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Isolation of human MHC class II-restricted T cell receptors from the autologous T-cell repertoire with potent anti-leukaemic reactivity

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Summary

Adoptive transfer of T cells genetically modified with tumour-specific T-cell receptors (TCR) is a promising novel approach in the treatment of cancer. We have previously isolated an allorestricted MHC class I-restricted TCR with specificity for Formin-like protein 1 (FMNL1) with potent activity against chronic lymphocytic leukaemia cells. $CD4^+$ T cells have been described to be highly important for tumour elimination although TCR derived from $CD4^+$ T cells with anti-tumour reactivity have been only rarely described. In this study we aimed to isolate MHC class-II-restricted $CD4^+$ T cells and TCR with specificity for leukaemia antigens. We used professional antigen-presenting cells pulsed with the leukaemia-associated and tumour-associated antigen FMNL1 for stimulation of autologous T cells *in vitro*. We isolated two $CD4^+$ HLA-DR-restricted T-cell clones and T-cell-derived TCR with so far unknown specificity but high reactivity against lymphoma cells and native malignant cells derived from HLA-matched patients with diverse leukaemias. Moreover, characterization of the TCR after TCR gene transfer revealed that specific characteristics of isolated TCR as reactivity in response to Toll-like receptors were transferable on effector cells. Our results have a major impact on the development of novel immunotherapies. They demonstrate that TCR with potent HLA-DR-restricted anti-leukaemic reactivity against so far undefined self-restricted antigens can be isolated from the healthy autorestricted $CD4^+$ T-cell repertoire and these TCR are highly interesting candidate tools for novel immunotherapies.

Keywords: leukaemia/lymphoma/myeloma; MHC/HLA; T-cell receptor (TCR); therapy/immunotherapy; toll receptors/Toll-like receptors.

Introduction

T cells have been demonstrated to play a fundamental role in anti-tumour responses. Strong anti-tumour effects have been especially shown in the allogeneic context [1] and alloreactive and xenoreactive peptide-specific T cells have been proposed to represent a source of high avidity T-cell receptors (TCR) with specificity for a number of tumour-associated antigens.[2–6] We have used this approach to target the self antigens HER2/neu and Formin-like protein 1 (FMNL1), the latter has been shown to be restrictedly expressed in haematopoietic cells as well as over-expressed in diverse malignant cell lines and primary tumour material derived from patients with leukaemia.[4,5,7,8] However, recent studies revealed a substantial autoreactive potential of cells transduced with alloreactive or xenoreactive TCR targeting diverse self antigens potentially indicating their limited usefulness.[5,9,10] Although autoreactivity is unlikely to occur in the autologous host, thymic negative selection has been assumed to be the major hurdle for isolation of autologous T cells with sufficient recognition of tumour-associated antigens representing mostly self antigens. Nevertheless, successful targeting of self antigens in the autologous host has been demonstrated to be feasible in some immunogenic tumours, such as melanoma.[11] Autologous tumour-reactive T cells may be also present in other malignancies including leukaemia although the recognized target antigens are mostly unknown.[12–14]

Adoptively transferred $CD4^+$ helper T cells may play a fundamental role in the induction and support of anti-tumour responses[15–17] although they may also be involved in tumour progression.[18] Characterization of MHC class II-restricted TCR with specificity for tumour-associated antigens has been only rarely performed.[19–21] Hence, the identification and characterization of human MHC class II-restricted TCR with tumour reactivity is an essential pre-condition for the further development of directed clinical applications of $CD4^+$ T cells and T cells transgenic for MHC class II-restricted TCR.

In this study we aimed to identify TCR derived from the autologous environment with specificity for leukaemia-associated antigens, such as FMNL1. We used dendritic cells (DC) pulsed with FMNL1 protein for repeated stimulation of unsorted peripheral blood mononuclear cells (PBMC) and sorted naive T cells. Stimulation of autologous T cells with protein-pulsed DC resulted in the isolation of two MHC class II-restricted TCR with undefined specificity but potent anti-leukaemic reactivity against HLA-matched leukaemic samples. Importantly, our data additionally provide insights about the complex interaction of effector and target cell as well as innate factors depending on distinct characteristics of the TCR determining the outcome of the anti-tumour immune response.

Materials and methods

Cells and cell lines

The PBMC were collected from healthy donors, patients with chronic lymphatic leukaemia (CLL) and acute myeloid leukaemia (AML) with the donors' informed consent following the requirements of the local ethical board and the principles expressed in the Helsinki Declaration. PBMC from healthy donors were isolated by density gradient centrifugation on Ficoll/Hypaque (Biochrom, Berlin, Germany). The donor selected for T-cell stimulation was typed positive for HLA-DRB1*0101 and HLADRB1*1101. $CD45RO^-$ cells were obtained by negative isolation using magnetic bead depletion (Miltenyi Biotech, Bergisch Gladbach, Germany). The DC were generated by plate adherence and cultured with interleukin-4 (IL-4; 20 ng/ml) and granulocyte–macrophage colony-stimulating factor (GM-CSF; 100 ng/ml) (Peprotech, Hamburg, Germany). On day 5, recombinant FMNL1 protein purified from 293T cells[4] was added to the culture and on day 6, DC were matured by a maturation cocktail containing interferon-γ (IFN-γ; 50 U/ml), tumour necrosis factor-α (TNF-α; 10 ng/ml), CD40L (1 μg/ml) in cocktail A or IL-6 (15 ng/ml), TNF-α (10 ng/ml), IL-1β (10 ng/ml) (Peprotech) in cocktail B. On day 7, DC were used for T-cell stimulation. For generation of type 1 or 2 macrophages (M1 and M2), monocytes were isolated by plate adherence. Adherent cells were cultured in medium complemented with either 20 ng/ml human M-CSF (Peprotech) or 100 ng/ml human GM-CSF for 6 days. Final maturation was induced by 100 U/ml IFN-γ and 1 ng/ml lipopolysaccharide (LPS; Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) in case of M1 and 20 ng/ml IL-4 for M2 differentiation.[22] The following targets were used: autologous Mini-LCL [B cells immortalized by latent Epstein–Barr virus (EBV)-genes[23]], autologous EBV^- B-cell lines cultured with IL-4 on CD40L-expressing feeder cells, 10 different wild-type EBV-transformed lymphoblastoid cell lines (LCL), C1R cells and the EBV^- Burkitt cell line BJAB. C1R and BJAB cells were transfected with HLA-DRB1*01 (HLA-DR1), HLA-DRB1*11 (HLA-DR11) or green fluorescent protein (GFP) encoded by the pINCO plasmid.[24] Other targets used were the human embryonic lung cell line WI-38 (ATCC CCL-75), the renal cell carcinoma RCC1·24[24] and the melanoma cell line Na-Mel 41.[25]

Antibodies

The following antibodies were used to characterize PBMC-derived cells, primary tumour cells and malignant cell lines: anti-CD3-FITC (UCHT1, BD, Heidelberg, Germany), anti-CD4-FITC and -Pacific Blue (RPA-T4, BD), anti-CD8-FITC (V5T-HIT8a, BD), anti-CD8-phycoerythrin (PE) and -Pacific Blue (RPA-T8, BD), anti-CD11c-PE (HL-3, BD), anti-CD14-PE (M5E2, BD), anti-CD19-FITC, -allophycocyanin and -PE (HIB19, BD), anti-CD25-PE (2A3, BD), anti-CD45RO-PE (UCHL1, BD), anti-CD56-PE (B159, BD), anti-CD80-PE (MAB104; Beckman Coulter, Krefeld, Germany), anti-CD83-FITC (HB15e, BD), anti-CD86-PE (2331, BD), hamster-anti-mouse TCR-$\alpha\beta$-FITC and -PE (TCRmu, H57-597, Beckman Coulter), goat anti-mouse IgG-PE (Jackson ImmunoResearch, Suffolk, UK), anti-HLA-A,B,C (W6/32, BD) and anti-HLA-DR (L243; Biolegend, San Diego, CA).

