**Avoidance of off target activation using a costimulation-only chimeric antigen receptor**

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**Contributor statements**

JF devised the initial concept, designed, performed and analyzed experiments. PA conceived, designed, performed and analyzed experiments. NDWD designed, performed and analyzed experiments. BF optimized and performed Cytometric Bead Array experiments. AC optimized the transduction methods, GWC performed experiments, KG provided immunological support, JA designed experiments, supervised the work and contributed to manuscript preparation in conjunction with JF and PA.

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**Abstract**

Chimeric antigen receptors (CARs) combine T-cell activation with antibody-mediated tumor antigen specificity, bypassing the need for T-cell receptor (TCR) ligation. A limitation of CAR technology is on-target off-tumor toxicity caused by target antigen expression on normal cells. Using GD2 as a model cancer antigen, we hypothesized that this could be minimized by using T cells expressing Vγ9Vδ2 TCR, which recognizes transformed cells in an MHC-unrestricted manner, in combination with a co-stimulatory CAR that would not bypass the TCR.

An anti-GD2 CAR containing the endodomain of the NKG2D adaptor, DAP10 was expressed in Vγ9Vδ2$^+$ T cells. Differential ligation of the CAR and/or TCR using antibody-coated beads showed that pro-inflammatory cytokine response depended on activation of both receptors. Moreover in killing assays, GD2-expressing neuroblastoma cells that engaged the Vγ9Vδ2 TCR were efficiently lysed whereas cells that express GD2 equivalently but do not engage the Vγ9Vδ2 TCR were untouched. Differentiation between "X-on-tumor" and "X-off-tumor" offers potential for safer immunotherapy and broader target selection.

**Abbreviations**

| | |
|---|---|
| TCR | T-cell receptor |
| γδTCR | Gamma-delta T-cell receptor |
| Vγ9Vδ2 or Vδ2 | V-gamma-9/V-delta22 gamma-delta T-cell receptor |
| CAR | Chimeric antigen receptor |
| CAR-T cell | T-cell expressing a chimeric antigen receptor |
| GD2-28-ζ | Chimeric antigen receptor targeting GD2, containing CD28 and CD3ζ endodomains |
| GD2-DAP10 | Chimeric antigen receptor targeting GD2, containing the DAP10 endodomain only |
| IPP | Isopentenyl-5-pyrophosphate |
| ZOL | Zoledronic acid |

**Summary (max 40 words)**

αβT cells expressing chimeric antigen receptors (CARs) potently kill tumor cells expressing target antigen but are limited by on-target off-tumor toxicity. This proof-of-concept paper describes a means of overcoming this toxicity by expressing co-stimulatory CARs in γδT cells rather than αβT cells.

## Introduction

Chimeric antigen receptor (CAR)-based immunotherapeutics have been developed to harness the cytotoxic potential of peripheral blood T cells. CARs comprise ectodomains, which recognize tumor-associated antigens, fused to endodomains, which provide co-stimulatory signals. The majority of CARs currently under investigation contain the CD3ζ endodomain, which provides TCR signalling, in addition to one (second generation) or two (third generation) costimulatory endodomains such as CD28, 4-1BB or OX40 (Chmielewski et al., 2013). When expressed in αβT cells, second and third generation CARs bypass the requirement for MHC-restricted antigen presentation, providing CD3ζ-mediated TCR signalling and costimulatory signals in the presence of the target antigen. $CAR^+$ T cells targeting CD19 have shown unprecedented clinical efficacy in lymphoid malignancy in adults and children (Maude et al., 2014; Lee et al., 2015). Progress in targeting solid tumors such as neuroblastoma has been more measured, though $CAR^+$ T cells are attractive as they offer the potential for sustained anti-tumor immunity. CARs targeting GD2 in neuroblastoma have shown early promise (Pule et al., 2008; Louis et al., 2011) but the potential for sustained neurotoxicity remains a concern, especially given the severe toxicities encountered in other $CAR^+$ T-cell trials as a result of unexpected off-tumor antigen expression (Lamers et al., 2006; Morgan et al., 2010). As a means to overcome this limitation we have devised a CAR design in which signals 1 and 2 of T-cell activation are provided by separate receptors.

GD2 is a ganglioside abundantly expressed on the surface of neuroblastoma cells and on several other cancer types. It is a highly ranked tumor associated antigen and an attractive target for immunotherapy (Cheever et al., 2009). In 2010, it was shown that adjuvant immunotherapy comprising an antibody (ch14.18-Sp2/0) targeting GD2, in combination with IL-2, GM-CSF and isotretinoin significantly improved the event-free and overall survival of high-risk neuroblastoma patients (Yu et al., 2010). However, the neurotoxicity and long-term survival benefits of anti-GD2 monoclonal antibody therapy are being questioned. The opiate requirements of patients receiving GD2-targeting antibody are substantial and a minority of patients develop long-term neurological sequelae (Yu et al., 2010; Simon et al., 2004). The degree of acute and chronic neurotoxicity that will emerge from ongoing trials using second and third generation GD2-targeting CARs is not yet known.

Comprising 1-5% of circulating T-cell numbers, γδT cells possess innate and adaptive immune functions. Their ability to recognize danger-associated molecular patterns to differentiate between healthy and tumor cells in an MHC-independent manner (Hayday, 2009) distinguishes them from αβT cells, which rely on MHC-restricted antigen presentation to detect altered-self. The most abundant γδT-cell subset in human peripheral blood expresses the Vγ9Vδ2 TCR (hereafter referred to as $V\delta2^+$ γδT cells), responding to increased phosphoantigen production in target cells (Vantourout and Hayday, 2013). Phosphoantigens such as isopentenyl-5-pyrophosphate are cholesterol biosynthesis intermediates, and their accumulation in response to metabolic dysregulation has been linked to aberrant EGFR activity (Poupot and Fournié, 2004; Asslan et al., 1999). Detection of phosphoantigen by the Vγ9Vδ2 TCR is indirect and, unlike antigen recognition by αβT cells, does not depend on MHC presented epitopes. Phosphoantigen detection is thought to occur through its binding to butyrophilin-3A1, although the mechanism by which TCR engagement is effected is a matter of intense debate, and other models have been proposed (Vavassori et al., 2013; Harly et al., 2012; Sandstrom et al., 2014; Silva-Santos et al., 2015).

