Introduction

Cellular engineering has offered many options for redirecting immune responses against cancer. In some cases the clinical responses have been remarkable{Maude:2014hh}{Maude:2015jx} but there are still many challenges to overcome. Broadly speaking, redirection of T-cell responses against specific tumour-associated antigens (TAAs) has most commonly been achieved in two ways – T-cell receptor (TCR) gene transfer or chimeric antigen receptor (CAR) expression. TCR gene transfer involves expression of a TCR derived from a tumour-reactive T cell derived from a tumour infiltrating lymphocyte (TIL), or from in vitro antigen-stimulated blood, into a polyclonal T-cell population. The alternative approach uses chimeric antigen receptors (CARs), which include an ectodomain that directly bind a cell surface molecule specific for the tumour cells (most commonly a single-chain variable fragment derived from a mononclonalantibody and and endodomains, which provide T cell signalling (most commonly CD3ζ in combination with one or more costimulatory endodomains such as CD28 or 4-1BB).

The majority of cellular engineering approaches have been applied to $\alpha\beta$T cells which are easy to expand and purify from peripheral blood. Notable media attention has been given to $\alpha\beta$T cells engineered to express second and third generation chimeric antigen receptors against targets such as CD19{Maude:2014hh}, and CAR-T cells targeting CD19 recently received FDA approval for sale in the United States. Engineered $\alpha\beta$T cells have numerous limitations however; whether they express a TAA directed TCR or a CAR. TCR transfer depends on the ability to isolate a HLA-matched TCR against an antigen expressed in tumour. It is also subject to tumour immune-evasion strategies such as down-regulation of MHC or loss of redundant neoantigens. Whilst CARs, by bypassing the $\alpha\beta$TCR entirely, remove the need for HLA-matching and antigen presentation on tumour MHC, antigen selection presents a challenge. CAR-T cells to target all cells expressing their cognate antigen; in the context of CD19, B-cell aplasia is considered an acceptable cost, but targeting of other antigens such as CAIX or ErbB2 have led to unexpected and sometimes fatal toxicity{Morgan:2010fy}{Lamers:2006cc}. In the same way, transferred TCR against tumour associatd antigens have also led to fatal and unexpected cross reactivity (MAGE 3A cardiotoxicity paper). Furthermore, the specificity of the response provides a prime opportunity for immune-evasion through antigen loss, which has proven to be a particular issue in anti-CD19 CAR-T therapy{Ruella:2016fl}.

Use of alternative cell types in cancer immunotherapy is not a novel concept. Adoptively transferred allogeneic NK cells or cytokine-induced killer (CIK) cells have generated clinical responses against metastatic melanoma{Gammaitoni:2017gc}, renal cell carcinoma, poor prognosis AML and Hodgkins lymphoma{Cheng:2013ft}. Whilst engineering of these cell types has lagged behind the that of conventional αβT cells, CAR transduced NK cell lines have been successfully directed against CD19 {Boissel:2009db}, CD20 {Muller:2008ht}, the disialoganglioside GD2{Esser:2012eb}, ErbB2{Schonfeld:2015jl} and other tumour associated antigens{Morvan:2016iv}. NK cell specificity to tumours has been engineered using bispecific antibodies that enhance synapse between NK cell and target (review PMID 28882429). NKT cells have been engineered to express CAR and trialled in humans (Metelista PMID 27183388)
This demonstrates the feasibility of using effector cells with an innate immune phenotype, possessing broader tumour recognition potential and having shorter lifespans in the patient circulation, potentially abrogating the need for "suicide genes" which are an essential safety feature of the much more long-lived CAR-αβT cells{Philip:2014ke}.

Properties of γδT cells

Whilst γδT cells comprise only 1-10% of the circulating T-cell compartment, they play a major role in mucosal and epithelial defence, where around 40% of lymphocytes are γδTCR$^+$ {Barisa:2017jt}. A correlation between the molecular profile of the tumour immune microenvironment and prognosis in over 5000 tumour samples indicated that the presence of γδT cells in the tumour microenvironment was the strongest predictor of positive outcome{Gentles:2015it}. This suggests that γδT cells retain some degree of anti-tumour activity beyond that possessed by other immune subsets, which may be subject to effective immune evasion.

