Bags versus flasks: a comparison of cell culture systems for the production of dendritic cell–based immunotherapies

Natalie Fekete,[1,2] *Ariane V. Béland*,[1] *Katie Campbell*,[2] *Sarah L. Clark*,[2] *and Corinne A. Hoesli*[1]

In recent years, cell-based therapies targeting the immune system have emerged as promising strategies for cancer treatment. This review summarizes manufacturing challenges related to production of antigen presenting cells as a patient-tailored cancer therapy. Understanding cell-material interactions is essential because in vitro cell culture manipulations to obtain mature antigen-producing cells can significantly alter their in vivo performance. Traditional antigen-producing cell culture protocols often rely on cell adhesion to surface-treated hydrophilic polystyrene flasks. More recent commercial and investigational cancer immunotherapy products were manufactured using suspension cell culture in closed hydrophobic fluoropolymer bags. The shift to closed cell culture systems can decrease risks of contamination by individual operators, as well as facilitate scale-up and automation. Selecting closed cell culture bags over traditional open culture systems entails different handling procedures and processing controls, which can affect product quality. Changes in culture vessels also entail changes in vessel materials and geometry, which may alter the cell microenvironment and resulting cell fate decisions. Strategically designed culture systems will pave the way for the generation of more sophisticated and highly potent cell-based cancer vaccines. As an increasing number of cell-based therapies enter the clinic, the selection of appropriate cell culture vessels and materials becomes a critical consideration that can impact the therapeutic efficacy of the product, and hence clinical outcomes and patient quality of life.

Cancer represents a significant socioeconomic burden, causing 8.2 million deaths worldwide in 2012.[1-4] By 2030, this burden is expected to nearly double, growing to 21.4 million cases and 13.2 million deaths.[2] In recent years, cell therapy has emerged as a novel, complex, and very promising therapeutic strategy for the treatment of diseases that do not respond to classical pharmaceutical or biopharmaceutical product-based treatments.[5,6] Targeting the immune system—and not the cancer itself—represents a paradigm shift in oncology and vaccinology. Similarly, chimeric antigen receptor (CAR)- and T-cell receptor–engineered T cells have resulted in a landslide transformation in immunology and adoptive cell transfer.[7] These sophisticated cell

ABBREVIATIONS: APC = antigen-presenting cell; CAR = chimeric antigen receptor; DC(s) = dendritic cell(s); FEP = fluorinated ethylene propylene; Mo-DC(s) = monocyte-derived DC(s); PFA = perfluoroalkoxy copolymers; PTFE = polytetrafluoroethylene.

From the [1]Department of Chemical Engineering, McGill University, Montreal, Canada; and [2]Saint-Gobain Ceramics & Plastics, Inc., Northboro R&D Center, Northborough, Massachusetts.

Address reprint requests to: Corinne A. Hoesli, Department of Chemical Engineering, McGill University, 3610 University Street, Montreal, QC, Canada, H3A 0C5; e-mail: corinne.hoesli@mcgill.ca.

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TABLE 1. Currently ongoing clinical trials in phase III using DCs for immunotherapy*

Study start date	Sponsor	Target indication	Product	ID
2016	Radboud University	Melanoma	nDC: natural DCs	NCT02993315
2014	Sotio a.s.	Metastatic castrate-resistant prostate cancer	DCVAC/PCa: autologous DCs	NCT02111577
2014	University Hospital, Erlangen, Germany	Uveal melanoma	Autologous DCs loaded with autologous tumor RNA	NCT01983748
2012	Argos Therapeutics,Inc.	Renal cell carcinoma	AGS-003: autologous DC immunotherapy	NCT01582672
2006	Northwest Biotherapeutics, Inc.	Glioblastoma multiforme	DCVax-L: autologous DCs pulsed with tumor lysate antigen	NCT00045968

* DC immunotherapy studies listed in the clinicaltrials.gov registry as Phase 3 "Recruiting" or "Active, not recruiting" with the search term "dendritic cell" accessed on December 28, 2017.

products are able to reengage the immune system's anticancer responses, replace damaged tissues, and heal chronic wounds.[8,9] Considering the magnitude of potential impact, the field of "cancer immunotherapy" was chosen as the "Breakthrough of the Year" by *Science* magazine in 2013.[10-12] The global market for cell therapy was valued at approximately US$2.5 billion in 2012 and is expected to reach US$8 billion by 2018.[5] The advance of improved technologies in coming years should further reduce production costs and enhance clinical efficacy.

Currently available cancer vaccines include cell-based, protein-based, recombinant live vector (viral or bacterial)–based and nucleic acid–based vaccines.[6,13] Detailed reports summarizing the state of the art and proposed mechanisms of action of both virus-based and DNA-based cancer immunotherapy products have been published recently elsewhere.[13-16] Currently, there are more than 1900 studies investigating the clinical efficacy of "cancer vaccines" registered with the US National Institutes of Health website (www.clinicaltrials.gov). Most of these trials are in the phase I/II testing safety/efficacy stage, with only a small number of trials in phase III (Table 1). Antitumor vaccination with viable, complex active ingredients such as cells is a complicated, multistep task.[17,18] The optimal clinical-scale production platform and culture modalities for guaranteeing an effective cell product have yet to be established.

