TALEN-mediated genetic inactivation of the glucocorticoid receptor in cytomegalovirus-specific T cells

Running title: Cytomegalovirus-specific T cells and glucocorticoid receptor inactivation

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Key points

- The GR gene can be inactivated in Streptamer®-selected CMV-specific CD8+ T cells using TALEN.
- The GR gene inactivation endows T cells with resistance to the immunosuppressive effects of corticosteroids in vitro and in vivo.

Abstract

Cytomegalovirus (CMV) infection is responsible for substantial morbidity and mortality following allogeneic hematopoietic stem cell transplantation (HSCT). T cell immunity is critical for control of CMV infection and correction of the immune deficiency induced by transplantation is now clinically achievable by the adoptive transfer of donor-derived CMV-specific T cells. It is notable, however, that most clinical studies of adoptive T cell therapy exclude patients with graft-versus-host disease (GvHD) receiving systemic corticosteroid therapy, which impairs cellular immunity. This group of patients remains the highest clinical risk group for recurrent and problematic infections. Here, we address this unmet clinical need by genetic disruption of the glucocorticoid receptor (GR) gene using electroporation of transcription activator-like effector nucleases (TALEN) mRNA. We demonstrate efficient inactivation of the GR gene without off-target activity in Streptamer®-selected CMV-specific CD8+ T cells (A2-NLV), conferring resistance to glucocorticoids. TALEN-modified CMV-specific T cells retained specific killing of target cells pulsed with the CMV peptide NLV in the presence of dexamethasone (DEX). Inactivation of the GR gene also conferred resistance to DEX in a xenogeneic-GvHD model in sub-lethally irradiated NOD-scid IL2rγnull (NSG) mice. This proof of concept provides the rationale for the development of clinical protocols for producing and administering high-purity genetically-engineered virus-specific T cells that are resistant to the suppressive effects of corticosteroids.
Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for high-risk hematological malignancies, including leukemias, lymphomas and myelodysplastic syndromes. A number of factors influence clinical outcomes, including history of exposure to cytomegalovirus (CMV). Human CMV is a β- herpesvirus with a seroprevalence of 30-70% in developed countries. CMV infection after HSCT can cause multiple complications including gastroenteritis, pneumonitis, hepatitis, retinitis or encephalitis. Pneumonitis is the most serious manifestation with a mortality rate of over 80% without antiviral chemotherapy, and of over 50% even with standard antiviral therapies.

Patients at the highest risk of clinically problematic CMV infection are serologically positive recipients, recipients of T-cell-depleted grafts and those receiving corticosteroids for the treatment of graft-versus-host disease (GvHD) post HSCT. Thus CMV infection occurs in 60-90% of CMV seropositive patients, dependent on whether T cell depletion is performed or not. Despite modern pre-emptive treatment strategies, potentially life threatening disease still occurs in 3-10% of hematopoietic stem cell transplant recipients. Effective antiviral drug therapies are available, but their use is associated with renal toxicity, myelosuppression and, less commonly, the emergence of drug resistance. There is therefore an important clinical need for therapies that speed the tempo of immune reconstitution to reestablish immunologic control of the virus.

Because T-cell mediated immunity has been shown to play a critical role in the sustained control of CMV infection, several groups have investigated strategies
to generate CMV-specific T lymphocytes for adoptive immunotherapy. These studies demonstrated that CMV-specific T cells can be expanded or directly selected from seropositive donors, administrated safely, and can restore functional T-cell immunity when the primary problem is numerical deficiency\(^{19-24}\). Importantly, virus-specific T cells engrafted, expanded and conferred protective immunity despite therapeutic levels of calcineurin inhibitors (e.g. cyclosporine, tacrolimus), suggesting that these agents are permissive to the desired therapeutic activity. Parallel advances have been made in cases where the donor is CMV seronegative, either by expanding naïve CMV-reactive T cells or by the use of third party donor cells\(^ {25,26}\). From a global perspective, however, most recurrent and refractory CMV infections occur in patients with significant GvHD\(^ {10}\), largely as a result of high dose glucocorticoid therapy. Despite advances in our understanding of the pathophysiology of GvHD, glucocorticoids remain the most effective and favored initial therapy for the treatment of both acute and chronic GvHD\(^ {27-29}\). This provides a major obstacle to adoptive antiviral immunotherapy since glucocorticoids suppress T cell numbers and function.

