T and NK cell immunity after hematopoietic stem cell transplantation

Lugthart, G.

Chapter 3

Simultaneous generation of multivirus-specific and regulatory T cells for adoptive immunotherapy

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Gertjan Lugthart
Sarah J. Albon
Ida Ricciardelli
Michel G.D. Kester
Pauline Meij
Arjan C. Lankester *and*
Persis J. Amrolia

Abstract

Previous studies have established that adoptive immunotherapy with donor-derived virus specific T-cells can prevent/treat viral complications post stem cell transplant and regulatory T-cells show promise as inhibitors of graft-versus-host disease. Based on flow cytometric analysis of upregulation of activation markers following stimulation with viral peptide pools, we have developed a rapid and clinically applicable protocol for the simultaneous selection of virus-specific T-cells (following stimulation with peptide pools for the immunodominant antigens of cytomegalovirus, Epstein-Barr virus and adenovirus) and regulatory T-cells using CD25 immunomagnetic selection.

Using tetramer staining, we detected enrichment of $CD8^+$ T-cells recognizing peptide epitopes from cytomegalovirus and Epstein-Barr virus antigens following CD25 selection in 6 of 7 donors. Enzyme-linked immunospot assays demonstrated the simultaneous presence of bi- or tri-virus specific cells in all evaluated donors, with a median 29-fold (6-168), 40-fold (1-247) and 16-fold (1-219) enrichment of cells secreting interferon-γ in response to cytomegalovirus pp65, adenovirus hexon and Epstein-Barr virus lymphoblastoid cells compared to unmanipulated PBMC from the same donors. Furthermore, the CD25 enriched cells lost alloreactivity in ^{3}H-thymidine proliferation assays and showed highly effective (median 98%) suppression of alloreactivity in all evaluated donors.

In summary, we have developed a rapid, simple GMP compliant methodology for the simultaneous selection of T-cells with multiple viral-specificities and regulatory T-cells. Adoptive transfer of T-cells generated using this strategy may enable restoration of cellular immunity to viruses post allogeneic stem cell transplant with a low risk of graft-versus-host disease. Because of the speed and simplicity of this methodology, this approach may significantly broaden the applicability of adoptive immunotherapy.

Introduction

Adoptive transfer of virus specific cytotoxic T-cells (CTL) generated by repetitive antigenic stimulation of donor T-cells, is a safe and effective method of restoring anti-viral immunity after stem cell transplant (SCT).[74-76;80;179-181] However, the use of specialized antigen presenting cells (APC), viral vectors, high workload and long culture period make this approach unfeasible for broad application. What is needed is a rapid, simple method for the generation of multi-virus specific cells. Currently two clinically applicable technologies are available to do this, but both have significant limitations. Multimer based magnetic isolation, which has been used for the selection of CMV-specific $CD8^+$ T-lymphocytes,[77] is rapid but is limited to certain HLA restrictions, does not select $CD4^+$ virus-specific T-cells and the production of tetramers under Good Manufacturing Practice (GMP) conditions is highly challenging. Interferon-γ (IFN-γ) capture Snables the rapid enrichment of both $CD4^+$ and $CD8^+$ virus-specific cells but only a subset of virus-specific T-cells secrete IFN-γ upon activation.[78;79] This method has been successfully used in the clinical setting to select T-cells specific for Cytomegalovirus (CMV), human adenovirus (HAdV) or Epstein-Barr Virus (EBV) singly.[80-82] A number of groups studied IFN-γ capture for the enrichment of multi-virus specific cells, but still employed viral vectors or nucleofection of specialized APC [182;183], precluding broader implementation of this strategy. An alternative to this is the use of recombinant viral peptide pools, which can be generated under GMP conditions and used without the need for professional APC. This approach has been used clinically for both HAdV[184] and EBV.[81] Since the immunodominant antigens from CMV (pp65), HAdV (hexon) and EBV (EBNA1,3 and LMP2) have been characterized,[184-187] it is now possible to stimulate T-cells with overlapping peptide pools from all these antigens simultaneously.

Given the limitations above, new methods for the selection of virus-specific T-lymphocytes are needed. The selection of T-cells upregulating cell-surface activation markers in response to stimulation with viral epitopes is a promising alternative. Activated T-cells express a variety of candidate surface markers including the IL-2 receptor α chain (CD25), the early activation marker CD69, the transferrin receptor (CD71) and tumor necrosis factor receptor superfamily member 9 (CD137). There is no data on the relative upregulation of these markers following stimulation with viral antigens. In this study, we compared the upregulation of cell surface markers and IFN-γ secretion upon stimulation with viral peptide pools. We have identified CD25 as the optimal marker for selection of virally-activated T-cells and have developed a clinically applicable CD25-based protocol for the enrichment of CMV, EBV and HAdV specific T-lymphocytes from a single culture. Selection of $CD25^+$ CTL should not only enrich for virus-specific CTL, but also deplete alloreactive T-cells, which will not be activated and therefore do not upregulate CD25. Further, since CD25 is also highly expressed on natural regulatory T-cells (Tregs), we rationalized that if these were also enriched by CD25-selection, adoptive transfer of T-cells generated using this strategy may enable both restoration of cellular immunity to viruses and prevention of GVHD post allogeneic SCT.

Materials and Methods

Viral antigens

The EBV peptide pool used was that described by Moosmann *et al.* [81] from JPT Peptide Technologies, (Berlin, Germany), consisting of 19 HLA class I restricted epitopes and 4 HLA class II restricted epitopes from 5 latent antigens (LMP2, EBNA1, EBNA3A, EBNA3B, EBNA3C), 4 immediate early/early antigens (BZLF1, BRLF1, BMLF1, BHRF1) and 2 late/structural antigens (BLLF1, BNRF1). The EBV epitopes were selected on the basis of being the immunodominant epitopes restricted by common HLA Class I and II alleles as shown by ELISPOT/tetramer analyses of peripheral blood from seropositive individuals and because they represent the main specificities that grow out in EBV CTL cultures. For CMV and HAdV, PepTivator peptide pools (Miltenyi Biotec, Bergisch Gladbach, Germany) for the immunodominant CMV-pp65 and HAdV5-Hexon were used, consisting of 15-mer peptides with 11–amino acid overlap covering the complete sequence of the CMV-pp65 and HAdV5-hexon protein. A list of peptides used is shown in Table 3.S1.

Donor cells and generation of EBV-transformed lymphoblastoid cell lines

For the comparison of different activation markers and IFN-γ capture, blood samples were drawn from 5 healthy CMV seropositive and EBV seropositive donors after informed consent. Peripheral blood mononuclear cells (PBMC) were separated using ficoll-paque density gradient centrifugation (Ficoll Paque Plus, GE Healthcare, Waukesha, CI, USA), washed twice and resuspended in serum free AIM-V cell culture media (Invitrogen/Gibco, San Diego, CA, USA) and a viability count was performed.

For the clinical grade CD25 enrichment, 7 single donor buffy coats (BC, National Blood Service, Colindale, UK) from CMV seropositive donors were used. Donors were tissue-typed for HLA Class I and II at medium resolution using Luminex technology. EBV-transformed lymphoblastoid cell lines (LCL) were generated by culturing PBMC with EBV containing supernatant of B95-8 cells as described.[188] LCL were maintained in RPMI 1640 (Gibco/Invitrogen) with 10% fetal calf serum (FCS, Sigma-Aldrich, St Louis, MO, USA). 1.2 x 10^8 PBMC were frozen in freezing medium (AIM-V medium with 10% Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, USA) and 10% Human Serum Albumin (HSA, Bio Products Laboratory, Herts, UK) and stored in liquid nitrogen for subsequent experiments, while the remainder was used for the CD25 enrichment.