γδTCR engagement alone is insufficient to induce $V\delta2^+$ γδT-cell cytotoxicity against neuroblastoma. A second signal is required, much in the same way that αβT cells require a costimulatory signal. Costimulation of γδT cells is known to occur in part through receptors such as NKG2D which are ligated by danger-associated molecules such as MIC-A and MIC-B, but can also be provided via Fcγ receptors within the context of an opsonized target cell as we have previously shown (Fisher et al.; 2014). Neuroblastoma cells have been reported to evade NKG2D-mediated immune destruction through down-regulation of NKG2D ligands or by secretion of receptor-blocking soluble ligands (Nedellec et al., 2010; Raffaghello et al., 2004). This has been demonstrated to be an important escape mechanism against NK cell-mediated killing of neuroblastoma (Raffaghello et al., 2005). Therefore, the NKG2D-adaptor protein, DAP10, was an attractive candidate for inclusion in a costimulatory CAR designed to target neuroblastoma. This approach is a development of previously reported strategies which divided the CD3ζ and co-stimulatory signals between two CARs targeting different tumor associated antigens (Lanitis et al., 2013; Kloss et al., 2013). By using the pre-existing danger-sensing specificity of the γδTCR, it is possible to escape the requirement for two distinct tumor associated antigens.

In this report we demonstrate that Vδ2$^+$ γδT cells are amenable to manipulation with a chimeric antigen receptor comprising a conventional ectodomain to target GD2 linked to an endodomain that provides DAP10 costimulation but no TCR signal. This design supplements but does not replace the endogenous γδTCR signal, capitalizing on the innate ability of γδT cells to differentiate between healthy and transformed self in an MHC-independent manner. Thus, "costimulation-only" CARs provide cytotoxic activation on encountering a cancer cell but not upon encountering non-transformed cells expressing the same target antigen.

## Materials and methods

### Isolation of PBMCs and T-cell expansion

20 ml of whole blood were diluted with 10 ml PBS + 500 µl 100 mM EDTA and layered on 20 ml Percoll. Interface PBMCs (20 min, 300xg, RT) were washed in PBS and re-suspended in 25 ml T-cell medium (RPMI-1640 medium supplemented with L-glutamine (2 mM, Sigma-Aldrich (Missouri, USA)), penicillin/streptomycin (100 IU/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich)) and 10% FCS (v/v, (Gibco, Massachusetts, USA)). In some experiments $CD56^+$ cells were depleted from PBMC preparations (day 1) by positive selection using CD56 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletions to less than 3% of $CD56^+$ cells were achieved regularly. For γδ T-cell expansion, 5 µM zoledronic acid (Actavis, New Jersey, USA) and 100 IU/ml IL-2 (Aldesleukin, Novartis, Frimley, UK) was added to PBMC suspension after PBMC isolation (day 1). IL-2 was replenished every 2-3 days by removing half of the media from the well and replacing with fresh media containing 200 IU/ml IL-2. αβ T-cell expansion was induced by addition of anti-CD3/CD28 Dynabeads (Gibco, Massachusetts, USA).

### Construction of retroviral constructs

The retroviral vector used in all constructs was the splicing oncoretroviral vector SFG (Rivière et al., 1995), pseudotyped with an RD114 envelope. DNA fragments were amplified using the Phusion HT II polymerase according to the manufacturer's instructions (Thermo Scientific, Massachusetts, USA). PCR was carried out in a PTC-200 DNA Engine (MJ Research, Massachusetts, USA). PCR products were extracted from 1% agarose gels using the Wizard SV Gel & PCR Clean-Up kit (Promega, Wisconsin, USA). Sample concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Massachusetts, USA). The CAR ectodomain comprised an scFv against human GD2 and a spacer derived from the human IgG4 CH2-CH3 portion has been described before in third generation format (Thomas et al., 2016) . The endodomain was generated using oligo-assembly PCR based on a codon optimised sequence of the human DAP10 endodomain (NCBI Gene ID 10870). In addition to the CAR construct, RQR8 which is a marker bearing a CD34 epitope (Philip et al., 2014) was included, separated from the CAR by a cleavable 2A peptide. This allows CAR expressing cells to be detected by flow cytometry by staining using the anti-CD34 antibody clone QBend10.

**Production of viral particles by transfection**

$1.5x10^6$ 293T cells/100 $mm^2$ dish (Nunclon Delta Surface, Thermo Fisher) were plated at day 1 in 293T medium (D-MEM, 10 % FCS (v/v)). γ-retroviral particles were produced by co-transfection of 293T cells at day 2 using GeneJuice Transfection Reagent (Novagen/Millipore, Massachusetts, USA) in accordance with manufacturer's directions. Viral particle-containing supernatants were harvested at day 4; medium was replenished, and harvested at day 5. Supernatants were pooled, filtered (0.45-µm filter, Millipore) and directly used for transductions or stored at -80°C.

**Transduction of T cells**

Transduction of T cells was carried out in Retronectin (Takara Bio, Tokyo, Japan) coated 24-well plates, which were pre-loaded with viral supernatant. $0.5x10^6$ T cells suspended in 0.5 ml T-cell medium + 400 IU IL-2 were combined with 1.5 ml viral supernatant and centrifuged for 40 min, 1000xg at RT. Timing of transduction differed between γδT cells and αβT cells due to expansion dynamics. γδT-cell expansion was stimulated with 5 µM zoledronic acid (Actavis, New Jersey, USA) and 100 IU/ml IL-2 (Aldesleukin,) and transduction performed at day 5. At day 8 of culture (day 3 after transduction) cells were pooled, washed and plated at $2x10^6$ cells/ml in T-cell medium + 100 IU IL-2/ml (24-well plates, Nunclon Delta Surface, Thermo Scientific, Massachusetts, USA). Transduction efficiency was determined by flow cytometry at day 10 (day 5 after transduction). In the case of αβ T cells, transduction was performed 72 h following stimulation with anti-CD3/CD28 Dynabeads (Gibco, Massachusetts, USA) in the presence of 100 IU/ml IL-2 (Aldesleukin), with re-plating 3 days after transduction.