Glucocorticoids exert potent immunosuppressive effects after binding to the glucocorticoid receptor (GR), a ligand-activated transcription factor\(^ {30}\). Sequestered by a heat-shock protein complex (hsp90, hsp70, FK506 binding protein 52), the GR translocates to the nucleus upon binding to glucocorticoids\(^ {31}\), where it exerts direct effects on gene expression (e.g., the induction of Annexin I and mitogen-activated protein kinases phosphatase 1) and indirect effects through interactions with other transcription factors (i.e., NF-κB and activator protein 1). Other evidence supports non-genomic mechanisms of action involving second-messenger cascades (e.g., the phosphatidylinositol-3-hydroxykinase (PI3K)–Akt–eNOS pathway)\(^ {32-34}\). It is notable that the GR is essential for the immunosuppressive effects of glucocorticoids in T
cells and that loss of GR function prevents all of their apoptotic actions. Despite the wide range of gene targets for the GR, only a single GR gene has been described, which generates several GR isoforms via alternative splicing. Based on these considerations, we hypothesized that selective inactivation of the GR gene in CMV-specific T cells would render them resistant to the suppressive effects of corticosteroids, thus providing a therapeutic option for the treatment of CMV infection in the context of glucocorticoid administration.

Powerful tools for genome editing are now available, including the Transcription Activator-Like (TAL) Effector Nucleases (TALEN). TALEN are attractive reagents for therapeutic applications because of their high specificity, conferred by the long targeting domains (17 base-pair target sequence) and the requirement for homodimerization of the associated FokI domain-containing nucleases. The endonucleases cause site-specific double-stranded DNA breaks (DSB) and trigger natural DNA repair mechanisms, notably non-homologous end joining recombination (NEHJ) that is error prone. The introduction of insertions or deletions can then shift the reading frame and inactivate the gene function. The success rate and activity of TALEN depends heavily on the cell type and delivery method. Transfection by electroporation with mRNA is an attractive method for therapeutic application because of its reproducibility and cost effectiveness.

We developed a highly efficient TALEN pair, specific for the exon 2 sequence of the human GR gene. We demonstrated that following mRNA transfection, GR-targeting TALEN induce disruption of the GR gene at high frequencies of 55% and 60% in primary human T cells and the human HLA-A*02 T cell lymphoma (T2) line respectively. After dexamethasone selection, the enriched subset of CMV-specific T
cells<sup>GR-Ex2</sup> (GR-Exon 2 targeted) were resistant to glucocorticoids, while maintaining their HLA-A*02-restricted cytotoxic activity towards CMV peptide-pulsed (pp65) targets. Finally, the in vivo resistance and functionality of the TALEN-modified CD8<sup>+</sup> T cells were confirmed in a preclinical murine model of xenogeneic-GvHD. These data demonstrate the feasibility of generating CMV-specific T cells resistant to glucocorticoids within 10 days and further show that their functional activity remains intact.

**Methods**

**Cell culture and transfection**

The HLA-A02 T2 lymphoma cell line was provided by Dr Martin Pule and maintained in RPMI-1640 supplemented with 10% Fetal Calf Serum (GIBCO), L-glutamine, Penicillin-Streptomycin (Sigma). CD8+T cells were selected by positive selection using human CD8 MicroBeads (Miltenyi) according to the manufacturer. The cells were stimulated with Dynabeads Human T-Activator CD3/CD28 (Invitrogen) at 37°C in 5% CO2 at 1×10<sup>6</sup> cells/ml for 4 days. Cells were kept at 1×10<sup>6</sup> cells/ml in complete RPMI-1640 supplemented with rhIL-2. HLA-A*0201, CMV-pp65-specific T cell clones were selected from healthy donors; peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation using Ficoll-Paque Plus ™ (GE Healthcare) and HLA-A*0201, CMV-pp65-specific T cell clones were selected with Strep-Tactin® Magnetic Nanobeads for MHC I Streptamer® HLA-A*0201, CMV-pp65: NLVPMVATV (IBA Life Sciences 6-7001-015) according to the manufacturer instructions. Clonal populations were then expanded in vitro using the REP protocol 41. 5x10<sup>4</sup> CD8+ T cells were added to cloning mix consisting of 5x10<sup>6</sup> irradiated TM-LCL (CD19+ EBV-transformed lymphoblastoid cell line), 25x10<sup>6</sup> irradiated allogeneic PBMCs, 30 ng/ml OKT3 (BioXcell) and 20
ng/ml rhIL-2. TM-LCL were generated using concentrated B95-8 virus supernatant and complete RPMI-1640 containing 1 μg/ml Cyclosporin A (Sigma). One week after REP, 5x10⁶ HLA-A*0201 CMV-pp65-specific T cells were resuspended in 200μl of Cytoporation medium T were electroporated with the same amount of GFP mRNA (mock transfection,) or with 10 μg of each TALEN mRNA in 0.4-cm gap MicroPulseur cuvettes (BioRad), using a BTX Agile Pulse electroporator (Harvard Apparatus BTX) delivering several square wave pulses. The cells were then transferred into complete RPMI 1640 medium with 20 ng/ml rhIL-2, 1% human serum. On day 2, the cells were resuspended, as previously described, to remove the Cytoporation medium T. On day 3, the cells were washed and the GR gene inactivated T cells were enriched by 5 days treatment with 10⁻⁴ M dexamethasone (DEX) (Sigma) in complete RPMI-1640 medium.

