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# 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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**ABSTRACT**

1  
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7 Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disease  
8 (PTLD) arises in up to 10% of organ transplant recipients and is fatal in ~50% of cases.  
9  
10 PTLD can be modelled in SCID mice using EBV+ve human B lymphoblastoid cell lines  
11 (BLCLs), and the current study investigated intraperitoneal (ip) inoculation of such  
12  
13 animals in experiments which assessed the effect of EBV-specific cytotoxic T  
14  
15 lymphocytes (CTLs) and cytokines on PTLD growth.  
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20  
21 Ip transfer of 1 dose of autologous CTLs, or CD8-enriched T cells, into ip  
22  
23 BLCL-inoculated animals significantly delayed tumour development (p=0.001) and  
24  
25 prevented tumour formation in a significant proportion (40%) of mice (p=0.001). A  
26  
27 combination of IL2, 7 and 15 conditioning of CTLs prior to ip injection significantly  
28  
29 delayed ip BLCL-derived tumour formation *in vivo* when compared to CTLs expanded  
30  
31 *in vitro* using only IL2 (p=0.04) and prevented tumour outgrowth in a significant  
32  
33 proportion (60%) of mice (p=0.02). Daily ip IL2 dosing of ip CTL-inoculated mice  
34  
35 significantly delayed tumour development *in vivo* (p=0.004) and prevented tumour  
36  
37 outgrowth in a significant proportion (78%) of mice (p=0.02) when compared to animals  
38  
39 dosed with vehicle only.  
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45 In SCID mice, autologous CTLs, and CD8-enriched T cells, have significant  
46  
47 capacity to hinder development of PTLD-like tumours. Whilst studies are needed to  
48  
49 delineate the role of cytokine conditioning and CD4-enriched T cells, the results suggest  
50  
51 that IL2 plays a key role in supporting CTL function *in vivo*.  
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## 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  
2 characterized by rapid onset, aggressive behaviour, and approximately 50% mortality  
3  
4 despite current treatment (Williams & Crawford, 2006). The major risk factors are the  
5  
6 degree and duration of immunosuppression required for graft maintenance and primary  
7  
8 EBV infection following transplantation (Thomas *et al*, 1995).  
9  
10

11 The majority of PTLD express all EBV latent antigens and the tumour is  
12  
13 generally susceptible to EBV-specific CTL destruction. Adoptive CTL immunotherapy  
14  
15 provides rapid, targeted treatment that is well tolerated and effective in preventing and  
16  
17 treating PTLD (Rooney *et al*, 1998; Haque *et al*, 2007). However, a better understanding  
18  
19 of the optimum CTL regime is required to realise its full potential. To this end, our  
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21 laboratory has used the SCID mouse to model PTLD (Johannessen *et al*, 2000).  
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25 Due to an autosomal mutation, SCID mice are unable to produce functional B  
26  
27 and T cell clones (Bosma *et al*, 1983) and, therefore, the animals cannot reject human  
28  
29 xenografts. Following transfer of human EBV+ve B cells, mice develop human PTLD-  
30  
31 like tumours (hu-SCID) that reflect patient lesions including expression of all EBV  
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33 latent antigens (Mosier *et al*, 1988; Johannessen *et al*, 2002). Various hu-SCID models  
34  
35 have been established, and it is not entirely clear which is most suitable for translational  
36  
37 treatment studies. Peripheral blood mononuclear cells (PBMCs) from EBV-seropositive  
38  
39 healthy donors give rise to PTLD-like lesions in a proportion of cases when cells are  
40  
41 transferred intraperitoneally (ip) into SCID mice (Picchio *et al*, 1992; Johannessen *et al*,  
42  
43 2000). However, inter-donor variability exists, and PBMCs from 25% of blood donors  
44  
45 do not form tumours in this hu-PBMC-SCID model. In contrast, *in vitro* EBV-infected B  
46  
47 lymphoblastoid cell lines (BLCLs) give rise consistently to PTLD-like lesions in the  
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49 animals (Cannon *et al*, 1990; Rowe *et al*, 1991) and such hu-BLCL-SCID models are  
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2 suited to answer questions of treatment efficacy. To this end, BLCLs can be inoculated  
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4 ip.  
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7 This study used the ip hu-BLCL-SCID model to assess the efficacy of CTLs *in*  
8  
9 *vivo* and the role of a panel of cytokines in supporting CTL function. The ip hu-BLCL-  
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11 SCID prevention model was a practical small (SCID mouse) animal model in which to  
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13 study CTL function. Using this model, CD8+ve T cell subsets prevent PTLD outgrowth  
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15 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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Buffy coats were obtained with ethical approval from the Scottish National Blood Transfusion Service (SNBTS), Edinburgh (UK). EBV serostatus was determined by anti-VCA IgG antibody titer using a standard indirect immunofluorescence assay (Henle & Henle, 1966). Donor PBMC MHC tissue typing was carried out at SNBTS (Prof M Turner) or the Anthony Nolan Research Institute (London; Prof A Dodi) using PCR-based methods.

### ***Establishment of BLCLs***

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Concentrated EBV was added to PBMCs in a total volume of 1 ml of culture medium [CM: RPMI 1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% v/v fetal calf serum (FCS)], incubated at 37°C for 1 hour, washed (in Hank's Buffered Saline Solution), resuspended ( $10^6$  cells/ml; with ciclosporin), and plated out in a 24-wells plate. During culture *in vitro*, cultures were fed CM every 2-3 days. At 4 weeks, cells were assessed directly under a light microscope for signs of immortalization, immunophenotyped, and % viability determined using trypan blue. A viability of  $\geq 70\%$  was considered acceptable.

