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SPOTLIGHT

INNOVATOR INSIGHT

The importance of material selection in the differentiation of monocytes into dendritic cells

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INTRODUCTION

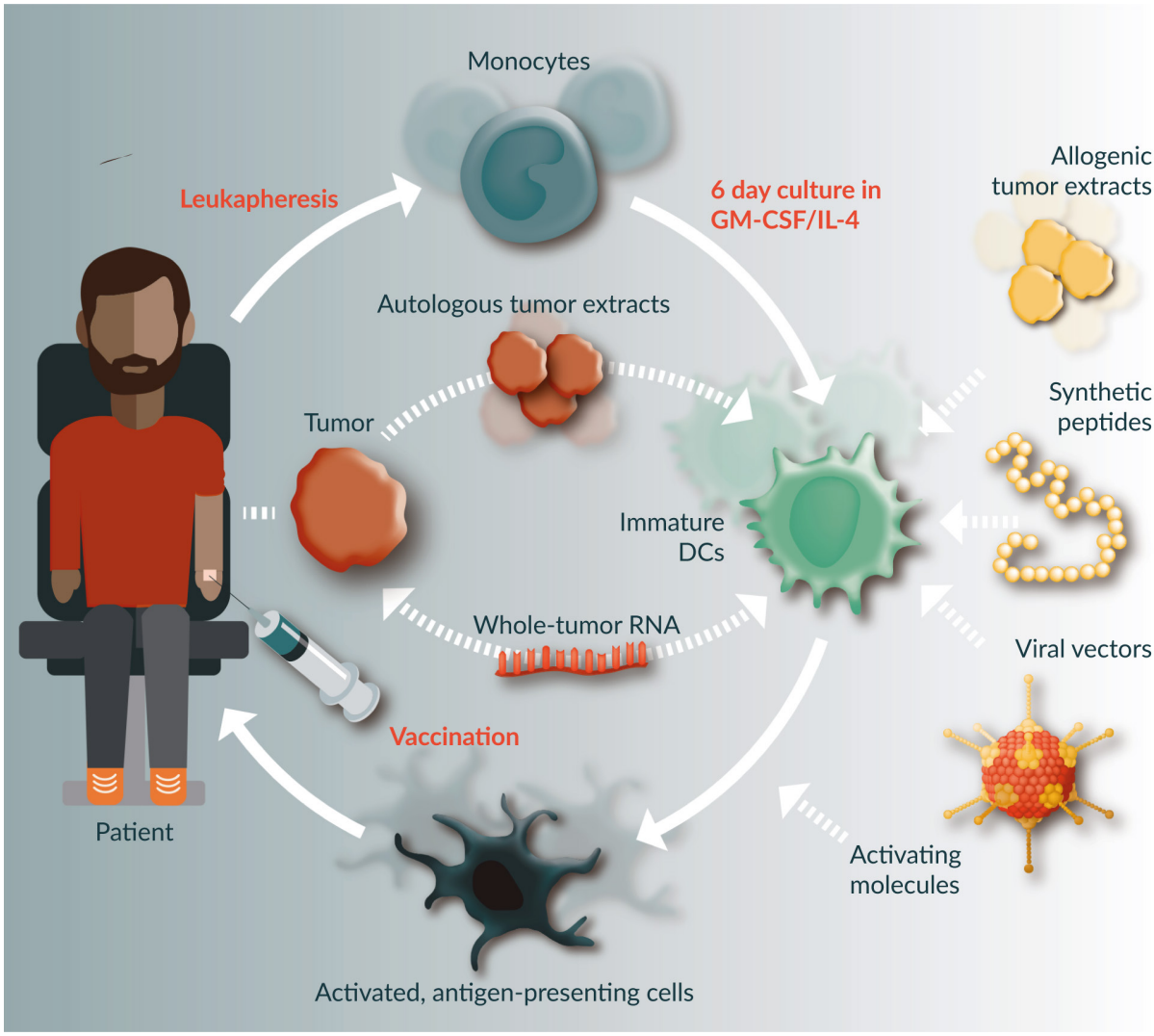
Cell therapy is an emerging market with the potential to shift the paradigm for how diseases are treated. In recent years, the 'cancer immunotherapy' subset of the market has seen rapid growth garnered from the efficacy of the often personalized treatment approach. Named 'Breakthrough of the Year' by Science magazine in 2013, the immunotherapy market has started to really come to fruition in the past few years, including the recent FDA approval of Novartis' CAR-T therapy, Kymriah™ (tisagenlecleucel) and Kite's CAR-T therapy, Yescarta™ (axicabtagene ciloleucel) [1–3].

