The inducible caspase-9 suicide gene system as a “safety switch” to limit on-target, off-tumor toxicities of chimeric antigen receptor T cells

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Immune modulation has become a central element in many cancer treatments, and T cells genetically engineered to express chimeric antigen receptors (CAR) may provide a new approach to cancer immunotherapy. Autologous CAR T cells that have been re-directed toward tumor-associated antigens (TAA) have shown promising results in phase 1 clinical trials, with some patients undergoing complete tumor regression. However, this T-cell therapy must carefully balance effective T-cell activation, to ensure antitumor activity, with the potential for uncontrolled activation that may produce immunopathology. An inducible Caspase 9 (iCasp9) "safety switch" offers a solution that allows for the removal of inappropriately activated CAR T cells. The induction of iCasp9 depends on the administration of the small molecule dimerizer drug AP1903 and dimerization results in rapid induction of apoptosis in transduced cells, preferentially killing activated cells expressing high levels of transgene. The iCasp9 gene has been incorporated into vectors for use in preclinical studies and demonstrates effective and reliable suicide gene activity in phase 1 clinical trials. A third-generation CAR incorporating iCasp9 re-directs T cells toward the GD2 TAA. GD2 is over-expressed in melanoma and other malignancies of neural crest origin and the safety and activity of these GD2-iCAR T cells will be investigated in CARPETS and other actively recruiting phase 1 trials.

Keywords: chimeric antigen receptor T cells, inducible caspase 9, AP1903, suicide gene, safety switch, cancer immunotherapy

INTRODUCTION TO CAR T-CELL IMMUNOTHERAPY FOR CANCER

Tumor initiation and progression is sculpted by host immunity (Shankaran et al., 2001; Fridman et al., 2012). Since recent US FDA approval of the cancer immunotherapeutic agents, sipuleucel-T and ipilimumab, for the treatment of prostate cancer and melanoma, respectively, immunotherapy has joined surgery, radiotherapy, and chemotherapy as a conventional modality of treatment for non-hematologic malignancies. Melanoma has been a particular target of immunotherapy because of its inherent immunogenicity, and early therapeutic approaches such as interleukin-2 (IL-2) and interferon-alpha2b (IFNα2b) aimed to stimulate these antitumor immune responses (Mocellin et al., 2010; Rosenberg, 2014). Recently, monoclonal antibodies (mAb) that inhibit immune checkpoint signaling molecules such as CTLA4 (via ipilimumab; Hodi et al., 2010; Wolchok et al., 2013), PD1 or PD-L1, (Hamid et al., 2013; Topalian et al., 2014) have shown recruitment of cytotoxic T-cell responses. Ex vivo expansion of tumor infiltrating lymphocytes (TIL) also aims to boost the melanoma-specific immune response and has shown impressive results for selected patients (Rosenberg and Dudley, 2004; Besser et al., 2013). While most of these therapies rely on a pre-existing immune response to tumor-associated antigens (TAA), the adoptive transfer of T cells genetically engineered to express novel TAA-specific receptors enables delivery of an antitumor immunotherapy even in the absence of intrinsic tumor-specific immunity (Restifo et al., 2012). The T cells may express either a transgenic TCR or a chimeric antigen receptor (CAR) and of the two types of gene modification, CAR T cells have shown the most promise in clinical trials, with over 51 CAR clinical trials currently registered in the US alone (clinicaltrials.gov).