### ***T Cell Subset Selection***

T cell subsets were positively selected from CTLs using EasySep<sup>®</sup> kits (StemCell Technologies, Grenoble, France) following the manufacturer's instructions. Briefly, 100 µl/ml EasySep<sup>®</sup> selection mix was added to  $1 \times 10^8$  cells/ml in EasySep<sup>®</sup> buffer, incubated for 15 minutes followed by addition of 50 µl/ml EasySep<sup>®</sup> magnetic dextran iron nanoparticles, a further incubation for 10 minutes, and increase of final sample volume to 2.5 mls (EasySep<sup>®</sup> 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  $1 \times 10^6$ /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  $1 \times 10^6$  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  $\mu$ l 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  $^{51}\text{Cr}$ -release assay was carried out as described previously (Wilkie *et al*, 2004). Briefly, target cells were labelled with 50  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  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  $^{51}\text{Cr}$  release measured in a  $\gamma$ -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):

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

### ***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

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2 complimentary to EBER1 and 2 was applied at 55°C for 1.5 hours, sections immersed in  
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4 a 55°C working solution (1:60 in pure water) of ‘Stringent Wash’ for 25 minutes, and  
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6 incubated with ‘Anti-FITC/AP’ for 30 minutes. Bound probe was visualized by an AP  
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8 ‘Substrate’ (BCIP/NBT) followed by counterstaining in Mayer’s Haemalum, mounting  
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10 and coverslipping.  
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### 13 14 15 16 **SCID Mice**

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18 CB.17 *scid/scid* mice were bred at the University of Edinburgh under specific  
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20 pathogen free conditions in individually ventilated microisolator cages and handled in  
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22 microbiological class 2 safety cabinets. All animal work was carried out under relevant  
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24 Home Office Project and Personal Licences in accordance with the Home Office  
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26 ‘Animals (Scientific Procedures) Act 1986’, and monitored by Home Office Inspectors  
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28 and Named University Veterinary Surgeons. Mice were observed twice daily and culled  
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30 if sick (defined as disinterest in their surroundings, hunched back, respiratory distress  
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32 and/or ruffled fur), or after 100 days. Tissues were harvested at necropsy and fixed in  
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34 neutral buffered formalin (NBF).  
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### 43 44 45 46 **Statistical Analysis**

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48 Advice on statistical analysis and presentation of data was sought from public  
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50 health departments of the London School of Hygiene & Tropical Medicine and the  
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52 University of Edinburgh. Tumour incidence was analysed by the Fisher’s exact test (2-  
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54 tailed p value). Time to tumour was assessed by the Kruskal, Mann-Whitney and  
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56 Spearman’s tests. ‘GraphPad Prism’ software (GraphPad Software Inc, La Jolla, USA)  
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2 was used for data analysis and  $p \leq 0.05$  was considered significant. Datapoints on survival  
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4 curves in Figures 2 and 4 reflect donor-derived results obtained as triplicates since  
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6 groups of 3 SCID mice were used for each test and control group. Standard Error (SE;  
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8 shown as error bars on graphs) was calculated as:  $SE = SD_{n-1} / \sqrt{n}$  (where SD is the  
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10 standard deviation and n denotes the number of samples analyzed). Similar to survival  
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12 curves, data presented in Tables 2 and 3 represent results obtained for each donor in  
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14 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  $1 \times 10^6$ ,  $2 \times 10^6$  or  $4 \times 10^6$  BLCL cells from our BLCL panel. All mice inoculated ip with either  $2 \times 10^6$  or  $4 \times 10^6$  BLCL cells developed macroscopic ip tumours in a median time of 6.5 and 6 weeks, respectively, whereas an inoculum of  $1 \times 10^6$  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  $2 \times 10^6$  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*

1  
2 (Johannessen *et al*, 2000). Therefore, we do not administer routinely an anti-NK  
3  
4 antiserum to our animals.  
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### 8 9 ***Analysis Of Tissues And Tumours***

10  
11 Paraffin wax-embedded tissue sections from lung, liver, spleen and tumour tissue  
12  
13 from a panel of 10 ip mice inoculated with BLCL from different donors (donors 1-10;  
14  
15 see Table 1) were screened using H&E staining, anti-human CD45 and CD20  
16  
17 immunostaining, *in situ* hybridization for EBERS, and immunostaining for EBV latent  
18  
19 (EBNA2, LMP1) and immediate early (IE) lytic (BZLF1) antigens (see Figure 1).  
20  
21 Macroscopic ip tumours formed primarily as solitary nodules at the undersurface of the  
22  
23 liver. (1) ***H&E***: Sections were scored for distortion of normal tissue morphology and  
24  
25 cellular infiltrations. Large immunoblastic tumour cells were detected consistently in ip  
26  
27 tumour tissue only. (2) ***Human CD45 and CD20***: Scattered human CD45+ve, CD20+ve  
28  
29 cells were found only rarely in lung, liver and spleen. Conversely, such cells formed ip  
30  
31 tumour tissue. (3) ***EBERS***: Scattered EBER+ve cells were found only rarely in lung,  
32  
33 liver and spleen. Conversely, such cells formed ip tumour tissue. (4) ***EBV Antigens***:  
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35 EBV+ve cells in tumour tissue were found to express the latent EBNA2 and LMP1  
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37 antigens with the occasional cell expressing the IE lytic BZLF1 lytic antigen.  
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### 47 48 ***Characterisation Of In Vitro Expanded T Lymphocytes***

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50 EBV-specific T cells were expanded from PBMCs from a panel of 25 EBV  
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52 seropositive donors (donors 1-25; see Table 1) by culture *in vitro*. Cells were harvested  
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54 after 7-17 weeks (median: 10 weeks) of culture at which time the percentage of cells  
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2 expressing the following markers were delineated by flow cytometry and the medians  
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4 calculated: CD4+ve median 6%, CD8+ve median 84%, CD56+ve median 1%, CD19+ve  
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6 <1%. In parallel, cytotoxic function was assessed by a 4 hour standard <sup>51</sup>Cr-release  
7  
8 assay with target cells including autologous and allogeneic (MHC mismatched) BLCLs  
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10 and K562. Median specific cell lysis at a 10:1 effector:target ratio were 36% for the  
11  
12 autologous BLCL, 6% for the allogeneic BLCL, and 7% for K562. The differences  
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14 between median % specific lysis for autologous *versus* allogeneic targets and the NK  
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16 cell target K562 were significant (Mann-Whitney test: p<0.0001 on both occasions).  
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### 20 21 22 23 **Model For Tumour Prevention**

