Title: Cardiovascular toxicity and titin cross-reactivity of affinity enhanced T cells in myeloma and melanoma

Short Title: Cardiovascular toxicity and titin cross-reactivity

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Scientific category: Clinical Trials and Observations
Key Points:

1. Engineered T cell receptors can have redundant recognition of alternative protein motifs, resulting in severe cardiac toxicity.
2. The use of induced pleuripotent stem cells (iPSC) is a promising approach to identify potential off target effects of engineered T cells.
ABSTRACT
An obstacle to cancer immunotherapy has been that the affinity of T cell receptors (TCRs) for antigens expressed in tumors is generally low. We initiated clinical testing of engineered T cells expressing an affinity-enhanced T cell receptor against HLA-A*01-restricted MAGE-A3. Open-labeled protocols to test the TCRs for patients with myeloma and melanoma were initiated. The first two treated patients developed cardiogenic shock and died within a few days of T cell infusion, events not predicted by pre-clinical studies of the high-affinity T cell receptors (TCR). Gross findings at autopsy revealed severe myocardial damage and histopathological analysis revealed T cell infiltration. No MAGE-A3 expression was detected in heart autopsy tissues. Robust proliferation of the engineered T cells in vivo was documented in both patients. A beating cardiomyocyte culture generated from induced pluripotent stem cells triggered T cell killing, which was due to recognition of an unrelated peptide derived from the striated muscle specific protein titin. These cases demonstrate that TCR-engineered T cells can have serious and not readily predictable off-target and organ-specific toxicities, and highlight the need for improved methods to define the specificity of engineered TCRs.

The protocols were registered at clinicaltrials.gov (NCT01350401 and NCT01352286).
Introduction

T cells can be genetically engineered to express TCRs with a high affinity and specificity for antigens. The MAGE-A3 antigen has been a leading target for immunotherapeutic approaches to cancer, due to its frequent expression in multiple tumor types and restricted expression in normal tissues. However, naturally-occurring T cell receptors (TCRs) to self-antigens are of lower affinity due to thymic selection, a fact that limits the ability of wild type (or natural) TCR to recognize the low antigen-HLA levels typically expressed on tumor cells. Therefore, we enhanced the affinity of a T cell receptor specific for the HLA-A*01 restricted MAGE-A3 peptide [EVDPIGHLY]. Following extensive molecular, biophysical, and immunologic testing in vitro and in mouse models of a library of TCR candidates, a moderately high-affinity TCR (MAGE-A3αa) was selected as the lead candidate for clinical evaluation.* Here we report that the adoptive transfer of autologous T cells engineered to express the MAGE-A3 TCR resulted in severe cardiac toxicity that was not due to off-tumor antigen expression, or to recognition of epitopes from related cancer testis antigens. Unexpectedly, toxicity was due to recognition of an epitope derived from an unrelated protein expressed by contracting normal cardiac tissue and one that would not be identified using typical preclinical screening strategies.

Materials and Methods

The affinity enhanced MAGE-A3αα TCR has 4 substitutions in the alpha chain of the CDR2 region and retained the wild type sequences in the beta chain. The preclinical testing of the TCR has been reported (http://oba.od.nih.gov/oba/RAC/meetings/June2012/RAC_Minutes_06-12.pdf).

Protocols to test the engineered TCR for patients with the HLA-A*01 allele and whose tumors expressed MAGE-A3 by RT-PCR analysis were initiated at Washington University and the University of Pennsylvania. The protocols were registered at clinicaltrials.gov (NCT01350401 and NCT01352286).

Manufacturing of the T cell products: T cell products were manufactured using CD25-depleted and monocyte-depleted leukopheresis product. T cells were stimulated with CD3/28 beads and transduced with lentiviral vector as previously described. Case 1 T cell product was in culture for 9 days, during which there was an expansion in total cell number of 7.5 population doublings. Case 2 T cell product was also in culture for 9 days, during which there was an expansion in total cell number of 5.1 population doublings. Characterization of the infused products is shown in Table 1.

