EPSTEIN-BARR VIRUS, B CELL LYMPHOPROLIFERATIVE DISEASE, T CELL IMMUNOTHERAPY AND SCID MOUSE MODELLING

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**EPSTEIN-BARR VIRUS, B CELL LYMPHOPROLIFERATIVE
DISEASE AND SCID MICE: MODELLING T CELL
IMMUNOTHERAPY *IN VIVO***

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Running Title: Modelling T Cell Immunotherapy in SCID Mice

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ABSTRACT

Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disease (PTLD) arises in up to 10% of organ transplant recipients and is fatal in ~50% of cases. PTLD can be modelled in SCID mice using EBV+ve human B lymphoblastoid cell lines (BLCLs), and the current study investigated intraperitoneal (ip) inoculation of such animals in experiments which assessed the effect of EBV-specific cytotoxic T lymphocytes (CTLs) and cytokines on PTLD growth.

Ip transfer of 1 dose of autologous CTLs, or CD8-enriched T cells, into ip BLCL-inoculated animals significantly delayed tumour development ($p=0.001$) and prevented tumour formation in a significant proportion (40%) of mice ($p=0.001$). A combination of IL2, 7 and 15 conditioning of CTLs prior to ip injection significantly delayed ip BLCL-derived tumour formation *in vivo* when compared to CTLs expanded *in vitro* using only IL2 ($p=0.04$) and prevented tumour outgrowth in a significant proportion (60%) of mice ($p=0.02$). Daily ip IL2 dosing of ip CTL-inoculated mice significantly delayed tumour development *in vivo* ($p=0.004$) and prevented tumour outgrowth in a significant proportion (78%) of mice ($p=0.02$) when compared to animals dosed with vehicle only.

In SCID mice, autologous CTLs, and CD8-enriched T cells, have significant capacity to hinder development of PTLD-like tumours. Whilst studies are needed to delineate the role of cytokine conditioning and CD4-enriched T cells, the results suggest that IL2 plays a key role in supporting CTL function *in vivo*.

INTRODUCTION

Novel therapeutic regimes require validation in suitable animal models to assess efficacy and safety before clinical studies can be undertaken. The severe combined immunodeficient (SCID) mouse is a small animal model that has been used extensively to study novel treatments for human cancer. For example, these humanized animals constitute an *in vivo* model in which the treatment of Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disease (PTLD) can be investigated.

EBV is a ubiquitous agent that infects persistently over 90% of adults worldwide (Henle & Henle, 1966). It is usually acquired in childhood when infection is generally asymptomatic although if deferred until young adulthood, infectious mononucleosis may result (Crawford *et al*, 2006). Following primary infection, EBV establishes a life long persistent infection in resting memory B lymphocytes (Babcock *et al*, 1998).

EBV immune control is mediated by CD4+ve and CD8+ve MHC-restricted EBV-specific cytotoxic T lymphocytes (CTLs) against latent and lytic viral proteins (Bogedain *et al*, 1995; Steven *et al*, 1997; Bickham *et al*, 2001; Moss *et al*, 2001) with CTL responses to the EBNA3 antigens being immunodominant in healthy EBV seropositive individuals (Murray *et al*, 1990, 1992) where up to 5% of circulating CD8+ve CTLs are committed to EBV immune control (Hislop *et al*, 2002).

In organ transplant recipients, iatrogenic immunosuppression reduces EBV-specific CTL activity, and this may result in EBV-driven B cell proliferation and, ultimately, PTLD. PTLD develops in up to 10% of organ graft recipients and is

1 characterized by rapid onset, aggressive behaviour, and approximately 50% mortality
2 despite current treatment (Williams & Crawford, 2006). The major risk factors are the
3 degree and duration of immunosuppression required for graft maintenance and primary
4 EBV infection following transplantation (Thomas *et al*, 1995).
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11 The majority of PTLN express all EBV latent antigens and the tumour is
12 generally susceptible to EBV-specific CTL destruction. Adoptive CTL immunotherapy
13 provides rapid, targeted treatment that is well tolerated and effective in preventing and
14 treating PTLN (Rooney *et al*, 1998; Haque *et al*, 2007). However, a better understanding
15 of the optimum CTL regime is required to realise its full potential. To this end, our
16 laboratory has used the SCID mouse to model PTLN (Johannessen *et al*, 2000).
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25 Due to an autosomal mutation, SCID mice are unable to produce functional B
26 and T cell clones (Bosma *et al*, 1983) and, therefore, the animals cannot reject human
27 xenografts. Following transfer of human EBV+ve B cells, mice develop human PTLN-
28 like tumours (hu-SCID) that reflect patient lesions including expression of all EBV
29 latent antigens (Mosier *et al*, 1988; Johannessen *et al*, 2002). Various hu-SCID models
30 have been established, and it is not entirely clear which is most suitable for translational
31 treatment studies. Peripheral blood mononuclear cells (PBMCs) from EBV-seropositive
32 healthy donors give rise to PTLN-like lesions in a proportion of cases when cells are
33 transferred intraperitoneally (ip) into SCID mice (Picchio *et al*, 1992; Johannessen *et al*,
34 2000). However, inter-donor variability exists, and PBMCs from 25% of blood donors
35 do not form tumours in this hu-PBMC-SCID model. In contrast, *in vitro* EBV-infected B
36 lymphoblastoid cell lines (BLCLs) give rise consistently to PTLN-like lesions in the
37 animals (Cannon *et al*, 1990; Rowe *et al*, 1991) and such hu-BLCL-SCID models are
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suited to answer questions of treatment efficacy. To this end, BLCLs can be inoculated ip.