**CFSE-based cytotoxicity assay**

5x10⁴, 5x10³ or 5x10² HLA-A*0201 CMV-specific effector T cells or CMV GR-Ex2 T cells were added to a 96-well round bottom plate (Thermoscientific) and incubated or not with 10⁻⁴M DEX for 72h in complete RPMI 1640. T2 GR-Ex2 cells, serving as target antigen-presenting cells, were pulsed with 1 μg/ml pp65 peptide or not in a cell-culture medium for 2/₄h at 37°C. T2GR-Ex2 pp65-pulsed (specific target) cells were labeled with 1μM CFSE (Molecular Probes) and the T2GR-Ex2 -non pulsed (unspecific target) cells were labeled with 10 μM CFSE for 10 minutes at 37°C. CFSE-labeled target cells were resuspended at 5x10³/100μl and co-cultured with the effector cells for 16h. A quantitative analysis of the cell populations was assessed by adding a fixed amount of (1x10⁵) Cell Sorting Set-up Beads. The percentage of specific lysis was calculated as follows: % specific lysis = 1-[absolute no. CFSE low cells (specific
target) /[absolute no. CFSE high cells (unspecific target)] × 100.

In vivo resistance and xenogeneic GvHD in NSG mice

5x10⁶ T2wt cells or 5x10⁶ T2GR-Ex2 cells were injected intravenously (IV) into sub-lethally irradiated (2Gy) NSG mice, treated daily with 15mg/kg DEX injected intraperitoneally (IP) or not for 12 days. 1x10⁶ CD8⁺wt T cells or CD8⁺GR-Ex2 T cells were injected IV into irradiated NSG mice. Mice received DEX treatment as previously described for 21 days. Spleens were harvested, weighed, and the absolute number of human CD8⁺ T was then quantified using beads. The human cells were selected from spleens using human CD2 MicroBeads (Miltenyi) and MS columns according to the manufacturer’s instruction. In all experiments, mice were monitored for the appearance of xeno-GVHD-like symptoms, including hair loss, hunched posture and reduced mobility.

For additional information, see supplemental data section

Results

Efficient inactivation of the GR in T2 cells after mRNA-mediated TALEN delivery

To inactivate the GR gene, 6 pairs of TALEN were designed to target different genomic sequences, corresponding to various domains of the GR protein (transactivation domain, DNA binding domain and ligand binding domain). Each pair was transfected into HEK-293 cells (2.5 µg of each half-TALEN) to allow high throughput sequencing analysis. Those targeting the exon 2 sequence caused up to 28.2% targeted mutagenesis in the GR gene (Table S1), without apparent cell toxicity.
and were selected for subsequent evaluation (Fig. 1A). TALEN were then delivered into the HLA-A*0201 T2 lymphoma cell line using an optimized mRNA-electroporation protocol. Indels (insertions/deletions) content analysis using the T7 endonuclease I assay revealed high TALEN efficacy, with mutation of up to 60% of the GR genomic sequence by 3 days after the transfection and in the absence of a selection marker (Fig. 1B). Targeted deep sequencing analysis (MiSeq) confirmed the high specificity of the TALEN for on-target mutagenesis with dominant presence of 4 to 18 base pairs deletions (Fig. 1C). Western blot analysis demonstrated a transient expression of the TALEN protein one and two days after transfection and a 50% decrease of the GR protein expression 72 hours after transfection. Finally, an in vitro selection in high dose (10^-4 M) of dexamethasone (DEX) for 7 days starting 72 hours after transfection, significantly enriched the population of T2^GR-Ex2 cells (Fig. 1D, Fig. S1A). It should be noted that culturing T2 wt cells (GFP mRNA mock transfected) in DEX resulted in too few viable cells for subsequent experiments (Fig. S1B).