Research sample draws and processing: Research sample processing, freezing, and laboratory analyses were performed in the Translational and Correlative Studies Laboratory at the University of Pennsylvania, which operates under principles of Good Laboratory Practice. Assay performance and data reporting conforms with MIATA guidelines. Samples (peripheral blood, bone marrow) were collected in lavender top (K2EDTA,) or red top (no additive) vacutainer tubes (Becton Dickinson). Lavender top
tubes were delivered to the laboratory within 2 hours of draw, or shipped overnight at room temperature in insulated containers essentially as described prior to processing. Samples were processed within 30 minutes of receipt according to established laboratory SOP. Peripheral blood and marrow mononuclear cells were purified, processed, and stored in liquid nitrogen as described. Red top tubes were processed within 2 hours of draw including coagulation time; serum isolated by centrifugation, aliquoted in single use 100 µL aliquots and stored at -80°C.

**Q-PCR analysis:** Whole-blood or marrow samples were collected in lavender top (K2EDTA) BD vacutainer tubes (Becton Dickinson). Genomic DNA was isolated directly from whole-blood and flash frozen autopsy tissues, and Q-PCR analysis on genomic DNA samples was performed in bulk using ABI Taqman technology and a qualified assay to detect the woodchuck post-translational regulatory element (WPRE) sequence present in the integrated lentivirus which contains the MAGE A3 transgene as described. To determine copy number per unit DNA, an 8-point standard curve was generated consisting of 5 to 10^6 copies lentivirus plasmid spiked into 100 ng non-transduced control genomic DNA. Each data-point (sample, standard curve) was evaluated in triplicate with a positive Ct value in 3/3 replicates with % CV less than 0.93% for all quantifiable values. A parallel amplification reaction to control for the quality of interrogated DNA was performed using 20 ng input genomic DNA from peripheral blood, and a primer/probe combination specific for non-transcribed genomic sequence upstream of the CDKN1A gene as described. These amplification reactions generated a correction factor (CF) to correct for calculated versus actual DNA input. Copies of transgene per microgram genomic DNA were calculated according to the formula: copies calculated from standard curve/input DNA (ng) x CF x
1000 ng x 0.5 (qualification process-established normalization factor to account for the efficiency of the WPRE-amplification). Accuracy of this assay was determined by the ability to quantify marking of the infused cell product by Q-PCR.

**Flow Cytometry reagents:** The following antibodies were used for these studies: anti-CD3-FITC, anti-C8α-PE-Cy7, both procured from e-biosciences, and anti-Vβ5.1 monoclonal antibody (Beckman Coulter PN IM2285). To detect the MAGE-A3 TCR α/β heterodimer a MAGE-A3-specific dextramer reagent (Immudex Corp) conjugated to APC was utilized (A*0101/EVDPIGHLY/APC, Cat# WA3249-APC).

**Multi-parameter flow cytometry:** Cells were evaluated by flow cytometry after thaw and overnight rest at 2 x 10⁶ cells/ml. Multi-parametric immunophenotyping was performed using approximately 1.0 x 10⁶ total cells/condition, and using fluorescence minus one (FMO) for dextramer and Vβ5.1 stains. Cells were stained in 100 µL PBS for 30 minutes on ice using antibody and reagent concentrations recommended by the manufacturer, washed, re-suspended in 0.5% paraformaldehyde and acquired using an Accuri C6 cytometer equipped with a Blue (488) and Red (633 nm) laser. Accuri files were exported in FCS file format and analyzed using FlowJo software (Version 9.5.3, Treestar). Compensation values were established using single antibody stains and BD compensation beads (Becton Dickinson) and were calculated by the software. The gating strategy for T cells was as follows: Live cells (FSC/SSC)> CD3+/SSC low.

**Soluble factor analysis:** Whole blood was collected in red top (no additive) BD vacutainer tubes (Becton Dickinson), processed to obtain serum using established laboratory SOP, aliquoted for single use and stored at -80°C. Quantification of soluble cytokine factors was
performed using Luminex bead array technology and kits purchased from Life technologies (Invitrogen). Assays were performed as per the manufacturer protocol with a 9 point standard curve generated using a 3-fold dilution series. The 2 external standard points were evaluated in duplicate and the 5 internal standards in singlicate; all samples were evaluated in duplicate at 1:2 dilution; calculated % CV for the duplicate measures were less than 15%. Data were acquired on a FlexMAP-3D by percent and analyzed using Xponent 4.0 software and 5-parameter logistic regression analysis. Standard curve quantification ranges were determined by the 80-120% (observed/expected value) range. Reported values included those within the standard curve range and those calculated by the logistic regression analysis.