T-cell priming and culture

$CD4^+$ T cells were generated from PBMC or a $CD45RO^-$ cell population and stimulated with matured DC. The effector : target (E : T) cell ratio was 10 : 1 for priming followed by weekly restimulations at a ratio of 100 : 1. Interleukin-2 (50 U/ml) (Chiron Vaccines International, Marburg, Germany), IL-7 (10 ng/ml) and IL-15 (10 ng/ml) (Peprotech) were added primarily after 3 days. T cells were then restimulated with autologous stimulated PBMC supplemented with cytokines and investigated weekly for reactivity against autologous Mini-LCL in mixed cell cultures. The supernatants were analysed by IFN-γ ELISA (BD). Reactive T-cell lines were cloned by limiting dilution and restimulated every 2 weeks using pooled allogeneic irradiated PBMC together with irradiated autologous Mini-LCL and addition of IL-2, IL-7 and IL-15.[26]

TCR analysis

TCR-specific PCR of T-cell clones was performed as previously described.[4,5] In brief, total RNA from T-cell clones and lines was extracted (Trizol reagent, Invitrogen, Darmstadt, Germany) and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) and oligo (dT) primers according to the recommendations of the manufacturer. Subfamily-specific TCR-PCR was performed followed by gel isolation (NucleoSpin; Macherey-Nagel, Düren, Germany) and DNA sequencing of the amplified products (Sequiserve, Vaterstetten, Germany). T-cell clone Aa2.2 used Vα 6*02 and Vβ 3-1*01 and Bb5.14 used Vα 29/DV5*01 and Vβ 6-4*01 variable chains according to the IMGT nomenclature.[27]

Cloning of reactive TCR

The TCR cloning was performed as previously described.[5] In brief, the specific TCR-α and TCR-β chain coding cDNA were amplified from isolated T-cell clones using primers containing a *Not*I and *Mfe*I or *Eco*RI restriction site. The TCR genes were cloned as bi-cistronic constructs separated by the Picorna virus-derived peptide element P2A.[28] Constant chains were murinized and an additional disulphide bridge was inserted to improve pairing of the transgenic TCR. Moreover, the whole murinized TCR was codon optimized to improve gene expression (Geneart, Regensburg, Germany).[29–32]

Retroviral TCR transfer into PBMC

The TCR-containing retroviral vector plasmids pMP71-TCR were co-transfected with plasmids carrying retroviral genes for gag/pol derived from Moloney murine leukaemia virus (pcDNA3.1-Mo-MLV) and env (pALF-10A1) into 293T cells using TransIT (Mirus, Göttingen, Germany). Activated PBMC were twice transduced with viral supernatant using RetroNectin (Takara, Saint-Germain-en-Laye, France) -coated culture plates. Thereafter, plates were spin-oculated with 800 ***g*** for 1·5 hr at 32°. Medium was replaced by fresh medium after 48 hr. Transduced PBMC were analysed by TCRmu expression as well as in functional assays at indicated time-points. The PBMC transduced with a GFP-containing MP71 vector were used as mock control.

Functional assays

Primary clones and TCR-transduced PBMC were analysed for cytokine secretion in response to different target cells. Therefore, effector T cells were incubated for 24 hr with selected target cells at diverse E : T ratios as indicated. The CLL, AML and epithelial cell lines were treated with 100 U/ml IFN-γ 3 days before the assay to induce or enhance HLA-DR expression and were washed before T-cell stimulation. Supernatants were analysed for IFN-γ concentration by ELISA (BD). The presence of additional cytokines was investigated by Flow Cytomix (Bender Med Systems, Frankfurt am Main, Germany) analysis. Diverse targets were additionally treated for 2 hr with different Toll-like receptor (TLR) ligands: 1 μg/ml LPS (Sigma Aldrich Chemie GmbH), 1 μg/ml Poly(I : C), 1 μg/ml Flagellin, 0·5 μM CpG oligodeoxynucleotides (CpG; Invivogen). Thereafter, target cells were washed twice before T-cell stimulation. For blocking, 1 μg/ml HLA-DR (L243; Biolegend, London, UK) or 1 μg/ml MHC I (W6/32) antibodies were used. For chromium-release assay, targets were incubated for 1 hr with ^{51}Cr for labelling, subsequently washed and co-incubated with TCR-transduced T cells. After 4 hr supernatants were harvested and analysed using a gamma-counter. For analysis of specific proliferation, T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) and analysed by flow cytometry.

Results

Isolation of TCR with self-reactivity derived from a healthy donor

We primarily aimed to identify leukaemia-specific CD4$^+$ T cells derived from the autologous environment demonstrating reactivity against haematopoietic and malignant cells over-expressing FMNL1. Monocytes were isolated from the donors' PBMC to generate DC, which were pulsed with FMNL1 protein in combination with two alternative maturation cocktails (Fig. 1a). Thereafter, two different autologous T-cell populations as PBMC or naive purified CD45RO$^-$ T cells were used for stimulation (Fig. 1a). T cells were repeatedly restimulated and tested for specific recognition of autologous Mini-LCL. T-cell lines demonstrating specific IFN-γ secretion were cloned by limiting dilution (Fig. 1a).

Stimulation of T cells with FMNL1-pulsed DC resulted in isolation of two T-cell clones with reactivity in response to autologous Mini-LCL (Fig. 1b,c). Clone Aa2.2 was derived originally from PBMC primed with protein-pulsed DC matured with cytokine cocktail A (Fig. 1b), whereas Bb5.14 was derived from the naive T-cell population primed with cocktail B-matured protein-pulsed DC (Fig. 1c). Since these T-cell clones did not further proliferate *in vitro*, TCR sequences were isolated and cloned into the retroviral vector MP71 for further analysis in transgenic effector T cells.

Selected TCR can be expressed in different effector T cells and demonstrate reactivity against Mini-LCL without any sign of T-cell fratricide

Successful transfer and expression of both TCR in different autologous (Fig. 2a) as well as allogeneic (see Supplementary material, Fig. S1) effector T-cell populations as PBMC,

