Brief Communication

Rapid Generation of EBV-Specific Cytotoxic T Lymphocytes Resistant to Calcineurin Inhibitors for Adoptive Immunotherapy

I. Ricciarelli[1,2,*], J. Brewin[1], G. Lugthart[1],
S. J. Albon[1], M. Pule[2] and P. J. Amrolia[1,3]

[1]Molecular Immunology Unit, Institute of Child Health, UCL, London, UK
[2]Department of Haematology, UCL Cancer Institute, London, UK
[3]Department of Bone Marrow Transplantation, Great Ormond Street Hospital for Children NHS Trust, London, UK
*Corresponding author: Ida Ricciarelli, i.ricciardelli@ucl.ac.uk

Epstein–Barr virus (EBV)-associated posttransplant lymphoproliferative disorder (PTLD) remains a major cause of morbidity and mortality after hematopoietic stem cell (HSCT) or solid organ transplant (SOT). Strategies to reconstitute immunity by adoptive transfer of EBV-specific cytotoxic T lymphocyte (CTL) therapy while highly effective in the HSCT setting where immunosuppression can be withdrawn have been less successful in the SOT setting where continued immunosuppression therapy is necessary. Additionally, the complexity and time taken to generate EBV-CTLs for adoptive transfer limit the clinical applicability. We have developed a system for the rapid generation of EBV-CTLs resistant to immunosuppression based on selection of interferon-gamma (IFN-γ) secreting EBV-CTLs and retroviral transduction with a calcineurin B mutant. With this methodology, EBV-CTLs resistant to the calcineurin inhibitor Tacrolimus (TAC) can be produced in 14 days. These CTLs show high specificity for EBV with negligible alloreactivity in both proliferation and cytotoxicity assays and are able to proliferate and secrete IFN-γ in response to antigen stimulation in the presence of therapeutic doses of TAC. This strategy will substantially facilitate clinical application of this approach for the treatment of PTLD in SOT recipients.

Keywords: Calcineurin inhibitors resistance, cytotoxic T lymphocytes, Epstein–Barr virus, IFN-γ selection, posttransplant lymphoma, solid organ transplant

Abbreviations: CTL, cytotoxic T lymphocyte; CsA, cyclosporin A; CyPA, cyclophilin; EBV, Epstein–Barr virus; eGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; FKBP-12,

FK binding protein-12; HSCT, hematopoietic stem cell transplant; IFN-γ, interferon-gamma; LCL, lymphoblastoid cell line; NFAT, nuclear factor of activated T cells; NK, natural killer; NKT, natural killer T cell; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PTLD, posttransplant lymphoproliferative disorder; SOT, solid organ transplant; TAC, Tacrolimus

Introduction

Epstein–Barr virus (EBV)-associated posttransplant lymphoproliferative disorder (PTLD) is a major complication of solid organ (SOT) or hematopoietic stem cell transplant (HSCT) arising because immunosuppression compromises virus-specific CTL immunosurveillance, allowing uncontrolled proliferation of EBV-infected B cells (1,2). Current therapies for PTLD are frequently ineffective and have significant toxicity. Reducing immunosuppression frequently results in graft rejection: indeed in one large series, death from graft rejection was as frequent as death from PTLD (3–6). Adoptive immunotherapy represents a logical approach to reconstitute EBV-CTL-mediated immunity and has been shown to be highly effective in the HSCT setting (3,4,7). In contrast, the application of this strategy for the treatment of PTLD in SOT patients, while feasible (5,6,8), is compromised by the ongoing immunosuppression required to prevent graft rejection (9,10).

Our group has developed (11) a strategy for genetically engineering EBV-CTLs to be resistant to calcineurin inhibitors, the most critical immunosuppressive drugs used after SOT. Cyclosporin A (CsA) and Tacrolimus (TAC) 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), preventing T cells activation. To neutralize the immunosuppressive effects of these drugs, we have developed calcineurin mutants disrupting binding of TAC–FKBP-12 and/or CsA–CyPA, without affecting the active site responsible for NFAT dephosphorylation. EBV-CTLs expressing such mutants

maintain their ability to proliferate and secrete interferon-gamma (IFN-γ) in response to stimulation with EBV in the presence of therapeutic levels of TAC and/or CsA (11).

The conventional methodology used to generate EBV-CTLs involves stimulation with autologous EBV-transformed B cells (lymphoblastoid cell line [LCL]) (12,13). This utilizes a live virus (B95-8) and takes at least 12 weeks limiting clinical applicability as during this time, the majority of patients with PTLD would have either succumbed or responded to alternative therapies. A simpler, more rapid system for the isolation of EBV-CTLs is therefore needed. Several groups have used the cytokine capture assay (14,15) based on selection of IFN-γ after antigenic stimulation to rapidly isolate virus-specific T cells. Recently, two groups (16,17) have isolated EBV-CTLs from normal donor blood after stimulation with pools of immunodominant EBV peptide epitopes. This approach was successfully used clinically with donor derived EBV-CTLs in HSCT recipients. We hypothesized that combination of this methodology with genetic modification of the isolated EBV-CTLs to render them resistant to calcineurin inhibitor would enable us to extend adoptive immunotherapy to the SOT setting.

Here we describe a simple, rapid and robust methodology for the generation of EBV-CTLs resistant to calcineurin inhibitors using IFN-γ capture after peptide stimulation, followed by retroviral transduction with the CNb30 mutant. This strategy may increase the efficacy of adoptively transferred EBV-CTLs in SOT patients developing PTLD and avoid the need for withdrawal of immunosuppression.

Materials and Methods

EBV peptides
The EBV peptide mix used (JPT Peptide Technologies, Berlin, Germany) consists of 23 immunodominant peptides (19 HLA class I restricted and 4 HLA class II) from 5 latent antigens (LMP2, EBNA1, EBNA3A, EBNA3B, EBNA3C), 4 immediate early/early antigens (BZLF1, BRLF1, BMLF1, BHRF1) and 2 late/structural antigens (BLLF1, BNRF1) as previously described (16).

