Genome-wide CRISPR–Cas9 screening reveals ubiquitous T cell cancer targeting via the monomorphic MHC class I-related protein MR1

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Human leukocyte antigen (HLA)-independent, T cell-mediated targeting of cancer cells would allow immune destruction of malignancies in all individuals. Here, we use genome-wide CRISPR-Cas9 screening to establish that a T cell receptor (TCR) recognized and killed most human cancer types via the monomorphic MHC class I-related protein, MR1, while remaining inert to noncancerous cells. Unlike mucosal-associated invariant T cells, recognition of target cells by the TCR was independent of bacterial loading. Furthermore, concentration-dependent addition of vitamin B-related metabolite ligands of MR1 reduced TCR recognition of cancer cells, suggesting that recognition occurred via sensing of the cancer metabolome. An MR1-restricted T cell clone mediated in vivo regression of leukemia and conferred enhanced survival of NSG mice. TCR transfer to T cells of patients enabled killing of autologous and nonautologous melanoma. These findings offer opportunities for HLA-independent, pan-cancer, pan-population immunotherapies.

Unconventional T cells do not recognize classical peptide-major histocompatibility complex (pMHC) ligands and can express αβ or γδ TCRs. The ligands recognized by many unconventional T cells remain unknown. Established unconventional T cell ligands include lipid antigens presented by the conserved CD1 family of molecules, as recognized by natural killer T (NKT) cells and germline-encoded mycolyl-lipid reactive T (GEM) cells. The human Vy9V82 T cell subset recognizes phosphorylated isoprenoid intermediates of lipid biosynthesis in the context of butyrophilin 3A1 (ref. 1). The concept of sensing intracellular biosynthetic pathways by T cells was recently extended by the discovery that mucosal-associated invariant T (MAIT) cells sense microbial metabolites bound to the evolutionarily conserved, monomorphic MHC class I-related protein MR1 (refs. 2,3). MAIT cell stimulatory antigens have been defined as riboflavin-derived derivatives produced by a range of bacteria and fungi4, notably 5-(2-oxopropylideneamino)-6-p-ribitylaminouracil (5-OP-RU)5. MAITs can rapidly clear pathogens through secretion of a range of cytokines that can be accompanied by granzyme and perforin expression on recognition of these antigens bound to MR1 (ref. 4). MAITs are defined by their semi-invariant TCR gene segment usage, consisting of TRAV1-2 rearranged with TRAJ33, TRAJ12 or TRAJ20, paired with a limited repertoire of TCR β-chains6,7,8, including, but not limited to, TRBV6 and TRBV20. More recent evidence shows that recognition of MR1-associated ligands can be accomplished by a wider range of TCR rearrangements, including those using TRAV14, TRAV21 and TRAV36 chains, and that MR1 can present a broader range of ligands than those from the riboflavin biosynthetic pathway, incorporating diverse chemical scaffolds, which includes drugs and drug-like molecules9–12. In combination, these data suggest that MR1 presents a wide range of metabolic intermediates at the cell surface, in much the same way that MHC molecules present arrays of peptides, and the CD1 family of molecules present various lipid antigens. T cell targeting of diseases via MHC I and MHC I-like molecules, such as CD1 and MR1, is especially attractive, as, unlike the highly polymorphic HLA targets of conventional T cells, these molecules are largely monomorphic in the human population. Indeed, MR1 is ubiquitously expressed and the currently known nonpeptide ligands that it presents cannot mutate, as they are essential biosynthetic intermediates to many microbes13. While most studies have indicated that MR1 is only expressed on the cell surface after an MR1-binding ligand has bound14,15, there is evidence that there is basal surface expression, including expression on cancer cells16,17. Intratumoral unconventional T cell infiltrations have been associated with favorable prognostic outcomes18, with MAIT cells also being shown to have a role in multiple myeloma19. Human MR1 has a very limited number of silent and intronic polymorphisms20,21 and natural isoforms22, highlighting its potential as a pan-population target. Currently, no self-ligands that bind to MR1 have been identified that induce a T cell response. However, there

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is increasing evidence that populations of MR1-restricted cells exist that likely respond to self-antigens. These MR1-restricted T cells are not classical MAIT cells, in that they do not appear to possess a TRAV1-2 TCR, nor do they react to bacterial antigens bound to MR1. Here we report a non-MAIT TCR that recognizes a non-bacterial antigen restricted by MR1, which results in lysis of cancer cells. This TCR does not respond to healthy cells but confers HLA-independent recognition to a wide range of cancer cells.

Results

Clone MC.7.G5 kills a broad range of cancer cells regardless of HLA allomorph. A T cell population that proliferated in response to A549 cancer cells was grown from peripheral blood mononuclear cells (PBMCs) from an HLA-mismatched healthy donor (Fig. 1a). Recognition of A549 cells by the αβTCR +γδTCR + CD8α-CD8β-CD44 + (Supplementary Fig. 1a) T cell clone MC.7.G5 grown from this line was not reduced by blocking MHC antibodies (Fig. 1b). TCR sequencing of MC.7.G5 confirmed expression of an αβTCR consisting of a TRAV38.2/DV8 TRAJ31 (Fig. 1b). MC.7.G5 killed the multiple cancer cell lines tested (lung, melanoma, leukemia, colon, breast, prostate, bone and ovarian) that did not share a common HLA (Fig. 1c). MC.7.G5 also killed minimally cultured primary ovarian and melanoma cancer cells, indicating that killing was not an artifact of long-term culture (Fig. 1c). MC.7.G5 remained inert to healthy cells (Fig. 1d) and showed high sensitivity to a melanoma target at low effector to target ratios (Fig. 1e). As MC.7.G5 preferentially killed cancer cells independently of classical MHC molecules, we set out to uncover its mechanism of action.