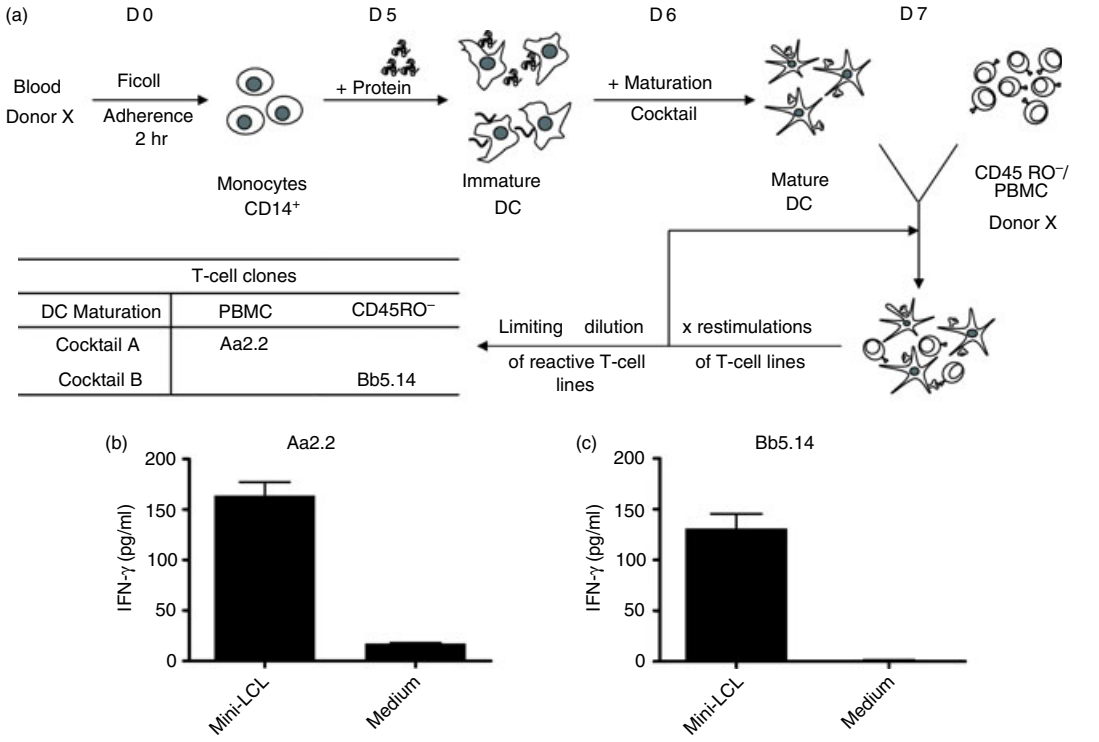

Figure 1. T-cell clones recognizing autologous antigen-presenting cells can be isolated from a healthy donor after stimulation with dendritic cells (DC) pulsed with FMNL1 protein. (a) Schematic view of the stimulation and analysis protocol: Autologous DC and peripheral blood mononuclear cells (PBMC) were pulsed with FMNL1 protein. $CD4^+$ T-cell clones were generated from PBMC or a sorted $CD45RO^-$ cell population. T cells were primed and weekly re-stimulated with protein-pulsed DC for three restimulations. Thereafter, these T-cell lines were restimulated bi-weekly with protein-pulsed autologous PBMC. T-cell lines were weekly investigated for their reactivity against autologous Mini-LCL in mixed cell cultures and supernatants were analysed by interferon-γ (IFN-γ) ELISA. T-cell clones Aa2.2 and Bb5.14 were recovered by limiting dilution procedures of reactive T-cell lines. Aa2.2 was isolated after eight and Bb5.14 after six restimulations. Cytokine secretion of isolated T-cell clones was analysed in a screening experiment after 24 hr co-incubation with autologous antigen-presenting cells. Reactivity of clone (b) Aa2.2 and (c) Bb5.14 in response to autologous Mini-LCL is shown. Supernatants were harvested and analysed by IFN-γ ELISA. An E : T ratio of 1 : 1 was chosen. Standard deviations (SD) of triplicates are shown.

$CD4^+$ and $CD8^+$ T cells was confirmed by staining of the constant murine TCR chains (TCRmu antibody). All effector T-cell populations transduced with TCR Bb5.14 (Bb5.14) showed high reactivity against Mini-LCL (Fig. 2b). In contrast, reactivity of effector T-cell populations transduced with TCR Aa2.2 (Aa2.2) was low. TCR-specific reactivity against Mini-LCL was distinguishable only in $CD4^+$ T cells (Fig. 2b) but not $CD8^+$ or PBMC populations. Enhanced background reactivity (> 50 pg/ml) observed in $CD8^+$ T-cell populations or PBMC may be caused to the presence of $CD8^+$ responses against latent EBV proteins (Fig. 2b). As activated T cells express MHC class II, we asked whether effector T cells transduced with these TCR may exert self reactivity potentially resulting in fratricide. Effector T-cell populations transduced with both TCR did not secrete IFN-γ in response to PBMC or activated PBMC of the same donor (Fig. 2b). To further investigate reactivity of these TCR against autologous T cells, total cell counts were calculated over a period of 3 weeks. There were no significant differences in proliferation of TCR-transduced, GFP-transduced and non-transduced effector T-cell populations as non-purified PBMC, $CD4^+$ or $CD8^+$ T cells (Fig. 2c, upper row). Moreover, no differences in growth of specific T-cell populations harbouring the transduced modified TCR with murinized constant chain ($TCRmu^+$) were observed in all three transduced effector T-cell populations (Fig. 2c, lower row). Similar results were observed in TCR-transduced allogeneic effector T-cell populations (see Supplementary material, Fig. S1).

As FMNL1 protein was pulsed on antigen-presenting cells used for T-cell priming and stimulation, we expected specific recognition of FMNL1-derived peptides. However, evidence of FMNL1-specific target recognition was not detectable using a peptide library derived from FMNL1 or FMNL1-transduced target cells (data not shown).

Effector T cells transduced with Aa2.2 or Bb5.14 show distinct differences regarding their reactivity, restriction element and target recognition patterns

Considerable reactivity against Mini-LCL prompted us to further characterize these two TCR. Therefore, effector