Flow cytometric comparison of different activation markers

The kinetics of IFN-γ secretion and upregulation of CD25, CD69, CD71 and CD137 on T-cells were compared flow cytometrically at serial time points after stimulation with viral peptides. 2 x 10^6/ml/well PBMC from 5 healthy CMV seropositive donors were cultured in serum free AIM-V medium in a 24-well cell culture plate (Greiner Bio-One, Kremsmünster, Austria), and stimulated with 1 µg/peptide/ml CMV-pp65 PepTivator mix or HAdV5-Hexon PepTivator mix. PHA (5 µg/ml) and medium were used as positive and negative controls. Cells were cultured at 37°C, and

at 0h, 6h, 24h, 72h and 120h after stimulation, were washed and resuspended in PBS. Half the cells were used for cell-surface staining with the following mAbs: anti-CD4 FITC, PE-labeled anti-CD25, anti-CD69, anti-CD71, anti-CD137 and anti-IgG and anti-CD3 APC-Cy7 (BD Biosciences, Franklin Lakes, NJ, USA). The remainder of the cells was assayed for IFN-γ secretion using the cytokine capture system (Miltenyi), as per manufacturer's instructions. ToPro3 was added to exclude dead cells and flow cytometric analysis was performed using the BD FACSArray flow cytometer (BD Biosciences) and FlowJo analysis software (Tree Star Inc, Ashland, OR, USA). The percentage of surface marker upregulation and IFN-γ secretion was calculated as percentage of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells after subtraction of IgG isotype control and unstimulated control.

Clinical grade enrichment of tri-virus specific cells

Normal donor PBMC from 7 buffy coats (see Table 3.1 for HLA typing and EBV/CMV serology) were stimulated with CMV, EBV and HAdV peptides, cultured for 3 days and $CD25^+$ cells enriched on the CliniMACS plus system (Miltenyi). At day 0, a median 4 x 10^8 PBMC were resuspended in 10 ml serum free AIM-V medium with 1 µg/peptide/ml CMV pp65 PepTivator mix, HAdV5 Hexon PepTivator mix and EBV peptide mix. After 1h incubation at 37°C / 5% CO_2 under continuous rotation, cells were diluted to 2 x 10^6/ml and cultured in T175 flasks. At day 3, cells were harvested and resuspended in CliniMACS buffer (PBS with 2mM EDTA + 0.5% HSA) and stained with 0.1 ml CD25 reagent (Miltenyi) per 10^8 cells for 30' at room temperature according to the manufacturer's protocol. Cells were washed, resuspended in 100ml CliniMACS buffer and enrichment performed under Good Manufacturing Practise (GMP) conditions on the CliniMACS PLUS instrument using Enrichment Program 3.2 and a TS600 research tubing set (Miltenyi). Enriched cells were washed, resuspended at 2 x 10^6/ml and sampled for flow cytometry. The remaining cells were rested for 3 days in 24-well plates with 40 IU/ml IL-2 (Genscript inc., Piscataway, NJ, USA) and irradiated (30Gy) autologous feeder cells at a ratio of 1:1 before assessment of anti-viral responses using tetramer staining, ELISPOT, cytotoxicity and proliferation assays and regulatory capacity in suppression assays.

Phenotyping and tetramer staining of CD25 enriched cells

The following mAbs were used to determine the phenotype of selected cells: FITC-labeled anti-CD4, PE-labeled anti-CD25, anti-CD45RA, anti-CD16, anti-CD56, anti-FoxP3 and anti-IgG, PerCP-labeled anti-CD8, APC-labeled anti-CD127 and anti-IgG, APC-Cy7-labeled anti-CD3 and PE-Cy5-labeled anti-CD25 and anti-IgG. All antibodies were purchased from BD Biosciences, except for the anti-CD127 (Miltenyi Biotec) and CD16 (eBioscience, San Diego, CA, USA) antibodies. For the intracellular staining of FoxP3, the FoxP3 staining set (eBioscience) was used, according to the manufacturer's protocol. Briefly, cells were stained with mAbs for cell surface staining, washed in FACS buffer and fixed in Fixation/Permeabilisation buffer. The following day, cells were washed thoroughly with permeabilisation buffer, blocked with 2% normal rat serum in permeabilisation buffer and stained with FoxP3 mAb or IgG isotype control.

	HLA restriction						Serology		EBV peptide pool match	
	A-allele		B-allele		DRB1-allele		CMV	EBV	HLA-alleles	EBV-antigens
BC1	0301		0702	4001	0101	1501	+	+	4	5
BC2	0101	2402	0801	1501	0401	1101	+	+	3	6
BC3	0201	3301	1402	4402	0701	1501	+	+	3	6
BC4	0201	1101	0702	3701	0101	1501	+	+	4	7
BC5	0101	0201	0801	1501	0407	1301	+	+	4	8
BC6	0201		1801	4402	1104	1301	+	+	4	8
BC7	0301	1101	1518	5701	0701	1301	+	+	3	4

Table 3. 1. Donor HLA restriction, serology and of EBV antigens.
HLA restriction, CMV and EBV serology of 7 buffy coats used for CD25 selection. For each donor, the number of HLA-alleles shared with the HLA-alleles of the EBV peptide pool and maximum number of EBV antigens that could be recognized is depicted.

The following PE-labeled major histocompatibility complex (MHC) tetramers were produced as described previously[189] and used to detect viral-specific CD8+ T-cells: *CMV-* pp65 specific HLA-A*0101- YSEHPTFTSQY (pp65-A1-YSE), HLA-A*0201-NLVPMVATV (pp65-A2-NLV), HLA-A*0301-VLCPKNMIIK (pp65-A3-VLC), HLA-A*2401 QYD (pp65-A24-QYD), HLA-B*0702-TPRYTGGGAM (pp65-B7-TPR) and HLA-B*0702-RPHERNGFTV (pp65-B7-RPH); *EBV-* BMLF-1-specific HLA-A*0201-GLCTLVAML (BMLF1-A2-GLC), LMP-2-specific HLA-A*0201-CLGGLLTMV (LMP2-A2-CLG), EBNA-3A-specific HLA-A*0301-RLRAEAQVK (EBNA3A-A3-RLR) and HLA-B*0702-RPPIFIRRL (EBNA3A-B7-RPP), and BZLF-1-specific HLA-B*0801-RAKFKQLL (BZLF1-B8-RAK): all of these teramers utilise peptides present in the peptide mix used in the manufacturing protocol for CD25-enriched CTL *Adenovirus* -hexon-specific HLA-A*2404-TYFSLNNKF (Hx-A24-TYF), HLA-A*0101-TDLGQNLLY (hx-A1-TDL), and HLA B*0702-KPYSGTAYNSL (Hx-B7-KPY). Cells were stained with tetramers appropriate to the donors' HLA restriction. 3 x 10^5 unmanipulated PBMC or CD25-enriched T-cells were stained per tube with PE-labeled tetramer for 15 minutes at room temperature and co-stained for cell surface markers with PerCP-labeled anti-CD8 and APC-Cy7-labeled anti-CD3 mAbs. PBMC from donors with known positive populations served as positive controls and PBMC from normal donors negative for the restricting HLA-type were used as additional negative controls. 4',6-diamidino-2-phenylindole (DAPI) staining was used to exclude dead cells and at least 20,000 events in the CD3+/CD8+ lymphocyte gate were analyzed. The percentage of tetramer$^+$ cells in the CD3+/CD8+ lymphocyte gate was expressed as a proportion of the CD8$^+$ cells with the unstained and HLA-mismatched control subtracted. For a population to be considered positive, a distinct population of > 25 CD3$^+$CD8$^+$ tetramer$^+$ events with the staining characteristics of the positive control population had to be acquired. Flow cytometric analysis was performed using the BD LSRII flow cytometer (BD Biosciences) and FlowJo analysis software.