**Flow cytometry**

For the analysis of intracellular cytokines, cells were incubated for 4 hours in T-cell medium supplemented with 1x 5 µg/ml Brefeldin A (BioLegend, California, USA). For exclusion of dead cells the Zombie Yellow Fixable Viability Kit (BioLegend) was used. For Fc-receptor blocking, cells were incubated in PBS supplemented with FcR Blocking Reagent (Miltenyi Biotec). For intracellular staining of cytokines the Inside Stain Kit (Miltenyi Biotec) was used. For absolute cell counting in secondary expansion experiments CountBright beads (Molecular Probes/Life Technologies, California, USA) were applied. The following antibodies were used to perform analysis by flow cytometry: anti-CD3-biotin (OKT3), anti-CD3-BV785 (OKT3), anti-CD3-APC (UCHT1), anti-CD56-FITC (HCD56), anti-CD69-APC-Cy (FN50), anti-

CD279/PD-1-APC-Cy7 (EH12.2H7), anti-CD366/Tim-3-PE-Cy7 (F38-2E2), anti-TCR Vδ2-PE (B6), anti-TCR αβ-APC (IP26), anti-IFN-γ-FITC (4S.B3), anti-TNF-α-BV711 (MAb11), anti-GD2-PE (14G2a) from BioLegend, anti-CD34-APC (QBEnd10) from R&D Systems (Minnesota, USA), anti-CD25-FITC (M-A251) from BD Pharmingen (San Jose, California, USA).

**$^{51}$Cr-killing assay**

To assess the killing capacity of T cells, 5,000 $^{51}$Cr-labelled target cells were co-cultured with 50,000, 25,000, 12,500 or 6,250 effector cells in 200 µl T-cell medium + 100 IU IL-2/ml. Cells were incubated in 96-well V-bottomed plates (Greiner Bio-one, Kremsmünster, Austria) for 4 h. 50 µl of supernatant were transferred into Isoplate-96 HB plates (PerkinElmer, Massachusetts, USA). After addition of 150 µl scintillation cocktail (Optiphase Supermix, PerkinElmer) samples were incubated overnight at RT. Counts were acquired using a 1450 MicroBeta TriLux scintillation counter (PerkinElmer).

**Activation of T cells using antibody-coated beads**

Anti-Biotin MACS iBeads (Miltenyi, Biotec) were labelled with 10 µg anti-CD3-biotin (OKT3, BioLegend), 10 µg anti-IgG (Fc)-biotin (Novex/Life Technologies), or 10 µg of anti-CD3-biotin and 10 µg anti-IgG (Fc)-biotin, respectively. After incubation (10 min, 4°C), beads were washed twice with PBS and re-suspended in T-cell medium. To assess T-cell functionality and act as a positive control, anti-CD3/CD28 Dynabeads were re-suspended in T-cell medium as well. Bead suspensions (100 µl) were plated in 96-well U-bottom plates (Thermo Scientific). Effector T-cell preparations were added ($0.2x10^6$ cells/well in 100 µl T-cell medium) and incubated with beads for 24 h. No exogenous IL-2 was added to the medium in either case. After 23 h 100 µl of supernatant were harvested for analysis of cytokine secretion by Cytometric Bead Array (CBA) and intracellular cytokine staining, i.e. data generated by CBA and intracellular cytokine staining are from the same experiments and are based on the same sample size.

**Cytokine secretion assay**

The BD CBA Flex Set Cytometric Bead Array (Becton Dickinson, New Jersey, USA) was used in accordance with manufacturers instructions to measure the concentrations of cytokines including IL-2, IL-4, IL-10, IL-17a, TNF-$\alpha$, IFN-$\gamma$ and

Granzyme B in the supernatant of γδT cells stimulated for 23 h with antibody-coated beads as above.

**Secondary expansion of transduced cells following antigen challenge**

γδT cells that had been transduced to express the GD2-DAP10 CAR were maintained for 3 weeks following transduction in T-cell medium and 100 IU/ml IL-2 (Aldesleukin). Following the 3-week culture with no further TCR stimulus, the γδT cells were co-cultured with irradiated (60 Gy) neuroblastoma cell lines (LAN-1 or SK-N-SH) at a 1:1 effector:target (E:T) ratio. Live cell numbers were determined using Trypan blue exclusion and the number of live TCRVδ2$^+$/CAR$^+$ γδT cells was determined by flow cytometry using the Zombie Yellow fixable viability dye (Biolegend) in addition to staining for TCR Vδ2 and the CD34 epitope which is co-expressed with the CAR. By multiplying the number of live cells by the percentage of live cells that were TCRVδ2$^+$/CAR$^+$ the absolute cell number could be determined and used to calculate a fold change based on the starting cell number.

**Cell lines**

Human neuroblastoma cells lines Kelly, SK-N-SH and LAN-1, and the murine colonic carcinoma cell line CT26 were originally obtained from the ATCC. The Ewing's sarcoma cell line TC-71 was obtained from DSMZ (Braunschweig, Germany). CT26-GD2 cells were generated by transducing wild-type CT26 with SFG gammaretrovirus encoding GD2 and GD3 synthase co-expressed via foot and mouth virus 2A self-cleaving peptide sequence, kindly provided by M Pule (UCL). After transduction, single GD2-expressing clones were generated by limiting dilution, and expanded.

**Statistical analysis**

Analyses were performed using GraphPad Prism version 6.0 (GraphPad, California, USA). In instances where comparison was made between two groups with one variable only, a t-test was used. Comparisons of more than two groups defined by one factor were done by 1-way ANOVA followed by Bonferroni post-hoc analysis. For comparisons of conditions where there was more than one variable (for example E:T ratio and transductions status) a 2-way ANOVA with Bonferroni correction was used to determine statistical significance. These results were deemed to be statistically significant if $P<0.05$. Where applicable, the following notation is used to denote significance: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

**Supplementary Figures**

In order to determine that the bead-based stimulation of cells would provide reliable and consistent stimulation we confirmed that staining by a directly fluorochrome-conjugated anti-CD3 or anti-Fc antibody gave equivalent results to a biotinylated antibody used in conjunction with fluorochrome conjugated streptavidin (Supplementary Figure S1), and that the anti-biotin bead binding to biotinylated antibody did not interfere with streptavidin binding to the same antibody (Supplementary Figure S2). Supplementary figures S3-S5 provide more detail on the exhaustion and activation phenotype of GD2-DAP10 and GD2-28-ζ transduced $V\delta2^+$ γδT cells. Supplementary Figure S3 shows differential expression of PD-1 which is significantly higher in transduced cells compared to non-transduced, and at day 16 was significantly lower in GD2-DAP10 $CAR^+$ $V\delta2^+$ γδT cells compared to GD2-28-ζ expressing αβT cells or $V\delta2^+$ γδT cells. Supplementary Figure S4 shows an additional time point in the overall analysis of exhaustion phenotype. Supplementary Figure S5 summarizes data on cellular activation in order to further characterize the γδT-cell phenotype after expansion and transduction.

