Universitat Autònoma de Barcelona

DEVELOPMENT AND CHARACTERISATION OF ADVANCED CELL THERAPIES BASED ON MULTIPOTENT MESENCHYMAL STROMAL CELLS AND VIRUS-SPECIFIC T LYMPHOCYTES

Marta Grau Vorster

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Tesi Doctoral

DEVELOPMENT AND CHARACTERISATION OF ADVANCED CELL THERAPIES BASED ON MULTIPOTENT MESENCHYMAL STROMAL CELLS AND VIRUS-SPECIFIC T LYMPHOCYTES

Tesi doctoral presentada per **Marta Grau Vorster**

Graduada en Biotecnologia per optar al grau acadèmic de doctora per la Universitat Autònoma de Barcelona

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Directors:

Francesc Rudilla Salvador

Joaquim Vives Armengol

Irene Oliver Vila

Tutor:

Francesc Gòdia Casablancas

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Francesc Rudilla Salvador, facultatiu especialista en Immunologia del Banc de Sang i Teixits de Barcelona, **Joaquim Vives Armengol**, facultatiu del Banc de Sang i Teixits de Barcelona i **Irene Oliver Vila**, directora científica de Cellab,

Certifiquem: Que la graduada **Marta Grau Vorster** ha dut a terme amb la nostra direcció, en el Servei de Teràpia Cel·lular del Banc de Sang i Teixits de Barcelona, el treball que amb el títol: Development and Characterisation of Advanced Cell Therapies Based on Multipotent Mesenchymal Stromal Cells and Virus-Specific T Lymphocytes, es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al grau de Doctora per la Universitat Autònoma de Barcelona.

I perquè en prengueu coneixement i tingui els efectes que correspongui, presentem davant de l'Escola de Doctorat de la Universitat Autònoma de Barcelona l'esmentada Tesi signant aquesta certificació a

Bellaterra, setembre de 2019

Dr. Francesc Rudilla Salvador (Director)	Dr. Joaquim Vives Armengol (Director)	Dra. Irene Oliver Vila (Directora)

Dr. Francesc Gòdia Casablancas (Tutor)	Marta Grau Vorster (Estudiant)

“Make no little plans,
they have no magic to stir men’s blood...
make big plans; aim high in hope and work.”

- *Daniel Burnham*

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ABSTRACT

Innovative therapies are being developed worldwide to tackle unmet clinical needs. In particular, progress in advanced therapy medicinal products (ATMP) has shown great promise for the treatment of diseases with no other option available. However, researchers and regulatory authorities deal with the sophisticated nature of these medicines, and struggle to standardise both production protocols and final product formulation. Challenges related to the living nature of these products include high donor intervariability and complex mechanisms of action, which are sometimes not completely understood. Additionally, these newly therapies need to demonstrate biological activity with potency assays.

This dissertation comprises the development and characterisation of two different ATMP based on multipotent mesenchymal stromal cells (MSC) and virus-specific T cells (VST).

On the one hand, assessment of identity and potency for product release of MSC isolated from Wharton's jelly (WJ) and bone marrow (BM), in the context of current good manufacturing practice (cGMP) production, is performed. In this regard, we aimed at proposing: a) a potency assay for assessing immunomodulation capacity of MSC; b) the revision of HLA-DR expression profile for MSC definition criteria; and c) the application of risk management methodologies in the assessment of product quality. The optimisation of an immunopotency assay, validated, and approved by the competent authority for product release is presented. Moreover, other quality attributes of MSC are addressed. Regarding BM-MSC, the apparently random expression of HLA-DR, a marker that was expected negative in expansion cultures of MSC, is studied in clinical grade productions. Our findings showed correlation of HLA-DR expression with levels of IL-17F and IL-33. Expression of HLA-DR did not affect MSC identity, differentiation potential nor immunomodulatory capacity. To further strengthen these outcomes, interlaboratory studies were performed obtaining similar results. Furthermore, the use of either human sera or platelet lysate supplements showed no differences in terms of HLA-DR expression. A risk management assessment methodology was also implemented as a tool for quality by design to detect weaknesses of an established bioprocess involving MSC products already in clinical trials.

On the other hand, regarding T lymphocytes, the development of a protocol for *ex vivo* expansion of VST was performed. VST therapy is intended for immunocompromised patients, which are susceptible of reactivation or *de novo* infection of herpesviruses among others. This is the case of cytomegalovirus (CMV) that undergoes a mild infection in healthy individuals but has been associated to a high morbidity and mortality in immunocompromised individuals. Unfortunately, available antiviral drugs can produce toxic side effects and are not always

effective. Adoptive immunotherapy offers an alternative approach for those patients in a critical situation with no other therapeutic option. Therefore, we developed a protocol for VST scale-up manufacture easily transferable to pharmaceutical standards. Following with the method proposed, we obtained large number of CMV pp65-specific T cells after 14-day co-culture with pp65 pulsed dendritic cells. Culture was based on G-Rex bioreactor technology and supplemented with IL-2, IL-7, IL-15, anti-CD3 and anti-CD28 antibodies. The final product was extensively characterised in terms of identity, purity and potency. VST product was comprised of both $CD4^+$ and $CD8^+$ T lymphocytes, and effector memory T cells represented the major subset, which are known to provide effector function. Most importantly, we successfully demonstrated pp65 specific cytotoxicity of the expanded cells. Interestingly, complete HLA mismatch alloreactivity resulted in less than 5% cell lysis. In summary, a feasible protocol transferable to cGMP was described for an *in vitro* safe and effective product, which remain functional after thawing, thus providing practical evidence for the generation of an allogeneic third-party bank. Future perspectives would include the manufacture of multivirus-specific T cells.

RESUM

El desenvolupament de noves teràpies s'està duent a terme arreu del món per poder fer front a les necessitats clíniques que actualment no disposen de tractament. En particular, els avenços en productes medicinals de teràpia avançada (ATMP) són una gran promesa per al tractament de malalties sense altres opcions terapèutiques. Tanmateix, els investigadors i les autoritats reguladores que implementen aquestes teràpies lluiten per estandarditzar tant els protocols com els productes finals. Entre els reptes s'inclouen l'elevada intervariabilitat de donants i mecanismes d'acció complexos. A més, és necessari demostrar l'activitat biològica d'aquestes teràpies mitjançant assajos de potència.

Aquesta tesi consisteix en el desenvolupament i caracterització de dos ATMP basats en cèl·lules multipotents estromals mesenquimals (MSC) i cèl·lules T específiques de virus (VST).

D'una banda, s'ha fet l'avaluació d'un test de potència i identitat per la producció de MSC aïllades de la gelatina de Wharton (WJ) i de la medul·la òssia (BM). L'objectiu proposat per MSC era realitzar: a) un assaig de potència per avaluar la capacitat immunomoduladora de les MSC; b) la revisió de l'expressió del HLA-DR pels criteris de definició de les MSC; i c) l'aplicació d'una eina per gestionar el risc. En aquest treball es presenta l'optimització d'un assaig d'immunopotència, validat i aprovat per l'autoritat competent per a l'alliberament de producte. S'ha estudiat l'expressió del HLA-DR, marcador suposadament negatiu, en les BM-MSC de grau clínic. Els resultats van mostrar una correlació entre l'expressió del HLA-DR i els nivells d'IL-17F i IL-33. L'expressió del HLA-DR no va afectar la identitat de les MSC, ni el potencial de diferenciació ni la capacitat immunomoduladora. Per reforçar aquests resultats, es van realitzar estudis interlaboratori obtenint resultats similars. L'ús de suplements basats en sèrum humà o lisat plaquetari no mostrava diferències en l'expressió de HLA-DR en MSC. També es va implementar la gestió de riscos com a eina de qualitat per detectar debilitats d'un bioprocés. Aquests enfocaments s'han dut a terme per MSC en assajos clínics.

D'altra banda, es va realitzar el desenvolupament d'un protocol d'expansió *ex vivo* de VST. La teràpia amb VST està destinada a pacients immunocompromesos, susceptibles de patir una reactivació o infecció *de novo* de l'herpesvirus entre d'altres. És el cas del citomegalovirus (CMV), que pot produir una infecció lleu en individus sans, però té una elevada morbiditat i mortalitat en individus immunocompromesos. Els medicaments antivirals disponibles poden produir toxicitat i no sempre són efectius. La immunoteràpia adoptiva ofereix una alternativa als pacients en una situació crítica i sense altres opcions terapèutiques. Per satisfer aquesta demanda es va elaborar un protocol de fabricació de VST fàcilment transferible als estàndards

farmacèutics. Amb el mètode proposat, després d'un cocultiu de 14 dies amb cèl·lules dendrítiques polsades amb pp65, es van obtenir un gran nombre de VST. El cultiu es basava en la tecnologia G-Rex i es suplementava amb IL-2, IL-7, IL-15, i anticossos anti-CD3 i anti-CD28. El producte final es va caracteritzar àmpliament i estava format per limfòcits T $CD4^+$ i $CD8^+$, on la subpoblació majoritària corresponia a les cèl·lules T efectores de memòria, població coneguda per proporcionar una funció efectora. Cal destacar la citotoxicitat de les cèl·lules expandides específicament enfront al pp65. Els estudis d'al·loreactivitat amb HLA totalment incompatibles, van mostrar una lisi cel·lular per sota del 5%. En resum, s'ha descrit un protocol transferible a les normes de correcta fabricació actuals i un producte segur i eficaç *in vitro*, funcional després de la descongelació. El darrer fet, facilitaria la generació d'un banc al·logènic de VST. Les perspectives de futur inclourien la fabricació de cèl·lules T específiques per a múltiples virus.

LIST OF PUBLICATIONS

I. Oliver-Vila I, Ramírez-Moncayo C, **Grau-Vorster M**, Marín-Gallén S, Caminal M, Vives J (2018). Optimisation of a potency assay for the assessment of immunomodulative potential of clinical grade multipotent mesenchymal stromal cells. *Cytotechnology,* 70(1), 31-44. https://doi.org/10.1007/s10616-017-0186-0

II. **Grau-Vorster M**, Rodríguez L, Torrents-Zapata S, Vivas D, Codinach M, Blanco M, Oliver-Vila I, García-López J, Vives J (2019). Levels of IL-17F and IL-33 correlate with HLA-DR activation in clinical-grade human bone marrow-derived multipotent Mesenchymal Stromal Cell expansion cultures. *Cytotherapy,* 21(1), 32-40. https://doi.org/10.1016/j.jcyt.2018.09.009

III. **Grau-Vorster M**, Laitinen A, Vives J, Nystedt. J (2019). HLA-DR expression in clinical-grade bone marrow-derived multipotent mesenchymal stromal cells: a two-site study. *Stem Cell Research & Therapy,* 10(1), 164. https://doi.org/10.1186/s13287-019-1279-9

IV. **Grau-Vorster M**, Rodríguez L, del Mazo-Barbara A, Mirabel C, Blanco M, Codinach M, Gómez S, Querol S, García-López J, Vives J (2019). Compliance with good manufacturing practice in the assessment of immunomodulation potential of clinical grade multipotent mesenchymal stromal cells derived from Wharton's jelly. *Cells,* 8(5), 484. https://doi.org/10.3390/cells8050484

V. **Grau-Vorster M**, López-Montañés M, Cantó E, Vives J, Oliver-Vila I, Querol S, Rudilla F. Characterization of a Cytomegalovirus-Specific T Lymphocyte Product Obtained Through a Rapid and Scalable Production Process for Use in Adoptive Immunotherapy. *Submitted.*

Additional publications not included in the thesis:

i. Oliver-Vila I, Coca M, **Grau-Vorster M**, Pujals-Fonts N, Caminal M, Ortega I, Reales L, Pla A, Blanco M, Garcia J, Vives J (2016): Evaluation of a cell banking strategy for the production of clinical grade Mesenchymal Stromal Cells from Wharton's jelly. *Cytotherapy,* 18(1), 25-35. https://doi.org/10.1016/j.jcyt.2015.10.001

ii. Vivas D, **Grau-Vorster M**, Oliver-Vila I, García-López J, Vives J: Formulation of a clinical grade injectable osteogenic product based on human mesenchymal progenitors. *Submitted.*

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Vives, J., Oliver-Vila, I., Vivas, D., **Grau-Vorster, M.**, García, J. (2017) 'Composition for regenerating bony tissue, method for preparation and use thereof', Ref. No.: EP17382614.0. Applicant: Banc de Sang i Teixits. *Submitted.*

CONTENTS

LIST OF ABBREVIATIONS

ADV	Adenovirus
AEMPS	Agencia Española de Medicamentos y Productos Sanitarios
APC	Antigen Presenting Cell
ATMP	Advanced Therapy Medicinal Products
BKV	BK virus
BM	Bone marrow
C	Constant
CAR	Chimeric Antigen Receptor
CAT	Committee for Advanced Therapies
CDR3	Complementary Determining Region 3
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CFU-F	fibroblast colony-forming units
cGMP	current good manufacturing practices
CHMP	Committee for Medicinal Products for Human Use
CMV	Cytomegalovirus
CTA	Clinical Trial Authorisation
CTL	Cytotoxic T lymphocyte
D	Diversity
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate-Buffered Saline
EBV	Epstein-Barr Virus
EMA	European Medicines Agency
ESC	Embryonic Stem Cell
EU	European Union
EV	Extracellular vesicles
FasL	Fas ligand
FDA	Food and Drug Administration
FMEA	Failure Mode and Effects Analysis
FMECA	Failure Mode, Effects and Critical Analysis
gB	glycoprotein B
GCP	Good Clinical Practices
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
GTMP	Gene Therapy Medicinal Product
GvHD	Graft Versus Host Disease
HHV-5	Human betaherpesvirus 5
HHV-6	Human betaherpesvirus 6
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSCT	Hematopoietic Stem Cell Transplantation
HSC	Hematopoietic Stem Cell
hSerB	Human Serum B
hTert	human telomerase reverse transcriptase
IDO	Indoleamine 2,3-dioxygenase
IE-1	Immediate early protein-1
IFN-γ	Interferon gamma
iPSC	Induced Pluripotent Stem Cell
J	Joining
MA	Marketing Authorisation
MAA	Marketing Authorisation Application
MCB	Master Cell Bank
mHA	Minor Histocompatibility Antigens
MHC	Major Histocompatibility Complex
MoA	Mechanisms of Action
MSC	multipotent Mesenchymal Stromal Cell
NK	Natural Killer
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cell
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
Pp65	65kDa phosphoprotein

PtS	Phosphatidylserine	Th9	T helper 9 cell
QbD	Quality by Design	Th17	T helper 17 cell
REDMO	Registro de Donantes de Médula Ósea	Th22	T helper 22 cell
RPMI	Roswell Park Memorial Institute	TIL	Tumour Infiltrating Lymphocyte
RWE	Real-World Evidence	TLR	toll-like receptors
SCID	Severe Combined Immunodeficiency	T_N	Naïve T Cell
sCTMP	somatic Cell Therapy Medicinal Product	TNF	Tumour Necrosis Factor
T_{CM}	Central Memory T Cell	TNF-α	Tumour Necrosis Factor α
TCR	T Cell Receptor	Treg	regulatory T cell
T_{EM}	Effector Memory T Cell	UC	Umbilical Cord
T_{EMRA}	Terminal Differentiated Effector Memory T Cell	V	Variable
TEP	Tissue Engineered Product	VST	Virus Specific T Cells
Tfh	Follicular helper T cell	WCB	Working Cell Bank
TGF-β	Transforming Growth Factor β	WJ	Wharton jelly
Th1	T helper 1 cell	WMDA	World Marrow Donor Association
Th2	T helper 2 cell		

PART I: INTRODUCTION AND AIMS

CHAPTER I: INTRODUCTION

1.1 Advanced therapy medicinal products

The development of experimental therapies based on human genes and cells within university hospital environments suffered a breakthrough in the 1990s, with the increase of gene-based therapies for severe combined immunodeficiency and haemophilia, and cell-based therapies for cornea and cartilage repair (Detela and Lodge, 2019).

In the European Union (EU), these pioneering investigational therapies were considered medicinal products, and therefore needed to comply with the Directive 2001/83/EC. This directive provides the legal framework that ensures the quality, safety and efficacy of medicines. It was not until 2007 that the EU Commission established a specific regulation: Regulation (EC) No 1394/2007, especially for this novel medicines defining them as advanced therapy medicinal products (ATMP).

1.1.1 Definition

According to the European Medicines Agency (EMA), ATMP are medicines for human use that are based on genes, tissues or cells and are classified into three main types (European Medicine Agency, 2019a):

- Gene therapy medicinal products (GTMP): refers to genes that lead to a therapeutic, prophylactic or diagnostic effect. They work by inserting 'recombinant' genes into the body.
- Somatic-cell therapy medicinal products (sCTMP): refers to cells or tissues that have been manipulated to change their biological characteristics or cells or tissues not intended to be used for the same essential functions in the body. They can be used to cure, diagnose or prevent diseases.
- Tissue-engineered medicinal products (TEP): refers to cells or tissues that have been modified so they can be used to repair, regenerate or replace human tissue.

ATMP can also be combined, when they contain one or more medical devices as an integral part of the medicine.

It is important to note that the definition of sCTMP does not include manipulation as: cutting, grinding, shaping, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilisation, irradiation, cell separation, concentration or purification, filtering, lyophilisation, freezing, cryopreservation and/or vitrification.

1.1.2 Drug development

The drug development process typically follows this workflow: a drug discovery phase, nonclinical studies, clinical trials and the later regulatory approval, distribution and biovigilance (**Figure 1**). Nonclinical studies, *in vitro* and *in vivo,* provide supportive data, regarding safety and mechanisms of action (MoA), which are necessary before the first use of the drug in humans. This pre-clinical work is maintained after drug discovery, and even when clinical trials start, in order to continuously improve the product. Nonclinical data support the first clinical trial authorisation (CTA) application. If after evaluation of these data, the CTA is granted, then the investigational medicine product can enter clinical trials. However, before each clinical trial phase can start, a CTA must be approved regarding previous clinical results. Moreover, scientific advice can be provided by the competent authority prior to each CTA. Clinical trials are divided into phase I: to test safety, phase II: to test safety and proof of concept (PoC) of the therapeutic mechanism as initial efficacy, and, phase III: to test efficacy. However, in many ATMP, healthy volunteer studies are not amenable for ethical reasons, and the evaluation of safety is combined with an early evaluation of efficacy in a phase I/II study design (Detela and Lodge, 2019). When the clinical trial ends with positive results, data demonstrating quality, safety and efficacy, is used for marketing authorisation application (MAA). If the product is granted marketing authorisation (MA), it can be commercialised, and post-MA studies and real-world evidence (RWE) must be collected in order to maintain the MA.

In the EU, the Committee for Advanced Therapies (CAT) of the EMA is in charge of evaluating MA applications for ATMP and provides scientific recommendations for the classification of ATMP among others (Hanna *et al.*, 2016). The CAT elaborates a draft opinion on the quality, safety and efficacy of the ATMP and then sends it to the Committee for Medicinal Products for Human Use (CHMP). Based on the expertise provided by the CAT, the CHMP adopts an opinion on whether to recommend the authorisation. The European Commission makes the final decision on the basis of the CHMP opinion. All ATMP in the EU are authorised centrally by the EMA or individually by each member state.

Similarly in other countries, the development of these drugs follow the regular procedure as any other pharmacological drug, which requires the approval by the competent authority: e.g. in Spain: the Agencia Española de Medicamentos y Productos Sanitarios (AEMPS); in Europe: the EMA; and in the USA: the Food and Drug Administration (FDA). However, according to sCMTP definition, any accredited clinical centre can readily adopt the use of “minimally manipulated” cell therapy without licensing requirements from the FDA or EMA (Galipeau and Sensébé, 2018).

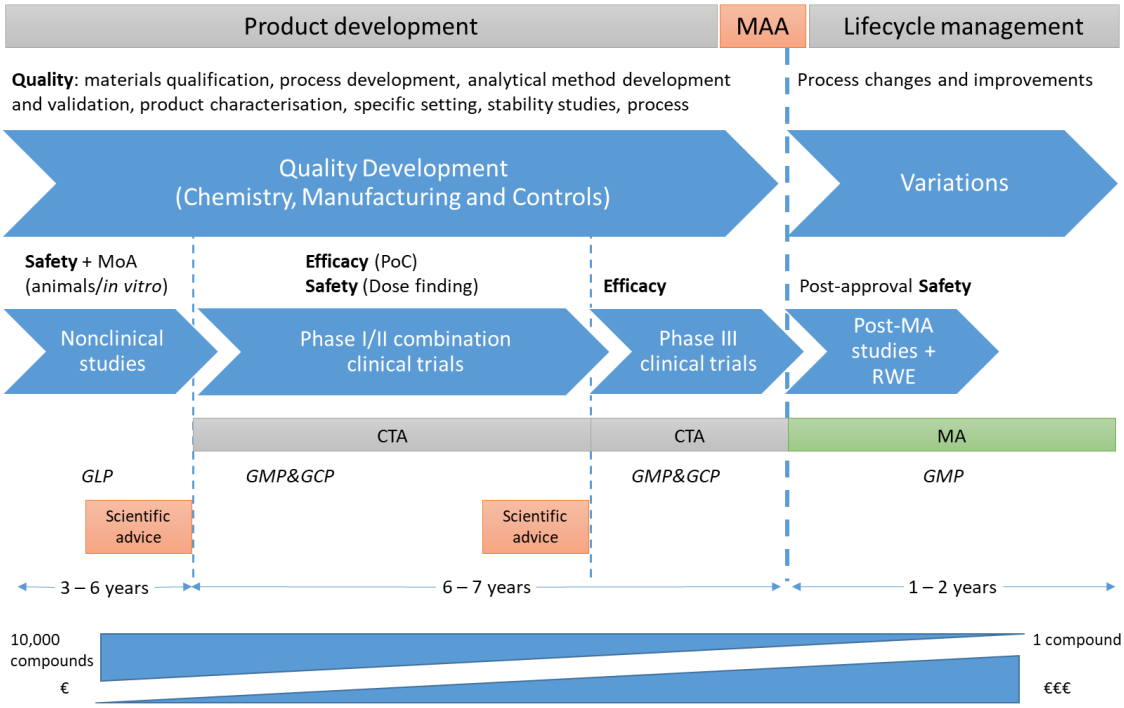

Figure 1. ATMP drug discovery and product development: nonclinical studies, clinical trials, and marketing authorisation. Description of the different phases of clinical studies. Here: MoA: Mechanism of Action, CTA: Clinical Trial Authorisation, MA: Marketing Authorisation, MAA: MA application, PoC: Proof of Concept, RWE: Real-world Evidence, GLP: Good laboratory practices, GMP: Good manufacturing practices, GCP: good clinical practices. This figure has been adapted from Detela and Lodge, 2019.

As illustrated in **Figure 1**, ATMP development faces two major challenges: it is a time-consuming process, where very few candidate drugs reach the regulatory approval, and it has intrinsic high development and production costs (Yu *et al.*, 2018). To accelerate this process, regulatory agencies, like the EMA, the FDA in the USA or the FDA in Japan, have implemented schemes to expedite clinical development as early as possible:

- The FDA in the USA has provided expedited programs including fast-track designation, priority review, accelerated approval, and designation as a breakthrough therapy (U.S. Food and Drug Administration, 2017; Marks and Gottlieb, 2018).
- In Japan, the regulatory requirements enable cell technologies and therapies a time span of 7 years of market approval to demonstrate scientifically sound efficacy, based on data from early trials that demonstrate safety and presumption of efficacy (Galipeau and Sensébé, 2018).
- The EMA has introduced the priority medicines (PRIME) scheme in 2016 for the development of priority medicines addressing unmet clinical needs and/or demonstrating therapeutic innovation (European Medicines Agency, 2018; Detela and Lodge, 2019).

Interestingly, another approach by the FDA is encouraging individual or small groups to collaborate in support for development of regenerative medicine products. Multiple manufacturers, follow the exact same protocol, and perform a multicentre clinical trial (Marks and Gottlieb, 2018).

Moreover, according to the EMA, developers of ATMP are responsible for ensuring compliance with standards set out in the EU legislation and guidelines for good laboratory practices (GLP), good manufacturing practices (GMP) and good clinical practices (GCP) for investigational medicinal products. GLP principles promote quality and validity of generated data. The manufacture of ATMP must be in compliance with the principles of GMP (Regulation (EC) No 1394/2007). ATMP must be prepared in adequate qualified clean room facilities, and throughout the whole process, controls must be performed to ensure the quality, safety and efficacy of the product. To ensure compliance with the regulations, routine inspections are conducted by the regulatory agency, EMA and/or FDA. Additionally, clinical trials worldwide must be performed in compliance with GCP.

To further ensure quality, competent authorities as the EMA and FDA encourage what is called quality by design (QbD). QbD is an approach to guarantee a robust production process and a product free of contaminations, paying special care to the design of the process and minimising the risks of failure to meet product specifications (Del Mazo-Barbara *et al.*, 2016). QbD is based on the principle that, quality should be designed into a product, and that most quality crises and problems relate to the way in which a product was designed in the first place (Yu *et al.*, 2014). More into detail, the EMA makes it clear in the Regulation (EC) No 1394/2007 article 14 (2) that a risk management system is required to be carried out in particular cases. Risk Management can be performed using Failure Mode and Effects Analysis (FMEA) and/or Failure Mode, Effects and Critical Analysis (FMECA). The two methodologies are designed to identify potential failure modes for a product or process before the problems occur in order to assess the risk (Lipol and Haq, 2011).

1.1.3 Applications

At present, emerging ATMP based on immunotherapy for cancer offer an enormous promise. On the one hand, targeting solid tumours such as melanoma, tumour infiltrating lymphocytes (TIL) pioneered by Steven A. Rosenberg and collaborators (Rosenberg, Spiess and Lafreniere, 1986) are being developed. And on the other hand, we find the chimeric antigen receptor (CAR) T-cell first engineered by Zelig Eshhar (Gross, Waks and Eshhar, 1989), based on T-cells that have

a genetically engineered cancer receptor (i.e. CD19 receptor for B-lymphomas and/or acute lymphoblastic leukaemia). Apart from these newly ATMP, we can also find a wide variety of potential ATMP based on dendritic cells, multipotent mesenchymal stromal cells and virus-specific T cells for the treatment of other diseases besides cancer. Dendritic cells are under development for potential ATMP targeting cancer and autoimmune diseases (Jansen *et al.*, 2018; Patente *et al.*, 2019). Moreover, multipotent mesenchymal stromal cells also show great promise for the treatment of degenerative or inflammatory disorders, and for bone regeneration (Sharma *et al.*, 2014; Naji *et al.*, 2019). Virus-specific T cells as a therapy for viral infections is another potential ATMP that has become of interest for many different centres (O'Reilly *et al.*, 2016; Houghtelin and Bollard, 2017; Tzannou *et al.*, 2017; Withers *et al.*, 2018).

