pos} patient derived ALL and CLL samples, DLBCL cell line TMD8, and MM cell line U266 (**Figure 5B**), although, TCR 6E2.18 Td T cells showed higher tetramer binding than TCR 6A2.18 Td T cells (**Figure 5A**).

Finally, BOB1 HLA-B35 restricted TCR 1C5.6 Td T cells efficiently recognized patient derived HLA-B35^{pos} ALL, CLL and MCL samples as well as HLA-B35 Td MM and DLBCL cell lines (**Figure 5B**). Based on these results TCRs 6B10.12 (FCRL5 HLA-A1), 6A2.18 (VPREB3 HLA-A24) and 1C5.6 (BOB1 HLA-B35) were selected to be the most potent TCRs identified. Since TCR 6B10.12 was the most potent FCRL5 HLA-A1 specific TCR, but safety of the parental T-cell clones had not been examined due to lack of *in vitro* expansion, safety screenings were performed using endogenous TCRαβ knock-out CD8 T cells Td with TCR 6B10.12 (**Figure S6**). Here, no cross-reactivity for TCR 6B10.12 was identified, therefore this TCR and TCRs 6A2.18 (VPREB3 HLA-A24) and 1C5.6 (BOB1 HLA-B35) are promising candidates to further investigate relevance for TCR gene therapy of B-cell malignancies.

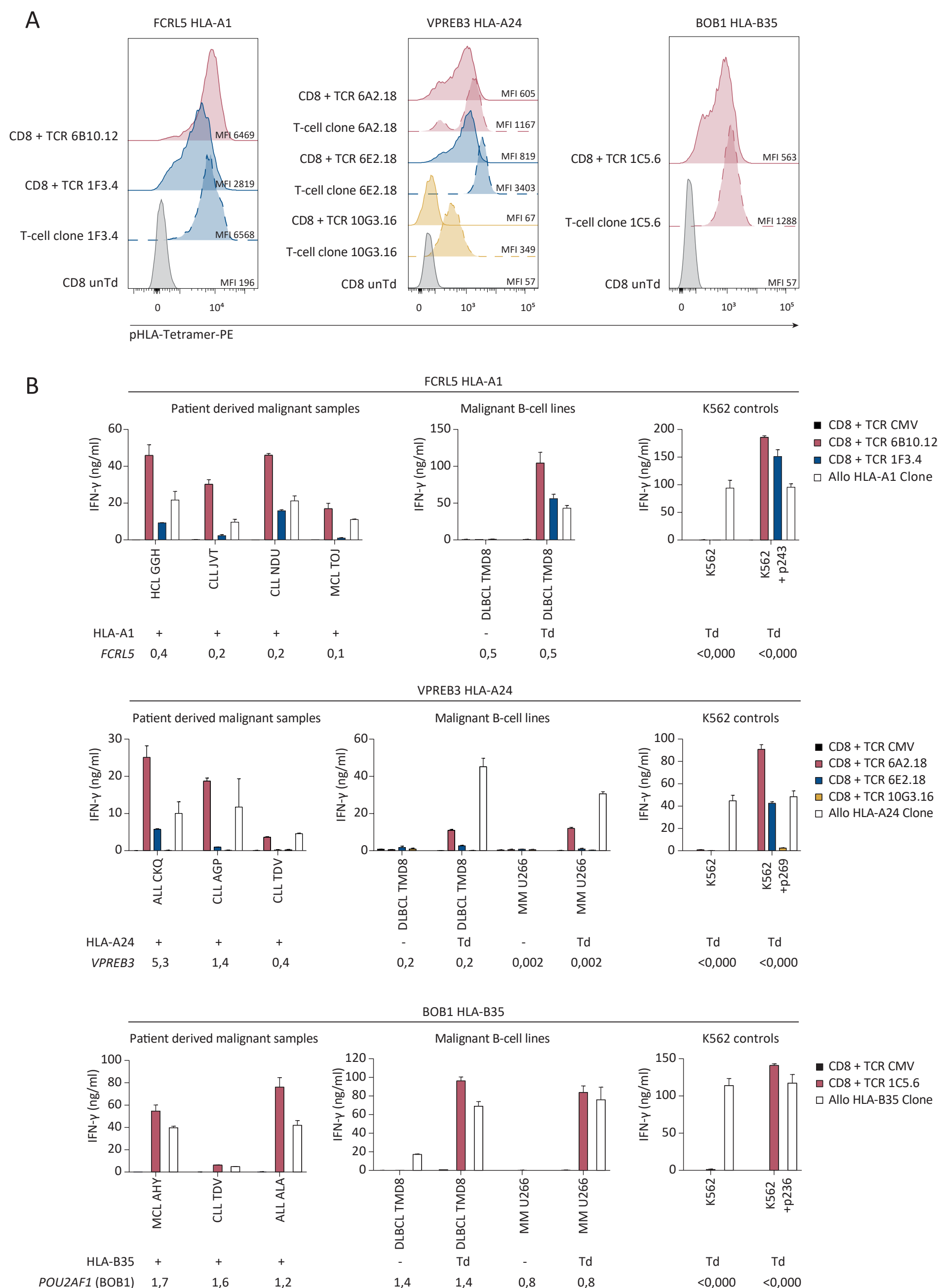

Figure 5. Target recognition by TCR transduced CD8 T cells. A) TCR transduced CD8 T cells and parental T-cell clones stained with pHLA-tetramer-PE, CD8 and mTCR. TCR transduced T cells were enriched for mTCR by MACS and gated on CD8 and mTCR expression. Parental T-cell clones were gated on CD8. Mean fluorescent intensity (MFI) of tetramer stains are depicted. Untransduced T cells were included as negative controls for tetramer staining. Left panel; FCRL5 HLA-A1 specific T cells, middle panel; VPREB3 HLA-A24 specific T cells, right panel; BOB1 HLA-B35 specific T cells. *[Legend continues on the next page]*

B) IFN-γ production after overnight co-culture of CD8 T cells Td with identified TCRs, CMV (pp65-NLV-HLA-A2) TCR as negative control and allo-HLA T-cell clones as positive controls. Target cells were patient derived B-cell malignancy samples, diffuse large B-cell lymphoma cell line TMD8 and multiple myeloma cell lines U266, HLA transduced K562 cells with (100nM) or without target peptide. Target cells were positive (+), negative (-) or transduced (Td) with target HLA. Target gene expression was measured by qPCR and is depicted below targets as expression relative to housekeeping genes (HKG set to 1). To correct for cell size different effector:target (E:T) ratios were used. E:T 1:12 for patient derived B-cell malignancies, E:T 1:6 for cell lines. Graphs are separated based on specificities, upper panels; FCRL5 HLA-A1 TCR T cells, middle panels; VPREB3 HLA-A24 TCR T cells, bottom panels; BOB1 HLA-B35 TCR T cells. Data is representative of two independent experiments, values and error bars represent mean and standard deviations of technical duplicates. Abbreviations: HCL, hairy cell leukemia; MCL, mantle cell lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia.

High avidity TCRs show strong promise for therapy of B-cell malignancies

To gain better insight in the value of the identified TCRs for TCR gene therapy of B-cell malignancies, TCR functionality was investigated further. Peptide titrations experiments demonstrated that the identified TCRs are of high avidity for the target peptide-HLA complexes, requiring between 364 and 1068 pg/ml peptide for a half-maximum response (**Figure S7**). Despite high-avidity interactions between the selected TCRs and target peptide-HLA complexes, TCR 6B10.12 (FCRL5 HLA-A1) and 1C5.6 (BOB1 HLA-B35) are dependent on expression of the CD8 co-receptor. Transfer into CD4 T cells induced pHLA-tetramer binding, but no IFN-γ was produced upon stimulation with antigen Td K562 cells (**Figure S8**). In contrast, TCR 6A2.18 (VPREB3 HLA-A24) Td CD4 T cells produced cytokine upon stimulation with antigen Td K562 cells in addition to pHLA-tetramer binding (**Figure S8**). However, stimulation with patient derived B-cell malignancy materials demonstrated reduced functionality of TCR 6A2.18 Td CD4 T cells compared to CD8 T cells (data not shown), therefore presence of the CD8 co-receptor was still beneficial for TCR 6A2.18 Td T cells.

To investigate the anti-tumor reactivity of the identified TCRs, *In vitro* cytotoxicity assays were performed with patient derived HCL, CLL, MCL and ALL samples, diffuse large B-cell lymphoma cell line TMD8 and multiple myeloma cell line U266. Demonstrating that TCR 6B10.12 (FCRL5 HLA-A1) Td T cells induced specific lysis of HCL, CLL, MCL and DLBCL (**Figure 6A**). Additionally, TCR 6A2.18 Td T cells (VPREB3 HLA-A24) mediated potent lysis of patient derived ALL, CLL, DLBCL and MM (**Figure 6B**) and TCR 1C5.6 Td T cells (BOB1 HLA-B35) efficiently killed ALL, CLL, MCL, DLBCL and MM (**Figure 6C**). No lysis of antigen negative target HLA Td K562 cells (**Figure 6**), or fibroblasts and keratinocytes endogenously expressing the HLA restriction alleles was observed (**Figure S9**). Demonstrating that no general *de novo* cross-reactivities were induced by transfer of the three selected TCRs into donor derived CD8 T cells.