A first-generation CAR typically comprises an antigen-binding ectodomain of a mAb-derived single chain variable fragment (scFv) and a signaling endodomain from the CD3ζ molecule. Following expression, the CAR re-directs the specificity of the T cell toward the cognate antigen. Unlike TCR, CAR are independent of MHC-restricted antigen presentation, which is a feature of immune evasion by many different cancers (Seliger et al., 1996; Seliger, 2008), and instead can bind directly to TAA, albeit those located at the cell surface. They can also include multiple intracellular signaling domains from costimulatory molecules. For example, second-generation CAR may contain CD3ζ and CD28 signaling domains, while third-generation CAR may contain CD3ζ, CD28 and either OX40 (CD134) or 4-1BB (CD137). T cells expressing these later generation CAR demonstrate enhanced activation and effector function (Pule et al., 2005). Autologous CAR T cells have shown promising objective responses in leukemia and neuroblastoma patients, including some complete and partial
CAR T-CELL-RELATED SAFETY CONCERNS

The major safety concern for CAR T-cell products is the risk of on-target but off-tumor effects resulting from T-cell activation in response to normal tissue expression of the TAA. Similar off-tumor side-effects have been reported for approved mAb therapies such as trastuzumab (anti-HER2), which has cardiomyocyte survival pathways, and cetuximab (anti-EGFR), which produces skin rashes and mucosal irritation related to EGFR expression on epithelial cells (Perez-Soler et al., 2005; Chien, 2006). In a more recent example, a phase 1 trial of an anti-EphA2 mAb/cytotoxic drug conjugate was discontinued due to serious AEs of clinical bleeding and coagulation probably because of endothelial-cell targeting (Annuziata et al., 2013). Transgenic TCR T cells likewise feature off-tumor side effects including one trial which reported MART-1- and gp100-specific T-cell killing of normal melanocytes, with the majority of patients experiencing widespread erythematous skin rash, transient uveitis or hearing loss, with most symptoms reversed upon local steroid treatment (Johnson et al., 2009). While in another trial, transgenic TCR T cells specific for the colorectal TAA CEA have resulted in severe transient colitis (Parkhurst et al., 2011). Thus there are multiple reports of toxicity by TAA-targeted cell therapies, and TAA-specific CAR T cells may also result in the targeting of healthy tissues.

There is also the theoretical risk of insertional mutagenesis and oncogenic expansion of transduced cells with retroviral-based gene therapy. Indeed, such an event has been reported in a gene-therapy trial for X-linked Severe Combined Immunodeficiency (X-SCID) in which patients received retrovirally transduced CD34+ bone marrow progenitor cells and four out of nine patients then developed acute T-leukemia. It is believed that insertional mutagenesis in the LMO2 proto-oncogene together with subsequent massive proliferation of the transduced CD34+ progenitor population in immunodeficient hosts contributed to T-leukemogenesis (Hacein-Bey-Abina et al., 2003, 2010). In contrast, similar AEs have not been observed in trials of adoptive transfer of autologous, polyclonal gene-modified mature T cells in immunocompetent hosts (Heslop et al., 2010; Scholler et al., 2012). However, there is evidence in murine model systems that monon- or oligo-clonal mature T-cell populations are more susceptible to insertional mutagenesis (Newrzela et al., 2008, 2011, 2012). Most CAR T cells to date have been manufactured from polyclonal peripheral blood T cells (Cooper et al., 2006), and even selected CAR T cells remain polyclonal in respect of their endogenous TCR repertoire (Wang et al., 2012). Nevertheless, insertional mutagenesis remains a concern and is another consideration in long-term clinical monitoring, which is a routine part of gene transfer trials and which helps to establish the safety profile of CAR T cells.

In addition, CAR T cells may also produce on-target, on-tumor toxicities more familiar with other cancer therapies. For example, the tumor lysis syndrome (TLS; Yang et al., 1999; Li et al., 2013), cytokine release syndrome (CRS) and the related macrophage activation syndrome (MAS) that are associated with some chemotherapies and targeted- or immuno-therapies (Suntharalingam et al., 2006; Teachey et al., 2013). Importantly these AEs may occur during the destruction of tumors, and thus even a successful, on-tumor CAR T-cell effect might result in toxicity that requires intervention.