Enzyme-linked Immunospot Assay

Enzyme-linked immunospot assay (ELISPOT) was used to determine the frequency of virus-specific interferon-γ (IFN-γ) producing cells as described previously.[190] The following stimulators were used to monitor anti-viral responses: autologous LCLs, autologous PBMC pulsed with

CMV-pp65 or HAdV5-Hexon PepTivator mix (1 µg/peptide/ml). Serial dilutions were performed for one donor to titrate the number of effector cells per well. Based on this, 2 x 10^5 unmanipulated PBMC or 2 x 10^4 CD25 enriched cells were cultured with 2 x 10^5 irradiated stimulators per well in 200 µl AIM-V in triplicates. After 18 hours the plates were stained with biotinylated detection mAbs and developed as described.[190] Plates were read using an AID EliSpot reader and counted with AID EliSpot system software (version 4.0, AutoImmun Diagnostika GmbH, Strassberg, Germany). The virus-specific cell frequency was expressed as the mean specific spot-forming cells (SFC) of triplicates after subtracting the mean frequency of spots from unstimulated responders and stimulators alone.

Proliferation and suppression assay

The ^{3}H-thymidine incorporation assay was used to assess the proliferative capacity of the enriched cells upon stimulation. CD25 enriched cells and PBMC from the same donor were plated in triplicate in round bottom 96 well plates (NUNC) at 2 x 10^5 cells per well in 200 µl AIM-V in triplicates and stimulated with 5 x 10^3 autologous LCLs or 2 x 10^5 autologous PBMC pulsed with CMV-pp65 or HAdV5-Hexon PepTivator mix (1 µg/peptide/ml), autologous PBMC alone or allogeneic PBMC. Medium and 5 µg/ml Phytohaemagglutinin (PHA) were used as negative and positive controls. To assess the enrichment of regulatory T-lymphocytes with the capacity to suppress alloreactivity, a suppression assay was performed. 2 x 10^5 autologous PBMC and 2 x 10^5 irradiated allogeneic PBMC were plated out in triplicate in a round bottom 96 well plate. As suppressor cells, CD25 enriched cells were added in serial dilutions (2 x 10^5 – 5 x 10^4). After 5 days incubation at 37°C / 5% CO_2, 1 µCi tritiated thymidine (^{3}H-thymidine, GE Healthcare) was added to each well for 18-20 hours. The plates were harvested and ^{3}H-thymidine incorporation was measured with a MicroBeta TriLux (Perkin-Elmer Weiterstadt, Germany). The data are presented as average of the triplicates after subtraction of unstimulated cells and stimulators and suppressors alone.

Cytotoxicity assay

A standard ^{51}Cr release assay was performed to determine the virus-specific cytolytic activity of the CD25 enriched cells. Autologous and allogeneic EBV-LCLs were used as targets. The target cells were labelled with 100 µCi ^{51}Cr (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 hours at 37°C. ^{51}Cr-labeled LCLs were plated at 5×10^3 cells per well, respectively, and cultured with CD25 enriched cells at different concentrations (effector to target ratios: 30:1, 5:1, and 1:1) in 96-well U-bottom plates. Target cells in complete media or lysed with 1% TritonX-100 (Sigma-Aldrich) were used to determine spontaneous and maximum release, respectively. After 6 hours of incubation at 37°C, plates were spun and 25-µl supernatant were harvested and transferred to 96-well Wallac isoplates (Perkin-Elmer, Weiterstadt, Germany) and mixed with 150-µl OptiPhase Supermix Cocktail (Perkin-Elmer). Counts were measured on a MicroBeta TriLux (Perkin-Elmer) and the percent-specific lysis was calculated as [(specific release-spontaneous release)/ (maximum release-spontaneous release)] x 100.

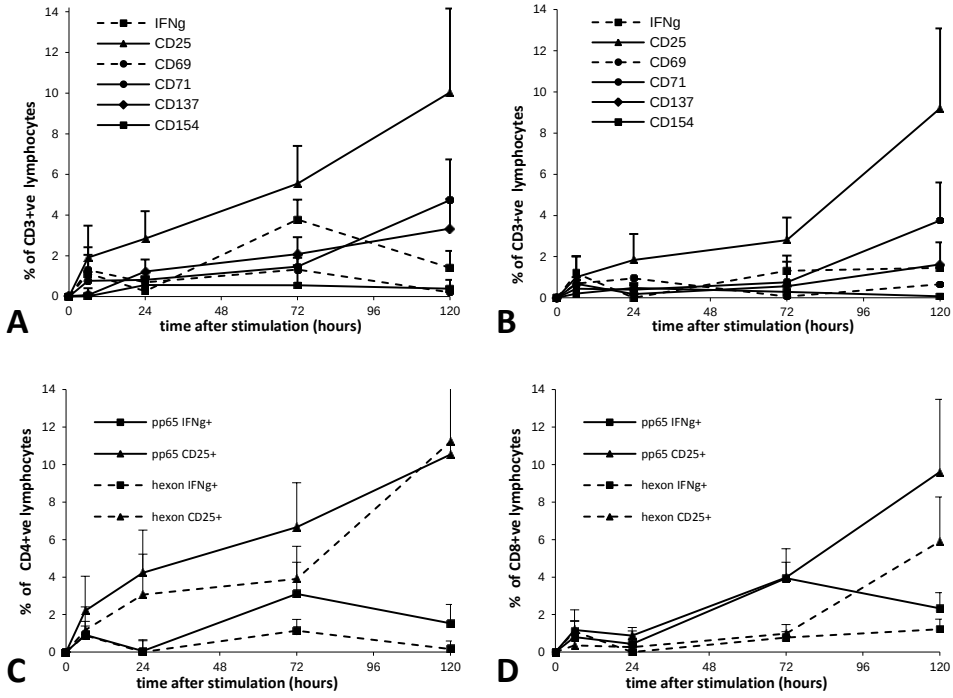

Figure 3.1. Flow cytometric analysis of activation marker expression.
Expression was evaluated 0, 6, 24, 72 and 120 hours after stimulation with CMV pp65 and HAdV5 hexon PepTivator mix at 1 µg/peptide/ml. **(A-B)** percentage of interferon- γ (IFN-γ) secreting and CD25, CD69, CD71 and CD137 expressing $CD3^+$ lymphocytes after stimulation with pp65 (A) and hexon (B) peptides.
(C-D) percentage of IFN-γ secreting and CD25 expressing $CD4^+$ (C) and $CD8^+$ (D) lymphocytes. Uninterrupted lines represent pp65 stimulated culture while dashed lines show hexon stimulated cultures. Square dots symbolize IFN- γ secreting cells while triangles show CD25 positive cells. Shown is the mean percentage (+SEM) of 5 healthy donors after subtraction of IgG isotype control and unstimulated control.

Statistical analysis

The upregulation of CD25 during culture with viral peptides, enrichment of CD25 and CD45RA expressing cells and the percentage of virus specific tetramer positive cells before and after enrichment were compared using the one-tailed paired t-test. This test was also used to compare virus specific IFN-γ secretion in the ELISPOT assays and alloreactivity and suppression in the proliferation assay. Prism statistical software (version 5.01; Graph-Pad Software, San Diego, CA, USA) was used to perform all statistical tests.

Ethics

Ethical approval for this study was obtained through the University College London non-NHS research ethics committee and blood samples were taken with informed consent.

