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EVALUATION AND OPTIMIZATION OF IMMUNOMAGNETIC METHODS FOR THE PURIFICATION AND SELECTION OF CMV-SPECIFIC CYTOTOXIC T LYMPHOCYTES FROM G-CSF PRIMED PERIPHERAL BLOOD MONONUCLEAR CELLS FOR ADOPTIVE IMMUNOTHERAPY

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Evaluation and optimization of immunomagnetic methods for the purification and selection of CMV-specific cytotoxic T lymphocytes from G-CSF primed peripheral blood mononuclear cells for adoptive immunotherapy

Memoria presentada por **Dª Lorea Beloki Bes** para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo mi dirección y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, ... de ..... de 2014.

Dr. Eduardo Olavarría López-Aróstegui Dra. Natalia Ramírez Huerto

To Ion

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ABBREVIATIONS

AdV	Adenovirus
AIDS	Acquired Immunodeficiency Syndrome
ALL	Acute Lymphoblastic Leukaemia
Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute Myeloid Leukaemia
APCs	Antigen Presenting Cells
APC	Allophycocyanin
ATMP	Advanced Therapy Medicinal Product
BM	Bone Marrow
BMT	Bone Marrow Transplantation
Bu	Busulfan
CAR	Chimeric Antigen Receptor
CBA	Cytometric Bead Array
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myeloid Leukaemia
CMV	Cytomegalovirus
CMV-T	Cytomegalovirus-specific T cells
CTL	Cytotoxic T Lymphocyte
CXCR	Chemokine Receptor
CY	Cyclophosphamide
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusion
dPBS	Dulbecco’s Phosphate Buffered Saline
EBV	Epstein-Barr Virus
EBV-LCL	EBV-transformed B-Lymphoblastoid Cell Lines
FITC	Fluorescein isothiocyanate

FoxP3	Forkheard Box P3
FSC	Forward Scatter
G-CSF	Granulocyte-Colony Stimulating Factor
GMP	Good Manufacturing Practice
GvHD	Graft versus Host Disease
GvL	Graft versus Leukaemia
HBV	Hepatitis B Virus
HLA	Human Leukocyte Antigen
HIV	Human Immunodeficiency Virus
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
IFN-γ	Interferon-gamma
IL	Interleukin
LFA-1	Lymphocyte Function-associated Antigen-1
MHC	Major Histocompatibility Complex
NK	Natural Killer
NKT	Natural Killer T cells
PBMCs	Peripheral Blood Mononuclear Cells
PBSCs	Peripheral Blood Stem Cells
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PHA	Phytohaemagglutinin
RIC	Reduced Intensity Conditioning
RPMI	Roswell Park Memorial Institute
SDF-1	Stromal-derived growth factor-1
SEB	Staphylococcal Enterotoxin B

SSC	Side Scatter
TBI	Total Body Irradiation
T_{CM}	Central Memory T cells
TCR	T Cell Receptor
T_{EM}	Effector Memory T cells
T_{EMRA}	CD45RA+ Effector Memory T cells
Th	T helper cells
Th1	T helper cell 1
Th2	T helper cell 2
TNF	Tumor Necrosis Factor
Tregs	T Regulatory Cells
TRM	Transplant Related Mortality
UCB	Umbilical Cord Blood
VCAM-1	Vascular Cell Adhesion Molecule 1
VLA-4	Very Late Antigen 4
vs.	Versus

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I. GENERAL INTRODUCTION

1. Allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a well-established clinical procedure introduced more than half a century ago, consisting on the infusion of genetically matched stem cells from a donor into a patient. It has evolved from the first experimental bone marrow transplantation (BMT) for rare cases of leukemia, combined immune deficiency, or aplastic anemia, to the standard care for patients with many congenital or acquired severe disorders of the hematopoietic system (Copelan, 2006; Gratwohl et al., 2013). Over the last 20 years allo-HSCT practice has increased almost ten times, becoming the main indication for many leukemias, lymphomas, myelodisplastic syndromes, and non-malignant disorders such as aplastic anemia (Copelan, 2006; Passweg et al., 2012). The procedure of allo-HSCT requires the total or partial elimination of the recipient's hematopoietic and immune systems through pre-transplant chemotherapy and/or radiotherapy. This conditioning treatment provides space for incoming cells to engraft, helps in preventing graft rejection, and kills most residual cancer cells.

1.1 Stem cell source

There are three main sources of hematopoietic stem cells (HSCs) for clinical transplantation: bone marrow (BM), peripheral blood stem cells (PBSC), and umbilical cord blood (UCB). The principal HSC source for both allogeneic and autologous HSCT are PBSCs collected by an apheresis procedure after donor or patient HSC mobilization by using drugs whose active substance is granulocyte-colony stimulating factor (G-CSF), known as filgrastim (Shaw et al., 2011). The European Group for Blood and Marrow Transplantation (EBMT) survey of over 650 centers about HSCT activity in Europe during 2011 reported a total of 32,075 HSCT and found that over 72% of allogeneic and 99% of autologous HSCT were performed using PBSCs (Passweg et al., 2013).

The original source of HSCs was BM, but its use in HSCT has decreased over the years due to the discomfort caused in the donor by BM collection procedure. Furthermore, several trials reported that allogeneic PBSC transplantation can produce a substantially faster engraftment than BM and with reduced rates of relapse, especially in patients with late-onset disease. Moreover, it improves overall and disease-free survival in patients with more advanced hematologic malignancies. However, although a similar correlation with acute graft versus

host disease (GvHD) incidence was found in some of the studies, all trials reported a trend towards more chronic GvHD with PBSC compared to BMT (Bensinger, 2012).

UCB is also a rich source of HSCs and is associated with a decreased incidence of GvHD and the benefits associated with reduced histocompatibility requirements. HSCT using UCB has largely been restricted to pediatric and small adults due to the dose limitations since UCB collections contain a significantly reduced total number of CD34+ cells compared with PBSCs, that can result in delayed or failed engraftment in adults. In more recent years, double unit cord blood transplantations have been carried out to improve survival in adult UCB transplantation, increasing CD34+ cell numbers and therefore reducing the time of engraftment in adult recipients (Rocha and Gluckman, 2009; Ballen et al., 2013).

1.2 Opportunistic cytomegalovirus disease in immunocompromised HSCT recipients

Human cytomegalovirus (CMV), also known as human herpesvirus 5, infects 30 – 70% of the population in developed countries. After primary infection, CMV establishes lifelong latency or persistence, predominantly within mononuclear leukocytes. Initial CMV infection induces a primary immune response that is able to restrain viral replication after reactivation from latency (Gandhi and Khanna, 2004). The immunological control of CMV exerted by the host requires a high proportion of the immune repertoire to be directed against this pathogen, with competent CMV-specific CD4+ and CD8+ T cell subpopulations involved (Khan et al., 2002; Pourgheysari et al., 2007).

However, this control is lost when the host suffers from immune suppression, condition present in patients undergoing HSCT. Susceptibility to viral, bacterial, or fungal infections in the HSCT recipient is the result of profoundly reduced innate and adaptive immunity in the immediate post-transplant period caused by the immunoablative effect of the host BM conditioning regimens (Winston et al., 1993; Zaia et al., 2009). CMV is a major opportunistic infectious agent among HSCT recipients; the incidence of CMV reactivation during the posttransplant period is approximately 70 – 80 % in adults and 30 – 40 % in pediatric allogeneic HSCT (Gerna et al., 2008), and primary infection following HSCT occurs in 20 - 40% of CMV seronegative patients whose donor is CMV seropositive (Sellar and Peggs, 2012). The most common clinical manifestations of CMV disease are pneumonia and gastrointestinal disease but retinitis, central nervous system disease and marrow suppression may also be observed (Busca, 2009).

1.3 Anti-CMV treatment post HSCT

Prophylactic or pre-emptive anti-viral strategies are widely used to control CMV infection following HSCT. Therefore the administration of anti-viral drugs, also able to restrain other herpesviruses, is nowadays the standard first line therapeutic treatment against primary or reactivated CMV infection and disease (Meijer et al., 2003). Ganciclovir and foscarnet are the most used drugs for preemptive therapy, showing similar efficacy although foscarnet is preferred when the patient suffers from neutropenia. Currently, new anti-CMV agents with evidence of clinical activity in CMV infection are being used, such as cidofovir, usually administered when a resistance to the previous treatments is developed (Boeckh and Ljungman, 2009; Busca, 2009).

However, even though they reduce morbidity and mortality associated with this virus, these treatments can lead to myelosuppression, thrombocytopenia, and other drug-related toxicities such as renal and metabolic damage, they show a high variability in efficacy, and can induce the emergence of resistant CMV strains (Busca, 2009). Furthermore, anti-viral therapies such as ganciclovir and foscarnet are associated with a delay in the reconstitution of virus-specific T cells and predispose the patient to develop late CMV disease (Li et al., 1994; Battiwalla et al., 2007). For all these reasons, many clinical trials have assessed the effects of the combination of anti-viral drugs with therapies based on infusion of virus-specific primed T cells (Bollard et al., 2004).

2. Adoptive immunotherapy against virus

The classical concept of adoptive immunotherapy is based on the infusion of donor unmanipulated bulk lymphocytes (DLI) at variable intervals following BMT, enhancing for example the antileukaemic effect of the graft (Perry and Mackinnon, 1996). This idea is supported by the cytogenetic remission that was observed in three patients receiving a buffy coat infusion of their original marrow donors after chronic myelogenous leukemia (CML) relapse following a conventional allograft transplant (Kolb et al., 1990). Moreover, in five patients who developed Epstein-Barr virus (EBV) lymphoma after T cell-depleted allogeneic BMT, nonirradiated DLI infusions resulted in clinical remissions that were achieved within 30 days after cellular treatment (Papadopoulos et al., 1994). Despite these promising clinical results, also corroborated by Heslop *et al.*, this type of cellular therapy presents a high risk for

the patient's health (Heslop et al., 1994). Indeed, there is evidence that administration of total lymphocytes is associated with high morbidity and mortality rates, mainly due to severe GvHD (Kolb et al., 1995). Alloreactive CD8+ cytotoxic T cells (CTLs) contained within the transferred bulk of leukocyte population are directly responsible for the aggressive GvH syndrome. Consequently, different protocols have been developed to limit the presence of these anti-host reactive subpopulations in order to obtain a safer cellular product.

In immunocompetent subjects, the exposure to viral antigens and their recognition by T cells trigger T cell receptor (TCR)-signal dependent activation, which drives their expansion, differentiation and regulates the magnitude of the T cell response (Lanzavecchia and Sallusto, 2002). Virus-primed T cells are generated during this process (Kaech et al., 2002). This physiological process can be replicated *ex vivo* in lymphocyte cultures exposed to viral antigens, and thus, cell proliferation and differentiation into diverse T cell subsets can be achieved (Wherry et al., 2003). This is the basis of the incipient adoptive immunotherapy strategy suggested by Ridell *et al.,* and adopted by many researchers nowadays (Riddell et al., 1992).

Antigen-specific T cell manufacturing strategies can be divided into 3 different main groups, depending on the method used for therapeutic product manufacture (Sellar and Peggs, 2012; Saglio et al., 2014) (Figure 1):

1. **Culture based techniques** to induce antigen-specific T cell expansion
2. **Genetic engineering techniques** for the redirection of T cell antigenic specificity
3. **Direct selection methods** to capture antigen-specific cells

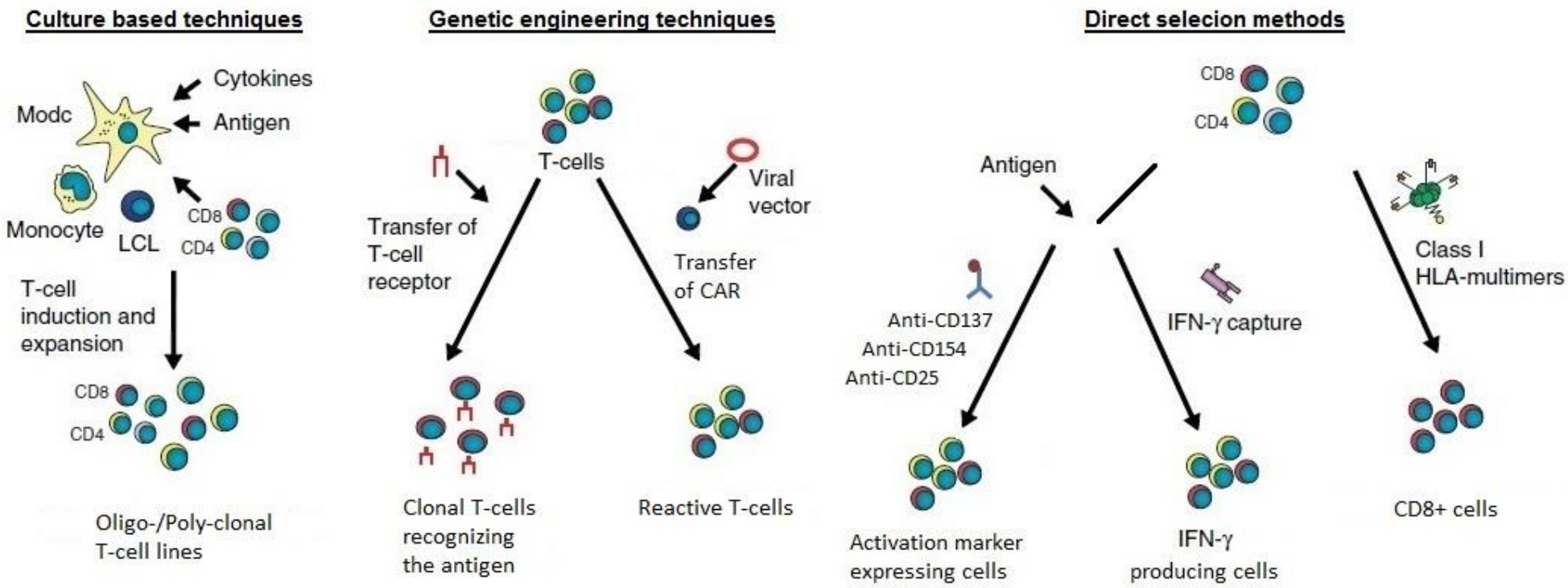

Figure 1: Strategies to generate antigen-specific T cells for adoptive immunotherapy. First methods used cell culture based techniques that induce antigen-specific T cell expansion through antigen presentation by B-cells, fibroblasts, DCs, or EBV-LCLs using peptides, peptide pools, or recombinant proteins. Genetic engineering approaches have recently been developed, which allow the redirection of the T cell antigenic specificity by the introduction of TCR genes or CAR constructs. Direct selection methods are faster and easier to obtain; MHC-multimers directly select antigen-specific CTLs, and for γ-capture method and for the isolation of virus-reactive cells expressing activation markers a previous antigenic stimulation is essential. Figure adapted from Sellar and Peggs (2012).

2.1 Culture based techniques

The first studies involving adoptive transfer of donor-derived CMV-specific T cells after allo-HSCT used CD8+ T cell clones expanded by co-culturing donor-derived peripheral blood mononuclear cells (PBMCs) with autologous virus-pulsed fibroblasts for 5 to 12 weeks (Riddell et al., 1992; Walter et al., 1995). Following these infusions no patient developed CMV-associated clinical disease or the side effects usually associated to the therapeutic treatment. The analysis of rearrangements of the TCR β-chain variable region demonstrated *in vivo* clonal expansion from the transferred clones, and these infused T cells exhibited a similar cytotoxic activity to that measured in the immunocompetent donors. However, as this *ex vivo* expansion required long culture periods and was undertaken in the presence of live virions, the methodology could put at risk the patient's safety, and therefore, it has been limited to a restricted number of cases (Peggs et al., 2001). But this first approach was the basis for the new protocols used for anti-viral cellular product manufacture.

Similar prophylactic application of CMV-reactive CD4+ T cell clones was also used to avoid CMV disease after allo-HSCT. The transfer was associated with the detection of both CD4+ and CD8+ CMV-specific responses shortly following transfer, and a reduction of CMV antigenemia was observed (Perruccio et al., 2005).

Dendritic cells (DCs), very potent professional antigen presenting cells (APC), have been extensively used for *in vitro* virus-specific T cell production to be used in adoptive immunotherapy (Bollard et al., 2007; Peggs et al., 2009; Blyth et al., 2013). The main advantages of using DCs is their high capacity for antigen processing and presentation of multiple epitopes in the context of major histocompatibility complex (MHC) class I and class II molecules and their applicability to patients of all human leukocyte antigen (HLA) types. It has also allowed the manufacture of polyclonal T cell subpopulations with the advantage of synergism between different immunological subpopulations (Peggs et al., 2001; Peggs et al., 2003). Otherwise, the immunodominant T cell epitopes present in a particular given antigen must be known in order to be able to expand the patients' T cells. In addition, it is possible to modulate the expression of their co-stimulatory molecules allowing the manipulation of the immunological synapsis that would enhance the activation or inhibition of the T cell response (Liechtenstein et al., 2013).

EBV-transformed B-lymphoblastoid cell lines (EBV-LCL) transduced with a retroviral vector encoding the immunodominant CMVpp65 have also been used as APCs to simultaneously expand memory EBV and CMV-specific CTLs. These CTLs showed class I and class II viral-bispecific restriction (Sun et al., 1999). Furthermore, EBV-LCL can be grown in large numbers, which would enhance the therapeutic potential of the protocol. Thus, Leen *et al*. have improved the system by using EBV-LCL transformed with a recombinant adenovirus expressing CMVpp65, with the purpose of obtaining T cell preparations with trivirus-specific activity; EBV-, Adenovirus (AdV)-, and CMV-specific CTLs. Following adoptive transfer CD4+ and CD8+ T cell expansion was observed, leading to *in vivo* resolution of virus-associated clinical symptoms within the first month of therapy (Leen et al., 2006).

Despite the clinical success of these techniques, culture periods needed for the generation of antigen-specific cells under good manufacturing practice (GMP) conditions require complex logistics that are both time consuming and costly, which limits the translation of this practice into clinical routine.

2.2 Genetic engineering based techniques

Several clinical trials are currently assessing the validity of new genetic engineering methodologies (Maus et al., 2014), consisting on the redirection of the T cell antigenic specificity by the introduction of TCR genes (variable α- and β-chains) or chimeric antigen receptors (CARs) followed by infusion in the patient. This strategy can be used to engineer fully functional virus-specific T cells, which would otherwise be "inactivated" after chronic viral antigen exposure (Dembic et al., 1986), like Hepatitis B virus (HBV)-specific T cells that are deleted or exhausted in chronic hepatitis B and HBV-related hepatocarcinoma patients.

a) TCR gene transfer

Gehring *et al.* obtained fully functional TCR re-directed HBV core (18-27) and surface (183-191; 370-379)-specific effector T cells after epitope-specific TCR cloning by retroviral transduction. These T cells exhibited cytotoxic activity toward HBV-infected cells (Gehring et al., 2011; Wood, 2011). Kessles *et al.* demonstrated the expansion and maintenance of TCR-modified transferred T cells up to 81 days after inoculation, clearly showing their *in vivo* proliferative capacity (Kessels et al., 2001). Currently, different tumors and viral

infections are being treated with this type of cell transfer therapies, in some cases demonstrating cancer regression in 30% of treated patients (Johnson et al., 2009).

A potential problem of this methodology is the association of the recombinant α/ß chains with the endogenously expressed TCR chains, generating T cells with unknown and undesired new autoantigen specificities (Heemskerk et al., 2004) and with reduced TCR surface expression. In this way, sporadic autoreactive events could potentially appear in the recipients, something to be taken into consideration. The introduction of cysteines on each recombinant TCR chain to form an extra interchain disulfide bond between both α- and ß-structures could prevent this intracellular molecular event, significantly boosting the effector activity (Cohen et al., 2007). Cohen *et al*. designed a murinization strategy based on the substitution of the human C regions with their murine counterparts (Cohen et al., 2006). However, these xenogenic sequences might also potentially trigger immunogenicity against the murinized TCR. Bialer *et al* minimally engineered human C regions with selected murine residues mediating superior chimeric TCR expression and improved activity, which would result in a more efficient pairing of the murine Cα and Cß1, decrease the formation of mixed TCR chain dimers, and minimize autoimmune manifestations (Bialer et al., 2010).

b) Chimeric antigen receptors

- **First CAR constructs**

Safety and feasibility of T cells transduced with CARs in adoptive cell therapy procedures are being evaluated in human clinical trials (Kandalaft et al., 2012). These constructs recognize tumor cell-surface molecules, consisting of the fusion between the antigen-recognition portion of a monoclonal antibody with an intracellular signaling domain capable of activating or enhancing T cell effector function by intensifying molecular signaling pathways in a MHC dependent and independent fashion (Johnson et al., 2009; Brenner and Heslop, 2010; Jena et al., 2010).

Consequently, Micklethwaite *et al.* engineered virus-specific T cells stimulated with multispecific-viral immunodominant antigens of CMV, EBV and AdV. These modified cells also exhibited antitumor activity that was conferred by their retroviral-mediated expression of CAR.CD19+, obtaining a bi-functional therapeutic harvest (Micklethwaite et al., 2010). This approach allows the expansion of multivirus-specific CAR-modified CTLs, which retain their

antiviral activities, but with significantly increased *ex vivo* antitumor activity against B-cell acute lymphoblastic leukemia (B-ALL) blasts from patients with hematological disease.

Recently, the monitoring of anti-CD19 CAR-modified T cells has been possible through the use of an antibody consisting of human CD19 extracellular domains and human immunoglobulin domain. Similarly to MHC-multimer technology, the fluorescent-labelling of this structure allows the direct visualization of CAR-expressing T cells by flow cytometry, which makes this approach very attractive (De Oliveira et al., 2013). However, only a few tumor-specific antigens expressed exclusively by cancer cells and susceptible of being targeted have been identified. This problem could be solved by the CAR-target replacement by a fluorescein isothiocyanate (FITC) molecule. The use of cetuximab, trastuzumab, or rituximab monoclonal antibodies conjugated with FITC would expand the applicability of this tool allowing the simultaneous recognition of a variety of tumor-associated antigens by a single therapeutic product (Tamada et al., 2012). Using this molecular approach, Louis *et al.* and Pule *et al.* achieved effective anti-tumor responses in patients with advanced-stage neuroblastoma. Treated patients exhibited partial and complete tumor responses respectively, and modified T cells showed a long-term persistence beyond 96 weeks. These T cells were engineered from EBV-specific cytotoxic T cells expressing the CAR.diasialoganglioside antigen-GD2 (Pule et al., 2008; Louis et al., 2011). These encouraging clinical results have demonstrated the high viability of these therapeutic T cells in cancer patients. However, these results have not been reproduced by other authors. Kershaw *et al.* observed a high level of therapeutic T cells during the first few days after infusion of autologous anti-α folate receptor CAR modified T cells in patients with metastatic ovarian cancer, but these T cells were undetectable after a month of monitoring (Kershaw et al., 2006). A poor choice of the pan-tumor antigens or a weak functional activity could explain these results.

- **Improved CAR constructs**

Following this approach, three different CAR-generations have been developed, which incorporate T cell co-stimulatory signaling molecules in their structure (CD28, CD3-zeta(ξ), OX40, 4-1BB (CD137)), improving the signaling capacities of modified T cells (Song et al., 2011; Shi et al., 2013). These molecular modifications have enhanced the *in vivo* expansion in more than 1000-fold, have increased the long-term maintenance for at least 6 months of the engineered CAR+ T cells into the patient, and have induced trafficking to the BM or cerebrospinal fluid. CAR+ T cells that targeted CD19 or *ERBB2* (HER-2/neu) and contained

costimulatory domains from CD28 or CD137 and the T cell receptor ξ chain signaling element were infused in patients with relapse or refractory chronic lymphocytic leukemia (CLL) (Brentjens et al., 2010; Kalos et al., 2011; Porter et al., 2011), acute lymphoblastic leukemia (ALL) (Grupp et al., 2013), and metastatic colon cancer (Morgan et al., 2010). A potent antitumor effect that induced tumor regression could be observed in all treated patients, in some cases associated with morphologic and molecular remission. However, unexpected clinical adverse events were noted in some patients including the occurrence of delayed tumor lysis syndrome accompanied by hemophagocytic syndrome, capillary leak syndrome, non-infectious fevers, hypotension, respiratory distress syndrome, grade 3/4 lymphopenia and loss of normal B cells. In most cases the administration of glucocorticoids or anti-cytokine therapy resolved these reversible systemic effects although hospitalization in intensive care units was necessary depending on the case. A cytokine-release syndrome or "cytokine storm" has been also observed in a limited number of patients, either after intensive lymphodepletion and before immunotherapy treatment, or after CAR-transduced T cell infusion itself (Kalos et al., 2011; Porter et al., 2011; Grupp et al., 2013). Most patients responded well to anti interleukin-6 (IL-6) (Tocilizumab) and/or anti tumor necrosis factor (TNF) (Etanercept) treatment, although in a few medically fragile patients cytokine blockade with drugs was not effective and resulted in multiple organ dysfunction and death (Brentjens et al., 2010; Morgan et al., 2010). However, the precise etiology of these patients' deaths remains uncertain.

2.3 Direct selection methods

a) Multimer technology

The development of multimer technology has provided an invaluable method for monitoring and purification of T cells with a known antigenic specificity. The basis for this technology resides in the recognition of antigen-specific TCRs by a recombinant class I or class II molecule complex bound to a certain immunodominant peptide. Identification of antigen-specific CTLs regardless of their biological activity allows the preparation of a heterogeneous T cell population, which avoids previous phenotypic characterizations required for the identification of primed subpopulations with long-term survival capacities. Consequently, this staining technology allows the isolation of T cells with a given antigen specificity from seropositive donors without any further manipulation (Ramirez and Olavarria, 2013).

Nowadays, there is a wide variety of available MHC multimer molecules such as dimers, tetramers (TM), pentamers (PM), streptamers (ST), dextramers, and octamers (Casalegno-Garduno et al., 2010). Tetramers are recombinant molecules made of four MHC subunits that have been biotinylated. They are folded with the peptide of interest and tetramerized by a fluorescence-labeled streptavidin molecule (Altman et al., 1996). The tetrahedral disposition of MHC molecules in the complex enables its binding to just three TCR molecules at once, in comparison to the pentamers, which can bind to five TCRs at a time. It is due to the disposition of its five MHC-peptide molecules facing the same direction and the multimerization by a self-assembling coiled coil domain (Figure 2A). This is the reason of the higher avidity of the pentamers compared to tetramers, although their sensibility is equivalent (Yao et al., 2008). More recently, Streptamer technology have been developed (Figure 2B), which allow the dissociation of the multimer from the antigen-specific cell after the addition of the competing molecule D-biotin. *Strep*-tags are fused to MHC-molecules forming MHC-I-*Strep* fusion proteins, which allow MHC oligomerization by joining to the *Strep*-Tactin structure. Multimers of MHC I-Streps complexed with either fluorescently or magnetically labeled *Strep*-Tactin form the Streptamer complex, and can be used for efficiently stain or isolate antigen-specific T cells (MHC Streptamer Manual, IBA, www.iba-lifesciences.com). The addition of the competitor molecule D-biotin allows the monomerization of the MHC molecules that are spontaneously released from cell surface (Knabel et al., 2002).

These multimer molecules have been extensively used to identify and select CMV, EBV and AdV-specific T cells from healthy donors, their transfer to immunocompromised hosts has shown excellent results, and they are being tested in various clinical trials. First, Cobbold *et al.* used tetramers to isolate CMV-specific CTLs, and they were infused into nine allo-HSCT patients. After the infusion, CMV reactivation resolved completely or was significantly reduced in all patients (Cobbold et al., 2005). Uhlin *et al.* treated 9 patients with CMV, AdV, or EBV cells isolated using pentamers, who showed insufficient treatment response to conventional antiviral treatment or drug-related toxicity. Successful results were found in the majority of patients. However, the only patient treated with Adv-specific CTLs was not able to respond to the treatment and later died from the adenoviral infection (Uhlin et al., 2009; Uhlin et al., 2012). Schmitt *et al.* adoptively transferred CMV-specific CTLs isolated using streptamers into two patients that underwent allo-HSCT, and showed that infused cells were able to expand *in vivo* and control CMV antigenemia (Schmitt et al., 2010).

A) B)

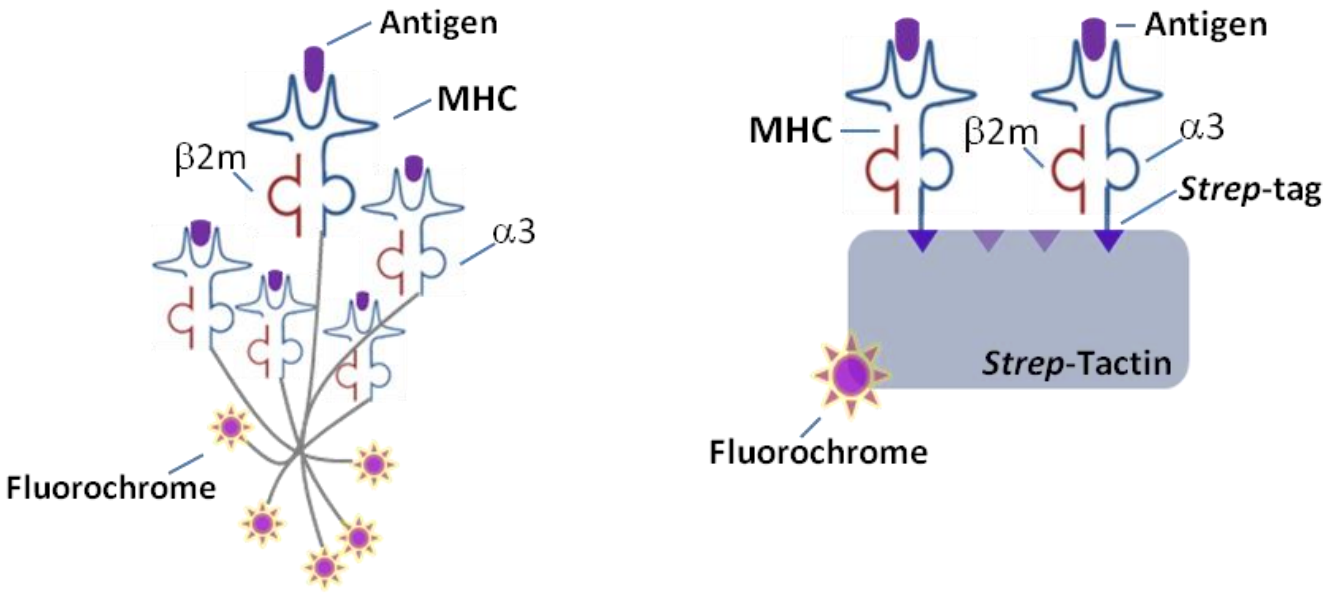

Figure 2: Structure of MHC-multimers. A) PM is composed of five MHC molecules facing the same direction and self-assembled by a coiled-coil domain. B) In the ST, MHC molecules are bound to the *Strep*-Tactin by the *Strep*-tag, generating the Streptamer complex.

- ***In vitro* modulation of the functional ability of viral-specific CTLs by multimer complexes**

Multimer technology developed by Altman *et al.* allows the identification and enrichment of viral-specific CTLs without altering their differentiation status (Altman et al., 1996). However, recent studies with soluble experimental MHC class I-tetramers have shown in a transgenic mouse model that continuous *in vivo* administration of a MHC multimer induces unexpected outcomes in the antigen-reactive CD8+ cells. An increase in the frequency of annexin V staining was observed that could be attributed to the induction of cellular anergy or activation of induced cell death (Maile et al., 2001). In the first case, anergy can be triggered by a strong "signal 1" provided by the binding of the experimental tetramer with its specific transgenic TCR, in the absence of "signals 2" (co-stimulation) and "signals 3" (cytokine priming) (Liechtenstein et al., 2013). Instead, a limited expansion of these CD8+ T cells and their effector activities are compromised by the continuous presence of the soluble tetramer within the transgenic mice, leading to either clonal exhaustion or anergy, resulting in T cell dysfunction.

In agreement with this, Neudofer *et al.* observed *in vitro* that both peptide-specific activation-dependent cytotoxic activity and proliferation capacity of primed T cells were impaired and decreased following staining with conventional tetramers (Neudorfer et al., 2007). Wang *et al.* corroborated these findings by reporting that, although CMV-specific CTLs selected with tetramers are able to proliferate and synthesize interferon-gamma (IFN-γ) and granzyme B, their proliferative capacity and ability to release cytokines is not as efficient as CMV-specific CTLs selected with Streptamers (Wang et al., 2013). Some authors have shown in experimental models that the interaction between the tetramer complex with the TCR of the antigen-specific CTL induces gene transcription (Daniels and Jameson, 2000; O'Herrin et al., 2001). It has also been demonstrated the loss of protective capacity to *Listeria monocytogenes* in BALB/c mice after transference of CTLs pre-treated with MHC-tetramers (Knabel et al., 2002). However, administration of CMV-specific CTLs in stem cell transplant recipients that had been previously selected using tetramer complexes efficiently contributed to the control of virus dissemination (Cobbold et al., 2005). In this study, the authors demonstrated the persistence of CMV-specific CTLs at least 110 days after infusion, even in patients without CMV-primed CTLs before cell transfer, which suggested expansion of the infused cells. This would discard anergy in the infused T cells, at least during the more than 100 days analyzed in the study.

Furthermore, it is currently unknown whether pentameric constructs would also induce anergy in these T cells. Pentamer and tetramer technologies are similar tools that use the same molecular approach, so it is theoretically possible that a similar impairment of T cell phenotype and function could happen *in vitro* using pentamers. Uhlin *et al* treated a patient diagnosed with post-transplant lymphoproliferative disease with EBV-specific CTLs isolated using pentamers. They demonstrated the presence of functional EBV-specific CTLs almost two months post-infusion, that induced complete regression of an EBV infection-associated lymphoma *in vivo* up to 189 days after infusion (Uhlin et al., 2009). They afterwards treated other 8 patients with antigen-specific CTLs isolated with the same methodology, against infections caused by CMV, AdV, or EBV. In this case, the antigen-specific cells could be detected after a month post-infusion in 6 of the 8 treated patients (Uhlin et al., 2012). The low number of patients studied makes it difficult to determine whether multimer binding induces anergy in CD8+ cells and its effect in *in vivo* functionality and maintenance of infused cells.

One possible explanation for the discrepancy between *in vivo* and *in vitro* results could be that T cell hyporesponsiveness negatively modulated by multimer engagement can be reversed by a cytokine storm generated in the lymphopenic recipient. That assessment was proposed by Brown *et al.* who demonstrated that anergic antitumor CD8+ T cells restore their function after transfer intro $RAG2^{-/-}$ immunodeficient recipients promoting tumor rejection (Brown et al., 2006). Specifically, Teague *et al.* showed that exogenous addition of IL-15 rescued and expanded previously tolerized cytotoxic T cells *in vitro* (Teague et al., 2006). However, there are no data in the literature concerning the association between cellular functional immunomodulation induced by the multimer complexes and the influence of the host microenvironment.

In order to address the possible influence of multimer binding to the T cell, a reversible human MHC-multimer, called Streptamer, was constructed by Neudorfer *et al.* (Neudorfer et al., 2007), using the molecular technology proposed by Knabel *et al*. (Knabel et al., 2002). Antigen-specific T cells can be obtained after multimer complex staining, and the reversible binding between the Streptamer and the TCR of the antigen-specific CTL allows its easy disengagement through exposure to a competitor molecule. Following dissociation, the Streptamer-treated CTLs are functionally indistinguishable from untreated T cells (Neudorfer et al., 2007). Wang *et al.* demonstrated that *in vitro* treatment of OT-I TCR-transgenic CTLs with peptide-loaded OT-I-streptamers markedly increased ^{3}H-thymidine incorporation and up-regulated early activation markers (Wang et al., 2008). The biochemical analysis of the signaling pathways in this assay identified several signaling molecules which were regulated after Streptamer engagement. Sustained phosphorylation of Akt and ERK1/2 was observed, possibly increasing Bcl-x_L expression, thus resulting in cell survival. According to these authors, Streptamer engagement is not silent, but this positive effect may be favorably used in adoptive immunotherapy protocols. The performance of cell enrichment methods using Streptamer complexes has significantly improved in just a few years (Schmitt et al., 2010; Wang et al., 2010; Terakura et al., 2012). Recently, this methodology has even been combined with genetic approaches, where *in vitro* analysis has shown excellent T cell dependent anti-viral and anti-tumor activities, thereby demonstrating the validity of the bi-specific system and the potential of this multimeric methodology (Terakura et al., 2012). However, it is unclear which signaling pathways may be activated by the Streptamer-TCR interaction, or whether this would have synergistic effects with other signal transduction cascades.