Regarding ATMP under development, almost half correspond to sCTMP, and the rest to gene therapies or TEPs in similar percentage, with small representation of combined products (Hanna *et al.*, 2016). Marketing authorisation for ATMP has been granted in different countries as the USA, Japan or the EU. In the USA, there are 17 MA for therapies based on cells, tissue-engineered and gene products (U.S. Food and Drug Administration, 2019), and, in Japan, 4 products hold MA based on cell therapy and TEP (Cuende *et al.*, 2018; Detela and Lodge, 2019). Focusing on the EMA, 8 ATMP are already on the market. Other countries like Australia, Canada, India, New Zealand and South Korea also have products based on cells, genes or tissue engineered, that hold market authorisation (Cuende *et al.*, 2018). So there is a global trend in the acceptance of this type of medicines for their clinical use.

Cell therapy products can be used in the autologous context: when donor and patient are the same individual; or in the allogeneic context: when donor and recipient are different individuals. Each use has certain advantages and disadvantages (Vives and Mirabel, 2019). Allogeneic products present a more sustainable model, allowing the generation of large numbers of doses that can be cryopreserved for a later use with most quality controls performed. However, it can present some difficulties in terms of compatibility that are nonexistent in the autologous context. Furthermore, autologous use increases the cost implied in the production of personalised medicines, is time-consuming and quality controls can present complications. According to Cuende and collaborators, cell, tissue, and gene products with MA are more frequently used worldwide in the autologous context, followed by the allogeneic use and next, the gene vectors (Cuende *et al.*, 2018).

If we look back into the number of cellular therapies in articles and clinical trials, there has been an exponential growth over the years (**Figure 2**). Although only a small number of cellular therapies are commercialised to date, there are high expectations that cell therapy is a promising growing field. The recent movements for streamlining drug development by the different competent authorities together with stakeholders' efforts can expedite ATMP to reach the market.

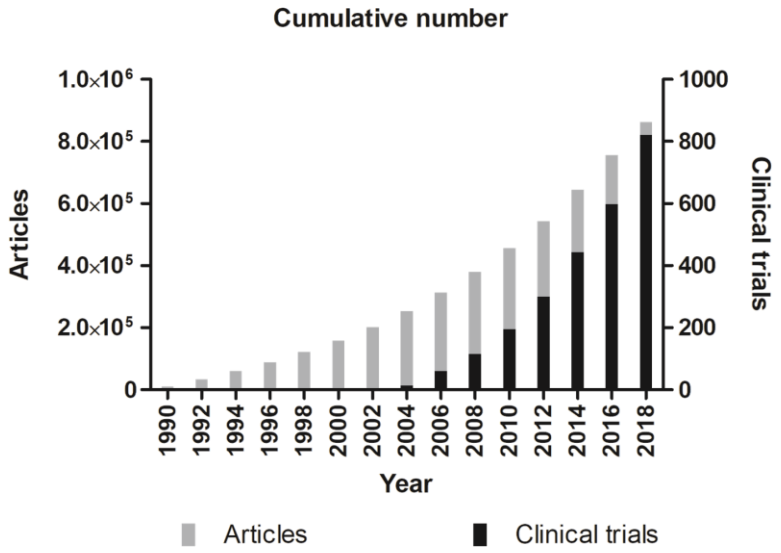

Figure 2. Number of cell therapies in PubMed publications (https://www.ncbi.nlm.nih.gov/pubmed/) and Clinical Trials (www.clinicaltrials.gov). Search in PubMed and Clinical Trials: "cell therapy OR cellular therapy". Date: 30/05/2019. *Clinical trials.gov first version was made available to the public in 2000.

1.1.4 Use

As mentioned before, the extremely long process for a candidate drug to reach the MA is a huge concern for both researchers and clinicians, and can be a hurdle for those patients that have no alternative treatment. To overcome these limitations, EU regulations allow patients in the EU region to also have access to the drugs in circumstances other than when an ATMP is approved or when enrolled in a clinical trial. These circumstances include:

1) Compassionate use according to article 83 of the Regulation (EC) Nº 726/2004 of the European Parliament (Regulation (EC) No 726/2004, 2004). Compassionate use is intended for patients with a chronically or seriously debilitating disease or whose disease is considered to be life threatening, and who can not be treated satisfactorily by an authorised medicinal product. The medicinal product concerned must either be the subject of an application for a marketing authorisation or must be undergoing clinical trials.

2) Another possibility is the hospital exemption, which is, according to article 3(7) of Directive 2001/83/EC (The European Parliament and the Council of the European Union, 2001) an ATMP prepared on a non-routine basis according to specific quality standards and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner. The manufacture of these products shall be authorised by the competent authority of the Member State. At present, at least 32 ATMP are available in individual EU member states via Hospital Exemption (Eder and Wild, 2019).

3) Moreover, the EMA facilitates the development and authorisation of medicines for rare diseases, called orphan drugs, for the diagnosis, prevention or treatment of life-threatening or very serious conditions (European Medicine Agency, 2019b). It is in this latter category, 6 out of 8 ATMP are found on the EU market.

This work is focused on two different ATMP, more specifically sCTMPs: multipotent Mesenchymal Stromal Cells (MSC) and Virus-Specific T cells (VST). In order to describe MSC it is necessary to first illustrate the stem cell classification.

1.1.5 Stem cell classification

According to Wagner and Ho, stem cells can be classified into four categories because of their differentiation capacity (Wagner and Ho, 2007):

a) Totipotent stem cells, that refers to the zygote, is produced by the fusion of egg and sperm cells and has the ability to develop into all specialised cells that make up the adult animal as well as extraembryonic tissue.

b) Pluripotent stem cells are derived from the blastocyst. Particularly, pluripotent cells are isolated from the inner cell mass. They are called embryonic stem cells and have the ability to differentiate into cells from the three germ layers: endoderm, mesoderm or ectoderm.

c) Multipotent stem cells are cells that can only differentiate into one of the three main germ layers. MSC are able to differentiate into different mesodermal cell lineages such as osteoblasts, chondroblasts and adipocytes.

d) Unipotent stem cells are cells that can only differentiate into one type of cells.

Human embryonic stem cells (ESC) were first reported in 1998 by Thomson and collaborators (Thomson *et al.*, 1998). ESC are derived from 5-day preimplantation embryos, more precisely

from the inner mass of the blastocyst. They are described as pluripotent cells capable of unlimited, undifferentiated proliferation *in vitro*. Although applicability of embryonic stem cells can offer new therapies, there are many ethical concerns. ESC research in Europe to generate human pre-embryos and embryos exclusively for experimental purposes is regulated by each member state yet forbidden in Spain by law 14/2007 (EuroStemCell, 2019). However, collection of ESC for therapy or research is allowed if that does not imply the creation of a pre-embryo or embryo for this purpose. Moreover, tumourigenicity of ESC is a major hurdle for the translation of this cells into the clinic (Ben-David and Benvenisty, 2011).

An alternative source of pluripotent stem cells was described in 2006 when Yamanaka discovered that mature cells can be reprogrammed into an immature pluripotent stem cell state (Takahashi and Yamanaka, 2006). He demonstrated the induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by introducing four factors: Oct3/4, Sox2, c-Myc and Klf4. This discovery and the later application to human cells has led Shinya Yamanaka to win the novel prize in 2012, together with John B. Gurdon, who discovered back in 1962 that cell specialisation was reversible (Nobel media AB, 2019b). Reprogrammed cells discovery, latter referred as induced pluripotent stem cells (iPSC), was followed by a plethora of publications of other researchers rushing to use the technique and further developing the reprogramming of mature cells. With the aim to explote the model of umbilical cord (UC) banking in our center, the Blood and Tissue Bank, the IPS-PANIA project was created in collaboration with the Center of Regenerative Medicine in Barcelona, as an investment in regenerative medicine, for the generation of an iPSC bank from UC (Alvarez-Palomo *et al.*, 2019). Many years later, the use of iPSC still has certain associated concerns. On the one hand, retro- or lentiviral vectors used for genes delivery poses many problems, including insertional mutagenesis, residual expression and re-activation of reprogramming factors, uncontrolled silencing of transgenes, apoptosis, cell senescence, and strong immunogenicity (Hu, 2014). On the other hand, ectopic transcription of these genes can lead to neoplastic development from cells derived from iPSCs, as the expression of these is associated with the development of multiple tumours known in oncogenetics (Medvedev, Shevchenko and Zakian, 2010). Although presenting some similarities with ESC in terms of pluripotency, self-renewal capacity and tumorigenic traits, ESC and iPSC are not identical (Ben-David and Benvenisty, 2011). Yet the development of new strategies to resolve these problems grants iPSC hopeful future ATMP. Although reprogramming techniques allow the induction of MSC from iPSC (Hynes *et al.*, 2014) and other cell types (Spitzhorn *et al.*, 2019), this has not reached the clinical setting yet and MSC are typically isolated from vascularised human tissues.

Part of this work is focused on multipotent mesenchymal stromal cells. The nomenclature of these cells was suggested in 2005 by the International Society of Gene and Cell Therapy (ISCT) (Horwitz *et al.*, 2005) a change from "multipotent mesenchymal stem cells", as initially referred, to "multipotent Mesenchymal Stromal Cells". This clarification was performed, as MSC do not seem to meet generally accepted criteria for stem cell activity (Horwitz *et al.*, 2005). The term stem implies cellular properties like long-term self-renewing with capacity of differentiation into specific, multiple cell types *in vivo*. On the one hand, MSC isolated from the BM are a heterogeneous cell population, with only a little fraction of adherent cells that can generate fibroblast colony-forming units (CFU-F) *in vitro.* On the other hand, *in vivo* evidence of long-term survival with self-renewal capacity and tissue repopulation with multi-lineage differentiation are proving to be far more challenging than for hematopoietic stem cells. Moreover, indication of MSC' source, as cells from mesenchymal origin, seems more appropriate. MSC are found *in situ* within the supportive stromal compartment of resident tissues. Using the nomenclature multipotent mesenchymal stromal cells for this population seems more accurate without implying unproven biologic or therapeutic potential (Horwitz *et al.*, 2005). Throughout this work, when referring to mesenchymal stem cells, we will be referring to multipotent mesenchymal stromal cells.

1.2 Multipotent mesenchymal stromal cells

MSC where first described by Friedenstein and collaborators (Friedenstein *et al.*, 1968), who isolated them from bone marrow (BM). In order to obtain BM-MSC, a puncture in the iliac crest is performed for the aspiration of the BM. This invasive procedure is painful and can be associated to infections. Therefore, and due to commercial interests in MSC that can be secured by patents, researchers put their efforts on finding alternative sources of MSC.

Thanks to those combined endeavours, nowadays, human MSC can be obtained from a wide range of different sources such as: BM, adipose tissue, UC blood and Wharton jelly (WJ) among others (Klingemann, Matzilevich and Marchand, 2008; Murrell *et al.*, 2015; Oliver-Vila *et al.*, 2016). Despite the existence of alternative MSC sources, BM-MSC are still the most studied and used in cell therapy since they were first developed (Guerrouahen *et al.*, 2019; Naji *et al.*, 2019). BM is also the most used source of MSC in clinical trials, followed by adipose tissue and, emerging MSC from puerperal discards (Galipeau and Sensébé, 2018). Included in this later source, we find the WJ-MSC, which are of interest due to the lack of somatic mutations and considered more primitive than adult MSC (Krause, Lozano and Lim, 2019).

1.2.1 Definition

With the increasing number of publications on MSC as indicative of related research, it became necessary to standardise the protocols of MSC procurement and to properly characterise those fibroblast-like morphology cells. In 2006 the ISCT defined MSC for their (Dominici *et al.*, 2006):

- capacity to adhere to plastic
- *in vitro* differentiation into osteoblasts, adipocytes and chondroblasts
- expression of markers: CD105, CD73, CD90 and lack of expression of: CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR

The latter surface antigen, HLA-DR, has been a matter of debate for its randomly expression in BM-MSC and is further discussed in Chapter III of this thesis. Furthermore, it has been under discussion if MSC require previous activation with cytokines, like interferon gamma (IFN-γ), with associated expression of HLA-DR, to induce the immunomodulation potential (Le Blanc *et al.*, 2003).

1.2.2 Multipotential capacity

As described by the ISCT, multipotentiality is the capacity of MSC to differentiate *in vitro* into adipogenic, chondrogenic and osteogenic lineages of the mesoderm. The differentiation of MSC into a number of mesodermal cell types is illustrated in **Figure 3** and is referred to as the mesengenic process (Murrell *et al.*, 2015). Although MSC can differentiate into many different cell types, there is certain scepticism on the differentiation capacity into lineages other than osteo-, chondro- and adipogenic cells (Schäfer, Spohn and Baer, 2016). It is believed, for the localisation of these cells *in situ*, in perivascular locations, and similarity in the markers expressed, that MSC are pericyte-derived cells (Caplan, 2008).

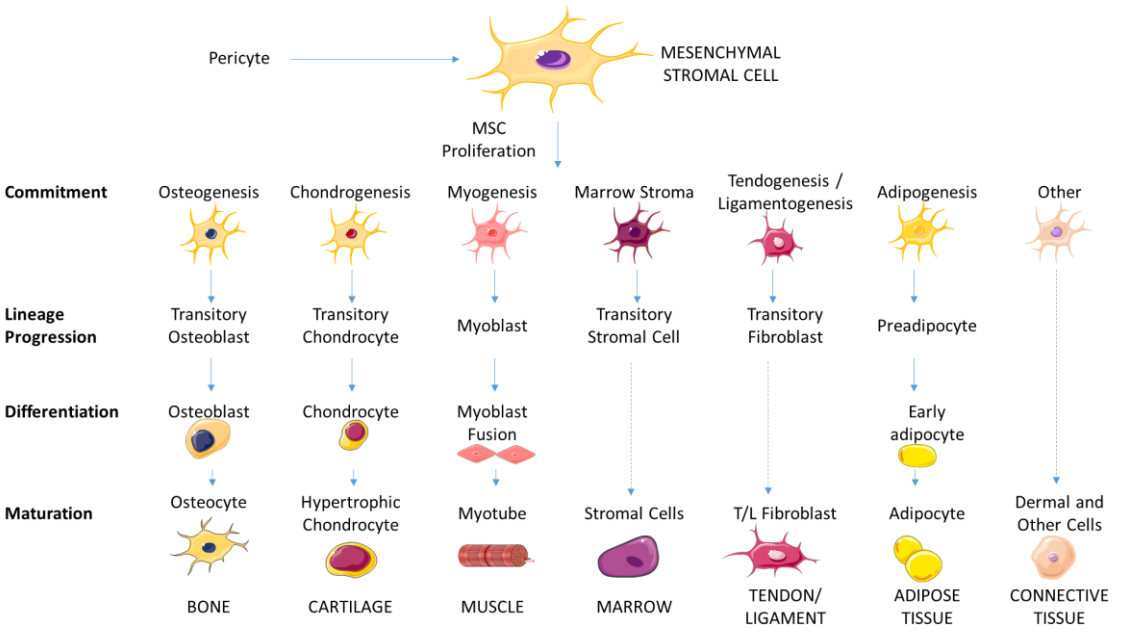

Figure 3. Mesengenic process. Source of multipotent mesenchymal stromal cells and differentiation into the different mesenchymal tissues. This figure has been adapted from Murrell et al., 2015. This figure has been produced using Servier Medical Art (http://smart.servier.com).

Multipotentiality was the feature that draw the attention to MSC in the first place, when efforts were focused on tissue repair by local engraftment and/or differentiation of MSC via direct injection into the target tissue or organ (Caplan and Correa, 2011; Caplan *et al.*, 2019). For example, MSC were infused in an *in vivo* model of osteonecrosis of the femoral head, where minor MSC persistence was found in the host tissue 3 months after the treatment (Caminal *et al.*, 2017). Approaches aiming at MSC engraftment were centred on MSC administration into a specific site rather than intravenous infusion (Hamidian Jahromi and Davies, 2019). However, MSC long term persistence *in vivo* seems rather unlikely to happen, and researchers have focused on other properties of MSC to exert their function, as the immunomodulation capacity of these cells (Caplan *et al.*, 2019).

1.2.3 Immunomodulation capacity

MSC function as cellular modulators gained more value and a broader application scenario (Caplan and Correa, 2011). The most extended theory today, is that MSC interact with the host tissue via direct contact or through paracrine factors, as extracellular vesicles (EV) and exosomes (Galipeau and Sensébé, 2018; Caplan *et al.*, 2019). This interaction with other cell types is what confers MSC the immunomodulation potential. It is now known that the immunosuppressive activity of MSC is not constitutive, but must be triggered by the inflammatory environment to which MSC are exposed. In this context, it is believed that patients receiving MSC play a determinant role in MSC clinical efficacy (Martin *et al.*, 2019).

Currently, MSC are of interest in regenerative medicine applications, not only for tissue regeneration but also for graft versus host disease (GvHD) treatment, among other immunological diseases (Horwitz, Andreef and Frassoni, 2006; Galipeau and Sensébé, 2018; Guerrouahen *et al.*, 2019). The immunomodulatory effects of MSC on immune system cells presented these cells as candidates for such applications.

The immunologic properties and signalling pathway of MSC, not only to T cells, but also to natural killer (NK) cells, B cells, dendritic cells (DC), monocytes, macrophages, and other T cell subsets, and the different soluble factors involved is summarised in **Table 1**.

Table 1. Immunomodulatory effects of MSC therapy on immune system cells and soluble factors involved. This table is taken from Amorin et al., 2014.

Cell type	Effects of MSC therapy	Soluble factors involved
T lymphocytes	Suppresses T-cell proliferation induced by cellular or nonspecific mitogenic stimuli Alters the cytokine secretion profile of effector T cells Promotes expansion and activity of regulatory T cells Induces apoptosis of activated T cells Regulatory T-cell generation	IL-1β TGFβ1 HGF PGE1 IDO LIF IGF HLAG CCL1
B lymphocytes	Inhibits B cell proliferation Affects the chemotactic properties of B cells Suppresses B cell differentiation	IFN-γ IL-6
NK cells	Alters the NK cell phenotype, suppresses NK cell proliferation, cytokine secretion, and cytotoxicity against HLA class I-expressing targets	TGFβ IDO HLAG5 PGE2
Dendritic cells	Influences the differentiation, maturation, and role of DCs differentiated from monocytes Suppresses DC migration, maturation, and antigen presentation	M-CSF
Macrophages	M2 macrophage recruitment Conversion of pro-inflammatory M1 macrophages into anti-inflammatory M2 macrophages Attenuates macrophage inflammatory response	CCL3 CCL12 CXCL2 PGE2 KYN

Related to effects of MSC in therapy, MSC showed remarkable therapeutic effects in preclinical disease models, safety and toxicity free treatment, offering an enormous potential (Salem and Thiemermann, 2010; Schäfer, Spohn and Baer, 2016). However, many hypotheses have been proposed on the exact MSC MoA. The latest one emerged from the observation that when MSC are infused intravenously they quickly generate emboli in the lungs, and are then cleared in a matter of hours (Bianco *et al.*, 2013). Although MSC have been found to initially accumulate in

the lungs, some authors have observed that a proportion of cells are able to escape and migrate to other organs, like the liver or the spleen, within 24-48h (Gholamrezanezhad *et al.*, 2011; Mukherjee *et al.*, 2012). However, these cells rarely engraft, and therefore whatever paracrine effect they exert must be short lived and may be related to cell death (Bianco *et al.*, 2013). In this sense, the efferocytosis theory has emerged and is gaining more and more weight (Galipeau and Sensébé, 2018). According to this hypothesis, as illustrated in **Figure 4**, infused MSC would send signals to the injured (inflammatory) environment promoting anti-inflammation and tissue regeneration. In order to act on other cells of the immune system, MSC are able to secrete a wide variety of soluble factors. Shortly after that, MSC would enter apoptosis and express certain molecules, such as phosphatidylserine (PtS), attracting MSC engulfment by phagocytic macrophages. Macrophages would in turn secrete factors that promote immune tolerance. Many studies are conferring more relevance to macrophages, suggesting that MSC require the presence of macrophages in order to have a therapeutic effect (Carty, Mahon and English, 2017). In this sense, MSC modulate inflammatory M1 macrophages, promote anti-inflammatory M2 macrophages, and can induce the conversion of monocytes or M1 macrophages into an M2-, IL-10-producing population (Carty, Mahon and English, 2017).

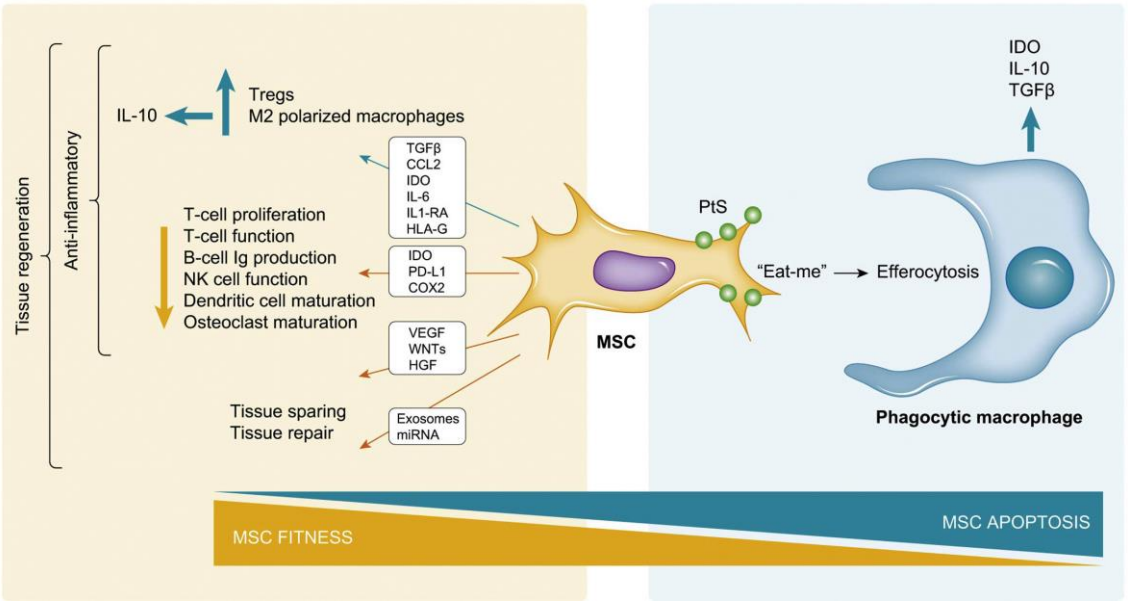

Figure 4. MSC function theory. MSC secrete anti-inflammatory paracrine factors that augment T-regulatory cell (Treg) function and M2 macrophage polarisation as well as suppress effector lymphoid cell function. MSC can also produce morphogens and exosomes that promote tissue repair. MSC progressing to apoptosis express "eat me" signals such as phosphatidylserine (PtS) and are susceptible to the alternate efferocytosis pathway, where their engulfment by phagocytic macrophages leads to expression of immune tolerance factors. The reciprocal relationship between fitness and apoptosis dictates whether MSC metabolism or their efferocytosis, respectively, is responsible for their *in vivo* biological effects. This figure is taken from Galipeau and Sensébé, 2018.

Galleu and colleagues (Galleu *et al.*, 2017) also suggested that MSC enter apoptosis in order to produce immunosuppression. They observed this in a mouse model of GvHD, where MSC entered apoptosis in the presence of cytotoxic T cells by the cytotoxic granules, to later be engulfed by phagocytes that produce indoleamine 2,3-dioxygenase (IDO) and then ultimately deliver MSC immunosuppressive activity.

Recently, MSC have also been described to communicate with other cell types by the secretion of EV (Katsuda *et al.*, 2013; Rani *et al.*, 2015; Wang *et al.*, 2016), which can promote tissue repair and modulate immune responses. The hypothesis is that paracrine mechanisms of MSC are responsible of the tissue repair and regeneration, mainly through EV secretion. EV are usually secreted with the aim to mediate intercellular communication. Similarly to the ISCT for cell and gene products, the International Society of Extracellular Vesicles (ISEV) was founded in 2012 with the aim to advance EV research globally (International Society for Extracellular Vesicles, 2019). Hu and collaborators tested EV isolated from UC blood into a senile osteoporotic mice model (Hu *et al.*, 2019). They observed that EV enhanced osteoblast formation and reduced osteoclast formation, indicating that UC-EV ameliorate age-related bone loss. These findings together with other studies leave open the broad spectrum of possible applications of EV.

1.2.4 Applications

MSC have been used in the treatment of a wide range of diseases, isolated from different sources and in both allogeneic and autologous context. When searching for “mesenchymal stem cells” in www.clinicaltrials.org [Date of search: 12/08/2018], the main topics divided by category and number of studies are: musculoskeletal diseases (n=168), immune system diseases (n=131), central nervous system diseases (n=119), and wounds and injuries (n=117), followed by a wide range of other diseases (U.S. National Library of Medicine, 2019), showing an excellent safety profile (Lalu *et al.*, 2012).

Current MSC applications under exploration in clinical trials are very broad (Salem and Thiemermann, 2010; Sharma *et al.*, 2014; Martin *et al.*, 2019). One of the most explored is osteogenesis imperfecta, leveraging MSC properties for tissue engineering applications like our approach with WJ-MSC for bone regeneration, described in another PhD project (Vivas Pradillo, 2018) whose patent was filed (Ref. No.: EP17382614.0) but not included as part of this work. Regarding the immunologic properties of these cells, they provide interesting applications for immune system diseases (Guerrouahen *et al.*, 2019): autoimmune diseases such as multiple sclerosis, and inflammatory diseases like spinal cord injury. Another major application that is

gaining interest due to the morbidity and mortality of the disease, and the immunomodulative properties of MSC, is for the GvHD treatment.