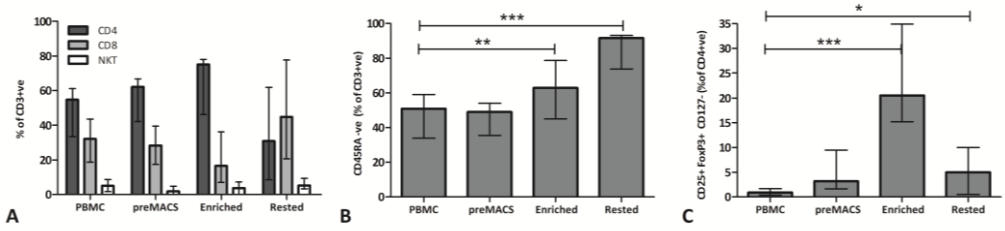

Figure 3.2. Immunophenotype of CD3$^+$ T-lymphocytes at 4 key time points during clinical grade CD25 selection: before procedure (PBMC), after 3 day culture (preMACS), immediately after CD25 selection (enriched) and 3 days after enrichment (rested). Shown is the median of 7 donors with error bars indicating highest and lowest value of: **(A)** percentage of CD4$^+$, CD8$^+$ and Natural Killer T-cells (NKT, CD3$^+$, CD16$^+$ /CD56$^+$) within CD3$^+$ lymphocyte gate. **(B)** percentage of memory T-lymphocytes (CD45RA$^-$) within CD3$^+$ lymphocyte gate. **(C)** percentage of regulatory T-cells (CD25$^+$, FoxP3$^+$ and CD127$^-$), within CD3$^+$ CD4$^+$ lymphocyte gate. * *p<0.05*, ****p<0.001* in paired t-test.

Results

Flow cytometric comparison of activation markers upregulation in response to viral antigens

To determine the optimal target for selection of viral peptide activated lymphocytes, we compared IFN-γ secretion with the upregulation of CD25, CD69, CD71 and CD137 in PBMC from 5 healthy CMV seropositive donors. For both hexon and pp65 stimulated PBMC, IFN-γ secretion rose by 6 hours and peaked at 72h after stimulation (pp65: mean 3.8% positive T-cells above unstimulated controls, hexon: mean 1.3%). CD25 (IL-2Rα) was the activation marker that was most upregulated with a mean 10.03% and 9.20% positive T-cells for pp65 and hexon stimulated PBMC at day 5 (Figure 3.1 A-B). CD69 upregulation was highest in the first 24 hour after stimulation (pp65: mean 1.30%; hexon: mean 0.95%) and the percentages of CD71$^+$ and CD137$^+$ T-cells rose progressively during culture to a peak 4.73% / 3.34% above background for pp65 and 3.76% / 1.20% for hexon stimulated PBMC after 5 days. In the unstimulated control, the basal expression of CD69 and CD137 was low (< 0.75%), whereas CD25, IFN-γ and CD71 showed significant expression in unstimulated T-cells (mean 1.3%, 1.2% and 2.45% of CD3$^+$ lymphocytes).

Similar data on up-regulation of IFN-γ and CD25 were obtained in separate experiments on normal donors PBMC (Figure 3.S1) following stimulation with EBV peptide mix. CD25 expression peaked at 7 days, with mean of 1.6% of CD25$^+$ T-cells above unstimulated controls, whereas, as with pp65 and hexon, peak IFN-γ secretion was rather lower (0.5% at 72 hours).

These studies indicated that IFN-γ is only secreted by a subpopulation of virus-specific T-cells and identified CD25 as a promising target for selection of virus-specific T-cells following stimulation with viral peptide epitopes. Comparison of the IFN-γ secretion and upregulation of CD25 in CD4$^+$ and CD8$^+$ lymphocytes showed that after stimulation with either pp65 or hexon peptides, the majority of cells upregulating CD25 but not secreting IFN-γ were in the CD4$^+$ compartment (Figure 3.1 C-D).

	Before enrichment		After procedure		3 Day rested
	Unmanipulated	3 Day cultured	Depleted	Enriched	Enriched
Viable Cell number (x 10^7)	40 (35-100)	27 (11-81)	12 (3.6-33.2)	2.2 (1.1-3.1)	1.7 (1.2-8.7)
T-lymphocyte (% PBMC)	63 (58-80)	79 (57-86)	82 (71-91)	71 (61-77)	84 (80-96)
NK (% of PBMC)	12 (6.6-16)	5.7 (4.7-7.2)	6.4 (4.7-9.0)	5.4 (1.5-8.3)	3.1 (0.5-5.3)
NKT (% of $CD3^+$)	5.1 (1.7-8.6)	1.8 (0.1-4.8)	1.4 (0.2-6.4)	3.8 (0.4-7.4)	4.3 (3.4-8.4)
Treg (% of $CD4^+$)	0.9 (0.35-1.7)	3.2 (1.7-9.5)	ND	21 (15-35)	5.0 (0.5-10)
$CD25^+$ (% of $CD3^+$)	1.4 (0.8-4.1)	6.4 (4.1-14)	0.0 (0.0-0.8)	44 (31-60)	35 (20-82)
$CD25^+$ (% of $CD4^+$)	2.9 (1.5-6.2)	7.1 (4.0-17)	0.2 (0.0-1.0)	31 (21-59)	35 (11-73)
$CD25^+$ (% of $CD8^+$)	0.0 (0.0-1.6)	7.8 (5.4-15)	0.0 (0.0-0.9)	97 (83-100)	68 (30-87)

Table 3. 2. Enrichment of tri-virus specific cells through clinical grade CD25 enrichment.
The median and range of 7 clinical grade CD25 enrichment experiments using PBMC from CMV seropositive buffy coats after 3 day culture with viral peptide mixes for CMV, HAdV and EBV is shown. T-lymphocyte: $CD3^+$ cells, Natural Killer (NK): $CD3^-$ $CD16^+/CD56^+$, Natural Killer T-cells (NKT) : $CD3^+$ $CD16^+/CD56^+$, Regulatory T-ceklls (Treg): $CD4^+$ $CD25^+$ $FoxP3^+$ $CD127^-$. ND: Not Done.

Clinical grade enrichment of tri-virus specific cells

Based on the expression pattern after stimulation with viral peptides, we next explored CD25 enrichment using an immunomagnetic positive selection as a potential methodology for the selection of multi-virus specific CTL. To limit non-specific activation of bystander cells, we chose to select for $CD25^+$ after 3 days of stimulation with viral peptides. CD25 selection was performed using clinical grade reagents using the CliniMACS system (Miltenyi) under GMP conditions. Seven enrichments (Table 3.2) were performed starting with a median 4.0 x 10^8 PBMC from CMV seropositive buffy coats. A median 1.4% of unstimulated PBMC at day 0 were $CD25^+$: most of these were in the $CD4^+$ subset, presumably reflecting Tregs. Following 3 day culture in serum-free medium with CMV, EBV and HAdV peptide pools, a median 6.4% of $CD3^+$ cells expressed CD25 (upregulation $p<0.01$). Following CD25 positive selection, 1.1 - 3.1 x 10^7 (median 2.2 x 10^7, 3.3% of starting PBMC dose) cells were collected in the enriched fraction, of which a median 44% were $CD25^+$ (Table 3.2).

As shown in Table 3.2, CD25 selection was highly efficient, as no $CD25^+$ cells were detected in the depleted fraction, but the percentage of $CD25^+$ T-cells was higher in $CD8^+$ (median 97%) than in $CD4^+$ T-cells (median 31% $CD25^+$, enrichment $p< 0.0001$ *and* $p< 0.001$ respectively). To optimize detection of functional anti-viral responses, CD25 selected T-cells were rested for 3 days following selection prior to restimulation. Immediately after selection, the majority of CD25-enriched cells were $CD4^+$, however as shown in Figure 3.2A, $CD8^+$ T-cells expanded preferentially during the resting period. Further, we observed a significant enrichment of the $CD45RA^-$ population during this period (Figure 3.2B), consistent with preferential expansion of antigen-stimulated memory T-cells.