CAR T-CELL-RELATED SERIOUS ADVERSE EVENTS

Specific examples of these toxicities have been observed in several CAR T-cell clinical trials (see Table 1). Interestingly, evident on-target, off-tumor toxicity does not require the incorporation of additional T-cell costimulatory domains into the CAR construct. In a trial of autologous T-cells expressing a first-generation CAR directed toward carbonic anhydrase IX (CAIX) in patients with metastatic renal cell carcinoma, NCI-CTC grade 3 or 4 liver function abnormalities were observed in 4 of 12 patients. This

Table 1 | Reports of life-threatening and fatal adverse events in CART-cell clinical trials.

| Trial | Event | Reference |
|-------|-------|-----------|
| CAIX CART T cells (first generation) | Grade 3 and 4 transient liver enzyme increases at 1–2 × 10^8 total cell dose as on-target toxicity related to CAIX expression on bile duct, preventable by pre-treatment with anti-CAIX monoclonal antibody. | Lamers et al. (2006, 2013) |
| CD19 CART T cells (second generation) | Tumor lysis syndrome at 3 × 10^9/kg total cell dose (1.46 × 10^9/kg CAR T cells), resolved by day 26 after infusion. | Porter et al. (2011), Grupp et al. (2013) |
| CD19 CART T cells (second generation) | Severe cytokine-release syndrome, reversible by monoclonal antibody blockade at 1 × 10^9/kg cell dose (1.2 × 10^7/kg CART cells). | |
| HER2/neu (ERBB2) CAR T cells (third generation) | Respiratory distress and death at 1 × 10^10 total cell dose, likely due to CART cell localization to the lung and “cytokine storm.” | Morgan et al. (2010) |
| CD19 CART T cells (second generation) | Renal failure, “sepsis-syndrome,” elevated cytokine levels and death at 1.2 × 10^7/kg, possibly due to a combination of sepsis, cyclophosphamide treatment and T-cell transfer. | Brentjens et al. (2010) |
toxicity was subsequently prevented by pre-treatment with an anti-CAIX mAb (Lamers et al., 2006, 2013).

Two fatal AEs have been reported in phase 1 clinical trials of an anti-HER2 CAR (Morgan et al., 2010) and of an anti-CD19 CAR (Park and Brentjens, 2010). In the third-generation (CD28.4-1BB.ζ) anti-HER2 CAR trial, a patient with pulmonary metastases of colorectal cancer received lymphodepleting chemotherapy followed by an intravenous dose of 10^6 HER2-CAR T cells. Within 4 h, the patient developed acute respiratory distress and died 5 days later with multi-organ failure. The investigators attributed her death to a “cytokine storm” hypothesized to result from T-cell activation on binding of CAR T cells to HER2-expressing bronchial epithelium. In the trial of a second-generation (CD28.ζ) anti-CD19 CAR, patients received lymphodepleting chemotherapy followed by 1.2–3 × 10^7 CD19-CAR T cells/kg. One patient developed persistent fevers, respiratory distress and progressive acute renal failure, and died 44 h post-infusion. Unlike other patients enrolled in this study, this patient’s serum cytokine levels were abnormally high after the chemotherapy and before the CAR T-cell infusion but no higher after the CAR T-cell infusion, and the patient’s death was thus attributed to undetected sepsis. However, the direct attribution of these CAR-related AEs to on-target effects is confounded by other factors such as high T-cell dose, first-pass through the pulmonary vasculature after intravenous administration, unintended consequences of conditioning treatments, and possible underlying infection.

