Objectives, benefits and challenges of bioreactor systems for the clinical-scale expansion of T lymphocyte cells

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Abstract

Cell therapies based on T cell have gathered interest over the last decades for treatment of cancers, becoming recently the most investigated lineage for clinical trials. Although results of adoptive cell therapies are very promising, obtaining large batches of T cell at clinical scale is still challenging nowadays. We propose here a review study focusing on how bioreactor systems could increase expansion rates of T cell culture specifically towards efficient, reliable and reproducible cell therapies.

After describing the specificities of T cell culture, in particular activation, phenotypical characterization and cell density considerations, we detail the main objectives of bioreactors in this context, namely scale-up, GMP-compliance and reduced time and costs. Then, we report recent advances on the different classes of bioreactor systems commonly investigated for non-adherent cell expansion, in comparison with the current "gold standard" of T cell culture (flasks and culture bag). Results obtained with hollow fibres, G-Rex® flasks, Wave bioreactor, multiple-step bioreactors, spinner flasks as well as original homemade designs are discussed to highlight advantages and drawbacks in regards to T cells' specificities.

Although there is currently no consensus on an optimal bioreactor, overall, most systems reviewed here can improve T cell culture towards faster, easier and/or cheaper protocols. They also offer strong outlooks towards automation, process control and complete closed systems, which could be mandatory developments for a massive clinical breakthrough. However, proper controls are sometimes lacking to conclude clearly on the features leading to the progresses regarding cell expansion, and the field could benefit from process engineering methods, such as quality by design, to perform multi parameters studies and face these challenges.

Keywords

Lymphocyte, T cells, bioreactor, clinical scale, expansion, proliferation, automation, scale-up

1. Introduction

Cell therapies involving T lymphocyte cells, or T cells, are a promising approach for the treatment of advanced cancers refractory to cytotoxic therapy or viral infections (Carswell and Papoutsakis, 2000; Donia et al., 2014; Janelle et al., 2015; Trickett et al., 2002). They are presented as

alternative to surgery, radiation or chemotherapy (Piscopo et al., 2018), in particular in the case of melanoma or renal cell carcinoma (Vacchelli et al., 2013) but also others such as prostate cancer (Vavrova et al., 2016) or chronic lymphocytic leukaemia (Hami et al., 2004; Hollyman et al., 2009). Reported as the third most investigated lineage for clinical trials behind hematopoietic and mesenchymal stem cells only 5 years ago (Heathman et al., 2015), T cells are now ranked first in several countries (https://ct.catapult.org.uk/clinical-trials-database and https://clinicaltrials.gov/ as consulted on 16/04/20). In particular, adoptive cell therapy (ACT) is based on the expansion of antigen-specific lymphocytes, autologous or genetically engineered to ensure the recognition of tumours after re-infusion at the location of the cancer (Klapper et al., 2009; Somerville et al., 2012). It is one of the first examples of gene transfer to human approved for therapy after pioneering works in the early 1990s (Bonini and Mondino, 2015; Kasid et al., 1990; Rosenberg et al., 1990). However, millions of such T cells are required for a single therapy, and this number cannot be obtained through direct harvesting from donors only (Somerville et al., 2012). An *in vitro* culture step dedicated to expansion is therefore needed in addition to T cell isolation, activation and transduction. The objective is the generation of T cells at the clinical scale with maximum efficiency and safety through increase in proliferation rate or cell survival (Bajgain et al., 2014) without increasing cost and time (Kalamasz et al., 2004). Recently, several CAR T cell therapy agents have been approved by the FDA, the first one being tisagenleucel (commercial name Kymriah™) in late 2017 for the treatment of refractory B-cell precursor acute lymphoblastic leukaemia (Maude et al., 2018; Prasad, 2018). It was rapidly followed by axicabtagene ciloleucel (Yescarta™) for the treatment of large B-cell lymphoma (Bouchkouj et al., 2019; Neelapu et al., 2017) and later by others such as brexucabtagene autoleucel (Tecartus™) against mantle cell lymphoma (Jain et al., 2021).

Expansion procedures developed over the last decades have led to excellent results for phase I/II developmental studies but it is not clear if they contributed specifically in moving forward to phase III nowadays or if progresses were made thanks to improvement in other steps of the overall CAR T cell generation process (Forget et al., 2016; Klapper et al., 2009). In particular, the use of bioreactors is a promising approach for the *in vitro* culture of T cells for adoptive transfer. A bioreactor is defined as a vessel containing cells, other organisms and/or bioactive substances in a controlled environment: temperature, nutrients, waste, pH, mechanical solicitations and flow rate are carefully monitored to achieve a large variety of biotechnological processes (Carpentier et al., 2011; Rauh et al., 2011; Yeatts and Fisher, 2011) (Figure 1). Numerous bioreactor designs were developed to fulfil the specific requirements of these different research fields.

Processes for the generation of Tumour infiltrating Lymphocytes (TIL) and state-of-the-art of T cell-based therapies have been extensively reported (Dai et al., 2018; Wang and Rivière, 2015), including reports of the clinical outcomes (Vacchelli et al., 2013). However, to the best of our knowledge, this is the first paper focusing specifically on T cell expansion procedures based on bioreactors and recent progresses on such biotechnologies. As seen in Figure 2, this approach is still in its infancy, with actually a small number of articles reported with the terms “T cell bioreactor” in title, abstract or keywords since the 1990s. Moreover, the number of articles seems to reach a plateau over the last 10 years compared to the general field of bioreactor research (Figure 2). This phenomenon highlights the crucial need for new approaches, findings and optimizations to go a step further towards clinical-scale production based on bioreactor systems.

In the present paper, general considerations on T cell culture and therapies, including a brief overview of the other steps besides expansion, are presented first. Specific T cell culture requirements

are then discussed to emphasize on the main objectives and approaches guiding the development of new expansion processes. Finally, current gold standards, *i.e.* culture flasks and moving culture bags, are reported and compared to the main bioreactor designs, namely hollow fibres, bag bioreactors and improved flask systems, along with original and recent proposals. We conclude on the ongoing challenges and technical issues still under investigations to move ACTs towards wider clinical application.

2. T Cells: general considerations

There are a range of methods used to generate a robust therapeutic action of the T cells after they are adoptively transferred. Manufacturing TILs for cell therapy usually requests standard *in vitro* expansion steps (Kaiser et al., 2015; Piscopo et al., 2018; Somerville et al., 2012): TILs are isolated from tumour biopsy, washed and concentrated and then expanded to reach clinically relevant population size. However, TILs are not always naturally present and can be generated only from some patients (Mellman et al., 2011; Rosenberg et al., 2008). Other strategies based on T-cell receptors (TCR) or chimeric antigen receptors (CAR) have therefore been developed. With such approaches, normal T cells from patients suffering from other cancers are isolated through apheresis and transduced to generate TCR or CAR T cells (Barrett et al., 2015) (Figure 3). Details on these methods and their development have been previously reported in review papers (Barrett et al., 2015; Dai et al., 2019, 2018; Field and Qasim, 2015; Mellman et al., 2011; Panagopoulou and Rafiq, 2019; Piscopo et al., 2018).

An activation procedure has to be performed to allow for transduction and proliferation of the manufactured T cells. In particular, Interleukin-2 receptor (IL-2R), a surface protein expressed on T cells, can be upregulated *in vitro*. Interleukin-2 (IL-2) has been used as the principal growth factor to trigger proliferation for primary culture and implantation since the pioneering works in the early 1990s (Carswell and Papoutsakis, 2000; Malone et al., 2001; Riddell and Greenberg, 1990; Rosenberg et al., 1990). The complete environment for T cell activation and proliferation involves anti-CD3 antibodies and exogenous IL-2, but also peripheral blood mononuclear as feeder cells (June, 2007; Klapper et al., 2009). This combination is reported as a standard “Rapid Expansion Protocol” (REP) (Dudley et al., 2003; J. Jin et al., 2012; Riddell and Greenberg, 1990; Sadeghi et al., 2011; Smith et al., 2015). Methods using co-culture with another cell lineage as a pre-stimulation step were also reported (Vavrova et al., 2016; Vera et al., 2010). How REP can be improved and refined is still investigated today, for instance with additional medium complements (IL-7, IL-15, anti-CD28 antibodies) but also pre-stimulation with a mix of peptides focusing on pp65 protein, leading to higher proliferation rates in the same culture vessel (Grau-Vorster et al., 2020) (G-Rex flask, see section 7.2). Recent alternatives also include for instance scaffolds mimicking the physiological signal of T cell activation (Zhang et al., 2020), DNA-based platforms to present anti-CD3 and anti-CD28 antibodies (Keskar et al., 2020) or bispecific antibodies (Guo et al., 2020).

To obtain optimal results in terms of rapid engrafting, antitumor activity and long-term protection thanks to memory T cells, it has been shown that a majority of CD8+ was needed but with a number of CD4+ cells above a threshold (studies in classic culture conditions as well as in Wave system as described below (Antony et al., 2005; Dudley et al., 2003; Moeller et al., 2005; Tran et al., 2007; Vacchelli et al., 2013)). Furthermore, CD4+ T cells have been suggested to be more effective *in vivo*: CD4+ T cells shared the same cytotoxic activity as CD8+ T cells; however the CD4+ subset was more persistent after TCR engagement (Yang et al., 2017). The most effective T cell subset for

therapeutic use has yet to be determined. This unknown is then further complicated by how bioreactors can influence the T cell subsets. For instance, CD8+ cells could grow faster in co-culture in stirred-tank bioreactors than CD4+ cells (Ou et al., 2019).

3. T cell activation in the setting of an expansion bioreactor.

In classic culture conditions, activation of T cells by CD3 and CD28 antibodies leads to robust activation, with the addition of CD28 specifically promoting: pro-survival factors such as increased IL-2 production (Boise et al., 2010); increased capacity of T cell respiration in mitochondria (Klein Geltink et al., 2017); and increased glucose uptake and glycolysis for activated T cells to further meet energetic and biosynthetic needs of an expanding population (Frauwirth et al., 2002). However, it has been reported that demonstrated other methods of T cell activation produced different beneficial effects. How these cues are presented plays a major role: putting the CD3 and CD28 antibodies onto scaffolds of different sizes and materials is known to affect how T cells respond in the consequent activation and expansion (Cheung et al., 2018; Wang et al., 2017). Further to this, the antibodies used also have a large effect on the therapeutic quality of the T cells produced. Zhang *et al* showed that a cell-based artificial antigen presenting cell expressing 4-1BB increased the expansion of CD8+ T cells compared to CD28 co-stimulation (Zhang et al., 2007). Furthermore, Bashour *et al* showed that CD3 and CD28 immobilised on a patterned surface allowed them to identify the forces that T cells could exert when binding to these antibodies (Bashour et al., 2014). Since there are a number of factors involved in T cell activation, it becomes apparent that any bioreactor involving the activation step should be evaluated on its effect on T cell interaction with the presentation of the antigen. For example, agitation to improve mass transfer in the bioreactor could significantly impact T cell activation. Although the development of the antigen presenting methods may initially be thought of as outside the scope of the cell expansion bioreactor, it is apparent that the method used for activation could have a strong effect on the expansion and therapeutic quality of the material produced.