One of the earlier areas of research and development in immunotherapy was in the use of dendritic cell (DC)-based treatments. DCs, often considered the most potent of the antigen presenting cells (APC), play a key role in connecting the innate and adaptive immune system [4,5]. Akin to a billboard, DCs process and then present antigens on their surfaces in order to appropriately activate T cells for immune attacks. This presentation and activation step is often the missing link in an immune response to cancer as the signal is not appropriately processed by the DC when the cancer is seen as 'self'. To tackle this, many researchers have looked to exploit the function of DCs through the culturing of monocytes, differentiation to DCs, and modifying DCs with appropriate tumor signals *ex-vivo*. This causes immune system stimulation upon re-infusion of activated, mature DCs (Figure 1) [6].

Although there are several early and late stage clinical trials leveraging this approach, there is currently only one, commercially available

FIGURE 1

Manufacturing of Dendritic Cell-Based Therapy [9].



DC: Dendritic cell; GM-CSF: Granulocyte-macrophage colony-stimulating factor

dendritic-based therapy in the USA [6]. Provenge® (Sipuleucel-T) was commercialized by Dendreon in 2010 and is still available for the treatment of advanced prostate cancer [7]. Recent market reports have anticipated an annual growth rate for the 'Global Dendritic Cell & Tumor Cell Cancer Vaccine' of 20.7% until 2030 [8].

With increasing interest in bringing cell-based products to fruition comes the increasing importance of selecting appropriate systems and materials for manufacturing. This paper will focus on reviewing material properties and important factors to consider when choosing a container for culture. With that, it also critical to consider process steps upstream (e.g., enrichment) and downstream (e.g., harvest) of culture as well as ancillary materials (e.g., medium and cytokines) when deciding on and developing a complete manufacturing process [10–12].

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TABLE 1.

Common Culture Systems For Monocyte to Dendritic Cell Culture.

Product	Company	Material Class	Type of System
T-Flask and Stacks	Multiple	Polystyrene	Rigid Flask
Evolve®	OriGen	Polyolefin	Flexible Bag
EXP-PAK™	Charter Medical	Polyolefin	Flexible Bag
PermaLife™	OriGen	Fluoropolymer	Flexible Bag
MACS® GMP Cell Differentiation Bag	Miltenyi	Polyolefin	Flexible Bag
VueLife®	Saint-Gobain	Fluoropolymer	Flexible Bag

Current landscape of systems for monocyte to dendritic cell culture

The use of disposable materials for cell culture dates back to the 1960s. What started out as a transition from glass petri dishes to more rigid plastic containers (T-Flask), the modern landscape of culture systems is now a dynamic mixture of rigid and flexible systems that are still evolving with new material innovations [13].

Table 1 outlines a high level landscape of common culture systems for monocyte to DC culture.

Critical material properties to consider when selecting system for cell culture

Oxygen permeability & water vapor permeability

There are many factors that impact the permeability of polymers, including, but not limited to:

- Size/physical state of penetrating molecule
- Morphology/properties of the polymer
- Solubility/diffusivity of the permeant
- Presence of fillers, humidity and plasticizers

Permeability of gasses, including water vapor, is arguably the most critical property when selecting a closed culture system. This is because the cells will depend on permeation through the material of the

FIGURE 2

High Level Reaction For Cellular Respiration [14–16].