Although second-generation CARs incorporating the CD28 or the 4-1BB costimulatory domains have generally been safe (Brentjens et al., 2011, 2013; Savoldo et al., 2011), other reports indicate that serious on-target toxicities including TLS and CRS have occurred (Brentjens et al., 2010; Kalos et al., 2011; Porter et al., 2011; Kochenderfer et al., 2012; Grupp et al., 2013). Four of 8 patients receiving second-generation (CD28.ζ)CD19-specific CAR T cells for progressing B-cell malignancies experienced acute and reversible toxicities, the severity of which correlated with serum cytokine levels (Kochenderfer et al., 2012). In a trial in chronic lymphoid leukemia (CLL) patients, one patient developed TLS after infusion of second-generation (4-1BB.ζ)CD19-CAR T cells requiring hospitalization on day 22 and treatment with fluid resuscitation and rasburicase, with symptoms resolving by day 26 (Porter et al., 2011). In a separate trial of CD19-CAR T cells in acute lymphoblastic leukemia (ALL) patients, two patients experienced CRS and for one patient this resulted in hospitalization on day 4 and transfer to intensive care on day 5 after the T-cell infusion (Grupp et al., 2013). The CRS was reversed by anti-cytokine therapy including tocilizumab (anti-IL-6 receptor mAb) and etanercept (decoy TNF receptor). This patient went on to develop a complete remission, while the other patient had a relapse of CD19-negative leukemia after 2 months. High levels of IL-10 and IL-6 were also detected in these patients, which suggests a MAS induced by non-physiologic T-cell activation (Maude et al., 2014).

As a general statement, early clinical investigation of any CAR T cell of novel specificity may result in unpredictable on-target, off-tumor toxicity. Hence, suicide gene technology can provide a valuable “safety switch” in these at-risk clinical scenarios, allowing for the targeted deletion of inappropriately activated CAR T cells. Also, although we have focused on CAR T-cell-related toxicities in this review, it is worth noting that two patient deaths have been reported for each of two different MAGE-A3-specific transgenic TCR T cells, which resulted from either neurologic toxicity when brain-expressed MAGE-A12 was cross-targeted (Morgan et al., 2013) or cardiac toxicity when heart muscle-expressed Titin was cross-targeted (Linette et al., 2013). The incidence of these toxicities, and of tumor burden-related CRS and MAS after CD19-CAR T cell therapy for B-leukemia (Maus et al., 2014), may be ameliorated by a “safety-switch” if the suicide gene system chosen is sufficiently rapid in onset.

**THE iCasp9 SUICIDE GENE**

The herpes simplex virus-thymidine kinase (HSV-TK) suicide gene system has long been used in cell therapy investigations as a method for depleting transduced cells in the case of AEs (Recchia et al., 2006; Ciceri et al., 2009). However, major disadvantages attend its use for this purpose. Activation of HSV-TK by ganciclovir is relatively slow, requiring 3 days to have a complete effect *in vitro* (Marin et al., 2012) and although mutant versions of TK have improved drug sensitivity and killing (Willmon et al., 2006) the viral TK gene product has intrinsic immunogenicity that may cause transduced cells to be rejected by the host immune system in immunocompetent individuals (Riddell et al., 1996; Berger et al., 2006). Additionally, if ganciclovir is used to treat CMV infections in immunocompromised recipients of hematopoietic stem transplants then the use of this suicide gene would result in the unwanted deletion of transduced cells (Bonini et al., 2007; Sangiolo et al., 2011).

An alternative suicide gene system is CaspaCIDe®, which consists of an inducible caspase 9 (iCasp9) gene together with the small-molecule, bio-inert, chemical induction of dimerization (CID) drug, AP1903. The iCasp9 gene contains the intracellular portion of the human caspase 9 protein, a pro-apoptotic molecule, fused to a drug-binding domain derived from human FK506-binding protein (Clackson et al., 1998; Straathof et al., 2005; see Figure 1). Intravenous administration of AP1903 produces cross-linking of the drug-binding domains of this chimeric protein, which in turn dimerizes caspase9 and activates the downstream executioner caspase 3 molecule, resulting in cellular apoptosis. The pharmacokinetics of AP1903 has been studied in a placebo-controlled, escalating-dose study in volunteers and the drug has been found to be safe and well tolerated to the highest dose of 1 mg/kg, with plasma levels directly proportional to administered dose and rapid reduction to 1% of the maximum plasma concentration by 10 h (Iulucci et al., 2001).

