Targeting pediatric cancers via T-cell recognition of the monomorphic MHC class I-related protein MR1

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ABSTRACT

Human leukocyte antigen (HLA) restriction of conventional T-cell targeting introduces complexity in generating T-cell therapy strategies for patients with cancer with diverse HLA-backgrounds. A subpopulation of atypical, major histocompatibility complex-I related protein 1 (MR1)-restricted T-cells, distinctive from mucosal-associated invariant T-cells (MAITs), was recently identified recognizing currently unidentified MR1-presented cancer-specific metabolites. It is hypothesized that the MC.7.G5 MR1T-cell clone has potential as a pan-cancer, pan-population T-cell immunotherapy approach. These cells are insensitive to healthy tissue while conferring T-cell receptor (TCR) dependent, HLA-independent cytotoxicity to a wide range of adult cancers. Studies so far are limited to adult malignancies. Here, we investigated the potential of MR1-targeting cellular therapy strategies in pediatric cancer. Bulk RNA sequencing data of primary pediatric tumors were analyzed to assess MR1 expression. In vitro pediatric tumor models were subsequently screened to evaluate their susceptibility to engineered MC.7.G5 TCR-expressing T-cells. Targeting capacity was correlated with qPCR-based MR1 mRNA and protein overexpression. RNA expression of MR1 in primary pediatric tumors varied widely within and between tumor entities. Notably, embryonal tumors exhibited significantly lower MR1 expression than other pediatric tumors. In line with this, most screened embryonal tumors displayed resistance to MR1-T-cell targeting. In vitro, MR1 susceptibility was observed particularly in pediatric leukemia and diffuse midline glioma models. This study demonstrates potential of MC.7.G5 MR1T-cell immunotherapy in pediatric leukemias and diffuse midline glioma, while activity against embryonal tumors was limited. The dismal prognosis associated with relapsed/refractory leukemias and high-grade brain tumors highlights the promise to improve survival rates of children with these cancers.

INTRODUCTION

Unconventional T-cell populations that recognize their ligand beyond the context of major histocompatibility complexes (MHC) are increasingly recognized for their potential in cellular cancer immunotherapy. Examples of unconventional T-cell populations include invariant NKT-cells (iNKT), γδ T-cells, mucosal-associated invariant T-cells (MAIT), and MR1T-cells. Unlike conventional T-cells, which rely on polymorphic MHC molecules for target recognition, these unconventional T-cells recognize their cognate target in the context of monomorphic MHC-like molecules.1 This majorly decreases the complexity of the generation of cell therapy products suitable for individuals with diverse human leukocyte antigen (HLA) backgrounds. One interesting unconventional T-cell population is the so-called MR1T-cell population, which recognizes their cognate target in the context of MHC class I-related protein 1 (MR1).

The MHC-I-like MR1 molecule is ubiquitously expressed in most nucleated human cells.2 Six different allelic MR1 variants are described, of which the vast majority of the population possess the wildtype MR1*01 allele.3 Unlike conventional MHC-I molecules, however, cell surface expression on healthy cells is low to undetectable.2 MR1 is retained in the endoplasmic reticulum until an antigen is loaded, resulting in transient cell surface expression after dimerization with β2M.4 MR1 complexes with intermediate metabolites produced by cells during metabolic stress, resulting in stress-induced MR1 translocation to the cell surface.5 Until recently, MR1 was mainly considered in
association with MAIT cells. MAITs recognize intermediate metabolites derived from bacterial vitamin B2 and B9 molecules presented in MR1 to detect bacterial infection in mucosal tissues.3,5,7

Interest in the field of cancer immunotherapy spiked after publication of two recent studies highlighting the discovery of a distinct subpopulation of cancer-specific MR1-restricted T-cells, distinctive from MAITs.3,6 These MR1T-cells recognize MR1-presented cancer-specific metabolite(s) on the cell surface of cancer cells, the exact nature of which remains unidentified.8 Crowther and colleagues isolated an MR1-restricted, tumor-reactive T-cell clone from peripheral blood of a healthy donor.5 The clone is irreversible to healthy tissue, while conferring HLA-independent cytotoxicity towards a diverse array of adult cancer cells. They emphasize the potential of this MC.7.G5 MR1T-cell clone as a pan-cancer, pan-population T-cell immunotherapy approach.9

The study conducted by Crowther et al. thus far focused exclusively on adult malignancies.6 Pediatric tumors exhibit distinct characteristics compared with adult tumors, including a scarcity of targetable molecules due to their low mutational burden. Consequently, there is a critical need to identify tumor-specific antigens in pediatric tumors. Even though both classified as ‘cancer’, it is now widely accepted that the pathobiology of pediatric tumors deviates from that of adult tumors.10 Therefore, it remains to be studied whether the MC.7.G5 MR1T-cell clone’s reactivity towards adult tumors translates to pediatric tumors. In the present study, we investigated the potential of MR1-targeted T-cell therapy in pediatric oncology using a comprehensive panel of pediatric tumor models.