- **Non-specific binding of MHC-multimers**

It is widely assumed that multimer complexes are only recognized by CD8+ T cells specific for the antigen of interest, but several authors have observed non-specific binding to CD8- cells after tetramer (Lee et al., 1999; Monsurro et al., 2001; Villacres et al., 2001), pentamer (Chiang et al., 2008; Milne et al., 2008; Cantisan et al., 2009; Sandalova et al., 2010) and streptamer (Wang et al., 2010) staining. These CD8- cells have been described as monocytes, B-lymphocytes, and CD4+ naïve T helper (Th) cells (Nagorsen et al., 2002). In this sense, the adhesion of multimeric complexes to non-target cells poses two different types of problems: overestimation of the targeted CD8+ CMV-specific subsets in quantitative assays and contamination of multimer complex-based selected cellular therapeutic products by activated cellular lineages. This second possibility could be confirmed by some studies were the purity of the obtained cellular product was limited, with cases of multimer+ cells in the product as low as 23% (Keenan et al., 2001; Uhlin et al., 2009; Schmitt et al., 2010; Wang et al., 2010; van Loenen et al., 2013). This issue can lead to a higher risk of GvHD development if the obtained cellular product is adoptively transferred to patients after allo-HSCT.

b) IFN-γ secreting T cell selection

The value of IFN-γ secreting antigen-specific T cells for the successful reconstitution of virus-specific immunity in allogeneic BMT recipients has been demonstrated (Morita-Hoshi et al., 2008), and some authors have been able to isolate antigen-specific T cells by the selection of IFN-γ secreting cells in response to *ex vivo* stimulation with viral proteins or peptides (Assenmacher et al., 2002; Bissinger et al., 2002). Manz *et al.* first developed a high-affinity physic matrix of cytokine-secreting cells that prevents cytokine spreading (Manz et al., 1995), and the method was improved by bispecific antibodies to capture released IFN-γ followed by staining and isolation of IFN-γ producing cells with anti-IFN-γ microbeads (Brosterhus et al., 1999). A number of groups have successfully used the γ-capture technique for the treatment of viral infections in immunocompromised patients, increasing viral clearance and avoiding the associated-disease (Feuchtinger et al., 2010; Moosmann et al., 2010). Peggs *et al.* used this strategy in the pre-emptive and prophylactic treatment of CMV infection in 18 patients that underwnent allo-HSCT, and received a cellular product with a median of 43.9% IFN-γ secreting cells which contained both CD4+ and CD8+ T cells that conferred protection in the majority of patients during the 6 month

monitoring (Peggs et al., 2011). Icheva *et al.* infused EBV-specific T cells isolated with the IFN-γ capture technique to be adoptively transferred into 10 patients after allo-HSCT to prevent from post-transplantation lymphoproliferative disease. The obtained cellular product was made of a mean of 57% IFN-γ producing cells composed of both CD4+ and CD8+ T cells. Results showed that EBV-specific cells were able to expand *in vivo* in 8 of 10 patients, correlating with clinical response. Only one patient developed grad I acute GvHD, but EBV-related mortality occurred in the two treated patients were no *in vivo* expansion was detected (Icheva et al., 2013).

However, several authors have questioned the homogeneity of the cellular product obtained with this γ-capture method, following the identification of non-specific natural killer (NK) cells, B-cells and monocytes in the harvest product (Brosterhus et al., 1999; Desombere et al., 2004; Jedema et al., 2007). The presence of some of these cell types could have important biosafety implications in immunocompromised patients.

c) Activation marker expressing cell selection

Antigen-specific T cells can also be identified and isolated through the use of early activation markers that are up-regulated after T cell activation. These approaches offer high sensitivity and their expression is not dependent of cytokine secretion, leading to the isolation of antigen-specific T cells with a more broaden repertoire (Thiel et al., 2004). These antigen-specific cell isolation methods, although being fast and simple, have not been used in clinical trials yet and studies applying them are in pre-clinical phase. Several activation markers have been characterized that allow detection and enrichment of antigen-specific T cells:

- **CD25**

CD25 is the IL-2 receptor alpha-chain, which is essential for T cell proliferation. On activation through signals derived by the TCR and costimulatory molecules, T cells upregulate CD25 and become highly IL-2 sensitive (Letourneau et al., 2009). Cross linking of the antigen-specific TCR is a key signal for CD25 expression, and that is the reason why it is a potential target for CMV-specific T cell selection. Gallot *et al.* demonstrated that virus-specific T cells can be isolated based on CD25 expressing cell selection after peptide stimulation (Gallot et al., 2001).

However, CD25 is also expressed in regulatory T cells (Tregs), and therefore identification of antigen-specific T cells through CD25 expression can be mixed by the similar CD25 expression pattern shown by Tregs. Tregs are a subset of CD4+ T cells that are suppressive and contribute to the maintenance of immunologic self-tolerance, and they constitutively express CD25 (Sakaguchi, 2004). The transcription factor forkhead box P3 (FoxP3) has been identified as a key molecule for the development and function of Tregs, and is used as a phenotypic marker of this T cell subset (Fontenot et al., 2003).

Some authors have analyzed the feasibility of CD4+CD25+ Treg removal from the original sample before antigen-specific T cell selection through CD25 marker, in order to boost the yield of antigen-specific T cell selection as well as to avoid negative immunoregulatory effects of Tregs *in vivo* that could suppress antigen-specific T cell activity (Powell et al., 2005). However, Melenhorst *et al.* have described that CD25 cell depletion has an unpredictable effect on the yield of antigen-specific T cell selection and that fails to eliminate a substantial population of Tregs (Melenhorst et al., 2008).

Lugthart *et al.* used CD25 as a target for clinical scale multi-virus specific T cell manufacture. A small proportion of generated T cells also expressed FoxP3 and was able to suppress T cell proliferation, indicating that a functional subset of Tregs was present in the product (Lugthart et al., 2012). They believed that there may be some benefit in a virus-specific T cell immunotherapy product containing a population of Tregs, especially after HSCT where patients have the risk of developing GvHD simultaneously with CMV reactivation. In this sense, clinical studies have demonstrated that adoptive transfer of Tregs followed by T cell infusion early after haploidentical HSCT prevented from GvHD in the absence of post-transplant immunosuppression, improved immunity to opportunistic pathogens, and did not weaken the graft versus leukemia (GvL) effect of infused T cells (Di Ianni et al., 2011). Murine studies have also demonstrated that co-infusion of Tregs with CD3+ T cells enhances virus-specific immune reconstitution and protects mice from lethal CMV infection after HSCT (Nguyen et al., 2008). These studies illustrate the importance of the Treg population to avoid GvHD and the unlike effect of Tregs in preventing proliferation of virus-specific T cells.

- **CD137**

CD137, also known as 4-1BB, is a cell-membrane glycoprotein that belongs to the TNF-receptor superfamily and is predominantly expressed on immune cells following activation, including CD8+ and CD4+ T cells, natural killer T cells (NKTs) and NK cells (Zhang et al., 2010), although a low expression on Tregs, monocytes, and neutrophils has also been described (Wang et al., 2009). It has been defined as a costimulatory receptor for T cell activation (Vinay et al., 2006). Its ligand, the transmembrane molecule CD137L, is present in APCs such as activated macrophages, B cells, and DCs (Melero et al., 2008). It represents a useful activation marker for antigen-specific T cell selection due to the fact that CD137 expression on resting PBMCs is minimal, which makes its expression specific for the cell subpopulation that recognizes the stimulation agent. Moreover, it is expressed in both CD4+ and CD8+ T cells after antigenic stimulation, offering the possibility of antigen-specific CD4+ and CD8+ T cell selection.

Recently, several groups have analyzed the feasibility of virus-specific CD4+ and CD8+ T cell isolation for antiviral adoptive immunotherapy through CD137 expression from non-mobilized leukapheresis samples after PBMC stimulation with the antigen of interest (Watanabe et al., 2008; Wehler et al., 2008; Han et al., 2009; Zandvliet et al., 2009; Leibold et al., 2012), all of them addressing the selection of highly specific T cells fully functional against the studied antigens. These results, added to the fact that CD137 antibodies can be purchased as GMP grade products, make CD137 the perfect target for antigen-specific T cell isolation to be used in clinical adoptive immunotherapy protocols.

- **CD154**

CD154, also known as CD40 ligand, is a member of the TNF ligand supergene family that is expressed as a homotrimeric type II transmembrane protein and as a soluble cytokine. Its only known ligand is CD40, a member of the TNF receptor family, expressed on B lymphocytes, monocytes, DCs, endothelial cells, and epithelial cells (van Kooten and Banchereau, 2000). CD154 is transiently expressed on CD4+ T cells and in a small percentage of CD8+ T cells in response to activation (Banchereau et al., 1994; Cron, 2003), and its expression identifies antigen-specific T cells with high specificity and sensitivity (Chattopadhyay et al., 2005; Frentsch et al., 2005; Samuel et al., 2013). French *et al.* showed that extracellular CD154 expression after antigenic stimulation can be preserved by the

addition of a CD40-specific blocking monoclonal antibody that inhibits the interaction of CD154 with its ligand CD40, enabling the detection of antigen-specific cells. They were also able to isolate and expand CMV-specific T cells based on CD154 expression from non-mobilized PBMCs (Frentsch et al., 2005). More recently Samuel *et al.* applied the same isolation and expansion protocol to generate CMV-specific T cells from G-CSF mobilized donors, showing functions comparable to specific cells obtained from steady-state leukapheresis (Samuel et al., 2013). Khanna *et al.* have also been able to isolate multipathogen-specific T cells through the activation-induced CD154 expression. They generated highly specific T-cell lines that, after subsequent culture, were mainly enriched in CD4+ T cells with a small number of CD8+ T cells present in the product (Khanna et al., 2011).

- **CD69**

CD69 is an early membrane receptor expressed following activation in all bone marrow-derived cells except erythrocytes (Testi et al., 1994). It is almost undetectable on resting T cells and transiently expressed on lymphocytes following activation, as early as 2 hours post stimulation. It might act as a regulatory molecule, mediating immune cell activation and exerting proinflammatory effects either directly or indirectly (Sancho et al., 2005). It has been shown that inducible CD69 expression measurement on lymphocytes, following stimulation with different agents, is an accurate method to screen *in vitro* lymphocyte response in normal subjects (Mardiney et al., 1996; Lindsey et al., 2007). Li *et al.* have analyzed the identification and isolation of *M. tuberculosis*-specific CD4+ T cell from pleural fluid cells of patients diagnosed with tuberculosis based on their CD69 expression (Li et al., 2011). However, its applicability for antigen-specific T cell selection has been limited probably due to the nonspecific upregulation of the marker and expression in various immune cells.

3. Importance of the therapeutic virus-primed T cell status

3.1 Effect of differentiation status in *in vivo* maintenance

So far, it is possible to reactivate the memory T cell pool through *ex vivo* antigen presentation by B cells-, fibroblasts-, DCs-, and EBV-LCL-based systems, by the addition of specific peptides, peptide pools, or recombinant proteins. The high prevalence of CMV, EBV and

AdV infections in the human immunocompetent population theoretically implies that memory rather than naïve T cells would be the best candidate to be selectively expanded *ex vivo*. However, there are clear evidences that prove how the *in vitro* culture period alters the differentiation status and phenotype of T cell clones, influencing notably in their ability to be maintained *in vivo* (Gattinoni et al., 2005b). Berger *et al.* demonstrated in a nonhuman primate model that antigen-specific T cell clones derived from effector memory T cells (CD8+CD62L-) were not able to migrate to lymph nodes and BM, and consistently failed to persist *in vivo*. If antigen-specific effector T cells were derived from central memory clones (CD8+CD62L+), they retained their ability to respond to CMV, expand *in vivo* and undergo phenotypic conversion to both central- and effector-memory T cells (Berger et al., 2008). Therefore, selection and infusion of more incipient primed cells would ensure that after the therapeutic procedure, virus-specific T cells would be able to permanently re-establish the immune memory response (Sallusto et al., 2004; Berger et al., 2008; Fiorenza et al., 2012). Otherwise, terminally differentiated T cells would be less effective at triggering disease regression *in vivo* (Gattinoni et al., 2005b; Speiser and Romero, 2005; Gattinoni et al., 2006).

3.2 New approaches using genetic modification of less differentiated cells

The combination of less differentiated T cell enrichment system with molecular engineering has a high therapeutic potential. Thus, Wang *et al*. isolated polyclonal T cells with a central memory-like phenotype (CD8+CD45RA-CD62L+) which exhibited anti-tumor activity after CD19-specific CAR expression (Wang et al., 2012). Recently, the enrichment of CD8+CD62L+ central memory T cells using magnetic microbeads technology and priming with peptide-pulsed APCs before transduction with a lentivector encoding CD19-CAR has allowed the engineering of CMV- and EBV-specific modified T cells. These cells were further selected with reversible streptamers and showed equivalent T cell responses to tumors and endogenous viral-bispecific TCRs (Terakura et al., 2012).

Experimental studies conducted by Hinrichs *et al.* went further by showing in a TCR transgenic murine model of adoptive immunotherapy that effector cells developed from the naïve compartment resist terminal differentiation and possess the highest expansion potential and anti-tumor activities (Hinrichs et al., 2009). They further analyzed the effect of T cell differentiation status in the efficiency of genetic modification using human PBMCs transduced with a retroviral vector encoding a mutant TCR. Genetic modification of subsets derived from naïve cells was improved compared to central memory-derived cells, leading to

a higher transduction efficiency and transgene expression. Similarly, killer cell lectin-like receptor G1 and CD57, implicated in T-cell exhaustion and replicative senescence, were expressed at lower levels in effector cells from naïve origin (Hinrichs et al., 2011).

Following this experience, Hanley *et al.* achieved extensive *ex vivo* expansion of naïve T cells isolated from cord blood using EBV-infected B cells as APCs, after their modification using EBV-infected B cells transduced with clinical-grade Ad5f35CMVpp65 adenoviral vector as sources of EBV, AdV, and CMV antigens. In this way, they were able to generate large numbers of CMV, EBV and AdV-specific T cells in a culture containing both CD4+ and CD8+ T cell subsets, with effector function against EBV, CMV, and AdV targets (Hanley et al., 2009).

3.3 Alloreactivity of naïve populations

Despite the functional benefits related to naïve T cells, data published by several research groups indicate that this T cell population may contain alloreactive precursors. These authors have found high frequencies of GvHD development in mice infused with naïve T cells in comparison with memory T cells (Anderson et al., 2003; Beilhack et al., 2005). In agreement with this observation, Distler *et al.* have demonstrated alloreactivity of sorted naïve T cells against single class I or class II mismatched MHC alleles, questioning the validity of naïve T cells as a substrate for adoptive immunotherapy in BM transplant recipients (Distler et al., 2011).

Recently, Gattinoni *et al.* have identified in humans a new lytic T cell memory subset with phenotypic (CD45RA+CD62L+CD95+CCR7+IL-7Rα+IL-2Rß+CXCR3+) and functional (IFNγ+IL-2+TNFα+) characteristics shared by naïve T and stem cells (Gattinoni et al., 2009; Gattinoni et al., 2011; Sallusto and Lanzavecchia, 2011).

Consequently, the use of T cells derived from naïve precursors requires an exhaustive analysis before it reaches routine application in human therapy.

4. Importance of the microenvironment in the recipient

4.1 Conditioning regimen

The purpose of the myeloablative conditioning regimen given to the recipient before transplantation is to eradicate the underlying disease and suppress the patient's immune system to allow engraftment of donor stem cells. More than 40 years ago the combination of cyclophosphamide (CY) and total body irradiation (TBI) was introduced (Aschan, 2006). As not all transplant centers have access to irradiation, and to avoid possible side effects of TBI, such as pneumonitis, cataracts, secondary tumors, endocrinological disturbances and decreased growth in children, treatment with busulfan (Bu) instead of TBI was implemented. Randomized trials comparing CY-TBI and Bu-CY in acute myeloid leukemia (AML) and CML patients found no differences in leukemia free survival and overall survival between this two conditioning regimens (Socie et al., 2001).

The high transplant related mortality (TRM) associated with the myeloablative conditioning regimens restricted the use of allo-HSCT to healthy young patients who are more tolerant to the high dose chemo-radiotherapy regimens. However, over the past 15 years the introduction of reduced-intensity conditioning (RIC) regimens before HSCT has allowed transplantation in elderly patients and in younger patients with comorbidities (Jimenez et al., 2007). RIC regimens minimize the toxicities associated with myeloablative regimens but require more profound immunosuppression to allow engraftment of the hemopoietic stem cells, as the conditioning is not intended to eradicate the endogenous bone marrow cells. Many different RIC regimens have been used, although the most widely used include a purine analogue (fludarabine) combined with melfalan or Bu. Moreover, an important part of these conditioning regimens is the use of post-grafting immunosuppression, the combination of cyclosporine and mycophenolate mofetil being one of the most frequently used schemes (Aschan, 2006; Blaise and Castagna, 2012).

4.2 Immunomodulatory effect of the immunosuppressive regimen

The host lymphoablative conditioning regimen essential for hematopoietic transplantation carried out before adoptive T cell transfer-based immunotherapy may enhance immune responses of infused cells by the modulation of the microenvironment through a range of mechanisms, including the inhibition of endogenous CD4+CD25+FOXP3+ Tregs,

upregulation of MHC class I proteins, increase in the pool of peptides available for presentation, T cell trafficking, potentiation of innate immunity and increase in homeostatic cytokines such as IL-2, IL-7, IL-15 and IL-21 (Dudley et al., 2002; Gattinoni et al., 2005a; Muranski et al., 2006; Dudley et al., 2008).

As described earlier, Tregs suppress effector T cells by a number of mechanisms; they induce an increase of the activation threshold of effector T cells, expression of inhibitory costimulatory molecules, promote anti-inflammatory biochemical pathways, direct or indirect killing, consumption of proinflammatory cytokines, or production of immunoregulatory cytokines (Brusko et al., 2008). The downregulation of Tregs by exogenous immunostimulatory agents could therefore potentially improve the migratory properties, engraftment and cytolytic activity of the transferred T cells. Similar actions have been demonstrated through combined therapy of Daclizumab (humanized anti-CD25 monoclonal antibody) with peptide vaccine administered to breast cancer patients. In this clinical trial, Treg eradication *in situ* and reprogramming induced robust increase of physiological CTL and Th responses (Morita et al., 2012; Rech et al., 2012). However, as it has been mentioned before, some studies demonstrate that Tregs do not affect the GvL function of adoptively transferred T cells (Di Ianni et al., 2011).

Common γ-chain cytokines, including IL-7 and IL-15, have been reported to induce vital cellular activity such as proliferation of human T cells in the absence of TCR stimulation, furthermore avoiding apoptosis and maintaining cell metabolism. Host lymphodepletion has been shown to actively induce the expression of cytokines regulating homeostatic expansion (Bracci et al., 2007; Dudley et al., 2008). The absence of some of these homeostatic cytokines could result in the metabolic atrophy of infused T cells, which could lead to delayed growth and proliferation following viral stimulation (Unutmaz et al., 1994; Jacobs et al., 2010). Thus, it is known that expression of IL-7 and IL-15 receptors is key to the establishment of resting memory cells in cellular therapy procedures, as both cytokines synergistically drive T cells through this crucial checkpoint in their differentiation process (Buentke et al., 2006). Shen *et al.* demonstrated that down-regulation of both IL-7R and IL-15R is likely to be a contributing factor for the poor survival of therapeutic influenza-specific memory CTLs in the respiratory tract. According to these authors, there could even exist molecular mechanisms that would condition their survival depending on the particular tissue (Shen et al., 2008). Therefore, the “cytokine storm” induced by chemotherapy and irradiation

could positively regulate immune responses (Bracci et al., 2007). Moreover, immunodepletion of the host before adoptive cell transfer reduces competing cell populations, called "cytokine sinks", enhancing the availability of homeostatic cytokines and increasing the activity of infused cells (Gattinoni et al., 2005a; Muranski et al., 2006).

Antigen-specific *in vivo* expansion is also thought to be driven by different factors such as self-peptides and other antigens. Furthermore, exposure to viral antigens during the period of profound lymphopenia results in a significant boost of cellular immunity (Hakki et al., 2003; Cobbold et al., 2005; Mackall et al., 2009) and it is a mandatory requirement for antigen-specific immunological recovery in the transplanted patient.

4.3 T helper cell requirement

To date, the essential role of Th cells for the maintenance of CTLs is a controversial issue and different studies have been published, that sometimes have shown inconsistent results.

Cobbold *et al.* observed no correlation between CMV-specific CD4+ T cells and the circulating level of CD8+ T cells after CMV-specific CTL infusion alone (Cobbold et al., 2005), but the low sample size could be the cause of this lack of correlation (n=5). In addition, a declining trend in both CD8+ and CD4+ subpopulations is observed in 4/5 patients 60 days from the time of infusion of the therapeutic product, and several studies confirmed this trend for a relatively long time. Riddell *et al.* demonstrated that the adoptive transfer of donor CMV-specific CTLs in transplant patients results in a fast therapeutic activity, although their long-term maintenance is hampered by the absence of the appropriate Th subpopulation (Riddell et al., 1992). In line with these observations, Rosenberg *et al.* described that human immunodeficiency virus (HIV)-1-infected subjects that did not develop acquired immunodeficiency syndrome (AIDS), showed a high virus-specific CD4+ proliferative response and an extremely vigorous CTL concomitant response (Rosenberg et al., 1997).

The particular mechanisms whereby CD4+ T cells maintain effective antiviral immunity are poorly understood, but they could be related to the regulation of CTL precursor activity. According to this, there are already studies raising tumor-specific CTL responses in which a simultaneous activation of the Th subpopulation is found after cellular vaccination with class I and II peptide-loaded DCs vaccines. With this strategy, bi-functional CD4+ activity is

generated, resulting in increasing CTL proliferation and Treg inhibition (Teramoto et al., 2013).

In order to examine the relationship between both protective immunological populations and their role during the immunological recovery process in the post-transplant period, the development of MHC class II multimer complexes is being encouraged by biotechnology companies (Ayyoub et al., 2010). In fact, more studies are incorporating them as a research tool (Braendstrup et al., 2013).

As a matter of fact, the use of Th cells as a therapeutic product has also been questioned, and the limited experience with these cells in adoptive cell therapy is insufficient to reach objective conclusions. Quezada *et al.* transferred tyrosinase-related protein 1-specific TCR CD4+ cells into irradiated $RAG2^{-/-}$ mouse model with advanced melanoma. Tumor eradication was mediated by cytotoxic CD4+ T cells, which appeared after acquisition of IFN-γ, TNF-α, IL-2, granzyme B and perforin expression. Other endogenous immunological subpopulations (helper- and cytotoxic- T cells, B cells and NK cells) did not play an essential role in the anti-tumor effect of these infused cells (Quezada et al., 2010). The lymphopenic microenvironment and the depletion of CD25+Foxp3+CTLA-4+CD4+ Tregs apparently played a crucial role in the *in vivo* priming, expansion and activation of the exogenous naïve CD4+ cells and their cytotoxic-like phenotype conversion (Vanasek et al., 2006). Furthermore, these conditions allowed the long-term establishment of a CD4+-memory subpopulation (Tanchot et al., 2001). On the other hand, Perruccio *et al.* infused CMV-specific CD4+ cell clones that prevented CMV reactivation and reduced CMV mortality. In this study CMV-specific CD8+ cells were detected shortly after infusing CMV-specific CD4+ clones, that could indicate that the adoptively transferred CD4+ cells had stimulated the clonal expansion of CMV-specific CD8+ T cells previously transferred in the graft (Perruccio et al., 2005).

5. Sources for antiviral T cell immunotherapy product manufacture

5.1 Mechanisms of stem cell mobilization with G-CSF

In order to be successfully mobilized, HSCs need to lose their adhesion interactions that keep them bound to BM stromal cells. HSCs express a diverse number of cell surface molecules that mediate their adherence in the BM microenvironment, such as very late antigen 4 (VLA-

4), chemokine receptor 4 (CXCR4), CXCR2, CD44, CD62L, lymphocyte function-associated antigen-1 (LFA-1), or CD117 (Rettig et al., 2012). VLA-4, together with its receptor vascular cell adhesion molecule 1 (VCAM-1) has a major role in anchoring HSCs to BM stromal cells and regulating HSC trafficking between the marrow and peripheral sites (Greenbaum and Link, 2011). The disruption of the interaction between these two molecules with anti-VLA-4 antibodies inhibits adhesion and promotes HSC mobilization (Papayannopoulou, 2000). On the other hand CXCR4, together with its major ligand stromal cell-derived growth factor-1 (SDF-1), provide a key retention signal for HSCs in the BM (Greenbaum and Link, 2011). G-CSF induces a severe reduction of SDF-1 within the BM due to degradation by proteolytic enzymes, and CXCR4 expression on mobilized cells in the circulation is decreased (Petit et al., 2002; Dlubek et al., 2006), essential for a successful mobilization. G-CSF treatment also induces a decrease in the expression of CD62L and LFA-1 molecules in mobilized HSCs (Bellucci et al., 1999).

5.2 Cellular effects of G-CSF

Pharmacologic treatment with this recombinant drug induces stem cell mobilization from the BM to the periphery, providing an increased concentration of HSCs in the peripheral blood that could afterwards be collected by leukapheresis. The recommended dose of G-CSF is 10 µg/kg/day for five days followed by apheresis collection, that allows the harvesting of large numbers of CD34+ HSCs (Reddy, 2005).

G-CSF mobilization is associated with a significant increase in the number of circulating lymphocytes compared to BM collections (Rutella et al., 1997; Hartung et al., 1999), and samples from G-CSF mobilized donors are enriched in multipotent progenitor cells, myeloid and lymphoid progenitor cells as well as in monocytes and neutrophils (Imamura et al., 2005; Christopher and Link, 2007; Anderlini and Champlin, 2008).

Some studies previously addressed that G-CSF induces a tolerogenic phenotype on T cells. It has been reported that the clinical use of this pharmacologic treatment induces a promotion of Tregs that produce IL-10 and TGFβ (Rutella et al., 2002), an altered T cell function, a tolerogenic dendritic cell differentiation, and a polarization toward T helper cells type 2 (Th2) differentiation while inhibiting T helper cells type 1 (Th1) differentiation (Rutella et al., 2005). T cells from G-CSF mobilized PBSC donors also present downregulation of genes associated with cytotoxicity, antigen presentation and GvHD (Toh et al., 2009). It has

recently been shown that G-CSF mobilization does not affect the frequency of antiviral T lymphocytes, but alters their functionality by reducing the capability to secrete IFN-γ and cytolytic molecules like granzyme B (Bunse et al., 2013). Moreover, G-CSF mobilization increases IL-10 carrying monocytes (Fraser et al., 2006) and reduces antigen presenting function of these cells (Sunami et al., 2001). Furthermore, G-CSF treatment of normal stem cell donors provokes an immunosuppressive DC differentiation (Arpinati et al., 2000; Rutella et al., 2004), generating T cell hyporesponsiveness. It also induces CD14 expression in neutrophils, while its expression remains unchanged in monocytes (Kerst et al., 1993; Spiekermann et al., 1997). On the other hand, G-CSF mobilization affects the expression patterns, clonality and distribution of genes encoding for TCR receptors of both $\alpha\beta^+$ T cells (TRAV and TRBV repertoire) (Xuan et al., 2012) and $\gamma\delta^+$ T cells (TRGV and TRDV repertoire) (Xuan et al., 2011), changes that might play a role in mediating GvHD.

5.3 Antiviral product generation from G-CSF mobilized vs. non-mobilized samples

The manufacture of antigen-specific T cells for clinical use post allo-HSCT has been published by many groups, showing excellent results. To date, the generation of antiviral T cells for adoptive transfer has predominantly used PBMC collected in an additional leukapheresis from the original allo-HSCT donor prior to starting or weeks after the G-CSF mobilization. The need for successive leukapheresis collections presents some difficulties, mainly associated with the logistical and regulatory issues of unrelated donors, together with the discomfort to the donor and the associated increased costs. An aliquot of the original G-CSF mobilized HSCT apheresis product would be a more readily available source of donor lymphocytes, which could offer great advantages when compared to the additional collection of non-mobilized PBMC and could widen the immunotherapy product availability to transplant recipients in the unrelated donor setting. However, previously described immunosuppressive effects induced by G-CSF on T cells could cause an alteration on the anti-viral efficacy of T cell products obtained from G-CSF primed PBMCs, although there is no peer-reviewed evidence supporting this lack of anti-viral activity.

In contrast, recent studies have supported the use of G-CSF-mobilized PBMC as a starting material for antiviral or antitumor immunotherapies. Samuel *et al.* and Clancy *et al*. have recently published that CMV-specific T cells generated from donors mobilized with G-CSF retain an antiviral cytokine profile and a strong cytotoxic activity against target cells

presenting CMV antigens, comparable to CMV-specific T cells generated from non-mobilized donor PBMCs (Clancy et al., 2013; Samuel et al., 2013). Clancy *et al.*, in addition, infused CMV-specific CTL lines generated from mobilized donors into seven patients diagnosed with acute myelogenous leukemia or myelodysplastic syndrome. Only one patient developed a low-level CMV reactivation episode, which resolved without intervention. None of the seven patients required treatment with ganciclovir or foscarnet and there were no cases of CMV disease. In addition, there were no immediate adverse events associated with the infusion, followed up to 17 months post-HSCT (Clancy et al., 2013). On the other hand, Otto *et al.* were able to develop a clinical-scale cell purification method for the enrichment of γδ T cells from leukapheresis products of G-CSF mobilized donors, and they obtained γδ T cells with strong anti-tumor activity, able to produce a variety of immunomodulatory cytokines and kill tumor cells (Otto et al., 2005).

II. HYPOTHESIS AND AIMS

CMV-reactive product generation from G-CSF mobilized donors would eliminate the need for consecutive blood collections, but the immunosuppressive effects associated to G-CSF treatment could affect the anti-viral functionality of the obtained cellular product. If it could be proved that CMV-specific T cells generated from G-CSF mobilized donors retain their anti-viral function, these products could be manufactured from the same G-CSF mobilized collection used for PBSC procurement, widening the clinical applicability of the adoptive immunotherapy protocols. Therefore, we assessed different approaches for CMV-specific T cell manufacture from G-CSF mobilized donors and compared the function of the generated cells to products obtained from non-mobilized samples.

These are the specific aims of this thesis:

1. To optimize different methods of CMV-specific CTL manufacture from G-CSF mobilized donor PBMCs using MHC-multimers, for the CMV-reactive cell quantification and isolation to be used in clinical adoptive immunotherapy protocols.

2. To analyze the feasibility of competent CMV-specific CTL manufacture by using a reversible MHC-multimer. Assessment of the specificity and functionality and comparison with CMV-specific CTLs obtained from non-mobilized PBMCs.

3. To examine the feasibility of competent CMV-specific CD4+ and CD8+ T cell manufacture through activation-induced CD137 expression. Analysis of the specificity and functionality and comparison to products obtained from non-mobilized PBMCs.

III. RESULTS

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The abrogation of TCR-independent interactions with human serum ensures a selective capture of therapeutic virus-specific $CD8^+$ T-cells by Multimer Technology in Adoptive Immunotherapy

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The abrogation of TCR-independent interactions with human serum ensures a selective capture of therapeutic virus-specific CD8$^+$ T-cells by Multimer Technology in Adoptive Immunotherapy

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Keywords: Pentamers, Streptamers, Fcgamma receptor I (FcγRI), CMV-specific CTL

Abbreviations

FcγRIA: Fc-gamma high-affinity receptor I A

PM: pentamer

ST: streptamer

TCR: T cell receptor

TM: tetramer

β2m: β2-microglobulin

Introduction

Adoptive Immunotherapy with Cytomegalovirus (CMV)-specific T cells is a recently developed method to enhance immune reconstitution against this virus after allogeneic stem cell transplantation (SCT) (Perales, 2011). Several methodologies have been tested to select and obtain different populations of T-lymphocytes with specificity against CMV epitopes. Amongst them, the use of multimer-based selection has shown promising results (Einsele et al., 2002; Cobbold et al., 2005; Schmitt et al., 2010). In all these studies, detection of circulating CMV-specific cytotoxic T cells ($CD8^+$) remains an integral part of the procedure. Moreover, multimer (Tetramer (TM), Pentamer (PM), or Streptamer (ST)) staining and quantification by flow cytometry has become a widely used standard method.

Multimer technology is based on complexes of recombinant MHC-class I molecules conjugated with antigenic immunodominant peptides and labeled with fluorochromes or magnetic microbeads. It is widely assumed that multimer complexes are only recognized by $CD8^+$ T cells specific for the antigen of interest, but several authors have observed non-specific binding to $CD8^-$ cells after TM, PM, and ST staining (Nagorsen et al., 2002; Wang et al., 2010), including B-cell, helper T cells, and monocytes (Nagorsen et al., 2002). In this sense, the adhesion of multimeric complexes to non-target cells poses two different types of problems: overestimation of the targeted $CD8^+$ CMV-specific sub-population in quantitative assays and contamination of multimer complex-based selected cellular therapeutic products by activated cellular lineages. In spite of this fact, guidelines for harmonizing multimer experiments have been developed, based on simultaneous use of antibody markers to identify unwanted multimer-positive subpopulations (Attig et al., 2011). Considering that such

identification does not imply the elimination of these cell subpopulations, it is necessary to pay special attention to methods that would avoid this non-specific staining. However, the mechanisms of these unspecific interactions remain unclear.

FcRs are thought to bind the Fc region of antibodies (Abs) not only *in vivo* but also during immunohistochemistry and flow cytometry-based experimental assays, leading to non-specific staining. In this latter case, usually the non-specific monoclonal Ab binding to FcRs is bypassed through the pre-incubation of the biological sample with same-species normal serum, avoiding the unwanted background noise induced by cross-linking of Fc portion of IgG to FcRs of the cells (Sedlmayr P, 2001).

Amongst the FcRs, Fc-gamma high-affinity receptors I A (FcγRIA/CD64) belong to the Immunoglobulin Superfamily whose natural ligand is IgG, and are widely expressed on many immunological cell types, including monocytes, macrophages, dendritic cells, neutrophils, and eosinophils. The main functions of the FcγR are the positive or negative regulation of immune-cell responses and clearance of immune cell complexes (Nimmerjahn and Ravetch, 2006).

In our laboratory, we studied the feasibility of using CMVspecific ($pp65^{495-503}$) HLA-A*02:01 restricted PM and ST to quantify the proportion of circulating CMV-specific cytotoxic CTLs and secondly to select cellular products enriched for CMV-specific CTLs. Our first approach was compromised by cross-contamination with non-specific $CD8^-$ cells that showed positivity for the multimer staining. Subsequently, we explored the use of human AB serum as a method for blocking this non-specific background.

In this study, we quantified the cross-contamination due to multimer non-specific binding, analyzed the subpopulations involved in it, and developed a method to abrogate this unwanted event in order to improve the specificity of the method.

1. Materials and Methods

2.1 Study subjects

The Navarra Government Institutional Review Board approved the research and all donors provided written informed consent before clinical and molecular determinations. 20 HLA-A*0201 healthy donors seropositive for CMV and without signs of viral reactivation were included in this study. HLA-I typing and serological analysis for CMV were done as routine clinical practice in the adjacent Complejo Hospitalario de Navarra (CNH).

