Landscape of Manufacturing Process of ATMP Cell Therapy Products for Unmet Clinical Needs

Ralf Pörtner, Shreemanta K. Parida,
Christiane Schaffer and Hans Hoffmeister

Additional information is available at the end of the chapter

Abstract

Immune cell therapies have been studied in numerous clinical trials using Advanced Therapy Medicinal Products (ATMP) against a number of diseases having no or inadequate alternative therapies available, for example, various cancer types, cerebral stroke, cardiac infarction, severe autoimmune disorders, or chronic infections. Despite the enormous number of positive observation in *ex vivo* or animal studies, convincing results in clinical studies remain scanty. The chapter presents a survey and reveals that the manufacturing of immune cells especially for clinical trials is until today primarily performed using archaic, scarcely controlled, and incomparable processes and methods. A deeper characterization of *ex vivo* expanded immune cells is urgently needed not only on the level of a few receptors and ligands on the cell surface but also with respect to the ever-contained subtypes in an expanded immune cell population, the pattern of secreted effector molecules, and their amounts over time and influences from *in vivo* components on them.

Keywords: immune cells, cell therapy, expansion technologies, T cells, TIL, NK cells, MSC, GMP production, ATMP

1. Introduction

Immune cells have been the key players as well as glamor of active clinical research of the current decade. T-lymphocytes (T cells), tumor-infiltrating lymphocytes (TIL), chimeric antigen receptor T cells (CAR-T cells), natural killer cells (NK cells), mesenchymal stem/stromal cells (MSC) from bone marrow, umbilical cord blood, umbilical cord tissue layers, placenta, and adipose tissues are the main objects studied in immune cell therapies for various diseases. Publications from numerous preclinical studies and developments on isolation, expansion,

IntechOpen

activation, and phenotyping of the different immune cells are increasing exponentially over the last years. Numerous clinical trials have been conducted or are running, evaluating immune cells as novel Advanced Therapy Medicinal Products (ATMP) therapy against number of diseases having no or inadequate alternative therapies available (see **Table 1**) [2]. There is a strong belief that progress for many severe, life-threatening diseases with bad prognosis that happens in various fulminant cancer types, cerebral stroke, cardiac infarction, severe autoimmune disorders, or chronic infections can either be effectively treated or even cured by immune cell therapy when applied optimally in combinations with other conventional therapies. It is not clear if this belief is justified at this juncture with adequate evidence. It is crucial to critically analyze this question for an insight to address the stumbling blocks to make significant advances in this field.

Isolated immune cells consistently display effectiveness against cancer cells, micro-organisms, inhibition of inflammation parameters, etc., in *ex vivo* test systems and often also in *in vivo* mouse models. The enormous number of positive observations are very encouraging and propel the immune cell research field and drives growing numbers of clinical trials. However, convincing results in clinical studies remain scanty over decades despite the wider engagement and R&D investments [3–6]. Several tumor-infiltrating lymphocytes (TIL) as well as treating therapy-resistant hematopoietic and solid tumor cancers with specific activated CAR-T cells show long-lasting benefits in otherwise grim cases [7, 8]. In cancer of the hematopoietic system, immune cell therapy has demonstrated its real potency and has become an effective standard therapy [9]. Leukemia and similar forms of cancer of the hematopoietic system can be cured with disease-free survival or without progression in a high percentage through the transplantation of bone marrow cells from healthy and genetically compatible (allogeneic) donors.

Immune cell type	Clinical trials in total	Open clinical trials	Clinical trials		
			Europe total	US total	China total
T-lymphocytes	2343	723	116	401	184
TIL	77	27	4	17	0
CAR-T-lymphocytes	170	123	7	40	78
NK cells	407	140	10	49	62
MSC	339	134	34	29	39
BM-MSC	104	46	12	12	7
UC-MSC	54	24	2	0	16
UCB-MSC	7	3	0	0	2
AT-MSC	20	6	1	1	2

Total number of clinical trials worldwide and in several geographical regions as registered on www.clinicaltrials.gov/, accessed on Feb 15, 2017 [1]. TIL: tumor-infiltrating lymphocytes; CAR-T-lymphocytes: chimeric antigen receptor lymphocytes; MSC: mesenchymal stromal cells; BM-MSC: bone marrow MSC; UC-MSC: umbilical cord MSC; AT-MSC: adipose tissue MSC.

Table 1. Current ongoing clinical studies with selected immune cells.

It is important to reflect into the realities of the limited clinical success in contrary to the promising *in vitro* or *ex vivo* findings. All the inadequate, inconsistent clinical outcomes observed using immune cells *in vivo* following *ex vivo* expansion and all the knowledge gained with immune cells in the numerous clinical trials and individual or small clinical studies using hospital exemptions might have to do with underestimating the key issues with respect to identity, quality, potency, and functionality of the used cells. All the characteristics of the cells as well as influences from production technology, characterization methods, etc. are seldom taken into broader account in robust ways before cells are infused as therapeutics. This will be highlighted in the following sections.

2. Requirements and existent challenges in producing immune cells for cell therapy

Looking on the methods, technologies, specific equipment, and analytical tools, it is obvious that cell expansion technologies suited for producing specific immune cells for individual immune cell therapy are the weak side of the story. The actual knowledge on the potentials of immune cells is so far only scarcely translated into practical technical solutions, broadly available methods, congruent with the capabilities. That is true for standardized and reproducible expansion of defined immune cell preparations as well as for estimates and methods of measuring specific functionalities of an expanded immune cell population against tumor cells, infections, or inflammations. Moreover, there are also missing generally available methods and techniques for fast and precise measurement of homogeneity of a cell population, of characteristics of sub populations, and single cells [10–13]. All these aspects appear to be widely responsible for the limited progress by these very promising new therapies. Several key issues are critical determinants for ATMP cell therapy which are enumerated below.

- Isolation of specific immune cells from blood or tissues and initial expansion steps are performed with broadly differing methods. The procedures need to be better standardized and harmonized with the validation of the composition of the starting populations of immune cells.

- Ideally, expansion of immune cells should be started with a pure population of specific immune cells. When a mixture of cells (like PBMC) or a piece of tissue are taken, isolation of cells should be performed in a defined procedure consistently.

- Immune cells for individual cell therapies must be produced in standardized, reproducible, and GMP conform processes. Amounts of 10^9 to more than 10^{10} cells of a pure immune cell population are assumed as a single optimal therapeutic dose. The manufacturing process with newer available technologies for each cell type is yet to be established coherently for the clinical trials.

- Standardized and consistent production processes need to be dynamically controlled and documented during the entire culturing process. Only a few production technologies are currently on the market fulfilling all of the abovementioned requirements and thus are capable of manufacturing individual cell therapeutics as ATMP.

- To ensure reproducibility of immune cell production for cell therapies, process conditions have to be controlled, evaluated, documented, and validated. Continuous dynamic control of temperature, pH, and pO_2 in the medium during the immune cell expansion process is indispensable. Glucose and lactate concentration as lead substances for substrates and metabolites should also be under steady control during processing.

- All immune cells can be expanded in a common basic medium. However, until now supplementing with serum or thrombocyte lysate is still indispensable. For optimized growth over a prolonged period of time and mass production of the different immune cell specimen, supply with nutrients, gases, and supplements should normally take place in a dynamic, homogenous, and stress-free process to maintain differentiation status, phenotype, and function of those cells within physiological ranges.

- Irradiated feeder cells are used to achieve an initial expansion with low numbers of isolated effector cells and to increase cell expansion when using insufficient technical equipment (e.g., wells or flasks with uncontrolled processing). Appropriate novel technologies can possibly eliminate the use of feeder cells.

- Important criteria of immune cells for therapies are a deeper marker profiling and standard estimates for chemokine and cytokine production efficiency (paracrine factors). Both together are decisive measures for the potency and functional power of immune cell populations/subpopulations for the intended effects. Standardized and comparable values as correlate measures should be mandatory for immune cell therapeutics which need to be established.

- Subpopulations or even monoclonal immune cells can be produced by guiding an *ex vivo* expansion process through specific activation/inhibition/triggering/priming the cells. Respective coating/fixed antibodies at bioreactor surface, O_2 concentration in culture conditions (hypoxic/normoxic/hyperoxic), flow dynamics of medium have to be explored, compared, and optimized for each condition.

- Immune cells for therapies have to fulfill all the conditions as well as national regulatory requirements and international standards for approval for clinical trial use as investigational medicinal product (IMP) and/or market authorization as ATMP. All the manufacturing steps of an individual immune cell preparation must still be performed in a Clean Room A containment for most processes used for production that consist of more or less open steps.