**TALEN-modified T2 cells are resistant to glucocorticoids**

The T2^GR-Ex2 enriched population of cells and control wild type (T2 wt) cells were incubated for 72 hours in vitro in the presence or absence of DEX (10^-4 M). Whilst 50% of the T2 wt cells were sensitive to DEX-induced apoptosis and secondary necrosis, the TALEN-modified T2^GR-Ex2 cells were DEX-resistant (Fig. 2A, Fig. S1B). A dose-response cell death assay with DEX or prednisone (10^-5M, 10^-4M, 10^-3M) confirmed that the T2^GR-Ex2 cells were specifically resistant to both glucocorticoids (Fig. S1B). Furthermore, the ability of T2 wt cells to proliferate in vitro, highlighted by a MTT metabolic assay, was significantly reduced in the presence of DEX, whilst T2^GR-Ex2 cells were DEX resistant, proliferating at levels equivalent to untreated T2 wt.
cells (Fig. 2B, Fig. S1C). To assess whether T2^{GR-Ex2} cells were also resistant to glucocorticoids in vivo, T2^{wt} and T2^{GR-Ex2} cells were injected intravenously into immunodeficient NSG mice that where either left untreated or treated with DEX for 12 days. After T cell infusion and in the absence of DEX, spleens were heavily infiltrated by human CD4^{+}/CD45^{+} T2^{wt} cells or T2^{GR-Ex2} cells. In contrast, in mice receiving DEX, splenic infiltration was significantly reduced in the T2^{wt} group, but remained unaffected in the group of mice injected with T2^{GR-Ex2} cells (Fig. 2C), supporting the in vitro data demonstrating resistance to glucocorticoid induced cell death. Finally, quantification of the absolute number of live human CD4^{+} cells in the spleens confirmed the macroscopic findings, further demonstrating the resistance of T2^{GR-Ex2} cells to DEX in vivo in comparison to T2^{wt} cells (Fig. 2D). These results show a potential proof of concept for the rapid production of glucocorticoid-resistant T cells, using an optimized mRNA-electroporation protocol and TALEN.

**TALEN-mediated GR inactivation in primary CMV specific-T cells**

Clinical application of CMV-specific T cell therapies following HSCT requires selection or selective expansion of virus-specific T cells in order to limit the number of potentially alloreactive T cells that are transferred. The necessity to avoid infusing alloreactive T cells is even more pertinent in patients who already have GvHD. Selection of CMV-specific T cells based on T cell receptor specificity using HLA-multimers (Streptamers®) delivers a high purity product, potentially mitigating risk in this setting. This technology depends on reversible binding of HLA-multimer-peptide complexes coupled to magnetic selection, isolating peptide-specific T cells that are phenotypically and functionally indistinguishable from untreated cells. We therefore selected primary CD8^{+}CMV-specific T cells from healthy donors using HLA-
Streptamers® directed against an immunodominant HLA-A*0201-restricted epitope derived from human CMV protein pp65 (NLV/A2) (Fig. 3A).

Pp65-specific CD8+ T cells were subsequently expanded in vitro for one week and then electroporated with 20 μg of GFP mRNA or 10μg of each half TALEN mRNA. The optimized protocol provided both high viability (median >60%), and a very high transfection efficacy (median 98%), overcoming the frequently reported limitations (toxicity and low transfection efficacy) of primary T cell engineering (Fig. 3B). Miseq analysis of the targeted exon 2 sequence showed 55% NHEJ-mutagenesis 3 days after transfection with the mRNA TALENs and characterized the different deletions induced at the specific on-target site (35 insertions were also reported in the 6046 reads; data not shown) (Fig. 3C, Fig. S2A). Analysis of the mutational rate by T7EI digestion before and after selection with DEX highlighted an increase in the percentage of mutated CMVGR-Ex2 cells from 60% to 89%, confirming the enrichment of GR gene-inactivated-T cells under DEX selection pressure. (Fig. S2B). Mirroring the data in the T2GR-Ex2 cells, protein analysis assessed by western blot demonstrated a transient expression of the TALEN proteins in the CMV GR-Ex2 cells. Similarly, 72 hours after transfection we observed a 50% decrease of GR protein expression and after 5 days of culture with DEX, no GR protein was detectable in the CMVGR-Ex2 cells by western blot (Fig. 3D, Fig. S2C). In addition, the potential off-target sites of the GR-Ex2 TALEN in the human genome were characterized in silico and the 14 most likely off-target cleavage sites, conserved in the seed region and containing 7–8-base mismatches were ranked (Table S3). We measured the frequencies of small indels at the on-target site and putative off-target sites using deep sequencing. Insertion and deletion events frequencies at the 14 potential off-sites in the TALEN-treated cells (CMVGR-Ex2 cells) were identical to the ones observed in the mock-transfected cells.
(CMV\textsuperscript{wt} cells) demonstrating the specificity of cleavage of the GR-Ex2 TALEN (Fig. 3E, Table S3-4).