Despite the apparition of such standardized procedures, more complex environments based on dedicated culture systems are still under investigation to obtain optimal proliferation and thus the generation of enough TILs or TCR/CAR cells for therapy. To go further, it is mandatory to understand the culture requirements of this lineage, the ongoing challenges and the specific objectives of the expansion in bioreactor.

4. Objectives of the expansion in bioreactor

4.1. Number of cells

As mentioned earlier, the main objective of developing bioreactor systems for culturing T cell is to improve further the *in vitro* proliferation rate compared to static flask culture, beyond REP and activation steps. The number of isolated cells remains too low to ensure efficient autologous immunotherapy (Bohnenkamp et al., 2002). In a pediatric and young adult CAR T trial a complex algorithm and method was developed to achieve an expected 3.24 billion CD3+ T cells from apheresis (Ceppi et al., 2018). Ceppi *et al* also describe how ill patients can also miss out on promising immunotherapies because of a failure to manufacture the CAR T therapy, which they suggest is partly due to obtaining sufficient cells from the apheresis (Ceppi et al., 2018). For the final therapeutic product, most studies report the range of 1 to 100 billion cells is needed for one single patient

(Bohnenkamp et al., 2002; Carswell and Papoutsakis, 2000; Foster et al., 2004; J. Jin et al., 2012) with at least 10 billion in average (Klapper et al., 2009; Malone et al., 2001). This number seems to be a physiological limit and is not related to progresses in the clinical procedures as it didn't decrease over the last decades (Jin et al., 2018; Knazek et al., 1990). Although it is possible to obtain such populations with multiple vessels and heavy labour work, patients will benefit from faster and more reliable processes to ensure cells will be ready on time and as soon as possible. REP are therefore commonly used in combination with bioreactor systems, regardless of specific designs and geometry (Bohnenkamp et al., 2002; Chakraborty et al., 2013; Donia et al., 2014; Klapper et al., 2009; Trickett et al., 2002), even if sometimes only partially (for instance only IL-2 or anti-CD3 antibodies addition and not a complete factor-enriched environment) (Knazek et al., 1990; Malone et al., 2001; Tran et al., 2007; Vera et al., 2010; Wang et al., 2015).

In the present paper, the maximum cell density and maximum yield that can be achieved in a specific type of device will be discussed. However, it has to be noticed that the differences in design and functionalities between systems, for instance hollow fibre bioreactors and stirred tank systems, make it difficult or nearly impossible to compare strictly maximum cell density without considering all other features and parameters. Therefore, it should not be the sole parameter to consider when assessing the potential of a bioreactor for T cell applications.

4.2. Reducing cost and volumes

Improvements in proliferation rate will help decreasing volume of solutions and labour time, and in turn costs of cell therapies. Indeed, T cell expansion usually lasts for 14 days in classic conditions (Donia et al., 2014; Dudley et al., 2003; J. Jin et al., 2012; Jin et al., 2018; Klapper et al., 2009) and tens of litres of culture medium can be needed to obtain the requested numbers due to low cell densities (Foster et al., 2004). Somerville *et al* reported volumes as large as 50 litres to achieve expected cell populations in open systems (Somerville et al., 2012). It is therefore crucial to optimize bioreactor designs to reduce volumes and to refine feeding schedules of T cell cultures to find a balance between nutrient support, waste removal and culture medium costs (Bajgain et al., 2014; Bohnenkamp et al., 2002). Moreover, it has been shown that partial medium changes could improve cell expansion as complete change leads to the removal of all beneficial autocrine factors, such as Interferon-γ, IL-1β and tumour necrosis factor-α, resulting in decreased proliferation (in particular in stirred systems (Bohnenkamp et al., 2002)). However, conversely, developing complex bioreactor systems could increase acquisition and starting costs, in particular for small academic structures involved in the early steps of design and optimization. This balance needs therefore careful considerations when evaluating the financial benefits of bioreactors.

4.3. Promotion of good manufacturing practice (GMP)-compliant protocols

Due to the high labour intensity of current T cell expansion protocols, it is important to promote processes ensuring GMP (Donia et al., 2014). Decreasing volumes and culture steps, as seen earlier, contributes positively to such approaches, along with the automation of routine procedures and the use of closed systems (Donia et al., 2014). Bioreactors should be designed to avoid multiple exposures of the cell population to the external environment over the expansion time, for feeding and sampling as well as transfer from one culture system to the other (Klapper et al., 2009; Sadeghi et al., 2011) removing the need of expansive facilities such as clean rooms (Hourd et al., 2014; Wang and

Rivière, 2016). GMP should also be applied to initial and side steps such as cryopreservation or transduction process for TCR/CAR cells and thus facilitate translation of research into clinical trials (Hollyman et al., 2009; Jin et al., 2018; Kaiser et al., 2015; Kalamasz et al., 2004; LEVINE et al., 1998).

As discussed in the next sections, it is possible for bioreactor systems to incorporate procedures performed at either side of the cell expansion phase, further reducing the number of open processing steps, which leads to compliant GMP processing within one unit.

5. Constant or increasing cell density: two different approaches

Although T cells can be attached to antibodies-functionalized surfaces, they are more commonly cultured in free expansion and non-adherent conditions (Piscopo et al., 2018). Cell density is therefore expressed as the number of cells per volume unit. To culture T cells over time, an optimal density of 1 to 3 million cells per mL should be maintained to benefit from a balance between cell-to-cell communication and available nutrients regardless of the culture vessel (Klapper et al., 2009; Somerville et al., 2012), a value consistent with the recommendations of the ATCC for T cell lines such as Jurkat cells (https://www.lgcstandards-atcc.org/products/all/TIB-152.aspx#culturemethod as consulted on 16/04/20).

Proliferation being the ultimate objective, a first approach is the continuous increase of available culture medium volume when cells proliferate to allow them to expand further while maintaining this optimal concentration stable, when suitable with the culture system design (Donia et al., 2014; Jin et al., 2018; Tran et al., 2007; Vavrova et al., 2016). For example, Tran *et al* doubled culture volume to decrease cell density to $5x10^5$ cells/mL as soon as it reached the limit of $1x10^6$ cells/mL (Tran et al., 2007). As a direct consequence, medium removal has to be avoided to prevent unwanted changes of cell concentration or disposal of a part of the cell population; moreover, for the latest stage of expansion, removal of waste with medium change would require extremely large volumes of fresh culture medium daily (Sadeghi et al., 2011). Culture and volume expansion then stop when the requested number of cells is achieved, or when cell concentration is not increasing anymore over time (Tran et al., 2007).

In contrast, a second approach focuses on maintaining a constant volume while cell density keep increasing until a plateau is reached, with up to 30 million cells per mL (Klapper et al., 2009; Sadeghi et al., 2011). Closed system bioreactors, which are relevant with GMP but don't allow for medium addition and volume expansion, could therefore benefit from this approach, in particular hollow fibre bioreactors. However, cell viability and functions have to be characterized carefully after expansion to confirm that they were not impacted by waste accumulation and cell concentration, necessarily higher than recommended values (Klapper et al., 2009). For instance, a large part of the study by Ou *et al*, conducted in a stirred-tank bioreactor, focused on assessing functionality, viability and quality of the produced T cells (Ou et al., 2019). Assessing extensively phenotype and functionality through anti-tumour activity in particular could actually be crucial to validate expansion procedures, as pushing forward proliferation rates has been showed to lead to T cell exhaustion in some cases (Ou et al., 2019; Roh et al., 2016; Wherry and Kurachi, 2015). In the next sections presenting the different bioreactor systems, phenotype considerations are reported when they are explicitly discussed in the studies.

In both cases, an important parameter to consider would be the initial cell density. Although a minimum concentration is required to ensure cell-to-cell communication and in turn rapid cell proliferation, the recommended density of around 1 million cells per mL are not usually required at

day 0 of culture (Bajgain et al., 2014; Vera et al., 2010) and lower seeding density has been shown not to affect proliferation rates (J. Jin et al., 2012). The same concern applies to the initial volume available for cell growth, as studied by Sadeghi *et al* who showed that small initial volumes led to best results (Sadeghi et al., 2011).

In summary, a balance has to be found between cell density over time, available volume, waste removal, concentration of beneficial autocrine factors, closed system features, GMP and costs. Some of these parameters may not be easily modified based on the culture system used for T cell expansion. Bioreactor systems are proposed to tackle this challenge in order to offer more reliable and faster proliferation rates than current gold standards. Traditional systems and new developments are therefore presented below.

6. Gold standard and limits

Standard plastic culture flasks (although sometimes maintained in unusual vertical position to increase available volume) and semi-permeable culture bags are known as the gold standards for T cell amplification *in vitro* (Bohnenkamp et al., 2002). Bags allow for better oxygen exchanges and easy sampling thanks to larger gas-permeable surface and dedicated ports, respectively. However, flasks are usually cheaper, they can offer large surfaces (175 cm^2) (Somerville et al., 2012) and higher proliferation rates can even be achieved according to some studies (Tran et al., 2007). They can also be used in conjunction (flasks first and then bags) (Somerville et al., 2012). In any case, the use of multiple replicates of such systems combined with REP allows for the generation of significant numbers of cells but at the expense of extended labour time, inhomogeneity and high consumable costs due to low overall cell density in a single product (Bohnenkamp et al., 2002). Frequent medium changes and cell transfers increase therefore the risk of contamination and interrupt optimal cell-to-cell contact conditions, crucial for therapy efficiency (Malone et al., 2001). They are indeed open systems and are therefore intrinsically not GMP-compliant (Sadeghi et al., 2011). Moreover, culture conditions are usually not controlled besides temperature and CO_2 concentration, leading to hardly repeatable experiments and culture schedules based on individuals' judgments and batch-to-batch variations (Bohnenkamp et al., 2002; Sadeghi et al., 2011).