This study used the ip hu-BLCL-SCID model to assess the efficacy of CTLs *in vivo* and the role of a panel of cytokines in supporting CTL function. The ip hu-BLCL-SCID prevention model was a practical small (SCID mouse) animal model in which to study CTL function. Using this model, CD8+ve T cell subsets prevent PTLD outgrowth and IL2 alone, or in combination with IL7 and 15, enhances this effect.

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MATERIALS AND METHODS

Blood Donations

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10 Buffy coats were obtained with ethical approval from the Scottish National
11 Blood Transfusion Service (SNBTS), Edinburgh (UK). EBV serostatus was determined
12 by anti-VCA IgG antibody titer using a standard indirect immunofluorescence assay
13 (Henle & Henle, 1966). Donor PBMC MHC tissue typing was carried out at SNBTS
14 (Prof M Turner) or the Anthony Nolan Research Institute (London; Prof A Dodi) using
15 PCR-based methods.
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Establishment of BLCLs

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28 Concentrated EBV was added to PBMCs in a total volume of 1 ml of culture
29 medium [CM: RPMI 1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin,
30 100 U/ml streptomycin, and 10% v/v fetal calf serum (FCS)], incubated at 37°C for 1
31 hour, washed (in Hank's Buffered Saline Solution), resuspended (10^6 cells/ml; with
32 ciclosporin), and plated out in a 24-wells plate. During culture *in vitro*, cultures were fed
33 CM every 2-3 days. At 4 weeks, cells were assessed directly under a light microscope
34 for signs of immortalization, immunophenotyped, and % viability determined using
35 trypan blue. A viability of $\geq 70\%$ was considered acceptable.
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T Cell Subset Selection

T cell subsets were positively selected from CTLs using EasySep® kits (StemCell Technologies, Grenoble, France) following the manufacturer's instructions. Briefly, 100 µl/ml EasySep® selection mix was added to 1x10⁸ cells/ml in EasySep® buffer, incubated for 15 minutes followed by addition of 50 µl/ml EasySep® magnetic dextran iron nanoparticles, a further incubation for 10 minutes, and increase of final sample volume to 2.5 mls (EasySep® buffer). Following 5 minutes on a magnet, supernatant was removed, 2.5 mls of buffer added to the selected cells followed by further magnetic separations (repeated thrice). The final cell fraction was resuspended in CM. The median purity of the CD4+ve and CD8+ve T cell subsets obtained was 53% and 88%, respectively.

Generation of EBV-Specific CTLs

EBV-specific CTLs were established as described previously (Wilkie *et al*, 2004). Briefly, a 40:1 PBMC:γ-irradiated (4,000 rads) BLCL mixture was cultured at 1x10⁶/ml in 20% v/v FCS CM. Ten days later, T cells were restimulated at a ratio of 4:1 T cells:γ-irradiated autologous BLCL followed four days later by addition of 20 IU/ml of recombinant interleukin (IL) 2 (rIL2). From this timepoint (14 days) onwards, T cell cultures were restimulated weekly with γ-irradiated autologous (4:1 ratio) BLCLs together with a thrice weekly dose of rIL2. At time of weekly stimulation, T cell concentration was adjusted to 1x10⁶ cells/ml to ensure optimum growth conditions.

Flow Cytometry

Flow cytometry was carried out using standard methods. Briefly, working dilutions of directly-conjugated monoclonal antibodies (mabs) were added to cells suspended in 50 µl of flow buffer (FB: phosphate-buffered saline containing 1% w/v bovine serum albumin, 5mM EDTA and 0.1% w/v sodium azide) and the mixture incubated at 4°C for 20 minutes in the dark. Following FB washes, immunostained cells were resuspended in x1 CellFIX™ (BD Biosciences, Oxford, UK). Flow cytometric acquisition (and analysis of 1,000-10,000 acquired events per sample) was performed using the 'CellQuest Software' on a FACSCalibur machine (Becton Dickinson, Oxford, UK).

Cytotoxicity Assay

A standard 4 hour ⁵¹Cr-release assay was carried out as described previously (Wilkie *et al*, 2004). Briefly, target cells were labelled with 50 µCi of ⁵¹Cr for 1 hour at 37°C, and plated out with effector cells at a 5:1, 10:1 and 20:1 effector-to-target ratio in triplicate wells. Cell mixtures were incubated for 4 hours at 37°C, and ⁵¹Cr release measured in a γ-counter. Spontaneous release was assessed by incubating target cells without effector cells, and maximum release determined by incubating target cells with 1% v/v Triton-X (Sigma, Gillingham, UK). The % specific cell lysis was calculated as follows (results were expressed as an average of readings from triplicate wells):

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% Specific lysis =
$$\frac{\text{Test release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

Histopathology and Immunohistochemistry

Paraffin wax-embedded tissues were dewaxed and rehydrated. Haematoxylin and eosin (H&E) staining was carried out using standard methods. Immunostaining included an initial antigen retrieval step (using ‘Target Retrieval Solution’ or ‘Antigen Retrieval Solution’; DakoCytomation, Ely, UK). Sections were immunostained with primary mabs (in ‘Antibody Diluent, Background Reducing’; DakoCytomation, Ely, UK) for 30 minutes at room temperature, washed in tris-buffered saline (TBS), and ‘EnVision™’ (DakoCytomation, Ely, UK) and ‘NovoLink™ Polymer Detection System’ (Novocastra, Newcastle Upon Tyne, UK) kits used to visualize bound antibody following manufacturers’ instructions. Sections were counterstained in Mayer’s Haemalum, dehydrated (if appropriate), mounted and coverslipped. For quantification, numbers of mab-labelled and -unlabelled cells were counted in 5 high power (x1,000 under oil) fields and results expressed as % mab+ve cells.