Genome-wide CRISPR–Cas9 screening revealed MR1 as the MC.7.G5 target on cancer cells. As MC.7.G5 killed a wide range of cancer cell lines originating from different tissues and organs, regardless of their HLA allomorph expression, its mode of target-cell recognition was unclear. A genome-wide CRISPR–Cas9 approach, using the GeCKO v.2 library, targets every protein-coding gene in the human genome with six different single guide (sg) RNAs, was used to identify genes essential for recognition of target cells by MC.7.G5 (Fig. 2a). Following two rounds of selection with MC.7.G5, the surviving transduced HEK293T cells exhibited reduced capacity to stimulate MC.7.G5, suggesting that key genes involved in their recognition had been ablated (Fig. 2b). Sequencing of the CRISPR sgRNAs in the lysate-resistant HEK293T cells showed that only six genes were targeted by more than one enriched sgRNA: β2M (five sgRNAs), MR1 (two sgRNAs), regulatory factor X (RXF, five sgRNAs), RXF-associated ankryin-containing protein (RXFANK, five sgRNAs), RXF-associated protein (RXFAP, three sgRNAs) and signal transducer and activator of transcription 6 (STAT6, two sgRNAs) (Fig. 2c). RXF, RXFANK and RXFAP are essential components of the protein complex responsible for transactivating β2M, MHC I and MHC II promoters. Combined with the fact that β2M and MR1 heterodimerize to form a monomorphic stable antigen-presenting molecule that is known to activate MAITs and other MR1-restricted T cells, these data strongly suggested that the MC.7.G5 T cell recognized cancer targets via the MR1 molecule. Accordingly, anti-MR1, but not MHC I or MHC II antibodies, blocked target-cell recognition by MC.7.G5 (Fig. 3a). CRISPR-mediated knockout of MR1 from A549 (ref. 31) and melanoma MM909.24 (frameshift deletion mutation shown in

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**Fig. 1** MC.7.G5 recognizes multiple cancer types through an HLA-independent mechanism. a, MC.7.G5 was cloned from T cells that proliferated in response to the cancer cell line A549. Performed once for this donor. b, MC.7.G5 did not recognize A549 through MHC I or MHC II. Overnight activation blocking antibodies and TNF ELISA. Bars depict the mean. c, MC.7.G5 killed a range of established (long-term culture) and primary cancer cell lines of different origin. Flow-based killing assay for 48–72 h at a T cell to target cell ratio of 5:1. Data combined from different experiments. Performed in triplicate. d, MC.7.G5 killed melanoma cells but not healthy cells. Flow-based killing assay at a T cell to target cell ratio of 5:1. Performed in triplicate or duplicate (fibroblasts). e, MC.7.G5 sensitively killed melanoma MM909.24 over 7 d. Performed in duplicate. Bars, horizontal lines and connecting lines depict the mean (b–e).
Supplementary Fig. 2a) protected against MC.7.G5-mediated recognition and lysis (Fig. 3b). Melanoma MM909.24 did not stain with anti-MR1 suggesting that very minimal levels of MR1 were required for target recognition (Supplementary Fig. 2b). Overexpression of MR1 resulted in strong recognition of the poorly recognized targets, HeLa and C1R (MR1 staining in Supplementary Fig. 2b), and slightly enhanced recognition of melanoma MM909.24 (Fig. 3c). Reintroduction of MR1 into CRISPR–Cas9 MR1 knockout A549 cells under a cytomegalovirus promoter restored recognition by MC.7.G5 (Fig. 3d), instilling further confidence that cancer cell
Fig. 4 | MC.7.G5 does not recognize MR1 by known mechanisms. a, MC.7.G5 did not stain with empty (K43A) or MR1 5-OP-RU tetramers. A canonical MAIT clone recognizes MR1 bound with 5-OP-RU. The MHC I-restricted clone was used as a positive control for the irrelevant MHC I tetramer. Performed twice with similar results. b, MC.7.G5 recognized target cells overexpressing wild-type MR1 (MR1+/+) but not K43A-altered MR1. Overnight activation performed in duplicate and TNF ELISA. c, Loading with MAIT-activating bacterium M. smegmatis (M. smeg) reduced MC.7.G5 recognition of A549 cells. Canonical MAIT clone used as a positive control. Staining for surface CD107a and intracellular TNF. Performed twice with similar results.

classification was MR1 dependent. In summary, whole-genome CRISPR screening effectively revealed MR1 as the restricting molecule on cancer cells for the HLA-agnostic T cell clone MC.7.G5.