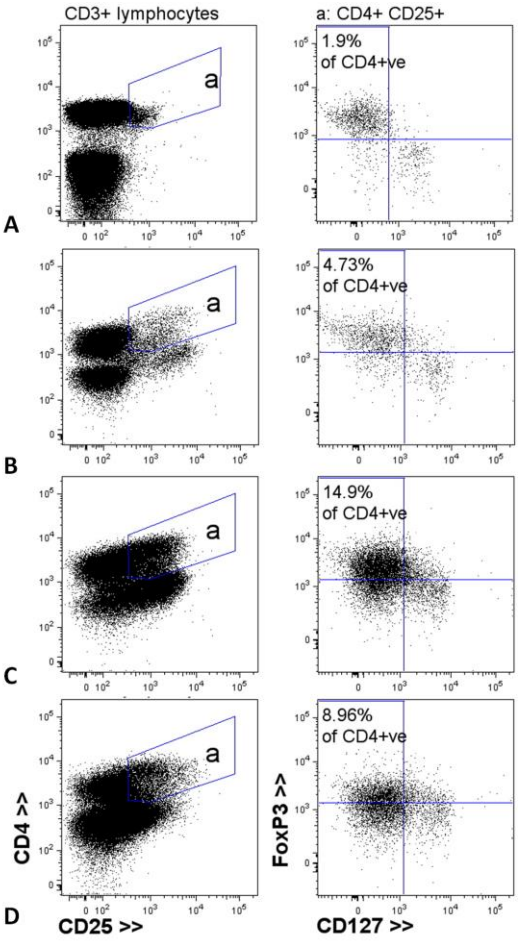

Figure 3.3. $CD4^+CD25^+$ T-cells with a Treg phenotype ($FoxP3^+$ $CD127^-$) are enriched during CD25 selection.

From one representative donor, FACS plots of $CD3^+$ T-lymphocytes are shown: **(A)** before procedure, **(B)** after 3 day culture, **(C)** immediately after enrichment and **(D)** 3 days after enrichment. In the left panel CD4 is plotted against CD25, whereas in the right panel FoxP3 is plotted against CD127 for $CD4^+$ $CD25^+$ cells. The percentage of cells with regulatory phenotype is shown in left upper quadrant as percentage of $CD4^+$ T-lymphocytes. Note the decrease in MFI of CD25 and FoxP3 during resting period, which may reflect transient expression in activated T-cells.

To determine whether Tregs are also enriched by CD25 based selection, we compared the frequency of $CD4^+$ $FoxP3^+CD127^-$ T-cells in unmanipulated PBMC with CD25 enriched cells immediately after selection and 3 days post enrichment. As shown in Figure 3.2C, in unmanipulated PBMC, a median 0.9% of $CD4^+$ cells were $CD25^+$ $FoxP3^+$ $CD127^-$. T-cells with this regulatory phenotype were enriched immediately after CD25 selection (median 21%, $p<0.001$) but declined to a median 5% of $CD4^+$ cells after resting ($p<0.05$). Figure 3.3 shows representative FACS plots for the CD25, FoxP3 and CD127 staining in unmanipulated, cultured, CD25-selected and rested cells.

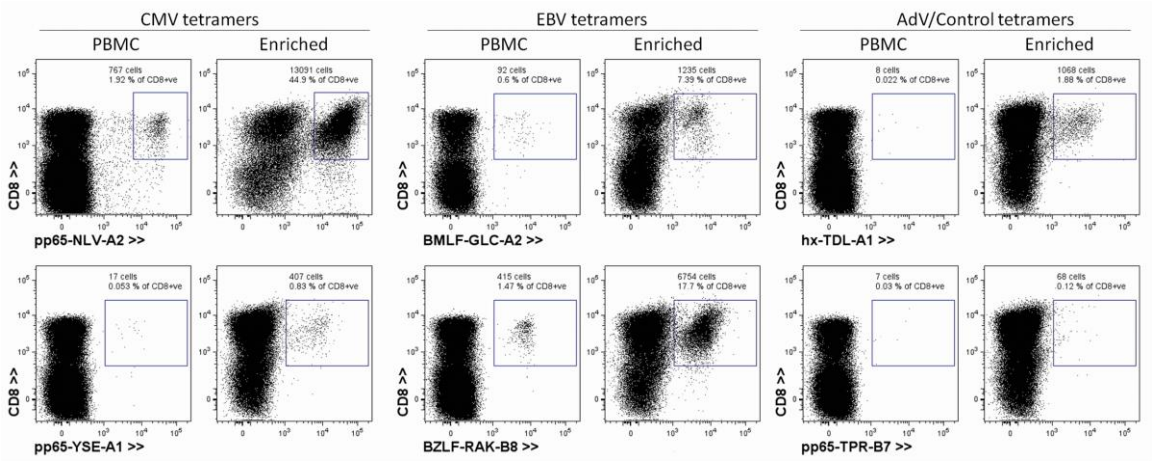

Figure 3.4. Simultaneous enrichment of CMV, EBV and HAdV- specific CD8$^+$ T-cells detected by tetramer staining.

Representative FACS plots (gated on live, CD3$^+$ T-cells) from donor 5 comparing unmanipulated PBMC and rested CD25 selected CTL are shown. Plotted are HLA class I tetramers for pp65-NLV-A2, pp65-YSE-A1, BMF-GLC-A2, BZLF-RAK-B8 and hexon-TDL-A2 against CD8. pp65-TPR-B7 was used as an HLA mismatched negative control. The percentage of tetramer$^+$ cells within the CD8$^+$ gate is shown.

Anti-viral responses of CD25-selected cytotoxic T-lymphocytes

In donors with the appropriate HLA-restriction, we analyzed the frequency of CMV, HAdV and EBV-specific CD8$^+$ T-cells in unmanipulated PBMC and the CD25 selected fraction using MHC-peptide tetramers. Immediately after enrichment, clusters of tetramer$^+$ cells were less distinctive than in unmanipulated cells, so results are presented comparing unmanipulated PBMC and CD25 selected T-cells that had been rested for 3 days (Table 3.3). In 6 out of 7 donors, populations of tetramer$^+$ CMV and EBV specific CTL could be detected in unmanipulated PBMC and 3 of the donors had tetramer$^+$ cells for more than one EBV epitope. Following CD25 selection, for CMV tetramers, a median 20-fold (4.4 – 53, $p< 0.01$) fold enrichment was seen, leading to a median 45% pp65 specific CD8$^+$ cells in the enriched rested fraction. For EBV tetramers, the median enrichment was 14-fold (4.3-38, $p<0.01$) and a median 5.3% of CD8$^+$ cells in the enriched rested fraction stained with EBV tetramers. Only 1 of 5 evaluable donors had a detectable population of HAdV tetramer+ cells in unmanipulated PBMC. In this donor (BC5), a population of 0.02% of unmanipulated CD8$^+$ T-lymphocytes positive for tetramer HAdV5 hx-TDL-A1 was enriched to 1.7% in the rested CD25 selected fraction. Figure 3.4 shows representative FACS plots illustrating the simultaneous presence of CD8$^+$ T-lymphocytes (from donor 5) recognizing peptides from CMV, EBV and HAdV.