EBER In Situ Hybridization (ISH)

‘Epstein-Barr Virus (EBER) PNA Probe/Fluorescein’ and ‘PNA ISH Detection Kit’ (DakoCytomation, Ely, UK) kits were used in accordance with the manufacturer’s instructions. Briefly, 0.1 mg/ml proteinase-K was applied to dewaxed and rehydrated paraffin wax-embedded sections for 30 minutes. A FITC-labelled PNA probe

complimentary to EBER1 and 2 was applied at 55°C for 1.5 hours, sections immersed in a 55°C working solution (1:60 in pure water) of 'Stringent Wash' for 25 minutes, and incubated with 'Anti-FITC/AP' for 30 minutes. Bound probe was visualized by an AP 'Substrate' (BCIP/NBT) followed by counterstaining in Mayer's Haemalum, mounting and coverslipping.

SCID Mice

CB.17 *scid/scid* mice were bred at the University of Edinburgh under specific pathogen free conditions in individually ventilated microisolator cages and handled in microbiological class 2 safety cabinets. All animal work was carried out under relevant Home Office Project and Personal Licences in accordance with the Home Office 'Animals (Scientific Procedures) Act 1986', and monitored by Home Office Inspectors and Named University Veterinary Surgeons. Mice were observed twice daily and culled if sick (defined as disinterest in their surroundings, hunched back, respiratory distress and/or ruffled fur), or after 100 days. Tissues were harvested at necropsy and fixed in neutral buffered formalin (NBF).

Statistical Analysis

Advice on statistical analysis and presentation of data was sought from public health departments of the London School of Hygiene & Tropical Medicine and the University of Edinburgh. Tumour incidence was analysed by the Fisher's exact test (2-tailed p value). Time to tumour was assessed by the Kruskal, Mann-Whitney and Spearman's tests. 'GraphPad Prism' software (GraphPad Software Inc, La Jolla, USA)

was used for data analysis and $p \leq 0.05$ was considered significant. Datapoints on survival curves in Figures 2 and 4 reflect donor-derived results obtained as triplicates since groups of 3 SCID mice were used for each test and control group. Standard Error (SE; shown as error bars on graphs) was calculated as: $SE = SD_{n-1} / \sqrt{n}$ (where SD is the standard deviation and n denotes the number of samples analyzed). Similar to survival curves, data presented in Tables 2 and 3 represent results obtained for each donor in triplicates (ie, each control and test group consisted of 3 SCID mice).

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RESULTS

The aim was to assess the ip hu-BLCL-SCID model for tumour prevention as a tool to assess safety and efficacy of novel CTL-based immunotherapy. The model was used to investigate the role of T cell subsets and cytokines in PTLD therapeutic regimes.

A panel of 25 BLCLs (and T cell subsets; see below) was generated by *in vitro* EBV infection of PBMCs from 25 healthy EBV-seropositive blood donors (denoted 'donor 1-25'; for MHC typing results, please refer to Table 1). BLCLs were transferred ip into SCID mice to study tumour prevention.

Establishment of Ip Hu-BLCL-SCID Model: Tumour Prevention

In this model, tumour prevention is used as experimental read-out. Firstly, the minimum cell number required for consistent BLCL-driven ip tumour formation *in vivo* was assessed by inoculating groups of 3 animals ip with 1×10^6 , 2×10^6 or 4×10^6 BLCL cells from our BLCL panel. All mice inoculated ip with either 2×10^6 or 4×10^6 BLCL cells developed macroscopic ip tumours in a median time of 6.5 and 6 weeks, respectively, whereas an inoculum of 1×10^6 BLCL cells produced ip tumours in 40% of animals in a median time of 6 weeks. Therefore, in this model, ip tumours were induced in all further experiments with a standard 2×10^6 BLCL dose. In parallel control experiments, PBMCs from 3 EBV-seronegative healthy donors did not give rise to ip tumours. Previously, the effect of depleting endogenous (murine) NK cells (using rabbit anti-mouse-ASGM1 antiserum) on ip tumour development in SCID mice has been investigated and found not to impact significantly on tumour formation *in vivo*

(Johannessen *et al*, 2000). Therefore, we do not administer routinely an anti-NK antiserum to our animals.

Analysis Of Tissues And Tumours

Paraffin wax-embedded tissue sections from lung, liver, spleen and tumour tissue from a panel of 10 ip mice inoculated with BLCL from different donors (donors 1-10; see Table 1) were screened using H&E staining, anti-human CD45 and CD20 immunostaining, *in situ* hybridization for EBERs, and immunostaining for EBV latent (EBNA2, LMP1) and immediate early (IE) lytic (BZLF1) antigens (see Figure 1). Macroscopic ip tumours formed primarily as solitary nodules at the undersurface of the liver. (1) **H&E**: Sections were scored for distortion of normal tissue morphology and cellular infiltrations. Large immunoblastic tumour cells were detected consistently in ip tumour tissue only. (2) **Human CD45 and CD20**: Scattered human CD45+ve, CD20+ve cells were found only rarely in lung, liver and spleen. Conversely, such cells formed ip tumour tissue. (3) **EBERs**: Scattered EBER+ve cells were found only rarely in lung, liver and spleen. Conversely, such cells formed ip tumour tissue. (4) **EBV Antigens**: EBER+ve cells in tumour tissue were found to express the latent EBNA2 and LMP1 antigens with the occasional cell expressing the IE lytic BZLF1 lytic antigen.