γδT cells diverge from αβT cells in the thymus; lineage commitment is thought to be complete by the DN3 stage of thymic development{Fahl:2014cl}. More is known about the distribution of γδT cells in mice than in humans. In mice, γδT cells are released from the thymus in waves during embryonic development. Each wave consists of γδT cells expressing different Vγ subtypes which home to particular immunological niches in the adult animal, for example at 2 weeks of gestation γδT cells expressing a Vγ5 receptor home to the epidermis, later in gestation Vγ4 γδT cells home to the spleen and pulmonary epithelium, and Vγ6 homes to reproductive epithelia and that in the airways. After birth, a process of extra-thymic selection occurs leading to an increase in γδT cells expressing Vγ4 receptors in the murine epithelium{Lefrancois:1990ur}. Such extra-thymic selection is also observed in

humans. The human fetal and neonatal circulation contains predominantly Vδ1$^+$ γδT cells but there is a gradual shift during the first years of life towards a predominantly Vδ2$^+$ circulating γδT cell compartment{Parker:1990uw}. This gradual expansion of the Vδ2$^+$ subset appears to have some form of genericism, as illustrated by the high degree of CDR3 homology in fresh Vδ2$^+$ γδT cells, those expanded using aminobisphosphonates and those which expand in response to co-culture with microbes such as *Escherichia coli*{Barisa:2017jt}. Furthermore, unlike αβT cells, the chances of finding CDR3 region homology between unrelated individuals following phosphoantigen exposure remains high{Fisher:2014gv}.

Human γδT cells express Vγ2-5, 8-9 and Vδ1-8{Lefranc:2001jz}, but most is known about the Vδ1$^+$ and Vδ2$^+$ subsets. The commonest subset in human peripheral blood expresses the Vγ9Vδ2 TCR, and is more commonly referred to as Vδ2. They are potent effectors of anti-tumour immunity in their own right, and demonstrate enhanced cytotoxicity against antibody-opsonised targets. Vδ2$^+$ γδT cells respond to many stimuli, not limited to those delivered via the γδTCR, which engages cells with a high endogenous phosphoantigen burden via an adaptor protein - butyrophilin 3A1 (BTN3A1){Vavassori:2013ds}{DiMarcoBarros:2016jk}. Other stimuli include those delivered through NK-cell receptors including NKG2D{Nedellec:2010hb}, DNAM-1{GertnerDardenne:2012km} and Fcγ receptors such as FcγRIII(CD16){GertnerDardenne:2009ii}{Fisher:2016cl}. Despite sharing many phenotypic similarities with αβT cells, emerging evidence suggests that the activation of Vδ2$^+$ γδT cells is more pleiotropic than was originally thought, demonstrating a sensitivity to multiple excitatory and inhibitory stimuli usually associated with NK-cells. Vδ2$^+$ γδT cells can be easily expanded from the blood of healthy donors and cancer patients using inexpensive and well validated compounds such as aminobisphosphonates in combination with low-dose IL-2. This approach is helpful as it allows production of large numbers of highly purified Vδ2$^+$ γδT cells using a relatively simple protocol. Aminobisphosphonates inhibit the mavalonate pathway enzyme farnesyl pyrophosphate synthase, leading to an intracellular accumulation of the phosphoantigen isopentenyl-5-pyrophosphate (IPP).

The Vγ9Vδ2 TCR detects cells with a high phosphoantigen burden in an MHC-independent manner. High phosphoantigen burden is associated with malignant transformation and disordered EGFR signalling{Poupot:2004ef}{Asslan:1999dh}.

