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

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# TCR-based Therapy for Multiple Myeloma and Other B-cell Malignancies Targeting Intracellular Transcription Factor BOB1

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Immunotherapy of hematological malignancies or solid tumors by administration of monoclonal antibodies or T cells engineered to express chimeric antigen receptors or T cell receptors (TCRs) has demonstrated clinical efficacy. However, antigen-loss tumor-escape variants and the absence of currently targeted antigens on several malignancies hampers the widespread application of immunotherapy. We have isolated a TCR targeting a peptide of the intracellular B cell-specific transcription factor BOB1 presented in the context of HLA-B*07:02. TCR gene transfer installed BOB1 specificity and reactivity onto recipient T cells. TCR-transduced T cells efficiently lysed primary B cell leukemia, mantel cell lymphoma and multiple myeloma *in vitro*. We also observed recognition and lysis of healthy BOB1-expressing B cells. In addition, strong BOB1-specific proliferation could be demonstrated for TCR-modified T cells upon antigen encounter. Furthermore, clear *in vivo* antitumor reactivity was observed of BOB1-specific TCR-engineered T cells in a xenograft mouse model of established multiple myeloma. Absence of reactivity towards a broad panel of BOB1-negative but HLA-B*07:02$^{pos}$ nonhematopoietic and hematopoietic cells indicated no off-target toxicity. Therefore, administration of BOB1-specific TCR-engineered T cells may provide novel cellular treatment options to patients suffering from B cell malignancies including multiple myeloma.

# Introduction

Immunotherapy of cancer based on the administration of chimeric antigen receptor (CAR)-modified T cells or monoclonal antibodies (mAbs) has demonstrated great clinical efficacy. In cases of hematological malignancies, CAR-engineered T cells specific for CD19 and CD20-targeting mAbs have led to complete remissions in patients suffering from chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), follicular and diffuse large B-cell lymphoma[1-5]. Therapeutic reactivity of mAbs and CAR-modified T cells is exerted by targeting extracellular cell surface antigens. In contrast to mAbs and CARs, T cell receptors (TCRs) recognize antigen-derived peptides that are bound to human leukocyte antigen (HLA) molecules on the cell surface. Since HLA molecules constantly sample the entire endogenous proteome of cells, both extracellular and intracellular antigens are presented. Therefore, the potential pool of antigens targeted by T cells via their TCR is greater than the pool of target antigens accessible for mAbs or CARs.

Similar to CARs, T cells can be modified with a TCR of choice to equip T cells with a specific reactivity towards a well-defined antigen[6-9]. Administration of such TCR-modified T cells is commonly referred to as TCR gene therapy and has already been successfully applied in the treatment of solid tumors[10, 11]. However, the widespread application of TCR gene therapy is hampered by lack of TCRs targeting common tumor-expressed self-antigens. Negative selection during thymic development deletes T cells carrying high affinity TCRs for self-antigens in the context of autologous (self) HLA in order to prevent autoimmune reactions. In contrast, self-antigens presented in the context of allogeneic (non-self) HLA (alloHLA) molecules can elicit strong immune responses as we have demonstrated by the isolation of PRAME-specific T cells after HLA-A*02:01-mismatched allogeneic stem cell transplantation[12]. Several strategies have been developed to generate T cell responses towards specific self-antigens by exploiting the immunogenicity of alloHLA molecules[13-17].

Despite the various technical advances in engineering T cells to recognize specific antigens, immunotherapy by administration of these modified T cells is lacking for many incurable malignancies such as multiple myeloma. Currently targeted antigens like CD20 and CD19 are not expressed by multiple myeloma. In this study, we isolated from the allorepertoire of healthy individuals TCRs specific for the intracellular B cell-specific transcription factor BOB1 (also known as Oct coactivator from B-cells (OCA-B) or OCT-binding factor-1 (OBF-1)). We demonstrate that TCR gene transfer of these BOB1-specific TCRs installs potent reactivity on recipient T cells resulting in lysis of primary B cell leukemia, mantle cell lymphomas and multiple myeloma in the absence of reactivity towards BOB1-negative cells. TCR-engineered T cells showed clear *in vivo* antitumor reactivity in a xenograft mouse model of established multiple myeloma. Administration of BOB1-specific TCR-engineered T cells can bring novel immunotherapeutic treatments to patients suffering from B cell malignancies including multiple myeloma.

# Results

## Identification of BOB1 T Cell Epitopes

We identified *POU2AF1* as a candidate gene with B cell lineage-restricted expression by comparing mRNA expression between $CD19^+$ B cells and healthy hematopoietic and nonhematopoietic cell samples in an in-house generated microarray gene expression database (Supplementary Figure S1). *POU2AF1* expression was high in primary $CD19^+$ B cells, Epstein-Barr virus (EBV)-transformed lymphoblastic B cell lines (B-LCL) and various B cell malignancies including chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), mantle cell lymphoma (MCL), and multiple myeloma (MM). No expression of *POU2AF1* was observed in $CD34^+$ hematopoietic progenitor cells (HPCs), T cells, professional antigen presenting cells (APCs), and other hematopoietic cell subsets not belonging to the B cell compartment. Furthermore, expression was absent in nonhematopoietic cells including fibroblasts, keratinocytes, melanocytes and samples of the gastrointestinal tract. mRNA expression measured by microarray analysis was verified for a selected set of cell samples by real-time quantitative polymerase chain reaction (RT-qPCR, data not shown). Since *POU2AF1* encodes for transcription factor BOB1, we mined the previously described B-LCL-derived HLA ligandome[18] for naturally processed and major histocompatibility (MHC)-presented peptides of BOB1. Four peptides were identified of which one was assigned to bind to HLA-A*02:01 (HLA-A2) and three peptides to HLA-B*07:02 (HLA-B7) using a public prediction algorithm[19, 20] (Supplementary Table S1). Efficient binding to their respective HLA-molecules and formation of stable peptide-MHC (pMHC) monomers was validated using UV-exchange technology (Supplementary Figure S2). Peptide $BOB1_{245}$ efficiently bound to HLA-A2 and prevented MHC degradation comparable to human Cytomegalovirus (CMV)-derived peptide $pp65_{NLV}$, which is known as a strong HLA-A2 ligand (Supplementary Figure S2A). Similarly, peptides $BOB1_{197}$, $BOB1_{44}$, and $BOB1_{14}$ prevented UV-induced MHC degradation of HLA-B7 comparable to peptide $pp65_{TRP}$, which is a strong HLA-B7 ligand (Supplementary Figure S2B). Stable pMHC tetramers for each peptide were generated by conjugating biotinylated pMHC monomers to fluorophore-labelled streptavidin.

## Isolation of BOB1-reactive T Cell Clones by Massive pMHC Tetramer Enrichment

T cell clones from $HLA\text{-}A2^{neg}/B7^{neg}$ individuals were isolated using the generated pMHC tetramers composed of BOB1-derived peptides bound to HLA-A2 or HLA-B7. Between 250 and $1000\times10^6$ PBMCs from six healthy $HLA\text{-}A2^{neg}/B7^{neg}$ individuals were labelled with the pool of pMHC tetramers. pMHC $tetramer^+$ cells were first enriched by magnetic-activated cell sorting (MACS) followed by single cell-sorting of pMHC $tetramer^+$ $CD8^+$ T cells. Thousands of T cells were clonally expanded for 2 weeks and screened in a high throughput fashion for peptide specificity. To this aim, GM-CSF secretion by the T cell clones was assessed after stimulation with the BOB1-negative K562 cell line expressing either HLA-A*02:01 (K562-A2) or HLA-B*07:02 (K562-B7) in the absence or presence of exogenously loaded BOB1 peptides.

The majority of T cell clones lacked BOB1 specificity and recognized K562-A2 or K562-B7 cells independent of the presence of BOB1 peptides as exemplified by T cell clones 3 and 29 (Supplementary Figure S3). Other T cells clones such as 227 showed no cytokine release under any stimulatory conditions indicating insufficient sensitivity to recognize MHC-bound BOB1 peptides. In contrast, T cell clones could be isolated demonstrating BOB1 specificity by recognizing either K562-A2 or K562-B7 cells when pulsed with BOB1 peptide. For example, T cell clone 3C10 specifically recognized K562-A2 loaded with BOB1 peptide but did not react towards unloaded K562-A2 cells. Similarly, T cell clone 4G11 reacted weakly but specifically towards K562-B7 cells pulsed with BOB1 peptides, whereas no reactivity against unloaded K562-B7 cells was observed. We performed pMHC tetramer staining to identify which of the four BOB1 peptides was recognized. No T cell clone stained with pMHC tetramer $BOB1_{14}$:B7 or $BOB1_{197}$:B7. However, 10 T cell clones specifically bound to pMHC tetramer $BOB1_{245}$:A2 (Figure 1A and Supplementary Figure S4A), and one clone (4G11) specifically bound to pMHC tetramer $BOB1_{44}$:B7 (Figure 2A). The identified T cell clones did not stain with a HLA-matched control pMHC tetramer containing an irrelevant peptide. Thus, from 6 HLA-$A2^{neg}$/$B7^{neg}$ healthy individuals, 11 T cell clones specific for BOB1 peptides presented either in HLA-A2 or HLA-B7 were selected for further analysis.



### Selection of T Cell Clones Specific for $BOB1_{245}$:A2 and $BOB1_{44:}$B7

Next, we aimed to identify high avidity clones among a set of T cell clones sharing same peptide specificity. Sensitivity to BOB1 peptides was determined by coincubating T cell clones with either K562-A2 or K562-B7 cells pulsed with titrated peptide. In addition, recognition of endogenously processed and presented peptide was assessed by coculturing T cell clones with BOB1-expressing HLA-$A2^{pos}$/$B7^{pos}$ B-LCLs. Varying degrees of peptide sensitivity could be observed among T cell clones classified as $BOB1_{245}$:A2 reactive (Figure 1B). Representative T cell clones 223, 7D1 and 3C10 demonstrated peptide specificity with increasing sensitivity. Increased sensitivity directly translated to better recognition of the three B-LCLs (Figure 1C). Clone 3C10 produced cytokine upon stimulation with all three B-LCLs while less peptide-sensitive clones 223 and 7D1 showed no or only weak recognition of the three B-LCLs. Supplementary Figure S4B-C summarizes the analysis of other $BOB1_{245}$-reactive T cell clones. T cell clone 4G11 exhibited $BOB1_{44}$ peptide-dependent recognition of K562-B7 cells (Figure 2B), and efficiently recognized all three B-LCLs (Figure 2C). This data indicated sufficient avidity of T cell clone 4G11 to recognize endogenously processed and presented peptide.