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25 Using 10 donors (donors 1-10; see Table 1), 2x10<sup>6</sup> BLCL were inoculated ip into  
26  
27 each mouse in groups of 3 SCID mice followed 1 hour later by inoculation ip of either  
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29 suspension medium (vehicle) or a median of 50x10<sup>6</sup> (range: 40x10<sup>6</sup>-50x10<sup>6</sup>) autologous  
30  
31 CTLs. Animals were culled when showing signs of sickness (see Materials and  
32  
33 Methods), or at a pre-determined time limit of 100 days (in line with previous  
34  
35 experience; Johannessen *et al*, 2000). On each occasion, a sick mouse was found to  
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37 harbour ip macroscopic tumour at necropsy. The percentages of mice in each group that  
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39 developed ip tumours are shown in Table 2. Individual results (together with Log-rank  
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41 test results) are shown in Figure 2. Tumours formed in 29 (97%) and 26 (87%) out of 30  
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43 mice injected with vehicle only and CTLs, respectively, and, thus, there was no  
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45 significant difference between the 2 groups of animals in terms of the proportion of mice  
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47 that developed ip tumours (Fisher's exact test: p=0.353). In contrast, mice inoculated  
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49 with CTLs survived significantly longer than control animals (Mann-Whitney test:  
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2 p=0.001; Log-rank test: p=0.003) and, therefore, their tumour development was delayed  
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4 when compared to control mice. However, this effect was lost when ip CTL inoculation  
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6 was deferred for 3 weeks following ip BLCL transfer (Log-rank test: p=0.60; Data not  
7  
8 shown). Furthermore, administration of mismatched CTLs did not have a significant  
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10 impact on tumour development *in vivo* (Log-rank test: p=0.77; Data not shown)  
11  
12 indicating that tumour prevention is MHC restricted.  
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### 19 ***Role of CD8+ve T Cell Subsets***

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21 Using the same 10 donor CTLs (donors 1-10; see above), we enriched for  
22  
23 CD8+ve T cells using magnetic beads. For each donor,  $2 \times 10^6$  BLCL were inoculated ip  
24  
25 into each mouse in groups of 3 SCID mice followed 1 hour later by inoculation ip of  
26  
27 CD8+ve enriched T cell populations. Thus, each of 3 mice received ip a median of  
28  
29  $37 \times 10^6$  (range:  $3 \times 10^6$ - $50 \times 10^6$ ) CD8+ve enriched T cells. Animals were culled when  
30  
31 showing signs of sickness (see Materials and Methods), or at a pre-determined time limit  
32  
33 of 100 days. On each occasion, a sick mouse was found to harbour ip macroscopic  
34  
35 tumour at necropsy. The percentages of mice in each group that developed ip tumours  
36  
37 are shown in Table 2. Individual results (together with Log-rank test results) are shown  
38  
39 in Figure 2A-C. Tumours developed in 18 out of 30 (60%) mice injected with CD8-  
40  
41 enriched T cells. Comparing the results with control mice inoculated with vehicle only  
42  
43 (see Table 2 and Figure 2), significantly fewer animals injected with CD8-enriched T  
44  
45 cells developed ip tumours (Fisher's exact test: 0.001). Furthermore, CD8-inoculated  
46  
47 mice survived significantly longer than controls (Log-rank test: p=0.0002). Whilst  
48  
49 groups of mice were also inoculated with a median of  $7 \times 10^6$  (range:  $3 \times 10^6$ - $67 \times 10^6$ )  
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2 autologous CD4-enriched T cells, it was not possible to compare the efficacy of CD4-  
3  
4 inoculated mice with that of CD8-injected ones since CD4-enrichment yielded fewer  
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6 cells *in vitro* than the CD8 one. Thus, although no significant difference was found  
7  
8 between mice inoculated with CD4-enriched T cells and control animals injected with  
9  
10 vehicle only when assessing tumour formation (Fisher's exact test:  $p=0.195$ ) and  
11  
12 survival (Log-rank test:  $p=0.101$ ), a valid comparison with CD8-injected mice could not  
13  
14 be carried out.  
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17  
18 Taken together, mice inoculated with CTLs, or CD8-enriched T cells, survived  
19  
20 significantly longer than control animals (Mann-Whitney test:  $p=0.001$  for both analysis;  
21  
22 Log-rank test:  $p=0.003$  and  $p=0.0002$ , respectively). Conversely, the use of CD4-  
23  
24 enriched T cells did not impact significantly on tumour development *in vivo* (Log-rank  
25  
26 test:  $p=0.101$ ). Since the proportion of CD4+ve cells in the transferred inoculum differed  
27  
28 between donors, we re-analysed the results using data obtained when using CD4-  
29  
30 enriched cells from the 5 donors whose CD4-enriched populations contained the highest  
31  
32 level of CD4+ve cells (a median of 74%; range: 70-97%; see Figure 2D). The transfer of  
33  
34 CD4-enriched cells from those individuals did not impact significantly on tumour  
35  
36 development *in vivo* (Log-rank test:  $p=0.35$ ) which is in line with the overall results  
37  
38 described above.  
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45 The *in vitro* cell separation manipulation *per se* did not affect significantly CTL  
46  
47 performance *in vivo* as determined in parallel control studies. For each of 3 donors,  
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49  $2 \times 10^6$  BLCLs were inoculated ip into each mouse in 2 groups of 3 SCID mice each.  
50  
51 Whilst  $50 \times 10^6$  unfractionated autologous CTLs were inoculated ip into each animal in  
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53 the control group, each mouse in a test group received  $50 \times 10^6$  CTLs that had been  
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2 fractionated into CD4+ve and CD8+ve T cell subpopulations using magnetic beads and  
3  
4 recombined in the original CTL subset proportions. Unfractionated CTLs and  
5  
6 fractionated/recombined CTLs had a similar effect *in vivo* (Log-rank test:  $p=0.71$ ; Data  
7  
8 not shown).  
9

10  
11 In the present study, the CD4- and CD8-enriched T cell populations were not  
12  
13 pure populations so calculations of the total number of these cells inoculated in each  
14  
15 experiment were carried out. A non-correlation was found between the numbers of  
16  
17 CD4+ve, or CD8+ve, T cells (included in the inoculated CTL, CD4-enriched, or CD8-  
18  
19 enriched T cells) and the average % survival (in days) beyond controls (Spearman's test:  
20  
21  $p>0.05$  for all analyzes). Similar results were obtained when analysing the CD8:CD4  
22  
23 ratio of cells inoculated (Spearman's test:  $p>0.05$  for all analyzes). Therefore, it does not  
24  
25 appear that survival of SCID mice treated with autologous CTLs, or T cell subsets, is a  
26  
27 direct function of the number of transferred CD4+ve or CD8+ve T cells or their ratio.  
28  
29 The level of CTL cytotoxicity against the autologous BLCL (as assayed *in vitro* by  $^{51}\text{Cr}$ -  
30  
31 release studies) did not correlate with survival of CTL-inoculated SCID mice  
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33 (Spearman's test:  $p=0.17$ ; Data not shown) and was therefore not a useful parameter for  
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35 predicting survival.  
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### 45 ***In Vivo* Trafficking of Human T Cells**