2.2 Laboratory procedures

PBMCs were isolated from peripheral blood samples anticoagulated with EDTA by Ficoll-Paque density gradient centrifugation (GE Healthcare Bio-Sciences, Uppsala, Sweden). Complete blood count and viability analysis was performed in Neubauer hemocytometer using 0.4% trypan blue staining (Gibco, Carlsbad, CA). 1 x 10^6 PBMCs in 100 μl were incubated with the same volume of heat-inactivated human AB serum (human serum type AB from male AB plasma, Sigma-Aldrich) for 10 minutes at room temperature. Cells not previously incubated with human AB serum were used as negative control. 5 μl of PE-labelled Pentamer (HLA-A*02:01/NLVPMVATV Pentamer, Proimmune, Oxford, United Kingdom) were added to the cells and incubation was carried out for 10 minutes at room temperature in the dark. In parallel, 0.75 μg of PE-labelled *Strep*-Tactin and 0.5 μg of antigen-specific MHC I-*Strep* (HLA-A*02:01/NLVPMVATV Streptamer, IBA GmbH, Göttigen, Germany) were incubated

for 45 minutes at 4ºC in the dark. The Streptamer was added at a concentration of 0.2 μg for 1 x 10^6 cells in a volume of 100 μl per test and it was incubated for 45 minutes at 4ºC in the dark. After cells were stained with multimer complexes and washed in Dulbecco's PBS (dPBS, Sigma-Aldrich, St. Louis, MO), 10 μl of anti-human CD3 HorizonV450, 10 μl of anti-human CD8 FITC, 10 μl of anti-human CD14 FITC, 5 μl of anti-human CD45 APC, and 5 μl of anti-human CD19 APC (Beckton-Dickinson (BD), San Jose, USA) were added, incubated for 15 minutes in the dark, washed and resuspended in 500 μl of dPBS before acquisition.

2.3 Flow Cytometry and Statistical analysis

For each sample, 500,000 total events were acquired in a FACSCanto II (BD). FACSDiva 6.0 software (BD) was used for acquisition process and Paint-A-Gate software (BD) for cellular analysis. Frequency of PM/$ST^+CD3^+CD8^+$, PM/ST^+CD14^+, and PM/ST^+CD19^+ cells was determined and data are presented as median (interquartile range). Wilcoxon signed-rank test was used for paired comparisons and Mann-Whitney for unpaired. The signification level was fixed to p=0.05. Statistical analysis was done using SPSS17 software package.

2. Results and Discussion

Multimer staining: All samples (n=20) were processed for the analysis of the frequency of CMV-specific $CD8^+$ cells. Using PM staining, the median frequency of total PM-positive cells was 0.18% (IQR: 0.05 to 0.57) and the frequency of CMV-specific $CD8^+$ cells was 0.01% (IQR: 0.00 to 0.20).

Using ST staining, the median frequency of total ST-positive cells was 0.50% (IQR: 0.17 to 1.20) and the frequency of CMV-specific $CD8^+$ cells was 0.00% (IQR: 0.00 to

0.29). These results show that a notable proportion of multimer-positive cells are likely to represent cross-contamination by cells lacking a TCR specific for pp65.

Characterization of non-specific staining: In previous studies, characterization of these cells demonstrated that, for both PM and ST staining, these unspecific events were $CD19^+$ B cells or $CD14^+$ monocytes. They did not show NK cell markers (Beloki L, *unpublished results, 2012*). In order to quantify cross-contamination of multimer staining with these cellular types, a particular subpopulation of multimer-stained $CD14^+$ or $CD19^+$ cells was also measured. In all samples, the median frequency of non-specific PM-positive $CD14^+$ or $CD19^+$ cells was 0.08% (IQR: 0.02 to 0.54). The median frequency of non-specific ST-positive $CD14^+$ or $CD19^+$ cells was 0.37% (IQR: 0.07 to 1.13), suggesting that ST complexes are more susceptible to non-specific binding than PM.

FcR Blockade with AB serum: We repeated the same analysis after AB serum incubation, to test the validity of our method. Following FcR blockade, the median frequency of total PM-positive cells was 0.10% (IQR: 0.01 to 0.88) and the median frequency of CMV-specific $CD8^+$ cells was 0.01% (IQR: 0.00 to 0.13).

The ST staining after FcR blockade showed a median frequency of total ST-positive cells of 0.41% (IQR: 0.14 to 1.16) and a median frequency of CMV-specific $CD8^+$ cells of 0.00% (IQR: 0.00 to 0.31).

Thus, after AB serum treatment the frequency of CMV-specific $CD8^+$ cells remained stable ($p>0.05$ in both cases), while the total frequency of multimer-positive cells was reduced significantly ($p=0.035$ for PM and $p<0.01$ for ST). In parallel, we observed a significant reduction of the frequency of multimer-positive $CD14^+$ or $CD19^+$ cells, to

0.02% (IQR: 0.00 to 0.27) for PM (p=0.045), and to 0.22% (IQR: 0.01 to 0.83) for ST (p<0.01) (Figure 1, Table 1). These results showed that AB serum treatment was efficient in the reduction of non-specific staining in both PM and ST techniques (Mann-Whitney test Z=-0.89, p=0.371).

These data support the notion that the use of AB serum with PM or ST staining is efficient in the elimination of non-specific cell binding in both techniques, while it does not impair the staining of CMV-specific cells. This is an essential aspect, as preservation of virus-specific CTL quantification is the main prerequisite for our FcR blockade method. These results show that the binding of PM or ST to non-CMV specific cells is at least partially due to cross-reaction between the multimer and the FcγR of the cells (Figure 2). In theory, non-specific binding of the multimer complexes to FcR-bearing cells could be a possible mechanism, since the α_3 extracellular domain of the heavy class I α chain and the non-MHC encoded polypeptide β2-microglobulin light chain (β2m) of MHC-I molecules show amino-acid sequences homologous to the constant domain of the human immunoglobulin and have tertiary structures resembling antibody domains (Bjorkman et al., 1987).

Our data allow us to conclude that abrogation of cross-contamination by cells expressing FcR in their surface is needed and can be achieved by the preincubation of the biological sample with AB serum. This allows a more accurate identification and quantification of CMV-specific cells in blood samples using multimeric staining. Furthermore, if a multimer-based strategy is to be used for selection of CMV-specific cytotoxic T-cells, the abrogation of cross-contamination by cells expressing FcR in their surface by a blocking agent is paramount. Therefore, this system could also be applied to improve outcomes of cellular selection strategies with multimer technology in

Adoptive Immunotherapy proceedings. The elimination of other cell subpopulations should increase the specificity of the selected product and minimise the risk of unwanted effects derived from the infusion of activated non-CMV-specific cells that could potentially trigger a graft versus host reaction in the context of adoptive immunotherapy after allogeneic SCT. Although promising, these approaches need to develop clinically oriented studies focused on the selection and parallel characterization of cellular products.

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Authorship

LB and MC performed experiments. LB and NR analyzed data. LB wrote the manuscript with NR and EO. CM, EB and MRC contributed to the drafting of the manuscript.

Conflict of Interest Disclosure

The authors declare no financial or commercial conflict of interest.

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Table 1. Multimer positive leukocyte subset stratification according to *in vitro* treatment.*

Subpopulations MM+ (%)	PM staining[1]: Med (IQR)			ST staining[1]: Med (IQR)		
	Unmanipulated	HAB Blocking	*P*	Unmanipulated	HAB Blocking	*P*
Overall	0.18 (0.05, 0.57)	0.10 (0.01, 0.88)	0.035	0.50 (0.17, 1,20)	0.41 (0.14, 1.16)	0.004
CD8+**	0.01 (0.00, 0.20)	0.01 (0.00, 0.13)	0.168	0.00 (0.00, 0.29)	0.00 (0.00, 0.31)	0.673
CD14+ and CD19+***	0.08 (0.02, 0.54)	0.02 (0.00, 0.27)	0.045	0.37 (0.07, 1.13)	0.22 (0.01, 0.83)	0.000

*Comparison by the Wilcoxon signed-rank test. [1]n=20. **CMV-specific cytotoxic T cells. ***Unspecific cells

Figure legends

Figure 1. Pentamer staining (A) and Streptamer staining (B). Effectiveness of the HAB serum blockade for quantification of CMVpp65-specific T lymphocytes.

The dot plots represent the multimer staining of CMV-specific $CD8^+$ cells (top right quadrants) and background events (top left quadrants) gated from CD $CD45^+$ cells from PBMCs of HLA-A*02:01CMV-seropositive donors. Unwanted cells cultured with HAB are completely removed or significantly decreased with HLA-A2/CMV pp65 Pentamer staining (A) and HLA-A2/CMV pp65 Streptamer staining (B). The numbers denote the percentage of multimer-positive $CD8^+$ or $CD8^-$ cells.

Figure 2: Specific and non-specific binding of PM and ST. A) In an unmanipulated sample without AB serum treatment multimers bind to CMV-specific T cells by their TCR, and also to cells bearing the Fc receptor in their surface. B) After HAB serum treatment, IgG binds to the FcR and blocks the unwanted binding of the multimers, alowing the multimers to bind the CMV-specific TCR.

Figure 1.

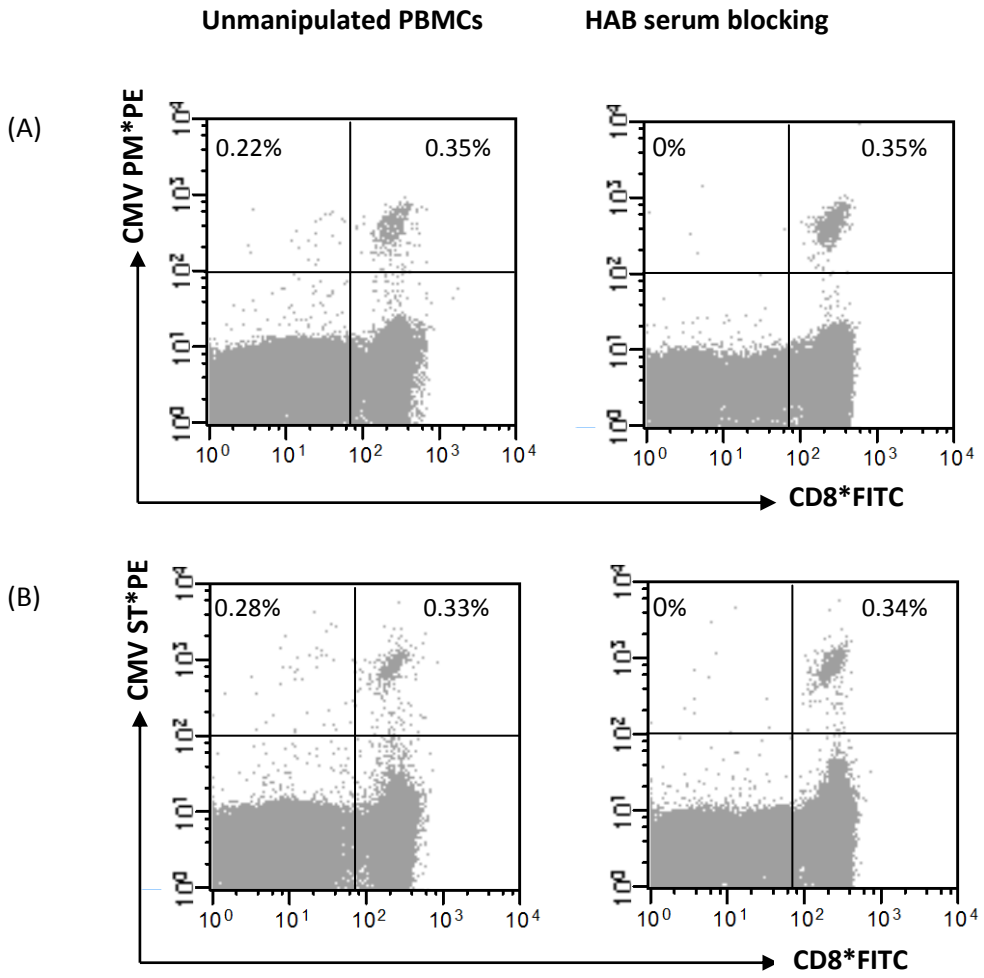
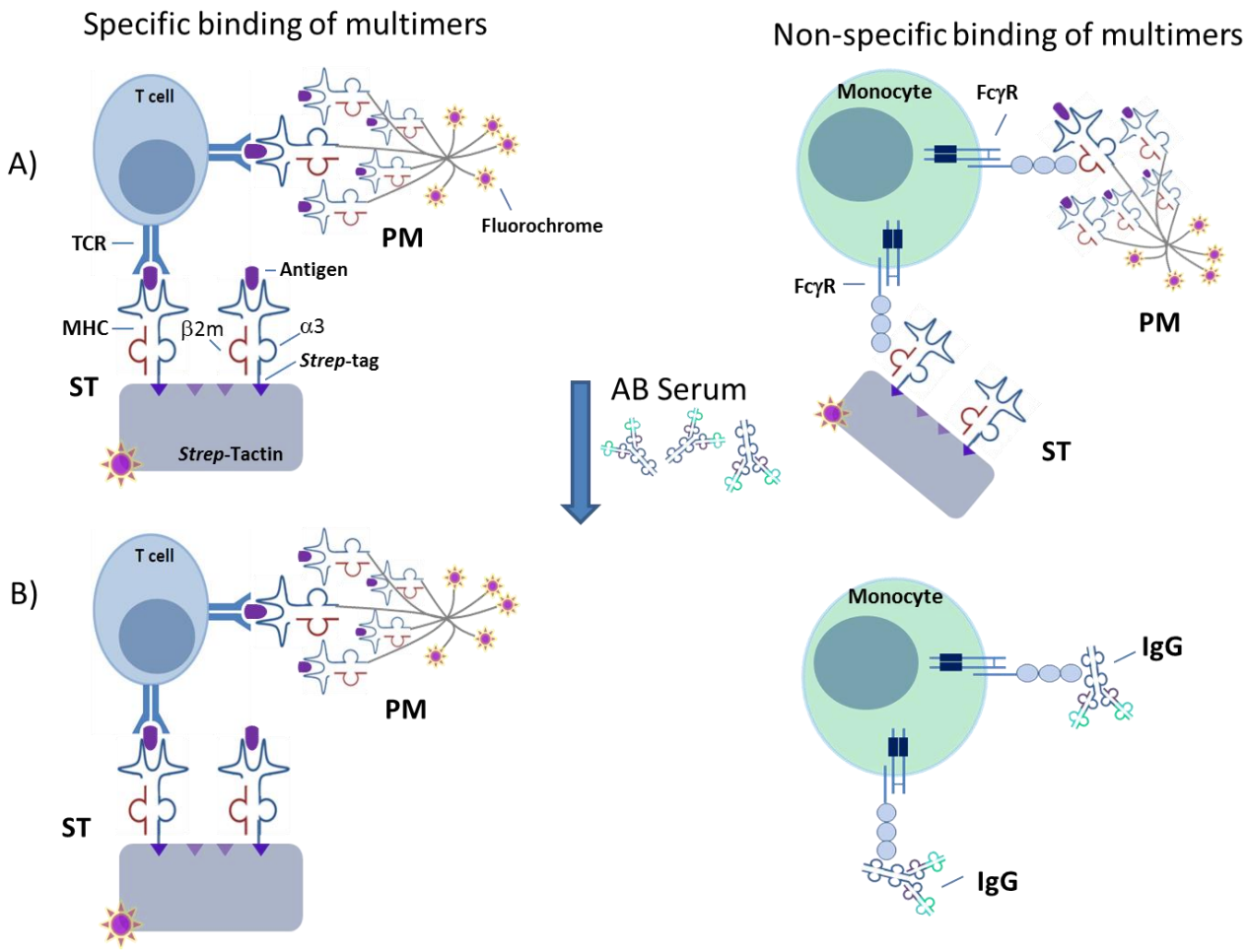

Unmanipulated PBMCs
HAB serum blocking
(A)
CMV PM*PE
0.22%
0.35%
0%
0.35%
CD8*FITC
(B)
CMV ST*PE
0.28%
0.33%
0%
0.34%
CD8*FITC

Figure 2.


Specific binding of multimers
Non-specific binding of multimers
A)
T cell
TCR
PM
Fluorochrome
Antigen
MHC
β2m
α3
Strep-tag
ST
Strep-Tactin
AB Serum
Monocyte
FcγR
FcγR
PM
ST
B)
T cell
PM
ST
Monocyte
IgG
IgG

CMV-specific T cell isolation from G-CSF mobilized Peripheral Blood: depletion of myeloid progenitors eliminates non-specific binding of MHC-multimers

Beloki L, Ciaurriz M, Mansilla C, Zabalza A, Perez-Valderrama E, Samuel ER, Lowdell MW, Ramirez N, Olavarria E

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CMV-specific T cell isolation from G-CSF mobilized Peripheral Blood: depletion of myeloid progenitors eliminates non-specific binding of MHC-multimers

Running title: Improving G-CSF primed sample for CMV-specific CTL isolation

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Conflict of interest: MWL is a shareholder in Cell Medica, a clinical-stage cellular therapeutics company. The authors have declared that there are no financial conflicts of interest in regard to this work.

Non-standard abbreviations:

GvHD	Graft versus host disease
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
PBSC	Peripheral blood stem cell

Abstract

MHC-multimers have been widely used for the quantification and selection of cytotoxic T lymphocytes (CTLs) with diverse specificities. This method has been used to isolate CMV-specific CTLs to be adoptively transferred to immunocompromised patients in an attempt to confer protection against this virus. In the allogeneic Hematopoietic Stem Cell Transplant (allo-HSCT) setting CMV-specific CTLs have been isolated from steady-state apheresis samples collected from the donor prior to G-CSF mobilization, and infused to the immunocompromised patient inducing a successful anti-viral response. However, in the case of unrelated donors two closely-timed cellular collections are not usually available, which limits the accessibility of anti-viral cellular product manufacture. CMV-specific CTL manufacture from the G-CSF mobilized collection offers great regulatory advantages, but when we tried to isolate these cells using MHC-multimers from the G-CSF mobilized donor sample our products were negatively influenced by the presence of non-specific cell subtypes. Therefore, in the present study we have developed an easy and fast method based on plastic adherence to be used for CMV-specific T cell isolation from G-CSF mobilized cellular products. The purity of the obtained cellular product improves and this could reduce the negative effects of undesirable alloreactive cells present in the final product.

Key words: allogeneic hematopoietic stem cell transplantation, cytomegalovirus-specific cytotoxic T cells, MHC-multimers, granulocyte-colony stimulating factor, immunotherapy

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) allows the recovery of a sick hematopoietic system affected by congenital or acquired severe disorders (1). The three main sources of hematopoietic stem cells (HSCs) are bone marrow (BM), umbilical cord blood, and peripheral blood stem cells (PBSCs) (2). HSCs are found in very low numbers in the peripheral circulation, and therefore recombinant granulocyte-colony stimulating factor (G-CSF; Filgrastim) is clinically used for their mobilization to the periphery. G-CSF mobilized PBSCs have significantly higher numbers of T-cell lymphocytes compared to BM collections (3), and samples from G-CSF mobilized donors are enriched in neutrophils, monocytes, and myeloid progenitor cells at different stages of maturation that will be differentiated into monocytic and granulocytic lineages (4, 5).

Studies of immune reconstitution after allo-HSCT have identified a decisive role for the recovery of CD8+ cytotoxic T lymphocyte (CTL) responses in preventing the development of viral diseases (6). In this sense, the adoptive transfer of antigen-specific T cells after allo-HSCT has been shown to prevent viral expansion and to protect the patient from virus-related diseases, avoiding collateral clinical toxic effects induced by anti-viral drugs (7). Some years ago HLA-multimer technology was implemented, which has allowed the direct selection of antigen-specific CD8+ T cells with no need for long-term *in vitro* culture (7). This method avoids functional damaging effects associated with *in vitro* expansion, preserving the survival potential and cellular properties of the therapeutic product (8-10). Furthermore, this technology offers a direct and fast selection strategy for a non-advanced therapy medicinal product (non-ATMP) generation, minimizing the regulatory requirements for its implementation.

Historically, the manufacture of virus-specific T cells for adoptive immunotherapy has involved the use of donor lymphocytes collected from a steady-state leukapheresis, obtained from an additional apheresis prior to the G-CSF administration for HSC mobilization. G-CSF has previously been shown to induce immunologic tolerance; it promotes T helper type 2 (Th2) and regulatory T cell (Treg) differentiation and downregulates genes associated with Th1 cells, cytotoxicity, antigen presentation and graft versus host disease (GvHD) (11-14). It also induces an increase in CD14 expression in neutrophils, while its expression remains unchanged in monocytes (15, 16). In spite of the above described immunosuppressive effects of G-CSF treatment, recently some authors have successfully generated competent CMV-specific T cells from G-CSF mobilized apheresis samples (17, 18). The CMV-specific T cell manufacture from G-CSF mobilized collections would abrogate the need for successive donations, assuring the availability of an anti-viral cell product in the unrelated donor setting while minimizing costs and discomfort for the donor.

Therefore, we aimed at isolating CMV-specific T cells from G-CSF mobilized donors using MHC-multimers. Some studies have characterized the ability of MHC-multimer molecules to bind non-specifically to activated B lymphocytes, naïve helper T cells or monocytes through unwanted interactions to Fc receptors (FcR) (19, 20). As a consequence, the isolation and subsequent adoptive transfer of potentially alloreactive cells could induce the development of harmful reactions such as GvHD.

In the present study, we have developed a method to avoid non-specific binding of multimers to potentially damaging cell subsets by using a physical procedure based on plastic adherence. In this way, we have managed to minimize the non-specific binding of multimers and eventually obtain a more pure cellular product safer for infusion.

Materials and methods

Donor population and ethical statement

This study was approved by the Institutional Review Board at Complejo Hospitalario de Navarra (CHN), and all donors gave informed consent prior to enrolment.

11 subjects who were stem cell donors at CHN for allo-HSCT were recruited. All were CMV-seropositive and carried the HLA-A*02:01 allele. HLA-I typing was done in the Immunology Unit of the CHN and the serological analysis for CMV was obtained from the Microbiology Service of the CHN.

PBSC mobilization and collection

Cells were collected from donors who received 10 μg/kg/day of recombinant G-CSF (Filgrastim, Sandoz Biopharmaceuticals, Paris, France) every 12 hours starting five days before collection. Leukapheresis were performed with a COBE Spectra continuous flow blood cell separator (COBE Spectra apheresis system, Caridian BCT, Lakewood, CO, USA). Cell products, anticoagulated with ACD-A, were collected with a 1.1 ml/min flux in a 500 ml container, from which an aliquot of 0.5 ml was used to perform the experiments. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare Bio-Sciences, Uppsala, Sweden) and counted in Neubauer hemocytometer using 0.4% trypan blue staining (Gibco, Carlsbad, CA).

Enrichment of lymphocyte populations by plastic adherence

2.25 x 10^7 cells were suspended in 45 ml of X-VIVO 15 Serum-free cell medium w/o supplements (Lonza, Basel, Switzerland) in a sterile 225 cm^2 A/N flask with CellBIND Surface (Corning, Corning, NY) for 1 hour at 37ºC and 5% CO_2. Non-adherent cells were carefully collected by aspiration to avoid the disruption of the adherent cellular

populations. Obtained cells were washed with Dulbecco`s phosphate buffered saline (dPBS, Sigma-Aldrich, St. Louis, MO) before quantification and cytometric analysis.

Phenotypic characterization of unmanipulated PBMCs and non-adherent cell product

Fresh G-CSF mobilized PBMCs and the cellular harvest obtained after adherent cell removal where phenotypically characterized.

Characterization of leukocyte subpopulations: 1 x 10^6 cells were stained with anti-human CD3-V450 (BD Biosciences, San Jose, USA), CD8-FITC (BioLegend, San Diego, USA), CD14-PE (BioLegend), CD45-PerCP-Cy5.5 (BioLegend), and CD4-APC (BioLegend). Incubation was carried out for 15 minutes in the dark, cells were washed once and resuspended in dPBS prior to cytometer acquisition.

CMV-specific CD8+ T cell quantification: 5 µl of PE-labelled Pentamer (PM; HLA-A*0201/CMV Pentamer, Proimmune, Oxford, United Kingdom) was added to 1 x 10^6 cells in a final volume of 50 µl. After an incubation of 10 minutes in the dark, cells were stained with CD3-V450, CD8-FITC, 7-AAD (BD), and CD45-APC-H7 (BD). Samples were incubated with monoclonal antibodies for 15 minutes in the dark, washed once and resuspended in dPBS prior to cytometer acquisition.

CMV-specific T cell selection

From 5 G-CSF mobilized PBMCs before and after the adherent cell removal, 1 x 10^7 cells were stained with 50 µl PE-labelled PM during 20 minutes at 4ºC in the dark. After a wash, 20 µl of anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to a final volume of 80 µl, followed by 20 minute incubation at 4°C in the dark. Afterwards, PM+ cells were isolated using a Possel_ds selection program on an AutoMACS Pro separator (Miltenyi Biotec).

Acquisition and analysis by Flow Cytometry

Samples were acquired in a FACSCanto II equipment (BD), FACSDiva 6.0 software (BD) was used for acquisition process and FlowJo version 10 (TreeStar Inc., Ashland, OR, USA) for cellular analysis. For the quantification of leukocyte subpopulations 50,000 total events were acquired, and CD3+CD8+, CD3+CD4+, and CD14+ frequencies were defined. For the CMV-specific T cell quantification 500,000 total events were acquired, and frequencies of total AAD+ and PM+CD3+CD8+ were determined. Only cells clustering with forward and side scatter properties of leukocyte subpopulations were included in the analysis, and the percentages were given from the CD45+ cell gate.

Statistical analysis

Data are represented as median (IQR). Wilcoxon signed-rank test was used for paired comparisons and the signification level was fixed to $p=0.05$. Statistical analysis was done using SPSS17 software package.

Results

Phenotypic characterization of the mobilized apheresis collection

Unmanipulated PBMCs of the apheresis products were characterized prior to adherence and their CD14+, CD3+, CD3+CD8+, and CD3+CD4+ cell subset percentages were determined. 47.76 % (35.99 – 54.98) of all PBMCs present in the mobilized apheresis expressed the CD14 marker. Furthermore, we could determine that 83.20 % (73.33 – 91.0) of cells with forward and side scatter properties of monocytes and granulocytes were CD14+, confirming that after G-CSF mobilization both monocytes and granulocytes express this marker. Additionally, total T lymphocytes represented the 24.04 % (18.0 – 37.11), from which 17.72 % (6.78 – 25.08) were CTLs and 12.54 % (11.38 – 19.38) helper T cells.

CTL enrichment and unwanted cell depletion after adherence

In the non-adherent cellular product, CD14+ cells were significantly reduced to 2.10 % (1.34 – 6.07) ($p=0.005$). Furthermore, T lymphocytes were significantly increased to 69.91 % (31.86 – 79.15) ($p=0.007$) and CTL subpopulation accordingly rose to 35.24 % (23.78 – 45.16) ($p=0.009$). Helper T lymphocyte percentage remained at 19.31 % (16.56 – 29.63), without reaching statistical significance ($p>0.05$) (Figure 1).

Percentages of different cell population recovery rates were calculated comparing the absolute number before adherence and the cells recovered in the non-adherent cellular fraction. On the one hand, 98.48 % (96.25 – 99.62) of CD14+ cells from the unmanipulated PBMC sample were lost after the adherence process. In comparison, 63.73 % (33.90 – 69.99) of the T lymphocyte population were collected in the non-adherent fraction, while 65.08 % (31.92 – 81.30) and 53.23 % (29.24 – 62.62) of the CTLs and helper T cells were recovered, respectively.

7-AAD dye was used to assess cell viability before and after adherence. In the original mobilized sample 98.03 % (95.6 – 98.54) of cells were viable, whereas after adherence the viability was decreased to 96.01 % (93.38 – 97.81) ($p>0.05$).

CMV-specific T cell enrichment and non-specific binding loss by plastic adherence

PM multimer was used to quantify CMV-specific CTL cell percentage. Prior to adherence 0.14 % (0.06 – 0.62) of PBMCs were specific for CMV, whereas after the adherence process this subpopulation was enriched to 0.65 % (0.24 – 1.51) ($p=0.003$) in the non-adherent fraction (Table I). The recovery rate of CMV-specific CTLs in the adherence process was 84.58 % (56.25 – 88.68).

In the same way, the non-specific binding of multimers to unwanted CD8- cells was diminished. In the original apheresis sample 0.56 % (0.41 – 0.86) CD8-PM+ were detected, while after adherence the non-specific CD8-PM+ cells were reduced to 0.16 % (0.12 – 0.37) ($p=0.003$).

Optimization of CMV-specific T cell isolation by magnetic selection

CMV-specific CTLs from G-CSF mobilized samples were isolated using an AutoMACS device from unmanipulated PBMCs or from non-adherent cells recovered after the adherence process. The purity of the obtained sample was determined as the percentage of PM+ cells in the product, and the yield was defined as the absolute number of PM+ cells present in the positive fraction as a proportion of the absolute number of PM+ cells in the sample before isolation. Using unmanipulated PBMCs, the median purity of the cellular product was 20.80 % (6.91 – 61.7) and the yield was 38.59 % (32.91 – 44.79). In comparison, the purity of the positive fraction using the sample after adherence was significantly increased to 76.00 % (32.65 – 83.7) ($p=0.043$) while the yield was 42.06 % (23.4 – 84.1) ($p>0.05$) (Figure 2, Table I).

Discussion

Multimer technology allows the quantification and selection of antigen specific CD8+ T cells (7, 21). Different multimer technologies have previously been used to isolate CMV-specific CTLs, the first ones using tetramers (22, 23), and more recently with the introduction of pentamer (24) or streptamer (25-28) technologies. However, in the majority of studies a significant proportion of undesirable non-specific cells are isolated in the cellular product, with the associated potentially harmful effects on the infused patient. Alloreactive donor effector cells have been identified as key players in the GvH reaction (29, 30), where B cells are also involved (31). In addition, donor derived monocytes have been found to be increased in bone marrow and peripheral blood of patients that developed GvHD (32) and their TNF-α production have been implicated in the pathophysiology clinical GvHD (33). In this regard, it is important to assure that the product to be adoptively transferred contains a purified virus-specific T cell population with high specificity.

Although it has been demonstrated that G-CSF administration induces an immunosuppressive state and T cells from G-CSF mobilized stem cell donors secrete less IFN-γ than those from untreated donors (11), CMV-specific T cells manufactured from this cell source maintain their anti-viral functions (17, 18, 34). Since the generation of anti-viral cell products from G-CSF mobilized apheresis samples offers great logistical advantages especially in the unrelated donor setting, we assessed the direct isolation of CMV-specific CTL from G-CSF mobilized PBMCs using MHC-multimers. However, in our first approaches we found a high proportion of non-specific cell binding and a low purity of the isolated cell product. In this sense, recent studies have shown that background levels of multimer staining are higher in G-CSF mobilized samples compared to non-mobilized ones (11). Multimers can join non-specifically to

FcRs (20), that are mainly expressed on monocytes, dendritic cells, neutrophils, and eosinophils (35). The up-regulation of FcRI and FcRIII in neutrophils and monocytes induced by G-CSF treatment (36, 37) could explain the high background levels described in MHC-multimer staining when G-CSF mobilized samples are used. This issue would lead to a limited yield and purity in the CMV-specific CTL cell isolation process, with potentially hazardous cells being acquired during the isolation process. Furthermore, the cost of the process would be increased due to the multimer wasted in non-specific binding and the larger quantities of multimer needed to obtain the same amount of specific cells. Therefore, we found the necessity to develop an approach that could avoid the non-specific binding of multimers and the subsequent isolation of potentially alloreactive cells.

Multimers have been described to bind non-specifically to CD14+ cells (19, 20), and G-CSF treatment in healthy individuals results in an increased expression of the CD14 antigen on neutrophils while maintaining its expression on monocytes (15, 16). It is well known that G-CSF mobilized samples are enriched in neutrophils, monocytes, multipotent progenitor cells, and myeloid and lymphoid progenitors (4, 5, 38). A simple process based on plastic adherence would remove all these cellular subsets from mobilized samples due to the fact that hematopoietic progenitors (39), monocytes (40), and neutrophils (15, 41) have the ability to adhere to plastic surfaces while T-cell lymphocytes do not.

Therefore, we have developed an adherent method to remove the majority of unwanted cells present in the apheresis, while the proportion of T lymphocytes and CTLs was significantly increased in the product. At the same time, the sample is enriched in CMV-specific CTLs and the binding of the multimers to CD8- cells is decreased, which consequently provokes an increase in the efficacy of the subsequent CMV-specific T

cell magnetic selection process. The purity of the obtained cellular product was significantly higher when a previous adherent process was carried out, while the recovery rate was able to reach a notable number of CMV-specific T cells.

Clinical protocols that infused CMV-specific CTLs obtained through MHC-multimer isolation to avoid CMV reactivation after allo-HSCT have described that an infusion of less than 1 x 10^4 cells/kg resulted in a considerable expansion of CMV-specific CTLs in vivo and was able to control CMV viremia (23, 24). These data demonstrate that the infusion of less than a total of 0.8 x 10^5 CMV-specific CTLs successfully prevent from viral disease, and these cell numbers could be manufactured from an aliquot of the original G-CSF mobilized PBSC graft. However, for the routine applicability of the method described in this study, the adherence process would be more easily performed using Hyperflask devices (Corning), with a median of 8 flasks (range 2 - 15) necessary to obtain the required CMV-specific CTL numbers. On the contrary, after the adherence process the multimer quantity required to isolate the same amount of specific cells would be reduced, with the associated decrease in the cost of the procedure.

In the present study we have addressed the high non-specificity present in the CMV-specific T cell isolation methods using MHC-multimers from G-CSF mobilized donors by using an easy, safe and cheap physical procedure. The use of mobilized PBMCs as starting material for the manufacture of anti-viral cellular products ensures a wider availability of this cellular treatment, especially in the unrelated donor setting when a second cellular collection is usually not an option.

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Tables

Table I: Phenotypic characterization and CMV-specific CTL isolation of unmanipulated PBMCs and the non-adherent fraction. Cell subsets, CMV-specific CTLs and non-specific PM staining were analyzed in the unmanipulated PBMC sample and in the non-adherent cellular product (*n=11*). CMV-specific CTLs were isolated from unmanipulated PBMCs and non-adherent fraction, and purity and yield of the obtained cellular product were determined (*n=5*). Comparison was done with the Wilcoxon signed-rank test and signification level was fixed to p=0.05.

	Unmanipulated PBMCs	**Non-adherent fraction**	**p**
	Leukocyte subpopulations		
CD14+ cells	47.76 % (35.99 – 54.98)	2.10 % (1.34 – 6.07)	0.005
T lymphocytes (CD3+)	24.04 % (18.0 – 37.11)	69.91 % (31.86 – 79.15)	0.007
CTLs (CD3+CD8+)	17.72 % (6.78 – 25.08)	35.24 % (23.78 – 45.16)	0.009
Helper T cells CD3+CD4+)	12.54 % (11.38 – 19.38)	19.31 % (16.56 – 29.63)	0.208
Viability (7-AAD-)	98.03 % (95.6 – 98.54)	96.01 % (93.38 – 97.81)	0.066
	PM staining: specific and non-specific binding		
CMV-specific CTLs (CD3+CD8+PM+)	0.14 % (0.06 – 0.62)	0.65 % (0.24 – 1.51)	0.003
Non-specific PM binding (CD8-PM+)	0.56 % (0.41 – 0.86)	0.16 % (0.12 – 0.37)	0.003
	CMV-specific CTL isolation		
Purity	20.80 % (6.91 – 61.7)	76.00 % (32.65 – 83.7)	0.043
Yield	38.59 % (32.91 – 44.79)	42.06 % (23.4 – 84.1)	0.893

Figure legends

Figure 1. Phenotypic analysis of cells before and after the adherence process. Plastic adherence method was applied to PBMCs from G-CSF mobilized donors (*n=11*) and CD3, CD8, CD4, and CD14 expression of the unmanipulated PBMCs and the non-adherent cells was analyzed by flow cytometry. Representative figure of the products before and after the adherent process. Cells are presented from the CD45+ cell gate.

Figure 2. CMV-specific CTL isolation using Pentamer. CMV-specific CTLs were isolated from unmanipulated PBMCs and the non-adherent fraction using PM (*n=5*) and the purity of the process was determined by PM staining. Representative histograms of CMV-specific CTL isolation. Displayed cells were previously gated on CD45+ cells.

