Leukemia (2014) **28**, 2355–2366

www.nature.com/leu

ORIGINAL ARTICLE

# Therapeutic targeting of naturally presented myeloperoxidase-derived HLA peptide ligands on myeloid leukemia cells by TCR-transgenic T cells

R Klar[1], S Schober[1], M Rami[1], S Mall[1], J Merl[2], SM Hauck[2], M Ueffing[2], A Admon[3], J Slotta-Huspenina[4], M Schwaiger[5], S Stevanović[6], RAJ Oostendorp[1], DH Busch[7,8], C Peschel[1,9] and AM Krackhardt[1,8,9]

T cells have been proven to be therapeutically effective in patients with relapsed leukemias, although target antigens on leukemic cells as well as T-cell receptors (TCRs), potentially recognizing those antigens, are mostly unknown. We have applied an immunopeptidomic approach and isolated human leukocyte antigen (HLA) ligands from primary leukemia cells. We identified a number of ligands derived from different genes that are restrictedly expressed in the hematopoietic system. We exemplarily selected myeloperoxidase (MPO) as a potential target and isolated a high-avidity TCR with specificity for a HLA-*B*07:02*-(HLA-B7)-restricted epitope of MPO in the single HLA-mismatched setting. T cells transgenic for this TCR demonstrated high peptide and antigen specificity as well as leukemia reactivity *in vitro* and *in vivo*. In contrast, no significant on- and off-target toxicity could be observed. In conclusion, we here demonstrate, exemplarily for MPO, that leukemia-derived HLA ligands can be selected for specific effector tool development to redirect T cells to be used for graft manipulation or adoptive T-cell therapies in diverse transplant settings. This approach can be extended to other HLA ligands and HLA molecules in order to provide better treatment options for this life-threatening disease.

*Leukemia* (2014) **28,** 2355–2366; doi:10.1038/leu.2014.131

## INTRODUCTION

Myeloid neoplasias are often very aggressive and difficult-to-treat diseases, with high morbidity and mortality. Remarkably, these diseases can be targeted and often eradicated by T cells. A direct proof of principle of the efficacy of T cells in these diseases are the durable remissions induced by the administration of donor lymphocyte infusions (DLI) to patients with acute and chronic myeloid leukemia (AML and CML, respectively) who have relapsed after allogeneic stem cell transplantation (SCT).[1] Those graft versus leukemia (GvL) effects are, however, unpredictable and often associated with graft versus host disease (GvHD), and currently little is known about how to target favorable structures for a GvL effect without GvHD. The advent of technology to redirect T cells through transgenic T-cell receptor (TCR) expression to defined leukemia antigens offers new opportunities for manipulating the GvL effect. This highlights the importance of defining suitable targets on leukemic cells in order to provide leukemia-reactive T cells for patients lacking GvL effects or without the option of allogeneic SCT.

Potential T-cell targets can be directly identified by analyzing the immunopeptidome of tumor cells.[2,3] Those peptides that have been eluted from tumor cells can be investigated for tumor specificity and immunogenicity and have been previously successfully used for peptide vaccination in patients with solid tumors.[4] Elution of peptides from leukemia cells has also been previously performed,[5] and eluted peptide mixtures were shown to be capable of inducing potent anti-leukemia effects in mice.[6] However, there is no information as to which peptides may serve as defined target antigens for T cells or characterization of TCR that might be used for adoptive T-cell therapies in humans.

Here we used leukemia samples from patients with diverse myeloproliferative neoplasias (MPN) and applied the immunopeptidomic approach to identify potential candidate epitopes for adoptive T-cell therapies using defined tumor-specific TCR. Our studies identified several human leukocyte antigen (HLA) ligands derived from genes that are restrictedly expressed in the hematopoietic system. One of these genes was myeloperoxidase (MPO) representing an essential and distinct marker for myeloid cells and AML.[7] We were subsequently able to isolate a T-cell clone and its TCR from the naive T-cell repertoire of an HLA-B7-negative donor with specificity against one selected HLA-B7-restricted MPO epitope. T cells transgenic for this TCR recognized $MPO^+$ leukemic cell lines and $MPO^+$ primary leukemic cells from patients with AML and MPN *in vitro*. Moreover, data also demonstrate efficacy *in vivo*. Extended investigation of potential on- and off-target toxicity suggests a favorable recognition profile as non-myeloid cells and healthy $CD34^+$ hematopoietic stem cells (HSC) were not recognized. By isolation and combination of more TCR specific for isolated HLA ligands derived from MPO or other genes restrictedly expressed in hematopoietic cells, this approach may be the base

[1]Medizinische Klinik III, Klinikum rechts der Isar, Technische Universität München, München, Germany; [2]Research Unit Protein Science, Helmholtz Zentrum München (GmbH), German Research Center for Environmental Health, Neuherberg, Germany; [3]Department of Biology, Technion Israel Institute of Technology, Haifa, Israel; [4]Institut für Allgemeine Pathologie und Pathologische Anatomie, Klinikum rechts der Isar, Technische Universität München, München, Germany; [5]Nuklearmedizinische Klinik, Klinikum rechts der Isar, Technische Universität München, München, Germany; [6]Department of Immunology, University of Tübingen, Tübingen, Germany; [7]Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, München, Germany; [8]Clinical Cooperation Group, Antigen Specific T Cell Therapy, Helmholtz Zentrum München (GmbH), German Center for Environmental Health, München, Germany and [9]German Cancer Consortium (DKTK), Munich, Germany. Correspondence: Professor AM Krackhardt, Medizinische Klinik III, Klinikum rechts der Isar, Technische Universität München, Ismaningerstr. 22, München 81675, Germany.
E-mail: angela.krackhardt@lrz.tu-muenchen.de
Received 22 October 2013; revised 1 April 2014; accepted 3 April 2014; accepted article preview online 16 April 2014; advance online publication, 13 May 2014

for a personalized therapeutic option for patients with relapsing leukemias.

## MATERIALS AND METHODS

### Primary material and cell lines

Blood from healthy individuals and patients with diverse hematopoietic malignancies as well as human healthy organ tissue samples obtained from diagnostic preparations were collected after informed consent following requirements of the local ethical board and principles of the Helsinki Declaration. Patient characteristics are shown in Supplementary Table S1. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation using Ficoll/Hypaque (Biochrom, Berlin, Germany). Granulocytes were purified from whole blood by differential centrifugation as described previously.[8] All target cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with Penicillin/Streptomycin (PAA, Pasching, Austria) and 10% fetal calf serum (Invitrogen) unless otherwise stated. Details regarding isolation of naive T cells and $T_{CM}$ as well as the cell lines used in the present study can be found in Supplementary Methods and Supplementary Table S2. Transduction of cell lines with HLA-B7-P2A-eGFP is indicated by the additional specification '-B7' to the name of the cell line; transduction with MPO-P2A-dsRed by the additional specification '/MPO'. For functional *in vitro* assays, CML and AML samples as well as non-lymphoid cell lines were treated as described in Supplementary Methods. Primary human renal glomerular epithelial cells (HRGEC) were purchased from ScienceCell (Carlsbad, CA, USA) and cultured according to the manufacturer's instructions. The hybridoma cell line HB-95 (ATCC, Manassas, VA, USA) was used for in-house production of the w6/32 antibody following the standard protocols.

### Immunopeptidomic identification of leukemia-derived HLA ligands

HLA I-restricted peptides were identified and analyzed by the immunopeptidomic approach as previously described.[5,9] After HLA class I immunoprecipitation and peptide elution, peptides were analyzed by nano-HPLC (Ultimate 3000, Dionex, Sunnyvale, CA, USA) coupled to a linear quadruple ion trap-Orbitrap (LTQ Orbitrap XL) mass spectrometer (Thermo Fisher, Waltham, MA, USA) equipped with a nano-electrospray ionization source as previously described,[10] allowing for the fragmentation of peptides with charges $+1$ to $+3$.

MS/MS (tandem mass spectrometry) data were analyzed using the software tools MASCOT, Peaks and Pep-Miner, and potential HLA ligands were further identified and selected as described in Supplementary Methods. To screen for HLA ligands derived from genes with suitable expression patterns for T-cell targeting, gene expression was determined by database and literature research (http://genome.ucsc.edu/cgi-bin/hgBlat, http://www.biogps.org, http://www.ncbi.nlm.nih.gov/pubmed/). For sequence validation of selected candidate ligands, synthetic counterparts (IBA, Goettingen, Germany) were analyzed by mass spectrometry. After normalization, spectra were matched using mMass.[11]

### MPO expression analysis by semi-quantitative real-time PCR (qPCR), immunohistochemistry and western blotting

To verify the expression profile of MPO in primary cells as well as diverse human tissues, qPCR, immunohistochemistry and western blotting were conducted as described in Supplementary Methods.

### Expansion of T cells specific for a HLA-B7-restricted MPO epitope

To expand T cells with specificity for the selected HLA ligands, we used a single HLA-mismatched priming approach as previously published,[12] with some modifications (Supplementary Figure S1 and Supplementary Methods). The HLA type of the donor is: HLA-*A*02/—*; *HLA-B*15/39*. Expanded T cells were analyzed and sorted on day 20 using HLA multimers on a high-performance cell sorter (MoFlo; Dako, Glostrup, Denmark) and cloned by limiting dilution.

### HLA multimers and antibodies

HLA multimers were synthesized as previously reported.[13] Antibodies used in the present study are listed in Supplementary Table S3.