Manufacturing and distribution challenges can significantly impact the commercial and clinical viability of personalized medicines such as cell-based immunotherapy products.[6,13-15] In the transition from preclinical to clinical studies, changes in scale and culture materials, which may at first sight appear trivial, can drastically change the quality and efficacy of the product. This review addresses the manufacturing challenges related to the production and the characterization of antigen-presenting cell (APC)-based cancer vaccines, with emphasis on the impact of cell culture vessel material selection on resulting cell products. Key bioprocess engineering and materials science considerations associated with the transition from standard polystyrene flask cultures to closed cell culture systems such as fluoropolymer-based cell culture bags are presented. This review summarizes translational efforts to improve current strategies in "'bench-to-bedside" applications for dendritic cell (DC)-based immunotherapies from a materials science and bioprocess engineering perspective.

APC-BASED CANCER VACCINES

The development of cell-based cancer vaccines originated with the concept of exploiting the potential of APCs to trigger the immune system. APCs are uniquely qualified to initiate a specific and targeted immune reaction against the presented tumor-associated antigens.[17,19] In vivo, DCs act as the "professional" and most potent APCs of the immune system.[20] The antigens used in cancer vaccines are typically derived either from the patient's own tumor or are presented to the cells as a recombinant tumor-associated antigen, which may be fused to an adjuvant protein for codelivery.[16]

Generating tumor-specific APCs in vitro

In vitro, several cell types can be used as progenitor cells to obtain APCs that are phenotypically similar to DCs.[21-25] Peripheral blood mononuclear cells (PBMCs) and CD34+ hematopoietic progenitor cells have been used as sources for DC generation, but monocytes remain the most commonly used progenitor cell type.[26-30] Monocytes can be induced to generate DCs via addition of differentiation-inducing cytokines, typically interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor,[31,32] during 3 to 7 days in culture.[33]

Cell maturation can be achieved via the addition of a cocktail of inflammatory cytokines and/or toll-like receptor agonists, such as tumor necrosis factor-α, prostaglandin E2, IL-6, lipopolysaccharide and polyinosinic:polycytidylic acid in the final 2 days of culture.[24,34-41] During this time period, patient-specific tumor-associated antigens may be added to

the cultures for uptake by the DCs.[33,42] Following maturation, DCs are then administered to the patient, usually in a series of repeated injections. The maturation phase of the DCs remains less standardized, providing an opportunity for optimization and patient tailoring of the manufacturing system. To this end, both biomedical (generation of DCs and their appropriate stimulation) and engineering (selection of culture vessels and microenvironment) considerations should be taken into account.

Sipuleucel-T

Sipuleucel-T was the first and so far only Food and Drug Administration–approved cell-based cancer vaccine. This product was developed by Dendreon Corp. Sipuleucel-T is intended as a first-line treatment of asymptomatic or minimally symptomatic metastatic castrate-resistant prostate cancer in patients that no longer respond to conventional hormone therapy.[8,18,43,44] Sipuleucel-T is generated from the patient's own peripheral blood mononuclear cellss, which are subsequently enriched for APCs[16] and exposed to a fusion protein comprised of prostatic acid phosphatase and granulocyte macrophage colony-stimulating factor.[17,18,45] The cells are reinfused into the patients within 3 days of the initial leukapheresis.[16,45] The efficacy of this cell-based immunotherapy was first reported in the landmark phase III IMPACT trial.[19,45] There, patients received a total of three infusions of the APCs generated in vitro using a fully closed fluoropolymer cell culture bag system. Patients treated with sipuleucel-T had a 4-month overall survival benefit compared to patients infused with the placebo. Despite this and other recent achievements,[16,17] the precise immunologic mechanisms of action underlying this therapeutic effect are challenging to assess and therefore still unclear.[8,18,46]

DC cultures: Suspension versus anchorage-dependent systems

Many traditional monocyte-derived DC (Mo-DC) culture protocols rely on the adherence of both monocytes and DCs to their culture substrates.[47-50] Several protocols use the plastic adherence of monocytes in an initial enrichment step before removing all nonadherent cells and proceeding to culture only the adherent cells as APCs.[51-53] This implied anchorage dependency of Mo-DCs in vitro contrasts with more recent protocols eschewing adherent cells for DCs cultured in suspension. Rouard and colleagues[54] cultured monocytes in hydrophobic bags before transferring them to polystyrene cultures for their maturation as adherent cells. Coulon and colleagues,[55] on the other hand, first cultured monocytes as adherent cell cultures on polystyrene surfaces before transferring them into suspension cultures in fluorinated ethylene propylene (FEP) bags at Day 5 for DC culture. An interesting approach proposing Mo-DC production in a novel closed-cell culture system utilizing a combination of hydrophobic cell culture bags and styrene copolymer microcarrier beads was proposed by Maffei and colleagues[56] in 2000 and since patented.[57] More recent reports document that Mo-DC culture and maturation can entirely be achieved in suspension cultures.[23,35,58-68] A number of studies have compared traditional polystyrene flask cultures for adherent cells to fluoropolymer-based bag systems, as summarized in Table 2. As a prominent example, sipuleucel-T was manufactured in fluoropolymer bags (Saint-Gobain) composed of an FEP copolymer.[45,73] Overall, a paradigm shift can be observed over the past decade, and current best practice methods no longer strictly associate the efficient clinical-scale production of both immature and mature DCs with their adherence to surfaces.