Figures

Figure 1

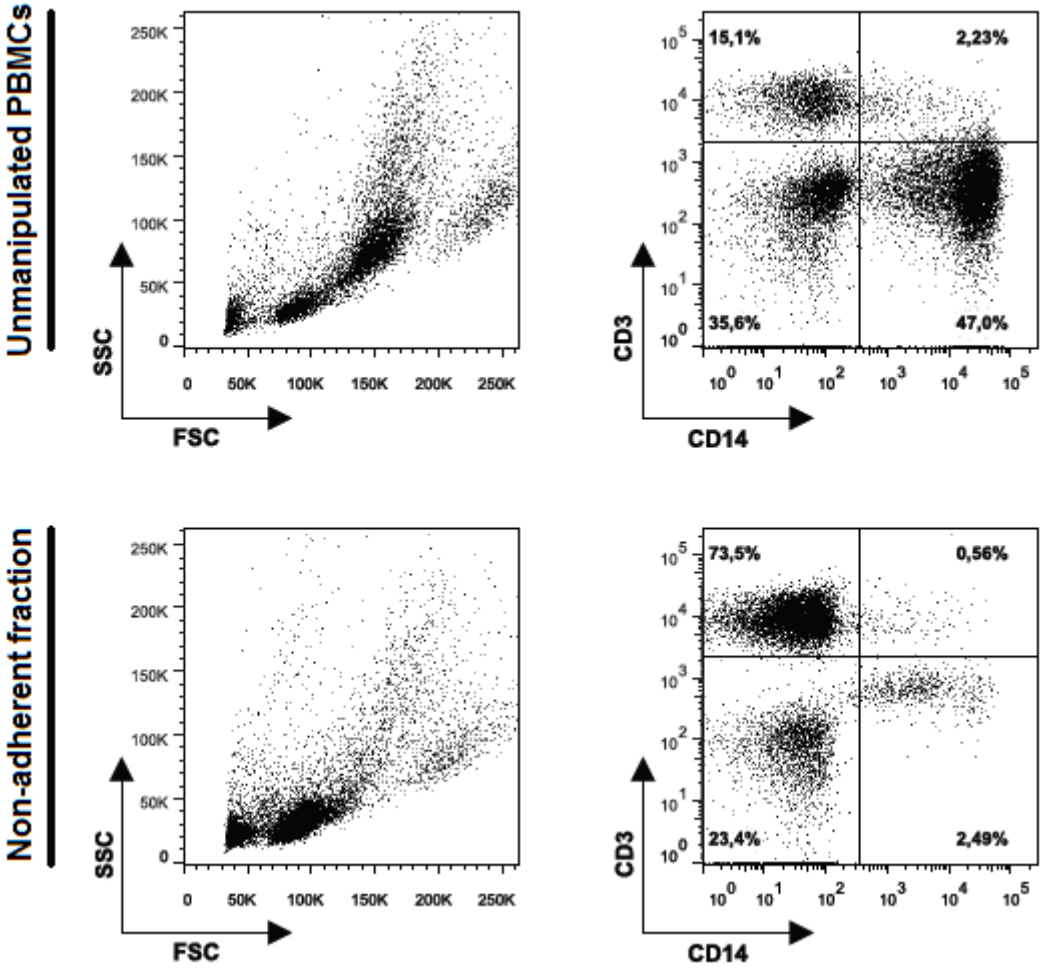
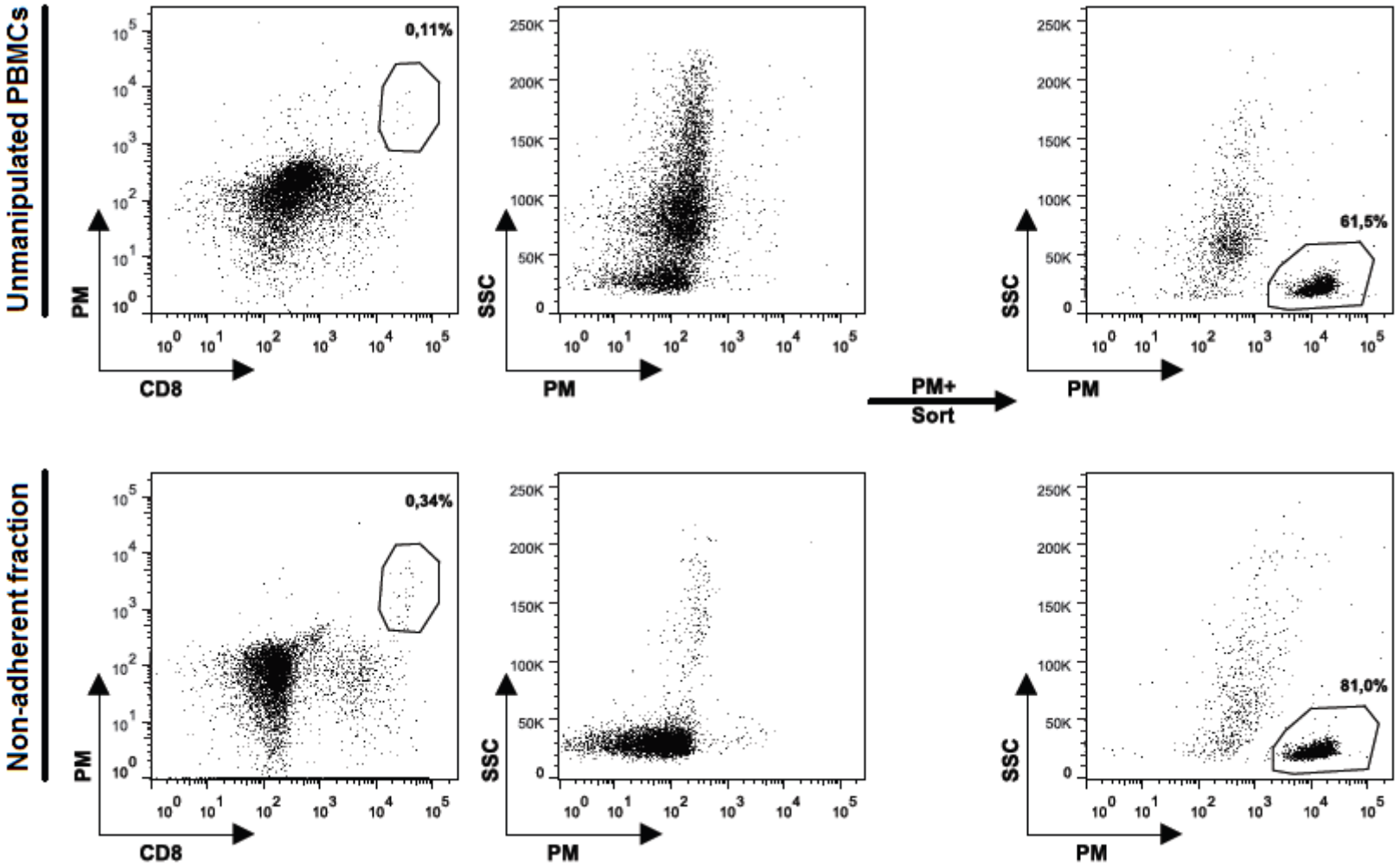


Figure 2


Unmanipulated PBMCs
0,11%
PM
CD8
SSC
PM
PM+
Sort
61,5%
Non-adherent fraction
0,34%
81,0%

CMV-specific CTLs isolated using MHC-multimers from G-CSF mobilized Peripheral Blood retain their effector function essential for a successful Adoptive Immunotherapy

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CMV-specific CTLs isolated using MHC-multimers from G-CSF mobilized Peripheral Blood retain their effector function essential for a successful Adoptive Immunotherapy

Running title: G-CSF mobilized donor samples for CMV-specific CTL isolation

Authors' affiliations

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Conflict of interest: MWL is a shareholder in Cell Medica, a clinical-stage cellular therapeutics company. The authors have declared that there are no financial conflicts of interest in regard to this work.

Non-standard abbreviations:

ATMP	Advanced therapy medicinal product
CMV-CTL	CMV-specific CTL
GvHD	Graft versus host disease
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
PBSC	Peripheral blood stem cell
ST	Streptamer

Abstract

Adoptive transfer of CMV-specific T cells has shown promising results in preventing the pathological effects caused by opportunistic CMV infection in immunocompromised patients following allogeneic hematopoietic stem cell transplantation. The majority of studies have used steady-state leukapheresis as the starting material for CMV-reactive product manufacture, using a collection obtained prior to G-CSF mobilization. The procurement of this additional sample is often not available in the unrelated donor setting due to regulatory and logistical problems. If the cellular product for adoptive immunotherapy could be generated from the same G-CSF mobilized collection, the problems associated with the additional harvest could be overcome. Despite the tolerogenic effects associated with G-CSF mobilization, recent studies have shown that CMV-primed T cells generated from mobilized donors retain their anti-viral properties. MHC-multimers are potent tools for direct isolation, allowing the rapid production of highly functional antigen-specific CTLs. Therefore, we have assessed the feasibility and efficacy of CMV-specific CTL manufacture from G-CSF mobilized apheresis samples using MHC-multimers. CMV-specific CTLs can be easily and efficiently isolated from G-CSF mobilized samples with Streptamer technology and retain functionality in response to antigenic stimulation, similar to that seen in non-mobilized cell products. The translation of streptamer technology for isolation of anti-viral T cells from G-CSF mobilized PBMCs into clinical practice would widen the availability of adoptive immunotherapy and allow a greater number of patients to benefit from this therapeutic strategy.

Key words: allogeneic hematopoietic stem cell transplantation, cytomegalovirus-specific cytotoxic T cells, Streptamer technology, granulocyte-colony stimulating factor, immunotherapy

IV. GENERAL DISCUSSION

Adoptive transfer of virus-specific T cells is a promising approach to promote the reconstitution of antigen-specific immunity that could control viral infection following allo-HSCT. The main objective of anti-viral adoptive immunotherapy is to provide a competent immunological response able to control the virus, but at the same time adverse events related to the infused cells need to be avoided. Therefore, the perfect cellular product to be adoptively transferred to an immunocompromised patient would consist of fully functional cells that are able to respond against the virus, with no contamination by cells with unknown specificities that could trigger unwanted alloreactive reactions and induce adverse events in the recipient such as GvHD.

Another key aspect of adoptive immunotherapy is the widespread applicability of the manufacture process; methods that require long expansion times and need to be produced in a GMP laboratory are time consuming, costly, and not applicable to the majority of transplant units. Thus, a direct selection method, fast, simple and cost-effective would be more easily introduced into routine practice. Accordingly, the procurement of a cellular product should be accessible to the majority of patients; in this case, the main studies of virus-specific T cell manufacture to be adoptively transferred following allo-HSCT have used donor PBMCs collected from an additional leukapheresis process prior to starting G-CSF treatment. Thus, two collections need to be carried out, the first one to isolate PBMCs from which to generate virus-specific T cells, and the second one after G-CSF mobilization to obtain PBSCs for the allo-HSCT. This double collection is often not possible due to regulatory or logistical problems, mainly in the unrelated donor setting. Furthermore, patients with unrelated donors whose samples are collected overseas are not eligible for immunotherapy due to the difficulties associated to collecting and transporting an additional blood donation. A possible solution to overcome these complexities would be to generate the cellular product to be adoptively transferred from the same G-CSF mobilized leukapheresis collection, using cells in excess of the amount required for allo-HSCT. However, it has been described that G-CSF treatment induces immunomodulatory effects on T cells or DCs (Rutella et al., 2004; Rutella et al., 2005) and that cells exposed to G-CSF have less proliferative and Th1 cytokine-producing capacity (Chen et al., 2004; Bunse et al., 2013). Whether this tolerogenic effect induced by G-CSF on global T cell responses also affects virus-specific memory T cells needs to be further studied.

The main objective of this thesis was to evaluate the feasibility of CMV-specific T cell manufacture from G-CSF mobilized donor samples compared to steady-state collections as starting material. In order to assess the effect of G-CSF mobilization in CMV-specific T cell generation and function, we used and compared two procurement methods; one based on CD137 activation marker expression in response to antigenic stimulation, and the second one by using reversible MHC-multimers. After isolation, antigen-specific cells were isolated and expanded during 21 days in order to increase cell numbers to be able to perform functional assays. We consider that the CMV-specific cells obtained directly after isolation is functionally equivalent to the expanded cellular product, and would even be less differentiated and exhausted than expanded cells, pontially more beneficial if adoptively infused. The expansion was done in the presence of homeostatic cytokines and CMV-loaded irradiated feeders, with no need to further antigen addition because it has been shown that antigen persistence is not required for the maintenance of memory CD8+ T cells (Bevan and Goldrath, 2000). IL-7 is important for T cell survival and maintenance and IL-15 necessary to mediate homeostatic proliferation, and their involvement in the maintenance of memory T cells has been confirmed by several groups (Surh et al., 2006; Tanel et al., 2009). Finally, the anti-viral function of the generated cellular product was assessed in response to antigenic re-stimulation.

CMV-specific T cell isolation by activation dependent CD137 expression

For the first approach, we used CD137 activation marker expression upon stimulation with CMVpp65 PepTivator to identify and isolate CMV-specific CD4+ and CD8+ T cells. Since the stimulation induces phenotypic changes in the cell, the obtained cellular product is considered an advanced therapy medicinal product (ATMP) with the regulatory restrictions associated with the GMP manufacture process. This procedure had been previously analyzed by other groups that used non-mobilized samples as starting material for the isolation, and showed the high functionality and specificity of the obtained cellular product (Wolfl et al., 2007; Wehler et al., 2008; Leibold et al., 2012). In our study we used G-CSF mobilized donor samples for the CMV-specific T cell generation, and compared the product with products obtained from non-mobilized samples. The obtained cellular product was made of both CD8+ and CD4+ cells, combination that would be more beneficial for adoptive transfer than infusing CTLs or helper T cells alone.

To assess the differentiation status of generated CMV-specific T cells, we first analyzed CD57 expression, a marker of functional immune deficiency and senescence of T cells (Focosi et al., 2010) that defines their limited proliferative capacity and survival (Brenchley et al., 2003; Palmer et al., 2005). CD57+ cells still maintain high cytotoxic properties (Le Priol et al., 2006) and expand upon diverse conditions of chronic immune activation like viral infections or malignancies (Focosi et al., 2010). In our analysis we could observe that CD57 expression was increased during the 3 week expansion in the majority of samples, but the overall expression was not significantly altered probably due to the small sample size and the variability between samples. This result illustrated that the generated cells retained their proliferative ability that was further demonstrated by the antigen-specific proliferation experiments where CMV-specific T cells proliferated in response to antigenic stimulation.

The cellular product obtained was composed predominantly of T_{EM} and T_{CM} cells, populations that would confer immediate effector properties as well as the capacity to efficiently differentiate to effector cells upon antigenic stimulation. In relation to these effector properties, generated CMV-specific T cells were able to re-express activation markers and produce pro-inflammatory cytokines such as IFN-γ, and lower levels of TNF-α and IL-2 after antigenic re-challenge, with no IL-10, IL-4, or IL-5 secretion detected. The secretion of type 1 cytokines illustrates the functional capacity to target the destruction of infected cells. CMV-specific T cells also produced granzyme B even after antigenic re-stimulation, showing that they are primed to secrete it in response to the antigen. The preformation of mature secretory lysosomes containing lytic proteins such as granzymes and perforin correlates with the killing ability of cytotoxic cells, and these two cytotoxic molecules are released upon interaction with target cells inducing their death (Bossi and Griffiths, 2005; Ewen et al., 2012). The type 1 cytokine secretion profile and the presence of cytolytic granules full of granzyme B demonstrates that generated CMV-specific T cells are mainly cytotoxic antiviral cells, a mixture of both CD8+ (Harty and Badovinac, 2008) and CD4+ (Appay et al., 2002) cytotoxic T cells in combination with a small percentage of Th1-polarized CD4+ Th cells (Luckheeram et al., 2012). This cytotoxic ability was further demonstrated by their capacity to lyse target cells pulsed with the CMVpp65 peptide, while they did not lyse cells that did not present the antigen. All these functional assays confirmed that anti-viral activity of CMV-specific T cells generated from G-CSF mobilized samples was comparable to CMV-specific T cells obtained from non-mobilized donors.

Finally, we used MHC-multimer staining to confirm the specificity of the cellular product in three G-CSF mobilized CMV-specific T cells. Two samples were stained with the Streptamer specific for the NLVAPVTMV peptide (HLA-A*02:01 restriction) and showed that the major part of the CMV-specific CTLs present in the product were specific against this peptide. On the other hand, the majority of the CMV-specific CTLs in the HLA-A*24:02 donor were not specific for the QYDPVAALFL peptide, showing that this peptide is not the most immunodominant in the HLA-A*24:02 setting, supporting the findings suggested by Morita *et al.* (Morita et al., 2005). A different explanation could be that the peptide pool composing the CMVpp65 PepTivator stimulates preferentially NLVPMVATV-specific cells while does not strongly stimulate QYDPVAALFL-specific subpopulation. Otherwise, it could be explained by the high donor variability, since the immunodominance of different epitopes can vary depending on the donor (Slezak et al., 2007).

Improving MHC-multimer staining for CMV-specific CTL quantification

Once determined that CMV-specific T cells obtained through CD137 expression from G-CSF mobilized donor samples are as functional as those generated from non-mobilized samples, we further wanted to characterize the effect of G-CSF mobilization on CMV-specific CTLs isolated using MHC-multimers. But before trying to isolate them from G-CSF mobilized samples, we aimed at improving CMV-specific CTL quantification from steady-state peripheral blood samples. Our first approaches were hampered by the high non-specific binding found. Using PBMCs obtained from peripheral blood we could determine, similar to the findings showed by Nagorsen *et al.* (Nagorsen et al., 2002), that both Pentamers and Streptamers bind to CD14+ and CD19+ cells not bearing the TCR specific for the peptide of interest. One possible explanation for this non-specific binding is that MHC-multimers could bind FcR molecules in the surface of CD8- cells, owing to the fact that the α_3 extracellular domain of the heavy class I α chain and the non-MHC encoded β2m of MHC-I molecules show aminoacid sequences homologous to the constant domain of the human immunoglobulin and have tertiary structures resembling antibody domains (Bjorkman et al., 1987). We have developed a method to remove unwanted binding of multimers to FcR by blocking these receptors with human AB serum; multimers loss the union to CD8- cells while remaining attached to antigen-specific CTLs. The described process is cheap and easily applicable for the quantification of antigen-specific CTLs from peripheral blood samples.

Improving the cellular product by removing non-specific binding of MHC-multimers in G-CSF mobilized samples

Looking into the clinical applicability of CMV-specific CTL production, we afterwards tried to isolate these cells from G-CSF mobilized donor samples using MHC-multimers, due to the regulatory benefits associated to CMV-specific CTL procurement from the mobilized collection. However, the purity of the cell product obtained in our first approaches was limited owing to the high non-specific binding of multimers. In fact, it has recently been described that background level of multimer staining is higher when mobilized samples are used (Bunse et al., 2013). This increased background could be explained by the up-regulation of FcRI and FcRIII on neutrophils and monocytes induced by G-CSF administration (Repp et al., 1991; Ohsaka et al., 1995), and the previously described ability of multimers to bind non-specifically to FcR on CD14+ cells, that in G-CSF mobilized samples is expressed by both monocytes and neutrophils (Kerst et al., 1993; Spiekermann et al., 1997). Therefore we found the necessity to implement a method, easily applicable into the clinic that would reduce unwanted cell subsets in the product. Accordingly, when the aim is not only quantification but also the isolation of CMV-specific CTLs to be adoptively transferred, a more feasible option than FcR blocking by human AB serum would be the implementation of a physical approach that does not induce any biochemical or functional alteration in the cell while preserves its integrity. Furthermore, if the method does not just block the non-specific binding of multimers but also removes the undesired cells from the collection, the sample will be enriched in the cells of interest and the purity of the subsequent isolation process will be more favorable.

G-CSF mobilization results in the enrichment of the leukapheresis sample in HSCs as well as in myeloid and lymphoid progenitor cells, monocytes, neutrophils, and lymphocytes (Hartung et al., 1999; Imamura et al., 2005; Anderlini and Champlin, 2008). Monocytes, neutrophils, and hematopoietic progenitors are capable of adhering to plastic surfaces (Ginis et al., 1992; Scott et al., 1995; Spiekermann et al., 1997; Elkord et al., 2005). Therefore, we considered to apply a straightforward physical process based on plastic adherence in order to remove the majority of unnecessary cell subsets present in the collection that, if non-specifically isolated and infused, could be harmful for the immunocompromised patient. We managed to eliminate the best part of CD14+ cells, while enriching the non-adherent sample in total lymphocytes and CTLs. Additionally, the CMV-specific CTL cell percentage was increased

in the non-adherent cellular product, while non-specific binding of multimers to unwanted cells was reduced. Furthermore, when CMV-specific CTLs were isolated from cells obtained after adherence, the purity of the obtained cellular product was significantly better when compared to total mobilized PBMCs. Here we have described a simple method that significantly improves the isolation process for CMV-specific CTL manufacture. For its routine applicability, the adherence process would be more asily performed in a closed system by using Hyperflask devices (Corning). This physical approach is easily and readily applicable into de clinic, does not alter the integrity of the cells while preserves the functionality of obtained cells.

CMV-specific CTL isolation using reversible MHC-multimers

Once we had managed to improve the CMV-specific CTL isolation process from mobilized apheresis samples, our next aim was to assess whether CMV-specific CTLs manufactured using MHC-multimers from samples collected from allo-HSCT donors treated with G-CSF were comparable to cells obtained from non-mobilized samples. The purity and yield of the isolation process were similar, but when selected CMV-specific CTLs were expanded during 3 weeks in the presence of CMV-loaded autologous feeders and IL-7 and IL-15, the expansion potential of G-CSF mobilized cells was reduced compared to non-mobilized samples. G-CSF treatment could impair proliferation potential of selected CMV-specific CTLs, or they could have lost their capacity to proliferate in the absence of CD4+ Th cell population. In spite of the reduced expansion potential, after 21 day culture the obtained cellular product was functionally equivalent to CMV-specific CTLs generated from non-mobilized samples; upon antigenic re-challenge they were able to re-express activation markers and produce high levels of pro-inflammatory cytokines such as IFN-γ, lower levels of TNF-α and IL-2, with no IL-10 production. They also synthesized granzyme B, even without antigenic stimulation, illustrating their cytotoxic potential. Moreover, almost all of them carried the TCR specific for the CMVpp65$_{495\text{-}503}$ peptide, confirming their specificity.

The CD57 T cell senescence marker was increased to high levels after 3 weeks expansion, in both non-mobilized and G-CSF mobilized CMV-specific CTLs. By the end of the expansion CD57 expression in non-mobilized samples was higher than G-CSF mobilized CMV-specific CTLs, which correlates with the higher fold expansion of non-mobilized cells observed during culture. The memory phenotype showed that both G-CSF mobilized and non-

mobilized samples were made mainly of T_{EM} and a noteworthy proportion of T_{EMRA}, during all the stages studied. These subpopulations are characterized by a rapid effector function that would confer immediate and effective protection against the virus (Sallusto et al., 2004).

We further analyzed the possibility of CMV-specific CTL isolation using Streptamers after a previous enrichment of the cells of interest in the original sample by stimulation and expansion. This process would be beneficial in cases where CMV-specific CTL percentage is low in the original sample, because a correlation between purity of the isolation with Streptamers and the frequency of virus-specific CTLs in the sample has been observed (van Loenen et al., 2013). When analyzing the proliferation potential of CMV-specific CTLs composing the whole PBMC sample during 21 days and comparing their fold expansion in G-CSF mobilized and non-mobilized samples, the expansion potential was similar. This suggests that the impaired expansion observed in directly isolated CMV-specific CTLs from G-CSF mobilized donors was not due to the G-CSF treatment but to the CD4 T helper cell lack.

CMV-specific CTLs generated by this alternative option remained functional, illustrated by re-expression of activation markers and synthesis of IFN-γ, TNF-α, IL-2, and granzyme B similarly to directly selected CMV-specific CTLs. However, since the expansion protocol induces some phenotypic and functional changes in the cells, this alternative method for CMV-specific T cell procurement would need to comply with the requirements and costs associated to advanced therapy medicinal product (ATMP) manufacture.

CMV-specific T cell manufacture from G-CSF mobilized donor samples: benefits and limitations of CD137 expressing cell or MHC-multimer isolation

In general, we have been able to demonstrate that G-CSF mobilized PBMC samples are suitable for CMV-specific T cell product manufacture. On the one hand, when generated through CD137 expression upon antigenic stimulation, the expansion potential and anti-viral function was similar to products generated from steady-state apheresis samples, with no disadvantages related to G-CSF treatment. On the other hand, when CMV-specific CTLs were isolated using MHC-multimers, the overall function was similar to non-mobilized cells but the *in vitro* expansion potential was reduced when G-CSF mobilized leukapheresis

samples were used as starting material, probably due to the absence of helper T cells in the product.

CMV-specific T cell isolation based on CD137 expression offers the main advantage of generating a product made of both CD4+ and CD8+ T cells. A cellular product that combines both CD4+ and CD8+ T cell subsets is potentially more beneficial to restore protection against the virus, because it is known that both CMV-specific CD8+ (Cwynarski et al., 2001) and CD4+ (Pourgheysari et al., 2009) T cell reconstitution is correlated with protection from viral replication and disease following HSCT. The infusion of a product made of a mixture of CMV-specific CD8+ and CD4+ cells would be more advantageous in the adoptive transfer setting than a product obtained with the MHC-multimer technology, which isolates antigen-specific CD8+ cytotoxic T cells alone (Ramirez and Olavarria, 2013). The lack of antigen-specific CD4+ T cell response has been associated with a nondetectable CD8+ T cell response (Boeckh et al., 2003), that would affect the preservation of the infused antigen-specific CTLs *in vivo*. In this sense, Riddell *et al.* observed that the adoptive transfer of CMV-specific CD8+ cells alone results in effective CMV-specific cytotoxic responses, even in patients without demonstrable CMV-specific CD4+ proliferative responses, but they were poorly maintained in the absence of recovery of CMV-specific T helper responses (Riddell et al., 1992). In contrast, Cobbold *et al.* infused CMV-specific CTLs isolated using Tetramers and described that CTLs were functional after transfer, were able to proliferate *in vivo*, and the CMV-specific CD4+ T cell response showed no correlation with the degree of CMV-specific CD8+ T cell expansion. However, the small number of patients involved (n=5) precludes making firm conclusions. Furthermore, a trend towards CMV-specific CD8+ and CD4+ declining can be inferred from these 5 studied patients (Cobbold et al., 2005). Similarly, it is well known that CD8+ T cell response is a fundamental effector mechanism in the immunologic control of CMV infection (Harari et al., 2004; Moss and Khan, 2004). This CD8+ CTL population is therefore essential for the immune reconstitution after transplantation, since the relationship between postransplant functional impairment of CD8+ T cells and failure to suppress CMV replication after solid-organ transplantation has recently been described (Crough et al., 2007; Mattes et al., 2008). In this case, the CMV-specific T cell product obtained according to antigen-induced surface expression of CD137 would potentially be more effective in the adoptive transfer setting than CMV-specific T cells generated based on CD154 activation marker, as CD154 is mainly expressed in CD4+ T cells

and the manufactured CMV-specific T cell product is predominantly composed of CD4+ cells (Chattopadhyay et al., 2005; Frentsch et al., 2005; Samuel et al., 2013).

A key point of MHC-multimer technology is that the approach is limited to patients that express the HLA alleles for which CMV peptides are available. However, many CMV epitopes have now been reported and more than 75 % of the transplant population would be eligible for treatment (Cobbold et al., 2005). In this sense, a limitation of the MHC-multimer technology is that only CTLs specific for a particular peptide are selected, obtaining a restricted part of the whole repertoire of the CMV peptides. In comparison, CD137 activation marker expressing cell isolation results in all of the T cells specific for the peptides composing the CMVpp65 protein to respond to the initial stimulation and express the activation marker, generating a product composed of a polyclonal CTL population that would target multiple epitopes. However, at least in HLA-A*02:01 patients, we have been able to determine that the majority of CTLs forming the expanded CMV-specific T cell product are specific for the CMVpp65$_{495\text{-}503}$ peptide, suggesting that a similar specificity would be obtained with both approaches in HLA-A*02:01 donors. Nevertheless, this results could not be reproduced in a HLA-A*24:02 donor. More samples, generated from patients with a variety of HLAs, would be needed to further investigate this issue.

Our results have shown that the CMV-specific T cell products obtained from CD137 expressing cell isolation are made of T_{CM} and T_{EM} subpopulations, whereas CMV-specific CTLs generated using MHC-multimers are mainly composed of T_{EM} and some T_{EMRA} cells. These results correlate with previous studies that isolated CMV-specific T cells through activation-dependent marker expression (Samuel et al., 2013) or using Streptamers (Schmitt et al., 2010). This difference could be related to the predominant presence in blood of T_{CM} in CD4+ cells, whereas T_{EM} are mainly in CD8+ cells (Sallusto et al., 2004). The less differentiated status of stimulation-dependent CD137 cells would suggest their longer maintenance *in vivo* compared to CMV-specific CTLs isolated by multimers (Gattinoni et al., 2005b).

CMV-specific T cells generated based on long cell culture methods have successfully prevented from viral reactivation after administration to patients undergoing allo-HSCT (Riddell et al., 1992; Walter et al., 1995; Einsele et al., 2002; Peggs et al., 2003; Leen et al., 2006; Mackinnon et al., 2008). However, despite being a very effective therapy, these methods are expensive, labor-intensive, time-consuming, and restricted to clinical units with

considerable expertise. Therefore, CMV-specific T cell generation by direct isolation methods offer great advantages compared to culture-based strategies. These protocols are fast, amenable to GMP standards, and hold greater promise in translation to a broader applicability to a greater number of transplant centers. Nevertheless, the isolation of CMV-specific T cells through CD137 expression requires a previous short stimulation with the CMVpp65 antigen, which induces some changes in the cells present in the sample and thus isolated cells are considered an ATMP. This process needs to be produced under GMP standards and comply with more strict regulatory requirements. In comparison, CMV-specific CTL isolation by using Streptamer technology presents favorable benefits; since Streptamers can be detached from cell surface after biotin addition, in some countries these constructs are not considered as drugs but as adjuvants (Fuji et al., 2011). This feature reduces the regulatory requirements for CMV-specific CTL manufacture, increasing the applicability of the method to a wider number of transplant centers while reducing production costs. Furthermore, we have been able to confirm previous results showing that the constant binding of multimers to the surface TCR impairs the functionality of the isolated cells (Neudorfer et al., 2007), demonstrating the need for reversible multimer implementation. In addition, the use of Streptamer technology offers further benefits compared to other isolation protocols based on non-reversible multimer techniques: first, the functional status of isolated CTLs is preserved, essential for virus clearance; second, since the Streptamer is detached from the cell surface prior to transfer, immune responses directed against the multimer are avoided; and third, the dissociation of the Streptamer from the selected cells may retain the capacity of infused cells to migrate *in vivo* to localizations were they are needed, while the multimer attached to the cell surface could target T cells non-specifically into certain tissues (Neudorfer et al., 2007).

General comparison and future perspectives

In conclusion, we have demonstrated the feasibility of CMV-specific T cell manufacture from G-CSF mobilized donor samples; cellular products maintain their anti-viral function and cytotoxicity, with no major differences between products obtained from non-mobilized or G-CSF mobilized samples. These findings demonstrate that the use of an aliquot from G-CSF mobilized donor samples is suitable for CMV-specific T cell product manufacture and that this approach could be widened to target other viruses associated with complications post allo-HSCT. The avoidance of successive donations, apart from extending the adoptive

therapy accessibility for patients with unrelated donors, would lead to lower costs and shorter manufacturing times, with the associated benefits for donors and patients.

The two described approaches for CMV-specific T cell manufacture, through CD137 expression after antigenic stimulation or using Streptamers, both offer fast and simple ways to directly isolate highly specific T cells. Moreover, the availability of GMP-grade Streptamers and CD137 monoclonal antibodies for clinical use makes these approaches readily available for clinical adoptive immunotherapy protocols. In general, both methods have great benefits but also some limitations. In the case of CD137 expressing cell isolation, the obtained cellular product is made of both CD4+ and CD8+ CMV-reactive cells, a polyclonal product composed T cells recognizing different peptides of the CMVpp65 protein. However, the phenotypic changes induced in the cell by the first stimulation require GMP a laboratory for the ATMP manufacture. In comparison, CMV-specific CTL isolation by MHC-multimers generates a product formed of only CD8+ cells, which recognizes only a peptide of the whole CMVpp65 protein, but the obtained cellular product is considered a non-ATPM and facilitates its applicability into clinical units without a GMP laboratory. Thus, as well as considering the availability of a GMP laboratory, clinical studies must answer whether the cellular products obtained from these two approaches offer protection in the infused patient, to be able to choose the method with a greater clinical applicability and benefits.

V. GENERAL CONCLUSIONS

1. MHC-multimers bind non-specifically to FcR expressing CD14+ and CD19+ cells.

2. Pre-treatment of non-mobilized PBMCs with human AB serum reduces non-specific binding of MHC-multimers to CD8- cells, improving CMV-specific CTL quantification using MHC multimers.

3. The elimination of immature myeloid progenitors from G-CSF mobilized samples using an adherent process diminishes non-specific binding of MHC-multimers and improves the purity of the CMV-specific CTL product after isolation.

4. If CMV-specific CTLs remain the MHC-multimer bound to their cell surface their proliferation potential and functionality is impaired, confirming the necessity of reversible MHC-multimer implementation.

5. CMV-specific CTLs isolated from G-CSF mobilized samples using Streptamers are functionally equivalent to CMV-specific CTLs obtained from non-mobilized samples, but their expansion potential in the presence of the antigen is reduced.

6. When CMV-specific CTL percentage in the original sample is low, PBMCs can be enriched in CMV-specific CTLs by stimulation and afterwards isolated using Streptamers, maintaining their functionality.

7. CMV-specific T cells generated from G-CSF donors based on activation-dependent CD137 expression are equivalent to those obtained from non-mobilized samples; CD137 expression upon activation is similar, they have comparable expansion potential, and expanded CMV-specific T cells are highly specific, functional and cytotoxic.

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VII. APPENDIXES

Impact of T cell selection methods in the success of clinical adoptive immunotherapy

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Title

IMPACT OF T CELL SELECTION METHODS IN THE SUCCESS OF CLINICAL ADOPTIVE IMMUNOTHERAPY

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Abstract

Chemotherapy and/or radiotherapy regular regimens used for conditioning of recipients of hematopoietic stem cell transplantation (SCT) induce a period of transient profound immunosuppresion. The onset of a competent immunological response, such as the appearance of viral-specific T cells, is associated with a lower incidence of viral infections after haematopoietic transplantation. The rapid development of immunodominant peptide virus screening together with advances in the design of genetic and non-genetic viral- and tumoral-specific cellular selection strategies have opened new strategies for cellular immunotherapy in oncologic recipients who are highly sensitive to viral infections. However, the fast development of cellular immunotherapy in SCT has disclosed the role of the T cell selection method in the modulation of functional cell activity and *in vivo* secondary effects triggered following immunotherapy.

1. Introduction

Susceptibility to viral infections in allogeneic haemopoietic stem cell transplantation (HSCT) is the result of profoundly reduced innate and adaptive immunity caused by the immunoablative effect of the bone marrow conditioning regimens, host-*versus*-graft and graft-*versus*-host interactions which take place in the immediate post-transplant period [1, 2]. Administration of anti-viral drugs is nowadays the standard first line therapeutic treatment and it is even used as prophylactic method in patients with high susceptibility to suffer viral primo-infection or reactivation. However, this treatment shows high regimen-related toxicity in different tissues, variability in efficacy and generation of viral resistant variants after treatment. Therefore, many clinical trials alternate or combine antivirals with prophylactic therapies based on infusion of virus-specific primed T cells [3]. Despite their success, there are many doubts about their application and efficacy after the reappearance of virus-associated clinical symptoms in some patients, correlating with the *in vivo* longevity of this therapeutic subpopulation and retention of virus reactivity. Interestingly, only few researchers have analysed in depth if the T cell cytolytic effector function is in fact dependent on their selection method, which would alter the persistence of the adoptively transferred cells.

This review summarizes the impact of classical and alternative protocols for purification of antigen-specific T cells on the establishment of immunological tolerance, the dynamics of *in vivo* T cell expansion and its clinical daily application. Our observations highlight the urgent need for standard technical approaches in Immunotherapy facilities which would induce minor alterations in the T cells, guaranteeing a long-lived and optimum repopulating potential.