- Current international regulatory standards are in process of evolution as ATMP cannot simply follow the standard of a chemical compound which is a fixed inert molecule, whereas a cell is a living dynamic entity with too many variables and dynamic potentials. It is being considered that regulations on individual cell therapeutics might be registered as distinct category and might be oriented on indications. This needs to mature in course of time to bring ATMP cell therapy for real clinical use in routine practice.

- Faster scientific progress, earlier availability, access, and affordable prices of immune cell ATMP can only be achieved when advanced production technologies can be utilized or further developed to realize these desired objectives.

3. Techniques and methods used for isolation and expansion of immune cells

Blood from patients or donors is a convenient starting material for cell purification and expansion. **Table 2** shows the number of different immune cells contained in a 200 ml sample of peripheral blood. A simple gradient centrifugation of a blood sample is normally taken to obtain "peripheral blood mononuclear cells" (PBMC). *Ex vivo* expansion of the total fraction of T cells, NK, and other cell types within PBMC is possible and is the most commonly used technique to provide starting material for subsequent *ex vivo* expansion of the different immune cell types.

Apheresis is a standard practice to obtain a larger number of CD3+ cells as a starting material for CAR-T cell production with the goal of obtaining a minimum of 0.6×10^9 to a target of 2×10^9 CD3+ T cells [24]. Apheresis has the advantage and choice of extracting one or more components of the whole blood with help of the appropriate device and return the rest of the donation to the donor. Standard leukapheresis and use of anti-CD25 magnetic bead resulted in a yield of 130×10^6 CD4+ CD25+ T regulator (T_{reg}) cells that could be expanded 8.3-fold over three weeks before a dose of 1×10^9 can be used for clinical trials designed to control HLA-mismatched GvHD or organ transplantation rejection [18]. Although the apheresis technology is available in clinical research, no advantages have been comparatively established to that of peripheral blood as a source. Routine production of pure immune cell specimen by apheresis is much more expensive (due to the costly procedure and the infrastructure required to carry out the intervention as well as subsequent large amounts of antibodies

Immune cell types#	Number of cells in 200 ml of human blood sample (×10⁶)	Clones of differentiation commonly determined for identification	Reference
Neutrophils	400–1300	CD11b+CD16+CD66b+	[14, 15]
Lymphocytes	200–600	T cells, B cells (CD3−CD19+)	[16]
T cells, total	100–400	CD3+CD4+CD8+CD56−	[17]
Naive T cells	40–160	CD3+CD25+CD45RA+CD45RO− CD127+CCR7+CD62L	[17]
Memory T cells	40–160	CD3+CD56−CD45RA− CD45RO+CCR7+CD62L+	[17]
T regulatory cells	5–20	CD3+CD4+CD25+CD127−	[18, 19]
NK cells	16–80	CD3−CD16+CD56+	[20]
NKT cells	0.4–5	CD1d+CD4+CD161+	[21]
Dendritic cells	4–12	CD11c+HLA−DR+CD3−CD19− CD80+CD86+	[22]
Monocytes	40–180	CD14+CD16+CD64+	[23]

Table 2. Prevalence of immune cells in blood of healthy donors and surface marker normally used for their identification.

needed for purification of specific cell types from the large number of cells). Another study from Germany reported that ~10^{10} PBMC from leukapheresis result in about 8% of NK cell yield using Clinimacs (Miltenyi) for further GMP therapeutic expansion [20]. Difference between *ex vivo* expanded cells for cytokine-induced cell therapy for hepatocarcinoma from apheresis and PBMC-derived cells has been reported [25], but this needs further evaluation for a comparative conclusion for various cell types. The initial procedure of cell collection may have an influence on the biological effects of the final cell product.

The whole PBMC fraction consists mainly of T cells (naive and memory CD3+/CD56− T cells, mostly of central T memory type). A minor part of the PBMC contained NK cells identified as CD3−/CD56+ cells. Isolation and *ex vivo* expansion of CD3+/CD56− T cells or CD3−/CD56+ NK cells (and even other specific cell types in patients' PBMC) to bigger numbers does not pose a big problem. It is crucial that T cells are only effective against a cancer cell type or an infectious agent when specifically activated prior to infusion. Hence, T cells, NK cells, and other PBMC-derived cells have to be separated in the first instance and then specifically activated/primed during the expansion process. Presentation of peptides or epitopes from tumor cells or micro-organisms to T cells *ex vivo*, directly or mediated by dendritic cells, and subsequent *ex vivo* expansion was evaluated over prolonged period of time with no or only limited success in *in vivo* efficiency. *Ex vivo* expanded NK cells showed similar negative results, even when applied to patients in greater numbers [26]. It was recognized that (in blood of patients contained) T regulatory cells do strongly inhibit lytic power of T cells and NK cells. However, this seems not the only reason for disappointing results with only T cell or NK cell fractions.

It is being increasingly recognized that particularly in cancer treatment, breakthrough can be achieved with specific activation and expansion of sub-clones of T cells and/or defined NK cells *ex vivo*. The PBMC-derived cells must be specifically stimulated during expansion which can only be realized by sophisticated integration of steps in the process (either positive or negative selection of unwanted cells through antibodies; enhanced growth of cells of interest through speeding up their mitotic division). In case of T cells as much as possible, effector T cell clones should be present at least in the expanded population with capabilities to persist and execute its desired functions [27]. Expanded NK cells should express high cytotoxicity against patient's tumor cells or infected cells. Specific activation has to be induced during the initiation steps by specific coatings of the surface of the culture flasks/container/bioreactor as well as by addition of different cytokines and growth factors in the medium during the subsequent expansion phase [6, 28, 29]. Cultivation of larger numbers of immune cells under those conditions is often challenging and can scarcely be realized in culture flasks.

A general belief is that a basic prerequisite for a therapeutic application of immune cell types is the provision of adequate number of cells. It is assumed that about 10^{10} of effective immune cells must be used for treating cancer or infections [1]. A tumor or metastasis of 1 cm^3 size yields around 10^9 cells. In the common cytotoxicity assays using effector versus target cells, five to tenfold more effector cells are often needed to destroy one tumor or infected cell. However, such *ex vivo* results might lead into wrong direction. It is crucial that the cells should have strong binding ability and be armed with specific cytotoxicity against the target cells. Meanwhile, it is apparent that it is important to know how to select, activate,

trigger, and/or prime and expand immune cells *ex vivo*. Single T cell clones being trained already *in vivo* against mutated patients' tumor cells or infected cells are normally present in PBMC only in very low numbers, but they can destroy tumor or infected cells manifold [30]. Concerning T cells, identifying and isolation of specific sub-clones and *ex vivo* expansion of those clones should get more attention in the future research. Besides there are strong hints that treatment with immune cells can be successful only when a systemic response of the total immune system in a patient is achieved along with the local response in the lesions [31]. These are extremely important aspects in the context of production technology enabling to manufacture adequate effective immune cell type for optimal treatment of a patient and a holistic analytical follow up of systemic profiling of the patient in order to gain further insights to iteratively make the process more efficient.

In recent times, the isolation of TIL from tissue and/or microenvironment has proven a much more promising way to get access to T cells being already specifically activated by antigens/epitopes shed from/presented by cells of solid tumors or metastases they are originating from. Generation of TIL seems no longer a problem. Resected parts of tumor tissues taken by tumor biopsies or from the microenvironment of a tumor is the method of choice, and different suited techniques have been published [32, 33]. It is yet not evident whether devices for standardized processing for cell suspension from the initial tumor tissue by mechanical dispersion and/or additional treatment with enzymes are of advantage [34, 35]. Expansion of TIL even in bigger numbers is possible; up to 10^{10} can be grown in common media supplemented with serum or thrombocyte lysate and specific cytokines (see **Table 4**). Long time expanded TIL often contain CD3+/CD4+/CD8+TIL. Deeper FACS analyses make likely that sub-clones are contained which are primed and directed against some single mutated clones [30].

Currently, there has been a major interest in genetically manipulated T cells, which can be transduced with chimeric antigen receptors (CAR). These CAR-T cells express this single chain antigen-binding domain (scFv), which ideally binds to a tumor-associated antigen (TAA). The CAR-T cell/tumor-binding reaction induces an activation signal in the T cells strong enough to destroy tumor cells completely, and contrary to normal T cells, the cytotoxic power of CAR-T cells is not suppressed when CAR-T cells are administered *in vivo*. However, CAR-T cells often elicit a dangerous cytokine storm. This potential adverse effect and the difficult complex and costly construction of efficient CAR-T cells seems to inhibit fast progress with this promising cell therapy [41–43].