METHODS
Bulk RNA sequencing

Parents of patients from whom tumors were RNA sequenced signed informed consent forms approved by the responsible authority. Sequencing library preparation and data preprocessing from fresh frozen primary tumor samples from the 812 included pediatric patients was done a priori according to the institute’s standardized pipelines and guidelines.11,12 In short, sequencing reads were aligned to the Genome Reference Consortium Human Build 38 assembly (GRCh38.p12) using STAR V.2.7.2d.13 Duplicates were marked using the Genome Analysis Toolkit’s (GATK) Picardtools V.2.20.1 MarkDuplicates. Base calling quality was reassessed with GATK V.4.2 BaseRecalibrator. Quality was assessed using Picard’s CollectMultipleMetrics, EstimateLibraryComplexity and MultiQC V.1.9 was used to aggregate all reports per sample. Exons based on the GENCODE V.31 gene annotation were quantified using FeatureCounts from the Subread V.1.6.4 package14 and normalized to gene counts per million (CPM). CPM for MR1, HLA-A, HLA-B, and HLA-C genes were retrieved via the Princess Máxima Center Biobank and Data Access Committee (BDAC) (EGAC00001001864). The code and data used in the analyses are available via https://github.com/VanHeeschLab/Cornel_et_al_2023.

Cell culture

In vitro pediatric cancer models, including neuroblastoma (NBL), acute myeloid leukemia (AML), B-cell/T-cell acute lymphoblastic leukemia (B-ALL, T-ALL), diffuse midline glioma (DMG), hepatocellular carcinoma (HCC), hepatoblastoma (HBL), and malignant rhabdoid tumors (MRT), were included; origin and culture conditions are summarized in table 1.13-24 The adult chronic myeloid leukemia K562 line and adult T-ALL Jurkat line were used as a positive control.6 MRI allelic variant expression of cell lines was derived from public databases (CCLE and Crown Bioscience). MR1 overexpressing cell lines were generated as described in the online supplemental methods. All cells were cultured under standard culturing conditions, refreshed and passaged biweekly, and checked every other month for mycoplasma infection and authenticity.

Generation and culturing of MC.7.G5 TCR+ T-cells

The MC.7.G5 TCR sequence was retrieved from the manuscript of Crowther and colleagues.9 MC.7.G5 TCR expressing T-cells were generated as described in the online supplemental methods.25-27 T-cells were stimulated once every other week using a rapid expansion protocol containing pooled irradiated peripheral blood mononuclear cells (35 Gy), LCL-TM (80 Gy), 1µg/mL PHA (Oxoid BV), and 50 U/mL IL-2 (Novartis) in RPMI supplemented with 5% pooled human serum and 1% P/S.28 T-cells were phenotyped by flow cytometry after expansion to confirm stable MC.7.G5 TCR expression. T-cells were used in functional assays 10–14 days after stimulation.

Functional T-cell assays

Cytotoxicity assay

The cytotoxic capacity of MC.7.G5 TCR+ T-cells was assessed using a flow cytometry-based cytotoxicity assay. Indicated target cells were labeled with Cell-Tracer Violet (CTV) (Life Technologies) according to manufacturer’s instructions. In cocultures with organoids and patient-derived xenograft cells, T-cells were labeled instead. Effects of labeling on the cytotoxic capacity of T-cells was ruled out. Cells were cocultured overnight (16–18 hours) at an effector-to-target ratio (E:T) of 10:1, unless otherwise indicated. After coculture, cells were spun down, supernatant was partly removed, and 7-AAD was added to evaluate cytotoxicity.

Cytokine secretion assay

Indicated target cells were co-cultured with MC.7.G5 TCR+ T-cells (or donor-matched, transduced controls) overnight at an E:T of 1:1. Supernatants were harvested to determine secretion of cytokines using Legendplex according to manufacturer’s instructions (Biolegend).
Table 1  List of utilized in vitro pediatric cancer models, culture conditions, and cell sources

<table>
<thead>
<tr>
<th>AML model</th>
<th>Type of model</th>
<th>Culture condition</th>
<th>Source (identifier)</th>
</tr>
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<tbody>
<tr>
<td>Kasumi-1</td>
<td>Cell lines</td>
<td>Standard RPMI</td>
<td>Princess Máxima Center (CVCL_0589)</td>
</tr>
<tr>
<td>SKNO1</td>
<td>RPMI+Glutamax supplemented with 20% FCS, 1% P/S and 10ng/mL GM-CSF (Miltenyi Biotec).</td>
<td>Princess Máxima Center (CVCL_2198)</td>
<td></td>
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<tr>
<td>PDX01</td>
<td>PDX material derived from primary t(8;21) pediatric acute myeloid leukemia was cocultured with human bone-marrow derived MSCs as feeder layer, MSCs were cultured in low glucose DMEM (Life Technologies) supplemented with 20% FCS, 8ng/mL FGF-2 (PeproTech) and 1% P/S and were seeded in 24 well plate at a density of 7500 cell/cm2 1 day prior to PDX culture.</td>
<td>Princess Máxima Center (CVCL_2198)</td>
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B-ALL

| NALM6     | Cell Lines   | Standard RPMI     | Princess Máxima Center (CVCL_0092) |
| RCH-ACV   |              |                   | Princess Máxima Center (CVCL_1851) |
| SUPB-15   |              |                   | Princess Máxima Center (CVCL_0103) |
| 697       |              |                   | Princess Máxima Center (CVCL_0079) |