Characterisation Of In Vitro Expanded T Lymphocytes

EBV-specific T cells were expanded from PBMCs from a panel of 25 EBV seropositive donors (donors 1-25; see Table 1) by culture *in vitro*. Cells were harvested after 7-17 weeks (median: 10 weeks) of culture at which time the percentage of cells

expressing the following markers were delineated by flow cytometry and the medians calculated: CD4+ve median 6%, CD8+ve median 84%, CD56+ve median 1%, CD19+ve <1%. In parallel, cytotoxic function was assessed by a 4 hour standard ^{51}Cr -release assay with target cells including autologous and allogeneic (MHC mismatched) BLCLs and K562. Median specific cell lysis at a 10:1 effector:target ratio were 36% for the autologous BLCL, 6% for the allogeneic BLCL, and 7% for K562. The differences between median % specific lysis for autologous *versus* allogeneic targets and the NK cell target K562 were significant (Mann-Whitney test: $p < 0.0001$ on both occasions).

Model For Tumour Prevention

Using 10 donors (donors 1-10; see Table 1), 2×10^6 BLCL were inoculated ip into each mouse in groups of 3 SCID mice followed 1 hour later by inoculation ip of either suspension medium (vehicle) or a median of 50×10^6 (range: 40×10^6 - 50×10^6) autologous CTLs. Animals were culled when showing signs of sickness (see Materials and Methods), or at a pre-determined time limit of 100 days (in line with previous experience; Johannessen *et al*, 2000). On each occasion, a sick mouse was found to harbour ip macroscopic tumour at necropsy. The percentages of mice in each group that developed ip tumours are shown in Table 2. Individual results (together with Log-rank test results) are shown in Figure 2. Tumours formed in 29 (97%) and 26 (87%) out of 30 mice injected with vehicle only and CTLs, respectively, and, thus, there was no significant difference between the 2 groups of animals in terms of the proportion of mice that developed ip tumours (Fisher's exact test: $p = 0.353$). In contrast, mice inoculated with CTLs survived significantly longer than control animals (Mann-Whitney test:

p=0.001; Log-rank test: p=0.003) and, therefore, their tumour development was delayed when compared to control mice. However, this effect was lost when ip CTL inoculation was deferred for 3 weeks following ip BLCL transfer (Log-rank test: p=0.60; Data not shown). Furthermore, administration of mismatched CTLs did not have a significant impact on tumour development *in vivo* (Log-rank test: p=0.77; Data not shown) indicating that tumour prevention is MHC restricted.

Role of CD8+ve T Cell Subsets

Using the same 10 donor CTLs (donors 1-10; see above), we enriched for CD8+ve T cells using magnetic beads. For each donor, 2x10⁶ BLCL were inoculated ip into each mouse in groups of 3 SCID mice followed 1 hour later by inoculation ip of CD8+ve enriched T cell populations. Thus, each of 3 mice received ip a median of 37x10⁶ (range: 3x10⁶-50x10⁶) CD8+ve enriched T cells. Animals were culled when showing signs of sickness (see Materials and Methods), or at a pre-determined time limit of 100 days. On each occasion, a sick mouse was found to harbour ip macroscopic tumour at necropsy. The percentages of mice in each group that developed ip tumours are shown in Table 2. Individual results (together with Log-rank test results) are shown in Figure 2A-C. Tumours developed in 18 out of 30 (60%) mice injected with CD8-enriched T cells. Comparing the results with control mice inoculated with vehicle only (see Table 2 and Figure 2), significantly fewer animals injected with CD8-enriched T cells developed ip tumours (Fisher's exact test: 0.001). Furthermore, CD8-inoculated mice survived significantly longer than controls (Log-rank test: p=0.0002). Whilst groups of mice were also inoculated with a median of 7x10⁶ (range: 3x10⁶-67x10⁶)

autologous CD4-enriched T cells, it was not possible to compare the efficacy of CD4-inoculated mice with that of CD8-injected ones since CD4-enrichment yielded fewer cells *in vitro* than the CD8 one. Thus, although no significant difference was found between mice inoculated with CD4-enriched T cells and control animals injected with vehicle only when assessing tumour formation (Fisher's exact test: $p=0.195$) and survival (Log-rank test: $p=0.101$), a valid comparison with CD8-injected mice could not be carried out.

Taken together, mice inoculated with CTLs, or CD8-enriched T cells, survived significantly longer than control animals (Mann-Whitney test: $p=0.001$ for both analysis; Log-rank test: $p=0.003$ and $p=0.0002$, respectively). Conversely, the use of CD4-enriched T cells did not impact significantly on tumour development *in vivo* (Log-rank test: $p=0.101$). Since the proportion of CD4+ve cells in the transferred inoculum differed between donors, we re-analysed the results using data obtained when using CD4-enriched cells from the 5 donors whose CD4-enriched populations contained the highest level of CD4+ve cells (a median of 74%; range: 70-97%; see Figure 2D). The transfer of CD4-enriched cells from those individuals did not impact significantly on tumour development *in vivo* (Log-rank test: $p=0.35$) which is in line with the overall results described above.

The *in vitro* cell separation manipulation *per se* did not affect significantly CTL performance *in vivo* as determined in parallel control studies. For each of 3 donors, 2×10^6 BLCLs were inoculated ip into each mouse in 2 groups of 3 SCID mice each. Whilst 50×10^6 unfractionated autologous CTLs were inoculated ip into each animal in the control group, each mouse in a test group received 50×10^6 CTLs that had been

fractionated into CD4+ve and CD8+ve T cell subpopulations using magnetic beads and recombined in the original CTL subset proportions. Unfractionated CTLs and fractionated/recombined CTLs had a similar effect *in vivo* (Log-rank test: $p=0.71$; Data not shown).