MC.7.G5 does not recognize MR1 by known mechanisms. MR1 is known to present intermediates of riboflavin synthesis at the cell surface to MAIT cells and is not considered to be expressed appreciably at the cell surface without a bound cargo13. MC.7.G5 did not stain with tetramers composed of MR1 containing the K43A substitution that allows MR1 refolding without bound ligand (Fig. 4a)13. Accordingly, MC.7.G5 did not recognize C1R cells transduced with the MR1 K43A substitution (Fig. 4b), despite high overexpression of surface MR1 K43A detectable by anti-MR1 staining (Supplementary Fig. 2b). This distinguishes recognition of target cells by MC.7.G5 from the previously described ‘MR1T’ cells, which do not require K43 for activation5. The requirement for ligand-binding K43 suggested that MC.7.G5 might recognize an MR1-bound ligand that was specifically expressed or upregulated in malignant cells. In agreement with this hypothesis, MC.7.G5 did not stain with tetramers assembled with MR1 presenting the microbial-derived T cell activator 5-OP-RU (Fig. 4a). Furthermore, recognition of target cells was reduced when loaded with either the MAIT-activating bacterium Mycobacterium smegmatis (M. smeg) or Salmonella enterica serovar Typhimurium (S. Typhimurium) (Fig. 4c,d) or the MR1 ligand acetyl-6-formylpterin (Ac-6-FP)11,12, despite a slight increase in surface expression of MR1 (Supplementary Fig. 2c). MC.7.G5 exhibited cancer specificity, unlike the majority of MR1T cells6, which require overexpression of MR1 for optimal target-cell recognition and also are activated in response to MR1 expression by healthy monocyte-derived dendritic cells. MC.7.G5 did not recognize immature or matured monocyte-derived dendritic cells (Fig. 5a), nor Langerhans cells (Fig. 5b). These results indicate that MC.7.G5 does not exclusively recognize MR1 per se, nor recognize MR1 by known mechanisms, but rather it recognizes...
MR1 with bound cargo that is specific to, or associated with, cancer cells. An MC.7.G5-like T cell clone was grown from a second donor, which was also dependent on K43 for target-cell recognition (Supplementary Fig. 3), suggesting that cancer-specific T cells capable of recognizing wild-type (WT) levels of MR1 may be present in multiple individuals.

MC.7.G5 remained inert to rest, activated, stressed or infected healthy cells from various tissues. To assess the safety of using the MC.7.G5 TCR for cancer immunotherapy we undertook further testing of healthy cells from various tissues. As an extension to the data shown in Fig. 1 (smooth muscle, lung fibroblast and liver cells) and Fig. 5a,b (dendritic and Langerhans cells), MC.7.G5 did not kill healthy cells from lung (alveolar and bronchus), skin (melanocytes), intestine, pancreas or kidney (Fig. 5c). In the same assay >95% of each cancer cell line from lung, skin (melanomas), blood, cervix and kidney were killed, whereas cancer cell lines rendered negative for MR1 using CRISPR–Cas9 were not killed (Fig. 5c). Next, we created conditions that may induce cellular upregulation of cell-surface MR1, or generate ligands bound to MR1. T or B cells that were sorted directly ex vivo and activated overnight with either phytohaemagglutinin or toll-like receptor 9 ligand, respectively (CD69 staining, Supplementary Fig. 4a), were untouched by MC.7.G5 (Fig. 6a). Lymphoblastoid cell lines, which are relatively poor targets of MC.7.G5, did not activate MC.7.G5 following treatment with tert-butyl hydroperoxide (tBHP) (Fig. 6b) to induce cell stress (detection of reactive oxygen species (ROS), Supplementary Fig. 4b). Furthermore, a normal renal epithelial cell line did not become a target when treated with tBHP or hydrogen peroxide (H₂O₂) (ROS detection, Supplementary Fig. 4b), which induce different pathways of cell stress, or by exposure to gamma irradiation (Fig. 6b). M. smegmatis infection of healthy lung epithelial cells did not lead to MC.7.G5 activation, whereas the infected cells were recognized by a MAIT cell line (Fig. 6c). Therefore, healthy cells are incapable of activating MC.7.G5, even when stressed or damaged.

MC.7.G5 controls leukemia in vivo. To examine the in vivo capacity of MC.7.G5 to target cancer, Jurkat leukemia cells were engrafted in NSG mice, followed by adoptive transfer of MC.7.G5. Bone marrow samples were analyzed for MC.7.G5 and Jurkat cell frequencies at days 12 and 18 following T cell transfer. MC.7.G5 appeared in the bone marrow at both time points, but the number of cells remaining on day 18 following the single transfusion was substantially reduced (Fig. 7a). Mice receiving MC.7.G5 had significantly fewer Jurkat cells than mice with no MC.7.G5 at days 12 and 18 (Fig. 7a). The difference in Jurkat cell burden was particularly striking at day 18, with mice receiving MC.7.G5 having 3.8%, 7.2% and 0.3% Jurkat cells in the bone marrow, compared with 83%, 78% and 85% for the mice without MC.7.G5 (Fig. 7a). The presence of Jurkat cells was also reduced in the spleen of mice that received T cells, with a similar drop in T cell numbers by day 18 (Supplementary Fig. 5). The in vivo targeting of Jurkat cells by MC.7.G5 was dependent on MR1 expression, as shown by cotransfer experiments with differentially labeled Jurkat wild-type and Jurkat MR1–/– cells to the same mice (Fig. 7b). The ability of MC.7.G5 to target Jurkat cells in vivo translated into a significant enhancement of survival for mice that received T cells (Fig. 7c). These data demonstrate that MC.7.G5 maintained its
reactivity towards cancer cells in an in vivo setting, thus reducing cancer burden and enhancing survival.

The MC.7.G5 redirects patient T cells to kill autologous cancer cells. To explore the therapeutic potential of targeting MR1 on cancer cells we purified T cells from the PBMCs of patients with stage IV melanoma and lentivirally transduced them with the MC.7.G5 TCR (≥85% expression, Supplementary Fig. 6a), which resulted in recognition and killing of autologous and nonautologous melanomas (Fig. 8a,b), but not of healthy cells (Fig. 8b). The killing was specific to MR1 as the MC.7.G5 TCR-transduced cells did not lyse MR1 knockout melanomas (Fig. 8b). We conclude that the MC.7.G5 TCR can redirect the T cells of patients to kill cancer cells without the requirement of a specific HLA.