Natural killer (NK) cells are getting more and more attention in the ATMP field, fighting cancer and infections since these innate immune cells can be successfully expanded not only in greater numbers but also in high degree of purity [41–43]. In contrast to T cells, NK cells do not feature immunological incompatibility when administered in haploidentical or even allogenic clinical trials. It is, however, important that NK cells in such settings are totally free from T cells. Advanced production technology makes NK cells attractive to use them in broader clinical perspective. Pure NK cells have shown nearly no unwanted side effects in clinical trials even when administered in high doses. The modern production processes deliver NK cells with enhanced functionalities (high cytotoxicity against many cancer cells in *ex vivo* tests, enhanced paracrine production). Pure NK cells can be manufactured in an

easy-to-handle closed system as ATMP in clinical settings near to patients [44, 45]. A particular advantage is that mass amounts of individual NK cells can be produced in a relatively inexpensive way due to low costs for selection, medium, activation, compared with other functionalized immune cells. NK cells can be expanded 2000–50,000-fold in designated perfusion bioreactors, whereas that has by far not been achieved in culture flasks. Adjuvant treatment of stem cell-transplanted patients with pure NK cells becomes a common clinical practice. NK cells isolated from donor blood and expanded effectively avoid infections and GvHD when applied immediately following transplantation.

Common sources for isolation of MSC are bone marrow aspirate, cord blood, and pieces of umbilical cord/placenta tissue/adipose tissue. MSC were originally identified in the 1970s from cellular suspensions from spleen and bone marrow by their capacity to adhere to plastic—which is still the standard form to culture MSCs and also by their ability to form colonies from single cells (explanted *ex vivo*), their fibroblast-like appearance and their capacity to differentiate into fat, cartilage, and bone. MSC are defined by surface markers of CD105, CD90, and CD73 expression, yet not CD45, CD34, and CD14 as of the consensus of ICSCT working group [46]. Recently, ICSCT also have defined a broad consensus of the international standards for harmonized potency assays to boost the clinical development of ATMP MSC therapy for many unmet clinical needs despite different tissue sources and disparate culture expansion protocols. Three preferred analytic methods in a matrix assay approach, namely, quantitative RNA analysis of selected gene products; flow cytometry analysis of functionally relevant surface markers, and protein-based assay of secretome have been proposed to reflect on the immunomodulatory potential of the ATMP cells for different clinical therapeutics as well as to evolve the regulatory landscape for the sake of the progress in the field [47].

Several techniques are employed for liposuction used for adipose tissue-derived stromal cell collections [48]. Processed lipoaspirate (LPA) contains multipotent cells that can be an alternate stem cell source to bone-marrow-derived MSCs. LPA contains stromal vascular fraction (SVF) containing a number of different cell types such as adipose stromal cells (ASC), pericytes, endothelial cells, fibroblasts, preadipocytes, and hematopoietic stem cells. ASC have differentiation potential to myogenic, osteogenic, chondrogenic, or adipogenic on culturing with specific induction media [49]. SVF contains a lot of vascular cells and hematopoetic cells that have to be eliminated before expansion of remaining MSC.

For the isolation of MSC adherence of these cells, plastic surfaces are used. The usual procedure is to put the starting material into culture flasks or discs. After 10–20 days, nonadherent cells are washed out, and the adhered MSC colonies are passaged, suspended in a fresh medium, and seeded in new flasks for further expansion. Thus, expanded MSC have been used in all clinical trials (see **Table 7**). The unavoidable detaching procedures of MSC at passaging influence the receptor quality of MSC. Long-term cultivation of big numbers of MSC in bioreactors is possible and the provision of large seeding areas avoids unwanted differentiation of expanded MSC [54–57]. Procedures have been worked out to proceed directly into the expansion phase of MSC. Outgrowth and isolation of MSC can be successfully performed by giving BM aspirate into the sterile plastic vessels of perfusion bioreactors. MSC from BM aspirate are not only diverse tumor tissue preparations but can also be placed directly in ZRP meander perfusion bioreactors. MSC colonies or outgrown TIL from tumor tissue pieces can then further be

expanded to big numbers of both cell types in the same bioreactor system under totally closed conditions (unpublished results with ZRP meander type bioreactors, Zellwerk, Germany).

The reports about the ever-used methods in the clinical trials do not always contain complete descriptions of the processes and show inconsistencies. However, in general, it can be seen that high-fold expansion as well as big numbers of cells have only been achieved when irradiated feeder cells are used. This might be tolerable during a developmental phase of immune cell therapeutics but should be overcome in case of ATMP manufacturing. In ZRP perfusion bioreactors it was shown that individual immune cell specimen can be expanded to therapeutic amounts without irradiated feeder cells.

4. Quantity and quality of T cells, TIL, and CAR-T used in acknowledged clinical trials

T cells are the most powerful immune cells. Despite the immense research on these cells enriching our deeper knowledge into T cell biology as well as their kinetics in health and diseases, the different T cell subsets are yet not routinely used as cell therapeutics. This is at least partly due to the complex nature of immune cells and many unsolved technical problems to produce and handle them. In **Tables 3–5**, some parameters have been compiled giving insight into methods and technologies as well as expansion success and purity of the different T cell types being used in clinical trials. The chosen examples in the tables are representative of the field and enlighten the diversity of production processes and produced cell specimen, and it explains probably in part the inconsistent and predominantly unsatisfying clinical results with more or less identical cell specimens.

Vaccination with dendritic cells being *ex vivo* treated with proteins or peptides from tumor cells or micro-organisms as well as present target-specific peptides to T cells *ex vivo*, directly

Cell source; supplements; activation	Expansion device used	Cultivation time (days)	Expansion by x-fold	Cell harvest	Cell purity (%)	Reference
PBMC/apheresis isolated by αCD3+ coated dynabeads, exp with IL2/αCD28	Wave system	13	101	1.37×10^{11} T cells	98.5% CD3+	[27]
PBMC	Flask	14	169	33×10^8	62%	[58]
PBMCs/apheresis CD25+Tregs depl, Stim with autol DC pulsed MART peptides, suppl IL2, IL7, IL21, CD8+ CTL sorted, exp Rapid Exp Prot	Flask	42	n.a.	n.a.	n.a.	[59]

Table 3. Manufacturing processes of T cells in clinical trials (n.a.= not available).

Cell source; supplements; activation	Expansion device used	Cultivation time (days)	Expansion by x-fold	Cell harvest	Cell purity (%)	Reference
TIL, IL2, serum, feeder cells	Bag	>14	1041	5×10^{10}	n.a.	[36]
TIL, IL2, serum, feeder cells, anti-CD3	Flask Wave	24	72 228	0.4×10^{10} 1.5×10^{10}	35 + 52 CD4 + CD8 35 + 63	[37]
TIL (6x GRex10) TIL, IL2,	GRex Flask	29	180 170	NA	62–72 CD3+ CD8+ 62	[38]
TIL, IL2, feeder cells, anti CD3	Bag Wave	>21	1259 1130	4.5×10^{10} 1.5×10^{10}	CD3+: F = W CD4+: F > W CD8+: F < W	[39]
TIL, IL2, feeder cells, anti CD3	Flask Wave	>14	1433 5576	NA	>97	[40]
TIL, serum, feeder cells, anti CD3, IL2, IL 15, IL21	ZRP Meander bioreactor	>20	5000	2×10^{9}	CD8: ~60% CD4: 38% (High% Tem)	Own data, not published

Table 4. Manufacturing processes of TIL in clinical trials (n.a.= not available).

Cell source; supplements, activation	Expansion device used	Cultivation time (days)	Expansion by x-fold	Cell harvest	Cell purity (%)	Reference
PBMC, anti-CD3, IL-2, feeder cells, serum	Well plates	14–24	n.a.	n.a. (infusion: $\leq 10^{10}$)	n.a.	[41]
PBMC, anti-CD3, anti-CD28	Bag	12	10.6	n.a.	CD3+: >95	[43]
PBMC, anti-CD3, IL-2, IL-15	WAVE	35	n.a.	10^{9}	n.a.	[33]
PBMC, serum, feeder PBMCs, IL-2	Bag	≤48	15.000	$\sim 3 \times 10^{11}$	CD4+: ≤82 CD8+: ≤85	[60]

Table 5. Manufacturing processes of CAR-T-cells in clinical trials (n.a.= not available).

or mediated by dendritic cells, and subsequent *ex vivo* expansion is used over a long time, and there are even a few cell therapeutics on the market. However, adjuvant treatment in different conditions of cancer in clinics show only limited success. In recent times, the isolation of TILs from tissue and/or microenvironment of solid tumors and expansion of TILs *ex vivo* has proven a much more promising way to get specific activated T cell in larger amounts.