**Beating cardiac myocyte iPSC-CM assay:** Induced-pluripotent stem-cell (iPSC)-derived cardiomyocyte cells (iPSC-CM) were thawed from liquid nitrogen storage and treated as per the manufacturer’s instructions. Prior to assay the cells were virally transduced with HLA-A*01 and confirmed positive. iPSC-CM and control target cells were plated at 5x10^4 per well of a 96 well plate and incubated for 24 hours with non-transduced or MAGE A3 transduced T cells at a ratio of 1:1. Quantification of IFNγ release in cell supernatants was performed using Luminex bead array technology with reagents purchased from Life Technologies. Samples were processed in triplicate and assays performed as per the manufacturer’s instructions.

**Results**

Clinical studies in stage III/IV melanoma and high risk or relapsed myeloma were initiated. The first 2 subjects enrolled on a cohort for patients with the HLA-A*01 allele and whose
tumors expressed MAGE-A3 by RT-PCR analysis are reported. The protocol schemas are shown in **Suppl Fig 1**. Both patients developed fever, progressive hypoxia and hypotension and expired 4 to 5 days after infusion (**Fig 1**).

**Case 1 (UPCC01611-100)**

The patient was a 63 year old man who was initially diagnosed with a 2.38 mm ulcerated melanoma of the left neck six years prior to enrollment. He was diagnosed with Stage IIIB (T3bN1aM0) melanoma after undergoing a wide local excision with sentinel lymph node sampling that was positive for melanoma. He received adjuvant interferon alpha but recurred at 1 year with mediastinal lymphadenopathy. His tumor tested negative for the *BRAF* V600E mutation. He was treated first on a protocol with a dendritic cell vaccine, then on a protocol with ipilimumab for 4 years. He had documented disease progression with increasing axillary and mediastinal adenopathy as well as left lung metastases. The 6cm axillary adenopathy was resected and histologically confirmed as metastatic melanoma. He was enrolled in UPCC-01611. His baseline screening was notable for a normal electrocardiogram and echocardiogram, and pulmonary function tests with a minimal obstructive ventilation defect. On protocol, a dose of 60 mg/kg/day of cyclophosphamide was administered for two days starting four days prior to the cell infusion since this has previously been shown to potentiate the effects of the infused engineered T cells.\(^3\) 5.3x10^9 MAGE-A3\(^{a3a}\) TCR cells were administered in the outpatient setting as a split infusion of 30% and 70% total cells on consecutive days (**Fig 1**). Three days following the first T cell infusion, he presented to the emergency department with chills, nausea, and abdominal pain. He was found to be neutropenic and febrile to 103.1°F and was treated with broad-spectrum antibiotics (cefepime and vancomycin). The next morning he was found to be
hypotensive, tachycardic, and hypoxic. He did not complain of chest pain, but EKG showed diffuse ST elevations and cardiac enzymes were elevated: troponin I was recorded at 54.4 ng/ml (normal range 0.00 – 0.24 ng/ml), and CK-MB at 65 ng/ml (normal range 0.7 ng/ml). Within 30 minutes, he became unresponsive and had a cardio-pulmonary arrest; he was initially resuscitated, but he had a second arrest approximately 90 minutes later from which he could not be resuscitated. The patient expired four days after the first T cell infusion. Analysis of 30 cytokines in blood samples collected after T cell infusion indicated a systemic inflammatory process that was consistent with T cell activation as interferon-γ, IL-5 and IL-8 were elevated approximately 100-fold above baseline (Fig 2).

The initial autopsy report concluded that the cause of death was a large acute myocardial infarction. The left anterior descending coronary artery showed severe atherosclerotic disease with 95% occlusion of the lumen. There were patchy full thickness hemorrhages in both ventricles and in the septum. Two scars in the anterior ventricle were noted as indicating prior myocardial infarctions. Of note, there was no indication of prior infarction by history or on the screening electrocardiogram and echocardiogram, indicating that the patient had a history of silent myocardial infarctions.

PCR analysis for vector sequences in blood indicated in vivo expansion of the MAGE A3α3a T cells, which reached over 100 cells per microliter of blood (Fig 3A). As expected, the total white blood cell count declined due to cyclophosphamide. Analysis of the biodistribution of the engineered T cells from tissues obtained at autopsy was also performed (Fig 3B). The highest levels of engineered T cells were found in spleen, blood and heart at nearly equivalent levels. Due to the hemorrhagic nature of the myocardial infarction, the T cell
distribution in the heart was initially attributed to infiltration with blood. Lymph node, liver, lung and liver metastases had similar numbers of engineered T cells, but few were found in brain or testis samples.