	Cytomegalovirus			Epstein-Barr Virus			Adenovirus		
Donor	Epitope	U	E	Epitope	U	E	Epitope	U	E
BC 1	pp65-VLC-A3	neg	neg	EBNA3A-RPP-B7	0.37%	1.6%	hx-KPY-B7	neg	neg
	pp65-TPR-B7	18%	80%	EBNA3A-RLR-A3	neg	neg			
BC 2	pp65-YSE-A1	0.03%	1.6%	BZLF1-RAK-B8	0.31%	6.6%	hx-TDL-A1	neg	neg
	pp6- QYD-A24	neg	neg				hx-TYF-A24	neg	neg
	pp65-VYA-A24	neg	neg						
BC 3	pp65-NLV-A2	0.99%	46%	BMLF1-GLC-A2	0.05%	2.2%	NA		
				LMP2-CLG-A2	neg	neg			
BC 4	pp65-NLV-A2	neg	neg	BMLF1-GLC-A2	0.44%	5.3%	hx-KPY-B7	neg	neg
	pp65-TPR-B7	1.3%	26%	LMP2-CLG-A2	neg	neg			
				EBNA3A-RPP-B7	0.09%	1.5%			
				EBNA3A-RLR-A3	0.16%	1.4%			
BC 5	pp65-YSE-A1	0.05%	0.7%	BMLF1-GLC-A2	0.59%	8.5%	hx-TDL-A1	0.02%	1.9%
	pp65-NLV-A2	1.8%	45%	LMP2-CLG-A2	0.05%	0.3%			
				BZLF1-RAK-B8	1.4%	17%			
BC 6	pp65-NLV-A2	3.10%	55%	BMLF1-GLC-A2	0.12%	3.0%	NA		
				LMP2-CLG-A2	0.10	5.8%			
BC7	pp65-VLC-A3	neg	neg	EBNA3A-RLR-A3	neg	neg	NA		

Table 3. 3. Enrichment of virus specific $CD8^+$ T-lymphocytes assessed by tetramer staining.
Shown is the percentage of living $CD3^+$ $CD8^+$ lymphocytes positive for the corresponding tetramer with unstained control subtracted. Unmanipulated PBMC (U) and CD25-enriched cells 3 days post enrichment (E) are compared. When no cluster of positive cells was detected, this donor was considered to be negative for this tetramer (neg) while NA indicates not applicable as not appropriate HLA type for tetramer analysis.

We next assessed the functional capacity of CD25 selected T-cells to secrete cytokines in response to viral antigens using IFN-γ ELISPOT assays. Unmanipulated PBMC and rested CD25 enriched cells from 5 donors were stimulated with irradiated PBMC pulsed with CMV pp65 or HAdV hexon PepTivator mix or irradiated autologous EBV-LCL. As shown in Figure 3.5 A-C, we observed a median 29-fold (6 - 168, $p<0.05$), 40-fold (1 - 247, $p=0.156$), and 16-fold (1 - 219, $p<0.05$) increase in cells secreting IFN-γ after stimulation with pp65 peptides, hexon peptides, or LCL respectively in CD25 selected cells compared with unmanipulated PBMC from the same donors.

Using this methodology, CD25 selected T-cells showed significant (> 25 SFC/ 10^5 cells) responses to CMV and EBV in all 5 donors and to HAdV in 3 donors. BC 1 and 4 had a low response to HAdV in both unmanipulated and enriched cells, suggesting these donors may not have had a pre-existing memory T-cell response to HAdV hexon.

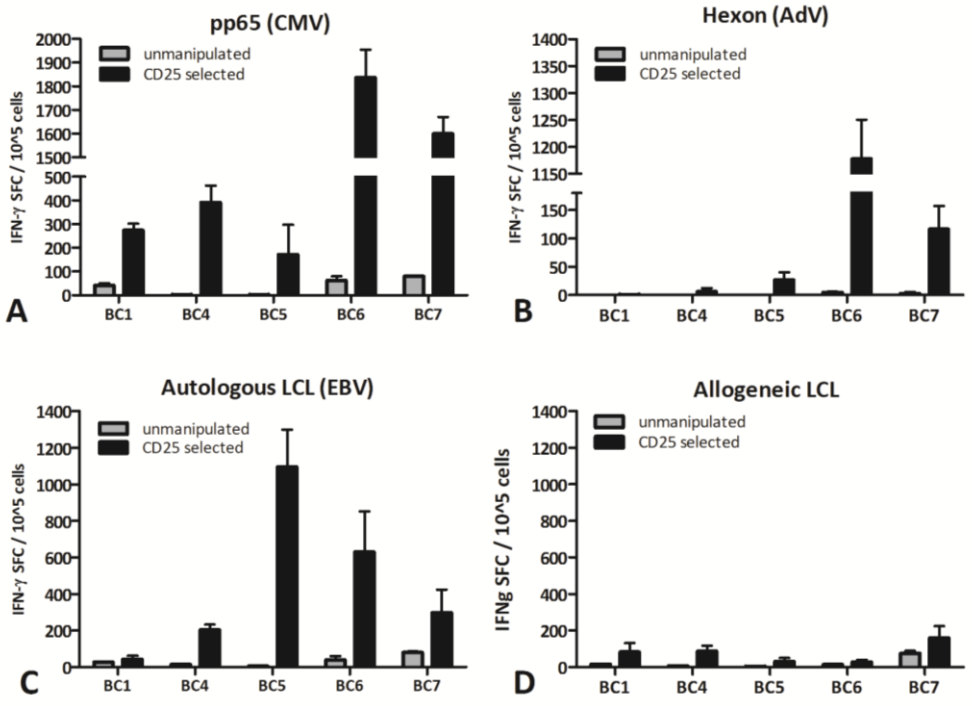

Figure 3.5. Enrichment of the IFN-γ secreting cells specific for all 3 viruses in the CD25 enriched fraction. ELISPOT assays comparing IFN-γ response of unmanipulated PBMC (grey) and CD25 selected, rested CTL (black) to **(A)** autologous CMV-pp65 peptide pulsed PBMC, **(B)** autologous HAdV5-hexon peptide pulsed PBMC and **(C)** autologous EBV-LCL in 5 donors. Shown is the mean number of spot forming cells (SFC) per 10^5 cells of triplicates after subtraction of response to autologous PBMC. Error bars represent standard error of the mean (SEM).

Given the numbers of CD25-enriched T-cells obtained, it was not possible to evaluate cytotoxic activity fully. Nonetheless, to investigate whether CD25 enriched T-cells were able to kill virally infected cells, in 2 donors we evaluated cytotoxic activity in a standard ^{51}Cr release assay against autologous and allogeneic EBV LCL. Our data show that the CD25 enriched T-cells show significant cytotoxicity against autologous EBV LCL but not allogeneic LCL (32.2% vs 11.5 % at a 30:1 ratio, Figure 3.6). Thus, our data demonstrate that CD25 enriched T cells are able to kill virally infected target cells in an MHC-restricted fashion.

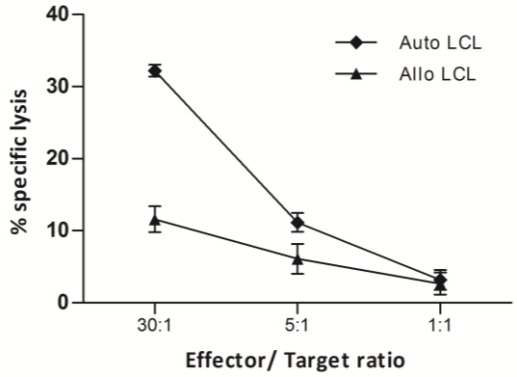

Figure 3.6. CD25 enriched T cells show EBV-specific cytotoxic activity.
A standard ^{51}Cr release assay was performed to determine the EBV virus-specific cytolytic activity of the CD25 enriched cells. The Figure 3.shows the percentage of specific chromium release from target cells. Autologous and allogeneic LCL were labeled with ^{51}Cr and incubated with CD25 cells for 6 h at the E:T ratios indicated.

