Towards gene therapy for EBV-associated Post-Transplant Lymphoproliferative disease: genetically modified EBV-specific Cytotoxic T Lymphocytes induce regression of autologous EBV-induced Lymphoproliferation despite immunosuppression.

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Running head:

Genetically modified EBV-specific CTLs induce regression of EBV-lymphoma despite immunosuppression.

Key Points:

- Immunotherapy with EBV-specific Cytotoxic T lymphocytes genetically engineered to be resistant to calcineurin inhibitors can mediate durable, potent anti-tumour responses despite the presence of ongoing immunosuppression in a murine model of PTLD.
- This approach may improve the efficacy of immunotherapy with EBV-CTL for PTLD arising after solid organ transplant and obviate the need for withdrawal of immunosuppression, thus reducing the risk of graft rejection.

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ABSTRACT

EBV associated post-transplant lymphoma (PTLD) is a major cause of morbidity/mortality after hematopoietic stem cell (SCT) or solid organ (SOT) transplant. Adoptive immunotherapy with EBV-specific cytotoxic lymphocytes (CTLs), whilst effective in SCT, is less successful after SOT where life-long immunosuppression therapy is necessary. We have genetically engineered EBV-CTLs to render them resistant to calcineurin inhibitor FK506 through retroviral transfer of a calcineurin A mutant (CNA12). Here we examined the ability of FK506-resistant EBV-CTLs to control EBV-driven tumour progression in the presence of immunosuppression *in vivo* in a xenogeneic mouse model. NOD/SCID/IL2rγ^{null} mice bearing human B cell lymphoma were injected with autologous CTLs transduced either with CNA12 or eGFP in the presence/absence of FK506. Adoptive transfer of autologous CNA12-CTLs induced dramatic lymphoma regression despite the presence of FK506 whereas eGFP transduced CTLs did not. CNA12-CTLs persisted longer, homed to the tumour and expanded more than eGFP-CTLs in mice treated with FK506. Mice receiving CNA12-CTLs and treated with FK506 survived significantly longer than control treated animals. Our results demonstrate that CNA12-CTL induce regression of EBV-associated tumours *in vivo* despite ongoing immunosuppression. Clinical application of this novel approach may enhance the efficacy of adoptive transfer of EBV-CTL in SOT patients developing PTLD without the need for reduction in immunosuppressive therapy.

INTRODUCTION

Epstein-Barr virus (EBV) is a human γ-herpes virus infecting and establishing latency in B-lymphocytes in more than 90% of adults. In healthy individuals EBV-specific Cytotoxic T Lymphocytes (CTL) prevent the outgrowth of the EBV-transformed B cells[1] . In hematopoietic stem cell (SCT) or solid organ transplantation (SOT) recipients, this T-cell immune surveillance is compromised by the immunosuppressive medication used to prevent GVHD/graft rejection. This can enable uncontrolled proliferation and malignant transformation of EBV-infected B-cells, resulting in post-transplant lymphoproliferative diseases (PTLD). The prevalence of this complication in SOT can vary from 1-30%, depending on the organ transplanted, patient age and the intensity of immunosuppression[2].

Therapies targeting EBV-infected B cells with monoclonal anti-CD20 antibodies (Rituximab), reduction of immunosuppressive drugs and chemotherapy are currently used[3,4] but are often ineffective and have substantial toxicity. Rituximab as monotherapy is associated with high rate of disease progression and relapse[5]; reduction of immunosuppression frequently results in graft rejection[6], and while chemotherapy results in better response rates, treatment related mortality is high in this patient population[7]. In the PTLD-1 trial[8] combining Rituximab with CHOP chemotherapy, 3 year progression-free survival was 54%. Thus novel therapies are clearly needed.

Adoptive transfer of *ex vivo*-derived EBV-specific cytotoxic T-cells (EBV-CTL) to reconstitute immunity to EBV[9-12] is a logical approach to the treatment of PTLD. However, the application of this approach for the treatment of PTLD in SOT patients, although feasible[10,13,14], has been challenging. This difference is likely to reflect the need for the ongoing immunosuppression to prevent graft-rejection post SOT which inhibits virus-specific T cell

responses[15,16]. While it is generally possible to withdraw other immunosuppressive medication (eg MMF) to facilitate CTL function in SOT recipients developing PTLD, reduction in calcineurin inhibitors, the most critical immunosuppressive drugs used after SOT, frequently results in graft rejection. Indeed, in a major study, graft rejection was as common a cause of mortality in PTLD patients as the disease itself[6].

To address this problem, we have previously developed a strategy for genetically engineering EBV-CTLs to be resistant to the calcineurin inhibitors, Cyclosporin A (CsA) and Tacrolimus (FK506)[17]. These drugs exert their immunosuppressive function by binding to Cyclophilin (CyPA) and FK binding protein-12 (FKBP-12), respectively. These complexes inhibit the calcium-sensitive phosphatase calcineurin from binding to the transcription factor Nuclear Factor of Activated T-cells (NFAT), thus preventing activation of cytokine genes in T cells. To enable CTL to function in the presence of immunosuppression, EBV-CTLs have been genetically engineered to express CN mutations which inhibit docking of either or both FK506/FKBP12 and CsA/CyPA complexes, but do not affect the active site. The mutant used in our current experiments, CNA12 has 2 mutations T351E and L354A which disrupt the binding between CNA and the charged surface residues H87-P88 of FKBP12 to the CN heterodimer but do not affect NFAT dephosphorylation.

EBV-CTLs expressing such mutants maintain their ability to proliferate and secrete IFN-γ in response to stimulation with EBV *in vitro* in the presence of therapeutic levels of FK506 and/or CsA[17].

To evaluate the functionality of calcineurin resistant EBV-CTLs and determine whether they can mediate rejection of EBV^+ B-lymphoma in the presence of immunosuppression with calcineurin inhibitors *in vivo*, in the current study we have assessed the efficacy of FK506

resistant EBV-CTLs in a xenograft tumor model in the immunodeficient mouse strain NOD/SCID/IL2rγ^{null} (NSG). NSG mice were engrafted with human EBV lymphoblastic cells (LCL) and then treated with autologous EBV-CTLs transduced with the calcineurin mutant CNA12 or control eGFP in the presence or absence of FK506. We demonstrate that EBV-CTL resistant to calcineurin inhibitors can mediate curative anti-tumour responses despite the presence of ongoing immunosuppression.

MATERIALS AND METHODS

Generation of LCLs and EBV CTLs

Peripheral blood was taken from healthy EBV-seropositive volunteers after informed consent and PBMCs were isolated by Ficoll centrifugation (GE Healthcare, Amersham, UK). EBV-LCLs were generated by infection of PBMCs with B95.8 supernatant as previously described[15]. EBV-CTLs were generated by repetitive stimulation of PBMC with autologous irradiated LCL as previously published[15].

Generation of retrovirus and transduction

High-titre stable SFG retroviral supernatants carrying either the eGFP transgene alone or expressed with the CNA12 mutant pseudotyped with the RD114 envelope were produced as described previously[17] and were used to transduce EBV-CTL. LCLs were transduced with an SFG retroviral vector encoding Firefly Luciferase (FLuc-eBFP) to facilitate imaging.