## Results

### Second generation CARs override the requirement of Vδ2$^+$ γδT cells for a second signal to induce cytotoxicity against neuroblastoma.

Vδ2$^+$ γδT cells are the most common subtype in human blood and are capable of direct interaction with tumor cells through the Vγ9Vδ2 TCR. However, we have previously demonstrated that Vδ2$^+$ γδT cells are incapable of significant direct cytotoxicity against neuroblastoma cell lines although capable of antibody-dependent killing following addition of an antibody recognizing GD2, which is brightly expressed on neuroblastoma. The degree of antibody-dependent cell-mediated cytotoxicity (ADCC) was proportional to the degree of antigen-antibody-CD16 interaction (Fisher et al.; 2014). To overcome this antibody dependence we expressed a second generation anti-GD2 CAR containing CD3ζ and CD28 endodomains (GD2-28-ζ, Figure 1A) in Vδ2$^+$ cells, and compared the antibody-dependent and -independent killing of GD2$^+$ and GD2$^-$ neuroblastoma cell lines. CARs were developed to bypass the need for TCR and costimulatory molecule engagement in T-cell immunotherapy, and will signal in the absence of a TCR ligand as shown in Figure 1A. Expression of GD2-28-ζ in the Vδ2$^+$ γδT cells was associated with increased antibody independent cytotoxicity (Figure 1B) against GD2$^+$ cell lines (Figure 1B & 1C). The benefit of CAR expression on γδT-cell cytotoxicity was highly significant (Figure 1C) indicating that CAR expression of zoledronate expanded Vδ2$^+$ is capable of transducing cytotoxic signals in these cells. It should be noted that the transduction efficiency and duration of stimulation differs between zoledronic acid induced CAR$^+$ γδT cells and the CAR$^+$ αβT cells stimulated with anti-CD3/28 beads. Therefore comparison of relative cytotoxic potential of these populations cannot be inferred from this experiment.

### Specific and selective activation of GD2-DAP10 CAR-expressing γδT cells

We speculated that it might be possible to retain full signal 2-dependent killing of neuroblastoma by Vδ2$^+$ cells whilst avoiding killing of non-transformed GD2-expressing cells by exploiting the ability of the Vγ9Vδ2 TCR to provide signal 1 following engagement by stressed but not by non-stressed cells. To this end we generated a chimeric antigen receptor comprising GD2-binding ectodomain and an endodomain containing the DAP10 endodomain hereafter referred to as GD2-DAP10 (Figure 2A). The DAP10 endodomain was chosen because DAP10 is the signalling

adaptor protein for NKG2D, an important activatory receptor found on NK cells and γδT cells and because neuroblastoma is known to demonstrate immune evasion through blockade of NKG2D signalling (Nedellec et al., 2010; Raffaghello et al., 2004).

To investigate the specific activation of GD2-DAP10 CAR$^+$ γδT cells in comparison to GD2-28-ζ CAR$^+$ γδT cells without the influence of additional confounding factors we established an *in vitro* assay using antibody coated anti-biotin beads (Supplementary Figures S1 and S2). These beads were labelled with biotinylated anti-CD3 antibody to engage the Vγ9Vδ2 TCR and/or anti-CAR antibody to engage the CH2CH3 linker in the respective CARs (Figure 2B). By coating the beads with only one of the two antibodies, γδTCR and CAR intracellular signals could be isolated from each other. Due to the changing presence or absence of either signal 1 or signal 2 in this system, differences in the cytokine production profile were expected between γδT cells expressing either the GD2-DAP10 or GD2-28-ζ CAR. Intracellular cytokine production was assessed by flow cytometry (Figure 2C) which demonstrated that, whilst stimulation of the CAR alone was sufficient to stimulate IFN-γ production in GD2-28-ζ CAR$^+$ Vδ2$^+$ γδT cells, CD3 and CAR stimulation was required to stimulate IFN-γ production in GD2-DAP10 CAR$^+$ Vδ2$^+$ γδT cells.

Incubation with unlabelled or anti-CD3 antibody-coated beads did not induce the production of IFN-γ in γδT cells transduced with either type of CAR. GD2-DAP10 CAR$^+$ Vδ2$^+$ cells showed increased production of IFN-γ compared to untransduced cells in the same culture when both the CAR *and* γδTCR were stimulated, consistent with both signals 1 and 2 being provided by the bead-stimulated receptors (Figure 2D). In contrast, incubation with beads that only engaged the CAR did not induce IFN-γ production in GD2-DAP10 Vδ2$^+$ cells, i.e. when the γδTCR is not engaged. GD2-28-ζ CAR$^+$ Vδ2$^+$ cells produced IFN-γ upon engagement of the CAR alone since both signals were provided by the CAR-endodomain structure. Hence, full IFN-γ response of GD2-DAP10 CAR$^+$ γδT cells only when engagement of both the TCR for signal 1 and of the CAR for signal 2 is provided, but not following engagement of either the TCR or the CAR alone. Interestingly, TNF-α was produced in the GD2-DAP10 CAR γδT following engagement of the CAR alone but not by engagement of the TCR indicating that the DAP10 derived signal 2 alone was sufficient to generate a TNF-α response (Figure 2E).

To further characterize γδT-cell function following ligation of either the GD2-DAP10 CAR or the GD2-28-ζ CAR we measured the concentration of cytokines in the supernatant following stimulation with beads as described above. Release of IFN-γ, TNF-α, IL-2, IL-4 and Granzyme B by GD2-DAP10 Vδ2$^+$ cells was only seen when both CD3 and the CAR were stimulated. If CD3 or the CAR were stimulated in isolation, cytokine release was minimal or absent. This was not the case for GD2-28-ζ Vδ2$^+$ cells which as expected produced substantial amounts of these cytokines following CAR ligation alone. Interestingly GD2-28-ζ Vδ2$^+$ cells also produced IL-10 following CAR stimulation, which was not seen in the GD2-DAP10 Vδ2$^+$ cells, even when CD3 and the CAR were stimulated (Figure 3).