The conclusions from the initial investigation were that myocardial ischemia followed by infarction was a result of underlying coronary disease and a combination of the metabolic demands of neutropenic fever, coupled with anemia, and a cytokine release syndrome. The patient had had a normal EKG and echocardiogram at study entry, which pointed to the need to add enhanced cardiac screening for study entry. As a result, the protocols were amended to include a nuclear cardiac stress test and troponin T for screen, and serial EKGs and troponin T to monitor cardiac status after infusion. Information regarding the death was added to the informed consents, and FDA and ethics committees approved proceeding with additional patients to receive the MAGE-A3\textsuperscript{a3a} engineered T cells. These studies were conducted in accordance with the Declaration of Helsinki.

**Case 2 (UPCC01411-100)**

The second patient infused with the engineered T cells was enrolled in the myeloma trial (Suppl Fig 1). The patient was a 57 year old man who was diagnosed with IgG kappa plasma cell myeloma 3 years prior to enrollment, when he presented with plasmacytomas of the T4 and T8 vertebral bodies. He was initially treated with radiation therapy, which was complicated by radiation pneumonitis. He completed one month of lenalidomide and dexamethasone but did not tolerate further lenalidomide. He was observed for two years, then he was treated with bortezomib and dexamethasone but his disease progressed. Three months prior to enrollment, he was treated with two cycles of D-PACE
(dexamethasone, cisplatin, doxorubicin, cyclophosphamide, and etoposide). He was assessed by a cardiologist because he had a history of hypertrophic obstructive cardiomyopathy that had been diagnosed at age 18 and he had had intermittent atrial fibrillation, requiring cardioversion on at least one occasion. His atrial fibrillation was rate-controlled with diltiazem and propranolol and he was anti-coagulated with aspirin in view of his underlying hematologic malignancy. His nuclear cardiac stress test and baseline troponin T were normal and his cardiologist concluded that his cardiomyopathy did not pose a contraindication to enrollment in this protocol.

On protocol the patient underwent stem cell mobilization and harvest, and received melphalan at 200 mg/m² followed by autologous stem cell transplant. A total of 2.4x10⁹ MAGE A3a3a T cells were infused two days later (Fig 1). That day he developed diarrhea and a stool sample tested positive for C. difficile toxin; he was treated with metronidazole. Two days after the T cell infusion, the patient became febrile to 102.4°F in the setting of neutropenia. There were no significant changes on EKG other than atrial fibrillation and tachycardia attributed to the fever. He was treated with broad-spectrum antibiotics. The next day he developed hypotension, hypoxia, altered mental status, and EKG changes. He was treated with oxygen, fluids, hydrocortisone, and vasopressors. An echocardiogram showed a large pericardial effusion and suggested pre-tamponade physiology concerning for cardiogenic shock. He was treated with aggressive supportive cardiac and medical intensive care, including high-dose corticosteroids given as hydrocortisone, then 1 gram methylprednisolone. Supportive care included placement of an intra-aortic balloon pump for his cardiogenic shock and continuous renal replacement therapy for acute renal failure. In the course of that day his troponin rose from 1.2 to 13.75 (normal range 0.0 – 0.03). Over
the following day he developed progressive metabolic acidosis and multi-system organ failure due to worsening cardiogenic shock. He expired five days after receiving T cells.

Analysis of 30 cytokines over time in blood showed evidence of immune activation, with IL-6 elevated >1000-fold above baseline (Fig 2).

PCR analysis for MAGE A3α3a TCR sequences in blood revealed robust in vivo expansion of the T cells which exceeded 400 cells per microliter of blood three days after T cell infusion (Fig 3A). Analysis of engineered T cell biodistribution by PCR revealed that the highest concentration of engineered T cells were in the blood and pericardial fluid (Fig 3B). Bone marrow, lung, heart and liver were the tissues with the next highest distribution of engineered cells. Of note, evaluated brain and testis specimens did not contain significant numbers of engineered cells, as was the case for the first subject. We also calculated the total number of T cells recovered from the total blood volume and from individual organs (Suppl. Table 1), and determined that at the time of autopsy, the transduced T cells expanded nearly 200-fold in Case 2 and 17-fold in Case 1.