7. Commercially available bioreactor systems

7.1. Hollow fibre membrane based bioreactor: Terumo Quantum

First hollow fibres cartridges were developed more than 40 years ago for dialysis systems (Klein et al., 1976; Lee, 1977) (Figure 4A) and were later proposed for cell culture and further applications (Huang et al., 2016; Patel et al., 2018; Yang et al., n.d.) and eventually as bioreactors (Nankervis et al., 2018; Yan et al., 2018). Some T-cells-related studies in hollow fibres, since the early years, are not focusing on the cell expansion but on the production of cytokines and other biomolecules by the cells inserted in the system (Lamers et al., 1999). Hollow fibres create a two-compartment culture environment as cells grow on one side of the small hollow fibres (200 µm diameter) while medium is perfused on the other side (Nankervis et al., 2018; Yan et al., 2018) (Figure 4B). Fibres are made of membranes permeable to specific components of interest (biomolecules, gas, nutrients, waste) and therefore allow for controlled exchanges between the cell compartment and the medium perfusion (Nankervis et al., 2018; Yan et al., 2018). The rationale behind applying such

systems to T cell culture is the avoidance of shear stress caused by flow perfusion thanks to the compartmented geometry, while maintaining medium movements like in shaking bags and stirring tanks (Nankervis et al., 2018), with opportunity to re-oxygenate and adjust medium pH before returning it back to the cells (Knazek et al., 1990). Moreover, closed loop of medium and concentration of nutrients in the cell compartment could help reduce the volume needed for feeding (Gramer and Poeschl, 2000; Nankervis et al., 2018). The approach regarding cell density is here an increasing concentration in a fixed volume (the cell compartment), however flow rate can be increased along with the number of cells (Knazek et al., 1990).

A potential concern with such systems is the size of the molecules to provide or remove, especially as cell density increases. Growth inhibitors gradually produced by T cells have to be easily removed to ensure continuous proliferation and expansion, which can be achieved with fibre membranes as shown in the early years (Knazek et al., 1990). In a similar way, growth factors should be able, in turn, to reach the cell area from the perfusion. The molecular weight of T-cell-specific activation factors required for expansion, namely IL-2 and antibodies as recommended to follow REP, would therefore guide the choice of the membranes to be used. For instance, Trickett *et al* noticed that polypropylene-based hollow fibres (0.3 – 0.5 µm molecular weight cut-off) were more suitable than 4 kDa cellulose cartridge for IL-2 diffusion (Trickett et al., 2002).

One commercial example of a hollow fibre-centred bioreactor system is the Terumo Quantum®. This system is capable of culturing T cells to a therapeutic dose within one single-use set in the system; it is capable of achieving 90-500 fold increase in cell numbers after an inoculation of $1x10^8$ lymphocytes (Nankervis et al., 2018; Startz et al., 2016). The starting number of cells is therefore quite high in experiments with the Quantum® system, and starting material quality can be a challenge in adoptive transfer of T cells (Juliano et al., 2018; Levine et al., 2017). The adaptability of this system to variabilities in starting material parameters such as cell number may need to be investigated further to confirm its full potential.

Co-culture of T cells with polymer particles known as Dynabeads is frequently used for the activation step in hollow fibre, wave bag and stirred tank bioreactor formats (Costariol et al., 2019; Hami et al., 2004; Startz et al., 2016). For the hollow fibre format, the CD4+:CD8+ ratio produced by the dynabead-activation and Quantum®-expansion of T cells was highly dependent on the donor material (Startz et al., 2016). Most samples tended to favour proliferation of CD8+ cells; however, for one donor, the ratio was close to 1:1. Improving upon the initial results, an optimized protocol in the Quantum® system found that, amongst changes in other parameters, increasing the number of dynabeads per T cell led to a higher fold expansion produced by the bioreactor (Nankervis et al., 2018). The effect on T cell phenotype was not reported in that study. The conclusion on the T cell subset produce by the bioreactor is that it may be reliant on factors not related to the bioreactor but for instance to donor variability, although the studies were lacking data related to control experiments using the gold standard methods discussed above.

A unique feature of the Quantum® and hollow fibre bioreactors is that they are capable of culturing adherent cells, and therefore to produce the lentiviral vectors used in transfection of CAR T therapies as well.

As mentioned earlier, T cell expansion in general needs specific composition of culture medium, based on REP but subject to changes depending on targeted application, bioreactor geometry and protocol. The use of hollow fibre bioreactors will require additional optimisation steps, as two different culture media can be used, in the cell compartment (the extracapillary space) and in the perfusion compartment (inside the fibres) (Gramer and Poeschl, 2000). Feeding schedules should

be adjusted as well and compared to static systems (Trickett et al., 2002). Large scale hollow fibre systems and commercial modules are not always suitable for such optimisation, but smaller bioreactors can be easily used to adjust composition before scale-up thanks to good correlation (Gramer and Poeschl, 2000). Dedicated micro-hollow fibre bioreactors were shown to be helpful to perform the optimisation with lower costs than full-scale systems and better reliability than flasks (Gramer and Poeschl, 2000). These preliminary steps would increase drastically the yields of the expansion culture (Nankervis et al., 2018), leading to proliferation rate far above T flask results (Gramer and Poeschl, 2000) and similar to semi-permeable bags while reducing costs and space needed (Knazek et al., 1990; Malone et al., 2001). Moreover, since hollow fibre system has been used for cell culture for producing cell secreted products, there is good industry infrastructure to support this system while acknowledging that the requirement to harvest cells at the end of culture may pose some challenge.

7.2. Improved flasks: G-Rex® system

As described earlier, although having many limitations, regular culture T-flasks have the advantage of being easy to use and affordable. An approach for the development of more relevant culture environment but with easy monitoring was therefore the design of "improved flasks" with additional features to increase proliferation rate. Although the bioreactor nature of such devices could rightfully be discussed, according to the strict definition of bioreactor, they can indeed be seen as a vessel with micro-controlled environment, in particular through gas exchange.

The G-Rex® system, for Gas Permeable Rapid Expansion, has been developed around 10 years ago by Wilson Wolf Manufacturing as an alternative to T-flask, spinner flasks and semi-permeable bags. The main feature of this cell expansion chamber is the presence of a semi-permeable membrane located at the bottom, in contact with the main cell culture surface area (Figure 5). The vessel can be filled with a very large volume culture medium compared to regular T flasks, where it is limited to 1 mL/cm^2 to avoid cell hypoxia (Bajgain et al., 2014; Vera et al., 2010). This device has been tested for suspension cells and can be combined with REP, including the addition of feeder cells (Chakraborty et al., 2013; J. Jin et al., 2012; Jin et al., 2018). It has therefore been considered for the amplification of T cells. According to the manufacturer's information, the large initial volume as well as the absence of specific requirements for cell passage and medium changes implies the approach of an increasing cell density in a constant volume for T cell expansion.

G-Rex® flasks have been studied with different protocols as semi-opened or closed systems. Following REP activation in regular flasks, regulatory T cells have first been successively expanded and transferred in increasing sizes/numbers of G-Rex® models, in a similar way as adherent cell passaging (Chakraborty et al., 2013; Vera et al., 2010). These studies confirmed that the proliferation rates were higher than in T flasks although using same protocols. The G-Rex® were then optimised to be used as a totally closed system (Bajgain et al., 2014) (besides periodic IL-2 addition) or for an approach with increasing volume to achieve even higher T cell proliferation rates (Jin et al., 2018). Eventually, the aim is to obtain a single closed system per patient, able to provide enough cells for the therapy while reducing drastically space, labour work and costs. Such a result was successfully reported by Bajgain *et al* (Bajgain et al., 2014) who reached a population of 1.5x10^9 cells in a single 1-litre G-Rex® flask with primary CAR cells after 10 days. According to this study, this was made possible by the oxygen exchanges combined to a virtually unlimited stock of nutrients provided by the large volume of medium. Above 10 mL of medium per cm^2 of the gas exchange membrane, there was no effect of

additional available volume on the cell behaviour (Bajgain et al., 2014). This high expansion potential was explained by a reduction in cell death overtime rather than an increased proliferation rate, therefore less divisions (and shorter culture time) were needed to achieve the expected cell number (Bajgain et al., 2014; Vera et al., 2010). This interesting approach, *i.e.* increasing cell survivability instead of focusing on proliferation rate only, could be investigated and optimised in other bioreactor designs as well.

Besides cell number and viability, the functionality of T cells expanded in G-Rex® systems was also assessed with regulatory T cells and TCR towards human papillomavirus (Chakraborty et al., 2013; Jin et al., 2018). After expansion, they successfully obtained cells that were potent and specific in their ability to kill the targeted tumour cells. Moreover, Forget *et al* showed that the phenotype of T cells expanded in G-Rex® flasks and semi-permeable bags ensured better survivability that could improve tumour control after implantation compared to regular T flasks, thanks to the better oxygen consumption during growth confirmed by mitochondrial respiration (Forget et al., 2016). They also showed the maintenance of a high polyclonal diversity of the TILs expanded in this system.

Regarding drawbacks, it was mentioned that cells cannot be visualized in G-Rex® during culture, in contrast with horizontal flasks (Z. Jin et al., 2012). Moreover, control groups for some analyses were T cells cultured in 24-well plates, an approach which is no longer considered as a gold standard (Bajgain et al., 2014; Vera et al., 2010). Finally, it has to be mentioned that most of the studies reporting G-Rex® findings disclosed conflict of interest with the manufacturer.

7.3. Improved culture bags: Wave bioreactor and GE Xuri system

The Wave bioreactor was initially developed by Singh *et al* in 1999 as an alternative bioreactor allowing for easy scale-up compared to previous systems (Singh, 1999). It was proposed for various applications, such as adherent and non-adherent cell culture as well as adenovirus production. It has been then more recently studied specifically for the expansion of T cells.