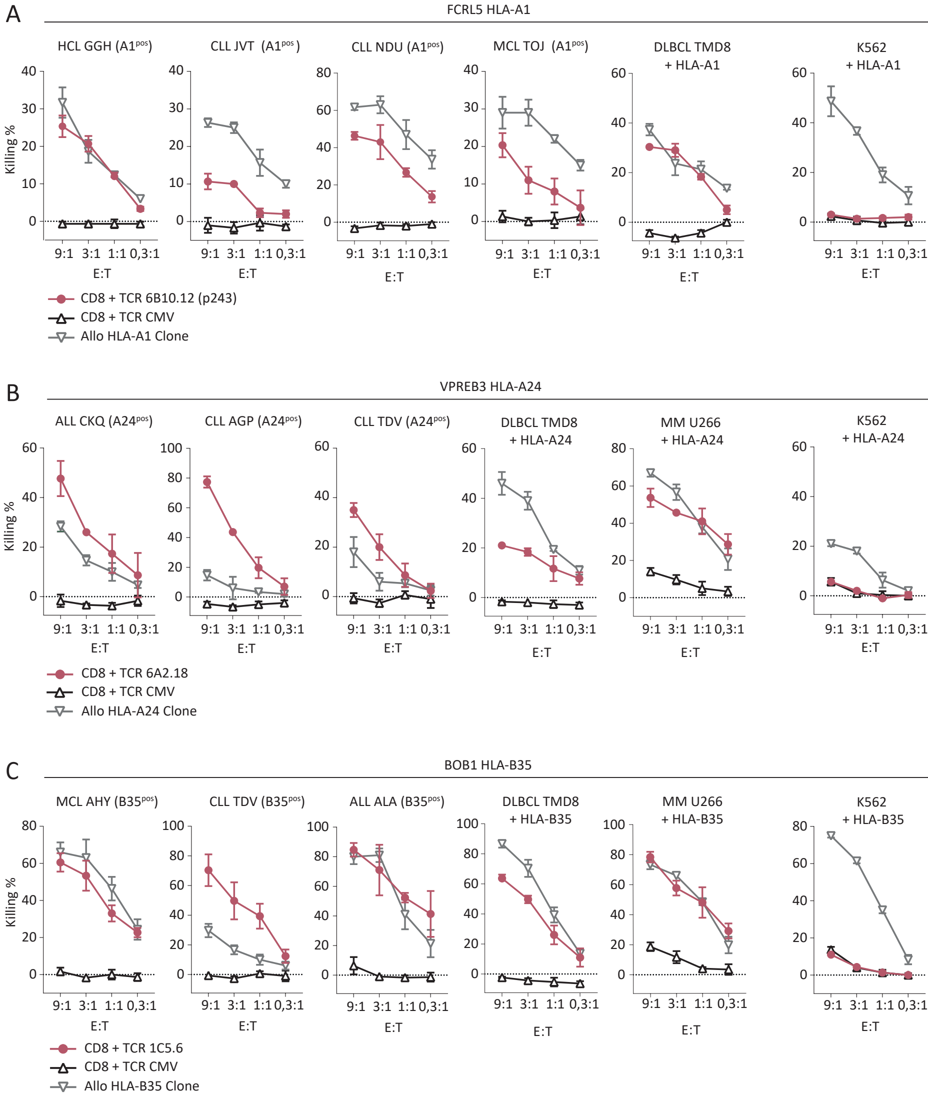

Figure 6. Antigen specific killing of B-cell malignancies by TCR transduced (Td) CD8 T cells. Killing by CD8 T cells Td with selected TCRs (in pink), CMV (pp65-NLV-HLA-A2) TCR Td CD8 T cells (in black) as negative control and allo-HLA-A1, A24 or B35 T-cell clones (in grey) as positive controls. Target cells were patient derived HCL, CLL, MCL and ALL, diffuse large B-cell lymphoma cell line TMD8, multiple myeloma cell line U266 and antigen negative target HLA Td K562 cells. Targets cells endogenously expressed target HLA (HLA^{pos}) or were transduced with target HLA alleles (+HLA). Killing was measured by 51CR release assay after 6-hour co-culture in different effector:target (E:T) ratios. Values and error bars represent mean and standard deviations of technical triplicates. Experiments are representative of two independent experiments. **A)** Killing by FCRL5 HLA-A1 specific TCR 6B10.12 Td T cells. **B)** Killing by VPREB3 HLA-A24 specific TCR 6A2.18 Td T cells. **C)** Killing by BOB1 HLA-B35 specific TCR 1C5.6 Td T cells. Abbreviations: HCL, hairy cell leukemia; MCL, mantle cell lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia.

Finally, we investigated the *in vivo* killing capacity of TCR 1C5.6 (BOB1 HLA-B35) Td CD8 T cells in a previously established xenograft model for treatment of established multiple myeloma.(16) NSG mice were inoculated with BOB1 expressing, HLA-B35 transduced multiple myeloma cell line U266. Upon treatment with BOB1 HLA-B35 restricted TCR 1C5.6 Td CD8 T cells a strong anti-tumor effect was observed (**Figure S10**). Tumors in TCR 1C5.6 treated mice reached their minimal size 6 days after T-cell infusion, when the mean tumor burden was 148-fold lower in 1C5.6 TCR treated mice compared to control TCR treated mice. Despite near complete tumor eradication, U266 regrows after day 6 post T cells likely due to absence of the required human cytokine environment.

In conclusion, TCR 6B10.12 (FCRL5 HLA-A1), 6A2.18 (VPREB3 HLA-A24) and 1C5.6 (BOB1 HLA-B35) are promising TCRs for further clinical development for application in TCR gene therapy of B-cell malignancies.

DISCUSSION

In this study we aimed to extend the options for cellular immunotherapy of B-cell malignancies. We performed a broad and stepwise approach to identify multiple TCRs specific for different target genes and HLA alleles. As a first step, target genes with restricted expression in B-cell malignancies and the B-cell lineage were selected. Twenty HLA-A1, A24, B8 or B35 binding peptides derived from these genes were identified by mass spectrometry based immunopeptidomics. T-cell clones were isolated for 11 of these 20 peptides and multiple efficacy and safety screenings revealed the 6 most promising T-cell clones for which TCRs were sequenced. Despite confirmed presentation of all selected epitopes, only the most potent FCRL5, VPREB3 and BOB1 specific T-cell clones were of sufficient avidity to recognize antigen levels naturally expressed by B-cell malignancies. Upon transfer to CD8 T cells, three TCRs directed against peptides derived from FCRL5, VPREB3 and BOB1, demonstrated potent lytic activity against multiple B-cell malignancies.

The first selected TCR was specific for a peptide derived from FCRL5 presented in HLA-A1. FCRL5 is a cell surface receptor involved in regulation of BCR signaling through binding to IgG.(28) In this study, we aimed to identify TCRs targeting selected B-cell malignancy antigens regardless of their cellular localization. However, the cell surface localization of FCRL5 would also permit CAR mediated targeting which could additionally be exploited as a therapeutic strategy. In healthy B cells, *FCRL5* is expressed by naïve and memory peripheral blood B cells as well as on plasma cells, while expression in germinal center B cells is low.(29) *FCRL5* is expressed in almost all cases of CLL and has previously been proposed as immunotherapeutic target for CLL.(29) Similarly, in our study *FCRL5*

expression was observed in CLL and was additionally expressed in HCL, MCL and DLBCL. The second TCR targets a peptide derived from VPREB3 in HLA-A24. The exact function of VPREB3 is still unknown, but VPREB3 has been suggested to play a role in the intracellular assembly of the pre-B-cell receptor and thus in B-cell development. In healthy B cells, *VPREB3* is expressed in precursor B cells as well as in a subset of germinal center B cells.(30) *VPREB3* was also reported to be expressed in all cases of Burkitt lymphoma and in a subset DLBCL. In DLBCL, *VPREB3* expression co-occurred with c-MYC abnormalities and was therefore associated with an aggressive phenotype.(30) In this study, we additionally observed *VPREB3* expression and TCR targeting of ALL and CLL. Furthermore, despite low *VPREB3* expression, DLBCL cell line TMD8 and MM cell line U266 were lysed by TCR Td T cells, indicating broad applicability for VPREB3 restricted TCRs.

The third TCR targets a peptide in HLA-B35 derived from *POU2AF1* which encodes the BOB1 protein. BOB1 is an intracellular transcription factor regulating both B-cell development as well as function and is broadly expressed throughout the healthy B-cell lineage.(31, 32) Additionally, *POU2AF1* (BOB1) is expressed in all types of B-cell malignancies and has been suggested to play a role in survival of malignant cells.(33, 34) We previously identified a TCR targeting a BOB1 derived peptide in HLA-B*07:02 which demonstrated specific lysis of B-cell malignancies of multiple origins, including MM.(16) Here we similarly observed potent lysis of B-cell malignancies of multiple origins and strong *in vivo* anti-tumor efficacy by CD8 T cells Td with the HLA-B35 restricted TCR.

All three candidate TCRs demonstrated promising safety profiles, as no cross-reactivities were identified using parental T-cell clones 6A2.18 (VPREB3 HLA-A24) and 1C5.6 (POU2AF1 HLA-B35) or TCR 6B10.12 (FCRL5 HLA-A1) Td CD8 T cells. Cross-reactivities with peptides presented in target HLA alleles were investigated in a safety screening including tumor cell lines derived from a variety of cellular origins, thereby ensuring broad gene expression profiles. However, as not all genes will be expressed in the included cell lines, potential cross-reactivities could have remained unidentified. To further investigate the safety profile of the identified TCRs, peptide library scanning remains to be performed in additional preclinical studies. In addition to TCR intrinsic cross-reactivities, *de novo* cross-reactivities could potentially occur when transferring TCRs into CD8 T cells, resulting from mixed dimer formation between chains of the introduced TCR and endogenous TCR.(35, 36) Here, TCR transfer into CD8 T cells confirmed a lack of general *de novo* cross-reactivity when stimulated with antigen negative K562, fibroblast and keratinocytes. As the risk for *de novo* cross-reactivities remains a valid concern for the safety of TCR gene therapy, future TCR gene-therapy is expected to shift focus to generating T-cell products with a knock out of the endogenous TCR, or insertion of the introduced TCR into the TCR locus. Deletion of the endogenous TCR will additionally increase the expression of the introduced TCR by eliminating competition for CD3 and thereby lead to more potent

antitumor efficacy.(37)

The three TCRs identified in this study were specific for peptides presented in the context of HLA-A1, A24 or B35. These HLA-alleles have relative allele frequencies in the worldwide population of 17%, 21% and 8% respectively. Future clinical application of the TCRs is restricted to individuals expressing these HLA genotypes. However, since the target genes are expressed in a broad range of B-cell malignancies, a large group of patients is still eligible for therapy with these TCRs.