At autopsy, the most significant finding was cardiac myonecrosis with an unusual lymphoid predominant infiltrate into the myocardium (Fig 4A). In contrast to the first case, there was no evidence of coronary artery disease or thrombosis. The histologic findings of myocardial necrosis were most consistent with immunologically-mediated damage.9 Immunohistochemistry for CD3 was performed and demonstrated that the lymphoid infiltrate was composed of T cells. A similar pattern of CD3+ cell infiltrate was not observed in all the other organs and tissues examined, including multiple sections of skeletal muscle. Therefore, CD3+ cells specifically infiltrated the myocardium, resulting in acute cardiac
injury and a histological pattern similar to allograft rejection.\textsuperscript{9,10} Analysis of 30 cytokines in blood and pericardial fluid obtained at autopsy from Case 2 at autopsy showed evidence local immune activation in addition to systemic immune activation (Fig 4B).

**Additional post-hoc investigation**

Because of the autopsy findings in Case 2 and the similarities in clinical courses, the cardiac tissue from the first case was re-examined. Immunohistochemistry of heart tissue from Case 1 revealed extensive CD3+ T cell infiltration (Fig 4A). Histopathologically, it was also noted that there was extensive myocyte necrosis and neutrophilic inflammation which was not fully explained by the distribution of atherosclerotic disease in the proximal LAD coronary artery, as the other coronaries had only minimal disease. The role of cardiac hypertrophy in the second patient is unclear. The data suggest that T cell mediated acute cardiac injury contributed to acute cardiac failure in both cases.

TCR-engineered T cells could cause injury to normal tissues by several mechanisms, including: (1) mispairing of introduced and endogenous TCR α/β chains resulting in novel specificities; (2) alloreactivity; (3) recognition of an unknown epitope expressed by normal tissue; (4) expression of the target MAGE A3 epitope by normal tissue. Mechanisms 1 – 3 would represent examples of off-target toxicity, while mechanism 4 would exemplify on-target off-tumor toxicity.

Mispairing of endogenous TCRs with exogenous TCRs could generate novel specificities and result in a graft versus host disease like syndrome.\textsuperscript{1} This possibility has been comprehensively studied in mice,\textsuperscript{11} but has not been described in any clinical trials of TCR-transduced T cells to date.\textsuperscript{3} Although precise quantification of mispaired TCRs in vivo is
difficult, the frequency of T cells expressing the Vβ5.1 chain used by the MAGE-A3 TCR can be compared to surface expression of the MAGE-A3 specific α/β heterodimer, which defines the correct α/β TCR pair. Using this method, an increase in the frequency of Vβ5.1 chain relative to the correct α/β TCR pair could indicate mispairing; this was not observed in either patient (Fig 5A).

We next evaluated each patients’ engineered T cell product against related epitopes from a comprehensive list of other MAGE family members as well as against an alloreactivity panel which covered >95% of known HLA types; no response was detected in either analysis.† It was also determined that there was no expression of MAGE A3, or potentially cross-reactive targets MAGE A6 or MAGE B18 by RT-PCR in either patients’ heart tissue or in autopsy samples from five other normal hearts.† Thus, we were unable to establish that alloreactivity or on-target off-tumor reactivity were responsible for the observed toxicity.

To evaluate the potential for off-target off-tumor reactivity being responsible for the observed toxicity we performed extensive in vitro analyses, both on the product that was administered to the second patient and with newly MAGE-A3a3a-transduced T cells. However, neither the patient’s cell product nor fresh MAGE-A3a3a-transduced T cells were activated in response to a large panel of HLA-A1+ cell lines, including cardiac myocytes and endothelial cells (Fig 5B). However, when an HLA-A1+ beating cardiomyocyte culture derived from induced pluripotent stem cells (iPSC-CM, or iCells) was exposed to MAGE-A3a3a-transduced T cells, the T cells did become activated (Fig 5C) and killed the

cardiomyocytes. Compared to T cells transduced with the wild-type MAGE-A3 TCR that served as the backbone of the a3a modifications, MAGE-A3a3a-transduced T cells were significantly more sensitive to activation both by iCells and the positive control (HLA-A1+ MAGE-A3+) cell line EJM. Having replicated the clinical findings in vitro, further studies demonstrated that a cross-reactive epitope derived from the human protein titin, presented in the context of HLA-A*01, could be potently recognized by the MAGE-A3-TCR engineered T cells.† Titin is a sarcomeric protein expressed in striated muscle,¹² and certain mutations in titin are known to cause dilated cardiomyopathy.¹³ Importantly, standard cultured primary cardiomyocyte cells do not express titin, while actively beating cardiac myocytes derived from induced pluripotent stem cells express the protein. Patient autopsy samples extracted from the heart were confirmed to be highly positive for titin by qRT-PCR, and presentation of titin was confirmed by mass spectrometry of peptides eluted from the surface of cells.†