Generation of EBV-specific T-lymphocyte lines
A total of 2–5×10^8 peripheral blood mononuclear cells (PBMCs) were stimulated with EBV pepmix at $1\,\mu g/mL$ in X-Vivo15 + 2% human AB Serum (Lonza, Slough, UK) as previously reported (16). After a 16-h stimulation period, IFN-γ-secreting cells were selected using the IFN-γ secretion assay according to the manufacturer's recommendations (Miltenyi Biotec, Bisley, UK). Briefly, PBMCs were labeled with a bi-specific anti-IFN-γ/CD45 antibody, and incubated for 45 min at 37°C to enable IFN-γ secretion. Cells that bound IFN-γ were labeled with magnetic beads conjugated with an IFN-γ-specific antibody, and IFN-γ positive secreting cells were selected using Miltenyi Mini-MACS columns.

EBV-CTLs were expanded in culture for 2 weeks. Selected EBV-specific T cells were resuspended at 3×10^5 cells/mL in plus 100 U/mL IL-2 (Proleukin; Chiron, Ratingen, Germany) in the presence of 5×10^6/mL autologous,

irradiated (30 Gy) PBMCs. After 7 days, T cells were re-plated at 5×10^5 T cells/well with autologous pepmix pulsed, irradiated PBMCs at a ratio of 1:8.

Generation of retrovirus
High-titer stable SFG retroviral producer lines carrying either the enhanced green fluorescent protein (eGFP) transgene alone or expressed with the CNb30 mutant pseudotyped with Gibbon Ape Leukemia Virus envelope (18) were produced as described previously (11). The titers of the retroviral vectors were, respectively, for CNb30 4.8×10^6 cells/mL, and for eGFP 9.3×10^6 cells/mL.

Transduction of EBV-CTLs
EBV-CTLs were transduced with CNb30 mutants or eGFP retroviral supernatants 3 days after the second peptide stimulation as previously described (11). Transduction efficiency was determined by expression of eGFP by flow cytometry.

For methodology for donors, immunophenotyping, tetramer staining, generation of LCLs, cytotoxicity assays, proliferation, enzyme-linked immunosorbent assay (ELISA) and statistical analysis, see Supplementary Material Online.

Results

Generation, expansion and transduction of EBV-CTLs
A mean of 3.5×10^6 EBV-CTLs (range 0.3×10^6 to 6.2×10^6 (n = 5) was obtained after selection of the IFN-γ secreting T cells. To obtain a suitable yield for clinical application, we expanded the isolated EBV-CTLs with autologous peptide loaded PBMCs as feeders. To render EBV–CTLs resistant to calcineurin inhibitors, cells were transduced on day 10 with retroviral vectors carrying eGFP transgene alone or expressed with the calcineurin mutant CNb30 (11). After 14 days of culture, the mean number of cells obtained was 46.9×10^6 (range 30–70×10^6) (Figure 1). Transduction efficiency assessed at day 14 was between 18% and 80.6% (CNb30 mean 37%, range 18–72%; eGFP mean 64.5%, range 46.3–80.6%). Thus, with this methodology we could achieve a 13.4-fold of the EBV-CTLs over 14 days.

EBV-CTLs are mainly CD8$^+$ with an effector memory phenotype
We next evaluated the immunophenotype of EBV-CTLs, and consistent with the fact that the EBV pepmix used to generate the EBV-CTLs contain mainly CD8 epitopes, flow cytometric analysis showed that the majority (mean 70%, range 4.1–97.7%) of the EBV-CTLs were CD8$^+$ but a significant proportion (mean 30%, range 2.3–95%), were CD4$^+$; 1.4% of the cells showed a natural killer (NK) phenotype (CD3$^-$CD56$^+$CD16$^+$) and 2.2% were natural killer T cells (CD3$^+$CD56$^+$CD16$^+$) (Figure 2A). The majority of the T cells in all five donors showed a CCR7$^-$ CD45RA$^-$ effector memory phenotype (mean 93.6%, range 76.3–99.6%) (Figure 2B), hence consisting mainly of T cells with the capacity for immediate effector function and durable memory responses. Comparison of the phenotype of untransduced and CNb30 transduced EBV-CTLs showed
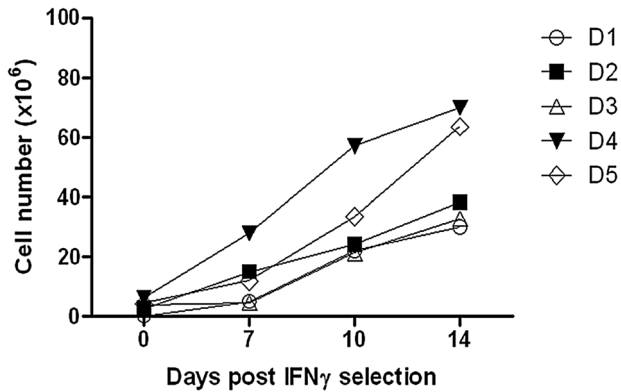
Figure 1: Growth kinetics of EBV-CTLs generated with IFN-γ capture. The graph illustrates the expansion rate of EBV-CTL lines after selection with IFN-γ capture. PBMCs from five EBV seropositive donors were stimulated with EBV pepmix. IFN-γ secreting T cells were selected and expanded with autologous, irradiated PBMCs as feeder cells. On day 7 after initial stimulation CTLs were restimulated using pepmix-pulsed autologous PBMCs. On day 10 EBV-CTLs were transduced with retroviral vectors carrying a control vector containing the enhanced green fluorescent protein transgene alone or expressed with the calcineurin mutant CNb30. The figure shows the expansion of CTLs from the beginning (day 0) to day 14, following a second stimulation at day 7 and transduction at day 10. CTLs expansion was evaluated using Trypan blue exclusion and results are shown as mean cell number ± SD. The total cell number of T cells obtained was 13.4-fold expanded over 14 days. CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; IFN-γ, interferon-gamma; PBMCs, peripheral blood mononuclear cells.

that transduction with calcineurin mutant did not alter the phenotype of EBV-CTLs (Figure 2B).