GvHD was first described by Billingham in 1966 (Billingham, 1966), who postulated that in order to develop GvHD: a) the donor must contain immunocompetent cells, b) the recipient must express tissue antigens not found in the donor and c) the recipient must be unable to reject the transplanted cells due to being immunologically suppressed. It is known that the immunological cells here are T cells (Choi, Levine and Ferrara, 2010). Therefore GvHD occurs when mature T lymphocytes from the donor react with recipient alloantigens. Thanks to the immunomodulatory properties of MSC they are a great promise to inhibit the T cells response in this context.

This work is focused on the development and characterisation of two products of advanced cell therapies. MSC can be applied in a wide variety of pathologies. For instance, in the context of post-HSCT complications, they can be used not only for GvHD, but also for bone regeneration in patients that have been treated with corticosteroids to avoid GvHD and have consequently suffered from avascular necrosis (Zhao *et al.*, 2012; Guerado and Caso, 2016; Im, 2017).

All clinical trials to date show a high heterogeneity in MSC treatments, with MSC obtained from different sources, through multiple manufacturing protocols and with different routes of delivery (Martin *et al.*, 2019), which difficult MSC study comparisions. These could explain the controversy in MSC results, and the fact that MSC is not yet a consolidated treatment. In the effort to standardise MSC as a cell therapy product, they have to match certain acceptance criteria as addressed later in this work. As an attempt to improve the product standardisation we have developed a quality control test to assess the immunomodulatory capacity of MSC that is being used as a product release criteria in our facility.

1.3 Lymphocytes

The field of immunotherapy is quickly evolving. Lymphocytes have been used for the treatment of numerous indications including cancers, autoimmune disorders and infectious disease (Dwivedi *et al.*, 2018). As already mentioned, an important example of these are the development of CAR T-cells, TIL or VST. Because of these and other discoveries the field of immunotherapy and cell therapy is growing fast, with more researchers developing new therapies. The second part of the thesis is centred on lymphocytes. In particular, the work is focused on virus specific T lymphocytes.

1.3.1 Lymphopoiesis

The most primitive mammalian blood-forming cell is designated as the hematopoietic stem cell (HSC), which has the capacity to: a) develop into all populations of blood cells (differentiation) and b) self-renewal. All T, B, and NK lymphoid cells ultimately develop from HSC (Beutler *et al.*, 2006). Precursor cells migrate from the bone marrow to the thymus to become T lymphocytes. T cells are divided in different types such as, cytotoxic T cells ($CD8^+$), natural killer T cells ($CD56^+CD8^+$), T helper cells ($CD4^+$), regulatory T cells ($CD4^+CD25^+FOXP3^+$) or gamma delta T cells ($\gamma\delta$ TCR^+) (K. Abbas, H. Lichtman and Pillai, 2008).

1.3.2 T cell receptor

The capacity of T cells to recognise different antigens derives from the recombination of VDJ region gene segments, known as variable (V), diversity (D) and joining (J) genes, which takes place in the thymus. The random assembly of different gene segments results in the generation of unique receptors contributing to the enormous diversity of antigen receptors that recognise a wide variety of molecules (**Figure 5**). The diversity of T cells' antigen receptor is restricted by thymus selection: T cells are selected in the thymus based on sufficient, but not excessive recognition of self-peptides presented on major histocompatibility complexes (MHC) (Schober, Buchholz and Busch, 2018). The recombination of these genes (V, D, J) enables the adaptive immune system to address a wide variety of infections. The antigen receptor of T cells, called T cell receptor (TCR), can be formed by two different chain dimers: alpha beta (95% of the T cells) or gamma delta (5% of T cells) (Rådestad *et al.*, 2014). Most common alpha beta (αβ) T cell receptors are formed by two different chains, the light chain or alpha, product of VJ recombination and the beta or heavy chain, product of VDJ recombination, with additional nucleotide deletions and insertions. The union between region V and constant (C) conform the complementary determining region 3 (CDR3), which plays an important role in the MHC recognition, plus it presents the greater variability. Albeit this huge diversity, there is a limited number of T cell clones present in an individual at a time to recognise the vast diversity of peptide-MHC ligands. The recognition of these peptides is explained not only by the TCR high level of antigen specificity but by its high level of plasticity (Joshi, Suresh and Chauhan, 2001). This phenomenon, the TCR binding cross-reactivity, is explained by a CDR3 loop flexibility caused by CDR3α which can undergo rearrangements to adapt to structurally different peptide residues, conferring T cells the ability to recognise almost any peptide within a time frame compatible with the speed at which infectious agents spread (Reiser *et al.*, 2003).

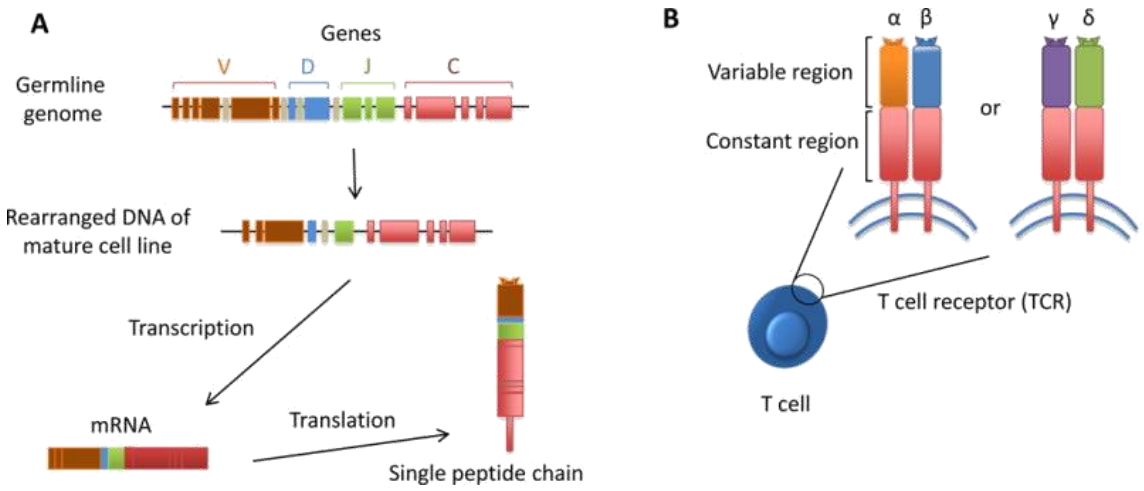

Figure 5. Recombination of variable (V), diversity (D) and joining (J) gene segments generates unique T cell receptors. A) TCR specificity and diversity is due to the VDJ recombination that takes place during lymphocyte development. Further diversity is produced by deletion and/or insertion of nucleotides at the junctions of those segments. B) TCR are heterodimeric and can be formed by the combination of either α and β chains (αβ TCR) or γ and δ chains (γδ TCR). Figure obtained from Matos et al., 2017.

Immunosequencing of the TCR is a rapidly progressing technique that allows sensitive and accurate identification and quantification of every distinct T-cell clone (Matos, de Rie and Teunissen, 2017). High-throughput sequencing of the TCR can be performed to identify TCR clones that expand upon antigenic stimulation, and the association of these TCR sequences with certain antigens (Emerson *et al.*, 2017). Another common application for TCR identification is to track T cells adoptively transferred and to determine their persistence after infusion (Keller *et al.*, 2019).

1.3.3 HLA system

The ability of the adaptive immune system to adequately address an infection relies on the generation of antigen-specific TCR through VDJ recombination as described earlier. T lymphocyte antigen recognition is restricted by the MHC, in humans called the human leukocyte antigen (HLA), and is conferred by the HLA genes.

The HLA genes were first described by Jean Dausset in 1958 (Dausset, 1958), who was awarded later in 1980 the Medicine Nobel prize jointly with George Davis Snell and Baruj Benacerraf “for their discoveries concerning genetically determined structures on the cell surface that regulate immunological reactions” (Nobel media AB, 2019a). HLA genes are closely linked on chromosome 6 and are inherited as haplotypes. It is now known that HLA is organised in class I (-A, -B, -C, -E, -F, -G) and class II genes (-DR, -DQ, -DP, -DM, -DO), and that the three forms (-DR, -DQ, -DP) of class II have two genes, A and B. HLA molecules present peptides to T cells: HLA

class I present antigens to $CD8^+$ cells and HLA class II to $CD4^+$ cells. Cells expressing HLA class I include all nucleated cells while HLA class II molecules are only expressed by antigen-presenting cells (APC) (i.e. dendritic cells). HLA molecules are characterised for being highly polymorphic, which in the field of transplantation and donor-receptor matching, makes it laborious to find a matching donor. Foreign HLA molecules can be highly immunogenic in the presence of T cells, leading to direct or indirect (through an antigen presenting cell) T-cell activation, and causing a strong immune response observed during rejection of allografts (Turner, 2004). The nomenclature of the HLA system is in general terms a HLA prefix (HLA-), followed by the gene (HLA-A), the allele group (HLA-A*02) and the specific HLA protein (HLA-A*02:101) (Anthony Nolan Research Institute, 2018). The identification of the HLA of an individual, called HLA typing, can be performed at low resolution, when it is only analysed at the antigenic level (i.e. HLA-A*02) or at high resolution typing when it is analysed at the allelic level (i.e. HLA-A*02:101). Every individual has its own HLA genotype with two haplotypes, one inherited from the mother and one inherited from the father. Moreover, the number of HLA alleles discovered for each gene is still increasing, being described to date 17,191 alleles for HLA class I and 6,716 alleles for HLA class II (European Bioinformatics Institute, 2019). In the context of HSCT, a HLA identical or the highest HLA compatibility is considered the optimal and first choice (Howard *et al.*, 2015). Therefore, given the high variability in the HLA alleles, it is useful to have registries that facilitate finding compatible donors, such as the "Registro de Donantes de Médula Ósea (REDMO)" in Spain, that consists of more than 350,000 BM HLA-typed donors (Fundació Josep Carreras contra la Leucèmia, 2019). Moreover, associations like the World Marrow Donor Association (WMDA) (World Marrow Donor Association, 2019), consisting of different organisations, including REDMO among them, were founded to promote global collaboration and best practices.

1.3.4 T cell subpopulations

T cell lymphocytes have different subpopulations according to their differentiation state: cells initiate as naïve T cells (T_N) and develop into central memory T cells (T_{CM}), effector memory T cell (T_{EM}) and terminal differentiated effector memory T cells (T_{EMRA}) (Gattinoni and Restifo, 2013; Flynn and Gorry, 2014; Golubovskaya *et al.*, 2016) (**Figure 6**). Specific cell markers are used for identifying these cell populations and each T cell subpopulation has different properties, as illustrated in **Figure 6**. Naïve cells have not yet encountered foreign antigens and have consequently no memory function. These cells are less specialised but show a higher capacity of self-renewal and proliferation. When naïve T cells meet an antigen through an APC, they activate, increase in number, and differentiate into effector cells to eliminate the pathogen

(Golubovskaya *et al.*, 2016). Along with T cell differentiation, T_N properties are diminished and others appear, as effector and memory function, and senescence. While effector cells are short-lived cells, memory cells have a long-term persistence. Among memory cells we can find some of them trafficking through lymphoid tissues (T_{CM}), which is a rare population in adult humans, or trafficking through non-lymphoid tissues and the blood (T_{EM} and the terminal senescence T_{EM}: T_{EMRA}), populations that correspond to the majority of non-naïve T cells in adult humans (Jameson and Masopust, 2018). Memory cells have a more specific and faster response upon pathogen re-exposure. It is this capacity and the following features that make T_{EM} interesting for adoptive immunotherapy: a) the presence of previous expansion and activation, b) persistence in the absence of antigen, and c) increased activity upon re-exposure to antigen (Golubovskaya *et al.*, 2016). There are many T cell subsets described, but another T cell subset worth mentioning is the tissue-resident memory T cell (T_{RM}), population that is maintained long-term *in situ* without trafficking the circulating pool and with surveillance on specific regions (Jameson and Masopust, 2018). The later stage of T cell differentiation is when the effector function is lost and we find T cell exhaustion. At this point, T cell subsets partially or completely lack the ability to produce large amounts of interferon gamma (IFN-γ) or beta-chemokines or to degranulate (Wherry, 2011).

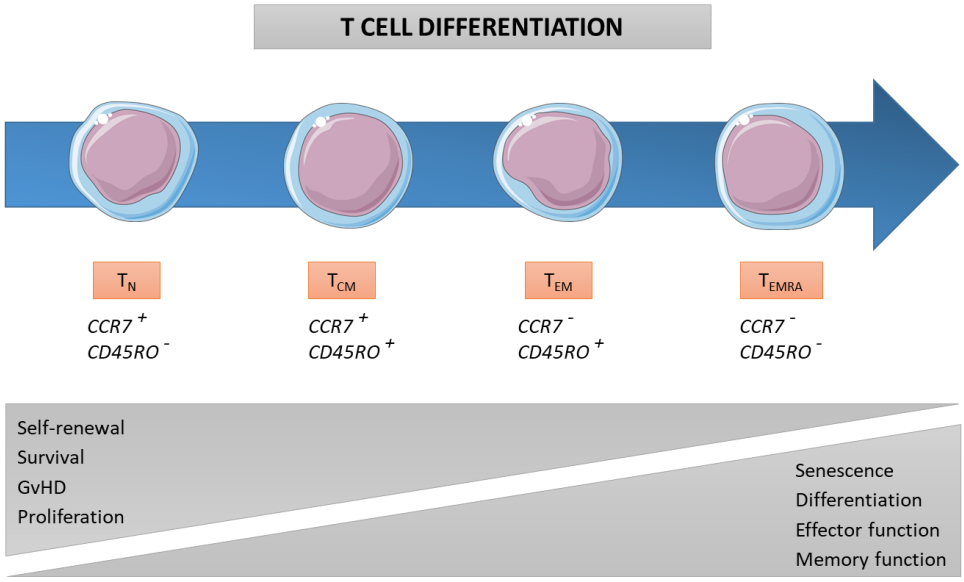

Figure 6. T cell differentiation: markers for T cell subset identification and properties of T cells among the differentiation state. Herein: naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) T cell subsets. This figure is adapted from Gattinoni and Restifo, 2013 and Golubovskaya et al., 2016. This figure has been produced using Servier Medical Art (http://smart.servier.com).

1.3.5 T cell activation, signalling and effector function

T lymphocytes carry out the cell-immune response in what is called the adaptive, acquired or specific immune system. This term is assigned because it has immune memory and acts specifically against each antigen, abilities conferred in part by the TCR recombination and T cell differentiation explained earlier. T lymphocytes recognise antigens, through the TCR, that are presented either by professional antigen presenting cell (APC) by means of MHC class II or by nucleated cells through MHC class I (**Figure 7**). For the first interaction with the antigen, T cells require co-stimulatory molecules expressed by the APC in order to proliferate and differentiate. The most characterised co-stimulatory signal is driven by the molecule CD28 in the surface of T lymphocytes, which binds to co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed in activated APC (K. Abbas, H. Lichtman and Pillai, 2008). Only dendritic cells, macrophages, and B cells are able to express both classes of MHC molecule and co-stimulatory cell-surface molecules that drive the clonal expansion of naive T cells and their differentiation into armed effector T cells (Janeway *et al.*, 2001). Upon cell activation, TCR signalling can take place through: 1) proximal signalling complex, 2) calcium influx or 3) Ras activation (Smith-Garvin, Koretzky and Jordan, 2009). In general terms, T cell activation and TCR signalling induces protein synthesis. T cells and other cells of the immune system secrete proteins, named cytokines, in order to mediate cell function. According to the profile of cytokines secreted by $CD4^+$ T cells they can be classified in general terms into T helper 1 cells (Th1) and T helper 2 cells (Th2). Th1 cells produce pro-inflammatory cytokines, IFN-γ, tumour necrosis factor α (TNF-α), and IL-2, which activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses; while Th2 cells, produce IL-4, IL-5, IL-10 and IL-13, which are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses (Romagnani, 1999; Golubovskaya *et al.*, 2016). Other $CD4^+$ T cell subsets characterised by different cytokine profiles include: T helper 9 cell (Th9), T helper 17 cell (Th17), T helper 22 cell (Th22), regulatory T cell (Treg) and follicular helper T cell (Tfh). All these subsets are differentiated from naïve $CD4^+$ T cells by specific cytokines and play an important role in the immune response. When these cells are differentiated they can secrete cytokines with pro- or anti-inflammatory functions, for example: Treg cell subset secretes IL-10, a cytokine with immunosuppressive function among other cells (Golubovskaya *et al.*, 2016). Treg cells inhibit the differentiation, function, and maduration of effector T cells and DC, in order to prevent autoimmunity and establish peripheral tolerance (Mattar and Bieback, 2015).

Regarding effector function, cytotoxic T lymphocytes (CTL) are specialised in killing virus-infected cells by the secretion of perforins and granzymes stored in cytotoxic granules. Perforin, is a protein that binds to the target plasmatic membrane and favours the entrance of granzymes in the target cell, which are enzymes that activate caspases in the proteolytic pathway in order to induce apoptosis (K. Abbas, H. Lichtman and Pillai, 2008). Another mechanism to kill infected cells follows the Fas/FasL pathway. $CD8^+$ and $CD4^+$ T cells can express Fas ligand (FasL) and are capable of inducing apoptosis by binding to Fas expressed by some target cells (Janeway *et al.*, 2001). Other CTL-killing mechanisms involve tumour necrosis factor (TNF) death ligand receptor-triggered apoptosis by activation of the TNF/TNFR, TRAIL, TWEAK and LTβ/LIGHT pathways (Paczesny *et al.*, 2010). Moreover, the secretion of interferon gamma (IFN-γ) of these cells inhibits the viral replication and is an important inducer of MHC class I expression and macrophage activation (Janeway *et al.*, 2001).

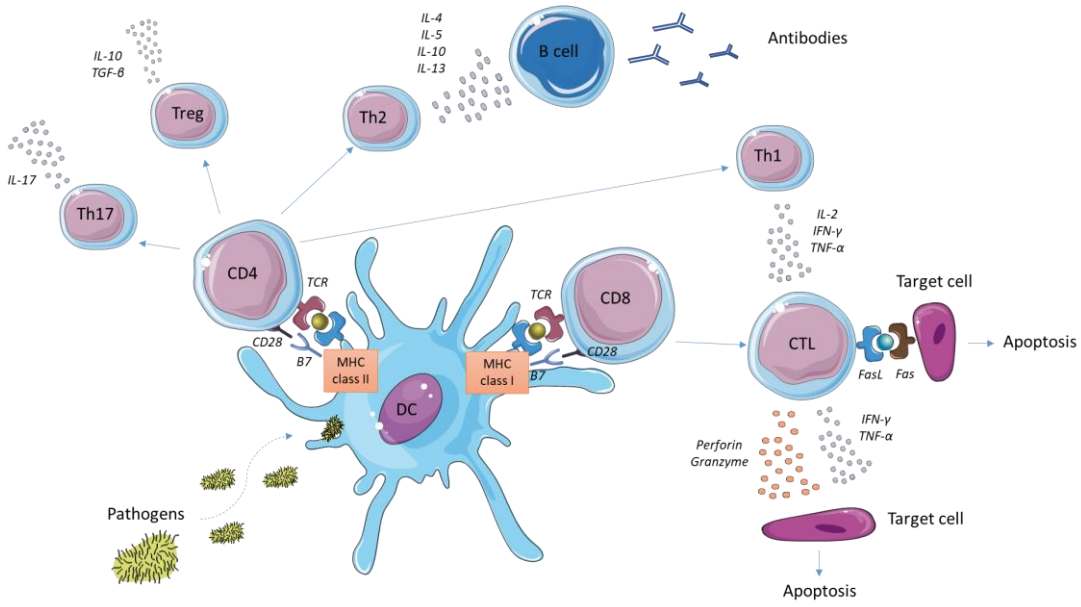

Figure 7. Cytotoxic T cell (CTL) effector function. Pathogen infection and presentation by dendritic cells (DC). DC peptide presentation through major histocompatibility complex (MHC) class II to $CD4^+$ T cells, and MHC class I to $CD8^+$ T cell. Activation and differentiation of effector cells. Apoptosis of target cells by CTL effector function represented through FasL-Fas pathway, release of perforin and granzyme granules or by other cytokines secreted. Here: B7 represents either B7-1 or B7-2 cell receptor, TCR: T cell receptor, Treg: regulatory T cell, Th: T helper cell. Data for figure design obtained from K. Abbas et al., 2008 and based on a figure from Miguel Neves et al., 2012. This figure has been produced using Servier Medical Art (http://smart.servier.com).

Many groups have leveraged the CTL property of T cells to develop cell products to treat viral infections as Cytomegalovirus (CMV), Epstein Barr virus (EBV), Adenovirus (AdV) or BK virus (BKV) among others. In this work we focus on the development of a T cell product against CMV.

1.4 Human cytomegalovirus

Human cytomegalovirus or human betaherpesvirus 5 (CMV/HHV-5), belongs to the order *Herpesvirales*, family *Herpesviridae* and subfamily *Betaherpesvirinae* (International Committee on Taxonomy of Viruses, 2019). CMV is estimated to have ~192 open reading frames (ORF) (Jean Beltran and Cristea, 2014). Like other herpesviruses, CMV is a lifelong latent infection, remaining silent in the host with periodic reactivation cycles that contribute to its efficient transmission (Jean Beltran and Cristea, 2014). The mechanisms by which CMV reactivates from latency are still a matter of intense study (Arcangeletti *et al.*, 2016). Risk factors for reactivation in HSCT include increasing recipient age, unrelated or HLA-mismatched donors, T-cell depletion, GvHD, and high-dose corticosteroids for GvHD treatment (Stern *et al.*, 2019). The prevalence of CMV seropositivity vary widely upon geographic, age, race and socioeconomic status, being generally 60% or more in people older than 50 (Cannon, Schmid and Hyde, 2010).

CMV infection is generally asymptomatic in immunocompetent individuals; however, the congenital infection or CMV infection in immunocompromised patients with severe combined immunodeficiency (SCID), human immunodeficiency virus (HIV) or under immunosuppression treatment can result in significant morbidity and mortality (Stern *et al.*, 2019). CMV infection has been reported to cause pneumonia, gastrointestinal disease, hepatitis, retinitis, cystitis, nephritis, myocarditis and pancreatitis among others (Ljungman *et al.*, 2017). CMV may cause febrile illnesses associated with pneumonia, hepatitis, and/or gastrointestinal tract ulcerations (Beutler *et al.*, 2006). Congenital CMV infection is associated with permanent hearing loss and neurological impairment (Colugnati *et al.*, 2007; Cannon, Schmid and Hyde, 2010). As illustrated in **Figure 8** the CMV infection is controlled by both innate and adaptive immunity. The virus infects and replicates in a wide variety of cells, such as epithelial cells, endothelial cells, fibroblasts and smooth muscle cells, to name a few (Sinzger, Digel and Jahn, 2008). Primary infection typically initiates with replication in mucosal epithelium (**Figure 8 A**). CMV cell tropism has been defined by only two complexes being essential for entry, glycoprotein B (gB) and gH-gL dimer (Griffiths, Baraniak and Reeves, 2015). The second complex being important for triggering gB fusion at the plasma membrane. Inside the cell, CMV initiates a cascade of temporally regulated gene expression that is a classic signature of herpesvirus lytic infection. CMV reservoir is the myeloid lineage (Kondo, Kaneshima and Mocarski, 1994; Traylen *et al.*, 2011; Jean Beltran and Cristea, 2014; Dupont and Reeves, 2016) including, $CD34^+$ progenitor cells and $CD14^+$ monocytes, which remain latently infected (Krishna *et al.*, 2017). Latently infected cells represent a very low percentage of the mononuclear cells in peripheral blood of

seropositive donors (0.004-0.01%) (Stern *et al.*, 2019) (**Figure 8 B**). CMV in these cells has restricted viral gene expression and in general encodes a number of immune evasion molecules throughout the viral life cycle (Griffiths, Baraniak and Reeves, 2015); thus limiting their immune recognition by effector cells. Differentiation of myeloid progenitor cells specifically to macrophage or dendritic cells not only reactivates viral lytic gene expression but also leads to the production of infectious virus particles (Traylen *et al.*, 2011; Dupont and Reeves, 2016) (**Figure 8 C**). Dendritic cells can process these particles and stimulate antigen-specific T cells (**Figure 8 D**), or when DC are activated through toll-like receptors (TLR), activate NK cells by the secretion of cytokines and chemokines. Macrophages also have the ability to directly stimulate antigen-specific T cells (**Figure 8 C**). Activated T cells and NK cells can directly lyse virus-infected cells by cytolysis both through cytotoxic granules filled with perforin and granzymes or through fas/fasL pathway (**Figure 8 E**). Both cell types also have the ability to block virus replication through the secretion of cytokines (i.e. IFN-γ, TNF) (**Figure 8 E**). Moreover, B cells activated by professional APC can control extracellular virus through antibody-mediated neutralisation (**Figure 8 F**).