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47 We analysed samples of lung, liver, spleen, and tumour harvested from the CTL-  
48  
49 treated hu-BLCL-SCID mice (see above) to look for human T cell trafficking within  
50  
51 these tissues. The percentage of specific antibody+ve cells on immunostained sections  
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53 was assessed by attributing a score of (-), (+), 1+, 2+ and 3+, indicating that none, <1%,  
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2 1-29%, 30-60%, or >60% of all cells counted were antibody+ve cells, respectively.  
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4 Tissues were obtained at necropsy from 32 ip [harvested at 17-73 (median: 32) days  
5 after ip CTL inoculation] hu-BLCL-SCID mice (see Figure 3). Human CD3+ve T cells  
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7 were found in 17 out of 32 (53%) tumour samples tested. Whilst 2 of these 17 (12%)  
8  
9 samples contained CD4+ve T cells, 11 (65%) had detectable CD8+ve T cells and 1 (6%)  
10  
11 contained CD4+ve and CD8+ve T cells. Three (18%) samples contained only CD3+ve  
12  
13 cells. Overall, T cells comprised <1-29% of each T marker-positive tumour section.  
14  
15 Human cells were not detected in control mice. The data suggest that human T cells are  
16  
17 able to home to human tumour tissue *in vivo*. Since human CD45+ve cells were detected  
18  
19 only very rarely in lung, liver and spleen tissue, these samples were not analysed further.  
20  
21 Immunostaining of the murine pan-nucleated cell marker MHC1 on tumour sections  
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23 showed no (or the very occasional) murine cell underlining further that the lesions were  
24  
25 human in origin (Data not shown).  
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33 Serial sections from the 17 ip tumours shown to contain CD3+ve T cells (see  
34 above) were stained for perforin and granzyme B cytolytic molecules (see Figure 3).  
35  
36 Six out of 17 (35%) tumours contained granzyme B+ve cells and a further 8 out 17  
37  
38 (47%) contained perforin+ve cells. Overall, 14 out of 17 (82%) tumour samples  
39  
40 expressed one or other cytotoxic granule molecule with no dual expression noted.  
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### 47 **Cytokine Conditioning**

48  
49 Cytokines that signal through the common  $\gamma$ -chain (IL2, 4, 7, 15 and 21) are  
50  
51 important for the homeostasis of CD4+ve and CD8+ve T cells with the ability to  
52  
53 enhance their homing and promote their expansion, function and survival *in vivo* (Ku *et*  
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2 *al*, 2000; Schluns *et al*, 2000). The effect on survival of CTL cytokine conditioning *in*  
3  
4 *vitro* prior to transfer *in vivo* into ip hu-BLCL-SCID mice was examined. CTL lines (day  
5  
6 -1) from 5 donors (donors 11-15; see Table 1) were cultured in medium containing 20%  
7  
8 v/v FCS and human IL2 (20 IU/mL) with the addition of IL7, 15, 21, 7 and 15, or 7 and  
9  
10 21, at a final concentration of 10 ng/mL for 24 hours prior to washes, reconstitution in  
11  
12 suspension medium, and inoculation into mice (day 0; Parada *et al*, 1998; Ayyoub *et al*,  
13  
14 2002).

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19 Two million cells from each donor BLCL were inoculated ip into each mouse in  
20  
21 groups of 3 SCID mice each. This was followed 1 hour later by inoculation ip of  
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23 suspension medium (vehicle) only into one group of 3 animals, and  $50 \times 10^6$  autologous  
24  
25 CTLs that had been conditioned with cytokines for 24 hours *in vitro* into further groups  
26  
27 of 3 SCID mice. Individual results showing the percentage survival over time (in days)  
28  
29 together with log-rank test results is shown in Figure 4A-E. When SCID mice were  
30  
31 inoculated with CTLs conditioned *in vitro* with IL2 together with IL7, 15, 21, 7 and 15,  
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33 or 7 and 21, animals that received CTLs conditioned with a combination of IL7 and 15  
34  
35 survived significantly longer than control mice inoculated with CTLs conditioned with  
36  
37 IL2 only (Log-rank test:  $p=0.04$ ). Furthermore, tumours developed in 6 out of 15 (40%)  
38  
39 mice that received such IL7/15-conditioned CTLs which is significant protection against  
40  
41 tumour development when compared with tumour formation in 13 out of 15 (87%)  
42  
43 control mice inoculated with IL2-conditioned CTLs (Fisher's exact test:  $p=0.02$ ; see  
44  
45 Figure 4D). In contrast, there was no significant difference between the survival curves  
46  
47 of animals that received CTLs conditioned *in vitro* with IL2 together with IL7, 15, 21, or  
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2 7 and 21, when compared with control mice (Log-rank test:  $p=0.96, 0.67, 0.52, 0.75,$   
3  
4 respectively).