From **Table 3**, it can be observed that the fold expansion in flasks and Wave bioreactor is similar, although the total number of T cells harvested differs enormously. Whether the (apheresis

derived) large number of generally stimulated T cells was of advantage for the treated patients could not be evidently established from published literatures. The example of CTL expansion and therapeutic use did not state details of the expansion process with missing information.

To get effective, functional T cells out of PBMC expansion process have to be "conditional" (activation of sub-clones by specific cancer/micro-organism-derived structures; naive cell/cell and cell/matrix contact; preferred or suppressed growth of a cell population/sub population by specific cytokine/growth factor/antibody stimulation; controlled gas and nutrient supply).

It is obvious from **Table 4** that the expansion of TIL even in bigger numbers is possible, up to more than 10^{10} TIL might be grown in flasks as well as in bags, G-rex flasks, Wave bioreactors, or ZRP perfusion bioreactors. Media and supplements are qualitatively similar. In all examples, use of feeder cells seem to be essential. Looking closer into **Table 4**, the fold expansion differs over a wide range. This implies that strongly differing numbers of TIL must have been isolated. G-Rex flasks is of advantage for TIL expansion by providing high O_2 concentration near to the sedimented TIL that enhances growth. In own studies (not published) of ZRP meander bioreactors, O_2 (Zellwerk, Germany) concentration can be up-regulated to find an optimal O_2 value in the medium for TIL growth.

Although a lot of clinical trials with TIL have been performed so far, there is not much information about clinical efficiency of TIL (with an exception of late-stage metastatic melanoma). Expanded TIL over a prolonged period often contains CD3+/CD4+/CD8+CD25+cells in different ratios. Deeper FACS analyses make likely that subclones that are contained are primed and directed against some single mutated clones. Again, it must be emphasized that even with TIL stronger standardized cell production processes, better characterization of contained clones, more comparable preclinical data as well as professional designed clinical trials are needed.

The expansion of several CAR-T cell specimen seems not to be a problem (**Table 5**). Blood-derived natural T cells were used for CAR transduction. Expansion to large numbers of the CAR-T cells was achieved by using the same interleukin activation and culturing procedures as in the case of normal T cells. Some of the advanced centers are engaged in optimizing newer technologies including Miltenyi's CliniMACS Prodigy as well as Zellwerk's ZRP technology system.

5. State-of-the-art with *ex vivo* expanded NK cells utilized in clinical trials

NK cells are getting more and more attention in fighting cancer and infections since it succeeded to expand these immune cells not only in huge amounts but also in pure quality [4–6, 61]. In contrast to T cells, NK cells do not show immunological incompatibility when administered in haploidentical or even allogenic clinical trials. It is, however, important that NK cells in such settings are totally free from T cells. In **Table 6**, a selection of clinical studies is listed, among them are those that are performed by known clinical research groups. It can be deduced that bigger numbers of NK cells can be produced in culture flaks as well as in some cell culture equipment consisting of plastic bags or vessels (VueLife static culturing in bags; wave system using different bags, mixing of medium by slow shaking movement of the bag). The bag systems are closed

systems, and some of the equipment are provided with regulating and/or steering elements for gasing, measurement of pH, pO_2, and medium temperature is obligatory. However, changing bag volumes and upscaling within a cultivation run is not easy and possible with these systems. One main advantage of NK cells is the good compatibility of this immune cell specimen. NK cells can be expanded up to 50,000-fold in suited perfusion bioreactors (**Table 6**), whereas this is by far not achieved in culture flasks. Adjuvant treatment of stem cell-transplanted patients with pure NK cells becomes common. NK cells isolated from donor blood and expanded avoids effectively GvHD when applied during the first phase after transplantation.

Advanced production technology makes NK cells attractive for use in broader bases: Pure NK cells have shown nearly no unwanted side effects in clinical trials even when administered in high doses. The modern production processes deliver NK cells with enhanced functionalities (high cytotoxicity against many cancer cells in *ex vivo* tests and enhanced paracrine production). Pure NK cells can be manufactured in an easy-to-handle closed system as ATMP in clinical settings near to patients. A particular advantage is that mass amounts of individual NK cells can be produced economically (due to low costs of selection, medium, and activation).

Cell source; supplements, activation	Expansion device used	Cultivation time (days)	Expansion by x-fold	Cell purity	Reference
PBMC fraction, serum; IL 2,	Stirred bioreactor (750 ml)	33	352	96%	[62]
PBMC fraction; serum; IL 2;IL 15; PHA	VueLife bag system (800 ml)	14	80–200	85–91%	[63]
PBMC fraction; serum; IL2/IL12/IL15; feeder cell line	T-flaks	10	40	62–95%	[64]
PBMC fraction; serum; IL 2; IL 15; feeder cell line K529; 4 BBL1	VueLife bag system	21	277	97%	[65]
PBMC fraction; medium; serum; IL 2	Wave bioreactor	21	12–354	37–54%	[66]
PBMC fraction; medium; serum; IL 2; feeder cell line K529;	G-Rex flask	8–10	442	54–79	[67]
PBMC fraction; medium; serum; IL 2;	ZRP type M single-use bioreactor	30	1000–2000	95–99%	Own data, not published
PBMC fraction; serum; IL 2/IL 21; coating with CD 16	ZRP typ M single-use bioreactor	30–35	1000–50,000	99%	Own data, not published

Table 6. NK cells for cell therapies. Different production methods.

6. State-of-the-art with *ex vivo* expanded MSCs utilized in clinical trials

With numbers of 10^9 MSC, therapeutic effects were reported in the treatment of autoimmune and infectious diseases [68, 69]. The issue on reliabilities and logistically practical methods for

production of MSC for adequate amounts of immune cells needs specific attention. Most of the current clinical trials use open culture system in flasks even though there have been ongoing efforts with newer technologies (compare **Table 7**).

Consistent and logistically practical methods for production of MSC for adequate numbers of immune cells need specific attention. In addition, there are many unresolved issues relating to the isolation, expansion technique, phenotyping characterization, mechanisms of action, and incomparability of study results due to different protocols and definitions. In most of the current clinical trials, open culture system in flasks is in use (compare **Table 7**) even though there have been ongoing efforts with newer technologies [56, 70, 71]. In contrast, MSC, in particular those derived from bone marrow, lead candidates to fight many important disease entities. It is believed that modulation of immune responses, remodeling of impaired tissues, pro-regenerative as well as antifibrotic effects can be attained with these immune cells. With numbers of 10^9 MSC, effects were reported in treatment of some autoimmune and infectious diseases [68, 69]. A lot of clinical trials have been performed or are ongoing on treating cardiovascular diseases (myocardial infarction, cardiomyopathy, critical limb ischemia, stroke [72–74]). Single or repeated doses of 10^7 to more than 10^9 are injected systemically or topically. Until now, some positive influences on cardiovascular disease could be ensured through cytokines and chemokines secreted from MSC. Avoiding GvHD is also under intense investigation due to increasing stem cell transplantation in cancer and organ transplant patients. In all these indications, autologous or allogenic MSC are being infused that are not really manufactured under controlled conditions.

Cell source; supplements	Expansion device used	Cultivation time (days)	Expansion by x-fold	Cell harvest	Cell purity	Reference
BM-MNC, serum	Flasks	30	n.a.	n.a.	99%	[50]
BM-MNC, serum	Flasks	30–45	6–52	$2.4–5.7 \times 10^7$	>60%	[51]
BM-E serum	Five layer flasks	10–28	n.a.	$6–27 \times 10^7$	n.a.	[52]
BM-W, serum 59 donors	Five layer flasks	28	5–145	2×10^7 to 5 $\times 10^9$	n.a.	[53]
UC blood, serum	FIve layer flasks	22–28	n.a.	$1–8 \times 10^8$	n.a.	[52]

Table 7. Manufacturing processes of MSC in clinical trials (n.a.= not available).

7. Newer techniques for characterization and production of immune cells therapeutics

Identification of different cell types, subpopulations, and even single subclones within a final cell therapeutics product can be a challenging exercise due to many constraints including the limited number of cells available. Immune cells are usually identified from its displayed surface receptors that also gives a hint on its characters to fight infections or cancer. Cell sorting by flow cytometry or magnetic beads are modern techniques allowing isolation and separation

of immune cell subtypes. These methods are still time-consuming and costly exercises due to the quantity of antibodies and reagents required in the manufacturing process when dealing with larger starting material such as cells derived through apheresis. GMP-grade antibodies are particularly costly making the ATMP cell therapy an unaffordable range.