T-ALL

| MOLT16    | Cell lines   | Standard RPMI     | Princess Máxima Center (CVCL_1424) |
| CCRF-CEM  |              |                   | Princess Máxima Center (CVCL_0207) |

HCC

| pHCC      | Organoid     | The culture medium was based on Salzman et al. with modifications as reported in. The medium consisted of the following: Advanced DMEM/F12 supplemented with 1% P/S, 1% GlutamAX, 10mM HEPES, 1x B27 supplement, 1x N2 supplement (all Life Technologies), 0.2% Normocin (Invivogen), 1.25mM N-acetyl-L-cysteine, 10% (vol/vol) RSPO1 conditioned medium, 10mM nicotinamide, 10mM recombinant human HGF (Both Peprotech), 10µM forskolin, 5µM AB301 (Both Tocris Bioscience), 10µM Y27632 (Abmole), and 0.5nM WNT surrogate. Cell culture and passing were performed as described by Kluiver et al. Baseline membrane extract (BME) (R&D systems) 3D-cultures were dissociated using dispase and TripleE (both Life Technologies). Cells were washed and resuspended in the culture medium above + 2% FCS. Plates were coated with collagen I (Corning) and cells were cultured in 2D-setting before assays were initiated. | Princess Máxima Center (CVCL_1520) |

NBL

| GIMEN     | Cell lines   | DMEM+Glutamax supplemented with 10% FCS, 1% P/S, 2% non-essential amino acids (Life Technologies). | Amsterdam Medical Center (CVCL_1232) |
| IMR32     |              |                   | Princess Máxima Center (CVCL_0346) |
| CHP212    | RPMI+Glutamax supplemented with 10% FCS, 1% P/S, 2% non-essential amino acids. | Dana Farber Institute (CVCL_1125) |
| SMSKAN    |              |                   | Dana Farber Institute (CVCL_7131) |
| SKNDZ     |              |                   | Dana Farber Institute (CVCL_1701) |
| NLF       |              |                   | Dana Farber Institute (CVCL_E217) |

691b Organoids

| 039       | Organoids    | NBL organoids were established, characterized and cultured as described previously. Organoids were maintained in DMEM low glucose, Glutamax-supplemented medium, supplemented with 25% HAM's F-12 Nutrient Mix, 2% B-27 supplement minus vitamin A, 1% N-2 Supplement, 1% P/S (all Life Technologies), 20ng/mL Animal-Free Recombinant Human-EGF, 40ng/mL FGF-basic, 200ng/mL IGF-I, 10ng/mL PDGF-AA, and 10ng/mL PDGF-BB (all Peprotech). | Princess Máxima Center |

MRT

Continued
MHC-I blocking assay

MHC-I independent cytotoxicity was confirmed in an MHC-I blocking assay. 697 target cells were preincubated with 50 ng/mL panHLA-ABC blocking antibody (Clone W6/32, Biolegend) for 1 hour.29 Cells were subsequently cultured with effector cells at an E:T of 10:1 for 5 hours. HLA-A2 restricted Wilms’ Tumor 1 (WT1)-specific T-cells30 acted as a positive control for MHC-I block. Isotype IgG antibody acted as a negative control.29

Flow cytometry analyses were performed on a BD FACSCanto (cytotoxicity and MHC-I blocking) or BD Fortessa (TCR expression & Cytokine Secretion Assay) (BD Biosciences). Results were standardized to cocultures with untransduced, donor-matched T-cells (online supplemental figure S3) using the formula: % cytotoxicity = (% dead cells in MC.7.G5 coculture − % dead cells in untransduced coculture)/(100 − % dead cells in untransduced coculture) × 100. Targeting was defined as >15% standardized killing. Data are shown as mean±SEM. K562 served as a positive control in all performed assays.

Quantitative real-time PCR analysis

Cells were pelleted, RNA was extracted with the RNeasy mini extraction kit (Qiagen), and cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit with random hexamer primers (Life Technologies). mRNA expression of MIRI was subsequently quantified using SYBR Select Master Mix (Life Technologies), the forward 5'-TAATGGGCTGACACCATCAAA-3' and reverse 5'-GGTCTTTCCCCATACTCAGAAATC-3' MIRI primers. Expression was determined relative to β-actin (forward primer: 5'-AGCCGGGAAATCGTGCGTGAC-3', reverse primer: 5'-CAATGTTGATGACCTTGCCTGTTGAC-3'). Expression analysis was performed using the QuantStudio 3 Real-Time PCR System (Life Technologies). Relative expression is visualized as 2^ΔCt, data shown as mean±SD.

Single cell RNA sequencing data analysis

Single cell RNA-seq data of HBL organoid models HB13E and HB13F were retrieved from and analyzed as in Kluiver and Lu et al.17 The immune activation score gene signature was based on Sengupta et al.31 and scores were calculated using the Seurat’s AddModuleScore function.32 Gene set enrichment analysis was performed using the fgsea package,33 with GO Biological Process gene sets downloaded from www.gsea-msigdb.org.