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6 The EBV-specific cytotoxicity *in vitro* of CTL lines on day 0 was assessed and  
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8 no significant difference found between median % specific lysis when comparing  
9  
10 autologous targets conditioned with IL2 alone, or IL2 combined with IL7, 15, 21, 7 and  
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12 15, or 7 and 21 (Kruskal-Wallis test:  $p=0.74$ ; Data not shown).  
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16 In order to assess further the role of IL2 for CTL function,  $2 \times 10^6$  BLCL cells  
17  
18 from one of 6 donors (donors 11-16; see Table 1) were inoculated ip into each mouse in  
19  
20 groups of 3 SCID mice each. One hour later,  $50 \times 10^6$  autologous CTLs that had been  
21  
22 conditioned for 24 hours *in vitro* with IL2 were inoculated ip into groups of 3 SCID  
23  
24 mice. This was followed by inoculations ip of either 250 IU of IL2 or vehicle only. Mice  
25  
26 were dosed daily for 14 days following ip transfer of BLCL cells. Individual results  
27  
28 showing the percentage survival over time (in days) together with log-rank test results is  
29  
30 shown in Figure 4F and the percentages of mice in each group that developed ip tumours  
31  
32 are shown in Table 3. CTL-inoculated animals that received IL2 daily survived  
33  
34 significantly longer than control mice dosed with vehicle only (Log-rank test:  $p=0.004$ ).  
35  
36 Tumours developed in 4 out of 18 (22%) such mice dosed with IL2 whereas 12 out of 18  
37  
38 (67%) control mice dosed with vehicle only developed ip tumours which is a significant  
39  
40 difference (Fisher's exact test:  $p=0.02$ ). Taken together, these results suggest a key role  
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42 for IL2 in supporting CTL function *in vivo*.  
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## DISCUSSION

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8 The importance of animal modelling has not diminished over the years despite  
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10 efforts to offer alternative *in vitro* options, and pre-clinical data obtained in suitable  
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12 animal models are still vital to guide translation into the clinical setting. Whilst SCID  
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14 mice are a small animal model that is widely used to study human cancer (including  
15  
16 PTLD), there is as yet no international standard defining how the model should be used  
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18 to ensure high quality data. In the current study, we used an ip hu-BLCL-SCID mouse  
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20 tumour prevention model because of its suitability for testing of novel immunotherapy  
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22 against PTLD.  
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26 Boyle *et al* (1993) engrafted SCID mice ip with BLCL and showed that  
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28 autologous CTL administered simultaneously (or 7 days later) delayed significantly  
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30 tumour formation whereas mismatched CTLs failed to do so, indicating the MHC  
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32 restriction of CTLs. In the present study, administration of autologous, but not  
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34 allogeneic, CTLs in the ip hu-BLCL-SCID model similarly delayed tumour development  
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36 and improved survival time, and further support for this tumour prevention model comes  
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38 from studies by DiMaio *et al* (1995) and Buchsbaum *et al* (1996).  
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43 The current study demonstrates that ip hu-BLCL-SCID mice give rise reliably to  
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45 macroscopic EBV+ve human B immunoblastic ip tumours portraying full (unrestricted)  
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47 latent virus gene expression with the occasional tumour cell in lytic cycle. In this  
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49 prevention model, ip tumour formation is the experimental read-out and autologous  
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51 CTLs can be detected in tumour tissue. In parallel studies, we have also investigated the  
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2 subcutaneous (sc) hu-BLCL-SCID model in which transfer of  $2 \times 10^6$  BLCL sc into  
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4 flanks of SCID mice gives rise consistently to visible sc PTLD-like tumours that can be  
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6 measured directly. Theoretically, such a model can be used to assess the efficacy of  
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8 novel PTLD immunotherapy when treatment is delivered by intravenous (iv)  
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10 inoculation. However, such a tumour regression model poses technical challenges that  
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12 undermine its use. In particular, in the sc model, sc tumours may arise at different  
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14 timepoints after inoculation making the experimental protocol for T cell therapy difficult  
15  
16 to plan. Furthermore, the transfer of large T cell inocula iv can be problematic and it is  
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18 difficult to ensure successful injection of the large T cell numbers required for analysis.  
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20 The ip hu-BLCL-SCID model used herein circumvents these difficulties and is  
21  
22 technically straight-forward to manage. However, since ip tumour development can not  
23  
24 be monitored clinically in this model, it is a prevention model with BLCLs and CTLs  
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26 given 1 hour apart, a situation that does not reflect accurately the clinical setting. Whilst  
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28 we have introduced MRI imaging of our animals to mimic better the clinical (patient)  
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30 situation with a view to provide a non-invasive, non-destructive method of monitoring ip  
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32 tumour development in longitudinal studies on the same animals, this approach has yet  
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34 to prove its value in face of technical challenges in demarcation of small lymphoid  
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36 lesions by MRI scanning of SCID mice. Taken together, translational data can be  
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38 obtained in the ip prevention SCID mouse model that is difficult to generate in other  
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40 SCID mouse models and such data can be used to inform clinical (patient) protocols.  
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49 Using the ip prevention hu-BLCL-SCID model, CD8+ve T cell subpopulations  
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51 from CTLs from across the donor panel delayed significantly tumour development, and  
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53 tumour formation was entirely prevented in 40% of mice (see Table 2). Boyle *et al*  
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1  
2 (1993) also used CD8-enriched CTL populations in this model with significant effect on  
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4 tumour development. Whilst inoculation of CD4-enriched T cells did not have a  
5  
6 significant impact on survival in this model (see Figure 2), the data is too limited to draw  
7  
8 conclusions from this set of experiments. Interestingly, in the hu-PBMC-NOD/SCID  
9  
10 model (NOD/SCID animals are deficient in NK as well as B and T cells), treatment of  
11  
12 mice with a depleting anti-human CD4 mab results in loss of both CD4+ve and CD8+ve  
13  
14 T cells (Wagar *et al*, 2000) suggesting at least a supporting role to CD8+ve T cells for  
15  
16 CD4+ve lymphocytes. Since the CD4-enriched T cell populations transferred into our  
17  
18 animals consisted of smaller total T cell numbers than the CD8-enriched inocula, and  
19  
20 there was no significant difference in the rate of ip tumour development between mice  
21  
22 inoculated with bulk CTLs and animals that received CD4-enriched cells only, the latter  
23  
24 may still have an anti-tumour effect or work to prevent CD8+ve T cell-mediated anti-  
25  
26 tumour mechanisms (see Results). Thus, the data must be interpreted with a certain  
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28 degree of caution.  
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35 CD4+ve T cells show cytotoxic function *in vitro* and *in vivo* (Appay, 2004), and  
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37 since PTLD cells express MHC2 molecules (Thomas *et al*, 1990), direct CD4+ve T cell-  
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39 mediated cytotoxicity is possible. However, CD4+ve T cells also have a variety of  
40  
41 helper functions and in a recent clinical trial using allogeneic 'best MHC match' CTL  
42  
43 treatment for PTLD, a significant trend for a better outcome with higher numbers of  
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45 CD4+ve T cells in infused CTL lines was interpreted as the effect of the few CD4+ve T  
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47 cells in the CTLs in enhancing the survival *in vivo* of the CD8+ve population (Haque *et*  
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49 *al*, 2007).  
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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 PTLD-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.