Discussion

MR1 is an attractive target for cancer immunotherapy due to its monomorphic, ubiquitously expressed nature. Recent advances in MR1 tetramers and ligand discoveries have progressed knowledge in this area, but there is still much to be discovered. Here, we confirmed cancer cell recognition by a T cell clone that responded to multiple cancer cell lines from diverse tissue types, resulting in killing of cancer cells in vitro and in vivo. The clone expresses a TCR that is not indicative of MAIT cells. Current MR1 antibodies are unable to detect low surface expression of MR1 on cancer cells, despite detectable expression of messenger RNA. Indeed, the level of MR1 surface expression required for cancer cell recognition by MC.7.G5 was often below the threshold required for staining with antibody, suggesting that the MC.7.G5 TCR might be capable of responding to a low copy number of the MR1 ligand, which is akin to T cells that recognize pMHC and MAIT TCR recognition of MR1 (ref. 25). Our results also demonstrate the immense power of genome-wide CRISPR–Cas9 screening as a discovery platform for unconventional T cell ligands, and we anticipate that the methodologies applied here will rapidly revolutionize the unconventional T cell field by revealing more ligands. Further work will be
required to establish the exact nature of the ligand recognized by the MC.7.G5 TCR. Knowledge of known MR1-restricted ligands suggests that the MC.7.G5 TCR ligand may be a cancer-specific or -associated metabolite. We failed to find hits in a metabolic pathway during our genome-wide CRISPR–Cas9 screens. This suggests that the MR1-associated ligand targeted by the MC.7.G5 TCR is part of a pathway essential for the basic survival of cancer cells, and therefore not amenable to the gene disruption required for CRISPR–Cas9 screening.

In summary, we describe a TCR that exhibits pan-cancer cell recognition via the invariant MR1 molecule, and, by equipping patients with melanoma T cells that lacked detectable cancer cell reactivity with the MC.7.G5 TCR, we rendered the T cells capable of killing autologous melanoma. Importantly, MC.7.G5 did not respond to healthy cells and caused no obvious pathology in the healthy donor cells that it was grown from. Since the MC.7.G5 TCR can recognize diverse cancer cell types, including primary cancer cells, irrespective of HLA, it opens up exciting opportunities for pan-cancer, pan-population T cell–mediated cancer immunotherapy approaches. Discovery of MR1-restricted ligands recognized by MC.7.G5-like T cells may further open up opportunities for therapeutic vaccination for many cancers in all individuals.

Online content
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Methods

Patient and human tissue. Patients with stage IV metastatic melanoma (MM909.11 and MM909.24) underwent rapid tumour infiltrating lymphocyte therapy at the Centre for Cancer Immunotherapy (CCIT), Herlev Hospital (ethics reference no. 13–507RC). Peripheral blood mononuclear cells were harvested through the Wales Cancer Bank (ethics reference WCB14/004) from a patient with stage 3 chemotherapy resistant (500031899) carcinoma at Velindre Cancer Centre. Blood was sourced from the Welsh Blood Service. The use of human blood was approved by the School of Medicine Research Ethics Committee (reference 18/56). All human blood was procured and handled in accordance with the guidelines of Cardiff University to conform to the United Kingdom Human Tissue Act 2004. All samples were taken with informed consent from participants.

Cell lines. Cell lines were regularly tested for mycoplasma, and cultured on the basis of ATCC guidelines; breast adenocarcinomas MCF-7 (HTB-22); prostate adenocarcinoma LNCaP (CRL-1740); cervical adenocarcinomas HeLa (CCL-2); and SiHa (HTB-36); acute lymphoblastic leukemia MOLT3 (CRL-1573); colorectal adenocarcinoma RXF101 (CRL-1552); chronic myeloid leukemia K562 (CCL-3344); myeloma/plasmacytoma U266 (TIB-196); osteosarcoma U-2 OS (HTB-96); immortalized embryonic kidney HEK293T (CRL-1573); acute monocytic leukemia THP-1 (TIB-202); lung carcinoma A549 (CCL-185); acute T cell leukemia Jurkat (TIB-152); colorectal adenocarcinoma COLO 205 (CCL-222); and ovarian carcinoma A2780 (ECACC 93112519) for culture guidelines. Melanomas FM-45, MM909.11 and MM909.24, and renal cell carcinoma RCC17 were sourced from the CCIT, and MEL 624 from in-house, with all being cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 100 μg ml⁻¹ streptomycin, and 2 mM l-glutamine (Life Technologies) at 37 °C as adherent monolayers, passaged when 50–80% confluent using 2 mM EDTA D-PBS to detach cells. CIR and lymphoblastoid cell line SAR26 were sourced or generated in-house and cultured in RPMI 10% FBS as suspension cells. The primary epithelial ovarian cancer cell line ECOC31 was generated from ascites following previous guidelines with the following amendments: the ascites were diluted 1:10 with RPMI 10% FBS and centrifuged to collect the cells, which were subsequently depleted of red blood cells and debris using standard density gradient centrifugation. DMEM-F12 media (Life Technologies) was supplemented as for RPMI with the addition of 5% human serum. Once cells had attached to the flask and grown for 3 d, fibroblasts were removed by incubation with trypsin/EDTA for 1 min leaving the ovarian cancer cells for assays. Primary melanoma lines MM909.11, MM909.20 and MM909.21 were sourced from the CCIT and used directly from cryopreserved samples for killing assays without prior culture. Normal/healthy cells and their proprietary culture media were obtained from Sciencell: SMCC (colonic smooth muscle); CIL-1 (nonpigmented bronchial ciliary epithelium); HH (hepatocyte); pulmonary alveolar epithelia; melanocytes; renal epithelia; and pancreatic stellate cells. MRC5s (fibroblast) were sourced locally and cultured as described by the ATCC. Intestinal epithelia and their media were sourced from Lonza. Dendritic cells and Langerhans cells were generated from CD14+ cells purified from PBMCs using magnetic beads (Miltenyi Biotech). Briefly, both immature dendritic cells and Langerhans cells were differentiated with GM-CSF (20 ng ml⁻¹) and IL-4 (10 ng ml⁻¹) sourced from Miltenyi Biotech, with Langerhans cells also receiving 20 ng ml⁻¹ of TGfβ (Miltenyi Biotech), for 7–10 d before maturation for 48 h with 20 ng ml⁻¹ TNFα, which was performed according to the manufacturer's instructions (R&D Systems). For intracellularly with antibodies for anti-TNF PE-Vio770 (clone c2A, Miltenyi Biotech) and anti-IFN-γ (clone 45–15, Miltenyi Biotech), was performed as described previously, following activation for 4 h at a T cell to target cell ratio of 1:1. Intracellular cytokine staining was performed according to the manufacturer's instructions using a Cytofix/Cytoperm kit, GolgiPlug and GolgiStop (BD Biosciences). For tetramer processing inhibitor TAPI-0 (Santa Cruz Biotechnology) assays, T cells and target cells were co-incubated for 4 h with 30 μM TAPI-0 and antibody directed against TNF (clone c2A, Miltenyi Biotech). CD107a antibody (clone HA43, Miltenyi Biotech Ltd.) was also included at the start of the assay to detect activation-induced degranulation of cytotoxic T cells. Following incubation, cells were washed and stained with fixable Live/Dead Dye and antibodies against T cell surface markers. Gating strategy and isotype antibody (as recommended by the manufacturer of the primary antibodies) control experiments for the TAPI-0 assay are shown in Supplementary Figure 7. Ac-6-FP (Schircks Laboratories) was reconstituted in DMSO to 50 mg ml⁻¹ and stored at −20 °C protected from light. For MR1 loading, Ac-6-FP was incubated overnight at 37 °C and 5% CO2 with target cells in their respective media.