Since tumors are often of heterogenous nature, tumor cells negative for non-essential antigens can be present. Upon single-antigen-targeting immunotherapy regimens this can result in escape of antigen-negative variants.(5) This limitation could be overcome by multi-antigen-targeting therapy.(7, 8) CAR T cells can be used to treat all malignancies expressing the target antigen, independent of HLA genotype of patients. However, the number of suitable CAR antigens is limited and might not suffice to prevent escape variants. Additionally, for MM the number of cell-surface antigens expressed by malignant cells but not by healthy cell types of non-B-cell origin is low.

In contrast to CARs, TCRs are not limited by the cellular localization of antigens. In this study cellular localization was not included as a selection criteria for target antigens, which resulted in identification of TCRs recognizing peptides derived from cell surface as well as intracellular proteins. Moreover, targeting intracellular proteins that are essential for cell survival, like the transcription factor BOB1 for which we here identified an HLA-B35 restricted TCR, could prevent escape through antigen-loss. The benefits of CAR and TCR therapy can complement each other and should be combined in multi-antigen-targeting therapies. Generating a library of CARs and TCRs will allow selection of multiple relevant receptors based on antigen expression and HLA genotypes of patients. Our research has contributed to this goal by identification of three clinically relevant TCRs. The approach described in this study can similarly be applied to identify TCRs for treatment of solid tumors. For these cancers, tumor associated antigens and neo-antigens are promising targets and TCR-based therapy will likely be the most valuable option as cell-surface expression of tumor specific antigens is generally absent.

In summary, we applied a broad and systematic approach to identify clinically relevant TCRs for the treatment of B-cell malignancies. This resulted in identification of three TCRs that induced potent lysis of B-cell malignancies of different origins and are therefore promising TCRs for future application in multi-antigen-targeting T-cell therapy for B-cell malignancies.

MATERIALS AND METHODS

Target gene selection, HT12 microarray

A detailed description of protocols followed for sample collection, purification, RNA isolation and Illumina HT-12.0 microarray measurements can be found in Pont *et al*.(25) Gene expression analysis was performed using gene expression in patient derived ALL, CLL and MM, ALL cell lines, EBV-LCLs, healthy B-cells, BMMCs, total PBMCs, multiple healthy hematopoietic and non-hematopoietic cell types (Figure S1).

Peptide elution and candidate peptide identification

To identify peptides derived from candidate genes that are presented in HLA on the cell surface, the HLA peptidome of primary B-cell malignancy samples of different origins was established as previously described.(38) In short, after informed consent, apheresis material was obtained at time of diagnosis. HLA typing of the material was performed and cell pellets were stored at -80 until use. Cell pellets (0.1 x 10^9 to 610 x 10^9 cells) were lysed and peptide-HLA complexes were purified by immunoaffinity using the anti HLA-I W6/32 antibody. Peptides were separated from HLA molecules using acid and the peptide containing fraction was obtained by size filtration. Peptide containing fractions were separated by strong cation exchange chromatography and freeze dried. Peptide fractions were lyophilized, dissolved in 95/3/0.1 water/acetonitrile/formic acid v/v/v and subsequently analyzed with nanoHPLC-MS/MS. Peptide and protein identification from tandem mass spectra was performed by proteome discoverer version 2.1 (Thermo Fischer Scientific) using the mascot node and the UniProt Homo Sapiens database. Synthetic peptides were ordered for potential target peptides meeting the following selection criteria: 1) peptides derived from one of the candidate genes; 2) for which a cross query between patient HLA type and predicted HLA binding according to NetMHC version 3.4 indicates binding to HLA-A1, A24, B8 or B35; 3) with a minimal Mascot Ionsscore ≥ 20; 4) with an amino acid length between 8-11 amino acids; 5) that were rank 1 peptides; 6) cysteine containing peptides were excluded; 7) the peptide sequence had to be unique for the candidate gene. For peptides meeting all selection criteria, synthetic peptides were ordered, measured by mass spectrometry and spectra of the synthetic and eluted peptides were compared to confirm peptide identification. Peptides for which the spectra did not match were excluded from the selection.

Generation of pHLA-tetramers

Synthetic peptides were generated in house using standard Fmoc chemistry. Recombinant HLA-A1, A24, B8 or B35 heavy chains (HCs) and human beta-2 microglobulin (B2M) were produced in house in *Escherichia coli.* PE-labelled pHLA-tetramers were produced as previously described with minor modifications.(39)

T-cell isolation and culture

Buffy coats were obtained from healthy donors negative for HLA-A1, HLA-A24, HLA-B8 and HLA-B35 after informed consent (Sanquin). PBMCs were isolated using Ficoll gradient separation and incubated with pHLA-tetramers for 1 hour at 4°C or 15 minutes at 37°C. Cells were washed and pHLA-tetramer bound cells were enriched by magnetic associated cell sorting (MACS) using anti-PE beads (Miltenyi Biotec). The positive fraction was stained with CD8 Alexa fluor 700 (Invitrogen/Catlag) and FITC labelled CD4, CD14 and CD19 (BD pharmingen). pHLA-tetramer^{pos}, CD8^{pos} cells were single-cell sorted using an Aria III cell sorter (BD Biosciences) in a 96 well round bottom plate containing 5x10^4 irradiated PBMCs (35Gy) and 5x10^3 EBV-JY cells (50Gy) in 100ul T-cell medium (TCM) with 0.8 µg/ml phytohemagglutinin (PHA; Oxoid Microbiology Products, Thermo Fischer Scientific). TCM contains IMDM (Lonza), 1% Penicillin/Streptomycin (Pen/Strep; Lonza), 1.5% glutamine (Lonza), 100 IU/ml IL-2 (Proleukin; Novartis Pharma), 5% fetal bovine serum (FBS; Gibco, Life Technologies) and 5% human serum. T-cell clones were restimulated every 10-15 days with irradiated feeder cells and PHA or cryopreserved until further use.

2

Target cell culture and generation

Cell lines were cultured in IMDM (Lonza), 1% Pen/Strep (Lonza), 1.5% Glutamine (Lonza) and 10% FBS (Gibco, Life Technologies). ALL cell lines were cultured as described. (40) Primary malignant samples were defrosted and rested overnight at 37°C in medium containing 10% human serum before use in experiments. HLA and target gene transduced (Td) target cells were generated by retroviral transduction with HLA alone or with target gene and HLA combined. Retroviral transduction was performed as previously described.(19) Candidate genes and HLA alleles were expressed in MP71 retroviral backbone vectors with marker genes truncated nerve growth factor receptor (NGF-R), CD34 or mouse CD19 (mCD19). Transduced cells were MACS or FACS enriched for marker gene and/or HLA-I expression using HLA-ABC FITC (serotec), NGF-R PE (BD/Pharmingen), mCD19 PE (BD) or CD34 PE (BD/Pharmingen).

T-cell recognition assay

Target cell recognition was determined by incubating 5,000 T cells with target cells in Effector:Target (E:T) ratio 1:6, unless indicated otherwise, in a 384-well flat-bottom tissue culture plate. T cells were washed before use in experiments to remove expansion-related cytokines. After overnight (O/N) incubation recognition was determined by measuring IFN-γ and/or GM-CSF production in supernatants by ELISA (Sanquin and R&D systems). Supernatants were tested undiluted, 1:5 diluted (Figure 2, Figure S4) or in multiple dilutions to calculate concentrations using OD values in the linear part of the standard curve (all other experiment). Supernatants were transferred using a *Hamilton Microlab STAR* Liquid Handling System (Hamilton company). Peptide loaded target cells were

loaded with 100nM per peptide or decreasing peptide concentrations starting at 1µM for peptide titration experiments. Initial screenings to determine peptide recognition of pHLA-tetramer^{pos}, CD8^{pos} sorted and expanded T cells were performed in a high throughput manner. The 96-well T-cell cultures were split into 4 wells of 384-well plate using a *Hamilton Microlab STAR* Liquid Handling System (Hamilton company). Peptide mix loaded or unloaded target cells were added manually. T-cell clones were defined reactive when IFN-γ and/or GM-CSF production was > 100 pg/ml upon target cell stimulation. T-cell clones were considered peptide specific when no cytokine production (< 30 pg/ml) was measured for unloaded target cells. T cells meeting these selection criteria were picked manually and transferred to a 24 well plate for restimulation and further expansion. T-cell mediated cytotoxicity was measured using ^{51}Cr-release experiments. Target cells were incubated 1 hour at 37°C with 100 µCi $Na_2{}^{51}CrO_4$ (PerkinElmer). Target cells were washed and co-cultured with T cells at various E:T ratios for 6 hours in 96-well U-bottom culture plates. Supernatants were harvested and transferred to 96-well LumaPlates (PerkinElmer). Spontaneous and maximum ^{51}Cr-release was determined using TCM alone or TCM containing 1% Triton-X 100 (Sigma-Aldrich), respectively. ^{51}Cr-release was measured in counts per minute (CPM) using a 2450 Microbeta2 plate counter (PerkinElmer). Percentage target cell killing was calculated using

$$\%killing=((CPM_{test}-averageCPM_{spon})/(averageCPM_{max}-averageCPM_{spon}))*100.$$

Quantitative RT-PCR

Total RNA was isolated from 0.5-5x10^6 cells using the Small Scale Kit or ReliaPrep RNA cell mini prep system according to manufacturer's protocol (Ambion, Promega respectively). Total RNA was converted to cDNA using Moloney murine leukemia virus reverse transcriptase and Oligo (dT) primer (Invitrogen by Thermo Fisher Scientific). Quantitative reverse transcription polymerase chain reaction (qPCR) was performed using Fast Start TaqDNA Polymerase (Roche) and EvaGreen (Biotum), gene expression was measured on the Lightcycler 480 (Roche). Forward and reverse primers used are depicted in Table S5. Target gene expression was calculated relative to the average expression of housekeeping genes: GUSB, PSMB4 and VPS29.