Enrichment of EBV antigen specificity

To demonstrate that our protocol enriched for EBV-CTLs, in three donors with the appropriate HLA restriction, we compared the frequency of CD8^{+}T cells specific for EBV using HLA-peptide tetramers in unmanipulated PBMCs and in selected, expanded, transduced CTLs from the same donor (Table 1). All three donors showed enrichment of EBV-tetramer-positive T cells in transduced CTLs compared with the starting PBMCs. In donor 2 we observed a fourfold increase of CD8^{+} T cells recognizing BZLF1 (RAK) in CTLs compared with PBMCs, in donor 3 a threefold increase for EBNA3A (RLR)-specific CD8^{+} T cells and in donor 5 eightfold increase for BMLF1 (GLC), twofold for LMP2 (CLG) and sixfold for BZLF1 (RAK)-specific CD8^{+} T cells (Figure 2C).

EBV-CTLs kill EBV-infected targets

In order to determine whether EBV-CTLs were able to lyse EBV-infected targets we performed ^{51}Cr release cytotoxicity assays. Both CNb30 and eGFP CTLs showed specific
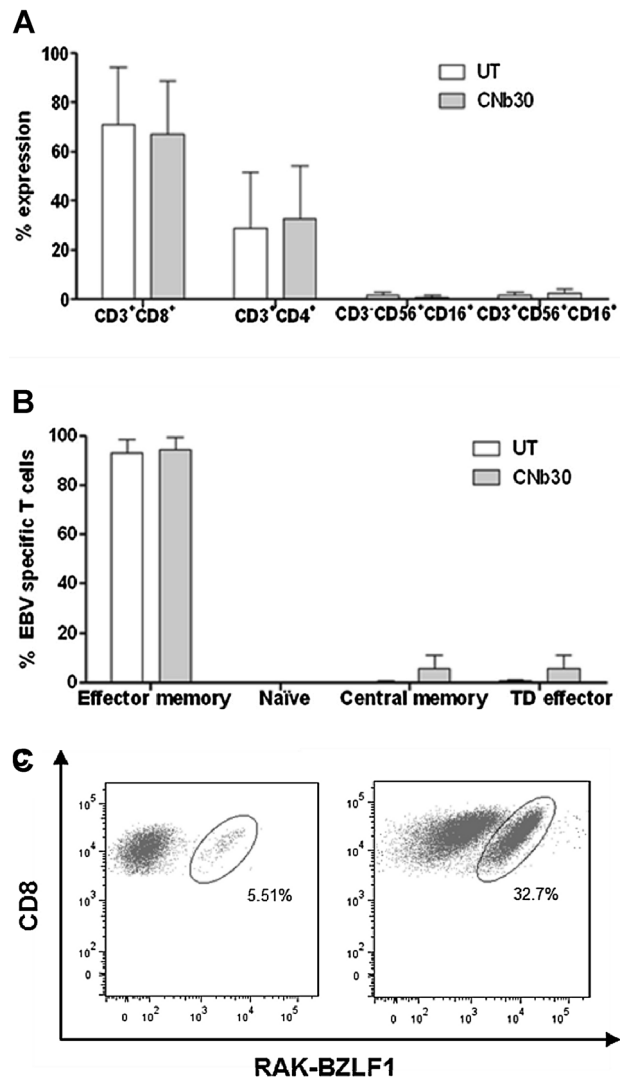

Figure 2: Phenotype and antigen specificity of EBV-CTLs. Flow cytometric immunophenotyping was performed on day 14 after expansion. (A) Untransduced and CNb30 transduced EBV-CTLs were analyzed for T and NK-cell marker expression. (B) Distribution of memory subsets in untransduced (UT) and CNb30 transduced EBV-CTLs. Effector memory (CCR7− CD45RA−), naïve (CCR7+CD45RA+), central memory (CCR7+CD45RA−), terminal-differentiated (TD) effector (CCR7−CD45RA+) T cells. Cells were gated on CD3+ cells. Mean expression ± SEM in EBV T cell lines generated from five donors are shown. (C) Enhancement of EBV specificity in CD8+ cells from one donor with appropriate HLA restriction. Flow cytometry profile of EBV-CTLs stained with anti-CD8 mAb and with relevant HLA class I/peptide tetramer. The left plot shows the frequency of tetramer-positive cells on peripheral blood mononuclear cells before the isolation and expansion. Right plot shows that isolated, expanded EBV-CTLs transduced with CNb30 mutant have an increased frequency of tetramer-positive T cells. CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus.

Table 1: Specificity of EBV-CTLs after selection and expansion analyzed by tetramer staining

Donor	HLA type	Tetramer	EBV antigen	PBMCs (%)	CNb30 CTLs (%)
D2	A01,31; B08,60	HLA-B*0801-RAKFKQLL	BZLF1	3.32	16
D3	A03,29; B39	HLA-A*0301-RLRAEAQVK	EBNA3A	5.07	16.1
D5	A01,02; B08	HLA-A*0201-GLCTLVAML	BMLF1	1.11	9.92
		HLA-A*0201-CLGGLLTMV	LMP2	2.5	4.8
		HLA-B*0801-RAKFKQLL	BZLF1	5.51	32.7