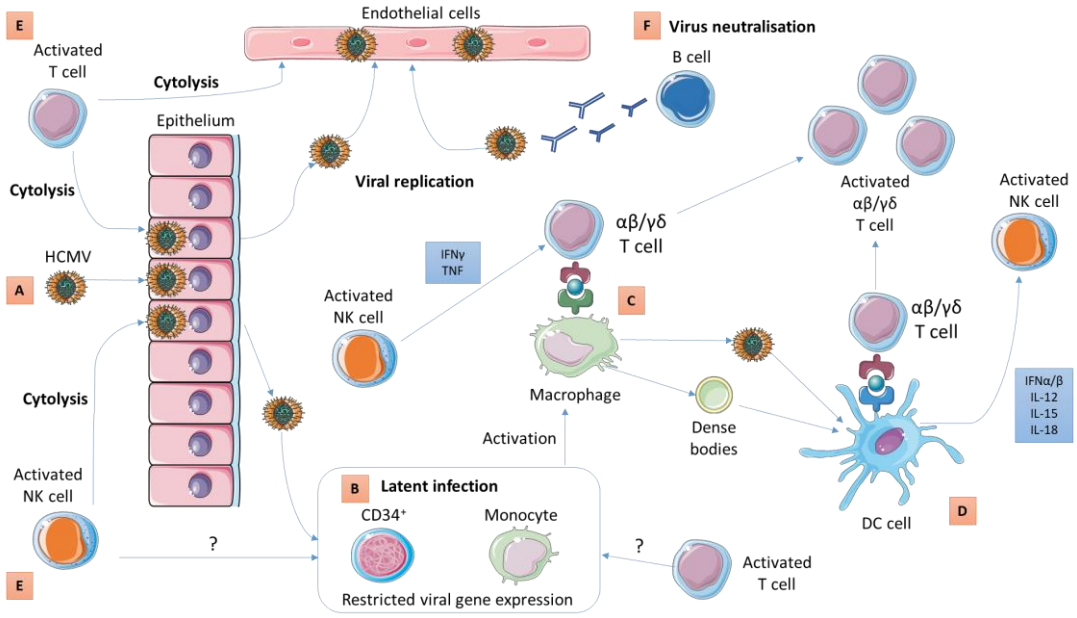

Figure 8. Activation of T cells by the infection of human cytomegalovirus (CMV). (A) Initial infection of epithelium, and (B) latent infection in $CD34^+$ progenitor cells and $CD14^+$ monocytes. (C) Activation of T cells through pathogen presentation by macrophages or (D) dendritic cells. (E) Activated T and NK cells can lyse infected cells by cytolysis. Activated B cells can induce virus neutralisation by antibodies. Figure adapted from Crough and Khanna, 2009. This figure has been produced using Servier Medical Art (http://smart.servier.com).

In virus infections, treatment includes antiviral drugs such as ganciclovir, or foscarnet. Both have shown efficacy in the treatment of CMV infection and are also effective against herpes simplex. However, they present certain toxicity, neutropenia in the case of ganciclovir and renal failure and/or electrolyte abnormalities in the case of foscarnet (Beutler *et al.*, 2006). In addition to the

two antiviral drugs mentioned, there are others also approved by the AEMPs and indicated for CMV like: valcanciclovir, valaciclovir, cidofovir or valganciclovir (Agencia Española de Medicamentos y Productos Sanitarios, 2017). Despite the existent treatments, drug resistance in human cytomegalovirus is a known issue (Chou *et al.*, 2003). Mutations in the UL97 and UL54 genes have been reported as a cause of ganciclovir resistance, and mutations in the gene UL54 are related to the resistance to cidofovir and foscarnet (Campos *et al.*, 2016). Moreover, drug interactions and toxicity associated to these treatments have been reported (Beutler *et al.*, 2006; Jacobsen and Sifontis, 2010). Therefore, approaches to overcome drug-resistant virus infections are necessary. In this work, we focus on the development of advanced therapies based on VST, mainly against CMV.

The major targets of the cellular immune response against CMV have been found to be 65kDa phosphoprotein (pp65) and immediate early protein-1 (IE-1) proteins (Slezak *et al.*, 2007). Among them, pp65, gene UL83 (UniProt, 2019), is the major immunodominant CMV antigen, and for this reason the protein pp65 is commonly used for experimental purposes. The CMV pp65 pepmix is a pool of 15-mer peptides with 11-amino acid overlap that covers the complete sequence of the pp65 (**Table 2**).

Table 2. Complete sequence of 65 kDa phosphoprotein (pp65) of human cytomegalovirus. Table taken from UniProt: https://www.uniprot.org/ [Date accessed: 08/08/2019]; search: "pp65" (ID: P06725).

10	20	30	40	50
MESRGRRCPE	MISVLGPISG	HVLKAVFSRG	DTPVLPHETR	LLQTGIHVRV
60	70	80	90	100
SQPSLILVSQ	YTPDSTPCHR	GDNQLQVQHT	YFTGSEVENV	SVNVHNPTGR
110	120	130	140	150
SICPSQEPMS	IYVYALPLKM	LNIPSINVHH	YPSAAERKHR	HLPVADAVIH
160	170	180	190	200
ASGKQMWQAR	LTVSGLAWTR	QQNQWKEPDV	YYTSAFVFPT	KDVALRHVVC
210	220	230	240	250
AHELVCSMEN	TRATKMQVIG	DQYVKVYLES	FCEDVPSGKL	FMHVTLGSDV
260	270	280	290	300
EEDLTMTRNP	QPFMRPHERN	GFTVLCPKNM	IIKPGKISHI	MLDVAFTSHE
310	320	330	340	350
HFGLLCPKSI	PGLSISGNLL	MNGQQIFLEV	QAIRETVELR	QYDPVAALFF
360	370	380	390	400
FDIDLLLQRG	PQYSEHPTFT	SQYRIQGKLE	YRHTWDRHDE	GAAQGDDDVW
410	420	430	440	450
TSGSDSDEEL	VTTERKTPRV	TGGGAMAGAS	TSAGRKRKSA	SSATACTSGV
460	470	480	490	500
MTRGRLKAES	TVAPEEDTDE	DSDNEIHNPA	VFTWPPWQAG	ILARNLVPMV
510	520	530	540	550
ATVQGQNLKY	QEFFWDANDI	YRIFAELEGV	WQPAAQPKRR	RHRQDALPGP
560				
CIASTPKKHR	G			

MHC compatibility is needed for peptide recognition. A phenomenon known as immunodominance skews the immune response towards certain antigen peptides. The part of the antigen that is recognised by the immune system is named epitope, which is a part of the peptide that is presented by the MHC molecules. A summary of pp65 protein epitopes that have been identified, and are associated to certain MHC molecules are represented in **Table 3**. Other groups have also reported CTL epitopes derived from pp65 protein (Kondo *et al.*, 2004; Slezak *et al.*, 2007; Lehmann *et al.*, 2019).

Table 3. HLA allelic restrictions for CMV protein pp65. Source: Immune Epitope Database and Analysis Resource: https://www.iedb.org [Date accessed: 01/08/2019]; search: "Epitope: any epitopes"; Antigen "Organism: Human herpesvirus 5 (ID10359, Human Cytomegalovirus)", "Antigen Name: 65 kDa phosphoprotein"; "MHC Restriction: Any MHC Restriction"; "Host: Human"; "Disease: Infectious Disease").

Epitope				MHC molecule		
ID	Description	Starting Position	Ending Position			
28061	IPSINVHHY	123	131	HLA-B*35:01	HLA-B*35:08	
44920	NLVPMVATV	495	503	HLA-A*02:01	HLA-A2	HLA-A*02:03
				HLA-A*02:06	HLA-A*02:11	HLA-A*02:12
				HLA-A*02:16	HLA-A*02:19	HLA-A*69:01
52886	QYDPVAALF	341	349	HLA-A*24:02	HLA-A24	HLA-C*04:01
55170	RPHERNGFTVL	265	275	HLA-B*07:02	HLA-B*35:01	HLA-B7
65748	TPRVTGGGAM	417	426	HLA-B*07:02	HLA-A*02:01	HLA-B7
75718	YSEHPTFTSQY	363	373	HLA-A*01:01	HLA-A1	

1.5 Virus-specific T lymphocytes applications

In the context of immunosuppression, viral infections can be a cause of morbidity and mortality. More specifically, viral infections from herpesvirus, such as CMV and EBV, or other viruses like AdV or BK virus, to name a few (Papadopoulou *et al.*, 2014; Tzannou *et al.*, 2018; Withers *et al.*, 2018; Kaeuferle *et al.*, 2019). These viruses can remain in a latent state within the body and reactivate under specific circumstances like immunosuppression, when T lymphocytes are inhibited. In the context of transplantation there are two possible scenarios: either the virus can reactivate when the patient had previously been exposed to it and starts an immunosuppressive treatment or the patient can be infected *de novo* if the donor is infected or a carrier and the patient is "naïve". As mentioned above, first-line treatments consist of antiviral drugs and reduction of the immunosuppressive therapy. However, these therapies are not always effective, and fail in a portion of patients (Kaeuferle *et al.*, 2019). In those cases, adoptive immunotherapy treatments are at stake when pharmacological agents generate toxicity and there is a refractory infection (Kaeuferle *et al.*, 2019). Allogeneic T lymphocytes from a specific virus reactive donor can efficiently kill virus-infected cells through specific pathways. Therefore

different strategies are being explored in many research and clinical centres. Some of them contemplate the direct selection of VST from fresh leukapheresis, notwithstanding, personalised medicines are consequently more expensive (Ottaviano *et al.*, 2019) and present the challenge of finding a donor that is available, and both HLA compatible and virus reactive. To overcome these limitations, different centres are generating VST through expansion cultures with the aim of creating VST third-party donor (allogeneic) banks (Papadopoulou *et al.*, 2014; Withers *et al.*, 2018). Thus, having a therapy ready to use, with its HLA typing registered for compatibility matching, and the manufacturing of multiple doses from one single donor results in an important cost reduction. Throughout this section, different VST for therapy are summarised, and classified based on three production methods: 1) selection, 2) genetic modification, and 3) expansion.

1) Selection

Selection method is based on the isolation of VST present in the body of the donor. This procedure is usually based on a leukapheresis (Peggs *et al.*, 2011; Tischer *et al.*, 2014), which involves the isolation of leukocytes and granulocytes, and return of erythrocytes and plasma. The sample is then processed to isolate peripheral blood mononuclear cells (PBMC) and a virus-specific stimulation is performed (usually with peptide mixtures). The direct selection of VST can be performed by two techniques: multimer based selection or direct selection. Both techniques are rapid but face the difficult and timely process of finding an available, virus-reactive and compatible donor.

The multimer approach is based on MHC molecules that specifically present a peptide to T cells. In order to be able to isolate and detect VST, MHC molecules are conjugated to a marker (i.e. fluorochrom, magnetic particle). The most common structure is a streptavidin that binds the monomers and the marker. There are different multimers according to the number of MHC molecules that form the multimer. Streptamer technology is used by Schmitt and collaborators for CMV VST selection (Schmitt *et al.*, 2011). The main advantage of this method is the rapid manufacturing, but the disadvantage is the restriction to certain HLA types.

Direct selection, specifically the gamma capture system (Miltenyi Biotec, 2019), is based on a double antibody that binds on one side to the $CD45^+$ molecule expressed on the lymphocyte membrane, and on the other side to the IFN-γ secreted by the cell. Afterwards a second antibody is added that binds to the IFN-γ and has a magnetic bead conjugated is added. The cell suspension flows through a column that captures the target cells ($IFN\text{-}\gamma^+$) (Mackinnon *et al.*, 2008; Moosmann *et al.*, 2010; Lindemann *et al.*, 2018; Kadauke *et al.*, 2019). The advantage of

direct selection over multimer selection is that it is not restricted to a HLA type. However, this method has the disadvantage that it does not select cells producing other cytokines.

2) Genetic modification

Additionally, since the advances in the development of CAR T-cells, the possibility of engineering antigen-specific T cells is becoming feasible from CMV-negative donors and, more remarkably, manufactured T cells can be HLA-independent (Proff *et al.*, 2018).

3) Expanded product

Another more extended approach is the *ex vivo* expansion of virus-specific T cells. The starting material most commonly used in this method is peripheral blood (Trivedi *et al.*, 2005; Papadopoulou *et al.*, 2014; Withers *et al.*, 2018).

All these processes have different advantages and disadvantages summarised in **Table 4**.

Table 4. Advantages and disadvantages of different virus-specific T cell (VST) obtaining methods. Table adapted from Bollard and Heslop, 2016.

Method	Advantages	Disadvantages
Multimer selection	Rapid manufacturing	Restricted to certain HLA types, few class II multimers available, not available when donor seronegative or has low frequency of circulating T cells specific for the peptides
Direct selection	Rapid manufacturing, not restricted to HLA, will select polyclonal T cells recognizing multiple epitopes	Large volume of blood required, will not select T cells producing other cytokines, not available when donor seronegative
Genetic modification	Not restricted to HLA	Manufacturing costs
Ex vivo expansion	Expand low frequency VST, not restricted by HLA type	Not possible when donor seronegative, time of culture

Ex vivo expansion offers other advantages besides the ones shown in **Table 4**. Not only low frequency VST can be expanded without restriction to HLA type, but also final product can be cryopreserved for later use. With the later approach, the limitation of manufacture time is reduced, as the product can be readily available in a third-party bank. Generation of VST lines with representative HLA would enable the possibility of creating a VST bank that covers the majority of the population. Many centres are starting to develop their own third-party banks in order to overcome virus-infection and centres like Baylor College (Tzannou *et al.*, 2017), Sloan Memorial Kettering (Koehne *et al.*, 2015) and Westmead Hospital in Sydney (Withers *et al.*, 2018) among others, have developed their VST expansion protocols.

Regarding VST expansion, time depends upon the protocol, comprised of a minimum of 9 days to up to 28 days. Supplements used for cell expansion are mostly based on IL-2. Different

expansion processes contemplate distinct stimulation methods, such as peptide pool stimulations (Papadopoulou *et al.*, 2014) or the traditional based antigen presenting cells (Kleihauer *et al.*, 2001; Withers *et al.*, 2018), like the most physiological stimuli. Recently other stimulations have been addressed such as the artificial antigen presenting cells or induced pluripotent stem dendritic cells (Hasan, Selvakumar and O'Reilly, 2015). As for the sources used, most of the protocols are based on PBMC from seropositive donors. According to Keller and collaborators, CMV-specific T cells can also be derived from CMV-naïve donors, although larger expansions (up to 28 days) are necessary (Keller *et al.*, 2018). Other groups are working towards the generation of VST from UC blood, which would offer advantages as being a source otherwise discarded, although it requires larger manufacture time (Abraham *et al.*, 2019).

Some centres opted for an alternative strategy: the generation of a registry with data from virus reactivity and HLA type. This is the case of the alloCELL registry generated in Hannover (Tischer *et al.*, 2014). Prof. Dr. Britta Eiz-Vesper group created a registry documenting each donor's: HLA type, virus serology (ADV, CMV, EBV), VST frequencies, best T-cell detection method, and results from functional and alloreactivity assays (Medizinische Hochschule Hannover, 2019). Similarly, the Blood and Tissue Bank, has also created a registry named REDOCEL with the aim of expediting donor selection for adoptive immunotherapy. The creation of registries would allow a vast amount of donors' sparing the sample cryopreservation, the occupied space and expenses. However, it has certain drawbacks attached, such as the reliability of an available donor, and the limitations associated to VST obtaining, either if the manufacturing involves selection or *ex vivo* expansion.

VST in clinical trials for the treatment of CMV infection are summarised in **Table 5**, showing a total of 33 clinical trials registered, with 22 belonging to different centres. Countries involved in the trials include: USA, UK, Switzerland, Belgium, France and China. Among the clinical trials that specify the method for VST manufacturing, 10 trials are based on selection and 14 on *ex vivo* expansion. Although being a possible treatment, and being explored by others (Proff *et al.*, 2018), no clinical studies with CAR T-cells for CMV indication were found. As indicated in **Table 5**, some studies are based on the treatment of multiple virus infection with VST.

Lately, some centres are focusing on the generation of multi-VST generation, rather than univiral VST (Tzannou *et al.*, 2018; Withers *et al.*, 2018). The general aim is the generation of third-party banks of VST to cover the main viruses frequently related to post-transplantation complications but also with other immunodeficiencies. Targets include CMV, EBV, AdV, BK and human herpesvirus 6 (HHV6) infections.

Table 5. Virus specific T cells use registered in clinical trials. Resource: https://www.clinicaltrials.gov [Date accessed: 02/08/2019]; search: “virus specific T cells”; trials included under the status: "not yet recruiting", "recruiting", "enrolling by invitation", "active, not recruiting", "terminated", "completed". Here NCT: clinical trial identifier, CMV: cytomegalovirus, AdV: adenovirus, EBV: Epstein-Barr virus, BKV: BK virus, HHV6: human herpesvirus 6. For center number reference see the footnote below the table.

Virus infection	Method	Antigen presentation	Status	Center	NCT
CMV, AdV, EBV	*Ex vivo* expansion	Synthetic viral peptide pools	Recruiting	1	NCT03475212
CMV, AdV, EBV	*Ex vivo* expansion	Not specified	Completed (n=2)	2	NCT00673868
CMV, AdV, EBV	*Ex vivo* expansion	CMV peptides	Completed (n=10)	3	NCT02313857
CMV, AdV, EBV	Cytokine Capture System	Not specified	Recruiting	4	NCT02007356
CMV, AdV, EBV	*Ex vivo* expansion	Dendritic cells transfected with AdV, CMV, and EBV genes	Completed (n=10)	3	NCT01070797
CMV	CliniMACS® Prodigy	MACS GMP PepTivator	Not yet recruiting	5	NCT03950414
CMV	CliniMACS® Prodigy	Not specified	Recruiting	6	NCT03798301
CMV	CliniMACS® Prodigy	Not specified	Recruiting	7	NCT03266640
CMV	*Ex vivo* expansion	Monocytes transducted with CMV gene and/or dendritic cells transducted with CMV gene	Completed (n=26)	3	NCT00078533
CMV	*Ex vivo* expansion	Pentadecapeptides of CMV pp65 / CMV peptide pp65	Completed (n=30)	8	NCT00674648
CMV	*Ex vivo* expansion	Not specified	Recruiting	8	NCT01646645
CMV	Donor Lymphocyte Infusion Other Name: Miltenyi Biotec	Not specified	Terminated (n=5)	9	NCT01274377
CMV	IFN-γ isolation of pp65-specific T cells	Viral antigen	Recruiting	10	NCT03067155
CMV, AdV	IFN-γ selection	Viral peptides	Completed (n=16)	11	NCT01325636
CMV, AdV	*Ex vivo* expansion	Dendritic cells transducted with CMVpp65 gene	Completed (n=9)	3	NCT00880789
CMV	Not specified	Not specified	Recruiting	12	NCT02985775
EBV, CMV, ADV, BKV	*Ex vivo* expansion	Not specified	Not yet recruiting	3	NCT04013802
CMV, AdV, EBV	Not specified	Not specified	Recruiting	13	NCT01535885
CMV	CliniMACS® Prodigy	Not specified	Recruiting	14	NCT02982902

Virus infection	Method	Antigen presentation	Status	Center	NCT
CMV	*Ex vivo* expansion	CMV antigen peptides	Recruiting	15	NCT03004261
CMV	Multimer selection	Not specified	Completed (n=52)	16	NCT01220895
CMV	*Ex vivo* expansion	Not specified	Recruiting	8	NCT02136797
EBV, CMV, ADV, or BKV	Not specified	Not specified	Recruiting	17	NCT02532452
EBV, CMV, ADV, HHV6 or BKV	*Ex vivo* expansion	Peptides	Completed (n=21)	3	NCT01570283
CMV, EBV, ADV, and BKV	*Ex vivo* expansion	Peptides	Recruiting	17	NCT02048332
CMV	Not specified	Not specified	Completed (n=89)	18	NCT01077908
CMV	Not specified	Not specified	Completed (n=6)	19	NCT02210065
CMV	CliniMACS® Prodigy	Not specified	Recruiting	20	NCT03665675
CMV, EBV, ADV, BKV or other	*Ex vivo* expansion	Dendritic cells with pathogen specific antigens	Recruiting	21	NCT03159364
CMV	Selection	Not specified	Recruiting	22	NCT02210078

1 City of Hope, Duarte|Children's Hospital Los Angeles|Stanford Lucile Packard Children's Hospital|UCSF Medical Center|Children's Hospital Colorado|Children's National Medical Center|Riley Hospital for Children - Indiana University|Tufts Medical Center|Columbia University Medical Center|Duke University Medical Center|Methodist Healthcare System of San Antonio (USA) 2 Penn State University (USA) 3 Texas Childrens Hospital|The Methodist Hospital system (USA) 4 Universitätsspital Basel (Switzerland) 5 University of Wisconsin School of Medicine and Public Health (USA) 6 University of Wisconsin Carbone Cancer Center (USA) 7 New York Medical College|Children's Hospital of Pennsylvania|Medical College of Wisconsin/Children's Hospital of Wisconsin (USA) 8 Memorial Sloan Kettering Cancer Center (USA) 9 Duke University Medical Center (USA) 10 Universitair Ziekenhuis Gent|ZNA Stuivenberg|AZ Sint-Jan Brugge|Institut Jules Bordet|Universitair Ziekenhuis Brussel|Cliniques Universitaires Saint Luc|Université de Liège|Heilig Hart Ziekenhuis Roeselare (Belgium) 11 Biotherapy department, Hôpital Necker - Enfants Malades (France) 12 Peking University People's Hospital & Peking University Institute of Hematology (China) 13 Medical College of Wisconsin (USA) 14 University Hospitals Cleveland Medical Center (USA) 15 Shanghai Jiao Tong University Affilated Shanghai General Hospital (China) 16 QEH Birmingham Hospital|Bristol Royal Hospital, Bristol|University College London Hospital|Kings College Hospital|Royal Free Hospital|Manchester Royal Infirmary|The Christie|Nottingham University Hospital - City Campus|Churchill Hospital (UK) 17 Cincinnati Children's Hospital Medical Center (USA) 18 Birmingham Heartlands Hospital (UK) 19 University of Texas MD Anderson Cancer Center (USA) 20 Ohio State University Comprehensive Cancer Center (USA) 21 Shenzhen Geno-immune Medical Institute (China) 22 M D Anderson Cancer Center (USA).

In this work, we opted for a rapid *ex vivo* expansion of VST, for the generation of large amount of cells, and with the objective of having a final product that is functional. The production of large numbers of VST would allow the cryopreservation of several doses, reducing costs and having the drug immediately available. The VST manufacture was designed bearing in mind the translation of the protocol into cGMP standards for later production and, with the future goal of generating a third-party bank of cryopreserved VST for allogeneic use.

CHAPTER II: HYPOTHESES AND AIMS

2.1 Hypotheses

Hypothesis 1:

Immunomodulation capacity of WJ and BM derived MSC can be quantified through a potency assay for product release, and this and other critical attributes of MSC are not altered in clinical grade BM-MSC HLA-DR$^+$ cells.

Hypothesis 2:

Large number of functional cytomegalovirus specific T cells can be obtained from a virus reactive donor through a short expansion culture.

2.2 Aims

The aim of the project presented here was to develop advanced cell therapies, for use mostly but not limited to the treatment of complications post-transplantation, based on MSC and VST.

In order to address the development of these cell therapies, the following specific objectives were established:

- Optimisation of a MSC potency assay based on their immunomodulation capacity
- Revision of HLA-DR expression in BM-MSC for MSC definition criteria
- Development of a rapid and scalable production process of CMV-specific T cells
- Extensive characterisation of CMV-specific T cells after expansion

PART II: RESULTS

CHAPTER III: Quality Attributes of Multipotent Mesenchymal Stromal Cells

3.1 Introduction

The work presented next is tightly related to the large experience of our laboratory in the development of MSC therapies, thanks to all the BM-MSC clinical studies presented (EudraCT 2009-016449-24, 2010-022909-18, 2010-023998-18, 2010-023999-12, 2010-024041-782011-006270-13, 2012-000734-19 and 2013-005025-23).

In line with the prior work in BM-MSC, the lab started to develop MSC isolated from WJ. Preceding work involves on the one hand, large-scale expansion and characterisation of WJ-MSC confirming genetic stability (this is, human telomerase reverse transcriptase (hTERT) activity, c-Myc expression and senescence) and multipotentiality and immunomodulation properties of the expanded cells, as it is shown in Appendix (Oliver-Vila *et al.*, 2016). On the other hand, WJ-MSC were also used for the creation of an osteogenic hydrogel for bone regeneration with WJ-MSC as active ingredient. Product and protocol were filed for an European patent (Ref. EP17382614.0). All this work as has led to the realisation of two clinical studies based on WJ-MSC (EudraCT 2015-005786-23 and 2018-001964-49).

This chapter includes studies that are focused on overcoming hurdles arising from these processes, more specifically regarding specifications of MSC final product. Acceptance criteria established for release of MSC prior to use in the clinics included a potency assay, required by the competent regulatory authority for cell-based ATMP in order to quantitatively measure the biological activity of the product. MSC efficacy was demonstrated through an immunomodulation assay, following a protocol that was described in a manuscript showing the optimisation process “3.2 Optimisation of a potency assay for the assessment of immunomodulative potential of clinical grade multipotent mesenchymal stromal cells” (Oliver-Vila *et al.*, 2018). The manuscript describes the optimisation of an immunomodulation assay for MSC isolated from BM, which is the potency assay protocol currently followed in our facilities, and can be used indistinctively for both WJ-MSC and BM-MSC. Another approach was made not only for WJ-MSC efficacy evaluation protocol earlier described, but also a risk analysis for WJ-MSC performed under cGMP. This work is detailed in the second manuscript “3.3 Compliance with Good Manufacturing Practice in the Assessment of Immunomodulation Potential of Clinical Grade Multipotent Mesenchymal Stromal Cells Derived from Wharton’s Jelly” (Grau-Vorster, Rodríguez, del Mazo-Barbara, *et al.*, 2019).

In an attempt to standardise the characterisation of MSC-based products, certain criteria were proposed by the ISCT: 1) plastic adherence, 2) capacity to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* and 3) phenotypic markers expression of CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules (Dominici *et al.*, 2006). In our hands, criteria for expression of phenotypic markers did not completely match to the characteristics of our products, in particular, the expression of HLA-DR in MSC isolated from the BM. The expression of HLA-DR has generated controversy between researchers: whether to use this marker as acceptance criteria for delivery into the clinic or just as an informative parameter. Therefore, we started to characterise BM-MSC to analyse if the randomly expression of HLA-DR protein was attributed to an external stimuli or whether it affected the identity, multipotentiality and immunopotency of the cells, all addressed in the third manuscript "3.4 Levels of IL-17F and IL-33 correlate with HLA-DR activation in clinical-grade human bone marrow-derived multipotent Mesenchymal Stromal Cell expansion cultures." (Grau-Vorster, Rodríguez, Torrents-Zapata, *et al.*, 2019). To move on one step further, a two-site study was performed in collaboration with the Finnish Red Cross Blood Service, which constitute the fourth, and last manuscript in this work regarding MSC "3.5 HLA-DR expression in clinical-grade bone marrow-derived multipotent mesenchymal stromal cells: a two-site study." (Grau-Vorster, Laitinen, *et al.*, 2019). The work proved once more, that MSC properties did not change regardless of HLA-DR expression and that either human sera or platelet lysate supplement resulted in similar results. Moreover, we studied if the expression of HLA-DR was dynamic, and if it could be induced or suppressed depending on the environment. With all the stated above, we concluded this work with a proposal to the ISCT to revise the identifying criteria for BM-MSC regarding HLA-DR expression.