In the present study, the CD4- and CD8-enriched T cell populations were not pure populations so calculations of the total number of these cells inoculated in each experiment were carried out. A non-correlation was found between the numbers of CD4+ve, or CD8+ve, T cells (included in the inoculated CTL, CD4-enriched, or CD8-enriched T cells) and the average % survival (in days) beyond controls (Spearman's test: $p>0.05$ for all analyzes). Similar results were obtained when analysing the CD8:CD4 ratio of cells inoculated (Spearman's test: $p>0.05$ for all analyzes). Therefore, it does not appear that survival of SCID mice treated with autologous CTLs, or T cell subsets, is a direct function of the number of transferred CD4+ve or CD8+ve T cells or their ratio. The level of CTL cytotoxicity against the autologous BLCL (as assayed *in vitro* by ^{51}Cr -release studies) did not correlate with survival of CTL-inoculated SCID mice (Spearman's test: $p=0.17$; Data not shown) and was therefore not a useful parameter for predicting survival.

***In Vivo* Trafficking of Human T Cells**

We analysed samples of lung, liver, spleen, and tumour harvested from the CTL-treated hu-BLCL-SCID mice (see above) to look for human T cell trafficking within these tissues. The percentage of specific antibody+ve cells on immunostained sections was assessed by attributing a score of (-), (+), 1+, 2+ and 3+, indicating that none, <1%,

1-29%, 30-60%, or >60% of all cells counted were antibody+ve cells, respectively. Tissues were obtained at necropsy from 32 ip [harvested at 17-73 (median: 32) days after ip CTL inoculation] hu-BLCL-SCID mice (see Figure 3). Human CD3+ve T cells were found in 17 out of 32 (53%) tumour samples tested. Whilst 2 of these 17 (12%) samples contained CD4+ve T cells, 11 (65%) had detectable CD8+ve T cells and 1 (6%) contained CD4+ve and CD8+ve T cells. Three (18%) samples contained only CD3+ve cells. Overall, T cells comprised <1-29% of each T marker-positive tumour section. Human cells were not detected in control mice. The data suggest that human T cells are able to home to human tumour tissue *in vivo*. Since human CD45+ve cells were detected only very rarely in lung, liver and spleen tissue, these samples were not analysed further. Immunostaining of the murine pan-nucleated cell marker MHC1 on tumour sections showed no (or the very occasional) murine cell underlining further that the lesions were human in origin (Data not shown).

Serial sections from the 17 ip tumours shown to contain CD3+ve T cells (see above) were stained for perforin and granzyme B cytolytic molecules (see Figure 3). Six out of 17 (35%) tumours contained granzyme B+ve cells and a further 8 out 17 (47%) contained perforin+ve cells. Overall, 14 out of 17 (82%) tumour samples expressed one or other cytotoxic granule molecule with no dual expression noted.

Cytokine Conditioning

Cytokines that signal through the common γ -chain (IL2, 4, 7, 15 and 21) are important for the homeostasis of CD4+ve and CD8+ve T cells with the ability to enhance their homing and promote their expansion, function and survival *in vivo* (Ku *et*

al, 2000; Schluns *et al*, 2000). The effect on survival of CTL cytokine conditioning *in vitro* prior to transfer *in vivo* into ip hu-BLCL-SCID mice was examined. CTL lines (day -1) from 5 donors (donors 11-15; see Table 1) were cultured in medium containing 20% v/v FCS and human IL2 (20 IU/mL) with the addition of IL7, 15, 21, 7 and 15, or 7 and 21, at a final concentration of 10 ng/mL for 24 hours prior to washes, reconstitution in suspension medium, and inoculation into mice (day 0; Parada *et al*, 1998; Ayyoub *et al*, 2002).

Two million cells from each donor BLCL were inoculated ip into each mouse in groups of 3 SCID mice each. This was followed 1 hour later by inoculation ip of suspension medium (vehicle) only into one group of 3 animals, and 50×10^6 autologous CTLs that had been conditioned with cytokines for 24 hours *in vitro* into further groups of 3 SCID mice. Individual results showing the percentage survival over time (in days) together with log-rank test results is shown in Figure 4A-E. When SCID mice were inoculated with CTLs conditioned *in vitro* with IL2 together with IL7, 15, 21, 7 and 15, or 7 and 21, animals that received CTLs conditioned with a combination of IL7 and 15 survived significantly longer than control mice inoculated with CTLs conditioned with IL2 only (Log-rank test: $p=0.04$). Furthermore, tumours developed in 6 out of 15 (40%) mice that received such IL7/15-conditioned CTLs which is significant protection against tumour development when compared with tumour formation in 13 out of 15 (87%) control mice inoculated with IL2-conditioned CTLs (Fisher's exact test: $p=0.02$; see Figure 4D). In contrast, there was no significant difference between the survival curves of animals that received CTLs conditioned *in vitro* with IL2 together with IL7, 15, 21, or

7 and 21, when compared with control mice (Log-rank test: $p=0.96$, 0.67 , 0.52 , 0.75 , respectively).

The EBV-specific cytotoxicity *in vitro* of CTL lines on day 0 was assessed and no significant difference found between median % specific lysis when comparing autologous targets conditioned with IL2 alone, or IL2 combined with IL7, 15, 21, 7 and 15, or 7 and 21 (Kruskal-Wallis test: $p=0.74$; Data not shown).