This system uses sterile disposable culture bags as main vessels on a platform ensuring heating and rocking motion (variable angles, magnitudes and rates) to induce “waves” in the culture medium (Donia et al., 2014; Sadeghi et al., 2011; Singh, 1999) (Figure 6). These specific movements provide improved oxygen exchanges, homogeneity and easy access to nutrients even at high cell density in small volumes (Donia et al., 2014) while reducing stresses to the cells compared to stirring systems (Singh, 1999). Culture medium can be perfused through the bag and standard tubing ports allow for sampling and inoculation (Sadeghi et al., 2011). This bioreactor has been successfully used in class I and II clinical trials (Donia et al., 2014; Hollyman et al., 2009). Compared to static and one-axis shaking bags, the main innovative feature of this system is therefore the combination of the complex wave loops with flow perfusion in a closed system. This combination provides a better homogeneity even at the cell scale as it avoids gradients of beneficial secreted factors with the highest concentration located in the close environment of the producing cells (Somerville et al., 2012). It is thus a more complex system than current gold standards from the technological point of view, but in practice it doesn’t increase labour time as monitoring and sampling are simple or even automated (Donia et al., 2014; Somerville et al., 2012; Tang et al., 2007). For instance, Sadeghi *et al* showed that adding IL-2 by inoculation in the bag daily or as a single initial loading in the perfusion reservoir led to the same results regarding T cell expansion (Sadeghi et al., 2011): REP procedures can be adapted in a simpler way with the use of the Wave bioreactor, which is also GMP-compliant (Donia et al., 2014; Hami et al., 2004). Hollyman *et al*, in particular, focused on biosafety checking and the respect of FDA and NIH

guidelines while using a Wave system (Hollyman et al., 2009). Moreover, all steps of the REP process can be performed directly in the system, while only late stages of REP are suitable to be ran in other designs such as hollow fibre membrane based bioreactors (J. Jin et al., 2012; Sadeghi et al., 2011). Besides increases in proliferation rate, this approach could also improve standard yields by making the culture process shorter thanks to easier handling and monitoring. The use of transparent culture bags is here an advantage itself, as they are disposable, can be sterilized, are optically clear and allows for microscopy observations and non-invasive optical measurements through culture medium (Tran et al., 2007).

Thanks to these features, the Wave Bioreactor has been used for T cell expansion with the approach of a constant volume and an increasing cell density, in combination with flow perfusion (Sadeghi et al., 2011), in a similar way as hollow fibres bioreactors. However, as the rocking motion alone provides mixing and oxygen exchanges, a hybrid approach can be investigated to adapt the parameters to the cell density: Sadeghi *et al* used a constant volume but started to perfuse culture medium only after a certain cell density was reached (Sadeghi et al., 2011). More importantly, they showed that the results of cell expansion were better when the process was initiated in a smaller volume. Another approach could therefore be developed by combining an increasing volume with flow perfusion based on cell density or nutrient levels (glutamine and glucose) (Somerville et al., 2012; Tran et al., 2007; Vavrova et al., 2016). Interestingly, besides available volume, all parameters can be adjusted over time while cells are proliferating, such as rocking rate, rocking angle, perfusion flow rate and etc. (Tran et al., 2007). Overall, proliferation rates obtained with the Wave bioreactor were similar (Somerville et al., 2012) or significantly higher than with static bag methods (Donia et al., 2014; Sadeghi et al., 2011) and required smaller volumes (Hollyman et al., 2009). It has been shown in other gas-permeable systems that high oxygen levels in the culture medium could play a strong role in the increase of T cell amplification (Forget et al., 2016). As the Wave bioreactor is expected to increase oxygen exchange and access even in the close environment of the cells, with a more stable environment than gas-permeable static bags requiring periodic refreshment, this could explained the higher proliferation rates (Forget et al., 2016; Somerville et al., 2012). It is important to note that control groups were mostly static bags and not non-wave shaking bags nor other forms of dynamic bioreactors. Such additional groups could have been included to highlight the specific role of the rocking motion / perfusion combination on the proliferation rate. There was even sometimes no other system used as control when the validation of the large scale expansion with maximum biosafety was the objective (Hami et al., 2004; Hollyman et al., 2009).

Besides the number of cells after expansion, T cell populations in Wave bioreactor were also analysed in terms of phenotype, with a focus on the balance between CD4+ and CD8+ cells (Hollyman et al., 2009; Sadeghi et al., 2011; Somerville et al., 2012; Tran et al., 2007; Vavrova et al., 2016). There are claims that: CD4+ cell numbers were too high in Wave-culture populations compared to static bags (Somerville et al., 2012; Vavrova et al., 2016); or that both groups were similar (Sadeghi et al., 2011); while others highlighted how the Wave system could help monitoring the balance (Tran et al., 2007). Further studies are therefore needed, but it is important to note that specific efforts have been made to analyse this aspect with Wave bioreactor, more than with other systems (although phenotype analysis can be monitored in other studies, in particular using G-Rex® flasks (Forget et al., 2016) or stirred-tank bioreactors (Ou et al., 2019)).

In addition to the direct effect of bioreactor technologies on cell phenotype, the effect of exogenous reagents used within the system to supply the cell culture with required components must

be considered. Dynabeads were used as a part of the activation process in some studies (Hami et al., 2004; Hollyman et al., 2009; Vavrova et al., 2016), before and after inoculation in the bag of the Wave bioreactor. This highlighted how the system can benefit from the use of a culture bag, a well-known and simple culture vessel, while being combined with other methods and complementary devices. The Dynabeads used in those studies activated T cells through engagement of the TCR by CD3, and co-stimulation by CD28 receptors. This method is known to produce a different ratio of CD4+ to CD8+ T cells depending on the dose of beads used (Cheung et al., 2018; Li and Kurlander, 2010; Startz et al., 2016). It is clear that in order to generate a further understanding of the cell expansion process, the effect of T cell activation, which has many effects as discussed earlier, and the effect of bioreactor need to be separated.

We established above that the wave system is compliant with GMP, however, it does not completely automate the expansion procedure. In a published protocol dated of 2020, addition of medium follows a manual procedure of taking a sample out of the bioreactor and performing an offline cell count, then using that cell count in combination with a reference table to set medium volume and perfusion rate (Swiech et al., 2020). The same reference book identifies that the Wave system does have built-in pH and dissolved oxygen sensors (Swiech et al., 2020). These types of soft-sensors are routinely used in larger scale bioreactors for culturing cell types other than mammalian cells (Chen et al., 2004).

Some limitations exist anyway for the use of this system. Many optimisation steps are required compared to static low-scale processes, regarding the basic culture parameters but also the different technical parameters specific to the Wave bioreactor. Such optimisation can be difficult as they can have various effects: for instance, rocking rate modifies shear stress but has also an influence on aeration and therefore oxygen exchanges (Tran et al., 2007). The initial purchase can be a bigger investment than other systems, but this can be nuanced as routine culture is then cheaper thanks to reduced medium volumes (up to half the consumption of other designs) (Sadeghi et al., 2011). As the system is a combination of multiple motions and perfusion, the risk of electrical and mechanical failure altering cell behaviour is more important and the set-up requires therefore strong redundancy.

7.4. One-system multiple-steps bioreactors: Miltenyi Biotec Clinimacs Prodigy.

We have so far discussed bioreactors that specifically focus on the cell expansion stage of the autologous, adoptive-transfer of T cell products. However, many of the processes to develop these products are a multi-stage linear process. Before cell expansion takes place, peripheral blood mononuclear cells would require extraction from blood apheresis products; followed by selection of T cells specifically; and then cell activation closely followed by possible genetic modification. Post-expansion, there are the tasks of formulating the cells into a suitable buffer for bio-preservation or infusion of the product, subject to it passing quality control measures (Wang and Rivière, 2016). Given that *bioreactor* is an umbrella term for a system that converts matter in biological process, a complete bioreactor system may be capable of performing multiple steps in the advanced therapy production line.

One such device is the Miltenyi Biotec Clinimacs Prodigy system. This system is capable of performing cell selection, activation, transduction, expansion, cell harvest, and formulation all within one piece of hardware and its related single-use tubing set (Mock et al., 2016). In the study by Mock *et al*, results achieved in the Prodigy were comparable to a process used in a GMP facility: the Prodigy results of cell number, viability and sterility neither improved upon, or more importantly, deteriorated

from those obtained from the comparative process (including culture bags and Wave bioreactor). This suggests the systems benefit lies in automation of multiple steps together in one system. Some parts of this system are not just automated by pre-programming when actions are to be performed, but also, on some level, controlled: pressure and liquid sensors can detect when a new task has completed; a camera can monitor the progress of cell separation during centrifugation steps; and a microscope can be used to assess the progress of cell culture (Tarnowski et al., 2017). The multiple steps enclosed in one device may reduce clean room space and allow production at satellite locations (Scibona and Morbidelli, 2019), which could help to realise the benefit of a decentralised manufacture process (Harrison et al., 2018).

A table drawn up by Mock *et al* shows that operator interaction is still required for a number of steps performed by the Prodigy, therefore, it is not an entirely automated system (Mock et al., 2016). Furthermore, there may be drawbacks to providing multiple capabilities in one-system hardware. Cell expansion is the longest phase of production of a T cell-based immunotherapy regardless of the culture system (Donia et al., 2014; Dudley et al., 2003; J. Jin et al., 2012; Jin et al., 2018; Klapper et al., 2009), assuming that the lentiviral vectors can be supplied efficiently (Levine et al., 2017). A long cell expansion step would mean that when a Prodigy is operating in the cell expansion phase, centrifugation and cell separation capabilities remain idle. This is not ideal, considering that these therapies are currently autologous, and therefore, that equipment could be used to process material for a separate patient. Allowing bottlenecks to develop at individual steps of advanced therapy manufacture, one-system multiple-step bioreactors may limit throughput of the bioprocessing chain by lack of availability of hardware. This would encourage future multiple-step bioreactors to be based on throughput analysis of all of the processing chain so that the effect of bottlenecks is minimised.

Another notable drawback of the system is its limited volume capacity (Swiech et al., 2020) that could limit its use in producing multiple doses of a therapy, and its use in producing an allogeneic therapy. The culture vessel is the same unit as used for centrifugation, suggesting that this multi-use may not be the best design for cell culture.

7.5. Stirring systems and homemade designs

Another class of bioreactors and dynamics vessels are spinner flasks and further stirred systems (Figure 7). Through mechanical or magnetic stirring, their main advantage is to offer simple geometries to provide homogeneity of culture conditions and excellent gas exchanges with ease of sampling (Bohnenkamp et al., 2002; Carswell and Papoutsakis, 2000). Different models are commercially available and scale-up can be performed easily by increasing volumes of the tanks (Kropp et al., 2016; Tran et al., 2007). Perfusion can increase cell densities achieved within stirred tanks, reducing volume required to scale up cell yields. However the perfusion investigated by Kropp *et al* required additional hardware to retain the cell-only stem cell aggregates within the bioreactor (Kropp et al., 2016). Stirred tanks also allow for the use of REP during culture (Bohnenkamp et al., 2002; Carswell and Papoutsakis, 2000; Foster et al., 2004) and are mostly used with a constant culture volume.