1.1 Adoptive immunotherapy precedents

The classical concept of adoptive immunotherapy is based on infusion of donor unmanipulated bulk lymphocytes (DLI) at variable intervals following bone marrow transplantation, enhancing, for example, the antileukaemic effect of the graft (GVL) [4]. This

idea is supported from the cytogenetic remission that was observed in three patients receiving buffy coat infusion of their original marrow donors after chronic myelogenous leukemia (CML) diagnosis and conventional allograft transplant [5]. Moreover, five patients receiving nonirradiated DLI developed Epstein-Barr virus (EBV) lymphoma after transplantation. Clinical remissions were achieved within 30 days after cellular treatment [6]. Despite these promising clinical results, also corroborated by Heslop and colleagues, this type of cellular therapy presents a high risk for the patient´s health [7]. Indeed, there is evidence that administration of total lymphocytes is associated with high morbidity and mortality rates, mainly due to severe graft-*versus*-host disease (GvHD) [8]. Alloreactive $CD8^+$ T cells (CTLs) contained within the transferred bulk leukocyte population are directly responsible for the aggressive GvH syndrome. Consequently, different protocols have been developed to limit the presence of these self-reactive subpopulations to obtain a safer cellular product.

In immunocompetent subjects, the exposure to viral antigens and their recognition by T cells trigger T cell receptor (TCR)-signal dependent activation, which drives their expansion, differentiation and regulates the magnitude of the T cell response [9]. Virus-primed T cells are generated during this process [10]. This physiological process can be replicated ex vivo in lymphocyte cultures exposed to viral antigens. Thus, cell proliferation and differentiation into different T cell subsets can be achieved [11]. This is the basis of the incipient adoptive immunotherapy strategy suggested by Ridell and colleagues, and adopted by many researchers nowadays, proving that autologous $CD3^+CD8^+CD4^-$ cytomegalovirus (CMV)-specific clones can be expanded by co-culturing donor-derived peripheral-blood mononuclear cells (PBMCs) with autologous virus-pulsed fibroblasts for 5 to 12 weeks [12]. Successful adoptive immunotherapy was later performed with these virus-specific T cells. None of patients developed CMV-associated clinical disease or side effects usually associated to the treatment. The analyses of the rearrangements of the TCR β-chain variable region demonstrated *in vivo* clonal expansion from the transferred clones. These expanded T cells exhibited the same cytotoxicity by the parental cells from the

immunocompetent donors for up to four weeks [13]. However, as this *ex vivo* expansion is undertaken in the presence of live virions, this methodology can jeopardize the patient's safety, and therefore, it has been limited to a restricted number of cases [14]. Nevertheless, the therapeutic potential of their findings, which have been corroborated in subsequent studies, represented a starting point for the designing of new cellular immunotherapy protocols [15, 16] (table S1).

1.2 Improvement of virus-specific T cell production and cell enrichment methods

In the meantime, other similar approaches have also been used to generate anti-viral T cell responses. For example, dendritic cells (DCs), one of the most potent professional antigen presenting cell (APCs) types, have been extensively used in immunotherapy protocols (table S2). They may be used as raw biological material for *in vitro* T cell production of both protagonist cells of the cellular therapy products. The main advantage of using DCs is their high capacity for antigen processing and presentation of multiple epitopes in major histocompatibility complex (MHC) class I and class II and applicability to patients of all HLA types. It has also allowed to obtain polyclonal T cell subpopulations with the advantage that this characteristic provide, synergism between different immunological subpopulations [14, 17]. Otherwise, the immunodominant T cell epitopes present in a particular given antigen have to be known to expand the patient's T cells. In addition, it is possible to modulate the expression of their co-stimulatory molecules allowing the manipulation of the immunological synapsis that would enhance the activation or inhibition of the T cell response [18]. Similarly, EBV-transformed B-lymphoblastoid cell lines (BLCL) which were transduced with a retroviral vector encoding the immunodominant CMV^{pp65} have also been used as APCs to simultaneously expand EBV and CMV-specific CTLs. These CTLs showed class I and class II viral-bispecific restriction. [19]. Furthermore, BLCL can be grown in large numbers, which would enhance the therapeutic protocol. Thus, Leen *et al*. has perfected the system, BLCL were transformed with a recombinant adenovirus expressing CMV^{pp65}, with the purpose of obtaining T cell preparations with trivirus-specific

activity (EBV-, Adenovirus (AdV)-, and CMV-specific CTLs). CD4 and CD8 T cell expansion was observed, leading to in vivo resolution of virus-associated clinical symptoms within the first month of the therapy [20].

In fact, several clinical trials are being carried out to validate a new genetic engineering methodology consisting on the redirection of the T cell antigenic specificity by the introduction of TCR genes (variable α- and β-chains) or chimeric antigen receptors (CARs) (Fig. 1) followed by infusion in the patient (table S3).

This strategy can be used to engineer fully functional virus-specific T cells, which would otherwise be "inactivated" after chronic viral antigen exposure [21]. For example, HBV-specific T cells are deleted or exhausted in chronic hepatitis B and HBV-related hepatocarcinoma patients. Gehring and colleagues have obtained fully functional TCR re-directed HBV core (18-27) and surface (183-191; 370-379)-specific effector T cells after epitope-specific TCR cloning by retroviral transduction. These T cells exhibited cytotoxic activity towards HBV-infected cells [22, 23]. Kessles and colleagues demonstrated the expansion and maintenance of TCR-modified transferred T cells up to 81 days after inoculation. This clearly showed their *in vivo* proliferative capacities [24]. At present, different kind of tumours and viral infections are treated with this type of cell transfer therapies, in some cases demonstrating cancer regression in 30% of treated patients [25]. A potential problem of this methodology is the association of the recombinant α/ß chains with the endogenously expressed TCR chains, generating T cells with unknown and undesired new autoantigen specificities [26] and with reduced TCR surface expression. In this way, sporadic autoreactive events could potentially appear in the recipients, something to be taken into consideration. The introduction of cysteines on each recombinant TCR chain to form an extra interchain disulfide bonds between both α- and ß-structures could prevent this intracellular molecular event even significantly boost the effector activity [27]. Cohen *et al*. designed a murinization strategy based on the substitution of the human C regions with their murine counterparts [28]. However, these xenogenic sequences might also potentially

trigger immunogenicity against the murinized TCR. Bialer *et al* minimally engineered human C regions with selected murine residues mediating superior chimeric TCR expression and improved activity, which would result in a more efficient pairing of the murine Cα and Cß1, decrease the formation of mixed TCR chain dimers and .minimize autoimmune manifestations [29].

Nowadays, human trials are in progress to evaluate the safety and feasibility of T cells transduced with CARs instead of αβTCR in adoptive cell therapy procedures [30]. Specifically, these constructs recognize tumor cell-surface molecules. They consist of the fusion of the antigen-recognition portion of a monoclonal antibody with an intracellular signalling domain capable of activating or enhancing T cell effector function by intensifying molecular signalling pathways in a MHC dependent and independent fashion [25, 31, 32]. Consequently, Micklethwaite *et al.* engineered virus-specific T cells stimulated with multispecific-viral immunodominant antigens of CMV, EBV and AdV. These modified cells also exhibited antitumor activity that was conferred by their retroviral-mediated expression of CAR.CD19+, obtaining a bi-functional therapeutic harvest [33]. This approach allows the expansion of multivirus-specific CAR-modified CTLs (which retain their antiviral activities), but with significantly increased *ex vivo* antitumoral activity against B-ALL blasts from patients with haematological disease. Recently, the monitoring of anti-CD19 CAR-modified T cells is possible through the use of an antibody consisting of human CD19 extracellular domains and human immunoglobulin domain. Similarly to MHC-multimer technology, the fluorescent-labelling of this structure allows the direct visualization of CAR-expressing T cells by flow cytometry, which makes this approach very attractive [34]. However, only a few tumour-specific antigens expressed exclusively by cancer cells and susceptible of being targeted have been identified. This problem could be solved by the CAR-target replacement by a fluorescein isothiocyanate (FITC) molecule. The use of cetuximab, trastuzumab, rituximab monoclonal antibodies conjugated with FITC would expand the applicability of this tool

allowing the simultaneous recognition of a variety of tumour-associated antigens by a single therapeutic product [35]. Using this molecular approach, Louis and colleagues and Pule *et al.* achieved effective anti-tumour responses in patients with advanced-stage neuroblastoma. The treated patients exhibited partial and complete tumour responses respectively and long-term persistence of modified T cells (beyond 96 weeks). These T cells were engineered from EBV-specific cytotoxic T cells expressing the CAR.diasialoganglioside antigen-GD2 [36, 37]. These encouraging clinical results have demonstrated the high viability of these therapeutic T cells in cancer patients. However, these results have not been reproduced by other authors. Kershaw and colleagues observed a high level of therapeutic T cells during the first few days after infusion of autologous anti-α folate receptor (FR) CAR modified T cells in patients with metastatic ovarian cancer. These T cells were undetectable after a month of monitoring [38]. A poor choice of the pan-tumor antigens or a weak functional activity could explain these results.

Following this approach, three different CAR-generations have been developed, which incorporate T cell co-stimulatory signalling molecules (CD28, CD3-zeta(ξ), OX40, 4-1BB (CD137)) in their structure, improving their signalling capacities in modified T cells [39, 40]. These molecular modifications have enhanced the *in vivo* expansion (>1000-fold), have increased the long-term maintenance of the engineered CAR+ T cells into the patient (for at least 6 months) and have induced trafficking to the bone marrow or cerebrospinal fluid. The infusion of CAR+ T cells that targeted CD19 or *ERBB2* (HER-2/neu) and contained costimulatory domains from CD28 or CD137 and the T cell receptor ξ chain signalling element in patients with relapse or refractory chronic lymphocytic leukemia (CLL) [41-43], acute lymphoblastic leukemia (ALL) [44] and metastatic colon cancer [45] generated tumor regression by a potent antitumor effect in all patients treated (in some cases associated with morphologic and molecular remission). However, unexpected clinical adverse events were noted in these patients including the occurrence of delayed tumor lysis syndrome accompanied by a hemophagocytic syndrome, capillary leak syndrome, non-infectious

fevers, hypotension, respiratory distress syndrome, grade 3/4 lymphopenia and loss of normal B cells. In most cases the administration of glucocorticoids or anti-cytokine therapy resolved these reversible systemic effects although hospitalization in intensive care units (ICU) was necessary depending on the case. A cytokine-release syndrome or “cytokine storm” has been also observed in a limited number of patients, either after intensive lymphodepletion and before immunotherapy treatment or after CAR-transduced T cells infusion itself [41, 42, 44]. Most patients responded well to anti IL-6 (Tocilizumab) and/or anti TNF (Etanercept), although in a few medically fragile patients cytokine blockade with drugs was not effective resulting in multiple organ dysfunction and death [43, 45]. However, the precise aetiology of these patients´ deaths remains uncertain.

Although the administration of corticosteroids in doses used to treat GvHD or antibodies specific to T cells such as alemtuzumab (CAMPATH-1H) would deplete the majority of circulating transduced cells [46], other alternative approachs are necessary. In this regard, the introduction of the herpes simplex thymidine-kinase (HSV-TK) suicide transgene in the viral construct has allowed the inducible apoptosis of transduced-cells through interference with DNA synthesis on exposure to ganciclovir administered in the event of GvHD after stem-cell transplantation in several phase I-II clinical trials [47-49]. Acute and chronic GvHD was successfully controlled with this suicide HSV-TK approach in the context of allo-HSCT and haplo-HSCT. However, a number of drawbacks need to be improved such as the immunogenicity of the TK protein, the restriction of killing to dividing cells and the elimination of transduced-cells when Ganciclovir is used for the treatment of CMV infection.

For this reason, other authors have investigated in a phase I-II clinical trial the suitability of an alternative suicide gene, inducible caspase 9 (iCasp9), showing >90% apoptosis of the modified T cells within 30 minutes of the administration of a specific chemical dimerizer drug [50]. This iCasp9 cell-suicide system did not change the antigen-

specific functionality [51, 52] even when combined with transgenic expression of IL-2 or IL-15 [53].

The development of multimer technology has provided an invaluable method for monitoring and purification of T cells with known antigenic specificities. The basis for multimer technology resides in the recognition of antigen-specific TCRs by a recombinant class I or class II molecule complex to a certain immunodominant peptide. Identification of antigen-specific cytotoxic T cells regardless of their biological activity allows the preparation of an heterogeneous T cell population which circumvents the previous phenotypic characterisations required for the identification of primed subpopulations with long-term survival capacities. Consequently, this staining technology allows the isolation of T cells with a given antigen specificity from seropositive donors without any further manipulation [54]. Nowadays, there is a wide variety of available MHC multimer molecules such as dimers, tetramers, pentamers, streptamers, dextramers and octamers which are key for studies of adoptive immunotherapy. These multimer molecules have been extensively used to identify and select CMV, EBV and AdV-specific T cells from healthy donors, and their transfer to immunocompromised hosts has shown excellent results [55-57] (table S4).

Other authors have demonstrated the value of IFNγ-secreted antigen-specific CD8+ T cells, for the successful reconstitution of virus-specific immunity in allogeneic bone marrow transplant recipients [58]. Manz *et al.* has developed a high-affinity physic matrix of cytokine-secreting cells that prevents cytokine spreading [59-61]. A number of groups have successfully used this technique for the treatment of viral infections in immunocompromised patients, increasing viral clearance and avoiding the associated-disease [62, 63]. However, several authors have questioned the homogeneity of the cellular product obtained with this capture method, following the identification of non-specific- NK-, B-cells and monocytes in the harvest product [64-66]. The presence of some of these cell types can have important biosafety implications in immunocompromised patients. In fact, Mutis and colleagues observed association between a high frequency of histocompatibility-minor antigen-specific

mismatch and the development of grade II-IV GvHD [67]. This pathological event after adoptive Immunotherapy was explained by the presence of allo-reactive clones in the infused product [68].

The establishment of good manufacturing practices (GMP)-based facilities and methodologies to achieve homogenous functional T cell preparations would favour the development of these techniques to a clinical scale improving the standardization of the process of cell selection methods for Immunotherapy applications. Thus, the above-mentioned results would justify an increased investment of funds and resources to develop a safe approach to implementing these promising genetic and cellular therapies in medical practice. To identify funding sources to support the viral constructs and cellular products manufactured under GMP conditions and to overcome the complex legal barriers should be a priority for the academia and the industry in we want to further develop these advanced technologies [69].

1.3 The therapeutic virus-primed T cell status determines its long-term persistence in the recipient

So far, it is possible to reactivate the memory CTL pool through *ex vivo* antigen presentation by B cells-, fibroblasts-, DCs- and BLCL-based systems. This is possible because of the high prevalence of CMV, EBV and AdV infections in the human immunocompetent population. Theoretically, this implies that memory rather than naïve T cells would be the best candidate to be selectively expanded *ex vivo*. However, there is no doubt about the notable influence of the *in vitro* culture period to obtain differentiation status-specific phenotype T cell clones and their permanence ability *in vivo* [70]. Few years later, this natural phenomenon was demonstrated by Berger and colleagues, which have shown in an experimental model of macaque with persistent CMV infection, that for its treatment the use of this *ex vivo* expansion system from effector memory T cell clones can hamper as both correct homing to lymph nodes (LNs) and bone marrow (BM) and their survival for a limited time *in vivo* [71]. On the other hand, if T cells are derived from central memory clones, they

retain their ability to respond to CMV, expand *in vivo* and undergo phenotypic conversion to both central- and effector-memory T cells. Therefore, selection and infusion of more incipient primed cells would ensure that after the therapeutic procedure, virus-specific cytotoxic T cells would permanently re-establish the immune memory response [71-73]. The combination of this enrichment system with molecular engineering would have a high therapeutic potential. Thus, Wang *et al*. isolated polyclonal T cells with a central memory-like phenotype (CD8+CD45RA-CD62L+) which exhibited anti-tumour activity after CD19-specific CAR expression [74]. Recently, the enrichment of CD8+CD62L+ T cells using magnetic microbeads technology and priming with peptide-pulsed APCs before transduction with a lentivector encoding CD19-CAR has allowed the engineering of CMV- and EBV-specific modified T cells. These cells were further selected with reversible streptamers and showed equivalent T cell responses to tumours and endogenous viral–bispecific TCRs [75]. These results have shown that the previous selection of specifically primed T cells significantly increases the efficiency of the harvest. The studies conducted by Hinrichs and colleagues went further by showing in a transgenic murine model of adoptive Immunotherapy, that T cells from the naïve compartment resist terminal differentiation and possess the highest expansion potential and anti-tumour activities. This finding implies that the pre-priming of lymphocyte subpopulations improves gene modification, leading to a high transduction efficiency and transgene expression [76, 77]. The use of these T cells would require *in vitro* priming and extensive expansion from both seropositive and seronegative donors indistinctly. Following this line, Hanley *et al.* has achieved extensive *ex vivo* expansion of naïve T cells isolated from cord blood utilising EBV-infected B cells as APCs, after their modification with adenoviral vectors. In this way, they have generated large numbers of CMV, EBV and Adenovirus-specific T cells [78]. Interestingly, expanded cytotoxic T cells from naïve precursors exhibited anti-leukemia activity after transplantation [79]. In addition, this methodology would bypass terminal differentiation as demonstrated by Gattinioni and colleagues. Otherwise, terminally differentiated T cells would be less effective at triggering disease regression *in vivo* [70, 80, 81]. However, data published by several research groups

indicate that naïve T cell populations contain alloreactive precursors. Thus, these authors have found high frequencies of GvHD in mice infused with naïve T cells in comparison with memory T cells [82, 83]. In agreement with this observation, Distler and colleagues have demonstrated alloreactivity of sorted naïve T cells against single class I or class II mismatched MHC alleles, questioning the validity of naive T cells as a substrate for adoptive Immunotherapy in BM transplant recipients [84]. Recently, Gattinoni and colleagues have identified in humans a lytic T cell memory subset with phenotypic (CD45RA+CD62L+CD95+CCR7+IL-7Rα+IL-2Rß+CXCR3+) and functional (IFNγ+IL-2+TNFα+) characteristics shared by naïve T and stem cells [85-87]. Consequently, the use of T cells derived from naïve precursors requires an exhaustive analysis before its routine application in human therapy.

1.4 Microenvironment of the recipient, a highlighted variable in the multivariate equation of biological immunotherapy

The host lymphoablative conditioning regimen essential for haematopoietic transplant carried out before adoptive T cell transfer-based Immunotherapy may enhance anti-tumour responses by the modulation of the microenvironment through a range of mechanisms, including the inhibition of endogenous CD4+CD25+FOXP3+ regulatory T cells (T_{regs}), upregulation of MHC class I proteins, increase in the pool of peptides available for presentation, T cell trafficking, potentiation of innate immunity and increase in homeostatic cytokines (IL-2, IL-7, IL-15 and IL-21) [88-92]. This subject has been the central issue of different experimental studies for over a decade in radiated or pharmacologic-treated recipients.

Furthermore, T_{regs} suppress effector T cells by a number of mechanisms including an increase of the activation threshold of effector T cells, expression of inhibitory costimulatory molecules, induction of anti-inflammatory biochemical pathways, direct or indirect killing, consumption of proinflammatory cytokines, or production of immunoregulatory cytokines

[93]. The downregulation of T_{regs} by exogenous immunostimulatory agents could therefore potentially improve the migratory properties, engraftment and cytolytic activity of the transferred T cells. Similar actions have been demonstrated through combined therapy of Daclizumab (humanized anti-CD25 monoclonal antibody) with peptide vaccine, administering to breast cancer patients. In this clinical trial T_{regs} eradication in situ and reprogramming induces robust augmented of physiological CTL and T helper response [94, 95]. Modulation of inducible Tregs as a consequence of the preparative regimen for transplant (immunosuppression) triggers inhibitory counterproductive cellular mechanisms that could limit the therapeutic potential of the infused cells.

Common γ-chain cytokines, including IL-7 and IL-15, have been reported to induce vital cellular activity such as proliferation of human T cells in the absence of TCR stimulation, furthermore avoiding apoptosis and maintaining cell metabolism after transfer into the lymphopenic host (homeostatic expansion). The absence of some of these homeostatic cytokines could result in the metabolic atrophy of infused T cells, which leads to delayed growth and proliferation following viral stimulation [90, 96]. Thus, it is known that expression of IL-7 and IL-15 receptors is key to the establishment of resting memory cells in cellular therapy procedures, as both cytokines synergistically drive T cells through this crucial checkpoint in their differentiation process [97]. Shen *et al.* demonstrated that down-regulation of both IL-7R and IL-15R is likely to be a contributing factor for the poor survival of therapeutic influenza-specific memory CTLs in the respiratory tract. According to these authors, there could even exist molecular mechanisms that would condition their survival depending on the particular tissue [98]. On the other hand, this *in vivo* expansion is also thought to be driven by different factors such as self-peptides and other antigens. Furthermore, exposure to viral antigens during the period of profound lymphopenia results in a significant boost of cellular immunity [55, 99, 100] and it is a mandatory requirement for antigen-specific immunological recovery in the transplanted patient.

To date, the essential role of T helper (Th) cells for the maintenance of CD8s is a controversial issue and different studies have sometimes shown inconsistent results.

Cobbold and colleagues observed no correlation between CMV-specific CD4+ T cells and the circulating level of CD8+ T cell [55]. However, this is likely due to the low sample size (n=5), so a declining trend in both subpopulations is observed in 4/5 patients 60 days from the time of infusion of the therapeutic product. Several studies confirmed this trend for a relatively long time. Riddell *et al.* demonstrated that the adoptive transfer of donor CMV-specific CTLs in transplant patients results in a fast therapeutic activity, although their long-term maintenance is hampered by the absence of the appropriate T helper subpopulation [12]. In line with these observations, Rosenberg and colleagues described that HIV-1-infected subjects without acquired immunodeficiency syndrome (AIDS) development, show a high virus-specific CD4+ proliferative response and an extremely vigorous CTL concomitant response [101]. The particular mechanisms whereby CD4+ T cells maintain effective antiviral immunity are poorly understood, but they could be related to the orchestration of CTL precursor activity. According to this, there are already studies raising tumour-specific CTL responses in which a simultaneous activation of the T helper subpopulation is found, after cellular vaccination with class I and II peptide-loaded DCs vaccines. With this strategy, bi-functional CD4+ activity is generated, resulting in increasing CTL proliferation and Treg inhibition [102]. In order to examine the relationship between both protective immunological populations and their role during the immunological recovery process in the post-transplant period, the development of MHC class II multimer complexes is being encouraged by biotechnology companies [103]. In fact, more studies are incorporating them as a research tool. As a matter of fact, the use of T helper cells as a therapeutic product has also been put in question, and the limited experience with these cells in adoptive cell therapy is insufficient to obtain objective conclusions. Quezada and colleagues transferred tyrosinase-related protein 1-specific TCR CD4+ cells into irradiated $RAG2^{-/-}$ mouse model with advanced melanoma. Tumour eradication was mediated by cytotoxic CD4+ T cells, which appeared after acquisition of IFN-γ, TNF-α, IL-2, granzyme B and perforin expression. Other endogenous immunological subpopulations (helper- and cytotoxic- T cells, B and NK cells) did not play an essential role in the anti-tumour effect of these infused cells [104]. The

lymphopenic microenvironment and the depletion of CD25+Foxp3+CTLA-4+CD4+ T_{regs} apparently played a crucial role in the *in vivo* priming, expansion and activation of the exogenous naïve CD4+ cells and their cytotoxic-like phenotype conversion [105]. Furthermore, these conditions allowed the long-term establishment of a CD4+-memory subpopulation [106].

Finally, it is important to mention that many of these cytokines may play a role in manipulating the microenvironment in the context of allogeneic hematopoietic stem cell transplantation and that there are ongoing clinical trials evaluating several modifications of the different conditioning regimens including non-chemotherapy based conditioning regimens for transplantation [107, 108].

1.5 *In vitro* modulation of the functional ability of viral-specific CTLs by multimer complexes

In contrast to the other T cell selection techniques, multimer technology (Fig. 2) developed by Altman *et al.* allows the identification and enrichment of viral-specific CTLs without altering their differentiation status [109]. However, recent studies with soluble experimental MHC class I-tetramers have shown that continuous *in vivo* administration of an MHC multimer, induces unexpected outcomes in the antigen-reactive CD8+. An increase in the frequency of annexin V staining was observed, and could be attributed to the induction of cellular anergy or activation of induced cell death [110]. In the first case, anergy can be triggered by a strong “signal 1” provided by the binding of the experimental tetramer with its specific transgenic TCR, in the absence of “signals 2” (co-stimulation) and “signals 3” (cytokine priming) [18]. Instead, a limited expansion of these CD8+ T cells and their effector activities are compromised by the continuous presence of the soluble tetramer within the transgenic mice, leading to either clonal exhaustion or anergy, resulting in T cell dysfunction. In agreement with this, Neudofer and colleagues have observed *in vitro* that both peptide-specific activation-dependent cytotoxic activity and proliferation capacity of primed T cells were impaired and decreased following staining with conventional tetramers [111]. So, gene

transcription after tetramer complex-TCR interaction has been previously showed in experimental models. Others authors have confirmed these observations [112, 113], and some have even demonstrated the loss of protective capacity to *Listeria monocytogenes* in BALB/c mice after transference of CTLs pre-treated with MHC-tetramers [114]. However, administration of CMV-specific CTLs in stem cell transplant recipients that had been previously selected using tetramer complexes efficiently contributed to the control of virus dissemination [55]. In this study, the authors demonstrated persistence of CMV-specific CTLs at least 110 days after infusion, even in patients without CMV-primed CTLs before cell transfer, which suggested expansion of the infused cells. This would discard anergy in the infused T cells, at least for this period of study.

Therefore, experimental and physiological microenvironments show conflicting results about the long-term cellular effect induced by the multimer on the virus-specific CTL. However, it is currently unknown whether pentameric constructs would also induce anergy in these T cells. Pentamer and tetramer technologies are similar tools that use the same molecular approach, so it is theoretically possible that a similar impairment of T cell phenotype and function could occur *in vitro* using pentamers. However, Uhlin *et al.* demonstrated the presence of functional EBV-specific CTLs that induced regression of an EBV-driven lymphoma *in vivo* up to 189 days after infusion. It is worthy to mention that complete regression of EBV infection-associated lymphoma occurred in a patient diagnosed with post-transplant lymphoproliferative disease after therapeutic treatment [56].

One possible explanation to this mismatch between *in vivo* and *in vitro* results is the possibility that T cell hyporesponsiveness negatively modulated by multimer engagement can be reversed by a cytokine storm generated in the lymphopenic recipient. That assessment is proposed by Brown and colleagues who demonstrated that anergic antitumor CD8+ T cells restore their function after transfer into $RAG2^{-/-}$ immunodeficient recipients promoting tumor rejection [115]. Specifically, Teague *et al.* has shown that exogenous addition of IL-15 rescued and expanded previously tolerised cytotoxic T cells *in vitro* [116]. However, no data in the literature is published concerning the association between cellular

functional immunomodulation induced by the multimer complexes and the influence of the host microenvironment.

As discussed above, the influence of tetramer and pentamer staining on T cell functional status remains an important unresolved issue. This could substantially limit their clinical applicability. In order to address this problem, a reversible human MHC/peptide multimer, called Streptamer, was constructed by Neudorfer and colleagues [111], using the molecular technology proposed by Knabel *et al*. [114]. “Naïve” antigen-specific T cells can be obtained after multimer complex staining. The reversible binding between both structures (Streptamer-TCR) allows its easy disengagement through exposure to a competitor molecule. Following dissociation, the streptamer-treated CTLs are functionally indistinguishable from untreated T cells. Wang and colleagues demonstrated that *in vitro* treatment of OT-I TCR-transgenic CTLs with peptide-loaded OT-I-streptamers markedly increased [^{3}H]thymidine incorporation and up-regulated early activation markers [117]. The biochemical analysis of the signalling pathways in this assay identified several signalling molecules which were regulated after streptamer engagement. Sustained phosphorylation of Akt and ERK1/2 was observed, possibly increasing Bcl-x_L expression, thus resulting in cell survival. According to these authors, Streptamer engagement is not “silent” thors. However, this positive effect may be favourably used in adoptive Immunotherapy protocols. The performance of cell enrichment methods using streptamer complexes has significantly improved in just a few years [68, 75, 118]. Recently, this methodology has even been combined with other genetic approaches, where *in vitro* analysis has shown excellent T cell dependent anti-viral and anti-tumoral activities, and thus demonstrating the bi-specific system validity and the potential of this multimeric methodology [75]. However, it is unclear which signalling pathways may be activated by the streptamer-TCR interaction, or whether this would have synergistic effects with other signal transduction cascades. Whether these molecular changes in T cells would affect their therapeutic behaviour once administrated to

the cancer patient is an open question. More detailed studies will be necessary to clarify this issue.

2. Concluding remarks: Interrelationship between *in vitro* and *ex vivo* potential of the virus-specific primed T cells

The particular physiological characteristics of the recipient's hematopoietic progenitors, the cellular composition and purity of the therapeutic product, and the selection method used to isolate or develop antigen-specific T cells will determine the long-term persistence of transferred lymphocyte populations. In some cases, the altered function and differentiation of these cells significantly affected the performance of adoptive immunotherapy used in SCT patients, making it difficult to predict clinical results. In the future, multivariate analysis will be necessary to understand the interaction between all these physiological variables and to determine the optimal method of achieving antiviral T cell immunity.

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Conflicts of interest

The authors declare no conflict of interest regarding the topics discussed in this manuscript.

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FIGURE LEYENDS

Figure 1. Genetic manipulation of the TCR

This figure shows two different methods of genetic manipulation of the therapeutic cell: Recombinant T-cell receptor (rTCR) transference and chimeric antigen receptors (CAR) cloning (**A**). This new genetic engineering methodology consists on the redirection of the T cell antigenic specificity by the introduction of CARs using viral vectors (**B**) or TCR genes (variable α- and β-chains) (**C**).

Figure 2. Multimer technology allows the identification and enrichment of viral-specific CTLs

This figure shows a diagrammatic representation of the structure of three different multimers: Tetramers; Pentamers and Streptamers together with the IFN-gamma (γ) catch reagent and their mechanism of action. The IFNγ-capture, Pentamers and Streptamers but not the tetramers are currently produced under GMP conditions.

FIGURES

Figure 1.

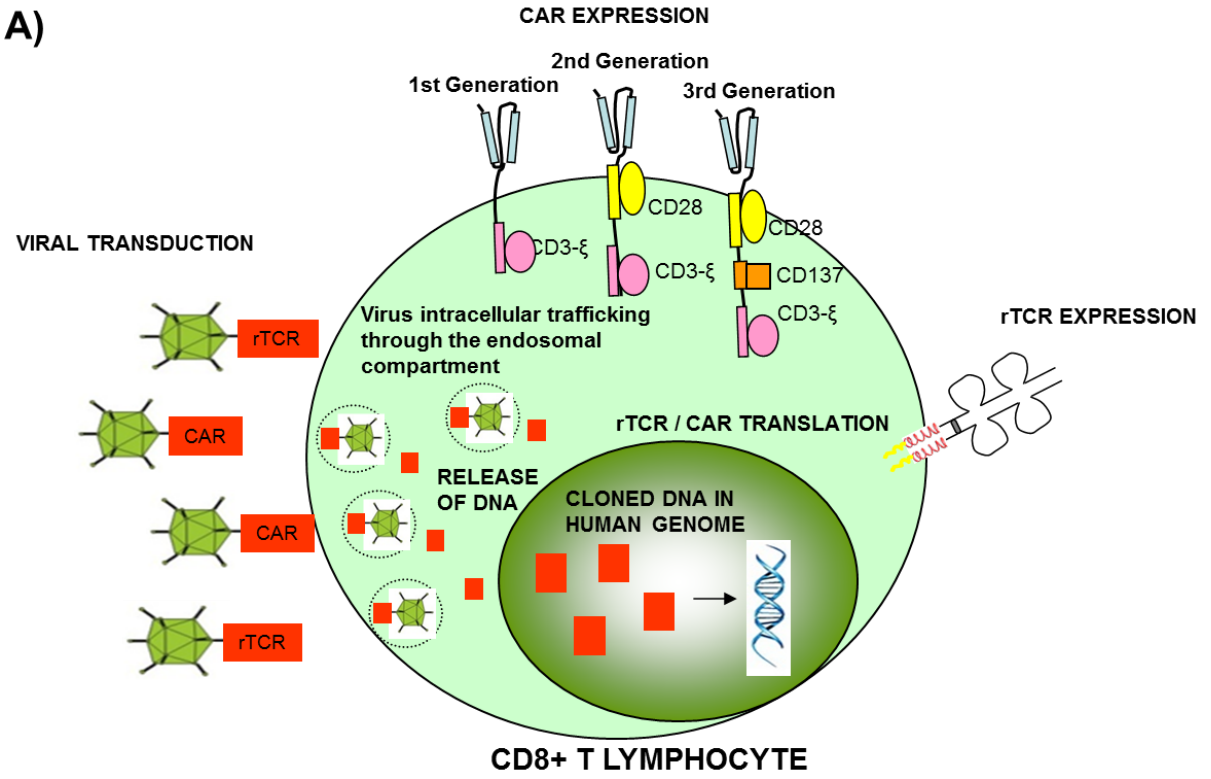
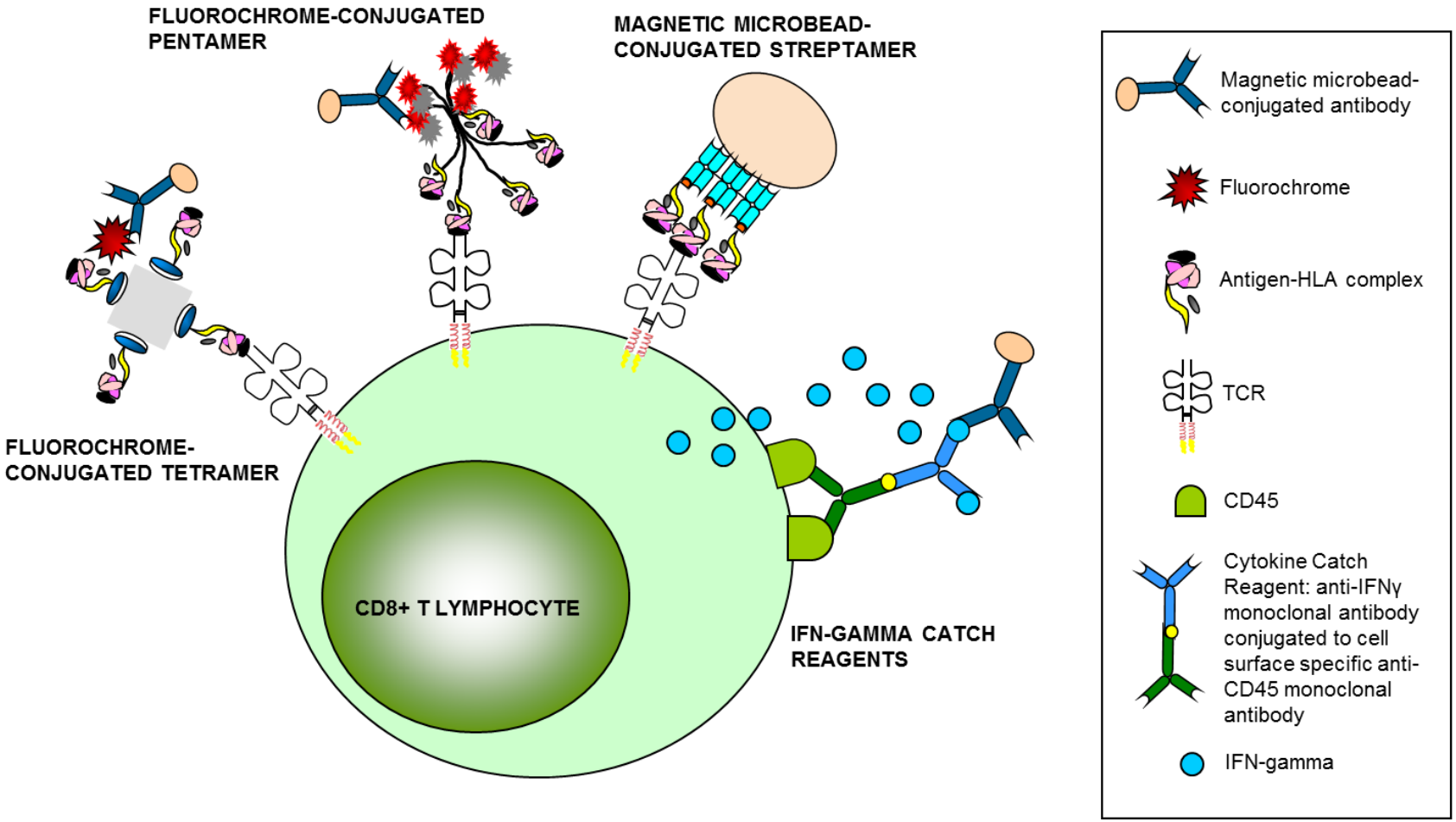


Figure 2.