Both for adherent and suspension cell cultures, the culture vessel selected may directly or indirectly impact the cues provided from the microenvironment via chemical and mechanical stimuli (Fig. 1). The gas permeability of the cell culture vessel can change parameters such as the dissolved oxygen concentration, the pH, and the osmolarity of the cell culture medium. The physicochemical properties of the culture material can affect the type, amount, and conformation of proteins and other medium components adsorbing to the culture vessel. The topography and stiffness of the culture vessel can change cell interactions with the surface, in addition to changing shear forces applied to cells during handling. All of these factors can significantly change the cell microenvironment, potentially synergistically, and alter product quality.

CLOSING THE MANUFACTURING PROCESS: SWITCHING Mo-DC CULTURE FROM POLYSTYRENE TO FLUOROPOLYMER SURFACES

Polystyrene-based flask or multiwell cultures

Polystyrene has been traditionally used for cell culture since the 1960s.[74,75] To support the culture of anchorage-dependent cells, the hydrophobic polystyrene must usually be surface treated to render it more hydrophilic and thereby facilitate cell adhesion and spreading in culture conditions.[76,77] This treatment is proprietary to each commercial manufacturer, but reportedly typically consists of a plasma treatment, which produces additional hydroxyl, carboxyl, and aldehyde groups on the culture surface.[74,75] Battiston and colleagues[78] compared tissue culture polystyrene surfaces from three different companies (Sarstedt, Wisent Corp., and Becton Dickinson) and found marked differences in protein adsorption, surface wettability, and monocyte retention following 7 days of culture. This study and others underline the frequently underreported

TABLE 2. Studies comparing flask to bag culture systems for DC immunotherapy

Reference	Bag system	vs.	Flask, multiwell system	Culture medium; supplements	Maturation factors	Potency assays	Outcome
Macke, 2010[66]	Polyolefin bags	vs.	Polystyrol-based cell culture flasks	CellGro (CellGenix); GM-CSF and IL-4	IL-1b, IL-6, TNF-α and PGE2 (48 h)	FC, SEM, MLR, DNA Microarray	No objective difference in the DCs generated in both systems
Tan, 2008[69]	Polyolefin bags	vs.	Surface-treated polystyrene flasks	CellGro; GM-CSF, IL-4, TNF-α and 10% autologous plasma	N/A	FC, allogeneic MLR	Reduced fraction of DCs in bags (4.7%) compared to flasks (40%) based on flow cytometry
Kurlander, 2006[70]	FEP bags	vs.	Polystyrene flasks	RPMI 1640 with autologous plasma or human AB serum; GM-CSF and IL-4	CD40L and IFN-γ or poly(I:C) and IFN-γ (24h)	FC, ELISA, DC migration assay, IFN-γ production of autologous T cells	FEP- and PS-cultured DCs are similar in phenotype and in some functional measures, but FEP markedly reduces DC production of IL-12 and IL-10
Elias, 2005[60]	Polyolefin bags	vs.	Polystyrene multiwell plates	CellGro DC for bags, X-VIVO + autologous serum for plates; GM-CSF and IL-4	LPS or TNF-α, IL-1b, IL-6 and PGE2 (48 h)	FC, MLR, autologous antigen presentation	No difference in viability; comparable phenotype with exception of CD1a
Wong, 2002[67]	FEP bags	vs.	Polystyrene flasks	X-VIVO 15; GM-CSF and IL-4	None	FC, MLR, autologous recall responses to tetanus toxoid and influenza virus	DCs were equivalent in yield, phenotype, and in vitro function
Guyre, 2002[71]	Polyolefin bags	vs.	Polystyrene flasks	AIM V; GM-CSF and IL-4	R848 or TNF-α (24-72 h)	FC, MLR, autologous antigen presentation	Bag-cultured DCs were superior to flask-cultured cells in terms of yield, viability, and function
Suen, 2001[72]	FEP bags	vs.	Polystyrene T175 flasks	X-VIVO 15; GM-CSF and IL-4	None	FC, MLR, dextran-uptake assay	DCs have similar viability, purity, phenotype, yield, and function

ELISA = enzyme-linked immunosorbent assay; FC = flow cytometry; FEP = fluorinated ethylene propylene; IFN = interferon; IL = interleukin; LPS = lipopolysaccharide; MLR = mixed lymphocyte reaction; PGE2 = prostaglandin-E2; R848 = resiquimod; TNF = tumor necrosis factor.