3.2 Optimisation of a potency assay for the assessment of immunomodulative potential of clinical grade multipotent mesenchymal stromal cells.

Irene Oliver-Vila, Carmen Ramírez-Moncayo, **Marta Grau-Vorster**, Silvia Marín-Gallén, Marta Caminal, Joaquim Vives.

Cytotechnology. 2018. 70(1)31–44.

Doi: 10.1007/s10616-017-0186-0

Optimisation of a potency assay for the assessment of immunomodulative potential of clinical grade multipotent mesenchymal stromal cells.

Irene Oliver-Vila[1], Carmen Ramírez-Moncayo[1], Marta Grau-Vorster[1], Silvia Marín-Gallén[1], Marta Caminal[1], Joaquim Vives[1,2,3].

[1]Servei de Teràpia Cel·lular, Banc de Sang i Teixits, Edifici Dr. Frederic Duran i JordàBarcelonaSpain
[2]Departament de MedicinaUniversitat Autònoma de BarcelonaBarcelonaSpain
[3]Musculoskeletal Tissue Engineering Group, Vall d'Hebron Research Institute (VHIR)Universitat Autònoma de BarcelonaBarcelonaSpain

Corresponding author:

Irene Oliver-Vila, PhD

e-mail address: irolvila@gmail.com

Joaquim Vives, PhD

e-mail address: jvives@bst.cat

3.3 Compliance with Good Manufacturing Practice in the Assessment of Immunomodulation Potential of Clinical Grade Multipotent Mesenchymal Stromal Cells Derived from Wharton's Jelly

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Marta Grau-Vorster, Luciano Rodríguez, Anna del Mazo-Barbara, Clémentine Mirabel, Margarita Blanco, Margarita Codinach, Susana Gómez, Sergi Querol, Joan García-López, Joaquim Vives.

Cells. 2019. 8*(5), 484*

Doi: 10.3390/cells8050484

Compliance with good manufacturing practice in the assessment of immunomodulation potential of clinical grade multipotent mesenchymal stromal cells derived from Wharton's jelly.

Marta Grau-Vorster [1,2],Luciano Rodríguez [1],Anna del Mazo-Barbara [1],Clémentine Mirabel [1],Margarita Blanco [1],Margarita Codinach [1],Susana G. Gómez [1],Sergi Querol [1],Joan García-López [1,3,*] andJoaquim Vives [1,4,5,*]

[1]Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Passeig Taulat, 116, 08005 Barcelona, Spain

[2]Transfusion Medicine Group, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

[3]Transfusion Medicine and Cellular and Tissue Therapies, Universitat Autònoma de Barcelona, Campus UAB, Cerdanyola del Vallès, 08035 Bellaterra, Spain

[4]Musculoskeletal Tissue Engineering Group, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

[5]Departament de Medicina, Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

Corresponding author:

Joan García-López, PhD

e-mail address: joangarcia@bst.cat

Joaquim Vives, PhD

e-mail address: jvives@bst.cat

3.4 Levels of IL-17F and IL-33 correlate with HLA-DR activation in clinical-grade human bone marrow-derived multipotent Mesenchymal Stromal Cell expansion cultures.

Marta Grau-Vorster, Luciano Rodríguez, Silvia Torrents-Zapata, Daniel Vivas, Margarita Codinach, Margarita Blanco, Irene Oliver-Vila, Joan García-López, Joaquim Vives.

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Doi: 10.1016/j.jcyt.2018.09.009

Levels of IL-17F and IL-33 correlate with HLA-DR activation in clinical-grade human bone marrow-derived multipotent mesenchymal stromal cell expansion cultures.

Marta Grau-Vorster[1], Luciano Rodríguez[2], Silvia Torrents-Zapata[2], Daniel Vivas[3], Margarita Codinach[4], Margarita Blanco[2], Irene Oliver-Vila[2], Joan García-López[5], Joaquim Vives[6].

[1]Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Barcelona, Spain; Transfusion Medicine Group, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain.

[2]Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Barcelona, Spain.

[3]Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Barcelona, Spain; Musculoskeletal Tissue Engineering Group, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain.

[4]Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Barcelona, Spain; Reconstructive Surgery of the Locomotor System Group, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain.

[5]Transfusion Medicine and Cellular and Tissue Therapies, Universitat Autònoma de Barcelona, Campus UAB Bellaterra, Cerdanyola del Vallès, Spain; Departament de Medicina, Universitat Autònoma de Barcelona, Barcelona, Spain.

[6]Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Barcelona, Spain; Musculoskeletal Tissue Engineering Group, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain; Departament de Medicina, Universitat Autònoma de Barcelona, Barcelona, Spain.

Corresponding author:

Joaquim Vives, PhD

e-mail address: jvives@bst.cat

3.5 HLA-DR expression in clinical-grade bone marrow-derived multipotent mesenchymal stromal cells: a two-site study.

Marta Grau-Vorster, Anita Laitinen, Johanna Nystedt, Joaquim Vives.

*Stem Cell Research & Therapy. 2019**10**:164*

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HLA-DR expression in clinical-grade bone marrow-derived multipotent mesenchymal stromal cells: a two-site study

Marta Grau-Vorster[1,2#], Anita Laitinen[3#], Johanna Nystedt[3*], Joaquim Vives[1,4,5*].

[1]Servei de Teràpia Cel·lular, Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Passeig Taulat, 116, 08005 Barcelona, Spain

[2]Transfusion Medicine Group, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

[3]Finnish Red Cross Blood Service, Advanced Cell Therapy Centre, Kivihaantie 7, FIN-00310 Helsinki, Finland

[4]Musculoskeletal Tissue Engineering Group, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

[5]Departament de Medicina, Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

[#]Marta Grau-Vorster and Anita Laitinen contributed equally to this work.

Corresponding authors:

Johanna Nystedt, PhD

e-mail address: Johanna.nystedt@gmail.com

Joaquim Vives, PhD

e-mail address: jvives@bst.cat

CHAPTER IV: Generation of virus-specific T lymphocytes

4.1 Introduction

The following work relates to the development of an ATMP composed of cytomegalovirus specific T cells and it follows up from previous work in our lab regarding the clinical use of virus-specific T cell direct selection with gamma capture system in two Phase I/II trials (EudraCT 2009-017100-81 and 2018-000911-25). Although being clinically effective, direct selection has certain limitations, herein: a) starting material based on leukapheresis; b) challenge of finding an available compatible and CMV seropositive donor; c) elevated costs associated to personalised therapy; and d) time, which is extremely critical.

In order to overcome these limitations, we designed an *ex vivo* expansion protocol for the generation of CMV-specific T cells from peripheral blood. An optimised protocol to obtain VST after 14-day coculture is aimed to constitute a cryopreserved VST bank to be used in the future as third-party treatment for CMV infections. With this process, the challenge of finding a donor would be reduced to a search through the HLA registry and thawing of the most closely matched sample, which would be readily available for administration. Moreover, the expansion protocol would allow the generation of several "clinical doses" at a reduced cost. The project's first step consisted in the creation of a protocol for the generation of VST cell lines, which is defined next in the submitted manuscript entitled "4.2 Characterization of a Cytomegalovirus-Specific T Lymphocyte Product Obtained Through a Rapid and Scalable Production Process for Use in Adoptive Immunotherapy".

In this work we developed a range of assays for product characterisation that could later be used for product release specificiations. Among them, we implemented an ELISPOT assay, a sensitive technique that allows the measure of IFN-γ secreting cells upon pp65 stimulation. Moreover, regarding functional assays we implemented the degranulation assay, based on CD107a detection in cytotoxic cells upon pp65 stimuli. Cytotoxicity assays were developed both to measure pp65-specific cell lysis of autologous and allogeneic cells, and alloreacitivity of nonpulsed cells, by flow cytometry. The assay was based on CFSE, a colorant that enters the cell passing through the plasmatic membrane and binds covalently to the amines inside. Phenotype characterisation was also performed for the analysis of different populations and T cell subsets, with additional detection of intracellular IFN-γ. Quantification of other cytokines in the supernatant after pp65 stimulation was performed to determine if the product was polyfunctional. All these techniques will be evaluated as quality controls and/or potency assay.

In this regard, the potency assay chosen would need to be robust, not limiting the product release by time and relatively easy to perform as a routine protocol.

The future perspective would involve the validation for cGMP grade production of VST and the establishment of a clinical trial to test their efficacy. Another future prospect would be the development of multiviral specific T cells with the same protocol or optimising the process if needed.

4.2 Characterization of a Cytomegalovirus-Specific T Lymphocyte Product Obtained Through a Rapid and Scalable Production Process for Use in Adoptive Immunotherapy

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Marta Grau-Vorster, María López-Montañés, Ester Cantó, Sergi Querol, Joaquim Vives, Irene Oliver-Vila, Francesc Rudilla.

Submitted

Characterization of a Cytomegalovirus-Specific T Lymphocyte Product Obtained Through a Rapid and Scalable Production Process for Use in Adoptive Immunotherapy

Marta Grau-Vorster[1,2], María López-Montañés[1,2], Ester Cantó[1,2], Sergi Querol[1], Joaquim Vives[1,3,4], Irene Oliver-Vila[1], Francesc Rudilla[1,2].

[1]Servei de Teràpia Cel·lular, Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Passeig Taulat, 116, 08005 Barcelona, Spain

[2]Transfusion Medicine Group, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

[3]Musculoskeletal Tissue Engineering Group, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

[4]Departament de Medicina, Universitat Autònoma de Barcelona, Passeig de la Vall d'Hebron 129-139, 08035 Barcelona, Spain

Corresponding authors:

Francesc Rudilla, PhD

e-mail address: frudilla@bst.cat

ABSTRACT

Immunosuppressed patients are susceptible to virus reactivation or *de novo* infection. Adoptive immunotherapy, based on virus-specific T lymphocytes (VST), can prevent or treat viral diseases. However, donor availability, HLA-compatibility restrictions, high costs and time required for the production of personalized medicines constitute considerable limitations to this treatment. *Ex vivo* rapid and large-scale expansion of VST, compliant with current good manufacturing practice (cGMP) standards, with an associated cell donor registry would overcome these limitations. This study aimed to characterize a VST product obtained through an expansion protocol transferable to cGMP standards. Antigenic stimulus consisted of cytomegalovirus (CMV) pp65 peptide pool-pulsed autologous dendritic cells (DCs) derived from monocytes. G-Rex technology, cytokines IL-2, IL-7, and IL-15, and anti-CD3 and anti-CD28 antibodies were used for culture. At day 14 of cell culture, the final product was characterized regarding T cell subsets, specificity, and functionality. The final product, comprised mainly $CD4^+$ and $CD8^+$ T lymphocytes (49.2±24.7 and 42.3±25.2, respectively). The culture conditions made it possible to achieve at least a 98.89-fold increase in pp65-specific $CD3^+$ IFN-γ^+ cells. These cells were specific, as pp65-specific cytotoxicity was demonstrated. Additionally, in complete HLA mismatch and without the presence of pp65, alloreactivity resulted in less than 5% cell lysis. In conclusion, a cGMP scalable process for the generation of a large number of doses of CMV-specific cytotoxic T cells was successfully performed.

KEY WORDS

Virus Specific T lymphocytes (VST), antigen presenting cells (APC), adoptive immunotherapy, specificity, cytotoxicity, peripheral blood mononuclear cells (PBMC), alloreactivity.

INTRODUCTION

Immunodeficient patients are susceptible to infection by cytomegalovirus (CMV) and other viruses. Herpesviruses, such as CMV, are often asymptomatic or mild in healthy individuals. Nevertheless, in the context of immunocompromised patients, viral infections can be a severe cause of morbidity and mortality.[1,2] CMV infection rates indicate that the virus is common in most of the population and that incidence increases with age.[3] The impaired immune systems of immunocompromised patients are unable to eradicate or limit the virus. Antiviral pharmacologic agents are effective against only some of these viruses; their use is costly, associated with significant toxicities and does not provide long-term protection.[4,5] Adoptive immunotherapy based on virus-specific T lymphocytes (VST) is therefore an attractive option, as T lymphocytes can confer long-term protection against the development of viral disease.[6–8]

Treatment with nonspecific donor lymphocyte infusions (DLI) has improved the clinical outcome of viral infection and leukemia relapse, but has been associated with a high risk of graft-versus-host disease (GvHD). Hence, it is not appropriate to treat infections with cells obtained from seronegative donors or those who have received an umbilical cord blood transplant.[9,10] Moreover, it has been shown that adoptive transfer of selected antigen-specific T cells is an effective and safe treatment option in these situations, as it does not increase the risk of GvHD and shows overall response rates of 90%.[11] Direct selection with peptide-multimers[12] or the cytokine capture system[13,14] are currently used to obtain VST. Both procedures present certain limitations, such as the challenge of finding an available compatible and seropositive donor, and the amount of final product obtained, which defines the number of doses. While multimer selection presents the disadvantage of being limited to certain HLA types, the cytokine capture system will only select T cells producing a specific cytokine. Another hurdle of these methodologies is the very low frequency of CMV-specific T cells present in peripheral blood. According to Gamadia et al., 2001, the frequency of $CD8^+$ interferon gamma (IFN-γ) producing cells ranged between 0.18% and 0.80%, with similar observed $CD4^+$ IFN-γ^+ frequencies.[15]

In order to create a VST bank, the cell expansion protocol must be well-established and must minimize the presence of alloreactive T cells in the final product, and the expanded T cell populations must contain both $CD8^+$ and $CD4^+$ cells to ensure an effective response to the infection.[16,17]

We describe a process for generating a single preparation of antiviral T lymphocytes ($CD4^+$ and $CD8^+$) that is consistently specific for immunodominant and subdominant antigens derived from CMV, a frequent cause of post-transplant morbidity or death. This approach uses a standardized mix of peptides focusing on pp65 protein, the major immunodominant CMV antigen, presented by mature monocyte-derived dendritic cells (moDC), and a combination of cytokines to promote the activation, survival, and expansion of T cells. After a bibliographic search of the media supplements most commonly used for lymphocyte expansion, cytokines IL-2, IL-7, and IL-15[18–20] were selected for VST activation and expansion. Protocol optimization focused on reducing production time and maximizing product functionality. We also aimed to generate large amounts of cells to ensure the availability of several doses for a single patient if needed. Importantly, the method proposed here is readily adaptable to clinical implementation in compliance with current good manufacturing practice (cGMP) legislation and the product may be used as a safe and effective antiviral agent for patients at high risk of disease due to CMV infection.

MATERIALS AND METHODS

PBMC Handling

Peripheral blood mononuclear cells (PBMC) were collected from the peripheral blood of CMV^+ seropositive healthy donors. PBMC isolation, cryopreservation and thawing were performed as described in previous studies[21]. Positive serology for CMV IgG was confirmed using chemiluminescence (Abbot, Abbot Park, Illinois, USA). PBMC from each donor were used to obtain CMV-specific VST, moDCs and phytohemagglutinin (PHA) lymphoblasts. The determination of cell concentration was performed by flow cytometry (Perfect-Count Microspheres™; Cytognos, Salamanca, Spain) or Neubauer chamber counting. The percentage of cell viability was determined either by 7-aminoactinomycin D (7AAD; BD Biosciences, San Jose, California, USA) staining or Trypan Blue (GE Healthcare, USA). Data was analyzed using CellQuest Pro software version 5.2.1 (BD Biosciences).

Obtaining and Pulsing Dendritic Cells

Freshly isolated PBMC were initially seeded for 2 hours to allow the adherence of monocytes. Nonadherent PBMC were then collected and cryopreserved. Monocytes were cultured for 8 days to obtain mature dendritic cells (DC), as described elsewhere.[22] Mature DC were pulsed for 1-2 hours at 37ºC, 5%CO_2, with 10µg/mL of PepTivator® CMV pp65 (Miltenyi Biotec, Bergisch Gladbach, Germany), referred to here as pp65. PepTivator CMV pp65 is based on peptide pools of mainly 15-mer peptides with 11-amino acid (aa) overlap, covering the complete sequence of the pp65 protein of human cytomegalovirus. Pulsed-DC were used for PBMC coculture.

Expansion Protocol

As shown in **Figure 1**, thawed PBMC were seeded at 0.25-1.0E+06 cells/cm^2 at a DC:PBMC ratio of 1:10 in a G-Rex culture system (Wilson Wolf Manufacturing, New Brighton, MN). Fresh medium was composed of Roswell Park Memorial Institute GlutaMAX media (RPMI, Gibco Laboratories, NY, USA) supplemented with 10% (v/v) human AB serum (hSerAB) (Banc de Sang i Teixits, Barcelona, Spain). After 5 days of culture, the medium was completely replaced with RPMI+10% hSerAB supplemented with IL-2 120 U/mL (Miltenyi Biotec), IL-7 4400 U/mL (R&D Systems, MN, USA), and IL-15 80 U/mL (Miltenyi Biotec). Moreover, monoclonal antibodies anti-CD3 (1 µg/mL) and anti-CD28 (2 µg/mL) were added (Biolegend, San Diego, California, USA). On days 7, 9, and 12, an aliquot of 100-300 µL was taken from each culture for cell counting and viability analysis, and fresh medium consisting of RPMI+10% hSerAB with the addition of IL-2 120 U/mL, IL-7 4400 U/mL and IL-15 80 U/mL was added. Culture split was performed when cell density reached 8E+06 cells/cm^2.

Obtaining and Pulsing Lymphoblasts

Fresh or cryopreserved PBMC were adjusted to 2E+06 cells/mL and stimulated with PHA 5µg/mL. After 24 hours, cells were washed twice with Dulbecco's phosphate-buffered saline solution (PBS, Gibco Laboratories) by centrifugation at 340g, RT, for10 minutes. The solution was then adjusted again to 2E+06 cells/mL with RPMI+10%hSerAB and supplemented with IL2 (100U/mL). At least 4 mL of this sample was seeded (2 mL/well) in a 24-well plate and the medium was replenished every 2-4 days. The culture was stored in the incubator at 37ºC, 5% CO_2 as long as G-Rex culture lasted. On the last day of culture, half of the blasts were pulsed with 1µg/mL of pp65 peptide pool (Miltenyi) for 1-2 hours at 37ºC 5% CO_2 and the other half were left nonpulsed. When blasts were used in an allogeneic context, samples were irradiated at 30 Gy for 15 minutes.

PBMC Immunophenotype

For the phenotype analysis, PBMC were collected and stimulated with 1 µg/mL of pp65 for 6 hours at 37ºC, 5%CO_2, at 1E+07 cells/mL. After 3 hours of stimulation, 1 µg/mL of brefeldin A was added. The cells were then washed and permeabilized using Cytofix/Cytoperm solution (BD). Staining consisted of Live/Dead and monoclonal antibodies: CD45, CD8, CD3, IFN-γ, CD19, CD20, CD56, CD4, FOXP3, CD25, C-C chemokine receptor 7 type (CCR7), CD45RO, CD45RA and CD62L (Miltenyi Biotech). Data acquisition was performed using a Miltenyi MACS Quant flow cytometer. Phenotype was analyzed using FlowJo software v10.

Kinetics of Cytokine Secretion

Cryopreserved expanded cells were stimulated with CMV pp65 peptide pool (Miltenyi) 50 ng/mL at a cell concentration of 1E+06 cells/mL in a 96-well plate. Supernatant samples were collected at 0, 6, 10, 24 and 48 hours. All samples were centrifuged at 1000 g for 15 minutes at 4°C. To remove aggregates or debris, samples were centrifuged again at 10,000 g for 10 minutes at 4°C and stored at -80ºC until analysis. Samples were thawed just once and kept on ice before assay. The Bio-Plex Pro™ Human Cytokine Th1/Th2 (Bio-Rad Laboratories, Hercules, CA, USA) was used for the determination of levels of 9 cytokines: IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, IFN-γ, GM-CSF and TNF-α in a multiplex assay using a Luminex 100IS analyzer (Luminex Corp. Austin. TX, USA) according to the manufacturer's instructions. Duplicates were tested for each sample. Data analysis was performed using Bioplex Manager Software v6.1 (Bio-Rad Laboratories Inc.).

Enzyme-Linked Immunospot (ELISPOT)

ELISPOT (MABTECH, Nacka, Sweden) assay was performed in order to determine the specificity of T cells against CMV pp65 peptide by means of interferon gamma secretion. Cells were stimulated with 50 ng/mL pp65 peptide pool (Miltenyi) and left overnight at 2E+05 cells/well. Plates were read in an AID ELISPOT reader (AID GMBH, Strassberg, Germany) following manufacturer's instructions.

CD107a Degranulation Assay

The final product was incubated for 4 hours at 37°C with 10% CO_2 in the presence of monensin (5 ng/mL, Sigma-Aldrich, Saint Louis, Missouri, USA) and anti-CD107a FITC (BD Biosciences Pharmingen) with PepTivator® CMV pp65 (Miltenyi). Stimulation with PHA (Sigma-Aldrich) was used as a positive control for degranulation. Unstimulated cells were used as a negative control for degranulation. Data acquisition was performed using a MACSQuant instrument (Miltenyi Biotech). Data analysis was performed using FlowJo software v10.

Cytotoxicity Assay Based on Flow Cytometry

A cytotoxicity assay was performed to detect the manufactured lymphocytes' ability to attack either autologous or allogeneic lymphoblasts with or without presentation of CMV pp65 peptides (Miltenyi). Lymphoblasts were labelled with CFSE (CellTrace™ CFSE Cell Proliferation Kit; Invitrogen, Waltham, MA, USA), as already reported.[21] Allogeneic and autologous nonpulsed lymphoblasts were stained with a high concentration of CFSE (2.5μM), while pp65 peptide pool-pulsed lymphoblasts were labelled with a low concentration of CFSE (0.25μM)[23]. Fluorescence loss in the target population was monitored by means of flow cytometry in order to quantify the

percentage of specific cytotoxicity.[24] Two different ratios of target (T) and effector (E) cells were tested: T:E 1:5 and 1:10, maintaining 1E+04 target cells in both conditions. Triplicates were performed for each condition. Samples were seeded in a 96-well plate and were incubated for 4 and 24 hours of coculture. Data acquisition was performed using a FACS Calibur instrument (BD). Data analysis was performed using FlowJo software v10.

Percentage of specific cytotoxicity was calculated using the ratio of pulsed and nonpulsed target cells without the presence of effector cells as a baseline, according to the following equation[25]:

$$\textbf{Equation 1.}\ \text{Cell lysis (\%)} = 100 - [100 * \frac{\text{Sample(CFSElow/CFSEhigh)}}{\text{Baseline(CFSElow/CFSEhigh)}}]$$

HLA typing

HLA typing for all samples was performed using the next generation sequencing method. Briefly, DNA samples were amplified by means of multiplex PCR using an in-house strategy. After DNA library preparation (GenDX, Utrecht, The Netherlands), pooled samples were paired-end sequenced using a Miseq (Illumina, San Diego, California, USA) following manufacturer's instructions. Data analysis was performed using NGSengine software (GenDX) and IMGT HLA data versions 3.29.0 - 3.35.0.

Data Analysis

Microsoft Office Excel® and GraphPad Prism 6 were used for the analysis of the results and for plot generation. Statistical significance was set at: *p<0.05, **p<0.01 and ***p<0.001, Mann-Whitney test.

RESULTS

Large Expansion of VST in 14-Day Coculture

Nine VST batches with pp65 specificity were manufactured from healthy CMV^+ donors. We obtained a median of 43.3E+06 cells (min., 4.5E+06 cells; max., 100.1E+06 cells) from an initial seed of 1E+06 PBMC, representing an average 42.2-fold total expansion within 14 days. The growth kinetics of these cells is shown in **Figure 2A**. Cells grow slowly up to day 7 and start proliferating exponentially at day 9, with the highest proliferation at day 12-14 of culture (**Figure 2A**). More specifically, the overall expansion factor of $CD3^+$ cells for PBMC cocultured with pulsed DC was an average of 49.3±48.6 (median, 13.6; min, 6.7; max, 127.2; n=9) while for PBMCs cocultured with nonpulsed DC, it was an average of 9.6±6.3 (median, 8.6; min, 4.1; max, 17; n=4).

Final Product Mainly Comprising T Cells

The presence of a variety of cell populations in the final product ($CD4^+$ T cells, $CD8^+$ T cells, NK cells, B cells and Treg cells) was evaluated. Expanded cells largely consisted of $CD3^+$ T cells (mean, 96.9%±1.9%) containing both $CD4^+$ (mean, 49.2%±24.7%) and $CD8^+$ (mean, 42.3%±25.2%) populations (**Figure 2B**). Compared to the phenotype of the initial product, significant differences were found in the $CD3^+$ cell subset, which had largely expanded (p=0.0004), with no changes among the $CD4^+$ cell subset (p=0.2891) but with a significant increase in the $CD8^+$ cell population (p=0.0315). B cells, NK cells and Treg cells did not expand and, consequently, their presence decreased to almost undetectable levels in the final product (B cells, 0.3%±0.6%; p=0.0013; NK cells, 0.5%±0.6%; p=0.0225; and Treg cells, 0.1%±0.1%; p=0.0395).

Expanded VST Cells Show Antiviral Specificity Using IFN-γ

In order to test the lymphocytes' CMV specificity, cells were re-exposed to pp65-pepmix and activation was measured using IFN-γ intracellular production by flow cytometry. A gating strategy to characterize IFN-γ secretion of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ populations is shown in a representative dot plot (**Figure 2C**). In the independent expansions performed, at day 14 of coculture with pp65-pulsed moDC, the total number of $CD3^+IFN\text{-}\gamma^+$, $CD3^+CD4^+IFN\text{-}\gamma^+$ and $CD3^+CD8^+IFN\text{-}\gamma^+$ cells specific for CMV-pp65 was significantly higher compared to day 0 (**Figure 2D**) (p=0.0002). Absolute values of $CD3^+IFN\text{-}\gamma^+$, $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ cells were calculated from day 14 with respect to day 0. The results for $CD3^+IFN\text{-}\gamma^+$ cells were min, 98.89; max, 12528; and median, 1480.18. The results for $CD4^+IFN\text{-}\gamma^+$ cells were min, 60.37; max, 6158.77; and median, 2082.53. The results for $CD8^+IFN\text{-}\gamma^+$ cells were min, 41.51; max, 32146.13; median, 562.41; n=9.

