TITLE

Anti-tumor activity without on-target off-tumor toxicity of GD2 Chimeric Antigen Receptor engineered T cells in neuroblastoma patients

SHORT TITLE (50 characters)

GD2-directed CAR T cell therapy for neuroblastoma

ONE SENTENCE SUMMARY

Neuroblastoma patients infused with GD2-directed CAR T cells experienced immune activation and transient tumor regressions without neurotoxicity.

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ABSTRACT

The re-programming of a patient's immune system through genetic modification of the T cell compartment with chimeric antigen receptors (CARs) has led to durable remissions in chemotherapy refractory B cell cancers. Targeting of solid cancers by CAR T cells is dependent on their infiltration and expansion within the tumor microenvironment, and thus far few clinical responses have been reported. Here we report a phase I study (NCT02761915) we treated twelve children with relapsed/refractory neuroblastoma with escalating doses of second generation GD2-directed CAR T cells and increasing intensity of preparative lymphodepletion. Of six patients receiving $\geq 10^8/m^2$ CAR T cells following fludarabine/cyclophosphamide conditioning, two experienced grade 2-3 cytokine release syndrome, and three demonstrated regression of soft tissue and bone marrow disease. Clinical activity was achieved without on-target off-tumor toxicity. Targeting neuroblastoma with GD2 CAR T cells appears a valid and safe strategy but requires further modification to promote CAR T cell longevity.

INTRODUCTION

Neuroblastoma is the most common extra-cranial solid tumor in childhood. Half of patients diagnosed have high-risk disease based on age, extent of disease and molecular features*(1)*. Treatment of high-risk neuroblastoma remains challenging; current multimodal treatment regimens achieve long term survival in <50% of patients and are associated with significant morbidity*(2, 3)*. Chimeric antigen receptor (CAR) T-cells can induce lasting responses in relapsed / refractory B-cell malignancy. Hence, we investigated treatment of relapsed/refractory neuroblastoma using second generation chimeric antigen receptor (CAR)-modified T cells directed against disialoganglioside (GD2).

The Ganglioside GD2 is an attractive target for CAR-based therapies since it is abundantly expressed on almost all neuroblastomas. However, GD2 is expressed at low levels on peripheral nerves and brain parenchyma*(4–6)*; Treatment with therapeutic antibodies targeting GD2 is frequently associated with an acute infusional pain syndrome and occasionally with central neurological syndromes*(7–9)*. A previous CAR T cell clinical study using a first generation CAR based on the 14.18 single chain variable fragment (scFv) did not cause neurological toxicity but resulted in minimal CAR T cell expansion and immune activation*(10–12)*. We developed a second generation GD2 CAR with CD28/CD3ζ signaling domains and humanized anti-GD2 scFv based on the K666 antibody*(13)*. We evaluated autologous T-cells expressing this receptor in a phase I clinical study (NCT02761915) of relapsed/refractory neuroblastoma. We aimed to investigate if these second generation GD2 CAR T-cells (1RG-CART) could be manufactured, and if they expand within the subjects. Further, we sought to determine if 1RG-CART cause immune or on-target toxicity and if any anti-tumour activity occurs.

In this study, both cell dose and preparative lymphodepletion regimen were escalated. As described below, 3 out of 6 patients receiving $\geq 10^8/m^2$ GD2 CAR T cells following fludarabine (Flu)/cyclophosphamide (Cy) lymphodepletion showed anti-tumor activity in bone marrow and

soft tissue sites of disease. These patients showed signs of immune activation (cytokine release and systemic immune activation). Critically, this was without on-target off-tumor neurotoxicity.

RESULTS

1RG-CART cell products derived from neuroblastoma patient peripheral blood show GD2-dependent effector function.

We tested a second generation GD2-directed CAR (1RG-CART) in a phase I study of patients with relapsed / refractory neuroblastoma. The CAR was constructed using a scFv from an intermediate affinity humanized GD2 antibody K666*(14)* linked via a human IgG1 Fc spacer (mutated to reduce Fc Receptor binding) to a CD28-Zeta endodomain*(15)* (Fig. 1A). The sort / suicide gene RQR8*(16)*, which renders T cells susceptible to Rituximab (Fig. S1), was co-expressed using a foot-and-mouth virus 2A peptide (Fig. 1B). RD114-pseudotyped gamma-retroviral vector encoding this bicistronic cassette was used to engineer autologous T cells harvested by leukapheresis.