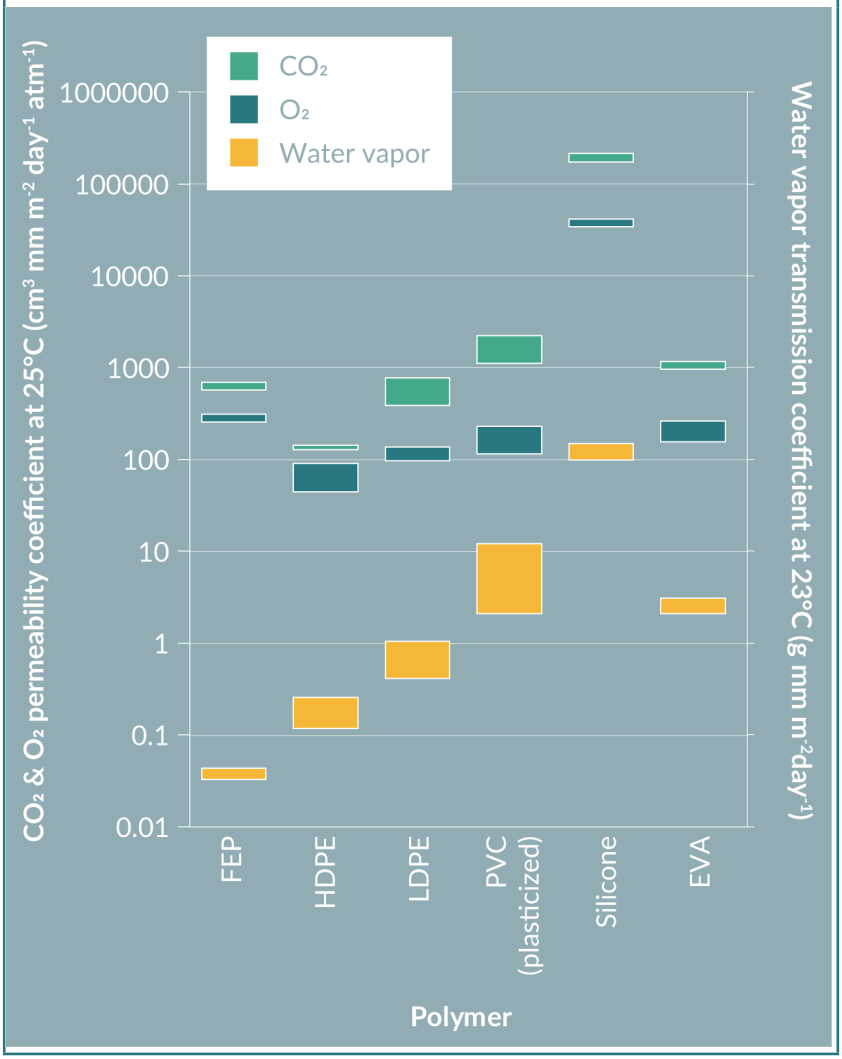


FIGURE 3

High Level Reaction For Cellular Respiration.

$C_6H_{12}O_6$ (Glucose) + $6O_2$ (Oxygen) ⟶ $6CO_2$ (Carbon dioxide) + $6H_2O$ (Water) + ATP (Energy)

system in order to maintain appropriate levels of oxygen (and carbon dioxide) and on a sufficient barrier to limit evaporation – all critical to the overall metabolic function of the cells. Humidified incubators are often required for cell culture to mitigate water loss from the cell environment (Figures 2–4).