Cytotoxicity assays. For cytotoxicity assays, target cells were labeled with chromium-51 (Perkin Elmer) then co-incubated with T cells at various T cell to target ratios for 6 or 18 h and specific lysis calculated, as described previously. For tetramer- and kilo-cell-labeled assays 5 × 10⁴ cancer or healthy cell lines were incubated in 96-well plates, and M7.4.G5 added to give the desired T cell to target cell line ratio (experimental wells). The cells were cocultured in 200 μl of target-cell media supplemented with 20 μl of IL-2 and 25 ng ml⁻¹ of IL-15. Target cells (control wells), M7.4.G5 and CFSE CIIs were also cultured alone to aid analysis. The cells were incubated for 4 h or 7 d and fed (50% media change) twice for each of the two before collection, either BD negative CompBeads (BD Biosciences) (1 drop in 100 μl of PBS then 25 μl per well) or 0.1% CFSE labeled (0.1 μM CFSE) were added to each well to allow the number of target cells that remained in experimental and control wells to be quantified. The cells were washed three times with chilled D-PBS EDTA (2 mM) then stained in the assay plates with Fixable Live/Dead Dye (VIVID, Thermo Fisher Scientific) then D3 PerCP (clone UCHT1, BioLegend) and/or anti-TRBV25.1 APC TCR (TRBV25.1 Arden nomenclature: clone C21, Beckman Coulter) to allow dead cells and T cells to be gated, leaving viable target cells for analyses (Supplementary Figure 7). Percentage killing was calculated using the following equation:

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\text{Percentage killing} = \left( \frac{\text{experimental target cell events} - \text{experimental background events}}{\text{experimental background events}} \right) \times 100
\]

Flow cytometry. Cells were stained with Fixable Live/Dead Violet Dye VIVID and the following surface antibodies: pan-αβ TCR PE (clone IF26, BioLegend), pan-γδ TCR PE (clone REA591, Miltenyi Biotech). CD3 PerCP (clone UCHT1, CD4 APC (clone VIT4, Miltenyi Biotech), CD8 PE (clone BW135/80, Miltenyi Biotech), rat CD2 PE (clone OX-34, BioLegend) and MR1 PE (clone 26.5, BioLegend). For staining with MR1 PE, Fc Block (Miltenyi Biotech) was used according to manufacturer's instructions and isotype antibody as described previously. To test tetramer staining, MR1 nn-213-12 (clone UCHT1, CD4 APC (clone VIT4, Miltenyi Biotech)) and/or and anti-TRBV25.1 APC TCR (clone 45–15, Miltenyi Biotech), was performed as described previously. The cells were stained on lymphocytes (FSC-A versus SSC-A), single cells (FSC-A versus FSC-H), then viable cells (marker of choice versus VIVID) as shown in Supplementary Figure 7. Data were acquired on a BD FACs Canto II (BD Biosciences) and analyzed with Flowjo software (Tree Star).

MR1 knockout and transgene expression. MR1 single guide (sg)RNA and CRISPR-Cas9 lentivirus was produced and used as described previously. The native MR1 transgene was cloned into the second generation pRRL.sin.cpt, pgk-gfp.wpre lentivector backbone developed by D. Trono’s laboratory (Addgene plasmid no. 12225) devoid of human PGK promoter and GFP cDNA. The codon-optimized MR1 K43A transgene was cloned into the third generation pELS vector (kindly provided by J. Riley, University of Pennsylvania) devoid of GFP cDNA. Lentiviral particles for native MR1 and MR1 K43A were produced by calcium chloride transfection of HEK293T cells, as described for MR1 sgRNA. Target cells were spininfected in the presence of 8 μg ml⁻¹ polybrene: 500g for 2 h at 37 °C (ref. 22). Anti-MR1 PE (clone 26.5, BioLegend)-positive cells were magnetically enriched using anti-PE magnetic beads according to the manufacturer's instructions (Miltenyi Biotech).