Data analysis

Flow cytometric analyses were performed with FlowJo V.10.7.1. Graphs were generated using Rstudio and Graphpad Prism V.9. Differences in expression between two (embryonal vs other tumors) or more groups (different tumor entities) were evaluated using a non-parametric Mann-Whitney U or Kruskall-Wallis test between separate groups, respectively. Correlation between two genes were calculated using Pearson correlation, correlation between killing and MIRI expression was calculated using linear regression. P values of<0.05 were considered significant.
RESULTS
Transcriptome analysis of primary pediatric tumors reveals variable MR1 RNA expression across tumor entities

MR1 RNA expression was analyzed using bulk RNA-seq in 812 primary pediatric tumors from newly diagnosed patients. Despite the large variation in expression within and between tumor entities, significant differences in MR1 expression were observed between tumor types (p<0.0001) (figure 1A). Across all tumors, low/high grade glioma (L/HGG), B-cell and T-cell acute lymphoblastic leukemia (B/T-ALL), acute myeloid leukemia (AML), and ependymoma expressed relatively high levels of MR1. Interestingly, tumors classified as embryonal (medulloblastoma (MB), atypical teratoid rhabdoid tumors (ATRT), neuroblastoma (NBL), malignant rhabdoid tumors (MRT), and hepatoblastoma (HBL)) expressed significantly lower levels of MR1 (p<0.0001) compared with other types of tumors (figure 1B).

Embryonal tumors present in early childhood are thought to develop due to neoplastic transformation of embryonal cell stages during fetal development.34 Many of these tumors exert low immunogenicity,29 35–38 among

Figure 1  MR1 mRNA expression across pediatric primary tumor entities. (A) MR1 mRNA expression levels (counts per million) across pediatric tumor entities around diagnosis. Primary tumor biopsies are routinely bulk RNA sequenced at time of diagnosis. Statistical differences between tumor entities were calculated using a Kruskal-Wallis test. (B) MR1 RNA expression differences (counts per million) between embryonal (MB, ATRT, MRT, NBL, and HBL) and other tumor entities. Statistical differences were calculated using a Mann-Whitney U test. (C) Correlation between MR1 and HLA-C expression in pediatric primary tumors. + : embryonal tumors, • : other tumors. Log2 transformed data are shown. Correlation was determined using Pearson correlation. AML, acute myeloid leukemia; ATRT, atypical teratoid rhabdoid tumors; B-ALL, B-cell acute lymphoblastic leukemia; EPN, ependymoma; HBL, hepatoblastoma; HCC, hepatocellular carcinoma; HGG, high-grade glioma; LGG, low-grade glioma; MB, medulloblastoma; MHC-I, major histocompatibility complex I; MR1, MHC-I related protein 1; MRT, malignant rhabdoid tumors; NBL, neuroblastoma; RMS, rhabdomyosarcoma; T-ALL, T-cell acute lymphoblastic leukemia.
others characterized by low expression of the MHC-I coding HLA-A/B/C genes, which is thought to be a derivative of their origin. We determined correlation of MR1 and HLA-A/B/C expression (figure 1C (HLA-C) and online supplemental figure S1 (HLA-A and HLA-B)) and observed a significant correlation between expression of these markers (r=0.71–0.72, p<0.001). This supports the hypothesis that low expression of MR1 is part of the low immunogenic phenotype of embryonal tumors.

**MC.7.G5 MR1T-cells target pediatric leukemias and glioma, but not neuroblastoma and malignant rhabdoid tumors**

We next screened available in vitro pediatric tumor models for their susceptibility to MR1T-cell targeting. MC.7.G5 MR1T-cells were generated by lentiviral TCR introduction and variable beta chain-based FACS sorting (online supplemental figure S2A), after which functionality and MHC-I independency was confirmed in line with previously confirmed susceptibility (online supplemental figure S2B, C).6 Overnight coculture experiments were conducted to assess the targeting capacity of MR1T-cells in cell lines and patient-derived organoids (figure 2A). Adult leukemia lines served as positive controls (K562 and Jurkat6). We observed a variable targeting capacity among screened pediatric cancer models. The largest potential of MR1-directed T-cell therapy was observed in DMG and leukemic models. Both screened patient-derived DMG models were targeted by MC.7.G5 MR1T cells. Similarly, the majority of screened leukemic models demonstrated susceptibility to MC.7.G5 MR1T targeting. Interestingly, each leukemic tumor entity included one resistant model. In contrast, most of the screened NBL models (8 out of 9) and all screened MRT models were resistant to MC.7.G5 targeting. Presence of cytokines and cytotoxic granular proteins in supernatants of targeted cells validated these findings (online supplemental figure S4). Consistent with the observed lower expression of MR1 observed in embryonal tumors (figure 1), our results demonstrated that embryonal tumor models displayed significantly reduced susceptibility to MC.7.G5 MR1T-cell targeting compared with other tumor entities (figure 2B). Targeting was not restricted to an allelic MR1 variant (online supplemental table S1).