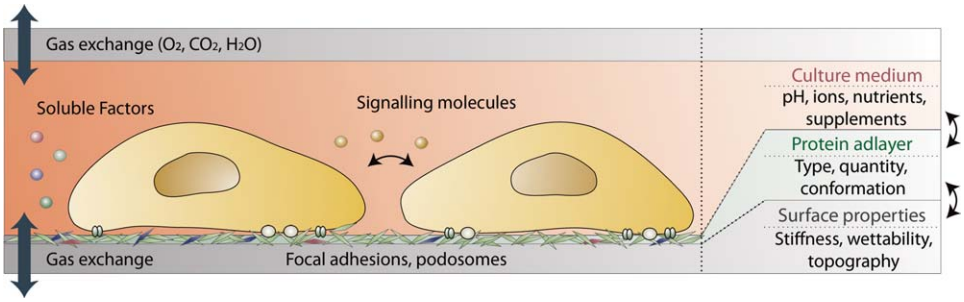

Fig. 1. Cellular microenvironment in vitro. During culture, cells interact with their microenvironment via a plethora of membrane-bound proteins. They are exposed to a multitude of physical, chemical, and biological cues including soluble (signaling or growth) factors in the culture medium, the protein adlayer adsorbed to the culture surface and other cells. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3. Examples of closed culture systems used for DC production

Material of fabrication	Format	Approximate standard capacity range (cm^2)*	Examples of commercial systems and manufacturers (currently available or used in published DC culture studies)
Polystyrene	Layered cell culture vessels	600-60,000	HYPERStack (Corning), Cell Factory (Nunc Thermo Scientific)
Silicone membrane bottom	Bottle with membrane bottom	50-500	G-Rex
Polyolefins	Bags	20-200	CellGro Cell Expansion Bags (Mediatech Corning), EXP-Pak Bio-Containers (Charter Medical), MACS GMP Cell Expansion Bag (Miltenyi Biotec), LifeCell X-Fold Cell Culture Container†, Opticyte, (all Nexell, Baxter Healthcare; discontinued)
Fluoropolymers (e.g., FEP)	Bags	20-5000	VueLife (Saint Gobain), PermaLife (OriGen Biomedical), SteriCell (DuPont; discontinued)
PVC, EVA, and other polymers or copolymers	Bags	100-1000	Evolve (OriGen Biomedical)

* The typical working volume is 0.2-0.5 mL/cm^2 for flasks or layered vessels, 4-11 mL for the membrane-bottom vessel system, or 0.5-1 mL/cm^2 for bags.
† Made from PL732 (polyolefin) coextruded with PL705 (high-impact polystyrene) on the inner surface.[83]
‡ Formerly manufactured by American Fluoroseal Corporation.
EVA = ethylene-vinyl acetate; FEP = fluorinated ethylene propylene; PVC = polyvinyl chloride.

observation that the choice of tissue culture polystyrene from different manufacturers can lead to significant disparities in terms of protein adsorption and cell spreading characteristics.[79-81] Given the ubiquitous presence of proteins in animal cell culture media, protein adsorption likely plays a key role in determining cell adhesion to surfaces. Cell surface adhesion is in fact thought to be largely mediated by proteins adsorbed to the surfaces, rather than the surfaces themselves.[74,82]

Polyolefin- and fluoropolymer-based bag cell culture systems

In contrast to the more rigid polystyrene-based cell culture plastics such as multiwell plates or T-flasks commonly found in most cell culture laboratories, cell culture bags are made of more flexible polymers, including polyolefins and fluoropolymers. The materials of fabrication of examples of closed systems used to scale up DC production are listed in Table 3. The most commonly known examples of polyolefins include polyethylene and polypropylene. Polyolefin-based cell containers have been tested for both in vitro culture and storage of CD34+ cells, platelets, DCs, and T cells.[60,66,67,72,84-86] In the case of monocyte-derived APCs, different monocyte isolation methods may alter the composition of "contaminating" cell populations and proteins entrained with the monocytes seeded, which may in turn impact the propensity of monocytes to adhere to surfaces and to differentiate into APCs.

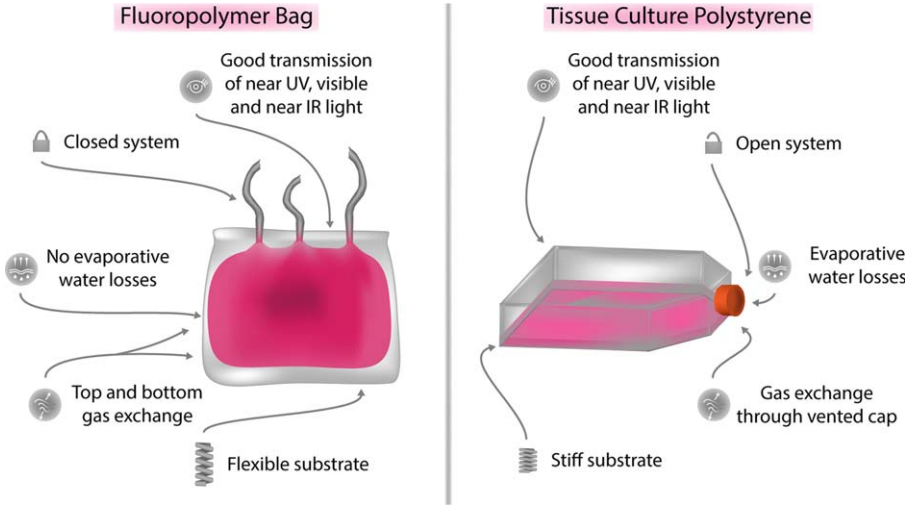

Fig. 2. Key similarities and differences between fluoropolymer bag and polystyrene flask cell culture systems. [Color figure can be viewed at wileyonlinelibrary.com]