Phenotypical and functional analysis of transduced CTLs.

CNA12eGFP or eGFP-EBV-CTL were assessed for phenotypic analysis by flow cytometry and for functionality using ^{51}Cr release, ^{3}H proliferation and IFN-γ ELISA assays in the presence/absence of 10ng/ml FK506 as detailed in Supplementary Materials and Methods.

Xenograft model and bioluminescence imaging.

NSG mice were obtained from Jackson Laboratory and raised under specific pathogen-free conditions. Mice were inoculated with 5×10^{6} EBV-LCLs engineered to express firefly Luciferase (F-Luc) subcutaneously on the nape of the neck on day 0 (4 mice/cohort in 2 separate

experiments). Expression of F-Luc allows non-invasive monitoring of xenografted tumours in mice by luminescent imaging *in vivo*. To assess antitumor activity of control and genetically modified human EBV-CTLs in the presence of FK506 *in vivo*, mice received 5×10^6 autologous EBV-CTLs intravenously (iv) via the tail vein after 7 days. In addition, mice also received intra-peritoneal (ip) injections of IL-2 (Proleukin; Chiron, Ratingen, Germany), 2500U, daily for 7 days post EBV-CTL. 10 mg/kg body weight of FK506 (Prograf, Astellas Pharma, US) was administered ip 5 days a week from 3-4 days after LCL injection. Pilot dose titration experiments in which trough FK506 concentration levels in the whole blood were measured daily by mass spectrometry after varying doses of FK506 demonstrated that a dose of 10mg/kg reliably resulted in blood levels within the human therapeutic range of 10-20ng/ml.

Tumor growth was evaluated using the IVIS imaging system (Xenogen; Caliper Life Sciences). Animals were imaged prior to T cell transfer and twice a week thereafter. Photon emission from $FLuc^+$LCLs expressed in photon/sec/cm^2/steradian (p/s/cm^2/sr) was quantified using the Living Image software (Xenogen) as previously described[18]. In addition to monitoring with bioluminescence imaging, the size of LCL tumors was also assessed twice weekly with caliper measurement. Tumours were resected immediately after euthanasia for immunofluorescence staining.

Immunofluorescence.

Double-immunofluorescence was performed on formalin-fixed, paraffin-embedded tumour sections. Staining was carried out for B and T cells using a 1:100 dilution of primary rabbit monoclonal antibody specific for human CD3 and 1:20 dilution of primary mouse monoclonal antibody for human CD20 (Abcam, Cambridge, UK). This was followed by

incubation with secondary antibodies, FITC anti-rabbit IgG and TRITC anti mouse IgG, respectively. Sections were viewed with epifluorescence using a Leica DM400B microscope and digitally captured using Metamorph software (Molecular Device, USA).

Statistical analysis

Mean ± SEM is depicted in all graphs. Statistical significance was determined by 2-way ANOVA followed by Bonferroni post-test for longitudinal data. Two-tailed Student's *t*-test for unpaired samples was applied to evaluate persistence of transferred T cells, CNA12 mutant expression, proliferation, cytokine secretion, and specific cytolysis at single time points. Survival curves were plotted according to the Kaplan–Meier method, and treatment groups compared using log-rank (Mantel-Cox) test analyses. Differences were considered significant for p values <0.05.

RESULTS

Characterisation of EBV-CTLs before infusion.

To investigate the ability of EBV-CTL genetically engineered to be resistant to calcineurin inhibitors to function *in vivo* despite on-going immunosuppression, we first transduced human EBV-CTLs with retroviral vectors encoding calcineurin mutant CNA12 or eGFP control. CTL transduction efficiency, as assessed by flow cytometry before infusion, was consistently high (mean 77.05%±0.6 for CNA12 and mean 89.5%±0.9 for eGFP). Before adoptive transfer of the EBV-CTLs into the mice we characterised the phenotype of transduced CTL. All CTL expressed CD3, the majority of them (97.7% ±0.1%; mean ± SD) were $CD8^+$, whereas 2%±0.1% expressed CD4 (**Figure 1a**). Because differentiation status can influence their expansion potential and persistence of the adoptively transferred T cells, we also examined memory subsets. We found that the majority of $CD8^+$ T cells (mean 88.7%±0.7%) showed an effector memory phenotype ($CCR7^-$ $CD45RA^-$) as shown in **Figure 1b**. Only 2-3% of the CTL were naïve ($CCR7^+CD45RA^+$), 5.3-5.7% were $CCR7^+CD45RA^-$ central memory T cells, and 2-2.5% were terminally differentiated effector cells ($CCR7^-CD45RA^+$). Transduction of EBV-CTLs either with eGFP control vector or CNA12 did not change the phenotype compared to untransduced CTL.

To assess the ability of CNA12 transduced CTLs to function in the presence of calcineurin inhibitors *in vitro* prior to injection, we investigated both their ability to proliferate and to secrete IFN-γ in response to antigenic stimulation with autologous B-LCLs (**Figure 1c and d**) in the presence of therapeutic doses (10ng/ml) of FK506. As previously described[17], CNA12 transduced CTL were able to proliferate and secrete IFN-γ *in vitro* in the presence of FK506 at comparable levels to in its absence. On the other hand, FK506 completely inhibited

proliferation ($p<0.0001$) and secretion of IFN-γ ($p<0.001$) of eGFP transduced CTLs. In cytotoxicity assays, as shown in **Figure 1e and f** respectively, both CNA12 and eGFP transduced CTLs showed equivalent specific cytotoxicity against autologous EBV-LCL targets (CNA12 transduced CTLs 25.3±1.8% lysis, eGFP transduced CTLs 22.9±1.0% lysis at effector-target ratio of 30:1, $n=2$). Cytotoxicity was MHC restricted as no lysis of allogeneic LCLs was observed and not NK mediated as significant cytotoxicity of the HSB2 cell line was not seen. Culture of either CNA12 or eGFP CTLs with FK506 before or during the cytotoxicity assay had no effect on the cytotoxicity of EBV-CTL against LCL targets (data not shown).

Taken together, these data show that the CNA12 transduced CTL infused in murine experiments were able to secrete cytokines/proliferate in response to EBV and lyse EBV infected targets *in vitro* despite the presence of FK506.

EBV-CTLs transduced with CN mutant lack alloreactivity.

If Tacrolimus resistant CTL are to be used clinically after organ transplant, it is critical that they are depleted of alloreactivity in order to avoid toxicity to the organ graft. We therefore assayed the proliferative responses of unselected and GFP^+ sorted CNA12-CTL to HLA-mismatched allogeneic PBMC stimulators, comparing this with the alloreactivity of unmanipulated PBMC from the same donor. As shown in **Figure 2**, unmanipulated donor PBMCs proliferated strongly in response to stimulation with irradiated, allogeneic PBMC ($p<0.001$). In contrast, the response of both unselected and GFP^+sorted CNA12-CTL to allogeneic PBMCs was negligible, indicating that the process of EBV-CTL generation, expansion and transduction with CNA12 depletes alloreactivity.

Adoptive transfer of autologous Calcineurin resistant EBV-CTLs induced rejection of EBV-lymphoma in xenografted NSG mice in the presence of FK-506.