**TALEN-modified CMV\textsuperscript{GR-Ex2} cells are resistant to DEX-induced immunosuppressive effects and retain cytolytic activity**

The resistance of CMV\textsuperscript{GR-Ex2} cells to DEX was quantified in an apoptosis assay. After 72 hours exposure to DEX no significant difference was observed in terms of AnnexinV labeling or absolute number of live cells between the CMV\textsuperscript{GR-Ex2} cells treated with or without DEX, in contrast to the impact of DEX on CMV\textsuperscript{wt} cells (Fig. 4A and B, Fig. S2D). We also analyzed both Granzyme B (GZB) expression and IFN-γ production in vitro. CMV\textsuperscript{wt} and CMV\textsuperscript{GR-Ex2} cells were treated with DEX for 30 hours (before too few viable cells remain for subsequent analysis), prior to overnight stimulation with TM-LCL cells pulsed with the NLV peptide. Whilst both GZB and cytokine expression were significantly reduced in CMV\textsuperscript{wt} cells by the addition of DEX, neither of these (Fig. S2E, G, H, I) nor the absolute number of cells (Fig. S2F) were significantly affected in CMV\textsuperscript{GR-Ex2} by the addition of DEX. In order to test the functional activity of the CMV\textsuperscript{GR-Ex2} cells in the presence or absence of DEX, we used the glucocorticoid-resistant T2\textsuperscript{GR-Ex2} cells generated in earlier experiments to assess target-specific cytolysis. T2 cells are deficient in the transporter associated with antigen transport protein (TAP) but still express low amounts of MHC class I (HLA-A\textsuperscript{*}02, -B\textsuperscript{*}51, and -C\textsuperscript{*}01) on their surface, which can be stabilized following loading with exogenous cognate peptides. Target T2\textsuperscript{GR-Ex2} cells were pulsed or not with the HLA-A\textsuperscript{*}0201-restricted pp65 NLV peptide, labeled with two different concentrations of CFSE (pp65 pulsed, CFSE-low; un-pulsed, CFSE-high) and mixed in a 1:1 ratio. The mixed population was incubated with an increasing number of either CMV\textsuperscript{wt} or...
CMV<sup>GR-Ex2</sup> cells treated or not with DEX prior to and during the co-culture. The NLV-specific lytic activity of the CMV<sup>GR-Ex2</sup> cells was comparable to that of the CMV<sup>wt</sup> cells, but was not affected by the DEX treatment in contrast to that of the CMV<sup>wt</sup> cells. High specific killing activity (60%) was maintained even at a 1:1 effector-target ratio (Fig. 4C, D).

**TALEN-modified CD8<sup>GR-Ex2</sup> T cells are resistant to DEX in a xeno-GvHD model**

To measure both glucocorticoid resistance and functional activity of polyclonal human CD8+ T cells in vivo, 1x10<sup>6</sup> CD8<sup>wt</sup> or CD8<sup>GR-Ex2</sup> T cells, generated as previously described, were injected intravenously into irradiated NSG mice treated or not with DEX for 21 days. These experiments also address a potential safety concern regarding the infusion of glucocorticoid-resistant alloreactive T cells. In human-mouse xenogeneic GvHD, human immune cell infiltration has been reported in peripheral tissues such as the skin 45-47, mimicking the human form of the disease 48. In human acute GvHD the cutaneous lesions often appear 2–3 weeks post HSCT, which is recapitulated in the xeno-GvHD model in NSG mice, where increasing infiltration by human cells is observed from day 14 to 28 42. Engraftment is associated with the development of acute xeno-GVHD syndrome, characterized by rapid weight loss (>10%), hunched posture, reduced mobility and signs of cutaneous GvHD pathology such as hair loss 45. We therefore evaluated development of xeno-GvHD by the occurrence of: hair loss, CD45+ (human) cell infiltration in the skin, spleen index, splenic engraftment of human CD8<sup>+</sup> cells and Granzyme B (GZB) expression. As expected, treatment with DEX for 21 days ablated engraftment and function of CD8<sup>wt</sup> T cells, consistent with glucocorticoid sensitivity. Hair loss, skin infiltration with human CD45<sup>+</sup> cells (score 0.5-1.5) and a low but consistent level of splenic
engraftment with the CD8+ population (range 2-4%) were observed in all mice receiving CD8+wt T cells or CD8+GR-Ex2 T cells in the absence of DEX, and in mice receiving CD8+GR-Ex2 T cells treated with DEX (Fig. 5A, Fig. S3). Extensive fibrosis and loss of normal splenic architecture, and the presence of a GZB+ CD8 population were also documented in these 3 cohorts (Fig. 5B, Fig. S3D). Human CD8+GR-Ex2 and CD8+wt T cells were selected from pooled-spleens using human anti-CD2 beads, analyzed for GR expression by western blot, and compared to CD8+GR-Ex2 at day 3 following transfection with TALEN. GR expression analysis at day 3 demonstrated a 50% decrease of GR protein, whilst the CD8+GR-Ex2 T cells engrafted in NSG mice treated with DEX or not did not express GR (Fig. 5C). Overall, the results demonstrate the ability to render polyclonal T cell populations resistant to glucocorticoids in vivo, but also the need to ensure that clinical development focuses on generating highly pure populations of virus-specific T cells that are devoid of alloreactive potential for application in the transplant setting.