In an early preclinical study of this system, it was shown that expression iCasp9 in, retrovirally transduced Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs) in the absence of CID had no effect on the phenotype or function of the transduced cells. However, a single 10 nM dose of CID killed 89–93% of transduced cells by day 7, as measured by GFP expression. These results were confirmed *in vivo* in a SCID mouse-human xenograft model where a single dose killed over 99% of circulating human GFP^+^ T cells by day 3. Importantly, killing via iCasp9 has been found to be extremely rapid with early apoptotic Annexin V^+^ cells.
Inducible caspase 9 homodimer (iCasp9) can be used to rapidly eliminate T cells and block an ongoing autoimmune attack. An important aspect of the iCasp9/CID system is that it enables an iCasp9 gene was reported by Hoyos et al. (2010) who developed second-generation CD19-CAR T cells that co-expressed IL-15 and iCasp9. The authors demonstrated that these iCasp9/CAR.19/IL-15 T cells had improved effector function and were effectively eliminated in vivo within 3 days of CID administration, as measured by live imaging of luciferase-positive CAR T cells (Hoyos et al., 2010). A third-generation, anti-CD20 CAR expressing iCasp9 has likewise demonstrated 90% reduction in peripheral blood CAR T cells in vivo 12 h after CID administration on two consecutive days (Budde et al., 2013). A study of anti-CD19 CAR T cells co-expressing a modified hygromycin resistance/HSV-TK suicide gene fusion found that transferred T cells did not persist in patients due to anti-transgene immune responses against the resistance or suicide genes, which highlights the need for close clinical monitoring of vector persistence and anti-transgene immunity in clinical trials of T cells expressing iCasp9 (Jensen et al., 2010).

The iCasp9/CID system has been successfully translated to a clinical setting. A trial of donor T-cell transfer in stem cell transplant patients demonstrated that iCasp9 could eliminate transferred T-cells and end GvHD in patients (Di Stasi et al., 2011). Five patients who had received CD34+ haploidentical stem cell transplants for relapsed leukemia were given allodepleted donor T-cells transduced to express iCasp9 and the truncated CD19 selectable marker. The sorted, CD19+ CD3+ transgenic T-cells were 90–93% pure, and were able to survive and expand when transferred to patients. Skin GvHD occurred in four of the five patients and liver GvHD occurred in one patient 14–42 days after T-cell transfer. Treatment of these patients with a single dose of AP1903 (CID) reduced the numbers of circulating transgenic T cells by 90% within 30 min of administration, but had no effect on endogenous T cells and did not cause any AEs. Within 24 h, the GvHD-associated skin abnormalities were terminated and did not return, however, non-alloreactive virus-specific transgenic T cells were subsequently identified in patient blood. A long-term follow up of this clinical study has confirmed that the patients have maintained immune reconstitution, including virus-specific donor T cells, without recurrence of GvHD 3.5 years after the initial T-cell infusion (Zhou et al., 2014). Other trials of iCasp9 gene transfer in haploidentical donor T cells are also ongoing (NCT01494103, ACTRN12614000290695).

An important aspect of the iCasp9/CID system is that it enables the specific killing of highly activated cells with high levels of transgene expression. As retroviral vectors preferentially integrate near transcription start sites and genes involved in proliferation (Wu et al., 2003; Recchia et al., 2006; Cattoglio et al., 2007), it is an inherent feature of the technology that actively dividing cells will have higher transgene expression and hence higher levels of iCasp9. Preclinical work suggests that transduced CTLs that are not killed by CID administration express insufficient iCasp9 to allow functional activation by CID (Straathof et al., 2005). However, it has been observed that activation of surviving T cells with down-modulated transgene expression can rapidly restore CID sensitivity (Tey et al., 2007). In the GvHD clinical trial, this incomplete depletion of transduced cells allowed for the re-expansion of the small population of non-alloreactive CD3+CD19+ T cells remaining after AP1903 treatment. These T cells were rapidly killed when activated and re-exposed to AP1903, indicating that the surviving
population remained sensitive to AP1903-mediated killing (Di Stasi et al., 2011). Notwithstanding these results, another in vitro study has found that immune-selected CTLs remaining after CID treatment have little or no proliferative potential or functional transgene activity. The study also found that immune-selected CTLs rested for 8–9 days after last antigen stimulation can be depleted significantly by CID treatment (Quintarelli et al., 2007). Hence, the functional significance of the iCasp9+T cells surviving CID treatment remains an open area of investigation. In the event of AP1903 administration for serious AEs related to either on-target, on-tumor or on-target, off-tumor effects, the persistence and reactivity of transgene-containing CAR T cells will need to be determined via studies of patient tissue samples, which may include attempted ex vivo re-expansion of transgene-containing T cells.