TCR identification

TCRα and TCRβ sequences of T-cell clones were identified as previously described with minor modifications.(41) mRNA was isolated from 1x10^6 cells using the Dynabeads mRNA DIRECT kit (Invitrogen by Thermo Fisher Scientific). TCR cDNA was generated using reverse primers in the TCR constant alfa and beta regions, SMARTScribe Reverse Transcriptase (Takara, Clontech) and a SA.rt template switching oligo forward primer.(42) Barcoded TCR PCR product was generated in two rounds of PCR. In the first PCR, alfa and beta TCR products were generated, in a second PCR the first PCR product was used to

include a barcode sequence that allowed discrimination between TCRs of different T-cell clones. PCR products of different T-cell clones were pooled after which TCR sequences were identified by HiSeq (GenomeScan). Hiseq data were analysed using MiXCR and ImMunoGeneTics (IMGT) database to determine the Vα /Vβ family. V(D)J segments of the TCRα and TCRβ were codon optimized and cloned into the modified MP71-TCR-flex retroviral vector. To increase expression and preferential pairing of the introduced TCRαβ chain, the MP71-TCR-flex vector contains codon-optimized and cysteine-modified murine TCRαβ constant domains and P2A sequence to link TCR chains.(43) Phoenix-AMPHO (ATCC) cells were transfected, after 48 and 72 hours virus supernatant was harvested and stored at -80°C.

TCR transfer to healthy donor T cells

CD8 and CD4 T cells were separately isolated from healthy donor PBMCs by MACS using anti-CD8 or anti-CD4 microbeads (Miltenyi Biotec). PBMCs were obtained after informed consent. T cells were activated with irradiated autologous PBMCs (35 Gy) and 0.8 µg/ml PHA. On day 2, retroviral supernatants were added to 24-well suspension culture plates (Greiner Bio-One) precoated with 30 mg/mL retronectin (Takara) and blocked with 2% human serum albumin (Sanquin). Plates were spun down for 20 min, 2000g at 4°C. Virus supernatant was removed and 0.3x10^6 activated T cells were transferred to each well. After O/N incubation T cells were transferred to a 24-well culture plate (Costar). On day 7 after T-cell activation TCR Td T cells were MACS enriched using anti-mouse TCR-Cβ (mTCR) APC antibody (BD Pharmingen) followed by anti-APC MicroBeads (Miltenyi Biotec) according to manufacturer's protocol. TCR Td T cells were functionally tested between day 10-12 after activation. For safety screening of TCR 6B10.12, endogenous TCRαβ knock out (KO) of healthy donor CD8 T cells was performed prior to TCR Td as described by Morton *et al*.(37) To asses TCR expression and tetramer binding cells were stained using mTCR APC antibody and PE labeled pHLA-tetramers. Cells were measured on the LSR II (BD Bioscience) and data were analysed with Flowjo software.

***In vivo* antitumor efficacy of BOB1 HLA-B35 TCR**

Female NSG mice (NOD.Cg-Prkdc(scid)Il2rg(tm1Wjl)/SzJ, The Jackson Laboratory) were injected i.v. with 2x10^6 U266 multiple myeloma cells, U266 were transduced with Luciferase- tdTomato Red and HLA-B35-NGFR and enriched to reach >98% purity. Tumor growth was measured 1-2 times per week after i.p. injection of 150 µl 7.5 mM D-luciferine (Cayman Chemical Co.) using a CCD camera (IVIS spectrum, PerkinElmer). On day 21 mice were i.v. injected with 5x10^6 T cells which were transduced with 1C5.6 (BOB1 HLA-B35) TCR (n=4) or irrelevant CMV TCR (n=3). The TCR transduced T cells were enriched for mTCR expression before infusion.

ACKNOWLEDGEMENTS

The authors thank the operators of the LUMC Flow cytometry Core Facility (Leiden University Medical Center, Leiden, The Netherlands) for providing expert technical assistance in flow cytometric cell sorting. The research in this study was funded by the Dutch Cancer Society (KWF kankerbestrijding, Amsterdam, The Netherlands) under grant number: KWF UL-2014-6831.

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SUPPLEMENTARY MATERIAL

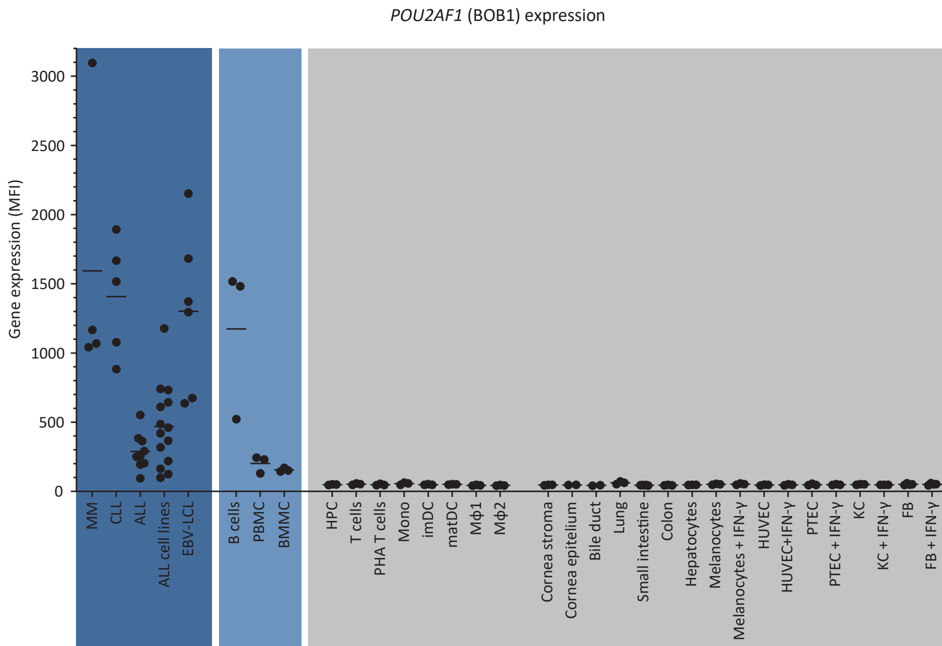


Figure S1. Example of a complete gene expression profile used to generate summary data in figure 1. Gene expression was retrieved from an Illumina HT12.0 microarray dataset.(25) *POU2AF1* (BOB1) gene expression (Mean Fluorescence Intensity; MFI) per cell type, individual samples and average (mean) gene expression is shown. Expression in patient derived B-cell malignancies or B-cell malignancy cell lines (dark blue), healthy B cells ($CD19^{pos}$) or B-cell containing PBMCs and BMMCs (light blue), healthy hematopoietic and non-hematopoietic cell types (grey). Abbreviations: MM, multiple myeloma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid cell lines; PBMC, peripheral blood mononuclear cells; BMMC, bone marrow mononuclear cells; HPC, hematopoietic precursor cells; Mono, monocytes; imDC, immature dendritic cells; matDC, mature dendritic cells; MΦ1, type 1 macrophages; MΦ2, type 2 macrophages; HUVEC, human umbilical vein endothelial cells; IFN-γ, interferon-γ; PTEC, proximal tubular epithelial cells; KC, keratinocytes; FB, fibroblasts.