While cognizant that the likely therapeutic dose would be $\geq 1x10^7/m^2$ and that lymphodepletion with both Fludarabine and Cyclophosphamide would be needed*(17)*, a cautious study design was selected to mitigate in particular against risks of target mediated neurotoxicity. The study incorporated escalation of both lymphodepletion regimen intensity and cell dose: starting with $1x10^7/m^2$ 1RG-CAR cells with no lymphodepletion, increasing to Cyclophosphamide only, then to Fludarabine/Cyclophosphamide with subsequent cell dose escalation to $1x10^8/m^2$ and $1x10^9/m^2$ (Table 1).

Seventeen patients with relapsed or refractory neuroblastoma (eligibility criteria and recruitment data in table S1 and S2) were enrolled on this phase I clinical study (Table 1). The median age was 7.5 years (range 2-15 years) and all patients had received multiple lines of previous treatment including anti-GD2 antibody therapies for 11/17 patients. Patients enrolled had disease in bone (n=13), bone marrow (n=11) and/or soft tissue sites (n=14).

Cell products at cell doses of up to $1x10^9$ cells/m^2 for dose level (DL) 5 were successfully manufactured for all 17 patients who underwent leukapheresis. Median transduction efficiency was 32.5% (range 24-56%). Cell products contained both CD4+ and CD8+ T-cells (median ratio CD4:CD8 = 0.72. (range 0.4 – 2.2) and showed a predominantly central and effector memory phenotype with a variable percentage of stem cell memory (scm)/naïve T cells (Fig. 1C, Table S3). For 4 patients for whom autologous bone marrow samples containing infiltrating neuroblastoma cells were available, cytolytic activity of 1RG-CART-cell products against autologous tumor cells was demonstrated (Fig. 1D).

Prior lymphodepletion is associated with measurable peripheral blood engraftment of 1RG-CART cells

Twelve patients were treated on DL1 (n=4), DL2 (n=1), DL3 (n=1), DL4 (n=3) and DL5 (n=3) respectively (Table 1). *In vivo* expansion and persistence of 1RG-CAR T cells were assessed in peripheral blood by transgene specific quantitative polymerase chain reaction (PCR) as well as flow cytometry using the QBend10 antibody specific for the CD34-epitope incorporated in RQR8. For patients receiving $1x10^7/m^2$ 1RG-CART either without lymphodepletion (DL1) or following administration of Cy (DL2) or Flu/Cy (DL3), 1RG-CART could not be detected. In this context, in the absence of dose limiting toxicity (DLT) as defined by trial protocol, at completion of cohort 1, an amendment to an accelerated dose escalation was made, with one patient treated on DL2 and one patient on DL3.

When higher CAR T cell doses of $1x10^8/m^2$ and $1x10^9/m^2$ were used with Fly/Cy conditioning (DL4 and DL5), expansion of 1RG-CART cells was seen in all 6 patients (Fig. 2A-C). Peak marking levels ranging from 3,500 to 48,000 copies (cp) per μg of DNA were reached at 7-14 days post 1RG-CART infusion (Fig. 2A). Following this the proportion of 1RG-CART cells within the peripheral blood declined, but 1RG-CART remained detectable at day +28 in 4 out of 5 evaluable patients in cohorts 4 and 5 (range 670 – 8,200 cp/μg of DNA).

Peripheral blood expansion of 1RG-CART cells is associated with immune activation but not neurotoxicity.

Immune activation, hematological and neurological toxicity data is summarized in Table 2**,** and blood cytokine measurements are listed in Table S4. No Grade ≥3 1RG-CART-related toxicity was observed in any patients. All six patients treated on DL4 and 5 developed signs of immune activation: 4 patients developed grade 1 cytokine release syndrome (CRS), 1 developed grade 2 and a further patient developed 3 CRS. Prolonged cytopenias developing after initial count recovery were seen in 3 patients, 2 of whom (patients 25/010 and 25/018) had significant immune activation syndrome (Fig. S2). Critically, using cell doses of up to $10^9/m^2$, no dose-limiting toxicity, including neurotoxicity, was seen.