To determine whether CNA12 transduced CTL could mediate rejection of EBV^+ lymphoma *in vivo* despite the presence of immunosuppression, we adoptively transferred CNA12 or eGFP CTL into NSG mice bearing autologous $EBV^+F\text{-}Luc^+$ LCL tumours in the presence or absence of FK506 (Experimental schema shown in **Figure 3**).

In vivo imaging showed tumour growth in the control mice treated with B-LCL within 20 days, requiring mice to be sacrificed **(Figures 4a and 4b)**. Mice treated with eGFP-CTL or CNA12-CTLs without FK506 showed reduced tumour growth: mean bioluminescence values were $1.2\pm0.3X10^5$ p/s/cm^2/sr (n=8) and $1.1\pm0.4X10^5$ p/s/cm^2/sr (n=8), respectively at day 40 compared with bioluminescence values of $6.7X10^7\pm6.6X10^6$ (n=8) in untreated control mice, (p<0.0001). Mice infused with resistant CNA12-CTLs showed tumour clearance from day 20 post EBV-CTL injection despite FK506 treatment with bioluminescence values reducing from a mean of $2.6\pm0.3X10^6$ p/s/cm^2/sr (n=7) at day 0 to $1.3\pm0.3X10^5$ by day 40 (p<0.05). In contrast, mice treated with control eGFP-CTLs in the presence of FK506 showed a progressive increase in tumour bioluminescence (mean $1.7X10^6\pm0.2$ p/s/cm^2/sr, at day 0 to $2\pm0.7X10^7$ p/s/cm^2/sr by day 40 (n=7) (p<0.01) (**Figure 4b).** Calcineurin inhibition *per se* led to minor tumour regression, as previously observed in another model, consistent with the ability of these drugs to induce apoptosis of human lymphoma and leukemic cells[19].

Tumour growth was also assessed by measuring the volume of the s.c. palpable tumour using calipers. In absence of EBV-specific T cell surveillance, challenge of NSG mice with EBV-LCLs induced the development of large tumors (mean volume 579.7 mm^3 ± 2.9 SD, n=8) at day 20. Mirroring our IVIS findings, adoptive transfer of either CNA12 or eGFP CTL led to

complete tumour regression in the absence of FK506. Similarly, we did not observe tumor growth in the mice treated with CNA12-CTLs in the presence of FK506 (mean volume 0 mm^3 at day 40, *n*=7). In contrast, mice infused with control GFP-CTLs in the presence of FK506 showed progressive increase in tumor volume from day 15 post CTLs infusion (mean volume 132.6 mm^3± 43.8 SD at day 20) (p<0.001) (*n*=8) (**Figure 4a**).

In order to make our approach more feasible for clinical application, we have developed a simpler, more rapid methodology for generation of tacrolimus-resistant EBV-specific T cells using IFN-γ selection after stimulation with immunodominant EBV peptides followed by transduction with a retroviral vector encoding the CNA12 mutant[20]. As shown in **Supplementary Figure 1**, we observed that adoptive transfer of FK506 resistant EBV-CTLs generated with this methodology also led to complete tumour rejection of B-cell lymphoma in NSG mice treated with CNA12CTLs in the presence of FK506, comparable to that seen with EBV CTL generated using conventional repetitive LCL stimulation.

Consistent with the control of tumor progression, injection of CNA12-CTLs in the presence of FK506 resulted in enhanced survival of these mice by day 40 post CTLs injection (P<0.0001) compared with mice receiving eGFP-CTLs with ongoing immunosuppression (**Figure 5b**). Similar results were observed in mice treated with CNA12 CTLs generated using IFN-γ selection which were able to survive beyond 60 days after CTLs infusion (data not shown).

Taken together, our results show that transfer of CNA12-CTLs into xenografted hosts was sufficient to induce complete regression of tumours despite on-going immunosuppression, resulting in prolonged lymphoma-free survival. In contrast, in mice treated with control eGFP-

CTL, the immunosuppressive environment compromised the function of the adoptive transferred CTLs leading to tumour progression.

Calcineurin resistant EBV CTLs show improved persistence and expansion.

One of the major barriers to the development of effective adoptive T cell therapy has been the inability of the transferred T cells to achieve prolonged persistence after infusion, required to mediate therapeutic efficacy and for long term tumour surveillance. In SOT patients, the immunosuppressed environment required to prevent graft rejection further limits the expansion and persistence of adoptive transferred T cells. We therefore examined whether the adoptively transferred CNA12-CTLs persisted *in vivo* despite the presence of FK506. To assess *in vivo* persistence, tail vein blood was collected from the mice the day of sacrifice (40 days after CTL infusion) and quantified the percentage human $CD3^+$ cells. We observed that the adoptively transferred EBV-CTLs showed peripheral expansion and persistence. The level of human T cell engraftment at 40 after infusion was reproducibly higher in mice treated with FK506 which received CNA12-CTLs (35.2±2.9%, mean ± SEM, n=7) than mice receiving eGFP-CTLs and FK506 (3.9±1.9%, n=7) (p<0.0001), as shown in **Figure 6a**, correlating with the improved anti-tumour responses seen with CNA12 transduced CTL. Further, mice receiving CNA12-CTLs showed improved expansion in the presence of FK506 (35.2±2.9%, n=7) than in its absence (16.2±4.3%, n=8) (p<0.01) whereas proliferation of GFP transduced CTL was inhibited by FK506 resulting in a reduced percentage of circulating human T cells. These results demonstrate that CNA12 transduced CTLs have a growth advantage in the presence of FK506 *in vivo* compared to eGFP-CTLs, resulting in their preferential expansion in circulation.

Next we looked at the percentage of circulating human T cells expressing eGFP to determine the fraction of persisting adoptive transferred EBV-CTLs expressing the transgene (**Figure 6b**). We observed that the transgene expression in the circulation 40 days after infusion was significantly higher (48.0±2%, *n*=7) in CNA12-CTLs from mice treated with FK506 compared with mice infused with CNA12-CTLs without FK506 (24.2±11.6%, *n*=8) ($p<0.001$). These data indicate a selective enrichment *in vivo* of CNA12 transduced CTL in the presence of FK506. In contrast, in mice receiving eGFP-CTL, there was no selective advantage in the presence of FK506, so that the percentage of human T cells expressing eGFP was stable with (89.4± 4.1%, *n*=8) or without (81.8±3.5, *n*=7) FK506, although the total number of circulating human T cells was reduced by FK506. The higher percentage of human T cells expressing GFP in mice receiving eGFP-CTLs compared to CNA12-CTL reflects differential transduction efficiency.

Similar results were observed in mice treated with CNA12 CTLs generated using IFN-γ selection which were able to persist *in vivo* beyond 60 days after adoptive transfer in the presence of FK506 (**Supplementary Figure 2a and b**).

These data suggest that EBV-CTL genetically engineered to express CNA12 showed improved expansion, growth advantage and are able to persist *in vivo* in the presence of FK506 beyond 2 months post adoptive transfer.