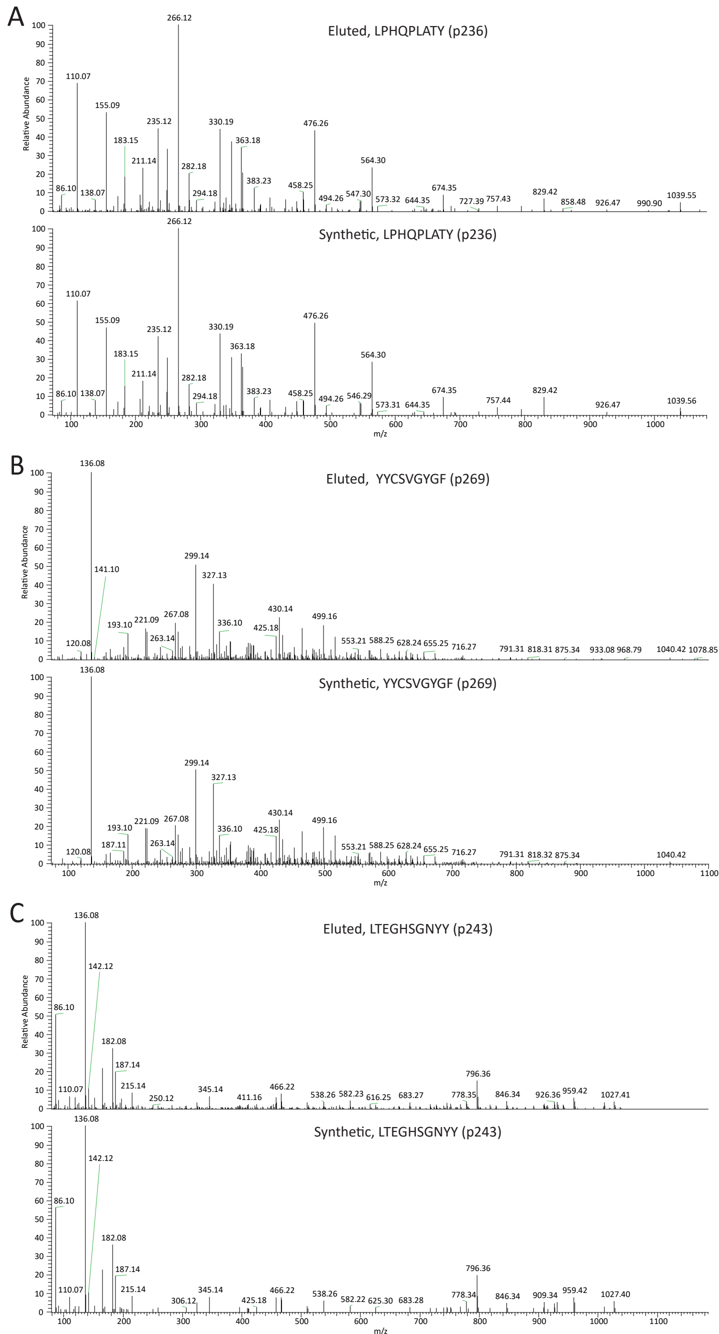


Figure S2. Examples of matching tandem mass spectra of eluted (top) and synthetic (bottom) peptides. A) Tandem mass spectra of peptide 236 (p236, LPHQPLATY) derived from BOB1 presented in HLA-B35. **B)** Tandem mass spectra of p269 (YYCSVGYGF) derived from VPREB3 presented in HLA-A24. **C)** Tandem mass spectra of p243 (LTEGHSGNYY) derived from FCRL5 presented in HLA-A1.

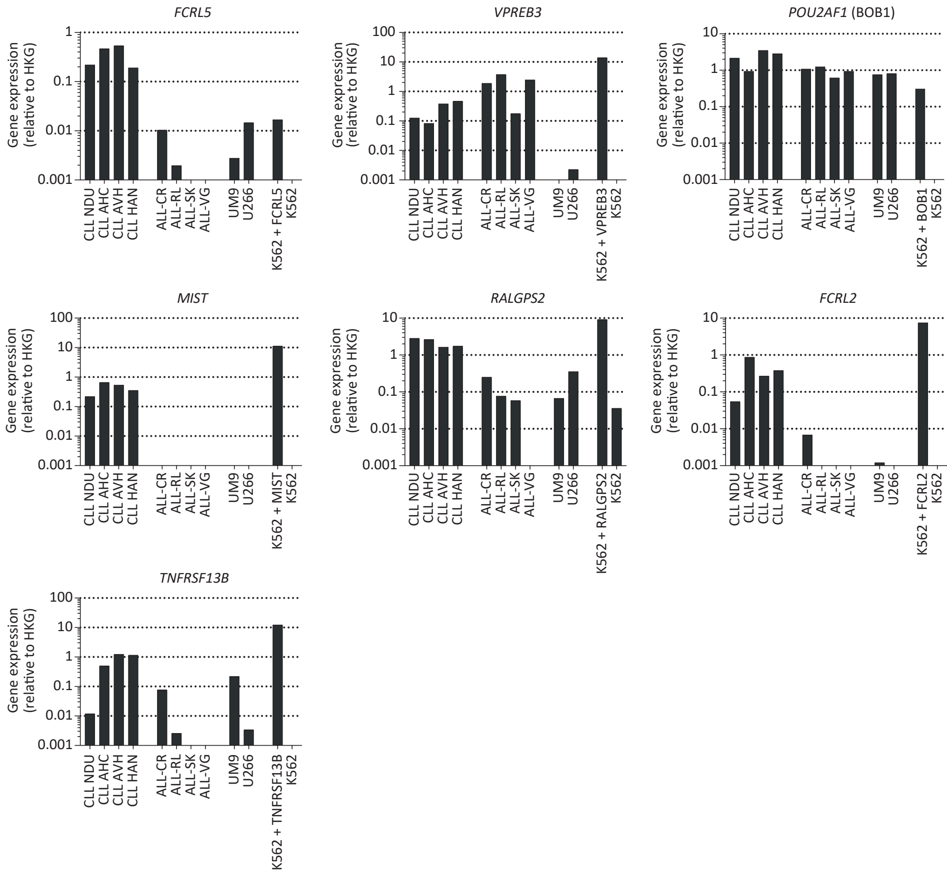


Figure S3. Target gene expression of targets used for T-cell selection. *FCRL5*, *VPREB3*, *POU2AF1*, *MIST*, *RALGPS2*, *FCRL2* and *TNFRSF13B* mRNA levels of patient derived chronic lymphocytic leukemia (CLL) samples, acute lymphoblastic leukemia (ALL) cell lines, multiple myeloma (MM) cell lines, target gene transduced K562 cells and K562 cells used in figure 4. Gene expression, measured by qPCR, depicted relative to housekeeping genes (HKG). Values not depicted are <0.001 relative gene expression.

Figure S4. Peptide titrations of T-cell clones selected to recognize gene transduced (Td) K562 cells. **[*Figure on the next page*]** IFN-γ (closed symbols) or GM-CSF (open symbols) production by T-cell clones from figure 3 after overnight stimulation with K562 cells Td to express target HLA, loaded with decreasing concentrations of target peptide in an effector:target ratio 1:6. Graphs are separated based on T-cell specificity and cytokine production. Values and error bars represent mean and standard deviations of technical duplicates.

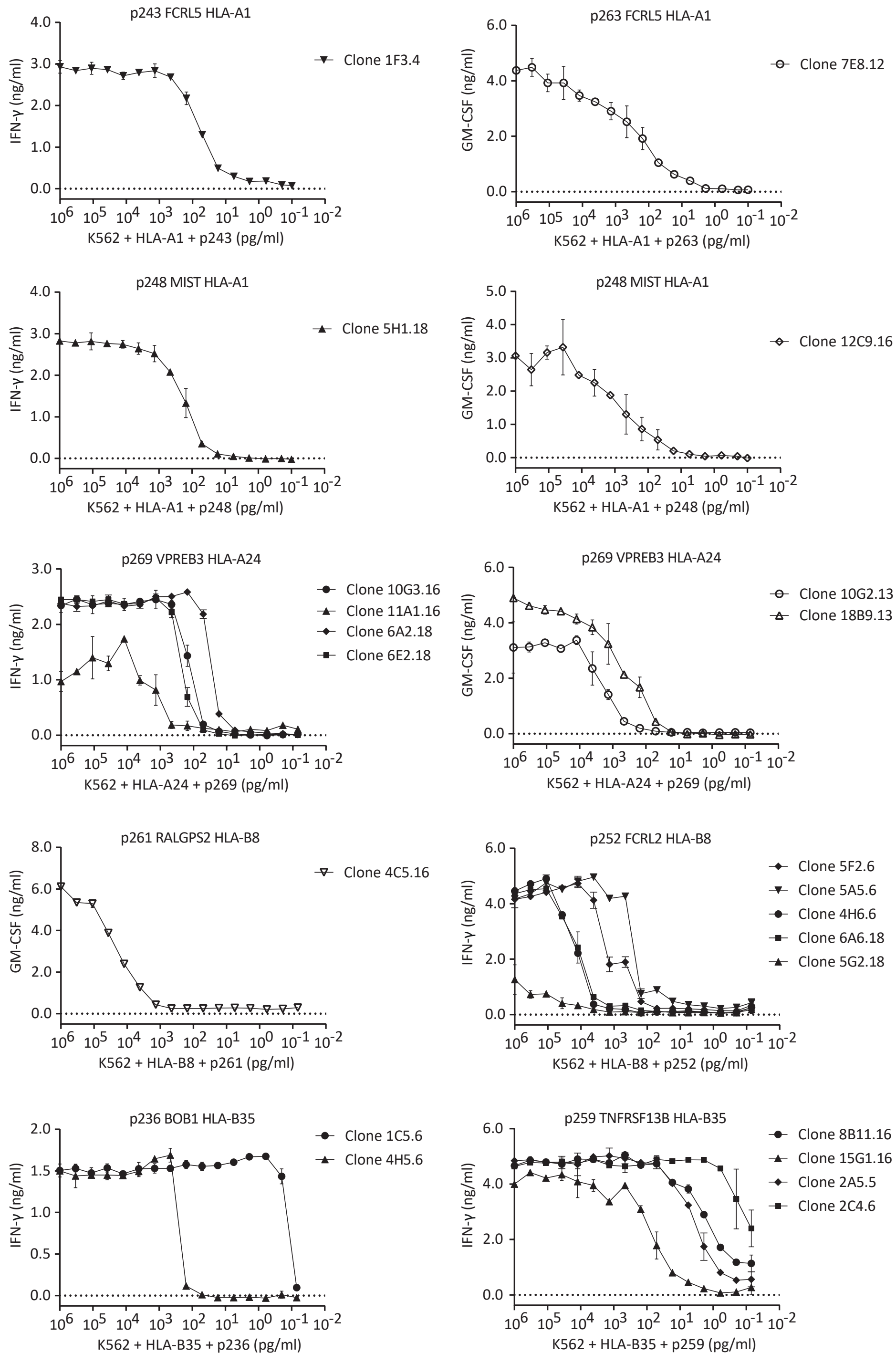


Figure S4 [*Legend on previous page*].