Whilst the clonal diversity within an expanded Vγ9Vδ2 population is large this represents a repertoire of TCR sequences many of which have varying affinity for the same stress marker – BTN3A1. Current understanding is that IPP, a phosphoantigen intermediate in the mavalonate pathway of cholesterol biosynthesis binds to the intracellular 30.2 subunit of BTN3A1, leading to a conformational change in the BTN3A1 ectodomain which is detected by the Vγ9Vδ2 TCR. This, in addition to stimulation via other stress-sensing receptors such as NKG2D and DNAM-1 allows the γδT cell to differentiate between healthy and cancerous cells. Less is known about the ligands for γδT cells from non-Vδ2 lineages; perhaps due to the diversity of targets and the MHC-independent activity of the γδTCR. Hence, i has been possible to identify numerous ligands without there being a clearly identifiable pattern. For example, Vδ1$^+$ γδT cells with reactivity against cells expressing lipids such as CD1c {Spada:2000uk}, CD1d-sulphatide {Bai:2012by}, CD1d-α-GalCer {Uldrich:2013jl}, but also against the MHC associated molecules MICA {Xu:2011dj} and MICB {Groh:1999tr}.

γδT cells are less well understood than αβT cells. As such, there must be a clear rationale for engineering them in preference to their more abundant cousins. γδT cells possess broad scope for MHC-independent tumour recognition, lack alloreactivity and show improved homing to epithelial tumour sites. There is also evidence that they are able to engage secondary immune responses through induction of dendritic cell maturation and professional antigen presentation in their own right{Himoudi:2012bz}{Anderson:2012ep}{Moser:2011kg}{Brandes:2005il}. Therefore, γδT cells and the γδTCR possess a range of useful characteristics which make them interesting candidates for cell engineers wishing to diversify beyond the conventional “CAR-T cell” paradigm. This engineering need can be met in a variety of ways, as outlined in Figure 1.

Transduction strategies for γδT cells

When engineering γδT cells it is important to select appropriate tools. In general, long-lasting transduction strategies which work well for αβT cells work well for γδT cells also. The predicted shorter lifespan of infused γδT cells offer the opportunity to use more transient engineering approaches as well, as the cells infused cells may not persist in the host long-term.

Many groups continue to use gammaretroviral vectors for transducing γδT cells. High transduction efficiencies are achievable using a Maloney murine leukaemia virus based vector, SFG{Riviere:1995bp}, pseudotyped with the envelope of the feline endogenous retrovirus RD114{Fisher:2017bv} or gibbon-ape leukaemia virus (GALV) envelopes. Gammaretroviral transduction has the advantage of allowing preparation of large, high titre batches of virus because of the availability of packaging cell lines which can be stably transduced to produce virus containing the construct of interest. Gammaretroviruses, lacking the machinery to penetrate the nucleus, require the cells to be actively cycling in order to achieve transduction as viral nucleic acids can enter through nuclear pores. This is not a restriction in the engineering of αβT cells, and the specific and rapid expansion of Vγ9Vδ2+ T cells in response to aminobisphosphonates allows for similar strategies to be applied. Transduction of other γδT cell subsets using gammaretroviruses following concanvaulin A driven expansion is less predictable however, with variable yield{Capsomidis:f425Mfo0}. There has been some concern regarding the potential for insertion-site mediated mutagenesis following gammaretroviral gene transfer, which has prompted some in the field to favour lentiviral vectors which have a safer insertional profile. There is little published data to compare lentiviral transduction techniques, though one group did find that the use of a VSVG pseudotyped simian immunodeficiency virus vector consistently provided higher transduction efficiency than a human immunodeficiency virus based vector with the same envelope (transduction 65% vs. 42%, p = 0.04) {Lamb:2013el}. New genome editing technologies such as CRISPR-CAS allow targeting ingegration of viral vectors with several potential advantages including avoidance of integration into oncogenic loci, and integration into loci that optimise CAR or TCR expression (Carl June recent paper on integration of CD19 CAR into TET2 locus and Sadelain TRAC locus paper PMID 28225754).

