Tackling major hurdles in cell therapy translational research:

Different products, common challenges

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ISBN: 978-94-93184-17-6

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Aanpak van grote hindernissen in translationeel onderzoek bij celtherapie: verschillende producten, gemeenschappelijke uitdagingen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
vrijdag 29 november 2019 des middags te 2.30 uur

door

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geboren op 3 februari 1979
te Jeita, Libanon

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This thesis was made possible with financial support from the European Commission and Institut Paoli-Calmettes.

A little knowledge that acts is worth infinitely more than much knowledge that is idle.

- Gibran Khalil Gibran

Man muss Geduld haben mit dem Ungelösten im Herzen, und versuchen, die Fragen selber lieb zu haben, wie verschlossene Stuben, und wie Bücher, die in einer sehr fremden Sprache geschrieben sind.

- Rainer Maria Rilke

Il senso della ricerca sta nel cammino fatto e non nella meta; il fine del viaggiare è il viaggiare stesso e non l'arrivare.

- Tiziano Terzani

Ce que l'on fait avec le temps,
Le temps le respecte.

- Auguste Rodin

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Chapter 1: General Introduction

Unmet need and definitions

The increasing prevalence of chronic diseases, particularly immune-mediated diseases, and certain cancers with low prognosis pose large direct health costs in the EU. Current therapies rarely cure, treating only symptoms and carrying significant side effects and diminished quality-of-life. To move forward and cure some of these conditions, there is a need to disrupt the paradigm of "treating symptoms" and put forward innovative "curative" options. Following long years of pre-clinical development, cell-based immunotherapies constitute a compelling alternative to conventional treatments for acute or chronic human diseases at a genetic, cellular or tissue level: from hemophilia (1), to diabetes (2) and multiple sclerosis (3), to control of graft rejection (4, 5), and hematological malignancies (6, 7).

Cell-based immunotherapies, designated as advanced therapy medicinal products by the European Medicines Agency, are defined as medicines for human use that are based on genes, tissues or cells. They are classified as:

- gene therapy medicines: containing genes that lead to therapeutic, prophylactic or diagnostic effect.
- somatic cell-therapy medicines: containing 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 medicines: contain cells or tissues modified to repair, regenerate or replace human tissues
- combined medicines: containing one or more devices as part of the medicine (https://www.ema.europa.eu/).

Activating or suppressive cellular immunotherapies

Focusing on cells destined to cure, diagnose or prevent diseases, a clear distinction relates to their immunological function. Cell-based immunotherapies designed to instigate or enhance an immune response are considered "activating immunotherapies" while those designed to repress an immune response are "suppressive immunotherapies" (8).

Activating cell-based immunotherapies include: tumor-infiltrating lymphocytes (TILs), chimeric antigen-transduced (CAR) T cells, T cell receptor-transduced (TCR) T cells, natural killer (NK) cells, cytokine-induced killer (CIK) cells, Gamma-delta T cells, and virus-specific T cells. Suppressing cell-based immunotherapies include: Mesenchymal stromal cells (MSC), Tolerogenic antigen-presenting cells (APC), and polyclonal or antigen-specific regulatory T cells (Tregs).

In cancer settings, adoptive transfer of ex vivo activated/expanded naturally occurring tumor-infiltrating/-specific T cells has shown significant clinical responses in melanoma patients over 20 years ago (9). An alternative to the process of isolating and expanding TILs is to genetically modify T cells isolated from the blood to express transgenic TCRs able to recognize tumor antigens and induce cancer cell killing. Treatment with TcR-transduced T cells has shown success in certain disease conditions (targeting NY-ESO-1, a cancer-testis antigen, in synovial cell sarcoma, melanoma, or myeloma) and not in others (targeting MART-1, gp100, CEA, and MAGE-A3); their safety profiles equally variable (10). To overcome these cells' dependence on MHC and external co-stimulation, an alternative approach is to genetically modify T cells to express chimeric antigen receptors (CAR-T cells). These hybrid receptors combine an antibody single-chain variable fragment for antigen recognition (extracellular domain), with activation signals that are derived from endogenous TCRs and costimulatory signals (intracellular domain). Autologous CAR-T cells directed against the B cell target CD19 have shown high rates of sustained complete responses in relapsed/refractory ALL, and significant responses in CLL and non-Hodgkin's lymphoma (11). CAR-T cells can cause

significant toxicities and their success has not yet reached solid tumors, issues that will be discussed in detail in a manuscript (12).

NK cells are attractive candidates for cancer immunotherapy owing to their cytotoxicity against tumor cells and virally-infected cells without prior exposure, without sensitization or MHC class I restriction (13). Ex vivo activated/expanded allogeneic NK cells have shown safety, feasibility and no graft-versus-host disease (GvHD) upon infusion alone or after hematopoietic stem cell transplantation (HSCT) (14). Novel methods are under clinical investigation to combine NK cells with bi-specific antibodies, to expand them starting from iPS cells or CAR-transduce them; these will be discussed in detail later in a review (15). Cytokine-induced killer cells (CIK), heterogeneous CD3+CD8+ cell populations, equally exhibit non-MHC-restricted cytotoxicity (16) and have shown some success in the few published trials (17). Gamma delta T (γδT) cells are attractive candidates owing as well to their non-MHC restriction for antigen presentation, thereby being not alloreactive or causing GvHD. Furthermore, they are potentially useful as CAR carriers (18). Since they represent <5% of circulating T cells, their ex vivo expansion proves challenging probably explaining the small number of trials testing them in solid tumors (19).

Combating viral susceptibility, whether in patients with primary immunodeficiency disorders or immunosuppressed patients after transplantation (HSCT or solid organ transplantation), is an unmet need tackled by virus-specific T cells (20). Ex vivo expanded T cells specific to cytomegalovirus (CMV) have first been shown to prevent CMV infection without causing GvHD upon adoptive transfer to an allogeneic HSCT recipient (21). The applications of virus-specific T cells have since expanded to cover: Epstein-Barr virus (EBV), adenovirus, BK virus and human herpes virus type 6 (22). Several challenges face this treatment: risks of T cell alloreactivity and GVHD after a costly manufacturing process (if used prophylactically) and deliverability and timeliness of manufacturing (if used as preemptive treatment) (23).

Many patients with autoimmune diseases, severe allergies and recipients of solid organ/stem cell transplants require lifelong treatment with immunomodulatory medication. These treatments partially alleviate symptoms via generalized immunosuppression and carry the risk of immediate or long-term severe adverse events. Therefore the need for tolerance-inducing treatments is justified which would establish "operational tolerance": used in the context of solid organ transplantation as being a maintained good graft function for prolonged periods despite discontinued immunosuppression (24). Mesenchymal stem cells possess immunomodulatory properties (among others) rendering them potential candidates for tolerance induction. They have shown safety and feasibility in the setting of GvHD control post-hematopoietic stem cell transplantation (HSCT) (25) and rejection of kidney transplants (26). Efficacy remains to be shown (27). Challenges pertain to their source, manufacturing, dosing, concomitant treatments… (28). Tolerogenic antigen presenting cells (TolAPC) are under clinical investigation in autoimmune diseases (diabetes, multiple sclerosis, rheumatoid arthritis and Crohn's disease) and kidney or liver transplantation to prevent graft rejection (29). In reported trials, they have shown safety and feasibility and to some extent efficacy, yet challenges remain in their dosing, timing and injection route, to name a few. Efforts to harmonize practices and reporting around TolAPC are commended for setting the minimal information required for manufacturing protocols to be able to compare products (30). Polyclonally expanded regulatory T cells (Tregs) and antigen-specific T regulatory Type 1 (Tr1) cells were initially tested in patients with GvHD post-HSCT. Both showed to be safe yet the former carried the risk of mild infections (31, 32). Candidates for treating autoimmune diseases, TRegs are under extensive clinical investigations: type 1 diabetes (2, 33), refractory Crohn's disease (34), and amyotrophic lateral sclerosis (35). TRegs are under continuous investigation as well in GvHD post-HSCT (prophylactic or curative strategies), liver transplantation (36), and kidney transplantation (37, 38). Clinical-grade protocols for manufacturing sufficient numbers of cells are variable in terms of method, target

cells, culture time, cryopreservation, need for cell sorting or immunomagnetic separation of starting material (39), yet efforts are under way to harmonize reporting on TReg expansion protocols (40).
The detailed discussion will focus on Tr1 cells, NK cells and CAR-T cells, being the cells described in the manuscripts presented in this thesis.

TR1 cells

Initially identified from a functional perspective, Tr1 cells predominantly produce IL-10 and transforming growth factor-beta (TGF-beta), conferring a suppressive function, independent from forkhead box P3 (FOXP3). Recently, surface markers LAG-3 and CD49b co-expression (with specific gating on CD4 memory T cells) was described as a phenotypic marker of Tr1 cells (41). As a mechanism of action, Tr1 cells perform their regulatory activity upon engagement of their TcR via antigen-specific suppression (42) and dampening non-antigen-specific bystander immune responses in their proximity within the tissue where interaction occurs (43). How Tr1 cells interact with immune cells involved in allogeneic or autoimmune responses remains an area of active investigation, it will be addressed in a manuscript within this thesis (44).
Studies in mice and humans identify several factors (cytokines, factors or cells) driving the induction of Tr1 cells in vivo, distinct depending on the microenvironment. IL-10 has been initially described as the key cytokine producing anergic T cells containing Tr1 cells starting from CD4+ T cells (45). TGF-beta plays a controversial role in Tr1 induction in vivo: on one hand TGF-beta and IL-10 additively induce the antigen-specific anergic T cells (46), while it is redundant on IL-10-induced generation of Tr1 cells upon TCR stimulation in the presence of APC, a setting where IFNα acts synergistically to IL-10 (47). DC have been shown in mice (48) and humans (49) as the key producers of IL-10 that drives Tr1-cell induction. Furthermore, such IL-10-producing DC have been shown to promote Tr1 cell differentiation from naive CD4+ T cells in vitro, serving as the basis for a proposed Tr1-cell manufacturing protocol (49). Hence mechanisms of Tr1 cell

induction in vivo (cells and cytokine environment) remain heterogeneous and not well understood. Although IL-10 and/or IL-27 can induce Tr1 cells in vivo, the same suppressive properties of these cytokines prevent Tr1 cells from proliferating. This may suggest that other factors and cells might be required for the differentiation and expansion of Tr1 cells. Furthermore, the site of conferring tolerance (site of inflammation or lymphoid tissue) adds another layer of complexity. Identification of a different cell population key in Tr1 cell induction in vivo will be addressed in a manuscript in this thesis (Mfarrej B et al, Submitted).

Several protocols have been proposed to generate antigen-specific Tr1 cells (or Tr1-enriched anergic T cells) ex vivo owing to their clinical utility in several immune-mediated diseases or graft rejection (Figure 1). Gregori S et al (49) proposed the maturation of monocytes into IL-10-producing DC (DC-10) and subsequent co-culture with allogeneic naive CD4+ T cell. Tr1 cells manufactured as such were tested in a proof-of-concept study for prevention of GvHD post-HSCT and shown to be safe and well tolerated (32). A new Phase I trial was recently initiated (NCT03198234) in the same indication with an ameliorated cell expansion method capable of producing higher proportions of Tr1 cells.

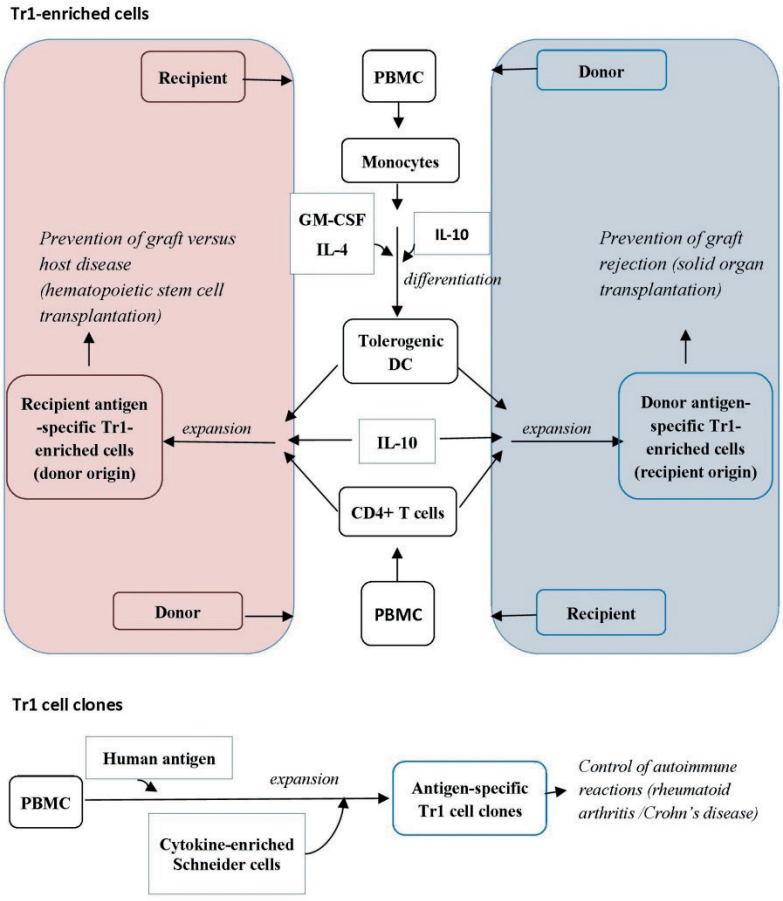

Figure 1: GMP-grade protocols for Tr1 cell manufacturing strategies (modified from *Gregori and Roncarolo Front Immunol 2018)*

Brun V et al (50) adopted a different approach to produce ovalbumin (OVA)-specific Tr1 cells using a T cell cloning method. Safety and tolerability were established in a first-in-human with patients affected by refractory Crohn's disease (34). The same method was adapted to produce joint-antigen collagen II-specific Tr1

cell clones intended for use in patients with severe refractory rheumatoid arthritis (51).

Studies using autologous polyclonal T regulatory cells to control inflammation or rejection have shown safety and feasibility (38, 52). Among the ongoing trials using regulatory cells, The ONE Study is a large-scale collaborative consortium entitled "A unified approach for evaluating cellular immunotherapy in solid organ transplantation" (37). Several immune-modulatory cells such as ex vivo isolated and in vitro expanded FOXP3+ TRegs, Tr1 cells, tolerogenic dendritic cells, and regulatory macrophages, are under evaluation for promoting graft tolerance after kidney transplantation. In preparation for use within The One Study consortium to prevent graft rejection in a living-donor kidney transplantation setting, adaptation of the Gregori protocol (49) needed to be done. Major changes were: source of monocytes (donor instead of recipient) and recipient-derived CD4+ cells instead of PBMC, since the proinflammatory conditions of dialysis patients prevented the successful expansion of Tr1-enriched anergic T cells (53), and GMP-grade reagents and methods. These modifications will be described in a manuscript in this thesis (54).

NK cells

NK were initially identified based on functional characteristics: their ability to exert cytotoxicity on tumor cells, stressed cells and virally-infected cells in a non-MHC restricted manner and without prior sensitization (55, 56). Phenotypically, NK cells are identified by their surface expression of CD56 in the absence of CD3. The balance of signals delivered by activating and inhibitory receptors on the surface of NK cells determines the killing or sparing of target cells (57), all the while sparing healthy "self" cells. Recognition of "self" MHC class I by inhibitory killer cell immunoglobulin-like receptor (KIR) is involved in the calibration of NK cell effector capacities during a developmental stage, allowing the subsequent recognition of the absence of "self" MHC class I during interaction with target cells (58).

During malignant transformation, a reduction of NK cell cytotoxicity and/or tilted balance towards increased expression of inhibitory NK cell receptors over activating receptors can occur, leading to immune evasion by tumors cells. Therefore, restoring NK cells' antitumor activity has been the focus of many treatment strategies (59). One strategy aims at activating circulating NK cells by 1) blocking the interaction of inhibitory receptors, or 2) targeting NK cell activating receptors (60). Another approach involves using drugs targeting immunosuppressive mechanisms within the tumor microenvironment (61).

Adoptive transfer of NK cells builds on the therapeutic potential NK cells hold conferring graft versus leukemia, while reducing the risk of GvHD. In fact, the first evidence comes from the seminal work from the Peruggia group showing that allogeneic, rather than autologous NK cells are more potent effectors owing to KIR-incompatibility in the graft versus host direction in patients receiving HLA-mismatched HSCT (62). NK cells can be expanded using feeder cells (K562 cells expressing mbIL-21, for instance) and cytokines that help expand and stabilize NK cells (IL-2, IL-15, IL-21, for instance) for variable periods of time (from overnight to 21 days) (Figure 2).

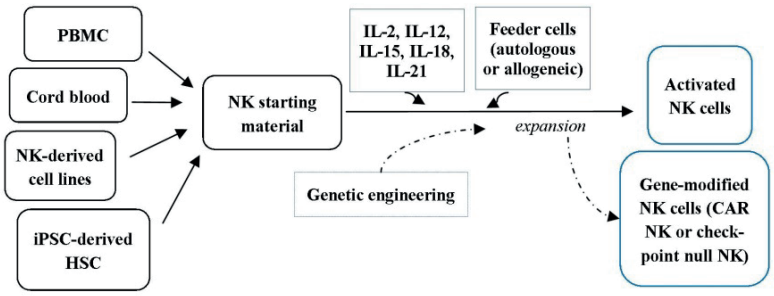

Figure 2: GMP-grade protocols for NK cell manufacturing strategies (modified from Souza-Fonseca-Guimaraes F et al, Trends in Immunol 2019)

Details pertaining to manufacturing NK cells as ATMPs will be addressed during the course of this thesis in a review (15) with an intent to highlight the need for harmonization. A number of trials were reported and are ongoing testing allogeneic NK cells in transplant setting (post-HSCT) or in non-transplant settings with variable clinical outcomes (reviewed in (17)). Reasons for that vary: differences in conditioning regimens, T cell depletion of NK cells before expansion, NK cell expansion protocols, immunosuppressive treatment, timing of NK cell infusion, target cell dose ... This points to the lack of consensus on what parameters are critical for the final NK-cell product, and further reinforcing proposals for harmonization (13).

CAR T cells

CAR T cell design was alluded to earlier as being ex vivo expanded T cells redirected to tumor cell surface epitopes by antibody-like tumor proteins. Several strategies are on-going to increase their safety and efficacy (63).

From a manufacturing perspective, autologous or allogeneic CAR T cell generation starts with a collection of mononuclear cells (MNC) from the patient (or donor in the case of allogeneic off-the-shelf CAR T cells), selection and activation of T cells, transduction to introduce the gene construct, expansion, formulation, and cryopreservation (Figure 3). Upon thawing, these cells are infused to the patient after a conditioning regimen is completed. Several hurdles are faced during this process, covering a large spectrum. The first being the variability of patient starting material due to the disease condition, prior lines of treatment and low MNC counts (64). Identification of the better T cell population to transduce/activate/expand, considering the superiority of less differentiated central memory and stem cell memory subsets over other T cell subsets is the next significant challenge (65).

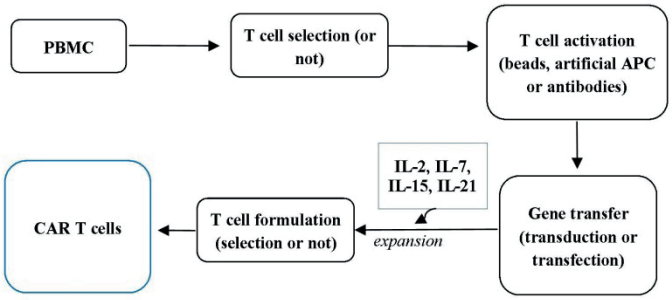

Figure 3: GMP-grade protocols for CAR T cell manufacturing (modified from Gomes-Silva and Ramos, *Biotechnol J* 2018))

Expensive viral vector production in a GMP-compliant manner (66), tedious supply chains (12) and quality control/release testing (67) are equally challenging aspects to manufacturing CAR T cells and deliver them to fairly sick patients in a timely manner.

After showing significant success in clinical trials (68), the first two CAR T cell products authorized by the FDA (2017) and EMA (2018) were:

- Kymriah® for diffuse large B-cell lymphoma (DLBCL) in adults and B-cell acute lymphoblastic leukaemia (ALL) in children and young adults
- Yescarta® for DLBCL and primary mediastinal large B-cell lymphoma (PMBCL) in adults

CAR T cells have shown modest success in solid tumors, limiting their potential. Many strategies are underway to expand their use, alongside managing their toxicities, different manufacturing models, off-the-shelf product development. All these challenges will be tackled in a manuscript in this thesis (12).

Cell therapy product development path

Cell therapy product development path starts with cell characterization, then technology transfer typically from academia to industry alongside regulatory activities, then process development to support large-scale commercial production.

Cell characterization is the process of identifying the relevant biological properties that define a population of cells. Defined as critical quality attributes (CQAs) of a product: identity, purity, safety and potency, need to be established (69). This process starts in the laboratory before clinical trials, so that understanding can improve throughout clinical development. A thorough understanding of a cell product's identity can help assure that the product remains substantially comparable to the laboratory product during development activities. Typically, identity specification is performed by flow cytometry, where a product must be >70% of a cell surface marker (70). Purity specification ensures that a cell therapy product is free of unwanted cells, endotoxin, or residual agents. Safety specification includes sterility (free from bacteria), free from mycoplasma, and adventitious viral agents. Potency is not meant as a direct measure of product efficacy in vivo (although it can be), it typically is quantitative assay for a relevant aspect of a product's mechanism of action (71). Definition of which assays are meant for release testing and which are meant "for information only" (FIO) is important.

If initial early studies show industry potential (particularly whether the process would be commercially viable), the technology and know-how is spun-off to a company, out-licensed to or acquired by a company. Technology transfer of laboratory standard operating procedures (SOP), process information and know-how takes place next. This involves effort on both academic and industrial sides to: clearly map the laboratory manufacturing process, perform risk assessments, design a new process, perform test runs, split runs and comparability runs, all the while training manufacturing staff. Choices on manufacturing strategy, logistics (particularly for autologous products), and a relevant business model initiate during this phase as well.

The company then launches the process development stage. In this phase, optimization of the manufacturing method and scaling-up occur, aiming at large-scale commercial production, lower cost of goods, automation of critical steps, and

establishment of a closed system. Changes that can come about in this phase must preserve the CQAs of the initial product.

From regulatory perspective at an EU-wide level, a continuous dialogue with EMA throughout the development of the ATMP (if classified as such) takes place and culminates in a marketing authorization application. This is evaluated and the outcome is communicated to the European Commission who grants or rejects an EU-wide marketing authorization.

(this section is a collection of information from the ISCT/ASBMT Cell Therapy Training Course, Seattle 2017 https://www.celltherapysociety.org/page/CTTC20172 and ISCT Commercialization signature series: https://www.celltherapysociety.org/page/CommSigSeries/Commercialization-Signature-Series.htm)

The aim of this thesis is to tackle some of the challenges facing the successful translation of cell-based immunotherapy products in order to move more products along the cell development path to reach more patients or cover more disease conditions:

a) Difficulties in definitively characterizing cell-based immunotherapy products: manufacturing process development relies on cells, which are inherently dynamic and dividing, hence, definitively characterizing these cells is challenging.

b) Feasibility of scaling-up of a laboratory-based manufacturing protocol: optimization of the manufacturing method requires a clear understanding of the manufacturing protocol and the impact of every step on the target CQAs.

c) Choice of timing for infusion: rationales for chosing the timing of cell infusion depend on several factors: disease settings, patient treatment or even preclinical data. This issue is particularly challenging when comparing efficacy of similar products.

d) Feasibility of automating manufacturing processes: identifying critical steps in a manufacturing process and establishing a long-term plan to automate these steps remains a significant challenge.

e) Need for harmonized and validated quality controls: the composition of the starting material is of utmost importance in target cell/volume collection and downstream processing, requiring validated analytical methods applicable to fresh and cryopreserved cell collections.

f) Heterogeneity in ATMP manufacturing: differences in cell manufacturing protocols prevent careful comparison of similar products. The challenge lies in harmonization efforts.

g) Bypassing proof-of-concept trials to achieve commercial success: choice of manufacturing strategy, mastering the supply chain, and managing toxicities are all relevant yet challenging strategies to reach larger patient populations.

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Chapter 2: The "unusual suspects" in allograft rejection: Will T regulatory-cell therapy arrest them?

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Curr Transpl Rep (2016) 3:221-226

Abstract

Several trials with adoptively transferred T regulatory cells (TRegs) have shown to be safe and – in some instances - to be effective in patients with hematological malignancies after allogeneic hematopoietic stem cell transplantation (to prevent graft-versus-host disease), with type 1 diabetes (to preserve residual beta cell function), and with refractory Crohn's disease (to control the disease). In solid organ transplantation, TReg cell therapy trials with TReg-supportive immunosuppression are currently being undertaken to control the well-documented T effector cells responsible for allograft rejection. Emerging reports show that many "unusual suspects" are involved in allograft rejection as well. Whether TReg cell therapy will also target them remains to be investigated. In this review we will shed some light on these underscored cells, the role TRegs would potentially have in controlling these unusual suspects, and the immune-monitoring strategies that would address potential TReg cell therapy efficacy.

Introduction

Murine thymus-derived regulatory lymphocytes were initially described in the seventies to be essential for tolerance induction and this tolerance was transferrable to naïve recipients (1). Years of active research led to the identification and characterization of T regulatory cells (TRegs). The best characterized TRegs, for their therapeutic immunomodulatory potential, are: CD4+CD25+ -forkhead box P3 (FOXP3)-expressing TRegs (2) and T regulatory type 1 (Tr1) cells (3). With promising preclinical data (4, 5) and the development of Good Manufacturing Practice (GMP)-grade protocols (6-8), clinical trials with TRegs have been completed with the aim to: (i) prevent graft-versus-host disease (GvHD) after hematopoietic stem cell transplantation (reviewed in (9)), (ii) preserve pancreatic □-cell function in patients with recent onset type 1 diabetes (T1D) (10, 11), (iii) control refractory Crohn's Disease (12), and (iv) induce tolerance after liver transplantation (13).

Among the ongoing trials, The ONE Study is a large-scale collaborative consortium entitled "A unified approach for evaluating cellular immunotherapy in solid organ transplantation" (14). Several immune-modulatory cells such as ex vivo isolated and in vitro expanded FOXP3+ TRegs, Tr1 cells, tolerogenic dendritic cells, and regulatory macrophages, will be evaluated for promoting graft tolerance after kidney transplantation (14). Our group participates in this consortium with in vitro expanded donor-specific Tr1-enriched cells (15). Aiming at inducing tolerance, the premises of such studies are: 1) cell therapy will alter the balance between graft-damaging T effector cells and immune-modulatory cells (16) and 2) regulatory cells will reduce the dependence of transplant recipients on immunosuppression with its long-term damaging effects on both the graft and the recipient (17).

With current immunosuppressive drugs targeting mainly T cells as the "usual suspects" (18, 19), new evidence is emerging that implicates other "unusual suspects" in the alloimmune responses. With this review we will attempt to shed some light on these often neglected players, while indicating the potential roles

TRegs may play in controlling undesired immune responses. Afterwards, we shall indicate some immune monitoring strategies that may help documenting this interaction.

A role for NK cells in ischemia-reperfusion injury of the allograft

Upon solid organ transplantation, the recipient's NK cells sense the damage initiated by the allograft's ischemia/reperfusion injury. These cells are equipped with activating and inhibitory receptors. Stress and danger signal molecules from the damaged allograft favor NK-cell responses inducing inflammatory cytokine production, T-cell costimulation, and cytotoxicity (reviewed in (20)). Evidence comes from the expression of NK-cell activating ligands MICA/MICB on renal and pancreatic allografts that correlates with rejection involving antibody-mediated NK-cell cytotoxicity (21, 22). In addition, NK cells can fine tune the balance between subtypes of host's dendritic cells (DC) (23). NK cells eliminate immature tolerogenic DC via the activating receptor NKp30 while they preserve mature DC via the inhibitory ligand NKG2A (24).

Since NK cells are spared by current immunosuppressive regimens, one would question whether the infused TRegs - after cell therapy - would target NK cells. Preliminary studies in mice show that CD4+CD25+ TRegs inhibit NK-cell effector functions in tumors (25, 26), transplantation (27) and inflammation (28, 29). An exhaustive review by Jacques Zimmer's group elaborated on these concepts while pointing out the contrasting phenomenon of an "activated NK-cell resistance" to TReg inhibition (30). A similar role for Tr1 cells in suppression of NK cells via TGF-beta/IL-10 production or IL-2 deprivation could be hypothesized yet this remains to be tested (5).

These studies raise the possibility that TReg cell therapy may prevent NK cell-mediated damage to the allograft, assuming a function that immunosuppressive regimens cannot.

An immunological role for endothelial cells in allografts

The endothelial-cell (EC) linings of the vascular-lymphatic circulatory system, in addition to acting as a physical barrier between tissues and the blood-lymph compartment, affect the immune responses by acting as non-professional antigen-presenting cells (APC) (31). EC constitutively express MHC-I and II, the antigen-processing machinery and co-stimulatory/co-inhibitory molecules (reviewed in (32)). Hence, EC are capable of mediating antigen-specific stimulation of primed CD4+ and CD8+ T cells but not naïve T cells since EC lack expression of CD80 and CD86 co-stimulatory molecules (33, 34). A recent in vitro study showed that anti-HLA-DR alloantibodies upon binding to EC induce IL-6 secretion leading to generation of Th17 cells and decreased expansion of functional FOXP3+ TRegs (35). In addition, non-HLA anti-endothelial cell antibodies (AECAs) play a pathogenic role in allograft rejection by increasing microvascular injury (36). These studies point to endothelial cells as "unusual suspects" in initiating alloimmune responses in an allograft setting.

In a murine in vitro setting, one study reported interaction between TRegs and endothelial cells: TRegs release TGF-ß that down-regulates the expression of adhesion molecules (E- and P-selectin) on endothelial cells, reducing the adhesiveness of T cells (37). Tested in vivo in a murine peritonitis model, adoptively-transferred TRegs adhere to the inflamed endothelium and reduced acute inflammation (37).

To date, the interaction between TRegs and EC in human grafts has not been directly addressed, yet two studies investigated the role TRegs play therein. Firstly, TRegs were detected in the skin of a hand allograft of one patient six years after transplantation (38). The authors demonstrated: 1) unresponsiveness of graft-infiltrating T cells to stimulation in vitro with donor mononuclear cells, 2) FOXP3 expression by immunohistochemistry in the skin and 3) increased mRNA expression of the anti-inflammatory cytokines TGF-beta /IL-10 and no expression of perforin in tissue homogenates. These data led to the conclusion that skin-residing FOXP3+

TRegs control donor-specific alloresponses after hand transplantation (38). Secondly, in patients after hearth transplant rejection, peripheral CD4+CD25brightFOXP3+ TRegs have an inadequate suppressive function in vitro while graft-infiltrating TRegs are competent and responsible for anti-donor "damage control". The authors concluded that these graft infiltrating TRegs are likely limited in numbers to avoid graft rejection (39, 40). Of note, in both these studies, only one mode of action for TReg was tested: in vitro suppression of T effector cell activation. Unfortunately, other mechanisms of TReg-mediated suppression, among which modulation of APC and EC, were not assessed.