After interferon-gamma selection and 14 days *in vitro* expansion, the EBV-specificity of the selected, expanded and CNb30 transduced cells was analyzed using tetramer staining. The frequency of T cells specific for an HLA-EBV peptide epitope was determined by staining T cells with CD3 APCCy7, CD8 Pacific Blue and Tetramer. Table shows the frequency of tetramer-specific T cells in the starting unmanipulated fraction (PBMCs) and in the selected, expanded and CNb30 transduced CTL (CNb30 CTL) populations in three donors with the appropriate HLA restrictions. The percentages refer to the proportion of the CD8^{+} cells positive for the corresponding tetramer with the isotype control subtracted. CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; PBMCs, peripheral blood mononuclear cells.

cytotoxicity against autologous LCL targets (Figure 3A and B). CNb30-CTLs showed a mean lysis of 24.45% ± SE 4.5% (at an effector:target ratio of 30:1, n = 4) and GFP-CTLs a mean lysis of 20.23% ± SE 2.6 (at a 30:1 effector:target ratio, n = 4). This lysis was MHC restricted as no lysis of allogeneic LCLs was observed (mean 1.8% ± SE 0.3%) and not NK-mediated as no significant cytotoxicity against the HSB2 cell line (mean 3.48%, range 3–6.5% at 30:1 ratio) was seen. To determine whether immunosuppression could have an effect on the cytotoxic ability of the EBV-CTLs, we cultured the CTLs in presence of therapeutic levels (10 ng/mL) of TAC before and during the cytotoxicity assay. We did not observe any difference in the cytotoxic activity against autologous LCLs for either CNb30 or eGFP-CTLs treated with TAC (CNb30 36.84%, eGFP 24.65% at 30:1 effector:target ratio; Figure 3C and D). These data demonstrate that the cytotoxicity of EBV-CTL lines is not affected by retroviral transduction with CNb30 and that the presence of calcineurin inhibitors has no effect on the cytotoxicity of EBV-CTL lines.
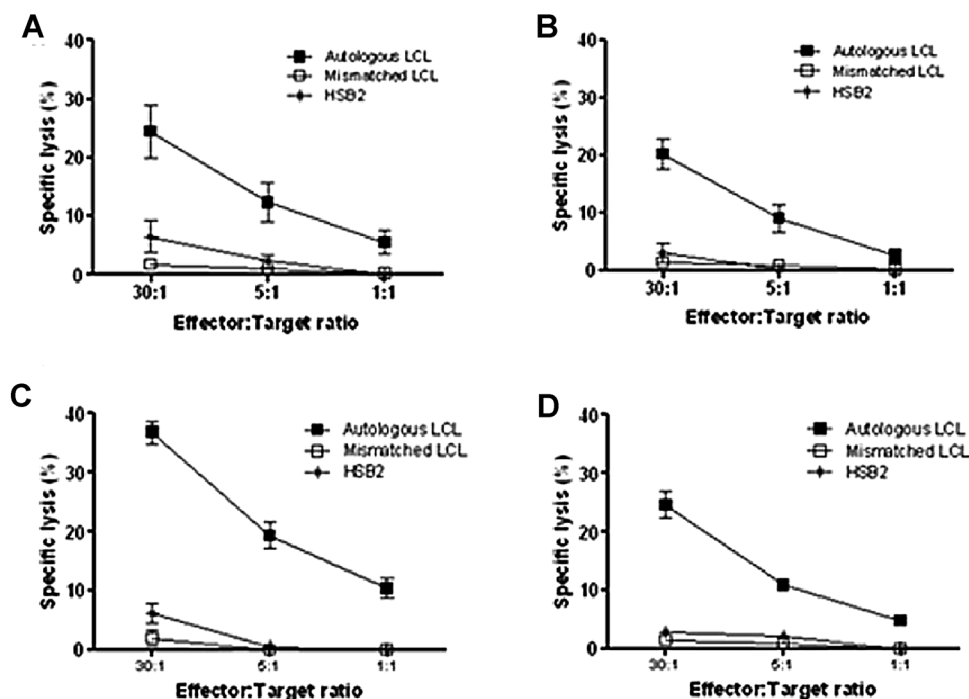

Figure 3: Cytotoxic activity of EBV-CTLs. A standard ^{51}Cr release cytotoxicity assay was performed to assess cytotoxicity of EBV-CTL lines against autologous, mismatched LCL targets or the T cell line HSB2. (A) Cytolytic activity of CNb30 transduced cells; (B) cytolytic activity of EBV-CTLs transduced with eGFP alone. Cytotoxic ability of EBV-CTLs transduced with CNb30 (C) or with eGFP alone (D) cultured in the presence of 10 ng/mL of Tacrolimus. No effect of calcineurin inhibitors was detected on cytotoxicity of either eGFP or CNb30 transduced EBV-CTLs. Both EBV-CTLs transduced with CNb30 or with eGFP alone show higher cytotoxic ability against autologous LCLs compared with mismatched targets or the HSB2 cell line. The mean values ± SEM in four donors tested are shown. CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; eGFP, enhanced green fluorescent protein; LCLs, lymphoblastoid cell lines.

EBV-CTLs transduced with calcineurin mutant secrete IFN-γ, proliferate in the presence of TAC and lack alloreactivity