A summary of the results for 9 donor-independent expansions is shown in **Figure 2E**. Our data confirmed that, with the expansion system used, the final product contains antigen-specific IFN-γ-producing T cells ($CD3^+IFN\text{-}\gamma^+$ 25.3%±16.2%) with no significant differences between compartments ($CD3^+CD4^+IFN\text{-}\gamma^+$, 15.7%±18.1%; $CD3^+CD8^+IFN\text{-}\gamma^+$, 29.3%±20.3%; p=0.3536). Moreover, significant differences were found regarding specificity gain in all subsets after 14-days of culture expansion ($CD3^+$ p-value<0.0001, $CD3^+CD4^+$ p-value<0.0001, and $CD3^+CD8^+$ p-value<0.0001).

CMV- Specific IFN-γ$^+$ T Cells Are Effector Memory T Cells

To thoroughly characterize the expanded product, $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations were identified and classified into 4 different subsets based on the expression of CCR7 and CD45RA markers, following the gating strategy shown in **Supplemental Figure 1**. Here, naïve T cells (T_N): $CD45RA^+CCR7^+$, central memory T cells (T_{CM}): $CD45RA^-CCR7^+$, effector memory T cells (T_{EM}): $CD45RA^-CCR7^-$, and terminally differentiated effector memory T cells (T_{EMRA}): $CD45RA^+CCR7^-$.

After expansion, the T_N subset decreased significantly in all T cell populations ($CD3^+$ p-value=0.0022, $CD3^+CD4^+$ p-value=0.0043 and $CD3^+CD8^+$ p-value=0.0022) (**Figure 3A**). T_{EM} increased significantly in $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ cells (p=0.0050, 0.0022, and 0.0022, respectively). In general, $CD3^+$ subpopulations T_{CM} and T_{EMRA} decreased significantly after expansion (p=0.0087). For $CD3^+CD4^+$ cells, the T_{CM} subset decreased significantly (p=0.0022) and T_{EMRA} showed no significant changes (p=0.1797). For $CD3^+CD8^+$ cells, the T_{CM} subset showed no significant changes (p=0.9372) and the T_{EMRA} subset showed a significant decrease (p=0.0022).

Interferon gamma, used here as a marker of CMV T cell specificity, was expressed by both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. The T cell subsets expressing IFN-γ corresponded overwhelmingly to the T_{EM} subset: $CD3^+$, 91.15±5.29%; $CD3^+CD4^+$, 90.95±5.25%; and $CD3^+CD8^+$, 89.03±7.62% (**Figure 3B**).

Functional and Specific Final Product Against pp65 CMV

Secretion kinetics of Th1 and Th2 cytokines after stimulation with pp65 of 2 cryopreserved batches of expanded cells at different time points (0 h, 6 h, 10 h, 24 h and 48 h) are shown in **Figure 4**. Cytokine secretion had high concentrations of IFN-γ and TNF-α detected with a secretion peak after 6 hours of stimulation. Furthermore, IL-2 was secreted in a similar manner but in much lower concentrations.

T cell response to pp65 was also assessed as a potency assay using IFN-γ ELISPOT for cryopreserved samples from day 0 and 14-day expanded product. Based on the number of spot forming colonies (SFC), a significant increase (p=0.0022) in reactivity was detected after expansion (**Figure 5 A**).

A CD107a degranulation assay was performed to check the functionality of the final product. The expression of $CD8^+CD107a^+$ was detected in the presence of CMV pp65 peptide pool (**Figure 5 B**), while only a slight expression was shown when cells were left unstimulated. However, this assay showed high variability between effector cell batches ($CD8^+CD107a^+$ 22.4±25.71, n=3, and $CD4^+CD107a^+$ 8.67±11.74, n=3 (**Supplementary Figure 2**).

We took advantage of the cytotoxicity assay to show that our product was able to induce cell death specifically in the target cell population (see gating strategy in **Supplemental Figure 3**). Both fresh and cryopreserved effector cells induced lysis among peptide-pulsed blasts, in a similar manner (fresh vs. cryopreserved: 4-h p-value=0.7, 24-h p-value=0.8). This further supports the feasibility of a ready-to-use cryopreserved cell-bank with verified post-thawing functional VSTs (**Figure 5 C**). The expanded product in the presence of autologous blasts pulsed with the peptide resulted in a high percentage of cytotoxicity at both 4 and 24 hours (23.45%±18.35% and 68.61%±19.76%, respectively).

We leveraged the cytotoxicity assay to assess *in vitro* safety by testing the alloreactivity and specificity of the final product. Supplementary Table 1 and Supplementary Table 2 show, respectively, the high resolution HLA typing for expanded cells (used here as effector [E] cells) and PHA blasts (used here as target [T] cells). Fresh and cryopreserved expanded cells were cocultured for 24 hours with allogeneic blasts (with 2-3 HLA matches) at ratios of (T:E) 1:5 and 1:10 to test for alloreactivity (**Figure 5 D**), which resulted in cell lysis below 5% in all cases. However, a correlation between the number of HLA mismatches and percentage of cell lysis was not observed (Pearson correlation: ratio 1:5, p=0.8211; ratio 1:10, p=0.9248; data not shown). Further characterization was performed by coculture of the final product with allogeneic blasts (2-5 HLA match), either pulsed with pp65 peptide pool or nonpulsed. Expanded T cells specifically induced cell lysis of the allogeneic blasts pulsed with pp65 regardless of the number of HLA matches (**Figure 5 E**). Similarly, a correlation between the number of HLA matches and the percentage of cell lysis was not observed (Pearson test, p=0.6460).

DISCUSSION

In recent years, expansion protocols for the creation of VST third-party banks for adoptive immunotherapy have been under development in different centers as an effective and feasible therapy for immunocompromised patients with herpesvirus infections.[1,5,26–29] We proposed a methodology based on *ex vivo* culture expansion of low-frequency VST to clinical numbers. The process is based on technology that allows scaling up and transfer to cGMP standards. Peripheral blood was used as starting material instead of leukapheresis, in order to facilitate donation. Physiological antigen presenting cells, DC, are used as potent stimulators for specific T cell activation. The protocol described in this study allowed us to produce *in vitro* safe and effective VST after a short expansion of only 14-days. The use of G-Rex technology enabled us to grow cells with a larger volume of media compared to the traditional plastic-based culture and therefore obtain a higher density. The final product was thoroughly characterized for IFN-γ expression among $CD8^+$ cells, which are the cells that most express this cytokine, although the $CD4^+$ subset is also essential for orchestrating the immune response.[16,17] According to the average obtained from the 9 samples, a single blood donation may yield up to 200 doses of 2E+07 VST/m^2, which involves a substantial cost reduction compared to other methodologies. Enhancement of final product reactivity against CMV pp65 peptides was also confirmed by means of IFN-γ ELISPOT assays. The presence of T helper 1 (Th1) $CD4^+$ cells ($CD4^+IFN\text{-}\gamma^+$), present in our final product, is associated with control of persistent infections.[30] The proliferation of NK and B cells was prevented and, despite the use of IL-2, regulatory T cells were not present in the final product, thus facilitating the activity of the VSTs generated.[31] Since naïve T cells are potentially alloreactive, it is also interesting that the final product contained low levels of T_N cells, thereby reducing the probability of GvHD.[32] The final product is mainly composed of T_{EM}, which provide the effector function needed. T_{EM} are known to mediate protective memory and they migrate to inflamed peripheral tissues and display immediate effector functions.[33] The highest percentage of IFN-γ secretion was attributed to this subset, which can therefore rapidly control the infection. Despite the low concentration of T_{CM} in the final product, we hypothesize that these cells may engraft and confer long-lasting protection with later differentiation to effector cells upon antigenic stimulation. *In vivo* T cell persistence of third-party partially HLA-matched VST has been confirmed by other authors for up to 12 weeks.[34] *Ex vivo* expanded cells could also be manufactured, in the case of hematopoietic stem cell transplantation, if available, from the same donor of the transplant, for which rates of functional VST engraftment have been shown to persist for up to 9 years.[35] The option of *ex vivo* expansion on purpose for a single patient or in an autologous context is also conceivable. However, although personalized expansions probably have some advantages, such as choosing the highest HLA matching, this would require manufacturing time that is otherwise absent in a third-party bank.

Final product polyfunctionality was demonstrated by the detection of several secreted cytokines after cell stimulation. Among the inflammatory cytokines tested, we found not only IFN-γ after stimulation with pp65 but also high concentrations of TNF-α. Products with production of multiple cytokines have been shown to provide a more effective immune response against a pathogen.[36]

We showed that the product has the potential to specifically kill pp65 peptide pool-pulsed autologous cells. To emulate allogeneic therapy conditions, we tested pp65-pulsed target cells with 2-3 match with our effector cells. The response varied from the HLA allele matched rather than with the number of HLA matching, in line with findings in the literature that certain pp65 epitopes are HLA restricted and some of them are immunodominant epitopes.[37–40] Moreover, the results shown by degranulation assays further confirmed that the VST obtained act as antigen-specific effector cells; cells degranulate, and therefore have the capacity to strike pp65 pulsed cells.

As for any other therapy it is essential that our product shows not only efficacy but safety. Alloreactivity assays were performed to characterize the *in vitro* safety of the product. When testing alloreactivity, we observed that effector cells did not lyse target cells that were not pulsed with the peptide pool, independently of the number of HLA matches. The similar functionality observed for both the fresh and cryopreserved product demonstrates the feasibility of generating a VST bank that maintains the product properties. The lack of correlation observed between HLA matching and specific cell lysis suggests that HLA restriction of immunodominant epitopes is more important than higher HLA compatibility between the donor and the patient in order to induce specific lysis. On the contrary, higher HLA matching would ensure engraftment of donor T lymphocytes as well as long-term protection. Alloreactivity is an important parameter to consider when using cell therapies as a treatment, as GvHD one of the major concerns when infusing allogeneic cells. Approaches to avoid GvHD include donor-patient HLA matching and reducing the number of T_N cells present in the final product. Moreover, alloreactivity *in vitro* is reduced by *ex vivo* culturing with virus-specific stimuli, as unspecific cell lysis induced by the expanded cells is less than 5%. Our results complement the cytotoxicity assays performed, which ensured the ability of VST to kill target cells without attacking those cells not loaded with the antigen. Some reports indicate that virus-specific memory T cells can exert allo-HLA reactivity.[41] Nevertheless, published studies report no increased incidence of GvHD, even when the donor had HLA mismatch, or toxicity related to VST infusion.[1,5,8,14,27,42]

Future perspectives include the translation of VST production and validation to cGMP standards. Future prospects also include the generation of other virus-specific T cells, such as EBV, AdV and BKV, or even multivirus-specific T cells.

CONCLUSIONS

The use of pp65 peptide pool pulsed DCs and the addition of anti-CD3 and anti-CD28 antibodies in the presence of IL-2, IL-7 and IL-15 resulted in greater than 98.89-fold $CD3^+$ IFN-γ^+ cell expansion at day 14. An average of 25.3% IFN-γ was achieved in 9 expansions, with very small amounts of $CD56^+$, $CD19^+$/$CD20^+$ or $CD4^+CD25^+FOXP3^+$ cells. T_{EM} cell subpopulations were increased after expansion, while T_N subpopulations were decreased in the final product. Moreover, the reactivity of the product against CMV peptide pp65 was enhanced after short culture expansion tested using the IFN-γ ELISPOT assay. In terms of effectivity and *in vitro* safety, strong cytotoxicity was shown when specific T cells were cocultured with pulsed pp65 autologous blasts. Final product degranulation was confirmed in line with cytotoxicity results. When expanded cells were cultured with nonpulsed allogeneic blasts, a maximum of 5% lysis was observed. Furthermore, cytotoxicity of expanded cells with pp65-pulsed allogeneic blasts showed specific cell lysis. In conclusion, we have shown a feasible and scalable process that can be easily transferred to cGMP standards and that generates a safe and functional product.

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FIGURE CAPTIONS

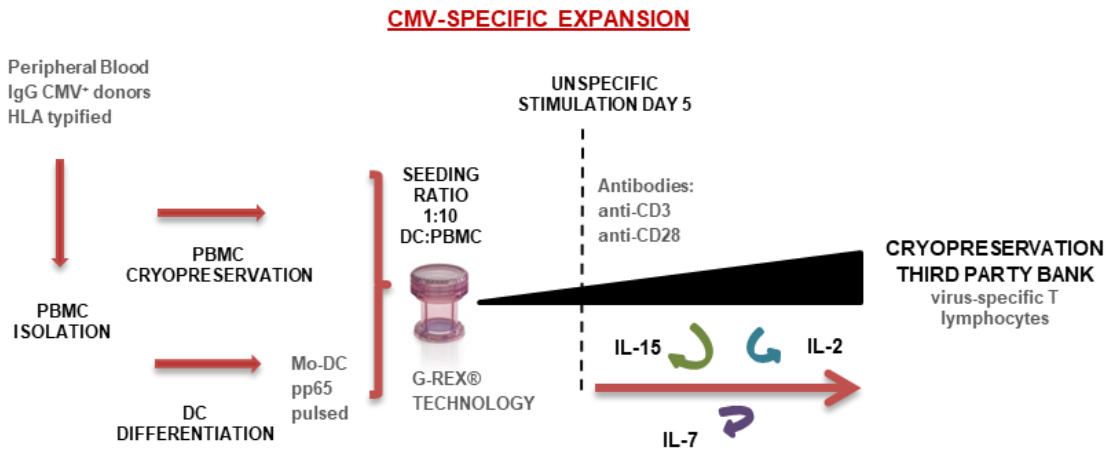


Figure 1. Expansion protocol schematic for the rapid generation of virus-specific T cells (VST). Starting material based on HLA typed peripheral blood IgG^{+} for CMV. Isolation of PBMC and selection of monocytes by plastic adherence. Non-adherent PBMC cryopreserved, and attached monocytes differentiated to DC. Seeding performed in a G-Rex device in a ratio 1:10 pp65-pulsed DC:thawed PBMC. Expansion lasted 14-days, with addition at day 5 of anti-CD3 + anti-CD28 antibodies, and IL-2, IL-7, IL-15. Renewal of cytokines was performed at days 5, 7, 9, and 12. At day 14 the final product was characterized and cryopreserved.

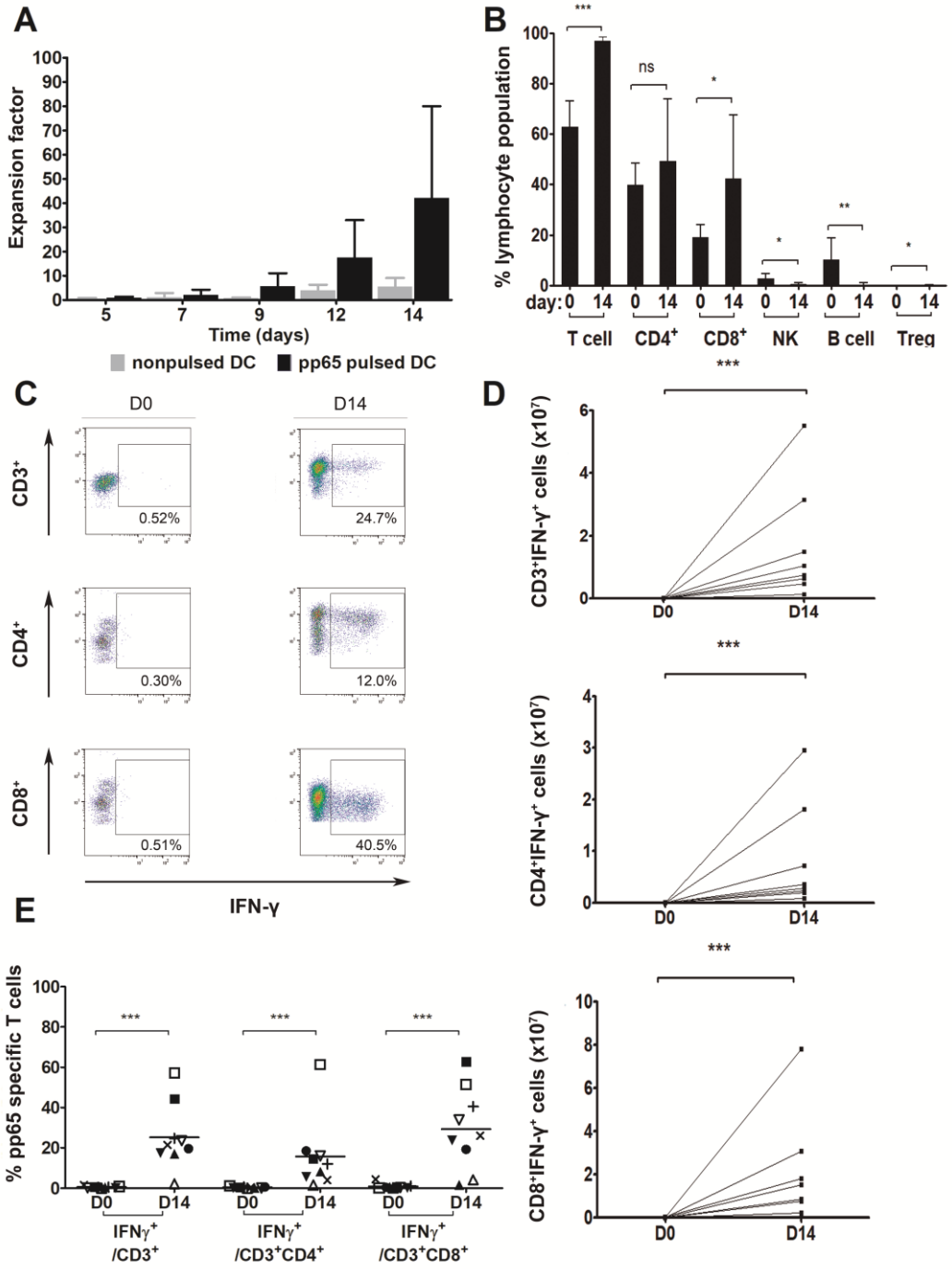
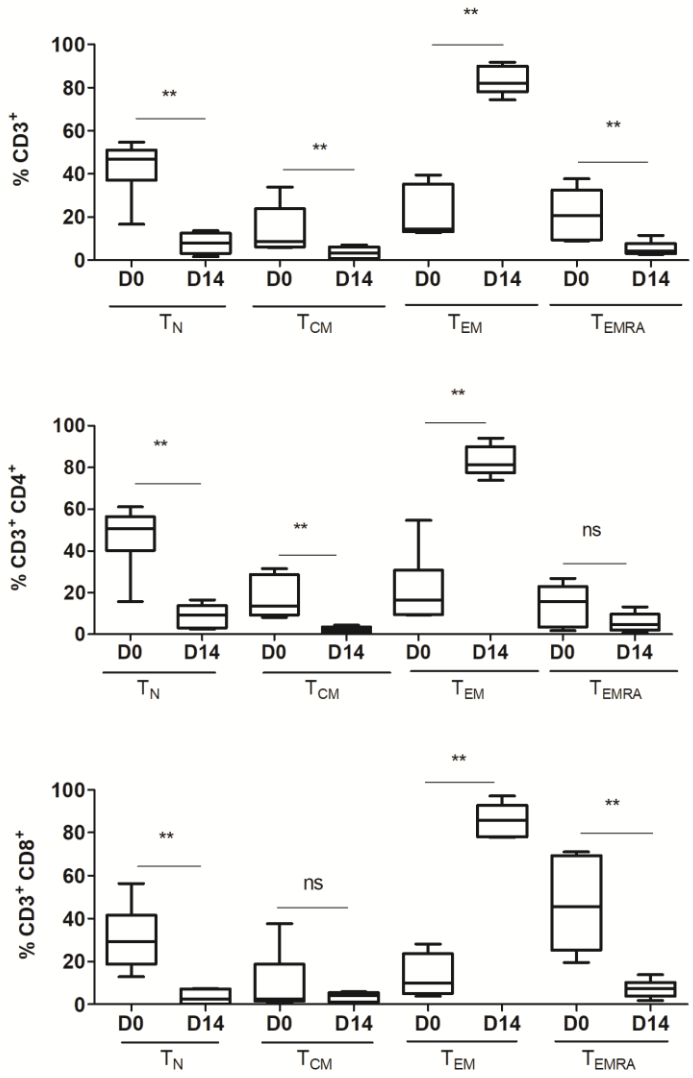


Figure 2. Pp65-specific T cell expansion. (A) Number of total cells at different time points during the 14-day culture. PBMC cocultured with: pp65 peptide pool-pulsed DC (black, n=9) and nonpulsed DC (grey, n=4). (B) Percentage of general cell subpopulations pre- (day 0) and post-expansion (day 14). T cells: $CD3^+$, $CD4^+$: $CD3^+CD4^+$, $CD8^+$: $CD3^+CD8^+$, NK: $CD3^-CD56^+$, B cell: $CD3^-CD19^+CD20^+$, Treg: $CD3^+CD4^+CD25^+FOXP3^+$ (n=9). (C) Gating strategy for IFN-γ secreting cells from one representative donor. Dot plots show the response of T lymphocytes against pp65 antigen stimulation. (D) Number of VST at day 0 and after the 14-day culture (n=8). (E) Percentage of IFN-γ secreting population cells at days 0 and 14 of culture (n=9).

A



B

	T_N	T_{CM}	T_{EM}	T_{EMRA}
$CD3^+$ IFN-γ^+	3.14 ± 2.79	3.61 ± 2.61	91.15 ± 5.29	2.11 ± 0.40
$CD3^+$ $CD4^+$ IFN-γ^+	3.65 ± 2.88	3.36 ± 2.67	90.95 ± 5.25	2.01 ± 0.87
$CD3^+$ $CD8^+$ IFN-γ^+	3.12 ± 3.13	3.62 ± 0.62	89.03 ± 7.62	4.24 ± 4.29

Figure 3. Frequency of different T cell differentiation subset phenotypes in the final product. (A) Comparison between pre-expansion and post-expansion for $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ subpopulations (n=6). (B) T cell differentiation phenotypes in post-expansion IFN-γ^+ T cells (n=4). T_N indicates naïve T cell ($CCR7^+CD45RA^+$); T_{CM}, central memory T cell ($CCR7^+CD45RA^-$); T_{EM}, effector memory T cell ($CCR7^-CD45RA^-$); and T_{EMRA}, terminally differentiated effector memory T cell ($CCR7^-CD45RA^+$).

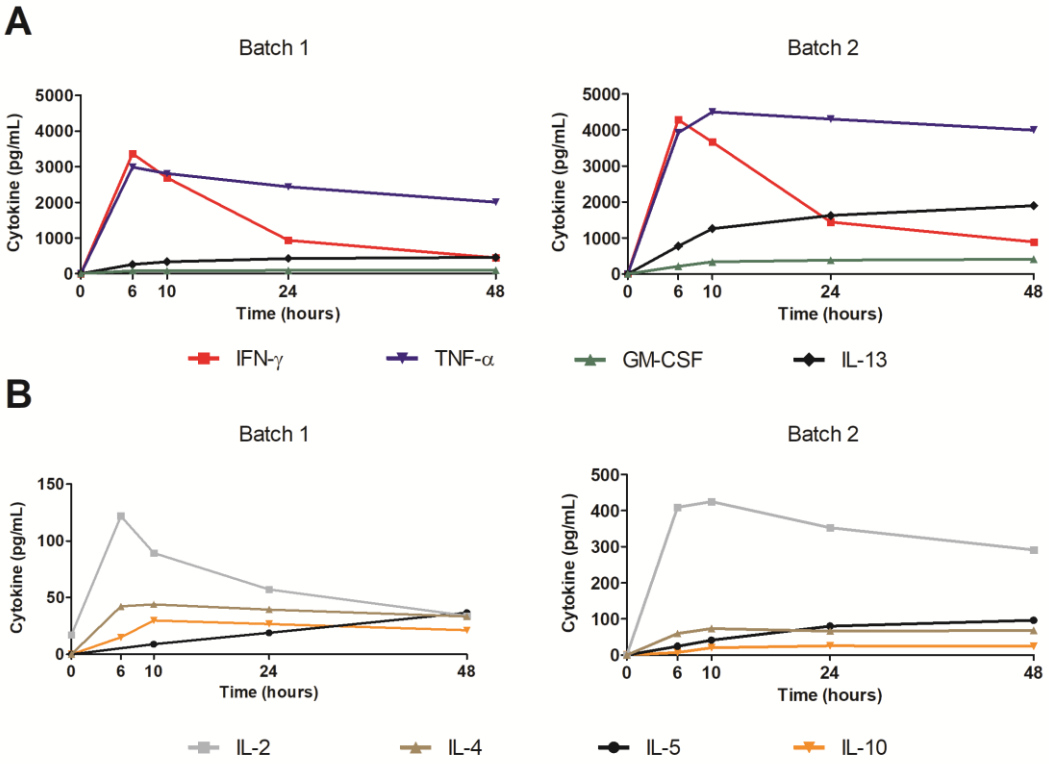
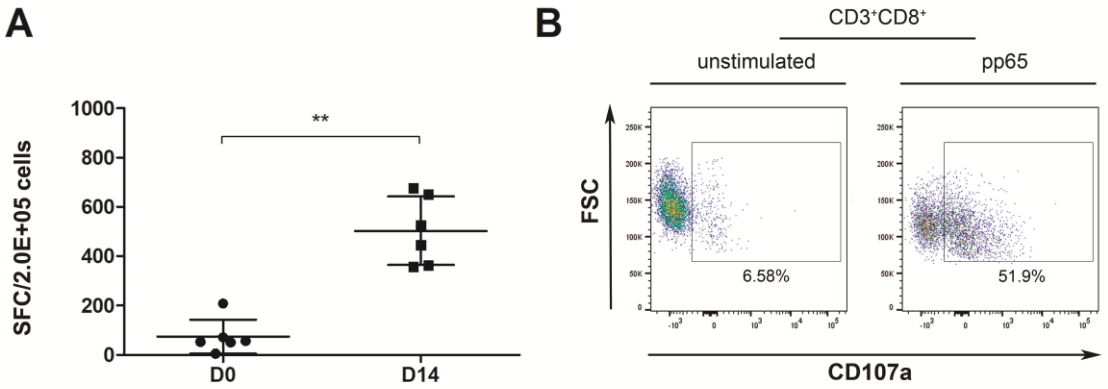
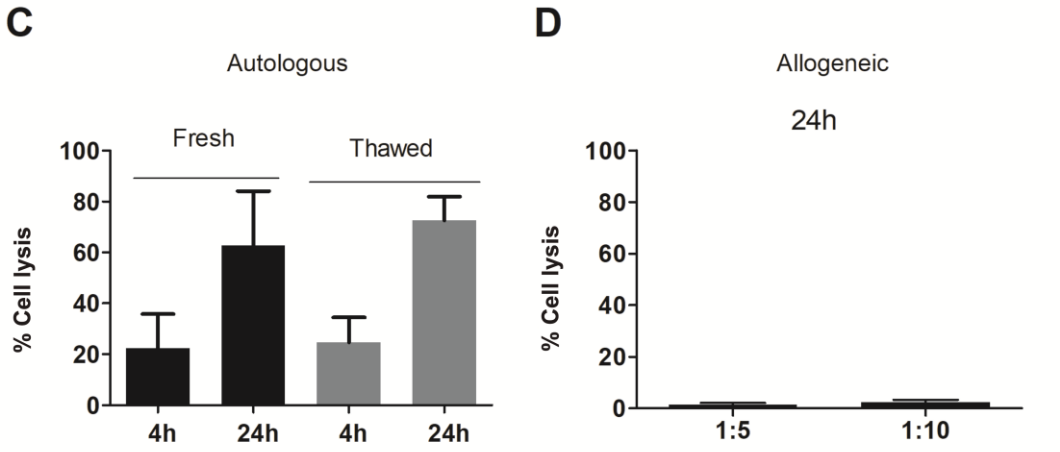


Figure 4. Polyfunctionality of the final thawed product tested using cytokine secretion kinetics after antigenic stimulation. Test performed with Luminex technology. Cytokines tested: IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, IFN-γ, GM-CSF and TNF-α. Cells were stimulated with pp65 50ng/mL at a cell concentration of 1E+06 cells/mL in a 96-well plate. Graphs show the secretion kinetics of the concentration of the cytokines present in the supernatant (pg/mL) at each time: 0h, 6h, 10h, 24h and 48h. (A) Representation of secretion kinetics for cytokines IL-13, IFN-γ, GM-CSF, and TNF-α from 2 batches of final product. (B) Representation of secretion kinetics for cytokines IL-2, IL-4, IL-5, and IL-10 from 2 batches of final product.