Fluoropolymers are a family of high-performance plastics consisting of fully or partially fluorinated monomers. Many of these polymers are linear and with a backbone of carbon-carbon bonds and pendant carbon-fluorine bonds. The carbon-fluorine bonds of fluoropolymers directly correlate to the unique physical and chemical properties associated with fluoropolymers including low surface free energy and coefficient of friction, chemical inertness, excellent electrical properties, high thermal stability, and maintenance of physical properties even at cryogenic temperatures—a combination of properties that make them interesting candidates for biomedical applications.[87] Polytetrafluoroethylene (PTFE) was the first fluoropolymer, discovered in 1938 by Dr Roy Plunkett of DuPont, and was marketed under the brand name Teflon.[88] Commonly used fluoropolymers today include the perfluoropolymers PTFE, FEP, and perfluoroalkoxy copolymers (PFA) as well as partially fluorinated materials such as ethylene tetrafluoroethylene and polyvinylidene fluoride.[89,90] PTFE, FEP, and PFA are all sold under the Teflon trademark from Chemours. PTFE is also sold under the Polyflon trademark and FEP and PFA under the Neoflon trademark from Daikin. Fluoropolymers have found extensive commercial application in the chemical, electronic, automotive, and construction industries and, with the exception of PTFE, are melt-extrudable thermoplastics that can be processed using traditional polymer processing methods.[88,90]

Similar to polyolefin-based bag systems, fluoropolymer-made bags are transparent, flexible, and permeable to oxygen, nitrogen, and carbon dioxide, allowing gas exchange in cell culture incubators. Fluoropolymers are highly resistant to almost all aggressive chemicals and biologics and remain flexible at temperatures ranging from −240°C to +205°C. Melt-processable fluoropolymers such as FEP and PFA offer an excellent alternative to polystyrene containers because of their thermal stability across a wide range of temperatures and the ability to be processed using melt extrusion or thermoforming methods.[91,92] Certain fluoropolymer bags are also suitable for cell cryopreservation.

BIOPROCESSING AND SCALE-UP CONSIDERATIONS IN BAG CULTURE SYSTEMS

Translating cell cultures to clinical scale

Manufacturing cells for immunotherapy at a clinical scale has traditionally been performed in "open" polystyrene-based vessels both for adherent or suspension cell cultures. Open cell culture systems require the "opening" of flasks or plates for media changes and other cell culture manipulations.[93,94] To decrease the risk of product contamination and by individual operators and to facilitate the scale-up or scale-out and automation of the cell production, "closed" culture systems are thus generally preferred by regulatory authorities to comply with current good manufacturing standards.[23,33,42]

Scaling up flask-based cultures often starts with T-flasks and then progresses to layered polystyrene culture systems, usually using up to eight units in one incubator per patient.[95] Closed or "functionally closed" culture systems that facilitate scale-up have been developed for certain cell types and applications of interest, such as Mo-DC production (Table 3), CAR–modified T cells, or mesenchymal stem/stromal cells for tissue regeneration or graft-versus-host disease therapy.[95,96] The implementation of current good manufacturing practices–compliant closed systems such as bioreactor bags reduces the risks of contamination and improves the overall connectivity between different units of the cultures at an industrial scale. Important considerations when transitioning from open systems