Tumor responses are associated with immune activation but are incomplete.

For 3 of the 6 patients treated with 1RG-CART cell doses of $\geq 10^8/m^2$ in DL4 and 5 there was evidence of 1RG-CART activity at soft tissue and bone marrow sites. These patients are described in detail below. The other 3 patients treated on these dose levels had clinical signs of progressive

disease confirmed with MRI and/or meta-iodobenzylguanidine (^{123}I-mIBG) scintigraphy at 3-4 weeks post 1RG-CART infusion (Table 3).

Patient 25/010 was an 8-year old girl with relapsed metastatic neuroblastoma after four lines of previous treatment. At trial entry she had widespread bone metastases and extensive bone marrow infiltration. She required opioid analgesia for pain and her performance score was 60%. She received $1x10^8/m^2$ 1RG-CART cells. On day +5 she developed fever, hypotension requiring fluid boluses and was supported with nasal canula oxygen. At this time, she had raised C-reactive protein (CRP) with serum interleukin-6 (IL-6) and IL-10 levels in keeping with Grade 3 cytokine release syndrome (CRS). These symptoms resolved following a single dose of tocilizumab (Fig. 3A). From day +7 she developed weight gain, tender hepatomegaly, and ascites. She had a low serum albumin, coagulopathy and raised soluble CD25 (16,100 pg/ml (normal <2,500)), raised triglycerides (1.75 mM), and raised ferritin (3,724 ug/ml, peak 17,208 ug/ml) (Fig. 3B). These symptoms of sustained immune activation resolved with supportive care by day +22. On day +21 she had an episode of acute back pain. Biochemical analysis of blood showed changes consistent with tumor lysis: raised potassium and phosphate serum levels and a peak LDH of 4,017 IU/L (Fig. 3C). Her symptoms resolved by day +24 and all analgesia were stopped. At disease reassessment on day +28, her performance status had markedly improved to 90%. ^{123}I-mIBG scintigraphy showed a reduction in avidity at multiple sites of bone/marrow disease. Histological assessment of the bone marrow which at baseline was heavily infiltrated with neuroblastoma showed extensive tumor necrosis (Fig. 3D-F).

Patient 25/013 was a 10-year-old girl with multiply relapsed localized neuroblastoma after 5 previous lines of treatment. At trial entry she had a locoregional collection of tumor nodules in the neck with no distant metastases (Fig. 4A). She received $1x10^9/m^2$ 1RG-CART. From day +9 signs of local inflammation were visible over the lateral component of the tumor mass with subsequent desquamation. From day +56 there was marked inflammation over the midline component, which resolved again by day +63 concurrent with reducing size of the midline tumor on clinical examination (Fig. 4B). On day +28, MRI showed reduction in size of the midline lesion with reduced avidity on ^{123}I-mIBG. However, the right sided lateral components increased in size and a new left sided lesion was visible (Fig. 4C). Two months post 1RG-CART, the disease in the right and left lateral tumor sites persisted but the midline lesion continued to reduce in size and now no longer showed ^{123}I-mIBG uptake. On day +63 post 1RG-CART administration biopsies were taken of the areas of disease persistence as well as the residual midline lesion. Whereas the biopsies of the lateral sites with persistent disease showed viable tumor throughout, the biopsy of the residual midline lesion showed extensive necrosis (Fig. 4D).