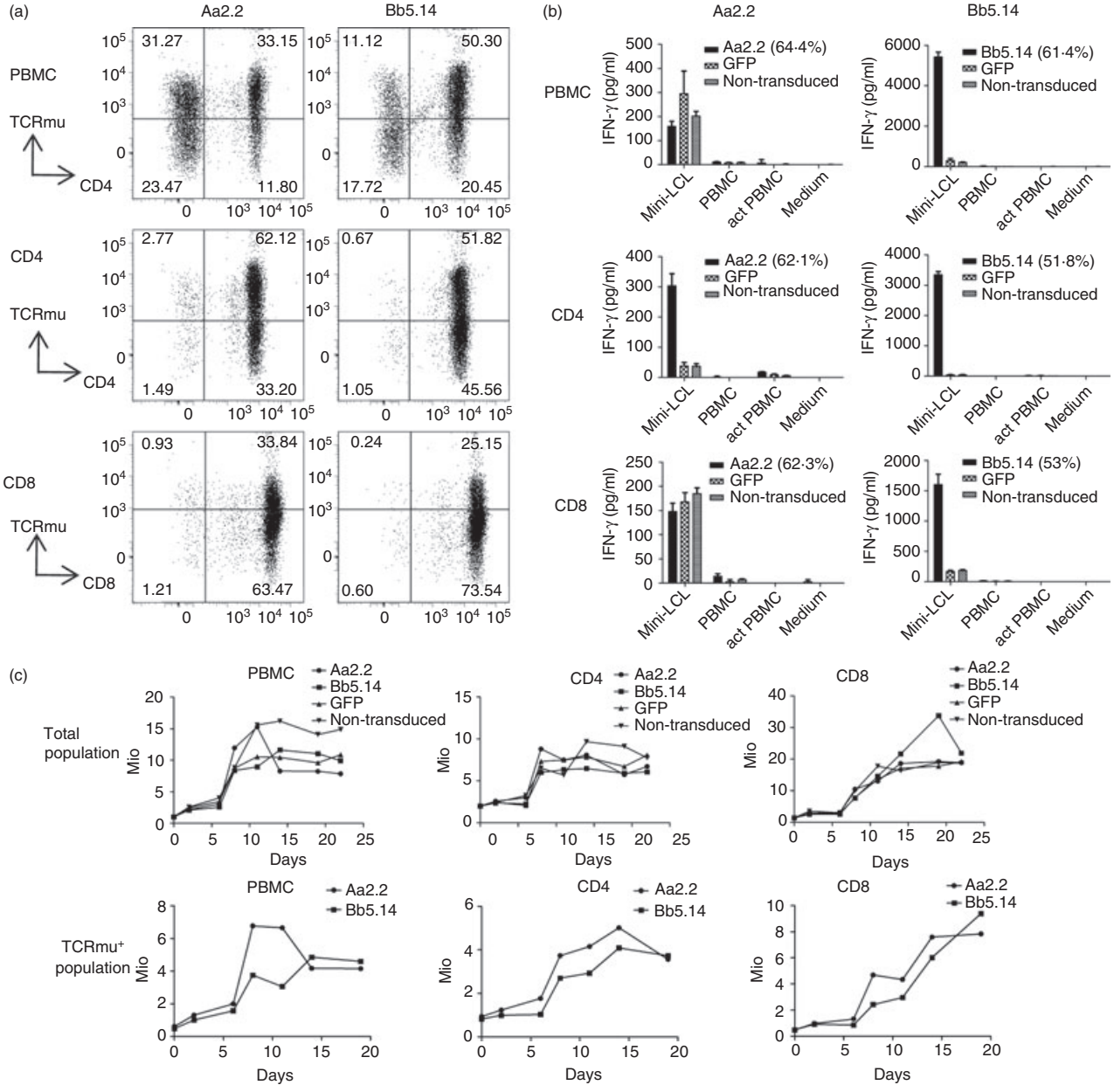

Figure 2. T-cell receptors (TCR) derived from isolated T-cell clones Aa2.2 and Bb5.14 are expressed and functional after genetic transfer into different effector T cells and show no sign of T-cell fratricide. TCR derived from isolated T-cell clones were retrovirally transduced in effector T-cell populations (PBMC, $CD4^+$ or $CD8^+$) of the same donor. (a) Expression levels of the transduced TCR were analysed 6 days after transduction by flow cytometry after staining with anti-human CD4 or CD8 and TCRmu-antibody. Non-transduced cells were identically stained and showed no $TCRmu^+$ population (data not shown). (b) TCR-transduced effector T cells [peripheral blood mononuclear cells (PBMC), $CD4^+$ or $CD8^+$] were co-incubated with diverse target cells (E : T ratio = 5 : 1) as autologous Mini-LCL, PBMC and PBMC activated with interleukin-2 (IL-2) and OKT3 (act PBMC). Supernatants of mixed cultures were removed after 24 hr and analysed by interferon-γ ELISA. Percentages of transduced $TCRmu^+$ cells at the time-point of the assay (6 days after transduction) are indicated. GFP-transduced and non-transduced effector T-cell populations were used as controls. SD of triplicates are shown. One representative out of 10 independent experiments (3×PBMC, 4× $CD4^+$ T cells, 1× $CD8^+$ T cells, 2× allogeneic PBMC) with transduced autologous effector cells is shown. (c) Proliferation of cells after retroviral TCR transfer was investigated by vital cell counting of total cell populations (upper row) and $TCRmu^+$ T cells (lower row). One representative out of six independent experiments (2× PBMC, 1× $CD4^+$ and 1× $CD8^+$ T cells, 2× allogeneic PBMC) with transduced autologous effector cells is shown.

T cells transduced with Aa2.2 and Bb5.14 were tested for their reactivity against various targets by co-incubation in 24-hr stimulation assays. The data shown in Fig. 3 were performed with autologous TCR-transduced $CD4^+$ T cells. Similar results were observed in experiments with other autologous ($CD8^+$, PBMC) as well as allogeneic