In order to assess further the role of IL2 for CTL function, 2×10^6 BLCL cells from one of 6 donors (donors 11-16; see Table 1) were inoculated ip into each mouse in groups of 3 SCID mice each. One hour later, 50×10^6 autologous CTLs that had been conditioned for 24 hours *in vitro* with IL2 were inoculated ip into groups of 3 SCID mice. This was followed by inoculations ip of either 250 IU of IL2 or vehicle only. Mice were dosed daily for 14 days following ip transfer of BLCL cells. Individual results showing the percentage survival over time (in days) together with log-rank test results is shown in Figure 4F and the percentages of mice in each group that developed ip tumours are shown in Table 3. CTL-inoculated animals that received IL2 daily survived significantly longer than control mice dosed with vehicle only (Log-rank test: $p=0.004$). Tumours developed in 4 out of 18 (22%) such mice dosed with IL2 whereas 12 out of 18 (67%) control mice dosed with vehicle only developed ip tumours which is a significant difference (Fisher's exact test: $p=0.02$). Taken together, these results suggest a key role for IL2 in supporting CTL function *in vivo*.

DISCUSSION

The importance of animal modelling has not diminished over the years despite efforts to offer alternative *in vitro* options, and pre-clinical data obtained in suitable animal models are still vital to guide translation into the clinical setting. Whilst SCID mice are a small animal model that is widely used to study human cancer (including PTLTD), there is as yet no international standard defining how the model should be used to ensure high quality data. In the current study, we used an ip hu-BLCL-SCID mouse tumour prevention model because of its suitability for testing of novel immunotherapy against PTLTD.

Boyle *et al* (1993) engrafted SCID mice ip with BLCL and showed that autologous CTL administered simultaneously (or 7 days later) delayed significantly tumour formation whereas mismatched CTLs failed to do so, indicating the MHC restriction of CTLs. In the present study, administration of autologous, but not allogeneic, CTLs in the ip hu-BLCL-SCID model similarly delayed tumour development and improved survival time, and further support for this tumour prevention model comes from studies by DiMaio *et al* (1995) and Buchsbaum *et al* (1996).

The current study demonstrates that ip hu-BLCL-SCID mice give rise reliably to macroscopic EBV+ve human B immunoblastic ip tumours portraying full (unrestricted) latent virus gene expression with the occasional tumour cell in lytic cycle. In this prevention model, ip tumour formation is the experimental read-out and autologous CTLs can be detected in tumour tissue. In parallel studies, we have also investigated the

subcutaneous (sc) hu-BLCL-SCID model in which transfer of 2×10^6 BLCL sc into flanks of SCID mice gives rise consistently to visible sc PTLT-like tumours that can be measured directly. Theoretically, such a model can be used to assess the efficacy of novel PTLT immunotherapy when treatment is delivered by intravenous (iv) inoculation. However, such a tumour regression model poses technical challenges that undermine its use. In particular, in the sc model, sc tumours may arise at different timepoints after inoculation making the experimental protocol for T cell therapy difficult to plan. Furthermore, the transfer of large T cell inocula iv can be problematic and it is difficult to ensure successful injection of the large T cell numbers required for analysis. The ip hu-BLCL-SCID model used herein circumvents these difficulties and is technically straight-forward to manage. However, since ip tumour development can not be monitored clinically in this model, it is a prevention model with BLCLs and CTLs given 1 hour apart, a situation that does not reflect accurately the clinical setting. Whilst we have introduced MRI imaging of our animals to mimic better the clinical (patient) situation with a view to provide a non-invasive, non-destructive method of monitoring ip tumour development in longitudinal studies on the same animals, this approach has yet to prove its value in face of technical challenges in demarcation of small lymphoid lesions by MRI scanning of SCID mice. Taken together, translational data can be obtained in the ip prevention SCID mouse model that is difficult to generate in other SCID mouse models and such data can be used to inform clinical (patient) protocols.

Using the ip prevention hu-BLCL-SCID model, CD8+ve T cell subpopulations from CTLs from across the donor panel delayed significantly tumour development, and tumour formation was entirely prevented in 40% of mice (see Table 2). Boyle *et al*

(1993) also used CD8-enriched CTL populations in this model with significant effect on tumour development. Whilst inoculation of CD4-enriched T cells did not have a significant impact on survival in this model (see Figure 2), the data is too limited to draw conclusions from this set of experiments. Interestingly, in the hu-PBMC-NOD/SCID model (NOD/SCID animals are deficient in NK as well as B and T cells), treatment of mice with a depleting anti-human CD4 mab results in loss of both CD4+ve and CD8+ve T cells (Wagar *et al*, 2000) suggesting at least a supporting role to CD8+ve T cells for CD4+ve lymphocytes. Since the CD4-enriched T cell populations transferred into our animals consisted of smaller total T cell numbers than the CD8-enriched inocula, and there was no significant difference in the rate of ip tumour development between mice inoculated with bulk CTLs and animals that received CD4-enriched cells only, the latter may still have an anti-tumour effect or work to prevent CD8+ve T cell-mediated anti-tumour mechanisms (see Results). Thus, the data must be interpreted with a certain degree of caution.

CD4+ve T cells show cytotoxic function *in vitro* and *in vivo* (Appay, 2004), and since PTLN cells express MHC2 molecules (Thomas *et al*, 1990), direct CD4+ve T cell-mediated cytotoxicity is possible. However, CD4+ve T cells also have a variety of helper functions and in a recent clinical trial using allogeneic ‘best MHC match’ CTL treatment for PTLN, a significant trend for a better outcome with higher numbers of CD4+ve T cells in infused CTL lines was interpreted as the effect of the few CD4+ve T cells in the CTLs in enhancing the survival *in vivo* of the CD8+ve population (Haque *et al*, 2007).