TCR sequencing and transduction. MC.7.G5 TCR was sequenced in-house using the SMARTer RACE kit (Takara Bio USA) and two-step polymerase chain reaction using universal forward primers and reverse primers specific for TCR-α and TCR-β constant regions. The TCR was then synthesized with codon optimization (Genewiz), with full-length α and β TCR chains separated by a ‘self-claving’ T2A sequence and cloned into the third generation pELNS lentiviral vector containing
Jurkat cell expressing DsRed-Express2 were generated using pELNS vector and Female JAX NOD scid A549 cells described above.

Cell stress assays. Cells were collected from culture then incubated with 100–200 μM of bH2P or H2O, for 1 h in R10, followed by staining with CellROX green reagent to detect ROS, according to the manufacturer’s instructions (Thermo Fisher Scientific). Cells were also stained with viability stain VIVID as above. Cesium source gamma irradiation of cells was performed using a GammaCell irradiator. M. smeg infection of healthy lung epithelial cells was performed as for A549 cells described above.

Mouse experiments. Female JAX NOD scid gamma (NSG) were purchased from Charles River at 6–7 weeks of age, housed under specific pathogen-free conditions and experiments initiated within 1 week of arrival. Experiments were performed under United Kingdom Home Office approved projects 30/3188 and P2F8675/AB conducted in compliance with the United Kingdom Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. Jurkat cell expressing DiRed-Express2 were generated using pELNS vector and lentiviral particles, as described above, then cloned. Before in vivo transfer Jurkat DiRed cells and MC.7.G5 were depleted of dead or dying cells by standard density gradient centrifugation. Jurkat cells (3 × 10⁷) were engrafted first, followed by 1.5 × 10⁷ MC.7.G5 cells 7 d later. Cells were infected into the tail vein of mice using a 29G BD microfine syringe in 100 μl of PBS. Mice that did not receive cells were injected with PBS. Each mouse (± T cells) received 5 × 10⁶ IU of IL-2 and 20 μg of IL-15 (details as above) via injection into the peritoneal cavity on the day of T cell transfer, and every 48 h for the duration of the experiment. Bone marrow was collected from the tibia and fibula, and spleens were prepared for staining using standard density gradient centrifugation. Cells were stained with the viable dye VIVID, followed by antibodies for human CD3 and CD8 (details as above), and anti-human CD45 APC-Cy7 (clone HI30, BioLegend) and anti-mouse/human CD11b PE-Cy7 (clone M170, BD Biosciences) as described previously. The gating strategy for analyses of flow cytometry data is shown in Supplementary Fig. 8. For Jurkat cointerfection experiments, MR1−/− DiRed-Express2 Jurkat cells were first generated as described above using the MR1 CRSIPR-Cas9, followed by cloning. Wild-type and MR1−/− (DiRed-Express2+) Jurkat cells (2 × 10⁶) transferred to the same mouse, then MC.7.G5 T cells (3 × 10⁷) were added 7 d later to the + T cell group. Splenocytes were collected at 25 d following T cell transfer, then incubated with mouse and human FcR block (Miltenyi Biotec), stained with VIVID and antibodies for CD3, CD8, CD45, as above, and also with mouse anti-human pan HLA class I (clone W6/32, BioLegend). Survival of mice with Jurkat cells was assessed by monitoring body weight; mice were killed when they had lost ≥15% of their initial body weight, according to United Kingdom Home Office stipulation.

Statistical analyses. Neither blinding nor randomization was performed for the in vivo studies. A two-sided nonparametric two-sample Kolmogorov–Smirnov test was used for Jurkat cell burden in NSG mice. The log-rank two-sided P value and hazard ratio were calculated using the MatSurv survival analysis function in Matlab, available at https://www.github.com/aebergl/MatSurv. The number of mice used in each group is indicated in the respective figure legend.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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Author contributions

A.K.S. and G.D. conceived project. M.D.C., G.D., M.E.C., S.A.E.G., M.A., A.L. and C.R. undertook the T cell experiments. M.D.C., M.L., C.P.F., B.S. and J.P. performed the genome-wide CRISPR experiments and/or analyses. A.F. generated lentiviral vectors and edited the manuscript. M.D. and I.M.S. supplied the patient PBMC and melanoma and renal carcinoma cell lines. A.A. provided advice on mouse experiments. A.I.P. provided expertise and ovarian cancer ascites. J.R. and J.M. provided the MR1 tetramer reagents. G.D. and A.K.S. supervised the work. M.D.C., G.D. and A.K.S. wrote and edited the manuscript.
Competing interests
Cardiff University has filed patents based on these findings.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41590-019-0578-8.

Correspondence and requests for materials should be addressed to A.K.S.

Peer review information Zoltan Fehervari was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement.
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- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons.
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. *Give P values as exact values whenever suitable.*
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings.
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes.
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated.

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- FACSDiva v. software for flow cytometry acquisition; MiSeq control software by Illumina for DNA sequencing; Perkin Elmer Microbeta Workstation Software.

Data analysis
- MAGeCK analysis of whole genome CRISPR data, as outlined in Li, W. et al. Genome Biol. 15, 554 (2014); FlowJo V10 for flow cytometry analysis; Prism v6 for graph production; Microsoft Excel. The logrank p-value and Hazard Ratio (HR) were calculated using the MatSurv survival analysis function in Matlab, available at https://www.github.com/aeberg/MatSurv.