One of the main parameters to be adjusted in order to optimise the controlled environment of culture in such systems is the rate of the stirring mechanism. Shear stresses created by high stirring rates could indeed jeopardize the beneficial effects of medium mixing on cell proliferation (Tran et al., 2007). The impact of rotation speed on T cell culture has been investigated for instance by Foster *et*

al (Foster et al., 2004) and Carswell *et al* (Carswell and Papoutsakis, 2000), the latter in combination with sparging-based aeration. A speed range of up to 120 RPM in spinner flasks or 180 RPM in large scale (2 litres) stirring bioreactor were successfully used without altering cell viability and proliferation (Carswell and Papoutsakis, 2000).

Besides speed rate, many other parameters such as osmolality, IL-2 concentration, pH, oxygen tension and feeding schedules can be monitored or controlled in stirred systems (Ou et al., 2019), if needed after optimization in static conditions (Bohnenkamp et al., 2002). The effect of shear stresses can be reduced by modifying the overall culture protocol as well as the stirring mechanisms such as cell retention filters or magnetic stirrers (Bohnenkamp et al., 2002). After studying different feeding strategies in stirred bioreactors, Bohnenkamp *et al* showed that refreshing a small fraction of culture medium (not more than half the total volume) on a daily basis could lead to higher proliferation rate than a larger change every 2/3 days, the worst approach being a complete renewal removing both waste and paracrine factors (Bohnenkamp et al., 2002). Therefore, a drawback of stirring bioreactors could be the need of frequent interventions for medium replacement, limiting the potential for closed systems. We could suggest the combination of stirred vessels with perfusion of medium but this approach is not widely investigated for T cell expansion to the best of our knowledge, although it has been recently reported for instance for stem cells (Kropp et al., 2016).

Indeed, stirred tank bioreactors have shown very good results for the expansion of other cell lineages, and in particular induced pluripotent stem cells (iPSC) from which immune cells can derive *in vitro* (Ackermann et al., 2018). Not only could such approaches pave the way to alternative *in vitro* expansion for immunotherapies, but T cell-based processes could also benefit from progresses made for other cell types. For example, optimization of feeding strategies elaborated by Kropp et al (Kropp et al., 2016) for human pluripotent stem cells could help to refine perfusion cycles and process monitoring. Although the present review is dedicated to systems primarily developed for T cell culture, such cross-analyses between cell types might be kept in mind for other bioreactor designs as well. Interestingly, Kropp et al (Kropp et al., 2016) confirmed with stem cells the results of Bohnenkamp et al (Bohnenkamp et al., 2002) and showed that continuous refreshment of culture medium through perfusion led to better results than repeated batch replacements (47% increase in cell yield as well as in stem cell specific parameters).

To date, T cell culture bioreactors don't show any consensus, and many different ways of improvement exist towards closed and automated systems. Although not widely reported recently, homemade and more original approaches could therefore be proposed as alternative to the main classes presented previously. A noticeable proposition by Klapper *et al* used a closed-system parallel plate bioreactor, made of two disk-shaped chambers (Klapper et al., 2009). Medium and gas compartments were separated by a membrane permeable to gas but impermeable to liquid (Figure 8), both phases being perfused during culture. Culture medium was provided by a 7-litre reservoir for the 14-day proliferation step, leading to a completely closed system. Flow rate was adjusted over time and based on batch-to-batch variations to maintain lactate concentration between 0.5 and 1 mg/mL with a slow and laminar flow, not harmful to cell suspension. This process allowed for the use of REP, in particular with the injection of IL-2 as a single bolus injection at day 0 (confirming the closed system potential) and low serum concentration, relevant with further clinical validation. We can mention here that in the past, studies have shown contradictory results on the effect of serum concentration in a stirred environment on cell development (Jager et al., 1988; Kunas and Papoutsakis, 1990). Further investigations on this specific parameter could be conducted. The limit of this approach was found to

be the surface area of the disk chamber, however higher proliferation rates compared to semi-permeable bags used as control were confirmed (Klapper et al., 2009). Novel designs can improve the expansion capability of bioreactors, although they still have limitations that may restrict their adoption into therapy manufacturing.

8. Discussion and outlooks: automation, and specifically process control, as an ultimate goal

In summary, as an overall result, the majority of investigated systems showed better results in terms or T cell proliferation compared to static standards; alternatively, in the case of similar results, improved ease of use and decreased costs and labour time (Table 1). As mentioned earlier, however, the maximum cell density should not be the sole parameter to consider when assessing the progresses made by a new system. To date, there is no consensus on an optimal bioreactor and different designs are still investigated and characterized to reach clinically relevant number of cells faster and with higher reliability. An issue to highlight the best approach is the use of various cell origins and control groups from one study to the other. There is a lack of direct comparison between different bioreactor systems and studies are mostly conducted between one specific system and standards only, usually in static conditions. Moreover, many parameters are involved (Trickett et al., 2002) and the method to calculate the expansion ratio is not always clearly reported: it is not always clear if proliferation rates are reported for the whole culture process or bioreactor steps only, and it is sometimes hard to distinguish the absolute number of cells seeded based on the initial cell concentration in the protocols. It could be useful to perform comparison studies between common geometries with the same parameters, in particular seeding density, medium composition, culture duration and feeding schedules, although intellectual property and proprietary processes and results could make such cross-analyses difficult to perform. Quality by Design (QbD) and Design of Experiment (DoE) methodologies presented in this review would be beneficial tools in performing these multiple parameter studies to truly understand the design space and operating conditions in autologous cell therapy production. These future studies also need to establish how the cell expansion bioreactor and their new capabilities can influence the QbD principles. In addition, as REP conditions are widely used in bioreactor approaches, it could be interesting to compare results with control groups where REP is not used (*i.e.* basic culture medium without factors triggering T cell activation and proliferation), in order to study possible synergies between REP and the specific bioreactor features. This could even highlight advanced systems where activation steps can be reduced to obtain the same range of expansion, leading to decreases in costs and labour work.

For bioreactors showing promising results, as mentioned by Jin *et al*, it would be mandatory to confirm quickly that the expansion rate found with cell lines and primary cells from healthy donors could be obtained again with samples from actual patients (Jin et al., 2018). In addition, the majority of the development studies reported here used T cells without modifications (no TCR or CAR-T processes). Feasibility and improvements in proliferation rate should be checked in combination with these transduction methods. Here again, how industrial and clinical data are shared or not with the scientific community will alter how the technology will move towards available, affordable treatments and large-scale manufacturing platforms.

By using a bioreactor, it is possible to better control the parameters in cell culture process as discussed above. However, due to the complex T-cell biology, the effect of the parameters used in T cell expansion on the resulting cell population are not always understood. We could therefore recommend taking a Design of Experiment (DoE) approach from the initial design and deployment of

bioreactors into bioprocesses. This approach would allow identification of all the parameters that influence the process a bioreactor performs, and development of an understanding of how the parameters can be changed to influence the output with efficient use of experimental runs (Montgomery, 2001). Further to this, it is apparent that a bioreactor can influence a number of quality attributes of a therapeutic cell. This multiple attribute affect would require an understanding of Quality by Design (QbD), considering that the cell product will have more than one factor that governs its success as a therapeutic, which should be considered from the initial design phase (Torres, 2015). This approach would extend bioreactor investigations to include all the therapeutic quality aspects of the cells produced by the bioreactor, not just the ease at which the reactor produces large quantities of cells.

Besides increasing proliferation, for various bioreactors, the ultimate goal was often to design a completely closed system (Klapper et al., 2009) to comply with GMP, but also to move towards a fully automated system (Maartens et al., 2017). It is for instance available as an optional module for Wave bioreactors to control medium exchanges (Sadeghi et al., 2011). An automated device reduces labour work and risk of contamination due to multiple interventions, but this is also the best approach to obtain standard results with batch-to-batch reliability (Kaiser et al., 2015). The standardised conditions of a bioreactor are indeed suggested to limit the differences in batches (Eaker et al., 2017). However, automation by pre-programming set tasks does not allow a system to respond to deviations. Improved systems may be produced by introducing analytical technologies and process control methods. Technological bottlenecks are the development of non-invasive and live analysis methods for cell culture parameters such as cell concentration, pH, lactate concentration or oxygen consumption that could be embedded in the humidified, 37°C atmosphere (Kaiser et al., 2015). Iyer *et al* showed that many promising methods exist that could provide in-line monitoring of the quality attributes of cell therapies, but there are challenges in: integrating the sensors in to the bioreactor, converting technologies to be suitable for single-use; and some methods requiring complex analysis to return the parameter of interest from a surrogate measure (Iyer et al., 2018). Measurements would be used to automatically adjust technical parameters such as rocking motion, speed rate, flow rate, medium renewal or injection in case of increasing volume. This moves the therapy-production bioreactor systems away from automation by pre-programming the tasks, currently the norm established by the review of systems above, and towards closed loop control, a more robust pillar of engineering. Sensors and actuators should not increase costs significantly or decrease safety and thus jeopardize the benefits of the bioreactors in terms of proliferation rate and GMP-compliance. With suitable methods to detect quality attributes available, there is then the challenge of implementing methods to provide feedback so that the cost of the monitoring equipment can be offset by improving aspects of the processing. A cost-benefit analysis of in-line sensing and control feedback in an autologous therapy setting needs to be performed to understand if the methods can produce a worthwhile improvement at the scale of autologous therapy manufacture.

Batch-to-batch variability is a known issue when cells form part of a therapeutic treatment, and from our review above, it is apparent that current generation bioreactors can stabilise parameters and automatically perform tasks in the same routine for each batch of cells expanded regardless of the exact design used. However, many of them lack closed-loop control to respond to a progressing cell expansion, the rate of progression can vary greatly from batch-to-batch, which is widely reported in the bioreactor studies referenced throughout this paper. The reason for a lack of control is related to a lack of sensor and established methodology to perform in-line sampling of the cell expansion process.

Closed systems could be promoted by the use of perfusion from a reservoir hosting enough medium for the whole culture duration, as proposed by Klapper *et al* (Klapper et al., 2009) in a homemade design. Perfusion is already used with hollow fibres bioreactors, and although additional steps to optimise flow rate would be needed, this could be combined with other systems such as G-Rex® flasks or stirred tanks to monitor better medium volumes and shear stress levels (Janas et al., 2015). Interestingly, there is a lack of study comparing the effect of periodic and continuous perfusion in T cell expansion bioreactors, and such analysis could be encouraged to obtain optimal protocols.