CD25 selected T-cells suppress alloreactivity

To determine the alloreactivity of CD25 enriched cells and their ability to proliferate in response to viral antigens, we next analyzed the proliferative response of CD25 selected CTL to irradiated allogeneic PBMC, autologous PBMC pulsed with CMV pp65/HAdV hexon PepTivator mixes or autologous LCL in ^{3}H-thymidine uptake assays. As illustrated in Figure 3.7A, in all 5 donors, CD25 enriched CTL showed a very marked depletion of proliferative responses to allogeneic stimulators compared to unmanipulated PBMC from the same donors ($p<0.05$), suggesting reduced alloreactivity. However, in 4 of 5 donors, CD25 enriched CTL also showed little proliferative response to viral antigens (pp65, HAdV and autologous LCL), in contrast to unmanipulated PBMC. Consistent with our flow cytometric data, this suggested that the CD25 selected fraction also contained a significant proportion of regulatory cells, which may inhibit proliferation in response to stimulation with viral antigens as well as alloantigens *in vitro*.
To elucidate whether Tregs within the CD25 selected CTL are functional, we assessed the capacity of CD25 selected CTL to suppress alloreactive proliferative responses. For this, we added CD25 enriched cells in different concentrations to autologous PBMC stimulated with irradiated allogeneic PBMC and measured proliferation in a standard allo-MLR. As shown in Figure 3.7B, our results demonstrate that CD25 selected CTL do indeed suppress alloreactive proliferative responses in a dose-dependent manner, with a median 98% reduction in proliferation at a suppressor :effector ratio of 1:1 ($p<0.01$).

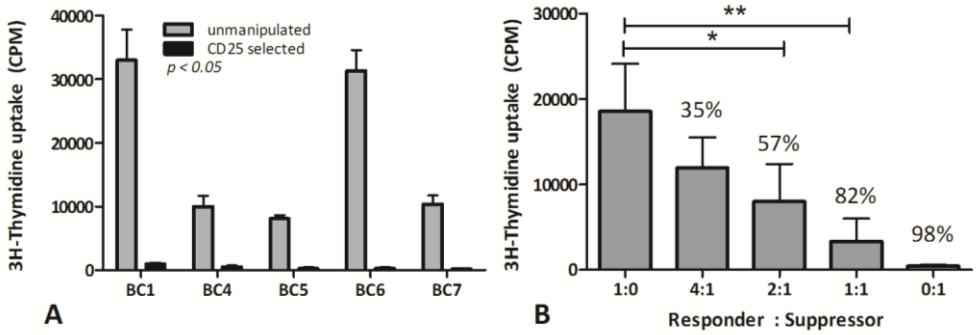

Figure 3.7. Suppression of alloreactivity by CD25 enriched cells.
(A) Proliferative response of unmanipulated PBMC (grey) and CD25 enriched CTL (black) to allogeneic PBMC stimulators in 5 donors. Bars represent mean ^{3}H-thymidine incorporation in counts per minute (CPM) of triplicates after subtraction of stimulators and responders alone. Error bars show SEM.
(B) Unmanipulated (responder) PBMC were cultured for 5 days with irradiated allogeneic PBMC and serial dilutions of CD25 enriched cells from the same donor (suppressor). Bars show mean ^{3}H-thymidine incorporation in CPM in 5 donors, error bars show SEM. Percentages reflect the mean percentage of suppression for the different responder: suppressor ratios. The assay was performed in triplicates and stimulators, responders and suppressors alone are subtracted.* *p<0.05*, ** *p<0.01* in paired t-test.

Discussion

The complexity of protocols needed for generation of CTL and Tregs has prevented broader application of adoptive immunotherapy beyond major academic centers. Here, we have developed a rapid (3 days), simple and clinically applicable method for the simultaneous selection of multi-virus specific CTL and Tregs for adoptive transfer.

We have shown CD25 (IL-2Rα) to be an excellent marker for the selection of virus activated T-cells. CD25 was upregulated upon activation with viral peptides on a larger proportion of virus-specific T-cells than IFN-γ or other activation markers tested. In this regard, it is clear that only a subset of virus-specific Th1 cells secrete IFN-γ,[79] so that CD25 based selection may enable the selection of activated virus specific T-cells that are not secreting IFN-γ, representing a significant HAdVantage over existing IFN-γ methodology. CD25 enrichment results in a mixed population of both CD4 and CD8 effector T cells. However, it should be noted that we have not fully characterized the cytokine secretion profile of virus-specific CTL obtained by CD25 enrichment and this requires further study.

Our approach represents a significant HAdVance over previous methodologies for generation of multi-virus specific CTL,[180;183;190] in that it avoids the necessity for viral vectors and generation of specialized antigen-presenting cells such as LCL or DC. Our protocol instead utilizes APC within PBMC to present exogenously pulsed peptide pools from immunodominant viral antigens. This approach has previously been shown to be effective in generation of CMV, HAdV and EBV-specific CTL singly.[81;184;191] We have demonstrated for the first time that following stimulation

with peptide pools from multiple viral antigens, it is possible to select CTL with specificity against multiple viruses, this is likely to reflect enrichment for discrete populations of CMV, Adenovirus and EBV-specific CTL. Using tetramer staining, we detected enrichment of $CD8^+$ T-cells recognizing CMV and EBV epitopes following CD25 selection in 6 of 7 normal donors. ELISPOT assays demonstrated the simultaneous presence of bi- (*n*=2) or trivirus (*n=3*) specific cells in all evaluated donors, with a median 29-fold (6-168), 40-fold (1-247) and 16-fold (1-219) enrichment of cells secreting IFN-γ in response to CMV pp65, adenovirus hexon and EBV LCL compared to unmanipulated PBMC from the same donors. Consistent with previous studies,[180;182] the magnitude of the response of CD25 selected CTL was highest for CMV, followed by EBV and then HAdV. Given the short stimulation period, we would not expect overgrowth of CTL with specificity for an individual virus, so that the differences in the frequencies of CTL specific for the 3 viruses in these assays are likely to reflect the different precursor frequencies for CMV, EBV and HAdV-specific CTL in donor blood. It is possible that the addition of epitopes to the EBV peptide pool could enhance the enrichment of EBV CTL - in our cohort, each donor could potentially recognize a median 6 of the 23 EBV epitopes. While tetramer-positive $CD8^+$ T-cells recognizing hexon were seen in only 1 evaluable donor, IFN-γ responses were detected in 3 of 5 donors after CD25 selection. This is likely to reflect the fact that T-cell response to HAdV is mainly mediated by $CD4^+$ lymphocytes[192] and the immunodominant hexon epitopes are not identified for many HLA restrictions as well as the lower frequency of HAdV-specific CTL.

It should be noted that multi-virus specific CTL cultures were generated only from CMV-seropositive donors. Previous studies have demonstrated the difficulty of generating CTL from seronegative donors, as existing protocols expand memory rather than prime naïve T-cell responses. As 90% of adult donors have been infected by adenovirus and EBV, this is generally not an issue for these viruses. However, only 60 % of the population is seropositive for CMV, which may limit the applicability of our approach in patients with CMV seronegative donors.

The peptide pools used for stimulation of virus-specific CTL were designed to stimulate both $CD4^+$ and $CD8^+$ virus-specific T-cells. Previous studies suggest that virus-specific $CD4^+$ T helper cells are needed to maintain responses *in vivo.*[193] In this regard, the presence of high proportion of $CD4^+$ (mean 67%) T-cells in our CD25 selected population may improve the durability of anti-viral T-cell responses following adoptive immunotherapy, as well as broadening their target specificity to include HLA Class II restricted antigens such EBNA-1 and hexon. As expected, the majority of CD25 selected CTL show a memory phenotype which may also favour sustained responses *in vivo.*

For safe adoptive transfer, virus-specific T-cells should have little alloreactivity. In our system, alloreactive T-cells should not be activated to express CD25 and selected CTL should therefore have reduced alloreactivity compared with unmanipulated PBMC. While our data from proliferation assays shows negligible proliferative responses of CD25-selected T-cells to 3rd party stimulators in all donors, this is complicated by the enrichment of Tregs in the CD25-selected fraction, which rendered our T-cells generally unresponsive to antigenic stimuli, so that it is difficult to be certain of the alloreactivity. However, recent data has shown that even when alloreactive T-cells are detectable *in vitro* in virus-specific CTL, this was not associated with the development of clinical GVHD when CTL were adoptively transferred post-SCT.[194] Further,

given that CD25-based selection of 450 ml blood draws gave a median yield of 2.2 x 10^7 T-cells, equivalent to a dose of 3 x 10^5/kg for a 70kg recipient, and that even with unmanipulated donor lymphocyte infusions in the HLA matched unrelated donor setting, doses of 10^6 $CD3^+$/kg rarely cause GVHD,[195] we believe that the likelihood of GVHD following adoptive immunotherapy with these doses of CD25-selected CTL is low. The presence of Tregs within our selected fraction may further reduce this risk.