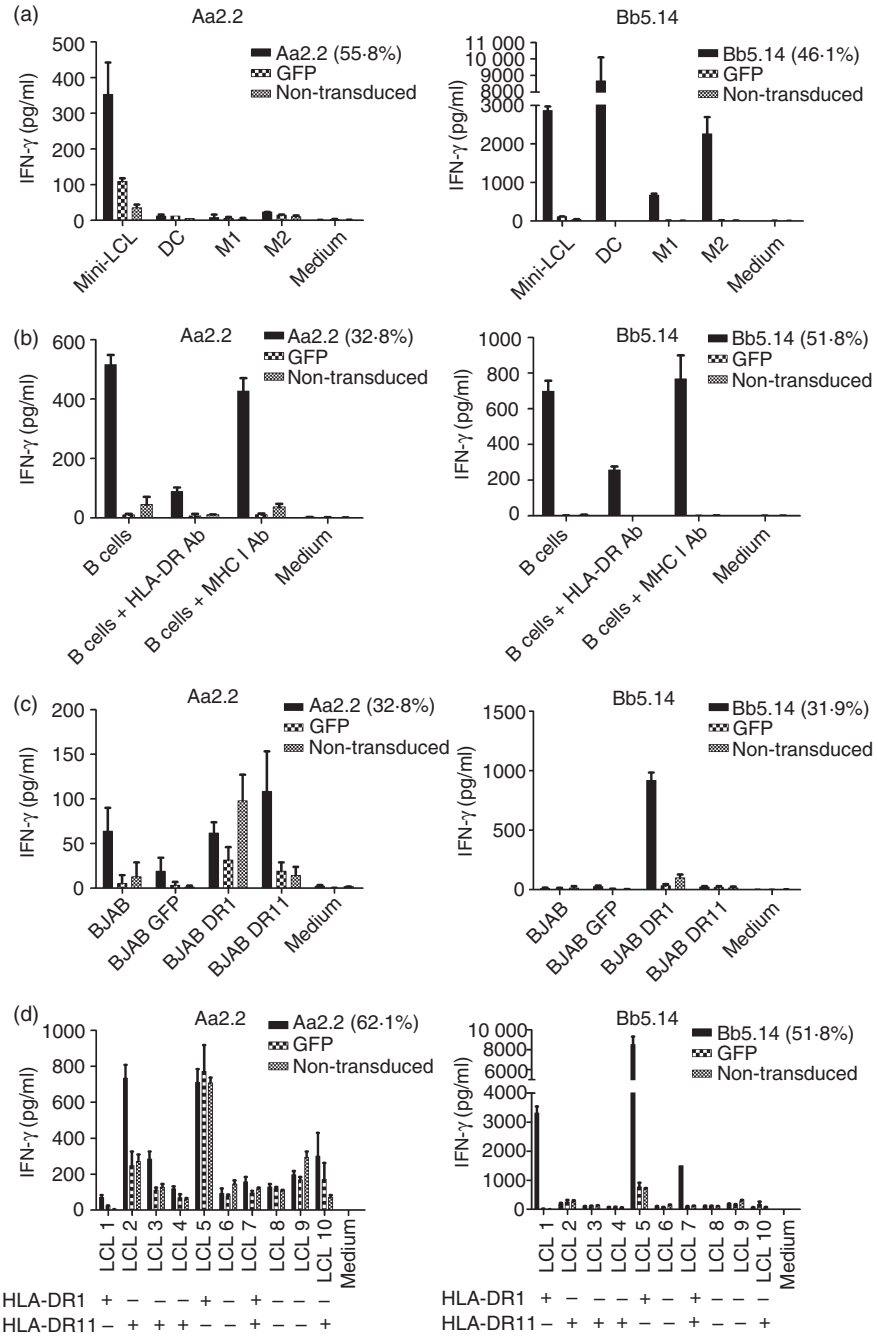

Figure 3. Aa2.2 and Bb5.14 reveal significant differences in functionality and reactivity pattern and recognize distinct MHC class II restriction elements. Both T-cell receptors (TCR) were analysed after transduction into autologous $CD4^+$ T cells and co-cultured with different target cells (E : T ratio = 5 : 1). Supernatants of mixed cultures were removed after 24 hr and analysed by interferon-γ (IFN-γ) ELISA. Percentages of $CD4^+$/$TCRmu^+$ cells at the time of the experiment are shown in each graph. GFP-transduced and non-transduced T cells were used as controls. SD of triplicates are shown. (a) Different professional antigen-presenting cells as Mini-LCL, dendiritic cells (DC) and macrophages differentiated into type M1 or M2 were used as targets 11 days after TCR transduction. One representative out of three independent experiments [1× peripheral blood mononuclear cells (PBMC), 1× $CD4^+$, 1× $CD8^+$ T cells] is shown. (b) Autologous B cells treated with anti-HLA-DR or anti-MHC class I antibodies were used as targets for determination of MHC restriction 6 days after TCR transduction. One representative out of five independent experiments (2× PBMC, 2× $CD4^+$, 1× $CD8^+$ T cells) is shown. (c) The distinct restriction element of both TCR was investigated by transfection of BJAB cells with donor-specific HLA-DR1, HLA-DR11 or GFP 12 days after TCR transduction. One representative out of three independent experiments (1× PBMC, 1× $CD4^+$ T cells, 1× allogeneic PBMC) is shown. (d) TCR-transduced effector T cells were incubated with partially HLA-DR-matched LCL and analysed for IFN-γ secretion 7 days after TCR transduction. One representative out of three independent experiments (1× PBMC, 1× $CD4^+$, 1× $CD8^+$ T cells) is shown.

effector T-cell populations (as indicated in detail in the figure legend of Fig. 3). Effector T cells transduced with Aa2.2 detected exclusively B-cell-derived cell lines, whereas Bb5.14-transduced cells secreted IFN-γ in response to different professional antigen-presenting cells as DC as well as M1 or M2 macrophages (Fig. 3a). Of note, reactivity of Bb5.14 was again much stronger compared with Aa2.2. Recognition of B cells by both TCR could be inhibited by treatment of target cells with HLA-DR blocking antibody whereas blocking with an MHC class I specific antibody was not effective (Fig. 3b). For definition of the restriction element and to exclude recognition of EBV-derived peptides, EBV$^-$ Burkitt BJAB cells were transfected with both DR-alleles of the donor, HLA-DR1 and HLA-DR11, or GFP (Fig. 3c). Recognition of EBV-derived peptides could be excluded as BJAB cells were recognized after transfection with the respective DR allele. Bb5.14 recognized BJAB after transfection of BJAB cells with HLA-DR1, whereas Aa2.2 specifically secreted IFN-γ in response to BJAB cells transfected with HLA-DR11 (Fig. 3c). Investigation of several partially HLA-matched LCL confirmed these results and excluded major alloreactivity of selected TCR (Fig. 3d) although reactivity of Aa2.2 was again low. Multifunctionality of TCR-transduced effector T cells was additionally tested demonstrating specific cytotoxicity and proliferation in response to target cells (see Supplementary material, Figs S2 and S3). Cytotoxicity of Bb5.14-transduced PBMC, CD4$^+$ and CD8$^+$ T cells was detected in response to B cells and Mini-LCL (Fig. S2). In contrast, Aa2.2-transduced effector cells recognized only B cells whereas no cytotoxicity of Aa2.2-transduced PBMC, CD4$^+$ and CD8$^+$ T cells could be observed against Mini-LCL (Fig. S2). Moreover, effector cells transduced with both TCR showed specific proliferation in response to B cells (Fig. S3).

Effector T cells transduced with Aa2.2 or Bb5.14 show distinct recognition of HLA-DR-matched primary leukaemia cells and non-haematopoietic transformed cell lines

We have demonstrated tumour reactivity of selected TCR by recognition of non-modified high-grade lymphoma BJAB cells. We further aimed to investigate recognition of primary leukaemic cells and transformed cell lines derived from non-haematopoietic tissue. We therefore tested the reactivity against diverse leukaemic cell samples from CLL and AML patients and diverse tumour cell lines matched for HLA-DR1 or HLA-DR11. Effector T cells transduced with Aa2.2 secreted substantial amounts of IFN-γ in response to all HLA-DR11-matched CLL cells, whereas Bb5.14 showed comparably low reactivity towards HLA-DR1-expressing CLL samples (Fig. 4a). In contrast, Bb5.14 secreted high amounts of IFN-γ in response to AML blasts expressing HLA-DR1, whereas Aa2.2 did not secrete IFN-γ in response to HLA-matched AML samples (Fig. 4b). Of note, treatment of leukaemic target cells with IFN-γ significantly increased recognition of leukaemic cells as untreated cells were only marginally detected (data not shown). Non-haematopoietic cells may also express MHC class II molecules after IFN-γ treatment and may be recognized by selected TCR. In fact, Bb5.14 secreted significant amounts of IFN-γ in response to IFN-γ-treated HLA-DR1-expressing tumour cell lines as RCC1.24 and Na-Mel 41 whereas Aa2.2 secreted no IFN-γ in response to matched WI-38 embryonic lung fibroblasts (Fig. 4c).

Cytokines secreted by TCR-transduced effector T cells show a mixed T helper lineage expression profile which is dependent on the target cells

Supernatants of TCR-transduced effector cells stimulated with selected target cells were additionally investigated by multi-cytokine analysis. Cytokine concentrations secreted by the GFP-transduced control T-cell populations were subtracted from values of Aa2.2 and Bb5.14 for reasons of clarity (Fig. 5) whereas primary data are listed in Table S1 (see Supplementary material). The cytokine profile secreted by PBMC transduced with both TCR represented a mixed lineage pattern in response to different target cells. However, differences in cytokine patterns were observed and seem to be dependent on characteristics of target cell, effector cell type and TCR. Whereas autologous PBMC or CD4$^+$ cells transduced with Aa2.2 showed a broad cytokine pattern in response to matched CLL cells, the same effector cell population transduced with Bb5.14 produced mainly IFN-γ at moderate levels and IL-10 by Bb5.14-transduced CD4$^+$ effector cells in response to matched CLL cells (Fig. 5a, and see Supplementary material, Fig. S4a). In contrast, incubation of matched AML blasts with Bb5.14 showed a broad cytokine response in both PBMC and CD4$^+$ cells whereas only a very limited set of cytokines was secreted by Aa2.2 in response to AML 4 (Fig. 5b, and see Supplementary material, S4b). This TCR mainly lacked specific reactivity against the other matched AML samples (Fig. 5b, and see Supplementary material, Fig. S4b). Otherwise, diverse samples derived from patients with identical diagnoses as CLL or AML showed differences in quality and quantity of secreted cytokines after stimulation of effector cells transduced by the same TCR although restriction to the defined MHC molecule was predominantly evident. Regarding non-haematopoietic targets, several cytokines were detected after co-incubation of Bb5.14 with RCC1.24 and Na-Mel 41 (Fig. 5c, and see Supplementary material, Fig. S4c). These data strongly suggest that cytokine patterns secreted by effector T cells transduced with these TCR seem to be dependent on characteristics of the distinct TCR, effector cell population and the target cell expressing the MHC restriction element and recognized peptide.