However, it is pertinent to analyze and to predict the potency of the cells that give a prior indication of the anticipated possible effects of the immune cells on the intended outcome. In case of T cells, it has been recently accomplished by tracking the fate and origin of clinically relevant adoptively transferred CD8+ T cells *in vivo* to identify and track single subclones specifically activated against few tumor cell mutants specific T memory cells [30]. By using high-throughput T cell receptor sequencing, the group has worked out a strategy to identify and track those very low frequency monoclonal T cells among the total bulk of polyclonal T cell pool with varying cancer-killing and fighting capabilities that have been given as adoptive cell therapy to 10 metastatic malignant melanoma patients being specifically activated against melanoma and correlated with the treatment response in patients. They were then also able to decipher the specific clonal population of extremely low density of T cells that were persisting and effective *in vivo* among two patients out of ten demonstrating complete remission. It is worth proving whether this new approach can be applied effectively in clinical practice and to prove its rationale in other cancer types. Only around 0.001% of all T cells in blood consisted of these active monoclonal T cells. This study also indicated that the younger T cells nearly in the phase of development had a better ability to fight tumors than older ones.

Further progress in the identification of subpopulations, primed monoclonal T cells, and information on functionality of cell preparations may be obtained by the spectra of single cells, their typical receptors/ligands/paracrine production. Raman microscopy has long been used in cell and metabolites analysis. However, the combination of this method with sophisticated software programs with in-depth analyses tools can lead to sharper, high-resolution Raman spectra enabling differentiating looks onto cells enabling subtype identification, quantification, analysis of functional status, etc. [10–13].

CyTOF, the latest novel format of flow cytometry combined with mass spectrometry, often referred as mass cytometry provides a measurement of >40 simultaneous cellular parameters at single-cell resolution, significantly augmenting the ability of cytometry to evaluate complex cellular systems and processes at any given point. This has been a greatest tool to unravel the mechanism of immune cells by studying kinetics before and after infusion. Currently, research on solid cancers has a strong focus on immune cells infiltrations in the microenvironment of tumors. In a mouse model of triple negative breast carcinoma, the new methods using CyTOF assessing immune cells at single cell level within the tumor in the microenvironment of the tumor as well systemically in different organs over time demonstrated a striking difference of the sustained response at systemic level in the effective treatment responsive group in comparison to the nonresponsive treatment group [31]. Local carcinoma treatment was followed by an infiltration of CD8+/CD4+ T cells into the tumor leading to death of tumor cells. Different immune cell specimens were expanded during the rejection phase not only in the microenvironment of the carcinoma but also in many lymphatic organs and blood to reflect on the changes taking place systemically. A systemic coordinated immune response of

CD4+ as well as CD8+ T cells, NK cells, DC cells, and activated B cells seems to be essential for achieving sustainable effects to eradicate tumors with immune cell therapy. This has much more connotation for the ATMP cell therapy to reach its full potential.

The ZRP platform of Zellwerk and the belonging bioreactor types provide sophisticated features not only for mass production of different immune cells but also for realizing isolation and culturing the cells in closed processes [54–57, 70, 75]. It enables controlled phases of selection/priming/activation of seeded cells by regulated medium flow, suited coating of seeding surfaces and/or fixed antibodies, followed by rapid expansion, all in a single run. This is due to the technical attributes:

Bioreactors of the ZRP system can be operated in the GMP breeder (**Figure 1**). The breeder combines a laminar flow sterile bench and an incubator. Controlling of the essential bioreactor and breeder functions is by the control unit (automatic regulation of pO_2, pH, medium temperature, medium feeding, mixing, and flow of gasses over a touch screen).

The cell cultivation platform and the belonging bioreactors enable the manufacturing of large quantities of individual immune cell preparations under GMP conditions. A series of ZRP systems can be driven in parallel in one clean room equipment due to the closed steps of the perfusion bioreactor processes. During a period of 1 year, several immune cell preparations from individuals amounting to ~100 can be undertaken in one B clean room thus reducing expenditures for production of immune cell therapeutics massive. Manufacturing of immune cells as ATMP is authorized by the national and regional German authorities.

Important features of the meander type bioreactors are as follows: A directed laminar flow of medium, which can be chosen over a wide range, makes an undisturbed cell/cell- and cell/surface-contact possible and minimizes cell stress. The ratio of medium circulation and fresh medium flow is automatically regulated over time by a chosen algorithm guaranteeing ananan consistent homogenous supply with nutrients and gasses as well as precise regulated pO_2, pH, and temperature in the medium. T cells, TILs, and NK cells can be expanded to more than a 10^{10} cells in one closed cultivation run. In parallel cultivation run with pure NK

Figure 1. Zellwerk's GMP Z®RP cell breeder with M type bioreactor for NK/T/TIL cells.

cells using the same medium and density of seeded cells, expansion in static flasks was not more than 50–100-fold, whereas in meander type bioreactor 5000–50,000-fold was achieved. Coating of seeding areas with specific matrices/antibodies, i.e., can be exploited to promote suppression or expansion of several immune cell specimen (e.g., Treg; CD56^{bright} NK cells).

8. Current advances and future prospects

Despite many clinical trials with immune cell therapy and much progresses in the field, the manufacturing of cells as ATMP for routine clinical use has not been realized in its full potential. Here we attempt to give an overview of different advanced technologies and devices currently available for cell expansion. These different devices need to be validated and compared in head-to-head comparison for different cell type to exploit the opportunities for unmet clinical needs.

Sterility, purity, identity, and potency are four cardinal requirements. Initially, cell expansions have been carried out in the conventional process of open flask culture system with gradual incremental innovations to meet the growing needs of regulatory requirements as well as better quality products in terms of safety (phenotype), consistency (reproducibility and controlled robust process), numbers, quality (structural and functional), and efficacy (functionality and potency) to qualify as ATMP to be used in patients/human subjects. From simple culture flasks, multilayered flask was made for ease of handling as well as scaling up with the numbers (such as Millicell, Millipore or BD Multiflask).

Different microcarrier system came into place to increase surface area to expand cells in 3D than in flasks in 2D [76–78]. G-Rex system is a gas permeable rapid expansion device with a silicone membrane at the base, allowing gas exchange to occur uninhibited by the depth of the medium above with high O_2 concentration stimulating cell expansion and simplify handling [99]. WAVE-type bioreactor systems [79] make use of sterile, transparent, and disposable culture bags with provision for media perfusion, harvest, sampling, and gas exchange, which greatly reduces the cross-contamination problem, one of the biggest challenge in conventional culture system [37, 38]. The culture bag is put on the device's temperature-controlled tray inside an incubator having the option of controlled rocking movement. Optional perfusion modules enable controlled addition and removal of culture media for optimized nutrient concentrations while disposing spent media resulting in higher cell densities with involvement of less time and effort for media exchange. Magnetic beads are used in the bag for various priming and activation of cells [80]. However, the chaotic medium flow causes cell stress [78, 79]. These factors have been well addressed in ZRP cell breeder that has controlled and directed medium flow and fulfills most of the regulatory requirements (see Section 7).

MILTENYI's CliniMACS Prodigy® [81] is an automated integrated sensor-controlled closed system device that uses single-use sterile disposable tubing set and can perform fractionation of cells, cell washing, cell separation, cell culture, and final product formulation in the workflow connected through sterile docking devices. TERUMO quantum system is a functionally

closed automated hollow fiber bioreactor system for GMP cell manufacturing that has been tried in MSC expansion [82]. Huge efforts are ongoing in Japan to build modular robotics-manipulated, automated high-throughput cell-processing machine to make off-the-shelf cell therapy products for wider application [83].

Product dictates the process development and optimization to ensure cost-effectiveness, well-controlled steps, high reliability, high cell density and viability, high product quality, easy recovery, high yield, and high safety for personnel. Perfusion system takes care of controlled dynamic gas exchange with a homogenous environment allowing high cell density with controllable and flexible process control, but demands complicated validation procedure.

Evolving regulatory landscape for ATMPs (cell therapy) [84–87] has the following two mandates to be fulfilled (quoted from EMA document [88]):

• Cells or tissues that have been subjected to substantial manipulation so that biological characteristics, physiological functions, or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor.

• Have properties for, or are used in or administered to human beings with a view to treating, preventing, or diagnosing a disease through the pharmacological, immunological, or metabolic action of their cells or tissues.