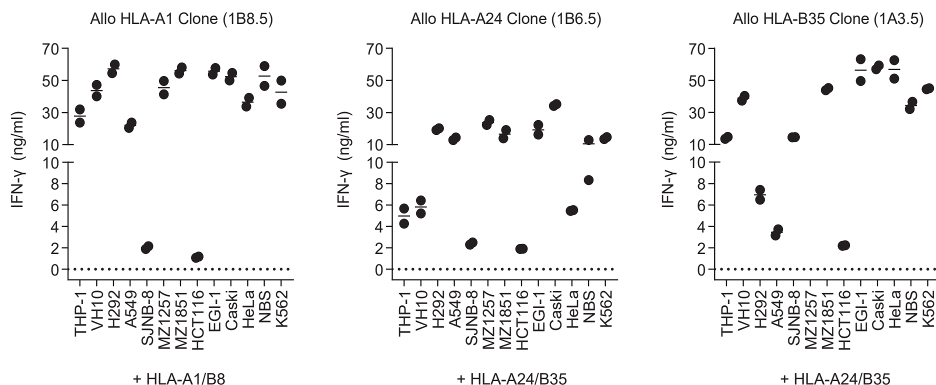


Figure S5. Recognition by allo HLA T-cell clones of cell lines used in safety screenings. IFN-γ production by allo HLA-A1 (left panel), A24 (middle panel) and B35 (right panel) T cell clones after overnight co-culture with HLA-A1 and HLA-B8 (+HLA-A1/B8) or HLA-A24 and HLA-B35 (+HLA-A24/B35) transduced cell lines. IFN-γ measured by ELISA, technical duplicates are depicted. Data obtained from the same experiment as shown in Figure 4.

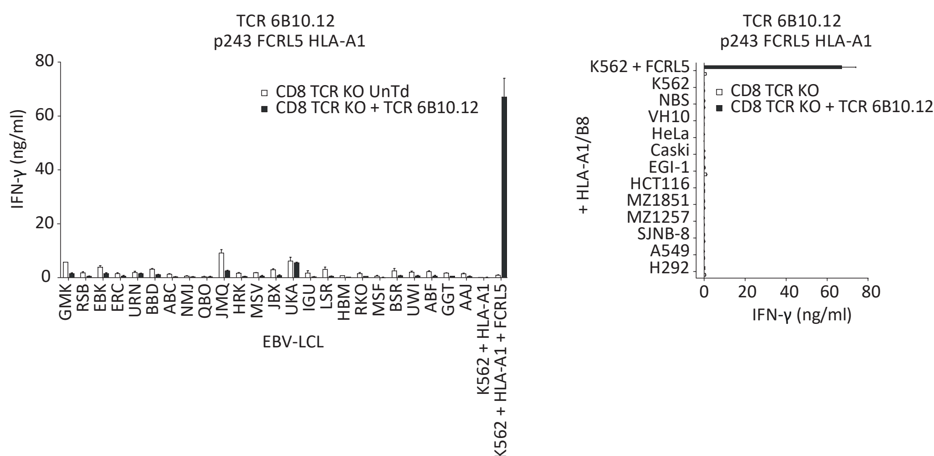


Figure S6. Safety screening of TCR 6B10.12 (FCRL5 HLA-A1). Endogenous TCR knock out CD8 T cells (5% residual huTCR^{pos} cells) untransduced or Td with TCR 6B10.12 (FCRL5 HLA-A1) were used because of lack of *in vitro* expansion of parental T-cell clone 6B10.12. Experiment performed as described in figure 5. Values represent means and standard deviations of technical duplicates.

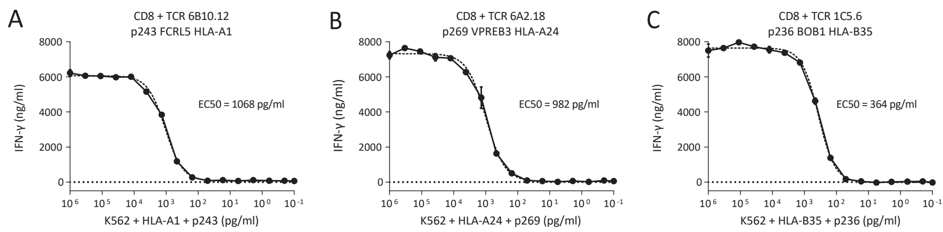


Figure S7. TCR avidity determined by peptide titrations. TCR transduced CD8 T cells were enriched for mTCR expression by MACS. IFN-γ production measured by ELISA after overnight co-culture with HLA transduced K562, loaded with decreasing concentrations of target peptide in an effector:target ratio 1:6. Sigmoidal curves (dotted lines) are plotted based on measured concentrations (solid lines). EC50 values were calculated based on sigmoidal curves and represent peptide concentrations required to induce 50% of the maximum cytokine production. Values represent means and standard deviations of technical duplicates. **A)** CD8 T cells transduced with FCRL5 HLA-A1 p243 restricted TCR 6B10.12. **B)** CD8 T cells transduced with VPREB3 HLA-A24 p269 restricted TCR 6A2.18. **C)** CD8 T cells transduced with BOB1 HLA-B35 restricted TCR 1C5.6.

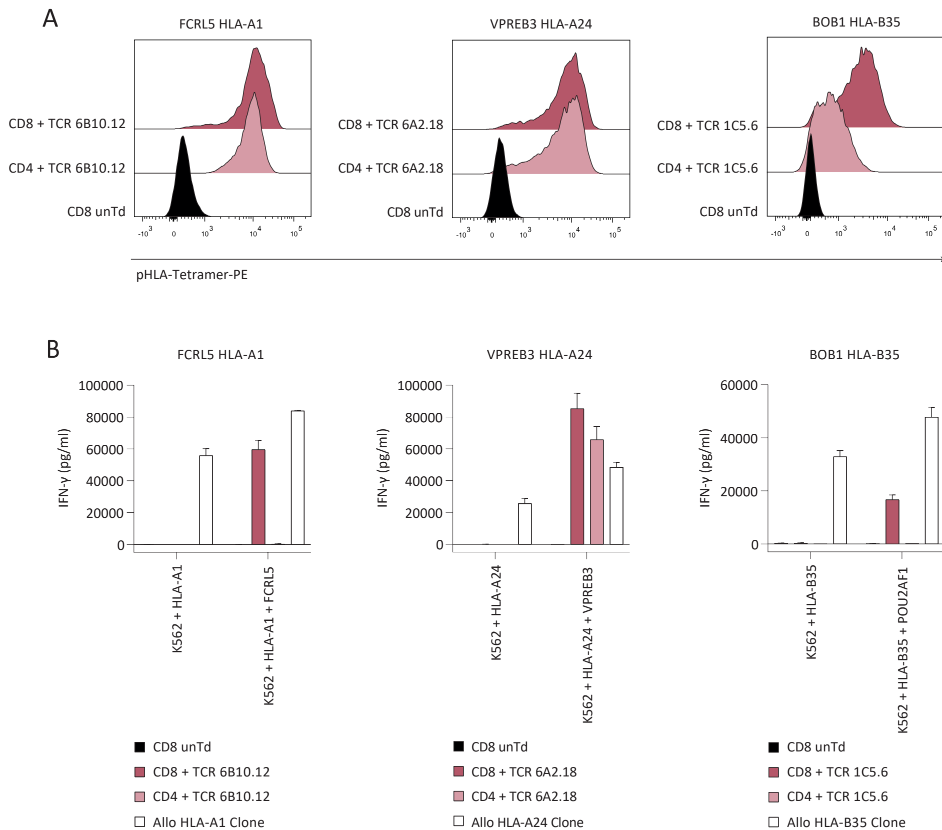


Figure S8. CD8 co-receptor dependence of identified TCRs. CD8 (dark pink) and CD4 (light pink) cells were isolated separately and transduced with identified TCRs or left untransduced (CD8 only, black). T cells were enriched for mTCR expression by MACS and used for analysis on day 10-12 after stimulation. T cells were transduced with FCRL5 HLA-A1 p243 restricted TCR 6B10.12. (left panels), VPREB3 HLA-A24 p269 restricted TCR 6A2.18. (middle panels) or BOB1 HLA-B35 restricted TCR 1C5.6 (right panels). **A)** FACS analysis of T cells stained with specific PE-labeled pHLA-tetramers, gated on CD8 or CD4 positive cells. **B)** IFN-γ production by T cells from experiment shown in A, after overnight co-culture with K562 cells transduced with target HLA alone or with target HLA and target gene combined in an effector:target ratio 1:6. Allo-HLA T-cell clones were included as positive control for HLA expression. Values represent means and standard deviations of technical duplicates.