Given the unknown MR1-presented ligand recognized by the MC.7.G5 TCR, we were unable to rely on presence of the metabolite in tumors to predict susceptibility to MC.7.G5 targeting. Therefore, we investigated whether MR1 expression could serve as a reliable marker to predict susceptibility. Since MR1 surface expression required for cancer cell recognition is below the threshold for antibody staining,6 we quantified MR1 mRNA expression levels and correlated this with susceptibility to MC.7.G5-mediated cytotoxicity. A significant but weak correlation (p<0.001, R²=0.385) was observed between MR1 expression and susceptibility to targeting (figure 2C). All tumor models with a high relative expression (>0.01) were targeted, models with a low relative MR1 expression (<0.002) were resistant to MC.7.G5-mediated cytotoxicity. Sensitivity of models with intermediate MR1 expression levels was less predictive (0.002–0.01). Overall, cells susceptible to MC.7.G5 MR1T-targeting exhibited higher relative MR1 expression compared with resistant cells (p<0.001) (figure 2D).

To further substantiate the hypothesis that low MR1 expression, and not availability of the targeted antigen, limits MC7.G5 targeting, we induced overexpression of MR1 in three resistant tumor models (CHP212, IMR32, and CCRF-CEM) and K562 as a positive control (online supplemental figure S5). MR1 overexpression significantly sensitized resistant models to MC.7.G5 MR1T-cell cytotoxicity (figure 2E). We next compared single cell sequencing data of the two screened HBL models that were established from the same patient, of which HB13F was susceptible to MC.7.G5 MR1T-cell targeting, while HB13E was resistant. While we observe major differences in both immunogenicity (figure 2F) and metabolic pathway enrichment (figure 2G) between the two models, we do not find enrichment of metabolic gene sets involved in nucleobase adduct generation and formation of reactive oxygen species, two processes recently suggested to be important in generation of MR1 presentable antigens (online supplemental figure S6).

**DISCUSSION**

The recent identification of T-cells that recognize an unknown cancer-specific metabolite presented by the monomorphic MHC-I-related protein MR1 posed the question of their potential as a pan-cancer, pan-population cellular immunotherapy approach.6 In this study, we investigated the potential of MR1-targeting cellular therapy strategies in pediatric cancer. Our findings revealed promise of targeting pediatric leukemias and brain tumors with MC.7.G5 T-cell-based immunotherapy, while targeting of embryonal cell-derived pediatric tumors was limited.

Pediatric tumors share a common low mutational burden, which critically limits the development of tumor-specific immunotherapeutic strategies.39 40 Metabolic dysregulation is observed across all pediatric tumors, making the targeting of aberrant metabolites presented in the context of MR1 of particular interest. It is hypothesized that tumor antigens with a tumor-supporting role have the highest potential as a therapeutic target, as this could minimize the risk of immune escape due to antigen loss.41 The CRISPR-Cas9 screen that identified MR1 specificity of the MC.7.G5 clone6 did not yield genes related to metabolic pathway(s) involved in the generation of the unknown MR1-presented metabolite. This observation suggests that the ligand of interest is likely part of a metabolic pathway essential for cancer cell survival. Interestingly, a more recent CRISPR-Cas9 screen using several other MR1T-cell clones, found that formation of nucleobase adducts, reactive oxygen species, and carbonyl species are all important in the formation of the MR1-presented antigenic metabolite(s).8
**Figure 2** Heterogeneity in MC.7.G5 MR1T-cell targeting of pediatric tumor models. (A) Standardized % killing after overnight coculture of MC.7.G5 MR1T-cells at an effector-to-target ratio of 10:1 (and 1:1 for Jurkats & Nalm-6, as aspecific killing was observed in controls at higher ratios). Killing was standardized to cocultures with untransduced, donor-matched T-cells. Striped bars represent patient-derived organoid or PDX samples, filled bars represent cell lines. Tumor types marked with * are considered embryonal. K562 cells were taken along as a positive control in every assay. 039, JD081T, JD041T, 78T2: n=2, rest: n≥3. (B) Standardized % killing in embryonal tumors (MRT, NBL, and HB) compared to the other screened tumor entities. Statistical differences were calculated using a Mann-Whitney U test. ***p<0.001 (C) Correlation between standardized killing % and relative MR1 expression levels (2−ΔCt) quantified by qPCR. Colors are matched to (A). Correlation was determined using simple linear regression. (D) Relative MR1 expression (2−ΔCt) in MC.7.G5 targeted and non-targeted pediatric cancer models. Susceptibility to targeting was defined as >15% standardized killing. Statistical differences were calculated using a Mann-Whitney U test. **p<0.01 (E) Standardized % killing after overnight coculture of MC.7.G5 MR1T-cells with wildtype or MR1 overexpressing target cells at an effector-to-target ratio of 10:1. Killing was standardized to cocultures with untransduced, donor-matched T-cells. K562 cells were taken along as a positive control. Colors are matched to (A). n=2, five replicates. Statistical differences were calculated using a Mann-Whitney U test. *p<0.05, **p<0.01 (F) Immune activation score calculated from single cell RNAseq data from MC.7.G5-resistant HB13E and MC.7.G5-sensitive HB13F HBL cells. Statistical differences were calculated using a Mann-Whitney U test. ***p<0.001 (G) Gene set enrichment analysis of gene sets involved in metabolism comparing single cell sequencing data from MC.7.G5 resistant HB13E and MC.7.G5 sensitive HB13F HBL organoids. Significantly enriched processes are shown (p<0.05). AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; DMG, diffuse-midline glioma (a type of high-grade glioma); HB, hepatoblastoma; MRTs, malignant rhabdoid tumors; NBL, neuroblastoma; PDX, patient-derived xenograft; T-ALL, T-cell acute lymphoblastic leukemia.
The current study indicates that embryonal tumors, characterized by a low immunogenic phenotype, also have low or absent MR1 expression. This limits the applicability of MR1-targeting immunotherapies in embryonal tumors. Nonetheless, the fact that two of the screened embryonal tumor models (GIMEN and HB13F) are susceptible to MC.7.G5 targeting does suggest the potential of inducing MR1 presentation in these tumor types. Interestingly, the GIMEN cell line is the only model out of the eight screened NBL models that reflects the mesenchymal NBL cell lineage, which is reported to be more immunogenic than adrenergic counterparts. In addition, we previously established two different hepatoblastoma organoid models using tumor tissue from the same patient. The HB13F organoid, which resembles the more differentiated fetal HBL tumor subtype, is susceptible to MC7.G5 targeting, while HB13E model, resembling the poorly differentiated embryonal HBL tumor subtype, is resistant to such targeting. The targeting potential in a subset of embryonal tumors indicates the opportunity to induce susceptibility to MR1-restricted immunotherapeutic strategies. The fact that both MR1-sensitive and MR1-resistant cancer cells can be isolated from the same patient does, however, warrant studying of MR1 downregulation as a resistance mechanism against MR1-based immunotherapies.