In summary, from a pool of T cell clones directed against different BOB1-derived peptides two T cell clones were selected by demonstrating specific recognition of peptide-pulsed targets cells as well as reactivity against endogenously processed and presented peptide. T cell clone 3C10 specifically recognized $BOB1_{245}$ in HLA-A2 whereas T cell clone 4G11 was specific for $BOB1_{44}$ presented in HLA-B7.

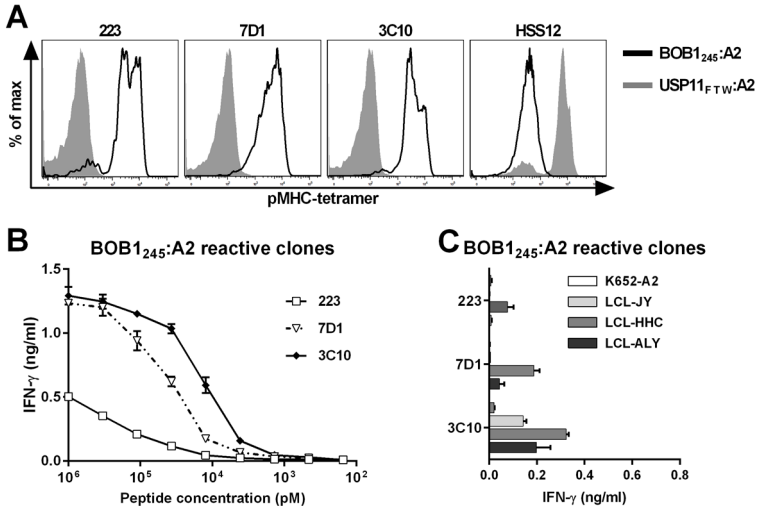


**Figure 1. BOB1-reactive T cell clones exhibit varying degrees of peptide sensitivity and avidity.** BOB1-reactive T cell clones recognizing peptide $BOB1_{245}$ presented in HLA-A2 were assessed for peptide sensitivity by peptide titration and their capacity to recognize endogenously processed peptide. (A) Shown are histograms of 3 representative T cell clones stained with pMHC tetramer $BOB1_{245}$:A2 (black line) or control pMHC tetramer composed of irrelevant USP11-derived peptide FTWEGLYNV bound to HLA-A2 ($USP11_{FTW}$:A2) (grey area). T cell clone HSS12 specific for $USP11_{FTW}$:A2 served as control. (B-C) BOB1-reactive T cell clones were coincubated with K562-A2 cells pulsed with titrated $BOB1_{245}$-peptide (B) or three BOB1-expressing HLA-$A2^{pos}$ B-LCLs (C). BOB1-negative K562-A2 cells were used as control. Shown are means with standard deviations of one representative experiment carried out in duplicate.

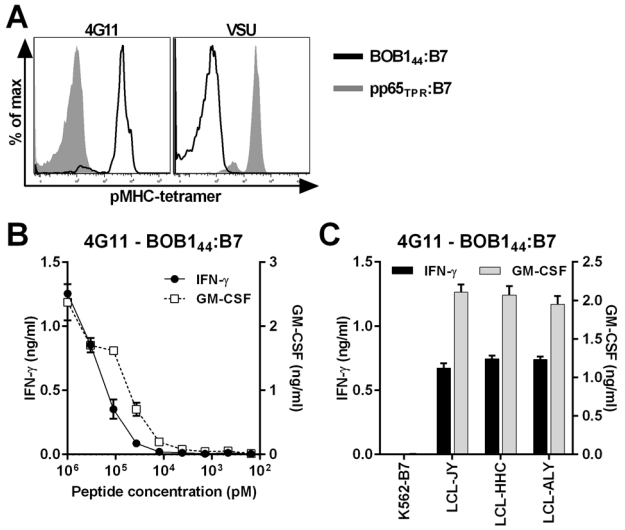


**Figure 2. T cell clone 4G11 recognizes endogenously processed and presented $BOB1_{44}$.** (A) Shown are histograms of T cell clone 4G11 or a control T cell clone VSU specific for CMV-derived peptide TPRVTGGGAM ($pp65_{TPR}$). T cell clones were stained with pMHC tetramer $BOB1_{44}$:B7 or $pp65_{TPR}$:B7. (B-C) T cell clone 4G11 was cocultured with K562-B7 pulsed with titrated concentration of $BOB1_{44}$-peptide (B) or three BOB1-expressing HLA-$B7^{pos}$ B-LCLs (C). Shown are means with standard deviations of one experiment carried out in duplicate.

## Specific Recognition of Malignant and Healthy B Cell Compartment

To further investigate the recognition profile of our selected BOB1-specific T cell clones we tested their reactivity towards primary BOB1-expressing B cell malignancies. T cell clone 4G11 efficiently recognized all HLA-B7$^{pos}$ primary B cell malignancies tested, including 5 chronic lymphocytic leukemia (CLL), 4 acute lymphoblastic leukemia (ALL), 3 mantle cell lymphoma (MCL) and 4 multiple myeloma (MM; Figure 3A). Furthermore, BOB1-dependent recognition of clone 4G11 could be demonstrated by introducing full length BOB1 into K562-B7 cells (Figure 3C). T cell clone 3C10 specific for $BOB1_{245}$ presented in HLA-A2 also recognized primary BOB1-expressing B cell malignancies including multiple myeloma. However, not all HLA-A2$^{pos}$ samples were recognized (Figure 3B). Clone 3C10 also failed to recognize K562-A2 when transduced with BOB1, indicating insufficient avidity to efficiently recognize endogenously processed peptide (Figure 3D). Two control T cell clones recognizing household gene-derived peptides in the context of HLA-B*07:02 or HLA- A*02:01 confirmed sufficient and correct HLA subtype expression of all tested malignant cell samples (Supplementary Figure S5A-B).

Since T cell clone 4G11 efficiently recognized all different B cell malignancies tested, we further investigated the recognition profile of this clone by coincubation with various HLA-B7$^{pos}$ nonhematopoietic and hematopoietic cell subsets. T cell clone 4G11 did not react with BOB1-negative fibroblasts even when cultured with IFN-γ to mimic inflammation (Figure 3E). Additionally, we tested clone 4G11 for the recognition of primary bronchial epithelial cells (PBECs), since a recent publication reported on the expression of *POU2AF1* in human airway epithelium.[21] *POU2AF1* mRNA could be detected at very low levels in air-exposed and non-air-exposed PBECs by RT-qPCR (data not shown). However, clone 4G11 did not produce IFN-γ upon stimulation with these cells (Figure 3F-G). Recognition was also absent when PBECs had been incubated with IFN-γ to mimic inflammation (Figure 3F-G). Clone 4G11 did not produce cytokine when stimulated with a panel of different BOB1-negative nonhematopoietic tumor cell lines (Figure 3H). Absence of BOB1 expression in tumor cell lines was confirmed by RT-qPCR (data not shown). Furthermore, reactivity towards hematopoietic cell subsets was restricted to the B cell compartment. BOB1-expressing primary and activated B cells were recognized whereas no reactivity was found against BOB1-negative resting and activated T cells, monocytes and monocyte-derived immature and mature dendritic cells derived from 2 different HLA-B7$^{pos}$ donors as well as CD34$^{+}$ HPCs (Figure 3I-J). A control clone CTZ, specific for a ubiquitously expressed antigen in the context of HLA-B7, efficiently recognized all cell samples tested, confirming the stimulatory capacity of these cells (Supplementary Figure S5C-H).

Finally, when tested against a panel of BOB1-expressing B-LCLs expressing 95 % of common and rare HLA class I and II alleles[22], T-cell clone 4G11 only reacted to HLA-B7$^{pos}$ B-LCLs, indicating HLA-B7 restriction without crossreactivity to all other HLA class I alleles tested (Figure 3K and Supplementary Table S2). In conclusion, T cell clone 4G11 showed potent antitumor reactivity as demonstrated by the recognition of malignant B cells, including multiple myeloma. Furthermore, absence of reactivity towards healthy hematopoietic non-B cells and nonhematopoietic cells indicated no off-target toxicity.