### Retroviral TCR transfer into lymphocytes

The sequence of the reactive TCR2.5D6 was determined by PCR and sequencing as previously described.[14,15] A bi-cistronic construct separated by a P2A element consisting of beta and alpha chains containing murinized constant chains and an additional disulphide bridge was generated *in silico* and codon-optimized (Invitrogen) as previously described.[16–18] The construct was cloned into the retroviral vector pMP71, retroviral supernatants were generated and lymphocytes or $CD8^+T_{CM}$ were transduced as previously described.[15,19]

### Functional characterization of T-cell clones as well as TCR-transgenic T cells

T cells were tested for their cytotoxic reactivity against $^{51}$Cr-labeled target cells in a standard 4-h $^{51}$Cr-release assay[14] or by a flow cytometry-based cytotoxic assay. For cytokine detection, effector and target cells were incubated at a ratio of 2:1 for 24 h. Supernatants were collected and cytokine levels were measured using interferon (IFN)-$\gamma$ enzyme-linked immunosorbent assay (ELISA) (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Alanine- and threonine-variants of the $MPO_5$ peptide (Genscript, Piscataway, NJ, USA) were used to define the residues critical for recognition by TCR2.5D6 as described previously.[20] The ScanProsite tool was used for identification of proteins that contain the motif 'XPRWDXXRL' as described previously.[21] HLA-B7 ligands analyzed for off-target reactivity were synthesized on an automated peptide synthesizer EPS221 (Abimed, Langenfeld, Germany) following the 9-fluorenylmethyl-oxycarbonyl/tertbutyl (Fmoc/tBu) strategy as described.[22]

### Establishment of human acute leukemia cells in an immunocompromized mouse model

C.Cg-*Rag2*$^{tm1Fwa}$ *Il2rg*$^{tm1Sug}$/JicTac mice on a BALB/c background (BRG) were purchased from Taconic (Germantown, NY, USA) and maintained in our animal facility according to the institutional guidelines and approval by local authorities. Mice were sublethally irradiated with 3.5 Gy on day 0 and $1 \times 10^6$ NB4-B7 cells were injected intravenously the day after. A schematic overview of the experimental setup is shown in Supplementary Figure S2A. Furthermore, Supplementary Figure S2B shows the typical manifestation of the disease in the lymph nodes of a killed animal (day 30). To provide additional information on tumor manifestations of this specific model, [$^{18}$F]-2-fluor-2-desoxy-D-glucose positron emission tomography analysis (see also Supplementary Methods) of a control mouse or a tumor-bearing mouse 30 days after injection of $1 \times 10^6$ NB4-B7 cells is shown in Supplementary Figure S2Ci and Movie S1 and Supplementary Figure S2Cii and Movie S2, respectively. The typical histopathology of resected tumors with positivity for MPO and human CD45 is depicted in Supplementary Figure S2D.

### Investigation of *in vivo* anti-leukemic reactivity of TCR2.5D6-transduced T cells

One day after tumor cell inoculation, mice received either 200 µl phosphate-buffered saline (PBS), $1 \times 10^7$ untransduced or $1 \times 10^7$ TCR2.5D6-transduced $CD8^+T_{CM}$ intravenously. To provide a systemic supply of human IL-15, $1.5 \times 10^7$, irradiated NSO-IL-15 cells (kindly provided by Stanley Riddell) were injected intraperitoneally twice per week as previously described.[23] Diseased mice (Criteria: Supplementary Methods) were killed and analyzed for tumor manifestations and T-cell engraftment by flow cytometry after organ collection and preparation of single-cell suspensions.

### Hematopoietic colony-forming assays

All hematopoietic progenitor assays were performed according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, BC, Canada). PBMC samples containing a total of 500 $CD34^+$ cells were co-incubated with either TCR2.5D6-transduced or untransduced lymphocytes. Incubation was performed in 250 µl alpha Minimum Essential Medium (PAA) supplemented with 2% fetal calf serum at different effector-to-target ratios for 15 min or 1 h at 37 °C. Subsequently, cell suspensions were mixed with 2.5 ml MethoCult H4435 and plated in duplicates of 1.1 ml in 35-mm petri dishes. After 12–14 days of incubation at 37 °C/5% $CO_2$ in a humidified atmosphere, plates were scored for colony growth under a light microscope.

## RESULTS

### Identification of leukemia-derived HLA ligands and selection of MPO as potential T-cell target

In order to identify novel target antigens expressed and presented by myeloid leukemias that could be targeted by T cells, we analyzed

the immunopeptidome of malignant cells obtained from patients with MPN. We identified, by mass spectrometry, 4386 unique peptides with adequate peptide length and proper anchor residues to serve as potential ligands of diverse HLA class I molecules. These peptides were then analyzed for their suitability as potential target structures for T-cell mediated anti-tumor immunotherapies. Nineteen of those peptides are derived from seven genes with presumably restricted expression to hematopoietic cells (Table 1). Interestingly, we detected, by three different data analysis tools, five naturally presented MPO-derived HLA ligands, which were restricted to four different HLA alleles, namely, HLA-*A*01:01*, HLA-*B*07:02*, HLA-*B*15:01* and HLA-*B*44:02* (Table 2). For sequence validation of MPO-derived HLA ligands, defined peptides were synthesized and reanalyzed by mass spectrometry. Resulting spectra were compared with the spectra of the naturally eluted counterparts validating four of the five peptides by highly overlapping fragmentation patterns (Figure 1a). For confirmation of restricted expression of MPO to myeloid cells, we performed qPCR and immunohistochemistry. High MPO expression was observed in most MPN and AML leukemia samples as well as fetal liver and healthy bone marrow. We observed very low MPO gene expression in mature PBMC subpopulations, including $CD14^+$ monocytes and granulocytes as well as solid organ tissues (Figure 1b). Immunohistochemistry was performed on healthy organ tissues demonstrating MPO protein expression only in single myeloid cells within diverse organs with the exception of bone marrow, where expression could be detected in myeloid progenitor cells (Figure 1c).

### Expansion of T cells specific for the HLA-B7-restricted peptide $MPO_5$ and confirmation of natural presentation of the epitope $MPO_5$

As HLA-B7 is a frequent phenotype,[24] we further focused on the two MPO-derived peptides presented by HLA-B7 ($MPO_2$ and $MPO_5$).

**Table 1.** Identified HLA ligands derived from genes with restricted expression to the hematopoietic system

| *Gene* | *Number of HLA ligands* | *HLA restriction* |
|---|---|---|
| *ELANE* | 1 | *B*15:01* |
| *HMHA1* | 1 | *B*07:02* |
| | 1 | *B*15:01* |
| *ITGA2B* | 1 | *A*03:01* |
| | 1 | *B*15:01* |
| *LAT2* | 1 | *A*01:01* |
| | 1 | *A*03:01* |
| *MS4A3* | 1 | *B*07:02* |
| | 1 | *A*03:01* |
| *MPO* | 1 | *A*01:01* |
| | 2 | *B*07:02* |
| | 1 | *B*15:01* |
| | 1 | *B*44:02* |
| *MYB* | 3 | *B*07:02* |
| | 1 | *B*15:01* |
| | 1 | *B*18:01* |

Abbreviation: HLA, human leukocyte antigen.

We determined whether high-affinity T cells specific for these two MPO peptides presented by HLA-B7 could be isolated in the single HLA-mismatched setting. The experimental procedure for priming of naive T cells is shown in Supplementary Figure S1. Staining of proliferated cells after restimulation on day 20 with an HLA multimer of $MPO_5$ demonstrated a small population of peptide-specific T cells (Figure 2a). The $MPO_5$ HLA multimer$^+$ T cells were sorted and cloned by limiting dilution. The resulting T-cell clones were tested for their cytotoxic activity against T2-B7 cells, pulsed with $MPO_5$ or an irrelevant peptide. Five clones were selected that only lysed T2-B7 cells pulsed with $MPO_5$ (Figure 2b). TCR analysis revealed that all five T-cell clones expressed the same Vα and Vβ chains, namely Vα1.1 and Vβ13.2. Murinized and codon-optimized TCR Vα and Vβ sequences (termed TCR2.5D6) were cloned into the retroviral vector pMP71 and transduced into lymphocytes of a healthy donor. Multimer binding as well as functionality after TCR-gene transfer was confirmed (Figures 2c and d). Functional avidity of the TCR was tested using T2-B7 cells pulsed with serial dilutions of $MPO_5$. As shown in Figure 2e, half maximal secretion of IFN-γ was observed at concentrations of 150 pM. Specific recognition of naturally processed and presented $MPO_5$ was analyzed using diverse B-cell lines originally negative for MPO. In fact, C1R-B7 and BJAB-B7 cells were recognized when additionally transduced with the MPO gene verifying recognition of endogenously processed peptide (Figure 2f).

### Reactivity of TCR2.5D6-transduced lymphocytes in response to MPO-expressing leukemia cell lines as well as primary leukemia samples

Reactivity of TCR2.5D6-transduced lymphocytes against different leukemia cell lines and primary leukemia samples was assessed by IFN-γ ELISA. As shown in Figure 3a, only leukemia cell lines that show expression of MPO (Supplementary Figures S3A and S3B) were recognized by TCR2.5D6-transduced lymphocytes. MPO-negative cell lines were only recognized when pulsed with $MPO_5$, demonstrating principal vulnerability of these cell lines by the defined TCR. No recognition by untransduced lymphocytes was observed.

We further analyzed IFN-γ production by TCR2.5D6-transduced lymphocytes in response to primary samples from patients with MPN and AML (Figure 3b). In contrast to healthy mature myeloid cells, leukemia cells demonstrate partially high MPO expression on both mRNA and protein level (Figure 1b and Supplementary Figures S3C and S3D). Reactivity of TCR2.5D6-transduced lymphocytes could only be observed against target cells expressing both HLA-B7 and MPO (MPN1, MPN2, MPN5, MPN6 and AML6). The MPO-positive but HLA-B7-negative sample MPN3 and the MPO-negative, HLA-*B*07:04*$^+$ sample AML2 were not recognized. Pulsing of AML2 with $MPO_5$ resulted in recognition of the sample by TCR2.5D6-transduced lymphocytes, indicating a potential binding of $MPO_5$ also to the HLA-B7 subtype *B*07:04*.