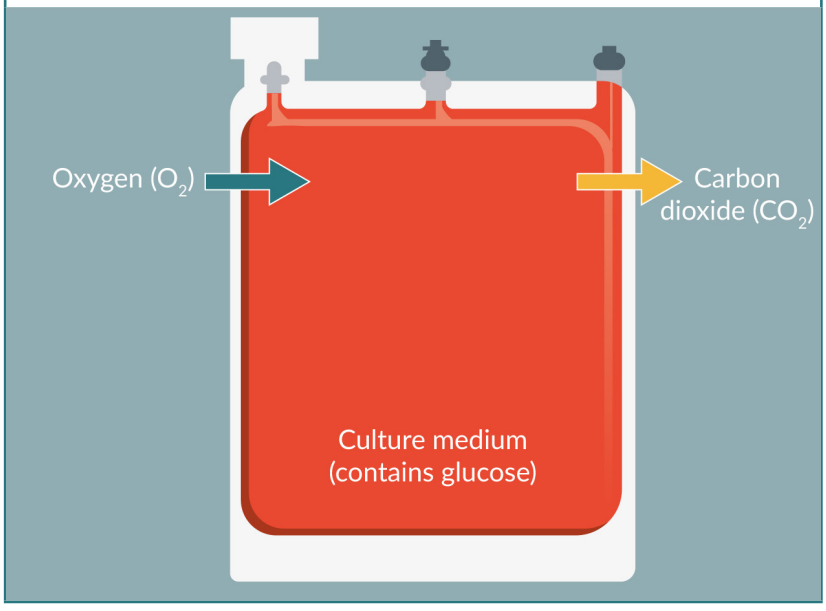
Transparency

Transparency is a physical property that is used to describe the ability of light to pass through a material. 'Light' can refer to a multitude of wavelengths in the ultraviolet, visible, and infrared spectrum.

Benefits of transparency to specific wavelengths depend on desired application. Some examples include, but are not limited to:

FIGURE 4

Static, Permeable Culture System.



Visible light transparency (400–700 nm)

- Optical microscope imaging
- Visual inspection of culture (e.g., for contamination or pH change)
- Fluorescence microscope imaging

UV-A light transparency (320–400 nm)

- Photopheresis
- Fluorescence microscope imaging

In any of these cases, the ability to leverage the transparency properties of the bag rather than taking samples or changing containers reduces labor, manipulation of the culture environment and, ultimately, chance for contamination.

Extractables & leachables

Extractables and leachables are terms used to describe migrating compounds in various conditions.

Extractables

Organic and inorganic chemical entities that can migrate from the contact surface under aggressive conditions. Aggressive conditions could include:

- Elevated temperature
- Extended contact time
- Aggressive solvents

Extractables have the potential to leach into a product under conditions of storage and use [17].

Leachables

Organic and inorganic chemical entities that migrate from the contact

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surface under application-specific or 'working' conditions [17]. These are generally considered a subset of extractables but not all leachables will be identified by typical extraction testing (Figure 5).

As leachables are extremely specific to a particular process, there is no generic test that can be performed for identification. In most cases, extractable testing with multiple solvents, alongside sufficiently sensitive analytical tools, can be used as a general proxy for migrating compounds. Some of the most common methods are shown in Table 2.

In general, it is imperative to quantitatively identify and understand potentially harmful impurities that may migrate from a plastic device and risk negatively impacting the cells during the culture period. In 2013, Amgen experienced, first-hand, the impact even small amounts of migrating compounds can have. At the time, Amgen investigated the effect of Irgafos® 168, a common antioxidant in polyolefins, on the growth of several Chinese Hamster Ovary (CHO) cell lines [18]. Eventually, and after thorough investigation of options, Amgen ended up working closely with their supplier to optimize the starting amount of Irgafos in the starting material which, as a result, lowered the leaching compound concentration and ultimately its detrimental effects [18].

Beyond their immediate impact on cell culture, there is also a risk of migrating compounds contaminating the downstream steps of drug manufacturing, especially in the manufacturing of cell-based therapeutics when there is no final filtration step.

Summary

Material properties like permeability, transparency, and extractables are all critical to consider when selecting a system for cell culture. With that said, there are several other material and non-material properties to bear in mind when choosing an appropriate solution. These can include, but are not limited to:

- Closed system options
- Size and shape configuration
- Type of tubing, ports, and connectors
- Sterility and shelf life

In the subsequent sections of this report, properties of fluorinated ethylene propylene (FEP), a common culture material, will be outlined alongside culture data in an

FIGURE 5

Relationship Between Extractables and Leachables.

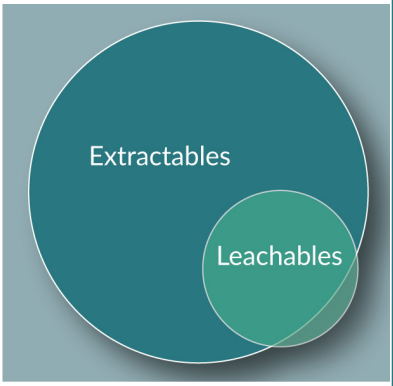

TABLE 2.

Overview of Common Extractables and Leachables analytical methods [17].