Patient 25/018 was a 10-year old boy with relapsed metastatic neuroblastoma with an extradural lumbar primary after 3 previous lines of treatment. At trial entry he had disease sites in the right cervical region with infiltration in the right ramus of the mandible, the left upper abdominal quadrant and the sacrum, but no bone marrow involvement. His performance score was 70%. He received $1x10^9/m^2$ 1RG-CART. He became febrile on day 0 and received tocilizumab on day +6 when he required fluid support to maintain normotension (Fig. 5A). He subsequently developed dyspnea secondary to fluid retention (ascites and pleural and pericardial effusions). This coincided with elevated levels of CRP, serum IL-6 levels and serum ferritin (1,597 ug/L, Fig. 5A, Table S4)

consistent with systemic immune activation. These symptoms resolved after further tocilizumab on day +16. While MRI performed on day +21 showed stable disease, disease reassessment at day +37 with ^{123}I-mIBG showed a mixed appearance in cervical and abdominal disease sites with clear areas of reduced radio-isotope uptake (Fig. 5B-C). Biopsies of the cervical mass showed viable tumor in one biopsy and large areas of necrosis with some viable tumor cells in a second biopsy from the area of ^{123}I-mIBG low avidity (Fig. 5D). The bone marrow remained clear of neuroblastoma but was hypocellular consistent with his prolonged pancytopenia. At this time the patient was pain free without use of regular analgesia.

Despite signs of anti-tumor activity, these 3 patients showed no clear evidence of residual 1RG-CAR T cells in post treatment biopsies, and subsequently had progressive disease: The bone marrow of patient 25/010 on d+45 was hypocellular with continued extensive tumor necrosis but also areas of infiltration of neuroblastoma (Fig. S3). She died on d+50 of pseudomonas septicemia. Patient 25/013 and 25/018 both died of progressive disease within 5 months post 1RG-CART. Expression of PD-L1 on was detected on recurring tumor for patient 25/010 and on myeloid cells/macrophages present in the tumor microenvironment (patient 25/013 and patient 25/018) indicating one potential cause of lack of persistence of CAR T cells at the tumor site (Table S5, Fig. S4). Post treatment tumor cells in bone marrow/biopsy samples showed continued GD2 expression in 3/3 evaluable patients and hence there was no evidence of antigen escape (Fig. S5).

CONCLUSIONS

The current study demonstrates that second-generation GD2 CAR T cells (IRG-CART) in a solid-tumor setting expand and can induce rapid regression of bulky and disseminated disease. Further, 1RG-CAR T cells cause immune activation syndromes, with similar clinical and immunological features as seen in patients with B cell malignancies treated with CD19 CAR T cells. In addition, targeting of GD2 with this second-generation receptor resulted in no on-target off-tumor toxicity despite CAR T-cell expansion and anti-tumor activity.

Autologous CAR T-cell manufacture was successfully performed in 17 out of 17 patients who underwent apheresis. This was despite multiple lines of previous treatment including high-dose chemotherapy and stem cell rescue in 14/17. In patients who had bone-marrow disease, cytolytic activity of the CAR T cell product against autologous neuroblastoma was demonstrated.

Immune activation syndromes, anti-tumor activity and detection of CAR T-cell expansion was dose-dependent. In all 6 patients treated with a cell dose of $10^7/m^2$ either without lymphodepletion or with Cyclophosphamide or Fludarabine/Cyclophosphamide conditioning, 1RG-CART in peripheral blood were below the level of detection. In contrast, treatment with a cell dose of $\geq 10^8/m^2$ following Fludarabine/Cyclophosphamide conditioning resulted in *in vivo* expansion of 1RG-CART. Of six patients treated with these higher cell doses, two experienced grade 2-3 CRS requiring intervention with IL-6 blockade, and in 3 patients activated CAR T cells resulted in anti-tumor activity.

A previous study*(10, 11)* in neuroblastoma using either unselected peripheral blood or Epstein Barr virus-specific T cells expressing a 14.18 GD2 antibody-based first-generation CAR reported a similar rate of response predominantly in patients with disease limited to a solitary bone lesion or bone marrow infiltration only. In contrast to the present study, there was more limited evidence

of CAR T cell expansion in peripheral blood during the two week period following infusion, consistent with the requirement for a costimulatory signal of a second generation CAR to effect T cell activation and proliferation.