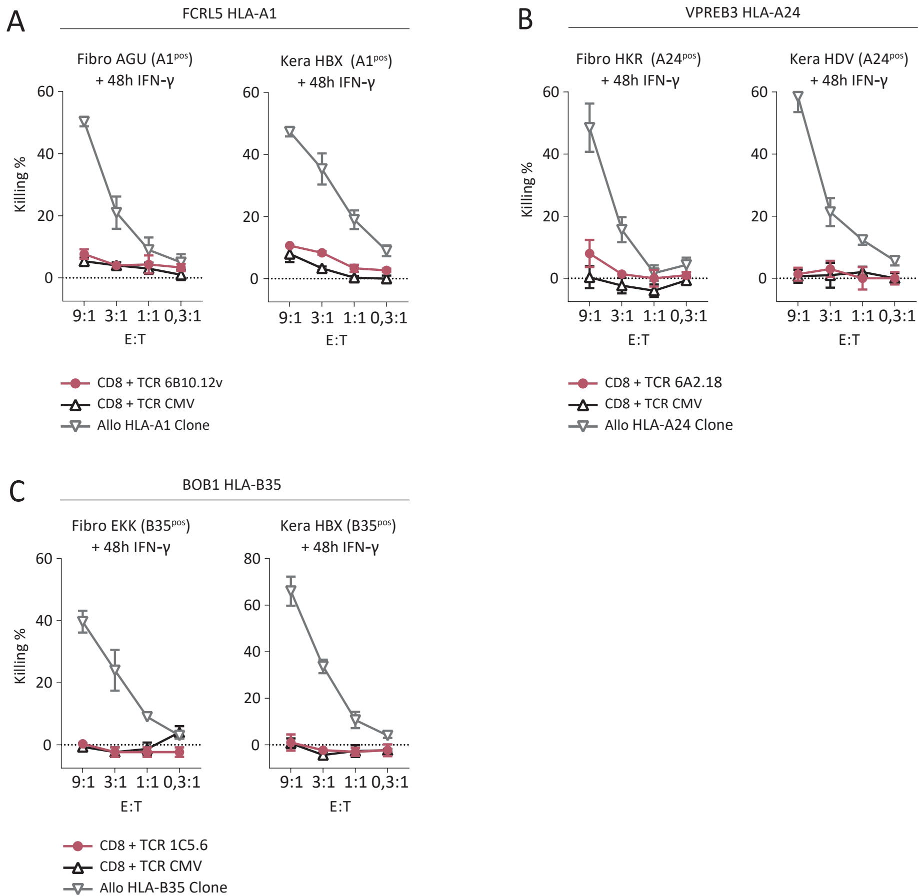
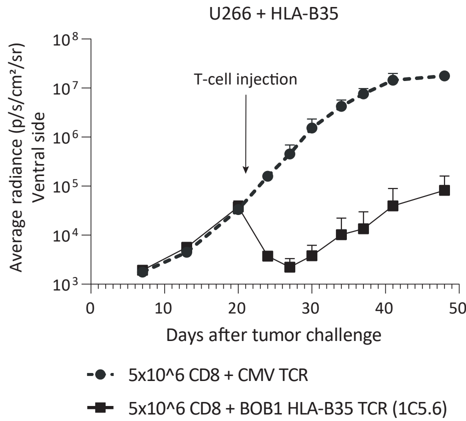


Figure S9. Cytotoxicity of antigen negative target cells by TCR transduced (Td) CD8 T cells. Killing by CD8 T cells Td with selected TCRs (pink), CMV (pp65-NLV-HLA-A2) TCR Td CD8 T cells (black) as negative control and allo-HLA-A1, A24 or B35 T-cell clones (grey) as positive controls. Target cells were fibroblasts and keratinocytes expressing target HLA alleles as indicated between brackets, pre-treated for 48 hours with 100 IU/ml IFN-γ to upregulate HLA expression. Killing was measured by 51CR release assay after 6-hour co-culture in different effector:target (E:T) ratios. Values and error bars represent mean and standard deviations of technical triplicates. Data was obtained from the same experiment as shown in figure 6. Fibroblasts and keratinocytes were negative (<0.0003 relative expression compared to house keeping genes) for *VPEB3*, *FCRL5* and *POU2AF1* expression measured by qPCR as described in figure S3. **A)** Killing by FCRL5 HLA-A1 specific TCR 6B10.12 Td CD8 T cells. **B)** Killing by VPREB3 HLA-A24 specific TCR 6A2.18 Td CD8 T cells. **C)** Killing by BOB1 HLA-B35 specific TCR 1C5.6 Td CD8 T cells. Abbreviations: Fibro, fibroblasts; Kera, Keratinocytes.

A



B

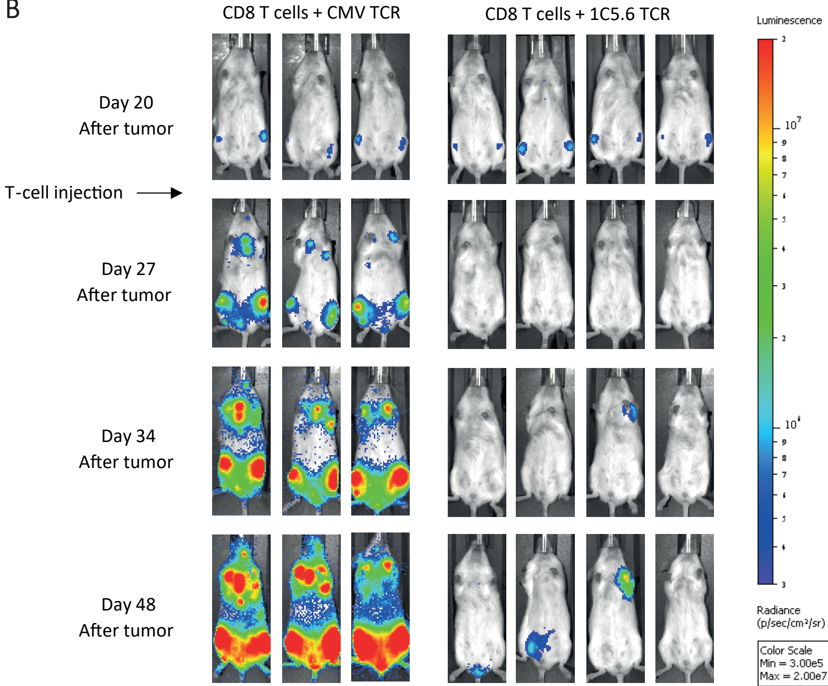


Figure S10. ***In vivo*** **antitumor efficacy of BOB1 HLA-B35 restricted TCR transduced CD8 T cells.** NSG mice engrafted with 2x10^6 U266 multiple myeloma cells transduced with luciferase and HLA-B35, were i.v. injected with 5x10^6 TCR transduced CD8 T cells after 21 days. T cells were transduced with BOB1 HLA-B35 restricted TCR 1C5.6 (n=4) or control CMV (pp65-NLV-HLA-A2) TCR (n=3) and enriched for mTCR expression by MACS. Tumor outgrowth was frequently tracked by bioluminescence imaging. **A)** Mean and standard deviations of tumor outgrowth over time on the ventral side of CMV TCR treated control mice (dashed line) and BOB1 HLA-B35 TCR (solid line) treated mice. **B)** Tumor outgrowth for individual CMV TCR (left) or BOB1 HLA-B35 TCR (right) treated mice measured on day 20, 27, 34 and 48 after tumor cell injection.

Table S1. Origin and HLA typing of HLA-A1, A24, B8 or B35^{pos} B-cell malignancies used for peptide elution and the number of unique peptides that were identified by mass spectrometry with Ion scores ≥20 or ≥35

Patient Code	Diagnosis	Material	HLA-A	HLA-B	HLA-C	Experiment Tag	Cells used for peptide elution (x10^9)	Unique Peptides Ion Score≥20	Unique Peptides Ion Score≥35
AGP	ALL	Peripheral blood	A*01:01- A*02:01	B*08:01- B*38:01	C*07:01- C*12:03	Exp1	233	36773	17449
CDT	HCL	Spleen	A*02:01- A*29:02	B*35:01	C*04:01	Exp2	500	63562	26981
SWD	CLL	Spleen	A*01:01- A*02:01	B*08:01- B*40:01	C*03:04- C*07:01	Exp3	180	68892	30168
						Exp15	0,1	1351	415
						Exp22	0,2	4981	1645
MWY	ALL	Peripheral blood	A*11:01- A*NT	B*35:01- B*40:02	C*02:02- C*04:01	Exp4	610	24873	13655
						Exp14	0,1	539	322
WSG	ALL	Peripheral blood	A*01:01- A*03:01	B*18:01- B*35:08	C*04:01- C*12:03	Exp5	62	44098	23211
SLE	ALL	Peripheral blood	A*01:01- A*32:01	B*08:01- B*45:01	C*06:02- C*07:01	Exp6	512	32830	11242
NBA	ALL	Peripheral blood	A*24:02- A*26:01	B*38:01	C*12:03	Exp7	210	35330	17375
HJS	FL	Peripheral blood	A*24:02- A*32:01	B*44:02- B*44:05	C*02:02- C*05:01	Exp8	243	2696	1296
KYE	ALL	Peripheral blood	A*02:01- A*24:02	B*18:01- B*40:02	C*03:04- C*07:01	Exp10	284	45721	13332
AHC	CLL	Peripheral blood	A*01:01- A*03:01	B*07:02- B*08:01	C*07:01- C*07:02	Exp16	0,2	3476	1002
ALA	ALL	Peripheral blood	A*02- A*11	B*07- B*35	C*04- C*07	Exp17	0,2	3628	1233
EMY	ALL	Peripheral blood	A*11- A*32	B*35- B*44	C*03- C*04	Exp18	0,2	3800	1662
HAN	CLL	Bone marrow	A*02:01	B*08:01- B*15:01	C*03:03- C*07:01	Exp20	0,2	3257	895
HBP	ALL	Peripheral blood	A*02:01- A*24:02	B*39:01- B*57:01	C*06:02- C*12:03	Exp21	0,2	7335	2373
UM9 + A2	MM	Celline	A*01:01- A*11:01 (A*02:01 Td)	B*07:02- B*55:01	C*03:03- C*07:02	Exp107	30	82504	34115