E

Effector	Target	Match	HLA match	Cell lysis (%)
1	II	2 match	*A*02:01, DRB1*07:01*	9.97
1	III	4 match	*A*03:01, B*18:01, DRB1*07:01, DQB1*03:03*	10.48
1	I	5 match	*A*02:01, B*18:01, C*05:01, DRB1*03:01, DQB1*02:01*	14.47
2	I	2 match	*A*02:01, B*35:03*	49.52
3	IV	2 match	*B*49:01, C*07:01*	5.39
3	V	3 match	*A*11:01, C*07:01, DRB1*13:02*	29.61

Figure 5. Potency and functional assays performed with the expanded product. (A) IFN-γ ELISPOT for D0 and D14 thawed cells (n=6). (B) Example of a degranulation assay ($CD8^+CD107a^+$) dot plot for effector cells (VST), unstimulated (left plot) and stimulated with pp65 (right plot). (C) Cytotoxicity assay representation for pp65-pulsed autologous blasts specific lysis (4-h ratio, 1:5 (T:E); n=3; 24-h ratio, 1:5 (T:E); n=2). (D) Cytotoxicity assay representation for irradiated allogeneic blasts unspecific cell lysis with 2-3 match (n=6, fresh n=2, thawed n=4). (E) Cytotoxicity assay representation for effector cells (E): VST, cocultured with target cells (T), pp65-pulsed allogeneic blasts. Percentage of cell lysis calculated from a ratio of 1:10 (T:E).

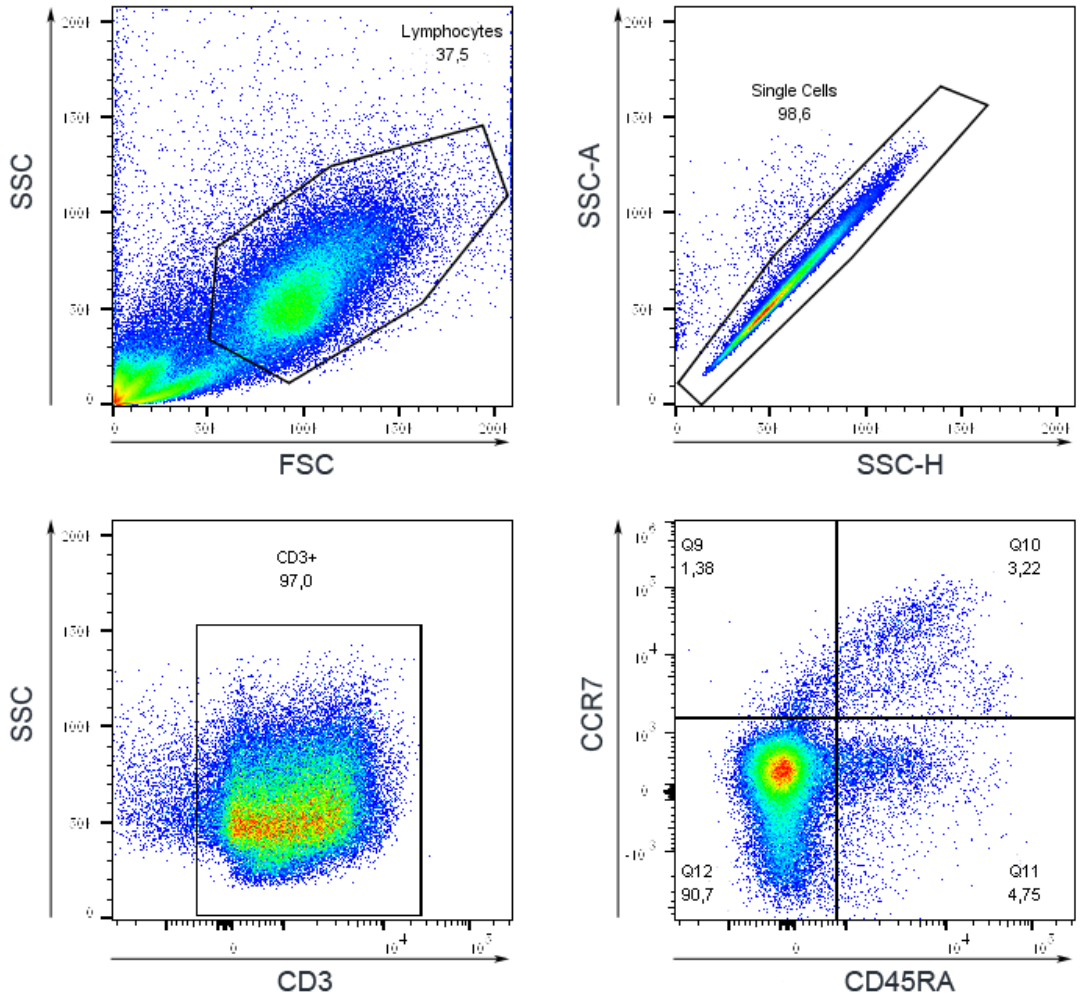
4.2.1 Supplementary material

Supplementary Table 1. HLA typing at high resolution for effector samples used in the alloreactivity assay.

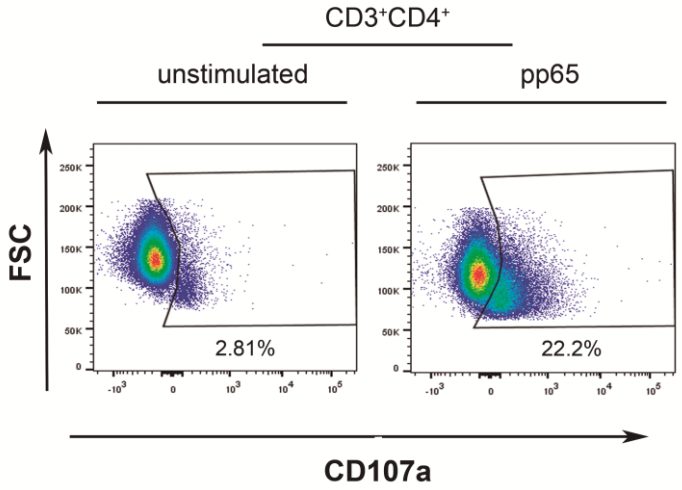
Batches	HLA type
1	*A*02:01, A*03:01, B*18:01, B*51:08, C*05:01, C*16:02, DRB1*03:01, DRB1*07:01, DQB1*02:01, DQB1*03:03*
2	*A*02:01, A*03:01, B*35:03, B*51:01, C*04:01, C*16:01, DRB1*07:01, DRB1*12:01, DQB1*02:02, DQB1*03:01*
3	*A*11:01, A*32:01, B*49:01, B*51:01, C*07:01, C*14:02, DRB1*13:02, DRB1*14:04, DQB1*05:03, DQB1*06:04*

Supplementary Table 2. HLA typing at high resolution for allogeneic PHA lymphoblasts used for alloreactivity assays.

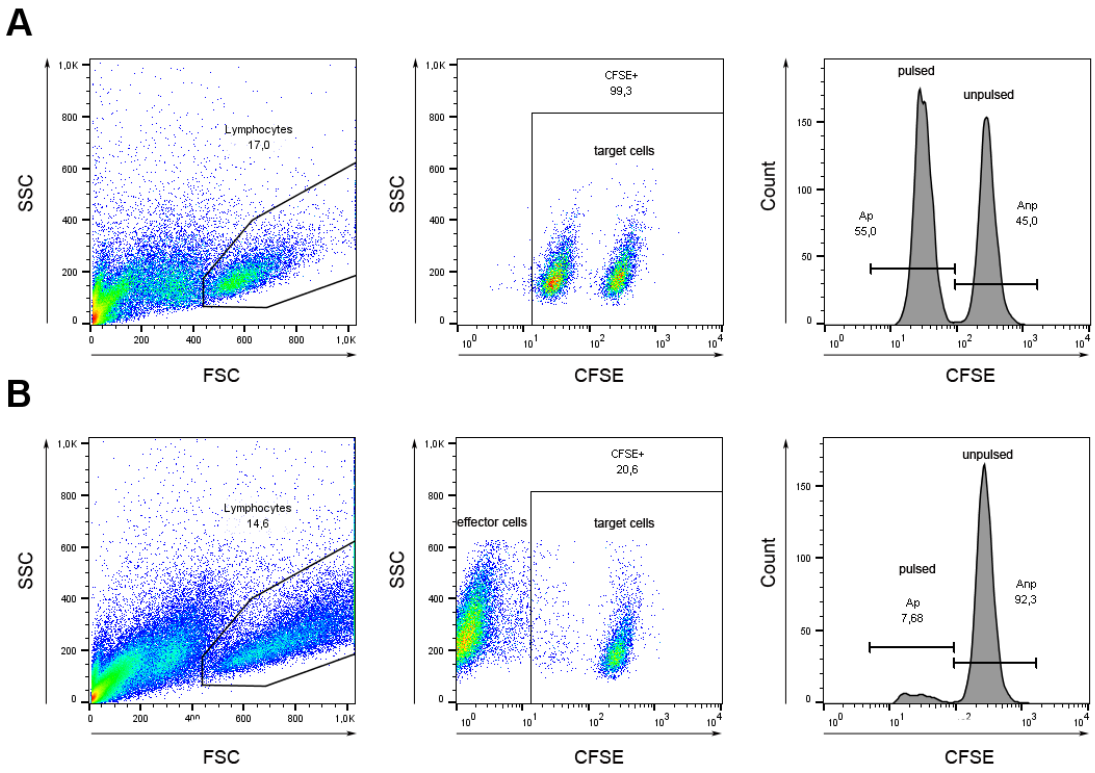
Blasts	HLA type
I	*A*02:01, A*23:01, B*18:01, B*35:03, C*05:01, C*12:03, DRB1*03:01, DRB1*04:08, DQB1*02:01, DQB1*03:04, DPB1*04:01, DPB1*04:01*
II	*A*01:01, A*02:01, B*44:03, B*56:01, C*01:02, C*04:01, DRB1*07:01, DRB1*11:01, DQB1*02:02, DQB1*03:01, DPB1*02:01, DPB1*04:01*
III	*A*03:01 A*24:02, B*18:01 B*35:08, C*04:01 C*07:01, DRB1*07:01 DRB1*13:02, DQB1*03:03 DQB1*06:04*
IV	*A*23:01, A*26:01, B*39:01, B*49:01, C*07:01, C*12:03, DRB1*01:01, DRB1*07:01, DQB1*02:02, DQB1*05:04*
V	*A*11:01, A*80:01, B*18:01, B*44:03, C*04:01, C*07:01, DRB1*13:02, DRB1*15:01, DRB3*03:01, DRB5*01:01 DQB1*06:02, DQB1*06:09, DPB1*04:01, DPB1*13:01*



Supplementary Figure 1. Flow cytometry gating strategy to identify naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) T cell subsets. A dot plot for the lymphocyte selection by FSC and SSC was performed, followed by doublet discrimination. Classification of the population was done after $CD3^+$ selection (from the singlets selection) by CD45RA and CCR7 gating, and determination of each cell population (T_N: $CD45RA^+CCR7^+$, T_{CM}: $CD45RA^-CCR7^+$, T_{EM}: $CD45RA^-CCR7^-$, and T_{EMRA}: $CD45RA^+CCR7^-$).



Supplementary Figure 2. Functional assays performed with the expanded product. Example of a degranulation assay ($CD4^+CD107a^+$) dot plot for effector cells (VST), unstimulated (left plot) and stimulated with pp65 (right plot).



Supplementary Figure 3. Gating strategy for cytotoxicity assay. Initial lymphocyte population selection by FSC-H vs SSC-H. CFSE stained lymphocytes correspond to target cells: autologous pp65-pulsed (low CFSE concentration) and autologous not pulsed target cells (high CFSE concentration). (A) Control sample containing only target cells, pulsed and nonpulsed. (B) Effector sample with pulsed and nonpulsed cells, ratio 1:5 (T:E).

PART III: DISCUSSION AND CONCLUSIONS

CHAPTER V: DISCUSSION

The breakthrough of sophisticated therapies, like sCMTP make drug development more complex than for other drug products. Cell therapy products are harder to characterise, plus they are intrinsically more variable than conventional drugs. Researchers and regulatory authorities face major challenges regarding standardisation of protocols, determination of potency assays, and description of the exact MoA among others. Taking into account these, in this work we attempted to address these issues by extensively characterising and developing cell products, with the additional potency assay establishment for MSC, as well as, the definition of a protocol to manufacture VST easily scalable to cGMP standards.

Rapid progress in the understanding of the biology of MSC and their unique properties, which include: multidifferentiation potential, tropism to injured tissues, immunomodulation capacity, and the release of growth factors and cytokines in response to environmental cues (Mirabel *et al.*, 2018), together with their considerable therapeutic success, has made MSC interesting for many applications. Consequently, researchers started to isolate MSC from a wide variety of tissues, for both scientific interest and better suitability for addressing specific pathologies, when the ISCT rushed to establish certain criteria for the characterisation of these cells (Dominici *et al.*, 2006). Additionally, the regulatory authorities like the FDA and the EMA established as a requirement to define a measure of potency to prove the product's MoA, as in the relevant therapeutic activity or intended biological effect (Galipeau *et al.*, 2016).

The formulation of MSC concept along with the criteria proposed by the ISCT generated ambiguity among researchers, whom already struggle to perform potency assays (Bianco *et al.*, 2013; Bravery *et al.*, 2013; Mattar and Bieback, 2015; Galipeau *et al.*, 2016; De Wolf, Van De Bovenkamp and Hoefnagel, 2017). Additionally, the lack of standardisation of protocols for potency assays plus variability associated to biological samples, in terms of MSC sources, manufacturing processes, route of delivery, donor, and *in vitro* and *in vivo* product characterisation, make clinical translation of MSC-based products difficult (Mendicino *et al.*, 2014; Caplan *et al.*, 2019; Martin *et al.*, 2019). This highlights the need of setting stakeholders' efforts towards generating product specifications that are consistent across batches and consensus assays for testing MSC potency. The proper definition would a) guarantee reproducible results; b) allow comparison across multiple studies; and c) facilitate potential clinical use. In this PhD project we contribute in the characterisation of MSC in two critical quality attributes, which are often a matter of debate: the MSC potency assay and regarding MSC phenotype, the HLA-DR expression.

In section 3.2 of Chapter III, we proposed a definition of a suitable and simple potency assay for product delivery into the clinic, in compliance with current quality and regulatory requirements, and based on the immunomodulative properties of MSC. The potency assay we developed is used as quality control for batch release in two clinical trials (EudraCT No.'s 2012-000734-19 and 2015-005786-23) for the treatment of multiple sclerosis and spinal cord injury, respectively. The potency assay, which is already challenging to define for ATMP, presents also the difficulty that it has to be designed according to the product application. In this case, the assay we developed is adequate for these applications, as the MSC MoA for the intended use does not require MSC differentiation but immunomodulation.

The *in vitro* potency assay we proposed is based on the stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin of allogeneic PBMC, labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE), co-cultured with MSC, with the measure of T cell proliferation as the read-out parameter.

In order to reduce variability in responder cells and provide consistency to the assay, we standardise the time of blood extraction (24-48h) and the storage (+4ºC). For PBMC isolation, we optimised the method with the use of SepMate^{TM} tubes instead of classical density gradients in conical tubes. This method reduces PBMC isolation variability among the technitian performing the assay. A major concern regarding CFSE labelling is the high mortality rate associated to the toxicity resulting from excessive surface protein substitution after CFSE coupling (Quah and Parish, 2012). To asses this issue, we tested CFSE labelling with Dulbecco's Phosphate-Buffered Saline (DPBS) and Roswell Park Memorial Institute (RPMI) supplemented with 10% human serum B (hSerB). We hypothesised that the use of supplemented medium could reduce toxicity. We tested different CFSE concentrations and compared the yields of the staining reactions with RPMI+10%hSerB and DPBS. Viability values were similar for all conditions, suggesting that the mortality observed by other authors may be attributed to the labelling procedure rather than CFSE itself. The optimum results were found after labelling PBMC with 0.625–2 μM CFSE in DPBS, as all the stainings performed in RPMI+10%hSerB showed very low fluorescence intensity. Remarkably, when labelling with 8 and 30 μM of CFSE in DPBS, fluorescence intensity was too high to correctly measure by flow cytometry.

The assay presented has the advantage of measuring the functionality of MSC on stimulated T cells, but is not restricted to $CD3^+$ T cells. Nevertheless, it can give misleading results due to cell death or functional alteration of cultured cells, which can be addressed with proper controls. Normalisation of data is therefore important for proper comparison of results, in this sense,

values of absolute proliferation (subtracting non stimulated average values) and normalised proliferation (considering single PBMC culture absolute proliferation as 100%) were calculated for each condition, thus reducing the impact of donor-dependent proliferation variability of PBMC.

We tested different polyclonal stimuli including PMA:ionomycin, phytohemagglutinin (PHA) as mitogens and monoclonal antibodies anti-CD3:anti-CD28 for the cross-linking and co-stimulation of TCR, respectively. From all stimuli tested, PMA:ionomycin induced the strongest response, with over 70% of proliferation in all conditions. One could argue about the stimuli chosen, which, ideally, should simulate the inflammatory environment that happens *in vivo,* however, this is practically impossible to determine, and it is accepted by the regulatory authorities to use approaches like this one. Another aspect to take into account is that this assay can present some variability due to the heterogeneity of the target population, herein PBMC. As reported by Wolf and collaborators, the presence of T regulatory cells can differ between donors, a cell type stimulated by MSC which is used as target in the assay, and this variability can lead to an underestimation or overestimation of MSC immunomodulatory potential (De Wolf, Van De Bovenkamp and Hoefnagel, 2017). An approach to avoid this and increase robustness of the assay is the generation of pools of PBMC from different donors (Ketterl *et al.*, 2015). However, the use of pools has intrinsic mixed lymphocyte reaction (MLR), and we preferred to rather deal with inter-donor variability with the corresponding controls, and take it into account in the validation of the technique. In addition, the PMA:ionomycin is technically easier and reproducible than MLR assay, and is more suitable for standardisation, as it avoids stimulator cells variability, which is an important issue in terms of a potency assay. Moreover, the ISCT addressed the issue of identification of functional potency markers and declared that it is still unknown whether a MLR truly reflects the MoA of human MSC *in vivo* (De Wolf, Van De Bovenkamp and Hoefnagel, 2017). However, if the goal of the immunopotency assay is to highlight the MSC MoA for application in the clinical setting, the cells involved in the disease pathogenesis rather than random PBMC from a healthy donor could be more informative (Galipeau *et al.*, 2016). Moreover, MSC should undergo the same conditions for the potency assay and for the patients (De Wolf, Van De Bovenkamp and Hoefnagel, 2017). Therefore, if MSC potency assay is performed with irradiated MSC, it is not adequate if MSC are infused without irradiation. Moreover, our results indicated that the inhibitory effect of non-irradiated MSC was higher than irradiated MSC, although differences were not statistically significant ($p = 0.2$).

Moreover, although the inhibition of T cell activation markers could be used *a priori* as read-out, this might not be appropriate for MSC, as the anti-proliferative effect of MSC seems not to be due to inhibition of T cell activation, but to an induction of cell cycle arrest in activated T cells without affecting the expression of early activation markers CD25 and CD69 (Glennie *et al.*, 2005).

In some cases, when a MSC product is indicated for different applications, the use of two different potency tests could be more adequate. As MSC have a wide variety of applications, potency assays should be performed according to the MoA intended for the treatment. Despite the different assays described, the assay of choice for MSC potency still appears to be T cell proliferation inhibition (De Wolf, Van De Bovenkamp and Hoefnagel, 2017).

In order to demonstrate the applicability of the potency test two examples were provided. The first example showed that thawed MSC had less immunomodulation potential than fresh cells. These results were in line with other groups that observed that cryopreservation impair MSC immunomodulatory properties (François *et al.*, 2012; Moll *et al.*, 2014). However, when the ratio of coculture with PBMC was increased, a similar inhibition of proliferation to the control was obtained. The maintenance of the immunomodulation capacitiy after cryopreservation, although diminished, is crutial for off-the-shelf use, as cryopreservation offers the advantage of having readily patients' samples for future distribution and use. Moreover, the ratio *in vivo* is impossible to simulate, and therefore the amount of cells infused might be enough to produce this effect.

The second example provided was regarding HLA-DR expression. We applied the potency test to assess if the immunomodulation capacity of BM-MSC was affected by HLA-DR expression. In order to do so, sorted HLA-DR$^+$ and HLA-DR$^-$ BM-MSC were tested. Interestingly, the immunomodulatory potential of MSC was not affected by HLA-DR expression, as HLA-DR$^+$ population was able to inhibit T cell proliferation. According to Le Blanc and collaborators results, either HLA-DR$^+$ or HLA-DR$^-$ MSC were found to inhibit PBMC proliferation (Le Blanc *et al.*, 2003).

HLA-DR expression is a marker that should be negative according to the ISCT minimal criteria, however, it is expressed spontaneously in culture, as we and others have observed (section 3.5 of Chapter III). In addition, HLA-DR is generally not used as release criteria, as product specifications need reliable markers that are consistent from batch to batch, and are therefore omitted by many groups and used only as an informative value at best (Mendicino *et al.*, 2014).

With the work presented in this thesis we encouraged others to present their data and propose a modification of this marker as MSC identifying criteria.

In section 3.4 of Chapter III we address our own experience in clinical grade BM-MSC produced under cGMP for patients in the autologous use. Initially, we increased the acceptance criteria of HLA-DR expression to 20%, to later use it only for informative purposes, in conformity with the competent regulatory authority. Nevertheless, since the median value of HLA-DR$^+$ cells was 17% in 81 batches, we decided to study what triggered this expression and if it had any effect on MSC identity and potency. The first hypothesis was that cytokines present in the human serum that supplemented the defined Dulbecco's Modified Eagle Medium (DMEM) affected this marker. In order to do so, we analysed both human serum, used in pools as a supplement for MSC culture media, and the culture supernatant. We had expected to find the presence of IFN-γ in the sera or supernatants, but according to our results HLA-DR expression was not related to the presence of this cytokine. Interestingly, a correlation was found with HLA-DR expression and pro-inflammatory cytokines IL-17F and IL-33 in the supernatant. They could potentially be used as biomarkers of MSC activation in culture. No correlation was found with sera and HLA-DR expression. Our results also show that BM-MSC identity and potency did not differ upon HLA-DR expression. MSC adipo-, chondro-, and osteogenic differentiation potential was shown not to be interfered by HLA-DR expression. In order to test pure populations of HLA-DR$^+$ cells, instead of heterogeneous populations, we sorted the activated fraction of BM-MSC that readily differentiated into the chondrogenic and osteogenic lineages. These data, in line with results from section 3.2 in Chapter III, was based on sorted HLA-DR$^+$ BM-MSC that were subjected to lymphoproliferation assay, and showed similar results for both cell fractions. In summary, we demonstrated that BM-MSC' identity and potency are not compromised regardless of HLA-DR expression.

Through the section 3.5 of Chapter III, we focused further on the study of HLA-DR expression in BM-MSC cultures, and demonstrated the unpredictable and dynamic expression of this molecule. We tested that BM-MSC HLA-DR expression can be triggered by IFN-γ (Le Blanc *et al.*, 2003). We also observed, that when IFN-γ was removed, the number of HLA-DR$^+$ cells gradually decreased over time. However, when characterising BM-MSC in terms of identity and potency, we observed that HLA-DR$^+$ expression had no effect on these parameters.