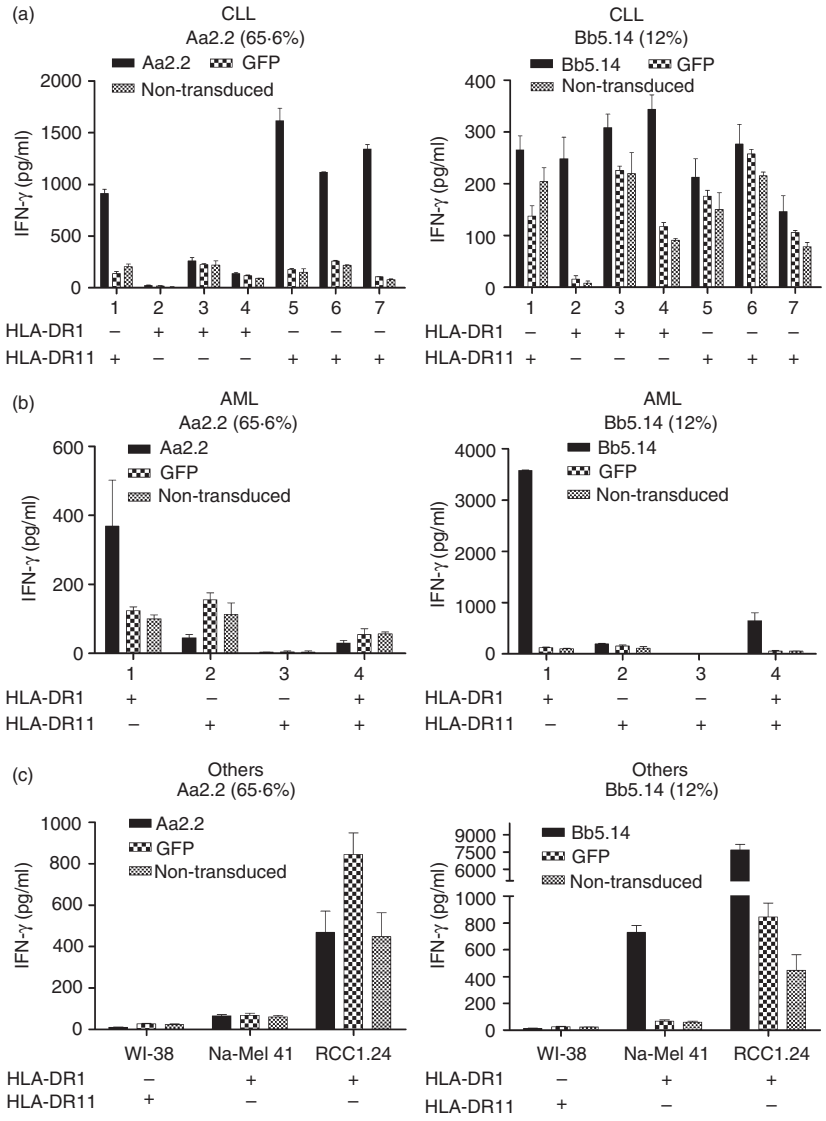

Figure 4. Aa2.2 and Bb5.14 show distinct tumour reactivity. Both T-cell receptors (TCR) were analysed after transfer into autologous effector T cells and co-cultured with different target cells (E : T ratio = 5 : 1). Supernatants of mixed cultures were removed after 24 hr and analysed by interferon-γ (IFN-γ) ELISA. Percentages of TCRmu$^+$ T cells at the time of the experiment are shown in each graph. GFP-transduced and non-transduced T cells were used as controls. SD of triplicates are shown. One representative out of two [1× peripheral blood mononuclear cells (PBMC), 1× CD4$^+$ T cells] independent experiments with transduced autologous effector cells is shown. (a) CD40L-activated, primary chronic lymphatic leukaemia (CLL) cell samples derived from seven patients (1–7) were used as targets for stimulation of TCR-transduced PBMC derived from the original donor 20 days after TCR transduction. (b) Primary acute myeloid leukaemia (AML) cells derived from four AML patients (1–4) were co-incubated with TCR-transduced PBMC derived from the original donor 13 days after TCR transduction. (c) Epithelial cell lines were co-cultured with TCR-transduced PBMC derived from the original donor 20 days after TCR transduction.

TLR ligand treatment of target B cells mediates the opposite effects on cytokine secretion by TCR-transduced effector T-cell populations depending on characteristics of the transgenic TCR

Toll-like receptor ligands are proposed as potential adjuvants for immunotherapies. Therefore, we investigated the influence of TLR treatment of target cells on TCR-transduced effector T cells. Unexpectedly, treatment of target cells with a number of different TLR ligands had a variable effect on the functionality of both TCR (Fig. 6). Interferon-γ secretion by Aa2.2 was greatly enhanced when target B cells were pre-treated with TLR ligand such as LPS, Poly(I : C), Flagellin and CpG (Fig. 6a). In contrast, Bb5.14 secreted less IFN-γ in response to the TLR-treated autologous B cells compared with untreated target cells (Fig. 6a). The same effect was observed regarding secretion of different cytokines analysed by Flow Cytomix (Fig. 6b). Similar effects on IFN-γ secretion were repeatedly observed