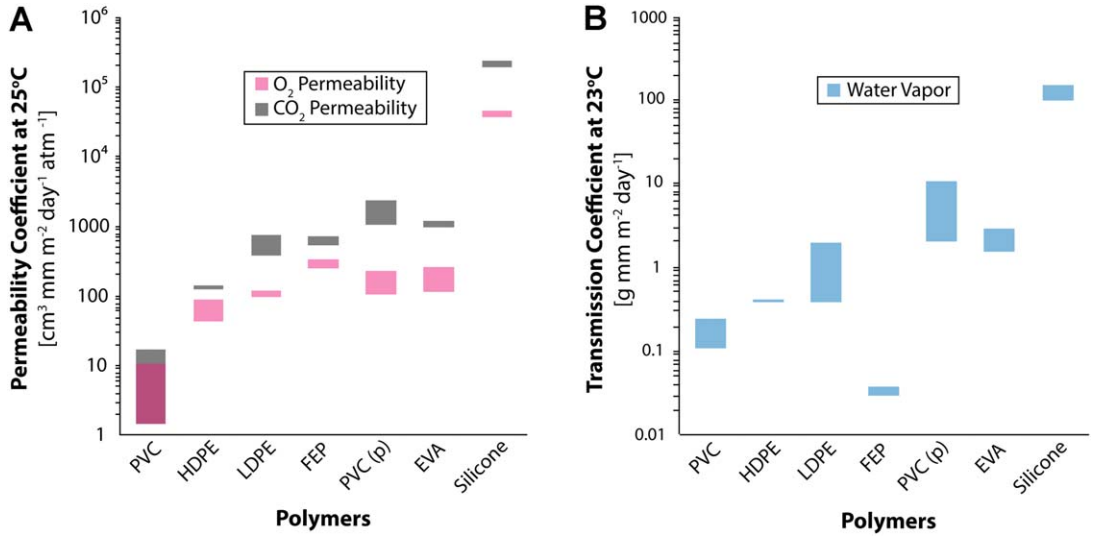

Fig. 3. Mass transfer considerations. (A) Gas permeability coefficient for O_2 and CO_2 and (B) water vapor transmission rates for polymers used to manufacture cell culture systems. EVA = ethylene-vinyl acetate; FEP = fluorinated ethylene propylene; HDPE = high-density polyethylene; LDPE = low-density polyethylene; PVC = polyvinyl chloride; PVC (p) = plasticized polyvinyl chloride. Permeability data were compiled from a variety of references.[104-106] [Color figure can be viewed at wileyonlinelibrary.com]

such as polystyrene flasks to bag-based closed systems are summarized in the following paragraphs and in Fig. 2, including mass transfer, handling, and processing.

Mass transfer considerations

The culture surface area in most commonly used tissue culture flasks ranges from 25 cm^2 to 225 cm^2. The recommended fill volume typically corresponds to a liquid height of 2 to 3 mm.[97] This gas/liquid interface is a limiting factor in the scale-up of cell culture systems, as gas exchange almost exclusively occurs via the vented screw cap of the flask.[98-100] To maintain this level of medium in the presence of evaporation, frequent media changes are necessary, which in turn increases costs of reagents and labor and the probability of contamination. Thus, a variety of gas-permeable bag cell culture containers with low water evaporation rates have been made available to the marketing sizes up to approximately 2 L fill volume (Table 3).[97,99,101,102] In addition, these devices allow mass transfer through both the upper and the lower surface area of the bags.[97,99,101-103]

The choice of plastic for a closed cell culture system, in which product is not exposed to the room environment, has a significant impact on mass transfer of oxygen, carbon dioxide, and water. As can be seen in Fig. 3, typical plastics used in life science applications have gas permeability and water permeability constants that span three orders of magnitude. Permeability constants are affected by two major materials science parameters: the free volume of a polymer and the chemical compatibility between the polymer and the permeant (e.g., oxygen or water). The free volume of a polymer is the molecular "space" between polymer chains. The effect of the free volume can be directly observed in Fig. 3 by comparing high-density polyethylene (HDPE) and low-density polyethylene. Manufacturers of polyethylene manipulate the free volume of their polyethylene resins by controlling the number and length of branches of the main polyethylene chain during synthesis. Low-density polyethylene has more and longer branches, leading to more free volume and, consequently, higher gas and water permeability than high-density polyethylene. Rubbers are also defined by their higher free volume and tend to have high permeability constants. The impact of chemical compatibility can be observed in Fig. 3 when considering the water permeability constants. Water is a polar solvent and is more chemically compatible with polar plastics such as ethylene-vinyl acetate, leading to higher permeability values. The presence of plasticizers and other fillers can also affect permeability and water vapor transmission through a polymer matrix as shown in Fig. 3 for polyvinyl chloride versus plasticized polyvinyl chloride. In instances where a closed cell culture system requires a plastic material with poor permeability, sterilizing-grade vent filters can be used, as typically employed in polystyrene T-flasks.

Leachables and extractables

In shifting from polystyrene-based flask cultures to bag systems, the profile of leachables and extractables is expected to change considerably due to the different types

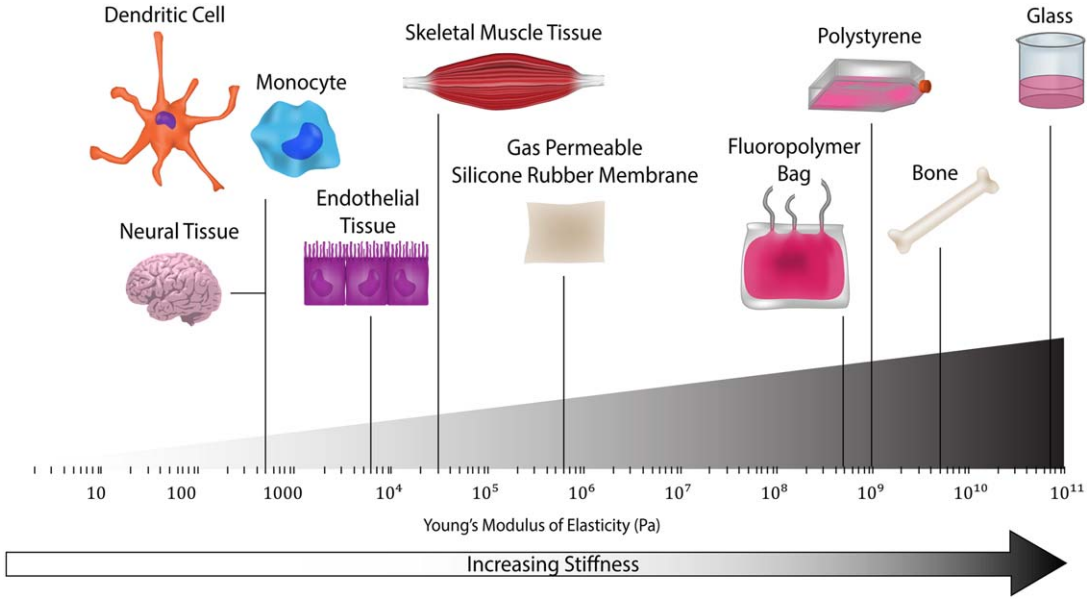

Fig. 4. Stiffness of polymers used to manufacture cell culture systems compared to that of tissues and cells found in the human body. Stiffness data originate from a variety of sources.[110,114,116-120] [Color figure can be viewed at wileyonlinelibrary.com]