Discrepancies exist between the murine and human situation. Furthermore, in the ip hu-BLCL-SCID model, the most effective combination of CD4+ve and CD8+ve T cells has not yet been identified. Interestingly, studies in our laboratory have shown that PBMC-inoculated mice (hu-PBMC-SCID) deplete of T regulatory (Treg) cells develop PTLN-like tumours at a similar rate to Treg-replete PBMC populations which conflicts with current ideas that Treg cells play a major role in tumour immune escape (Data not shown). These results highlight further the discrepancies that exist between the murine and human situation.

Human T cells were detected in T cell treated animals for up to 64 days which is in line with observations by others underlining that T cells can home to PTLN-like tumours in SCID mice. Thus, Lacerda *et al* (1996) showed preferential homing of CD8+ve T cells to autologous (and not MHC mismatched) tumour tissue as did imaging studies by Koehne *et al* (2003) using radioactive labelling of human EBV-specific T cells. Whilst our immunostaining results support a role for the cytotoxic molecules perforin and granzyme B, the data must be interpreted with a certain degree of caution.

A combination of IL7 and 15 enhanced the ability of CTLs from across the donor panel to mediate tumour prevention *in vivo* although individually IL7, 15, and 21 did not (Figure 4). Furthermore, a combination of IL7 and 21 did not have an impact *in vivo*. It is postulated that a combination of IL7 and 15 improved the ability of the *in vitro* conditioned T cells to home to, and mediate destruction of, human PTLN-like tumour cells *in vivo* which is in line with recent *in vivo* data using IL15 (Klebanoff *et al*, 2004). However, since a correlation between the effects of the cytokines with expression of their relevant receptors by CTLs at the time of *in vitro* cytokine conditioning was not

carried out, the results should be interpreted with a degree of caution. Whilst *in vitro* cytokine conditioning did not affect significantly CTL cytotoxicity *in vitro*, additional results from this study (as well as data from clinical studies in our laboratory; Haque *et al*, 2007) suggest that *in vitro* cytotoxicity does not correlate with CTL function *in vivo*.

Previous studies have given conflicting results when examining a possible role of cytokine administration in sustaining transferred T cells *in vivo* (Boyle *et al*, 1993; Rencher *et al*, 1994; Baiocchi *et al*, 1994, 2001). However, in line with data from other laboratories (Baiocchi *et al*, 1994, 2001), the current study underlines a supporting role for IL2 in facilitating CTL survival and function *in vivo* (see Figure 4F and Table 3) although the results do not exclude possible contribution from IL2-mediated activation of murine NK cells. Whilst the results suggest inclusion of IL2 in immunotherapeutic regimes using CTLs against PTLD, a degree of caution must be exercised in the use of the cytokine in a patient population due to its ability to cause side effects.

Our laboratory has successfully infused PTLD patients with EBV-specific CTLs on a 'best MHC match' basis (Haque *et al*, 2002, 2007). Although this *proof-of-principle* trial showed that CTL therapy could succeed, a mechanism for bypassing MHC restriction would facilitate its dissemination to the clinic. A chimeric T cell receptor (cTCR) can be constructed that directs T cells against a novel surface antigen in a non-MHC restricted manner (Schumacher, 2002; Mansoor *et al*, 2005), and currently our laboratory employs such an approach to re-target CTLs to EBV-associated cancers. This novel therapeutic approach will be tested in the pre-clinical ip hu-BLCL-SCID model presented in the current study.

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FIGURE 1
Immunophenotyping Of SCID Mouse Tumours:
Human Leukocyte Markers And EBV Transcripts
(Paraffin Wax-Embedded Sections)

Figures 1A,B: Photographs of the peritoneal cavity of ip inoculated control (Figure 1A: suspension medium only) and test (Figure 1B: BLCL) SCID mice taken at necropsy. Arrow indicates ip BLCL-derived tumour in test mouse (Figure 1B). **Figures 1C** (x100), **D** (x400): Photomicrographs of H&E staining of tumour sections showing large lymphoblastoid tumour cells with large nuclei (blue haematoxylin staining) and scant cytoplasm. **Figures 1E,F** (x400): Photomicrographs of immunostaining of the human pan-leukocyte marker CD45 on tumour sections using an alkaline phosphatase (AP) label. Figure 1E shows CD45+ve human cells (red membrane staining) counterstained with Mayer's haemalum. Figure 1F shows the conjugate control with counterstained cells only. **Figures 1G,H** (x400): Photomicrographs of immunostaining of the human pan-B cell marker CD20 on tumour sections using a peroxidase (HRP) label. Figure 1G shows CD20+ve human cells (brown membrane staining) counterstained with Mayer's haemalum. Figure 1H shows the conjugate control with counterstained cells only. **Figures 1I,J** (x400): Photomicrographs of *in situ* hybridization for EBERs on tumour sections using an alkaline phosphatase (AP) label. Figure 1I shows EBER+ve cells (dark blue/black nuclear staining) on a probed tumour section which was counterstained with Mayer's haemalum (blue nuclear staining). Figure 1J shows the counterstained unprobed (negative) control tumour section.

The panels show a single mouse experiment but are representative of several experiments.

1A: Necropsy (Normal) **1B: Necropsy (Ip Tumour)** **1C: H&E** **1D: H&E** **1E: CD45** **1F: CD45 Control** **1G: CD20** **1H: CD20 Control**

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1I: EBERs

IJ: EBERs Control

For Peer Review

FIGURE 2
% Survival Using CTLs And T Cell Subsets

(Datapoints reflect results in triplicates; Standard error, SE, is shown on each column as a vertical bar.)