**γδT cells expressing GD2-DAP10 CARs show cytotoxicity against GD2$^+$ neuroblastoma and Ewing sarcoma *in vitro***

To evaluate possible therapeutic efficacy of the GD2-DAP10 CAR in Vδ2$^+$ γδT cells we evaluated specific cytotoxicity against representative cell lines derived from the childhood cancers neuroblastoma and Ewing sarcoma, which we and others have previously demonstrated to express GD2 uniformly (Kailayangiri et al., 2012; Fisher et al. 2016). Expression of the GD2-DAP10 CAR in Vδ2$^+$ γδT cells yielded significantly enhanced cytotoxicity against the GD2$^+$ neuroblastoma cell line LAN-1, which was equivalent to the cytotoxicity imparted by the GD2-28-ζ CAR expressed in Vδ2$^+$ γδT cells (Figure 4A). This effect was also seen against GD2$^+$ Ewing sarcoma cell lines such as TC-71 (Figure 4B) but was not seen against GD2 non-expressing neuroblastoma cell line SK-N-SH (Figure 4C). To demonstrate that the GD2-DAP10 CAR was not providing sufficient signal to elicit killing independently of the γδTCR we expressed the same construct in αβT cells. Expression of the GD2-DAP10 CAR had no effect on αβT-cell cytotoxicity against GD2$^+$ neuroblastoma cell lines unlike the conventional GD2-28-ζ CAR which significantly enhanced αβT-cell cytotoxicity against LAN-1 GD2$^+$ neuroblastoma cells (Figure 4D).

**γδT cells expressing GD2-DAP10 CARs do not show toxicity against GD2$^+$ cells that do not engage the γδTCR**

Phosphoantigens such as isopentenyl-5-pyrophosphate (IPP) are produced in increased quantities by cancer cells and, in human cells, are detected by the Vγ9Vδ2

γδTCR via mechanisms involving intermediate molecules such as butyrophilin (Vavassori et al., 2013). This process has been linked to aberrant EGFR activity which is seen in many tumor types (Poupot and Fournié, 2004; Asslan et al., 1999). Healthy cells do not produce increased levels of phosphoantigen and therefore do not engage the γδTCR. When using GD2 as a model antigen, culture of "healthy" human GD2$^+$ cells is not technically or ethically feasible, given that GD2 expression is restricted to peripheral neurons. MHC-independent phosphoantigen recognition by the Vγ9Vδ2 γδTCR is species-specific (Kato et al., 2003; Green et al., 2004); therefore we developed a murine model of a GD2$^+$ cell line that would not engage the human Vγ9Vδ2 γδTCR by transducing the murine colon carcinoma cell line CT26 to express GD2, as murine cells do not engage the human Vγ9Vδ2$^+$ TCR (Figure 5A)

First, in order to demonstrate the suitability of the antigen model, we determined that the expression of GD2 alone was sufficient to increase the susceptibility of CT26 to killing by αβT cells expressing the GD2-28-ζ CAR (Figure 5B). Vδ2$^+$ γδT cells expressing the second generation GD2-28-ζ CAR retained the ability to kill CT26-GD2 cells; in contrast, GD2-DAP10 CAR$^+$ Vδ2$^+$ γδT cells did not significantly kill CT26-GD2 cells, consistent with failure of γδTCR engagement and lack of the necessary signal 1 (Figure 5C). The killing by GD2-DAP10 CAR$^+$ Vδ2$^+$ γδT cells was dependent on the presence of the target antigen because wild type CT26 elicited no killing response from these effectors (Figure 5C, right panel). Hence, just as lack of engagement of the TCR in GD2-DAP10 CAR$^+$ Vδ2 cells leads to failure of IFN-γ production in the bead-based system (Figure 2D), failure to engage the TCR by a cellular target, results in a failure to elicit cytotoxicity.

**γδT cells expressing GD2-DAP10 CARs have a favorable phenotype for adoptive transfer**

The ability to exert specific cytotoxicity against tumor cells must be accompanied by the ability to mount an antigen-specific secondary expansion of CAR$^+$ T-cell populations. Failure to do this has been linked to T-cell exhaustion and obstructs the efficacy of CAR-T cells used in clinical trials (Long et al., 2015). Unfortunately, testing in-vivo persistence in animal models is complicated by the potential for a xeno-response to drive T-cell expansion, which would confound results. Therefore an in-vitro model of rested CAR$^+$ cells was used. Vδ2$^+$ cells expressing the GD2-DAP10 CAR which had been left for 3 weeks without antigenic challenge following

initial zoledronic acid stimulation were re-challenged with an irradiated $GD2^+$ cell line (LAN-1) or a $GD2^-$ control (SK-N-SH) or simply maintained in a target-free state. Co-culture with the $GD2^+$ neuroblastoma cell line LAN-1 led to a sustained secondary expansion over a 3-week period, whereas incubation with the $GD2^-$ neuroblastoma cell line SK-N-SH produced no significant difference from cells incubated with no targets (Figure 6A).

T-cell exhaustion is associated with phenotypic and functional changes. Exhausted T-cells are less able to mount a secondary expansion when challenged, and will express cell surface markers such as TIM-3 and PD-1. Exhaustion attenuates the response of adoptively transferred CAR-T and reduces their clinical efficacy, an effect which can be reduced through choice of CAR endodomain (Long et al., 2015). To determine the susceptibility of γδT cells expressing either GD2-28-ζ CARs or GD2-DAP10 CARs to exhaustion we determined their expression of TIM-3 and PD-1 over the course of a prolonged culture (Figure 6B). For the purposes of statistical analysis, "exhaustion" was defined as the expression of at least one of the markers TIM-3 and PD-1. Expression of either CAR promoted a more exhausted phenotype, which was associated with greater PD-1 expression in $CAR^+$ T-cells. (Figure 6B-C, Supplementary Figure S3). After 10 days of culture (5 days after transduction), no differences were seen in the percentages of exhausted cells between the two $CAR^+$ groups (Figure 5C), but by day 16 (11 days after transduction), the GD2-DAP10 $CAR^+$ population contained significantly fewer exhausted cells than the GD2-28-ζ $CAR^+$ population (Figure 6D). This tendency was also seen at day 24 (Supplementary Figure S3).. Moreover, comparison of frequencies of "activated" events, i.e. cells that express activation markers CD25 and/or CD69, indicated a tendency towards a more activated phenotype ($CD69^+/CD25^+$) in GD2-28-ζ CAR+ γδT cells compared to GD2-DAP10 $CAR^+$ γδT cells, in the absence of further antigenic stimulus (Supplementary Figure S5). Thus, whilst CAR expression appears to impart a degree of exhaustion, it appears that this effect is less pronounced when the GD2-DAP10 CAR is expressed compared to the GD2-28-ζ CAR, perhaps related to lower levels of background activation in GD2-DAP10 $CAR^+$ γδT cells.