To move further towards clinics, it will also be important to consider side steps, for instance how yield and viability in bioreactor could be altered when using cryopreserved cells. Additional recovery periods could have to be included in the culture schedule (Sadeghi et al., 2013). In addition, to reach very high throughputs, bioreactors could be used for continuous culture as proposed by Knazek *et al* (Knazek et al., 1990) in hollow fibre bioreactors. When harvesting the expanded T cells, a fraction of the cell population would remain in the system to be re-expanded from the same cell source. Further investigations on such an approach would be interesting to develop multi-cycles bioreactors and emergency secondary batches of cells for patients.

9. Conclusions

In conclusion, there is currently no consensus on an optimal bioreactor approach to obtain clinically relevant numbers of T cells, although most of the reported systems showed higher proliferation rates than current gold standards. However, optimal cell density and proliferation rates are not the only objectives to ensure translational progresses: automation, process control and complete closed systems are still unachieved ultimate goals but could be progressively implemented if considered from the early steps of development, as it was done previously to comply with GMP and REP, now widely used. Process engineering methods were suggested to help overcome the challenges in implementing a new generation of bioreactor technologies.

Conflict of interest statement

There is no conflict of interest to disclose.

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SYSTEMS	YIELD COMPARE TO STANDARDS	COST AND VOLUME REDUCTION	GMP-COMPLIANT
Hollow fibres (Quantum)	Equal or slightly higher	Yes	Yes, closed system
Improved flasks (G-Rex)	Same final density, faster process	Yes	Protocol-dependant
Improved bags (Wave)	Equal or higher, easier process	Yes, but initial investment	Yes, closed system and automation potential
One-system multiple-step bioreactors (Prodigy)	Equal, but volume limited.	Yes, but initial investment	Yes, closed system and automation potential
Stirred tanks	Higher after careful optimisation	Not without design modifications	Automation potential but standard designs needs interventions.

Table 1: Summarized comparison of the main approaches in terms of proliferation rate, cost and volume reduction, and compliance with GMP.

Figure 1: Main concept of a bioreactor chamber including the most common elements.

Figure 2: Number of articles found for a search of the words "bioreactor" or "T cell bioreactor" in title, abstract and keywords from 1983 to 2019 in scopus.com database. Results as consulted on 21/04/20.

Figure 3: Generation of T cells targeting tumours through autologous TILs harvesting or CAR and TCR processes.

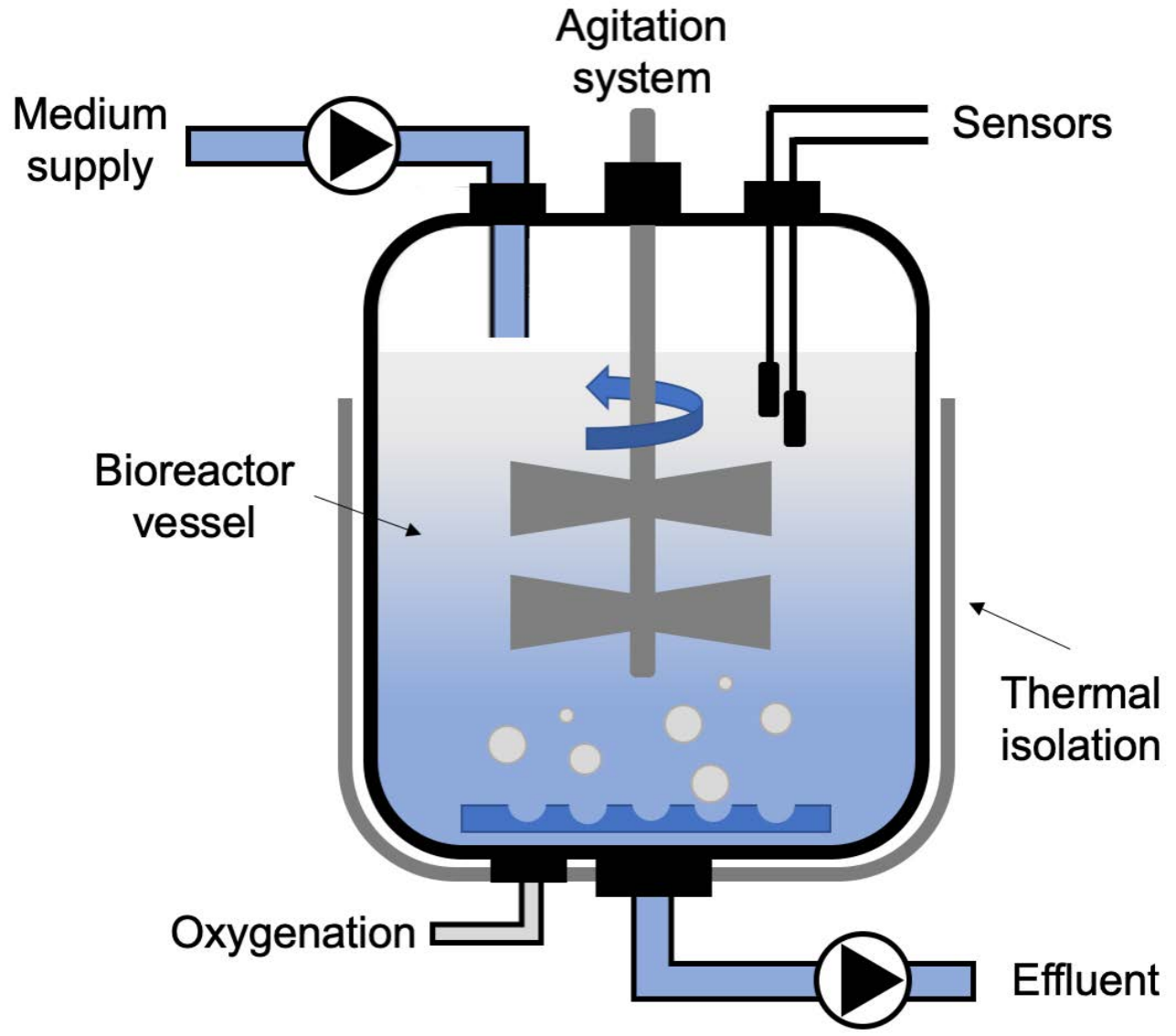
Figure 4: Schematic representation of hollow fibres used as a bioreactor for cell culture and bioproduction, reprinted from Patel et al with permission from Elsevier (Patel et al., 2018).

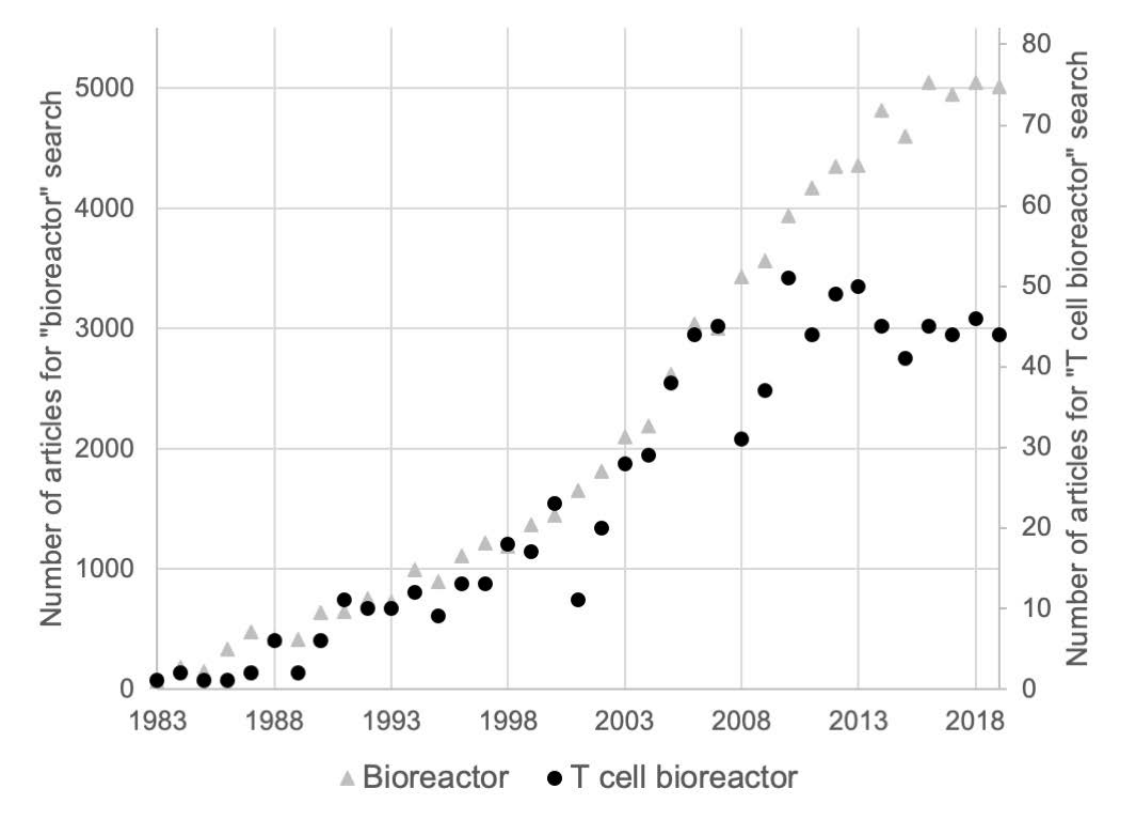
Figure 5: Geometry of different sizes of G-Rex (Gas Permeable Rapid Expansion) flask models with respectively 5-cm^2, 100-cm^2 and 500-cm^2 culture surfaces. Gas exchange (O_2, CO_2) occurs through the bottom semi-permeable membrane. Reprinted from Bajgain et al (Bajgain et al., 2014) *with permission from Elsevier and the ASGCT.*