2A) CTLs

(Log-rank test: $p=0.003$)

2B) CD4-Enriched Cells

(Log-rank test: $p=0.101$)

2C) CD8-Enriched Cells

(Log-rank test: $p=0.0002$)

2D) CD4-Enriched Cells (Subset)

(Log-rank test: $p=0.35$)

FIGURE 3
Immunophenotyping Of SCID Mouse Tumour Tissue:
Human Leukocyte and Cytotoxic Molecule Markers

Figures 3A,B (x400): Photomicrographs of immunostaining of the human pan-leukocyte marker CD45 on tumour sections using an alkaline phosphatase (AP) label. Figure 3A shows CD45-positive human cells (red membrane staining) counterstained with Mayer’s haemalum. Figure 3B shows the conjugate control with counterstained cells only. **Figures 3C,D** (x400): Photomicrographs of immunostaining of the human pan-T cell CD3 marker on tumour sections using an alkaline phosphatase (AP) label. Figure 3C shows CD3-positive human cells (red membrane staining) counterstained with Mayer’s haemalum. Figure 3D shows the conjugate control with counterstained cells only. **Figures 3E,F** (x400): Photomicrographs of immunostaining of the human T helper cell CD4 marker on tumour sections using an alkaline phosphatase (AP) label. Figure 3E shows CD4-positive human cells (red membrane staining) counterstained with Mayer’s haemalum. Figure 3F shows the conjugate control with counterstained cells only. **Figures 3G,H** (x400): Photomicrographs of immunostaining of the human T cytotoxic CD8 marker on tumour sections using an alkaline phosphatase (AP) label. Figure 3G shows CD8-positive human cells (red membrane staining) counterstained with Mayer’s haemalum. Figure 3H shows the conjugate control with counterstained cells only. **Figures 3I,J** (x400): Photomicrographs of immunostaining of the cytolytic granule molecule perforin on tumour sections using a peroxidase (HRP) label. Figure 3I shows perforin-positive human cells (brown cytoplasmic staining) counterstained with Mayer’s haemalum. Figure 3J shows the conjugate control with counterstained cells only. Arrows point to examples of specific antibody+ve cells.

3A: CD45 **3B: CD45 Control** **3C: CD3** **3D: CD3 Control** **3E: CD4** **3F: CD4 Control** **3G: CD8** **3H: CD8 Control**

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3I: Perforin

3J: Perforin Control

For Peer Review

FIGURE 4**% Survival Using Cytokine Conditioned CTLs**

(Datapoints reflect results in triplicates; Standard error, SE, is shown on each column as a vertical bar.)

4A) IL2 vs IL7 Conditioning (Log-rank test: $p=0.96$)**4B) IL2 vs IL15 Conditioning** (Log-rank test: $p=0.67$)**4C) IL2 vs IL21 Conditioning** (Log-rank test: $p=0.52$)**4D) IL2 vs IL7/15 Conditioning** (Log-rank test: $p=0.04$)**4E) IL2 vs IL7/21 Conditioning** (Log-rank test: $p=0.75$)**4F) IL2 vs Vehicle Daily Dosing** (Log-rank test: $p=0.004$)

TABLE 1
MHC Profile of 25 BLCL and T Cell Sample Sets

Donor No	A		B		C		DR	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
1	03	31	27	45	01	06	NT	NT
2	24	68	14	15	03	08	NT	NT
3	23	23	44	49	04	07	NT	NT
4	02	29	44	44	NT	NT	01	07
5	02	32	07	14	NT	NT	07	15
6	01	31	08	44	NT	NT	07	17
7	01	03	07	14	07	08	NT	NT
8	01	02	35	73	04	15	NT	NT
9	03	68	40	44	03	08	NT	NT
10	01	29	07	08	07	07	NT	NT
11	01	03	07	07	NT	NT	04	15
12	02	68	50	60	NT	NT	04	17
13	02	11	27	44	NT	NT	01	14
14	03	03	51	60	NT	NT	04	15
15	11	32	27	62	NT	NT	01	11
16	02	32	44	44	05	16	04	07
17	02	02	08	44	05	07	03	13
18	01	02	08	44	05	07	04	15
19	02	31	35	51	NT	NT	07	17
20	02	03	40	44	03	05	NT	NT
21	23	25	44	45	NT	NT	04	12
22	01	32	08	35	NT	NT	01	17
23	02	02	37	44	NT	NT	01	07
24	01	02	07	57	NT	NT	07	09
25	01	02	08	57	NT	NT	01	07

BLCL: B lymphoblastoid cell line; MHC: Major histocompatibility complex; No: Number; NT: Not tested;

TABLE 2
Ip Tumour Development

Inoculum	Medium Only	CTL	CD4+	CD8+
Number of Tumours/ Number of Mice Injected (%)	29/30 (97)	26/30 (87)	25/30 (83)	18/30* (60)

*: Statistically significant difference (p=0.001) when compared with mice inoculated with medium only; CTL: Cytotoxic T lymphocytes; Ip: Intraperitoneal; Data reflects results obtained for each donor in triplicates (ie, groups of 3 SCID mice).

TABLE 3
Ip Tumour Development

Inoculum	Medium Only	CTL	CTL+IL2
Number of Tumours/ Number of Mice Injected (%)	12/18 (67)	12/18 (67)	4/18* (22)

*: Statistically significant difference ($p=0.02$) when compared with mice inoculated with medium or CTL only; CTL: Cytotoxic T lymphocytes; IL: Interleukin; Ip: Intraperitoneal; Data reflects results obtained for each donor in triplicates (ie, groups of 3 SCID mice).