A role for B cells in antibody-mediated rejection

B cells can behave as antigen-presenting cells (APC) as well as antibody-producing cells. Owing to their expression of MHC and costimulatory molecules they can bind T cells. The cytokines produced by T cells in this cluster affect B-cell activation, differentiation and antibody production (reviewed in (41)). B cells are implicated both in cellular- and antibody-mediated rejection after kidney transplantation as evidenced by antibodies, B cells and B-cell clusters detected in biopsies of recipients experiencing rejection (42-44). Importantly, in addition to this effector role, an immunomodulatory function for B cells has been described in tolerant renal transplant recipients (45, 46).

Human FOXP3+ TRegs were reported to indirectly suppress antibody production by B cells via T-helper cell suppression in vitro (47) or to directly inhibit cell-cell contact with B cells in T-B areas of germinal centers of tonsils (48). Human iTRegs (ex vivo TGF-beta-induced TRegs) were also shown to directly suppress activation, proliferation and antibody production by B cells in vitro (49). In addition, murine antigen-specific Tr1 cells induced in vivo by nanoparticles in several mouse models of autoimmune diseases drive the formation and expansion of B regulatory cells from cognate B cells (50). All the above-mentioned interactions between Tregs and B cells remain to be investigated in the transplant settings, particularly after TReg cell therapy.

A role for complement in mediating local inflammation

One effector mechanism for antibody-mediated rejection involves complement-binding donor-specific antibodies (DSA) (51). Since circulating components of the complement system are fixed to tissue surfaces during activation, they represent a durable marker of inflammation. Robert Colvin's group has pioneered exploiting such a characteristic by staining for C4d deposits in graft tissues to diagnose antibody-mediated rejection (52). Renal transplant recipients with post-transplant complement-binding DSA have a 5-year risk of graft loss four times higher than recipients with non-complement-binding DSA (53). Hence clinical trials testing the efficacy of complement-targeted strategies are in progress (reviewed in (54)).
Blocking the receptors of complement components C3a and C5a on T cells favors induction and stability of murine and human induced TRegs (55). However, to the best of our knowledge, whether and how TRegs directly influence complement has not been addressed. It can be speculated that TRegs can indirectly control complement components by inhibiting complement-binding antibody production by B cells (47, 48).

A role for tertiary lymphoid organs within allografts as local immune response amplifiers

Kerjaschki and colleagues were the first to describe a "massive lymphatic neoangiogenesis" in human renal transplant biopsies (56), later known as tertiary lymphoid organs (TLO). Similar to secondary lymph nodes in cellular organization, TLOs are composed of T and B cell zones, active germinal centers, high endothelial venules and lymphatic vessels (57). These structures were detected in explanted human heart and kidney grafts upon chronic rejection and intragraft alloimmune responses were different from those in the circulation (58, 59). Other studies showed TLOs to be associated with donor-specific tolerance or to act as amplifiers of the prevailing immune response, whether tolerant or alloreactive (60, 61).

In several murine cancer and transplantation studies accelerated tumor growth or allograft tolerance were associated with FOXP3+ T cell accumulation in TLO (reviewed in (62)). Whether TLOs will harbor the infused TRegs and accordingly lead to better transplant outcome remains to be determined.

A role for memory T cells in mediating transplant rejection

Memory T cells are capable of mediating transplant rejection by rapidly trafficking into allografts and releasing inflammatory cytokines, which in turn recruit other cytolytic immune cells (63). Memory T-cell generation is favored during homeostatic proliferation (lymphopenia-induced compensatory proliferation) (64) and heterologous immunity (cross-reactivity due to prior antigen exposure) (65). Moreover, around 50% of alloreactive T cells reside in the memory compartment, indicating that heterologous immunity is the major contributor to the expansion of this compartment (66). Peter Heeger's group revealed a role for memory T cells (primed by environmental stimuli/antigens) in the recipient that cross-reacted with donor alloantigens leading to acute rejection (67). Additionally, pre- (67) and post-transplant (68) donor-specific memory cells correlate with risk of acute rejection.
It is thought that memory T cells generated by homeostatic proliferation or heterologous immunity after transplantation are resistant to tolerance induction (69). Memory T cells can evade /regulation by FOXP3+ TRegs in both mouse and human transplant settings (70, 71). However, a recent study in mice showed that depletion of graft-infiltrating neutrophils restores the suppressive function of donor-reactive CD8+ TRegs on memory T cells (72). If applicable in the transplant setting in humans, therapeutic manipulation of innate cells may support transferred FOXP3+ TRegs in suppressing memory T cells. Alternatively, the memory barrier needs to be overcome using other therapeutic strategies (reviewed in (73)). Of note, it was demonstrated by Maria Grazia Roncarolo's group that human Tr1 cells inhibit the proliferative responses of both naive and previously activated memory T cells in vitro (74). With The One Study trials ongoing, it will get clearer whether Tr1 cells will inhibit the proliferation of memory T cells in vivo as they do in vitro.

Immune-monitoring strategies to assess the "crime scene"

All the above-mentioned interactions between the "unusual suspects" in transplantation and the transferred TRegs could remain speculative unless precise immune monitoring strategies are devised. We believe the main challenges, as per today, are: tracking the adoptively transferred TRegs and availability of samples collected from pertinent organs. Different labeling strategies have been used. One is the detection of TReg-specific HLA markers by flow cytometry but this is limited to allogeneic settings (75). Another strategy is direct cell labeling that proved to be feasible with TRegs and regulatory macrophages (MRegs) (11, 76). Collection of samples relevant to the disease is not always feasible. Pancreas biopsies from patients with T1D are, for example, ethically and practically challenging. On the contrary, graft biopsies from some solid organ transplants can be collected longitudinally. One can then envisage, for instance, to analyze the interaction between the "unusual suspects" and the transferred TRegs by laser capture microdissection followed by gene expression analysis (77-79).

So far, to the best of our knowledge, only one TReg-cell therapy trial after solid organ transplantation has been completed and reported (13). This study involved the collection of longitudinal samples (including liver graft biopsies) and immune monitoring. Unfortunately the transferred TRegs were not labelled and assessing potential interactions with other cells was not possible. Alternatively, in a different trial, TRegs were labeled yet it was not possible to collect pancreas biopsies from T1D patients (11).

Conclusions

We believe that - to have a better understanding of the possible in vivo mechanisms of action of adoptively transferred TRegs - we need to design clinical trials in which both cell labeling and collection of samples relevant to the disease are possible. The usefulness of such an approach lies in complementing clinical manifestations of operational tolerance or rejection, while identifying new mechanisms of TReg-mediated suppression.

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Chapter 3: Key role of macrophages in the induction of T regulatory type 1 (Tr1) cells in vivo

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Clinical and Experimental Immunology *(under review)*

Abstract

Background T regulatory type 1 (Tr1) cells are a class of regulatory T cells participating in peripheral tolerance, hence the rationale behind their testing in clinical trials in different disease settings. One of their applications is tolerance induction to allogeneic islets for long-term diabetes-free survival. Currently the cellular and molecular mechanisms that promote Tr1-cell induction in vivo remain poorly understood.

Methods We employed a mouse model of transplant tolerance where treatment with G-CSF/Rapamycin induces permanent engraftment of allogeneic pancreatic islets in C57BL/6 mice via Tr1 cells. The innate composition of graft and spleen cells in tolerant mice was analyzed by flow cytometry. Phagocytic cells were depleted *in vivo* at different time points during G-CSF/Rapamycin treatment, to identify their role in graft survival and in Tr1-cell induction. CD11c-Cre DTA mice were used to discriminate the role of dendritic cells (DC) from that of macrophages in tolerance induction.

Results In the spleen, the site of Tr1 cell induction, no differences in the frequencies of macrophages or DC were observed. In the graft, the site of antigen uptake, a high proportion of macrophages and not DC was detected in tolerant but not in rejecting mice. Tr1 cell induction was perturbed upon *in vivo* depletion of phagocytic cells leading to graft loss suggesting that either macrophages or DCs were involved in tolerance induction. Experiments in DC-deficient CD11c-CreDTA mice showed that DCs are dispensable for Tr1-cell induction, strongly supporting that macrophages instructed Tr1-cell development in vivo in this model.

Conclusions/interpretation Taken together, in this mouse model of Tr1-cell induced tolerance to allogeneic islets, macrophages infiltrating the graft upon G-CSF/rapamycin treatment, are key in inducing Tr1 cells in the spleen.

Introduction

Tr1 cells are a subset of $CD4^+$ T cells that induce immune tolerance. They were first discovered in 2 severe combined immunodeficiency (SCID) patients exhibiting mixed chimerism after successful treatment with HLA-mismatched fetal liver hematopoietic progenitor cell transplantation (1, 2). Tr1 cells were detected in the peripheral blood of healthy donors and were correlated with better glucose control in type 1 diabetic (T1D) patients, if detected at disease onset (3). Additionally, absence or lack of induction of Tr1 cells has been associated with disease progression in autoimmune conditions (T1D, psoriasis, multiple sclerosis, and celiac disease) and allergic diseases, while their presence in HIV/HCV infections or different tumor conditions has been associated with disease progression (reviewed in (4)).
Upon activation of their T cell receptor (TcR) by the cognate antigen, Tr1 cells mediate direct suppression of effector T cells and bystander suppression of professional antigen presenting cells (APC) mainly by secretion of IL-10, granzyme B and TGF-beta (5). Their ability to confer immune tolerance lies behind the rationale for their *ex vivo* expansion and administration in clinical trials of different disease settings: control of autoimmunity, control of graft versus host disease (GvHD), or prevention of graft rejection in solid organ transplantation (6, 7).

Tr1 cells can be generated in vitro from naïve CD4+ T cells by repetitive TCR stimulation in the presence of high doses of IL-10 (8, 9). Early studies have shown that IL-10-producing DC promote Tr1 cell development in vivo (10). Later, IL-10 has been shown not to play a crucial role in vivo while DC-derived IL-27 induced Tr1 cell generation, with TGF-beta amplifying this process (11, 12). IL-27 produced by conventional dendritic cells (DC) (13, 14) and T-bet and donor-macrophage-derived IL-27 (15) have also been reported in different mouse models of inflammation. Hence mechanisms of Tr1 cell induction in vivo (cells and cytokine environment) remain heterogeneous and not well understood. Although IL-10 and/or IL-27 can induce Tr1 cells in vivo, the same suppressive properties of these

cytokines prevent Tr1 cells from proliferating. This may suggest that other factors and cells might be required for the differentiation and expansion of Tr1 cells.

We have previously established a murine model of streptozotocin (STZ)-induced diabetes whereby allogeneic islet transplantation was tolerated upon treatment with granulocyte colony-stimulating factor (G-CSF)/rapamycin. Long-term tolerance was associated with increased Tr1 cell generation, detected at the time using their cytokine secretion profile $CD4^{+}IL10^{+}IL4^{-}$ T cells (16, 17).

With surface marker LAG-3 identifying putative Tr1 cells (18, 19), we confirmed our previous findings and reported on their ability to transfer and maintain tolerance in an antigen-specific manner (20). While we reported splenic Tr1 cells mediating peripheral tolerance, Yu et al described Tr1 cell migration from the gut to the site of inflammation, the pancreatic islets of diabetic mice, to manifest tolerance (21). Therefore, the site of conferring tolerance (site of inflammation or lymphoid tissue) adds another layer of complexity.

Here, we investigate using our stringent mouse model of islet transplantation: which phagocytic cells are mobilized during Tr1-promoting G-CSF/rapamycin treatment, their location and role in Tr1 cell induction and consequently graft survival.

Methods

Mice BALB/c and C57BL/6 mice were purchased from Charles River (Calco, Italy). CD11c-CreDTA mice (C57BL/6 background), a generous gift from M. Falcone (IRCCS San Raffaele Scientific Institute, Milan, Italy) were bred in-house. Blood glucose levels were monitored twice per week with a monitoring system (OneTouch Ultra; LifeScan, Milpitas, CA, USA). Diabetes onset and graft rejection were defined as blood glucose values above 250 mg/dL on two consecutive days. All animals were housed under specific pathogen-free conditions in compliance with guidelines of the San Raffaele Institutional Animal Care and Use Committee (protocol no. 860).

Islet transplant and *in vivo* treatments Pancreatic islets from BALB/c donors were transplanted under the kidney capsule of STZ-induced diabetic recipient mice (C57BL/6 or CD11c-CreDTA), as previously described (16). G-CSF (Myelostim, rHuG-CSF, Italfarmaco) was diluted in PBS and injected s.c. for 30 consecutive days at 200 mg/kg from the day of transplant. Rapamycin (Rapamune; Wyeth Europe, Taplow, UK) was diluted in water and administered by gavage for 30 consecutive days once a day at 1 mg/kg. Clodronate liposomes were purchased from clodronateliposomes.org (Vrije Universiteit, Netherlands), reconstituted in PBS at the concentration of 1 mg/ml and injected i.p. at 10mg/kg 3 times at 3-day intervals.

Flow cytometry Antibodies to CD45 (clone 30F11, dilution 1/100), IA(b) (clone AF6-120.1, 1/100), CD11b (clone M1/70, 1/100), CD223/LAG3 (clone c9b7w, 1/50), were obtained from BD Pharmingen (San Diego, CA, USA), antibody to F4/80 (clone CI:A3-1, 1/200) from Bio-Rad Laboratories (Hercules, CA, USA), antibody to CD11c (clone N418, 1/100) from eBiosciences (San Diego, CA, USA) and antibody to CD4 (clone RM4-5, 1/1000) from Miltenyi Biotec (Bergisch-Gladbach, Germany). All antibodies were titrated before use. A 2.4G2 (Fc) blocking step preceded surface staining. Cell acquisition was performed on FACS Canto II cytometer (Beckman Coulter) and analysis was done using FCS Express 4.0 (De Novo Software, Glendale, CA, USA).

Statistical analysis All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Graft survival curves were plotted using the Kaplan-Meier method. Comparisons between groups were performed using Mann-Whitney test.

Results

Macrophages infiltrate the grafts of tolerant mice

Tr1 cells accumulate in the spleens of tolerant mice in a model of allogeneic islet transplantation to treat diabetes (16) and in a mouse model of inflammatory bowel disease (19). To dissect the cells key in the in vivo Tr1 cell induction we analyzed antigen presenting cell (APC) changes after transplantation and during a Tr1-cell–inducing treatment. Macrophages and DC are the two major known phagocytic APC subsets in inflammatory environments that dynamically interact to establish the balance between immune activation and tolerance (22). Furthermore, they mature into tolerogenic cells in response to rapamycin and/or GM-CSF (23, 24). Splenocytes of transplanted treated and untreated mice were analyzed by flow cytometry to determine APC content. The frequency of macrophages and DC was not different between treated and untreated mice immediately following the transplant and during the 30 days of G-CSF/rapamycin treatment (Fig. 1a, b). Islets are transplanted under the kidney capsule, the tissue where APC are likely to uptake antigens and transport them to secondary lymphoid tissues (25, 26). Islet allografts were therefore analyzed as well. Macrophages infiltrate the graft as early as 5 days after transplant and are significantly more frequent (p=0.0021) in the graft of G-CSF/rapamycin-treated animals as compared to that of control rejecting mice (Fig. 1c). This graft infiltration gradually decreases to reach the same levels as control mice at day 30. On the contrary, DC infiltrating the grafts are not different in G-CSF/rapamycin-treated compared to untreated mice (Fig. 1c).

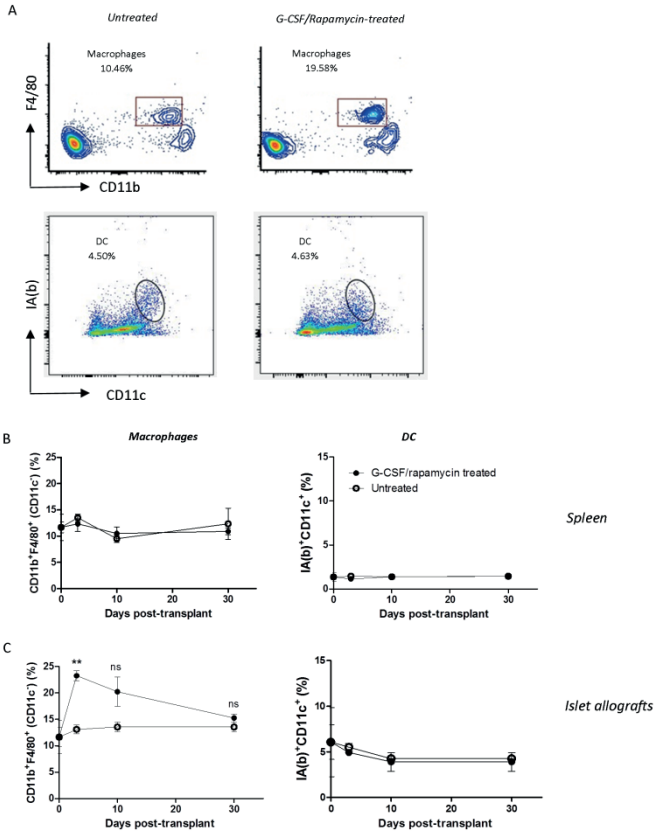


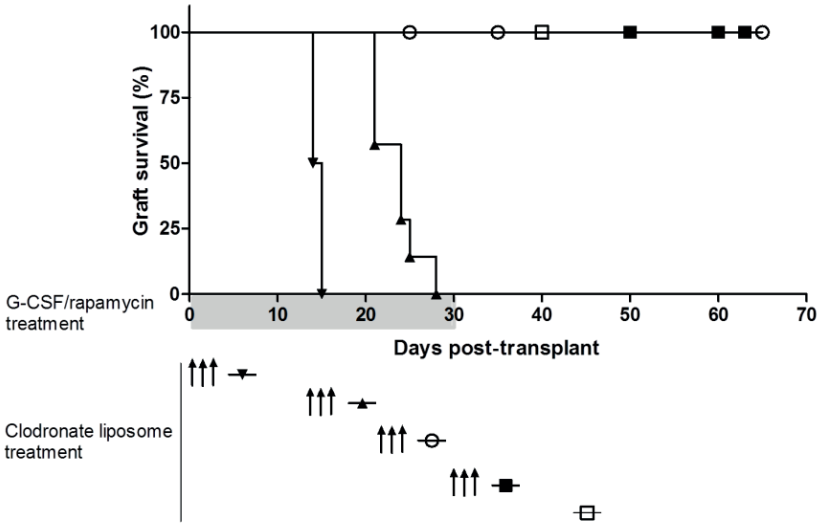
Figure 1: **Early surge in phagocytic cells in the grafts of G-CSF/rapamycin treated and transplanted mice.** Diabetic C57BL/6 mice were transplanted with Balb/c-derived pancreatic islets and treated for 30 days with G-CSF/rapamycin. Grafts and spleens were harvested and cells were collected and detected by flow cytometry. One representative dot plot (out of 4 per group) of flow cytometry staining of graft-infiltrating macrophages gated on CD11c- cells (untreated mice: *upper left panel* **a**, treated mice: *upper right panel* **a**) and DC gated on live cells (untreated mice: *lower left panel* **a**, treated mice: *lower right panel* **a**) infiltrating the spleens (**b**) and the grafts (**c**) of G-CSF/Rapamycin-treated (closed circles) and untreated (open circles) diabetic C57BL/6 mice over time. Data is presented as mean ± SEM, n=4-6/group, $^{**}p<0.01$, *ns*= non-significant, Mann-Whitney test.

These data demonstrate that macrophage frequencies increase upon a Tr1-cell inducing treatment in vivo and localize during the first days after treatment in the graft, thus suggesting their important role for in vivo Tr1 cell induction.

Early in vivo removal of phagocytic cells prevents transplant tolerance

We next tested whether the early surge of macrophages in the grafts of G-CSF/rapamycin-treated mice contributes to graft survival. Consequently, clodronate liposomes that are known to deplete circulating and tissue-infiltrating phagocytic cells (27), were administered *in vivo* starting at different time points after islet transplantation in G-CSF/rapamycin treated mice. We first confirmed that 3 i.p. injections of clodronate liposomes at 3-day intervals deplete graft- and spleen-residing macrophages and DC (Supplementary Fig. 1 a, b). Of interest, liposome-mediated phagocytic cell depletion - from the day of islet transplant until 14 days after - leads to graft loss and lack of tolerance induction (Fig. 2). No such effect was observed upon depleting phagocytic cells at 21 or 30 days after allogeneic transplant and G-CSF/rapamycin treatment initiation. This data shows that phagocytic cells are indispensable for tolerance induction upon G-CSF/rapamycin treatment during the first 2 weeks after transplant.

Figure 2: **Depletion of early and not late phagocytic cells leads to graft loss.** Phagocytic cell-depleting clodronate liposomes were administered to transplanted mice at several timepoints during the course of G-CSF/rapamycin treatment. Graph shows graft survival curves without () or with clodronate liposome treatment at d0 (), d14 (), d21 (), d30 (). n= 4-6/group.

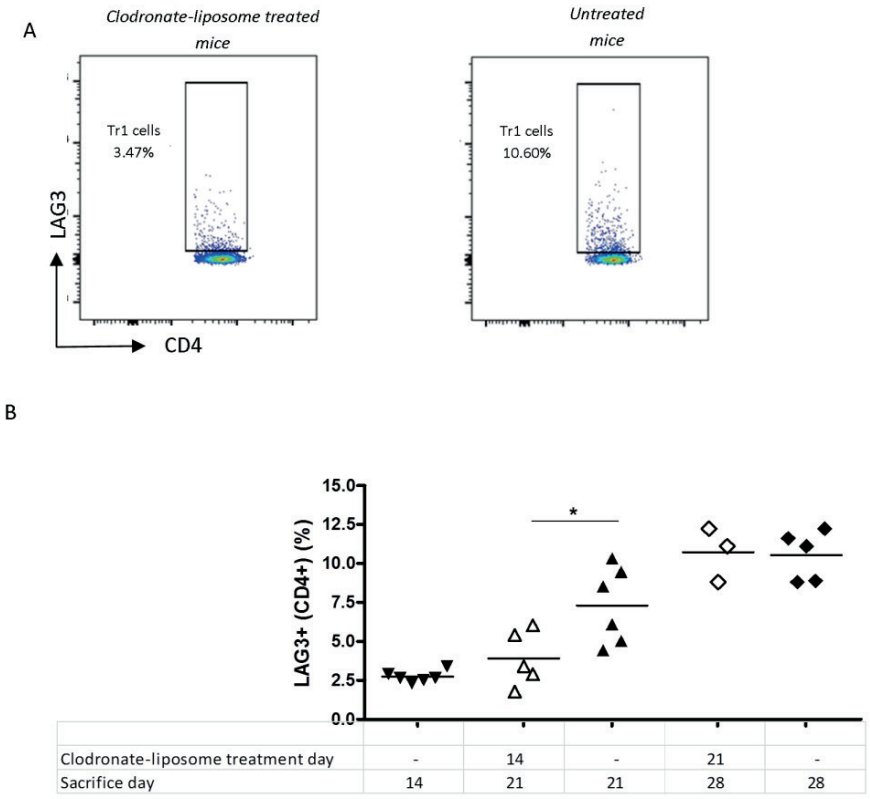


Tr1 cell induction in vivo is inhibited by early depletion of phagocytic cells

We previously demonstrated that Tr1 cells are responsible for graft tolerance in this murine model and that their blockade leads to loss of transplant tolerance (17, 20). We next investigated their number in relation to the early and late depletion of phagocytic cells. To give time for Tr1 cell induction to take place, clodronate liposomes were administered at 14 and 28 days during the course of G-CSF/rapamycin treatment and mice were sacrificed one week later to monitor splenic Tr1 cell presence/frequency. A gradual increase over time in Tr1 cell frequency was observed in the absence of clodronate liposome administration. A discontinuation in Tr1-cell induction was observed upon phagocytic cell depletion starting at 14 days after transplantation, consistent with graft loss upon clodronate liposome treatment at this timepoint. Conversely, Tr1-cell induction continued

unaffected by phagocytic cell depletion at 21 days post-transplant (Fig. 3 a, b). A statistically significant difference in Tr1-cell frequencies was observed between mice treated with clodronate liposomes at 14 days and untreated mice, strongly suggesting that the phagocytic cells present early during G-CSF/rapamycin treatment are necessary for a continued in vivo Tr1-cell induction, which in turn mediates transplant tolerance.

Figure 3: **Early phagocytic cell depletion leads to a halt in Tr1 cell induction.** Clodronate liposomes were administered at 14 or 21 days during the course of G-CSF/Rapamycin treatment of transplanted mice. Mice were sacrificed one week after clodronate treatment, their spleens were harvested and frequency of Tr1 cells was detected by flow cytometry. One representative dot plot (out of 6) of flow cytometry staining of Tr1 cells gated on $CD4^+$ cells in mice treated (*left panel* **a**) or untreated (*right panel* **a**) with clodronate liposomes (CLO) at day 14 or 28 post-transplant (open forms) or no treatment (closed forms) (**b**). Data is presented as mean ± SEM, n=3-6/group, *p<0.05, Mann-Whitney test.



DCs are not required for the in vivo Tr1-cell induction

Clodronate liposomes deplete all phagocytic cells including macrophages and DCs. To better dissect which phagocytic cells act as Tr1-cell inducers, a targeted approach was undertaken by using CD11c-CreDTA mice on a C57BL/6 background as transplant recipients. Due to constitutive DC depletion, CD11c-CreDTA mice develop spontaneous autoimmunity in steady-state conditions (28). These mice were used by other groups to show that DC are dispensable for antigen presentation and can be replaced by other APC in mouse models of autoimmune CNS inflammation (29, 30). We first confirmed that CD11c-CreDTA mice lacked >90% of their DC yet maintained their macrophages (Supplementary Fig. 2). Next, diabetes was

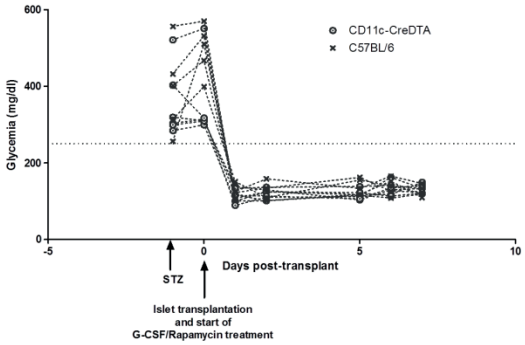
successfully induced by STZ in CD11c-CreDTA mice as detected by an increase in glycemic levels similar to C57BL/6 mice. Upon transplantation with Balb/c allograft islets, glycemia normalized in both CD11c-CreDTA mice and C57BL/6 (Fig. 4a). Glycemia continued to be under control throughout G-CSF/rapamycin treatment in both groups, while graft rejection occurred in transplanted yet untreated mice from both groups. Time to graft rejection was similar between CD11c-CreDTA mice and C57BL/6 mice. Of interest, CD11c-CreDTA transplanted and treated mice maintained a functional allograft at least until 60 days post-transplant, demonstrating that these mice develop transplant tolerance despite lacking DC (Fig. 4b).

Overall these data indicate that DCs are dispensable for in vivo allograft tolerance induction via G-CSF/rapamycin treatment, implicating macrophages as the key phagocytic cells mediating tolerance.

Given our previous findings that Tr1 cells are fundamental for G-CSF/rapamycin-mediated in vivo tolerance induction and that absence of phagocytic cells abrogate Tr1-cell mediated in vivo tolerance, we assume that macrophages are the key cells for the in vivo Tr1-cell induction in this mouse model of transplantation.

Figure 4: **Macrophages and not DC are essential for Tr1 induction.** CD11c-CreDTA mice (deficient in DC) and C57BL/6 diabetic mice were transplanted and treated with G-CSF/Rapamycin. a) Glycemia level fluctuation upon diabetes induction (STZ at day -1), transplantation and throughout G-CSF/Rapamycin treatment in CD11c-CreDTA mice () and in C57BL/6 mice (). b) Graphs represent graft survival of G-CSF/Rapamycin-treated CD11c-CreDTA mice () or C57BL/6 mice () versus transplanted but untreated CD11c-CreDTA mice () or C57BL/6 mice (). N=6-8/group.

A



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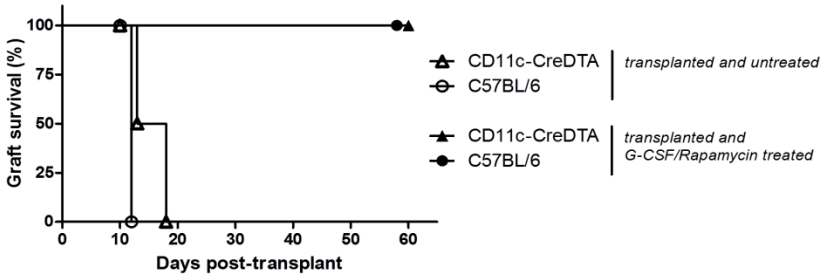

Discussion

Building on our previous observations that Tr1 cells are key players in maintaining tolerance in a STZ-induced diabetes mouse model of transplantation of allogeneic islets (16, 31, 32), we report on macrophages constituting the early phagocytic cells infiltrating the grafts of tolerant mice. The early infiltration of grafts plays a crucial role in Tr1 cell generation and maintaining tolerance. Furthermore, using mice deficient in DC, tolerance could be achieved owing to the preservation of macrophages, implicating them as the probable inducers of Tr1 cells.

Our mouse model was used to dissect the events building up to tolerance induction by Tr1 cells upon short-term treatment with G-CSF/Rapamycin. This model allows

not only to identify the role of early graft-infiltrating macrophages but also to suggest the requirement for a continuing generation of Tr1 cells to establish tolerance. This could be inferred from the data showing that graft loss occurs when Tr1 cell induction discontinues. A strength of our study is the ability to use CD11c-CreDTA mice lacking most of their DC on one hand to successfully induce tolerance to allogeneic grafts and on the other to rule-out DC and point to macrophages as being the most probable Tr1-inducing cells.

Our results implicating macrophages in Tr1 cell induction are partly in agreement with the observations reported by Zhang *et al.* where donor-derived macrophages produced IL-27, stimulating Tr1 cell development in a mouse model of GvHD (15). On the other hand, the requirement for recipient DC for initial stimulation, observed by Zhang *et al.*, was not confirmed by our data where a role for DC was limited/excluded. Differences in the modes of action of the administered compounds could explain this conclusion: therapeutic induction of Tr1 cells via nasal anti-CD3 leads to the production of IL-10, TGF-beta and IL-27 by $CD11c^+$ DC and not by $CD11b^+$ macrophages, while G-CSF/Rapamycin stimulates $CD11b^+F4/80^+$ macrophages. The cytokine profile of these macrophages and their classification (possibly alternatively-activated macrophages) remains to be investigated.

Although our group and others have identified DC as potent inducers of Tr1 cells *in vitro,* Tr1 cell expansion in healthy humans or mouse models of autoimmunity (8, 21, 33), data from our current in vivo transplant model in diabetes do not confirm such findings. The use of the CD11c-CreDTA mouse model rules out DC and implicates macrophages as possible candidates for Tr1 induction in mice in vivo. Furthermore, tolerance could be induced using G-CSF/Rapamycin treatment in these mice with comparable graft survival curves, despite their predisposition to develop spontaneous autoimmunity (28).

One weakness to this study is the inability to localize the interaction between macrophages and Tr1 cells. Yet, one can hypothesize from the data that migration of macrophages from the graft to the spleen can be excluded since no surge in splenic

macrophage infiltration was noted when graft-infiltrating macrophages started to decrease during G-CSF/Rapamycin treatment. Alternatively, a role for a macrophage-derived factor or a change in cytokine profile of splenic macrophages can be proposed. This remains an effort we are currently pursuing.