FLUOROCHROME-CONJUGATED PENTAMER
MAGNETIC MICROBEAD-CONJUGATED STREPTAMER
FLUOROCHROME-CONJUGATED TETRAMER
CD8+ T LYMPHOCYTE
IFN-GAMMA CATCH REAGENTS
Magnetic microbead-conjugated antibody
Fluorochrome
Antigen-HLA complex
TCR
CD45
Cytokine Catch Reagent: anti-IFNγ monoclonal antibody conjugated to cell surface specific anti-CD45 monoclonal antibody
IFN-gamma

Impact of T cell selection methods in the success of clinical adoptive immunotherapy

Supplementary material

Table S1. Major studies published about immunotherapy treatments performed with different molecular and cellular approaches

Authors	Methodology	Results
Doubrovina E, *et al.* (15)	- Patients received either donor leukocyte infusions (DLI) or EBV-specific T cells (EBV-CTLs) from donors. EBV-CTLs were generated by enrichment of T cells from peripheral blood mononuclear cells (PBMCs) by depletion of monocytes by adherence to plastic and natural killer (NK) cells by adsorption to anti-CD56 immunomagnetic beads. T cells were sensitized in vitro with irradiated autologous EBV transformed B cells.	- Patients were monitored during 4 months - The rate of response to therapy for each group were equivalent - Infusions of EBV-CTLs resulted in much higher EBV-CTL precursor levels which were sustained in the circulation for longer periods of time than those detected after DLIs. - Some patients receiving EBV-CTLs did not respond to the treatment because these CTLs did not lyse spontaneous EBV-BLCLs.
Peggs KS *et al.* (16)	- PBMCs were incubated with recombinant human cytomegalovirus (HCMV) pp65 or pool of overlapping peptides from pp65. CMV-specific activated T cells were isolated with interferon-γ (IFN-γ) capture system.	- Patients were monitored during 6 months - *In vivo* expansion of CMV-reactive T cells was observed in patients who received cells preemptively
Heslop HE *et al.* (17)	- PBMCs were stimulated with irradiated autologous lymphoblastoid cells lines (LCL), expanded with interleukin 2 (IL-2) and transduced with G1-Na retroviral vector to obtained Epstein-Barr virus (EBV)-specific T cells.	- None of the patients who received cytotoxic T lymphocytes (CTL) prophylaxis developed EBV-associated lymphoproliferative disease (LPD) - It is demonstrated the persistence of functional CTLs for up 9 years
Melenhorst JJ *et al.* (18)	- Hematopoietic stem cell transplant recipients were treated with EBV-specific CTLs, bivirus-specific CTL cultures (EBV, AdV), trivirus-specific CTLs (CMV, AdV, EBV).	- Virus-specific T–cell can have cross-reactivity with allo-human leukocyte antigen (HLA)-mismatched targets *in vitro*, but adoptive transfer of partially HLA-mismatched CTLs is safe
Jones K *et al.* (20)	- EBV-nuclear antigen-1 (EBNA1) and BZLF1-specific T cells were expanded using EBNA1 and BZLF1 overlapping peptide pools. IFN-γ-producing polyclonal EBNA1-specific T cells were isolated.	- EBNA1-specific CD8+ effector T cells were successfully generated in 9/14 patients and 16/19 controls
Scheinberg P *et al.* (21)	- PBMCs were stimulated with CMV_{pp65} and CMV_{IE-1} peptide pools.	- The infusion of donor lymphocytes was not necessarily associated with an increase in CMV-specific T cells at the time of analysis after hematopoietic stem cell transplantation (HSCT) - A decrease in the CMV-specific $CD8^+$ T-cell in 2/2 recipients - After HSCT, almost all responding T cells exhibited a more differentiated phenotype associated with a restricted functional profile skewed toward the production of proinflammatory cytokines
Peggs KS *et al.* (22)	- CMV-specific $CD4^+$ and $CD8^+$ T cell lines were generated by short-term *ex vivo* culture of donor lymphocytes with donor monocyte-derived dendritic cells pulsed with virus lysate.	- Massive *in vivo* expansions of CMV-specific T lymphocytes - $CD8^+$-specific T-cell enhanced in all recipients
Brestrich G *et al.* (23)	- Autologous PBMCs were stimulated with overlapping IE-1/pp65 peptide pools to generate a T-cell line	- After the first infusion, the frequency of CMV-specific T-cells increased and viral load decreased - After 4 weeks, the infection reappeared and persisted at a low level even after a second T cell infusion
Hill GR *et al.* (24)	- PBMCs from patient were stimulated with irradiated autologous PBMCs coated with a mixture of HLA class I restricted HCMV-peptide epitopes to expand HCMV-specific T cells.	- HCMV replication in peripheral blood became persistently negative within 3 months of adoptive T-cells transfers - 6 months after adoptive transfer, complete resolution of HCMV disease
Ohira M *et al.* (25)	- Liver lymphocytes were cultured with human recombinant IL-2 for 3 days. One day before the infusion, 1 μg/ml of OKT3 was added in order to opsonize the $CD3^+$ fraction. $CD56^+$ and $CD56^-$ fractions were isolated with anti-human CD56 microbeads.	- The lymphocytes in the peripheral blood of LT recipients who received immunotherapy in the early postoperative period showed significantly enhanced cytotoxicity as compared with those who did not receive the therapy in the same period - The number of IFN-γ–secreting cells in the peripheral blood of LT recipients who received adoptive immunotherapy was significantly higher than that who did not receive immunotherapy during the trial period -
Leen AM *et al.* (26)	- PBMCs were infected with Ad5f35 vector to generate bivirus-specific CTL cultures (EBV and Adenovirus). Then, these cells were stimulated with irradiated autologous LCL.	- 3/13 patients showed initially increased levels of EBV DNA, but without additional CTL infusions, virus load decreased - EBV-specific CTLs expanded *in vivo* and persisted for more than 12 weeks - Adenovirus-specific component only expanded *in vivo* in the presence of concominant adenoviral infection, although, adenovirus-specific T cells could be detected for at least 8 weeks -
Louis CU *et al.* (27)	- PBMCs were stimulated with irradiated autologous LCLs and then EBV-specific CTLs were transduced with the neo-containing G1Na retroviral vector.	- Patients were evaluated for disease 8 weeks after CTL infusion - One patient had complete response more than 24 months; 2 patients had stable disease 8 weeks after CTL infusion

Authors	Methodology	Results
Micklethwaite KP *et al*. (28)	- Dendritic cells (DCs) were differentiated from donor monocytes and were transduced with Ad5f35pp65 vector. Mature DCs were used to stimulate autologous PBMCs.	- After a median follow-up of 218 days after T-cell infusion, 6 episodes of CMV reactivation occurred in 4 patients - In 5/12 participants, rapid reconstitution to pp65 occurred with minimal or no reconstitution of IE1-specific immunity. In 3 patients, IE1-specific reconstitution either matched pp65 or dominated the immune response. In the 4 remaining participants, CMV-specific immunity remained unchanged or decreased after T-cell infusion
Bollard CM *et al.* (29)	- Immature dendritic cells (DCs) were harvested and transduced with Ad5f35LMP2 vector and were cocultured with nonadherent PBMCs. Responder T cells were restimulated with irradiated LCLs transduced with the same LMP2 vector to generate EBV-specific CTL	- LMP2-specific T cells increased up to 5-fold following infusion of the first infusion of T cells - 10/16 patients increase in LMP2-specific T cells in the peripheral blood 1 week after infusion; 9/10 had complete remission for up to 37 months after CTL infusion - 4/6 had complete clinical responses, and complete remission for up to 15-34 months after CTL infusion
Gandhi MK *et al.* (30)	- Allo-CTL were grown by stimulation weekly with autologous EBV-LCLs from EBV-seropositive blood donors	- After allo-CTL there was a restoration of EBV-specific $CD8^+$ T cells presented by a shared HLA allele - One patient died at day 11 after allo-CTL from a respiratory/renal failure. Another died at day 113 but autopsy confirmed complete remission. The third patient had complete remission 17 months after following first infusion
Haque T *et al.* (31)	- PBMCs from donor were stimulated with irradiated autologous LCLs to generate EBV-specific CTLs in order to treat post transplantation lymphoproliferative disease (PTLD)	- Tumor response was recorded 5 weeks and 6 months after CTL therapy (14 patients had complete remission, 3 showed a partial response a and 16 had no response)
Amrolia PJ *et al.* (32)	- PBMCs from donor blood were cocultured with irradiated recipient EBV-LCLs. Alloreactive cells were eliminated using an immunotoxin.	- Immune reconstitution was studied monthly for 9 months - By 6 months after stem cell transplantation (SCT), 1/5 at dose level 1 had normal T-cell counts but none had normal CD4 counts. But at dose level 2, 3/5 had normal CD4 counts
Perruccio K *et al.* (33)	- PBMCs and plasma were collected to generate pathogen-specific T-cell clones through incubation with CMV and *Aspergillus* antigens.	- CMV-specific T-cell clones and high IFN-□□production were detected in PBMCs, 3 weeks after infusion and their frequencies have remained stable for as long as they were monitored (6 months)
Comoli P *et al.* (34)	- Autologous EBV-specific CTLs reactivated and expanded *ex vivo* from peripheral blood lymphocytes through stimulation with EBV-LCL.	- Cell therapy with EBV-targeted autologous CTLs induces LMP-2-specific immunologic responses, and is associated with objective responses and control of disease progression in patients with stage IV NPC resistant to conventional treatments.
Bollard CM *et al.* (35)	- PBMCs were cocultured with γ-irradiated autologous LCLs to generate EBV-specific CTLs	- Gen marking studies showed that infused effector cells could expand up to at least 100-fold *in vivo*, contribute to the memory pool (persisting up to 12 months) and homing to tumor sites, T cells expanded in blood after infusion
Einsele H *et al.* (36)	- PBMCs were incubated for 10 days in RPMI medium with CMV lysate. Live cells were restimulated with irradiated autologous PBMCs, CMV antigen and IL-2.	- CMV-specific CD8+ T-cell remained in the circulations for at least 8 weeks
Savoldo B *et al.* (37)	- PBMCs were stimulated with autologous LCLs irradiated and administered intravenously	- 6-36 months follow up. Resolution of fatigue and malaise, disappearance of fever, and regression of lymphadenopathy and splenomegaly
Lin CL *et al.* (38)	- Autologous monocyte-derived DCs of the advanced nasopharyngeal carcinoma patients, matured with cytokine, pulsed with HLA-A*1101-, A*2402-, or B*40011-restricted epitope peptides from EBVLMP2	- Patients were evaluated for overall tumor response every 3 months after the fourth injection during 1 year
Haque T *et al.* (39)	- Autologous PBMCs were stimulated with X-irradiated LCLs to generate EBV-specific CTLs.	- CTLs were given about every 2 weeks, until tumor either had completely regressed or was enlarging - They recorded outcome at 6 months after the last infusion - No GVHD and 3 patients had complete remission - EBV load in peripheral blood to undetectable levels in patients who responded to the treatment, but was more variable in those who did not
Mitsuyasu RT *et al.* (40)	- CD4+ and CD8+ T cells are genetically engineered with HIV specificity by inserting a gene, CD4ζ, containing the extracellular domain of human CD4 linked to the ζ chain of the T-cell receptor	- Follow-up was extended to 1 year in 18/24 subjects - CD4ζ-modified T cells were detected in the peripheral blood of all patients following infusion during 8 weeks in both treatment arms. - Extended follow-up through 12 months in 18 patients demonstrated sustained persistence of CD4ζ-modified T cells in the blood of 17 of 18patients
Gustafsson A *et al.* (41)	- PBMCs from donors were stimulated with autologous LCLs to generate EBV-specific CTLs	- Monitoring was initiated within 2 to 3 weeks after transplantation and was continued at regular intervals for at least 100 days and up to 450 days - The EBV-DNA load showed a rapid increase, reaching 1 genome in 6.4 cells, 1 genome in 32 cells, and 1 genome in 160 cells within 31 to 130 days after grafting.

Authors	Methodology	Results
Rooney CM *et al.* (43)	- PBMCs were stimulated with irradiated autologous LCLs and were transduced with the neo-containing G1Na retroviral vector	- PBMCs were collected weekly post-infusion for 6 weeks, monthly for 1 year and then every 3 months for 2 years - Gene-marked T cells were found in the peripheral blood of patients for a median of 11 weeks - CTL precursor frequency showed a median 32-fold increase 1 month after completion of infusion
Rooney CM *et al.* (44)	- EBV-specific CTL lines were generate by coculture of PBMCs with irradiated autologous EBV-LCLs.	- 3 patients received CTLs as treatment; 2 had complete remission and 1 died , 25 days after CTL infusion - Patients who received CTL that had been gene marked could be followed by PCR and CTL persisted for up 18 weeks in blood after infusion. If EBV-specific CTL were expanded, marked T cells could be detected for up to 3 years post-infusion
Roskrow MA *et al.* (45)	- EBV-specific CTLs were activated from the peripheral blood of patients and normal donors by coculturing PBMC with irradiated autologous LCLs and they were genetically marked by transduction with the G1Na retroviral vector.	- CTL infusion increased the proportion of circulating EBV-specific cytotoxic precursor cells showing an enhanced cell-mediated immune response to EBV - The level of EBV DNA decreased dramatically after CTL infusion and was undetectable after 4 weeks.
O`Reilly RJ *et al.* (46)	- Adoptive transfer of small numbers of PBMCs or HLA-partially matched T cells from *in vitro* expanded EBV-specific CTLs with irradiated autologous/donor LCLs.	- Induce durable regressions of bulky, widely metastatic EBV lymphomas in a high proportion of cases
Rooney CM *et al.* (47)	- PBMCs from donors were used to generate EBV-specific CTL through previous incubation with LCLs.	- Patients were followed up for 1 year - 3/10 patients had EBV reactivation, (EBV DNA concentrations increased 1000-fold or more and returned to the control range within 3-4 weeks of immunotherapy) - A patient had resolution of immunoblastic lymphoma - CTL persisted for 10 weeks after administration
Bex F *et al.* (48)	- PBMCs were activated with gp160 purified from recombinant vaccinia virus WR89-infected cells.	- After second transfer with activated cells, there was an increase in total lymphocytes and in proliferative responses to HIV antigens - The patient was followed up during 60 days
Ho M *et al*. (49)	- Autologous $CD8^+$ cells were first isolated selectively from leukapheresis products. Then, they were cultured and expanded with phytohemaglutinin and recombinant IL-2 before infusion.	- Patients were followed up 26 weeks - After infusion, in-labeled $CD8^+$ cells quickly accumulated in the lungs, with less than 10% of the labeled cells remaining in the circulation. After 24 hours, labeled $CD8^+$ cells were reduced in the lungs, but increased and persisted in liver, spleen, and bone marrow. - 4/5 have improved or remained clinically stable

Table S2. Clinical trials performed using *in vitro* long-term expansion in presence of viral-specific antigen (peptides, proteins, lysates, dendritic cells…) (http://ClinicalTrials.gov/) for the therapeutic treatment of viral infections in immunocompromised patients.

ClinicalTrials.gov Identifier	Phase	Coordinator	Sponsor	Main Objective	Methodology
NCT00001353	I / II	Not available	National Institute of Allergy and Infectious Diseases (NIAID)	Adoptive transfer of genetically marked syngeneic lymphocytes in HIV-Infected Identical Twins.	T cells from each seronegative twin are obtained by periodic apheresis, induced to polyclonal proliferation with anti-CD3 and rIL-2 stimulation, transduced with distinctive neoR retroviral vectors, and expanded 10-1,000 fold during approximately 2 weeks of culture.
NCT00110578	I	Stanley Riddell	NIAID	Autologous $CD8^+$ HIV-specific CTLs combined with IL-2 for HIV seropositive individual.	CD8 cells are isolated from the blood of HIV infected patients and are allowed to multiply in the laboratory.
NCT00000756	I	J Lieberman, H Standiford, D Stein	NIAID	Autologous HIV-specific CTLs in HIV-Infected Patients.	*Ex vivo* expanded HIV-specific CTLs.
NCT00431210		Lori J. Wirth	Dana-Farber Cancer Institute	EBV-specific imunotherapy for nasopharyngeal carcinoma.	EBV immunotherapy product is prepared during 12 weeks from peripheral blood collected intravenously.
NCT00693095	I	Duane A Mitchell	John Sampson	CMV-autologous lymphocyte transfer (CMV-ALT) in patients with newly-diagnosed glioblastoma multiforme (GBM) with CMV-DC vaccines.	ALT with CMV pp65-activated T-cells from PBMC and autologous DC pulsed with pp65 RNA.
NCT00000875		Not available	NIAID	Autologous antiviral CTL infusion following combination antiretroviral drug therapy for asymptomatic HIV-1 infection.	*Ex vivo* expanded anti-HIV CTL.
NCT00779337	I	Maher K Gandhi	Queensland Institute of Medical Research	Autologous EBV-specific T cells as therapy for relapsed/refractory EBV-positive lymphomas.	AdE1-LMP-specific CTL expanded *ex vivo* from the blood of EBV-positive lymphoma patients.
NCT00000824		Not available	NIAID	Allogeneic HIV-specific CTLs for HIV-infected patients.	HIV-specific CTLs generated from HIV-negative sibling-supplied DCs and lymphocytes.
NCT00001064		Not available	NIAID	HIV-1 antigen pulsed allogenic DCs for HIV-infected asymptomatic patients.	DCs from an HIV-negative sibling are obtained and treated with various viral proteins (HIV vaccines) or immunomodulators.
NCT00058604	I	Helen E Heslop	Baylor College of Medicine	Autologous EBV-specific CTLs for prophylaxis and therapy of EBV lymphoma post solid organ transplant.	T cells are stimulated *in vitro* with EBV-LCLs (a EBV infected B cell line) made from patients blood.
NCT00002673	II	Stan Riddell	Fred Hutchinson Cancer Research Center	Adoptive immunotherapy as prophylaxis for CMV disease after allogeneic bone marrow or peripheral stem cell transplantation.	CD8+ CMV-specific CTL clones and CD4+ CMV-specific T-helper (Th)-cell clones harvested and cultured *in vitro* at least 2 weeks.
NCT00159055	I/II	Shimon Slavin	Hadassah Medical Organization	Prevention and treatment of CMV disease by adoptive immunotherapy with immune donor lymphocytes.	Donors are immunized with CMV-specific peptides for induction of CTLs *in vivo*, following subcutaneous inoculation of peptides with adjuvant or donor APC pulsed with relevant peptides.
NCT00402142	I/II	Felipe García	Hospital Clinic of Barcelona	Autologous myeloid DC as a therapeutic HIV-1 vaccine in early stage HIV-1 infected patients.	Monocyte-derived DCs pulsed *ex vivo* with high doses of heat-inactivated autologous HIV-1.
NCT00058591	I	Helen E Heslop	Baylor College of Medicine	Autologous EBV-specific CTLs for therapy of severe chronic EBV infection.	T cells grown from the patient's blood are stimulated with EBV infected cells.
NCT00006100	I	Kenneth G. Lucas	Milton S. Hershey Medical Center	Allogeneic EBV-specific T lymphocytes for the treatment of relapsed/refractory Hodgkin's disease.	EBV-specific CTLs are cultured *in vitro*.
NCT00590083	I	Catherine Bollard	Baylor College of Medicine	Allogeneic AV-specific CTLs for the prophylaxis and therapy of AV infection post allogeneic HSCT.	T lymphocytes are stimulated by monocytes transferred with AV, generating AV-specific T lymphocytes that are stimulated with AV-infected monocytes and B lymphoblasts from the donor.
NCT00058773	I	Catherine Bollard	Baylor College of Medicine	Autolotous EBV-specific CTLs as therapy in patients receiving autologous BMT for relapsed EBV-positive lymphoma.	Autologous EBV-LCL are used to stimulate T cells from patient's blood and thus generate EBV-specific T cell line.
NCT00058617	I	Catherine M Bollard	Baylor College of Medicine	Autologous EBV-specific CTLs in patients with relapsed EBV-positive lymphoma.	Autologous EBV-LCL are used to stimulate T cells from patient's blood and thus generate EBV-specific T cell line.
NCT00058812	I/II	Helen E Heslop	Baylor College of Medicine	Allogeneic EBV-specific CTLs in BMT recipients at high risk.	Autologous EBV-LCL are used to stimulate T cells from patient's blood and thus generate EBV-specific T cell line.

Identifier	Phase	Coordinator	Sponsor	Main Objective	Methodology
NCT00674648	I	Richard O'Reilly	Memorial Sloan-Kettering Cancer Center	Allogeneic T cells sensitized with pentadecapeptides of the CMV-pp65 protein for the treatment of CMV infections following allogeneic HSCT.	T-cells are grown *in vitro* and immunized against the CMV virus by sensitization with pentadecapeptides of the CMV-pp65.
NCT00796770	I	Jacques Banchereau	Baylor Research Institute	Autologous DC vaccination of HIV-1 infected patients in addition to antiretroviral treatment.	DCs generated *ex vivo* using GM-CSF and interferon-alpha (IFN-α) are loaded with HIV-1 lipopeptides and activated with lipopolysaccharide.
NCT00856154	I	Anders Fomsgaard	Statens Serum Institut	Vaccination with autologous DC pulsed with HIV-antigens in patients with chronic HIV-infection.	Macrophage-derived maturated DCs gnerated *in vitro* are pulsed with 10 HIV peptides.
NCT00002956	I	Kenneth G. Lucas	University of Alabama at Birmingham	Allogeneic EBV-specific CTLs for the treatment of EBV-associated lymphoproliferative diseases in organ transplant recipients.	EBV specific T lymphocytes cultivated *in vitro*.
NCT00510497	I/II	Sharon A Riddler	Sharon Riddler	Autologous HIV-1 ApB DC vaccine.	A therapeutic vaccine derived from autologous dendritic cells loaded with autologous inactivated HIV-1 infected apoptotic cells.
NCT00609219	I	Helen E. Heslop	Baylor College of Medicine	Autologous EBV-specific CTLs in patients with active nasopharyngeal carcinoma.	EBV-specific cytotoxic T-cell lines generated in the laboratory.
NCT00690872	II	Toh Han Chong	National Cancer Centre, Singapore	EBV-specific CTLs in patients with metastatic or locally recurrent EBV-positive nasopharyngeal carcinoma.	T cells purified from PBMCs are co-cultured with irradiated autologous EBV-specific CTLs and expanded in vitro for the establishment of cytotoxic T-cell lines.
NCT00110578	I	Stanley Riddell	NIAID	Autologous $CD8^+$ HIV-specific CTLs combined with IL-2 for HIV seropositive individuals.	CD8 cells isolated from the blood of HIV infected patients are expanded *in vitro*.
NCT00407836	II	Klaris Riesenber	Soroka University Medical Center	CD4-specific T cell vaccination for HIV infected patients.	The T cell vaccine are prepared from autologous T cells that responded by specific proliferation to recombinant CD4, further expanded *in vitro* by IL-2, and finally fixed by glutaraldehyde.
NCT00000680	I	M Ho, R Herberman, J Armstrong	NIAID	Autologous $CD8^+$ lymphocytes infused with or without recombinant IL-2 to HIV-infected patients receiving zidovudine (AZT).	Expanded and activated autologous $CD8^+$ lymphocytes are grown *in vitro* for 21 days.
NCT00509691	I	Kenneth G. Lucas	Milton S. Hershey Medical Center	CMV pp65 specific CTLs for recipients of allogeneic HSCT with persistent or therapy refractory infections.	CMV pp65-specific CTLs generated using pp65 peptides.
NCT00062868	I	Catherine Bollard	Baylor College of Medicine	Autologous or allogeneic LMP-specific CTLs in patients with relapsed EBV-positive Hodgkin's and non-Hodgkin lymphoma.	LMP-specific cytotoxic T-lymphocyte lines.
NCT00005606	II	Ann Traynor	Northwestern Univ ersity	EBV-specific CTLs for EBV-induced lymhoproliferative disease.	EBV reactive autologous and allogeneic lymphocyte clones generated *ex vivo*.
NCT00547235		Thomas Manley	Fred Hutchinson Cancer Research Center	CMV-specific T cells after donor BMT of an infant with immunodeficiency syndrome and CMV infection.	$CD8^+$ and $CD4^+$ CMV-specific T cells collected from the donor are used to generate T-cell lines.
NCT01274377	I	Nelson Chao	Nelson Chao	CMV Specific T cell infusion following nonmyeloablative allogeneic HSCT.	Specific T cell clones.
NCT00078546	I	Stephen Gottschalk	Baylor College of Medicine	Autologous EBV-Specific CTLs after anti-CD45 monoclonal antibody in patients with EBV-positive nasopharyngeal carcinoma.	EBV-specific CTL lines are generated by co-cultivation of irradiated EBV-LCL with patients PBMCs.
NCT00611637	I	H. Kim Lyerly, Michael A Morse	Duke University	Allogeneic CMV-specific T cells in patients who have undergone allogeneic HSCT for malignant disease.	PBMC from CMV-seropositive donors were cultured CMV pp65(495-503) peptide for the CMV pp65 specific T cell generation.
NCT00769613	I	Kenneth G. Lucas	Milton S. Hershey Medical Center	CMV pp65 and IE-1 specific CTLs for recipients of allogeneic HSCT with persistent CMV infections.	CMV pp65- and IE-1 specific CTL culture by CTL stimulation with peptide mixes for pp65 and IE-1.
NCT01570283	I/II	Helen E Heslop	Baylor College of Medicine	Administration of donor-derived multi-virus-specific CTLs in patients at risk of developing CMV, AV, EBV, HHV6, or BK virus infections post allogeneic HSCT.	Donor cells are stimulated with peptides from AV, CMV, EBV, BKV, and HHV6, to rapidly generate the multi-virus-specific CTLs (mCTLs).

Identifier	Phase	Coordinator	Sponsor	Main Objective	Methodology
NCT01498484	II	Richard O'Reilly	Memorial Sloan-Kettering Cancer Center	EBV immune T-lymphocytes derived from third-party donor in the treatment of EBV lymphoproliferative disorders and EBV-associated malignancies.	HLA restricted EBV-specific CTLs expanded from the donor.
NCT01447056	I	Carlos A Ramos	Baylor College of Medicine	HLA-matched allogeneic LMP1/2-specific CTLs for treatment of patients with relapsed EBV-associated diseases.	LMP specific CTL generation by stimulation of donor cells with DCs or monocytes with AV that carries the LMP gene.
NCT00953420	II	Chrystal U Louis	Baylor College of Medicine	Carboplation and docetaxel treatments followed by EBV-CTLs in patients with refractory/relapsed EBV-positive nasopharyngeal carcinoma.	EBV-CTLs grown in the laboratory.
NCT01555892	I	Helen E Heslop, Cliona Rooney	Baylor College of Medicine	Rapidly generated LMP, BARF1 and EBNA1 specific CTLs to patients with EBV-positive lymphoma	LMP/BARF1/EBNA1-specific cytotoxic T-lymphocyte lines.
NCT01646645	II	Richard O'Reilly	Memorial Sloan-Kettering Cancer Center	Primary transplant or third party donor derived CMVpp65 specific T-cells for the treatment of CMV infection or persistent CMV viremia after allogeneic HSCT.	Pp65-CTLs are grown *in vitro*, from blood of CMV seropositive donors.
NCT00706316	I	Lillian Siu	University Health Network, Toronto	EBV-specific CTLs to patients with recurrent or metastatic EBV-positive nasopharyngeal cancer (NPC)	EBV-specific CTL lines

Table S3. Clinical trials performed using CARs, TCR or therapeutic-gene transference using viral vectors (http://ClinicalTrials.gov/) for the simultaneous treatment or viral infections and oncologic diseases.

ClinicalTrials.gov Identifier	Phase	Coordinator	Sponsor	Main Objective	Methodology
NCT00840853	I/II	Catherine Bollard	Baylor College of Medicine	Allogeneic multi-virus-specific cytotoxic T lymphocytes (CTLs) expressing CD19 chimeric receptors for prophylaxis or therapy of relapse of CD19 positive malignancies post HSCT.	CMV, Epstein-Barr virus (EBV) and AV (tri-virus) specific CTLs genetically modified to express artificial T-cell receptors (CAR) targeting the CD19 molecule (CD19CAR).
NCT01460901	I	Doug Myers	Children's Mercy Hospital Kansas City	Treatment post-allogeneic HSCT of patients with relapsed/refractory neuroblastoma expressing the mesenchymal tumor marker GD2.	Tumor redirected, multi-virus specific cytotoxic T-cells (tV-CTL), retrovirally transduced to express a chimeric antigen receptor (CAR) specific for disialoganglioside, GD2, expressed on neuroblastoma.
NCT00085930	I	Malcolm K Brenner	Baylor College of Medicine	Peripheral blood T-cells and EBV-specific CTLs transduced to express GD-2 specific chimeric T cell receptors to patients with neuroblastoma.	Peripheral blood CTLs and EBV-specific cytotoxic CTLs, both transduced with retrovirus to express the 14g2a.zeta chimeric receptor (GD-2 specific chimeric T cell receptor). CTLs are generated by co-cultivation of EBV transformed lymphoblastoid cell line (EBV-LCL) with patien peripheral blood mononuclear cells (PBMCs).
NCT00709033	I	Carlos Ramos	Baylor College of Medicine	Autologous peripheral activated T-Cells or EBV Specific CTLs expressing CD19 chimeric receptors for advanced B-cell Non-Hodgkin's lymphoma and Chronic Lymphocytic Leukemia.	Peripheral blood CTLs and EBV-specific cytotoxic CTLs (generated by stimulation of CTLs with EBV-LCLs), both genetically modified to express artificial T-cell receptors targeting the CD19 molecule (CD19CAR).
NCT01109095	I	Nabil Ahmed	Baylor College of Medicine	CMV-specific CTL expressing CARs targeting HER2 in patients with HER2-positive Glioblastoma Multiforme (GBM).	CMV-specific CTLs genetically modified to express CAR targeting the HER2 molecule (HER2.CAR CMV-specific CTLs).
NCT00889954	I	Stepehen Gottschalk	Baylor College of Medicine	Autologous TGFBeta-resistant CTLs directed to EBV through their native receptor and HER2 through their CAR in patients with advanced HER2-positive lung tumors.	EBV CTLs are manufactured using autologous EBV-LCL as stimulator cells and the patients' peripheral blood cells as responder cells. These EBV-specific T cells are transduced with vectors that encode TGFBeta dominant negative receptor (DNR) or the HER2 CAR, and expanded *ex vivo* on LCLs and stimulated with IL-2.
NCT01195480	I/II	Persis Amrolia	University College, London	CD19ζ gene-modified allogeneic EBV-specific CTLs after HSCT in children with high-risk acute lymphoblastic leukaemia.	Donor-derived EBV-specific cytotoxic T-cells, generated by EBV-LCL stimulation, transduced with the retroviral vector SFGalpha-CD19-CD3zeta. In some cases, an additional vaccine of CD19-zeta-transduced irradiated EBV-LCL will be administered.
NCT01430390	I	Nancy Kernan	Memorial Sloan-Kettering Cancer Center	EBV-specific cytotoxic T lymphocytes (EBV-CTLs) genetically targeted to the B-cell antigen CD19 for patients with CD19+ acute lymphoblastic leukemia after allogeneic HSCT.	EBV-specific CTLs expanded *in vitro* and genetically modified to target the B-cell antigen CD19.
NCT01192464	I	Helen E Heslop	Baylor College of Medicine	Autologous EBV CTLs expressing CD30 chimeric receptors for relapsed $CD30^+$ Hodgkin's Lymphoma and $CD30^+$ Non-Hodgkin's Lymphoma (CAR CD 30).	EBV-specific CTLs expressing a CAR targeted to the CD30 antigen (CAR.CD30).
NCT01013415	I	Naomi Aronson	University of Pennsylvania	Autologous CD4-zeta gene modified T cells and/or recombinant interleukin-2 (IL-2) for anti-HIV treatment.	Introduction of a gene for HLA-unrestricted "universal" receptors specific for the gp120 HIV envelope protein, the CD4-zeta gene.
NCT00991224	I	Pablo Tebas	University of Pennsylvania	Autologous T cells genetically modified for the treatment of HIV.	T cells modified with lentiviral vectors expressing high affinity gag-specific TCRs, WT-gag-TCR and α/6-gag-TCR transduced T cells.
NCT00131560	II	Tessio Rebello	VIRxSYS Corporation	Autologous CD4 modified T cells for anti-HIV treatment.	CD4 T cells modified with the vector VRX496, a viral vector expressing a genetic antisense targeting HIV.
NCT00622232	II	Tessio E Rebello	VIRxSYS Corporation	VRX496-transduced autologous CD4 T cells for HIV treatment.	CD4 T cells modified with the vector VRX496. The study has concluded its 9-month active phase (NCT00131560).
NCT01070797	I/II	Helen E Heslop	Baylor College of Medicine	Rapidly generated multivirus-specific CTLs for the prophylaxis and treatment of EBV, CMV, and AV infection post-allogeneic HSCT.	Trivirus specific CTL lines generated using using plasmid-nucleofected DCs. DNA plasmids express protective and immunogenic viral antigens of EBV (EBNA1, LMP2, and BZLF1), CMV (pp65) and AV (hexon and penton). Nucleofected DCs are used to stimulate virus specific T cells from the PBMC.
NCT00295477	I/II	Pablo Tebas	University of Pennsylvania	Autologous T cells transduced with VRX496 for HIV infected subjects.	T cells are transduced *ex vivo* with the VRX496 vector.