To assess the ability of CNb30-CTLs to function in the presence of TAC, we measured IFN-γ release and proliferation in response to antigenic stimulation. As shown in Figure 4A and B, addition of TAC to eGFP-CTLs completely inhibited secretion of IFN-γ ($p < 0.05$, $n = 5$) and abrogated proliferation ($p < 0.05$) after stimulation with EBV pepmix. In contrast, all five CNb30-CTLs were able to secrete IFN-γ ($p = 0.42$) and proliferate ($p = 0.4$) in presence of TAC at comparable levels to CNb30-CTLs in the absence of TAC. Neither CNb30 nor eGFP CTLs secreted IFN-γ or showed any proliferation when cultured with AdV5, a control irrelevant peptide, demonstrating the antigen specificity of our CTL lines. These data demonstrate that CNb30-CTLs are able to secrete effector cytokines and to proliferate in response to antigen stimulation in the presence of TAC.
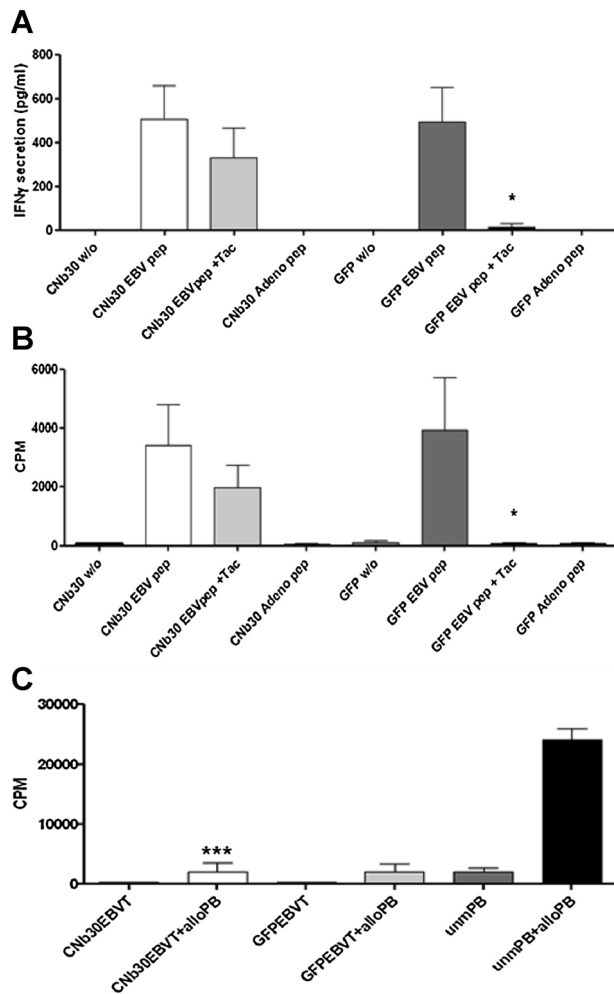
If TAC resistant CTLs are to be used in the allogeneic setting, it is critical that they are depleted of alloreactivity by the process of selection and culture. We therefore measured the alloreactivity of the eGFP/CNb30-CTLs in a mixed lymphocyte reaction, and compared this with the alloreactivity of unmanipulated PBMCs from the same donor. Donor PBMCs or selected EBV-CTLs were cultured with irradiated, HLA mismatched PBMCs for 6 days. As shown in Figure 4C, unmanipulated donor PBMCs proliferated strongly in response to stimulation with irradiated, allogeneic PBMCs ($p = 0.0001$). In contrast, the response of CNb30-CTLs to allogeneic PBMCs was negligible. The absence of proliferation in response to allogeneic PBMCs indicates that the process of IFN-γ selection, expansion and transduction of EBV-CTLs diminish their alloreactivity.

Generation of TAC resistant EBV-CTLs from SOT patients receiving immunosuppression

To ensure that the approach we describe would be feasible using T cells from SOT patients with PTLD, we isolated EBV-CTLs using our methodology above from three SOT patients (two heart transplants, one small bowel transplant) with PTLD and transduced them with the CNb30. All three patients were receiving TAC.

Figure 4: EBV-CTLs transduced with CNb30 secrete IFN-γ retain proliferative ability in the presence of TAC and lack of alloreactivity. Selected, expanded and transduced EBV-CTLs were stimulated in the presence of EBV pepmix with or without TAC and IFN-γ secretion was assessed by ELISA 24 h after stimulation. (A) EBV-CTLs transduced with CNb30 were able to secrete IFN-γ in the presence of EBV pepmix plus 10 ng/mL TAC at comparable levels to that seen with CNb30 transduced T cells stimulated with EBV pepmix alone ($p = 0.42$). eGFP transduced EBV-CTLs did not produce IFN-γ in the presence of EBV pepmix plus 10 ng/mL TAC compared with eGFP transduced T cells stimulated with EBV pepmix alone ($p < 0.05$). Results also show that both CNb30 and GFP transduced EBV T cells do not produce IFN-γ when stimulated with an irrelevant peptide (Adeno hexon). Proliferation ability of EBV-CTLs was evaluated 4 days after stimulation with EBV pepmix with or without TAC and was tested by H^3-thymidine uptake for 21 h. (B) Proliferation of eGFP transduced EBV-CTLs after stimulation with EBV pepmix in the presence of TAC was significantly inhibited (*$p < 0.05$) compared with CNb30 transduced EBV-CTLs ($p = 0.4$). These data also show that both CNb30 and GFP transduced EBV-CTLs do not proliferate in the presence of an irrelevant peptide (Adeno hexon). (C) Alloreactive potential of unmanipulated PBMCs as well as selected, expanded and transduced EBV-CTLs from the same donor using a primary mixed lymphocyte reaction. Unmanipulated unselected PBMCs proliferated significantly (***$p < 0.0005$) in response to allogeneic, irradiated PBMCs compared to the proliferation of the selected EBV-CTLs. Data show the mean values and SEM of experiments from five donors. CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; eGFP, enhanced green fluorescent protein; IFN-γ, interferon-gamma; PBMCs, peripheral blood mononuclear cells; TAC, Tacrolimus.

As these patients were children, we started with 30–60 mL of blood. EBV-CTLs were generated by stimulating PBMCs with the pepmix for 16 h followed by IFN-γ capture assay. A mean of 0.14×10^6 EBV-CTLs (range 0.08×10^6 to 0.18×10^6) was obtained after selection. The isolated EBV-CTLs were expanded and transduced using the method above. After 14 days of culture, the mean number of cells obtained from three patients was 8.92×10^6 (range 3.16–17.8×10^6) (Figure 5A). Transduction efficiency assessed at day 14 was between 56.7% and 84.2% (CNb30 mean 62.35%, range 56.7–68%; eGFP mean 83.25%, range 82.3–84.2%). Thus, we could achieve a 63.5-fold expansion of the EBV-CTLs over 14 days even in PTLD patients.