These data offer insight into one mechanism of *in vivo* induction of Tr1 cells in a murine model of allogeneic transplantation in diabetic mice. Macrophage modulation could be explored therapeutically, as an alternative to adoptive transfer of *ex vivo* expanded Tr1 cells, currently under active clinical investigation to control autoimmune diseases (5). Understanding mechanisms of in vivo Tr1-cell induction can help us design different approaches to induce tolerance by mobilizing/stimulating the Tr1-inducing cells.

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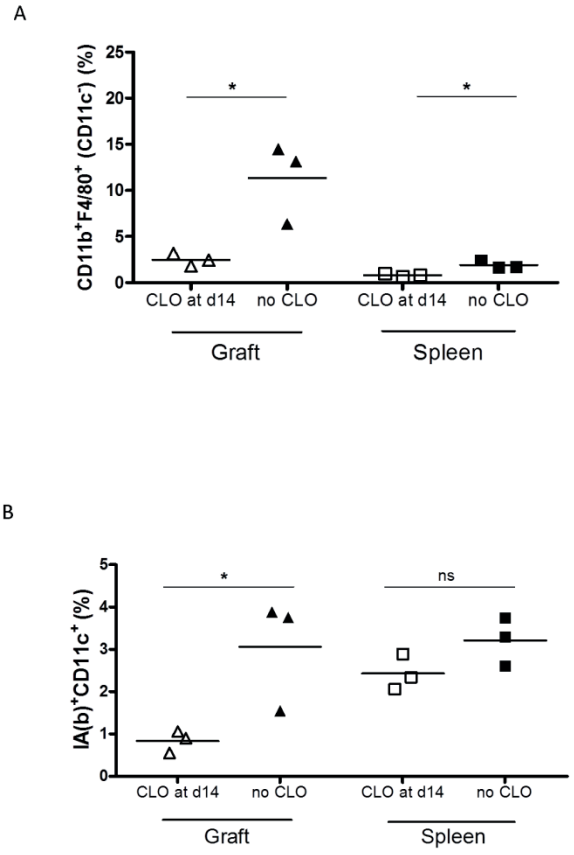
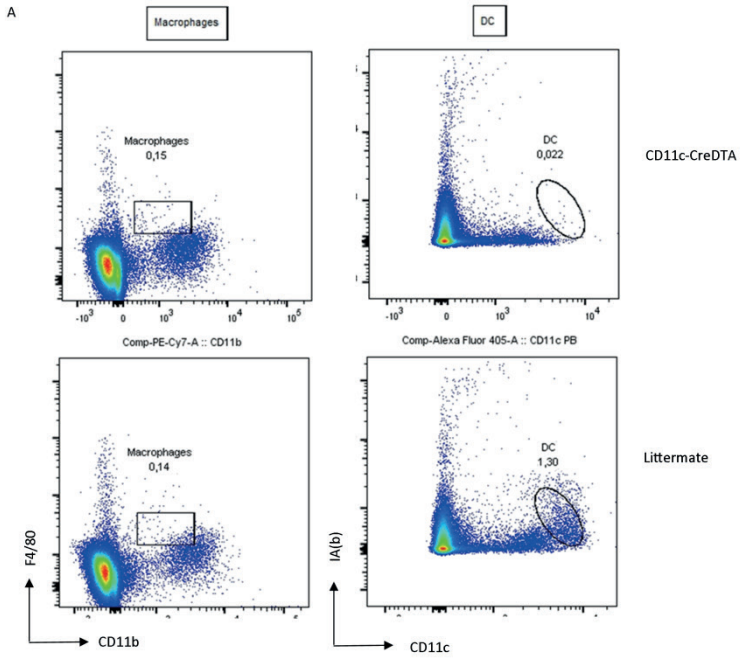
Supplementary Figure 1: **Macrophages and DC are the phagocytic cells sensitive to depleting treatment by clodronate liposomes.** Transplanted diabetic mice were treated with clodronate liposomes at 14 days during the course of G-CSF/Rapamycin treatment. Grafts and spleens were harvested 1 week later, tissue-residing cells were collected and identified by flow cytometry. Graphs represent the means of macrophage (**a**) and DC (**b**) frequencies in the grafts or spleens of clodronate liposome-treated mice (CLO) compared to untreated mice (no CLO). n=3/group, **p*<0.05, *ns*= non-significant, Mann-Whitney test.

Supplementary Figure 1



Supplementary Figure 2: **CD11c-CreDTA mice lack DC while maintaining macrophages.** CD11c-CreDTA mice were phenotyped to confirm their lack of DC. One representative dot plot of splenic DC and macrophages from C57BL/6 (*upper panel*) and CD11c-CreDTA (*lower panel*) mice as detected by flow cytometry is shown.



Chapter 4: Generation of donor-specific Tr1 cells to be used after kidney transplantation and definition of the timing of their in vivo infusion in the presence of immunosuppression

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J Transl Med (2017) Feb 21;15(1):40

Abstract

Background: Operational tolerance is an alternative to lifelong immunosuppression after transplantation. One strategy to achieve tolerance is by T regulatory cells. Safety and feasibility of a T regulatory type 1 (Tr1)-cell–based therapy to prevent graft *versus* host disease in patients with hematological malignancies has been already proven. We are now planning to perform a Tr1-cell–based therapy after kidney transplantation.

Methods: Upon tailoring the lab-grade protocol to patients on dialysis, aims of the current work were to develop a clinical-grade compatible protocol to generate a donor-specific Tr1-cell–enriched medicinal product (named T_{10} cells) and to test the Tr1-cell sensitivity to standard immunosuppression *in vivo* to define the best timing of cell infusion.

Results: We developed a medicinal product that was enriched in Tr1 cells, anergic to donor-cell stimulation, able to suppress proliferation upon donor- and not third-party stimulation *in vitro*, and stable upon cryopreservation. The protocol was reproducible upon up scaling to leukapheresis from patients on dialysis and was effective in yielding the expected number of T_{10} cells necessary for the planned infusions. The tolerogenic gene signature of circulating Tr1 cells was minimally compromised in kidney transplant recipients under standard immunosuppression and it eventually started to recover 36 weeks post-transplantation, providing rationale for selecting the timings of the cell infusions.

Conclusions: These data provide solid ground for proceeding with the trial and establish robust rationale for defining the correct timing of cell infusion during concomitant immunosuppressive treatment.

Background

Circulating T regulatory type 1 (Tr1) cells with an alloantigen-specific regulatory function have been consistently associated with operational tolerance after transplantation (1). Alloantigen-specific Tr1 cells can be induced *in vitro* in the presence of exogenous IL-10 or by tolerogenic IL-10-producing dendritic cells (DC-10) and they are hyporesponsive (anergic) to the alloantigen used for their generation (2, 3). These IL-10-anergized T cells have been tested as medicinal product in a proof-of-concept trial in patients undergoing haploidentical hematopoietic stem cell transplantation (HSCT) to provide immune reconstitution in the absence of severe graft *versus* host disease (G*v*HD) (the ALT-TEN trial) (4).

The ONE Study – a European Commission FP7-funded consortium - aims to test several distinct haematopoietic immunoregulatory cells as therapies after kidney transplantation from living donors by initiating a series of independent clinical trials based on the same general design (5). Our group participates in this consortium to test donor-specific Tr1 cells. The ALT-TEN trial already performed was certainly instrumental although the know-how that was developed in this first clinical experience did not necessarily allow performing The ONE Study faster and more efficiently. We overcame the first hurdle of tailoring the Tr1-cell generation protocol to patients on dialysis, yet only at a lab-scale (6). In this study, we aimed at: (i) defining a reproducible and clinical-grade compatible protocol for the generation of a Tr1-cell–enriched medicinal product for kidney transplant recipients, (ii) characterizing the final cell product and (iii) testing the sensitivity of circulating Tr1 cells to immunosuppressive therapy to determine the ideal timing of the medicinal product infusion.

Methods

Healthy donors and patients

Peripheral blood, buffy coat or leukapheresis were obtained from healthy donors or renal transplant recipients enrolled in The ONE Study Reference Group Trial (i.e.,

control group in which patients were treated with standard immunosuppressive therapy) (NCT01656135) after written informed consent in accordance with the Declaration of Helsinki under the protocol approved by the San Raffaele Hospital's Ethics Committee (IRB #OSR-TheOne).

Generation and characterization of dendritic cells

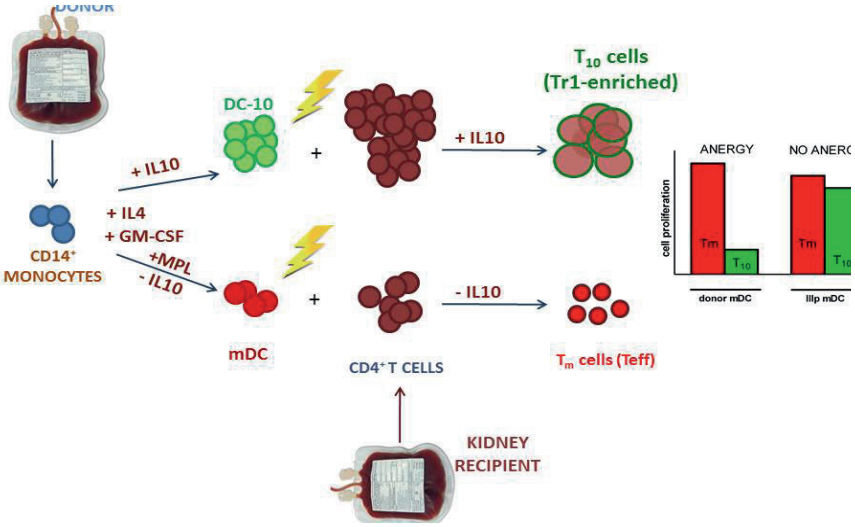
Peripheral Blood Mononuclear Cells (PBMC) were isolated by density-gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). IL-10-producing dendritic cells (DC-10) and mature DC (mDC) were generated from healt hy donors (7). Monocytes were isolated by harvesting the adherent fraction of PBMC or by selection with CD14+ microbeads using the AutoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) and following manufacturer's instructions. Monocytes were cultured with 10 ng/ml rhIL-4 (GMP-grade, Miltenyi Biotec) and 100 ng/ml rhGM-CSF (GMP-grade, Miltenyi Biotec) for 7 days in the presence (DC-10) or absence (mDC) of 10 ng/ml rhIL-10 (GMP-grade, CellGenix GmbH, Freiburg, Germany). The culture medium was supplemented with GMP-grade fetal bovine serum (FBS) (Lonza, Basel, Switzerland) or GMP-grade human AB sera (Lonza). mDC were matured during the last two days of culture with 5 μg/ml of rMPL-A (GMP-grade, Invivogen, Toulouse, France). At the end of the 7-day cultures, DC-10 and mDC were harvested and irradiated at 60Gy with a Cs137 source Biobeam 2000 irradiator (Gamma-Service Medical GmbH, Leipzig, Germany). DC-10 yield was measured as: 100x[no. of generated DC-10 cells/no. of plated cells].

Supernatants were collected 48 hours after culturing DC-10 in the presence or absence of stimulation by lipopolysaccharide (LPS) from E.coli (5 μg /ml, Sigma Chemicals, St. Louis, MO). IL-10 released into the supernatant was quantified by ELISA (BD Pharmingen, San Diego, CA). The detection limit of IL-10 was 15 pg/ml.

Generation and characterization of Tr1- cell enriched product: T_{10} cells

$CD4^+$ T cells were isolated from donors different from those used to generate DC by $CD4^+$ microbeads using the AutoMACS system (Miltenyi Biotec) following manufacturer's instructions. Purified $CD4^+$ T cells were cultured with irradiated allogeneic DC-10 or mDC (10:1 ratio) in the presence or absence of exogenous rhIL-10 (10 ng/ml) for 10 days to generate T_{10} or control Tm cells, respectively (Figure 1) [8].

Figure 1. **Graphical representation of the protocol for generating T_{10} cells to be used in kidney transplanted patients**. $CD14^+$ cells are selected from the kidney donor leukapheresis and cultured with GM-CSF and IL-4 in the presence (DC-10) or absence (mDC) of IL-10. At day 5, mDC are activated with monophosphoril MPL. Upon harvest at day 7 of culture, DC-10 and mDC are irradiated and co-cultured for 10 days with $CD4^+$ T cells selected from the recipient leukapheresis and exogenous IL-10 to generate T_{10} cells. mDC cultured with $CD4^+$ T cells without IL-10 generate the control Tm cell population. T_{10} cells are donor-specific anergic $CD4^+$ T cells, yet they respond to third party mDC stimulation (IIIp mDC) similar to the control population as shown by the representative cell-proliferation bars (simulating what one should expect when measuring T_{10}-cell proliferation toward donor mDC or toward third party unrelated mDC).



T_{10}-cell yield was measured as: 100x [no. of T_{10} cells generated/no. $CD4^+$ T cells plated]. To test the generation of donor-specific anergic T cells, T_{10} and Tm cells were cultured with the original-donor mDC (previously frozen) and cell proliferation was monitored via ^{3}H-thymidine (PerkinElmer, Waltham, MA) incorporation (counts per minute, cpm) in the last 16-18 hours of a 3-day culture. Anergy was calculated as: cpm [(T_{10}+mDC)/(Tm+mDC)] x100. Supernatants were collected before ^{3}H-thymidine addition and quantification of IFNgamma or IL-10 by ELISA (BD Pharmingen) was done. The detection limit of IFNgamma was 15 pg/ml.
Ability of T_{10} cells to suppress the proliferation of autologous $CD4^+$ T cells upon donor or third party mDC stimulation was assessed by ^{3}H-thymidine incorporation in the last 16-18 hours of a 5-day culture.

Flow cytometry

The immune phenotype of *in vitro* generated DC, T_{10} and Tm cells was tested by flow cytometry as previously described (8). The TCR Vbeta repertoire was

determined with the IOTest® Beta Mark TCR V beta Repertoire Kit (Beckman Coulter, Inc, Brea, CA) following manufacturer's instructions.
Cells were analyzed with the BD FACS Canto II (Beckton Dickinson, San Jose, CA) within few hours after staining. Data was analyzed using FCS 3.0 (DeNovo Instruments, Los Angeles, CA).

Dual IFNgamma/IL-10 ELISPOT

Dual IFNgamma/IL-10 ELISPOT (Diaclone, Besancon, France) was performed according to manufacturer's instructions with a slight modification: visualization of IL-10 was performed using Vector Blue Alkaline Phosphatase substrate kit (Vector Labs, Burlingame, CA) and the A.EL.VIS 4-Plate ELISPOT Reader (A.EL.VIS GmbH, Hannover, Germany) was used. Analysis was performed using ImageJ (version 1.48, NIH, USA) to quantify IFNgamma-producing cells (red spots), IL-10-producing cells (blue spots) or dual IFNgamma/IL-10-producing cells (purple spots).

Transcript analysis of purified Tr1 cells

Peripheral blood was collected and PBMC were frozen from patients enrolled in The ONE Study Reference Group Trial at our center at the following time points: 4-weeks pre-transplant, 8- 36- and 60-weeks post-transplant. PBMC were thawed and Tr1-cell sorting was performed using MoFlo Legacy Cell Sorter (Beckman Coulter, Indianapolis, IN). To verify the expression of anti-inflammatory genes characteristic of Tr1 cells (as previously described [1]) and pro-inflammatory genes characteristic of T effector cells (i.e., *il-17a, il-1b, tnf, il-6* and *ifngamma*), QuantiGene 15-plex assay (Affymetrix, Santa Clara, CA) was performed following manufacturer's instructions. Mean fluorescence intensity from the measured beads per gene was reported using Bio-Plex 200 system (BioRad Laboratories, Hercules, CA). Probe set information is provided in Additional file 1.

Statistical analysis

Comparisons between groups were performed using Student's t test, Mann-Whitney test or Wilcoxon matched pairs test depending on the experiments. For all analyses,

a two-tailed p value ≤ 0.05 was considered significant. Comparison of variances was performed using the F-test. Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA).

Results

DC-10 generation in compliance with clinical-grade manufacturing

The generation of donor-specific Tr1 cells is contingent to the production of donor-derived DC-10 (7). The first step in defining a clinical-grade compatible protocol was therefore establishing an efficient and reproducible method for DC-10 generation. Based on the MLR/DC-10 protocol used in the ALT-TEN trial (7), DC-10 were generated from the PBMC adherent fraction in medium supplemented with Fetal Bovine Serum (FBS) in wells. Due to the availability of GMP-compatible human serum (HS) and guidelines from the regulatory authorities on the use of bovine-derived sera (EMEA/CHMP Guideline on the use of bovine serum in the manufacture of human biological medicinal products - original version EMA/CPMP/BWP/1793/02, and revised version EMA/CHMP/BWP/457920/2012 rev 1), we compared FBS- and HS-supplemented medium during DC-10 generation. The same batch of FBS was used throughout the experiments described throughout the manuscript. Alongside a certificate of analysis, we confirm the availability of a TSA Certificate of Suitability, issued by EDQM via the process of Certification of Suitability of Monographs of the European Pharmacopoeia. DC-10 recovery was better when medium supplemented with FBS was used, yet the intra-experiment variability – determined by the coefficients of variation (CV) - was high (Figure 2). This high variability could be attributed to the non-specific monocyte selection method, which leads to unpredictable monocyte recovery (9). Thus, provided methods for clinical-grade monocyte selection are available, monocytes were purified by CD14+ magnetic beads and selected on magnetic columns (10). DC-10 recovery was less variable upon starting from bead-selected monocytes than from adherence and FBS was necessary to generate sufficient DC-10 numbers (as shown

in Figure 2 and also supported by our unpublished results generated from independent unrelated experiments).

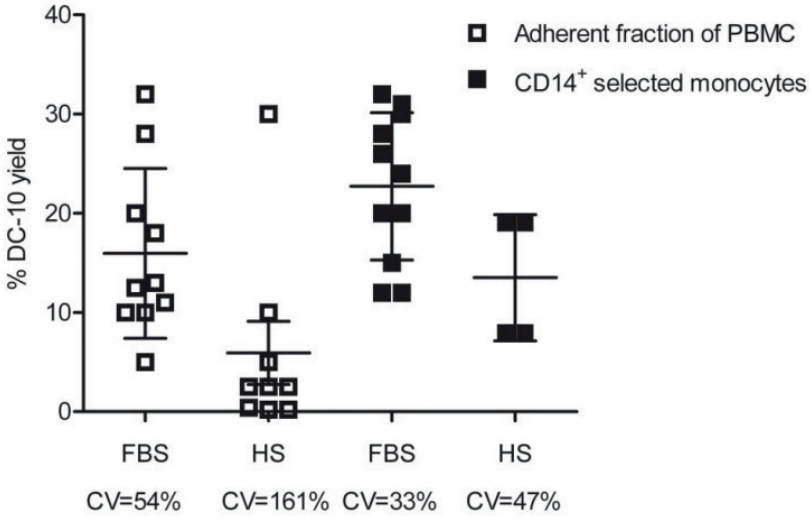


Figure 2. **CD14$^+$ bead-selected monocytes cultured in FBS-supplemented medium represent an optimal clinical-grade compatible approach for DC-10 generation**

Adherent fraction of PBMC (□) or CD14$^+$ selected monocytes (■) were cultured in FBS- or HS-supplemented medium for seven days to generate tolerogenic Tr1-cell-inducing DC-10. DC-10 yield is shown, measured as: 100x[no. of generated DC-10 cells/no. of plated cells]. One square represents one experiment, lines represent mean value of each data set ± SD. Coefficient of variation (CV) is shown for each data set.

DC-10 generated from purified CD14+ monocytes in FBS-supplemented medium had the anticipated phenotype (i.e., CD14+CD86+CD16+) and they produced IL-10, either at steady state or upon LPS activation (Figure 3) (7). Based on these data, we concluded that peripheral blood monocyte selection by CD14+ magnetic beads and culture media supplemented with FBS is the optimal clinical-grade compatible approach for DC-10 generation. This conclusion was drawn based on the limited number of DC-10 generated with HS that was incompatible with the clinical need. In addition, DC-10 will be irradiated and kept in culture with recipient CD4+ T cells for 10 additional days in the absence of any bovine-derived products.

T_{10} cell generation in compliance with clinical-grade manufacturing

A protocol for the generation of cell products to be infused into patients has to be solid and highly reproducible to have the highest chance to be used in clinical trials. Accordingly, buffy coats from eight healthy donors were used for DC-10 generation and buffy coats from eight more donors were used for the isolation of $CD4^+$ T cells. Flasks were used as clinical-grade compatible culture containers to generate T_{10} cells.

Monocytes isolated from buffy coats with lab-grade magnetic columns (AutoMACS-Miltenyi) had a mean purity of 95±3% (mean±SD). DC-10 yield in flasks (13±6, mean±SD) was, as expected, lower than that in wells (23±7, mean±SD) (Table 1) but all DC-10 preparations in flasks had a tolerogenic phenotype (Figure 3).

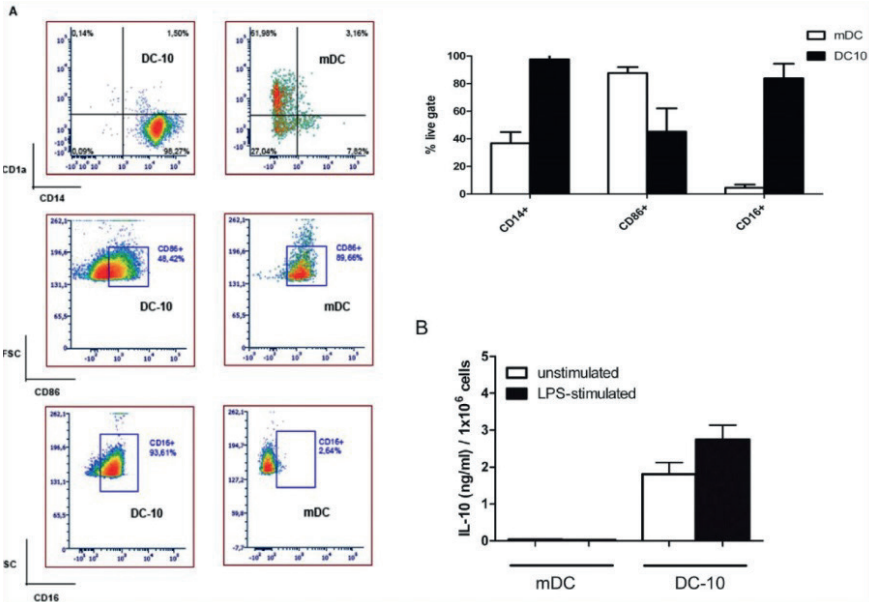


Figure 3. **Tolerogenic phenotype of DC-10 cells: CD14$^+$CD86$^+$CD16$^+$ cells that produce IL-10 (whether stimulated or unstimulated).** One representative flow cytometry staining (out of eight) of DC-10 cells and control mDC gated on live cells. Percentages of CD14+ cells (upper left panel **A**), CD86+ cells (middle left panel **A**) and CD16+ cells (lower left panel **A**) are shown. Bars represent mean value of each data set ± SD for DC-10 and mDC (upper right panel **A**). Bars represent mean value of IL-10 (quantified by ELISA) ± SD secreted by DC-10 or mDC into the supernatant of a 48-hour culture in the presence or absence of LPS stimulation (panel **B**).

Table 1. **Protocol reproducibility with the optimized clinical-grade compatible conditions using buffy coats from eight donor pairs**

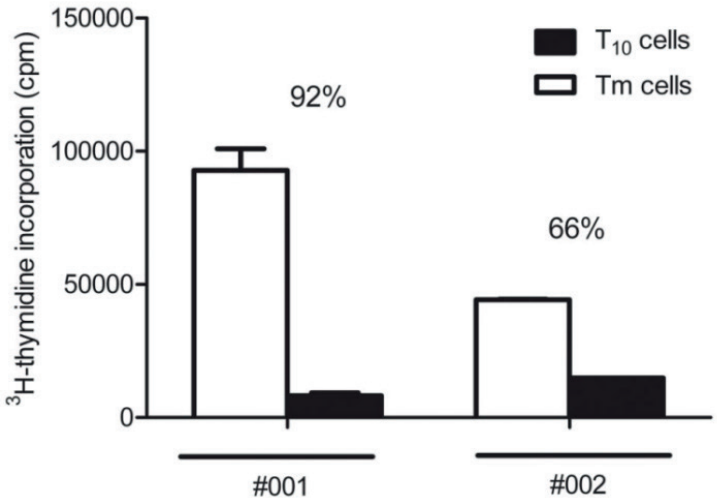
	$CD14^+$ purity (%)	DC-10 yield (%)	$CD4^+$ purity (%)	T_{10}-cell yield (%)	Donor-specific anergy (%)
Mean	95	13	98	53	80
SD	3	6	1	31	10
Range	90-99	4-21	97-99	17-123	63-94
CV	3,3	47,3	0,9	59,0	14,6

DC-10: tolerogenic dendritic cells; SD: standard deviation; CV: coefficient of variation

$CD4^+$ T cells isolated from buffy coats with lab-grade magnetic columns had a mean purity of 98±1% (mean±SD). Average T_{10}-cell yield after 10 days of co-culture with allogeneic DC-10 was 53±31% (mean±SD) and T_{10}-cell donor-specific anergy was 80±10% (mean±SD) (Table 1). The T_{10}-cell product was constituted of 96±4% (mean±SD) $CD4^+$ T cells; the remaining non-$CD4^+$ T cells were donor-derived DC-10 cells that were irradiated and therefore dead or prone to die (Additional File 3). Coefficients of variation were high for both DC-10 and T_{10}-cell yield, probably due to intrinsic differences among donors. The cut off anergy value for classifying T_{10} cells as anergic towards donor antigens was determined utilizing the "mean minus 2xSD" as statistical method (4, 11). This method was chosen based on our previous experience in the ALT-TEN trial (4). Based on the eight T_{10}-cell preparations generated from eight different donor pairs, the anergy cut off of T_{10} cells was 60%. Thus, T_{10}-cell products for clinical-grade-compatible use will be considered anergic when the value is $\geq 60\%$.

Based on our previous murine studies we plan to infuse a total of $2x10^6$ T_{10} cells/kg (12). Given the DC-10 and T_{10}-cell yield observed, buffy coats would fail to provide sufficient numbers of monocytes and $CD4^+$ T cells to reach the required number of T_{10} cells. Up scaling from buffy coats to leukapheresis was therefore a necessary step (13). As a proof-of-concept, leukapheresis from two healthy donors were used as sources of $CD14^+$ cells for the generation of DC-10 cells. To generate T_{10} cells, leukapheresis from two patients with kidney failure on dialysis were used as sources of $CD4^+$ T cells. This setting mimics exactly the clinical situation that we will face during the future clinical trial. The yield of T_{10} cells from both donor pairs surpassed the minimum number required for the planned infusions ($2x10^6$/kg): $11x10^6$ cells/kg (#001) and $19x10^6$ cells/kg (#002). T_{10} cells displayed donor-specific anergy higher than 60% (Figure 4). In addition to this, 3 more donor pairs were recruited in the study and T_{10} cells were successfully generated from leukapheresis in a GMP-compatible facility (Battaglia et al., manuscript in preparation), further proving protocol up scaling. These data demonstrate that the protocol for the generation of clinical-grade-compatible T_{10} cells defined by using buffy coats is also applicable to leukapheresis. Additionally, these data confirm our previous work tailoring the protocol to patients on dialysis (8). A statistically significant positive correlation existed between the expression of the activation marker CD86 on DC-10 and T_{10}-cell yield (Additional File 4). These data suggest that for DC-10 to generate antigen-specific T_{10} cells *in vitro*, they need to have an appropriate level of activation.

Figure 4. **Protocol for T_{10}-cell generation is reproducible upon up scaling from buffy coats to leukapheresis.** $CD4^+$ T cells purified from leukapheresis from two patients with kidney failure on dialysis were co-cultured with allogeneic DC-10 cells (generated from leukapheresis from two healthy donors) to generate T_{10} cells. Counts per minute (cpm) of incorporated ^{3}H-thymidine in the last 16-18 hours of a 3-day co-culture of T_{10} or Tm cells with donor-mDC are shown. Bars represent mean value of cpm of T_{10} or Tm cells (in triplicates) generated from patient #001 and #002. Percentage of anergy of T_{10} cells is indicated over the bars.



In vitro characterization of the medicinal product

Additional important functional features of T_{10} cells that go beyond the development of the clinical-grade-compatible protocol were also tested to better characterize the cells that will be infused into patients. As we had previously reported, the medicinal products generated *in vitro* with DC-10 are enriched in Tr1 cells and are comprised not only of Tr1 cells, but also $CD4^+$ memory T cells that respond to nominal antigens (7). To test the Tr1-cell content in the T_{10} cells generated with the clinical-grade-compatible protocol described above, the frequency of $CD4^+CD45RA^-CD49b^+LAG\text{-}3^+$ cells (i.e., Tr1 cells (14)) was tested by flow cytometry. T_{10} cells contained an average of 6±3% (mean ± SD) Tr1 cells, while control Tm cells lacked Tr1 cells (Figure 5A). Tr1-cell content in T_{10} cells generated from patients on dialysis using leukapheresis products was comparable to that from T_{10} cells generated from healthy donors using buffy coats (open *vs* closed squares, respectively Figure 5A). The Tr1-cell content in T_{10} cells was irrespective of the frequency of Tr1 cells originally present in the starting $CD4^+$ T-cell population supporting the notion of *de novo* generation of donor-specific Tr1 cells rather than the expansion of circulating Tr1 cells (Figure 5B).

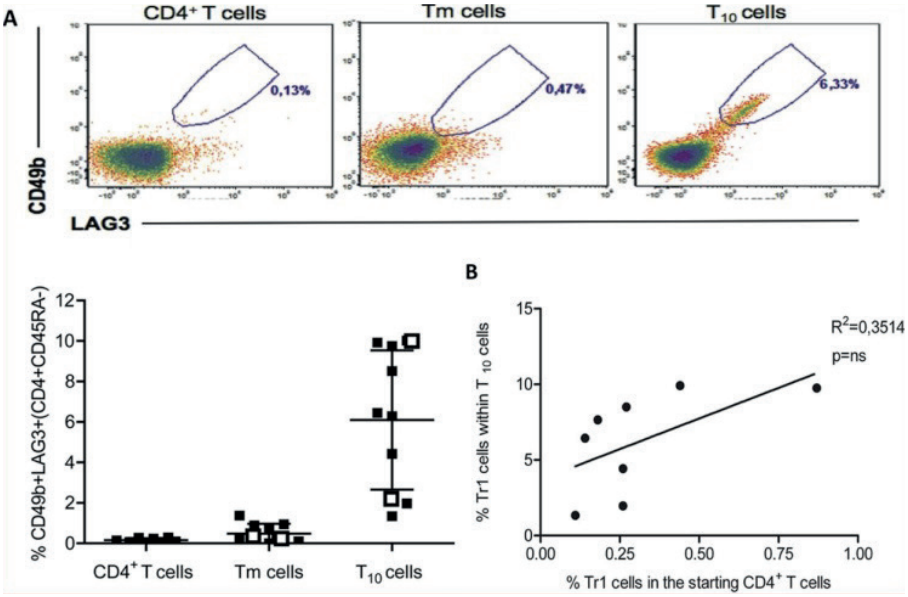
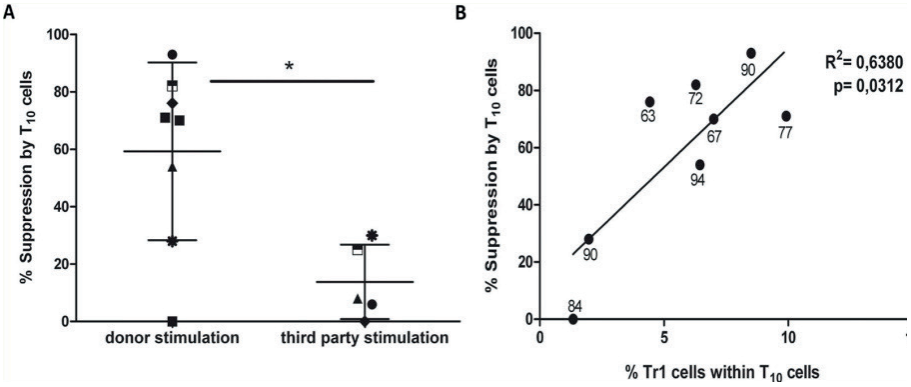


Figure 5. **T_{10} cells are enriched in Tr1 cells.** One representative flow cytometry staining (out of eight) of $CD49b^+LAG\text{-}3^+$ cells gated on $CD4^+CD45RA^-$ of $CD4^+$ T cells, control Tm cells and T_{10} cells is shown (upper panel **A**). Percentages of $CD49b^+LAG\text{-}3^+$ (within $CD4^+CD45RA^-$) Tr1 cells are shown. Each closed square represents one experiment with buffy coats from healthy donors; each open square represents one experiment with leukapheresis from patients on dialysis. Lines represent mean value of each data set ± SD (lower panel **A**). Percentages of Tr1 cells in the starting $CD4^+$ T cells are plotted against percentages of Tr1 cells within the corresponding T_{10} cells. One dot represents one experiment. Line represents linear regression (**B**).