Since CD25 is highly expressed on Tregs,[196] these are enriched by our selection methodology. Consistent with this, a significant proportion of CD25 enriched T-cells (median 21% of $CD4^+$ T-cells immediately after selection) showed a $CD4^+$ $CD25^+$ $FoxP3^+$ $CD127^-$ Treg phenotype. While flow cytometric distinction between Tregs and activated T-cells that transiently upregulate CD25 and FoxP3[197] is difficult and some down regulation of FoxP3 was indeed seen when cells were rested, a proportion of selected T-cells retained this Treg phenotype. More importantly, CD25 selected T-cells from all donors were able to effectively prevent the proliferation of autologous PBMC in response to allogeneic stimulators in suppression assays, demonstrating the presence of functional Tregs in CD25 enriched T-cells. Further studies are needed to determine if restimulation of CD25 enriched T-cells with viral antigens *in vitro* would enhance CTL responses at the expense of Tregs. The infusion of Tregs in murine models had a protective effect against the development of GVHD[198;199] and early clinical studies[200;201] provide support for such an approach. Potentially therefore, CD25 enriched CTL may have a beneficial effect in terms of GVHD. However, previous studies suggest high Treg : T-effector ratios are required for this effect[199] and it may be that the numbers of Tregs produced using our methodology are too small to confer significant clinical benefit.

One key issue is whether the presence of Tregs within the CD25 enriched fraction will influence the function of virus-specific CTL. Our data demonstrate that enriched CTL are able to secrete IFN-γ in response to stimulation with viral antigens and lyse EBV infected target cells. The observed absence of proliferation in response to viral antigens may reflect the *in vitro* system used, since suppression by Tregs is contact dependent.[202] In the absence of such cell-cell contact when enriched cells are adoptively transferred *in vivo*, it is unlikely that infused Tregs will prevent proliferation of virus-specific CTL. In support of this, data from murine models suggest that the co-infusion of Tregs and conventional T-cells enhances rather than abrogates recovery of virus-specific immune reconstitution through prevention of GVHD.[203]

Our protocol is GMP compliant and scale-up experiments were performed under GMP conditions. Clinical grade PepTivator mixes for CMV pp65 and HAdV5 hexon are available (Miltenyi), while the EBV peptide mix has already been used in a clinical study.[81] Cultures were performed in serum-free medium and selection performed in a closed system using clinical grade CD25 reagent which is CE marked for clinical application. The selection methodology used (CliniMACS) is already in widespread clinical use, so that our approach could be broadly applicable in many transplant centers. Starting with 450 ml blood, we routinely generated enough CD25 enriched cells for adoptive transfer of $10^7/m^2$ multi-virus specific CTL, a dose similar to that used in previous clinical studies of immunotherapy with CTL generated by repetitive antigenic stimulation[75;180;181] and significantly higher than those previously achieved using IFN-γ capture.[80]

Further, particularly in the presence of viral reactivation, infused T-cells are likely to expand significantly post-infusion.[77;181]

In conclusion, we have developed a rapid, simple methodology for the simultaneous selection of T-cells with multiple viral-specificities and Tregs in a single 3 day culture. Potentially this approach could be extended to other pathogens where immunodominant antigens have been identified. Because of the speed and simplicity of this methodology, this approach may significantly broaden the applicability of adoptive T-cell transfer to restore cellular immunity to viruses post-SCT. However, the clinical utility of this approach will require evaluation in well-designed clinical studies.

Funding and Disclosures

All authors approved the final manuscript for publication. The authors reported no potential conflicts of interest.

Supplementary data

Type	Short name	Amino acid sequence	Antigen	HLA restriction
Class I & II	11-amino acid overlapping 15-mer peptide pool covering complete sequence of protein		CMV- pp65	All
Class I & II	11-amino acid overlapping 15-mer peptide pool covering complete sequence of protein		AdV5-hexon	All
Class I	CLG	CLGGLLTMV	EBV- LMP2	A*0201
	GLC	GLCTLVAML	EBV- BMLF1	A*0201
	YVL	YVLDHLIVV	EBV- BRLF1	A*0201
	RLR	RLRAEAQVK	EBV- EBNA3A	A*0301
	RPP	RPPIFIRRL	EBV- EBNA3A	B*0702
	QPR	QPRAPIRPI	EBV- EBNA3C	B*0702
	QAK	QAKWRLQTL	EBV- EBNA3A	B*0801
	RAK	RAKFKQLL	EBV- BZLF1	B*0801
	IVT	IVTDFSVIK	EBV- EBNA3B	A*1101
	DEV	DEVEFLGHY	EBV- BMLF1	B*1801
	RYS	RYSIFFDYM	EBV- EBNA3A	A*2402
	TYS	TYSAGIVQI	EBV- EBNA3B	A*2402
	TYP	TYPVLEEMF	EBV- BRLF1	A*2402
	RRIY	RRIYDLIEL	EBV- EBNA3C	B*2702/04/05
	YPL	YPLHEQHGM	EBV- EBNA3A	B*3501
	EPL	EPLPQGQLTAY	EBV- BZLF1	B*3501
	IED	IEDPPFNSL	EBV- LMP2	B*4001
	KEH	KEHVIQNAF	EBV- EBNA3C	B*4402
	VEI	VEITPYKPTW	EBV- EBNA3B	B*44
Class II	PYYV	PYYVVDLSVRGM	EBV- BHRF1	DRB1*04
	FGQL	FGQLTPHTKAVYQPR	EBV- BLLF1	DRB1*13
	IPQC	IPQCRLTPLSRLPFG	EBV- EBNA1	DRB1*13/14
	TDAW	TDAWRFAMNYPRNPT	EBV- BNRF1	DRB1*15

Table 3.S1. Peptide pools used for activation of lymphocytes with specificity for Cytomegalovirus, Adenovirus and Epstein-Barr virus

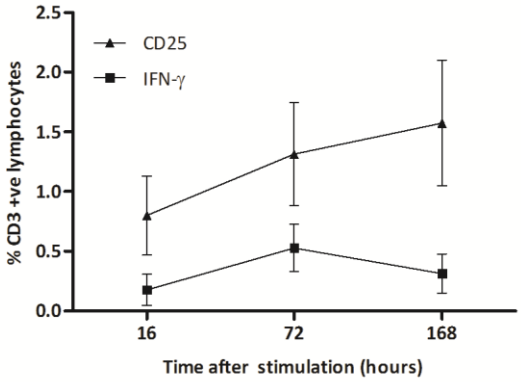

Figure 3.S1. Flow cytometric analysis of EBV-specific $CD3^+$ T cells after stimulation *in vitro* with EBV immunodominant peptides for 16, 72 and 168 hours. Percentage of IFN-γ producing and CD25 expressing in total $CD3^+$ T cells in peripheral blood of 7 EBV-seropositive normal donors. The frequency of $CD3^+/CD25^+$ or $CD3^+$/IFN-γ T cells is shown as the mean percentage (+SEM) after subtraction of IgG isotype and unstimulated controls. Squares show IFN-γ secreting cells while triangles show CD25 positive cells.