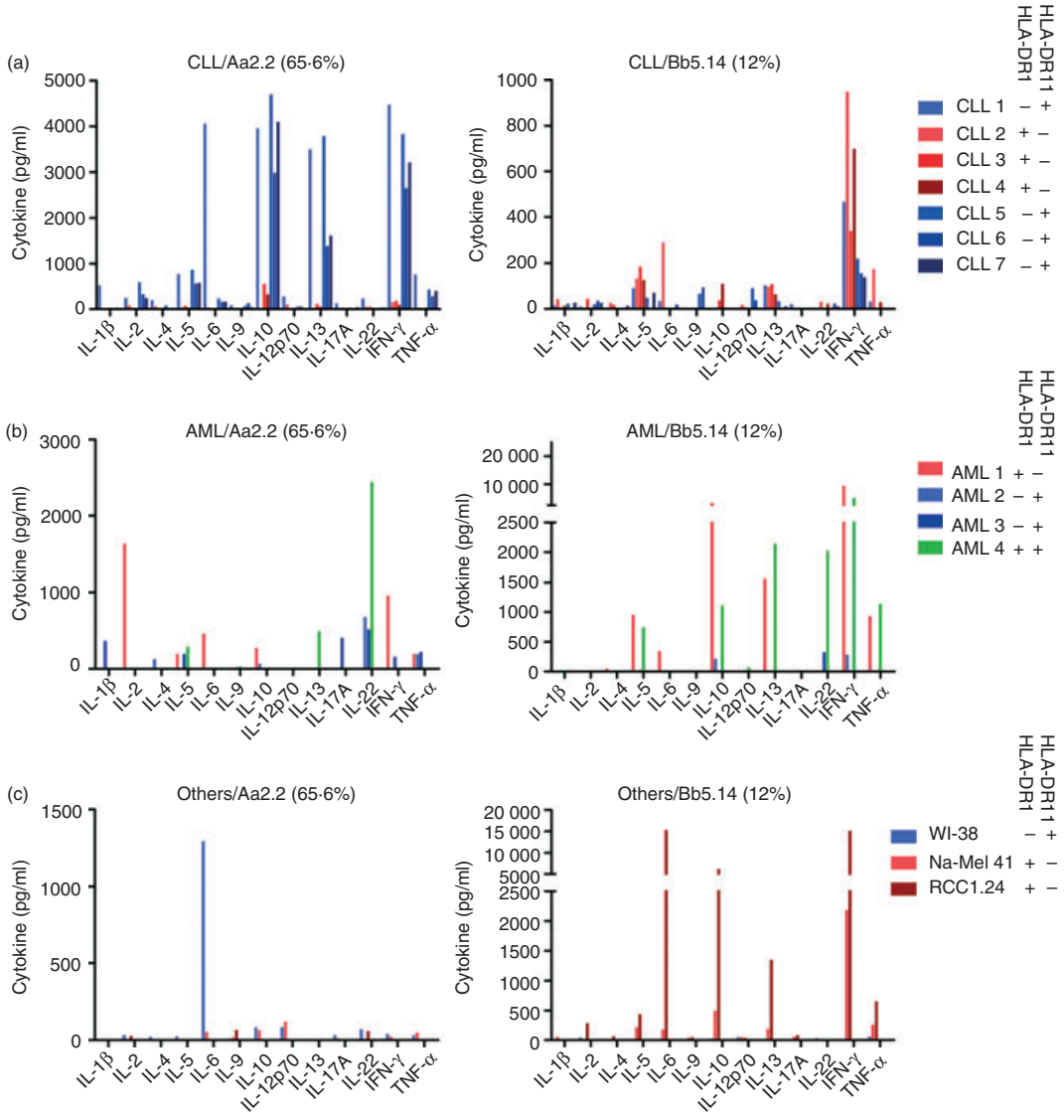

Figure 5. Cytokines secreted by T-cell receptor (TCR) -transduced effector T cells reveal a mixed T helper lineage pattern that is dependent on the TCR as well as the target cell. Supernatants collected from 24 hr stimulation assays as shown in Fig. 4 were investigated by Flow Cytomix. One representative out of two independent experiments [1× peripheral blood mononuclear cells (PBMC), 1× $CD4^+$ T cells] with transduced autologous effector cells is shown. Percentages of $TCRmu^+$ cells at the time-point of the assays are indicated in each graph. GFP-transduced effector T-cell populations were used as controls. The cytokine concentrations secreted by GFP-transduced effector T-cell populations were subtracted from the cytokine concentrations secreted by the TCR-transduced T-cell populations. The corresponding primary data are shown in Table S1 (see Supplementary material). (a) Chronic lymphatic leukaemia (CLL) cells were used as targets for stimulation of TCR-transduced PBMC derived from the original donor 20 days after TCR transduction. (b) Acute myeloid leukaemia (AML) blasts were co-incubated with TCR-transduced PBMC derived from the original donor 13 days after TCR transduction. (c) Epithelial cell lines were co-cultured with TCR-transduced PBMC derived from the original donor 20 days after TCR transduction.

in case of TCR-transduced autologous and allogeneic effector T cells (see Supplementary material, Fig. S5a), using TLR ligand-pulsed autologous B cells (see Supplementary material, Table S2). In contrast, treatment of T cells with TLR ligands before co-culture with untreated B cells showed no significant modulation of cytokine secretion by TCR-transduced effector T cells, suggesting an indirect effect on TCR function mediated by TLR ligand-treated B cells (see Supplementary material, Figs S5b and S6). The effect could be blocked by HLA-DR antibodies confirming association to TCR signalling (Fig. S5). Interferon-γ secretion of effector T cells transduced with both TCR was altered in a similar way in response to LPS-treated C1R cells transfected with the matching HLA-DR molecule further confirming TCR-dependent immune modulation (see Supplementary material, Fig. S7). Similar results regarding

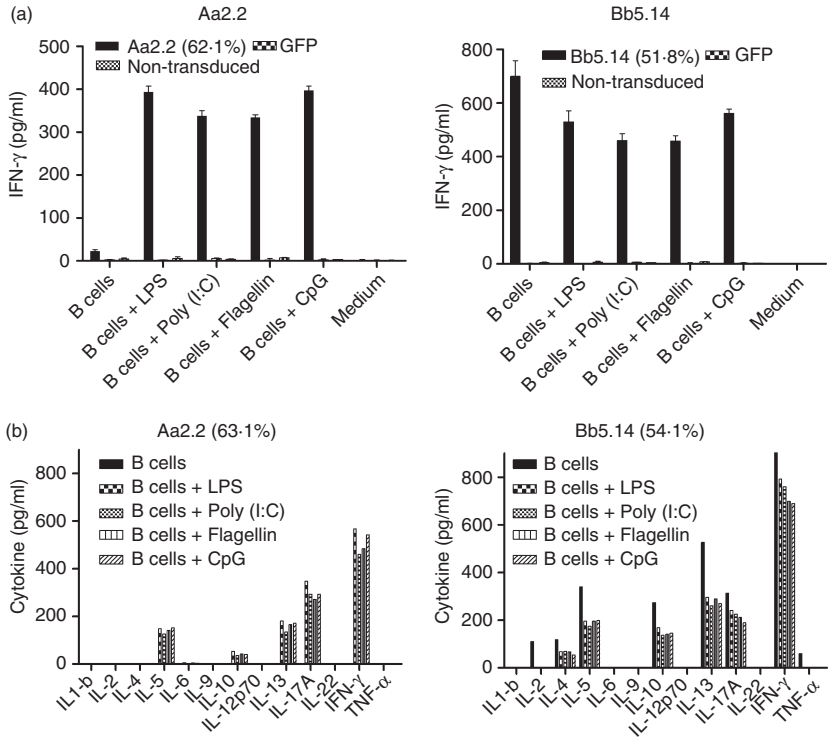

Figure 6. Toll-like receptor (TLR) ligand treatment of target B cells mediates opposite effects on cytokine secretion by T-cell receptor (TCR) -transduced effector T-cell populations depending on characteristics of the transgenic TCR. The effect of TLR ligand treatment of target B cells on the functionality of TCR-transduced autologous $CD4^+$ T cells and peripheral blood mononuclear cells (PBMC) was examined. Supernatants of mixed cell cultures (E : T ratio = 5 : 1) were collected after 24 hr. GFP-transduced and non-transduced effector T-cell populations were used as controls. One representative out of four independent experiments (1× PBMC, 1× $CD4^+$, 1× $CD8^+$ T cells and 1× allogeneic PBMC) with transduced effector cells investigating four TLR ligands in parallel is shown. (a) B cells were treated with different TLR ligands for 2 hr followed by intensive washing. B cells and B cells previously pulsed with different TLR ligands were used as targets for TCR-transduced autologous $CD4^+$ T cells and interferon-γ (IFN-γ) concentration was determined by IFN-γ ELISA. SD of triplicates are shown. $CD4^+$ T cells were used as effector T cells and percentages of $CD4^+$/$TCRmu^+$ cells at the time-point of the assays (5 days after transduction) are indicated in each graph. (b) Supernatants from similar stimulation assays using TCR-transduced PBMC were investigated by Flow Cytomix for secretion of multiple cytokines. Percentages of $TCRmu^+$ cells at the time-point of the assays (5 days after transduction) are indicated in each graph. Insignificant background levels for interleukin-2 and interleukin-13 were observed in GFP-transduced effector T cells (data not shown).

modulation of the T-cell responses by treatment of B cells with TLR ligands were observed investigating cytotoxicity of Aa2.2 and Bb5.14 TCR-transduced PBMC (see Supplementary material, Fig. S2). The Aa2.2-transduced effector PBMC and $CD8^+$ T cells showed significant cytotoxicity against autologous B cells only after pre-treatment of target cells with LPS and enhanced cytotoxicity in the case of transduced $CD4^+$ T cells (Fig. S2). In contrast, Bb5.14-transduced effector cells showed significant cytotoxicity against B cells and LPS-treated B cells (Fig. S2). Cytotoxicity was not enhanced but slightly reduced when B cells were pre-treated with LPS (Fig. S2).