Treg-cell function is commonly tested *in vitro* by means of suppression assays (14, 15). These assays are cumbersome and difficult to be used as standard tests in clinical-grade labs. However, the demonstration that T_{10} cells have *in vitro* suppressive activity further supports their potential efficacy *in vivo* in patients. The *in vitro* ability of T_{10} cells to suppress proliferation of autologous $CD4^+$ T cells in response to donor-mDC was therefore tested. To prove donor-specific regulation, suppression of autologous $CD4^+$ T cell responses towards third party allogeneic mDC was also tested when numbers of T_{10} cells permitted. Priority was given to anergy testing, being most relevant in the upcoming Phase 1 trial testing the safety of T_{10} cells. All, except one T_{10}-cell preparation, suppressed *in vitro* proliferation of autologous $CD4^+$ T cells in response to donor-mDC but not to third party-mDC stimulation proving their antigen-specific regulatory properties (Figure 6A). Importantly, a positive correlation between Tr1-cell content and the suppressive capacity of T_{10} cells was observed thus further suggesting that the *in vitro* regulation of T_{10} cells is mediated by Tr1 cells (Figure 6B). Interestingly, a strong correlation was observed when Tr1-cell content exceeded 5% indicating that T_{10} cell preparations - to have a good suppressive capacity - need to contain at least 5% of Tr1 cells: $CD4^+CD45RA^-CD49b^+LAG\text{-}3^+$. To note, one preparation resulted in T_{10} cells with no suppressive capacity and low Tr1-cell content. However, the anergy level of these T_{10} cells was 84%, suggesting that even with a low Tr1-cell content these cells remain anergic towards donor stimulation, hence complying with the safety requirement of this medicinal product.

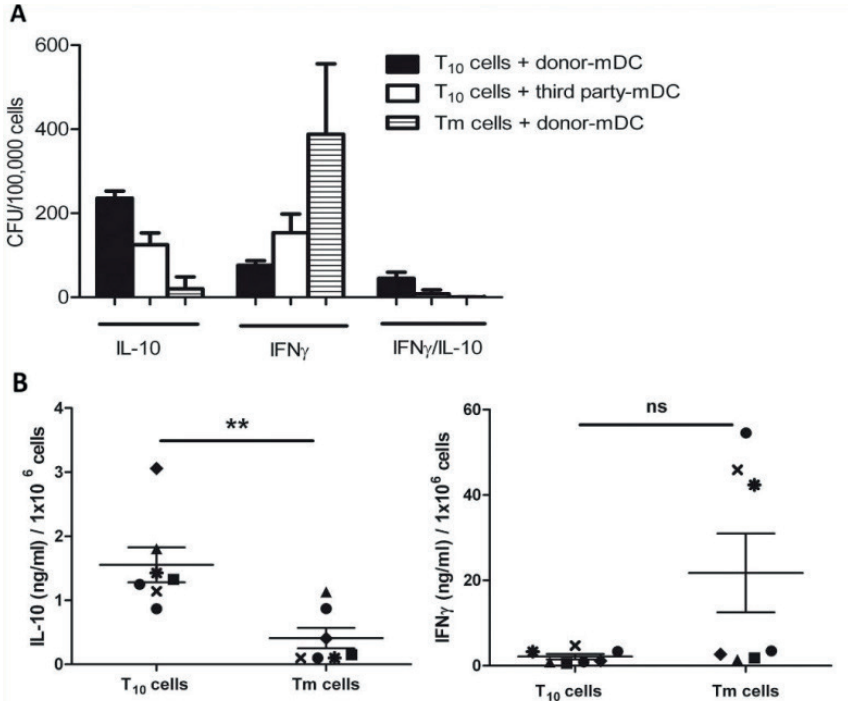
Figure 6. **Donor-specific suppression of $CD4^+$ T cell proliferation by T_{10} cells correlates with Tr1-cell content.** $CD4^+$ T cells were stimulated with donor mDC (or third party mDC - when numbers of T_{10} cells permitted) in the presence or absence of autologous T_{10} cells. Percentages of suppression by T_{10} cells are shown. One dot represents one experiment with buffy coats from healthy donors (each symbol depicts one donor). Lines represent mean value of each data set ± SD (* $p \leq 0.05$ Wilcoxon matched pairs test) (**A**). Percentages of Tr1-cell content within T_{10} cells is plotted against percentage of suppression by T_{10} cells. One dot represents one experiment and different shapes represent different donor pairs. Numbers represent anergy levels measured on each T_{10}-cell preparation. Line represents linear regression (**B**).



A high number of IL-10-producing cells and a low number of IFNgamma-producing cells in response to donor-mDC stimulation was detected by dual ELISPOT in T_{10} cells, as compared to those detected in control Tm cells (Figure 7A). This was confirmed by the levels of IL-10 and IFNgamma by ELISA in the supernatant of co-culture of T_{10} cells with donor-mDC (Figure 7B).

Figure 7. **T_{10} cells produce more IL-10 and less IFNγ than control Tm cells in response to donor- and not third party-mDC stimulation**

T_{10} or control Tm cells were stimulated with donor-mDC or third party-mDC. Cytokine-forming units (CFU) were detected by dual ELISPOT for IFNgamma and/or IL-10. Number of CFU/100,000 cells is shown. Bars represent mean value of each dataset ± SD (n=3 experiments) (**A**). T_{10} or control Tm cells were stimulated with donor-mDC. IL-10 and IFNgamma measured in the supernatant 48 hours after culture by ELISA is shown. One dot represents one experiment with buffy coats from healthy donors (each symbol depicts one donor and different shapes represent different donor pairs). Lines represent mean value of each data set ± SD (ns, ** $p<0.005$ Mann-Whitney test) (**B**).

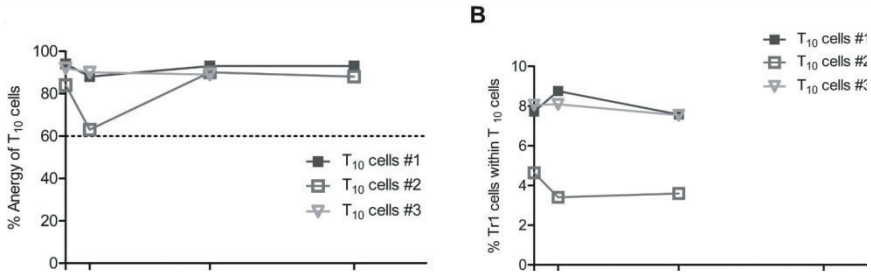


T_{10} cells maintained a polyclonal TCR-Vbeta repertoire proving lack of a skewed immune response *in vitro* (Additional File 5) supporting previous findings (4).

We anticipate the infusion of T10 cells in patients only upon obtaining all safety data (i.e., quality controls (QC) and anergy values) on the medicinal product. Thus, cryopreservation of T10 cells is inevitable. However, not all cell products are suitable for freezing and thawing, requiring additional manipulation to restore their functionality upon thawing (16, 17). We thus aimed at testing cell product viability and safety upon various cryopreservation time-points. All T10 cells had a viability ≥70% upon thawing (after 1, 6 or 12 months of cryopreservation) and preserved their Tr1-cell content (data not shown). All T10-cell preparations tested, remained anergic upon donor-mDC stimulation and preserved Tr1 content when thawed (Figure 8). For limited cell number availability, the suppressive ability and/or the cytokine production profile of thawed products could not be tested. We gave priority to the anergy test, given that it provides a clear answer on the safety of thawed

medicinal products. These data provide evidence that T10 cells can be cryopreserved up to 12 months without losing their viability, stability and donor-specific anergy upon thawing and do not require further manipulation prior to in vivo infusion. Similar data were obtained with the 3 GMP-grade generated medicinal products (Battaglia et al., manuscript in preparation).

Figure 8. **Freezing has little effect on donor-specific anergy and Tr1 content of T_{10} cells.** T_{10} cells were thawed after 1, 6 or 12 months of freezing and immediately stimulated with donor-mDC. In parallel, flow cytometry staining for Tr1 cells was performed. Percentages of donor-specific anergy (**A**) and Tr1 cell content (**B**) are shown. One symbol represents one T_{10} cell preparation starting from buffy coats from healthy donors. Lines represent the time course of the percentage of anergy or Tr1-cell content of individual T_{10} cells thawed at different timepoints. Dotted line represents the set cutoff anergy level of 60%. Tr1-cell content 12 months after freezing was not available.



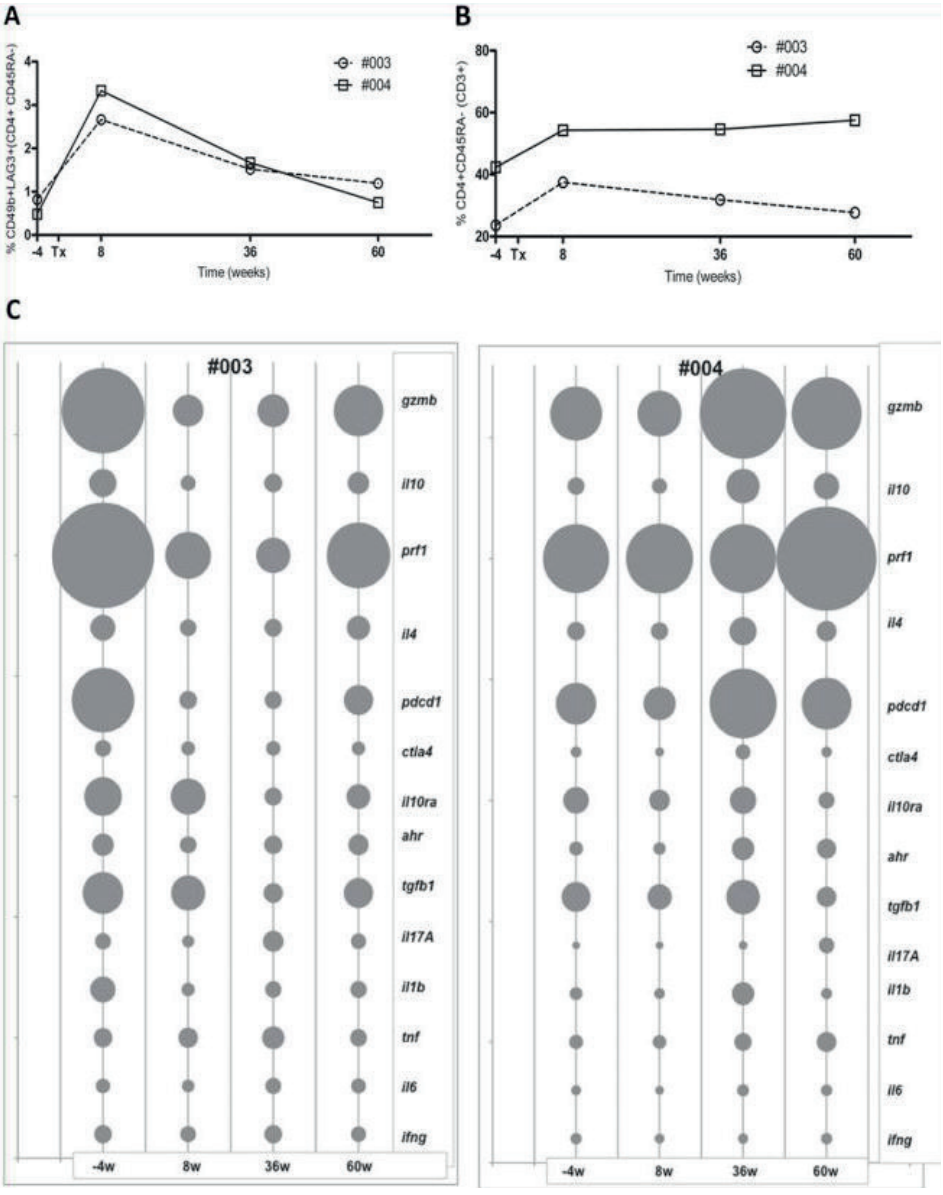
Gene signature of circulating Tr1 cells is transiently affected by immunosuppressive drugs

The ONE Study trial with donor-specific Tr1 cells infused into patients undergoing kidney transplantation envisages the concomitant administration of immunosuppressive drugs (5). Thus, the obvious question on administering cell therapy under active immunosuppression is the effect of the selected drugs on Tr1-cell viability and stability/function in vivo (18). The ONE Study also included a parallel clinical trial with no cell therapy but standard immunosuppressive treatment, to be used as a reference group for the analysis of cell therapy trials (Additional File 6, detailing immunosuppressive regimen). We aimed at monitoring whether circulating endogenous Tr1 cells preserve their gene signature under standard immunosuppressive therapy: *il10*^{hi}, *il4*^{lo}, *il17*^{lo}, *tgfβ*$^{hi/int}$, *pd1*^{hi}, *granzyme b*^{hi}, (reviewed in (1)). To this aim, circulating Tr1 cells of two patients enrolled in the Reference Group Trial at our clinical site were studied (Additional File 2 detailing immunosuppression dosages and trough levels). The frequency and gene expression profile of circulating Tr1 cells purified from patients after kidney transplant and under active immunosuppression (8, 36, and 60 weeks post-transplant) was compared to those of circulating Tr1 cells purified from the same patients before transplant (4 weeks pre-transplant) and in the absence of any drug treatment. Tr1

cells were detectable in the circulation and were increased in frequency and absolute numbers (Figure 9A). Tr1-cell frequency peaked at 8 weeks post-transplant then returned to pre-transplant levels in both patients (Additional File 7). This increase was likely due to homeostatic proliferation after induction therapy, as observed in memory $CD4^+$ T cells (Figure 9B) (19). Tr1 cells were isolated by flow cytometry-based cell sorting (Additional File 8 for gating strategy). The gene signature typical of Tr1 cells (14) was slightly diminished (patient #003) or remained unchanged (patient #004) at 8 weeks post-transplant and then returned to pre-transplant levels or heightened at 60 weeks post-transplant (in the two patients, respectively) (Figure 9C).

These data suggest that Tr1 cells expand along with the $CD4^+$ T-cell memory population and that the Tr1-cell tolerogenic gene expression profile remains stable even under active immunosuppressive treatment. Data are limited to two patients (being the only patients we enrolled in the reference group trial) but they attempted to dissect the Tr1-cell sensitivity to immunosuppression *in vivo*, a relevant concern that was - to our knowledge - never addressed before. Importantly, these data suggest that the best timing of *ex vivo*-generated Tr1-cell infusion could be right at the moment of transplant (to reduce inflammation and control alloreactivity) and around 36 weeks post-transplant, when the Tr1-cell signature starts to recover.

Figure 9. **Gene signature of circulating Tr1 cells of two kidney transplant recipients is transiently affected by immunosuppression.** Percentages of Tr1 cells before (4 weeks pre-transplant) and after kidney transplantation (8, 36, 60 weeks) are shown. One line represents one patient (**A**). Percentages of circulating $CD4^+CD45RA^-$ T memory cells (within $CD3^+$ T cells) in the two patients are shown (**B**). Transcript analysis of the indicated genes was performed on purified circulating Tr1 cells from patient #003 and #004. Bubble chart represents the gene expression signals for each patient over time: 4 weeks pre-transplant, 8-, 36- and 60-weeks post-transplant (normalized to housekeeping gene *hprt1*). Bubble size represents gene expression signal. Gene symbols are represented on the right (**C**).


A
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Discussion

Optimization of a clinical-grade compatible protocol for generating donor-specific Tr1-enriched cell medicinal products is a pre-requisite for the planned clinical trial in kidney transplant recipients (5). In this study we described a clinical-grade compatible protocol that enabled the production of donor-specific Tr1-cell enriched medicinal product (named T10 cells) by coculturing recipient CD4+ T cells with tolerogenic donor DC-10 in the presence of exogenous IL-10 for 10 days. The generated T10 cells are anergic and suppressive towards donor stimulation in vitro, maintain a stable function upon cryopreservation and are successfully produced in clinically sufficient amounts starting from leukapheresis from patients on dialysis. We also demonstrated that circulating Tr1 cells have a limited sensitivity (in terms of viability and gene expression profile) to standard immunosuppressive treatment in vivo.

Several hurdles are encountered when attempting to perform cell therapy clinical trials (18). First, the protocol for generating the medicinal product needs to be clinical-grade. To that end, we surpassed all the obstacles. Second, a sufficient number of cells to be infused is a prerequisite. Up scaling to leukapheresis could, in some instances, represent a hurdle in terms of sample collection and protocol adaptability (20). Here we proved that leukapheresis can be collected with no medical contra-indications from patients on dialysis and that both tolerogenic DC-10 and T10 cells can be generated from leukapheresis products.

Another key aspect is the definition of lab tests that ensure safety of the cell product. Within The ONE Study consortium, some groups are using polyclonal Tregs. We decided to invest in the donor-specific Tr1-cell-based therapy in an attempt to promote antigen-specific tolerance. However, the generation of T10 cells with donor-derived DC, although they are tolerogenic and well characterized (3, 7), contains an intrinsic risk of generating alloreactive T cells that, once infused, could be potentially risky for the patient leading to graft rejection. The ONE Study Cell Therapy Trials are feasibility and safety trials (5). Thus, in vitro assays that prove

medicinal-product safety are mandatory. Donor-specific anergy is an optimal assay to test T10-cell safety, while the suppression assay provides indications on the possible efficacy of Tr1 cells in vivo. Accordingly, T10 cells that show no suppressive capacity in vitro but retain donor-specific anergy are considered safe and therefore will be infused in patients participating in The ONE Study cell therapy trial at our institute.

Some groups working with FOXP3+ Tregs as cell therapy products, reported problems with cryopreservation, thus requiring - for instance - further cell manipulation upon thawing (16, 17). The demonstration that T10 cells are stable and conserve their Tr1 content and donor-specific anergy properties upon cryopreservation, allows for flexibility in their preparation and feasibility for more than one infusion.

Whether infused T10 cells retain their viability and function in vivo under treatment with immunosuppressive drugs remains an important open question. One approach to address this issue was by testing the effect of immunosuppressive drugs in vitro on the ex vivo-expanded human FOXP3+ Tregs or by using a humanized mouse model (21). This showed detrimental dose-dependent effects of immunosuppressive treatments on viability and proliferative capacity while sparing the immunosuppressive function of FOXP3+ Tregs. We approached this issue by analyzing the frequency and the gene signature of circulating Tr1 cells collected from renal transplant recipients under active immunosuppressive treatment. Our data suggest that immunosuppressive drugs do not affect Tr1 cells since the cells remain in circulation and a transient change in the intensity of the gene signature is observed. Based on these data, we chose two different timings of T10-cell infusion to increase the chance of obtaining in vivo immune regulation. The first dose will be infused at the time of transplant (for Tr1-cell enrichment just around the transplant period). The second dose will be infused at 36 weeks post-transplant, timepoint in which the Tr1-cell signature is recovering.

Taken together, our results demonstrate the reproducibility of an optimized clinical-grade-compatible protocol for generating Tr1-enriched T10 cells. The necessary following steps for performing the trial in patients are underway.

Conclusion

We describe the steps undertaken to achieve and validate a reproducible optimized clinical-grade compatible protocol capable of generating donor-specific Tr1 cells in sufficient numbers. Additionally, selecting the timing of infusion of Tr1 cells to patients under immunosuppression remains an open question. We provide data assessing the viability and gene signature of circulating Tr1 cells in the presence of active immunosuppression thus supporting our rationale for selecting the timing of the planned infusions. We believe that this study highlights the importance of optimizing and validating Tr1 cell manufacturing protocols to bring them closer to the bedside.

Declarations

Ethics approval and consent to participate: Written informed consent in accordance with the Declaration of Helsinki under the protocol approved by the San Raffaele Hospital's Ethics Committee (IRB #OSR-TheOne) was obtained.

Availability of data and materials: All data generated or analysed during this study are included in this published article (and its supplementary "Additional files").

Competing interests: The authors declare that they have no competing interests

Funding: This project was funded through a grant to MB from the 7th framework programme of the EU (FP7-HEALTH-2010 The ONE Study; grant agreement 260687). BM was supported by the 7th framework programme of the EU (Marie Curie Initial Training Network -FP7-PEOPLE-2011-ITN), under the Marie Skłodowska-Curie grant agreement No 289903: EUTRAIN.

Acknowledgements

We thank Maria Grazia Roncarolo, Rosa Bacchetta (Stanford University, CA) and Silvia Gregori (San Raffaele Telethon Institute for Gene Therapy, Milano) for helpful scientific discussion. We equally thank members of the lab of Lorenzo Piemonti (Diabetes Research Institute, San Raffaele Hospital) and FRACTAL facility (San Raffaele Hospital) for technical assistance. We also thank all the donors who actively participated to this study.

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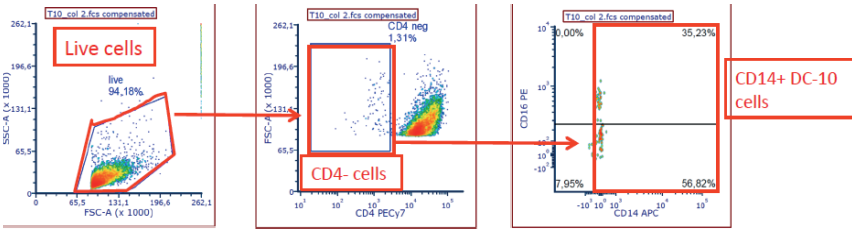
Additional File 1. Information on probe set used for defining the Tr1 cell signature

Accession Number	Symbol	Other Names	Sequence Length	Probe Set Region	Specificity designed specifically to hybridize:
NM_004131	GZMB	HLP; CCPI; CGL1; CSPB; SECT; CGL-1; CSP-B; CTLA1; CTSGL1	941	425-916	human **Granzyme B**
NM_002190	IL17A	CTLA8; IL-17; IL17-A	1859	980-1731	human **IL17A**
NM_005041	PRF1	P1; PFP; HLPH2; MGC65093	2529	728-1180	human **Perforin1**, both transcript variants
NM_000572	IL10	CSIF; TGIF; IL-10; IL10A	1629	44-654	human **IL10**
NM_000576	IL1B	IL-1; IL1F2; IL1-BETA	1498	24-659	human **IL1β**
NM_000589	IL4	BSF1; IL-4; MGC79402	921	12-558	human **IL4**, both transcript variants
NM_005018	PDCD1	PD1; SLEB2; hPD-1; hPD-I	2115	255-796	human Programmed cell death protein 1 (**PD1**)
NM_005214	CTLA4	CD152; CTLA-4	2033	726-1307	human Cytotoxic T-lymphocyte-associated protein 4 (**CTLA4**)
NM_000194	HPRT1	HPRT; HGPRT	1435	673-1404	human Hypoxantine phosphoribosyltransferase 1 (**HPRT1**)
NM_000594	TNF	DIF; TNFA; TNFSF2; TNF-alpha	1669	812-1230	human Tumor necrosis factor alpha (**TNFα**)
NM_001558	IL10RA	IL10R; CDW210A; HIL-10R; IL-10R1	3672	255-653	human **IL10RA**, both transcript variants
NM_000600	IL6	HGF; HSF; BSF2; IL-6; IFNB2	1201	3-669	human **IL6**

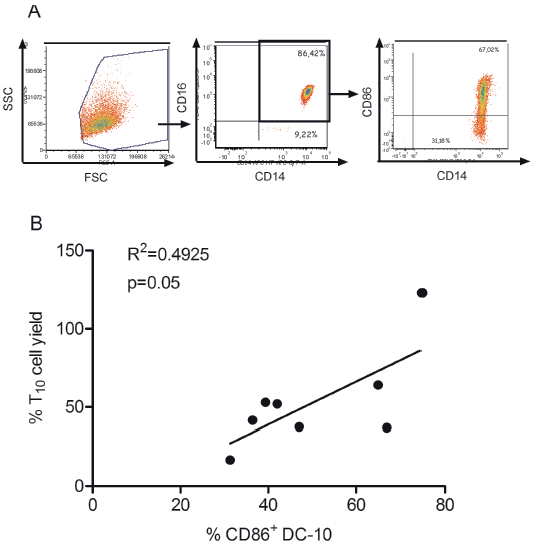
NM_001621	AHR		6247	1289-1990	human Aryl hydrocarbon receptor (**AHR**)
NM_000660	TGFB1	CED; DPD1; TGF-beta	2217	1284-1649	human Transforming growth factor, beta 1 (**TGFb1**)
NM_000619	IFNG	IFG; IFI	1240	53-438	human interferon gamma (**IFNg**)

Additional File 2. Dosages of immunosuppressive drugs per patient enrolled in The ONE Study reference group at each time point

	- 4 weeks PRE transplant		+ 8 weeks POST transplant		+ 36 weeks POST transplant		+ 60 weeks POST transplant	
	#003	#004	#003	#004	#003	#004	#003	#004
Tacrolimus trough levels (ng/ml)	-	-	10.6	10.6	9.9	5.8	9.1	4.3
target levels (ng/ml)	-		3-10		3-8		3-6	
Prednisolone (mg/day)	-		10		0		0	
MMF (g/day)	-		1.5		1.5		1.5	
Basiliximab (mg)	-		0		0		0	



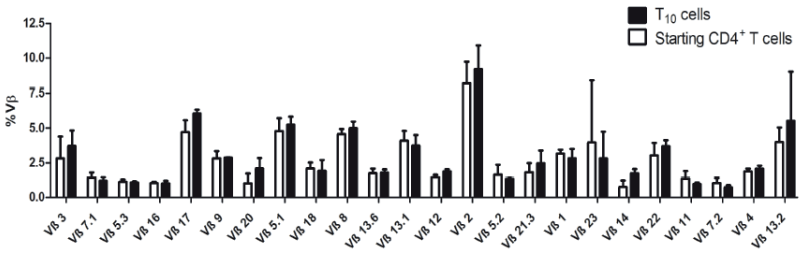
Additional File 3. DC-10 cells represent the non-CD4+ cell population within the T10 cell preparations. One representative dot plot of eight of T10 cells. Percentage of $CD4^{-}CD14^{+}$ cells is shown. Gating was done on live T10 cells.



Additional File 4. Frequency of CD86$^+$ DC-10 cells correlates with T_{10} cell yield.

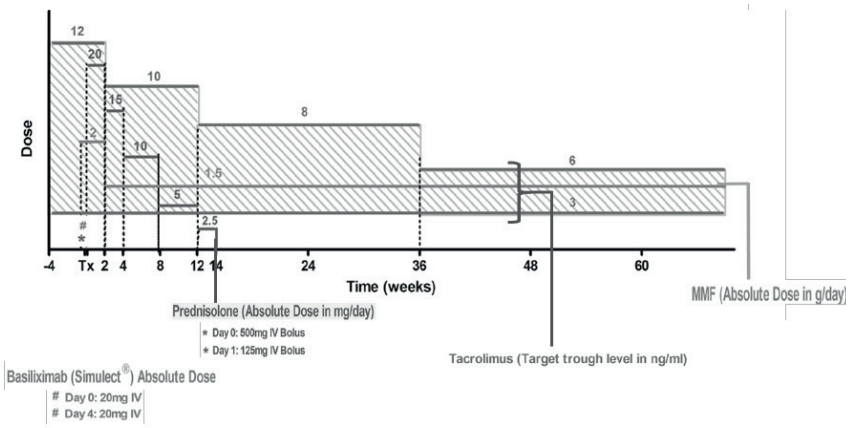
Phenotype of one representative preparation (out of eight) of DC-10 is shown (**A**).

Percentages of CD86$^+$ DC-10 (CD14$^+$CD16$^+$) are plotted with yield of the corresponding T_{10} cells. Line represents linear regression (**B**).

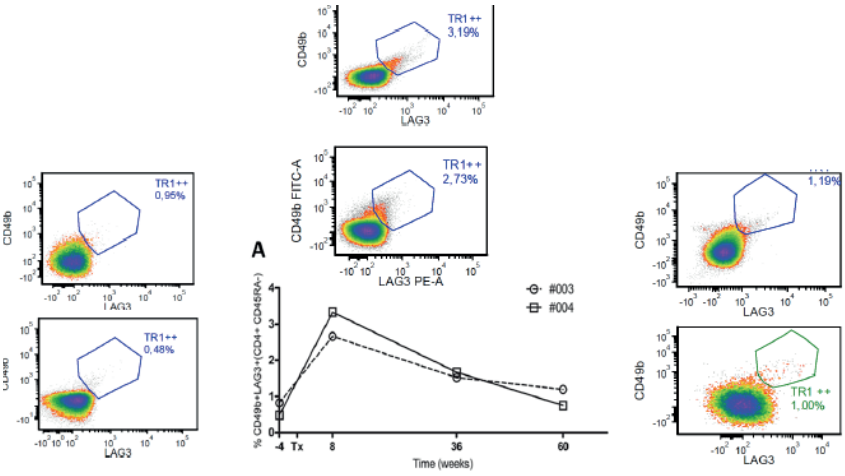


Additional File 5. The TCR-Vβ repertoire of T_{10} cells is similar to that of the starting CD4$^+$ T cells

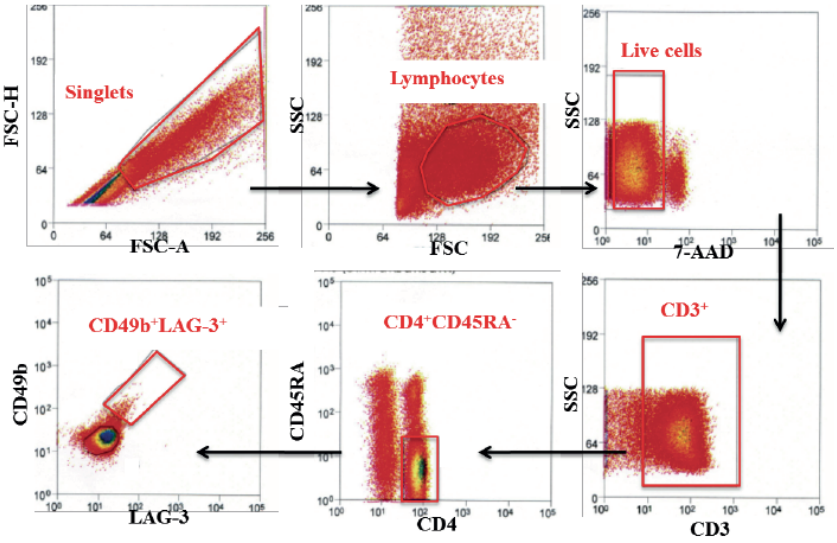
Percentages of Vβ usage in T_{10} cells (■) and in the corresponding CD4$^+$ T cells before culture (□) are shown. Bars represent mean value of each dataset ± SD (n=5 T_{10}-cell preparations)



Additional File 6. Detailed immunosuppressive regimen as foreseen by The ONE Study Reference Trial



Additional File 7. Flow cytometry dot plots of circulating Tr1 cells in two kidney transplant recipients under active immunosuppression.
Percentages of Tr1 cells (CD49b⁺LAG3⁺) are shown pre- and post-transplantation. Gating was performed on live CD3⁺CD4⁺CD45RA⁻ cells.