Non-viral methods of transduction have provided particular advantages when engineering γδT cells. The Sleeping Beauty Transposon system {Aronovich:2011gc} uses enzymes originally derived from fish to insert new genetic material into host cells. Cells must be electroporated in order for this to occur, but do not require a specific proliferative stimulus. Deniger et al used this to good effect to express an anti-CD19 CAR (CD19RCD28) in a polyclonal repertoire of γδT cells which were subsequently expanded using CD19+ artificial antigen presenting cells (aAPCs) {Deniger:2013bp}{Suhoski:2007cv}. On the day following electroporation of freshly isolated PBMC, γδT cells were isolated by paramagnetic bead negative selection and

cultured with irradiated CD19+ aAPCs which were refreshed weekly. The resultant γδT cell population showed low expression of exhaustion markers such as CD57 and contained a heterogeneous mixture of memory phenotypes. Unlike proliferation-driven transduction techniques, these was no "skewing" of the γδT cell population towards a particular Vγ/Vδ subset; aAPC-based expansion has been shown to preserve the distribution of Vδ1+, Vδ2+ and Vδ1-/Vδ2- γδT cell subsets within a donor PBMC sample{Deniger:2014ft}{Fisher:2014gv}. This may be of particular interest if engineered γδT cells were to be directed against epithelial tumours, as non-Vδ2+ γδT cells are enriched in epithelial surfaces, a tropism which could be harnessed. It has not yet been established that the SB-transposon system is more efficacious than lentiviral transduction, and there is an unknown potential for insertion mutagenesis at present {Levine:2017ew}.

The previously described techniques generate stable construct expression over time by integrating into the transduced cell genome. This has been considered important in the CAR-T cell field as it allows for persistence of CAR-T cells for weeks or months periods in the circulation. More transient CAR expression strategies have been suggested as a means of reducing toxicity following CAR-T cell infusion. mRNA transfection using electroporation has been used to generate γδT cells expressing invariant NKT-cell receptors {Shimizu:gz} and, more recently, HLA-A2/gp100 specific TCR or CARs targeting melanoma-associated-chondroitin-sulphate-proteoglycan (MCSP). MCSP is a tumour associated antigen expressed on melanona{Campoli:2004tn}, glioma{Yadavilli:2016hl}, triple negative breast cancer{Wang:2010du} and sarcomas{Godal:1986wu}. Expression peaked at around 24h after transfection, returning to baseline by around 72h{Harrer:2017hj}. Similar techniques have been tested clinically in the context of CAR+ αβT cells, where they were used to transfect cells with a construct targeting mesothelin{Beatty:2014dz}, though repeated infusions of CAR-T cells were required, presumably because of the need to "top-up" the reservoir of circulating CAR-T cells as expression was lost.

T-cell receptor gene transfer

T-cell receptor transfer was initially demonstrated in the context of αβTCRs isolated from tumour infiltrating lymphocytes. Transfer of specificity through the transfer of murine α and β T-cell receptor genes was first used to target the hapten molecule, fluorescein{Dembic:1986ki}, an approach which has subsequently been used to redirect αβT cell immunity against antigens from viral{Cooper:2000ue} and

tumour{Clay:1999uq} targets, notably in highly immunogenic tumours such as melanoma. Transferring a new αβTCR gene construct into an αβT cells runs the risk of TCR chain mis-pairing unless the endogenous α and β chains are suppressed {Berdien:2014ho}. Mis-pairing can lead to inefficient expression of the novel construct and may lead to the generation of self-reactive TCR clones leading to off-target toxicity (Bendle, Schumacher 2010 Nature med "lethal graft versus host disesae ….). By using γδT cells, which lack endogenous TCRα or β chains as the recipients of αβTCR gene transfer, this can be avoided. Dorrie et al {Dorrie:2014jc}demonstrated that γδT cells could be induced to express a HLA-A*0101 restricted αβTCR targeting a peptide derived from an adenovirus hexon protein. Engineered γδT cells produced more IFNγ and TNFα than CD8+ αβT cells expressing the same TCR and had equivalent cytotoxicity against autologous adenovirus-infected dendritic cells. Similar antigen-specific cytokine release was demonstrated by Harrer et al, when γδT cells expressing a gp100/HLA-A2 restricted αβTCR were exposed to gp100^{+} melanoma cells{Harrer:2017hj}.