Method	Description
Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP/AES)	Quantitative analysis for metal ions
Direct-injection Gas Chromatography / Mass Spectrometry (DI GC/MS)	Semi-quantitative analysis for semi-volatile and volatile organic compounds
Liquid Chromatography / Ultraviolet / Mass Spectrometry (LC/UV/MS)	Semi-quantitative analysis for non-volatile organic compounds
Ion Chromatography (IC)	Quantitative analysis for ions
Total Organic Carbon (TOC)	Concentration of total organic carbon analysis

TABLE 3.

Oxygen Permeability of FEP and EVA.

Temperature (°C)	OTR (cc/(m²·day·atm)	
	FEP	EVA
25	~2,000	~1,500
37	~2,900	~2,600

EVA: Ethylene vinyl acetate; FEP: Fluorinated ethylene propylene; OTR: Oxygen transmission rate.

TABLE 4.

Water Vapor Permeability of FEP and EVA.

Temperature (°C)	Humidity	WVTR (g/(m²·day·atm)	
		FEP	EVA
37.8	100%	~0.5	~5

EVA: Ethylene vinyl acetate; FEP: Fluorinated ethylene propylene; WVTR: Water vapor transmission rate.

effort to empirically understand the relationship between key material properties and performance in cell culture.

DETAILED MATERIAL PROPERTIES OF FLUORINATED ETHYLENE PROPYLENE

FEP is a fully fluorinated fluoropolymer with several inherent material properties that make it suitable for many cell therapy applications, including cell culture. In this section, data on FEP is presented to align with the aforementioned critical properties for cell culture systems.

TABLE 5.

Water loss in FEP bag.

Temperature (°C)	# of days	Average water loss (%)	Standard deviation (±%)
40	14	0.21%	0.01%

FEP: Fluorinated ethylene propylene;

All properties data provided are based on measurements of 5 mil (0.127 mm) film, a common thickness used in FEP culture containers. For permeability properties, Ethylene vinyl acetate (EVA) film was also tested to help establish a point of comparison given it is also commonly used in cell culture applications. In these cases, measurements were performed on 8 mil (0.203 mm) film, an ordinary thickness for the EVA culture container.

Oxygen permeability

Oxygen transmission rate (OTR) was measured using a MOCON OxTran 220 OTR Analyzer following ASTM D3985 at both 25°C and 37°C (Table 3).

The oxygen permeability of FEP allows for a substantial transmission of oxygen, sufficient for metabolism in many cell culture processes.

Water vapor permeability

Water vapor transmission rate (WVTR) was measured using a MOCON Permatran W700 Water Vapor Analyzer following ASTM F1249 (Table 4).

While FEP is substantially permeable to oxygen, it is an effectively strong barrier to water vapor, usually eliminating the need for humidification for prevention of significant water loss in the incubator. This can be seen from the data in table 5. The data shows average water loss over a total of 14 days for six water-filled FEP bags in a 40°C non-humidified oven. (Table 5).

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TABLE 6.

Summary of Extractables Profile in FEP.

Analyte type	Analytical method	Extractables in water	Extractables in 70% ethanol/ 30% water by volume
Metals	ICP/AES	Ca (0.01 mg/l)	Not detected
Semi-volatile and volatile compounds	Direct injection GC/MS	Not detected	Not detected
Semi-volatile and non-volatile compounds	LC/UV/MS	Not detected	Not detected
Ions	IC	Not detected	Not detected
Organic carbon	TOC	0.30 mg/l (0.00010 mg/cm2)	N/A

GC/MS: Gas chromatography mass spectrometry; IC: Ion Chromatography; ICP/AES: Inductively coupled plasma atomic emission spectroscopy; LOD: Limit of detection TOC: Total organic carbon.

Extractables

Unlike most polymers, FEP film is extruded without the use of any additives (e.g., antioxidants, plasticizers, processing aids, etc.). 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 (Table 6).

Results are based on the pooled analysis of two, sterile 2PF-0290 VueLife® FEP Bags. Extraction was performed in either water or 70% ethanol/30% water by volume at 70°C for 24 h at a 3 cm^2:1 ml extraction ratio.

Transparency

FEP light transmission was measured on a Perkin Elmer UV-Vis-NIR spectrophotometer.