**FUTURE DIRECTIONS: iCasp9-CAR CLINICAL STUDIES**

Autologous iCasp9-expressing CAR T cells are being clinically evaluated in actively recruiting trials. Our imminent phase 1 trial will investigate GD2-specific and iCasp9-expressing CAR (GD2-iCAR) T cells in advanced melanoma patients (CARPETS, ACTRN12613000198729). Autologous patient T cells will be evaluated in an open phase 1 trial for phase 1 clinical evaluation in neuroblastoma patients (GD2-iCAR) T cells in advanced melanoma patients (CARPETS, ACTRN12613000198729). Autologous patient T cells will be transduced with a retroviral vector encoding a third-generation GD2-specific CD28.OX40.t CAR (Pule et al., 2005), which was developed by the Brenner group in a first-generation CAR format for phase I clinical evaluation in neuroblastoma patients (Pule et al., 2008; Louis et al., 2011), and then modified further to encode iCasp9 (Gargett et al., 2014). This third-generation GD2-iCAR is also currently being investigated in an open phase I trial in neuroblastoma patients (GRAIN, NCT01822652), sarcoma patients (VEGAS, NCT01953900) and other GD2+ solid tumors (NCT02107963).

GD2 is a disialoganglioside upregulated on the cell surface of tumors of neuroectodermal origin. The GD2-specific ch14.18 mAb, which has been included in standard treatment regimens for high-risk neuroblastoma (Yu et al., 2010), has the same antigen-binding domain as the 14g2a mAb, which donated the scFv for the GD2-CAR (Pule et al., 2008; Louis et al., 2011). Ch14.18, 14g2a and other GD2-specific mAb can elicit toxicities such as fever, rash, hypotension, and painful peripheral neuropathy (Saleh et al., 1992; Murray et al., 1994), which probably results from complement activation (Sorkin et al., 2010). Although such toxicities are possible in any clinical trial of GD2-CAR T cells, no significant AEs were observed in the clinical study of the first-generation GD2-CAR (Pule et al., 2008; Louis et al., 2011). Compared to earlier generation receptors, the improved proliferation, CTL activity and cytokine secretion of the third-generation GD2-iCAR (Pule et al., 2005) may add to the risk of toxicity either from enhanced tumor lysis, or on-target, off-tumor effects on healthy tissues, and thus inclusion of iCasp9 offers the means to mitigate these risks.

CAR T cells have shown great initial promise in the clinic but this has been accompanied by some severe and even fatal side-effects. By significantly improving its safety profile, the iCasp9/AP1903 suicide gene technology can complement CAR T-cell technology and advance its more widespread adoption in the clinic.

**ACKNOWLEDGMENTS**

Funding for this study was provided by NHMRC Project Grant APP1010386 and Therapeutic Innovation Australia Research Access Scheme.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 August 2014; accepted: 07 October 2014; published online: 28 October 2014.

Citation: Gargett T and Brown MP (2014) The inducible caspase-9 suicide gene system as a “safety switch” to limit on-target, off-tumor toxicities of chimeric antigen receptor T cells. Front. Pharmacol. 5:235. doi: 10.3389/fphar.2014.00235

This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology.

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