To analyze the reactivity of TCR2.5D6 against colony-forming leukemic precursor cells, we incubated either untransduced or

**Table 2.** Identified naturally presented HLA class I ligands of MPO

| *Ligand* | *Sequence* | *HLA restriction* | *Patient sample* | *Pep-Miner*[a] | *Mascot*[b] | *Peaks*[c] | *Validation*[d] |
|---|---|---|---|---|---|---|---|
| $MPO_1$ | EEAKQLVDKAY | *B*44:02* | MPN6 | √ | | √ | |
| $MPO_2$ | TPAQLNVL | *B*07:02* | MPN1,5 | | | √ | √ |
| $MPO_3$ | NQINALTSF | *B*15:01* | MPN2 | √ | √ | | √ |
| $MPO_4$ | FVDASMVY | *A*01:01* | MPN3,4,5,7 | √ | | √ | √ |
| $MPO_5$ | NPRWDGERL | *B*07:02* | MPN2,5 | | √ | √ | √ |

Abbreviations: HLA, human leukocyte antigen; MPO, myeloperoxidase. [a]Score ⩾ 70. [b]Score ⩾ 30. [c] – LogP10 ⩾ 15. [d]Validation by overlapping fragmentation patterns with synthetic counterparts.

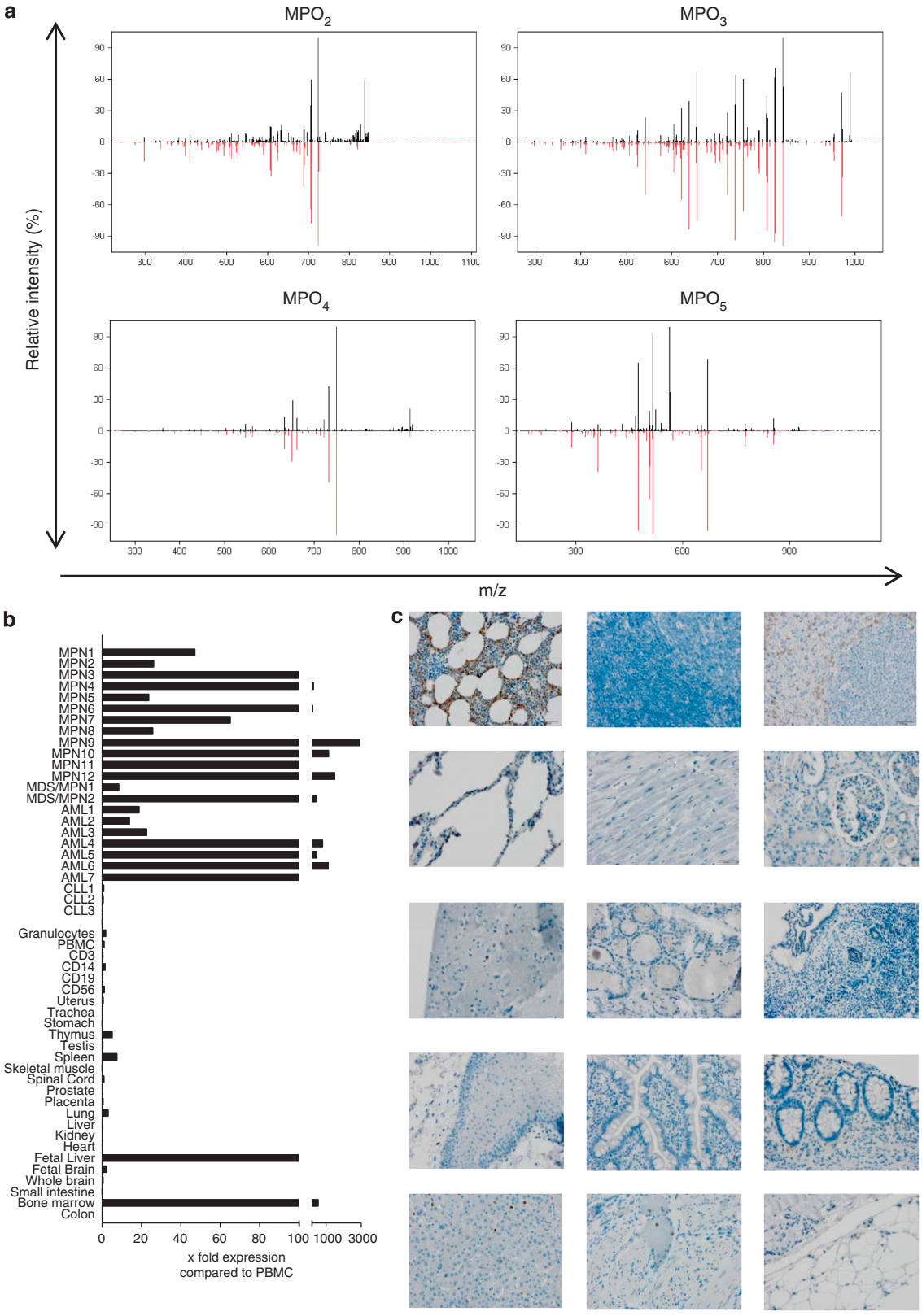


**Figure 1.** Selecting MPO as potential T-cell target antigen in myeloid leukemia. (**a**) MPO-derived HLA ligands identified by the immunopeptidomic approach were synthesized, sequenced by mass spectrometry and matched to the spectra of eluted peptides after normalization with the mMass tool for sequence validation. Spectra of synthetic peptides are shown in red (lower) and spectra of eluted peptides in black (upper). (**b**) mRNA expression of MPO in relation to PBMC was analyzed by qPCR on primary material and diverse healthy tissues. Ct values were normalized against the geometric mean of three housekeeping genes (GAPDH, HPRT1, HMBS) using the ddCT method. (**c**) MPO immunohistochemistry in diverse healthy tissue samples (from left to right and top to bottom): bone marrow, lymph node, spleen, lung, heart, kidney, brain, thyroid gland, endometrium, esophagus, intestinal mucosa, colon mucosa, liver, vessels, soft tissue.