Regulatory landscape for ATMP cell therapy at this juncture is quite diverse in various regions of the world and is evolving though in a gradient [89–95]. It is a daunting task, but is an absolute necessity to harmonize all the harmonizers (regulatory bodies) across the globe with an unified coherent process to make ATMP cell therapy possible for unmet clinical needs. The regulatory bodies have the responsibilities to assure the safety and rights of patients and to ensure quality of the nonclinical and clinical evidences to allow appropriate evaluation of the safety and effectiveness of the cell therapy product through clinical trials for market authorization. FDA and EMA have distinct guidelines, whereas there are distinct guidelines in other parts such as Australia, Brazil, Japan, Korea, Singapore, and Taiwan just to give a flavor of the unique differences that pose constraints in the current clinical development of ATMP cell therapy. While such efforts to converge on an unified regulatory process are underway through engagements, two critical issues have been identified to be addressed, the concept of potency assessment as well as *in vivo* tumorigenicity studies [96].

FDA has developed a number of expedited programs to facilitate ATMP cell therapy use in patients when no satisfactory alternative therapies are available while ensuring the standards of the products for safety and efficacy. EMA made a new regulation in 2009 for all 28 member countries in EU with obligatory centralized market authorization process. Eight cell therapy ATMPs have been authorized in EU [97], namely ChondroCelect (withdrawn in January 2017) and Maci (suspended from July 2014 due to closure of European manufacturing unit), both the products are autologous cartilage cells grown *ex vivo* for cartilage repair; Provenge (autologous PBMC activated with fusion protein of prostatic acid phosphatase attached to GM-CSF *ex vivo*) for treatment of advanced prostate cancer, withdrawn in May

2015; Holoclar, autologous limbal stem cells to repair damaged corneal epithelium, this has orphan drug status due to rare condition. A great deal of effort are underway in EMA to refine and execute adaptive regulatory pathway to foster rapid development and accelerated assessment for innovative cell therapies. Korea has 14 cell products authorized including four stem cell products with 46 ongoing clinical trials with other cell therapy products. New adaptive regulatory framework has been enacted under the Pharmaceuticals, Medical Devices and Other Therapeutic Products Act (PMD Act) in Japan in late 2014 to facilitate access of promising ATMP cell therapy to the patients with limited treatment options as well as creating conducive regulatory environment to give accelerated conditional and time-limited authorization to stimulate further clinical development. Two products namely, MSC for GvHD second-line therapy and skeletal myoblast sheets for ischaemic heart failure have been authorized under the new scheme in 2015 and 2016.

Since cell culture uses animal-derived serum or growth factors, the sources need to be certified and proven free of any adventitious agents, consistent in quality and free of risk of any possible infections.

First ATMP cell therapy product to get market approval was in Canada for Prochymal (Remestemcel-L), adult MSC for IV infusion for acute GvHD in May 2012. Allogeneic adipose tissue-derived MSC expanded *ex vivo* have been shown efficacious in Phase 3 clinical trial when given intra-lesionally in complex perianal fistulas in Crohn's disease patients and a decision is awaited in 2017 for market authorization by EMA. This product named as Cx601 by Takeda and TiGenix has received orphan status by the Swiss Agency for Therapeutic Products (Swissmedic) for the rare disease. While these innovations are taking place at immense pace, there is growing requirement of policymakers to be engaged along with patients' community to see how best a value-based frameworks and be drawn as rational approaches to use these expensive novel therapeutic modalities in patient care [98].

9. Conclusion

The manufacturing of immune cells is until today primarily performed using archaic, scarcely controlled, incomparable processes and methods. These issues need to be better harmonized and put into standard practice. When looking into the processes of immune cell production used in clinical studies, it is obvious that the cells in most settings are expanded totally or partly in conventional culture flasks or similar vessels. That is due to the fact, that all immune cell types can be grown in this simple and cheap way without special skills. However, deeper characterization of *ex vivo* expanded immune cells is urgently needed not only on the level of a few receptors and ligands on the cell surface but also with respect to the ever-contained subtypes in an expanded immune cell population, the pattern of secreted effector molecules, their amounts over time and influences from *in vivo* components on them.

More research on aspects of modern cell therapy might be qualified as too costly, but will be more targeted and will at least avoid expensive and unjustified clinical studies maximizing the best use of the available R&D resources for better outcomes.

Conflict of Interest

All authors RP, SKP and CS do not have any conflict of interest. HH is the CEO of Zellwerk GmbH.

Author details

Ralf Pörtner[1]*, Shreemanta K. Parida[2], Christiane Schaffer[1] and Hans Hoffmeister[3]

*Address all correspondence to: poertner@tuhh.de

1 Hamburg University of Technology, Institute of Bioprocess and Biosystems Engineering, Hamburg, Germany

2 Independent Global Health and Translational Medicine Consultant, Berlin, Germany

3 Zellwerk GmbH, Oberkrämer, Germany

References

[1] ClinicalTrials Registry. USA: NIH; 2017

[2] Hanna E, Remuzat C, Auquier P, Toumi M. Advanced therapy medicinal products: Current and future perspectives. Journal of Market Access & Health Policy. 2016;**4**: 31036. DOI: 10.3402/jmahp.v4.31036

[3] Galluzzi L, Zitvogel L, Kroemer G. Immunological mechanisms underneath the efficacy of cancer therapy. Cancer Immunology Research. 2016;**4**:895-902. DOI: 10.1158/2326-6066.CIR-16-0197

[4] Koepsell SA, Miller JS, McKenna DH. Natural killer cells: A review of manufacturing and clinical utility. Transfusion. 2013;**53**:404-410. DOI: 10.1155/2011/676198

[5] Levy EM, Roberti MP, Mordoh J. Natural killer cells in human cancer: From biological functions to clinical applications. Journal of Biomedicine & Biotechnology. 2011;**676198**. DOI: 10.1155/2011/676198

[6] Miller JS. Therapeutic applications: Natural killer cells in the clinic. Hematology. ASH Education Book. 2013;**1**:247-253

[7] Yang JC, Rosenberg SA. Adoptive T-cell therapy for cancer. Advances in Immunology. 2016;**130**:279-294

[8] Wang Z, Wu Z, Liu Y, Han W. New development in CAR-T cell therapy. Journal of Hematology & Oncology. 2017;**10**:53

[9]   Universal CAR T cells treat leukemia. Cancer Discovery. 2017;**7**(4):342. DOI: 10.1158/2159-8290.CD-NB2017-023

[10] Leelatian N, et al. Single cell analysis of human tissues and solid tumours with mass cytometry. Cytometry. Part B, Clinical Cytometry. 2017;**92**:68-78

[11] McReynolds N, Cooke FG, Chen M, Powis SJ, Dholakia K. Multimodal discrimination of immune cells using a combination of Raman spectroscopy and digital holographic microscopy. Scientific Reports. 2017;**7**:43631

[12] Ichimura T, et al. Non-label immune cell state prediction using Raman spectroscopy. Scientific Reports. 2016;**6**:37562

[13] Next Generation Cell Analysis. In: www.horizon2020projects.com, editor; 2016

[14] Oh H, Siano B, Diamond S. Neutrophil isolation protocol. Journal of Visualized Experiments. 2008;**17**:745

[15] Lakschevitz FS, et al. Identification of neutrophil surface marker changes in health and inflammation using high-throughput screening flow cytometry. Experimental Cell Research. 2016;**342**:200-209

[16] Boyum A. Isolation of lymphocytes, granulocytes and macrophages. Scandinavian Journal of Immunology. Supplement 1976;**5**:9-15

[17] Golubovskaya V, Wu L. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. Cancers (Basel) 2016;**15**:8(3). pii: E36. DOI: 10.3390/cancers8030036

[18] Cherai M et al. Generation of human alloantigen-specific regulatory t cells under good manufacturing practice-compliant conditions for cell therapy. Cell Transplantation. 2015;**24**:2527-2540

[19] Haase D, et al. Large-scale isolation of highly pure "untouched" regulatory T cells in a GMP environment for adoptive cell therapy. Journal of Immunotherapy. 2015;**38**:250-258

[20] Torelli GF, et al. A good manufacturing practice method to *ex vivo* expand natural killer cells for clinical use. Blood Transfusion. 2015;**13**:464-471

[21] Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. Nature Reviews. Immunology. 2012;**12**:239-252

[22] Reardon DA, Mitchell DA. The development of dendritic cell vaccine-based immunotherapies for glioblastoma. Seminars in Immunopathology. 2017;**39**:225-239

[23] Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. Nature Reviews. Immunology. 2017;**17**(5):306-321. doi: 10.1038/nri.2017.11