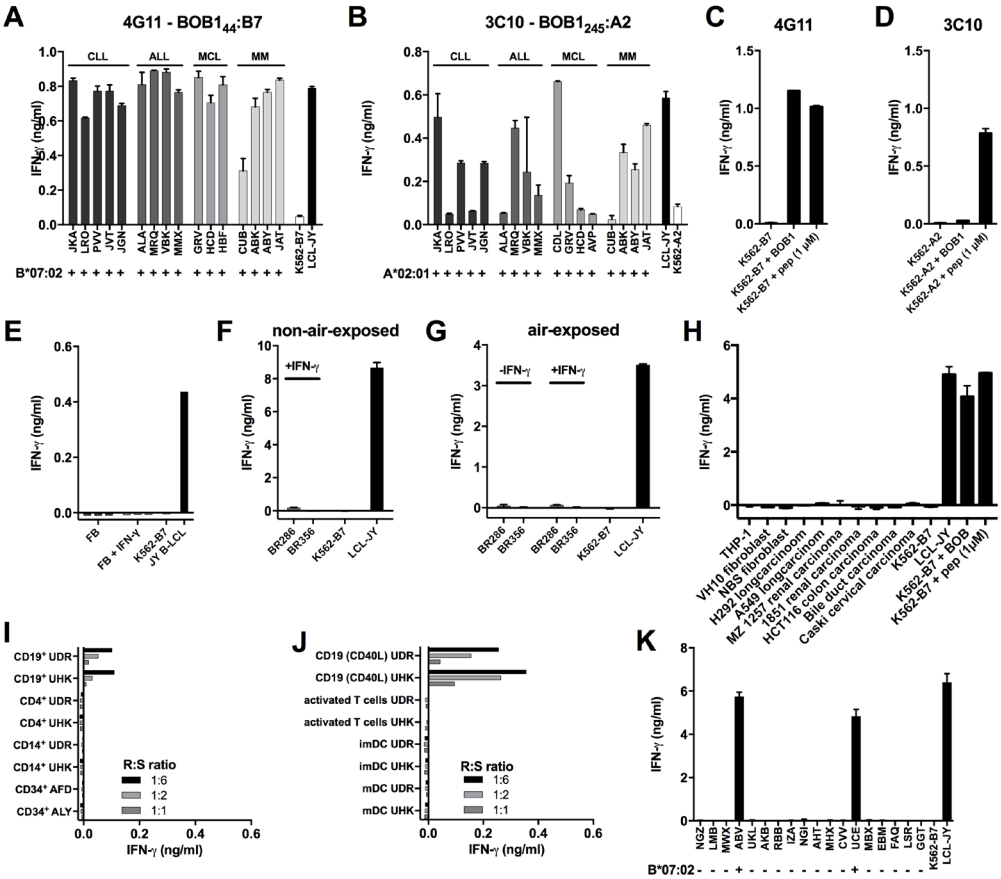


**Figure 3. T cell clone 4G11 efficiently recognizes B cell malignancies while sparing non-B cell lineages.** (A-B) T cell clones 4G11 and 3C10 were co-incubated with primary samples of malignant B-cells including chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), mantle cell lymphoma (MCL) and multiple myeloma( MM). HLA allotype of each sample is indicated. Controls included BOB1-negative K562-B7 cells and BOB1-expressing LCL-JY. Shown is one representative experiment of two independent experiments. (C-D) T cell clones 4G11 and 3C10 were incubated with K562-B7 and K562-A2 cells alone, or transduced to express BOB1 (K562-B7 + BOB1 or K562-A2 + BOB1, respectively) or K562-B7 and K562-A2 cells pulsed with 1 µM BOB1$_{44}$ or BOB1$_{245}$ peptide, respectively. (E-J) T cell clone 4G11 was cocultured with different HLA-B7$^{pos}$ healthy primary or activated cell subsets. Fibroblasts (FB) from three different individuals were either left untreated or cultured for four days in 200 IU/ml IFN-γ (FB + IFN-γ) before coculture (E). Primary bronchial epithelial cells were cultured under non-air-exposed (F) or air-exposed condition (G) before being used as stimulator cells. Cells had also been cultured in the absence (-IFN-γ) or presence (+IFN-γ) of 100 IU/ml IFN-γ for two days before coculture. (H) Clone 4G11 was coincubated with various HLA-B7$^{pos}$ BOB1-negative nonhematopoietic tumor cell lines. (I-J). Primary CD19$^{+}$, CD4$^{+}$, CD14$^{+}$ and CD34$^{+}$ cells were isolated from PBMCs of healthy individuals (UDR, UHK, AFD, ALY) and cocultured in different responder:stimulator (R:S) ratios (I). Activated B cells (CD19 (CD40L)) were generated by stimulating CD19$^{+}$ B cells with CD40L. Activated T cells were generated from PHA-stimulated PBMCs. Immature (imDC) and mature (mDC) dendritic cells were monocyte derived (J). (K) T cell clone 4G11 was cocultured with a panel of BOB1-expressing B-LCLs expressing a wide variety of HLA class I and II molecules. HLA-B7 status of each B-LCL is indicated with + (positive) or – (negative). IFN-γ production was measured after 18 hours of coculture. Shown are means with standard deviations of one experiment carried out in duplicate. Representative data from two or more independent experiments.

### TCR Gene Transfer Installs BOB1 Reactivity onto Recipient T Cells

Next, we investigated whether gene transfer of the TCR of clone 4G11 (TCR-4G11) could install BOB1 reactivity onto recipient cells. TCR-4G11 was sequenced, codon-optimized, modified with a disulfide bond to increase preferential pairing of the TCRα and TCRβ chain, and cloned into the MP71 vector expressing NGF-R as a marker gene. Expression of NGF-R was used to enrich TCR-transduced T cells to high purity (> 98%) by MACS-guided isolation of NGF-R expressing cells. Retrovirally transduced $CD8^+$ T cells from a HLA-$B7^{pos}$ healthy individual expressed TCR-4G11 on the cell surface indicated by their capacity to bind pMHC tetramer $BOB1_{44}$:B7 (Figure 4A). More intensive staining with pMHC tetramer correlated with higher NGF-R expression suggesting that cells expressing higher levels of the introduced TCR bound pMHC tetramer more efficiently. Tetramer binding was not observed for mock-transduced T cells. Although $CD4^+$ T cells could be transduced as indicated by the expression of NGF-R, no binding to $BOB1_{44}$:B7 tetramer was observed (Figure 4A). TCR-transduced $CD8^+$ T cells readily recognized BOB1-expressing HLA-$B7^{pos}$ stimulator cells such as multiple myeloma cell lines UM9 and U266, LCL-JY, and two ALL cell lines ALL-BV and ALL-VG mirroring the reactivity profile of T cell clone 4G11 (Figure 4B). In contrast, TCR-transduced $CD4^+$ T cells failed to recognize any of these cell lines. No recognition for mock-transduced $CD8^+$ T cells was observed, indicating that recognition was mediated by the introduced TCR-4G11.
Furthermore, TCR-transduced $CD8^+$ T cells efficiently lysed primary HLA-$B7^{pos}$ malignant cell samples (Figure 5A and Supplementary Figure S6A). Complete or nearly complete lysis was observed for primary ALL and MCL samples and MM cell lines at equal effector-to-target ratio and even when targets cells exceeded effector cells threefold. Efficient lysis by TCR-transduced T cells could also be observed for both tested primary CLL samples as well as both ALL cell lines. In addition, both purified HLA-$B7^{pos}$ primary multiple myeloma samples were readily lysed by TCR-transduced T cells at low effector-to-target ratios. In all cases, no lysis was observed for mock-transduced T cells, indicating that lysis was mediated by the introduced TCR-4G11. BOB1 expression in healthy B cells also led to lysis by autologous TCR-transduced $CD8^+$ T cells. (Figure 5B) Lysis was specific as the BOB1-negative K562-B7 cell line was not lysed (Figure 5A). In addition, TCR-transduced $CD8^+$ T cells did not lyse autologous activated T cells or $CD14^+$ monocytes, indicating a safe reactivity profile (Figure 5B). Finally, TCR-transduced $CD8^+$ T cells proliferated upon stimulation with various BOB1-expressing HLA-$B7^{pos}$ primary samples, including ALL, CLL, MCL, multiple myeloma cell lines and autologous activated B cells (Figure 6A-B and Supplementary Figure S6B). In contrast, absence of any stimulus or stimulation with antigen-negative cell line K562-B7 or autologous activated T cells did not lead to proliferation of TCR-transduced T cells. Proliferative capacity of TCR- and mock-transduced T cells was confirmed using CD3/CD28 T cell activator beads.
In summary, TCR gene transfer of TCR-4G11 installed BOB1 reactivity onto recipient $CD8^+$ T cells. TCR-transduced T cells efficiently lysed primary ALL, CLL, MCL and multiple myeloma at low effector-to-target ratios while sparing non-B cells. Furthermore, TCR-transduced T cells readily proliferated upon antigen encounter.

## *In Vivo* Efficacy of BOB1-specific TCR-transduced T Cells

Using a multiple myeloma xenograft mouse model, we investigated the *in vivo* efficacy of our TCR-transduced T cells. TCR-4G11 expressing T cells demonstrated strong antitumor reactivity against U266 cells that were allowed to expand in NSG mice for three weeks before treatment. Mock-transduced T cells failed to control tumor growth, although in later stages tumor expansion was curbed probably caused by the alloreactivity of mock-transduced T cells. In contrast, significant tumor reduction was observed in mice after injection of TCR-4G11 T cells (Figure 7A-B). When bone marrows of mice were investigated for the presence of tumor cells at the end of the experiment, mock-treated mice contained on average an order of magnitude more tumor cells than could be found in TCR-treated mice (Figure 7C).
In conclusion, BOB1-specific TCR-transduced T cells showed potent *in vivo* antitumor reactivity in an *in vivo* xenograft model of established multiple myeloma.

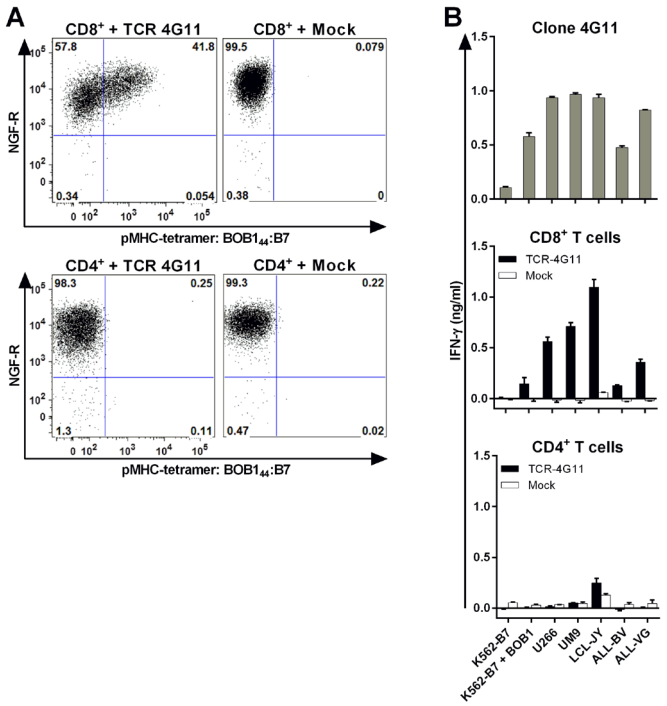


**Figure 4. Transfer of TCR-4G11 installs BOB1 reactivity on recipient CD8⁺ T cells.** CD4⁺ and CD8⁺ T cells were isolated from a healthy HLA-B7$^{pos}$ individual using MACS. T cells were transduced with retroviral supernatant to express TCR-4G11 together with NGF-R. Transduction with an empty vector (Mock) containing only the NGF-R marker gene served as control. Transduced T cells were purified based on the expression of marker gene NGF-R using MACS. Level of purity exceeded 98% in all cases. (A) Shown are FACS plots of purified T cells after transduction. CD8⁺ (top row) or CD4⁺ (bottom row) T cells were stained with pMHC tetramer BOB1$_{44}$:B7 and an antibody against NGF-R. Numbers in corners indicate percentage cells per quadrant. FACS plots are shown with biexponential axes. (B) T cell clone 4G11 or purified transduced CD8⁺ or CD4⁺ T cells were coincubated with various HLA-B7$^{pos}$ cell lines. Cell lines included K562-B7 transduced to express BOB1 (K562-B7 + BOB1), two multiple myeloma cell lines UM9 and U266, LCL-JY, and two ALL cell lines ALL-BV and ALL-VG. IFN-γ concentration was assessed after 18 hours of coculture. Shown are means with standard deviations of one experiment carried out in duplicate.