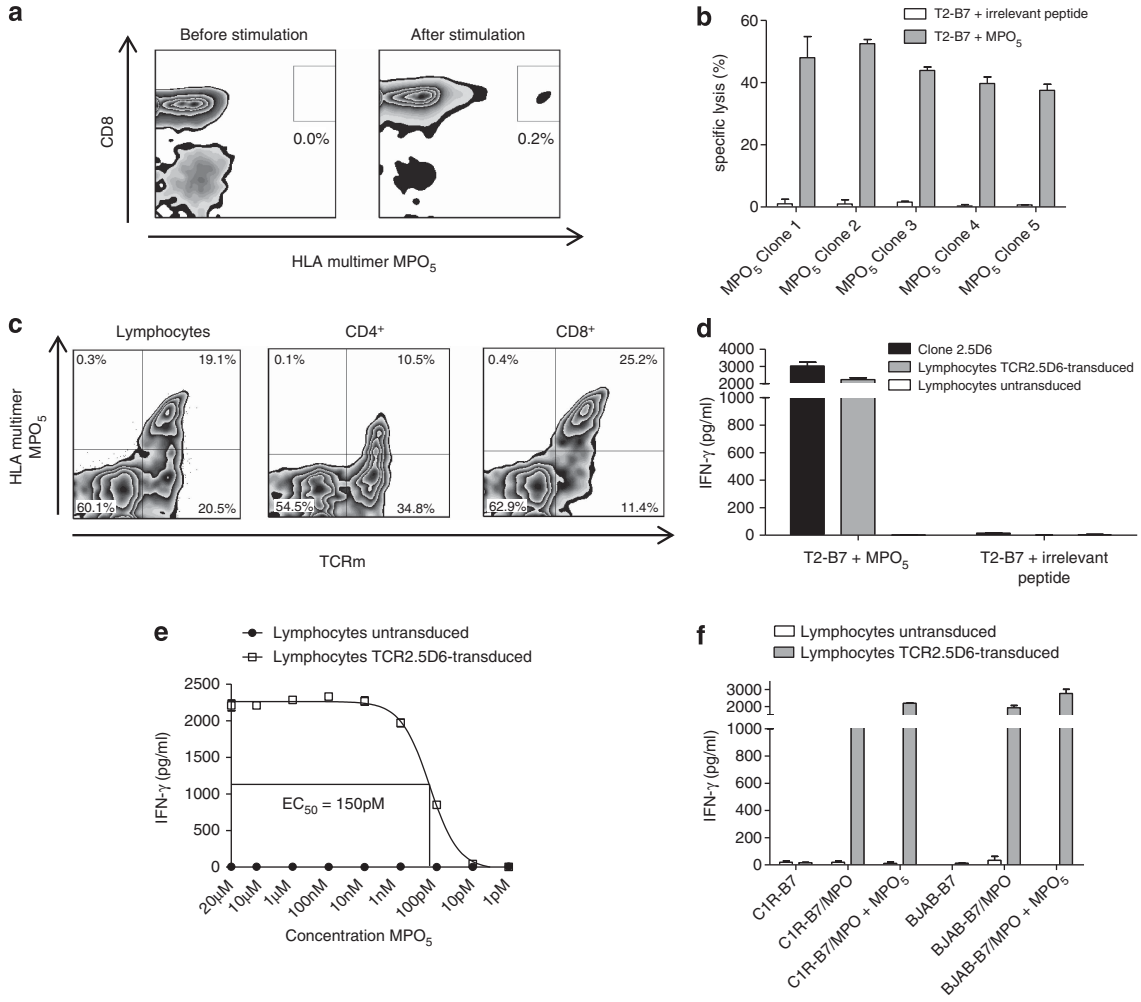


**Figure 2.** Expansion of T cells specific for the HLA-B7-restricted peptide $MPO_5$. (**a**) $MPO_5$ peptide-specific T cells were expanded after stimulation of naive T cells from an HLA-B7 negative donor with peptide-pulsed (0.1 μM) single HLA-mismatched DC. Peptide specificity of expanded T cells was investigated with an $MPO_5$-specific HLA multimer by flow cytometry. (**b**) Peptide-specific, lytic capacity of sorted and cloned T cells at estimated effector-to-target ratios (25:1) was tested by $^{51}Cr$ assay (the mean of duplicates is shown). (**c**) Stimulated peripheral lymphocytes from a healthy donor were transduced with TCR2.5D6 and stained with anti-CD4, anti-CD8, the $MPO_5$-specific HLA multimer and the TCRm antibody, which is directed against the murinized constant region of the transgenic TCR. Binding of $MPO_5$-specific HLA multimer and expression of TCRm is shown in total lymphocytes as well as T-cell subpopulations positive for CD4 and CD8. (**d**) For functional characterization, transduced lymphocytes were co-incubated for 24 h with peptide-pulsed T2-B7 cells at an effector-to-target ratio of 2:1. Supernatants were collected and IFN-γ secretion was measured by ELISA (s.d. of the mean of triplicates is shown). (**e**) For characterization of the functional avidity of the TCR2.5D6, transduced lymphocytes were co-incubated with T2-B7 cells pulsed with graded amounts of $MPO_5$ at effector-to-target ratios of 2:1. Supernatants were collected 24 h later, and IFN-γ secretion was measured by ELISA (s.d. of the mean of triplicates is shown). (**f**) Antigen-specific IFN-γ release by TCR2.5D6-transduced lymphocytes was determined in response to the MPO-negative cell lines C1R and BJAB, transduced with HLA-B7 alone or HLA-B7 and MPO, respectively. Cell lines transduced with both constructs were additionally pulsed with $MPO_5$ as positive control; untransduced lymphocytes were used as negative control (s.d. of the mean of triplicates is shown). One representative experiment out of at least three is shown in panels (**c**–**f**).

TCR2.5D6-transduced lymphocytes with bulk PBMC of the HLA-B7-positive patient MPN2 at different effector-to-target ratios. We observed a decrease in colony-forming units (CFU), when cells of MPN2 were incubated with TCR2.5D6-transduced lymphocytes at effector-to-target ratios of 100:1 and 1000:1 corresponding to an effective ratio of 20:1 and 200:1, respectively, for TCR2.5D6-transduced T cells (Figure 3c), whereas no reduction was detectable when cells were incubated with untransduced lymphocytes. As a control, we incubated untransduced and TCR2.5D6-transduced lymphocytes with cells of patient MPN3 and did not observe a difference in the number of CFU (Figure 3d). Taken together, these data demonstrate strong $MPO_5$-specific reactivity of TCR2.5D6-transduced lymphocytes against leukemic cells, including leukemic precursors *in vitro*.

### TCR2.5D6-transduced $CD8^+ T_{CM}$ increase survival in a xenogeneic mouse model of human AML

*In vivo* tumor reactivity of TCR2.5D6-transduced $CD8^+ T_{CM}$ was analyzed in a xenogeneic mouse model of human AML using NB4 cells[25] (See Material and Methods section and Supplementary Information). We injected $1 \times 10^6$ NB4-B7 cells into BRG mice, followed by injection of $10 \times 10^6$ untransduced or TCR2.5D6-transduced $CD8^+ T_{CM}$ or PBS alone. TCR expression and functionality of injected T cells before application is shown in Supplementary Figure S4. Mice were killed when suffering from health problems as described in Supplementary Methods. At end point analysis, infiltration of bone marrow with $CD45^+CD8^-$ tumor cells was significantly reduced in mice that were treated with TCR2.5D6-transgenic $T_{CM}$ compared with the untreated

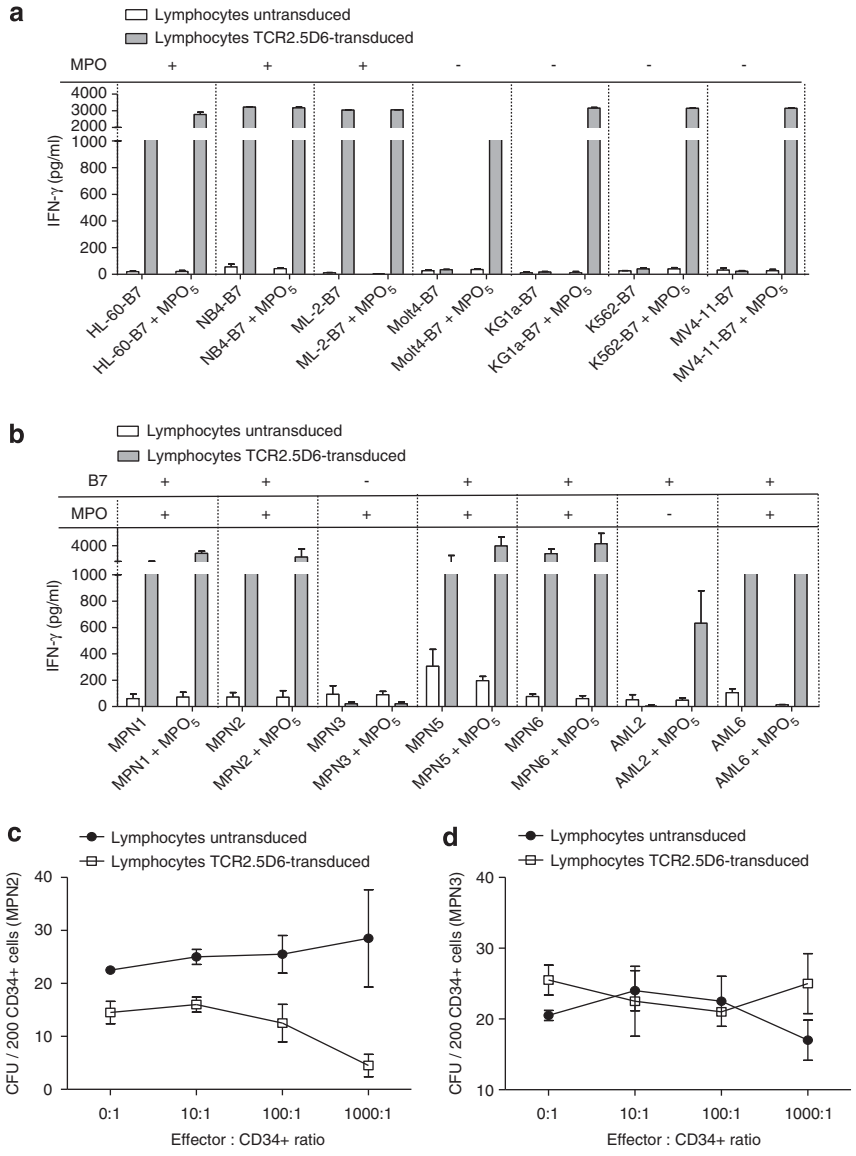


**Figure 3.** Reactivity of TCR2.5D6-transduced lymphocytes in response to leukemic cell lines and primary leukemia samples. Lymphocytes either untransduced or transduced with the TCR2.5D6 (transduction rate: 19%, Figure 2c), were stimulated with target cells for 24 h at an effector-to-target ratio of 2:1. Supernatants were collected, and IFN-$\gamma$ secretion was analyzed by ELISA. S.d. of the mean of triplicates is shown. (**a**) Myeloid leukemia cell lines with different MPO expression (Supplementary Figures S3A and S3B) were transduced with the retroviral construct HLA-B7-P2A-eGFP. Cell lines were additionally pulsed with $MPO_5$ at a concentration of 1 μM as positive controls. (**b**) Primary samples of patients with MPN and AML were pretreated as described in Supplementary Methods. MPN1, MPN2, MPN5, MPN6 and AML6 express MPO and HLA-B7, MPN3 expresses MPO but not HLA-B7 and AML2 is positive for HLA-*B*07:04* but does not express MPO (Supplementary Figures S3C and S3D). As a positive control, cells were pulsed with $MPO_5$ at a concentration of 1 μM. One representative out of at least two experiments is shown in panels (**a**) and (**b**). Reactivity of TCR2.5D6-transduced lymphocytes in response to colony-forming leukemic cells was analyzed by CFU assays. Therefore, $CD34^+$ cells (500 cells within a PBMC bulk population) of the leukemic sample MPN2 ($MPO^+$, HLA-$B7^+$) (**c**) and MPN3 ($MPO^+$, HLA-$B7^-$) (**d**) were incubated with untransduced (black circles) or TCR2.5D6-transduced (open squares) lymphocytes at different effector-to-target ratios for 15 min and plated in duplicates in methylcellulose medium. Colonies were counted 14 days later under a light microscope. The mean of duplicates in one representative out of two experiments is shown in panels (**c**) and (**d**).

group ($P = 0.0043$) as well as the group that received untransduced $T_{CM}$ ($P = 0.0247$) (Figure 4a; gating strategy: Supplementary Figure S5A). We could also observe a significant prolonged overall survival of mice treated with TCR2.5D6-transduced $CD8^+T_{CM}$ compared with mice that received untransduced $CD8^+T_{CM}$ ($P = 0.0048$), with median survival of 44 days versus 34 days, respectively. There was no significant difference in median survival between the control group that received no T cells at all compared with the group that was treated with untransduced $CD8^+T_{CM}$ (37 days versus 34 days, $P = 0.2229$; Figure 4b). All visible tumors from killed mice were resected, single-cell suspensions were obtained and pooled tumor cells from each individual mouse were analyzed for expression of human CD45 and enhanced green fluorescent protein (eGFP; gating strategy: Supplementary Figure S5B). Tumors consisted of $>95\%$ of $CD45^+CD8^-$ NB4 cells (Supplementary Figure S2D). Interestingly, we observed $CD45^+CD8^-$ tumors lacking eGFP in four out of five mice in the group that was treated with TCR2.5D6-transduced $CD8^+T_{CM}$ (Figure 4c). Concomitant loss of HLA-B7 was confirmed in one animal, whereas MPO expression was preserved (Supplementary Figure S6). One mouse in the TCR2.5D6-treated group did not develop tumors and was killed at the end of the