Some researchers have highlighted the restrictions inherent in αβTCR transfer, in particular the restriction to particular HLA types and the possibility of antigen-negative escape variants. This prompted exploration of γδTCR transfer into αβT cells which may overcome these limitations through their broader recognition of danger-associated molecular patterns. The Vγ9Vδ2 TCR clone G115{Allison:2001di} was expressed in αβT cells by Marcu-Malina et al {MarcuMalina:2011ej} using antibiotic-based selection of successfully transduced cells. They demonstrated that both the γ9 and δ2 chains were required for either to be detected, indicating that mis-pairing with endogenous α or β chains was not occurring. The γδTCR-expressing αβT cells showed similar functional properties to "native" Vγ9Vδ2 cells in terms of cytotoxicity against the Daudi cell line, release of TNFα and IFNγ, enhancement of cytotoxicity following target pre-treatment with aminobisphosphonates and the ability to induce dendritic cell maturation. Vγ9Vδ2 transduced αβT cells showed a surprising lack of alloreactivity, linked to a down-regulation of their endogenous αβTCRs, and were able to mount responses against a broad panel of tumour cell lines. Different Vγ9Vδ2 T cell clones show varying anti-tumour responses, linked to small differences in the γ9 and δ2 CDR3 regions; no correlation was found between the expression of NKG2D, CD158a, NKAT-2 or NKB-1 and anti-tumour reactivity {Grunder:2012cs}.

Because each Vγ9Vδ2 TCR has the ability to detect multiple cancer types through its common ligand, butyrophilin, opportunities to enhance the functional avidity of the chosen Vγ9Vδ2 TCR are a rich area for optimisation. Using CD4+ αβT cells as the recipient cells, Gründer et al selectively replaced amino acids between δ2-$G115_{L109}$ and δ2-$G115T_{113}$ with alanine residues, and also inserted 0-12 alanine repeats between these residues. By co-expressing the mutated δ2-G115 chains with γ9-$G115_{wt}$ and undertaking the same process between γ9-$G115_{E108}$ and γ9-$G115_{E111.1}$, co-expressed with δ2-$G115_{wt}$, they were able to demonstrate that the length and sequence between δ2-$G115_{L109}$ and δ2-$G115T_{113,}$ and to demonstrate that the individual sequence between γ9-$G115_{E108}$ and γ9-$G115_{E111.1}$ were critical for ligand interaction, with particular importance being placed on γ9-$G115_{A109,}$ in addition to the J-region residues δ_{109} and δ_{117}{Grunder:2012cs}.

Such detailed knowledge of Vγ9Vδ2 avidity control provides excellent opportunities for γδTCR engineering, as highly optimised γδTCRs can be expressed in more readily available αβT cells. γδTCR-engineered αβT cells prevented tumour growth in an immunodeficient (irradiated $Rag^{-/-}\gamma_c^{-/-}$) murine model of Burkitt lymphoma (Daudi) and multiple myeloma (OPM2) and also protected mice who had responded to initial treatment from re-challenge with OPM2 performed 120 days after the first tumour and T-cell injection{Straetemans:2015jd}. The down-regulation of the αβTCR in the transduced cell population allows for facile selection of cells by αβTCR depletion, rather than positive selection of the transduced cells. This “untouched” cell product does not require co-expression of a marker gene and can be processed using pre-existing αβT cell depletion techniques currently used before some bone-marrow transplants, making it highly amenable to GMP-compliant manufacture. As such, this technology appears to offer a chance of making $\gamma\delta TCR^+$ cells an important contributor to cellular immunotherapy.

γδT cells expressing chimeric antigen receptors

Whilst harnessing the innate potential of the γδTCR is a highly attractive option, manipulating cellular behaviour in an antigen-specific manner remains one of the mainstays of modern immunotherapeutics. This is most commonly achieved through use of a chimeric antigen receptor (CAR). Compared to the substantial body of literature on αβT cells expressing CARs, there are relatively few reports of CAR-γδT cells. First described in 2004{Rischer:2004hu}, γδT cells expressing the first