Discussion

We demonstrate efficient and rapid inactivation of the GR gene in CMV-specific T cells using electroporation of TALEN mRNA, leading to glucocorticoid resistance but retention of functionality in both in vitro assays and in vivo models. The processes described here are compatible with scaling to Current Good Manufacturing Practice (cGMP) regulatory standards, providing a potential strategy for treating patients with CMV infection and concurrent glucocorticoid therapy following HSCT. The transient expression of TALEN following mRNA electroporation offers a potential advantage for clinical applications, as the system does not require integration of the delivery vector, avoiding perceived risks associated with insertional mutagenesis 49.
Furthermore, the process has already been established under clinical-scale cGMP conditions. Transient expression also reduces the risks of off-target gene modification. For the GR-Ex2 TALEN, the 14 most likely off-target sites were identified by in silico analysis and deep sequencing analysis confirmed the high specificity of cleavage. Whilst this work focuses on the use of TALEN, other strategies for genome engineering such as CRISPR/Cas9 could be applied in a similar fashion. Future studies may directly compare these technologies in terms of relative benefits with respect to clinical application. The specificity of targeting remains critical, and this is highly dependent on the design of targeting moieties. It is possible that no single technology will be superior for all applications.

Beyond the immediate translational application outlined for CMV, the approach could prove beneficial in other settings in which T cell therapies are considered, since glucocorticoids are commonly used in medical practice. Application for other viral infections that cause a significant burden of disease following HSCT such as adenovirus and Epstein-Barr virus in patients receiving corticosteroids are logical extensions, as adoptive T cell therapies targeting these pathogens have demonstrated sustained antiviral efficacy and clinical benefits in patients not receiving corticosteroids. Particular consideration must be given to product purity in the setting of GvHD post HSCT. Whilst CMVGR-Ex2 T cells would have a selective survival advantage over any remaining CMVwt T cells, the presence of alloreactive GR-deleted T cells would be a potential safety concern. This potential is highlighted by our results in the murine xeno-GvHD model. In the case of CMV, the use of a Streptamer®-selection strategy allows rapid selection of highly pure populations of antigen-specific T cells. Attaining such high purities of other virus-specific T cells, such as adenovirus-specific T cells, remains more challenging. Because of these
considerations, incorporation of a suicide gene may be justified in these translational applications.

Cell persistence will be important for clinical efficacy. This issue cannot be addressed definitively in a xenogeneic model in which longer term engraftment of human cells is not adequately modelled, and will be most readily resolved within the context of a clinical trial. Memory phenotype of the infused cells may influence persistence. The recent demonstration that the distribution between naïve, effector, and memory subsets of dKO-CART19 cells were indistinguishable from unmodified cells suggests that TALEN can efficiently target all of these T cell subpopulations. The strategy may also be applicable to T-cell-based anti-cancer therapies. Many glioma and glioblastoma tumor cells express potential targets for chimeric antigen receptor (CAR)-based therapies, such as the IL-13 receptor or EGFRvIII. Clinical application of CAR T cells is, however, hampered by the fact that patients with these malignancies receive glucocorticoids to reduce inflammation and cerebral edema following tumor resection. Glucocorticoids are also used as part of primary therapy in the clinical management of several other solid tumors, either because of their effectiveness in treating the malignancy, or for managing tumor-associated symptoms (such as pain or edema). In all of these examples, GR-resistance would both protect adoptively transferred cells from the effects of glucocorticoids, and provide a selective survival advantage over endogenous T cells.

In conclusion, we show that glucocorticoid-resistant CMV-specific T cells can be rapidly generated in vitro using GR-TALEN and these GR-inactivated T cells maintain a specific cytolytic activity in the presence of DEX. Furthermore, when transferred into NSG recipients, TALEN-modified CD8+ T cells retained functionality and induced xeno-GvHD in the presence of DEX, providing evidence
for their functional activity and resistance in vivo. Our findings have important implications for the transplantation field as it represents the first proof of concept for the development of improved adoptive immunotherapy, using targeted genome modifications in T cells.

Authorship:

Contribution: LM, MM, KB and AG performed experiments, LM analyzed results; AG and JS provided TALEN GR and TALEN antibody; LM, SAQ and KSP designed the research and LM wrote the manuscript with input and approval from the coauthors. Conflicts-of-interest disclosure: AG and JS are Cellectis employees.