and quantities of plasticizers used in the manufacture of "plastic" disposable systems for cell culture. Leachables and extractables found in polystyrene or ethylene-vinyl acetate polymers can have unknown, and both negative or positive effects on cell processing.[107,108] Because extractables' profiles may vary from lot to lot for some materials, extractables can reduce cell culture process reproducibility. FEP is extruded as a virgin resin and does not contain any additives or plasticizers. As a fully fluorinated polymer, it has very high inherent stability, and there are no modifiers or other content to leach out in water or other solvents. Consequently, extractables are typically at or below detection limits for FEP.

Handling and processing

Cell culture bags can be used as stand-alone units or as single-use, wave-rocking programmable bioreactor systems.[33,103] Cell feeding or splitting is made redundant by higher volumes of media in increasing bag sizes, and cell manipulation events are minimized by aseptic tubing connection technologies (e.g., Luer lock, sterile docking, tubing welding).[42] Typical unit operations used to manufacture autologous cell therapy products include (1) cell isolation/enrichment; (2) cell culture and, if applicable, modification; (3) concentration/washing; (4) product purification; (5) formulation and filling; and (6) cryopreservation.[42,94] In the case of genetically modified APCs or other cell therapy products such as CAR-T cells, closed culture systems decrease risks of contamination and exposure of personnel to viral constructs and other potentially pathogenic agents. Cell culture bags and other functionally closed systems can also facilitate the automation of most of these processing steps. Sterile docking (using tube welders) is commonly used in order to infuse or withdraw fluids from cell culture bags using pumps. Alternatively, fluid handling can be performed manually (e.g., using syringes) through a variety of ports such as needle access ports, spike ports, or fluid loss valves.

Effect of culture surfaces on cell fate in vitro

The effect of culture surfaces on cell fate during an extended period of time ex vivo is still largely unknown. Several reports document the loss of progenitor cells and cellular properties related to a cell's "stemness" on planar surfaces displaying nonphysiologic properties.[109-111] Interestingly, Yang and colleagues[112] showed that stem cells not only respond to mechanical cues presented to them by their microenvironment, but also possess a mechanical memory that plays a role in governing cell fate decisions.[113] Polystyrene, the most commonly used material for cell culture vessels, has an elastic modulus of approximately 3 GPa, more than five orders of magnitude stiffer than most cell types.[110,114] The FEP used in fluoropolymer bags such as those manufactured by Saint-Gobain has an elastic modulus of approximately 500 MPa. By comparison, the elastic modulus experienced by cells in situ in most tissues is four to six orders of magnitude lower.[111,114,115] In Fig. 4, the stiffness of materials commonly used to fabricate cell culture vessels is compared to the stiffness of DCs and other cells or tissues DCs may interact with in vivo.

In addition to mechanical properties, the chemical makeup and wettability of culture surfaces directly impact protein adsorption to surfaces and cell-surface

interactions. In general, in the presence of proteins present in blood and most cell culture media (e.g., insulin, transferrin, albumin), anchorage-dependent cells attach more readily to hydrophilic materials.[121] The extent of cell adhesion and spreading depends on the type and on the conformation of proteins present on surfaces—a topic that has been extensively reviewed elsewhere.[122] Cell culture vessel surfaces can be surface treated, typically using plasma treatments, to introduce charged functional groups on surfaces, increase surface hydrophilicity, and promote cell adhesion. For example, polystyrene flasks as well as FEP bags are available in "untreated" (hydrophobic) or "treated" (more hydrophilic) versions. Cell culture vessels can also be preincubated with media containing extracellular matrix proteins that mediate cell adhesion. Extracellular matrix precoating of culture vessels can improve antigen uptake[123] and/or maturation[124] of Mo-DCs.

Studies comparing Mo-DC cultures in bag versus flask systems

Most studies comparing Mo-DC cultures in polystyrene flasks (adherence) to hydrophobic bags (suspension) report no marked difference between DCs generated in either system (Table 2). Kurlander and colleagues[70] reported that DCs generated in FEP bags yielded cells with a surface marker expression profile that was comparable to adherence cultures on polystyrene surfaces. However, the DCs generated in the FEP bags produced significantly less IL-10 and IL-12 during their maturation, and these differences persisted upon rechallenge after harvest.[70] Elias and colleagues reported that after 6 days of culture in polyolefin coextruded with polystyrene cell culture containers (Opticyte, Baxter International, Inc.; see Table 2), DCs no longer expressed CD1a, although they otherwise exhibited a surface phenotype that was comparable to DCs cultured on polystyrene surfaces. These results confirmed a previous observation by Thurner and colleagues.[53,60] Furthermore, Guyre and colleagues[71] reported that DC cultured in hydrophobic bags (polyolefin coextruded with polystyrene; see Table 2) were phenotypically different from those cultured in flasks. However, this did not affect their capacity to present antigens, as expression of both major histocompatibility complex class I and class II molecules was consistently high. While the implications of these findings have yet to be determined, these minor differences should be considered when selecting the culture vessel for immunotherapy. Not all cell container systems may be equally suited for generating the optimal cell-based product targeting a specific clinical indication. Stringent potency assays will be necessary to define the essential quality control and release criteria ensuring the generation of efficacious cell therapy products.