[24] Allen ES, Stroncek DF, Ren J, Eder AF, West KA, Fry TJ, Lee DW, Mackall CL, Conry-Cantilena C. Autologous lymphapheresis for the production of chimeric antigen receptor T cells. Transfusion 2017;**57**:1133-1141

[25] Liu H, et al. Comparative study of different procedures for the separation of peripheral blood mononuclear cells in cytokine-induced killer cell immunotherapy for hepatocarcinoma. Tumour Biology. 2015;**36**:2299-2307

[26] Parkhurst MR, Riley JP, Dudley ME, Rosenberg SA. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumour regression. Clinical Cancer Research. 2011;**17**:6287-6297

[27] Hami LS, et al. GMP production and testing of Xcellerated T cells for the treatment of patients with CLL. Cytotherapy. 2004;**6**:554-562

[28] Konjevic G, Vuletic A, Mirjacic MK. Natural killer cell receptors. Alterations and therapeutic targeting in malignancies. Immunologic Research. 2016;**64**:25-35

[29] Huber CM, Doisne JM, Colucci F. IL-12/15/18-pre activated NK cells suppress GvHD in a mouse model of mismatched hematopoietic cell transplantation. European Journal of Immunology. 2015;**45**(6):1727-1735

[30] Chapuis AG, et al. Tracking the fate and origin of clinically relevant adoptively transferred CD8+ T cells *in vivo*. Science Immunology. 2017;**2**(8):eaal2568

[31] Spitzer MH, et al. Systemic immunity is required for effective cancer immunotherapy. Cell. 2017;**168**:487-502, e415

[32] Ellebaek E, et al. Adoptive cell therapy with autologous tumour infiltrating lymphocytes and low-dose Interleukin-2 in metastatic melanoma patients. Journal of Translational Medicine. 2012;**10**:169. DOI: 10.1186/1479-5876-10-169

[33] Wang X, Riviere I. Manufacture of tumour- and virus-specific T lymphocytes for adoptive cell therapies. Cancer Gene Therapy. 2015;**22**:85-94

[34] Donia M, et al. Characterization and comparison of 'standard' and 'young' tumour-infiltrating lymphocytes for adoptive cell therapy at a Danish translational research institution. Scandinavian Journal of Immunology. 2012;**75**:157-167

[35] Topalian SL, Muul LM, Solomon D, Rosenberg SA. Expansion of human tumour infiltrating lymphocytes for use in immunotherapy trials. Journal of Immunological Methods. 1987;**102**:127-141

[36] Itzhaki O, et al. Establishment and large-scale expansion of minimally cultured "young" tumour infiltrating lymphocytes for adoptive transfer therapy. Journal of Immunotherapy. 2011;**34**:212-220

[37] Sadeghi A, et al. Large-scale bioreactor expansion of tumour-infiltrating lymphocytes. Journal of Immunological Methods. 2011;**364**:94-100

[38] Jin J, et al. Simplified method of the growth of human tumour infiltrating lymphocytes in gas-permeable flasks to numbers needed for patient treatment. Journal of Immunotherapy. 2012;**35**:283-292

[39] Somerville RP, Devillier L, Parkhurst MR, Rosenberg SA, Dudley ME. Clinical scale rapid expansion of lymphocytes for adoptive cell transfer therapy in the WAVE(R) bioreactor. Journal of Translational Medicine. 2012;**10**:69

[40] Donia M, Larsen SM, Met O, Svane IM. Simplified protocol for clinical-grade tumour-infiltrating lymphocyte manufacturing with use of the Wave bioreactor. Cytotherapy. 2014;**16**:1117-1120

[41] Morgan RA, et al. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Molecular Therapy. 2010;**18**:843-851

[42] Wang X, Riviere I. Clinical manufacturing of CAR T cells: Foundation of a promising therapy. Molecular Therapy Oncolytics. 2016;**3**:16015

[43] Tumaini B, et al. Simplified process for the production of anti-CD19-CAR-engineered T cells. Cytotherapy. 2013;**15**:1406-1415

[44] Bouwmans EE, et al. NK cells expanded with the ZRP cultivation system keep cytotoxic properties and remain alloreactive. NK Cell Meeting. 2013. Heidelberg, Germany

[45] Friedrichs B, et al. Expansion of CD3-ve CD56+ve NK Cells for Repeated Application in a GMP Compliant Process Using a Novel Bioreactor System. Geneva, Switzerland: EMBT; 2012

[46] Krampera M, et al. Immunological characterization of multipotent mesenchymal stromal cells—The International Society for Cellular Therapy (ISCT) working proposal. Cytotherapy. 2013;**15**:1054-1061

[47] Galipeau J, et al. International Society for Cellular Therapy perspective on immune functional assays for mesenchymal stromal cells as potency release criterion for advanced phase clinical trials. Cytotherapy. 2016;**18**:151-159

[48] Heymans O, Castus P, Grandjean FX, Van Zele D. Liposuction: Review of the techniques, innovations and applications. Acta Chirurgica Belgica. 2006;**106**:647-653

[49] Zuk PA, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Engineering. 2001;**7**:211-228

[50] Baron F, et al. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GvHD) without abrogating graft-versus-tumour effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. Biology of Blood and Marrow Transplantation. 2010;**16**:838-847

[51] Emadedin M, et al. Long-term follow-up of intra-articular injection of autologous mesenchymal stem cells in patients with knee, ankle, or hip osteoarthritis. Archives of Iranian Medicine. 2015;**18**(6):336-344

[52] Capelli C, Pedrini O, Valgardsdottir R, Da Roit F, Golay J, Introna M. Clinical grade expansion of MSCs. Immunology Letters. 2015;**168**:222-227

[53] Lechanteur C, et al. Clinical-scale expansion of mesenchymal stromal cells: a large banking experience. Journal of Translational Medicine. 2016;**14**(1):145. DOI: 10.1186/s12967-016-0892-y

[54] Diederichs S, et al. Dynamic cultivation of human mesenchymal stem cells in a rotating bed bioreactor system based on the Z RP platform. Biotechnology Progress. 2009;**25**:1762-1771

[55] Lavrentieva A, Hatlapatka T, Neumann A, Weyand B, Kasper C. Potential for osteogenic and chondrogenic differentiation of MSC. Advances in Biochemical Engineering and Biotechnology. 2013;**129**:73-88

[56] Neumann A, Lavrentieva A, Heilkenbrinker A, Loenne M, Kasper C. Characterization and application of a disposable rotating bed bioreactor for mesenchymal stem cell expansion. Bioengineering. 2014;**1**:231-245

[57] Suck K, et al. A rotating bed system bioreactor enables cultivation of primary osteoblasts on well-characterized Sponceram regarding structural and flow properties. Biotechnology Progress. 2010;**6**:671-678

[58] Subramani B, et al. Autologous immune enhancement therapy: A case report of a stage IV colonic cancer. Oncology Letters. 2013;**5**:1611-1614

[59] Chapuis AG, et al. T-cell therapy using interleukin-21-primed cytotoxic T-cell lymphocytes combined with cytotoxic T-cell lymphocyte antigen-4 blockade results in long-term cell persistence and durable tumour regression. Journal of Clinical Oncology 2016,**34**,31:3787-3795. DOI: 10.1200/JCO.2015.65.5142

[60] Kershaw MH, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clinical Cancer Research. 2006;**12**:6106-6115

[61] Koehl U, Brehm C, Huenecke S, et al. Clinical grade purification and expansion of NK cell products for an optimized manufacturing protocol. Frontiers in Oncology. 2013;**3**:118. DOI: 10.3389/fonc.2013.00118

[62] Pierson BA, Europa AF, Hu WS, et al. Production of human natural killer cells for adoptive immunotherapy using a computer-controlled stirred-tank bioreactor. Journal of Hematotherapy. 1996;**5**(5):475-483

[63] Luhm J, Brand JM, Koritke P, Hoppner M, Kirchner H, Frohn C. Large-scale generation of natural killer lymphocytes for clinical application. Journal of Hematotherapy & Stem Cell Research. 2002;**11**:651-657

[64] Torelli GF, Guarini A, Maggio R,et al. Expansion of natural killer cells with lytic activity against autologous blasts from adult and pediatric acute lymphoid leukemia patients in complete hematologic remission. Haematologica. 2005;**90**(6):785-792

[65] Fujisaki H, Kakuda H, Shimasaki N, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Research. 2009;**69**(9):4010-4017. DOI: 10.1158/0008-5472.CAN-08-3712

[66] GE Healthcare. Application note 28-9936-25 AA. Perfusion culture of human natural killer cells in the WAVE Bioreactor 2/10 system. 2011. http://www.cellgenix.com/fileadmin/published_content/3_downloads/4.1_application_notes/GE_paper_NK_cells.pdf