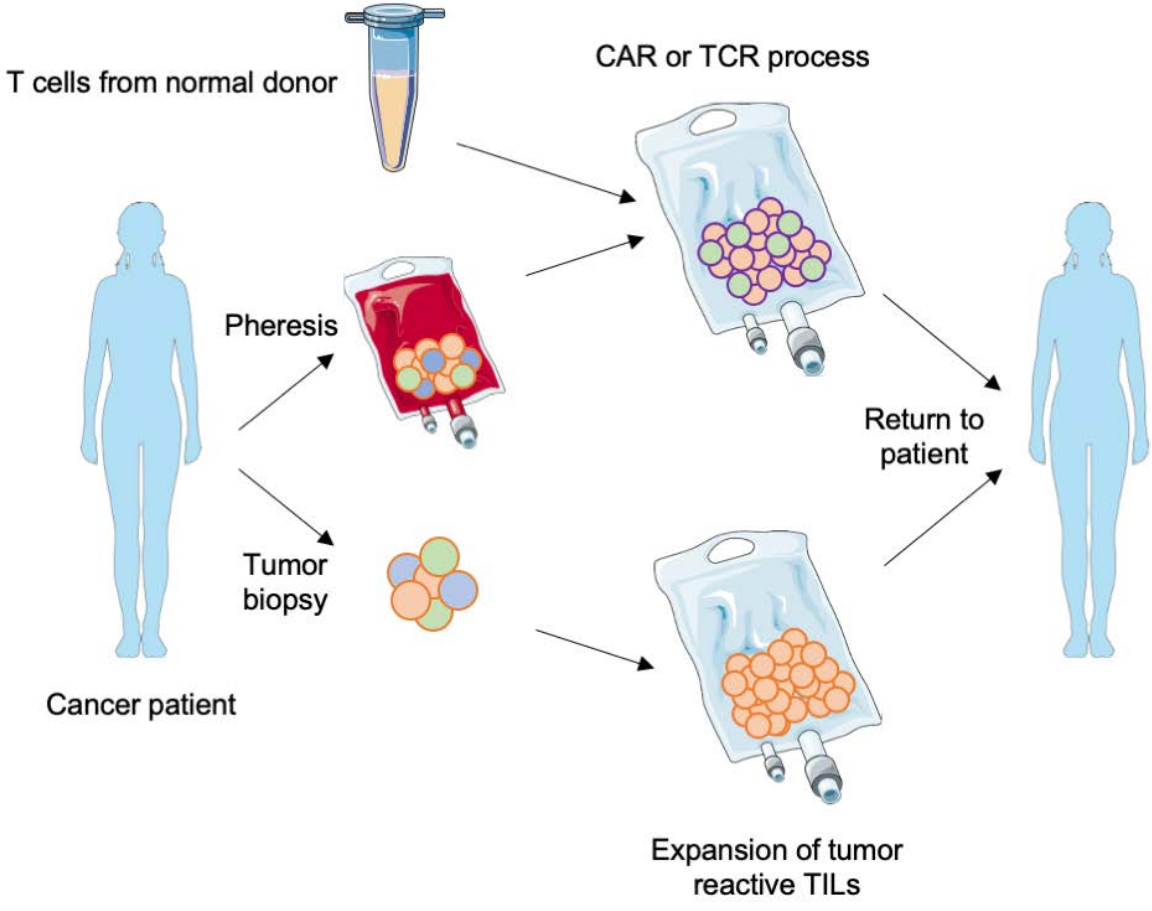
Figure 6: Geometry of the Wave Bioreactor with perfusion system. Reprinted from Tang et al (Tang et al., 2007) *with permission from Wiley.*

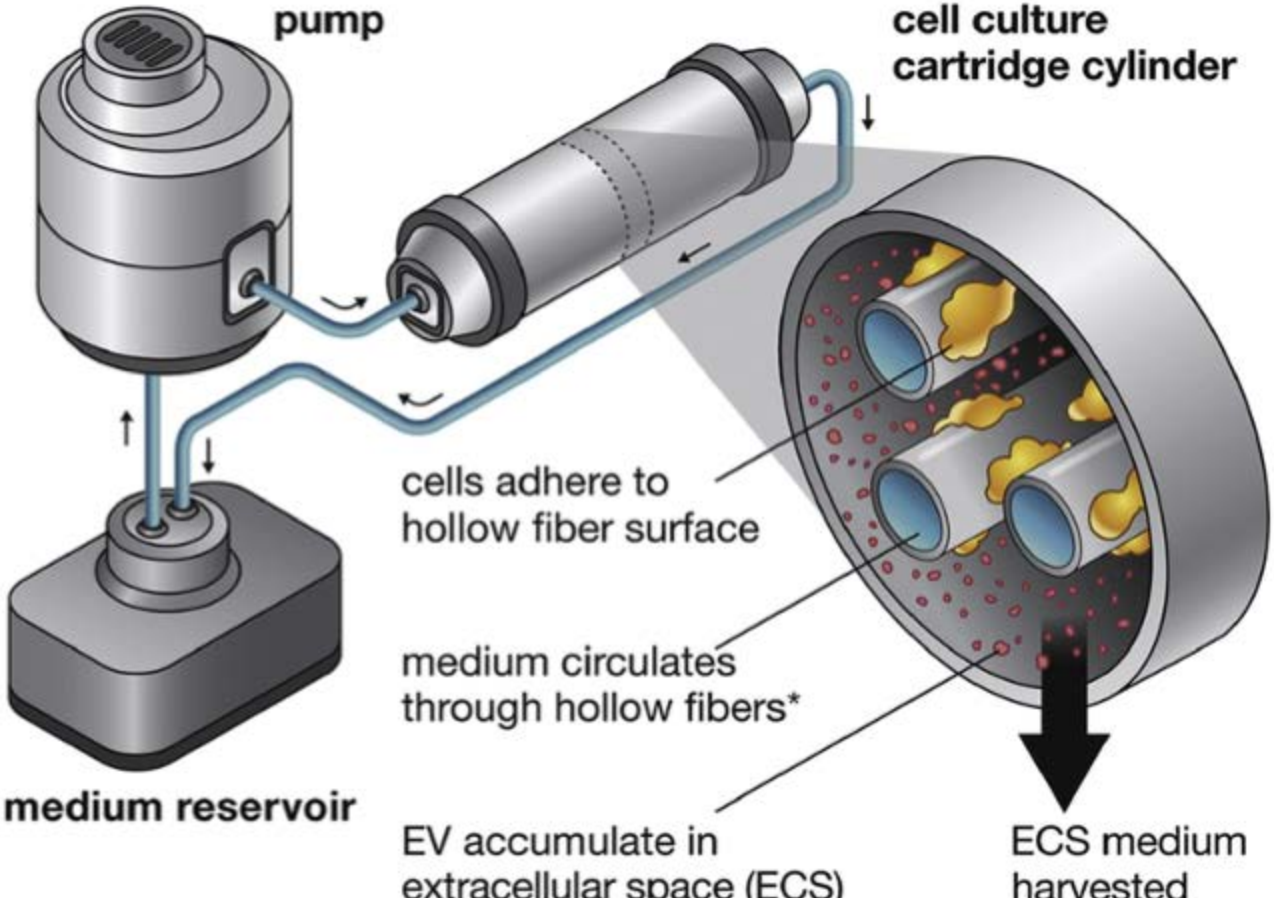
Figure 7: Standard design of a spinner flask with mechanical stirring. Based on Foster et al (Foster et al., 2004).

Figure 8: Example of an original homemade design. A disk-shaped chamber is made of two compartments, culture medium and gas, separated by a membrane permeable to gas but not to liquid. The medium reservoir can perfuse the system based on lactate concentration over the total culture time (14 days) to ensure a complete closed-system (Klapper et al., 2009). *Reprinted from Klapper et al with permission from Elsevier* (Klapper et al., 2009).


Agitation system
Medium supply
Sensors
Bioreactor vessel
Thermal isolation
Oxygenation
Effluent

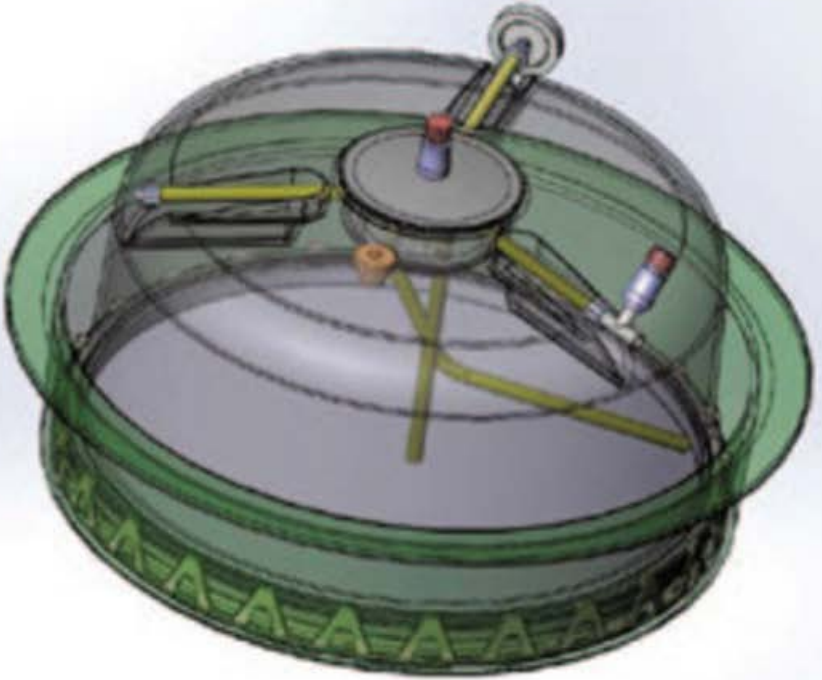

Number of articles for "bioreactor" search
Number of articles for "T cell bioreactor" search
0
1000
2000
3000
4000
5000
0
10
20
30
40
50
60
70
80
1983
1988
1993
1998
2003
2008
2013
2018
Bioreactor
T cell bioreactor


T cells from normal donor
CAR or TCR process
Pheresis
Return to patient
Tumor biopsy
Cancer patient
Expansion of tumor reactive TILs