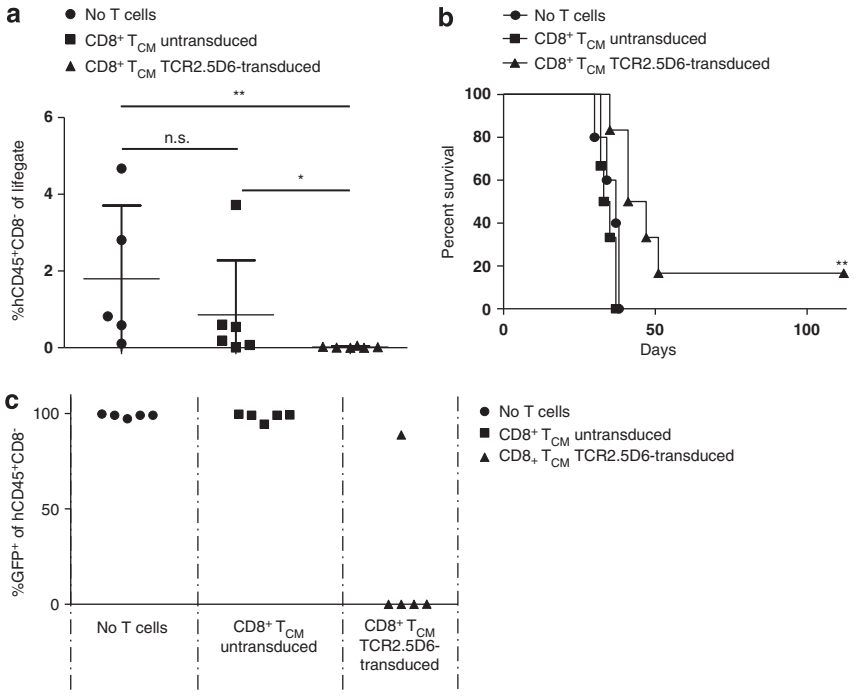


**Figure 4.** TCR2.5D6-transduced $CD8^+T_{CM}$ increase survival in a xenogeneic murine AML model. BRG mice were irradiated with 3.5 Gy and inoculated intravenously with $1 \times 10^6$ NB4-B7 leukemia cells 24 h later. T cells were transferred 24 h thereafter by intravenous injection of $10 \times 10^6$ untransduced ($n = 6$) or TCR2.5D6-transduced ($n = 6$) $CD8^+T_{CM}$ (Experimental design and tumor model: Supplementary Figure S2, Phenotype and functionality of transferred T cells: Supplementary Figure S4). Control mice ($n = 5$) received 200 µl PBS only. Continuous support of human IL-15 was provided by biweekly intraperitoneal injection of $15 \times 10^6$ IL-15-producing γ-irradiated NSO cells. (**a**) Infiltration by human $CD45^+CD8^-$ tumor cells in the bone marrow was analyzed using flow cytometry in diseased mice that had to be euthanized between days 30–51 (gating strategy: Supplementary Figure S5A). Statistical analysis was done by using Mann–Whitney test (*$P \leqslant 0.0247$, **$P = 0.0043$). Similar results have been observed in an additional *in vivo* experiment under inferior conditions (Supplementary Figure S8B). (**b**) Kaplan–Meier curves are shown for all three groups. Statistical analysis was done between animals receiving untransduced $CD8^+T_{CM}$ and $CD8^+T_{CM}$ transduced with TCR2.5D6 using the Mantel–Cox test (**$P = 0.0048$). A trend to increased survival has been observed when unselected lymphocytes (Figure 2c) were injected under inferior conditions (higher leukemia cell load, later T-cell transfer, Supplementary Figure S8C) (**c**) Visible tumors of diseased euthanized mice were resected and cells of each individual animal were pooled. The percentage of $CD45^+CD8^-eGFP^+$ cells was analyzed by flow cytometry (gating strategy: Supplementary Figure S5B). Loss of eGFP has not been observed in the alternative mouse experiment under inferior conditions (Supplementary Figure S8D). Of note, one mouse treated with untransduced T cells did not develop lymph node manifestations of tumor, but infiltration of the bone marrow was present.

experiment on day 112. In contrast, all animals treated with untransduced T cells or no T cells developed tumors with preserved eGFP expression (Figure 4c). As shown in Supplementary Figure S7, we could specifically detect human $TCRm^+$ T cells within the bone marrow, tumor, lung and spleen in the group that received TCR-transgenic $T_{CM}$. An additional experiment has been performed with clearly less favorable conditions as higher tumor load and later transfer of unselected lymphocytes with reduced TCR transduction rate. Treatment still resulted in statistically significant reduction of bone marrow infiltration by leukemic cells as well a non-statistically significant trend to improved survival. In these animals, loss of eGFP expression has not been observed (Supplementary Figure S8). Taken together, these mouse experiments demonstrate *in vivo* efficacy of TCR2.5D6-transduced $T_{CM}$ against leukemic cells with expression of both MPO and HLA-B7.

### On-target reactivity of lymphocytes transduced with the $MPO_5$-reactive TCR2.5D6

As it has been previously shown that on-target reactivity may induce unpredicted serious side effects,[26] we investigated potential reactivity of TCR2.5D6-transduced lymphocytes against a number of different target cells, including different $HLA\text{-}B7^+$ PBMC subsets. As shown in Figure 1 and Supplementary Figures S9A and S9B, granulocytes as well as monocytes demonstrate relevant MPO protein but very low mRNA expression. Regarding recognition by TCR2.5D6-transduced lymphocytes, IFN-γ production was only detected at very low levels in co-cultures of normal PBMC subsets with TCR2.5D6-transduced lymphocytes unless the target cells were pulsed with $MPO_5$ (Figure 5a). We additionally investigated the reactivity of TCR2.5D6-transduced lymphocytes against healthy, $CD34^+$ HSC of a $HLA\text{-}B7^+$ donor in CFU assays. As shown in Figure 5b, the potential for formation of CFU by granulocyte-colony-stimulating factor -mobilized HSC was not reduced when HSC were incubated for 1 h before plating in methylcellulose medium with TCR2.5D6-transduced lymphocytes compared with untransduced lymphocytes. Similar results were obtained when HSC were incubated for a longer period (24 h) with TCR2.5D6-transduced or untransduced lymphocytes (Supplementary Figure S10A). In contrast, pulsing of target cells with $MPO_5$ resulted in a strong reduction in CFU when incubated with TCR2.5D6-transduced but not untransduced lymphocytes (Figure 5c). These data indicate the absence of reactivity of TCR2.5D6 against mature myeloid cells as well as normal HSC.

### Off-target reactivity of lymphocytes transduced with the $MPO_5$-specific TCR2.5D6

Recently, fatal side effects due to unpredicted off-target reactivity have been observed after transfer of tumor-reactive transgenic T cells expressing an affinity-enhanced TCR.[21,27] Although TCR2.5D6

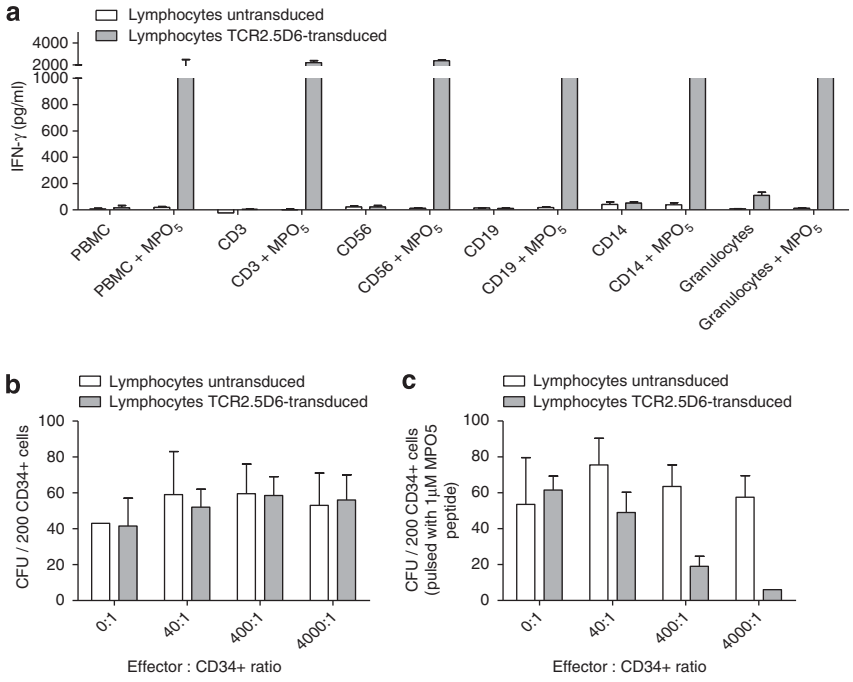


**Figure 5.** On-target reactivity of lymphocytes transduced with the TCR2.5D6. (**a**) Lymphocytes transduced with TCR2.5D6 (transduction rate: 19%, Figure 2c) were tested for potential on-target toxicity. IFN-γ secretion was measured by ELISA after stimulation with isolated $CD3^+$, $CD56^+$, $CD19^+$, $CD14^+$ cells and granulocytes of a HLA-B7-positive healthy donor (MPO expression data: Supplementary Figures S9A and B) at effector-to-target ratios of 2:1 for 24 h. As a positive control, cells were pulsed with 1 μM $MPO_5$. S.d. of the mean of triplicates in one representative out of three experiments is shown. Reactivity of TCR2.5D6-transduced lymphocytes against HLA-$B7^+$, $CD34^+$ HSC was assessed in CFU assays. $CD34^+$ cells (500 cells within a PBMC bulk population) were incubated at different effector-to-target ratios with untransduced or TCR2.5D6-transduced lymphocytes for 1 h (a similar experiment is shown in Supplementary Figure S10A with co-incubation for 24 h) (**b**). As a positive control, target cells were pulsed with 1 μM $MPO_5$ (**c**). Cells were plated in duplicates in methylcellulose medium. Colonies were counted 14 days later under a light microscope. The mean of duplicates is shown.