[67] Lapteva N, Durett AG, Sun J, et al. Large-scale *ex vivo* expansion and characterization of natural killer cells for clinical applications. Cytotherapy. 2012;**14**(9):1131-1143. DOI: 10.3109/14653249.2012.700767

[68] Skrahin A, et al. Autologous mesenchymal stromal cell infusion as adjunct treatment in patients with multidrug and extensively drug-resistant tuberculosis: An open-label phase 1 safety trial. The Lancet Respiratory Medicine. 2014;**2**:108-122

[69] Panes J, et al. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: A phase 3 randomised, double-blind controlled trial. Lancet. 2016;**388**:1281-1290

[70] Reichardt A, et al. Large scale expansion of human umbilical cord cells in a rotating bed system bioreactor for cardiovascular tissue engineering applications. Open Biomedical Engineering Journal. 2013;**7**:50-61

[71] Gnecchi M, editor. Mesenchymal Stem Cells. Methods and Protocols. 2nd ed. Humana Press, Springer Protocols; 2016

[72] Skorska A, Müller P, Steinhoff G, et al. GMP-conformant on-site manufacturing of a CD133+ stem cell product for cardiovascular regeneration. Stem Cell Research & Therapy. 2017;**8**(1):33

[73] Karantalis V, Hare JM. Use of mesenchymal stem cells for therapy of cardiac diseases. Circulation Research. 2015;1413-1430

[74] Heldman AW, DiFede DL, et al. Transendocardial mesenchymal stem cells and mononuclear bone marrow cells for ischemic cardiomyopathy. The TAC-HFT randomized trial. JAMA. 2014;**311**(1):62-73

[75] Egger D, Spitz S, Fischer M, Handschuh S, Glösmann M, Friemert B, Egerbacher M, Kasper C. Application of a Parallelizable Perfusion Bioreactor for Physiologic 3D Cell Culture. Cells Tissues Organs. 2017;**203**(5):316-326. doi: 10.1159/000457792

[76] Cierpka K, Elseberg CL, Niss K, Kassem M, Salzig D, Czermak. hMSC production in disposable bioreactors with regards to GMP and PAT. Chemie Ingenieur Technik. 2013;**85**:1-2, 67-75. DOI: 10.1002/cite.201200151

[77] Schirmaier C, Jossen V, Kaiser SC, Jüngerkes F, Brill S, Safavi-Nab A, et al. Scale-up of adipose tissue-derived mesenchymal stem cell production in stirred single-use bioreactors under low-serum conditions. Engineering in Life Sciences. 2014;**14**(3):292-303. DOI: 10.1002/elsc.201300134

[78] Jossen V, Schirmer C, Mostafa Sindi D, Eibl R, Kraume M, Pörtner R, Eibl D. Theoretical and practical issues that are relevant when scaling up hMSC microcarrier production processes. Stem Cells International. 2016;**2**:1-15. DOI: 10.1155/2016/4760414

[79] Jossen V, Pörtner R, Kaiser SC, Kraume M, Eibl D, Eibl R. Mass production of mesenchymal stem cells—Impact of bioreactor design and flow conditions on proliferation and differentiation. In: Eberli D, editor. Cells and Biomaterials in Regenerative Medicine. InTech, Rijeka, Croatia; 2014. DOI: 10.5772/59385

[80] Neurauter AA, Bonyhadi M, Lien E, Nokleby L, Ruud E, Camacho S, Aarvak T. Cell isolation and expansion using Dynabeads. Advances in Biochemical Engineering/Biotechnology. 2007;**106**:41-73. DOI: 10.1007/10_2007_072

[81] Apel M, Brüning M, Granzin M, Essl M, Stuth, J, Blaschke J, et al. Integrated clinical scale manufacturing system for cellular products derived by magnetic cell separation, centrifugation and cell culture. Chemie Ingenieur Technik. 2013;**85**:1-2, 103-110. DOI: 10.1002/cite.201200175

[82] Barckhausen C, Rice B, Baila S, Sensebe L, Schrezenmeier H, Nold P, et al. GMP-compliant expansion of clinical-grade human mesenchymal stromal/stem cells using a closed hollow fiber bioreactor. Methods in Molecular Biology (Clifton, N.J.). 2016;**1416**:389-412. DOI: 10.1007/978-1-4939-3584-0_23

[83] Kami D, Watakabe K, Yamazaki-Inoue M, Minami K, Kitani T, Itakura Y, et al. Large-scale cell production of stem cells for clinical application using the automated cell processing machine. BMC Biotechnology. 2013;**13**:102. DOI: 10.1186/1472-6750-13-102

[84] Parolini O, Alviano F, Bergwerf I, Boraschi D, De Bari C, De Waele P, Dominici M, Evangelista M, Falk W, Hennerbichler S, Hess DC, Lanzoni G, Liu B, Marongiu F, McGuckin C, Mohr S, Nolli ML, Ofir R, Ponsaerts P, Romagnoli L, Solomon A, Soncini M, Strom S, Surbek D, Venkatachalam S, Wolbank S, Zeisberger S, Zeitlin A, Zisch A, Borlongan CV. Toward cell therapy using placenta-derived cells: Disease mechanisms, cell biology, preclinical studies, and regulatory aspects at the round table. Stem Cells and Development. 2010;**19**(2):143-154. DOI: 10.1089/scd.2009.0404

[85] Culme-Seymour EJ, Davie NL, Brindley DA, Edwards-Parton S, Mason C. A decade of cell therapy clinical trials (2000-2010). Regenerative Medicine. 2012;**7**(4):455-462. DOI: 10.2217/rme.12.45

[86] Maciulaitis R, D'Apote L, Buchanan A, Pioppo L, Schneider CK. Clinical development of advanced therapy medicinal products in Europe: evidence that regulators must be proactive. Molecular Therapy. 2012;**20**(3):479-482. DOI: 10.1038/mt.2012.13

[87] Ancans J. Cell therapy medicinal product regulatory framework in Europe and its application for MSC-based therapy development. Frontiers in Immunology. 2012;**3**:253. DOI: 10.3389/fimmu.2012.00253

[88] www.ema.europa.eu/docs/en_GB?document_library/Scientific-guideline/2015/06/WC500187744.pdf/)

[89] European Medicines Agency. Advanced Therapies. 2017. http://www.ema.europa.eu/ema/

[90] Advanced Therapy Medicinal Products (ATMPs)—ICH: 2017

[91] http://www.ich.org/fileadmin/Public_Web_Site/Training/ASEAN_Q5C_workshop_ May_2011/SESSION_IVb_ATMPs.pdf

[92] ATMP Manufacturing Community. 2017. https://atmpmanufacture.org/

[93] Advanced Therapy Medicinal Products - Parenteral Drug Association. 2017. https://www. pda.org/global-event-calendar/event-detail/advanced-therapy-medicinal-products

[94] ATMP Regulation - LFB CellForCure. 2017. www.cellforcure.com/atmp-regulation/

[95] Advanced therapy medicinal products: Regulation and licensing—Gov.uk.2017. https:// www.gov.uk/guidance/advanced-therapy-medicinal-products-regulation-and-licensing

[96] Hayakawa Y, Kawada M, Nishikawa H, Ochiya T, Saya H, Seimiya H, Yao R, Hayashi M, Kai C, Matsuda A, Naoe T, Ohtsu A, Okazaki T, Saji H, Sata M, Sugimura H, Sugiyama Y, Toi M, Irimura T. Report on the use of non-clinical studies in the regulatory evaluation of oncology drugs. Cancer Science. 2016;**107**(2):189-202. DOI: 10.1111/cas.12857

[97] CAT monthly report of application procedures, guidelines and related documents on advanced therapies. March 2017 Meeting. http://www.ema.europa.eu/docs/en_GB/doc-ument_library/Committee_meeting_report/2017/03/WC500224413.pdf

[98] Lawler M, French D, Henderson R, Aggarwal A, Sullivan R. Shooting for the moon or flying too near the sun? Crossing the value rubicon in precision cancer care. Public Health Genomics. 2016;**19**(3):132-136. DOI: 10.1159/000446530

[99] Vera JF, Brenner LJ, Gerdemann U, Ngo MC, Sili U, Liu H, Wilson J, Dotti G, Heslop HE, Leen AM, Rooney CM. Accelerated production of antigen-specific T cells for preclini-cal and clinical applications using gas-permeable rapid expansion cultureware (G-Rex). Journal of Immunotherapy. 2010;**33**:305-315