**Discussion**

On-target off-tumour toxicity is one of the major impediments to successful cancer immunotherapy. It is typically approached in two ways – either by avoiding antigens which may be expressed on healthy tissues, restricting antigen choice, or by accepting non-tumor antigen expression and the potentially severe toxicities that accompany it. Examples of this approach include anti-CD19 and anti-CD20 immunotherapies which have been used against lymphoid malignancies (Jackson et al., 2016; Batlevi et al., 2016). The elimination of healthy $CD19^+$ B-cells is considered to be an acceptable toxicity in this instance, as patients can be supported long-term with intravenous immunoglobulin infusions as required. Neurotoxicity associated with the CD19 CAR T cells is an emerging concern (Maude et al., 2014; Davila et al., 2014; Lee et al., 2015). In other cases such as neuroblastoma targeted with anti-GD2 monoclonal antibodies, the toxicity is more severe with pain being experienced presumably secondary to engagement of GD2 expressed on peripheral nerve fibres. Fatal off target toxicity has also been reported using CAR T cells targeting EGFR (Morgan et al., 2010). The ability to differentiate between "X-on tumour" and "X-on healthy" would solve this problem; not only reducing toxicity but also making previously overlooked antigens available for targeting.

Here, we have demonstrated that by providing supplementary DAP10 signals via a "co-stimulation only" CAR, $V\delta2^+$ $\gamma\delta$T cell anti-neuroblastoma cytotoxicity can be induced. Neuroblastoma and DAP10 were chosen for this model because of low background killing of neuroblastoma by unmanipulated $V\delta2^+$ $\gamma\delta$T cells, and because of previously documented immune evasion of NKG2D/DAP10 signalling in this disease (Raffaghello et al., 2004). In tumor systems which do not evade killing through down-regulation of membrane bound or production of soluble NKG2D ligands, supplementing DAP10 signals to tumor-targeting T cells may not be of benefit. Anti-tumor cytotoxicity is associated with production of IFN-$\gamma$, which is not produced by GD2-DAP10 $CAR^+$ $V\delta2$ when the CAR or the $\gamma\delta$TCR are stimulated in isolation (Figures 2 and 3). The lack of pro-inflammatory cytokine production in the absence of a second signal in the neuroblastoma model is consistent with our previously published findings which demonstrated the need to coat neuroblastoma cells with antibodies that sensitize to ADCC to generate an optimal IFN-$\gamma$ response in $V\delta2$ T cells (Fisher et al., 2014). It is interesting however that the IFN-$\gamma$ signal provided by concurrent $\gamma\delta$TCR and "co-stimulation only" CAR stimulation is still lower than that induced by engagement of a second generation CAR with CD3$\zeta$-CD28,

suggesting that inclusion of other co-stimulatory molecules in the CAR design may be of benefit. In addition, future characterization of other co-stimulatory endodomains within the costimulation-only CAR architecture will explain if intracellular detection of TNF-α upon engagement of the GD2-DAP10 CAR only is due to the endodomain chosen here, i.e. DAP10, which has previously been associated with TNF-α production after NKG2D activation (Rincon-Orozco et al., 2005).

We have demonstrated that the GD2-DAP10 CAR can induce Vδ2$^+$ γδT-cell cytotoxicity only against cell lines that engage both the γδTCR and the CAR, providing more precise control than conventional CD3ζ-containing CARs which induce cytotoxicity in the presence of target antigen alone. This precision of control is robust in cell-based and bead-based assays indicating mechanistic consistency. Unfortunately, due to the restriction of human GD2 expression to primary neurons, it is not feasible to perform cytotoxicity assays against healthy GD2$^+$ cells. We have previously demonstrated that peripherally administered Vδ2$^+$ γδT cells will invade and kill subcutaneous neuroblastoma in a murine model (Fisher et al. 2016). However, it would not be possible to generate a murine model of healthy neuronal tissue which would have any possibility of engaging the human Vγ9Vδ2 TCR due to the species-specificity of this interaction. The majority of γδT cells that penetrate epithelial surfaces are of non-Vδ2$^+$ subsets, which have greater antibody-independent anti-tumor ability (Fisher et al., 2014) and therefore are less amenable to precise control. Vδ2$^+$ γδT cells are generally restricted to the bloodstream and are much less abundant in the tissue space.

Adoptively transferred cells used in cancer immunotherapy must be able to survive in the host and mount a secondary expansion in response to their target antigen. T-cell exhaustion due to over-stimulation or tonic CAR signalling can render a cellular immunotherapy short lived or ineffective (Long et al., 2015; Wherry and Kurachi, 2015). There is no sustained significant difference in the exhaustion phenotype of "co-stimulation only" CAR Vδ2$^+$ γδT cells and those transduced to express conventional second generation CARs, though there is a tendency towards a less exhausted phenotype. Whilst GD2-DAP10 Vδ2$^+$ cells are able to produce IL-2 in response to simultaneous CD3 and CAR stimulation (Figure 3), the secondary expansion seen after re-challenge with target antigen expressing tumor cells is modest, suggesting that further stimulus may be required. It would be perhaps unfair to compare the secondary expansion potential of Vδ2 γδT cells with that of CAR

transduced αβT cells however, as the baseline dynamics of these cell types are different. New light will be shed on the applicability of γδT-cell subsets in future immunotherapy approaches as novel insights underpin the important role of specific tissues in modelling their local γδT-cell compartments (Di Marco Barros et al., 2016).