The majority of the EBV-CTLs generated from these patients were CD8$^+$ (data not shown). Comparison of the frequency of CD8$^+$T cells specific for the immunodominant EBV epitope RAKFKQLL from BZLF1 in one evaluable donor showed a marked (17.7-fold) enrichment of tetramer-positive cells in selected, expanded, transduced CTLs compared with unmanipulated PBMCs from the same donor (Figure 5B).

To assess the ability of CNb30-CTLs from PTLD patients to function in the presence of TAC; we measured IFN-γ release and proliferative ability in response to antigenic stimulation. Our data show (Figure 5C and D) that addition of TAC to eGFP-CTLs completely inhibited secretion of IFN-γ (p < 0.05, n = 3) and abrogated proliferation (p < 0.05) after stimulation with EBV pepmix. In contrast, CNb30-CTLs were able to secrete IFN-γ (p = 0.0884, n = 3) and to proliferate (p = 0.18) in presence of TAC at comparable levels to CNb30-CTLs its absence. Neither CNb30 nor eGFP
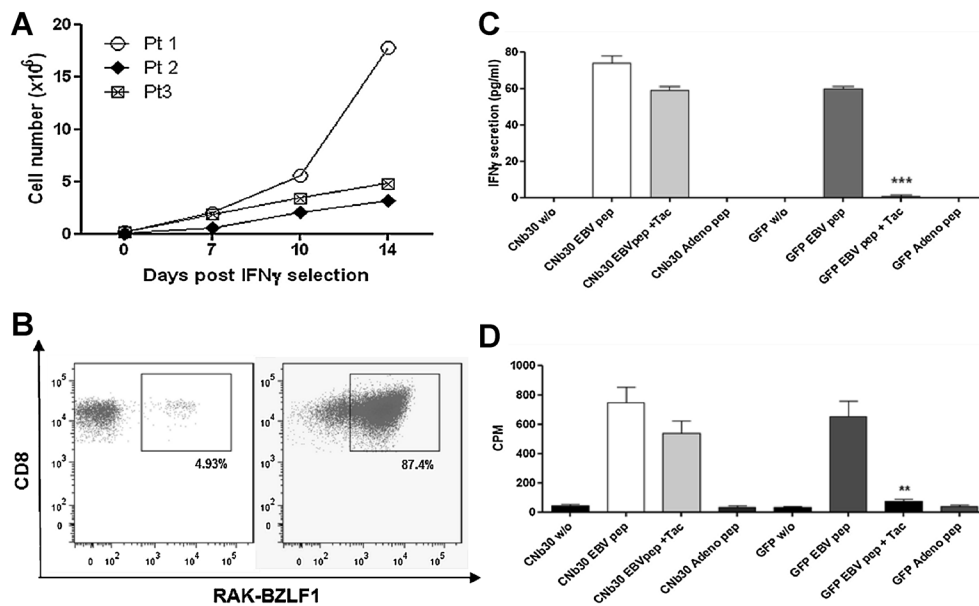

Figure 5: Expansion, antigen specificity and function *in vitro* of EBV-CTLs from SOT patients with PTLD. The growth kinetics, and antigen specificity by tetramer staining of the EBV-CTLs isolated with IFN-γ capture, expanded *in vitro* and transduced with CNb30 from our cohort of SOT recipients are shown. (A) Expansion of CTLs from the beginning (day 0) to day 14, following a second stimulation at day 7 and transduction at day 10. CTLs expansion was evaluated using Trypan blue exclusion and results are shown as mean cell number ± SD. The total cell number of expanded T cells obtained was 63.5-fold over 14 days. (B) Enhancement of EBV specificity in CD8$^+$ cells from one PTLD patient with appropriate HLA restriction. Flow cytometry profile of EBV-CTLs stained with anti-CD8 mAb and with relevant HLA class I/ peptide tetramer. The left plot shows the frequency of tetramer-positive cells on PBMCs before the isolation and expansion. Right plot shows that isolated, expanded EBV-CTLs transduced with CNb30 mutant have an increased frequency of tetramer-positive T cells. Selected, expanded and transduced EBV-CTLs were stimulated in the presence of EBV pepmix with or without TAC and IFN-γ secretion was assessed by ELISA 24 h after stimulation. (C) EBV-CTLs transduced with CNb30 were able to secrete IFN-γ in the presence of EBV pepmix plus 10 ng/mL TAC at comparable levels to that seen with CNb30 transduced T cells stimulated with EBV pep mix alone (p = 0.08). eGFP transduced EBV-CTLs did not produce IFN-γ in the presence of EBV pepmix plus 10 ng/mL TAC compared with eGFP transduced T cells stimulated with EBV pepmix alone (***p < 0.05). (D) Proliferation was tested by H^3-thymidine uptake for 21 h after 4 days of stimulation with EBV pepmix with or without TAC. Proliferation of eGFP transduced EBV-CTLs after stimulation with EBV pepmix in the presence of TAC was significantly inhibited (**p < 0.05) compared with CNb30 transduced EBV-CTLs (p = 0.18). These data also show that both CNb30 and GFP transduced EBV-CTLs from PTLD patients do not secrete IFN-γ or proliferate when stimulated with an irrelevant peptide (Adeno hexon). Data show the mean values and SEM of experiments from three patients examined. CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; eGFP, enhanced green fluorescent protein; IFN-γ, interferon-gamma; PBMCs, peripheral blood mononuclear cells; PTLD, posttransplant lymphoproliferative disorder; SOT, solid organ transplant; TAC, Tacrolimus.

CTLs secreted IFN-γ or showed proliferation when cultured with a control irrelevant peptide, demonstrating the antigen specificity. Taken together these results show that CNb30-CTLs isolated from PTLD patients have an effective immunological response to EBV that is not abrogated in the presence of TAC.