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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 PTLD-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.

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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 PTLD-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

1  
2 carried out, the results should be interpreted with a degree of caution. Whilst *in vitro*  
3  
4 cytokine conditioning did not affect significantly CTL cytotoxicity *in vitro*, additional  
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6 results from this study (as well as data from clinical studies in our laboratory; Haque *et*  
7  
8 *al*, 2007) suggest that *in vitro* cytotoxicity does not correlate with CTL function *in vivo*.  
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11 Previous studies have given conflicting results when examining a possible role of  
12  
13 cytokine administration in sustaining transferred T cells *in vivo* (Boyle *et al*, 1993;  
14  
15 Rencher *et al*, 1994; Baiocchi *et al*, 1994, 2001). However, in line with data from other  
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17 laboratories (Baiocchi *et al*, 1994, 2001), the current study underlines a supporting role  
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19 for IL2 in facilitating CTL survival and function *in vivo* (see Figure 4F and Table 3)  
20  
21 although the results do not exclude possible contribution from IL2-mediated activation  
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23 of murine NK cells. Whilst the results suggest inclusion of IL2 in immunotherapeutic  
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25 regimes using CTLs against PTLN, a degree of caution must be exercised in the use of  
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27 the cytokine in a patient population due to its ability to cause side effects.  
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33 Our laboratory has successfully infused PTLN patients with EBV-specific CTLs  
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35 on a 'best MHC match' basis (Haque *et al*, 2002, 2007). Although this *proof-of-*  
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37 *principle* trial showed that CTL therapy could succeed, a mechanism for bypassing  
38  
39 MHC restriction would facilitate its dissemination to the clinic. A chimeric T cell  
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41 receptor (cTCR) can be constructed that directs T cells against a novel surface antigen in  
42  
43 a non-MHC restricted manner (Schumacher, 2002; Mansoor *et al*, 2005), and currently  
44  
45 our laboratory employs such an approach to re-target CTLs to EBV-associated cancers.  
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47 This novel therapeutic approach will be tested in the pre-clinical ip hu-BLCL-SCID  
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49 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.

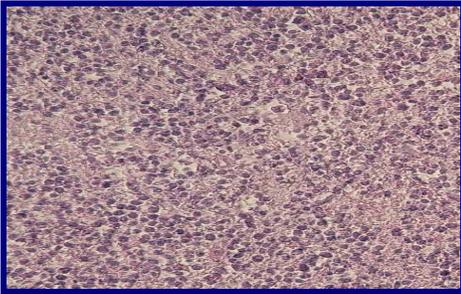
1A: Necropsy (Normal)



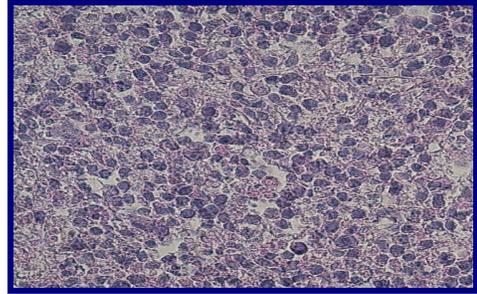
1B: Necropsy (Ip Tumour)



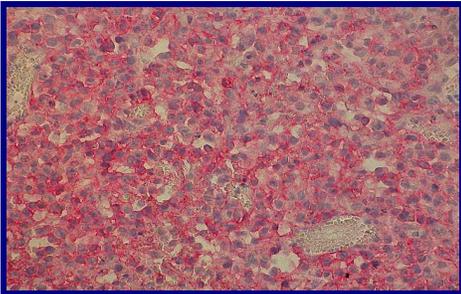
1C: H&E



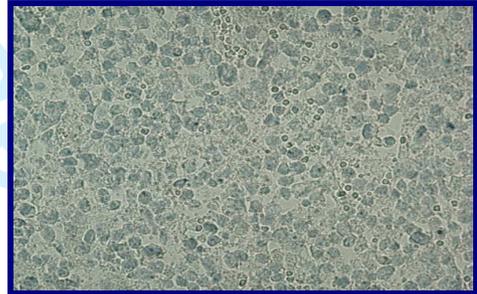
1D: H&E



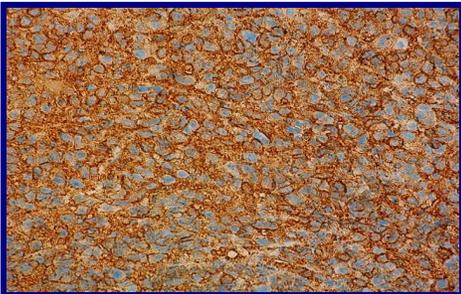
1E: CD45



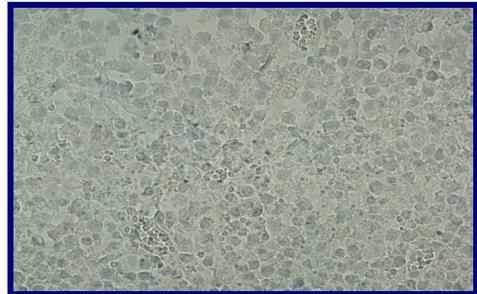
1F: CD45 Control



1G: CD20



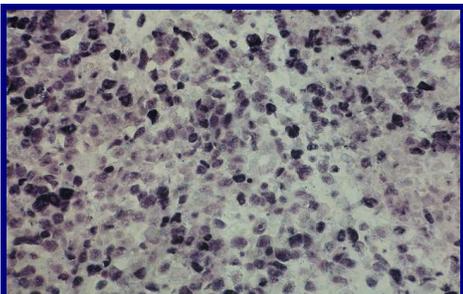
1H: CD20 Control



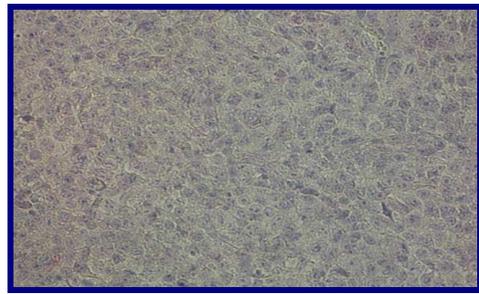
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**1I: EBERs**