generation CARs targeting GD2 (14.G2aζ), a sphingolipid expressed on the surface of neuroblastoma and Ewing sarcoma cells{Kailayangiri:2012im}{Schulz:1984wh} showed enhanced antigen-specific tumour reactivity. Following co-culture with the $GD2^+$ neuroblastoma cell line LAN-1, $14.G2a\zeta^+V\gamma9^+$ cells showed greater production of the Th1 cytokine IFNγ compared to non-transduced zoledronate expanded $14.G2a\zeta^-V\gamma9^+$ γδT cells. This effect was mirrored in the expression of the T-cell activation marker CD69, which was also upregulated the presence of the tumour cells. In the absence of $GD2^+$ cells, $14.G2a\zeta^+V\gamma9^+$ γδT cells showed only 1.5 ± 0.5% $IFN\gamma^+CD69^+$ but following co-culture with $GD2^+$ targets this rose to 33 ± 3%, with a background level of 5.7 ± 1.2% in $14.G2a\zeta^-V\gamma9^+$ γδT cells in response to LAN-1. Similar results were seen when γδT cells expressing the CD19ζ CAR were co-cultured with $CD19^+$ cell lines Daudi, Raji and Reh{Rischer:2004hu}, with substantial increases in IFNγ production by mixed populations of $CD19\zeta^{+/-}$ γδT cells upon co-culture with targets. Whilst Daudi is known to engage the γδTCR and is highly susceptible to γδT cell mediated killing in its own right, Raji is usually considered to be a γδT cell resistant cell line{LFaqihi:1999tf}, and it was in this model that the highest IFNγ production was seen, suggesting that CAR expression could overcome some of the immune-escape mechanisms shown by the target cells.

Since the publication of this work, progress in γδT cell adoptive transfer immunotherapy was largely restricted to unmanipulated γδT cells, the ex vivo expansion of which has been an area of research interest. A series of papers eventually demonstrated the possibility for expanding γδT cells with a broad range of γδTCR subsets using either plant-derived T-cell mitogens such as concanavalin A{Siegers:2012im}{Siegers:2011is} or artificial antigen presenting cells (aAPC) engineered to express co-stimulatory ligands and membrane-bound IL-15{Suhoski:2007cv}. Two groups used the aAPC system to expand $V\delta2^-$ γδT cells, initially demonstrating that the repertoire of γδT cells produced could be influenced by the loading of anti-γδTCR antibodies to the CD64 expressed on the aAPC{Fisher:2014gv}{Deniger:2014ft} in addition to showing that this approach could be used to specifically propagate anti-CD19 CAR^+ γδT cells{Deniger:2013bp}. Whereas previous studies had used zoledronic acid to push $V\delta2^+$ cells into exponential growth prior to transducing them with a gamma-retroviral vector {Rischer:2004hu}{Riviere:1995bp}, Deniger and colleagues used non-viral gene transfer via the *Sleeping Beauty* (SB) transposon, removing the selection pressure

introduced by aminobisphosphonate treatment. This yielded a $CAR^+\gamma\delta TCR^+$ population containing a broad range of Vγ and Vδ chain combinations. By using negative selection following electroporation with the SB transposon/transposase system{Ivics:1997tp}, γδT cells were isolated from the transduced PBMC population and then propagated on $CD19^+CD64^+CD86^+CD137L^+IL\text{-}15^+$ aAPCs in the presence of IL-2 and IL-21. The group had previously demonstrated that culture using this aAPC system enforced CAR expression in αβT cells{Singh:2011cs} resulting in >90% CAR^+ αβT cells after 28 days of co-culture. Interestingly, this effect was reduced when γδT cells were selected, presumably due to the inherent reactivity of non-transduced γδT cells against the aAPC leading to non-specific proliferation. The authors noted that this expansion technique may be particularly advantageous for γδT cells due to their expression of CD28 and CD137 which interact with CD86 and CD137L on the aAPC. Furthermore, the $CAR^+\gamma\delta TCR^+$ cells also expressed CCR7, and CD62L suggesting that they would have the capacity to home to the bone marrow and lymph nodes where $CD19^+$ leukaemia is known to reside. The CAR^+ cells produced IFNγ, TNFα, MIP-1α, MIP1β and RANTES following CAR activation through co-culture with a $huCD19^+$ murine cell line which does not engage the γδTCR due to inter-species differences, and killed human $CD19^+$ cell lines with much greater efficacy than $CAR^-\gamma\delta TCR^+$ cells{Deniger:2013bp}. Immunodeficient mice xenografted with $CD19^+ffLuc^+$ NALM6 B-cell leukaemia showed enhanced survival following CAR-γδT cell treatment compared to untreated, though a non-transduced or irrelevant CAR control was not included in the in-vivo study so the in-vivo activity is harder to dissect.