Discussion

Adoptive transfer of *ex vivo* derived EBV-CTLs prevents or ameliorates PTLD by reconstituting the EBV-specific T cell immunity after HSCT (4,19) but in the SOT setting has been more challenging (5,6,20,21). First, CTL generation requires the use of live, replication-competent EBV virus (B95-8) for LCL generation and is time-consuming (12). Second, SOT patients require ongoing immunosuppression to prevent rejection and this limits the proliferation, function and persistence of adoptively transferred CTLs (9,10,22). Here, we have overcome these two barriers by developing a methodology for the rapid generation of EBV-CTLs, which are resistant to immunosuppression with calcineurin inhibitors.

For the rapid generation of virus-specific CTLs, several groups have utilized IFN-γ capture to select antigen-specific T cells (14,15,23,24). Recently, two groups (16,17) have reported the clinical application of EBV-CTLs generated from donor blood using IFN-γ capture in the HSCT setting. In our work, we have used the same peptide pool described by Moosmann et al (16) that contains 23 immunodominant peptides from a range of EBV latent and lytic antigens.

In order to generate EBV-CTLs resistant to calcineurin inhibitors, we have developed a methodology involving restimulation of CTLs isolated by IFN-γ capture with EBV-peptide loaded autologous feeders followed by retroviral transduction with CNb30. This approach avoids the use of live virus and shortens the time taken to generate CTLs to 14 days, thereby reducing regulatory complexity and facilitating clinical application. Further, this culture process has the additional advantage of reducing the potential for alloreactivity, which has been seen with CTLs isolated directly after γ-capture from HLA-mismatched donors (25).

EBV-CTLs generated using this approach showed a similar phenotype to those generated by conventional LCL stimulation with a predominance of CD8^{+} T cells with an effector memory phenotype but also a significant proportion of helper CD4^{+} T cells, which are important for the maintenance of durable antigen-specific responses after adoptive transfer (26). Using HLA-peptide tetramers, we have demonstrated significant enhancement of antigen specificity of EBV-CTLs compared with unmanipulated PBMCs from the same donor. We need to assess alloreactivity against graft donor in further studies.

Clearly in the SOT setting, donor blood is often not available and is generally HLA-mismatched, so that donor derived CTLs can be rejected and may not recognize tumor B cells, which are almost always of recipient origin. Rejection of adoptively transferred CTLs may limit their persistence and efficacy particularly when partially HLA-mismatched third-party EBV-CTLs are used (21). Thus, it is critical to evaluate the feasibility of generating autologous CTLs from SOT patients receiving immunosuppression. Similar to previous studies using conventional LCLs (5,8,13), we have shown that it is possible to generate EBV-CTLs from SOT patients on immunosuppression using our methodology. Moreover, we show that the function and antigen specificity is similar to those generated in healthy donors and we found that the effect of the immunosuppressive drugs has no effect on the *ex vivo* EBV-CTLs as they do not show anergy by keeping their ability to respond *in vitro* to viral antigens.

To enable CTLs to function in the presence of immunosuppression, our group has previously engineered CTLs to be resistant to CsA and TAC. Binding of these drugs with their chaperone proteins to the calcineurin heterodimer sterically blocks entry and subsequent activation of NFAT. We have designed calcineurin mutations that inhibit docking of either or both TAC/FKBP12 and CsA/CyPA complexes, but do not affect the active site. The mutant used in our current experiments, CNb30, has two mutations (L124T point mutation and insertion K125-LA) that disrupt binding of FKBP12/CyPA to the calcineurin heterodimer but do not affect NFAT dephosphorylation. Consistent with our previous study (11), here we show that EBV-CTLs generated using our novel methodology, when transduced with CNb30, are able to proliferate and secrete the Th1 effector cytokine IFN-γ in response to stimulation with EBV peptides even in the presence of TAC, whereas EBV-CTLs transduced with a control vector were not. Cytotoxicity is unaffected by calcineurin inhibitors, and both CNb30-CTLs and CTLs transduced with a control vector were able to lyse autologous EBV-infected targets effectively in the presence of TAC. Importantly, CNb30-CTLs were devoid of *in vitro* alloreactivity, suggesting they are unlikely to cause graft rejection. Likewise, since transduced EBV-CTLs are terminally differentiated, retroviral gene transfer is extremely unlikely to result in leukemogenesis: indeed there are no reported cases of this in over 200 patients treated with retrovirally transduced T cells.

The methodology described here will greatly facilitate translation of this approach to clinical use in the SOT setting by enabling generation of autologous EBV-CTLs resistant to immunosuppression without the need for replication-competent EBV in a timely fashion. Our approach is animal serum-free; the EBV pepmix is recombinant and has been used in two previous clinical studies, and the IFN-γ capture approach has been used clinically by a number of groups to generate virus-specific CTLs for adoptive transfer (16,17,24). We are currently scaling up our approach for clinical use under good manufacturing practice

conditions. As shown by our data in SOT recipients, one challenge will be to generate an adequate cell dose of CNb30-EBV-CTLs from patients on immunosuppression. Previous studies with EBV-CTLs in the HSCT setting have shown efficacy at a cell dose of $2 \times 10^7/m^2$. While we are routinely able to generate such doses from a 500 mL blood draw from normal donors, this may not be feasible in SOT patients, particularly children. We are currently investigating alternate cytokine regimens and culture in gas-permeable bioreactors (27) to optimize CTL expansion.