Table S2. Peptides derived from selected genes identified by mass spectrometry from B-cell malignancy samples

				RALGPS2-LTDSEKGNSY-A1	FCRL5-LTEGHSGNYY-A1	FCRL5-TTENSGNYY-A1	MIST-ESEYADTHY-A1	TLR10-YLDHNSFDY-A1	IGJ-YTAVVPLVY-A1	BLK-AYIERMNSI-A24	VPREB3-YYCSVGYGF-A24	RALGPS2-QYIEELQKF-A24	TLR10-ELFKRTIQL-B8	TLR10-LPHLKTLIL-B8	IGLL1-HGLLRPTAA-B8	KLHL14-DMNTKRAIHTL-B8	FCRL2-IVKIKVQEL-B8	FAM129C-LPALRAQTL-B8	FAM129C-YLRLLDAL-B8	RALGPS2-TLKIRAEVL-B8	TNFRSF13B-SADQVALVY-B35	POU2AF1/BOB1-APAPTAVVL-B35	POU2AF1/BOB1-LPHQPLATY-B35
Patient Code	Diag-nosis	Target HLA expressed	Experi-ment Tag	p 242	p 243	p 263	p 248	p 265	p 268	p 246	p 269	p 258	p 247	p 256	p 251	p 239	p 252	p 255	p 271	p 261	p 259	p 233	p 2 36
AGP	ALL	A*01:01, B*08:01	Exp1					34,2	44,2						35,9			23,0		36,8			
CDT	HCL	B*35:01	Exp2																			39,9	40,0
SWD	CLL	A*01:01, B*08:01	Exp3	39,2	34,9	32,3	45,2	33,5	49,3				32,3	39,1		25,7			43,3	50,4			
SWD	CLL	A*01:01, B*08:01	Exp15						41,8											26,2			
SWD	CLL	A*01:01, B*08:01	Exp22	36,2					35,6											29,3			
MWY	ALL	B*35:01	Exp4																		21,8		35,7
MWY	ALL	B*35:01	Exp14																				27,5
WSG	ALL	A*01:01	Exp5	40,0				25,2															
SLE	ALL	A*01:01, B*08:01	Exp6					35,5	43,3				25,1				32,4			30,6			
NBA	ALL	A*24:02	Exp7							32,0		46,5											
HJS	FL	A*24:02	Exp8									44,6											
KYE	ALL	A*24:02	Exp10									22,1											

Table S2. Continued.

Patient Code	Diag- nosis	Target HLA expressed	Experi- ment Tag	RALGPS2-LTDSEKGNSY-A1 p 242	FCRL5-LTEGHSGNYY-A1 p 243	FCRL5-TTENSGNYY-A1 p 263	MIST-ESEYADTHY-A1 p 248	TLR10-YLDHNSFDY-A1 p 265	IGJ-YTAVVPLVY-A1 p 268	BLK-AYIERMNSI-A24 p 246	VPREB3-YYCSVGYGF-A24 p 269	RALGPS2-QYIEELQKF-A24 p 258	TLR10-ELFKRTIQL-B8 p 247	TLR10-LPHLKTLIL-B8 p 256	IGLL1-HGLLRPTAA-B8 p 251	KLHL14-DMNTKRAIHTL-B8 p 239	FCRL2-IVKIKVQEL-B8 p 252	FAM129C-LPALRAQTL-B8 p 255	FAM129C-YLRLLDAL-B8 p 271	RALGPS2-TLKIRAEVL-B8 p 261	TNFRSF13B-SADQVALVY-B35 p 259	POU2AF1/BOB1-APAPTAVVL-B35 p 233	POU2AF1/BOB1-LPHQPLATY-B35 p 2 36
AHC	CLL	A*01:01, B*08:01	Exp16	34,7	23,9	27,1											32,4	21,8		30,5			
ALA	ALL	B*35	Exp17																				32,1
EMY	ALL	B*35	Exp18																				
HAN	CLL	B*08:01	Exp20																	31,6			
HBP	ALL	A*24:02	Exp21								22,3												
UM9	MM	A*01:01	Exp107						45,6														

* For each material, the patient code, diagnosis, expressed target HLA alleles and experiment tag as used in supplementary table 1 is shown. Columns indicate different identified target peptides. Values represent the highest detected Ion score per material, when a peptide was not detected in a material, no value is shown.

Table S3. HLA typing of healthy donor PBMCs used for T-cell isolation

Donor number	HLA-A	HLA-B	HLA-C
3	A*01- A*30	B*08- B*39	C*07- C*07
4	A*03- A*03	B*07- B*07	C*07- C*07
5	A*02- A*03	B*07- B*27	C*01- C*07
6	A*02- A*03	B*07- B*44	C*05- C*07
7	A*03- A*32	B*15- B*44	C*03- C*05
8	A*02- A*02	B*44- B*62	unknown
12	A*02- A*02	B*07- B*55	C*03- C*07
13	A*02- A*23	B*07- B*44	C*05- C*07
14	A*03- A*03	B*07- B*07	C*07- C*07
15[a]	A*24- A*29	B*07- B*55	C*03- C*03
16	A*23- A*30	B*44- B*49	C*04- C*07
17	A*02- A*03	B*07- B*44	C*01- C*07
18	A*03- A*11	B*07- B*56	C*01- C*07

[a] When target HLA is expressed, tetramers with this HLA restriction are excluded from experiment

Table S4. HLA typing of EBV-LCLs used in EBV-LCL panels

EBV-LCL	HLA-A	HLA-B	HLA-C
GMK	23:01:01- 02:01	41:01- 40:01	17:01:01:01- 03:04:01:01
RSB	02:01- 03:01/03:03/03:04	44:02- 57:01	06:02- 07:04/07:12/07:11
EBK	02:05- 02:05	58:01- 58:01	unknown
ERC	02:01- 02:01	13:02- 44:02	05:01- 06:02
BBD	02:01- 02:05	15:01- 45:01	01:02- 06:02
ABC	02:01:01- 11:01:01:01	44:05:01 - 51:01:01:01	02:02:02- 14:02:01
NMJ	02:01- 66:01/66:04	40:01/40:11/40:14 - 41:02	03:04/03:08/03:09- 17
HRK	03:01- 25:01	15:17 - 18:01/18:03/18:05	07:01/07:05/07:06- 12:03/12:06
MSV	03:01- 33:01	07:02- 14:02	07:02- 08:02
JBX	02:01- 30:02	15:01- 39:01	03:03- 12:03
IGU	03:01- 26:01	07:02:01- 14:01	07:02- 08:02
LSR	32:01- 68:01	35:03- 52:01	12:02- 12:03
HBM	02:01:01- 02:01:01	15:01:01:01 - 51:01:01	03:03:01- 15:02:01
RKO	02:05- 29:02	27:05- 44:03	01:02- 16:01:01
MSF	03:01/03:03/03:04- 30:01	07:02- 38:01	07:02/07:03/07:05- 12:03/12:06
BSR	02:01- 68:01	35:03- 37:01	04:01- 06:02
ABF	30:04- 68:02	38:01- 55:01	03:03- 12:03
GGT	26:01/26:08/26:02 - 31:01/31:02/31:06	14:01- 49:01	07:01/07:05/07:06- 08:02/08:07
AAJ	03:01/03:03/03:04 - 11:01/11:02/11:03	40:02/40:35/40:37 - 56:01	01:02/01:06/01:07 - 02:02/02:04/02:08
QBO	24:02:01:01- 31:01:02	07:02/07:61 - 35:08:01	04:01- 07:02

Table S4. Continued.

EBV-LCL	HLA-A	HLA-B	HLA-C
MMG	01:01:01- 32:01	35:08- 35:08	04:01- 04:01
UKA	03:01- 25:01	18:01- 35:01	04:01- 12:03
JBZ	01:01- 02:01	07:02- 18:01	07:01- 07:02
JMQ	02:01- 24:02:01:01	35:02- 44:02	04:01- 05:01
UJE	01:01:01:01- 33:03:01	44:03:02- 51:01:01	07:06/07:18- 14:02:01
UWI	02:01- 24:02	07:02:01- 40:02:01	02:02:02- 07:02:01
CAA	02:01- 02:01	40:02- 40:02	02:02- 02:02
URN	02:01- 03:01	08:01:01- 50:01:01	06:02:01- 07:01
AKB	01:01- 02:01	37:01- 39:01	06:02- 07:02
APZ	01:01- 68:01	44:02- 44:02	05:01- 07:04

Table S5. Primers used to measure target gene expression by qPCR

Gene	Forward primer	Reverse primer
GUSB	ACTGAACAGTCACCGACGAG	GGAACGCTGCACTTTTTGGT
PSMB4	GTTTCCGCAACATCTCTCGC	CATCAATCACCATCTGGCCG
VPS29	TGAGAGGAGACTTCGATGAGAATC	TCTGCAACAGGGCTAAGCTG
FCRL5	TGCAAATCCTAGAGGAGAAAATGTG	TAGGGGAACCCTTGTTCCTGA
VPREB3	GGGGACCTTCCTGTCAGTTTC	CGTAGTCCCTGATGGTGACG
POU2AF1	GACATGTATGTGCAGCCCGT	GAGCTTCTTGTCGTGACATTGG
MIST	GGACTCAGAGGAGATGAGAAGTT	GTTTCCTGTGGACCCCAGTT
RALGPS2	GCTGACTGACTCTGAGAAAGGAAA	CAGGCTGCACTCAAATGCTT
FCRL2	TCTCTGGGGACTGTTTGGTGT	GAAGCCCCTCTGGGTTCATTAGT
TNFRSF13B	GTGGCTATGAGATCCTGCCC	CAGCTGAGTGACCTGCAGAA