The beneficial properties of γδT cells must be considered against their relative scarcity in the circulating blood. A recent study indicated that γδT cells transduced with 2nd generation anti-GD2 CARs (GD2-28ζ) which show efficient and specific anti-GD2 cytotoxicity retain the ability to cross-present tumour associated antigens leading to a clonal expansion of αβT cells. Using a 25 amino acid fragment of the melanoma antigen MART-1 which encompasses a 10 amino acid epitope but is too long to be presented in MHC in its un-processed form, Capsomidis et al demonstrated that $HLA\text{-}A201^+V\delta2^+GD2\text{-}28\zeta^+$ cells pulsed with the long peptide were able to elicit secondary expansions in αβT cells expressing a HLA-A201 restricted MART-1 αβTCR{Capsomidis:2018vv}. This assay is a refinement of previous work by the same group which used lysate from MART-1+ tumour cell lines to

demonstrate the ability of Vδ2+ γδT cells to act as professional APCs{Himoudi:2012bz}. Vδ2$^+$GD2-28ζ$^+$ cells also retained the ability to migrate towards tumour cell lines; GD2-28ζ expression had no effect on migration in either Vδ1$^+$ or Vδ2$^+$ subsets in an in-vitro trans-well assay. Whether the antigen-presenting capacity of γδT cells has relevance in-vivo remains to be seen.

Engineering strategies which harness the innate properties of γδT cells would seem to be the best justification for using them as an alternative "chassis" for CAR-T cell therapy. CARs were initially developed to bypass the αβTCR, limited as it is by MHC restriction and a requirement for specific TAA epitopes to be presented. The γδTCR is not subject to these limitations; through its MHC-unrestricted detection of moieties associated with cellular stress. As such, there is an opportunity to "tune" the CAR-T cell response by modulating the level of stimulus delivered by the CAR. So far, this has been demonstrated in the context of neuroblastoma. Vδ2$^+$ γδT cells have minimal innate cytotoxicity against neuroblastoma cell lines, in part due to the tumour shedding soluble NKG2D ligands which block NKG2D activation. If further stimulus is provided to the γδT cell, this cytotoxicity can be restored, either via a conventional 2nd generation CAR{Capsomidis:2018vv}{Fisher:2017bv}, opsonisation of the target cell{Fisher:2014gv}{Fisher:2016cl} or, as was recently shown, by restoring the NKG2D signal using a co-stimulatory CAR that lacks CD3ζ (GD2-DAP10). This approach, using the endodomain from the NKG2D adaptor protein DAP10, enhanced killing of neuroblastoma cells but did not induce cytotoxicity against GD2$^+$ cells that did not engage the γδTCR. Cytokine release was also controllable using this "AND gate" system; IL-2, IFNγ, and TNFα were only released from GD2-DAP10$^+$Vδ2$^+$ cells when they received both CD3 and CAR stimulus, whereas in GD2-28ζ$^+$Vδ2$^+$ cells, only CAR stimulus was required{Fisher:2017bv}.

The potential implications of this for the CAR-T cell field are far-reaching. At present, target selection is a balance between expression on tumour cells and expression on healthy tissue. It is no surprise that CD19 CAR-T therapies have been more successful than others, as depletion of healthy CD19$^+$ B-cells is considered an acceptable toxicity. In other cases, on-target off-tumour toxicity has been severe or fatal, experiences which have shaped the way that target antigens are chosen{Morgan:2010fy}{Lamers:2006cc}. If on-target off-tumour toxicity could be avoided, the range of available targets would be substantially increased and constructs previously deemed to be unsafe may be re-purposed. It remains to be

seen whether this approach will provide benefit in tumour models where this type of toxicity is a limiting problem.