Discussion

Through an unfortunate series of clinical events, we discovered that T cells engineered to express an affinity-enhanced TCR directed to an epitope of MAGE A3 are also able to effectively recognize a similar peptide epitope derived from the entirely unrelated protein titin which is expressed in cardiac tissue. MAGE A3 has been prioritized by the National Cancer Institute as the top rated cancer testis antigen immunotherapy target due to its frequent expression in many types of cancer and lack of expression on adult cells that express HLA class I antigens.¹⁴,¹⁵ Therefore, infusions with engineered T cells expressing
TCRs with enhanced affinity to another cancer testis antigen, NY ESO-1, have been safe and demonstrated clinically evident antitumor responses.\textsuperscript{16}

The observation that the striated muscle-specific protein titin could be targeted by a TCR specific for a cancer testis antigen is the first clinical example of off-target activity mediated by engineered TCRs. It has been previously reported that affinity modified TCRs can show lack of specificity, including self-reactivity.\textsuperscript{17} Titin is the largest gene in the human genome, and is related neither structurally nor functionally to MAGE A3 nor to other cancer testis antigens.\textsuperscript{12} Titin itself is immunologically interesting, as patients with autoimmune diseases such as myasthenia gravis, particularly when associated with thymoma, often have antibodies directed to Titin.\textsuperscript{18} Whether this is a result of antigen-spreading in a neuromuscular disease or whether the association with thymoma is mechanistically related to the development of anti-titin antibodies remain unanswered questions. We specifically evaluated but, interestingly, did not observe any T cell infiltration in skeletal muscle sections in the second case. Although we cannot fully explain this finding, it appears that skeletal muscle may be immunologically distinct from cardiac muscle; for example, skeletal muscle can be immunologically silent when transduced with adeno-associated viral vectors\textsuperscript{19} whereas liver is not.\textsuperscript{20} Furthermore, cardiac muscle may be particularly susceptible to dysregulated T cell activation, as mice deficient in the T cell inhibitory receptor PD-1 develop an enhanced T cell-mediated myocarditis in some experimental models.\textsuperscript{21,22}

The parental TCR from which the MAGE A3\textsuperscript{43a} TCR was derived was originally isolated from a patient with melanoma that expressed the MAGE antigen.\textsuperscript{23} It is likely that the low affinity
of the parental MAGE-A3-specific TCR as a consequence of the natural thymic selection process, allowed for expansion of the original T cells in a patient without cardiac toxicity. MAGE A3 may be a particularly difficult target because another group using an independently derived TCR that was specific for MAGE A3 peptides presented on HLA A*02 has reported severe on-target off-tumor toxicity. In that study, neurologic toxicity was observed due to the unexpected expression of other members of the MAGE cancer testis family in the central nervous system. Other instances of on-target off-tumor toxicity have been reported in cases with T cells engineered with a TCR specific for the carcinoembryonic antigen resulting in colitis and TCRs targeting melanoma antigens destroyed normal melanocytes in the skin, ears and eyes.