In summary, we have developed a simple, robust and potentially clinically applicable methodology for the rapid generation of EBV-CTLs resistant to immunosuppression. Potentially, adoptive transfer of autologous calcineurin inhibitor resistant EBV-CTLs could be used as prophylaxis for PTLD in high-risk groups, such as in patients undergoing pediatric small bowel transplantation, where the risk of PTLD may be as high as 30% (28). In cohorts at lower risk of PTLD, resistant EBV-CTLs could be used as adjunctive therapy for established PTLD with Rituximab. In this situation, first-line therapy with Rituximab could be used to establish disease control during the time required for generation of the EBV-CTLs, with subsequent transfer of resistant CTLs to maintain remission and overcome the significant rates of partial response and relapse associated with Rituximab monotherapy (29) without the toxicity associated with chemotherapy (30). Critically, such a strategy would obviate the need for reduction in immunosuppression with calcineurin inhibitors, which is a frequent cause of rejection and treatment failure (31). Such an approach could be of major benefit to PTLD patients by reducing the morbidity and mortality without the need for withdrawal of immunosuppression with calcineurin inhibitors.

Acknowledgments

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

References

1. Murray RJ, Kurilla MG, Brooks JM, et al. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): Implications for the immune control of EBV-positive malignancies. J Exp Med 1992; 176: 157–168.
2. Bollard CM, Rooney CM, Heslop HE. T-cell therapy in the treatment of post-transplant lymphoproliferative disease. Nat Rev Clin Oncol 2012; 9: 510–519.
3. Gustafsson A, Levitsky V, Zou JZ, et al. Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: Prophylactic infusion of EBV-specific cytotoxic T cells. Blood 2000; 95: 807–814.
4. Heslop HE, Slobod KS, Pule MA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood 2010; 115: 925–935.
5. Khanna R, Bell S, Sherritt M, et al. Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. Proc Natl Acad Sci USA 1999; 96: 10391–10396.
6. Comoli P, Maccario R, Locatelli F, et al. Treatment of EBV-related post-renal transplant lymphoproliferative disease with a tailored regimen including EBV-specific T cells. Am J Transplant 2005; 5: 1415–1422.
7. Heslop HE, Ng CY, Li C, et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. Nat Med 1996; 2: 551–555.
8. Savoldo B, Goss JA, Hammer MM, et al. Treatment of solid organ transplant recipients with autologous Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs). Blood 2006; 108: 2942–2949.
9. Savoldo B, Goss J, Liu Z, et al. Generation of autologous Epstein-Barr virus-specific cytotoxic T cells for adoptive immunotherapy in solid organ transplant recipients. Transplantation 2001; 72: 1078–1086.
10. Zhan X, Brown B, Slobod KS, Hurwitz JL. Inhibition of ex vivo-expanded cytotoxic T-lymphocyte function by high-dose cyclosporine. Transplantation 2003; 76: 739–740.
11. Brewin J, Mancao C, Straathof K, et al. Generation of EBV-specific cytotoxic T cells that are resistant to calcineurin inhibitors for the treatment of posttransplantation lymphoproliferative disease. Blood 2009; 114: 4792–4803.
12. Rooney CM, Smith CA, Ng CY, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. Blood 1998; 92: 1549–1555.
13. Comoli P, Labirio M, Basso S, et al. Infusion of autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. Blood 2002; 99: 2592–2598.
14. Rauser G, Einsele H, Sinzger C, et al. Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. Blood 2004; 103: 3565–3572.
15. Feuchtinger T, Matthes-Martin S, Richard C, et al. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol 2006; 134: 64–76.
16. Moosmann A, Bigalke I, Tischer J, et al. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. Blood 2010; 115: 2960–2970.
17. Icheva V, Kayser S, Wolff D, et al. Adoptive transfer of epstein-barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. J Clin Oncol 2013; 31: 39–48.
18. Cosset FL, Takeuchi Y, Battini JL, Weiss RA, Collins MK. High-titer packaging cells producing recombinant retroviruses resistant to human serum. J Virol 1995; 69: 7430–7436.
19. Hislop AD, Taylor GS, Sauce D, Rickinson AB. Cellular responses to viral infection in humans: Lessons from Epstein-Barr virus. Annu Rev Immunol 2007; 25: 587–617.

20. Subklewe M, Marquis R, Choquet S, et al. Association of human leukocyte antigen haplotypes with posttransplant lymphoproliferative disease after solid organ transplantation. Transplantation 2006; 82: 1093–1100.

21. Haque T, Wilkie GM, Jones MM, et al. Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: Results of a phase 2 multicenter clinical trial. Blood 2007; 110: 1123–1131.

22. Plosker GL, Foster RH. Tacrolimus: A further update of its pharmacology and therapeutic use in the management of organ transplantation. Drugs 2000; 59: 323–389.

23. Gerdemann U, Keirnan JM, Katari UL, et al. Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. Mol Ther 2012; 20: 1622–1632.

24. Feuchtinger T, Opherk K, Bethge WA, et al. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood 2010; 116: 4360–4367.

25. Qasim W, Gilmour K, Zhan H, et al. Interferon-gamma capture T cell therapy for persistent Adenoviraemia following allogeneic haematopoietic stem cell transplantation. Br J Haematol 2013; 161: 449–452.

26. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med 1995; 333: 1038–1044.

27. Vera JF, Brenner LJ, Gerdemann U, et al. Accelerated production of antigen-specific T cells for preclinical and clinical applications using gas-permeable rapid expansion cultureware (G-Rex). J Immunother 2010; 33: 305–315.

28. Finn L, Reyes J, Bueno J, Yunis E. Epstein-Barr virus infections in children after transplantation of the small intestine. Am J Surg Pathol 1998; 22: 299–309.

29. Choquet S, Oertel S, LeBlond V, et al. Rituximab in the management of post-transplantation lymphoproliferative disorder after solid organ transplantation: Proceed with caution. Ann Hematol 2007; 86: 599–607.

30. Choquet S, Trappe R, Leblond V, Jager U, Davi F, Oertel S. CHOP-21 for the treatment of post-transplant lymphoproliferative disorders (PTLD) following solid organ transplantation. Haematologica 2007; 92: 273–274.

31. Webber SA, Naftel DC, Fricker FJ, et al. Lymphoproliferative disorders after paediatric heart transplantation: A multi-institutional study. Lancet 2006; 367: 233–239.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supplementary Material and Methods