In clear contrast with the first generation GD2-CAR study*(10, 11)*, in this current study significant immune activation was induced. All three patients with evidence of anti-tumor activity had a syndrome which resembled cytokine release syndrome observed in patients treated with CAR T cells directed against lymphoid malignancies. IL-6 was elevated, and the two patients treated with IL-6 blockade responded. Induced cytokine release and inflammatory response may be essential to achieve sustained T cell function within the tumor microenvironment. Two patients had prolonged symptoms of immune activation with fluid retention and biochemical features consistent with ongoing systemic immune activation. This is may suggest a difference to the more typically curtailed immune activation seen in patients with hematological malignancies. As seen in leukemia CAR T cell studies*(18, 19)*, we observed prolonged cytopenia in 2 patients. While this may in part be due to heavy prior treatment and lymphodepletion, both patients had had sustained immune activation, which may have been contributory.

As GD2 is expressed at low levels in pain fibers and brain parenchyma*(5)*, an important safety consideration is the increased sensitivity to antigen of CARs incorporating co-stimulation*(20)*. GD2 therapeutic monoclonal antibody therapy is associated with frequent peripheral and central neurotoxicity*(8, 21)*. Further, severe CNS inflammation was observed in a small animal model of neuroblastoma treated with a high affinity second generation GD2 CAR*(6)*. With this in mind, the CAR used in 1RG-CART used an intermediate affinity GD2 binder*(22)*, and the suicide gene RQR8 was co-expressed*(16)*. In our study, despite the presence of clear anti-tumor activity and CAR T cell activity, no neurotoxicity was observed, with exception of transient headache with

normal neuroimaging (patient 25/010) and hallucinations whilst febrile (patient 25/018). Hence, an important conclusion from this study is that tumor-directed activity could be achieved with a CD28-CD3ζ second generation GD2-directed CAR without on-target off-tumor toxicity.

The progression of disease following initial response mirrored the decline of CAR T cells in peripheral blood. Disease progression was evident when 1RG-CART level decreased to less than 2,000 cp/μg of DNA, suggesting that future refinements to convert induced anti-tumor activity into sustained clinical responses should be focused on maintaining CAR T cell persistence and function. Several factors could explain the short-lived nature of 1RG-CART following adoptive transfer. CAR T-cell product phenotype can impact on clinical activity. However, here we found no difference in T cell memory or T cell "activation/exhaustion" marker phenotype of the infused 1RG-CAR T cells in patients with or without immune activation and anti-tumor activity albeit that patient numbers are small (Fig. S6). Alternatively, the hostile tumor immune inhibitory microenvironment, as has been well described in neuroblastoma*(23–25)*, may overwhelm T cell proliferation. Expression of PD-L1 was present on recurring tumor or myeloid cells/macrophages within the tumor microenvironment in some of the post treatment biopsies available and may indicate one mechanism of T cell inhibition. Similarly, at the tumor site sustained antigenic stimulation may either help to drive an exhaustion phenotype of CAR T cells*(26, 27)*, or may drive effector cells to activation induced cell death*(28, 29)*. Despite efforts to phenotype the CAR T cells *in situ*, detailed flow cytometric and immunohistochemical analysis of the biopsies performed at day +28 could not detect residual CAR T cells so further characterization was not possible.

An emerging observation in CAR T cell therapy is modulation or loss of the target antigen. For instance, a frequent cause of CD19 CAR T cell treatment failure is CD19 negative escape due to mutational disruption of the CD19 gene*(30)*. CD22 directed CAR T-cell therapy is associated with

down-modulation of CD22*(31)*. In theory, GD2 may be more susceptible to loss given that its expression requires a chain of synthetic enzymes. Flow cytometric analysis for GD2 expression was performed in all patients who responded, this included analysis of biopsies taken from multiple sites and at different time-points after 1RG-CART. No GD2 negative or GD2 dim neuroblastoma cells were observed. This includes serial and bilateral samples from patient 25/010 who had almost complete clearance of marrow disease with recrudescence, suggesting considerable selective pressure on disease expression of GD2.