Acknowledgments: The authors thank Dr Ida Ricciardelli (Institute of Child Health, UCL) for provision of B95-8 virus supernatant, James Motta (Universidad Andres Bello, Chile) for optimizing the ICH staining, UCL core facilities: Cancer Genome Engineering (CAGE) Facility, Catherine King, Bill Lions Informatics Centre, Dr Herrero Javier, UCL/UCLH BioBank for Health and Disease and UCL Biological Services. Funding: S.A.Q. is funded by a Cancer Research UK Career Development Fellowship and a Cancer Research Institute Investigator Award. K.S.P. receives funding from Cancer Research UK, Leukaemia and Lymphoma Research, and the NIHR Blood and Transplant Unit (BTRU) for stem cell transplantation and immunotherapy. This work was undertaken at University College London Hospitals/University College London, which received support from the Department of Health and Cancer Research United Kingdom funding schemes for National Institute for Health Research Biomedical Research Centres and Experimental Cancer Medicine Centres.
References and Notes


56. Kelly WK, Curley T, Leibretz C, Dnistrian A, Schwartz M, Scher HI. Prospective evaluation of hydrocortisone and suramin in patients with


**Figures and legends**

**Fig. 1. Efficient TALEN-mediated editing of glucocorticoid receptor gene in the human HLA-A02 T lymphoma (T2 cell line).** (A) Schematic representation of the *GR* genomic locus (ch. 5q31-q32) and TALEN targeted sequence (exon 2). (B) T7EI assay showing efficient NHEJ-mediated mutagenesis (60%) at the intended target site on the *GR* gene. 10μg of mRNA from each TALEN were used to transfect the T2 cell line with BTX Agile Pulse electroporator. At day 3, the gDNA was amplified by PCR and subjected to a mismatch-sensitive T7 Endonuclease digestion, prior to a separation on a 10% polyacrylamide TBE gel. Gene modification (indels) quantification was based on relative band intensities (Image J) (ND: non-digested; D: digested). (C) Representative sequences of the human *GR* on targeted site using Miseq analysis. (D) Western blot analysis of TALEN and GR expression before and after dexamethasone enrichment of mutated cells. At day 3, the T2*GR-Ex2* (TALEN modified) cells were treated with dexamethasone [10^{-4}M] for 7 days.

**Fig. 2. TALEN-modified T2 cells resistance to dexamethasone in vitro and in vivo.** (A) Apoptosis of T2*wt* cells and T2*GR-Ex2* cells measured by Annexin V and PI staining. Cells were cultured with dexamethasone [10^{-3}M] for 72h and the proportion
of cell death was determined as positivity (right quadrants) for PI, Annexin V or both. 

(B) Time-response curves for cell proliferation of T2<sup>wt</sup> or T2<sup>GR-Ex2</sup> (open) treated with DEX by using a typical MTT assay. Data are represented as means ± SD from 3 independent experiments, statistical analysis was performed by one-way ANOVA (**P < 0.001). (C) Splenic engraftment of T2<sup>wt</sup> or T2<sup>GR-Ex2</sup> cells, stained by human anti-CD45 and anti-CD4 and pictures depicting the smaller size of harvested spleens from DEX treated T2-injected recipients. 5.10⁶ T2<sup>wt</sup> or T2<sup>GR-Ex2</sup> cells were IV injected into irradiated NSG mice, daily treated with DEX IP at 15mg/kg or vehicle for 12 days. (D) Total number of human CD4<sup>+</sup> cells/spleen. Data depict means from 2 independent experiments with 4 mice per group, statistical analysis was performed by one-way ANOVA (**P < 0.01) with Bonferroni's multiple comparisons (a non-parametric t test between T2 and T2 DEX groups reveals a p= 0.0034).

Fig. 3. TALEN-mediated GR editing in Streptamers<sup>®</sup> selected-CMV specific CD8<sup>+</sup> T cells. (A) MHC I Streptamers<sup>®</sup> (HLA A02) allow isolation of CMV-specific (NLV peptide) CD8<sup>+</sup> T cells in high purity from donor blood leukocytes. (B) Primary T cells transfection efficacy (>98%) and viability (>60%), using electroporation with IVT mRNA. (C) Representative TALEN-induced NHEJ mutagenesis in Streptamers-selected primary T cells by Miseq analysis. (D) Western blot analysis of TALEN and GR expression before and after DEX enrichment of mutated cells. (E) Off-target site analysis from in silico prediction after next generation sequencing.

Fig. 4. Resistance to DEX and cytolytic activity of TALEN-modified CMV specific CD8<sup>+</sup> T cells. (A) Apoptosis of CMV-specific T cells (CMV<sup>wt</sup>) and CMV-specific TALEN-modified T cells (CMV<sup>GR-Ex2</sup>) measured by Annexin V and PI
staining, after 72h in the presence of $[10^{-4}\text{M}]$ DEX. (B) Absolute number of live cells after 72h of DEX treatment measured by anti-CD8 staining and cell counting beads. (C) Antigen-specific killing assay using CFSE labeled target cells. The T2$^{\text{GR-Ex2}}$ cell line was pulsed with the pp65 (NLV) viral peptide for 1h and CFSE labeled prior to co-culture with CMV–specific T cells under DEX for 72h. (D) Dose response of specific cytolytic activity under DEX. Data are represented as means ± SD from 3 independent experiments, statistical analysis was performed by one-way ANOVA ($**P<0.001$).