Identifier	Phase	Coordinator	Sponsor	Main Objective	Methodology
NCT00672191	II	JP Routy, M Loutfy, C Tremblay, J Gill, JG Baril, S Vezina, JB Angel, S Walmsley, F Smaill, A Rachlis, J Montaner, J Jacobson	Argos Therapeutics	AGS-004 therapy antiretroviral therapy (ART)-Treated Subjects Infected With HIV.	AGS-004 is an immunotherapeutic agent made from autologous DCs co electroporated with amplified *in vitro* transcribed RNA encoding CD40L and three or four autologous HIV-1 antigens. The HIV-1 RNA is derived from the plasma sample taken immediately prior to the initiation of ART.
NCT00295477	I/II	Pablo Tebas	University of Pennsylvania	Autologous T cells transduced with VRX496 for HIV infected subjects.	T cells are transduced *ex vivo* with the VRX496 vector.
NCT00711035	I	Helen E Heslop	Baylor College of Medicine	Most closely HLA-matched allogeneic multivirus specific CTL lines (CHM-CTLs) to treat persistent reactivation or infection with AV, CMV and EBV after HSCT.	To initiate the trivirus-specific CTL line, PBMC are transduced with an adenovirus vector (Ad5f35-pp65). The monocyte fraction of PBMC will express CMV-pp65 peptide epitopes and the virion proteins from the AV vector and present them to the virus-specific CTLs. These trivirus-specific T cells are expanded with EBV-LCLs transduced with Ad5f35-pp65, which presents CMV-pp65 and AV virion peptides to the T cells as well as endogenously expresses EBV antigens.
NCT00070226	I	Helen E Heslop	Baylor College of Medicine	Autologous LMP2A-specific CTLs in patients with relapsed EBV positive lymphoma.	Dendritic Cells (DCs) generated from the patient blood are transduced with recombinant AdLMP2A and matured with GM-CSF, TNFa, PGE-1, and IL-4 over 2 days to stimulate cytotoxic T-lymphocytes (CTL).
NCT00368082	I	Catherine M Bollard	Baylor College of Medicine	Autologous or allogeneic TGFbeta-resistant LMP2A-specific CTLs in patients with relapsed Hodgkin's or non-Hodgkin's lymphoma.	The *in vitro* expanded LMP-specific CTL are transduced with a retrovirus vector expressing the dominant TGFb receptor II (DNRII).
NCT00001409	I	Not available	NIAID	Adoptive transfer of syngeneic gene-modified CTLs in HIV infected identical twins.	Introduction of a gene for HLA-unrestricted "universal" receptors specific for the gp120 HIV envelope protein, the CD4-zeta gene. These genetically modified T-cells are expanded *ex vivo*.
NCT01205334	I/II	Nabil H Ahmed	Baylor College of Medicine	Autologous CMV-specific T cells for patients with GBM.	Patient’s blood DCs are transduced with a specially produced adenovirus that carries one CMV gene. These cells are used to generate the CMV-specific cells, that are grown by more stimulations with Epstein-Barr virus (EBV) infected cells from the patient's blood, which also contain the adenovirus with the CMV gene.
NCT00516087	I		Baylor College of Medicine	LMP1- and LMP2-specific CTLs to patients with EBV-positive nasopharyngeal carcinoma.	Irradiated DCs transduced with an AV that carries the LMP1 and LMP2 gene are used to stimulate the T cells and produce the LMP1- and LMP2-specific CTLs. The specific CTLs are grown by more stimulation with irradiated EBV infected cells also transduced with AV that carries the specific genes.
NCT00082225	I	Catherine M Bollard	Baylor College of Medicine	LMP2a-specific CTLs following CD45 antibody to patients with relapsed EBV-positive Hodgkin’s or non-Hodgkin’s lymphoma	Irradiated DCs transduced with an AV that carries the LMP2a gene are used to stimulate the T cells and produce the LMP2a-specific CTLs. The specific CTLs are grown by more stimulation with irradiated EBV infected cells also transduced with AV that carries the LMP2 gene.
NCT00880789	I	Catherine Bollard	Baylor College of Medicine	Allogeneic CTLs specific for CMV and AV given to patients with or at risk for CMV and AV disease after cord blood (CB) transplant.	T cells specific for adenovirus and CMV are reactivated *in vitro* from CB by a single stimulation with DC transduced with the Ad5f35pp65 vector, followed by 1-2 stimulations with EBV-LCL transduced with the same vector.

Identifier	Phase	Coordinator	Sponsor	Main Objective	Methodology
NCT01153646	0	John A. Zaia	City of Hope Medical Center	T-Cell immunotherapy using lentivirus vector-expressed RNAi in autologous T-cells of HIV-1 infected patients who have failed anti-retroviral therapy.	The lentivirus vector induces 3 forms of anti-HIV RNA: RNAi in the form of a short hairpin RNA (shRNA) targeted to an exon in HIV-1 tat/rev (shI), a decoy for HIV TAT-activated RNA (TAR), and a ribozyme that targets the host T cell CCR5 cytokine receptor (CCR5RZ). The vector is called rHIV7-shI-TAR-CCR5RZ and is used in the transduction and expansion of CD4-enriched T cells.
NCT00078533	I	Catherine M Bollard	Baylor College of Medicine	Virus specific CTLs for the treatment of CMV after allogeneic HSCT.	Stimulation of PBMCs with monocytes or DCs with AV that carries part of the CMV gene (pp65) and *ex vivo* expansion of the generated CMV-specific CTLs.
NCT00001535	I	Not available	National Human Genome Research Institute (NHGRI)	Activated, genetically engineered, syngeneic CD4+ T lymphocytes obtained from HIV-1 seronegative identical twins and then will be infused into the seropositive twin.	Lymphocytes transduced with retroviral vectors containing HIV-1 antisense TAR and transdominant Rev protein genes. These engineered T cell populations will be expanded during 1-2 weeks of culture.
NCT01640301	I/II	Merav Bar	Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium	Adoptive immunotherapy after allogeneic HSCT with virus specific CD8+ T cells transduced to express Wilms Tumor (WT1)-specific TCR, for patients with high risk or relapsed AML, MDS, or CML.	Virus-specific CD8+ T cells genetically-modified to express a high affinity WT1-specific TCR (WT1-sensitized T cells).
NCT01475058	I/II	Cameron Turtle	Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium	Cellular immunotherapy with donor derived virus-specific CD8+ T-cells engineered to target CD19 for CD19+ malignancies after allogeneic HSCT at risk post-HCT relapse.	*Ex vivo* expanded CMV- or EBV-specific T cells derived from donor CD62L+ central memory T cells and genetically modified to express a CD19-specific CAR

Table S4. Clinical trials performed using multimeric complexes or gamma-capture techniques (http://ClinicalTrials.gov/ or https://drks-neu.uniklinik-reiburg.de/drks_web/).

Identifier	Phase	Coordinator	Sponsor	Main Objective	Methodology
NCT01077908	III	Karl S Peggs	Cell Medica Ltd	Prophylactic CMV-specific adoptive cellular therapy following T cell depleted allogeneic HSCT for reducing recurrent CMV reactivation.	Cells selected by either the Gamma Catch or Multimer Selection techniques.
NCT01220895	II	Karl S Peggs	Cell Medica Ltd	Pre-emptive cytomegalovirus (CMV)-specific adoptive cellular therapy following T cell depleted allogeneic hematopoietic stem cell transplantation (HSCT) for reducing recurrent CMV reactivation. Based on the earlier study NCT01077908	Multimer Selection techniques.
NCT00986557	II	Frederick Chen	University Hospital Birmingham	CMV-specific CTL after allogeneic HSCT in Patients at Risk of CMV Disease.	Selected CMV-specific CTLs. *In vitro*-treated peripheral blood lymphocyte therapy.
DRKS00000205	I/II	Götz-Ulrich Grigoleit	Stage Pharmaceuticals GmbH	CMV specific T cells for treatment of CMV infection refractory to antiviral chemotherapy in patients after allogeneic bone marrow or peripheral blood stem cell transplantation	CMV-specific T cells selected with the Streptamer technology.
NCT01325636	I/II	Marina Cavazzana-Calvo	Assistance Publique - Hôpitaux de Paris	$CD4^+$ and $CD8^+$ T Cells anti-CMV or anti-Adenovirus for the treatment of viral infections occurring after allogenic HSCT.	Donor memory T cells selected on the basis of their ability to produce interferon-gamma (IFN-g) after stimulation with viral peptides.

Isolation of Highly Suppressive CD25+FoxP3+ T Regulatory Cells from G-CSF-Mobilized Donors with Retention of Cytotoxic Anti-Viral CTLs: Application for Multi-Functional Immunotherapy Post Stem Cell Transplantation

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Isolation of Highly Suppressive CD25+FoxP3+ T Regulatory Cells from G-CSF-Mobilized Donors with Retention of Cytotoxic Anti-Viral CTLs: Application for Multi-Functional Immunotherapy Post Stem Cell Transplantation

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Abstract

Previous studies have demonstrated the effective control of cytomegalovirus (CMV) infections post haematopoietic stem cell transplant through the adoptive transfer of donor derived CMV-specific T cells (CMV-T). Strategies for manufacturing CMV immunotherapies has involved a second leukapheresis or blood draw from the donor, which in the unrelated donor setting is not always possible. We have investigated the feasibility of using an aliquot of the original G-CSF-mobilized graft as a starting material for manufacture of CMV-T and examined the activation marker CD25 as a targeted approach for identification and isolation following CMVpp65 peptide stimulation. CD25+ cells isolated from G-CSF-mobilized apheresis revealed a significant increase in the proportion of FoxP3 expression when compared with conventional non-mobilized CD25+ cells and showed a superior suppressive capacity in a T cell proliferation assay, demonstrating the emergence of a population of Tregs not present in non-mobilized apheresis collections. The expansion of CD25+ CMV-T in short-term culture resulted in a mixed population of CD4+ and CD8+ T cells with CMV-specificity that secreted cytotoxic effector molecules and lysed CMVpp65 peptide-loaded phytohaemagglutinin-stimulated blasts. Furthermore CD25 expanded cells retained their suppressive capacity but did not maintain FoxP3 expression or secrete IL-10. In summary our data indicates that CD25 enrichment post CMV stimulation in G-CSF-mobilized PBMCs results in the simultaneous generation of both a functional population of anti-viral T cells and Tregs thus illustrating a potential single therapeutic strategy for the treatment of both GvHD and CMV reactivation following allogeneic haematopoietic stem cell transplantation. The use of G-CSF-mobilized cells as a starting material for cell therapy manufacture represents a feasible approach to alleviating the many problems incurred with successive donations and procurement of cells from unrelated donors. This approach may therefore simplify the clinical application of adoptive immunotherapy and broaden the approach for manufacturing multi-functional T cells.

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Introduction

Cytomegalovirus (CMV) reactivation continues to be a significant cause of morbidity and mortality following allogeneic haematopoietic stem cell transplantation (aHSCT) [1,2] with the incidence of CMV disease being reported to be as high as 70% [3]. Several strategies have been employed in the manufacture of donor-derived CMV-specific T cells (CMV-T) for adoptive transfer over the last two decades that have successfully demonstrated both safety and efficacy in restoring antiviral immunity [4–10]. More recently the direct selection of γ-secreting (IFN-γ) cells in response to CMVpp65 peptide stimulation [11,12] has simplified generation of CMV-T, significantly reduced the manufacturing time and has also been successfully used to select T cells specific for adenovirus (AdV) and Epstein Barr virus (EBV) for clinical use [13,14]. Isolation of antigen-specific T cells through the identification of activation markers that are up-regulated after T cell activation is also a promising alternative. T cell activation markers offer an increased sensitivity over approaches such as IFN-γ secretion as they are independent of cytokine secretion and therefore could allow the isolation of increased numbers of antigen-specific T cells. Several T cell activation markers have

been identified including CD25, CD69, CD137 and CD154 [15–20] with differing temporal dynamics that allow for simultaneous detection and enrichment. The availability of good manufacturing practice (GMP) compliant CD25 antibodies for clinical use makes the selection of CMV-T through CD25 selection a feasible option. Indeed several groups have investigated CD25 in large scale clinical manufacture for potential use in adoptive transfer [21,22] due to the commercial availability of CD25 reagents.

To date models for CMV-T manufacture have focussed primarily on using peripheral blood mononuclear cells (PBMCs) collected by leukapheresis from the original HSCT donor. The procurement of an additional apheresis for CMV-T manufacture is associated with some degree of difficulty especially in the unrelated donor setting where donor refusal, registry refusal and scheduling difficulties can prevent collection. The prospect of manufacturing antigen-specific T cells from an aliquot of the original HSCT obtained by leukapheresis after mobilization by recombinant human granulocyte-colony stimulating factor (G-CSF) as an alternative PBMCs source is attractive. Murine and human studies have suggested that G-CSF-mobilization inhibits type 1 cytokine production by T cells, through inhibition of secretion at a single cell level as well as reducing the fraction of cytokine-secreting cells in the periphery, arguing against the use of these cells for adoptive immunotherapy [23–27]. Furthermore extensive gene expression profiling in G-CSF-mobilized PBMCs has revealed the up-regulation of genes related to T helper cells type 2 (TH_2) and Treg cells and down-regulation of genes associated with T helper cells type 1 (TH_1), cytotoxicity, antigen presentation and GvHD [28]. However there has been little published data with regard to the effect of G-CSF on the anti-viral T cell response and the influence of G-CSF in this regard, beyond the time of apheresis. The clinical use of G-CSF-mobilized donor lymphocytes administered for therapy of relapse post aHSCT in acute myeloid leukaemia (AML) has demonstrated efficacy with a similar graft versus leukaemia (GvL) response when compared with conventional non-mobilized donor lymphocytes [29,30]. These results alleviate some of the major concerns surrounding the feasibility of using G-CSF-mobilized lymphocytes as a starting material for the manufacture of anti-viral immunotherapies. We have published our previous findings that demonstrated the feasibility of employing the use of G-CSF-mobilized PBMCs as an effective strategy for manufacture of CMV-T with the retention of

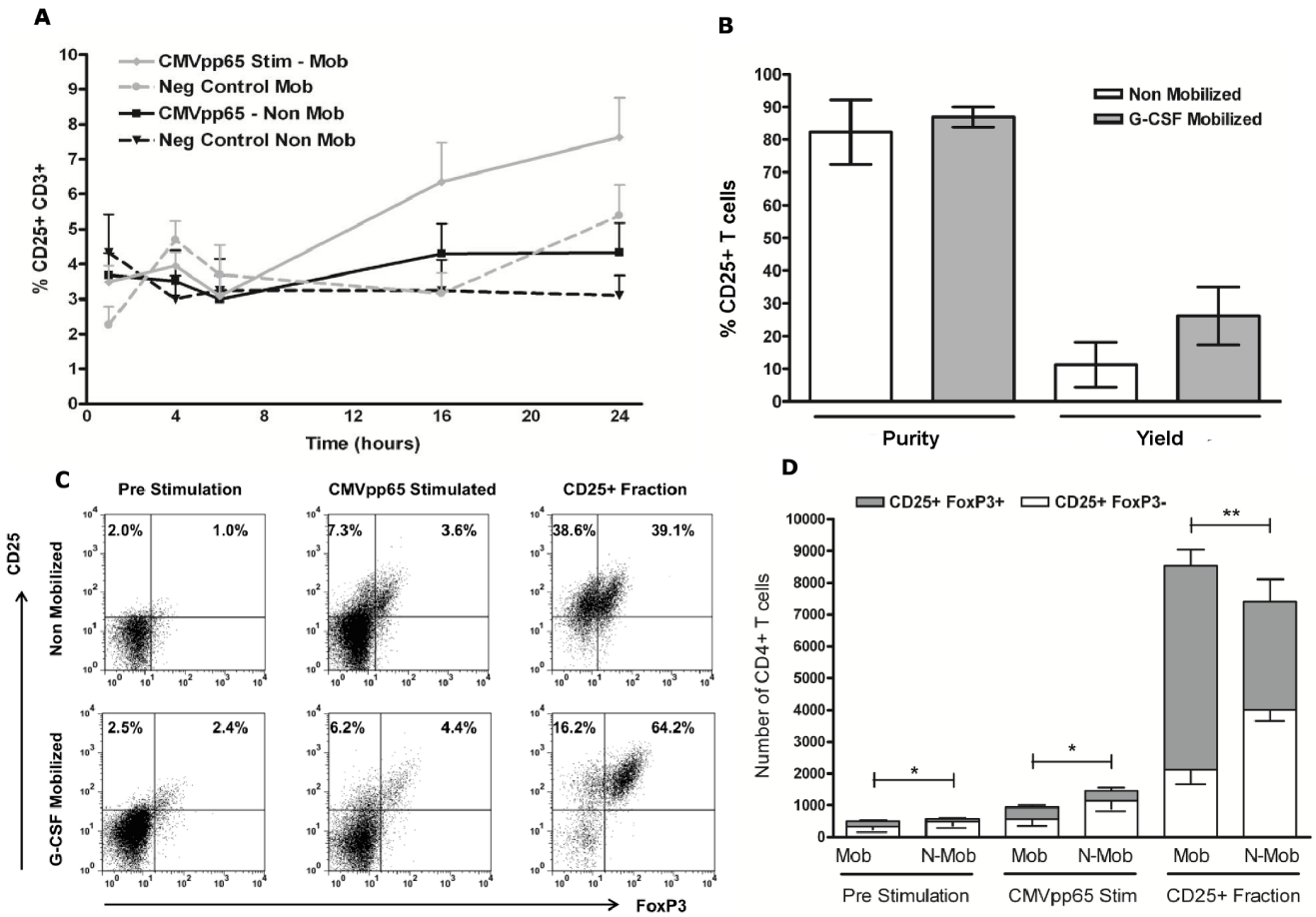

Figure 1. Identification and Isolation of CD25+ CMVpp65-specific T cells in G-CSF-mobilized and non-mobilized PBMCs. G-CSF-mobilized ($n = 6$) and non-mobilized PBMCs ($n = 5$) were stimulated for 24 hours or left untouched (negative control) and samples taken at 1, 4, 6, 16 and 24 hours for analysis of CD25 expression amongst CD3+ T cells (A). Purity and yield of CD25 expressing cells was determined from within the CD3+ population following selection (B). Evaluation of FoxP3 expression in CD4+ CD25+ T cells in G-CSF-mobilized and non-mobilized PBMCs was assessed in pre-stimulated, CMVpp65 stimulated and post CD25+ selection after gating on CD3+ CD4+ T cells (C). Cumulative data of FoxP3 expression was assessed by analysing CD25+FoxP3+ and CD25+FoxP3− populations per 10,000 CD4+ T cells in both G-CSF-mobilized and non-mobilized PBMCs (D). $^{**}p<0.01$ and $^{*}p<0.05$ in an unpaired t test comparing the absolute number of CD25+FoxP3+ cells. Stim, stimulated; Mob, mobilized; N-Mob, non-mobilized; Neg, negative.
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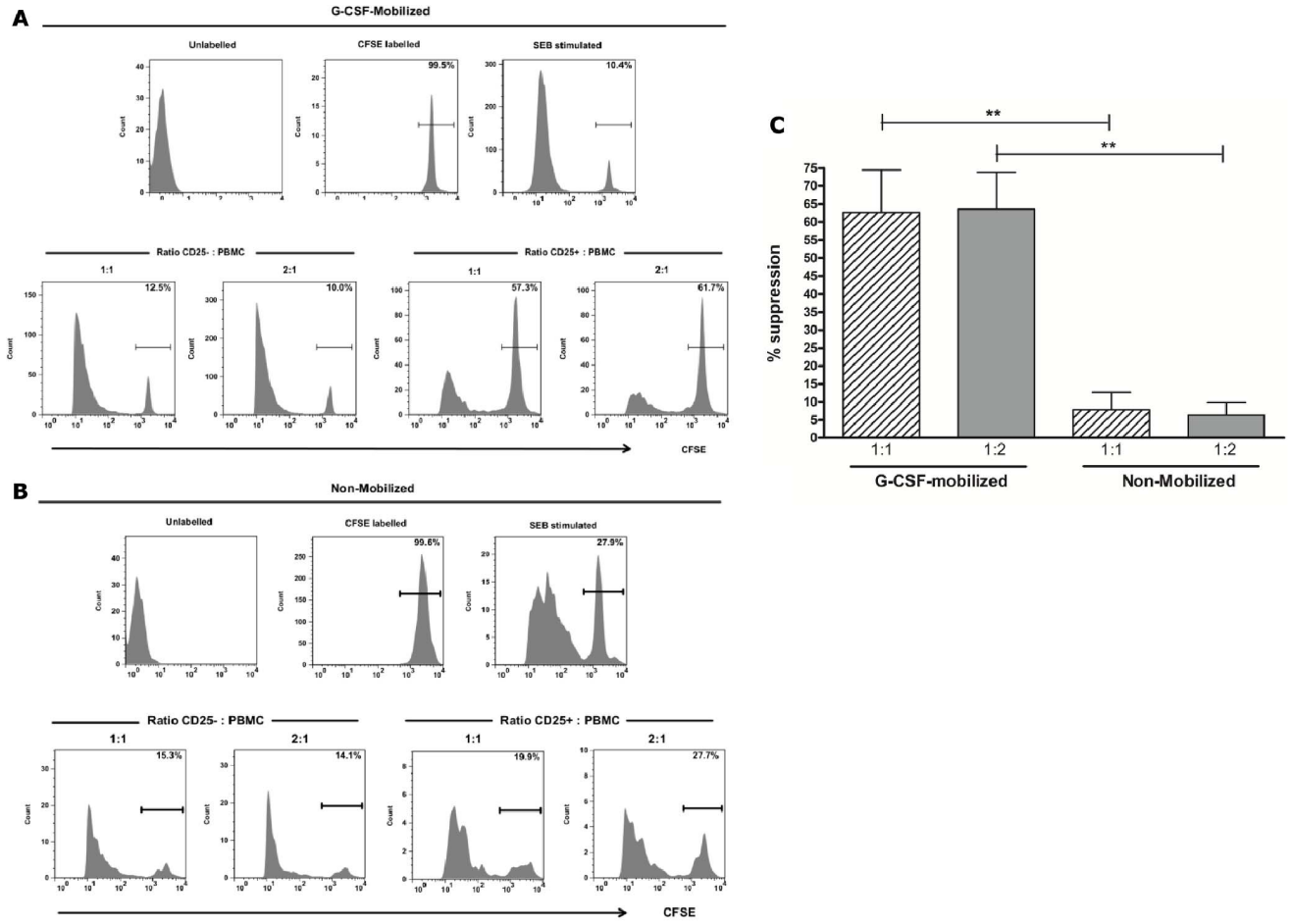

Figure 2. Suppression of autologous PBMCs by CMV-stimulated CD25+ cells isolated following CMVpp65 stimulation. Suppressive capacity was assessed in CD25-positive and CD25-negative fractions after magnetic selection following CMVpp65 stimulation between G-CSF-mobilized (A) and non-mobilized (B) donors. CFSE-labelled PBMCs were cultured at two ratios in the presence of SEB for 5 days. Cumulative data in G-CSF-mobilized ($n = 5$) and non-mobilized ($n = 5$) donors are shown at a ratio of 1:1 (diagonal bars) and 1:2 (shaded bars) PBMCs to CD25+ selected T cells after calculating percentage of suppression as indicated in the Material and Methods section. Data are presented as mean values with standard deviation (C). Unlabelled, PBMCs alone (CFSE) and PBMCs with SEB were cultured as experimental controls. $^{**}p<0.01$, in an unpaired t test.
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functional CMV-specific cytotoxicity comparable with non-mobilized PBMCs, using CD154 based selection [31]. But the translation of this particular approach is currently restricted by the non-availability of a GMP-compliant anti-CD154 antibody. More recently the generation and adoptive transfer of CMV-T cell lines expanded from G-CSF-mobilized PBMCs in short term culture has been demonstrated in a phase I/II clinical trial, where seven patients received CMV-T cell products [32]. However, the widespread application of short-term culture manufacturing processes can be complex to translate into cellular therapy laboratories, from a both a regulatory and GMP compliant perspective.

In this study we explore the feasibility of using CD25-based enrichment of CMV-T from G-CSF-mobilized PBMCs due to the relative simplicity and rapidity of current direct selection methods. Identification of antigen-specific T cells through CD25 expression can be confounded by the similar CD25 expression pattern exhibited by regulatory T cells (Tregs). Tregs are a subset of CD4+ T cells that are suppressive in nature and regulate responses towards tumour, foreign and allo-antigens that constitutively express CD25 [33,34]. The transcription factor forkhead box P3 (FoxP3) has also been identified to play a role in the development and function of Tregs and is also used as a phenotypic marker of Tregs [35]. Ukena and colleagues [36] in their studies report the application of G-CSF resulted in a significant increase in Treg yield and that cells retain a cytokine profile and phenotype associated with Treg characteristics and remained highly suppressive. Previous reports in non-mobilized PBMCs have identified populations of both antigen-specific T cells and Tregs following CD25 enrichment and argue that the adoptive transfer of both these populations in a single immunotherapy product could prove beneficial to recipients of aHSCT who are at risk of both CMV reactivation and GvHD [21].

We have therefore investigated the phenotype and function of CMV-T isolated through CD25 expression from G-CSF-mobilized PBMCs with regard to FoxP3 expression and suppressive capacity and explored the impact of removal of CD4+ CD25+ Tregs from starting populations to augment selectivity of antigen-specific T cells for adoptive immunotherapy. CD25+ CMV-T were expanded in short term culture and subsequently analysed for both CMV-specificity through cytokine profiling and cytotoxicity together with suppressive function and FoxP3 expression to ascertain the retention of Tregs.

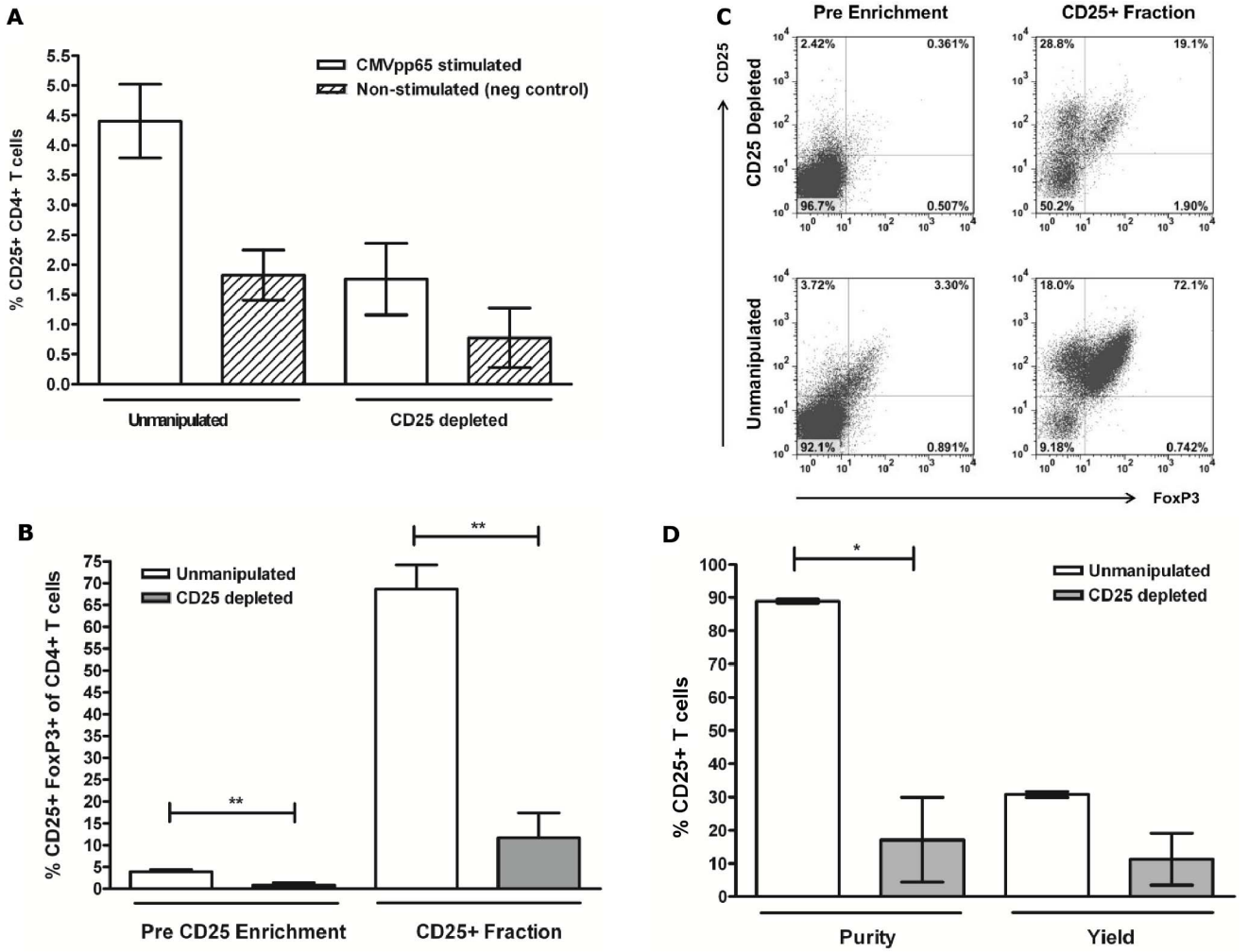

Figure 3. Effect of CD25 immunomagnetic depletion on CD25+ CMVpp65 CMV-T and CD25+ FoxP3+ Tregs. CD25 expression was assessed in paired unmanipulated and CD25-depleted G-CSF-mobilized PBMCs following CMVpp65 peptide stimulation (A). FoxP3 expression was analysed both pre and post CD25-enrichment amongst CD4+ T cells (B–D) and CD25 purity and yield determined following CD25 enrichment (D). $^{**}p<0.01$ and $^{*}p<0.05$ in a paired *t* test. Neg, negative.
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Materials and Methods

Blood Donors and Cell Preparation

Prior to sample collection ethical approval was given by the National Health Service (NHS) Health Research Authority (North West London Research Ethics Committee) and permission obtained from the institutional research and development office of Royal Free London Foundation Trust. After obtaining signed written informed consent, fresh blood samples were taken from both G-CSF-mobilized and non-mobilized CMV-seropositive healthy donors, after a 3–5 hour collection on the COBE Spectra apheresis system (Caridian BCT, Lakewood, CA). PBMCs were isolated from all donor samples after density gradient separation using Lymphoprep (Axis Shield Diagnostics, Dundee, UK) and subsequently cultured in RPMI 1640 Medium supplemented with 1% antibiotic (Both Life Technologies, Paisley, UK) and 10% heat inactivated human AB serum (Biosera, Ringmer, UK) at a concentration of 1×10^7/ml. PBMCs were stimulated for up to 24 hours in flat bottom 6 well culture plates (NUNC, Roskilde, Denmark) with 1 μg/peptide/ml of CMVpp65 peptide pool spanning the entire CMVpp65 protein (CMVpp65 Peptivator. Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated at 37°C/5% CO_2. In some experiments freshly isolated PBMCs were cryopreserved at 1:1 with human serum albumin (HSA) 4.5% (Bio Products Laboratory Ltd, Elstree, UK) containing 20% DMSO (WakChemie, Steinbach, Germany) according to standard protocols, for use in future experiments as well as a source of feeder cells.

Flow Cytometric Analysis

Flow cytometry experiments consisted of four to six colour panels where a minimum of 50,000 CD3+ events were acquired after gating of viable lymphocytes using FSC and SSC signals on a FACScan flow cytometer (Cytek UK) and data analysed using FlowJo version 7.6 (TreeStar Inc. Ashland, OR). For control staining of cytokines and activation markers we used PE-conjugated mouse antibodies of matching isotype and supplier of the retrospective antibodies. Cells were stained for 15 minutes in the dark, washed in 2 ml of HBSS for 5 minutes and resuspended in 200 μl of FACS Flow (BD Bioscience, Franklin Lakes, NJ) before acquisition.

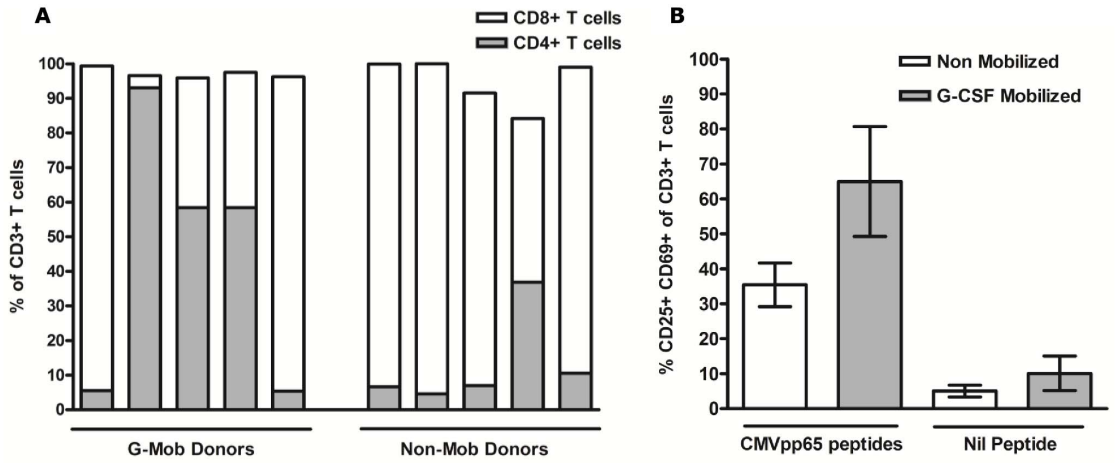

Figure 4. Expansion of CD25+ CMVpp65-specific T cells in short term culture. 0.25×10^6 CD25+ T cells were expanded in culture for up to 24 days in the presence of IL-7, IL-15 and 12.5×10^6 irradiated autologous feeder cells (ratio of 1:50) in G-CSF-mobilized and non-mobilized donors. CD25+ expanded cells were analysed for the proportion of CD8+ (open bars) and CD4+ (grey bars) T cells amongst CD3+ T cells in both G-CSF-mobilized and non-mobilized donors (A). Expanded cells were assessed for co-expression of CD25 and CD69 following CMVpp65 re-challenge in both G-CSF-mobilized ($n=5$) and non-mobilized ($n=5$) expansions (B). Expanded cells were also challenged with control feeders (without CMVpp65 peptides). G-Mob, G-CSF-Mobilized; Non-mob, Non-Mobilized.
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Time Course Assay of Activation Marker Kinetics

PBMCs isolated from mobilized and non-mobilized donors were stimulated in 96 well plates at a concentration of 1×10^7/ml for 24 hours with either CMVpp65 Peptivator or 1 μg/ml of Staphylococcal Enterotoxin B (SEB. Sigma-Aldrich, Gillingham, UK) or left untouched. Samples were taken at 1, 4, 6, 16 and 24 hours and stained with APC-conjugated anti-CD3, FITC-conjugated anti-CD4, PerCP-conjugated anti-CD8 and PE-conjugated anti-CD25 (BD Bioscience).

Separation Using Anti-CD25-Microbeads

CD25-Microbeads (Miltenyi Biotec) were used for both the isolation and depletion of CD25+ cells dependent upon the experiment being performed. For the isolation of antigen-specific T cells following CMVpp65 stimulation, cells were labelled with CD25-microbeads after 16 hour incubation. Labelling was performed for 15 minutes using 10 μl of microbeads per 10^7 cells in 90 μl of CliniMACS buffer. Following incubation and washing the cell suspension was enriched using MS columns on a MiniMACS (all Miltenyi Biotec). For CD25 depletion experiments cells were labelled using 20 μl of microbeads per 10^7 cells in 80 μl of CliniMACS buffer. Depletions were performed using LD columns and sorted on a VarioMACS separator (both Miltenyi Biotec). All incubation steps were performed at 4–8°C in the dark. Both enriched and depleted cells were stained with CD3-APC, CD4-FITC, CD8-PerCP, CD69-APC Cy7 (All BD Bioscience) and CD25-PE (Miltenyi Biotec).

Identification of T Regulatory Cells by FoxP3 Staining

1×10^6 PBMCs from pre stimulation, CMVpp65 stimulated and CD25 positive fractions were stained with CD3-APC, CD25-PE, CD4-APC Cy7 and CD8-PerCP then fixed and permeabilized using the FoxP3 staining kit (BD Bioscience) before staining with either FoxP3-Alexa Fluor 488 monoclonal antibody or matched IgG isotype control. Samples were acquired on the FACScan flow cytometer with a minimum of 50,000 CD4+ events recorded.

CFSE Based Proliferation Assay

The carboxyfluorecein diacetate succinimidyl ester (CFSE) based suppression assay was used to assess the suppressive capacity of CMV-T isolated through the activation marker CD25 after 16 hour stimulation with CMVpp65 peptides and compared directly against the CD25 negative fraction. PBMCs were labelled with 1 μM of CFSE (CellTrace CFSE, Life Technologies) and cultured in round bottom 96 well plates (NUNC) with a total of 2×10^5 cells per well. Depending on the number of effector cells available CD25+ CMV-T and CD2− PBMCs were added to the cultures at a ratio of 1:1 and 1:2 of CFSE labelled PBMCs to effectors. Cell cultures were stimulated with 1 μg/ml of SEB (Sigma-Aldrich) or 2 μg/ml of purified anti-CD3 antibody (Clone HIT3a, Biolegend, San Diego, CA). In control experiments CFSE labelled and unlabeled PBMCs were cultured alone. Cultures were incubated for 5 days at 37°C/5% CO_2 before harvesting and cells stained with CD3-APC and CD69-APC Cy7 (Both BD Biosciences) antibodies. Samples were acquired on the FACScan flow cytometer and a minimum of 10,000 CD3+ CFSE+ events recorded. The frequency of suppression was analysed by gating on the CD3+ CFSE+ population and the percentage of undivided cells ($CFSE^{high}$) determined using the CFSE labelled and unlabelled PBMCs control samples. Percentage of suppression was calculated as follows: [100– (%$CFSE^{low}$ of CD3+ in the presence of effectors/%$CFSE^{low}$ of CD3+ in the absence of effectors) x 100].

Expansion of Antigen-Specific T Cell Lines

After isolation of CD25+ CMV-T, up to 0.25×10^6 cells were cultured in the presence of 12.5×10^6 (50:1) γ-irradiated (30 Gy) autologous PBMCs to act as feeder cells in 24 well plates with RPMI 1640 medium containing 10% human AB serum, 1% antibiotic and supplemented with 10 ng/ml of IL-7 and IL-15 (CellGenix, Freiburg, Germany). Culture medium was replenished every 2–3 days and cells split when necessary. Cells were expanded up to a maximum of 24 days before harvest.