Based on our findings, modifications to enhance T cell persistence systemically but particularly within the tumor, will likely be required to induce more sustained responses. Possible approaches include additional T cell engineering to provide cytokine signal,*(32)* transcriptionally re-program to enhance persistence,*(33–36)* or create resistance to the inhibitory microenvironment*(37–39)*. Notwithstanding future developments, the evidence we present indicates the capacity for GD2-directed CAR T cells to enter the solid tumor environment, expand with associated but manageable CRS, and induce extensive local tumor necrosis without off-target toxicity. GD2 is expressed on a wide range of cancers including medulloblastoma, diffuse midline glioma, osteosarcoma, melanoma, and small cell lung cancer giving this work broad applicability. Future studies are therefore warranted to determine the clinical effects of GD2-targeted CAR T cells armed with engineering approaches to promote their sustained proliferation.

MATERIAL AND METHODS

Study design. The 1RG-CART trial (NCT02761915) was a single-center non-randomized, open-label phase I clinical study in which eligible patients from throughout the United Kingdom were recruited. Eligible patients were children with relapsed or refractory neuroblastoma with at least one site of measurable disease. Inclusion and exclusion criteria for the study are given in Table S1. The study was approved by the UK Medicines and Healthcare Products Regulatory Agency (clinical trial authorization no. 21106/0260/001-0001), the London – West London & GTAC Research Ethics Committee (REC ref no. 15/LO/1510) and the research and development of department of Great Ormond Street Hospital NHS Foundation Trust. Written informed consent was obtained from patients or their caregivers prior to study entry.

Generation of retroviral RQR8_2A_GD2-CAR cassette. A single chain variable fragment was derived from the fully humanized anti-GD2 monoclonal antibody K666 as previously described. The CAR contains a human IgG1 hinge-Fc spacer with deleted FcγR binding domains, the CD28 transmembrane domain and CD28 and CD3z signaling domains in cis. Using the Foot-and-mouth Disease 2A sequence (FMD-2A)*(40)* sort/suicide gene RQR8*(16)* and the GD2-CAR were co-expressed in retroviral vector SFG designated MP10413.*(41)*

Generation of retrovirus packaging cell line. The RD114 pseudotyping 293Vec-RD114 packaging cell line was used for vector production (Fig. S7). A high titer RD114 pseudotyping producer clone was produced by multiple co-transfection. Two packaging cell lines were used for this production: 293Vec-GALV to produce transient GALV pseudotyped supernatant and 293Vec-

RD114 to generate the final clone. Both cell lines were supplied by BioVec Pharma. GALV pseudotyped retroviral supernatant was generated by transient transfection of the packaging cell line 293Vec-GALV with the MP10413 plasmid. This supernatant was then used to multiply transduce a RD114 pseudotyping packaging cell line 293Vec-RD114 to generate a bulk producer. This 293Vec-RD114 bulk producer was single-cell cloned by limiting dilution and a high-titer clone selected by titration of the supernatant generated from each clone.

Manufacture of 1RG-CART cell product. Products were generated from autologous PBMCs after leukapheresis of the patient. PBMCs were washed and activated with anti-CD3 (1 µg/mL) and anti-CD28 (1 µg/mL) GMP antibodies (Miltenyi Biotec). On day 3, retroviral transduction was performed in cell differentiation bags (Miltenyi Biotec) coated with retronectin (Takara). On day 6, T cell were removed from the retronectin-coated bags and cultured in fresh media. The cell product was cryopreserved on either day 7 for DL 1-3 or day 10 for DL 4 & 5. Throughout manufacture, cells were cultured in X-VIVO15 medium (Lonza) supplemented with 5% human AB serum (Life Science Production) and IL-2 (120 IU/mL Clinigen Healthcare Ltd). Release assays performed prior to infusion included assessments of sterility (bacterial culture, mycoplasma PCR), endotoxin levels (LAL), viability and transduction efficiency by flow cytometry.

Flow cytometry. Flow cytometry acquisition was performed with a BD LSR II, Aria or Canto II (BD Biosciences) or FortessaX20. Data analysis was performed using FlowJo v10 (Tree Star, Inc., Ashland OR), or FACs DIVA 8.0.1.