It should be noted that the MC.7.G5 sensitivity of leukemic models was variable. This may be resulting from the stage of hematopoiesis from which the leukemic cells arose. The resistant Kasumi-1 line, for example, arose from very early myeloid stem cells that may lack immunological features like MR1 expression. This further substantiates the hypothesis that the low immunogenicity of embryonal tumors causes a lack of susceptibility to MR1-mediated cytotoxicity. Future studies into cancer-specific MR1-targeting immunotherapy strategies should focus on stratification of subtypes of tumor entities susceptible to MR1 targeting. This will help to identify the specific tumor subtypes that would benefit from MR1-targeting immunotherapies.

We studied the correlation between MR1 mRNA levels and sensitivity to targeting by the MC.7.G5 TCR to determine whether targeting efficacy could be predicted. Although the correlation was significant, the data indicate that mRNA expression of MR1 is not the only prerequisite for efficient targeting. Overexpression of MR1 in resistant cell lines, however, did sensitize cells to MC.7.G5 cytotoxicity, indicating that the targeted antigen is present in these resistant models. Unraveling the presented metabolite(s) to MR1 is key to efficiently predict, and potentially pharmacologically increase, sensitivity to MR1-cell cytotoxicity.

Recent studies found several single nucleotide polymorphisms (SNPs) in the MR1 gene, generating six different allelic MR1 variants. One of these SNPs, present in the MR1*04 allelic variant represented in 1% of the population, diminishes loading of the MAIT ligand 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil. This indicates that MR1 is not as monomorphic as previously assumed, which may have implications for the pan-population applicability of MR1-based immunotherapies. A recent study suggests that a murinized version of the MC.7.G5 TCR may preferentially recognize ligand loaded MR1*04 complexes, also on healthy B-cells and monocytes of MR1*04 individuals. Consequently, it is important to study on-target and off-target reactivity of MR1-based immunotherapies in tumor and healthy tissue of individuals with the several MR1 allelic variants. The fact that we and/or Crowther and colleagues show reactivity of the MC.7.G5 clone against MR1*01 (71% population), MR1*02 (25% population), and MR1*04 (1% population) expressing cancer cells indicates on-target reactivity of the clone across several allelic variants.

In conclusion, while MR1T-cell therapy may not be universally applicable to all pediatric cancers, it holds significant potential for the treatment of various pediatric tumor types. Future studies should particularly focus on the potential of MR1 targeting therapy in patients with relapsed/refractory AML and T-ALL, DMG, and ependymoma, as these patients currently have a dismal prognosis. The unique features of MR1, including its relatively monomorphic nature, widespread expression in various pediatric tumors, and the tumor-specific metabolic disruption targeted by MR1T-cells, highlight the promise of MR1-based cellular therapy as a cost-effective approach to improve the survival rates of children with cancer.

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Contributors
The manuscript was written by AMC with critical comments of SN, WCP, OTH, SHv, JvdE, EH, JK, ZS, LNN. The pediatric cancer models were cultured by LvdS, MA, Mvh, ED, AMC, NMMD, JMvH, DAmhVdB, JLB, Auc, FB, YL, and AKH. Functional assays and qPCR were performed and supervised by LvdS, MA, AMC, ED, Mvh, NMMD, LD. RNA sequencing analysis was performed by Jvd, TAK, APFL. Figures were generated by AMC, JvD, TAK, LvdS. Conceptualization was performed by AMC and SN.