Calcineurin resistant EBV CTLs infiltrate the tumour in the presence of FK506

We next investigated the ability of transduced EBV-CTL to home to LCL tumours *in vivo* using immunofluorescence analysis 40 days after CTL administration. In the absence of FK506, both CNA12 and eGFP transduced CTL homed well to the tumour, mediating regression (**Figure**

7a). Consistent with the increased persistence of CNA12-CTLs in the peripheral blood in the presence of FK506, we observed robust accumulation and infiltration of tumour infiltrating T lymphocytes (human $CD3^+$) in regressed B-cell lymphoma, whereas very few human $CD3^+$ T cells were detected in tumours resected at the same time from mice that received eGFP-CTL cells.

Calcineurin resistant EBV-CTLs do not infiltrate and do not induce toxicity in organs.

Histological examination showed no human T-cell infiltration or histological evidence of toxicity in brain, lungs, heart, kidneys, intestine and liver of the mice sacrificed 40 days after injection of CNA12-CTL (**Figure 7b**), demonstrating that FK506 resistant CTL do not mediate off target organ toxicity in this model.

DISCUSSION

Adoptive immunotherapy with T-cell directed against tumor- specific or tumor-associated antigens represents a novel strategy for the treatment of human malignancies with a great clinical potential[21-25]. Adoptive transfer of *ex-vivo* derived EBV specific CTLs to reconstitute EBV-specific T cell immunity[3,26] is a logical approach for patients developing PTLD. This disease represents an ideal target for adoptive T-cell therapy because of the high level expression of immunogenic antigens from EBV presented by excellent antigen-presenting cells. The application of this approach has been shown to be extremely effective for PTLD arising after SCT, where immunosuppressive therapy can be reduced or suspended completely before EBV-CTLs administration[11,27,28]. In contrast in the SOT setting, although possible[10,13,14], the approach has been limited by the need for life-long immunosuppressive therapy to avoid rejection of the transplanted organ which inhibits the proliferation, expansion and persistence of transferred EBV-CTL[15,16]. For example in the study of Savoldo *et al*[10], where autologous EBV CTL were infused in 12 SOT patients with EBV viremia only 1 patient cleared viremia, the *in vivo* expansion of infused EBV-CTL was poor (1.5-4.8 fold) and the frequency of circulating EBV-CTL returned to pre-infusion levels in all patients within 2 months of infusion.

To overcome this problem, we have previously generated EBV-specific CTLs which are resistant to the Calcineurin inhibitors Tacrolimus and Ciclosporin and have demonstrated their ability to function *in vitro* despite the presence of immunosuppression[17].

In order to translate novel T-cell therapy approaches from bench to bedside, *in vivo* modelling is critical. Here we have studied the anti-tumour effects of human CNA12 transduced EBV-CTL against established EBV-lymphoma in an immunodeficient NSG xenotransplant mouse model in the presence of immunosuppression. Previous studies *in vivo* have demonstrated

that inoculation of EBV-transformed B cell lines rapidly induces human EBV-induced B lymphoproliferation with characteristics comparable to those observed in immunocompromised patients developing PTLD[29,30]. In this study, we have demonstrated that adoptive transfer of human EBV-specific CTL resistant to Tacrolimus in NSG mice results in eradication of the lymphoma *in vivo* even in the presence of immunosuppression with therapeutic levels of FK506. Our data show that FK506 resistant EBV-CTLs are able to home to the tumour site and persist in the circulation beyond 60 days after transfer, resulting in markedly improved survival of mice despite ongoing immunosuppressive treatment. We demonstrate that EBV-CTLs transduced with CNA12 are selectively enriched *in vivo* by immunosuppression with FK506, resulting in a growth advantage over untransduced EBV-CTL. The potent anti-tumour effects of CNA12 transduced EBV-CTL were durable even in the face of immunosuppression and no off-target toxicity was observed. This approach may thus overcome the major barrier to application of chemotherapy with EBV-CTL in the SOT setting.

An alternative strategy to rendering CTL resistant to FK506 using small interference RNA (siRNA) knockdown of native FKBP12 has been proposed by De Angelis *et al*[31]. However, this methodology results in only partial resistance to FK506 *in vitro*. Similarly *in vivo*, mice receiving EBV CTL in which FKBP12 had been knocked down by siRNA, while showing delayed tumour progression, still had residual tumor detectable whereas in our study CNA12 transduced EBV-CTL resulted in complete tumor clearance even in the presence of FK506. This may reflect the fact that siRNA knockdown is relatively inefficient and very high transgene expression is needed. Moreover, there is little experience of siRNA knockdown clinically, down-regulating endogenous FKBP12 may have unpredictable sequelae on T-cell function and introduction of siRNA can potentially result in off-target suppression of other genes. Thus we

believe our approach is both more effective in fully restoring CTL function in the presence of FK506 and more clinically feasible.

Reduction in immunosuppression is commonly used in SOT patients developing PTLD patients but this frequently results in graft rejection: indeed in the study reported by Webber *et al* rejection was as common a cause of mortality in PTLD patients as disease itself[6]. .

Obviating the need for reduction in immunosuppression thus represents a major advance. Following SOT, post-transplant immunosuppression with a variety of drugs including FK506, low dose steroids and mycophenolate mofetil (MMF) is used. Clearly, our strategy only confers resistance to calcineurin inhibitors. However, these are the most critical component of post-SOT immunosuppression: MMF and steroids can generally be withdrawn without toxicity in the context of PTLD but when FK506 is reduced or withdrawn, rejection is common. Therefore our strategy overcomes the key issue of graft rejection by enabling clinicians to treat PTLD with EBV-CTL whilst maintaining immunosuppression with FK506 at therapeutic levels.

One of the major barriers to broader the application of this approach is also the complexity of generation of EBV-CTL. We have recently simplified the generation of resistant CTL by retroviral transduction of EBV-specific T-cells selected on the basis of secretion of IFN-γ in response to EBV immunodominant antigens[20]. Further, we have shown that it is possible to generate FK506 resistant CTL from SOT patients on immunosuppression who develop PTLD[20]. Currently we are in process of validating production of FK506 resistant EBV-CTL using this methodology under GMP conditions. We envision resistant CTL could be used as adjuvant therapy following Rituximab to improve response rates and prevent rejection without the need for chemotherapy which is poorly tolerated in SOT patients. Importantly, such an approach would obviate the need for reduction in immunosuppression, reducing the risk of graft rejection

which is a major cause of mortality in PTLD patients. Given that the cost of generating transduced EBV-CTL is approximately $15,000; such an approach could potentially have major health economic benefits by avoiding the need for prolonged admission for chemotherapy and reducing the risk of graft rejection. In terms of toxicity, we have shown that CNA12 transduced EBV-CTL have negligible alloreactivity against HLA mismatched targets, suggesting they should have no detrimental effect on the organ graft. This is likely to reflect depletion of alloreactive T cells during the process of CTL culture and our data suggest that selection of retrovirally transduced CTL is not necessary to abrogate alloreactivity. Indeed, no graft toxicity was observed in previous clinical studies with either autologous or 3rd party CTL[12,32]. Since CTL are autologously derived, they should not cause off target toxicity to the host and, consistent with this, in our experiments no organ toxicity was observed in mice receiving FK506 resistant EBV-CTL. Importantly, since transduced EBV-CTLs are terminally differentiated, retroviral gene transfer is extremely unlikely to result in leukaemogenesis. As a safety strategy, in our model we have genetically modified EBV-CTLs with a CN mutant conferring resistance to Tacrolimus only, allowing suppression of the infused T cells with Cyclosporin in case of toxicity.