**1J: EBERs Control**

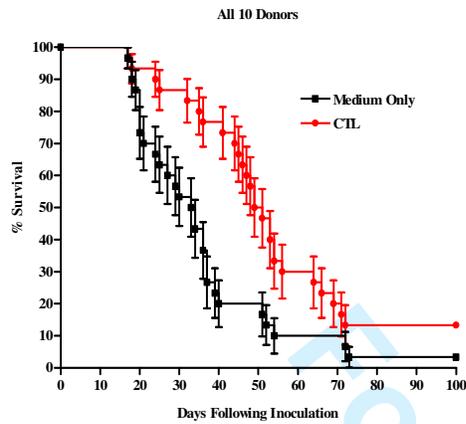


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**FIGURE 2**  
**% Survival Using CTLs And T Cell Subsets**

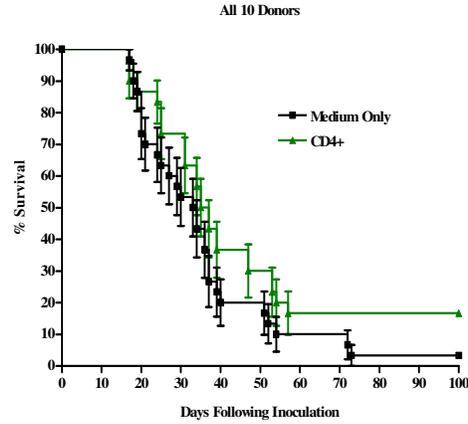
(Datapoints reflect results in triplicates; Standard error, SE, is shown on each column as a vertical bar.)

**2A) CTLs**



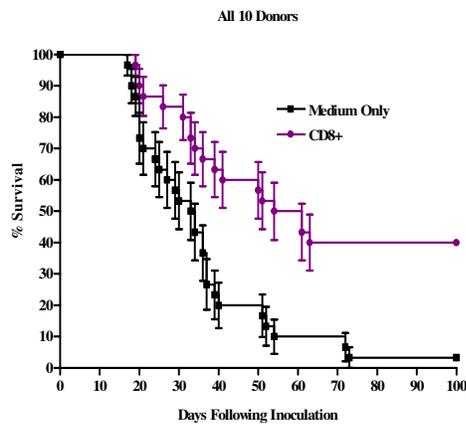
(Log-rank test:  $p=0.003$ )

**2B) CD4-Enriched Cells**



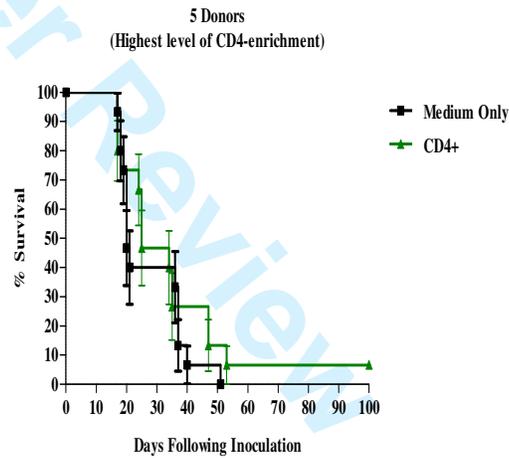
(Log-rank test:  $p=0.101$ )

**2C) CD8-Enriched Cells**



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

**2D) CD4-Enriched Cells (Subset)**



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

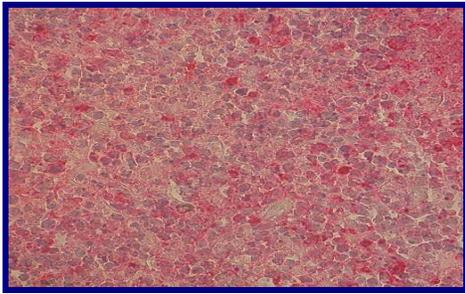
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**FIGURE 3**  
**Immunophenotyping Of SCID Mouse Tumour Tissue:**  
**Human Leukocyte and Cytotoxic Molecule Markers**

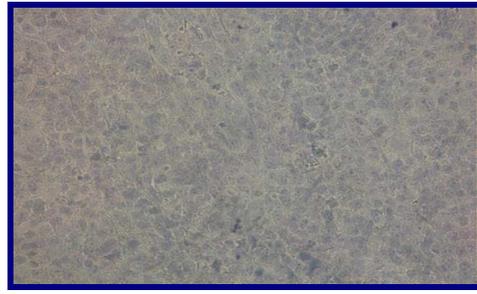
**Figures 3A,B** (x400): Photomicrographs of immunostaining of the human pan-leukocyte marker CD45 on tumour sections using an alkaline phosphatase (AP) label. Figure 3A shows CD45-positive human cells (red membrane staining) counterstained with Mayer's haemalum. Figure 3B shows the conjugate control 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.

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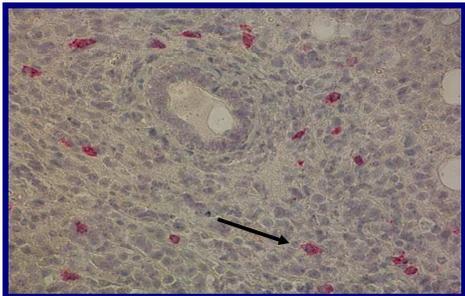
**3A: CD45**