Data exist suggesting that MSC require certain activation by pro-inflammatory cytokines in order to exert immunosuppression (Ghannam *et al.*, 2010), or that even immunomodulation activity was enhanced by these (Le Blanc *et al.*, 2003). Interestingly, they found that HLA-DR was

expressed first intracellularly, after 2 days of IFN-γ exposure, but required 7 days for extracellular expression. On the contrary, we observed HLA-DR expression after only 48 hours of IFN-γ stimulation and slowly downregulation after removal of IFN-γ. This fact reveals that HLA-DR expression can come from a previous exposure to stimuli that might not be related to the short expansions for clinical grade MSC obtaining. It also highlights that HLA-DR expression is dynamic, and since quality controls are only performed at specific time points, these can only provide a snapshot of the cell expression.

The major concern regarding HLA-DR expression in BM-MSC is the possible rejection of these cells. However, Le Blanc and collaborators demonstrated *in vitro* that MSC are not inherently immunogenic and can therefore be transplanted between HLA-incompatible individuals (Le Blanc *et al.*, 2003). They tested that both differentiated and undifferentiated MSC inhibit mixed lymphocyte cultures, and that their suppressive activity increased when the cells were exposed to IFN-γ. The latter cytokine was used to induce the expression of HLA-DR, whose upregulation on the cell surface after differentiation diminished. Later in 2004, Le Blanc and collaborators transplanted haploidentical BM-MSC to a patient with severe treatment-resistant grade IV acute GvHD of gut and liver. They obtained a striking clinical response, and concluded from these results that although MSC express HLA class I and, in some cases HLA class II (i.e. expression induced by cytokines, depending on the source (BM)), these cells can be infused safely regardless of HLA-DR expression without rejection (Le Blanc *et al.*, 2004). Moreover, the co-administration of allogeneic MSC and hematopoietic stem cells to patients with haematological malignancies is currently used and has resulted in an increment of the engraftment and reduced incidence and severity of GvHD (Liu *et al.*, 2017; Barrett and Galipeau, 2019).

According to the literature and with our results regarding potency and identity of MSC, we suggested the removal of HLA-DR expression of BM-MSC quality control panels as product identifying criteria. This would allow a better standardisation of the product, and reduce the variability observed among different laboratories. As future prospects it would be interesting to further investigate the triggers of HLA-DR expression in BM-MSC. Moreover, further clinical studies could discard the secondary effects of HLA-DR$^+$ BM-MSC and their therapeutic effect.

Moreover, the use of MSC is being explored for a wide variety of diseases. MSC immunomodulation potential is of huge interest for the treatment of GvHD, autoimmunity and transplant rejection. Nowadays, the majority of MSC in clinical development based on immunomodulatory properties are indicated for acute GvHD, either in the autologous, haploidentical, or HLA mismatched MSC (Amorin *et al.*, 2014; De Wolf, Van De Bovenkamp and

Hoefnagel, 2017). Although allogeneic MSC can and do generate both innate and humoral responses from the immune system, many pre-clinical studies indicate that MSC efficacy is often independent of the immune rejection of donor MSC, either due to MSC activity occurs prior to immune recognition or because the MoA include the host immune system (Caplan *et al.*, 2019). Moreover, no malignant potential has been reported *in vitro* nor *in vivo* in studies of clinical grade MSC (Caplan *et al.*, 2019). Approaches to reduce tumorigenic transformation include careful monitoring of cultures, short expansion *in vitro*, and evaluation of cytogenic aberration when concerns rise (Caplan *et al.*, 2019). Along MSC research advances, immunomodulation capacity of MSC is extensively reported but very few reports exist regarding the mechanisms by which they induce immunosuppression. Galleu and collaborators reported that MSC enter apoptosis after cell infusion and do not engraft (Galleu *et al.*, 2017). Apoptosis is mediated in this case by recipient cytotoxic cells and is necessary for MSC activity. Apoptotic MSC are engulfed by phagocytic macrophages, by a process named efferocytosis, which in turn secrete molecules affecting immunosuppression like IDO, IL-10 and transforming growth factor β (TGF-β) (Galipeau and Sensébé, 2018). Altogether, these data support the efferocytosis (or hit and run theory) based on MSC paracrine effects (Galipeau and Sensébé, 2018). Remarkably, the unpredictable benefit of MSC for therapy could be explained. Patients with GvHD that responded to MSC infusion, were those that had a high cytotoxic activity against MSC, leaving it open but uncertain if the future application of MSC would be the infusion of apoptotic MSC generated *ex vivo* (Galleu *et al.*, 2017). Response rates to MSC occur in 50-60% of GvHD patients, although it is difficult to compare studies due to the challenge of identifying the exact MoA, and failure to consider issues of dosing, timing, route and viability (Carty, Mahon and English, 2017; Martin *et al.*, 2019).

Yet, MSC can be used in the same context for tissue engineering applications, in the treatment of avascular necrosis due to GvHD treatment with corticosteroids (Zhao *et al.*, 2012; Im, 2017). Making special notice of MSC treatment of GvHD due to the morbidity and mortality associated to this disease but also because of being a post-transplantation complication after HSCT.

The laboratory experience was initially focused on BM-MSC, in line with the literature, until we developed protocols for WJ-MSC expansion and switched to use the UC as an alternative source of MSC, as shown in the Appendix (Oliver-Vila et al., 2016). The protocol developed in our laboratory was based on a banking model of UC, with WJ-MSC derivation followed by the generation of a two-tiered system of Master Cell Bank (MCB) and Working Cell Bank (WCB), compatible with cGMP production environment. The extensive expansion in culture of WJ-MSC

and their survival after cryopreservation make these cells suitable for the off-the shelf format (Mirabel *et al.*, 2018).

WJ-MSC are interesting for their lack of somatic mutations, non-invasive procedures for tissue collection, standardisation of donor age, and for the UC being a tissue that is otherwise discarded (Krause, Lozano and Lim, 2019). Reports indicate that WJ-MSC have a higher immunomodulation capacity than BM-MSC, which makes them interesting for the applications in the inflammatory context (Li *et al.*, 2014; Donders *et al.*, 2018). Additionally, WJ-MSC have a higher proliferation capacity than BM-MSC (Li *et al.*, 2014; Batsali *et al.*, 2017). Larger comparisons of MSC potency regarding source, as well as results from different centres, would be facilitated by standardisation of protocols (Mattar and Bieback, 2015). Moreover, although HLA-DR expression does not seem to have any effect limiting HLA-DR$^+$ BM-MSC use, WJ-MSC do not express HLA-DR. Additionally, WJ-MSC do not completely replace the use of BM-MSC, as the latter still have higher differentiation potential towards osteocytes and adipocytes (Batsali *et al.*, 2017). Researchers are approaching this low differentiation potential, for instance, by improving it by treatment of WJ-MSC with recombinant human WNT-associated molecules WISP-1 or sFRP4 (Batsali *et al.*, 2017). All these data suggest that, although the use of either type of MSC can be interchangeable at the moment, MSC release criteria should match the hypothesised MoA, which depends on the tissue source, type of disease and the characteristics of the recipients (Martin *et al.*, 2019).

In the work exposed in Section 3.3 of Chapter III, we presented the successful manufacture under cGMP of 8 batches of WJ-MSC for clinical use. In the context of MSC manufacturing, it is important to perform extensive analysis that ensures quality, efficacy, and safety of the final product (Sensebé, Bourin and Tarte, 2011). Therefore, we used the tool of QbD to integrate the risk management into our production process and the potency test, to pin the main points that may affect the quality of our final product, and increase the consistency as well as to decrease the possibility of products out of specifications.

The bioprocess design and the immunomodulation assay, earlier developed as presented in section 3.2, has the authorisation of the competent authority. Since the regulatory environments have established stringent requirements to ensure product safety and efficacy, we proposed the immunomodulation assay for quantification of WJ-MSC potential, since the application of these cells require an immunomodulatory activity. The assay proposed covers some of the MoA *in vivo*, regarding T cell proliferation inhibition. An interesting future

perspective would involve the study of a correlation between the clinical results and the quantitative attribute derived from our potency assay.

Recently described paracrine functions of MSC through EV put at stake MSC therapy, proposing the use of EV obtained during MSC culture and offering a cell-free therapeutic application (Rani *et al.*, 2015; Hu *et al.*, 2019). However, these nanoparticles also need proper characterisation. Current results on EV effects show some incongruences, which can be probably explained by variability in the preparation of EV that can lead to contamination with high quantities of non-EV proteins (Monguió-Tortajada *et al.*, 2017), fact that highlights the importance of purity in the EV product. Despite the advances in EV field, they still present certain challenges, as the isolation protocol, study and characterisation, to confidently use them with clinical purposes (Ramirez *et al.*, 2018).

Since the clinical experience of MSC has largely preceded the basic knowledge of their biology, future perspectives of MSC involve a better understanding of MSC MoA as well as product standardisation of this therapy (Martin *et al.*, 2019). In this context, quality control criteria standardisation is necessary as new MSC reach the clinical setting and comparability studies are required to define efficacy for new drug candidates (Cuende *et al.*, 2018).

It is both somatic cell medicinal products presented in this work, MSC and VST that offer great promise to overcome the main transplant-related mortality causes, which are GvHD (17% in match related-donor and 19% in unrelated-donor) and infections (12% in match related-donor and 17% unrelated-donor) (Henig and Zuckerman, 2014).

Another major complication post HSCT includes infections, which is addressed in the second part of this work. Many factors contribute to the vulnerability of HSCT recipients: a) cytotoxic drugs administered during conditioning, b) the delay in endogenous immune recovery after transplant, and c) the immunosuppressive therapies given to prevent or treat GvHD (Papadopoulou *et al.*, 2014). The patients' immunocompromised conditions, suppose a risk of opportunistic infections (bacterial, fungal or viral). CMV reactivation is the major viral infectious complication after allogeneic HSCT (Stern *et al.*, 2019), and can either be endogenous (reactivation of latent virus) or exogenous (infection or reinfection with a new strain of CMV) (Zuhair *et al.*, 2019). In most patients CMV infection and/or reactivation remain subclinical, presumably with virus replication controlled by effective immune surveillance (Stern *et al.*, 2019; Zuhair *et al.*, 2019). The high prevalence of CMV seropositivity with rates of 45-100% in the general adult population (Lindemann *et al.*, 2018), increased with age, can be an issue in the context of HSCT when the

individual is immunosuppressed. In these cases, CMV reactivation develops in over 60% of seropositive recipients, with the highest incidence with CMV-naïve donors, and in approximately 10% of seronegative recipients transplanted from seropositive donors (Stern *et al.*, 2019).

CMV infection is not only limited to transplanted patients, after HSCT or solid organ transplantation, but also to immunodeficient patients. Moreover, not only CMV is frequent, but other viral infections, including BKV, EBV, AdV and HHV6 can also contribute to substantial transplant-associated disease or death (Papadopoulou *et al.*, 2014). The majority of infections are due to viruses of the herpesvirus family. Related to the infection of CMV, like in other infections, virus-specific T cells are crucial to erradicate and control *de novo* viral infections or reactivations. Nevertheless, the frequencies in peripheral blood of healthy individuals of CMV specific T cells are very low (range 0.18-0.80% $CD8^{+}IFN\text{-}\gamma^{+}$) (Gamadia *et al.*, 2001), and negligible in the case of immunodeficient or immunosuppressed patients.

The first approach to treat CMV infection is the use of antiviral pharmacological agents. However, this treatment has relevant drug toxicity associated and, as described earlier, CMV can be refractory to treatment due to certain mutations presented by the virus (Chou *et al.*, 2003; Campos *et al.*, 2016; Lindemann *et al.*, 2018; Kaeuferle *et al.*, 2019; Ottaviano *et al.*, 2019). Since some patients are refractory to antiviral treatment, other alternatives have emerged giving rise to adoptive T cell therapy. Approaches using lymphocytes for treatment include those selecting VST by tetramers or cytokines, and *ex vivo* expansion. Approaches based on direct selection include the isolation of $IFN\text{-}\gamma^{+}$ cells, mentioned before and currently used in our facilities under clinical trials. Other centres, developed expansion protocols for the creation of VST third-party banks for therapy for immunocompromised patients with herpesvirus infections among other infections (Wilkie *et al.*, 2004; Haque *et al.*, 2007; Barker *et al.*, 2010; Leen *et al.*, 2014; Papadopoulou *et al.*, 2014; Withers *et al.*, 2018).

In this PhD project, in the section 4.2 of Chapter IV, we proposed an *ex vivo* expansion method from low-frequency VST to clinical numbers. The main advantages of the protocol we developed include: a) the short expansion time; b) a single specific stimulation; c) the high-fold expansion, and d) reduced costs.

The large expansion of cells, ease the donation, as the donor initial sample does not need to come from a sample with high number of lymphocytes, like the leukapheresis for direct selection (Peggs *et al.*, 2011; Tischer *et al.*, 2014), but can be obtained from peripheral blood. Expansion of specific cells was possible with the G-Rex technology, which allowed having a larger density

of cells in culture due to the gas permeable membrane and having consequently a larger expansion than with the traditional culture system. CMV pp65 peptide pool was used instead of IE-1 for: a) being the most immunogenic CMV protein, and b) inducing T cell responses of the greatest magnitude and in the highest proportion of individuals compared to other proteins (Koehne *et al.*, 2015). For specific expansion, although DC require manufacturing time, they were preferred for stimulation over other systems as being potent stimulators for specific T cell activation (Houghtelin and Bollard, 2017). Although the culture had little requirements regarding the use of feeders and other supplements, it required cytokines and an unspecific stimuli (IL-2, IL-7, IL-15 and antibodies anti-CD3 and anti-CD28). Nevertheless, large numbers of VST were obtained in a short expansion of only 14-days, and from just a single specific stimulation based on pp65-pulsed DC. This is a clear advantage over other protocols that perform 3 to 4 specific stimulations with DC (Mackinnon *et al.*, 2008; Koehne *et al.*, 2015). We were also able to achieve a higher expansion of VST compared to other groups that stimulated either directly with peptides (Papadopoulou *et al.*, 2014) or with DC (Withers *et al.*, 2018). In detail, according to the average of 42.2 fold-expansion, we estimate to produce under cGMP up to 200 doses of 20 million VST/m^2 from a single peripheral blood donation. This fact would substantially reduce the costs of manufacture and make this therapy economically feasible, having multiple doses available for the same, if required, and/or different patients.

Regarding our results we performed different techniques for extensive product characterisation. On the one hand, we performed characterisation by immunophenotying the expanded cells. On the other hand, we perfomed functional assays that were based on secretion kinetics of Th1 and Th2 cytokines, IFN-γ ELISPOT, degranulation, cytotoxicity and alloreactivity assay. Namely, cytotoxicity and alloreactivity assays will be referred to as *in vitro* efficacy and safety assays.

The final product phenotype was extensively characterised for IFN-γ expression, as a marker of specificity. Both $CD8^+$ and $CD4^+$ cells secreted IFN-γ in the presence of pp65 stimulation. The presence of $CD4^+$ cells enhances the memory and effector response and supports persistence and expansion of the cytotoxic T cells (Novy *et al.*, 2007; Pourgheysari *et al.*, 2009). Purity of the final product was also assessed by flow cytometry phenotype. Results showed no presence of NK, B or regulatory T cells in the final product. Furthermore, T cell subsets mainly consisted of T_{EM} subpopulation, which confer protective memory and immediate effector functions (Sallusto, Geginat and Lanzavecchia, 2004). The highest percentage of IFN-γ secretion was attributed to the T_{EM} subset. Low levels of T_N cells were present after expansion, thereby reducing the

probability of GvHD (Distler *et al.*, 2011). Minor presence of T_{CM} in the final product was found, although we hypothesise that if these cells engraft they could confer long-lasting protection.

Final product polyfunctionality was demonstrated by the detection of several secreted cytokines after cell stimulation. The secretion of pro-inflammatory cytokines like TNF-α, which attracts other cell types into the infectious focus and activates them to eradicate the antigen, or IFN-γ, which happens to activate other effector cells and has antiviral activity, together provide a more effective immune response against a pathogen (K. Abbas, H. Lichtman and Pillai, 2008; Foley, 2012).

According to IFN-γ ELISPOT assay results obtained from the initial and the final product, CMV pp65 reactivity was tremendously increased after expansion. Further functional assays performed were based on T lymphocytes' physiologically mechanisms to kill, here MoA, which are long and well described (Lowin *et al.*, 1994): secretion of granules filled by perforins and granzymes, or expression of Fas ligand, through which they are capable of inducing apoptosis by binding to Fas molecule expressed by some target cells.

In order to test the cytotoxicity capacity of our effector cells we performed two functional assays: degranulation assay and cytotoxicity assay. The results obtained with the degranulation assays show that VST act as antigen-specific effector cells and generate granules upon pp65 stimulation. To further demonstrate the potential to specifically kill pp65 pulsed cells, we used the CFSE labelling technique developed previously in this work. The expanded product was tested for specific cytotoxicity, and was demonstrated to induce specific cell lysis on pp65 pulsed lymphoblasts. To emulate "third party VST conditions", we tested pp65-pulsed allogeneic target cells, with 2-3 matches with our effector cells. Pp65 pulsed cell lysis varied for the HLA allele matched rather than for the number of HLA matching. Many groups set efforts to describe CTL epitopes derived from CMV-pp65 as well as, to identify the antigen restriction by HLA alleles (Kondo *et al.*, 2004; Slezak *et al.*, 2007; Lehmann *et al.*, 2019). Indeed, as with our results, VST cytotoxicity will take place through shared HLA molecules. In the nonidentical HLA setting, due to peptide immunodominance, antigen presentation will be driven through certain HLA molecules. In this context, if the HLA presenting the peptide in the recipient is not recognised by the VST, these will be ineffective in treating the host infected target (Barrett, Prockop and Bollard, 2018). This problematic translated to the clinical setting, can be solved either by testing the HLA restriction before infusion or by characterising manufactured VST HLA response with tetramers. Nevertheless, large registries of VST lines would also allow the possibility of having the highest HLA match between donor and recipient.

In an attempt to measure the *in vitro* safety we also performed cytotoxicity assays with complete mismatch. Interestingly, alloreactivity *in vitro* resulted in very low percentage of cell death even when cells were completely mismatched. Although some reports indicate that memory T cells can produce allo-HLA reactivity (Amir *et al.*, 2010), no cases of GvHD have been reported due to VST infusions when the donor was mismatched (Koehne *et al.*, 2015; Bollard and Heslop, 2016). Our results, proved VST efficacy and safety *in vitro*, adding an extra value to the final product with this extensive characterisation.

As with MSC, VST do not escape regulatory setting, and would also require, by the competent authorities, a potency assay for product delivery. The most extended assay to measure quantitatively the biological activity of VST is the IFN-γ ELISPOT (Papadopoulou *et al.*, 2014). This technique is sensitive (can detect a single cell out of a million cells), relatively fast (results in 24h) and highly efficient. All these makes the ELISPOT a strong potency assay candidate for product release into the clinic.

Patients with these infections that are refractory to antiviral therapy, require rapid treatment due to the aggressive nature of these infections. The readily availability can be possible by the creation of a VST third-party bank (Barrett, Prockop and Bollard, 2018). Moreover, the generation of VST is not only limited to the third-party bank, but VST products can also be manufactured individually for a patient, either autologous or allogeneic, and cryopreservation can be performed later for the remaining doses. However, although personalised expansions probably have some advantages, such as choosing the highest HLA matching, this would require the need of an available donor and manufacturing time that is otherwise absent in a third-party bank. Moreover, for the generation of a bank, the product needs to be functional after thawing. Cytotoxicity assays performed, and final product polyfunctionality tested, show that cells remain functional after cryopreservation and demonstrates the feasibility of a VST bank.

Moreover, other groups are sequencing the TCR clones to identify unique Vβ gene sequences, which contributes to characterise the product TCR repertoire, track the persistence of VST infused and, to identify which clones expanded *in vivo* (Koehne *et al.*, 2015; Withers *et al.*, 2018). Regarding persistence of third-party VST, infused VST in HSCT recipients are able to proliferate *in vivo* for 1-4 weeks, yet they do not achieve long-lasting engraftment (O'Reilly *et al.*, 2016). Nevertheless, Tzannou and collaborators, were able to track *in vivo* third-party partially HLA-matched persistence for up to 12 weeks (Tzannou *et al.*, 2017). However, small numbers of VST can persist long enough at infection sites and control infection until the immune system reconstitution is established (Barrett, Prockop and Bollard, 2018). Further experiments in our

group could involve the sequencing of TCR in order to follow-up clones. However, expanded VST would probably need to be tested in preclinical animal models prior to entering clinical trials. Bearing in mind the translation to cGMP standards, this process was developed focusing on the future scale up and validation of the protocol in the clean room.

Furthermore, the development of anti-viral treatments with more optimum results is ongoing, as for Letermovir (Marty *et al.*, 2017), or the development of CMV vaccines (Anderholm, Bierle and Schleiss, 2016) albeit presenting some challenges. Although the promising results, to date there is no licensed CMV vaccine and antiviral drugs are still limited by drug resistance and toxic side-effects (Stern *et al.*, 2019). Additionally, the product developed in this project, based on VST for CMV could also be extended to other viruses where pharmacological treatment is less advanced, like in the case of BK polyomavirus infections (Chong *et al.*, 2019). As future prospects, the generation of other VST, such as for EBV, AdV and BKV, or even multivirus-specific T cells is being considered.

Nowadays, efforts of many research groups are put into the generation of multivirus-specific T cells banks. With the success of prophylaxis and treatment of individual viral infections with VST, targeting multiple viruses with a single product is the future perspective for post-HSCT patients susceptible of multiple viral infections (Houghtelin and Bollard, 2017). Moreover, the generation of banks for use in third-party multivirus-specific T cells would offer immediate protection against even up to 5 viruses (EBV, AdV, CMV, BKV, and HHV6) in some cases like the one described by the Baylor College of Medicine (Papadopoulou *et al.*, 2014), or against 3 viruses (CMV, EBV and AdV) like the one described by the Westmead Hospital in Sydney (Withers *et al.*, 2018). Nevertheless, antigenic competition among these viruses plus the role that is played by the HLA between donor and patient can present great challenge. The production of multi-VST can result in lines dominated by responses to a single virus or restricted to a spectrum of viral antigens. Antigenic competition occurs between high and low frequency T cells, as well as multiple antigens expressed at different levels, and competing for presentation on shared antigen presenting cells (Gerdemann *et al.*, 2012). Consequently, this could limit the antiviral coverage provided by a single multi-VST product. Nonetheless, this approach is being developed by groups with optimistic results (Tzannou *et al.*, 2017; Withers *et al.*, 2018).

Another possibility that would overcome the challenge of HLA-matching would be engineering cells against CMV epitopes. Generation of CAR T-cells directed towards viruses has already been approached by Proff and collaborators (Proff *et al.*, 2018). CAR T-cells present a potential solution for many diseases but it is costly and preclinical and clinical studies are still needed. This makes them, at least in this application, far from the clinical application yet.

For both ATMP presented in this work, discordance among cell doses exist. Cell dose used is a parameter that would also need standardisation to make clinical response more comparable. Different doses are being used as reported in the literature. For MSC, doses range from 1 to 2 million cells/kg and never more than 12 million cells/kg, some patients receiving single and others multiple doses (Galipeau and Sensébé, 2018). For VST, it is reported in the literature a range of 10,000 to 1 million VST/kg (O'Reilly *et al.*, 2016), although the most common dose is 20 million VST/m^2. In the case of VST, multiple infusions have also been used (Tzannou *et al.*, 2017).

Another concern is the route of administration. For VST, intravenously route was chosen without controversy. However, MSC depends on the indication, and has multiple delivery routes suggested. Just over half of submissions surveyed proposed intravenous, while the other half proposed a range of routes, including but not limited to: injection directly into the heart, intramuscular injection, and topical application (Mendicino *et al.*, 2014). Indeed, different clinical indications will require different routes for optimal therapeutic efficacy, nevertheless, selecting a suitable delivery route should also include a consideration of the desired MoA (Caplan *et al.*, 2019).

Cellular therapies follow regulation for their manufacture, like cGMP, in order to have robust data as well as evidence of the quality of a product that is manufactured consistently (Bedford *et al.*, 2018). In spite of complying regulations and following robust protocols, ATMP's complicated nature still makes them need further standardisation, as they can have multiple MoA and their MoA are harder to study and have donor-associated variability.

In summary, the work presented here is an assessment and development of the manufacture of cell therapies, based on *ex vivo* expanded MSC and VST, in order to have solid protocols that ensure robust products with proven biological activity. The later property is tested by potency assays, already validated or apt to validation for product release. The methods elaborated are feasible to be escalated to cGMP standards, and products tested resulted safe and effective *in vitro*, for therapy targeting diseases that have no other alternative treatment, with the preceding of successful results from other groups indicating great promise on these ATMP.

CHAPTER VI: CONCLUSIONS

1. Optimisation of parameters affecting the performance of lymphocyte proliferation assay has been performed and a robust, reproducible and versatile method for determining immunomodulation potency of MSC has been successfully established and currently used for product release.
2. Robust production of clinical-grade WJ-MSC under pharmaceutical standards has been assessed by a detailed risk management method, and the use of a robust protocol for immunomodulation potency testing.
3. Regardless of HLA-DR expression, BM-MSC' identity, differentiation potential, and immunomodulatory capacity was demonstrated not to be compromised.
4. The use of HLA-DR as a negative marker of BM-MSC does not add any additional value to quality control panels and its expression does not affect other attributes, such as phenotype and functionality *in vitro*, as demonstrated with a two-site study. The expression of HLA-DR was shown to be dynamic in MSC cultures.
5. Feasibility of a protocol for rapidly generation of CMV-specific T cells, with a single stimulation with specificity against pp65 CMV, in clinical relevant numbers was demonstrated, showing a scalable process to cGMP standards, providing an *in vitro* safe and functional product.

CHAPTER VII: REFERENCES

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APPENDIX

Evaluation of a cell-banking strategy for the production of clinical grade mesenchymal stromal cells from Wharton's jelly

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Irene Oliver-Vila, Maria Isabel Coca, **Marta Grau-Vorster**, Noèlia Pujals-Fonts, Marta Caminal, Alba Casamayor-Genescà, Isabel Ortega, Laura Reales, Arnau Pla, Margarita Blanco, Joan García, Joaquim Vives.

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