By introducing a novel, more precise approach to cellular cancer immunotherapy, "co-stimulation only" CARs expressed in Vδ2$^+$ γδT cells present a choice. Vδ2$^+$ γδT cells are less abundant, harder to expand and less well understood than αβT cells, but they offer a means of specifically targeting tumors without harming healthy antigen-positive cells. The balance between force and precision of the anti-tumor response will depend on the context, but this approach at least offers an opportunity to target abundant and immunogenic molecules that were previously placed out-of-bounds by toxicity.

**Figure Legends**

**Figure 1. Chimeric antigen receptor expressed in expanded gamma delta T lymphocytes enhances antigen specific cytotoxicity. (A)** 2nd generation anti-GD2 Chimeric Antigen Receptor containing the CD3ζ and CD28 endodomains allows activation of T cells in the absence of TCR engagement**. (B and C)** $^{51}$Cr-release assay demonstrating cytotoxicity of Vδ2$^+$ γδT cells expressing an anti-GD2-28ζ CAR against GD2$^+$ and GD2$^-$ neuroblastoma cell lines. In (B) antibody dependent cytotoxicity at E:T ratio of 10:1 was measured after coating the target cells with ch14.18/CHO anti-GD2 antibody or isotype control antibody (n=3 independent experiments). In (C) statistical differences shown for 10:1 E:T ratio Error bard indicate mean values ± SEM; **P<0.01, ****P<0.0001 after Bonferroni post-hoc analysis. The bar chart on the right shows 10:1 E:T ratio with analysis by unpaired t-test; n=3 independent donors for GD2-28-ζ and untransduced αβT cells, n=6 for GD2-28-ζ CAR$^+$ γδT cells, n=14 for untransduced γδT cells.

**Figure 2. CAR-mediated co-stimulation is essential to allow functional activation of γδT cells upon TCR engagement. (A**) Vδ2$^+$ γδT cells are activated in an MHC independent manner, by indirectly sensing increased phosphoantigen levels on tumors through engagement of the TCR with cognate ligand. Tumors evade γδT-cell cytotoxicity by suppressing the co-stimulatory signals required. CARs containing costimulatory domains can bypass this evasion whilst retaining tumor specificity. Healthy tissues do not produce increased phosphoantigen levels so that the TCR is not engaged and the CAR alone is insufficient to provide full activation**. (B)** Schematic representation of the experimental design. Anti-CD3 antibodies coupled to the surface of beads were used to activate TCR (signal 1); anti-CAR (anti-Fc) antibodies activate the CAR (signal 2) through binding the CH2CH3 linker of the CAR. **(C)** Representative dot plots of γδT-cell analysis by flow cytometry after incubation with beads labeled with anti-CD3 and/or anti-CAR antibodies. The binding and crosslinking of a second generation CAR is sufficient to allow full T-cell activation since both signal 1and 2 are provided by the CAR, i.e. by the CD3ζ chain and the CD28 endodomain, respectively. According to the co-stimulation only CAR concept the engagement of a CAR providing only signal 2, e.g. by a DAP10 endodomain, does not lead to γδT-cell activation. Only if signal 1, e.g. due to TCR activation, is provided in parallel γδT cells become activated and respond by functional changes,

e.g. IFN-γ production. **(D)** The engagement of a second generation CAR, i.e. GD2-28-ζ, is sufficient to induce a profound production of pro-inflammatory IFN-γ by γδT cells. In contrast, γδT cells expressing a co-stimulation only CAR (GD2-DAP10) produce IFN-γ only upon both TCR and CAR engagement. **(E)** No differences in TNF-α production were found for respective anti-CD3 and/or anti-CAR antibody-stimulated γδ T cells expressing the GD2-DAP10 or the GD2-28-ζ CAR. (D, E) Bonferroni post-hoc analysis after 1-way ANOVA. Graphs display merged results from two independent experiments (n=4-6 independent donors). Error bars represent mean values ± SD.

**Figure 3. Cytokine secretion by GD2-DAP10 Vδ2$^+$ cells is dependent on CD3 and CAR engagement.** Cytokine production by Vδ2$^+$ γδT cells expressing GD2-DAP10 or GD2-28-ζ CARs following 23 h stimulation with antibody-coated beads engaging CD3, the CAR or both. Non-transduced γδT cells denoted "no virus" are included for comparison, and CD3/CD28 bead stimulation was included as a positive control. Error bars indicate SEM of 3 independent donors.

**Figure 4. Vδ2$^+$ γδT cells expressing GD2-DAP10 CARs kill GD2$^+$ tumor cells.** Specific killing of (A) Neuroblastoma line LAN-1 (n=14 independent donors for untransduced and (B) GD2$^+$ Ewing sarcoma cell line TC-71 (n=3 independent donors) by GD2-DAP10 CAR$^+$. (C) This effect is not seen against the GD2$^-$ neuroblastoma cell line SK-N-SH (n=3 independent donors). (D) The GD2-DAP10 CAR does not function in αβT cells where signal 1 is absent indicating that the signal is restricted to the co-stimulatory pathway (n=3 independent donors for each subgroup). Frequencies are shown as mean ± SEM; significance by Bonferroni post-hoc correction after 2-way ANOVA, with only E:T 10:1 annotated for clarity.

**Figure 5**. **Target cells not engaging the Vγ9Vδ2 are not killed by GD2-DAP10 CAR Vδ2$^+$ γδT cells. (A)** The murine colonic carcinoma cell line CT26 was transduced to express human GD2 and to derive the cell line CT26-GD2. Expression of GD2 was as homogenous as that detected for LAN-1. **(B)** $^{51}$Cr-release assay demonstrating the ability of αβT cells expressing the GD2-28-ζ CAR to kill CT26-GD2 cells in an antigen specific manner. Killing of wild-type CT26 and background killing by untransduced αβT cells is minimal (n=3 independent donors for each subgroup). **(C)** $^{51}$Cr-release assay demonstrating that Vδ2$^+$ γδT cells expressing the GD2-28-ζ CAR kill CT26-GD2 cells in an antigen-specific manner even though CT26 does not

engage the Vδ2$^+$ γδTCR, whereas Vδ2$^+$ γδT cells expressing the GD2-DAP10 CAR do not (n=7 independent donors for each subgroup). A difference for killing of CT26 target cells was also determined at E:T 10:1 using GD2-28-ζ CAR$^+$ cells compared to GD2-DAP10 CAR$^+$ cells (n=7 independent donors for each subgroup). Frequencies are shown as mean ± SEM, with significance by Bonferroni post-hoc correction after 2-way ANOVA,; only E:T 10:1 annotated for clarity.