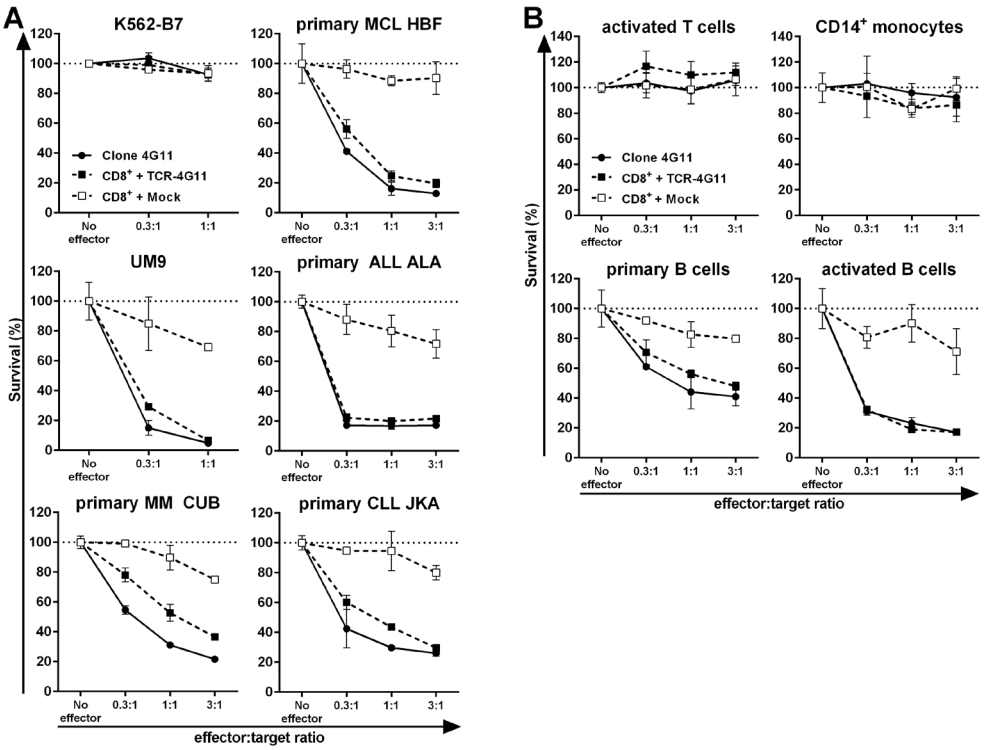


**Figure 5. TCR-transduced CD8⁺ T cells efficiently lyse primary B cell malignancies including multiple myeloma.** (A-B) T cell clone 4G11 or purified TCR- or mock-transduced CD8⁺ T cells were tested for their lytic capacity of HLA-B7$^{pos}$ target cells. PKH-labelled target cells were cocultured at various effector:target ratios with effector T cells. After 18 hours of coculture, the number of live targets cells was assessed by flow cytometry and percent survival calculated. (A) Malignant cell samples included multiple myeloma cell line UM9, primary multiple myeloma (MM), mantle cell lymphoma (MCL), acute lymphoblastic (ALL) and chronic lymphocytic leukemia (CLL). Controls included BOB1-negative cell line K562-B7. (B) Healthy hematopoietic cells were of same origin as transduced T cells (autologous setting) and included PHA-activated T cells, CD14⁺ monocytes, CD19⁺ primary B cells and CD40L-activated B cells. Shown are means with standard deviations of one experiment carried out in triplicate.

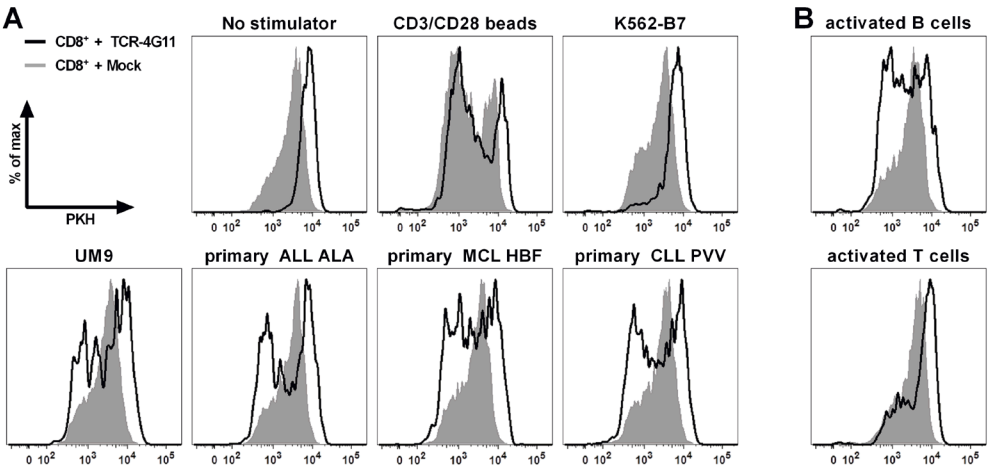


**Figure 6. TCR-transduced CD8$^+$ T cells proliferate upon antigen encounter.** (A-B) PKH-labelled transduced CD8$^+$ T cells were cocultured with irradiated HLA-B7$^{pos}$ stimulator cells. Histograms show TCR-transduced (black line) or mock-transduced (grey area) CD8$^+$ T cells after 5 days of co-culture. (A) Stimulator cells included cell-line UM9 and primary ALL, CLL and MCL. Negative controls included culture in absence of stimulator cells (No stimulators) or coculture with BOB1-negative K562-B7 cells. Positive control included stimulation in the presence of CD3/CD28 T cell activator beads (CD3/CD28 beads). (B) Autologous CD40L-stimulated B-cells or PHA-activated T cells were used as stimulator cells.



# Discussion

TCR gene transfer is an attractive strategy for cancer therapy by equipping T cells with a TCR of defined antigen specificity to eradicate antigen-positive malignant cells. However, TCR gene therapy is still unavailable for many solid tumors and hematological malignancies since no suitable targets or antigen-specific TCRs have been identified. Here, we describe the isolation of TCRs recognizing peptides derived from the intracellular transcription factor BOB1 presented in the context of HLA-A2 or HLA-B7.

BOB1 is highly expressed in B cell malignancies such as ALL, CLL and MCL. Moreover, BOB1 is also highly expressed in multiple myeloma for which no curative treatment is currently available. BOB1 is an octamer-binding B cell-specific coactivator. By binding to the $POU_H$ and $POU_S$ subdomains of OCT-1/2 proteins it increases their affinity for octamer-containing DNA and further increases octamer-dependent gene transcription[23, 24]. In addition, cytosolic BOB1 has been implicated to regulate early events in intracellular signaling of B cells via its interaction with tyrosine kinase SYK[25]. A drastically reduced B cell count due to increased apoptosis of B cells in the bone marrow and complete lack of formation of germinal centers