Percentage transduced cells was determined by staining with QBEND10 detecting the CD34 epitope within the RQR8 marker/suicide gene. Co-staining with anti-human Fc was performed to demonstrate co-expression of the RQR8 and the GD2-CAR. The following reagents were used for phenotypic analysis of 1RG-CART: QBEnd10-APC (R&D Systems), CD3-APC-Cy7 (Biolegend), CD45-FITC (BD Biosciences), CD45RA BV480 (BD Biosciences), anti-CCR7 PE-Dazzle (BD Biosciences), TIM-3 BV711 (BD Biosciences), LAG-3 FITC (BD Biosciences), PD-1 Pe-Cy7 (BD Biosciences) and 7-AAD (BD Biosciences) or Fixable viability stain 780 (BD Biosciences). Fluorescence minus one (FMO) controls were used to determine expression thresholds where required. To assess GD2 expression on neuroblastoma infiltrating bone marrow, bone marrow aspirates were incubated with ammonium chloride lysis buffer to lyse red cells for 10 minutes, washed and then directly stained. For the same analysis in tumor biopsies, tissue was mechanically disaggregated, filtered, washed and then directly stained. Antibodies used included CD45 V450 (BD Biosciences), CD33 APC (BD Biosciences), CD56 (BD Biosciences), GD2 PE Cy7 (Biolegend) and 7-AAD viability dye (Beckman coulter).

Lymphodepletion and 1RG-CART infusion. Patients treated on DL1 did not receive prior lymphodepletion whereas patients treated on DL2 received cyclophosphamide only at 300 mg/m^2 /day on days -4 to -1 (total of four doses), followed by 1RG-CART on Day 0. Patients treated on DL3-5 received 300 mg/m^2/day of cyclophosphamide on each of days -7 to -4 (total of four doses) and 25 mg/m^2 /day of fludarabine on each of days -8 to -4 (total of five doses), followed by 1RG-CART on Day 0 (or on Day 0 and Day 1, requiring a split dose).

1RG-CART detection in peripheral blood. 1RG-CAR T cells were detected using a validated qPCR assay detecting a transgene-specific sequence. Genomic DNA was isolated and sequencing reactions carried out with transgene- specific primers and Taqman probes (Applied Biosystems), using an input of 0.5µg genomic DNA where possible. A control qPCR assay using primers and probes for albumin was carried out in parallel to quantify amplifiable DNA present per sample. Results were reported as copies per 10^6 peripheral blood cells, with a validated detection limit of 10,000 copies per 10^6 PBMC. Circulating 1RG-CART in blood and bone marrow were also analyzed by flow cytometry using QBEND10 antibody. Absolute T cell numbers were obtained using a Trucount method (BD) and staining for viable, CD45 + CD3 + cells. Reagents used were Anti-human CD3 (clone UCHT1, BioLegend), Anti-human CD45 (Clone 2D1, BD), Anti-human CD34 (QBEnd10, clone ab8536s, R&D Systems FAB7227A), 7-AAD (BD) and BD Wash (BD). The assay was validated for performance across a range of 10 – 320 cells/µL.

Cytokine bead array. Serum cytokine measurements were assessed on days including 0, 1, 2, 3, 5, 7, 10 and 14 post 1RG-CART infusion by an International Organization for Standardization-accredited method using cytometric bead array analysis of IL-2, IL-4, IL-6, IL-10 TNF-α and IFN-γ (BD Biosciences).

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections of bone marrow trephines and tumour biopsies were subjected to immunostaining. Conventional immunohistochemistry was performed to detect PD-L1 using the 22C3 monoclonal antibody (Dako UK Ltd) and to detect PHOX2B using the EPR14423t monoclonal antibody (Abcam). In brief, sections were de-waxed and re-hydrated prior to the immunostaining according to an

established protocol previously described*(42)*. To establish optimal staining conditions (i.e. antibody dilution and incubation time, antigen retrieval protocol, suitable chromogen) the antibody was tested and optimized on 2-4 um cut tissue sections of (human tonsil and lung tissues for PD-L1 staining) by conventional single immunohistochemistry. Assessment of specificity of PD-L1 staining and scoring was performed by a histopathologist in a manner blinded to patient case and time point pre/post 1RG-CART treatment.

LIST OF SUPPLEMENTARY MATERIALS

Supplementary Figures 1, 2, 3, 4, 5, 6 and 7

Supplementary Tables 1, 2, 3, 4 and 5.

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