Additional File 8. Tr1 cell sorting gating strategy

Tr1 cells were sorted from thawed PBMC. Gating strategy is shown: from singlets (FSC-A, FSC-H), to lymphocytes (FSC, SSC), live cells (SSC, 7AAD$^-$), T cells (SSC, CD3$^+$), memory CD4$^+$ T cells (CD4$^+$CD45RA$^-$), and Tr1 cells (CD49b$^+$LAG3$^+$).

Chapter 5: Preclinical assessment of the Lovo™ device for DMSO removal and cell concentration in thawed hematopoietic progenitor cell grafts

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Cytotherapy 2017 Dec;19(12)

Abstract

Background: Cryopreserved hematopoietic progenitor cell (HPC) grafts are widely infused to patients with malignant and nonmalignant conditions. Despite reduction of immediate side effects linked to DMSO- and cell debris-containing grafts, and comparable hematopoietic engraftment between washed and unwashed cryopreserved products, bedside infusion of thawed HPC grafts is still preferred. Introduction of automated devices is important for standardization and consistency of graft manipulation. Additionally, these techniques are likely to be useful for the delivery of innovative cell-based medicinal products that are currently under development.

Methods: In this study, we evaluated three consecutive versions of the Lovo™ device (Fresenius Kabi) for automated washing of thawed HPC products. A total of 42 HPC products intended for destruction were used. Measured outcomes included viable $CD34^+$ cell recovery, viability, total processing time, and post-washing stability.

Results: Preliminary data using the prototype Lovo™ 0.0 to process a single HPC unit, shows better recovery and viability of $CD34^+$ cells using a 2-cycle than 3-cycle wash, with >95% DMSO elimination. The Lovo™ 1.0 performed equally well. When simultaneously processing two HPC units, the upgraded Lovo™ 2.0 device demonstrated comparable $CD34^+$ recovery, DMSO elimination efficiencies, and time-saving capacity. Furthermore, washed cell products were stable for four hours at room temperature.

Discussion: Lovo™ device satisfies clinically-relevant issues: minimal contact of thawed cells with DMSO and compatibility with transport to nearby transplant centers.

Introduction

Autologous hematopoietic stem cell transplantation (HSCT) after high dose chemotherapy remains a standard of care for the management of lymphoma and myeloma patients (1). Mobilized peripheral blood is the unique source of autologous hematopoietic progenitor cells (HPC) obtained from patients mobilized with rhG-CSF alone or following myelosuppressive chemotherapy; plerixafor can be added to this regimen for patients who mobilize poorly (2). In the autologous setting, cryopreservation of HPC is mandatory before infusion; CD34+ progenitor cell count in the cryopreserved product(s) is the most important predictor of successful engraftment (3, 4). Therefore, cryopreservation procedures must preserve the highest proportion of these progenitors (5, 6). Several cryoprotective agents exist, of which dimethyl sulfoxide, or DMSO which is by far the most widely used; yet once the cells are thawed, it is recommended to limit the duration of contact with DMSO. Bedside thawing by trained nurses and physicians and immediate infusion of the thawed cell product fulfills this requirement and allows for documented neutrophil and platelet engraftment (7); immediate adverse reactions can occur and are attributed to DMSO itself, to red cell lysates or to damaged granulocytes (8, 9). Alternatively, techniques exist that allow for thawing and washing of cryopreserved cell products by trained technologists in a processing laboratory, thus allowing for elimination of most of the DMSO and cell debris before reinfusion to the recipient of a ready-to-use final cell product (10). In addition, the technique allows for the implementation of quality controls on the product that is actually reinfused.

We previously reported a retrospective comparison of two matched cohorts of patients who received either washed or unwashed autologous HPC products, showing comparable hematopoietic engraftment (11). Switching from bedside thawing to laboratory thawing thus provides an opportunity for better standardization of this procedural step, improved stability of the thawed product, precise dosing of infused viable CD34+ progenitor cells, and improved risk-efficacy profile of the graft (12, 13). However, the workload is transferred from the clinical

ward to the processing facility, and additional resources including the costs of necessary reagents and disposables are consumed. Further benefits – including reduction of the process duration - are expected while evolving from mostly manual techniques to fully automated processes. To that end, several approaches have been developed: washing and enrichment using centrifugation techniques (supernatant extraction whether manual or automated with Sepax-2, Biosafe GE Healthcare instruments) (12), or filtration by spinning membrane (Cytomate, discontinued since 2010) (8).

A recent addition is the LovoTM device (Fresenius Kabi), an automated closed cell processing system that performs washing and enrichment using non-pelletizing spinning membrane filtration, adopting the same separation technology as the Cytomate with novel algorithms enabling the processing of up to 21000 ml. The system works by performing a series of volume reductions by supernatant (i.e. DMSO, plasma, platelets, cell debris) removal during passage through the spinning membrane. This wash procedure results in a final product containing the original cells suspended in a reduced volume of fresh solution.

In this present study, we performed a preclinical evaluation of the LovoTM device aiming at: 1) using LovoTM 0.0 and 1.0 to process single thawed HPC bags, 2) using the upgraded LovoTM 2.0 to process two bags, and 3) testing the stability of processed HPC products at room temperature.

Methods

Collection and cryopreservation

A total of 42 rHuG-CSF-mobilized peripheral blood HPC products collected and cryopreserved between 2005 and 2016 at our JACIE-accredited institution were identified as intended for disposal (deceased patients; informed consent had been taken at the time of collection for use of the HPC products for research purposes (14)). Characteristics of the HPC products used are detailed in Supplementary Table 1. After collection, volume reduction to 100mL, and distribution into two bags

(Macopharma), 50mL of cryopreservation solution (Voluven supplemented with 20% DMSO) was slowly added to each bag at +4-10°C, manually (30ml/min) or using the Smart-Max (BioSafe, GE Healthcare) system (5ml/min), before placing in a controlled rate freezer (Minidigitcool, Cryo Bio System) and final storage in gas-phase liquid nitrogen tanks. All products tested negative for microbiological contamination (aerobic and anaerobic).

Thawing and DMSO quantification

Thawing was performed on Smart-Max (BioSafe, GE Healthcare) using the thawing protocol: 9 min static + 1 min dynamic. For DMSO quantification [13], using P/ACE capillary electrophoresis system (Sciex, Beckman Coulter), 1 ml of thawed product was collected before and after washing on LovoTM device. Percentage of DMSO elimination was calculated as (1 – (value after washing/value before washing))*100 = DMSO elimination %.

Washing/enrichment with LovoTM device

Prior to washing, percentage of packed cell volume (% PCV) calculation was performed as follows per the LovoTM operator's manual:

((Total volume of cellular material: RBC, WBC and Platelets)/(total source product volume)) x100. The HPC bags selected covered the low, medium and high ranges of the spectrum of cell content (PCV range 3.4-19.9%), representing the variation intrinsic to these products and met in our daily routine (Supplementary Table 1C).

Whether LovoTM 1.0 or LovoTM 2.0 software was used, the procedure, as detailed in Figure 1, involves a pre-wash step (setting up the instrument and kit), source prime (with sequential priming of multiple source bags by adding an equal volume of buffer being unique to LovoTM 2.0 and single source priming being used on LovoTM 0.0 and 1.0). For multiple bag processing, LovoTM 2.0 was used (2-cycle or 3-cycle washes, volume reduction and final product "labelled retentate" collection). A cycle refers to one pass through the spinning membrane, dilution of the product, and rinsing of the tubing set. Washing was performed using +4-10°C 6% hydroxyethylstarch 130/0.4 (Voluven, Fresenius Kabi). The LovoTM device

settings were customized for this application, as detailed in Supplementary Table 2 with a final volume being as minimum as possible (mean = 148 ml; range: 108-171 ml). An aliquot was taken immediately after final retentate collection for DMSO quantification, flow cytometry testing, and for any observations of aggregate formation. Total processing time was calculated as the time taken from protocol selection on the LovoTM device to the completion of final retentate collection.

Flow cytometry testing

Total nuclear cell count was performed on Sysmex XP300 (Sysmex). The sample was diluted in PBS /0.5% human Ig/EDTA to target a concentration of 20 x 106 cells/ml. Viable CD34+ and CD45+ cell counts were determined by single platform flow cytometry assay using Stem-Kit (Beckman Coulter) according to the modified International Society of Hemotherapy and Graft Engineering protocol (15). Sample acquisition was performed on FC500 Flow Cytometer (Beckman Coulter). CD34+ cell recovery (%) was calculated as follows:

((Post-wash viable CD34+ cell count/kg) / (pre-cryopreservation viable CD34+ cell count/kg)) x 100.

The products processed on the LovoTM device were initially counted pre-freezing/post apheresis collection. The source material information (PCV%) was input into the LovoTM device based on the pre-freezing counts.

Stability testing

The final washed product was kept on a rocking surface at +18-24°C, simulating the transport conditions before infusion to patients (according to FACT-JACIE International Standards Accreditation Manual, Sixth Edition, ideal transporting temperatures may range from +2-24°C). Sampling was performed at 2 and 4 hours post-wash for flow cytometry testing. CD34+ recovery and viability results were reported relative to post-wash sample (T0).

Statistical analysis

Data were expressed as median ± standard deviation (SD). Two-tailed Mann-Whitney test or Student's t-test was performed when comparing two unpaired or

paired groups, respectively. P values ≤ 0.05 were considered significant. Statistical analyses were done using GraphPad Prism 5 (GraphPad, La Jolla, CA).

Results

We and others had shown that bedside thawing and automated washing of HPC products lead to similar engraftment, yet a choice needs to be made between immediate infusion to avoid cell loss – an empirical "advantage" of the former – and removal of DMSO and cell debris – an advantage of the latter (11, 16). In an effort to remove DMSO and cell debris from two thawed HPC bags, we evaluated the LovoTM device. This evaluation was performed in three steps. First, two versus three-washing cycles on single-HPC bags were compared using LovoTM prototype 0.0. Next, LovoTM 1.0 was used to wash single-bag HPC products. Finally, lessons learned from LovoTM 1.0 guided the development of the LovoTM 2.0 that was set up to simultaneously process more than one HPC bag.

Lovo™ 0.0: 2-cycle versus 3-cycle wash of one HPC bag

The HPC bags that were used at this stage originated from the same apheresis product cryopreserved in two identical bags. Paired bags were run on each of the wash cycles using Lovo™ 0.0. The bags had a frozen PCV of 3.8 ± 1.0% (median ± SD). As shown in Figure 2, post-wash recovery of $CD34^+$ cells was higher using a 2-cycle wash process as compared to a 3-cycle wash process for single HPC bags (n=15). Both cycles had comparable DMSO elimination efficacies. Source prime and processing was performed in 21 ± 3 min (median ± SD). Total processing time could not be reported since the "pre-wash" part of the workflow was not yet available on this prototype version (Figure 1). Since both cycles eliminated DMSO with comparable efficiencies, the rationale for choosing the 2-cycle wash for the subsequent version of Lovo™ device is its superior recovery of viable $CD34^+$ cells.

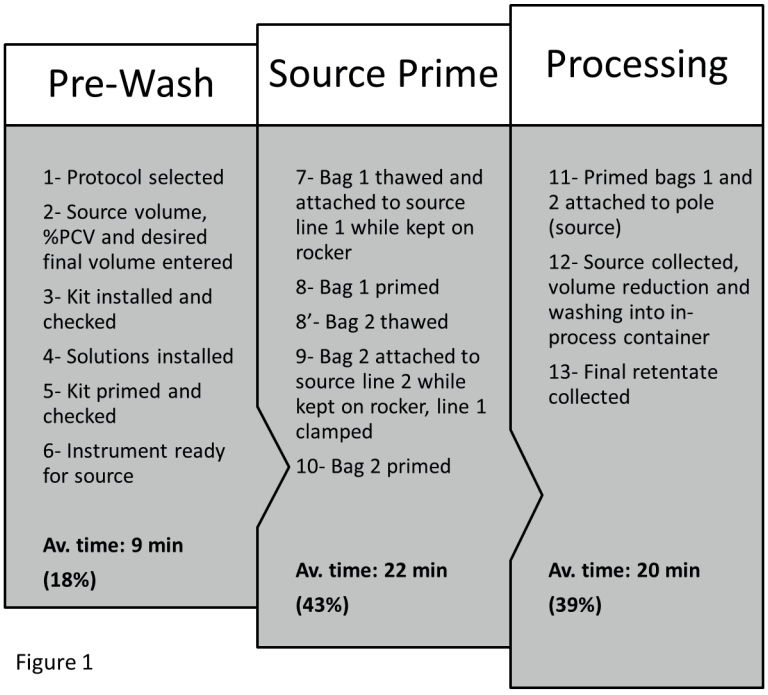
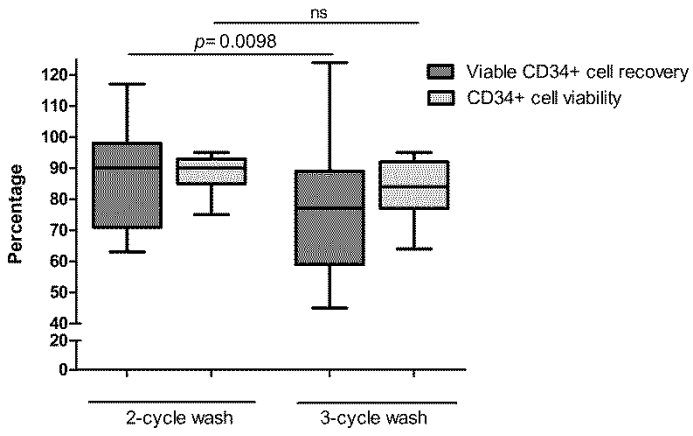


Figure 1

Figure 1. **Description of the workflow using Lovo™ device for washing and enrichment of CD34+ progenitor cells in thawed HPC products.** Percentage of the total processing time is reported for each section.

Figure 2



Number of runs	15	15	p=ns
Median viable CD34+ cell recovery	90%	77%	p=0,0098
Median CD34+ cell viability	90%	84%	p=ns
DMSO elimination	96%	95%	p= ns

Figure 2. **Viable CD34+ cell recovery and viability from thawed HPC products using the 2-cycle versus the 3-cycle wash on Lovo™ 0.0.** The values for the absolute counts of viable CD34+ cells and viability were measured by single platform flow cytometry assay (n=15) for each wash cycle. Values are shown as box-and-whiskers Tukey plots representing median with interquartile range (IQR), whiskers representing outliers. Paired Student *t*-test was performed for group comparisons since the same units were assessed with either 2- or 3-cycle wash. *p*-value ≤ 0.05 was considered significant.

Lovo™ 1.0: 2-cycle wash of one HPC bag

Following this first evaluation, the upgraded Lovo™ 1.0 software was used, yet with a faster source priming rate (50ml/min instead of 20ml/min), targeting shorter processing times. Single HPC bags (n=12) with low PCV (4.6 ± 0.02%, median ± SD) were washed, resulting in comparable viable CD34+ cell recoveries than the LovoTM 0.0 (81 ± 14% versus 90 ± 17%, respectively), as well as comparable CD34+ cell viabilities (91 ± 12% versus 90 ± 8%, respectively) and DMSO elimination efficacies (96% versus 96%, respectively) (Table 1). The fast source priming rate was re-evaluated for its efficacy in terms of CD34+ cell recovery versus time-saving advantage and accordingly the initial slow source priming rate was re-adapted for the following upgraded version.

Table 1			
	Lovo 1.0 (2-cycle)	Lovo 2.0 (2-cycle)	Lovo 2.0 (3-cycle)
Number of runs	12	5	6
Number of bags per run	1	1-2	1-2
PCV% (median, IQR)	4.0% (2.9-6.85)	7.6% (3.9-17.8)	8.4% (6.9-11.4)
Viable CD34+ cell recovery (median, IQR)	81% (74-99)	72% (60-87)	84% (61-93)
CD34+ cell viability (median, IQR)	91% (95-96)	95% (74-97)	92% (81-94)
DMSO elimination (median, IQR)	96% (93-98)	98% (93-99)	97% (97-98)
Total processing time	15 min*	51 min	62 min
* processing time excludes the instrument / kit preparation, and bag thawing			

LovoTM 2.0: 2-cycle and 3-cycle wash of one or two HPC bags

When the upgraded version of LovoTM 2.0 was released, 2-cycle and 3-cycle wash were tested, yet using 1 or 2 HPC bags. HPC bags with higher PCV% (10.1 ± 5.2%) (mean +/- SD) were selected in order to evaluate the LovoTM device for washing high cell content products that are collected in patients with high WBC counts following mobilization and high numbers of platelets (Supplementary Table 1). The 3-cycle procedure allowed for more gradual removal of platelets, debris and original supernatant, compared to the 2-cycle procedure, while still targeting similar total washout. This was confirmed during this study as no high pressure alarms occurred with the 3-cycle procedures as opposed to some 2-cycle runs (Supplementary Table 2). Viable CD34+ cell recovery was comparable when using 3-cycle wash compared to 2-cycle wash (84 ± 17% versus 72 ± 15%, respectively, p=0.64), with comparable CD34+ cells viabilities (92 ± 10 versus 95 ± 13, p=0.46) and DMSO elimination efficacies (97% versus 98%, respectively, p=0.50) between both wash cycle configurations. Total processing times were 62 min using a 3-cycle wash and 51 min using a 2-cycle wash. No statistically significant correlation was seen between viable CD34+ cell recovery and pre-cryopreservation viable CD34+ cell count, PCV% or Cell pellet volume of source material (Supplementary Figure 1). It is important to note, the 2 cycle procedures were designed to target a lower final volume for pediatric indications and were more exploratory in nature. While the data indicates slight superiority in a 3-cycle procedure (yet not statistically significant) it is noted the 3-cycle procedure using a packout percentage increase demonstrated its effectiveness in our study.

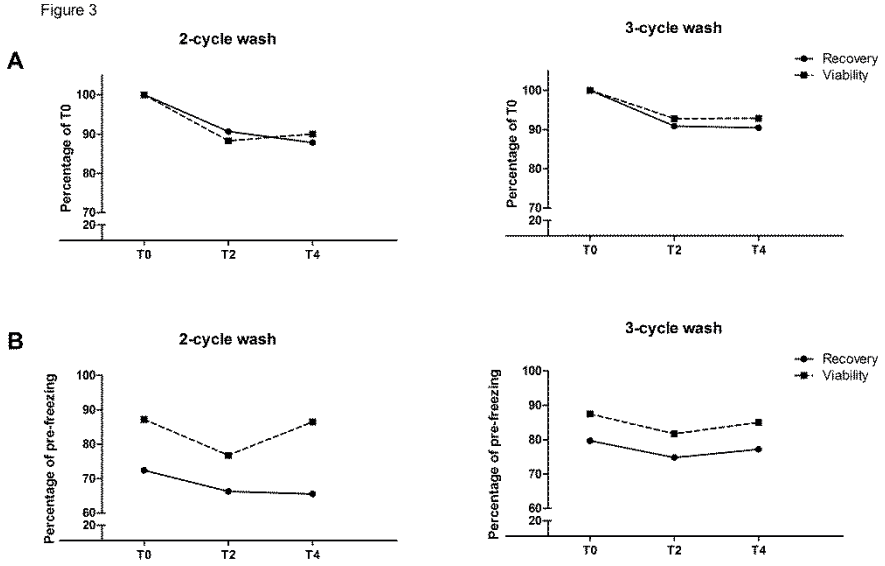
As described in Figure 1, the effective processing time of two simultaneously thawed HPC bags using LovoTM 2.0 was 42 min on average (82% of the total processing time), with instrument setup and kit preparation taking an additional 9 min on average, for a mean total processing time of 51 min for a 2-cycle wash or 62 min for a 3-cycle wash.

Washed HPC product stability at four hours post-washing at room temperature

Of clinical relevance is the stability of the thawed product at room temperature to allow for transport and delivery to nearby transplant centers (17). Accordingly, CD34+ cell recovery and viability were assessed using the five HPC bags thawed using the 2-cycle wash and six HPC bags thawed using the 3-cycle wash, at two and four hours post-washing while the products were placed on a rocking surface at +18-24°C. As shown in Figure 3, both CD34+ cell recovery and viability decreased to 90% of the starting percentages at two hours post-washing, yet remained stable for two additional hours. Product stability was comparable between the two wash protocols. Aggregate formation was evaluated in all samples and was not found in any of the 11 samples evaluated. The samples did not contain anti-coagulant.

Figure 3. **Stability of the washed HPC products in terms of $CD34^+$ cell recovery and viability using the 2-cycle versus the 3-cycle wash on Lovo™ 2.0.** Data is expressed as a percentage of the median $CD34^+$ cell recovery and viability compared to recovery and viability at the end of washing (T0).

Figure 3



Discussion

In the present study, we evaluated 3 consecutive versions of the LovoTM device for washing thawed HPC products with the aim to efficiently remove DMSO, while preserving CD34+ cell viability and recovery prior to infusion to patients. Our study uncovered several clinically relevant advantages of using automated devices such as the LovoTM for post-thaw processing of HPC grafts. First, it is capable of simultaneously washing more than one HPC product and pooling into a single bag to reach a final specified volume. While maintaining the priming strategy of slow volume to volume dilution of the thawed product prior to filtration through the spinning membrane, it reduces the toxicity associated with the HPC's exposure to 10% DMSO at room temperature. Second, washing the product in 2 or 3-cycles results in a high recovery of viable CD34+ progenitor cells, while efficiently eliminating DMSO responsible for adverse events associated with infusion of unwashed products. Third, the one-hour total processing time of two bags, a more

systematic event in cell therapy facilities than processing a single bag – due to cryopreservation of collected products in two equal bags, stored in separate containers for safety reasons – helps deliver a washed, precisely-dosed product faster to patients.

As shown in previous reports (5, 6, 18, 19), CD34+ progenitor cell recovery and viability upon freezing and thawing are variable, hence QC testing is necessary to properly assess the product (20). At our institute, we currently wash thawed HPC products using Sepax-2, performing sequential washing of two bags: our data of CD34+ cell recovery, viability and DMSO elimination efficacy are comparable to those pertaining to the 2- or 3-cycle wash of LovoTM 2.0, yet the advantage the latter holds is the reduced total processing time for two bags: 60 min using LovoTM device versus 75 min using Sepax-2. Considering the current processing schedules in our facility, the gain of 15 min that LovoTM provides over Sepax-2 could help accommodate distributing two instead of one washed HPC product before mid-day. The LovoTM performance met our QC requirements for infusion, which are based on FACT-JACIE standards. Our group reported no statistically significant difference in using the LovoTM and Sepax-2 in terms of CD34+ cell recovery, viability, and DMSO removal (poster presentation at EMBT Meeting 2016, Valencia, Spain). Previously, centrifugation followed by manual supernatant extraction was performed; although less resource-consuming, its variability and inability to be standardized rendered automation a natural evolution. Abonnenc M. et al reported an estimated operating time of 105 min of sequential processing of two bags using Sepax-2 (12). We and others have previously reported a washing time (including source priming) of one hour for two bags using the Cytomate system (21, 22), an automated device based on the same principle than the LovoTM device, that is however no longer manufactured. Therefore from a time-consumption and ability to process larger volumes of source material point of view, LovoTM device holds an advantage over other currently available automated cell processing devices or manual supernatant extraction.

Pre-cryopreservation transport time and temperature (23), rHuG-CSF mobilization and disease condition (24), length of cryopreservation time (25, 26), or conditions of flow cytometry testing (27) were comparable between HPC bags used during this study and do not account for the slight differences observed (or not) between groups. A difference noted in the CD34+ cell recovery between LovoTM 0.0 and 1.0 is possibly related to the thawed HPC bag source prime volume addition rate which was fixed at 50 mL/min. Additionally, the LovoTM 1.0 would allow users to target a final volume without a final rinse of the tubing line leading into the retentate bag. Therefore, it is suggested these two differences in the software led to differences in CD34+ cell recovery between LovoTM 0.0 and 1.0.

We and others have adopted a protocol for transport of washed HPC products at +18-24°C thanks to a proven stability up to four hours post-washing and in compliance with FACT-JACIE standards (17). LovoTM 2.0 device shows stability of washed HPC products under such conditions, further establishing its ability to produce stable products fit for transport.

In summary, our data demonstrated that LovoTM device is an advantageous option for efficient washing and enriching of more than one thawed HPC product, simultaneously. Our evaluation focused on DMSO removal before infusion, DMSO dosage being a marker of cell debris and supernatant removal, yet applications of LovoTM device extend beyond that to wash and concentrate ex vivo-expanded immunotherapy cell products, owing to its capacity to process volumes up to 21000ml.

Acknowledgements

We would like to acknowledge the cell therapy facility staff for their availability and Mrs. Carine Malenfant for her coordination efforts. We would also like to acknowledge the LOVO R&D team of Dolores Juarez and Christopher Wegener for their contributions.

Disclosure of Interest

Mathieu Mercier and Steven Binninger are employees of Fresenius Kabi. Fresenius Kabi provided the disposables to support the studies identified in this publication.

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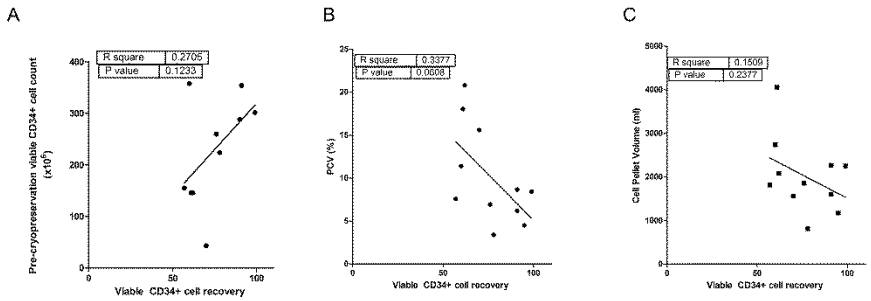
Supplementary Figure 1: Correlations between viable $CD34^+$ cell recovery and precryopreservation viable CD34+ cell count (A), PCV% (B), or Cell pellet volume of starting material (C), calculated as source material volume x PCV%.



Chapter 6: Validation of a flow cytometry-based method for quantitative lymphocyte immunophenotyping in hematopoietic cellular products

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Work in preparation

Abstract

Adoptive cellular therapy with immune effector cells (NK, T, B, regulatory T and dendritic cells) has shown remarkable toxicity against tumor cells and potential in immune regulation. The starting material to manufacture all these cells is a leukapheresis (fresh or frozen) from patients which carries significant variabilities necessitating a validated method for quantifying viable lymphocyte immunophenotypes. We hereby report on the validation parameters: accuracy, precision (intra- and inter-assay), specificity, linearity of dilution, sensitivity, robustness, stability and carry-over for quantification of viable CD3+, CD4+ T cells, CD8+ T cells, CD3-CD56+CD16+/- NK cells, CD19+ B cells and CD14+ monocytes of relevance to leukapheresis products and immune effector cell (IEC) products on the Cytomics FC500 Beckman Coulter cytometer. The acceptance criteria were all met. Additionally, the method complies with FACT Standards for IEC requirement for an "appropriate and validated assay", FACT-JACIE Hematopoietic Cell Therapy Standards, ICH Q2(R1) and ISO15189 standards. Furthermore, it complies with validation recommendations/guidelines (LBAFG-AAPS, ICSH/ICCS and European Bioanalysis Forum) applicable to such a method.
The implications of this effort include, but are not limited to: standardization of input cellular material for cell manufacturing, cell selection, or dosing of donor lymphocyte infusions. Furthermore, meaningful changes in data collected throughout the processing of a product, particularly during cryopreservation or over longitudinal studies with IEC can be assessed using this method.

Introduction

Adoptive cellular therapy with immune effector cells (IEC) faced several challenges: manufacturing processes, logistic and coordination aspects and toxicity profiles. Accordingly, the consensus from academic cellular therapists, transplant programs, commercial cell manufacturers, and regulatory agencies was towards the creation of adapted guidelines that meet many of these above-mentioned challenges. Therefore the Immune Effector Cell Task Force was created and it formulated Standards and a corresponding accreditation program for IEC (1).

FACT Standards for Immune Effector Cells, First Edition 2017 have the major objective to promote quality practice when administering IEC destined to therapeutically modulate, elicit or mitigate an immune response (2).

Going into the specifics of the guideline, a relevant and validated assay should be employed to evaluate cellular therapy products undergoing manipulation that alters the nature/function of the target cell population. IEC currently comprise cells that confer broad cytotoxicity against tumors, for example ex vivo activated/expanded, engineered or selected natural killer (NK) cells (3), or targeted cytotoxicity against tumor-associated antigens, for example chimeric antigen receptor (CAR)-transduced T cells (4), T-cell receptor-engineered T cells (5), tumor-peptide directed cytotoxic T cells (6). Cells that induce tolerance by suppressing inflammatory responses, for example regulatory T cells (7), tolerogenic dendritic cells (DC) (8), regulatory macrophages (9), or regulatory B cells (10) or enhancing immune recognition, for example peptide-loaded or genetically-engineered DC (11) are equally included. Whether these cellular therapy products undergo manipulation or not, their identity, enumeration, and viability are critical information needed on both sides of the process: initiating manipulation or releasing the products for infusion.

Therefore a relevant and standardized assay for quantifying the initial and final cell population needs to be established and validated. To that end, multi-color flow cytometry is the technology of choice in cell manufacturing facilities for cell surface marker detection, viability and enumeration (12). Measurement of viable absolute

counts of cells is additionally performed using single-platform panels – recommended by ISHAGE and JACIE standards-- that include a cell viability dye and counting beads with a lyse-no-wash preparation using commercial kits for CD34+ cells, and for lymphocyte subpopulations, yet without a viability dye for the latter (13-15). The challenging aspect is validating laboratory-developed flow cytometry methods, hence recommendations/guidelines ensued in three waves: the first formulated in 2011 by the Flow Cytometry Subcommittee of the Ligand Binding Assay Bioanalytical Focus Group (LBAFG) of the American Association of Pharmaceutical Scientists (AAPS) (16, 17), the second in 2013 by the International Council for Standardization of Hematology (ICSH) and the International Clinical Cytometry Society (ICCS) (18-22) and last in 2017 by the European Bioanalysis Forum (23).

In this work, we focus on fresh and cryopreserved mononuclear cell (MNC) and hematopoietic progenitor cell (HPC) products and the cell manufacturing of IEC products by reporting on the validation of the analytical method: quantification of viable absolute counts of lymphocyte immunophenotypes using Cytomics FC-500 (Beckman Coulter) in compliance with 1) FACT Standards for Immune Effector Cells, 2) FACT-JACIE Hematopoietic Cell Therapy Standards, 3) International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human Use (ICH): Validation of Analytical Procedures: Text and Methodology, Q2(R1), and 4) International Organization for Standardization and Standard for Clinical Laboratory Accreditation (ISO15189) standards. The intended use of the data generated by this assay is decision-making and not exploratory.