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- A list of figures that have associated raw data
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Overall: all raw data can be provided if needed. Figure 1: A, raw data displayed. B, raw absorbance available. C, some raw data in supplementary to show the methodology, other can be provided. D, flow data can be provided. E, flow data can be provided. Figure 2: B, raw absorbance available. C, MAGeCK data available. Figure 3: A, B, and D, raw absorbance available. B and C, raw C51 data available. Figure 4: A, C and E, flow data displayed. B and D, raw absorbance available. E, raw flow data available. Figure 5: A and B, raw absorbance available. C, raw flow data available. Figure 6: A, flow data available. B and C, flow data displayed. B, raw flow available. Figure 7: A, representative flow data displayed in the supplementary figures; all other available. B, raw data displayed. Body weights can be provided. Figure 8: A, raw data displayed. B, raw flow data available.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- **Sample size**: We based our approach on our previous studies of duplicate or triplicate samples within in vitro assay. In vivo design was based on our previous work to give group sizes amenable to statistical testing.

- **Data exclusions**: Data exclusion was not pre-established: confirmation of CD14a restriction of clone 40E.22 not shown due to future publication of data. Data exclusion was pre-established: whole genome sequencing data where hits were <2 were not included due to lack of relevance regarding enrichment.

- **Replication**: All experiments were performed in either duplicates or triplicates. Experiments were repeated multiple times as indicated in the legends as requested. All data was repeatable. Not possible to repeat whole genome sequencing due to high cost, robustness provided by high throughput design.

- **Randomization**: Mice were allocated randomly into treatment groups.

- **Blinding**: No blinding was performed as only one author performed the experiments.

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | Antibodies            |
| ✔   | Eukaryotic cell lines |
| ✔   | Palaeontology         |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChiP-seq              |
|     | Flow cytometry        |
|     | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

Pan-αβTCR PE (clone IP26, BioLegend, Catalogue number 306708, 1:50), pan-γδ TCR FITC (clone REA591, Miltenyi Biotec, cat 130-113-511, 1:50), CD3 PerCP (clone UCHT1, BioLegend, cat 300428, 1:100), CD4 APC (clone VI74, Miltenyi Biotec, cat 130-113-210, 1:50), CD8 PE (clone BW135/80, Miltenyi Biotec, cat 130-113-158, 1:100), rat CD2 PE (clone OK34, BioLegend, cat 201305 1:200) and MR1 (clone 26.5, BioLegend, PE cat 361106: 1:25 for staining, cat 361109: blocking details in the methods section), CD69 APC (clone FN50, BioLegend, cat 310910, 1:50), pan HLA class I (clone W6/32, BioLegend, APC cat 311410: 1:50 for staining, cat 311427: blocking details in the methods section), HLA DR DP DQ antibody (clone Tu39, cat 361702: blocking details in the methods section), CD45 APC-Cy7 (clone HI30, BioLegend, cat 304014, 1:50), TNF PE-Vio770 (clone CA2/ Miltenyi Biotec, cat 130-120-492, 1:60), CD107a PE (clone H4A3, Miltenyi Biotec, cat 130-119-872, 1:83), IFNγ APC (clone 45-15, Miltenyi Biotec, cat 130-113-490, 1:50) and CD11b PE-Cy7 (clone M1/70, BD Biosciences, cat 552850, 1:100).

**Validation**

All antibodies were validated by manufacturers for flow cytometry, with positive and negative staining provided in appropriate data sheets available at the following links: IP26 (https://www.biolegend.com/en-gb/products/pe-anti-human-alpha-beta-t-cell-receptor-antibody-773); UCHT1 (https://www.miltenyibiotec.com/GB/en/products/macs-flow-cytometry/antibodies/primary-antibodies/anti-terg-d-antibodies-human-reas91-1-50.html#fitc-for-100-tests); VI74 (https://www.biolegend.com/en-gb/products/percp-anti-human-cd3-antibody-4213); VIT4 (https://www.miltenyibiotec.com/GB-en/products/macs-flow-cytometry/antibodies/primary-antibodies/cd4-antibodies-human-vit4-1-50.html#apc-for-100-tests); BW135/80 (https://www.miltenyibiotec.com/GB-en/products/macs-flow-cytometry/antibodies/primary-antibodies/cd8-antibodies-human-bw135-8d-1-50.html#pe-for-100-tests); OK34 (https://www.biolegend.com/en-gb/products/pe-anti-rat-cd2-antibody-2379); 26.5 (staining: https://www.biolegend.com/en-gb/products/pe-anti-human-mouse-rat-mr1-antibody-92811; blocking: https://www.biolegend.com/en-gb/products/ultra-leaf-purified-anti-humanmouserat-mr1-antibody-17174; PMID 28632133); FN50 (https://www.biolegend.com/en-gb/products/apc-anti-human-cd69-antibody-1674); W6/32 (staining: https://
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Breast adenocarcinomas MCF-7 (HTB-22); prostate adenocarcinoma LnCAP (CRL-1740); cervical adenocarcinomas HeLa (CCL-2) and SiHa (HTB-36); acute lymphoblastic leukemia MOLT3 (CRL-1552); chronic myeloid leukemia K562 (CRL-3344); myeloma/plasmacytoma U266 (TIB-196); osteosarcoma U-2 OS (HTB-96); immortalized embryonic kidney cell HEK293T (CRL-1573); acute monocytic leukemia THP-1 (TIB-202); lung carcinoma A549 (CCL-185); acute T-cell leukemia Jurkat (TIB-152); colorectal adenocarcinoma COLO 205 (CCL-222); ovarian carcinoma A2780 (ECACC 93112519 for culture guidelines); melanomas FM-A5, MM590.21 and MM590.24; renal cell carcinoma RCC17, MM590.20 and MM590.21 were sourced from the CCT, and MEL 624 from in-house, CIR and lymphoblastoid cell line (LCL) SAR26 were sourced or generated in-house. Epithelial ovarian cancer cell line EOC301 was generated from ascites. SMCC (colonic smooth muscle); CIL-1 (non-pigmented bronchial ciliary epithelium); HH (hepatocyte); pulmonary alveolar epithelia; melanocytes; renal epithelia; and pancreatic stellate cells were all sourced from Sciencell (Carlsbad, CA, USA). MRCSS (fibroblast) were sourced locally. Intestinal epithelia were sourced from Lonza (Basel, Switzerland). Dendritic cells and Langerhans were generated from CD1+ cells purified from PBMCs from the Welsh Blood Service, as were healthy T- and B-cells.