Discussion

$CD4^+$ T cells have been demonstrated to be capable of inducing direct as well as indirect anti-tumour effects in the autologous environment and adoptive transfer of $CD4^+$ T cells resulted in efficient tumour rejection in murine models as well as primary clinical trials in humans.[15–17] In this project we aimed to isolate autologous $CD4^+$ T-cell-derived TCR with specificity for leukaemia-associated target antigens such as FMNL1. Identification of FMNL1 by SEREX suggests the presence of such FMNL1-specific $CD4^+$ T-cell responses although they might not be relevant for tumour targeting.[7] The DC are haematopoietic cells expressing haematopoiesis-specific antigens potentially useful for targeting acute and chronic leukaemias. Moreover, DC have been shown to express FMNL1.[4] We therefore used DC pulsed with recombinant FMNL1 protein. After repeated stimulation of T cells with FMNL1 protein-pulsed autologous DC, we were able to isolate two T helper clones and respective TCR with potent anti-leukaemic reactivity although specificity for FMNL1 was not evident. Both TCR demonstrated distinct reactivity patterns suggesting different recognized target epitopes undefined so far. Beside

FMNL1, DC present a high number of self antigens [33] which might potentially represent attractive tumour-associated antigens.[34] The TCR derived from unselected PBMC (Aa2.2) showed preferential recognition of B-cell-derived targets whereas other target cells were not detected, or were only marginally detected, suggesting that the recognized antigen may be preferentially presented by B cells. This TCR demonstrated strong reactivity in response to matched leukaemic cells derived from patients with CLL. In contrast, the other TCR, Bb5.14, derived from a $CD45RO^-$ naive T-cell population represented a TCR with high functionality in response to diverse MHC class II-expressing targets. There was a strong recognition of matched AML blasts inducing a broad panel of cytokines. In addition, other HLA-DR-matched transformed cell lines as renal cell carcinoma and melanoma were also detected. Hence, our data clearly demonstrate that TCR derived from autorestricted $CD4^+$ T cells may recognize potentially attractive tumour-associated self antigens resulting in anti-leukaemic and anti-tumour reactivity. Activated T cells normally expressing MHC class II are obviously not recognized as determined by absence of any cytokine secretion in response to these cells. T cells transduced with these TCR acquired the ability to kill target cells; however, we have not observed any sign of fratricide during expansion of TCR-transduced effector T cells suggesting that peptides derived from ubiquitously expressed genes are not targeted by these TCR. Of special interest, these TCR were successfully expressed in allogeneic cells showing no fratricide and being functional (see Supplementary material, Fig. S1). Hence, these TCR are highly interesting candidate tools for novel immunotherapies in patients with leukaemia and might be especially useful for patients with haematopoietic malignancies after stem cell transplantation with a haplo-identical or single MHC mismatched donor.

The cytokine profile secreted from T cells transduced with both TCR represented a mixed lineage pattern in response to different target cells including T helper type 1 (Th1) and Th2-associated cytokines. Our data correlate well with the mixed cytokine pattern secreted by T-cell clones derived from cancer patients after vaccination, which was described as being not compatible with the distinctive Th1/Th2 paradigm.[35] Secretion of IL-10 was often observed in our experiments, which may reflect a self-regulatory pathway.[36] In contrast, relevant levels of IL-4 were mostly not detected. The transfer of different MHC class II restricted TCR in identical effector T-cell populations allowed us to investigate the influence of the specific TCR on cytokine secretion. Interestingly, differences in cytokine patterns in response to different target cells seemed to be attributed to target, effector cell population and TCR. Both TCR demonstrated different quality and quantity of cytokine secretion in response to identical cellular targets. Otherwise, we observed variable secretion of cytokines in different effector populations transduced by a defined TCR after stimulation with distinct leukaemia samples belonging to the same disease entity. Although these results need to be further confirmed by investigation of additional patient samples, they potentially reflect distinct characteristics of individual leukaemic cells.[14,37,38] Hence, the specific interaction between target cell, effector cell population and TCR will decide the outcome after potential clinical application and it might be beneficial to test the secreted cytokine pattern before therapeutic application.

Innate immune modifiers such as TLR ligands can further modulate and enhance innate and adaptive anti-tumour effects and are intensively investigated as adjuvants in pre-clinical and clinical studies with, however, limited success.[39,40] We aimed to investigate if the reactivity of both TCR can be further enhanced by addition of TLR ligands to the target cells. Interestingly, effector T cells transduced with both TCR reacted differently. Whereas cytokine secretion of the functionally weaker Aa2.2 was highly enhanced after treatment of target B cells and C1R cells with diverse TLR ligands, cytokine secretion of the strong Bb5.14 was substantially reduced by the same treatment using the same effector population for TCR transfer. TLR ligand treatment of target B cells induced similarly an enhancement of cytotoxicity of Aa2.2-transduced T cells and a slight reduction of cytotoxicity by Bb5.14-transduced T cells. It has been previously reported that TLR agonists may mediate suppressive effects on T cells and several mechanisms are proposed as induction of IL-10, stimulation of regulatory T cells and indoleamine-2,3-dioxygenase-dependent immune suppression.[41] Our data suggest an indirect effect by B-cell modulation but demonstrate that the TCR itself plays an essential role if TLR ligands mediate stimulating or inhibitory effects. The exact pathway of enhanced stimulation of Aa2.2-transduced or suppression of Bb5.14-transduced T cells by TLR ligand treatment of target cells observed in our experiments has not been clarified yet. PD-L1 and regulatory T cells seem not to play a major role in preliminary experiments. Reduced reactivity of effector T cells may also originate from an excess of T-cell activation as described for CD28 co-stimulation.[42] This would explain the predominantly inhibiting effect of TLR ligands on the strong Bb5.14 and additionally the inconsistency in a few experiments (see Supplementary material, Table S2) potentially reflecting differences in T-cell condition. Although the underlying mechanism needs to be further clarified, these data clearly show that the effect of innate co-stimulation by TLR ligands depends on characteristics defined by the specific TCR. As TLR ligands might potentially be used as immune adjuvants in the clinical setting of an adoptive T-cell therapy, it will be of high interest to further investigate the here-observed TCR-dependent effects of TLR ligands.

In conclusion, we have isolated two distinct TCR from the autologous environment with reactivity against MHC class II expressing leukaemic cells. Moreover, characterization of these TCR point to a complex peripheral immune regulation influenced by effector cell, TCR and tumour cell. Hence, individualized effector T-cell-dependent and tumour-cell-dependent directed immune modulation may be of essential importance for the successful development of personalized anti-cancer immunotherapies. Further investigations will have to identify the exact recognized epitopes of both TCR and future *in vivo* experiments will further determine the potential of these attractive candidates for clinical application.

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Disclosures

There is no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. T-cell receptor (TCR) derived from isolated T-cell clones Aa2.2 and Bb5.14 are expressed after genetic transfer into allogeneic effector T cells and show no sign of T cell fratricide.

Figure S2. Diverse T-cell receptor (TCR) -transduced effector populations mediate specific cytotoxicity modulated by Toll-like receptor ligand treatment.

Figure S3. B cells have a stimulating effect on proliferation of effector cells transduced with both T-cell receptor (TCR).

Figure S4. Cytokines secreted by T-cell receptor (TCR) -transduced effector T cells reveal a mixed T helper lineage pattern which is dependent on the TCR as well as the target cell.

Figure S5. Opposite effects induced by Toll-like receptor (TLR) ligand treatment of target B cells are associated to characteristics of the transgenic T-cell receptor (TCR) additionally demonstrated by transduction of allogeneic effector cells.

Figure S6. Toll-like receptor (TLR) treatment of T-cell receptor (TCR) -transduced effector cells has no effect on functionality of transduced autologous T cells.

Figure S7. Toll-like receptor (TLR) ligand treatment of C1R cells transfected with selected HLA restriction elements mediates opposite effects on cytokine secretion by T-cell receptor (TCR) -transduced effector populations.

Table S1. Cytokine Response of Aa2.2 and Bb5.14 transduced effector cells in response to (A) chronic lymphatic leukaemia (CLL), (B) acute myeloid leukaemia (AML) and (C) non-haematopoietic cell lines.

Table S2. Immune modulation of T-cell receptor (TCR) -transduced T cells by Toll-like receptor (TLR) -ligand treated B cells.

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