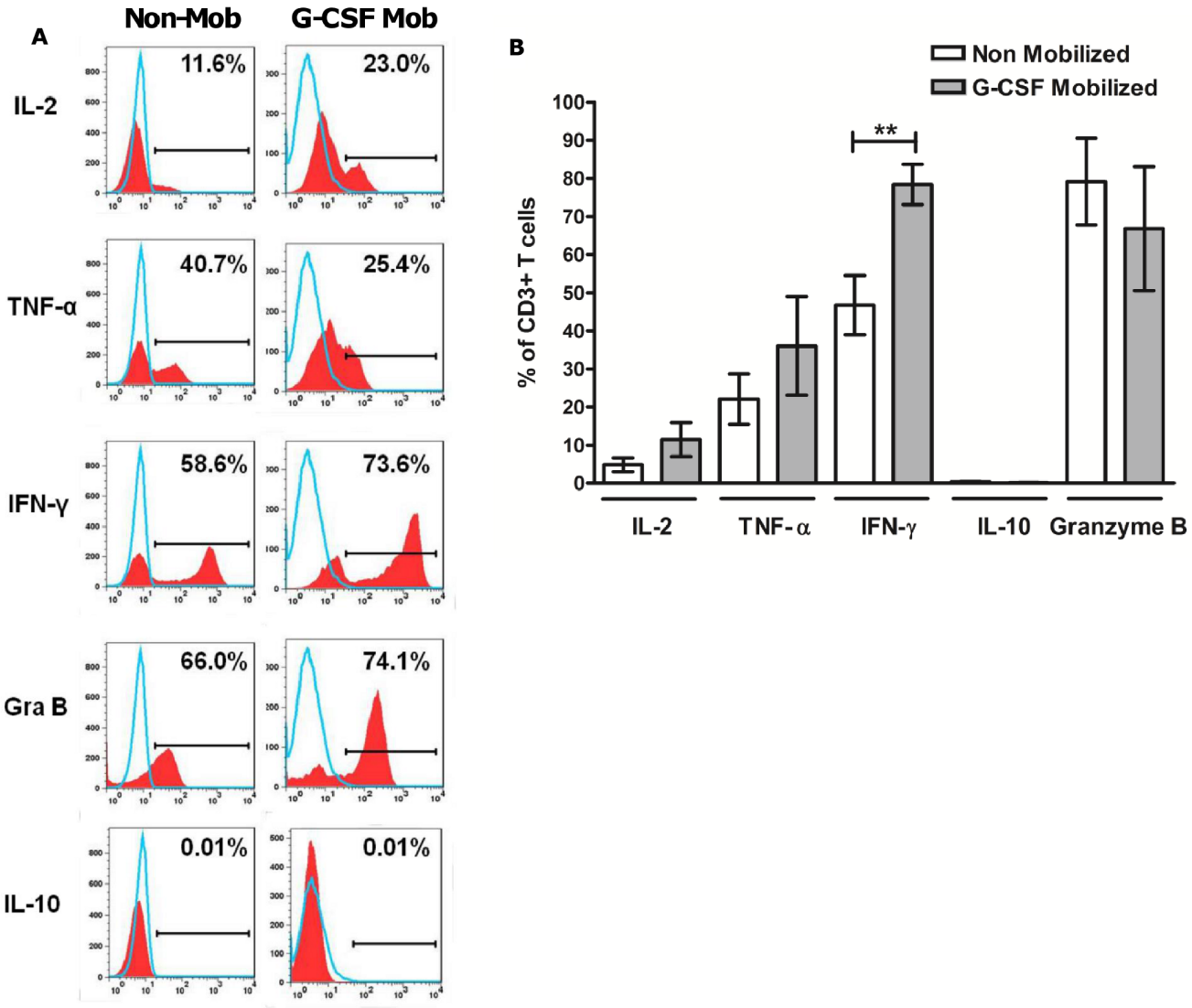

Figure 5. Qualitative and quantitative assessment of cytokine secretion by expanded cells after antigenic re-challenge. Expanded CD25+ cells isolated from both G-CSF-mobilized and non-mobilized donors were stimulated with CMVpp65 peptide loaded autologous PBMCs in the presence of Brefeldin A and purified anti CD28-antibody. Histograms illustrate CMVpp65 rechallenge experiments in representative CD25+ expansions from a G-CSF-mobilized and non-mobilized donor, analysed for IL-2, TNF-α, IFN-γ, Granzyme B and IL-10. Open peaks represent isotype matched controls (A). The combined assessment in G-CSF-mobilized ($n = 5$) and non-mobilized ($n = 5$) donors are summarized (B). $^{**}p<0.01$, in an unpaired *t* test. Gra, granzyme; Mob, mobilized.
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Re-stimulation of Expanded Antigen-Specific T Cell Lines

Expanded cells were restimulated for a period of 5–6 hours with either CMVpp65 Peptivator loaded autologous PBMCs or untouched autologous PBMCs as a control, all labelled with 1 μM CFSE (Sigma-Aldrich) at a ratio of 5:1 at a concentration of 1×10^7/ml in 48 well plates. For analysis of intracellular cytokines, cells were incubated in the presence of anti-CD28 antibody (BD Bioscience) and 1 μg/ml of Brefeldin A (Sigma-Aldrich) added after 2 hours. Cells were fixed and permeabilized using Intrastain (DakoCytomation, Ely, UK) according to the manufacturer's instructions and stained with CD25-APC, CD4-PerCP either PE-conjugated anti-IL-2, anti-TNF-α, anti-Granzyme B, anti-IL-10 or anti IFN-γ and CD69-APC Cy7 (all BD Biosciences) monoclonal antibodies. For surface staining cells were stained for 15 minutes with CD4-FITC, CD25-PE, CD8-PerCP, CD3-APC and CD69-APC Cy7 (all BD Biosciences) monoclonal antibodies.

Cytotoxicity Assay

Autologous PBMCs were stimulated with 3 μg/ml of PHA (Sigma) for 24 hours and then 20 U/ml of IL-2 for a further 72 hours (Miltenyi Biotec) at a concentration of 1×10^6/ml in RPMI 1640 with 10% AB serum, in 24 well flat bottom plates (NUNC). PHA blasts were then used as target cells in the killing assay and loaded with CMVpp65 Peptivator, or left untouched. Loaded target cells were labelled with Calcein-AM (Life Technologies) at a concentration of 10 μM and incubated for 1 hour at 37°C. After four washes in complete medium cells were adjusted to 7×10^4/ml and added to effector cells at E: T ratios ranging from 20:1 to 0.5:1, in triplicate, in U bottom 96 well plates (NUNC). Triplicate wells were also set up to measure spontaneous release (target cells only), maximal release (target cells plus 2% Triton X-100) and medium alone. After incubation at 37°C/5% CO_2 for 4 hours, 100 μl of supernatant was harvested and transferred into new

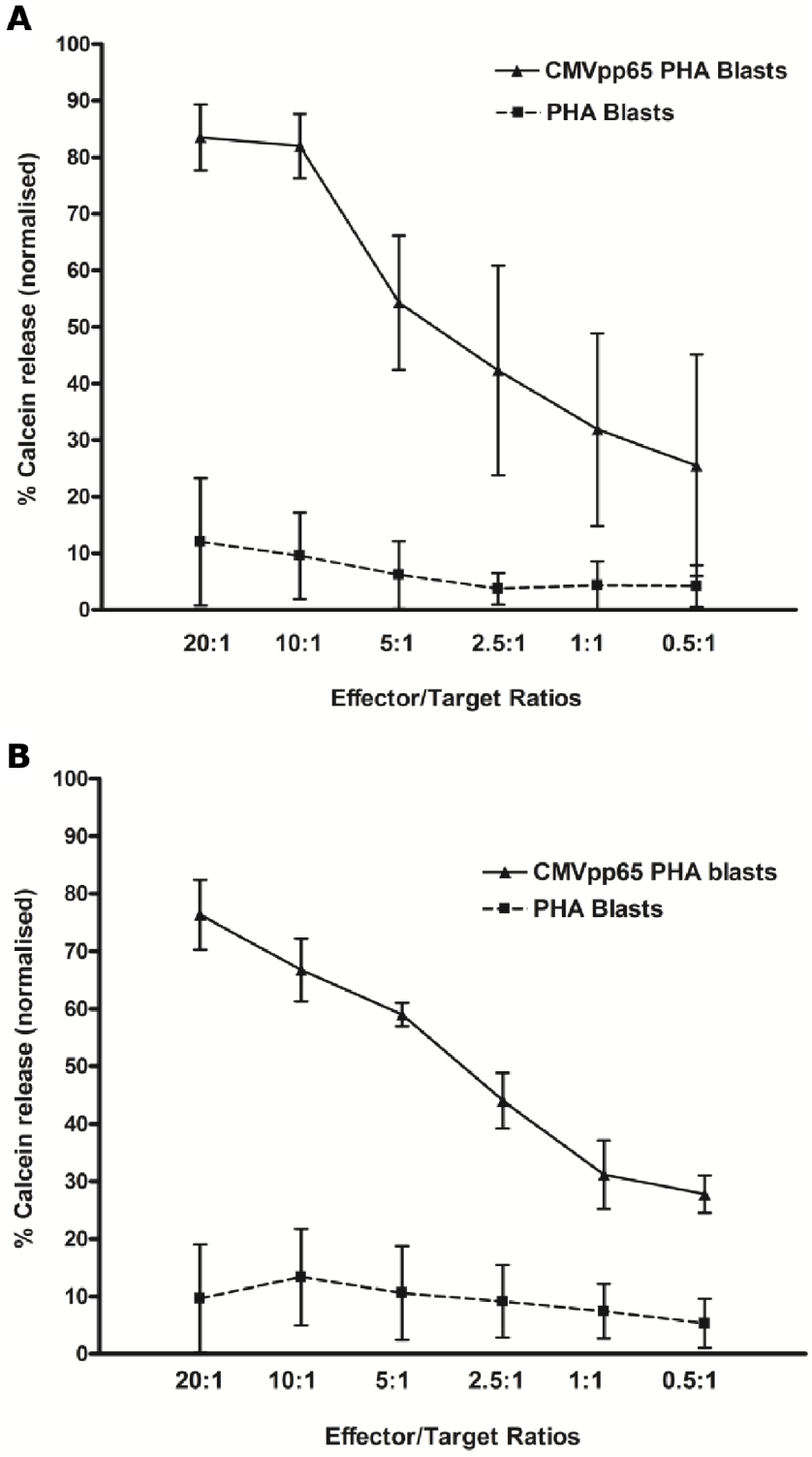

Figure 6. Cytotoxicity of CD25+ expanded CMV-T. Specific lysis of autologous PHA blasts loaded with CMVpp65 peptides at E:T ratios from 20:1 to 0.5:1 was determined using fluorescent dye Calcein-AM cytotoxicity assay in CD25+ expanded cells. Graphs show pooled data from all cytotoxicity experiments in G-CSF-mobilized (A; $n = 4$) and non-mobilized (B; $n = 4$) CD25+ expanded CMV-T.
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plates. Samples were measured using a BMG FLUOstar Galaxy microplate fluorescence spectrophotometer (MTX Lab Systems Inc. Vienna, VA) (excitation filter: 485±9 nm: band-pass filter: 530±9 nm). Data were expressed as arbitrary fluorescent units (AFU) and percent lysis was calculated using the formula [(test release-spontaneous release/maximal release-spontaneous release) x 100].

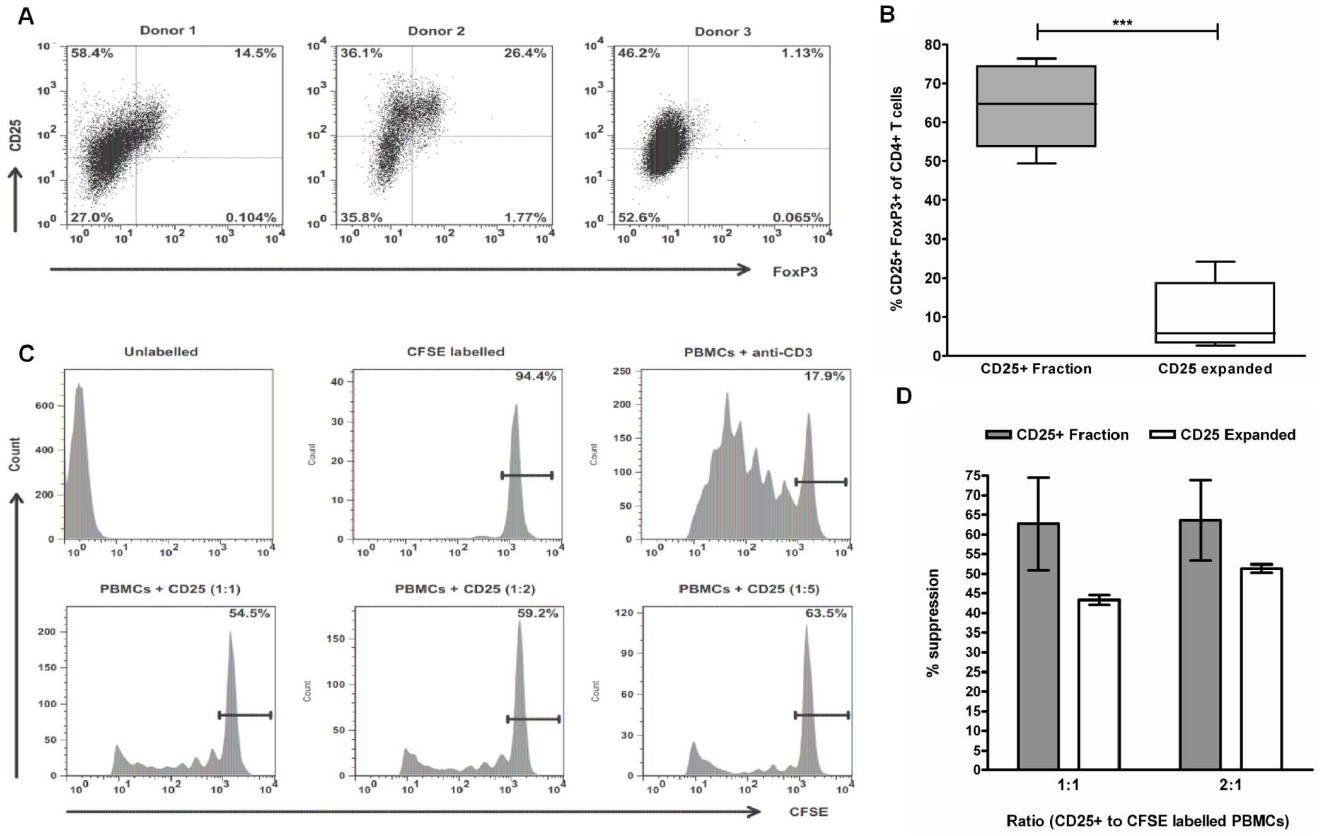

Figure 7. Assessment of FoxP3 expression and suppressive capacity of CD25+ expanded cells in G-CSF-mobilized PBMCs. FoxP3 expression was assessed in G-CSF-mobilized CD25+ expanded cells after 14–24 days in culture. FACS plots illustrate results in 3 expansion experiments (A). FoxP3 expression was compared between CD25+ expanded cells ($n = 5$) and CD25+ fractions following magnetic enrichment ($n = 5$) (B). Suppression of CFSE-labelled PBMCs proliferation by CD25+ expanded cells ($n = 3$) was determined at three ratios in the presence of anti-CD3 antibody cultured for 5 days (C). PBMCs alone (CFSE) and PBMCs incubated with anti-CD3 antibody were cultured as experimental controls. Assessment of suppressive capacity was determined by comparing CD25+ expanded cells with CD25+ fractions (D). ***$p<0.0001$, in an unpaired t test.

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Statistical Analysis

Analyses were conducted using GraphPad Prism 4.0 (Graph Pad Software Inc. La Jolla, CA). An unpaired t test was used to determine the statistical significance between G-CSF-mobilized and non-mobilized PBMCs which followed a normal distribution. A paired t test was used for analysing the effect of CD25-depeletion on CD25 and FoxP3 expression. Statistical significance was considered to be achieved when p was less than 0.05. Data are presented as mean ± SD.

Results

Identification and Isolation of CD25+ CMV-T from G-CSF-mobilized and Non-mobilized PBMCs

The kinetics of activation induced CD25 expression on CMVpp65 peptide stimulated PBMCs was assessed in G-CSF-mobilized donors to determine the optimal time for maximal expression and compared directly against conventional non-mobilized PBMCs. Baseline CD25 expression was assessed prior to CMVpp65 stimulation amongst CD3+ T cells with no significant difference observed between G-CSF-mobilized (4.5% ±0.59) and non-mobilized (4.3% ±0.59; $p = 0.83$) PBMCs. PBMCs were stimulated over 24 hours, sampled at 1, 4, 6, 16 and 24 hours and analysed for CD25 expression by flow cytometry (Fig. 1A). The mean antigen-dependent expression of CD25 was maximal at 24 hours in G-CSF-mobilized PBMCs (7.63% ±1.12) and was significantly elevated when compared to non-mobilized PBMCs (4.33% ±0.84; $p = 0.04$). Further analysis of CD25 expression in G-CSF-mobilized PBMCs revealed the optimal time of expression to be at 16 hours after subtraction of the unstimulated control when compared with 24 hour stimulation (3.20% ±1.43 vs. 2.25% ±0.76). On the basis of the results from expression kinetics, isolation of CD25+ CMV-T was investigated following 16 hour CMVpp65 peptide stimulation. A single enrichment step was performed using magnetic-bead based cell separation in six G-CSF-mobilized and five non-mobilized healthy CMV-seropositive donors. No significant difference was observed in CD25+ purity (86.9% ±3.1 vs. 82.3% ±9.9; $p = 0.32$) or yield (26.1% ±8.8 vs. 11.3% ±6.9; $p = 0.12$) when comparing G-CSF-mobilized and non-mobilized PBMCs (Fig. 1B). Yield was defined as the absolute number of CD25+ T cells in the positive fraction as a proportion of the absolute number of CD25+ T cells in the pre-sort sample. Analysis of CD3+ T cell subsets in CD25 positive fractions revealed a predominantly CD4+ population in both G-CSF-mobilized (97.6% ±1.8) and non-mobilized (92.3% ±4.9) PBMCs.

FoxP3 Expression in CD25+ CMV-T

To evaluate the suitability of CD25 as a target for isolation of CMV-T from G-CSF-mobilized PBMCs, the level of Treg enrichment was determined by analysing the proportion of CD25+FoxP3+ cells amongst CD4+ cells at three time points: (1) pre-CMVpp65 stimulation; (2) post-CMVpp65 stimulation; and (3) post-CD25 enrichment. Figure 1C illustrates a representative experiment in both a G-CSF-mobilized and non-mobilized donor showing the increased level of CD25+FoxP3+ expression present in G-CSF-mobilized PBMCs post CD25 enrichment. Figure 1D summarizes experiments from six G-CSF-mobilized and five non-mobilized donors where analysis revealed that the proportion of FoxP3+ cells contained within the CD4+CD25+ population was significantly increased in G-CSF-mobilized PBMCs in resting PBMCs (41.35% ±8.41 vs. 17.5% ±6.5; $p = 0.04$) and following CMVpp65 stimulation (42.79% ±4.07 vs. 20.84% ±5.46; $p = 0.012$), when compared with non-mobilized PBMCs. The proportion of CD25+FoxP3+ cells after CD25 enrichment was also shown to be significantly increased in G-CSF-mobilized (73.64% ±4.88) vs. non-mobilized (43.82% ±5.64; $p = 0.004$) PBMCs.

Assessment of Suppressive Capacity of CD25+ enriched CMV-T in G-CSF-mobilized PBMCs

Having demonstrated that CD25+ cells enriched after CMVpp65 stimulation from G-CSF-mobilized PBMCs contain a significant proportion of FoxP3-expressing cells, their suppressive activity was assessed using a CFSE-based T cell proliferation assay. The suppressive capacity of CD25+ T cells enriched after CMVpp65 stimulation was compared to the CD25− fraction, in both G-CSF-mobilized ($n = 5$) and non-mobilized PBMCs ($n = 5$). Both fractions were cultured at ratios of 1:1 and 2:1 and in some experiments at 5:1 (effectors: responders), with CFSE-labelled PBMCs, in the presence of the superantigen SEB. Figure 2A illustrates a representative experiment from a G-CSF-mobilized donor, where T cell proliferation in the presence of CD25− cells was similar to PBMCs alone (10.4% undivided CD3+ T cells) at both 1:1 (12.5% undivided CD3+ T cells) and 2:1 (10.0% undivided CD3+ T cells). In contrast, T cell proliferation was greatly reduced when CFSE-labelled PBMCs were cultured in the presence of CD25+ CMV-T at both 1:1 (57.3% undivided CD3+ T cells) and 2:1 (61.7% undivided CD3+ T cells), highlighting a strong suppressive capacity. Suppression of T cell proliferation by CD25+ CMV-T isolated from non-mobilized PBMCs was shown to be reduced when compared with G-CSF-mobilized PBMCs. Figure 2B shows the results from a representative experiment using non-mobilized PBMCs, where proliferation of CFSE-labelled PBMCs in response to SEB stimulation was comparable when cultured with CD25− and CD25+ cells at both ratios of 1:1 (15.3% vs. 19.9% undivided CD3+ T cells) and 2:1 (14.1% vs. 27.7% respectively). Cumulative analysis of CD25+ enriched T cells following CMVpp65 stimulation revealed a significant difference in the mean suppressive capacity of G-CSF-mobilized ($n = 5$) compared to non-mobilized ($n = 5$) PBMCs (Fig 2C). G-CSF-mobilized PBMCs were highly suppressive of autologous PBMCs proliferation following SEB stimulation at both ratios of 1:1 (62.7% ±11.75 suppression) and 2:1 (63.58% ±10.27). This was significantly reduced in non-mobilized PBMCs at both ratios of 1:1 (7.90% ±4.79; $p = 0.007$) and 2:1 (6.37% ±3.48; $p = 0.002$) PBMCs.

Depletion of CD25-expressing Cells in Resting G-CSF-mobilized PBMCs

To determine the role of Tregs in CD25+ T cell responses to CMVpp65 peptide stimulation following isolation from G-CSF-mobilized PBMCs, we depleted CD25 expressing cells prior to stimulation. CD25-depleted G-CSF-mobilized PBMCs were compared to unmanipulated PBMCs to assess the impact on CD25 purity, yield and FoxP3 expression following CMVpp65 stimulation and CD25 magnetic enrichment. Mean CD25 expression amongst CD3+ T cells in CD25 depleted G-CSF-mobilized PBMCs, pre stimulation, was 0.57% ±0.13 compared to 2.85% ±0.69 ($p = 0.05$) in the unmanipulated paired samples. CD25 expression post CMVpp65 stimulation amongst CD4+ T cells (Fig. 3A) was reduced when comparing unmanipulated (4.40% ±0.61) and CD25-depleted G-CSF-mobilized PBMCs (1.75% ±0.59). CD25+ FoxP3 expression was assessed pre and post CD25 enrichment to determine the impact of CD25−depletion on the significant proportion of Tregs identified in CD25+ fractions following immunomagnetic CD25-enrichment. CD25-depletion significantly reduced FoxP3 expression (Figs. 3B–C) both pre CD25 enrichment (3.88% ±0.51 vs. 0.74% ±0.55; $p = 0.002$) and post CD25 enrichment (68.60% ±5.56 vs. 11.59% ±5.75; $p = 0.001$) in G-CSF-mobilized PBMCs. Assessment of CD25 expression following CMVpp65 stimulation and CD25 enrichment revealed that CD25-depeletion had a significantly negative impact on CD25 purity when comparing depleted versus unmanipulated (17.08% ±12.78 vs. 88.91% ±0.57 respectively; $p = 0.03$) in G-CSF-mobilized PBMCs (Figure 3D). However, analysis of mean CD25+FoxP3− T cells amongst CD4+ T cells following enrichment revealed no significance ($p > 0.05$) between CD25-depleted (16.93% ±6.34) and unmanipulated (22.20% ±7.18) G-CSF-mobilized PBMCs, suggesting that CD25 depletion does not remove CMVpp65-specific T cells.

Expansion of in-vitro Expanded CD25+ CMV-T and Assessment of CMV Specificity

CD25+ CMV-T were cultured for up to 24 days in complete medium supplemented with IL-7 and IL-15 in the presence of autologous irradiated feeder cells. The mean proliferative capacity of G-CSF-mobilized CD25+ (90.3-fold expansion ±30.0; $n = 5$) was reduced ($p = 0.07$) when compared with non-mobilized CD25+ CMV-T (237.0-fold expansion ±63.8; $n = 5$) Expanded cells from G-CSF-mobilized PBMCs revealed a higher proportion of CD4+ T cells compared with non-mobilized PBMCs (44.13% ±17.0 vs. 13.9% ±5.9) and a reduction in the proportion of CD8+ T cells (53.0% ±17.3 vs. 79.1% ±8.7). In two of the five CD25 expansion experiments performed from G-CSF-mobilized donors, the CD8+ subset at day 22 accounted for >90% of CD3+ T cells compared with <5.5% CD4+, which was contradictory to the results seen in the remaining three CD25 expansions and illustrates a high level of donor variability in cultured cells, not seen in the non-mobilized setting (Fig. 4A). Cultures showed specificity for CMVpp65, determined by up-regulation of CD25+ and CD69+ expression following re-challenge with autologous CMVpp65-loaded PBMCs in contrast to re-challenge with autologous PBMCs without peptide (Fig. 4B). The mean level of CD25+ CD69+ co-expression in CD25+ expanded CMV-T from G-CSF-mobilized (64.9% ±15.7) PBMCs was elevated when compared to non-mobilized (35.4% ±6.2) PBMCs. In control experiments, re-challenge with autologous PBMCs alone produced a low mean level of CD25+ CD69+ co-expression in CD25+ expanded CMV-T from G-CSF-mobilized (10.0% ±4.9) and non-mobilized (5.0% ±1.7) PBMCs.

CD25+ Expanded Cells Synthesize and Secrete an Effector Cytokine Repertoire and Effectively Lyse CMVpp65 Targets

Antigen specificity was assessed through intracellular cytokine staining (ICS) following CMVpp65 re-challenge (G-CSF-mobilized, $n = 5$; non-mobilized, $n = 5$). CD25+ expanded cells revealed low to undetectable mean levels of IL-10 secretion after CMVpp65 re-challenge from both G-CSF-mobilized (0.11% ±0.05) and non-mobilized (0.41% ±0.10) PBMCs. Expanded cells synthesized and secreted low levels of IL-2 and significant levels of TNF-α, IFN-γ and Granzyme B (Figs. 5A–B), indicating that cells possessed the effector molecules necessary for cytotoxic activity. Cytokine secretion was comparable between G-CSF-mobilized and non-mobilized CD25+ expanded cells, apart from IFN-γ where G-CSF-mobilized CD25+ CMV-T revealed a significant increase ($p = 0.009$) in secretion compared with non-mobilized CD25+ CMV-T (78.4% ±5.2 vs. 46.7% ±7.7). In experiments where CD25+ expanded cells were re-challenged with unstimulated or CMV IE-1 peptide loaded autologous PBMCs minimal cytokine secretion was observed (data not shown).

Cytotoxic activity was evaluated using autologous PHA blasts loaded with CMVpp65 peptides and labelled with Calcein-AM dye as targets. CD25+ expanded cells from both G-CSF-mobilized ($n = 4$) and non-mobilized ($n = 4$) PBMCs effectively lysed CMVpp65 targets at all E: T ratios in a dose-dependent manner (Figs. 6A–B), with minimal lysis of PHA blasts without CMVpp65 peptides. Comparison between G-CSF-mobilized and non-mobilized CD25+ CMV-T revealed no significant difference in lysis of CMVpp65 PHA blasts at any of the E:T ratios.

Assessment of FoxP3 Expression and Suppressive Capacity of CD25+ Expanded CMV-T from G-CSF-mobilized PBMCs

Previous experiments identified that CD25+ cells enriched from G-CSF-mobilized PBMCs following CMVpp65 peptide stimulation contained a significant proportion of FoxP3 expressing cells. Assessment of FoxP3 expression in CD25 expanded cells after short term culture is illustrated in three expansions (Fig. 7A) and revealed a significant reduction ($p = <0.0001$) in expression compared to pre-expansion. Mean FoxP3 expression amongst CD4+ T cells after culture was 9.89% ±4.02, compared to 64.24% ±4.88 at pre-expansion (Fig. 7B). The suppressive capacity of CD25+ expanded cells from G-CSF-mobilized PBMCs were assessed in a 5-day CFSE proliferation assay (Fig. 7C) as previously described. G-CSF-mobilized CD25+ expanded cells were shown to retain their suppressive capacity at all ratios at a level comparable with CD25 enriched cells post CMVpp65 stimulation (Fig. 7D). Mean suppression of autologous PBMCs when co-cultured at 1:2 with CD25+ expanded cells was 51.35% ±1.05 compared with 63.58% ±10.27 with CD25+ enriched cells.

Discussion

CD25 expression has been reported as an optimal marker for the identification of antigen-specific T cells following peptide stimulation [17–21], enabling the magnetic enrichment of activated anti-viral T cells that would otherwise be undetectable using current IFN-γ secretion methods. Generation of anti-viral T cells has been largely restricted to non-mobilized steady state leukapheresis or a single blood draw for manufacture. More recently, studies have investigated the use of G-CSF-mobilized PBMCs from the original aHSCT graft as a potential source for CMV-T manufacture [31;32]. Here, we expand on these studies to investigate the activation marker CD25 as a possible target and investigate the nature of CD25+ enriched cells following CMV peptide stimulation with regard to Treg characterisation, due to their increased number following G-CSF-mobilization [36].

We have shown that CD25 expression is up-regulated in G-CSF-mobilized PBMCs after CMVpp65 peptide stimulation at a level comparable to that seen in non-mobilized PBMCs and that magnetic enrichment of CD25 expressing cells results in a high level of purity. However further analysis revealed that the CD25 positive fractions isolated from G-CSF-mobilized PBMCs co-expressed FoxP3, a population that was largely absent in the CD25 positive fraction isolated from non-mobilized PBMCs. Functional studies revealed they were highly suppressive *in vitro*, demonstrating a significant population of Tregs. Several studies have indicated the existence of anti-viral Tregs, revealed following antigenic stimulation [37–42] and indeed Ltjens and colleagues concluded that CD4+CD25+CD127−FoxP3+ Tregs contained a population of CMV-specific T cells [38], which is consistent with the results seen here. This suggests that CMVpp65 stimulation in G-CSF-mobilized PBMCs induces a population of antigen-specific CD25+ Tregs that are able to recognize CMVpp65 peptides and possibly proliferate in response to engagement of their cognate antigen. Support for this hypothesis is evident in a murine model of *Leishmania* infection, in which Tregs proliferated in response to recognition of parasite derived antigen [40]. Previous studies [21] have suggested that the co-infusion of Tregs and anti-viral T cells could be advantageous in terms of an immunotherapy that inhibits GvHD on one hand and promotes ant-viral immune reconstitution on the other. It has also been argued that it is unlikely that infused Tregs will prevent proliferation of virus-specific T cells; a hypothesis supported by studies in mice demonstrating co-infusion of Tregs and conventional T cells (Tcons) enhances virus-specific immune reconstitution and protected mice from lethal CMV infection in the HSCT setting [43]. Clinical studies have since demonstrated that early infusion of freshly isolated donor Tregs followed by Tcons at the time of full-haplotype-mismatched aHSCT prevented GvHD in the absence of any post-transplant immunosuppression and improved immunity to opportunistic pathogens [44]. However in this study Tregs were generated from steady-state leukapheresis and did not use viral antigen-specific Tregs which appear to be preferentially generated from G-CSF-mobilized PBMCs.

In the context of manufacturing CMV-T for adoptive immunotherapy the emergence of a population of CD25+FoxP3+ cells from G-CSF-mobilized PBMCs with a highly suppressive capacity suggests that CD25 will be an unsuitable target for CMV-T isolation. We therefore investigated the impact of depleting CD25-expressing cells prior to CMVpp65 peptide stimulation in order to eliminate the CD25+FoxP3+ cells identified following enrichment from G-CSF-mobilized PBMCs. Depletion of CD25 expressing cells in G-CSF-mobilized PBMCs, prior to CMVpp65 peptide stimulation, resulted in a reduction in CD25 expression post stimulation which in turn had a negative impact on both yield and purity following CD25 enrichment, confirming previous studies by Melenhorst and colleagues in non-mobilized PBMCs (39). The reduction in CD25 expression is partly explained by the elimination of contaminating Tregs prior to CMVpp65 stimulation, as indeed was the aim, but could also be attributed to a population of CMV-T that are in an activated state and express CD25 prior to CMVpp65 stimulation *in vitro*. Although a significant reduction in FoxP3 expression was observed in CD25-depleted PBMCs post-CMVpp65 stimulation, FoxP3 expression post-CD25 enrichment was still detectable. These

results suggest either the emergence of newly expressing FoxP3 cells or persistence of FoxP3+CD25– cells following the depletion step and comparable to results seen in non-mobilized cells [39–42] and supports the concept of CMV-specific Tregs.

To determine the functionality of CD25+ CMV-T and the possible impact of Tregs on the anti-CMV response, we analysed whether these cells were capable of secreting inflammatory cytokines in response to antigenic re-challenge following short-term culture. CD25+ expanded cells from G-CSF-mobilized PBMCs secreted high levels of IFN-γ and Granzyme B, and to a lesser extent TNF-α and IL-2, in the absence of IL-10. This cytokine profile is in keeping with anti-viral CTL rather than antigen-specific Tregs, which was confirmed by their ability to lyse autologous CMV-pulsed target cells. Further analysis of FoxP3 expression also revealed a significant reduction following expansion. Nonetheless expanded CD25+ CMV-T retained a capacity to suppress polyclonal T cells proliferation suggesting the presence of a population of FoxP3– IL10– Tregs. However it must be noted that a limitation of this study was the failure to procure paired donor samples before and after G-CSF-mobilization for subsequent comparison of both the CMV immune response and Treg numbers and potency. The use of paired samples could have allowed for stronger statistical analysis to be made when analysing the effect of G-CSF-mobilization given the knowledge of high donor variability that exists, in terms of the CMV immune response.

Although FoxP3 generally identifies natural thymus-derived Tregs, adaptive Tregs may or may not express this transcription factor [45]. Studies have also indicated that FoxP3 expression can be induced in human CD4+ effector T cells after activation as a normal consequence of CD4+ T cell activation [42–46] and that IFN-γ and IL-2 production are not suppressed in FoxP3+ effector T cells [47,48], thus bringing into doubt its validity as an exclusive marker of Tregs. The characterisation of the pool of CD25+FoxP3+ Tregs identified post CD25 enrichment following CMVpp65 stimulation and the subsequent loss of FoxP3 expression following short-term culture highlights the need for future studies. Most notably to extensively characterise these highly suppressive cells with regard to disseminating between inducible/adaptive and antigen-specific subsets of Tregs, that may be committed to regulating specific arms of the immune response.

In conclusion our data indicates that CD25 enrichment post CMV stimulation in G-CSF-mobilized PBMCs results in the simultaneous generation of a functional mixed population of CMV-T and Tregs and outlines a potential therapeutic strategy for the treatment of both GvHD and CMV reactivation following HSCT. The use of G-CSF-mobilized PBMCs represents a feasible approach to alleviating the many problems incurred with successive donations and procurement of cells from unrelated donors and thereby simplifying the clinical application of cellular therapy manufacture in the aHSCT setting. Questions remain as to whether these cells are capable of both conferring protection from CMV and minimising the GvH response *in vivo* and whether the infused Treg population will prevent the persistence and proliferation of CMV-T, both of which can only be answered by clinical trials utilizing this approach, in recipients of aHSCT.

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Author Contributions

Conceived and designed the experiments: ERS MWL KN. Performed the experiments: ERS LB. Analyzed the data: ERS LB MWL KN. Contributed reagents/materials/analysis tools: ERS KN. Wrote the paper: ERS MWL SM.

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