Authentication
Normal/healthy cells from Sciencell (via Cultag) or Lonza were purchased for the purpose of this study and came with validation certification. The normal cell lines undergo senescence if kept in culture. Melanomas and renal cell carcinomas from the CCT in Copenhagen and ovarian primary cancer cell line EOC301 were generated using established methods as described in previous publications. This includes removal of fibroblasts, other non-cancer cell subsets [such as T-cells] and passage to ensure immortalisation. EOC301 was used as a primary cancer cell line, but a passaged cancer cell has been established, which divides as avidly as other cancer cell lines and could be used for further validation if required. HLA-typing confirmed patient origin after the cancer cells were generated as lines. Lymphoblastoid made in-house cell lines undergo repeated division and stain for CD19 (CD3- CD8- CD4+) and activate EBV specific T-cells. Langerhans and dendritic cells were phenotyped for standard markers of differentiation and maturation (CD14 CD209 CD86 CD80 CD83). Purified T-cells and B-cells stained with CD3 or CD19 respectively. All other cell lines are checked regularly for morphology based on imagery, and growth habit descriptions, according to information available from the ATCC. Additionally, surface Ab staining and functional testing was performed for ongoing validation of cell lines; of MCF-7 (HLA A1, A2, A24, A33, HLA A1, A2, A3, A24, SiHa (HLA A1, A2, A3, A24), Daudi (HLA A1, A2, A3, A24), MOLT3 (HLA A1, A2, A3, A24), K562 (HLA null), U266 (HLA A2, A24), U-2 OS (HLA A2, A24), HEK293T (able to produce lentivirus, THP-1 (HLA A1, A2, A3, A24), A549 (able to take up bacteria and activate MALT1), Jurkat (CD3+, alpha beta TCR+ CD4+), and able to express transduced T-cell receptors), COLO205 (HLA A1, A2, A3, A24), CIR (HLA null, and able to activate EBV specific T-cells when transduced with HLA A2), MEL624 (HLA A1, A2, A3, A24, and able to activate Melan A specific T-cells through HLA A2) and A2780 (HLA A1, A2, A3, A24).

Mycoplasma contamination
All cell lines tested negative for mycoplasma contamination.

Commonly misidentified lines
(See ICAC register)
No commonly misidentified cell lines were used (according to ICAC).

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Female JAX NOD scid gamma (NSG*) were purchased from Charles Rivers (Wilmington, MA, US) at 6-7 weeks of age, housed under specific pathogen free conditions and experiments initiated within one week of arrival.

Wild animals
Study did not involve wild animals.

Field-collected samples
Study did not involve field collected samples.

Ethics oversight
United Kingdom Home Office approved projects 30/3188 and P2F675AB.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Human research participants

Policy information about: studies involving human research participants

Population characteristics
- Metastatic melanoma patients prior to immunotherapy: age (MM909.11 41 years, MM909.24 56 years), gender (MM909.11 and MM909.24 male) and genotypic profile not critical for the study. Ovarian cancer patient with active disease: age, gender and genotypic profile not critical for the study. Healthy donors: age (18-65 year donation criteria for the Welsh Blood Service), gender (unknown) and genotypic profile not critical for the study.

Recruitment
- Melanoma patients: selected due to the availability of PBMCs collected prior to immunotherapy, and because an autologous melanoma line was available. Patients MM909.11 and MM909.24 were selected from a cohort of banked samples at the CCT in Copenhagen. Ovarian cancer patient: selected due to the local availability of ascites fluid, removed from the patient as part of palliative care, and that a primary cancer line could be generated from the ascites. Healthy donors: selected due to the availability of donated blood, as purchased from the Welsh Blood Service from donations not needed for hospital use.

Ethics oversight
- Ethical agreement to cover the work performed in Cardiff with human samples: School of Medicine Research Ethics Committee (reference 18/56). Melanoma patients: ethics reference EuDrACT no. 2008-008141-20. Ovarian patient: Wales Cancer Bank ethics reference WC814/004.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
- Cells were harvested and washed in PBS before fixable live/dead staining. Antibodies were added and incubated at 4 degrees celcius for 20 minutes, cells were washed in PBS and resuspended in appropriate volume for analysis. Cells were either from PBMCs or cancer cell lines as described in 'Eukaryotic Cell Lines' described above.

Instrument
- BD FACS Canto II 8 colour, BD Biosciences Cat No.:338962; BD FACS Aria (https://www.cardiff.ac.uk/central-biotechnology-services).

Software
- BD FACS DIVA v6.0.

Cell population abundance
- Cell numbers were provided by FACS DIVA sorting software. Confirmation not possible as cells were immediately placed into T-cell expansion conditions with irradiated feeder cells.

Gating strategy
- Cells were gated on FSC/SSC for lymphocytes, followed by single cell gating on FSC-A/FSC-H, followed by alive gating using a fixable live/dead stain. Positive and negative gating based on FMO staining.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.