was not affinity-enhanced, we sought to screen for peptide-dependent or -independent recognition of diverse common HLA molecules. We therefore tested different lymphoblastoid cell lines (LCL) (Supplementary Table S2) either unpulsed or pulsed with $MPO_5$ (Figure 6a). LCL1 is HLA-$B7^+$ and served as positive control. We did not observe any reactivity against cell lines without pulsing of $MPO_5$. Following peptide pulsing, reactivity against LCL6 was observed. *In silico* analysis of binding motifs for HLA molecules expressed by LCL6 revealed potential binding of $MPO_5$ to HLA-*B*42:01* (HLA-B42). We therefore generated the cell lines C1R-B42 as well as C1R-B42/MPO. C1R cells were only recognized after transduction of both HLA-B42 and MPO, verifying on-target reactivity against $MPO_5$ also when presented by HLA-B42 (Figure 6b). We further tested off-target reactivity against different cell lines of non-hematopoietic origin as well as primary HRGEC not expressing MPO (Supplementary Figure S9C). As shown in Figure 6c, reactivity of TCR-transgenic lymphocytes could only be observed after pulsing of target cells with $MPO_5$, which further strengthens the high specificity of the TCR2.5D6. In order to investigate potential peptide promiscuity of our TCR,[20,21] we performed experiments with alanine- and threonine-variants of $MPO_5$ loaded onto the HLA-$B7^+$ cell line LCL1. As shown in Figure 6d, the residues at position 2, 3, 4, 5, 8 and 9 seem to be essential for recognition by TCR2.5D6. Similar results have been observed using two other HLA-$B7^+$ LCL (Supplementary Figures S10B and S10C). *In silico* analysis using the ScanProsite tool revealed MPO being the only human protein containing the motif 'XPRWDXXRL'. Finally, we tested crossreactivity against 65 peptides, divided into 6 pools, which have been eluted from HLA-B7 molecules. As shown in Figure 6e, no reactivity could be observed at all, except against $MPO_5$. Thus, off-target reactivity could not be observed by these experiments.

## DISCUSSION

The adoptive transfer of TCR-transgenic T cells that target HLA-A2-presented peptides derived from melanoma-associated differentiation antigens as GP100 and MART1 has proven to be clinically efficacious in a subset of patients with malignant melanoma.[26,28] Interestingly, therapeutic activity of TCR-transgenic T cells is not restricted to melanoma, as targeting a broader expressed cancer testis antigen such as NY-ESO-1 also demonstrated clinical efficacy in patients with sarcoma.[29] However, the choice of the target antigen, its affinity for major histocompatibility complex, as well as the specificity and affinity of the defined TCR are all important parameters to ensure selective tumor cell targeting sparing healthy tissue and to provide complete tumor eradication.[30,31]

Target antigens with restricted expression in hematopoietic cells may represent important recognition elements for the GvL effect in patients with leukemia undergoing hematopoietic SCT. However, there is limited knowledge regarding these potential antigens. Those that have been identified to date are mostly restricted to HLA-A2 and many show broader tissue expression than desired.[32,33] WT-1 may be a candidate target antigen in leukemia patients for TCR-transduced T cells,[34] and transfer of WT-1-specific T-cell clones after transplantation demonstrated some clinical activity without any signs of GvHD.[35] In addition, PR1, a peptide derived from neutrophil elastase and proteinase 3, has been proposed to be an attractive target in patients with CML and AML, and T cells transgenic for PR1-specific constructs are under investigation.[36,37] Peptides carrying tumor-related mutations, not present in healthy tissue, may be the ideal target for peptide-specific T cells.[38,39] However, these mutations and their HLA-restricted epitope presentation are often patient-specific and therefore not relevant for the majority of patients. Thus, in order to provide therapeutic tools for a broad patient population

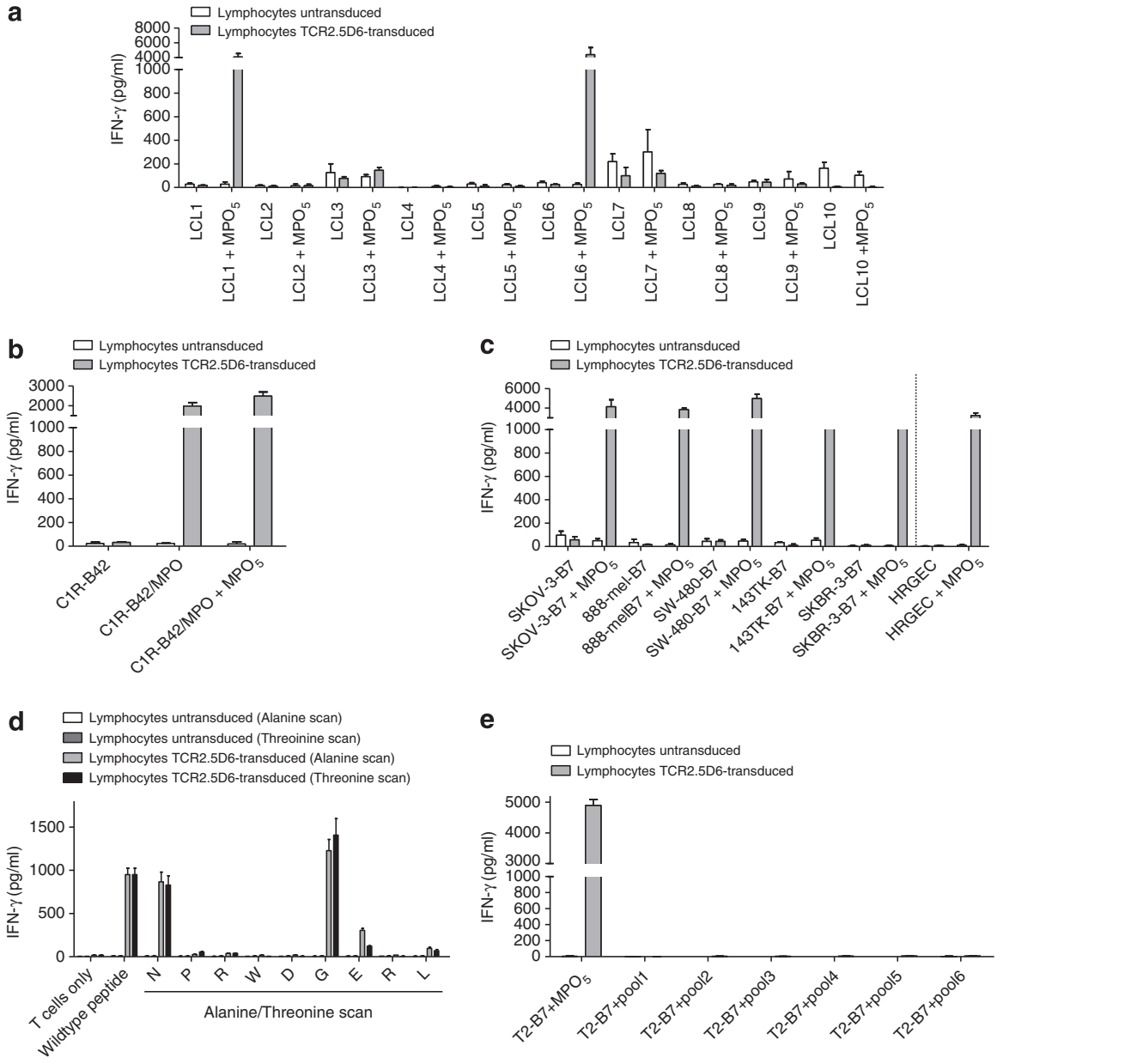


**Figure 6.** Off-target reactivity of lymphocytes transduced with the TCR2.5D6. Lymphocytes transduced with TCR2.5D6 (transduction rate: 19%, Figure 2c) were tested for potential off-target toxicity. IFN-γ secretion was measured by ELISA after stimulation with different target cells at effector-to-target ratios of 2:1 for 24 h. S.d. of the mean of triplicates is shown. (**a**) Peptide-dependent and -independent allo-HLA reactivity was measured by IFN-γ secretion after stimulation of TCR2.5D6-transduced or untransduced lymphocytes with LCL expressing common HLA alleles (Supplementary Table S2). Binding of $MPO_5$ to diverse HLA alleles was investigated by pulsing LCL with 1 μM $MPO_5$. LCL1 is HLA-B7$^+$ and served as a positive control. (**b**) Lymphocytes untransduced or transduced with TCR2.5D6 were stimulated with C1R cells transduced with HLA-*B*42:01* alone or additionally transduced with MPO. As a positive control, cells were additionally pulsed with $MPO_5$. (**c**) Recognition of HLA-B7-transduced, non-myeloid cell lines SKOV-3, 888mel, SW480, 143TK$^-$, SKBR-3 (transduction rate 25–50% without sorting and cloning) and primary HRGEC (HLA-B7$^+$) by TCR2.5D6-transduced lymphocytes was tested (MPO protein expression: Supplementary Figure S9C). (**d**) To determine the $MPO_5$ amino-acid residues critical for recognition by TCR2.5D6, alanine- and threonine-variants of $MPO_5$ were loaded onto the HLA-B7$^+$ cell line LCL1 at a concentration of 1 μM. The wild-type $MPO_5$ peptide was used as a positive control, unpulsed target cells as a negative control (Results for the two HLA-B7$^+$ cell lines LCL11 and 12 are depicted in Supplementary Figures S10B and C). (**e**) Sixty-five naturally presented HLA-B7 ligands were divided into six peptide pools. The pools were loaded onto T2-B7 cells at a final concentration of 1 μM for each peptide. T2-B7 cells pulsed with $MPO_5$ were used as a positive control. One representative experiment out of at least two is shown in panels (**a**–**e**).

and to reduce the risk for tumor escape,[40–42] TCR specific for multiple HLA ligands derived from diverse antigens and presented on various HLA molecules need to be defined.