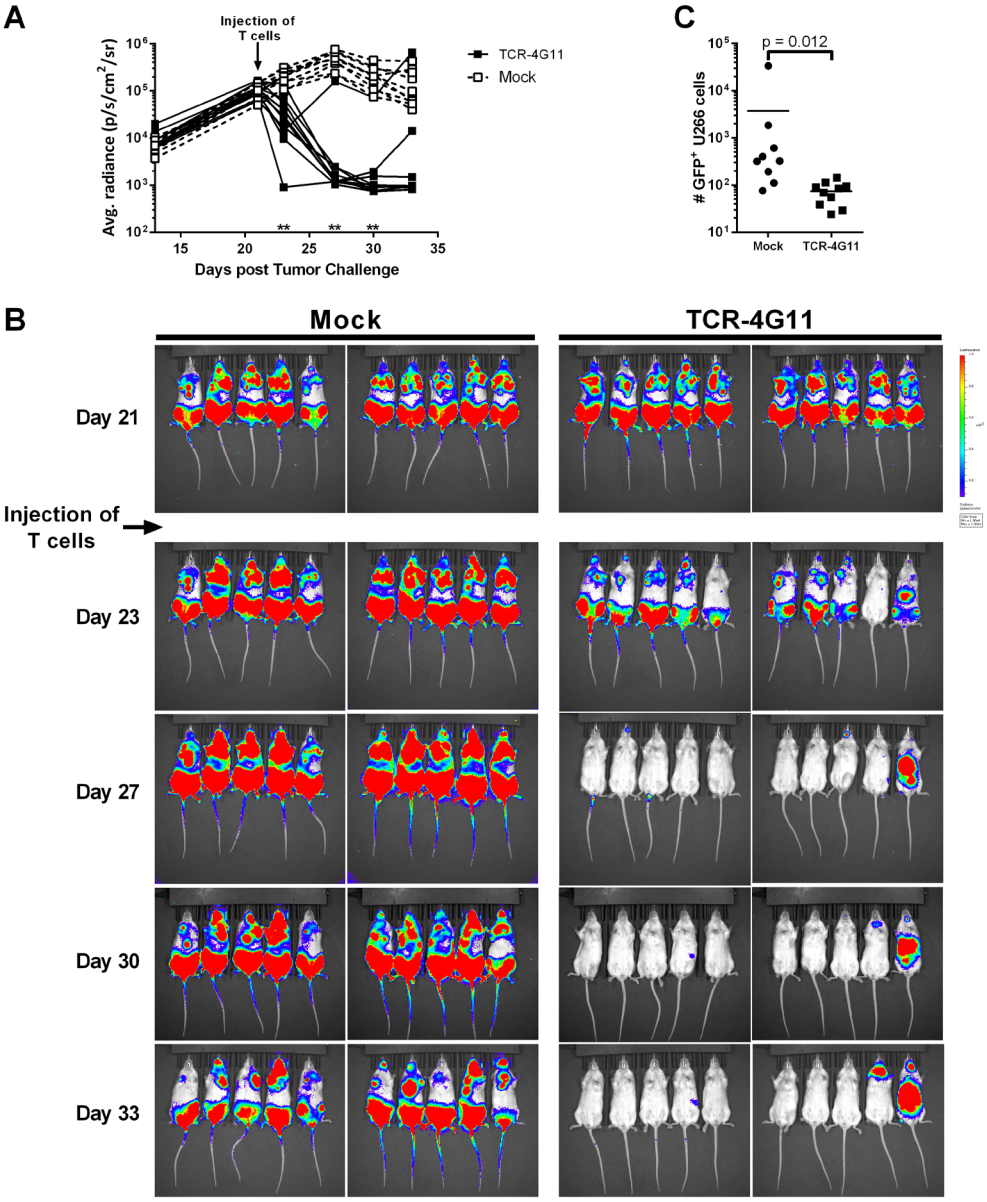


**Figure 7. TCR-transduced CD8⁺ T cells demonstrate *in vivo* antitumor efficacy in model of established multiple myeloma.** GFP⁺ U266 multiple myeloma cells expressing luciferase were allowed to engraft in NSG mice. Mice were intravenously treated with TCR-transduced (TCR-4G11) or mock-transduced (Mock) T cells 21 days after tumor injection. (A-B) Tumor growth was monitored by bioluminescence. Each treatment group contained 10 mice. Results from unpaired 2-tailed Mann-Whitney test comparing Differences in mean bioluminescence between treatment groups is shown in A (**$p < .01$). (C) Mice were sacrificed on day 33, bone marrow was harvested and analyzed for presence of GFP⁺ U266 cells. Result from unpaired 2-tailed Mann-Whitney test is shown.

in BOB1-knockout mice has established a broad role of BOB1 in B cell development and function throughout all B cell stages[26-30]. The *POU2AF1* gene locus has been identified as a target of gene amplification in a subset of multiple myeloma[31]. Furthermore, in multiple myeloma cell lines showing gene amplification of the *POU2AF1* gene locus, it has been shown that proliferation can be hampered by BOB1 knockdown or knockdown of downstream targets of BOB1. Based on its role in B cell development and involvement in proliferation in a subset of multiple myeloma, it is tempting to speculate that BOB1 is a vital player in B cell survival and therefore BOB1-loss tumor escape variants may not likely occur in the course of BOB1-targeting therapy as has been observed when targeting other antigens[2, 32]. However, whether B cell malignancies indeed lack the capacity to escape from immune surveillance by shutting down BOB1 expression should become clear in clinical trials in which BOB1 is specifically targeted.

By exploiting the immunogenicity of allogeneic (non-self) HLA, we were able to identify a high affinity TCR targeting the self-antigen BOB1. Although several T cell clones directed against different BOB1-derived peptides were isolated, only T cell clone 4G11 demonstrated sufficient peptide sensitivity as well as stringent antigen specificity. Endogenously processed peptide was efficiently and reproducibly recognized on HLA-B7$^{pos}$ B-LCLs as well as B cell malignancies. In contrast, T cell clone 3C10 specific for $BOB1_{245}$ presented in HLA-A2 demonstrated similar peptide sensitivity but failed to recognize all HLA-A2$^{pos}$ B-LCLs and B cell malignancies. This data may suggest superior affinity of TCR-4G11 for $BOB1_{44}$:B7 than TCR-3C10 for $BOB1_{245}$:A2. However, the different recognition profiles of the T cell clones may also stem from more efficient processing and greater abundance of $BOB1_{44}$:B7 on the cell surface as compared to $BOB1_{245}$:A2. If true, efficient targeting of $BOB1_{245}$ bound to HLA-A2 requires expression of a higher affinity TCR than expressed by clone 3C10.

The various reactivity patterns of the different T cell clones resemble our previous experience when using pMHC tetramers for enrichment of specific T-cell populations[17, 33, 34]. Reactivity against antigen-negative K562 cell-line was often observed and an indication of potential harmful off-target toxicity. Of note, T cell clone 17A5, demonstrating recognition of an irrelevant peptide in HLA-A2 when stimulated with BOB1-negative K562-A2, also recognized HLA-A2$^{pos}$ fibroblasts and healthy hematopoietic cell subsets (Supplementary Figure S7).

T cell clone 4G11 specific for $BOB1_{44}$ presented in the context of HLA-B7 strongly recognized various B cell malignancies including multiple myeloma. Furthermore, T cells engineered to express TCR-4G11 successfully controlled tumor growth in an *in vivo* model of established multiple myeloma. No reactivity against a wide panel of BOB1-negative but HLA-B7$^{pos}$ stimulator cells and no crossreactivity with other HLA class I and II alleles was observed for TCR 4G11. From a standard protein BLAST search, no protein could be identified that shared 100% amino acid (aa) sequence similarity with peptide $BOB1_{44}$. Proteins with partial aa sequence similarity did not contain peptides covering this partial homology that would strongly interact with HLA-B7 as predicted by a public prediction algorithm[19, 20]. Additionally, proteins sharing a partial aa sequence with the $BOB1_{44}$ epitope are expressed in T-cells or monocytes, which we have demonstrated not to be recognized by clone 4G11. However,

the possibility remains that unwanted off-target or on-target off-tumor toxicity may occur once TCR-modified T cells are administered in patients[35, 36]. Therefore, TCR-engineered T cells should additionally be equipped with a suicide switch to abolish their reactivity in case of adverse effects[37-42]. Furthermore, since also healthy B cells expressing BOB1 will be depleted in the course of therapy, specific depletion of BOB1-reactive TCR-modified T cells after successful therapy may be desirable to enable restoration of the healthy B cell compartment. Nonetheless, long-term B cell aplasia is tolerable from a clinical perspective and can be managed as has been demonstrated by other immunotherapeutic interventions targeting B cell-expressed antigens[3, 43].

Transfer of TCR-4G11 installed BOB1-reactivity onto recipient $CD8^+$ but not $CD4^+$ T cells. We speculate that TCR-4G11 requires expression of coreceptor CD8 for sufficient binding to cognate pMHC molecules as has been observed for other high affinity TCRs[44, 45]. However, BOB1-specific CD4 T cell help could be provided by cotransduction of TCR-4G11 and coreceptor CD8[45, 46].

In conclusion, we expect BOB1-targeting TCRs to be a valuable addition to current immunotherapies by broadening their application to yet incurable diseases like multiple myeloma. Furthermore, because of broad expression of BOB1 throughout the entire B cell lineage, BOB1-specific T cells could be of use in patients suffering from B cell malignancies in which standard therapy or other immunotherapies have failed. Moreover, targeting monomorphic (nonmutated) peptides in the context of common HLA molecules, BOB1-targeting TCRs are widely applicable by bringing novel therapeutic strategies to patients suffering from tumors in which the occurrence of an endogenous immune response directed against mutated neoantigens is less likely[47].



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# Materials and Methods

## Culture Conditions and Cells

Peripheral blood was obtained from different individuals after informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-gradient centrifugation and were cryopreserved. T cells were cultured in T cell medium consisting of IMDM (Lonza, Basel, Switzerland) supplemented with 100 IU/ml IL-2 (Proleukine; Novartis Pharma, Arnhem, The Netherlands), 5% fetal bovine serum (FBS; Gibco, Life Technologies, Carlsbad, California) and 5% human serum. Isolation, generation, and culture of various hematopoietic and nonhematopoietic cell subsets and lines is described in supplemental Materials and Methods.

## Isolation of BOB1-reactive T Cell Clones

T cells binding to BOB1-specific peptide-MHC (pMHC) tetramers were isolated from PBMCs of healthy HLA-$A2^{neg}/B7^{neg}$ individuals. PBMCs were first incubated with phycoerythrin (PE)-labeled pMHC tetramers for 1 h at 4 °C. Cells were washed twice and incubated with anti-PE-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4 °C. PE-labeled cells were isolated on an LS colomn (Miltenyi Biotec) according to manufacturer's instruction. Positively selected cells were stained with an antibody against CD8 in combination with antibodies against CD4, CD14, and CD19. pMHC $tetramer^+$ $CD8^+$ T cells were single cell-sorted into round-bottom 96-well plates containing $5\times10^4$ irradiated (35 Gy) feeders in 100 µl T cell medium supplemented with 0.8 µg/ml PHA.

## TCR Gene Transfer to Tecipient $CD4^+$ and $CD8^+$ T Cells

TCRAV and TCRBV usage of clone 4G11 was determined using reverse transcriptase (RT)-PCR and sequencing[48]. A retroviral vector was constructed on a MP71 backbone with a codon-optimized and cysteine-modified TCRα and TCRβ chain joined by the T2A sequence in combination with the truncated nerve growth factor receptor (NGF-R) and ordered from GenScript (Piscataway, New Jersey). For *in vivo* experiments, the optimized TCRα and TCRβ chains joined by the T2A sequence were cloned and expressed on the SFG retroviral backbone[49].

Purified $CD4^+$ or $CD8^+$ T cells were activated using irradiated autologous PBMCs and PHA. T cells were transduced using retroviral supernatant and high purity TCR-transduced T cell populations were obtained as described in supplemental methods.