**Figure 6. Expression of GD2-DAP10 CAR in Vδ2$^+$ γδT cells leads to less exhaustion and activation marker expression than GD2-28-ζ CAR. (A)** Vδ2$^+$ T cells expressing the GD2-DAP10 CAR were transduced and then maintained in medium containing 100 IU/ml IL-2 for 3 weeks. They were then washed thoroughly and co-cultured with irradiated GD2$^+$ LAN-1 or GD2$^-$ SK-N-SH neuroblastoma cells in the absence of exogenous IL-2. A combination of live-cell counting by Trypan Blue exclusion and FACS analysis for CAR$^+$/Vδ2$^+$ cells was used to determine fold change over a 3-week co-culture. Only in the presence of GD2$^+$ targets did the CAR$^+$ cells mount a secondary expansion (n=3 independent donors, fold change compared by paired t-test at day 14 and day 20). **(B)** Gating to determine phenotype of CAR transduced γδT and αβT cells over prolonged culture in medium containing 100 IU/ml IL-2. Transduction efficiencies as well as the expression of TIM-3 and PD-1 were assessed by flow cytometry at each time point. Note, that analysis of TIM-3 and PD-1 expression for conventional αβ T cells was based on gating on Vδ2$^-$ events. Cells expressing TIM-3 and/or PD-1 were considered to have an "exhausted" phenotype. **(C)** After 10 days of culture (5 days after transduction) no significant differences were found by comparing the frequencies of exhausted events in all subgroups. Exhausted cells were defined as those expressing at least one of the exhaustion markers TIM-3 or PD-1. **(D)** At day 16 of culture (10 days after transduction) αβT or γδT cells expressing GD2-28-ζ CARs were more exhausted than either untransduced αβT or γδT cells. Furthermore, γδT cells expressing the GD2-28-ζ car were significantly more exhausted than γδT cells expressing the GD2-DAP10 CAR. As in (C), for the purposes of this analysis, cells were considered to be exhausted if they expressed TIM-3, PD-1 or both. (C) and (D) display combined results from two independent experiments (n=5 for γδT cells, n=6 for αβT cells) subjected to Bonferroni post-hoc analysis after 1-way ANOVA. Columns represent mean values ± SD.

**Supplementary Figure S1 Detection of CD3 and CAR expression using biotinylated primary antibodies.** Human αβT cells were expanded using anti-CD3,

anti-CD28-coated beads. **(A)** Using flow cytometry, frequencies of cells staining positive for expression of CD3 or CAR were determined, using the human Fc derived CH2CH3 linker of the CAR as a target (shown: cells stained with anti-Fc-biotin and Streptavidin-PE). **(B)** By staining T cells with anti-CD3-FITC antibody 97.8% of events were found to be $CD3^+$. In comparison, 93.6% of $CD3^+$ events were detected using Streptavidin-PE after staining of cells with anti-CD3-biotin. **(C)** To determine the frequency of CAR-expressing T cells the anti-CD34-APC antibody was used. 73.5% of T cells were $CAR^+$. By using anti-CAR-biotin and Streptavidin-PE 76.4% of events were found to express the CAR.

**Supplementary Figure S2 Efficient labeling of anti-biotin beads upon incubation with biotinylated antibodies and Streptavidin-PE. (A)** The specific loading of beads with biotinylated antibodies was detected by flow cytometry. **(B)** First, beads were labeled with anti-CD3-biotin, anti-CAR-biotin or anti-CD3-biotin plus anti-CAR-biotin antibodies followed by incubation with Streptavidin-PE. **(C)** Overlays of histograms showing successful binding of antibodies by beads in comparison to controls.

**Supplementary Figure S3 γδT cells and αβT cells transduced to express GD2-DAP10 or GD2-28-ζ CARs express significantly more PD-1 than untransduced γδT or αβT cells.** At day 16, a reduced frequency of PD-$1^+$ GD2-DAP10-expressing γδT cells was found compared to PD-$1^+$ GD-28-ζ CAR-expressing γδT and αβT cells. For analysis 2-way ANOVA followed by Bonferroni post-hoc analysis was applied. The graph displays merged results from two independent experiments (n=5 for γδT cells, n=6 for αβT cells). Columns represent mean values ± SD.

**Supplementary Figure S4 Expression of TIM-3 and PD-1 by CAR-expressing T cells at day 24.** At day 24 of culture (19 days after transduction) of γδT or αβT cells the expression of TIM-3 and PD-1 were analyzed as a follow-up of day 10. A higher frequency of exhausted GD2-28-ζ CAR expressing γδT and αβ T cells was found in comparison to untransduced γδT cells in the GD2-DAP10 system. Graphs display merged results from two independent experiments (n=5 for γδT cells, n=6 for αβT cells) subjected to Bonferroni post-hoc analysis after 1-way ANOVA. Columns represent mean values ± SD, *$P<0.05$ after post hoc analysis.

**Supplementary Figure S5: Expression of CD25 and CD69 by CAR-expressing T cells in the course of expansion after transduction.** At day 10 and 18 after induction of γδT-cell expansion the expression of activation markers CD25 and CD69 were analyzed. **(A)** No significant differences in frequencies of "activated" events, i.e. events positive for CD25 and/or CD69, were found for GD2-28-ζ CAR expressing γδT cells and GD2-28-ζ mock-transduced γδT cells in comparison to GD2-DAP10 CAR γδT cells and GD2-DAP10 mock-transduced γδT cells. **(B)** Additionally, at day 18 of γδT-cell expansion, no significant differences in frequencies of "activated" events were found comparing CAR-expressing γδT cells and mock-transduced γδT cells. Graphs display results from one experiment (n=4 at day 10, n=3 at day 18). Bonferroni post-hoc analysis was performed after 1-way ANOVA. Both the overall ANOVA and the post-hoc analysis showed no significant differences. Columns represent mean values ± SD.

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