Overall, our findings provide strong support for the clinical application of calcineurin inhibitor resistant EBV-CTL as adjuvant therapy for SOT patients developing PTLD. Based on this work, we are now proceeding to a "double marking" clinical study in which such patients will be treated with both autologous CNA12 transduced and control EBV-CTL generated using our simplified methodology[20] without reduction in FK506 as adjuvant therapy following Rituximab. The *in vivo* persistence/expansion of EBV CTL transduced with the 2 vectors will be compared. We hypothesize that CNA12 transduced CTL will show preferential expansion and prolonged persistence compared with control CTL because of ongoing FK506 therapy. The

incidence of graft rejection and PTLD relapse will be compared to historical data on patients receiving Rituximab alone. This study will determine if this strategy improves *the in vivo* persistence and efficacy of adoptively transferred EBV-CTL. Such an approach could potentially be of major clinical benefit to organ transplant patients developing PTLD by avoiding the need for chemotherapy and reduction in immunosuppression. It remains possible however, that other factors such as the absence of a lymphopenic environment resulting in reduced homeostatic expansion may contribute to the limited persistence and expansion of infused EBV CTL observed in previous studies in SOT patients, in which case addition of lymphodepleting chemotherapy prior to adoptive T cell transfer may be required. More broadly, this represents a generic approach enabling T-cells directed against other pathogens (e.g. Cytomegalovirus, BK and Adenovirus) and tumor antigens to function in patients on immunosuppression.

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Authorship:

Contribution: I.R, P.J.A and M.P designed the work; I.R, M.B and J.B. performed experiments and analysed data; I.R, M.P, and P.J.A wrote the manuscript and all authors read and commented on the paper.

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FIGURE LEGENDS

Figure 1

Immunophenotypic and functional analysis of transduced EBV-specific CTLs before adoptive transfer.

A. CNA12 and eGFP transduced EBV-CTLs were analyzed for T-cell markers expression by flow cytometry. Percentages of $CD3^+$, $CD4^+$ and $CD8^+$ T cells are shown. **B**. Distribution of memory subsets in CNA12 and eGFP transduced EBV-CTLs. Effector memory ($CCR7^-$ $CD45RA^-$), Naïve ($CCR7^+CD45RA^+$), Central memory ($CCR7^+CD45RA^-$), Terminal differentiated (TD) effector ($CCR7^-CD45RA^+$) T cells. **C)** Proliferation ability of EBV-CTLs was evaluated after stimulation in vitro with autologous LCLs with or without therapeutic dose (10ng/ml) of FK506 and was tested by H^3-thymidine uptake for 21h Proliferation of eGFP transduced EBV-CTLs after stimulation with LCLs in the presence of FK506 was significantly inhibited (p<0.0001) compared with CNA12 transduced EBV-CTLs. **D**) EBV-CTLs transduced with CNA12 were able to secrete IFN-γ in the presence of autologous LCLs plus 10ng/ml of FK506 at comparable levels to that seen with CNA12 transduced T cells stimulated with LCLs alone. eGFP transduced EBV-CTLs did not produce IFN-γ in the presence of LCLs plus 10ng/ml FK506 compared with eGFP transduced T cells stimulated with LCLs alone (P<0.001).

A standard ^{51}Cr release cytotoxicity assay was performed to assess cytotoxicity of EBV-CTL lines against autologous, mismatched LCL targets or the T cell line HSB2 in the presence of 10ng/ml of FK506. Cytolytic activity of eGFP (**E**) and CNA12 (**F**) transduced cells at different effector-to-target ratios one week after fifth stimulation. Mean expression ± SEM of two CTL lines are shown.

Figure 2

CNA12 EBV-CTLs lack alloreactivity versus HLA mismatched donors.

Alloreactive potential of unmanipulated PBMCs as well as CNA12 transduced EBV-CTLs from the same donor using a primary mixed lymphocyte reaction (MLR). Unmanipulated PBMCs proliferated significantly ($P<0.001$) in response to allogeneic, irradiated PBMCs compared to the proliferation of CNA12 EBV-CTLs unselected or GFP^+ selected. Data show the mean values and SEM of experiments from 4 donors.

Figure 3

Experimental design of PTLD xenograft mice model.

In order to perform analysis on the Calcineurin resistant EBV-CTLs *in vivo*, we investigated their functionality as adoptive therapy in a NSG xenograft model. Firstly, we started generating human EBV-LCLs by infecting PBMCs from EBV seropositive donor with EBV-producing marmoset B-cell line (B95.8) *in vitro*. Once B-LCL was established, it was used to do repetitive stimulation of autologous PBMCs to generate EBV-CTLs. For *in vivo* purposes we transduced B-LCLs with a retroviral vector encoding firefly luciferase to allow us to monitor tumour development *in vivo*.

Mice were inoculated with 5×10^6 EBV-LCLs subcutaneously on the nape of the neck on day 0 (4 mice/cohort in 2 separate experiments). To assess antitumor activity of control and genetically modified human EBV-CTLs in the presence of FK506 *in vivo*, mice received 5×10^6 autologous EBV-CTLs *iv* via the tail vein after 7 days. In addition, mice also received *ip* injections of 2500 U of IL-2 daily for 7 days post EBV-CTL. 10 mg/kg body weight of FK506 was also

administered *ip* 5 days a week from 3-4 days after LCL injection. Tumor growth was evaluated using the IVIS imaging system. Photon emission from $FLuc^+$ LCLs was quantified using the Living Image software.

Figure 4

Adoptive transfer of Calcineurin resistant EBV-CTLs induces regression of EBV B cell lymphoma *in vivo* in a xenogeneic mouse model despite the on-going immunosuppression

A, To evaluate *in vivo* antitumor activity, light emission by tumour cells was monitored as an indication of tumour growth using the IVIS *in vivo* imaging system in 4 NSG mice per group. Mice with the B-cell lymphoma cells (LCLs) show tumour progression and were sacrificed after 20 days. Mice receiving autologous CNA12 or eGFP-CTLs without FK506 showed a decrease of tumour formation. CNA12-CTLs mediate tumour clearance despite the presence of FK506 as observed by reduction of $F\text{-}Luc^+$ bioluminescence signal. On the contrary, mice receiving eGFP-CTLs showed tumour development in the presence of FK506.

B, Graph shows kinetics of tumour growth. Photon emission from $FLuc^+$ tumor cells was quantified and measured as maximum photon/sec/cm^2/steradian (p/s/cm^2/sr). Figure shows that tumour growth was significantly ($p<0.05$) greater in mice receiving EBV-CTLs expressing eGFP than in mice receiving CNA12-CTLs in presence of FK506. Lines represent cumulative results of light emission values ± SEM bioluminescence signal determined in 2 separate experiments, $n=8$.

Figure 5

Adoptive transfer of Calcineurin resistant EBV-CTLs induces reduction of tumour growth and improves survival of the mice despite the presence of on-going immunosuppression.