In addition, not all cells cultured in hydrophobic bags remain in suspension. For example, Kurlander and colleagues[70] reported that some Mo-DCs adhered to the surfaces of FEP bags. However, these cells were not used for their analyses, as they represented only a minor percentage of all cultured cells. Guyre and colleagues[71] performed mixed lymphocyte reaction assays in either polystyrene or polypropylene round-bottom multiwell plates to determine if cell adherence was necessary for DC function, but found only slightly higher proliferative responses in polystyrene wells compared to polypropylene. These observations and the large variety of Mo-DC culture protocols present a yet unsolved conundrum: Is cell adherence during culture beneficial for an efficient differentiation and maturation of Mo-DCs? From a bioprocessing and handling perspective, suspension cultures are preferred when compared to adhesion cultures. Moreover, are the adherent cells mature DCs or do they present a different cell type, such as monocyte-derived macrophages? Systematic studies characterizing DCs obtained via adherent and suspension cell cultures using different bag containers will be necessary to investigate the identity and function of the cell products generated within these systems.

OUTLOOK: HOW TO IMPROVE CLINICAL EFFICACY OF CELL-BASED IMMUNOTHERAPY PRODUCTS?

The approval of sipuleucel-T by the Food and Drug Administration in 2010 was recognized as an important proof of concept that paved the way for industrial production of cell-based cancer vaccines.[12] Patients infused with sipuleucel-T demonstrated a significant increase in median survival time by 4 months compared to placebo controls.[45] However, no differences in the time to disease progression were observed. In addition, no tumor regression or reduction in tumor burden could be measured in treated patients, resulting in novel investigations of the mode and mechanism of action of sipuleucel-T.[14] Recent findings demonstrated that patients receiving sipuleucel-T showed increased levels of secondary self-antigens, an immunologic response known as antigen spread.[125] Several approaches are being investigated to achieve the full potential of DC-based vaccines, including increasing the potency of the DCs, targeting the DCs to the tumors, and inhibiting endogenous mechanisms that limit tumor-specific immune responses.[126]

Recent findings also raised questions related to the migratory capacities of the administered APCs to home toward the lymph nodes, a crucial step for the efficient activation of CD4+ and CD8+ T lymphocytes. Reportedly, only 5% of the injected cells reached the lymph node, which may have contributed to the suboptimal success of pioneer immunotherapy products.[126] The reasons for this impaired homing capacity remain unclear. However, the type, number, and source used for APCs, as well as the site and frequency of injection, may be cornerstones

for improving the performance of cell-based products.[13,14,43,44,93,126,127] In addition, the in vitro cell culture may adversely regulate cell adhesion and migratory surface receptors, further highlighting the importance of understanding cell-material and cell-surface interactions. Previous studies aimed to identify the optimal culture system (bags vs. flasks), culture surface (polystyrene vs. polyolefins vs. fluoropolymers), and time of culture ("fast" DCs vs. conventional).[25,128]

Rather than stand-alone therapies, combinatorial approaches employing both cell-based vaccines and potent but safe adjuvant molecules suppressing the tumor's tolerance mechanisms will be essential for future cancer care.[12,130] Improved immune-monitoring strategies and an extended repertoire of predictive biomarkers will be necessary to yield a better understanding of the optimal timing and sequence of administering immunomodulatory agents.[8,18]

CONCLUSIONS

The combination of conventional treatments with novel products such as cell-based therapeutics may augment current strategies for patient healthcare. Currently, a wide range of cell types, including hematopoietic stem cells, multipotent progenitors, and fully differentiated effector cells, is manufactured and tested for cell therapy applications. The results of many recent clinical trials, however, remain ambivalent as to the efficacy and potency of these cell products. Additive or synergistic effects between the administered cell products and existing drug-based therapies that lead to desirable clinical outcomes are being explored, but the mechanisms that may lead to these effects remain little understood. Researchers have only recently started to explore the impact of changing cell culture "plastics" for clinical-scale production of cell therapy products. Differences in mass transfer and mechanical and chemical properties can have drastic effects on cell fate. Selecting the appropriate cell cultivation system is thus fundamental in the design and development of cell production strategies. Although this review focused on studies in the immunotherapy field, other therapeutic cell types are being manufactured in the same or similar culture vessels. The handling, processing, and material properties considerations presented in this review will impact the outcome of other therapeutic cell manufacturing processes. Strategically designed cell culture systems will pave the way for the generation of potent cell-based cancer vaccines and other therapeutic cell types.

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