## FACS Analysis

FACS was performed on a LSRII (BD Biosciences, Franklin Lakes, New Jersey) and analyzed using Diva Software (BD Biosciences) or FlowJo Software (TreeStar, Ashland, Oregon). Isolated T cell clones were analyzed for binding to specific pMHC tetramers and CD8 expression by

staining with PE- or allophycocyanin (APC)-labelled pMHC tetramers, and an Alexa700-conjugated antibody against CD8 (Invitrogen/Calteg, Buckingham, United Kingdom) combined with FITC-labelled antibodies against CD4, CD14, and CD19 (BD Pharmingen, San Jose, California). 20,000 cells of a T cell clone were first incubated with 2 µg/ml pMHC tetramers for 15 min at 37 °C before antibodies were added and incubated for an additional 15 min at 4 °C. Similarly, 25,000 TCR-transduced or mock-transduced T cells were incubated with 2 µg/ml pMHC tetramers for 15 min at 37 °C before antibodies against CD8, CD4 and CD271 (NGF-R, Sanbio, Uden, The Netherlands) were added and incubated at 4 °C for 15 min. PBMCs, purified hematopoietic cell subsets or activated cells were stained with antibodies against CD3, CD4, CD14, CD19, CD34 (BD Pharmingen) for 4 °C for 15 min.

### Functional Analysis and Cytotoxicity Assay

T cells and stimulator cells were coincubated for 18 h before cell viability was assessed or supernatants were harvested and IFN-γ or GM-CSF production was measured by enzyme-linked immunosorbent assay (ELISA, Sanquin Reagents or R&D Systems, Minneapolis, Minnesota, respectively).



### *In vivo* U266 Multiple Myeloma Model for Antitumor Efficacy

NSG mice (Jackson Laboratory, Bar Harbor, Maine) were intravenously injected with $2\times10^6$ HLA-B7$^{pos}$ U266 cells expressing green fluorescent protein (GFP) and luciferase. On day 21 after tumor inoculation, NSG mice were treated intravenously with $5\times10^6$ TCR-4G11 or mock-transduced T cells. Tumor growth was monitored using bioluminescence. On day 34, mice were sacrificed and bone marrow was harvested and analyzed for the presence of GFP$^+$ U266 cells by FACS analysis. Treatment groups were compared using unpaired 2-tailed Mann-Whitney test in GraphPad Prism (Version 6.05, GraphPad Software, Inc., La Jolla, CA).

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# Supplementary Information

**Supplementary Table S1.** Sequences and properties of BOB1-derived peptides.

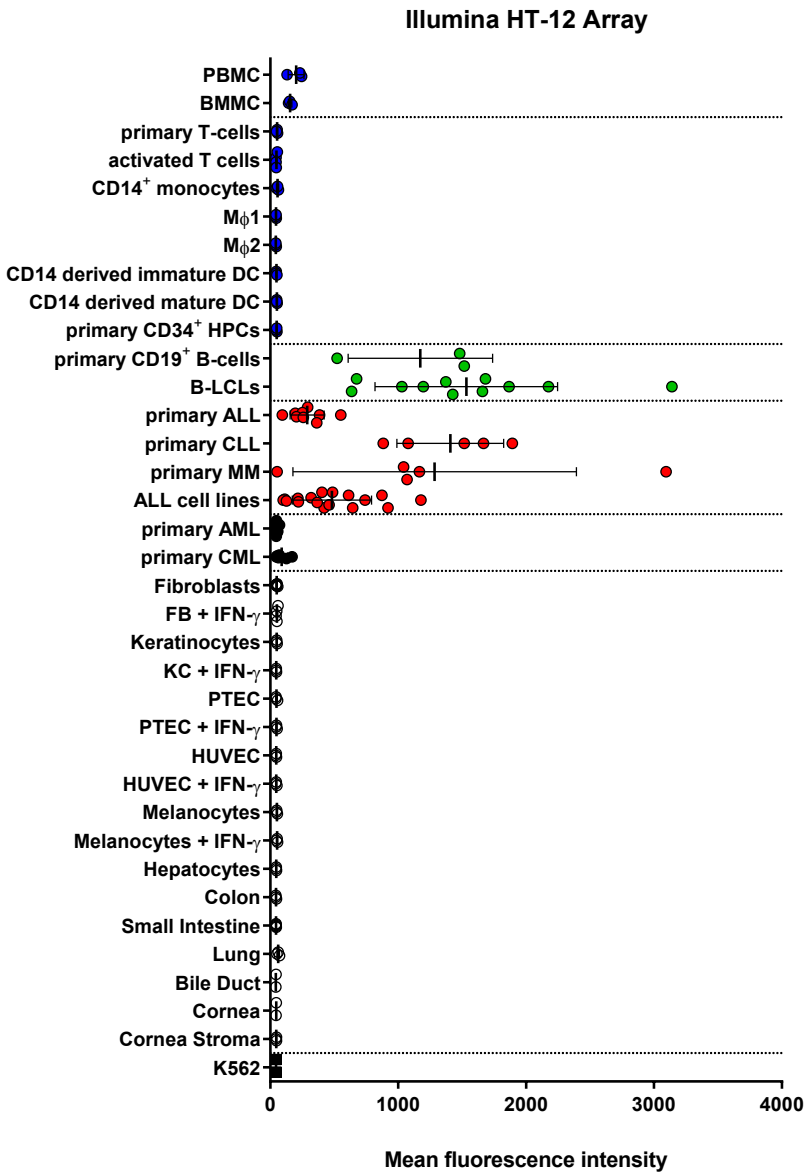
| | Peptide sequence | HLA-restriction | netMHC affinity (nM)* |
|---|---|---|---|
| $BOB1_{245}$ | YALNHTLSV | A*02:01 | 15 |
| $BOB1_{197}$ | APALPGPQF | B*07:02 | 23 |
| $BOB1_{44}$ | APAPTAVVL | B*07:02 | 15 |
| $BOB1_{14}$ | APARPYQGV | B*07:02 | 49 |

*netMHC server 3.4[19, 20]

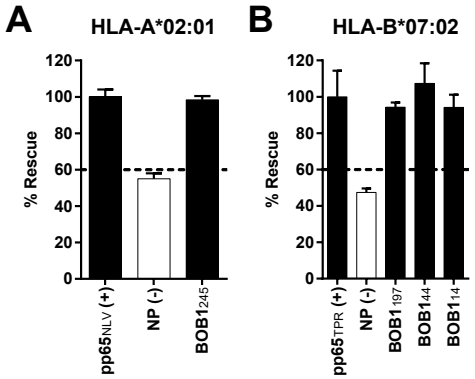
**Supplementary Table S2.** HLA typing of B-LCL panel used in this study (Adapted from van Loenen *et al.*[22])

| | HLA class I | | | HLA class II | | |
|---|---|---|---|---|---|---|
| **B-LCL** | **A** | **B** | **C** | **DR** | **DQ** | **DP** |
| JY | 2 | 7 | 7 | 4, 13 | 6, 7 | 1*02:01 |
| HHC | 2 | 7, 44 | 5, 7 | 4, 15 | 6, 7 | 1*04:01 |
| ALY | 2, 25 | 7, 41 | 7, 17 | 7, 13 | 6, 9 | 1*04:01, 1*14:01 |
| NGZ | 11, 24 | 53, 61 | 2 | 1, 11 | 5, 7 | 1*0201, 1*0301 |
| LMB | 29 | 44, 51 | 14, 16 | 7, 8 | 2, 4 | 1*0401, 1*110101 |
| MWX | 1, 34 | 15, 35 | 4, 12 | 1, 15 | 5, 6 | 1*0601, 1*1301 |
| ABV | 3, 29 | 7, 44 | 7 | 7, 14 | 5, 2 | 1*0401, 1*1101 |
| UKL* | 1, 30 | 8, 13 | 6, 7 | 4, 17 | 2, 7 | 1*0401 |
| AKB | 1, 2 | 37, 39 | 6, 7 | 1, 10 | 5 | 2*0102, 1*0401 |
| RBB* | 2, 26 | 8, 27 | 1, 7 | 17, 52 | 2 | 1*0401 |
| IZA | 2, 24 | 8, 60 | 3, 7 | 13, 17 | 2, 6 | 1*0401, 1*1401 |
| NGI | 11, 24 | 8, 39 | 7 | 17, 8 | 2, 4 | 1*0101, 1*1401 |
| AHT | 24, 25 | 55, 62 | 3 | 8, 15 | 5, 6 | 1*0401 |
| MHX | 1, 0205 | 18, 50 | 6, 7 | 7, 9 | 2, 9 | 1*0201, 1*0301 |
| CVV | 11, 31 | 57, 62 | 3, 6 | 4, 7 | 7, 9 | 1*0201, 1*0401 |
| UCE* | 3, 11 | 7, 27 | 2, 7 | 11, 14 | 5, 7 | 1*0201, 1*1601 |
| MBX | 1 | 8, 63 | 7 | 12, 17 | 2, 7 | 1*0101, 1*0401 |
| EBM | 23 | 64 | 8 | 4 | 8 | 1*0201 |
| FAQ | 23, 68 | 14, 38 | 8, 12 | 13 | 7, 6 | 1*0201 |
| LSR | 32, 68 | 35, 52 | 12 | 15, 16 | 5, 6 | 1*0401, 1*1401 |
| GGT | 26, 31 | 14, 49 | 7, 8 | 1, 7 | 2, 5 | 1*0402, 1*110101 |

*B-LCL not used in previous study and added in this panel.