Materials and methods

Samples

Fresh leukapheresis products from healthy donors previously collected at the apheresis unit and processed/cryopreserved at the cell processing facility of Institut

Paoli-Calmettes were used. All studies were performed according to institutional and Helsinki guidelines regarding human ethics. All consent forms are available on record. Leukapheresis products are collected in Anticoagulant Citrate Dextrose (ACD) solution A (Terumo BCT Europe N.V, Arcueil, France). The samples used for this validation were: Status Flow®, flow cytometry control tube, is a stable preparation of human peripheral blood, with expected values used to daily monitor the immunophenotyping panels (R&D Systems Inc, MN USA, EuroCell Diagnostics, Noyal Chatillon/Seiche, France). The sample is diluted in PBS /0.5% human Ig/EDTA to reach a target concentration of 20 x 10^6 cells/ml.

Antibodies, reagents and buffers

Antibodies are supplied by Immunotech SAS (Beckman Coulter, Marseille, France). Tube 1: IOTest conjugated antibody anti-CD45-FITC (clone J33), anti-CD3-PE (clone UCHT1), anti-CD4-ECD (clone SFCI12T4D11), 7AAD viability dye, anti-CD8-PC7 (SFCI21Thy2D3). Tube 2: anti-CD45-FITC (clone J33), anti-CD3-PE (clone UCHT1), anti-CD16-ECD (clone 3G8), 7AAD viability dye, anti-CD56-PC7 (clone N901, NKH-1). Tube 3: anti-CD45-FITC (clone J33), anti-CD19-PE (clone J3-119), anti-CD14-ECD (clone RMO52) and 7AAD viability dye. All antibodies contain 0.1% sodium azide. All lot numbers are retained on record.

For sample erythrolysis IOTest3 Lysing Solution (PN IM3514, Immunotech SAS, Beckman Coulter, Marseille, France) diluted at 1X is used. For determination of absolute counts of cells Flow-Count Fluorospheres (PN 7507992-MD, Beckman Coulter, CA, USA) were added to each tube, and their concentration inserted into the CXP acquisition software of the Cytomics FC500 cytometer.

Lyse-no-wash single-platform viable cell enumeration

A 100µl aliquot of sample (concentration adjusted to 20 x 10^6 cells/ml) is added per tube containing 80µl of the corresponding antibody mix and incubated for 20 min at room temperature in the dark. Afterwards, 2ml of 1X working lysis solution is added to fresh products followed by a 10 min incubation period at room temperature in the dark. For thawed products, 2ml of PBS replaces the 1X working lysis solution

bypassing the incubation period. Finally, 100µl of Flow-Count fluorospheres is added to each tube and acquisition on the flow cytometer followed immediately or the tube is stored at 4ºC for a maximum of 10 min before acquisition. The sample is acquired on Cytomics FC500 (compliant with the EU in vitro diagnostic medical device directive 98/79/EC) operated through the CXP Acquisition software version 2.2 (Beckman Coulter, CA, USA). The resulting data are saved as listmode (LMD) files. CXP software is linked to a database of patient details enabling automated reporting of absolute counts of cells.

Assay validation procedure

Accuracy, precision (intra- and inter-assay), specificity, linearity of dilution, sensitivity, robustness, stability and carry-over were performed for quantification of viable CD3+, CD4+ T cells, CD8+ T cells, CD3-CD56+CD16+/- NK cells, CD19+ B cells and CD14+ monocytes. Table 1 provides an overview of the evaluation of these parameters, the set acceptance criteria and the relevance to apheresis products (as starting material) and immune effector cell products.

Statistics

Coefficient of variation (CV) is reported as percentage of standard deviation (SD) to mean. Bland-Altman graphs were used to compare absolute counts of lymphocyte immunophenotypes between FC500 and FACS Canto. Standard deviation index is reported as: (Mean value at our center – Mean value of group of centers)/ SD of the group. Precision index is reported as being the ratio of our center's %CV to the group %CV. All analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Table 1: **Validation plan with relevance to cell therapy products**

Relevance to cell therapy product	Parameter	Description	Experiment	Acceptance criteria
Accurate quantification of viable absolute numbers of cells in starting material from patients as well as healthy donors Stability of reported results over time are necessary in longitudinal studies and multicenter trials	**Accuracy** • Comparison with another method/instrument/kit •External quality assurance/ proficiency testing*(EEQ)*	Closeness of the measured result to the true value for the sample	• Staining using stabilized blood samples (n=33) or DLI / apheresis (n=11 healthy donors) and acquisition on FC500 or FACS Canto (TBNK kit) on the same day for both methods • 3 yearly campaigns of staining and analysis of stabilized blood at 3 concentrations, distributed to 38 QC labs	• ≥90% agreement between methods • -2.0 ≤ SDI ≤ 2.0
Confidence in meaningful changes during product processing (in-process monitoring) Confidence in reporting cell recovery before and after cryopreservation and in consecutively-collected specimens	**Precision** • Intra-assay • Inter-assay	Closeness of individual measures of an analyte when the test or procedure is applied repeatedly (testing for variations introduced by analyst, reagent or instrument, single or multiple)	• Staining using stabilized blood samples (n=30) or DLI (n=30), acquisition and analysis in a single batch • Staining using stabilized blood (normal or sensitive levels) samples (n=30), acquisition and analysis in batches (5 replicates per run on 6 occasions)	• CV ≤ 10% • CV ≤ 10%
Confidence in quantifying cell composition of starting material from different sources	**Specificity**	Ability of a test or procedure to correctly identify or quantify an entity in the presence of interfering substances	Assay optimization (Panel design, antigen marker selection, mAB clone selection, fluorochrome selection, gating strategy, anticoagulant, interferences)	Inclusion of cell subset of interest while excluding other subsets and non-specific events
Ability to report efficacy of cell depletion and selection	**Linearity of dilution** **Sensitivity**	Determination of the lowest quantity of cells measured by acceptable accuracy and precision	Staining using serially-diluted stabilized blood samples (n=8), acquisition and analysis	• R^2>0.95 between predicted and measured viable absolute cell counts • Exploratory
Ability to use the same test or measurement despite variations in room temperature on daily basis	**Robustness**	Measurement of susceptibility of the test or procedure to changes	Staining using stabilized blood samples (n=10) at 4°C or 37°C, acquisition and analysis	CV ≤ 15%
Eliminating risk of over-estimating cell content	**Carry-over**	Of relevance in Cytomics FC500 with carousel design	Staining and analyzing sequentially : replicates of stabilized blood then water specimens	Carry-over ≤ 1%

Results

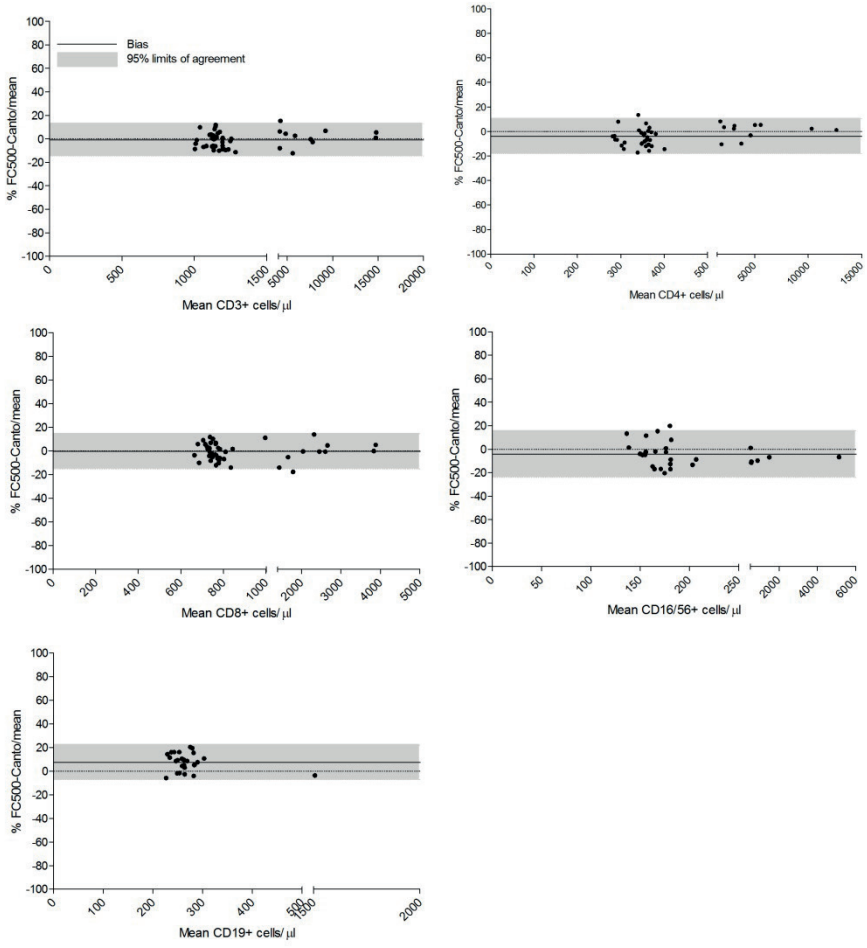
Accuracy

Accuracy of the quantification of viable lymphocyte immunophenotypes is defined by closeness of the measured result to the true value for the sample (22). To assess accuracy of our method, we followed the two strategies recommended by the ICSH/ICCS guidelines: comparison with another method and acceptable performance in College of American Pathologists (CAP) proficiency testing (17). In comparison to the TBNK panel performed on FACS Canto II cytometer, the bias for all subpopulations is close to zero percent (92%-98% agreement) between both methods: -0.95% for CD3+ cells, -3.88% for CD4+ cells, -0.37% for CD8+ cells, -4.31% for CD56+/16+ cells and +7.43% for CD19+ cells, indicating closeness of the reported results (Figure 1A). The bias values are constant for all measured concentrations (low, medium and high values) falling within the 95% limits of agreement. Data for CD14+ cells were not reported since only the Beckman Coulter panels include reagents to stain for these cells. The acceptance criteria our lab set at ≥90% agreement between methods, were satisfied for all subpopulations. When fresh and cryopreserved samples were assessed, setting the bias and acceptance criteria of fresh samples, >50% of frozen products had their bias outside the limits of agreement (Figure 1B). This discrepancy can be explained by the fact that the TBNK panel reports for absolute values of CD45+ cells and not individual sub-populations as the FC500 method we are reporting. This plays in favor of adopting the new method particularly when using cryopreserved products.

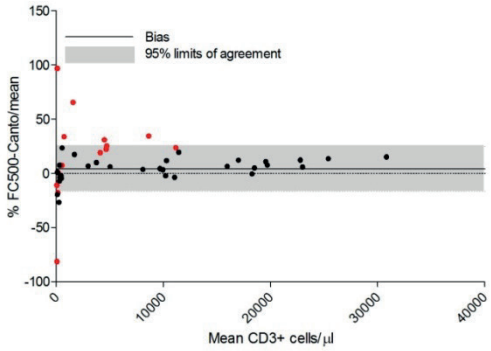
To assess our lab's results compared to other labs, we participate in triannual French campaigns of External Quality Assurance (EQA)/ Proficiency testing held by the Centre Toulousain pour le Contrôle de qualité en Biologie clinique (CTCB) (Figure 1C). The results fall within the acceptable values identified by the CTCB (-2< SDI <+2). Therefore, both approaches to check the accuracy of the quantification of viable lymphocyte immunophenotypes using the Beckman Coulter platform indicate that this criterion is met, at all levels of cell absolute counts.

Figure 1: **Accuracy of viable lymphocyte immunophenotype quantification method was established.** Stabilized blood Eurocell Status Flow (n=33) and leukapheresis products from healthy donors (n=11) were stained with each of the three panels and acquired on Cytomics FC500 or stained using the Beckton Dickinson Multitest 6-Color TBNK kit and FACS Canto II cytometer. **A)** Bland-Altman plots represent the comparison data from *fresh* products using both techniques. Line represents the bias, region between the limits of agreement at 95% is shown in gray **B)** Similar comparison using fresh (black dots) and frozen products (red dots) assessed using both techniques. Bias and limits of agreement of fresh products are represented. **C)** External Quality Assurance (EQA)/ Proficiency testing results reported with no discrimination between platforms of cell quantification from 38 French labs. Standard deviation index (SDI) is shown on the graph. Results from the last two years are reported as mean standard deviation indices of the three campaigns per year. Dashed lines represent ideal values, dotted lines represent acceptable values.

A


Bias
95% limits of agreement
% FC500-Canto/mean
Mean CD3+ cells/ µl
Mean CD4+ cells/ µl
Mean CD8+ cells/ µl
Mean CD16/56+ cells/ µl
Mean CD19+ cells/ µl

B


Bias
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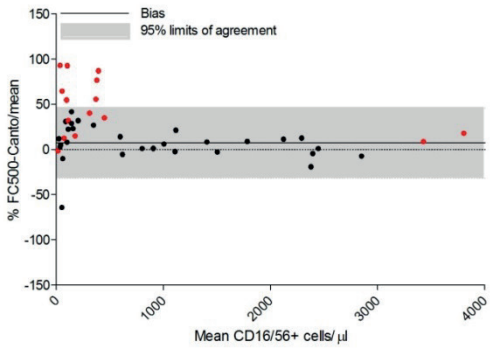
Mean CD3+ cells/µl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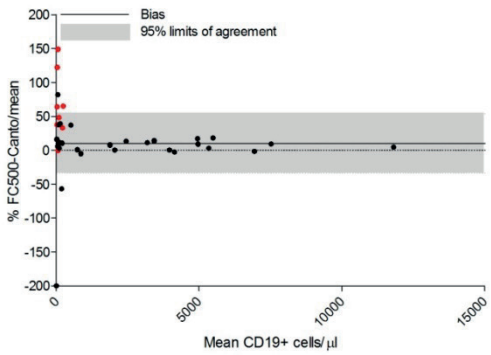
Bias
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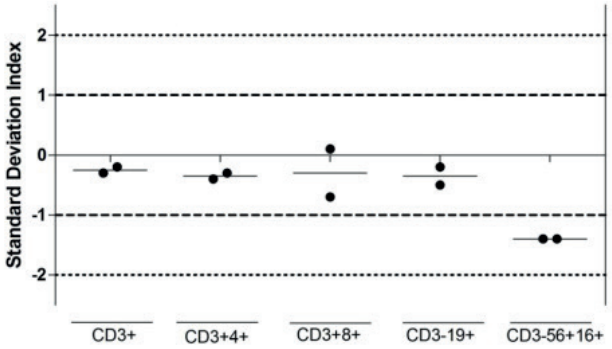
Mean CD19+ cells/µl

C



Precision

Precision describes the closeness of individual measures of an analyte when the assay is repeatedly applied: in a single batch by the same operator (intra-assay) or in batches on several occasions by different operators (inter-assay) (24). The percentages of CV of both starting materials falls within the set acceptance criteria of <10% for all subpopulations (Table 2). All in all, this data shows an acceptable assay precision, giving confidence in reporting meaningful changes in viable immunophenotypes of immune effector cell products' starting material.

Table 2: **Precision assessment meets acceptance criteria**. Precision was assessed by staining and acquiring samples on Cytomics FC500 for all 3 mixes in a single batch or on several occasions using stabilized blood (normal or low values) or leukapheresis products. A) **intra-assay** is reported as mean ±SD of viable absolute counts of cells performed in a single batch by the same operator. The coefficient of variation (CV) percentage is equally reported. B) **inter-assay** was performed over several batches by different operators. N=30 per assessment.

rial	CD3 (cells/ul)		CD4 (cells/ul)		CD8 (cells/ul)		CD19 (cells/ul)		CD16/56 (cells/ul)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)
s Flow	1198 ± 49,6	4,1	772,5 ± 36,1	4,8	377,9 ± 17,5	4,7	235,7 ± 7,0	3,1	180 ± 10,8	6,1
apheresis	10459 ± 939	9	6607 ± 546	8,4	3482 ± 334	9,7	ND	ND	ND	ND

0

sion - **Inter-assay** experiments, measured values

rial	CD3 (cells/ul)		CD4 (cells/ul)		CD8 (cells/ul)		CD19 (cells/ul)		CD16/56 (cells/ul)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)
s Flow	1170,3 ± 47,6	4,07	765,9 ± 32,2	4,2	349,6 ± 14,6	4,2	279,2 ± 14,5	3,1	153,7 ± 12,4	8,1
s Flow Lo	896 ± 36,9	4,12	137,9 ± 5,0	3,6	645,3 ± 22,5	3,5	417,4 ± 22,2	5,3	393,4 ± 23,2	5,9

0

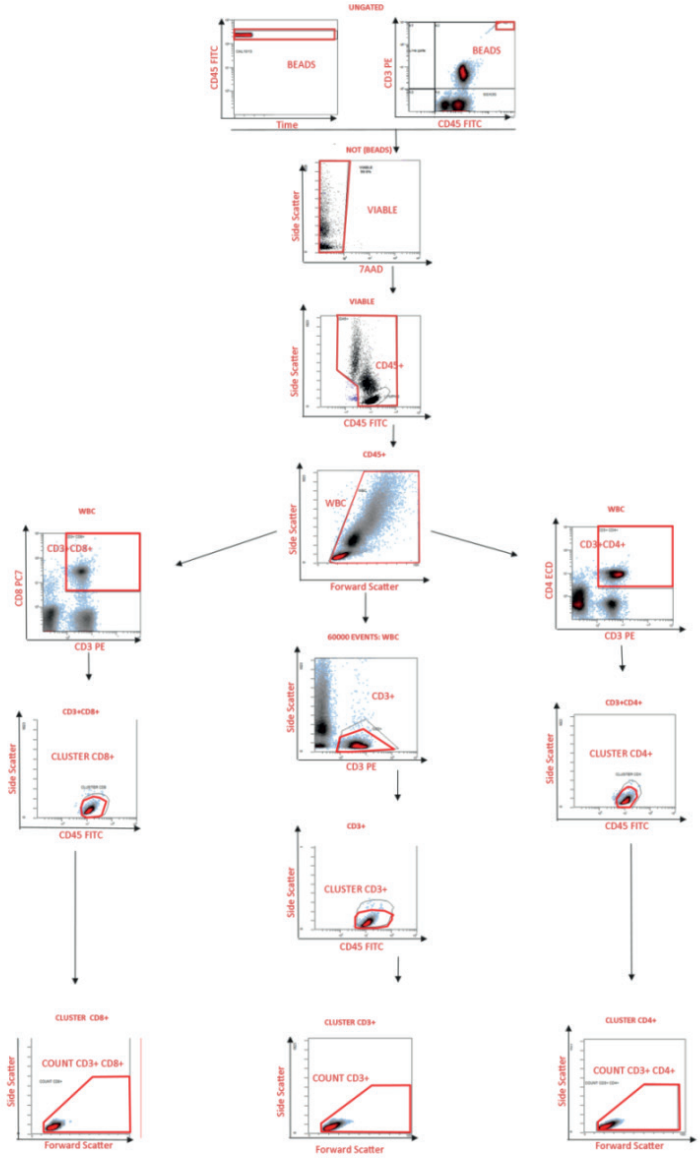
Specificity

Specificity refers to the degree to which only the true component is measured rather than a mistaken component, addressed by establishing cross-reactivity of the reagents (23). The choice of monoclonal antibodies (mAb) was based on the Tetra panel (Beckman Coulter) in addition to integrating 7-AAD as viability dye. The fluorochrome-mAb pairing, lysis using ammonium chloride solution and gating strategy (Figure 2 A, B, C) followed the same logic behind the ISHAGE recommendations for stem cell enumeration and a seminal work by Koehl and colleagues (13).

Possible causes for interference in flow-based immunophenotyping are: erythrocytes, fat, OKT3 and fluorescein, as listed in Beckman Coulter's antibody product sheets. The use of lysis solution and CD45 gating eliminates the effects erythrocytes and/or lipids could have on fluorescence signals. The validation runs have been performed on leukapheresis products or lymphocytes of healthy donors, hence OKT3 and fluorescein were not tested.

Figure 2: **Gating strategy allows for specificity in reporting viable lymphocyte counts.** Stabilized whole blood or apheresis sample was diluted to 20 x10^6 cells/ml and stained, lysed without washing, beads added and acquired on Cytomics FC-500. **A) Mix 1:** For $CD3^+$, $CD4^+$ and $CD8^+$ absolute T cell counts, gating starts with "no beads", followed by viable cells, then $CD45^+$ cells (to remove debris), lower limit of FSC/SSC gate, then $CD3^+$ cells gated, clustered (to isolate a clear signal) and counted (absolute counts), same applies for $CD3^+CD4^+$ cells and $CD3^+CD8^+$ cells. B) **Mix 2**: the same strategy is followed for $CD3^-$ $CD56^+CD16^{+/-}$NK cells yet gating on lymphocytes instead of $CD45^+$ cells. C) **Mix 3**: the same strategy is followed for $CD14^+$ monocytes and $CD19^+$ B cells with gating on $CD45^+$ cells. Limit of acquisition is set at 60,000 – 70,000 events in WBC gate (viable $CD45^+$ cells excluding beads)

A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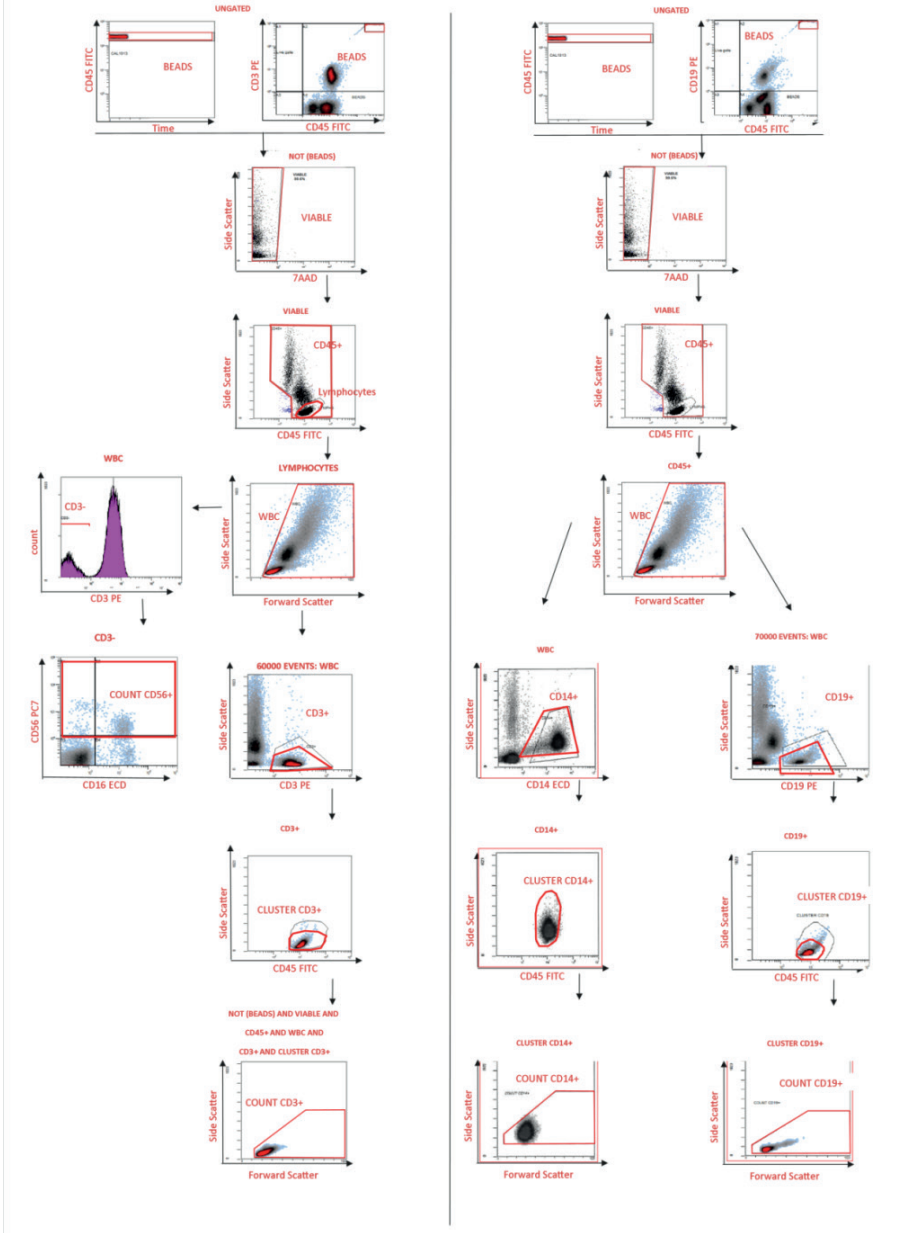
B C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CD19 PE
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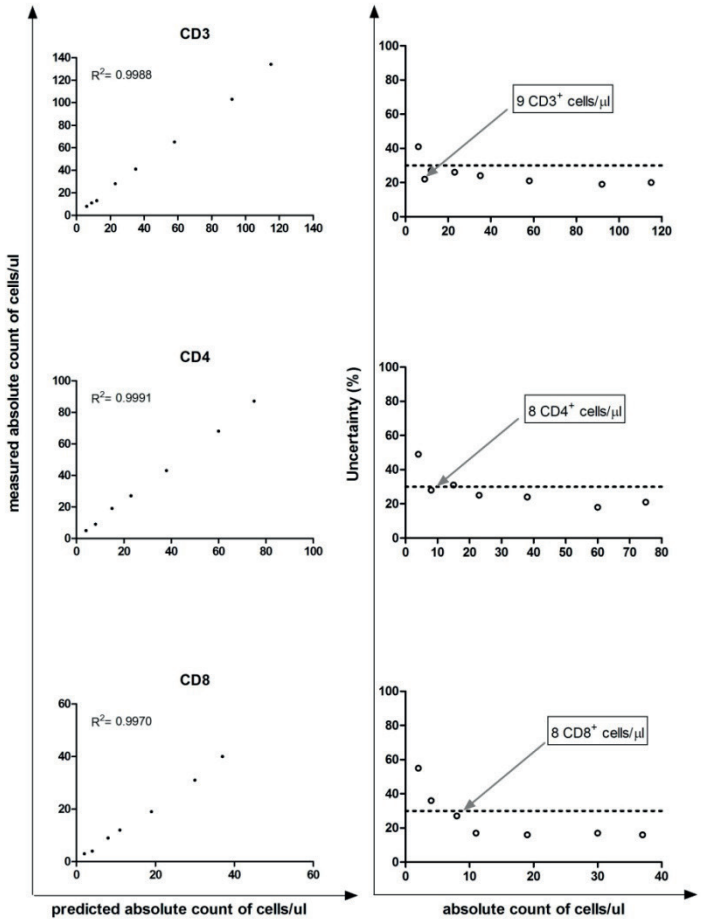
Dilution linearity and sensitivity

Several cell therapy products will undergo cell selection or depletion, hence of relevance in these situations is setting the lower limit of quantification, whose total error meets the set acceptable criteria for accuracy (22). We explored the linearity of the method using serially diluted samples. A high goodness of fit for a linear regression for predicted versus measured absolute counts (R2 >0.99) was revealed (Figure 3A). Using the same data set, the lower limit of quantification with an acceptable uncertainty (variation from theoretical values) was identified for CD3+, CD4+ and CD8+ cells: 9, 8 and 8 cells/µl, respectively (Figure 3B).

Figure 3: **Linearity of the viable lymphocyte immunophenotype quantification method and lower limit of sensitivity were established.** Linearity and lower limit of quantification of the method were established using serially diluted samples, acquired ten times using two lots of stabilized whole blood on several days. A) Predicted absolute counts are plotted against measured absolute counts and goodness of fit (linear regression) is elucidated. B) Lower limit of quantification with an acceptable CV% is identified for $CD3^+$, $CD4^+$ and $CD8^+$ cells. Dashed line represents the acceptable CV%.



A B

Robustness

A method needs to withstand changes in assay conditions, to accommodate to a reality in cytometry core labs. From our experience, temperatures fluctuate in our room which could have an influence on the results. Considering that our procedure required a staining at 18-25°C according to manufacturer's recommendations, testing the robustness of our method at two extremes of staining temperature (4°C and 37°C) was required by the accreditation authority. Samples of stabilized whole

blood were stained at both temperatures and acquired in four batches at different days (n=10 runs per temperature). The method proved to be robust at both temperatures, with a CV% ranging from 3 to 13% falling within our set acceptance criteria (<15%) (Table 3). This parameter provides further assurance as to the resistance of the assay to temperature changes.

Table 3: **The quantification method is robust at different staining temperatures**. Stabilized blood was stained at two temperatures (4°C or 37°C) and acquired on Cytomics FC500. N=10 per assessment.

Staining temperature	CD3 (cells/ul)		CD4 (cells/ul)		CD8 (cells/ul)		CD19 (cells/ul)		CD16/56 (cells/ul)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)
37°C	1041,2 ± 43,3	4,2	667,6 ± 21,5	3,2	327,8 ± 11,6	3,5	149,3 ± 9,9	6,7	254,9 ± 10,2	4
4°C	1157,6 ± 115,5	10	767,4 ± 81,4	10,6	338,3 ± 31,3	9,2	198,4 ± 25,4	12,8	266,9 ± 25,9	9,7

CD14 (cells/ul) are not reported on the product insert
n=10 per run

Carry-over

The design of Cytomics FC500 cytometer risks carry-over between samples, being a 32-tube circular rack. This is equally recommended in a dedicated guideline (Clinical and laboratory standards institute CLSI-H52-A, 2001) when an automatic sample loader is used routinely. Carry-over was assessed by staining and acquiring triplicate samples (H1, H2, H3), followed immediately by triplicate blank samples (B1, B2, B3) repeated 5 times. Accordingly carryover percent is calculated according to the formula:
((mean B1-mean B3)/(mean H-mean B3))x100

Set at less than 1% contamination, the method proved that no carry-over takes place (0.0056%) rendering further confidence in proper assessment of individual samples.

Discussion

What started off as being a back up method for quantifying lymphocyte immunophenotypes on another cytometer, turned into the method of choice when evaluating cryopreserved/thawed products. We report on the validation of a method

to enumerate viable lymphocyte immunophenotypes on the Cytomics FC500 Beckman Coulter cytometer. The acceptance criteria for accuracy, precision, specificity, linearity, sensitivity, robustness and carry-over were all met. The method complies with FACT Standards for IEC (first edition) requirement for an "appropriate and validated assay", FACT-JACIE Hematopoietic Cell Therapy Standards, ICH Q2(R1) and ISO15189 standards. Furthermore, it complies with validation recommendations/guidelines (LBAFG-AAPS, ICSH/ICCS and European Bioanalysis Forum) applicable to such a method. The method can help manufacturers draw meaningful comparisons during the process of scaling-up a laboratory-based manufacturing protocol. It is equally useful in reporting on variability in cell content of collected leukapheresis products, a challenge manufacturers face and need to adapt their manufacturing protocols to.

Since for the time-being cellular therapy products that have obtained a marketing authorization are being manufactured and released by pharmaceutical stakeholders, starting from leukapheresis products collected in hospitals, the applicability of the method is limited to quality control of the starting material, with variability inherent to such collections (25). For example, target cell number of CD3+ T cells is one of the criteria for collecting starting material for CAR T cell manufacturing. Nonetheless, manufacturing IEC in a point-of-care manner would benefit from such a validated quantification method (3, 26-28).

The gating strategy adopted for NK cell absolute counts differs from that adopted by Koehl et al in their inclusion of CD14+ monocytes in the mix and initially gating on live CD45+ cells (29). We argue that we excluded monocytes (via size and granularity) by gating on live lymphocytes instead of CD45+ cells. Furthermore, we agree with Koehl et al on the absence of CD14+ monocytes in expanded NK cell products, despite their detection upon selection/prior to expansion (Institut Paoli-Calmettes, NCT01853358).