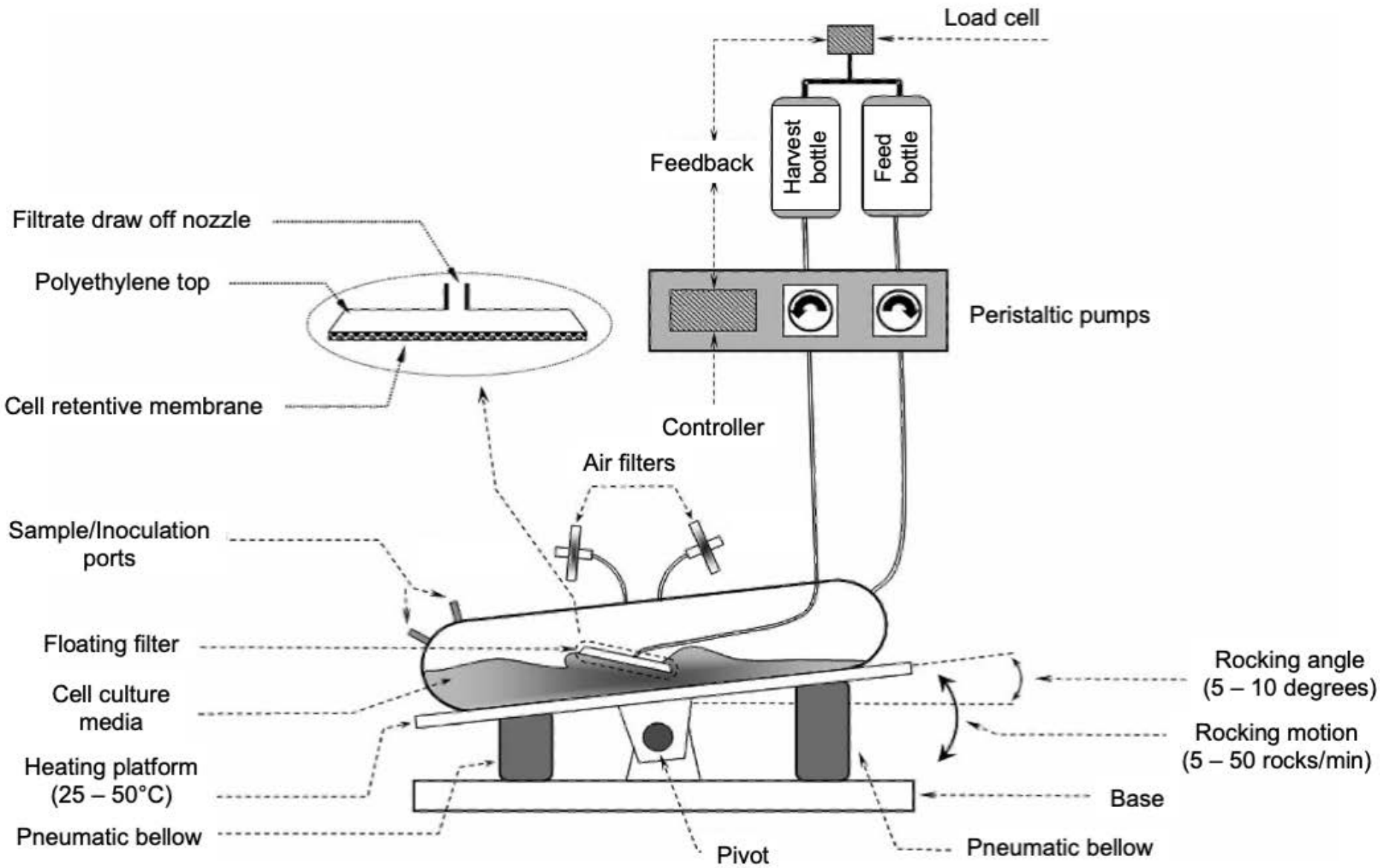

pump
cell culture
cartridge cylinder
cells adhere to
hollow fiber surface
medium circulates
through hollow fibers*
EV accumulate in
extracellular space (ECS)
ECS medium
harvested
medium reservoir

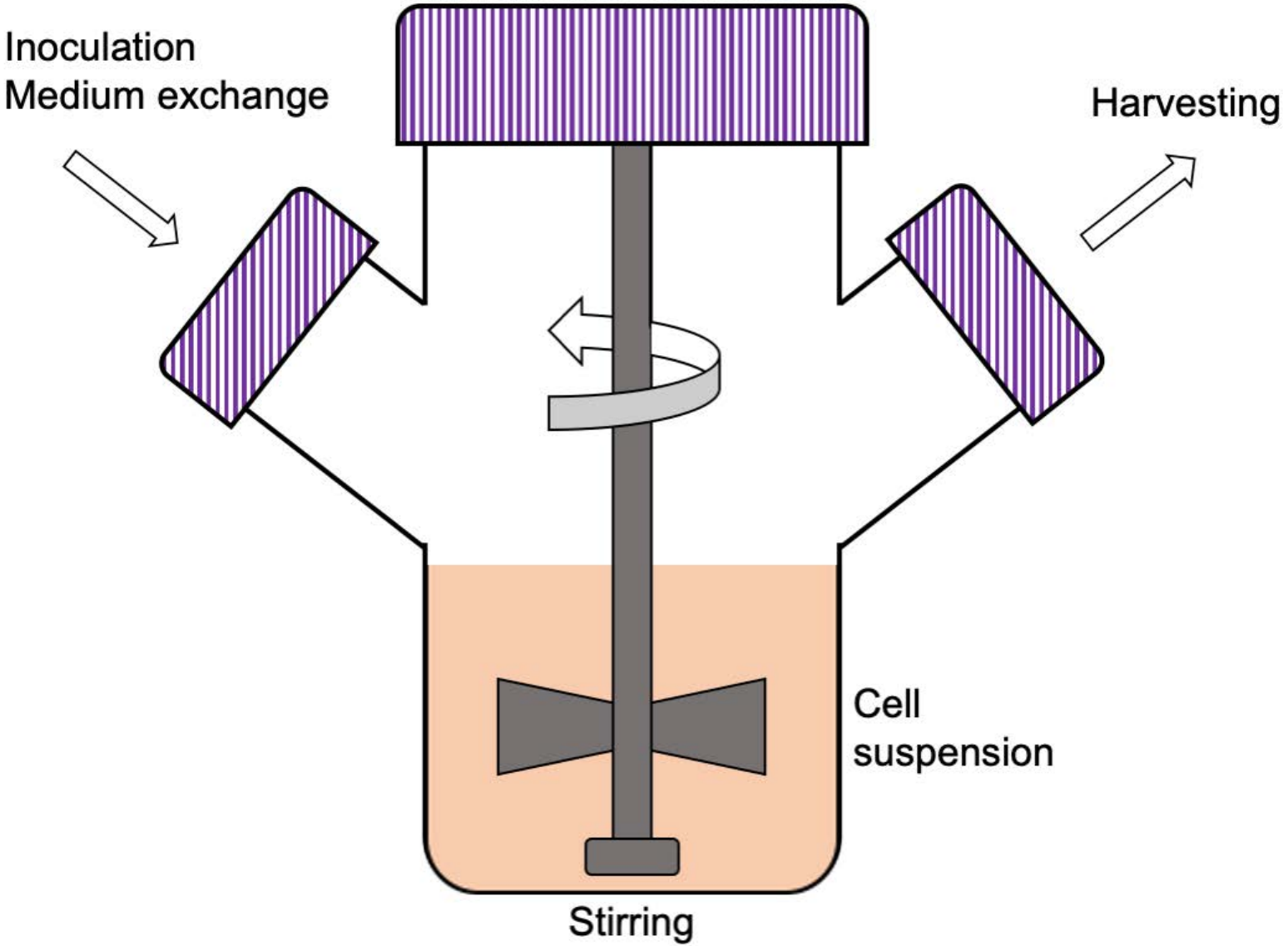
G-Rex 5

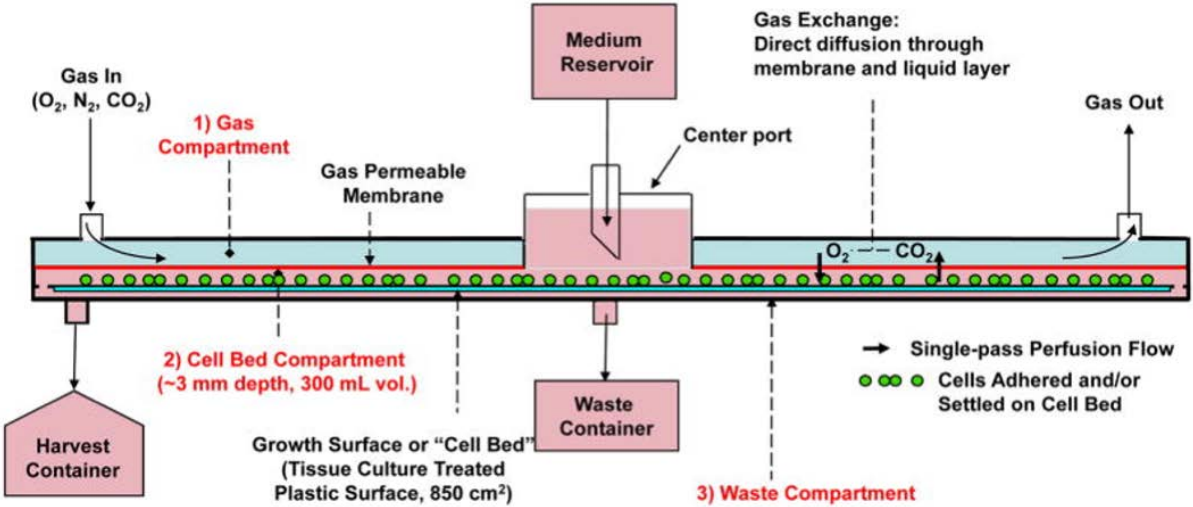
G-Rex 100



G-Rex 500


Load cell
Feedback
Harvest bottle
Feed bottle
Filtrate draw off nozzle
Polyethylene top
Peristaltic pumps
Cell retentive membrane
Controller
Air filters
Sample/Inoculation ports
Floating filter
Cell culture media
Rocking angle (5 – 10 degrees)
Rocking motion (5 – 50 rocks/min)
Heating platform (25 – 50°C)
Base
Pneumatic bellow
Pivot
Pneumatic bellow


Inoculation
Medium exchange
Harvesting
Cell
suspension
Stirring


Medium Reservoir
Gas Exchange:
Direct diffusion through membrane and liquid layer
Gas In
(O_2, N_2, CO_2)
Gas Out
1) Gas Compartment
Center port
Gas Permeable Membrane
O_2
CO_2
2) Cell Bed Compartment
(~3 mm depth, 300 mL vol.)
Single-pass Perfusion Flow
Cells Adhered and/or Settled on Cell Bed
Harvest Container
Waste Container
Growth Surface or "Cell Bed"
(Tissue Culture Treated Plastic Surface, 850 cm²)
3) Waste Compartment