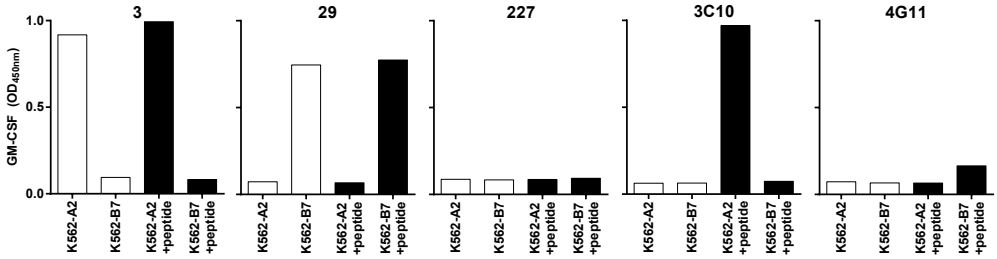



**Supplementary Figure S1. *POU2AF1* gene expression is restricted to the B cell compartment**. *POU2AF1* mRNA expression was measured using the Illumina HT-12 chip array platform. Shown are samples of healthy hematopoietic origin (blue dots), healthy B cells (green dots), B cell malignancies (red dots), myeloid leukemia (black dots) and samples of nonhematopoietic origin (open dots). Dots indicate individual samples. Vertical bars indicate mean with standard deviation. PBMC, peripheral blood mononuclear cells; BMMC, bone marrow mononuclear cells; Mø1 and Mø2, macrophages type 1 and 2; DC, dendritic cells; HPCs, hematopoietic progenitor cells; B-LCL, EBV-transformed lymphoblastic B cell lines; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; FB, fibroblasts; KC, keratinocytes; PTEC, proximal tubular epithelia cells; HUVEC, human umbilical cord epithelial cells; +IFNγ, cells were cultured in medium supplemented with IFNγ for 4 days.

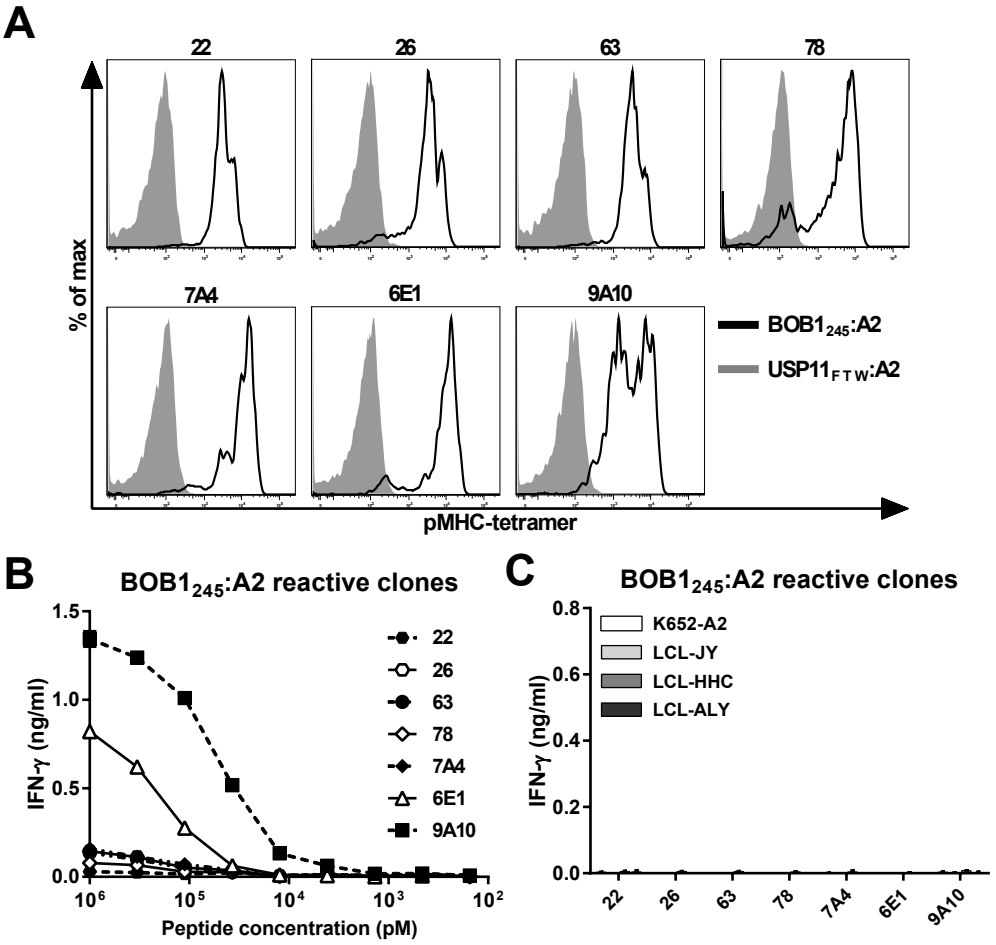



**Supplementary Figure S2. BOB1-derived peptides form stable peptide-MHC monomers**. Peptides of BOB1 were tested for their capacity to inhibit UV-mediated degradation of MHC monomers. (A-B) HLA-A*02:01 monomers (A) or HLA-B*07:02 monomers (B) loaded with UV-sensitive peptides were subjected to UV-light in the presence of indicated peptide or no peptide (NP). Rescue was calculated by normalizing mean fluorescence intensity (MFI) of indicated peptide to MFI of HLA-A*02:01 rescued in the presence of strong binding peptide $pp65_{NLV}$ for HLA-A2 binding peptides or MFI of HLA-B*07:02 in the presence of strong binding peptide $pp65_{TPR}$ for peptides binding to HLA-B7. Rescue for reference peptides $pp65_{NLV}$ and $pp65_{TPR}$ was set to one hundred. Dotted line at 60% indicates rescue threshold. Experiment was carried out in triplicate. Error bars indicate standard deviation.