An accurate quantification of viable absolute numbers of cells is a prerequisite for several activities: standardization of input cellular material for CAR-transduced T or

NK cell manufacturing (30, 31), cell selection (3, 14, 32, 33), or dosing of donor lymphocyte infusions (34). As reported by others, participation in EQA indeed helped improve the accuracy of the method [35, 36]. Our data on high accuracy across a range of values of lymphocyte immunophenotypes gives assurance of the method's capacity to quantitate them in starting material from lymphopenic patients as well as from healthy donors (37).

In cell therapy, the precision aspect of a viable lymphocyte immunophenotype quantification assay gives confidence in meaningful changes in data collected throughout the processing of a product (38, 39), particularly during cryopreservation (40) or over longitudinal studies with IEC (41). All in all, this successful validation renders the method ready-available for adaptation by quality control labs processing, cryopreserving or manipulating leukapheresis products for IEC manufacturing.

A limitation to this method is its inability to respond to quality controls required for extensively-manipulated products like TCRαbeta/CD19-depleted progenitor cell grafts (42). To that end, a more advanced 10-color single-platform method has been validated for graft composition and is currently used in an ongoing multi-center phase I/II trial (Miltenyi Biotec, EudraCT-No.:2011-005562-38) (14).

Since many labs worldwide use Cytomics FC500 for CD34 enumeration via CXP software, adapting to this validated single-platform method may prove less challenging to implement if processing IEC –or even collecting and shipping leukapheresis to pharmaceutical manufacturers-- is foreseen in their short-term plans.

Acknowledgement

We would like to acknowledge the cell therapy facility staff for their availability and Mrs. Carine Malenfant for her coordination efforts. We equally acknowledge the support of Olivier Jaen, PhD (Beckman Coulter, Marseille) throughout the setting up of the method and the validation process.

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6

Chapter 7: Manufacturing natural killer cells as medicinal products

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Front Immunol 2016 Nov 15;7:504

Abstract

Natural Killer (NK) cells are Innate Lymphoid Cells (ILC) with cytotoxic and regulatory properties. Their functions are tightly regulated by an array of inhibitory and activating receptors, and their mechanisms of activation strongly differ from antigen recognition in the context of HLA presentation as needed for T-cell activation. NK cells thus offer unique opportunities for new and improved therapeutic manipulation, either *in vivo* or *in vitro,* in a variety of human diseases, including cancers. NK cell activity can possibly be modulated *in vivo* through direct or indirect actions exerted by small molecules or monoclonal antibodies. NK cells can also be adoptively transferred following more or less substantial modifications through cell and gene manufacturing, in order to empower them with new or improved functions and ensure their controlled persistence and activity in the recipient. In the present review, we will focus on the technological and regulatory challenges of NK cell manufacturing, and discuss conditions in which these innovative cellular therapies can be brought to the clinic.

Introduction

Cellular therapies are nowadays increasing in numbers and in diversity. Manufacturing of various types of immune cells is likely to provide additional therapeutic resources and complete the portfolio of immunotherapies, along with chemical molecules and engineered monoclonal antibodies. It is envisioned that combination of these different medicinal products, tailored to disease characteristics as well as to the host (immune) environment (1), will contribute to precision medicine and expectedly to higher rates of success in the cure of a variety of health disorders, including but not restricted to cancers. Among immune effectors amenable to cell and genetic manipulation prior to adoptive transfer, NK cells present with appealing biological characteristics. NK cells are ILC and contribute to innate immunity (2). Their activities are regulated through the biological modulation of a large array of both inhibitory and activating receptors, including Killing Inhibitory Receptors (KIR), NKp44 and NKp46. These receptors do not bind specific antigens on target cells as do T cells, but rather molecules induced by cellular stress that provide an activating signal, or HLA molecules that predominantly provide inhibitory signals; already published material provides in-depth description of these pathways. Here, we review the existing literature that describes the rationale for various technological approaches to NK cell manufacturing, either autologous or allogeneic, as a prerequisite to adoptive transfer and clinical evaluation of these peculiar populations of immune effectors.

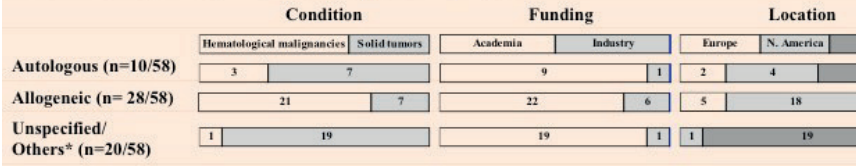
Medical applications of NK cell manufacturing and adoptive transfer

Autologous NK cells

Adoptive transfer of NK cells engineered to express new or augmented functions represent an interesting avenue for the treatment of various high-risk malignancies in which conventional options have failed (Figure 1). Examples include B-chronic lymphocytic leukemia (B-CLL) (3), multiple myeloma (4) but also tumors of non-

hematopoietic origin such as breast cancer (5), melanoma or renal cell carcinoma (6).

Figure 1: Ongoing clinical trials that evaluate NK cell-based cellular therapies. Data were collected at https://clinicaltrials.gov/ in September 2016, using the search terms: « recruiting studies », « NK », or « natural in the « Title » field. Raw data available in Supplementary Table.



** unspecified= 18 studies with no designation whether NK cells are autologous or allogeneic ; others= 1 study tests 1*

Early studies reporting the adoptive transfer of $CD56^+$ bead-selected autologous NK cells into patients with metastatic cancers, in combination with high doses of IL-2 demonstrated feasibility yet poor clinical efficacy (7). Patients experienced severe toxic side effects due to the high doses of IL-2. In addition to activation-induced NK cell death, NK cell function may have been inhibited due to regulatory T cell expansion in response to high IL-2 doses (8, 9). Reducing the daily IL-2 doses after NK cell transfer resulted in limited clinical success (10). Several strategies are under investigation to overcome this hurdle (reviewed in (11)). Another mechanism by which autologous NK cells are inhibited is by self-HLA molecules. Therefore "releasing the brakes" with anti-KIR antibodies such as Lirilumab that targets the inhibitory KIR receptors on NK cells could be one approach (12-14).

Allogeneic NK cells

A number of arguments support an important role for donor-derived NK cells in the context of allogeneic hematopoietic stem cell transplantation (allo-HSCT). Following the administration of either myelo-ablative (15) or reduced-intensity (15-17) conditioning regimen and allo–HSCT, the rapid reconstitution of high numbers of circulating and phenotypically-defined NK cells is associated with better clinical outcome. The recovery of various functions for donor-derived cells may be further modulated and improved in vivo with additional intervention (18).

Transplantation of high-doses of immune-selected CD34+ cells collected from haplo-identical donors after myelo-ablative conditioning regimen has provided a setting in which to demonstrate that "KIR-incompatibility" was associated with lower incidence of disease relapses, at least for AML (19). Transplantation of T-replete marrow or blood cell grafts obtained from haplo-identical donors, using modified immune-suppressive conditioning regimen such as those including post-transplant cyclophosphamide, represent a more widely applicable procedure, in which to further explore the potential contribution of alloreactive NK cells in post-transplant clinical events. Unexpectedly, a recently published report suggests that in this context, the presence of recipient class I ligands to donor KIR receptors confers some protection to the recipient against leukemia relapse, an observation that needs further confirmation and would imply a role for Killer Activating Receptors (KAR) as much as for KIR (20). The role of alloreactive NK cells remains more elusive in the context of HSCT performed from other categories of donors. Expression of specific KIR receptors in HLA-matched unrelated donors was demonstrated to produce superior or inferior clinical outcomes in recipients, depending on donor-recipient combinations (21-23).

Adoptive transfer of allogeneic NK cells either with a stem cell graft ex vivo depleted of immune effectors, or as a substitute to post-transplant "Donor Lymphocyte Infusions" (DLI) is thus appealing as a way to improve engraftment, immune reconstitution and anti-tumor activity with reduced chances of triggering GVHD (24). Results of a small number of clinical trials have been reported so far, demonstrating the feasibility of manufacturing allogeneic NK cells from matched related, matched unrelated or mostly from haplo-identical donors (25-29). Although allogeneic NK cell infusions were generally reported as safe, a recent publication describes the clinical outcome of a small cohort of pediatric patients treated for non-hematological high-risk malignancies and a high proportion of aGVHD triggered by HLA-matched donor-derived NK cells (30). Mostly, these limited clinical results suggest that additional improvements are needed either during the ex vivo

manufacturing process (31) or after infusion of manufactured NK cells (25) to improve long-term persistence and activity in vivo.

Factors affecting NK cell-product manufacturing

Many variables contribute to an efficient NK-cell generation protocol (Table 1). Donor-recipient combinations, the source of starting material and culture conditions are factors that can be carefully selected to optimize the manufacturing process and potentially the clinical efficacy of the resulting medicinal product upon administration to the recipient.

Cell source
Bone marrow
Umbilical cord blood
Embryonic stem cells
Induced pluripotent stem cells
NK cell lines
Culture conditions
Cytokines (IL-2, IL-15, IL-12, IL-18)
Feeder cells (autologous PBMC, EBV-TCT-LCL, K562-mb15-41BBL)
Antibodies (anti-CD3, anti-CD52)
Genetic manipulation (retro- or lentiviral-based transduction, mRNA transfection)
Culture containers
Standard culture flasks
Culture bags
Gas-permeable static cell culture flasks
Bioreactors
Final product evaluation
Viability (live/dead)
Identity and contamination (CD56, CD16, CD3, CD14, CD45, CD19)
Yield
Phenotype (KIR, NKp44, NKp46, NKG2A, NKG2C)
Functionality (degranulation, cytokine release, target cell lysis, activation)

Donor Selection

In the setting of allogeneic NK-DLI, donor selection can affect the clinical outcome of NK cell therapy, since certain KIR, HLA and FcgammaR polymorphisms influence NK cell function (32, 33). KIR typing can be genotypic, classifying donors on the basis of gene expression of activating and inhibitory KIR (34), thereby assigning them scores to select "preferable donors" (35-37). Additionally, KIR

typing can be phenotypic assessing surface protein expression of KIRs (38), adding another layer to selecting "preferable donors". KIR “allele-typing” is a recent addition to the donor selection algorithm, whereby alleles that possess better functional properties (stronger licensing capability and more durable surface expression upon ligand binding) are favored (22, 39). Typing FcgammaR polymorphism is relevant in NK cell therapy settings that use monoclonal antibodies to enhance NK cell activation and consequently empowering their antibody-dependent cytotoxicity (ADCC) properties (40, 41). All these strategies have helped define "preferable donor" profiles.

Source of cells

NK-cell therapies can be manufactured from a variety of sources: these include peripheral blood, either steady-state or taking advantage of apheresis performed to collect hematopoietic stem and progenitor cells mobilized with growth factors such as G-CSF, bone marrow and cord blood.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) can be collected in large numbers using apheresis. It is nowadays the preferred source for allogeneic hematopoietic stem cell transplantation; donor apheresis is collected after receiving a mobilization treatment that increases the percentage and number of circulating progenitor and stem cells (as evaluated by the number of circulating CD34+ cells); G-CSF is the only marketed agent for CD34+ cell mobilization that can be used in donors; the use of other mobilizing agents such as acutely myelo-suppressive drugs or plerixafor is restricted to patients undergoing autologous collection. Since CD34+ cells represent only a small proportion of collected PBMC, these collected cell products may also represent a source of immune effectors, and thus either an alternative to PBMC collected in homeostatic conditions for standard DLI, or a starting material for further immune cell manufacturing, including NK cell manufacturing. One caveat to this approach is that studies looking at the effects of G-CSF on NK cell function

have produced controversial results; some studies suggest minimal consequences (42, 43), while others suggest significant changes (44).
Non-mobilized apheresis products contain 5-15% NK cells. To isolate NK cells, the strategy commonly used is CD3+ cell depletion of PBMC followed by CD56+ cell enrichment using immune-magnetic bead separation with medical devices and clinical-grade reagents.

Bone marrow

Since bone marrow is nowadays used as the source of stem cells for a minority of recipients – in part due to the increased resource needed for the logistics of BM collection compared to aphereses - there is little pre-clinical or clinical experience in the manufacturing of NK cells from this starting material.

Cord blood cells

The use of cord blood (CB) as a source of stem cells has raised great hope in the field 30 years ago, when the first clinical transplants were reported (45). CBU can be used even when not fully matched to the recipient, offering the opportunity to identify a "donor" even for patients who had no HLA-matched related or unrelated donor. CB transplantation is nowadays facing tough competition from the rapidly emerging field of related haplo-identical transplantation, and is further hampered by the lengthy immune reconstitution and the lack of possibility to use pre-emptive or curative DLI post-transplant.
However the more than 600,000 CBU that are stored in public banks worldwide – not to mention the unknown cumulative number of CBU preserved in private cord blood banks – represent a unique source of human material to start with the manufacturing process. Indeed, pre-clinical validation studies reported the production of significant numbers of functional NK cells either from a complete CBU (46), or even from a minute sample of a CBU (47), the latter opening the way to post-transplant immune cellular therapies in recipients of CBU transplantation while the former offers the promise of "off-the-shelf" allogeneic NK cellular therapies.

Initial attempts to use CD56+ bead-selected NK cells from CB followed by culture on mesenchymal stromal cells in the presence of cytokines resulted in modest yields incompatible with therapeutic needs (48). Starting from immune-selected CB CD34+ cells and using refined protocols produced more interesting results (49-51). Such efforts culminated in a novel GMP-compliant technique that mimics the extra-cellular bone marrow environment: stromal cell-free/ serum-free medium, heparin and cytokine supplements (52, 53). Consequently, Glycostem Therapeutics (Oss, the Netherlands) and Radboud University Medical Center (Nijmegen, the Netherlands) are currently conducting a Phase I/II clinical trial in elderly AML patients using NK-cell products generated using this method (CCMO nr. NL31699 and Dutch Trial Register nr. 2818).

ES and iPS cells

Manufacturing of clinical grade NK cells from either embryonic (ESC) or induced pluripotent stem (iPSC) cells nowadays appear as a futuristic option, although pre-clinical demonstrations that NK cells can be differentiated from these sources of pluripotent stem cells were already published (54-56). Several factors affect the procurence of pluripotency: choice of target donor somatic cell type, reprogramming factors and method of delivering transcription factors into somatic cells (57).

An important step in the specific hematopoietic lineage-differentiating protocols starting from ESC and iPSC is the generation of CD34+ hematopoietic precursors, particularly CD34+CD45+ cells, preferred for their high content of hematopoietic progenitors (58). A 30-day culture of sorted ESC-derived CD34+ cells with feeder cells (murine fetal-liver-derived stromal cell line) and cytokines generates NK cells with characteristic maturation markers and target cell lysis capabilities (58, 59). Another expansion method has been described for PSC using embryonic body assay followed by culture with feeder cells and cytokines (60, 61).

Although advantaged by low immunogenicity, being patient-derived, the main hurdle that remains is the need for safe, efficient and standardized clinical-grade manufacturing protocols.

Similarly to what has been mentioned for CBU, progress along this avenue will offer an opportunity to develop off-the shelf personalized cellular therapies.

NK cell lines

Ex vivo expanded primary NK cells persist in vivo for short periods of time after adoptive transfer. In an attempt to take advantage of the long lifetime of established cell lines, several groups have evaluated their therapeutic potential, particularly for the NK-92 cell line (NantKWest Inc, Culver City, CA) characterized by good cytotoxicity and expansion kinetics (62, 63). Although other cell lines exist (NKG, YT, NK-YS, YTS cells, HANK-1 and NKL cells), NK-92 cell line has been employed the most in preclinical investigations and clinical trials (NCT00900809 and NCT00990717) (64). It has been tested in a small number of clinical contexts, yet with minimal efficacy (65-67). Recently, chimeric antigen receptor (CAR) modification by gene transfer for NK cellshas opened a new avenue to explore (68, 69). NK cell lines represent a more homogeneous population compared to the other candidate for CAR modification, peripheral blood NK cells, but this advantage comes hand-in-hand with their inability to expand in vivo due to the necessary irradiation before infusion and the need for transfecting CD16 to gain ADCC function. The choice of CAR construct adds another layer to NK cell line modification (69).

Culture conditions: medium, cytokines and cell culture systems

NK-cells are generally isolated through immune-selection techniques, using the canonical CD3-/CD56+ phenotype (42, 70), then cultured for functional activation and possibly expansion. Furthermore, NK cells can be genetically engineered to express natural or chimeric molecules empowering them with improved immune functions (5, 64).

Expansion and activation of potent cytotoxic NK cells require several signals for survival, proliferation and activation. Culture conditions thus incorporate media and serum supplements, together with clinical-grade cytokines, monoclonal antibodies or

other soluble molecules, and possibly native or engineered cell feeders. Culture conditions can further be improved through the substitution of bioreactors to static conditions.

As already mentioned, and since most protocols that use only cytokines result in limited NK cell expansion, the introduction of feeder cells in the culture protocol has been extensively tested. Feeder cells provide additional stimulatory signals necessary for NK cell proliferation. Monocytes provide humoral signals and cell-to-cell contacts hence can serve as feeder cells (71); irradiated autologous PBMC have been used as feeder cells to produce sufficient numbers of NK-cells with acceptable purity (6, 72, 73). Alternatively, irradiated allogeneic cells have been evaluated: Epstein-Barr Virus-transformed lymphoblastoid B cell lines (EBV-TM-LCL), K562 cells (leukemic cell line) engineered to express a membrane-bound form of IL-15 fused to T-cell receptor CD8alpha and the 41BB ligand (74-78) or K562 cells transduced with IL-21 (79). Such feeder cells proved effective in preclinical validation of the production of clinically relevant numbers of NK cells however raise regulatory issues when it comes to manufacture medicinal products.

Since the presence of residual feeder cells in the final product is of major concern for clinical applications (80), alternative approaches are evaluated as substitutes. Anti-CD3 (OKT3) antibodies in addition to IL-2, with or without IL-15, produced substantial although lower fold expansion of CD3-/CD56+ enriched cells (81-86) than protocols that use feeder cells.

Expansion strategies of clinical-grade NK cells usually require 7 to 21 days of culture; up to 28 days of culture have been reported (87). There is an incentive to substitute animal or human serum-replete medium with animal and human component-free medium. The most commonly used media for CB-derived NK cells is Glycostem Basal Growth Medium (Clear Cell Technologies, Beernem, Belgium), preferred for being free of animal-derived components (52, 53). For PBMC-derived NK cells, preferred media are: X-Vivo serum-free media (Biowhittaker, Verviers, Belgium), AIM V serum-free medium (Thermo Fisher Scientific, Grand Island, New

York), Stem Cell Growth medium (Cell Genix, Freiburg, Germany), or complete Roswell Park Memorial Institute 1640 (Biowhittaker, Verviers, Belgium).
Media supplements still being used by some groups include GMP-grade human AB serum, pooled human AB plasma or fetal bovine serum (FBS). GMP-grade cytokines (recombinant human IL-2 and IL-15), antibodies (anti-CD3-OKT3) and other ancillary reagents (nicotinamide-NAM) commonly serve as medium supplements for PBMC-derived NK cell generation. Additional growth factors and cytokines are necessary for CB-derived NK cells since the starting material is commonly CD34+ stem cells (SCF, IL-7, IL-15, IL-2, IL-6, Flt3L, TPO, G-CSF, LMWH, GM-CSF, LIF, MIP1α).

In addition to using T75 cell culture flasks, several groups have used culture bags (Baxter LifeCell or VueLife) (76, 83). On larger scales, gas-permeable static cell culture (G-Rex) flasks (Wilson Wolf Manufacturing, New Brighton, MN) (78) or WAVE Bioreactor™ (GE Healthcare Life Sciences, Chicago, IL) (83) served as expansion platforms.

Amplitude of NK cells expansion and definition of an optimal cell dose
Numbers of infused NK cells in clinical trials range from 5 to 50x106 NK cells/kg, but infusion of as many as 108 NK cells/kg has been reported (88). Based on percentages of NK cells in the starting materials, manufacturing the higher doses implies significant expansion during in vitro cultures. This raises a practical issue, since in the absence of feeder cells, NK cells expansion is modest if any. Using autologous irradiated PBMC as feeder cells, up to 2,500-fold expansion of functionally active NK cells at day 17 has been reported (89). The use of genetically modified cell lines as feeder leads to a 30,000-fold expansion of NK cells after 21 days of culture (79).

A recent study took advantage of the introduction of anti-CD3 and anti-CD52 monoclonal antibodies over a period of 14 days, and reports a median 1500-fold increase in NK cell numbers; however it must be emphasized that T cells represent

up to 40% of the final cell product and that NK cells were not obtained through a cGMP protocol (90).

Quality controls and release criteria for engineered NK cell cells

Tools for assessing the efficacy of NK-cell generation protocols are necessary for comparing technical results from different NK-cell therapy studies. Furthermore, EMA and other guidelines require the characterization of the final product to define release criteria in order to ensure safety and efficacy.

Basic yet essential criteria are generally used to characterize the final product: these include purity and viability of the target cell population, contamination with undesirable cells such as residual T and B cells, and sterility. These are commonly used as release criteria although their relevance may vary for different clinical conditions: T cell contamination for instance is most important in an allogeneic, but not so much in an autologous setting. More sophisticated testing may provide additional information: a reduction in telomere length indicates cell senescence due to extensive long-term culturing.

Phenotype and function (tumor cytotoxicity) are additional characteristics that should help identify the most effective NK cell products. When expanded NK cells were compared with freshly isolated and IL-2-activated NK cells, a higher expression of NKG2D, TRAIL and natural cytotoxicity receptors NKp30, NKp44 and NKp46 was reported (91), in addition to higher cytotoxicity to K562 cells. Efforts are however much needed to harmonize technical protocols and identify a panel of phenotypic and functional biomarkers that would allow comparisons between protocols that evaluate adoptive transfer of NK cells (92). It is essential to mention that such a panel needs to be run within a reasonably short time to release the product in time for a "fresh infusion".

Cryopreservation and conservation of cytolytic activity of thawed NK cells would render multiple rounds of adoptive NK cell infusions feasible. Lapteva et al and Berg et al reported that an overnight activation with IL-2 would rescue the reduced

cytolytic activity of thawed NK cells, yet at the cost of a diminished recovery (74, 78). Efforts to optimize cryopreservation and thawing methods are in progress.

Regulatory status of engineered NK cells & commercial perspectives

Autologous and allogeneic NK cells engineered from primary human cells are individually produced for a unique and designated individual, rather than manufactured as batches. Since manufacturing incorporates ex vivo culture and activation of immune-selected cells from the primary material, these will be considered as substantially manipulated or more-than minimally–manipulated cell products, and thus will qualify as "Advanced Therapy Medicinal Products" (ATMPs) and somatic cell therapy products as defined in EC regulation 1394/2007. Before the regulation was released, such cell therapies were engineered by cell processing facilities, usually supported and operated by academia, no differently from minimally manipulated cell transplants. Since the regulation has been published, the view is that somatic cell therapy products and gene therapy products will be manufactured in compliance with good manufacturing practices (GMP) (85, 86, 93), and eventually marketed by industry, when a marketing authorization is granted by competent authorities at a European level, i.e. by the European Medicine Agency (EMA) or by the Food and Drug Administration (FDA) in the US of America.

NK cells have now aroused the interest of a significant number of Pharma companies (94), although no NK-based cellular therapy has so far been authorized as an ATMP. However, in December 2014, orphan designation (EU/3/14/1395) was granted by the European Commission to Glycostem, for allogeneic ex vivo-generated natural killer cells from CD34+ CB progenitor cells for the treatment of acute myeloid leukemia: GCT-NK cell product (EMA/COMP/730059/2014 Committee for Orphan Medicinal Products).

Conclusion

Over the last 30 years, enormous progress has been made in our understanding of the biology of NK cells. New agents targeting their activity in vivo have been evaluated, and technological improvements in NK cell manufacturing have been introduced in the clinic. However, the demonstration that modulation of NK cell activity by any of these means can achieve therapeutic activity over a wide range of diseases is still awaited. The ability to follow and image in vivo adoptively transferred autologous or allogeneic NK cells would represent a major advantage to understand the "pharmacokinetics" and mechanisms of action of these immune effectors, as illustrated in pre-clinical (95) as well as in clinical (96) studies. It would help to understand the consequences of culture conditions on in vivo persistence and activity. Ongoing developments for innovative cellular therapies in the academic sector as well as in the commercial sector suggest that such progress may result in broader clinical applications in the near future.

Funding statement

This work was supported in part by Institut Paoli-Calmettes, by grant INCa-DGOS-Inserm 6038 (Site Intégré de Recherches et d'Innovations en Cancérologie, SIRIC Marseille) from Institut National du cancer (INCa), by Fondation ARC pour la recherche sur le cancer and by Cancéropôle PACA. The E.V. laboratory is supported by the European Research Council (THINK Advanced Grant), the Ligue Nationale contre le Cancer (Equipe Labellisée) and by institutional grants from INSERM, CNRS and Aix-Marseille University to CIML. E.V. is a scholar of the Institut Universitaire de France. S. U. is supported by grants from the European Research Council, l'Agence nationale de la recherche (ANR), la Fondation ARC pour la recherche sur le cancer, la région PACA.

Acknowledgements

This work was supported in part by Institut Paoli-Calmettes, by grant INCa-DGOS-Inserm 6038 (Site Intégré de Recherches et d'Innovations en Cancérologie, SIRIC Marseille) from Institut National du cancer (INCa), by Programme Hospitalier de Recherche Clinique (PHRC, to DB), by Fondation ARC pour la recherche sur le cancer and by Cancéropôle PACA. EV's laboratory is supported by the European Research Council (THINK Advanced Grant), the Ligue Nationale contre le Cancer ("Equipe Labellisée") and by institutional grants from INSERM, CNRS and Aix-Marseille University to CIML. EV is a scholar of the Institut Universitaire de France. SU is supported by grants from the European Research Council, l'Agence nationale de la recherche (ANR), la Fondation ARC pour la recherche sur le cancer, la région PACA.

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Chapter 8: From clinical proof-of-concept to commercialization of CAR-T cells

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Drug Discov Today 2018 Apr;23(4):758-762

Abstract

The development of CAR-T Cells currently represents an exciting opportunity to convert the already published clinical successes observed in clinical trials into commercially-available efficient therapies. However, the path towards successful commercialization is still hindered by many hurdles. Here, we review such issues: the need for structured collaborations between hospital collection/ clinical facilities and industry manufacturing facilities to streamline the supply chain, necessity for uniform and efficient medical procedures to cope with severe toxicities associated with CAR-T cells, and absolute need to define an economical and sustainable model for both manufacturers and payers. The fast pace at which the field is evolving requires careful assessments for the benefit of patients.

Of great clinical potential was the first successful combination of antibody specificity with T-cell mediated target cell lysis (1). "Upgraded" generations of chimeric antigen receptors (CAR) directed at tumor antigens in a major histocompatibility class (MHC)–independent fashion followed. The design of CAR implies the recognition of unprocessed antigens and provision of appropriate co-stimulatory signals, means by which genetically-engineered T cells would fight immune system-evading tumors (2). The design was further optimized to confer cytokine-secreting and ligand-modifying capacities to CAR-T cells, to overcome the immunosuppressive tumor microenvironment (3). Ongoing efforts aim to diversify targets, broaden clinical applications and improve CAR-T cell safety, capacity to traffic to tumor sites, in vivo persistence and use of combination therapies (4).

Clinical manufacturing of CAR-T cells represents a challenging undertaking that many investigators and companies are working to resolve. Initial manufacturing took advantage of existing academic organizations and infrastructures, complying with Good Manufacturing Practices (GMP). To date, most published studies have used autologous CAR-T cells; this complex process generally starts with obtaining a patient's peripheral blood mononuclear cells (PBMC) harvested through apheresis. T cells are then selected and activated prior to gene modification with viral / non-viral vectors. Expansion of modified T cells precedes final formulation and administration into the patients. Choice of T cell subpopulation, vector, CAR target and structure, activation method, expansion bioreactor, duration of culture, cryopreservation, final formulation and release testing (5, 6) remain too complex to reliably and cost-effectively upscale to an industrial level without additional tools using quality-by-design principles for instance (7). Ultimately, CAR-T cells need to be "sold to customers", hence one should question which business model would fit the unique nature of cellular therapies: single-center co-located manufacturing model, hub-and-spoke model, franchise model or traditional pharma model (8).

Towards multicenter trials and commercialization

Small-scale manufacturing, with a local organization involving experienced academic facilities strongly backed up and supported by industry, could be extended and robustly adapted to a multicenter network. The currently dominant multicenter model proposes an organization where starting material collected at local hospitals converge towards a central manufacturing facility, before the manufactured human cell derived medicinal products are returned to these hospitals and administered to patients. Identification of challenges associated with such an organization that replicates the historical model used by the pharma industry however encourages exploring alternative logistic solutions for the production of these medicinal products, with the hope to offset some of the costs.

Controlling the supply chain

Most published clinical trials investigated autologous CAR-T cells that were prepared from apheresis products. For autologous CAR-T cell manufacturing, the process starts with PBMC collection. The process remains relatively ineffective at precisely predicting the characteristics of the collected cell product from patient features or procedural parameters, in some cases not reaching the target CD3+ T cell dose (9). Variability attributed to the patients (pediatric or adult), disease conditions and prior treatments/stem cell transplants requires careful assessment and adaptation of protocol for an efficient cell collection (10). Cell isolation for manufacturing initiation can be adapted as well to remove potential contaminating cells (leukemic or myeloid cells) (11). It is currently unclear whether cell separator manufacturers will commercialize improved instruments to suit the needs of their customers in the near future.

The heterogeneity in practices and procedures of hospital-based or blood bank-based apheresis facilities has long been identified in this context. Professional associations of the FACT-JACIE accreditation process have partially resolved this lack of harmonization (www.jacie.org & http://www.factwebsite.org). Nevertheless,

procedural variations continue to exist and can significantly impact the outcome of CAR-T cell manufacturing. The extent to which liability and responsibility will be split in case of failure of the manufacturing process will need to be carefully defined. This raises the question of centralizing the cell procurement step through the creation of dedicated cell collection facilities. Although this could resolve procedural problems, it will raise logistic, financial and possibly medical challenges for patients in need of these treatments, and who will be required to travel to a different city or country. Alternatively, this will help resolve issues associated with long distance transfer of living cells from the collection site to the manufacturing facility. Lessons learned from shipping and receiving hematopoietic cell grafts collected worldwide from unrelated donors could be applied and refined to become part of critical supply chains. This includes minimizing time to destination to protect the products' biological integrity.

Most CAR-T cell manufacturers plan to distribute their gene therapy medicinal products as a cryopreserved product. Transportation of human cells in a cryopreserved state rather than as cell suspensions kept at positive ranges of temperatures is biologically less challenging, but raises security issues that contribute to the high costs of such shipments. Furthermore, a number of questions related to conditions in which the cryopreserved product should be delivered for on-site administration require solutions such as: harmonizing conditions for temporary storage onsite, thawing and administration of the thawed product to the recipient, assigning responsibilities for the hospital pharmacy and cell processing facilities. The latter are equipped with validated and monitored cryopreservation containers while the former rarely are. Temporary storage in validated dry shippers originating from the central manufacturing facility will raise safety concerns in the clinical ward. While not only affecting healthcare personnel who are not specifically trained to deal with this equipment and its associated hazards, the product itself is at risk since autonomy of these cryogenic containers is limited. Conversely, temporary storage in cell processing facilities that are fully equipped with permanently

monitored cryopreservation containers offers increased safety, however restricts collaborations to hospitals that operate such infrastructures, i.e. housing organ, tissue and cell transplant programs. Similarly, bedside thawing using conventional water baths offers low possibility of standardization for the last processing step before infusion. This can hardly be achieved in environmentally-controlled conditions, and contrasts with more stringent measures implemented by cell processing facilities for minimally-manipulated cell products compliant with national or international regulations.