The cardiac toxicity that we observed was severe, and in some respects resembled graft versus host disease limited to a single organ. In the second case, the process was refractory to immunosuppression with corticosteroids. It should be noted that the toxicity was delayed in onset for several days and was not primarily due to a cytokine storm, as has been reported with T cells expressing chimeric antigen receptors. We have recently observed that toxicity from chimeric antigen receptor modified T cells can be aborted with blockade of IL-6 signaling, and it is possible that tocilizumab may be applicable to other instances of toxicity from engineered TCR therapy, given that our patients had high levels of IL-6 in the serum and pericardial fluid. However, this would not have been expected to abate the primary off-target cardiac toxicity. Another approach that may be useful would be the incorporation of a conditional suicide gene. This approach effectively ablated alloreactive T cells in a clinical trial after human hematopoietic cell transplantation, and may prove useful to abrogate on-target or off-target toxicities of TCR-engineered T cells.
The development of engineered, affinity-enhanced TCRs is emerging as a powerful strategy to effectively target tumors and expands the opportunities for engineered TCR-based adoptive T cell based therapies.\textsuperscript{31,32} Although MAGE-A3 remains an attractive target due to its restricted normal tissue expression, the challenge for targeted T cell therapy remains the identification of suitable epitopes to ensure on-target specificity. Here we show the need for improved preclinical testing methods to better enable prediction of specificity and illustrate the application of more elaborate cell culture systems which identified the previously unknown cross-reactivity of the MAGE TCR. Finally, this study highlights the potency of engineered T cells if harnessed appropriately, and suggests that reactivity to unknown epitopes expressed on normal essential tissues may represent the gravest safety risk with the use of engineered T cells.
Acknowledgements

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Author Contributions

MVM and CHJ wrote the manuscript. The protocols were written and designed by GPL, EAS, APR, and GB-S. CHJ was the regulatory sponsor with help from MVM. Adaptimmune Ltd engineered the MAGE-A3 TCR and was the commercial sponsor. LE, LL, AB, BMC and PJC conducted clinical research. MK supervised the laboratory analyses of clinical samples. NH and BKJ devised experiments uncovering the role of cross-reactivity to titin. BLL supervised cell manufacturing. All authors discussed and interpreted results and vouch for the data and analyses.

Disclosure of Conflicts of Interest

NH, JH, and JD are employees of Immunocore Ltd and GB-S, DPS, ABG, NJP, ADB, JEB, HKT-M and BKJ are employees of Adaptimmune Ltd. The other authors declare that they have no conflicts of interest.
References


Table 1. Characterization of infused products.

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<th>Criteria</th>
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<th>Case 2</th>
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<td># of cells infused</td>
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<td>5.09E+09</td>
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<td>% Transduction efficiency</td>
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* As per protocol, Case 1 was infused using a split-dose regimen with 30% and 70% of the dose infused on day +5 and day +6, respectively.
FIGURES AND LEGENDS

Figure 1. Timeline of events after infusion of engineered T cells. Abbreviations: ED, emergency department. T, temperature. SBP, systolic blood pressure. 100% on RA, 100% oxygen saturation on room air by pulse oximetry. CXR, chest x-ray. LLL, left lower lobe. PNA, pneumonia. Wnl, within normal limits. HR, heart rate. STE, ST elevations on electrocardiogram. Trop, Troponin. CP, chest pain. ICU, intensive care unit (transfer). ASCT, autologous stem cell transplant. Afib, atrial fibrillation. ΔMS, altered mental status. TTE, transthoracic echocardiogram. Cath lab, cardiac catheterization laboratory. CRRT, continuous renal replacement therapy. EF, ejection fraction. IABP, intra-aortic balloon pump. MSOF, multi-system organ failure.

Case 1

Nausea, abdominal pain, chills at home

ED at 2 am: T 103.1; SBP 110; 100% on RA; neutropenia

CXR: LLL PNA. Heart wnl.