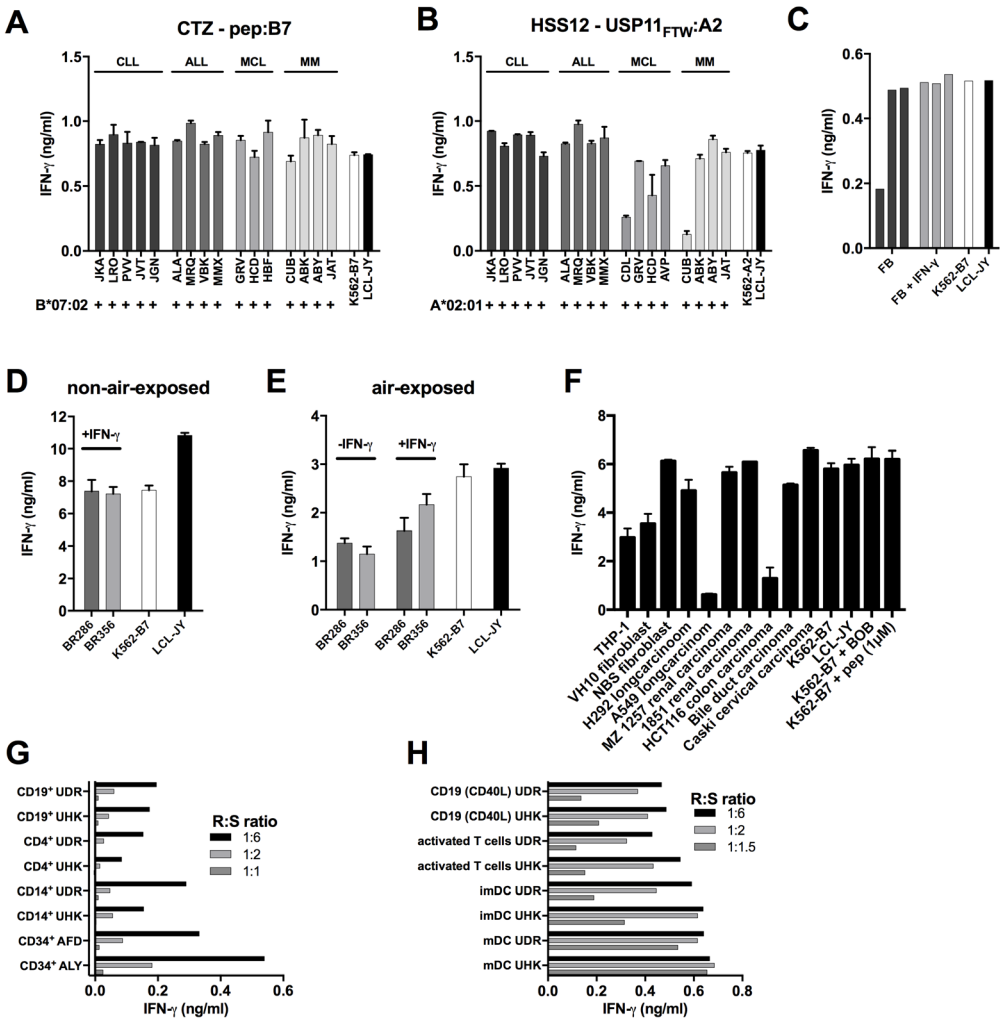




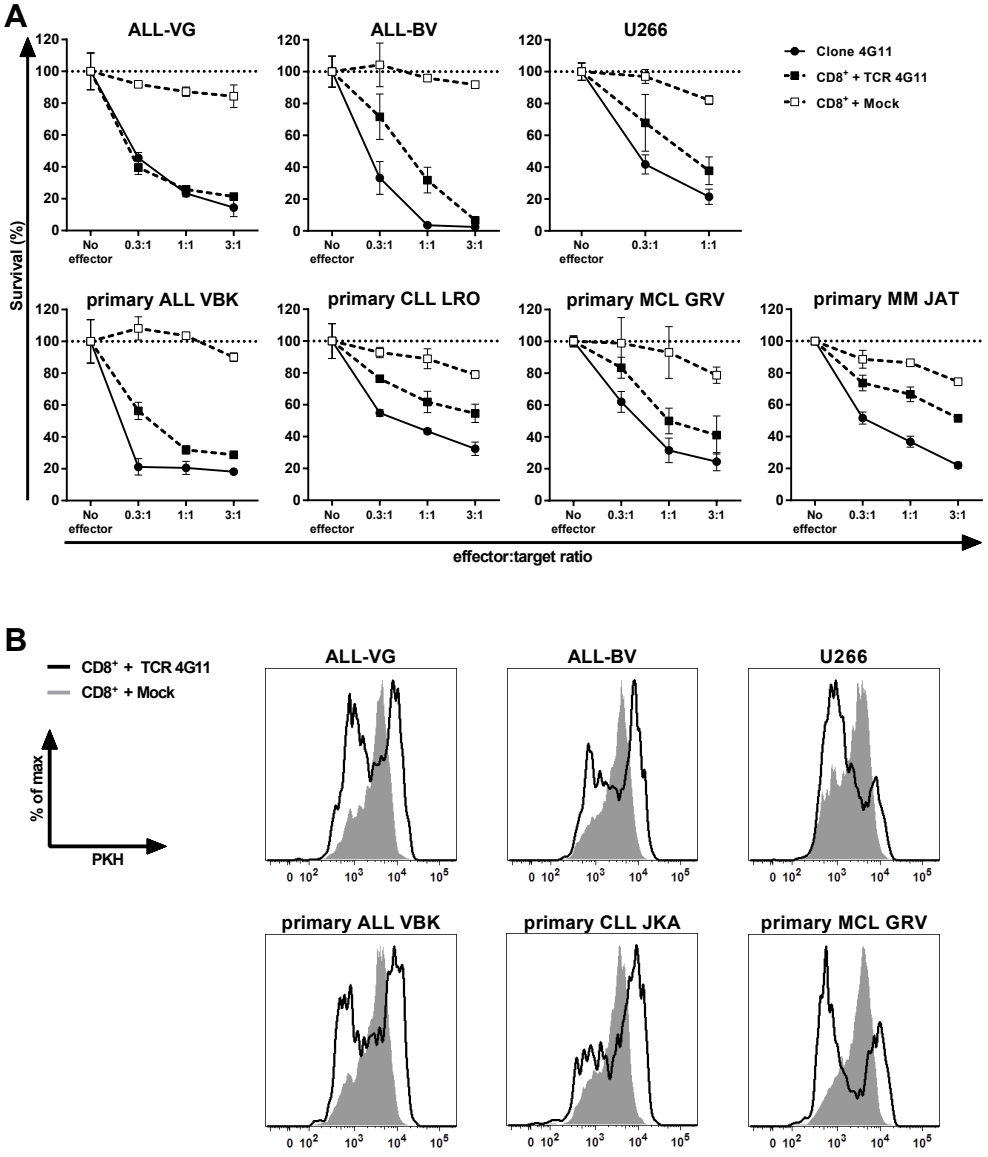

**Supplementary Figure S3. Reactivity profiles of clonally expanded T cells isolated using pMHC tetramers.** From HLA-$A2^{neg}/B7^{neg}$ healthy individuals, $CD8^{+}$ T cell clones were isolated using pMHC tetramers composed of BOB1-derived peptides bound to HLA-A*02:01 or HLA-B*07:02. T cell clones were stimulated with K562 cells transduced with HLA-A*02:01 (K562-A2) or HLA-B*07:02 (K562-B7) and either left unloaded (empty bars) or pulsed with 50 nM BOB1-derived peptides (+peptides; filled bars).



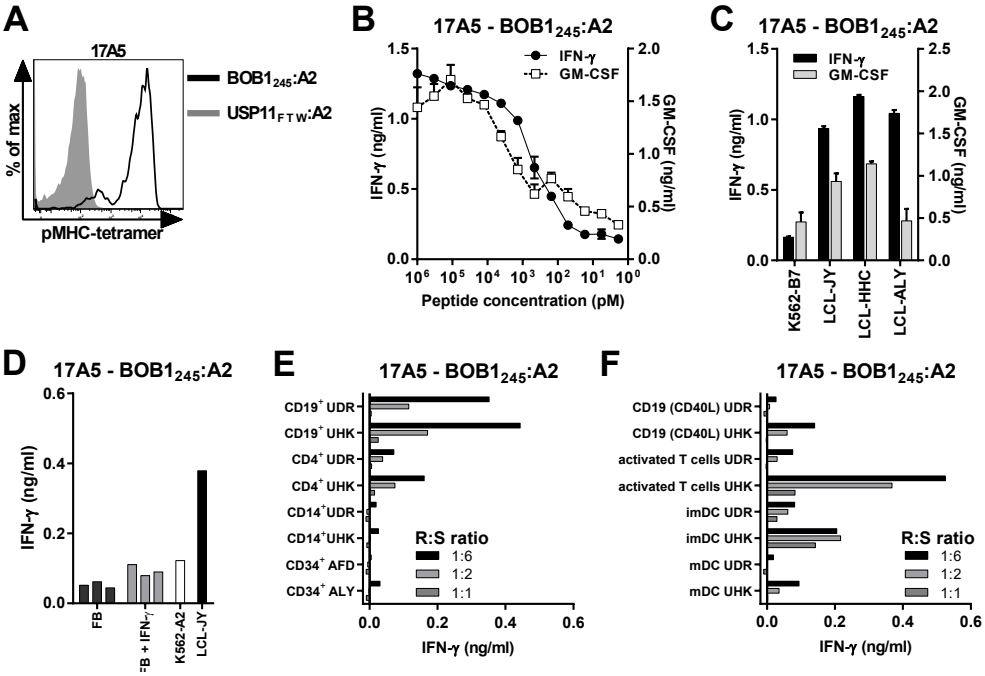

**Supplementary Figure S4. $BOB1_{245}$-reactive T cell clones exhibit varying degrees of peptide sensitivity and avidity.** BOB1-reactive T cell clones recognizing peptide $BOB1_{245}$ presented in HLA-A2 were assessed for peptide sensitivity by peptide titration and their capacity to recognize endogenously processed peptide. (A) Shown are histograms of T cell clones stained with pMHC tetramer $BOB1_{245}$:A2 (black line) or control pMHC tetramer composed of irrelevant USP11-derived peptide FTWEGLYNV bound to HLA-A2 ($USP11_{FTW}$:A2) (grey area). (B-C) BOB1-reactive T cell clones were coincubated with K562-A2 cells pulsed with titrated $BOB1_{245}$-peptide (B) or three BOB1-expressing HLA-$A2^{pos}$ B-LCLs (C). BOB1-negative K562-A2 cells were used as control. Shown are means with standard deviations of one representative experiment carried out in duplicate.




**Supplementary Figure S5. Stimulatory capacity of fibroblasts, primary and activated hematopoietic cells.** T cell clone CTZ recognizing a household gene-derived peptide presented in HLA-B7 and T cell clone HSS12 recognizing USP11-derived peptide FTWEGLYNV ($USP11_{FTW}$) presented in HLA-A2 were used to demonstrate the stimulatory capacity of cells used in stimulation assays belonging to Figure 3A,B and 3E-J. (A-B) Clone CTZ (A) and clone HSS12 were cocultured with primary samples of malignant B-cells including chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), mantle cell lymphoma (MCL) and multiple myeloma( MM). HLA allotype of each sample is indicated. Shown are means with standard deviations of one experiment carried out in duplicate. (C-H) Clone CTZ was co-cultured with fibroblasts (FB) either left untreated or cultured for four days in 200 IU/ml IFN-γ (FB + IFN-γ) before co-culture (C). Primary bronchial epithelial cells were cultured under non-air-exposed (D) or air-exposed condition (E) before being used as stimulator cells. Cells had also been cultured in the absence (-IFN-γ) or presence (+IFN-γ) of 100 IU/ml IFN-γ for two days before co-culture. (F) Clone CTZ was cocultured with HLA-$B7^{pos}$ BOB1-negative tumor cell lines. Shown are means with standard deviations of one experiment carried out in duplicate. (G-H) Primary $CD19^+$, $CD4^+$, $CD14^+$ and $CD34^+$ cells were isolated from PBMCs and cocultured in different responder:stimulator (R:S) ratios (G). Activated B cells (CD19 (CD40L)) were generated by stimulating $CD19^+$ B cells with CD40L. Activated T cells were generated from PHA-stimulated PBMCs. Immature (imDC) and mature (mDC) dendritic cells were monocyte derived (H).




**Supplementary Figure S6. TCR-transduced CD8⁺ T cells lyse primary B cell malignancies including multiple myeloma and proliferate upon antigen encounter.** The same T cells as in Figure 5-6 were used for this experiment. (A) T cell clone 4G11 or purified transduced CD8⁺ T cells were tested for their lytic capacity. HLA-B7$^{pos}$ PKH-labelled target cells were cocultured at various effector-to-target ratios with effector T cells. After 18 hours of coculture, the number of live targets cells was assessed by flow cytometry and percent survival was calculated. Malignant cell samples included ALL cell lines ALL-VG and ALL-BV, multiple myeloma cell line U266, primary multiple myeloma (MM), mantle cell lymphoma (MCL), acute lymphoblastic (ALL) and chronic lymphocytic leukemia (CLL). Shown are means with standard deviations of one experiment carried out in triplicate. (B) Purified transduced CD8⁺ T cells were tested for their proliferative capacity upon antigen encounter. PKH-labelled transduced CD8⁺ T cells were cocultured with irradiated HLA-B7$^{pos}$ stimulator cells. Histograms show TCR-transduced (black line) or mock-transduced (grey area) CD8⁺ T cells after 5 days of coculture. Stimulator cells included cell lines ALL-VG, ALL-BV and U266 and primary ALL, CLL and MCL.




**Supplementary Figure S7. Off-target recognition of isolated T cell clone 17A5.** T cell clone LJD 17A5 was isolated from an HLA-A2$^{neg}$/B7$^{neg}$ individual using pMHC tetramer BOB1$_{245}$:A2. (A) Shown is a histogram of T cell clone 17A5 stained with specific pMHC tetramer BOB1$_{245}$:A2 or irrelevant pMHC tetramer USP11$_{FTW}$:A2. (B-C) T cell clone 17A5 was cocultured with K562-A2 pulsed with titrated concentration of BOB1$_{245}$-peptide (B) or three BOB1-expressing HLA-A2$^{pos}$ B-LCLs (C). Shown are means with standard deviations of one experiment carried out in duplicate. (D-F) Clone 17A5 was cocultured with the same stimulator cells as in Figure 3E, 3H and 3I (D, E and F, respectively) at different responder-to-stimulator (R:S) ratios.