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Competing interests
ZS and JK are inventors on different patents for γδ TCR sequences, recognition mechanisms and isolation strategies. JK is scientific co-founder and shareholder of Gadeta (www.gadeta.nl). The remaining authors declare no competing interests.

Patient consent for publication
Not applicable.

Ethics approval
Not applicable.

Provenance and peer review
Not commissioned; externally peer reviewed.

Data availability statement
Data are available in a public, open access repository. CPM for MR1, HLA-A, HLA-B, and HLA-C genes were retrieved via the Princess Máxima Center Biobank and Data Access Committee (BDAC) (EGAC000001001864). The code and data used in the analyses are available via https://github.com/VanHeeschLab/Cornel_et_al_2023. The single cell RNA sequencing data from two analyzed hepatoblastoma organoids are available via ref.6. Data of functional assays is available upon request.
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REFERENCES


**Supplementary Methods**

**Generation of MR1 overexpressing cell lines**

The MR1 transgene was synthesized (Genscript) and cloned into the pCCL-cPPT-hPGK-ORF-bPRE4-SIN lentiviral transfer vector[36]. Lentiviral particles were produced using standard calcium phosphate transfection (Clontech) of HEK-293T-cells with the pMDL-g/pRRE, pMD2-VSVg, and pRSV-Rev plasmids[37,38]. Transducing units per mL of concentrated vector batches were determined using serial dilution on Jurkat cells followed by flow cytometric analysis of MR1 expression (anti-MR1 APC (8F2.F9, Biolegend)). Cells were transduced at a multiplicity of infection (MOI) of 2.5 and FACS-sorted on MR1 expression (Sony SH800S Cell Sorter) (**Supplementary Figure 5**).

**Generation of MC.7.G5 TCR expressing T-cells**

The MC.7.G5 TCR sequence was obtained from the manuscript of Crowther and colleagues[5]. A furin cleavage site was inserted upstream of a T2A sequence linking the TCR beta and alpha chains. The gene was synthesized (Genscript) and cloned into the pCCL-cPPT-hPGK-ORF-bPRE4-SIN lentiviral transfer vector[36]. Lentiviral particles were produced using standard calcium phosphate transfection (Clontech) of HEK-293T-cells with the pMDL-g/pRRE, pMD2-VSVg, and pRSV-Rev plasmids[37,38]. Transducing units per mL of concentrated vector batches were determined using serial dilution on Jurkat cells followed by flow cytometric analysis of Vβ11 expression (anti-Vβ11 FITC (KT11, Biolegend)).

Cord blood (CB) from healthy donors was collected under informed consent in accordance with the Declaration of Helsinki, and collection protocol approved by the Ethical Committee of the UMC Utrecht. CB mononuclear cells were isolated through Ficoll-Paque PLUS separation (GE Healthcare Biosciences AB), after which CD8⁺ T-cells were separated using magnetic bead separation (Miltenyi Biotec). CD8⁺ T-cells were stimulated and transduced at an MOI of 10 according to a previously described protocol[36]. 7-10 days after transduction, cells were FACS-sorted on Vβ11 expression (BD FACSAria II), using a panel of anti-Vβ11 FITC, anti-CD3 BV510 (Biolegend, OKT3), anti-CD8 APC (RPA-T8, BD Biosciences), and 7-AAD (BD Biosciences).
Supplementary Figures

Supplementary Figure 1. MR1 expression correlates with HLA-A and HLA-B expression in primary pediatric tumor samples.

Correlation between MR1 and HLA-A (A) and HLA-B (B) in pediatric primary tumors. +: embryonal tumors, #: other tumors. Log2 transformed data is shown. Correlation was determined using Pearson correlation.

Supplementary Figure 2. Generated engineered CD8+ MC.7.G5 MR1T-cells are functional.

(A) TCR expression in transduced, FACS-sorted T-cells (grey) compared to untransduced, donor-matched cells (white). TCR expression was determined using variable beta chain 11 (Vβ11) expression. (B) Left: TCR functionality was determined in an overnight killing assay at an effector-to-target (E:T) ratio of 10:1. Standardized % of killing of MC.7.G5 MR1T-cells (grey) and untransduced, donor-matched counterparts (white) during co-culture with 697 and K562 target cells. Killing was standardized to target only wells, 697: n=3, K562: n=4. Right: TNFa and IFNγ secretion (in pg/mL) was determined in overnight cultures at an E:T of 1:1. Secretion in co-cultures with...
untransduced T-cells was under the detection limit, n=2 per cell line. (C) Standardized killing % of MC.7.GS T-cells in a 5h co-culture at an E:T of 10:1 when MHC-I mediated cytotoxicity is blocked using a panHLA-ABC blocking antibody. MHC-I dependent WT1$_{37}$-specific T-cells function as a positive control for MHC-I block, an IgG isotype antibody acted as a negative control.