A. Tumour volume (mm^3) was decreased in tumour bearing NSG mice adoptively transferred with CNA12-CTL, CNA12-CTL +FK506 and eGFP-CTL compared with the mice receiving eGFP-CTL plus FK506 (*n*=8).

B. Kaplan-Meier tumor-related survival curve showed an increased long term survival of mice adoptively transferred with CNA12-CTLs in the presence of FK506 ($P<0.0001$) compared with mice receiving eGFP-CTLs in the presence of immunosuppression (*n*=7).

Figure 6

EBV-CTL genetically engineered to express CNA12 showed improved expansion and persistence *in vivo* in the presence of FK506.

A.Persistence *in vivo* of genetically engineered human EBV-CTLs transduced with the CNA12 or eGFP. Peripheral tail vein blood was collected from the mice the day of sacrifice (40 days after CTLs infusion) and quantified by flow cytometry for the persistence of human $CD3^+$. Mean cell count ± SEM is shown (*n*=8). **B.** eGFP expression on human $CD3^+$ T cells from peripheral blood of treated mice measured by flow cytometry. Mean $eGFP^+$ expression frequency ± SEM per group is shown (*n*=8).

Figure 7

EBV-CNA12- CTLs infiltrate B-cell lymphoma despite the presence of FK506.

A. Tumours were collected 40 days post CTL infusion and stained by double immune fluorescence for human T cell infiltration (CD3 expression, green) into B-cell lymphoma (CD20, red). Tumours treated with CNA12-CTLs show a substantial T cell infiltration despite the presence of FK506. Representative sections are shown at ×100 magnifications.

B. Calcineurin resistant EBV-CTLs do not infiltrate and do not induce toxicity in organs. Organs were collected 40 days post CTL infusion and stained for human T cell infiltration (CD3 expression). Organs from mice treated with CNA12-CTLs do not show any T cell infiltration in the brain (A), intestine (B), heart (C), kidney (D) lungs (E) or liver (F). Representative sections are shown at ×20 magnifications.

FIGURES

Figure 1

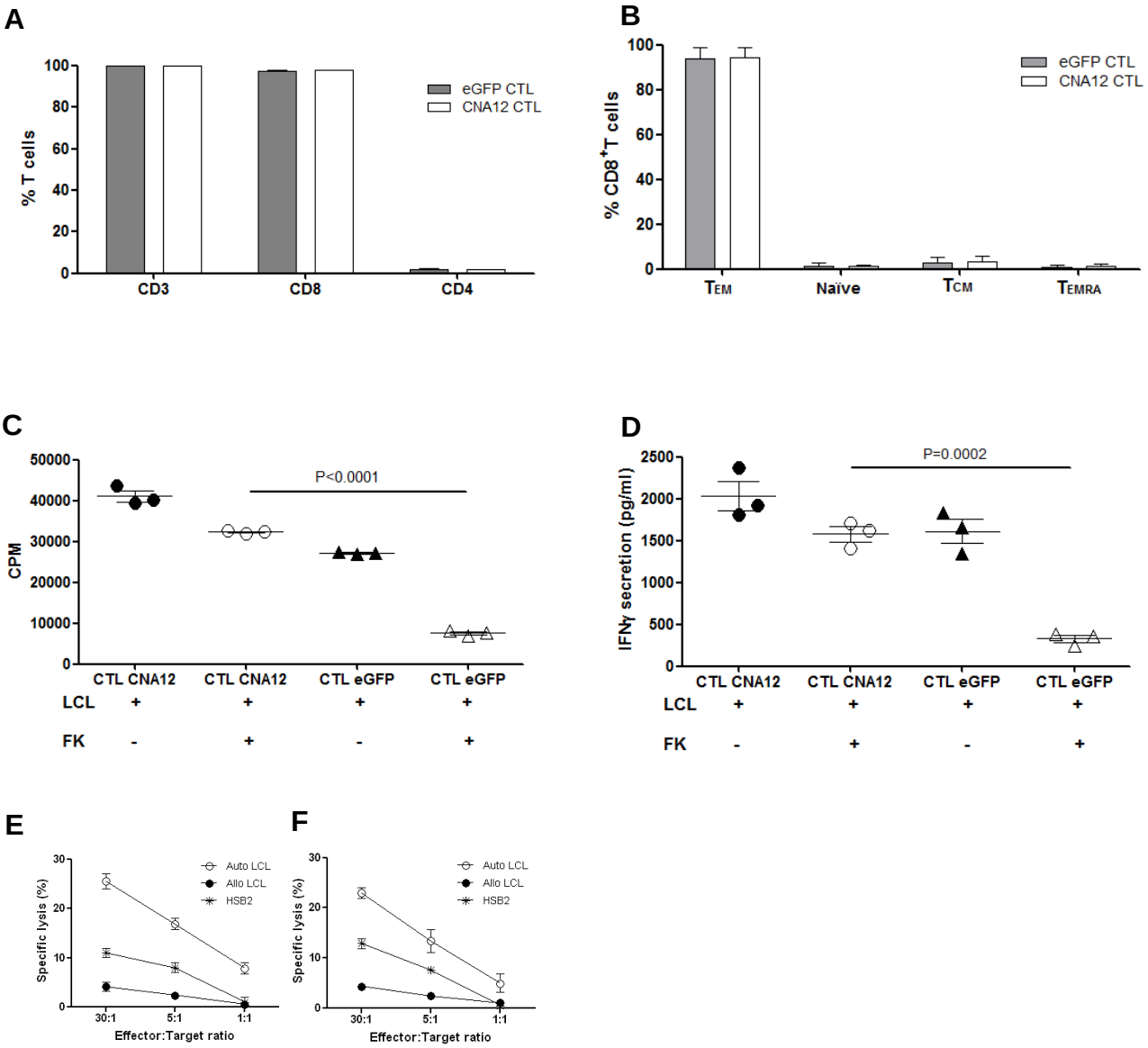


Figure 2

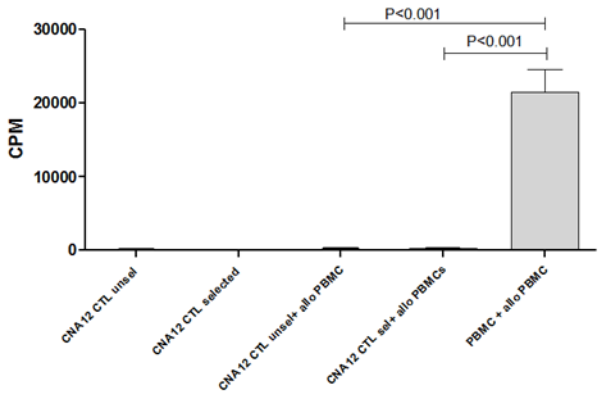

P<0.001
P<0.001
30000
20000
10000
0
CPM
CNA12 CTL unsel
CNA12 CTL selected
CNA12 CTL unsel+ allo PBMC
CNA12 CTL sel+ allo PBMCs
PBMC + allo PBMC