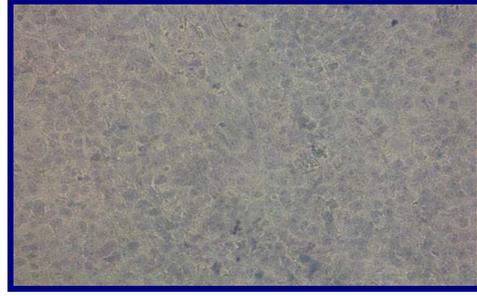
**3B: CD45 Control**



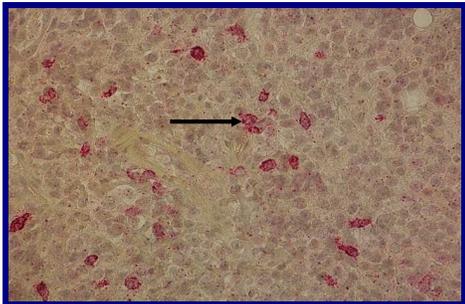
**3C: CD3**



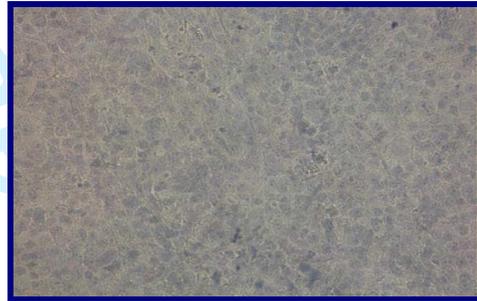
**3D: CD3 Control**



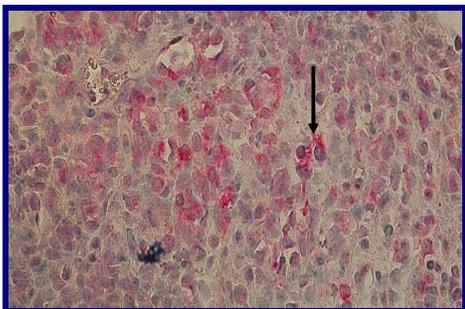
**3E: CD4**



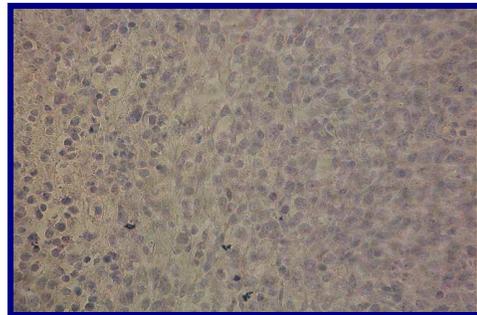
**3F: CD4 Control**



**3G: CD8**

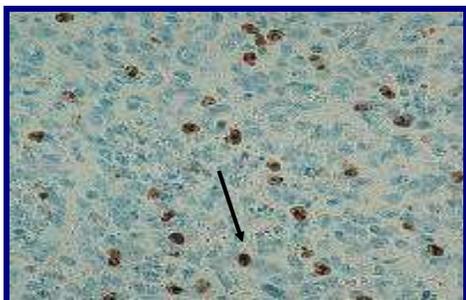


**3H: CD8 Control**

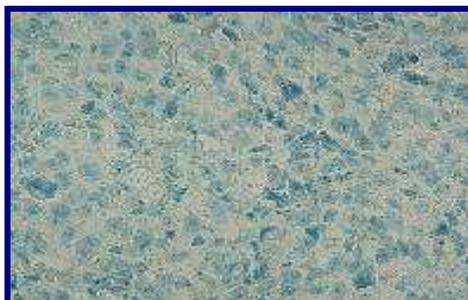


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**3I: Perforin**



**3J: Perforin Control**

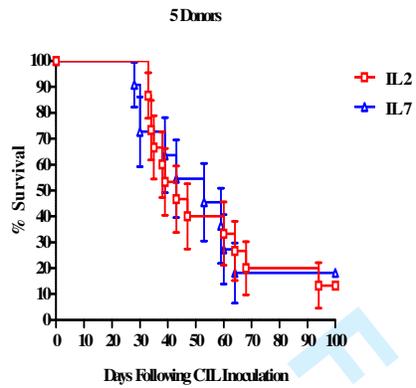
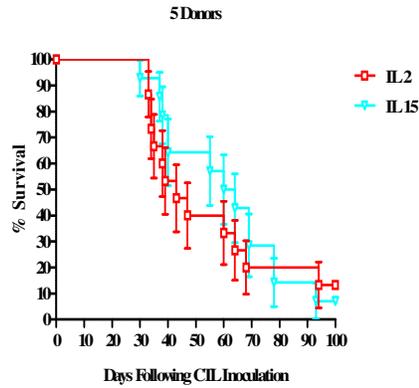
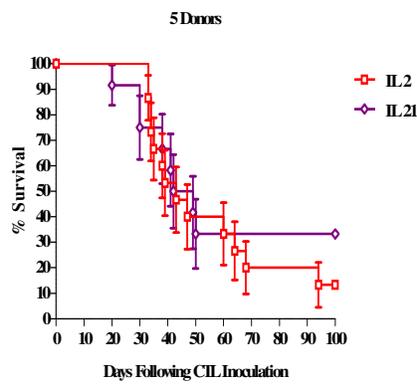
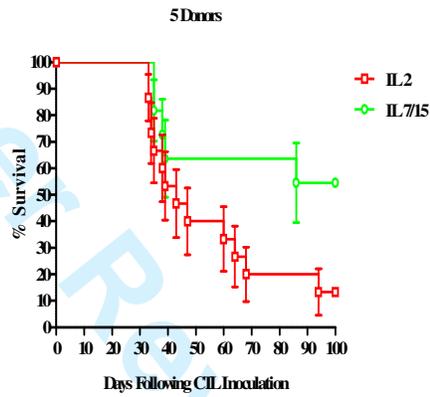
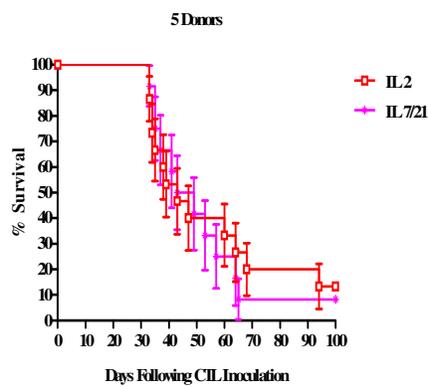
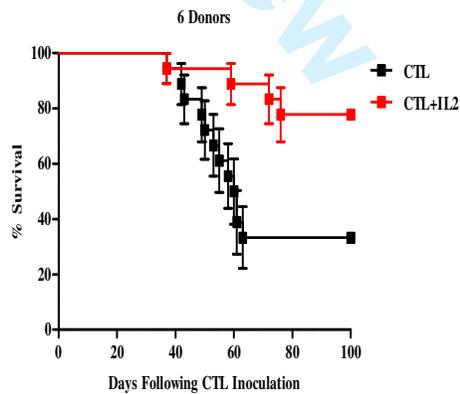


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FIGURE 4

**% 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						
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).

For Peer Review