Supplementary Figure 3. Non-specific targeting of untransduced T-cells

Standardized % of killing after overnight co-culture of untransduced T-cells, matched to the utilized MC.7.GS MR1T-cells, at an effector-to-target ratio of 10:1 (and 1:1 for Jurkats & Nalm-6, as aspecific killing was observed in controls at higher ratios). Killing was standardized to wells with target only. Striped bars represent patient-derived organoid or PDX samples, filled bars represent cell lines. Co-culture with K562 cells was taken along as a positive control in every assay. O39, JD081T, JD041T, 78T2: n=2, rest: n≥3. NBL: neuroblastoma, MRT: malignant rhabdoid tumors, HBL: hepatoblastoma, DMG: diffuse-midline glioma (a type of high-grade glioma), AML: acute myeloid leukemia, T-ALL: T-cell acute lymphoblastic leukemia, B-ALL: B-cell acute lymphoblastic leukemia.
Supplementary Figure 4. MC.7.G5 MR1T-cells secrete cytokines and cytotoxic granular proteins in co-cultures with targeted cell lines.

Secretion of TNFα (red), IFNγ (green), granzyme B (GZMB) (orange), Perforin-1 (PRF1) (blue), and Granulysin (GNLY) (purple) in pg/mL in co-cultures after overnight incubation at an effector-to-target ratio (E:T) of 1:1, standardized to untransduced control cells. Two MC.7.G5 sensitive cell lines (697 and NALM6) and two resistant cell lines (IMR32 and CHP212) were analyzed in duplicate, K562 co-cultures were taken along as a positive control. Relative concentrations per secreted factor are depicted by circle size, absolute concentrations per secreted factor is shown by color gradient.

Supplementary Figure 5. Efficient lentiviral induction of MR1 overexpression.
MR1 was overexpressed in K562 (purple, adult positive control) and three MC.7.GS-mediated targeting resistant cell lines (1 T-ALL model (Grey, CCRF-CEM) and 2 NBL models (Blue, CHP212 & IMR32). Open: wildtype, filled: MR1 overexpressing.

Supplementary Figure 6. No enrichment of metabolic gene sets implied in formation of antigens recognized by MR1T-cells between targeted HB13F and resistant HB13E organoids.

Differential gene set enrichment analysis was performed on single cell RNA sequencing data[40] of MC.7.GS targeted HB13F and MC.7.GS resistant HB13E HBL organoids grown from the same patient. Enrichment analysis in gene sets containing “Reactive Oxygen Species” and “Nucleobase” did not reveal any significant differences
**between targeted and resistant cells. Size: number of genes included in gene set. GOBP: gene ontology biological process, NES = normalized enrichment score; PAdj = Adjusted P-value.**

**Supplementary Tables**

**Supplementary Table 1.** Allelic MR1 variants observed in utilized cell lines.

Allelic MR1 variants, extracted from the EnaraBio and CCLE databases. Targeting defined as >15% standardized killing.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>MR1 Mutation</th>
<th>MR1 Allelic Variant</th>
<th>Targeted?</th>
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</thead>
<tbody>
<tr>
<td>K562</td>
<td>-</td>
<td>MR1*01</td>
<td>Yes</td>
</tr>
<tr>
<td>Jurkat</td>
<td>-</td>
<td>MR1*01</td>
<td>Yes</td>
</tr>
<tr>
<td>CHP212</td>
<td>p.H39R</td>
<td>MR1*02</td>
<td>No</td>
</tr>
<tr>
<td>Kasumi-1</td>
<td>p.H39R</td>
<td>MR1*02</td>
<td>No</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>p.H39R</td>
<td>MR1*02</td>
<td>No</td>
</tr>
<tr>
<td>SUP-B15</td>
<td>-</td>
<td>MR1*01</td>
<td>Yes</td>
</tr>
<tr>
<td>RCH-ACV</td>
<td>-</td>
<td>MR1*01</td>
<td>No</td>
</tr>
</tbody>
</table>

**Supplementary Table 2.** Characteristics of previously established organoid models.

Characteristics of models established with the respective disease-specific organoid establishing- and culture protocols from which basic characteristics are not yet present in literature. HCC: hepatocellular carcinoma, NBL: neuroblastoma, MRT: malignant rhabdoid tumor

<table>
<thead>
<tr>
<th>Organoid</th>
<th>Disease</th>
<th>Source</th>
<th>Location</th>
<th>Age of patient</th>
<th>Establishing Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHCC</td>
<td>HCC</td>
<td>Primary Tumor, Biopsy</td>
<td>Liver</td>
<td>10 years</td>
<td>[14,40]</td>
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<tr>
<td>039</td>
<td>NBL</td>
<td>Relapse, Resection</td>
<td>Lymph Node</td>
<td>2 years</td>
<td>[17]</td>
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<tr>
<td>JD041T</td>
<td>MRT</td>
<td>Primary tumor, Resection</td>
<td>Eye</td>
<td>3 years</td>
<td>[18,19]</td>
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<tr>
<td>JD081T</td>
<td>MRT</td>
<td>2nd relapse, primary tumor site, resection</td>
<td>Rectum</td>
<td>4 years</td>
<td>[18,19]</td>
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</tbody>
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