**Fig. 5.** Resistance to DEX and functional activity of TALEN-modified CD8+ T cells in a xenogeneic GvHD model. (A) Hair loss and skin infiltration with human CD45+ cells counterstained with Haematoxylin from irradiated NSG mice, injected with $1.10^6$ CD8$^{\text{wt}}$ or CD8$^{\text{GR-Ex2}}$ T cells and treated daily or not with DEX IP at 15mg/kg for 21 days. (B) Absolute number of human granzyme B-expressing (GZB) CD8$^{\text{wt}}$ or CD8$^{\text{GR-Ex2}}$ T cells in the spleens of previously described NSG mice. (C) Western blot analysis of GR expression from human-selected cells in pooled-spleens ex vivo and at day 3 after transfection with TALEN. Data are representative means from 2 independent experiments with 5 mice per group, statistical analysis was performed by one-way ANOVA (**$P<0.001$) with Bonferroni's multiple comparisons.
Fig. 1

Transactivating DNA-binding Ligand-binding

NH2 1 2 3 4 5 6 7 8 9a 9b COOH

CT T
G A
A T C
CT T
A A C
A G T
A A C
T A A
A C A
A T T
A C T
A A C
A G T
N FokI

N
FokI

A

C

T2wt T2GR-Ex2

ND D ND D

150

300

D1 D2 D3

TALEN

GR

ACTIN

WILD-TYPE

Δ-4

Δ-7

Δ-18

Count

Location

TATTCACTGATGGACTCCAAAGAATCATTAACTCCTGGTAGAGAAGAAA

TATTCACTGATGGACTCCAAAG----ATTAACTCCTGGTAGAGAAGAAA

Δ-4

TATTCACTGATGGACTCCAA------ATTAACTCCTGGTAGAGAAGAAA

Δ-7

TATTCACTGATGG-----------------ACTCCTGGTAGAGAAGAAA

Δ-18

60%

+DEX

T2wt d1 d2 d3

D10

TALEN

GR

ACTIN

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Fig. 2

A

Propidium iodide

Annexin V

B

Absorbance A570 nm

Days

C

CD45

CD4

1 cm

T2wt

T2wt DEX

T2GR-Ex2

T2GR-Ex2 DEX

D

Number of Hu CD4+ cells/spleen

0

4 x 10^6

8 x 10^6

1.2 x 10^7

1.6 x 10^7

T2wt

T2wt DEX

T2GR-Ex2

T2GR-Ex2 DEX
Fig. 3

A

Unselected

Selected

CD8

HLA-A02-NLV

0.041

96.1

24

0.20

94.3

3.64

1.23

4.25

B

Non electroprated

Electroprated

FSC-A

Viability dye

GFP

62.8

0.11

98.4

C

Counts

Deletion size

55%

D

CMVGR-Ex2

+DEX

CMVwt

CMVGR-Ex2

TALEN

GR

ACTIN

E

Indels %

Left sequence

Right sequence

TATGACACAGATGGCT...CTGTTATAGAAGAAA
TTTCTCTATTATACCTG...TTCTATTATTTAATA
TCTTCAATAATACAAA...GGCCATCATTGCATA
TATCTGCAAAACAAG...CTTGTTGAGAAGAAA
TATTCACAAAAGCCA...GTCCATCAGTGGATA
TATACAAGGATAGAC...CTGGTAGATAAGTCA
TTTCTTCTCAACAAC...TTTTCTCAGTGTATA
5'-TTTCTTCTCTACCAG...GTCCATCAGTGAATA-3'
3'-AAAGAAGAGATGGTC...CAGGTAGTCACTTAT-5'

Control

Stained

0.041

0.24

0.20

0.24

1.23

4.25

25

200

400

600

62.8

98.4
Fig. 5

A

| CD8<sup>wt</sup> | CD8<sup>wt</sup> DEX | CD8<sup>GR-Ex2</sup> | CD8<sup>GR-Ex2</sup> DEX |

B

![Bar graph showing the number of CD8<sup>+</sup> GZB<sup>+</sup> cells in the spleen for different groups.](chart)

C

![Western blot analysis showing GR and ACTIN expression.](blot)
TALEN-mediated genetic inactivation of the glucocorticoid receptor in cytomegalovirus-specific T cells

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