7 am: HR 138; SBP <90; STE; trop 54; no CP

8:40 am: Hypoxia; ICU

9:02 am Cardiac arrest

10:20 am Death

Case 2

Diarrhea, C.diff+

Fever, neutropenia

T 102.4; Afib

Hypotension, hypoxia, ΔMS; STE

ICU; intubation; 100 mg hydrocortisone, levophed

TTE: large pericardial effusion, bradycardia

Cath lab: no tamponade

CRRT

1 gm solu-medrol

Trop 1.2->13.75

TTE: new low EF

Cath lab: IABP

Acidosis, MSOF

Death
**Figure 2. Peripheral blood cytokines elevated in both subjects.** Peripheral blood samples were collected in both cases at pre-determined time points; specific levels of thirty cytokines were quantified and compared to baseline (pre-infusion) samples. Only cytokines that were elevated at least 10-fold over baseline at any time point are shown. Case 2 received G-CSF as part of standard of care therapy following the autologous stem cell transplant. Baseline (pre-infusion) serum values of the 9 analytes shown in Case 1 were (pg/ml): IL-6 (12.5), IL-15 (30.8), IL-5 (1.82), IFN\(\gamma\) (1.68), IL-1Ra (245), IP-10 (99.9), IL-2R (322), MIG (30.7), IL-8 (11.6). Baseline levels of all cytokines analyzed in the serum in case 2 are detailed in the legend to Figure 4B.
Figure 3. Fate of the infused engineered T cells. (Panel A). Expansion of the engineered T cells over time in both subjects. Left y-axis shows cell count per microliter of blood (total white blood cell count in red, engineered (marked) cells in green). Right y-axis shows the number of copies of DNA used in the engineered T cells as a function of total genomic DNA in peripheral blood mononuclear cells (PBMC) over time. (Panel B). Distribution of engineered T cells in tissue samples obtained post-mortem in both subjects. Quantitative PCR for vector sequences was performed from total genomic DNA obtained from flash-frozen tissues. Note different scale on y-axis for each case.
**Figure 4. Analysis of cardiac-specific toxicity. (Panel A).** Pathologic and immunohistochemical analysis of myocardium from both subjects. Hematoxylin and eosin (H&E) stained sections of myocardium shown at two magnifications for each subject (top panels), demonstrating lymphocytic infiltrate and diffuse myocyte necrosis. Immunohistochemical (IHC) staining with anti-CD3 (lower panels) demonstrates that lymphocytic infiltrates are T cells, shown at two magnifications. (Panel B). Cytokines in the peripheral blood and the pericardial fluid in Case 2 obtained post-mortem show evidence of T cell activation. Thirty cytokines were assayed; each cytokine level shown is normalized to the concentration of that cytokine in a peripheral blood sample obtained at baseline. The patient was given injections of filgrastim (G-CSF) as per standard of care post-ASCT. Baseline levels of cytokines in blood were (pg/ml): VEGF (1.73), IL-1β (0.5), G-CSF (10.51), EGF (23.13), IL-10 (2.54), HGF (357.83), FGF-Basic (4.45), IFNα (70.76), IL-6 (3.42), IL-12 (93.34), RANTES (12.717), Eotaxin (76.1), IL-13 (0.53), IL-15 (59.94), IL-17 (0.16), MIP1α (14.85), GM-CSF (0.77), MIP1β (36.44), MCP-1 (953), IL-5 (0.34), IFNγ (2.49), TNFα (1.26), IL-1Ra (37.66), IL-2 (0.56), IL-7 (15.36), IP-10 (23.14), IL-2R (205.81), MIG (10.51), IL-4 (9.91), IL-8 (10.68).
Figure 5. Elucidation of mechanism of clinical cardiac toxicity. (Panel A). Analysis for expression of the correctly paired MAGE-A3 engineered TCR (MAGE dextramer staining, y-axis) of input (untransduced) T cells, transduced T cell product, and T cells recovered from PBMC in both subjects at time of death. The x-axis shows staining for specific TCRβ chain (Vβ5.1) utilized by the engineered MAGE-A3 TCRs. Numbers shown in each quadrant indicate percentages of the gated CD3+ cells. Cell populations that stain only for Vβ5.1 but not dextramer are the sum of (1) endogenous TCR that utilizes that beta chain (i.e. population shown in input T cells) and (2) mispaired MAGE-A3 engineered TCR. No expansion of T cells with mispaired TCRs was detected after infusion in the subjects. (Panel B). A sample of the T cell product infused into the second patient, along with fresh MAGE-A3a3α-transduced T cells and untransduced T cells, was tested for IFNγ production by ELISPOT when co-cultured with a large panel of HLA-A1+ cell lines, including one that was MAGE-A3+ (EJM) as a positive control. Bars indicate mean +/- SEM of 3 replicates. (Panel C). Activation and cytokine production of MAGE-engineered T cells incubated in vitro with HLA-A*01 positive, titin positive, MAGE-A3 negative beating cardiac myocyte cells derived from induced pluripotent stem cells (iPS-CM or iCells). The EJM plasmacytoma cell line expresses HLA-A*01 and MAGE-A3 (positive control), and the colo205 cancer cell line expresses HLA-A*01 but not MAGE-A3 or titin (negative control). Controls for the effector cells are non-transduced (ntd) T cells, or no T cells (targets only). Bars indicate mean +/- SEM of 3 replicates.* indicates p<0.0001 by t-test.
A.

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MAGE dextramer

B.

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Cardiovascular toxicity and titin cross-reactivity of affinity enhanced T cells in myeloma and melanoma