Figure 3

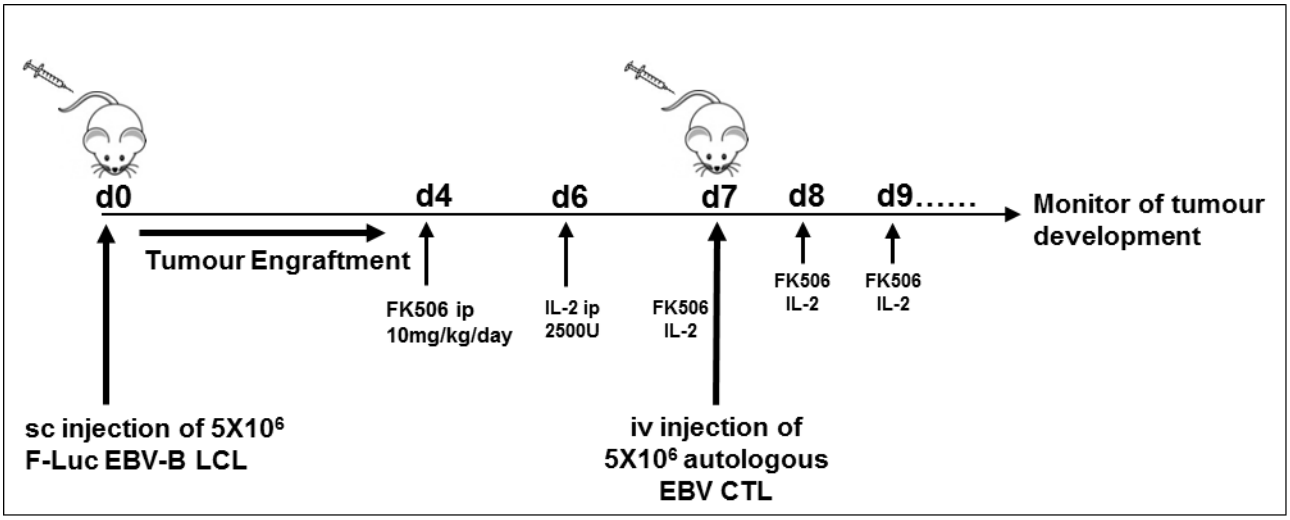

d0
d4
d6
d7
d8
d9……
Monitor of tumour development
Tumour Engraftment
FK506 ip 10mg/kg/day
IL-2 ip 2500U
FK506 IL-2
FK506 IL-2
FK506 IL-2
sc injection of 5X10⁶ F-Luc EBV-B LCL
iv injection of 5X10⁶ autologous EBV CTL

Figure 4

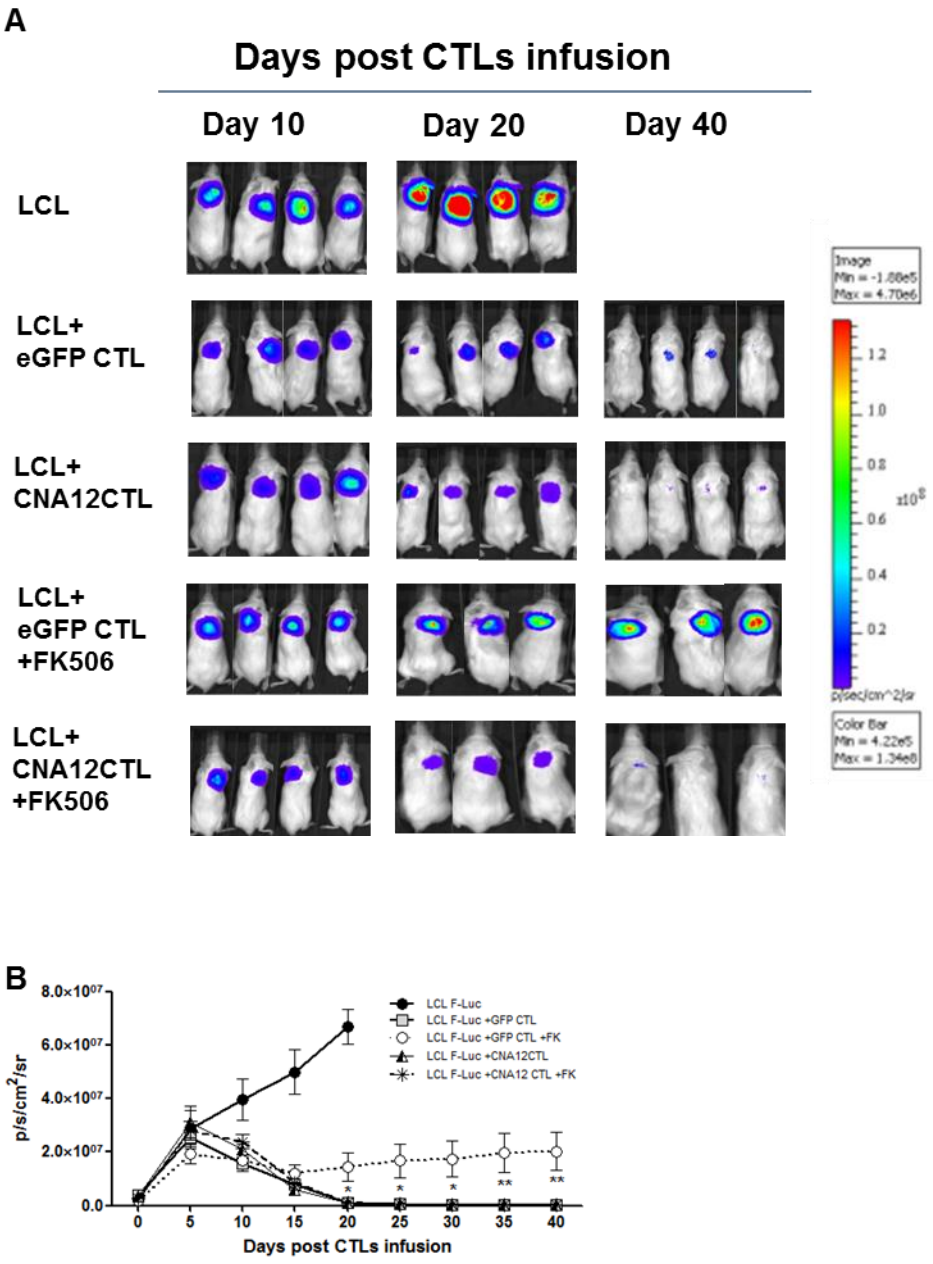

A
Days post CTLs infusion
Day 10
Day 20
Day 40
LCL
LCL+ eGFP CTL
LCL+ CNA12CTL
LCL+ eGFP CTL +FK506
LCL+ CNA12CTL +FK506
Image
Color Bar
p/sec/cm^2/sr
B
LCL F-Luc
LCL F-Luc +GFP CTL
LCL F-Luc +GFP CTL +FK
LCL F-Luc +CNA12CTL
LCL F-Luc +CNA12 CTL +FK
p/s/cm2/sr
Days post CTLs infusion