By investigating the immunopeptidome of seven patients with MPN, we identified four naturally presented HLA ligands that are derived from MPO representing a differentiation antigen with restricted expression in myeloid precursors, mature neutrophils, monocytes and macrophages.[43] MPO is additionally an essential and distinct marker for myeloid leukemias[7] and thereby may provide a highly suitable candidate target for the treatment of patients with this disease. By using the single HLA-mismatched setting for stimulation, we isolated the TCR2.5D6 that recognizes a

MPO-derived peptide presented by HLA-B7. The TCR shows high reactivity against leukemia cells endogenously expressing MPO. Moreover, we were able to show in animal experiments significantly decreased infiltration of leukemic cells into the bone marrow and prolonged survival in BRG mice inoculated with NB4-B7 tumor cells after the adoptive transfer of TCR2.5D6-transduced $CD8^+T_{CM}$ compared with the control groups. However, the majority of mice treated with TCR2.5D6-transduced lymphocytes developed delayed progression of myeloid tumors and had to be euthanized. In the experiment shown in Figure 4, animals treated with TCR2.5D6-transduced lymphocytes developed human $CD45^+$ tumors that were negative for eGFP in four of the five mice, and loss of HLA-B7 expression was confirmed. This may indicate genetic or epigenetic instability of the transgene, which has been described to occur frequently[44] resulting in loss of transgene expression. As all control mice developed tumors with preserved eGFP expression and loss of the transgene has not been observed in our model under inferior experimental conditions, the immunogenic pressure of TCR2.5D6-transduced $T_{CM}$ is most likely involved in this effect. Interestingly, a similar observation has recently been published by Klippel *et al.*[45] using a murine model of NY-ESO-$1^+$ multiple myeloma where two out of the six mice demonstrated loss of heterozygosity for HLA-A2 in residual tumor manifestations after treatment with an NY-ESO-$1_{157-165}$-specific TCR. As clinical efficacy of this TCR has been previously shown in patients with melanoma and sarcoma,[29] it has yet to be clarified whether these models reflect the clinical situation. However, loss of mismatched HLA in leukemic cells has been described to be responsible for relapse in patients after haploidentical SCT.[41] Thus, our model may, although not in that frequency, still simulate a realistic tumor escape scenario. Of note, loss of MPO has not been observed in $CD45^+$ tumor cells in our animal model, and MPO has been confirmed to be expressed in $CD34^+/CD38^-$ leukemia stem cells of MPO-high-expressing leukemias.[46–48] This suggests that those cells would be equally eliminated by MPO-directed T-cell targeting. In fact, we have observed reactivity of TCR2.5D6-transduced lymphocytes against malignant HSC of the HLA-$B7^+$ patient MPN2 as shown by CFU assays. As diverse MPO-derived peptides have been eluted from different HLA molecules, isolation and application of TCR recognizing these other presented peptides as well as peptides derived from other proteins with restricted hematopoietic expression may reduce the risk for escape by loss of HLA or potentially leukemia-associated antigen.

The development of effective immunotherapies has been demonstrated to also include the risk for autoimmunity. On-target toxicity against normal tissues that express low levels of targeted antigen has been previously observed.[26] We reanalyzed expression of MPO by qPCR, western blotting and immunohistochemistry and confirmed restricted protein expression to myeloid cells, whereas high mRNA expression was not detected in mature myeloid cells as granulocytes and monocytes. This is consistent with previous publications demonstrating high MPO mRNA expression only in myeloid precursor cells but not in mature myeloid cells.[49,50] Although the protein expression data suggest that on-target toxicity may affect human myeloid precursors as well as neutrophils, monocytes and tissue macrophages, we did not observe reactivity against monocytes and only very low reactivity against granulocytes. As the major source for substrates used by the transporter associated with antigen processing seems to be newly synthesized proteins,[51] mature myeloid cells with low MPO protein turnover may, in fact, not be targeted by MPO-specific T cells. In addition, we have not observed reactivity against healthy $CD34^+$ HSC, as shown by CFU assays, which is consistent with previous publications reporting that MPO expression is absent or low in healthy $CD34^+$ HSC.[46,47] Recent efforts to target MAGE-A3 with T cells transgenic for affinity-enhanced TCR constructs were compromised by fatal clinical outcomes owing to unexpected toxicity against off-target epitopes recognized on neurons and contracting cardiac tissue.[21,27,52] Although we did not perform affinity maturation, crossreactivity against other peptides presented by HLA molecules allogeneic for the donor of the TCR or minor histocompatibility antigens presented by HLA-B7 or other HLA alleles of the donor cannot be completely ruled out. However, off-target reactivity could not be observed against several LCL with diverse HLA types, various HLA-B7-transduced cell lines of non-myeloid origin as well as a high number of peptides that have been redundantly eluted from HLA-B7. Furthermore, the recognized motif of the TCR2.5D6, identified by the alanine- and threonine-scan experiments, is only present in the MPO protein. Taken together, our data suggest that adoptive T-cell therapy with T cells transgenic for this TCR may be safe in the haploidentical or HLA-B7 allele mismatch allogeneic SCT setting, where donor myeloid progenitor cells lack the HLA-B7 restricting allele. The fact that we have not observed reactivity against monocytes and granulocytes as well as healthy $CD34^+$ stem cells is tempting to consider application of this TCR even apart from the HLA-mismatched transplant setting. In this regard, inclusion of suicide mechanisms to deplete TCR-transduced T cells posttreatment[53] may be important in order to potentially allow regeneration of healthy myeloid cells in the bone marrow. This might be also desirably to limit unpredictable on- and off-target toxicity not detectable in preclinical experiments. Similar scenarios are currently considered for a CD123-specific chimeric antigen receptor.[54] In addition, selective transfer of TCR in predetermined effector populations such as $CD8^+T_{CM}$ or $CD8^+$ cells with defined virus specificity may further increase safety in combination with other advantages, such as enhanced long-term survival and virus defense.[55,56]

In conclusion, we identified MPO as a novel target antigen in patients with AML and MPN and isolated a HLA-B7-restricted TCR recognizing this antigen from an allogeneic repertoire. This TCR has a strong and specific anti-leukemic reactivity *in vitro* and also demonstrated efficacy in experiments *in vivo* suggesting that both, target antigen and TCR are highly promising for novel therapeutic strategies in these life-threatening diseases. By extension of this approach to other isolated HLA ligands derived from genes restrictedly expressed in hematopoietic cells and presented by several frequently expressed HLA molecules, it might be possible to provide efficient treatment options for a substantial part of patients suffering from refractory leukemia.

## CONFLICT OF INTEREST

A patent application is currently ongoing for peptide and TCR sequences. Apart from that the authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We thank Stanley Riddell and Hans-Jochem Kolb for the helpful discussions and critical review of the manuscript; Josef Mautner, Ralph Mocikat, Steve Marsh and Stanley Riddell for providing cell lines; Eilon Barnea for data analysis with Pep-Miner; Ciara Utsch for language revision as well as Stephanie Rämisch, Ciara Utsch and Ines Nachtigal for excellent technical support. This work was supported by grants to AMK from the Deutsche José Carreras Leukämie-Stiftung (DJCLS R11/23), BayImmuNet (Nr. F2-F5121.7.3-10c/23932), Deutsche Krebshilfe (110281) and the Deutsche Forschungsgemeinschaft (DFG), SFB824/C10.

## AUTHOR CONTRIBUTIONS

RK, S Schober, MR, SM, JM, JS-H and AA did experiments; RK, S Schober, MR, SMH, MU, JS-H, CP and AMK analyzed the data; DHB provided the HLA multimer technology; S Stevanović provided HLA-B7 ligands for off-target toxicity studies; MS supported PET analysis of mouse models; S Stevanović and RO provided technical support; AMK conceived and supervised the study; and RK and AMK planned experiments and composed the manuscript.