Despite these uncertainties and following the encouraging results of phase I trials that used upgraded academic cell processing facilities as manufacturing facilities for a single clinical site (results reviewed in 12), several phase II trials evaluating anti-CD19 CAR-T cells were started. Partial results were recently communicated (Figure 1), and demonstrate the feasibility of a central manufacturing facility serving up to 25 centers across 4 continents with a turnaround time of 2 to 3 weeks (13). These trials -- and the large-scale collaborative efforts behind-- sponsored by three of the major players in the field provide a starting point from which to improve and refine the logistics for transportation of patient-specific cell products.

trial	ELIANA[1]	TRANSCEND NHL 001[2]	ZUMA-1[3]
product	CTL-019	JCAR-017	KTE-C19
costimulatory domain	4-1BB	4-1BB*	CD28
vector	lentiviral	lentiviral	retroviral
indication	pediatric and young adult R/R ALL	adult R/R NHL	adult R/R DLBCL
patients	63	55 DLBCL	101
efficacy	83% molecular CR at 3 months	61% CR at 3 months	51% CR at 3 months
severe neurotoxicity	15%	14%	31%
deaths from CRS or neurotoxicity	0	0	2

[1] EHA meeting, June 2017
[2] ASH meeting, Dec 2017
[3] Gilead/Kite corporate FDA approval announcement, October 2017
* defined composition of CD4/CD8 T cells

Figure 1: Major CAR T cell clinical trials sponsored by industry

Managing toxicities

CAR-T cell administration is associated with significant toxicities (14). Management of the awaited B-cell aplasia is feasible, yet caring for the cytokine release syndrome (CRS), macrophage activation syndrome (MAS) and neurological disorders requires special resources and organization that currently limit the number of hospitals that could safely administer such treatments (15). In particular, a strong and proactive collaboration between hematology or transplant wards on one hand and intensive care departments on the other hand is mandatory.

Most of the patients receiving CAR-T cells experience various degrees of cytokine-mediated toxicities, from fever to life-threatening symptoms such as respiratory distress or severe neurotoxicity, which may necessitate off-label use of agents such as the anti-IL6-R tocilizumab in addition to resuscitation measures. Causal

mechanisms for neurological syndromes are under investigation (16) leading to mostly observational and empirical approaches to supportive care. Treatment-related death rate can be as high as 13%, as observed in the Juno Rocket trial, where five deaths from cerebral edema lead to the arrest of the clinical trial in March 2017 and discontinued development of the JCAR-015 CAR-T cell product. Several approaches were adopted to reduce toxicities: selective depletion of CAR-T cells via an integrated suicide gene (17), granting dual-input regulation via an integrated ON-switch (18) or generating CAR-T cells responsive to hypoxic environments (19), to cite a few. Despite the latter approach only being in preclinical testing, it promises economic viability. Given the expected high price tag of manufactured CAR-T cells, one would rather modulate their therapeutic activity than proceed to complete elimination to manage toxicities. The FDA's clinical hold on the UCART123 studies in September 2017 following the death due to severe neurotoxicity (although lifted in November 2017), raises questions about the safety attribute conferred by the suicide gene and the commitment to safety testing by cell therapy developers. Suitable and relevant animal models that predict safety, and algorithms for dosing a "live drug" are two of the more pertinent issues that can help deliver safer products (20).

Complying with a complex regulatory environment

Wording of regulations are significantly different across the Atlantic, although they incorporate similar principles. Within European member states, CAR-T cells are classified as gene therapy medicinal products, and belong to the wider category of Advanced Therapy Medicinal Products (ATMPs), as per regulation EC 2007/1394. The regulation is intended to foster the development of this new category of medicinal products, ensure their efficacy and safety, as well as facilitate access to these treatments. In contrast to cell transplants, ATMPs are medicinal products and destined to be placed on the market and commercialized. Criteria that allow distinguishing cell-based products as cell transplant or ATMPs unambiguously identify CAR-T Cells as belonging to the second category. Nevertheless, the

regulatory framework provided by regulation EC 2007/1394 imposes an organizational model that mimics the central manufacturing model long used by the pharma industry to produce and distribute conventional drugs or biologicals. Early attempts by the industry to obtain a centralized marketing authorization for ATMPs have led to little (if any) commercial success; this is in part because this model largely ignores the specificities of medicinal products manufactured from human living cells, and that its translation to the production of personalized somatic cell medicinal products or gene therapy medicinal products results in skyrocketing costs. Combination of these logistic and financial constraints with niche markets, or limited clinical advantages over existing or emerging therapeutic tools turned out to be commercially deadly for most of the first ATMPs that received marketing authorization, since no sustainable reimbursement model could be established with healthcare authorities in most member states (21). A point-of-care manufacturing model of CAR-T cells (alluded to later) would adopt a "hospital exemption" regulatory framework, limiting the authorization, manufacturing and distribution of these products to the individual EU member states and their corresponding health authorities. Consequently, to harmonize the different requirements for implementation between the states, the Voluntary Harmonization Procedure (regulation 536/2014 EC) was established in 2009 and modified/improved in 2014.

Figure 2: Major pharmaceutical industry players currently contributing to developments in the CAR-T cell field

Avenues to increase the likelihood of commercial success

Conditions surrounding the development of CAR-T cells are far different from conditions that surrounded the development of the first authorized ATMPs. It is nowadays remarkable that not only small and medium-sized enterprises (SMEs) but also big pharma (Figure 2) are partnering and investing large resources to support the development of CAR-T cells. Accordingly progressive strides are achieved in the design and tools to engineer CAR-T cells, technological and logistical improvements in the supply chain are tested at a rapid pace, and industrial strategies that take advantage of alternative organizational schemes such as the possibility of point-of-care manufacturing are elaborated. Simultaneously, new clinical applications and thus potential markets are explored.

Point-of-care technologies as alternatives to central manufacturing

Semi-automation of the CAR-T cell manufacturing process was introduced to support one of the published phase I trials (22). Availability of laboratory devices, such as the CliniMACS prodigy (Miltenyi Biotech) further allows for the generation of CD19-targeting CAR-T cells using a fully-closed automated clinical scale cell manufacturing system (23), one patient at a time. As currently designed, several

devices running in parallel would be necessary to accommodate the needs of a typical hematology program in major US and European hospitals. Different organizations where each step of the manufacturing process uses a different device may emerge in the future, the string of devices making up a production line, similar to organizations seen in other industry activities (24). For both scenarios, up scaling the manufacturing process with minimal operator intervention or supervision, is likely to become easier and cheaper. Alternatively, fully integrated instruments offer an opportunity to move away from the central manufacturing model, and evaluate the possibility of point-of-care manufacturing. Gene therapy medicinal products would then be manufactured close to the site of administration, thus limiting the need for costly shipment of the starting material and the manufactured medicinal product. Such approaches would greatly benefit from improved safety in terms of gene transfer technologies, as well as manufacturing technologies resulting in shorter processes. As for the former aspect, the use of viral-vectors to engineer CAR-T cells will inevitably raise more safety and regulatory issues than non-viral approaches. Along this strategy, Ziopharm and Juno Therapeutics are developing point-of-care platforms with shortened manufacturing processes.

Off-the-shelf products for rapid and timely access for patients eligible for treatment with CAR-T cells

Development of "off-the-shelf" products bears potential advantages over manufacturing of patient-specific CAR-T cells (25). Being manufactured from donors and engineered to suit the needs of many subsets of potential recipients, they open avenues for batch manufacturing. Since manufacturing is limited to small batches because primary cells constitute the starting material, they are variable yet to a lesser extent than individualized CAR-T cells. They still require intensive pre-clinical and clinical work and monitoring on safety and efficacy, including determination of their maximally tolerated dose, full validation of clinical lots prior to administration, immunogenicity and anti-host reactivity. Being able to produce allogeneic CAR-T cells from pluripotent stem cells or T cell precursors will further

favor the translation of manufacturing activities towards a centralized manufacturing model, still farfetched but one step closer to the historical organization for the production of conventional medicinal products. Nonetheless, increased risks of graft-versus-host disease (GvHD) and graft rejection are intrinsic to such allogeneic products; hence several strategies were explored to circumvent these risks: *ex vivo* depletion or anergization of alloreactive T cells, recipient immuno-suppression/-modulation while sparing adoptively-transferred cells, and reconsidering the degree of HLA matching or homozygosity at HLA loci for donor selection (26). Cellectis was the first biopharmaceutical company to receive Investigational New Drug (IND) approval from the US-FDA for its off-the-shelf CAR-T cell product UCART123 targeting acute myeloid leukemia (AML) and blastic plasmacytoid dendritic cell neoplasms (BPDCN) and a special license from the UK-Medicines & Healthcare products Regulatory Agency (MHRA) for its UCART19 to treat a young leukemia patient. The death of a UCART123-infused patient triggered rethinking the safety profile of these cells and a consequent 10-fold reduction in dose for upcoming patients along with a cautious stringent strategy of inclusion in this trial placed on clinical hold (from September to November 2017).

New Indications to broaden potential markets and increase the likelihood of commercial success

The early clinical success of CD19-targeting CAR-T cells in leukemias and lymphomas will in the best case scenario address the needs of a relatively small patient population, due to the low frequency of $CD19^+$ hematological malignancies. The ability to target other diseases will open doors for more sizeable markets with the potential for wider commercial successes. These expectations are fueling active investigations to develop CAR-T cells for new indications (27). To date, the evaluation of CAR-T cell therapy in solid tumors has been hindered by several factors, the first of which being the identification of a tumor-associated antigen (TAA) expressed on the surface of target cells: unique or shared tumor antigens (tumor-specific antigens, differentiation antigens or overexpressed antigens) that can

be robustly targeted by engineered T cells on tumor cells, while sparing normal tissues. Homing to tumor sites and overcoming an immunosuppressive tumor microenvironment are equally challenging issues. Clinical trials infusing CAR-T cells to patients with metastatic renal cell carcinoma or neuroblastoma have not been encouraging (28, 29). Hence, different CAR-T cell designs destined to overcome these obstacles are under investigation in phase I or I/II trials in gastric cancer, colorectal cancer, pancreatic cancer and pancreatic ductal adenocarcinoma, hepatocellular carcinoma and liver metastases (30). Apart from a case report of death following infusion of ERBB2-targetting CAR-T cells in a patient with colon cancer, metastatic to the lung and liver (31), mesothelin-targeting CAR-T cells (32) and carcinoembryonic antigen (CEA)-targeting CAR-T cells (33) showed safety and (some) efficacy in advanced malignant pleural mesothelioma (MPM) and CEA^+ liver metastases, respectively. Recently a phase I trial using multiple intracranial infusions of autologous CAR-T cells targeting TAA interleukin 13 alpha 2 to patients with recurrent multifocal glioblastoma reported promising results with manageable therapy-related toxic effects (34).

Conclusion

All actors interested in the development of human cell- and tissue-based therapies scrutinize the fate of CAR-T cell developments with much attention. They may represent the first example of a long awaited achievement to bring a commercially-viable cellular therapy to the market. Yet, if no sustainable economic model arises, industry investment may recede, and leave academic actors facing alone the financial and regulatory burden of developing innovative cell-based therapies.

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Chapter 9: General Discussion

The first advanced therapy medicinal product (ATMP) was approved in 2009 in the EU, 11 other products followed yet only 8 maintain their marketing authorizations (1). The timing is right for an assessment of the challenges facing cell-based immunotherapy translational research at the pre-clinical level, development level, and commercialization level, considering recent -- almost simultaneous-- approval of 2 ATMPs and withdrawal of 4 others in the last 5 years. Considering their potential as curative or disease-delaying strategies, many more products are in pre-clinical development than on the market. Translational research in this field is facing many challenges in reaching more patients.

Cell characterization and use

Defining the CQAs of Tr1 cells at the laboratory level (identity, purity, safety and potency) was successfully achieved. Whether these CQAs will be preserved during manufacturing process development at a later stage remains to be seen. Nevertheless, several points play in favor of the ability of these CQAs to withstand changes: using leukapheresis products from patients on dialysis (the target patient population) as starting material, eight successful validation runs with comparable attributes and stability upon cryopreservation/thawing.

Open questions remain to be answered throughout process development in general:

- what degree of purity is minimally required for safe clinical use, for instance in terms of contaminating DC-10 cells for Tr1 cells, debris and DMSO for HPCs (2), donor-derived T cell contamination for NK cells (3)?
- will the selected potency assays correlate with clinical efficacy (4)?
- from a safety perspective, can patients susceptible to toxicities be stratified and their management anticipated? For instance, cytokine release syndrome or on-target/off-tumor toxicity reported with CAR T cell treatments (5)?

- how feasible will it be to reliably measure cell persistence in vivo as a correlate to clinical efficacy (6)?

We have described other mechanisms (in mice and/or humans) by which regulatory T cells can suppress alloreactive responses. Since tracking these cells in vivo remains challenging, a comprehensive and exhaustive immunomonitoring strategy of patients receiving Tr1-enriched T10 cells can serve two purposes: validating such interactions and guiding the reduction of immunosuppression (7, 8). This strategy has shown efficacy in the One Study consortium where harmonization of immunomonitoring in the reference group arm is paving the path for data-driven decisions on reducing immunosuppression for some regulatory T cell-infused patients (9).

In vivo pharmacological induction of Tr1 cells in a tolerance induction mouse model can be an alternative strategy to infusion of ex vivo expanded Tr1 cells. Validation of our study in other transplant models and feasibility of administering G-CSF and rapamycin to patients on dialysis are prerequisites to a corresponding proof-of-concept trial.

Understanding the clinical setting and concomitant treatments can serve as a basis for choosing the timing of cell infusion. In tolerance-inducing cell therapy clinical trials, in solid organ transplantation or prevention of GvHD post-HSCT, the first dose of regulatory T cells is infused around the day of transplant (10, 11), or as in the One Study consortium, ranging between -7 and +7 days relative to the day of transplant (12). Other groups reasoned similarly to infuse mesenchymal stromal cells at +7 days post-transplant (13). On the other hand, an efficient immunomonitoring strategy can be used to identify the favorable time frame during which to infuse cells (14).

Therefore, 3 strategies go hand-in-hand: well-defined CQAs, established immunomonitoring scheme and strong rationale for dose/timing.

Technology transfer

Beyond the readiness of the clinical-grade laboratory manufacturing protocol, technology transfer to a GMP facility was undergone that culminated with 3 successful runs using leukapheresis products from patients on dialysis (Battaglia et al, manuscript in preparation). Thinking beyond technology transfer, one needs to evaluate which manufacturing strategy to use upon scaling-up. Two manufacturing models currently exist for cell-based therapies: centralized and decentralized manufacturing. Centralized manufacturing indicates few facilities handle the manufacturing for all the market's demand, while decentralized manufacturing indicates that products are manufactured in --or nearby-- hospitals where the patient's leukapheresis product is collected (15). Manufacturing donor-specific Tr1-enriched T10 cells is different from polyclonal T regulatory cell manufacturing and is complicated by the need for two asynchronous leukapheresis collections. The complexity of the manufacturing protocol for Tr1-enriched product may benefit from a decentralized manufacturing strategy, requiring an automated closed-system cell selection and co-culturing. Resolving comparability between products across different sites in an eventual multi-center trial and overcoming regulatory challenges remain to be resolved. NK cell manufacturing may profit from a decentralized strategy as well, yet much work is needed to define CQAs and harmonize manufacturing approaches to achieve them at points-of-care. Furthermore, if they will be used in combination with checkpoint inhibitors, bispecific antibodies, or proliferation-promoting cytokines, CQAs need to be reassessed. Automated closed-system solutions exist and have shown feasibility, yet several regulatory hurdles still exist (16).

Process development

We reported an automated solution to washing DMSO, dead cells, debris and plasma upon thawing of cryopreserved hematopoietic stem cell products to substitute the highly variable manual process at this critical step. This highlights the need for a long-term process development strategy aiming at automation. Key in this effort was

working together with an industrial partner building the technology up from the prototype, through ameliorated versions then to the final version of what is now a CE marked medical device (Fresenius Kabi website, Lovo Automated Cell Processing System). Despite being a time and resource investment, validating a new technology can be useful in multiple processes (17). We continue to work with our industrial partner to use the same device (in a closed system) for leukapheresis product washing, volume reduction, incubation with immunomagnetic beads and wash-out before cell selection on a magnetic column. These steps are to some extent uniform to manufacturing processes of cell-based immunotherapies. Furthermore, we are planning to use the device to wash and concentrate large volumes of cultured ex vivo expanded NK cells in preparation for infusion to patients in a planned follow-up NK cell trial as prophylactic treatment post-HSCT in patients with hematological malignancies (NCT01853358).

We reported on the establishment and validation of a flow cytometry-based method to quantitate viable lymphocyte subpopulations, of relevance to frozen and fresh mononuclear cell collections as starting material of cell-based product manufacturing. This method responds to the urgent need of harmonizing the analytical methods around quantifying the cell populations in the starting material (18). A natural evolution of this method is the use of automated algorithms for gating and reporting (19), a process we are currently developing via an interdisciplinary collaboration (20). In retrospect, this quality control method would be applicable in the manufacturing of the Tr1-enriched T10 cell product to assess identity during cell selection initially or purity during release. Alternatively, non-destructive quality controls are being sought by both academic and industrial developers, the likes of an array of biosensors that continuously monitor a protein biomarker in the media, or in vivo imaging technologies coupled with cell phenotype recognition software (machine-learning platforms) (21).

From an academic perspective, we reviewed the literature on NK cell manufacturing as ATMPs (22) with the purpose to propose a new role for academic developers:

establishing platforms, via large consortia, to harmonize manufacturing and quality controls of clinical-grade NK cells, both allogeneic and autologous, using point-of-care strategies (23). This would require introduction of process analytic technologies (PAT) into manufacturing processes to ensure a quality product that passes release testing. Additionally, having harmonized immune monitoring of patients is necessary for comparability of trials and products (24). Furthermore, introduction of quality-by-design concepts into academic mindsets would open new venues and attract industry investments into academia-developed products, serving the overarching goal of reaching larger patient populations (25).

Reaching a larger patient population

Achieving commercial success and reaching larger patient populations is a long road to take. Hurdles here involve: management of toxicities associated with cell-based immunotherapies, reduction of cost of goods, global availability, availability of biomarkers to support/predict efficacy or to stratify responders from non-responders, and further automation for reproducibility. Tackling these challenges is riskier at this stage than during early development and requires significant capital to purchase equipment, train dedicated staff, increase facilities' capacity utilization, and perform comparability studies on process improvements (26). The scene may be complicated by the regulatory dimension; developers may be faced with regulators with limited experience in assessing process improvements (27). Additionally, adapting practices and training critical care physicians to properly manage toxicities provide an additional burden.

One of the first challenges facing ATMPs even once they reach the marketing authorization stage: small size of the target patient population and high price. EMA experience has shown that 4 products were withdrawn so far from the market for commercial failure and not safety issues (28). If upcoming treatments were to target larger patient populations, their manufacturing cost and consequently their price would need to decrease, leading to affordable life-changing therapies for patients, while maintaining the incentive to invest in developing new products (29).

Conclusion and recommendations

The high pace of basic discoveries in biomedicine is not matched by the slow pace of translating these discoveries into tangible improvements in patients' lives. Several challenges hindering this translation process were tackled in this thesis. Some other hurdles are albeit important and relevant, yet could not be tackled in this thesis: business models for point-of-care manufacturing, reimbursement, insufficiency of skilled workforce, handling out-of-specification products and most importantly the role of patients and patient advocacy groups in ATMP development processes.

FDA commissioner Scott Gottlieb predicted in January 2019 that "by 2025, the FDA will be approving 10 to 20 cell and gene therapy products a year based on an assessment of the current pipeline and the clinical success rates of these products". Many more efforts are needed to overcome the hurdles facing cell-based translational research in order to prove this prediction right.

This thesis documents a unique career path that has covered basic, clinical and translational research.

Therefore, I would like to close with a set of practical recommendations that could have helped me personally throughout my career. I hope they help current or aspiring academic developers of cell-based immunotherapies:

- Get introduced to pharmaceutical quality culture early on: defining CQAs of final products, release criteria, clinical utility, technology transfer, proper documentation of initial data, regulatory affairs, business model development, establish safety not only efficacy in pre-clinical phases (if possible), find other providers for critical reagents/instruments (if possible)
- Freeze cells from pre-clinical runs to be used during technology transfer for comparisons
- Interact/collaborate with GMP facilities and pharma industry to investigate feasibility of your manufacturing process and its cost

- Interact with patients and their families to understand side effects/toxicities, in general quality-of-life, do not aim only at overall survival or disease-free survival
- Participate in efforts to harmonize practices; although less scientifically challenging, they will turn useful in multi-center trials and later clinical trial phases
- Understand the difference in roles between cell-based product developer and basic researcher
- Understand the needs of industry for regulatory compliance and successful commercialization to better develop your product at the laboratory level
- Get exposed to other cell therapy fields, they are facing (or have faced) many of your current hurdles

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Addendum

English summary

Cell-based immunotherapies address a number of unmet medical needs and some have shown success. All cell-based immunotherapy products undergo extensive preclinical and proof-of-concept investigations for only a few to reach the bedside. Thus, several hurdles need to be overcome in cell therapy translational research for wider access to such innovative treatments. Major hurdles include, but are not limited to: definitive characterization of cell therapy products, feasibility of upscaling a laboratory-grade cell manufacturing protocol, automation of critical manufacturing processes, standardized quality controls, choice of therapeutic window, comparability between clinical trials and commercial success. The aim of this thesis is to tackle these common challenges faced by different products during preclinical, translational and clinical phases. With several market authorizations and withdrawals by the EMA for advanced therapy medicinal products, it is timely to evaluate the translational activity around cell-based immunotherapy products.

Adoptive transfer of ex vivo expanded T regulatory cells is currently under clinical investigation in solid organ transplantation, autoimmunity, and graft-versus-host disease, yet definitive characterization of their many modes of action remains to be identified. Such mechanisms are reviewed in **Chapter 2.** In **Chapter 3,** a *preclinical* investigation dissects one mechanism by which T regulatory type 1 (Tr1 cells) are therapeutically-induced in vivo in a mouse model of Tr1-induced tolerance to allogeneic islet graft transplantation, identifying macrophages as key players in this induction. In a *translational* context, feasibility of upscaling a laboratory-grade Tr1 cell manufacturing protocol using reagents and procedures compatible with good manufacturing practices (GMP) is reported along with data supporting the choice of therapeutic window for cell infusion in the upcoming clinical trial **(Chapter 4)**. In an effort to reduce variations in manufacturing and cost-of-goods, feasibility of automating a critical step in hematopoietic stem cell transplantation (HSCT), washing and concentrating thawed cells is established **(Chapter 5)**. To address the

need for standardized and accredited quality controls, validation of a quantitative immune-phenotyping method is reported in **Chapter 6**, of relevance during collection of apheresis products, manufacturing and release of cell-based immunotherapy products. Heterogeneity in manufacturing NK cells for *clinical* use is reviewed in **Chapter 7** while expanding on tools for harmonization of processes and quality controls to address the challenge of comparability between clinical trials. The hurdle of reaching larger patient populations and achieving commercial success is dissected for CAR-T cells in **Chapter 8.** Management of toxicities, investigating new indications and target tumor antigens, different manufacturing options and off-the-shelf products are addressed.

This thesis documents a unique career path covering the spectrum from bench to bedside to provide arguments to many hurdles facing cell therapy translational research. Others surely remain, for instance role of patients in product development. Activating and suppressive cell-based immunotherapies alike face these challenges nevertheless possible solutions can be extrapolated from one product to another for the benefit of larger patient populations.

Nederlandse samenvatting

Op cellen gebaseerde immunotherapieën zijn gericht op een aantal onvervulde medische behoeften en sommige hebben succes aangetoond. Alle celgebaseerde immunotherapieproducten ondergaan uitgebreide preklinische en proof-of-concept-onderzoeken voor slechts enkelen om het bed te bereiken. Verschillende hindernissen moeten dus worden overwonnen in translationeel translationeel onderzoek voor een bredere toegang tot dergelijke innovatieve behandelingen. Belangrijke hindernissen omvatten, maar zijn niet beperkt tot: definitieve karakterisering van celtherapieproducten, haalbaarheid van opschaling van een laboratoriumclassificatieprotocol, automatisering van kritieke productieprocessen, gestandaardiseerde kwaliteitscontroles, keuze van therapeutisch venster, vergelijkbaarheid tussen klinische proeven en commercieel succes . Het doel van dit proefschrift is om deze gemeenschappelijke uitdagingen waarmee verschillende producten worden geconfronteerd tijdens preklinische, translationele en klinische fasen aan te pakken. Met verschillende markttoelatingen en intrekkingen door de EMA voor geneesmiddelen voor geavanceerde therapie, is het tijd om de translationele activiteit rond celgebaseerde immunotherapieproducten te evalueren.
Adoptieve overdracht van ex vivo geëxpandeerde T-regulerende cellen wordt momenteel onderzocht in het kader van solide orgaantransplantatie, auto-immuniteit en graft-versus-hostziekte, maar de definitieve karakterisering van hun vele werkingswijzen moet nog worden vastgesteld. Zulke mechanismen worden besproken in hoofdstuk 2. In hoofdstuk 3 ontrafelt een preklinisch onderzoek één mechanisme waarmee T-regulatorische type 1 (Tr1-cellen) in vivo therapeutisch worden geïnduceerd in een muismodel van Tr1-geïnduceerde tolerantie voor allogene transplantatie van eilandjes en transplantaten, identificerend macrofagen als hoofdrolspelers bij deze inductie. In een translationele context wordt de haalbaarheid gerapporteerd van opschaling van een Tr1-celproductieprotocol van laboratoriumkwaliteit met behulp van reagentia en procedures die compatibel zijn met goede productiepraktijken (GMP), samen met gegevens die de keuze van een

therapeutisch venster voor celinfusie ondersteunen in het komende klinische onderzoek (hoofdstuk 4).). In een poging om variaties in productie en kosten van goederen te verminderen, wordt de haalbaarheid van het automatiseren van een kritische stap in hematopoietische stamceltransplantatie (HSCT), wassen en concentreren van ontdooide cellen vastgesteld (hoofdstuk 5). Om de behoefte aan gestandaardiseerde en geaccrediteerde kwaliteitscontroles aan te pakken, wordt de validatie van een kwantitatieve methode voor immuunfenotica gerapporteerd in hoofdstuk 6, van belang tijdens het verzamelen van afereseproducten, de productie en afgifte van op cellen gebaseerde immunotherapieproducten. Heterogeniteit in de productie van NK-cellen voor klinisch gebruik wordt in hoofdstuk 7 besproken en uitgebreid met instrumenten voor harmonisatie van processen en kwaliteitscontroles om de uitdaging van vergelijkbaarheid tussen klinische onderzoeken aan te pakken. De hindernis om grotere patiëntenpopulaties te bereiken en commercieel succes te behalen, wordt in hoofdstuk 8 ontleed voor CAR-T-cellen. Beheer van toxiciteiten, onderzoek naar nieuwe indicaties en target tumor-antigenen, verschillende productiemogelijkheden en kant-en-klare producten komen aan bod.

Dit proefschrift documenteert een uniek carrièrepad dat het spectrum van bank tot bed bestrijkt om argumenten aan te dragen voor veel hindernissen voor translationeel onderzoek op het gebied van celtherapie. Anderen zullen er zeker blijven, bijvoorbeeld de rol van patiënten bij productontwikkeling. Zowel activerende als onderdrukkende celgebaseerde immunotherapieën staan voor deze uitdagingen, maar desalniettemin kunnen mogelijke oplossingen van het ene product naar het andere worden geëxtrapoleerd ten behoeve van grotere patiëntenpopulaties.

Acknowledgements

First and foremost, I would like to thank my wife and children for their patience, support and sacrifices, as well as our friends distributed around the globe.

I would like to thank as well my parents, brother and sister and their families, and my mother-in-law.

I would namely like to thank my supervisors/people I worked with:

C. Chabannon, B. Calmels, C. Lemarie, S. Thevenet, technicians of the Cell Therapy facility and administrative staff at Institut Paoli-Calmettes (*Marseille*)

M. Battaglia, S. Gregori, M.G. Roncarolo, Eleonora, Tatiana, Nicola, Georgia, Angela, Alessandra, Vittoria, members of Piemonti Lab and Falcone Lab at San Raffaele Hospital (*Milano*)

A. Habicht, M. Guba and M. Fischereder from Klinikum der Universität München (*Munich*)

M. Sayegh, A. Chandraker, S. Khoury, Y. Morrison, I. Guleria, N. Najafian, S. De Serres, R. Abdi, A. Ritchie, T. Erceg, O Boenisch, J Azzi, L. Riella, M. Jurewicz, F. D'addio, P. Fiorina, M.J. Ansari, and X. Yuan from Brigham and Women's Hospital, Boston Children's Hospital and Harvard Medical School (*Boston*)

I would like to thank my fellow early stage researchers from EUTRAIN, and B.J. Prakken, E. Roks, SJ Vastert, and W. de Jager, my fellow "young scientists" from the Lindau Nobel Laureate Meeting 2014, my fellow colleagues from EUREKA, my fellow scholars from the ISCT-ASBMT Cell Therapy Training Course 2017, and our partners in the FRAMINK consortium and collaborators from the cell therapy industry sector.

Curriculum vitae

Mr. Bechara Mfarrej was born on February 3, 1979 in Jeita, Lebanon. He graduated in 1999 from Haigazian University (Beirut, Lebanon) with a Bachelor of Sciences. He graduated in 2004 with a Masters of Sciences from the Lebanese American University (Byblos, Lebanon) while working full-time as an underwriter in a life insurance company. He moved to Boston in 2007 to manage the Sayegh Lab's animal work at Harvard Medical School. Soon after, he became a research technologist with projects in basic research focusing on transplantation immunology and autoimmunity, along with managing the alloantibody and alloreactivity core lab within a clinical trial consortium in solid organ transplantation. He moved in 2011 to Munich, Germany and briefly worked in a basic research lab on ischemia/reperfusion always in the context of transplantation. He moved with his family in 2013 to Milan, Italy after being selected as an Early Stage Researcher within a Marie Skłodowska-Curie initial training network: European Translational tRaining for Autoimmunity & Immune manipulation Network (EUTRAIN) under Prof. Berent Prakken's coordination to work in the Battaglia Lab and Roncarolo Lab at Ospedale San Raffaele in the field of cell therapy for tolerance induction in a transplantation setting (basic and translational research). During this time, he was selected as one of 600 young scientists to participate in the 64th Lindau Nobel Laureate Meeting on Physiology/Medicine. He then moved again with his family in 2016 to Marseille, France to join the Center for Cell Therapy at Institut Paoli-Calmettes. Soon after the start of his employment, he was selected as one of 13 scholars to participate in the ISCT/ASBMT Cell Therapy Training Course in Seattle. He currently holds the position of Cellular Immunotherapy Research and Development Engineer under the supervision of Prof. Christian Chabannon, and continues to work in cell therapy in a hematological setting, translational research and on the establishment/maintenance of a European network of excellence focusing on harmonization of practices around NK cell manufacturing.

Together with his wife, they have two children: Marko and Lea.

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