Figure 5

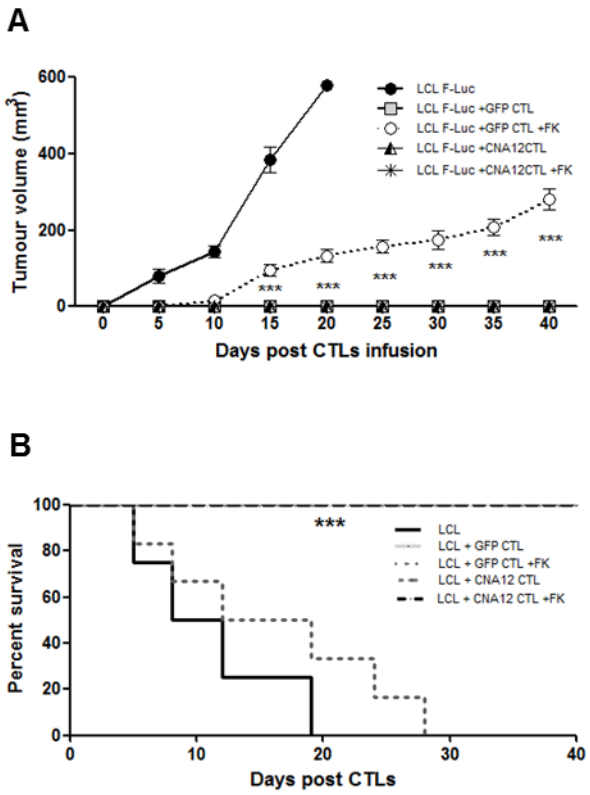

A
Tumour volume (mm3)
600
400
200
0
0
5
10
15
20
25
30
35
40
Days post CTLs infusion
LCL F-Luc
LCL F-Luc +GFP CTL
LCL F-Luc +GFP CTL +FK
LCL F-Luc +CNA12CTL
LCL F-Luc +CNA12CTL +FK
***
B
Percent survival
100
80
60
40
20
0
0
10
20
30
40
Days post CTLs
***
LCL
LCL + GFP CTL
LCL + GFP CTL +FK
LCL + CNA12 CTL
LCL + CNA12 CTL +FK

Figure 6

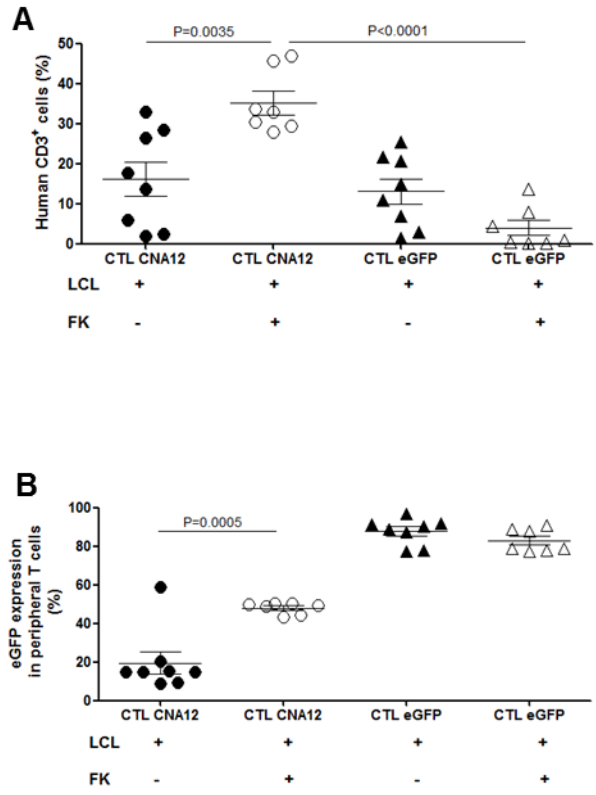
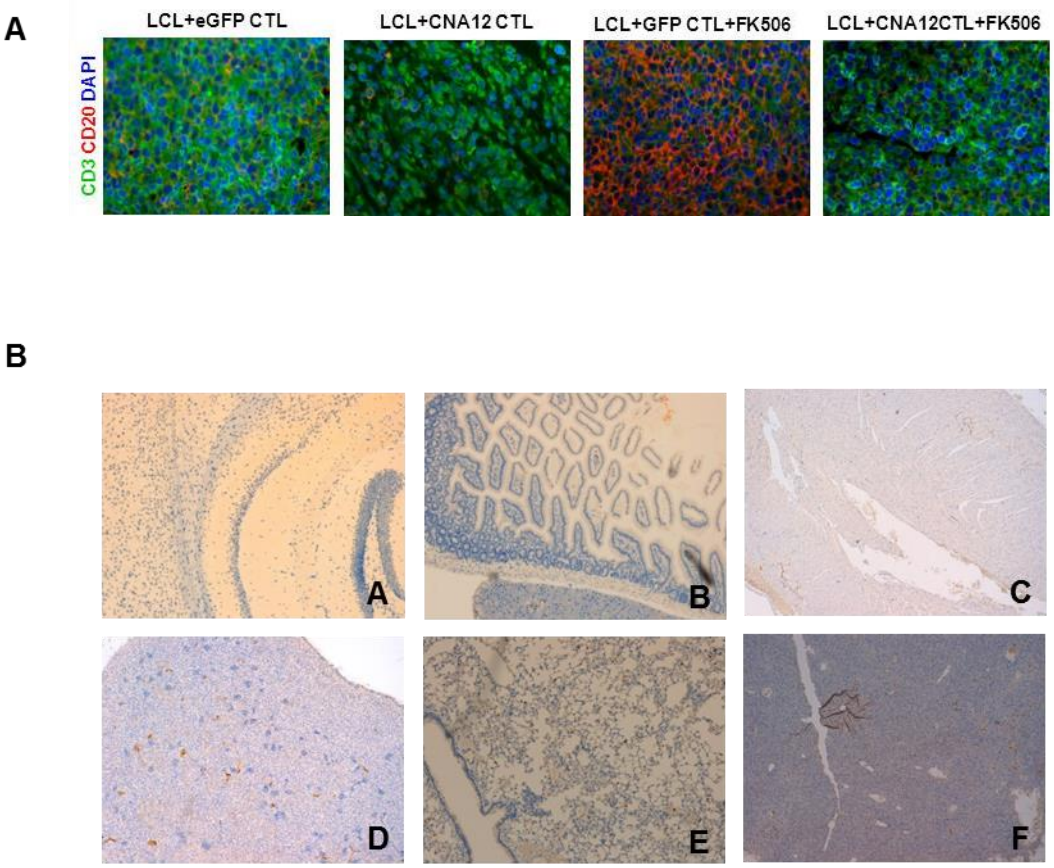

A
P=0.0035
P<0.0001
Human CD3+ cells (%)
50
40
30
20
10
0
CTL CNA12
CTL CNA12
CTL eGFP
CTL eGFP
LCL + + + +
FK - + - +
B
P=0.0005
eGFP expression in peripheral T cells (%)
100
80
60
40
20
0
CTL CNA12
CTL CNA12
CTL eGFP
CTL eGFP
LCL + + + +
FK - + - +

Figure 7


A
LCL+eGFP CTL
LCL+CNA12 CTL
LCL+GFP CTL+FK506
LCL+CNA12CTL+FK506
CD3 CD20 DAPI
B
A
B
C
D
E
F