## REFERENCES


1 Kolb HJ, Mittermuller J, Clemm C, Holler E, Ledderose G, Brehm G *et al.* Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 1990; **76**: 2462–2465.
2 Rotzschke O, Falk K, Deres K, Schild H, Norda M, Metzger J *et al.* Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* 1990; **348**: 252–254.
3 Hunt DF, Michel H, Dickinson TA, Shabanowitz J, Cox AL, Sakaguchi K *et al.* Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science* 1992; **256**: 1817–1820.
4 Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C *et al.* Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med* 2012; **18**: 1254–1261.
5 Bassani-Sternberg M, Barnea E, Beer I, Avivi I, Katz T, Admon A. Feature Article: Soluble plasma HLA peptidome as a potential source for cancer biomarkers. *Proc Natl Acad Sci USA* 2010; **107**: 18769–18776.
6 Delluc S, Tourneur L, Fradelizi D, Rubio MT, Marchiol-Fournigault C, Chiocchia G *et al.* DC-based vaccine loaded with acid-eluted peptides in acute myeloid leukemia: the importance of choosing the best elution method. *Cancer Immunol Immunother* 2007; **56**: 1–12.
7 Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A *et al.* The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 2009; **114**: 937–951.
8 Eggleton P, Gargan R, Fisher D. Rapid method for the isolation of neutrophils in high yield without the use of dextran or density gradient polymers. *J Immunol Methods* 1989; **121**: 105–113.
9 Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 1991; **351**: 290–296.
10 Hauck SM, Dietter J, Kramer RL, Hofmaier F, Zipplies JK, Amann B *et al.* Deciphering membrane-associated molecular processes in target tissue of autoimmune uveitis by label-free quantitative mass spectrometry. *Mol Cell Proteomics* 2010; **9**: 2292–2305.
11 Niedermeyer TH, Strohalm M. mMass as a software tool for the annotation of cyclic peptide tandem mass spectra. *PLoS One* 2012; **7**: e44913.
12 Wilde S, Sommermeyer D, Frankenberger B, Schiemann M, Milosevic S, Spranger S *et al.* Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity. *Blood* 2009; **114**: 2131–2139.
13 Knabel M, Franz TJ, Schiemann M, Wulf A, Villmow B, Schmidt B *et al.* Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med* 2002; **8**: 631–637.
14 Schuster IG, Busch DH, Eppinger E, Kremmer E, Milosevic S, Hennard C *et al.* Allorestricted T cells with specificity for the FMNL1-derived peptide PP2 have potent antitumor activity against hematologic and other malignancies. *Blood* 2007; **110**: 2931–2939.
15 Weigand LU, Liang X, Schmied S, Mall S, Klar R, Stotzer OJ *et al.* Isolation of human MHC class II-restricted T cell receptors from the autologous T-cell repertoire with potent anti-leukaemic reactivity. *Immunology* 2012; **137**: 226–238.
16 Cohen CJ, Zhao Y, Zheng Z, Rosenberg SA, Morgan RA. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer Res* 2006; **66**: 8878–8886.
17 Kuball J, Dossett ML, Wolfl M, Ho WY, Voss RH, Fowler C *et al.* Facilitating matched pairing and expression of TCR chains introduced into human T cells. *Blood* 2007; **109**: 2331–2338.
18 Scholten KB, Kramer D, Kueter EW, Graf M, Schoedl T, Meijer CJ *et al.* Codon modification of T cell receptors allows enhanced functional expression in transgenic human T cells. *Clin Immunol* 2006; **119**: 135–145.
19 Wang X, Naranjo A, Brown CE, Bautista C, Wong CW, Chang WC *et al.* Phenotypic and functional attributes of lentivirus-modified CD19-specific human CD8+ central memory T cells manufactured at clinical scale. *J Immunother* 2012; **35**: 689–701.
20 Liang X, Weigand LU, Schuster IG, Eppinger E, van der Griendt JC, Schub A *et al.* A single TCR alpha-chain with dominant peptide recognition in the allorestricted HER2/neu-specific T cell repertoire. *J Immunol* 2010; **184**: 1617–1629.
21 Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L *et al.* Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood* 2013; **122**: 863–871.
22 Schirle M, Keilholz W, Weber B, Gouttefangeas C, Dumrese T, Becker HD *et al.* Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach. *Eur J Immunol* 2000; **30**: 2216–2225.
23 Wang X, Berger C, Wong CW, Forman SJ, Riddell SR, Jensen MC. Engraftment of human central memory-derived effector CD8+ T cells in immunodeficient mice. *Blood* 2011; **117**: 1888–1898.
24 Brown J, Poles A, Brown CJ, Contreras M, Navarrete CV. HLA-A, -B and -DR antigen frequencies of the London Cord Blood Bank units differ from those found in established bone marrow donor registries. *Bone Marrow Transplant.* 2000; **25**: 475–481.
25 McCormack E, Micklem DR, Pindard LE, Silden E, Gallant P, Belenkov A *et al. In vivo* optical imaging of acute myeloid leukemia by green fluorescent protein: time-domain autofluorescence decoupling, fluorophore quantification, and localization. *Mol Imaging* 2007; **6**: 193–204.
26 Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS *et al.* Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 2009; **114**: 535–546.
27 Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z *et al.* Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother* 2013; **36**: 133–151.
28 Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM *et al.* Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006; **314**: 126–129.
29 Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME *et al.* Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 2011; **29**: 917–924.
30 Engels B, Engelhard VH, Sidney J, Sette A, Binder DC, Liu RB *et al.* Relapse or eradication of cancer is predicted by peptide-major histocompatibility complex affinity. *Cancer Cell* 2013; **23**: 516–526.
31 Zhong S, Malecek K, Johnson LA, Yu Z, Vega-Saenz de Miera E, Darvishian F *et al.* T-cell receptor affinity and avidity defines antitumor response and autoimmunity in T-cell immunotherapy. *Proc Natl Acad Sci USA* 2013; **110**: 6973–6978.
32 Heemskerk MH, Hoogeboom M, de Paus RA, Kester MG, van der Hoorn MA, Goulmy E *et al.* Redirection of antileukemic reactivity of peripheral T lymphocytes using gene transfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved alpha joining region. *Blood* 2003; **102**: 3530–3540.
33 Amir AL, van der Steen DM, van Loenen MM, Hagedoorn RS, de Boer R, Kester MD *et al.* PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. *Clin Cancer Res* 2011; **17**: 5615–5625.
34 Xue SA, Gao L, Hart D, Gillmore R, Qasim W, Thrasher A *et al.* Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood* 2005; **106**: 3062–3067.
35 Chapuis AG, Ragnarsson GB, Nguyen HN, Chaney CN, Pufnock JS, Schmitt TM *et al.* Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. *Sci Transl Med* 2013; **5**: 174ra27.
36 Yong AS, Rezvani K, Savani BN, Eniafe R, Mielke S, Goldman JM *et al.* High PR3 or ELA2 expression by CD34+ cells in advanced-phase chronic myeloid leukemia is associated with improved outcome following allogeneic stem cell transplantation and may improve PR1 peptide-driven graft-versus-leukemia effects. *Blood* 2007; **110**: 770–775.
37 Sergeeva A, Alatrash G, He H, Ruisaard K, Lu S, Wygant J *et al.* An anti-PR1/HLA-A2 T-cell receptor-like antibody mediates complement-dependent cytotoxicity against acute myeloid leukemia progenitor cells. *Blood* 2011; **117**: 4262–4272.
38 Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J *et al.* Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* 2013; **19**: 747–752.
39 van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B *et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol* 2013; **31**: e439–e442.
40 Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR *et al.* Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* 2013; **368**: 1509–1518.
41 Vago L, Perna SK, Zanussi M, Mazzi B, Barlassina C, Stanghellini MT *et al.* Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med* 2009; **361**: 478–488.
42 DuPage M, Mazumdar C, Schmidt LM, Cheung AF, Jacks T. Expression of tumour-specific antigens underlies cancer immunoediting. *Nature* 2012; **482**: 405–409.
43 Prokopowicz Z, Marcinkiewicz J, Katz DR, Chain BM. Neutrophil myeloperoxidase: soldier and statesman. *Arch Immunol Ther Exp (Warsz)* 2012; **60**: 43–54.
44 Frank O, Rudolph C, Heberlein C, von Neuhoff N, Schrock E, Schambach A *et al.* Tumor cells escape suicide gene therapy by genetic and epigenetic instability. *Blood* 2004; **104**: 3543–3549.

45 Klippel ZK, Chou J, Towlerton AM, Voong LN, Robbins P, Bensinger WI *et al.* Immune escape from NY-ESO-1-specific T-cell therapy via loss of heterozygosity in the MHC. *Gene Ther* 2014; **21**: 337–342.
46 Kim YR, Eom JI, Kim SJ, Jeung HK, Cheong JW, Kim JS *et al.* Myeloperoxidase expression as a potential determinant of parthenolide-induced apoptosis in leukemia bulk and leukemia stem cells. *J Pharmacol Exp Ther* 2010; **335**: 389–400.
47 Majeti R, Becker MW, Tian Q, Lee TL, Yan X, Liu R *et al.* Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. *Proc Natl Acad Sci USA* 2009; **106**: 3396–3401.
48 Gal H, Amariglio N, Trakhtenbrot L, Jacob-Hirsh J, Margalit O, Avigdor A *et al.* Gene expression profiles of AML derived stem cells; similarity to hematopoietic stem cells. *Leukemia* 2006; **20**: 2147–2154.
49 Fouret P, du Bois RM, Bernaudin JF, Takahashi H, Ferrans VJ, Crystal RG. Expression of the neutrophil elastase gene during human bone marrow cell differentiation. *J Exp Med* 1989; **169**: 833–845.
50 Tobler A, Miller CW, Johnson KR, Selsted ME, Rovera G, Koeffler HP. Regulation of gene expression of myeloperoxidase during myeloid differentiation. *J Cell Physiol* 1988; **136**: 215–225.
51 Reits EA, Vos JC, Gromme M, Neefjes J. The major substrates for TAP *in vivo* are derived from newly synthesized proteins. *Nature* 2000; **404**: 774–778.
52 Cameron BJ, Gerry AB, Dukes J, Harper JV, Kannan V, Bianchi FC *et al.* Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. *Sci Transl Med* 2013; **5**: 197ra03.
53 Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L *et al.* HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 1997; **276**: 1719–1724.
54 Mardiros A, Dos Santos C, McDonald T, Brown CE, Wang X, Budde LE *et al.* T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. *Blood* 2013; **122**: 3138–3148.
55 Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* 2008; **118**: 294–305.
56 Heemskerk MH, Hoogeboom M, Hagedoorn R, Kester MG, Willemze R, Falkenburg JH. Reprogramming of virus-specific T cells into leukemia-reactive T cells using T cell receptor gene transfer. *J Exp Med* 2004; **199**: 885–894.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)