Light transparency of FEP is among the highest for plastics, allowing for easy viewing through the film itself. This is important for morphology characteristics as well as highlighting drastic changes in cell environment through markers such as phenol red (Table 7).

CULTURE & DIFFERENTIATION OF MONOCYTES TO DENDRITIC CELLS UTILIZING FEP CULTURE SYSTEM

Method

Monocyte enrichment

The Elutra (Terumo) cell processing system was used as per manufacturer's instructions. In short, HBSS (Lonza) supplemented with 1% HSA (CSL) was connected to the media line, 0.9% sodium chloride (Baxter) was connected to the secondary media line and fresh volunteer apheresis (Key Biologics, TN)

TABLE 7.

Transparency Properties of FEP.

Wavelength type	Transmission (%)
Visible	94.8%
UV-A	93.1%

TABLE 8.

Elutra Separation Program Details.

Step	Flow rate (ml/min)	Fraction volume (ml)	Centrifuge speed (rpm)
Fraction 1	37	900	2400
Fraction 2	97.5	975	2400
Fraction 3	103.4	975	2400
Fraction 4	103.9	975	2400
Fraction 5	103.9	250	0

Other Settings left as default. Flow ramp 0.1 ml/min/s, centrifuge ramp 528 rpm/s and cell:media ratio 1:1.

was attached to the sample input line of the Elutra disposable kit.

The Elutra separation program was run as per manufacturer's instructions, shown in Table 8.

Monocyte culture/ differentiation

The fraction containing the majority of monocytes was centrifuged at 800 × g for 5 min, and resuspended in complete CellGenix® GMP DC Medium (supplemented with 500 U/ml GM-CSF and 500 U/ml IL-4 (both CellGenix)). 1 million cells/ml were transferred to a VueLife® 160-C1 bag (Saint-Gobain). The contents of the bag were mixed thoroughly by rocking/inversion, then placed in a standard humidified tissue culture incubator (37°C, 5% CO_2) for 7 days.

On Days 1, 3, 5 and 7 the bags were removed from the incubator, viewed under an inverted microscope, and a sample taken after thoroughly mixing. On days 1 and 3, fresh DC medium was supplemented to ensure there was no change in volume after sampling. On day 5, the cultures were supplemented with DC medium and additionally 500 ng/ml tumor necrosis factor α (TNFα) (CellGenix). On day 7, the cultures were stopped and cells were collected for analysis.

Dendritic cell yield is calculated as a percentage in relation to the starting number of monocytes.

TABLE 9.

FACS Panel Dendritic Cell Analysis.

FL1	HLA-DR FITC*
FL2	CD66b PE
FL4	7-AAD
FL6	CD83 APC#
FL8	CD14 APC-Cy7

*For additional markers of DC maturation in the FL1 channel, FITC-conjugated antibodies to CD80 and CD40 may be used.

#For additional markers of DC maturation in the FL6 channel, APC-conjugated antibodies to CD1a and CD86 may be used.

Analytics

Immediately after taking a sample, acid/base and respiratory parameters (pCO_2, pO_2 and pH) were measured on the Nova pHOx bioanalyzer.

A second sample was frozen prior to thawing and running lactate, glucose, glutamine and ammonium analytics on the Cedex Bioanalyzer (Roche). A complete blood count (CBC) was completed on the AC•T DIFF™ Hematology Analyzer, Beckman Coulter.

Cell count and viability were conducted on the NucleoCounter-200, Chemometec prior to running flow cytometry (FACS) samples on Gallios, Beckman Coulter with the below panels (Table 9).

Compensation and analysis was performed using FlowJo software.

Results

Cell viability & yield

The use of FEP bags for culture show comparable yields (60%), Figure 6, left panel and viability (92%), Figure 6, right panel to that expected in monocyte to DC culture [19–21]. Tuyaerts' *et al.* review

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highlights that DC yield and purity is highly variable based on monocyte enrichment methodology with report values ranging from 4–100% and 1–20% respectively [10]. With the use of elutriation, the recovery and purity of monocytes tends to be about 90%, although the differentiation process into DCs is variable with Eyrich reporting 47% yield with a standard deviation of 32% [11], and Adamson reporting 42% with 13% standard deviation [12].

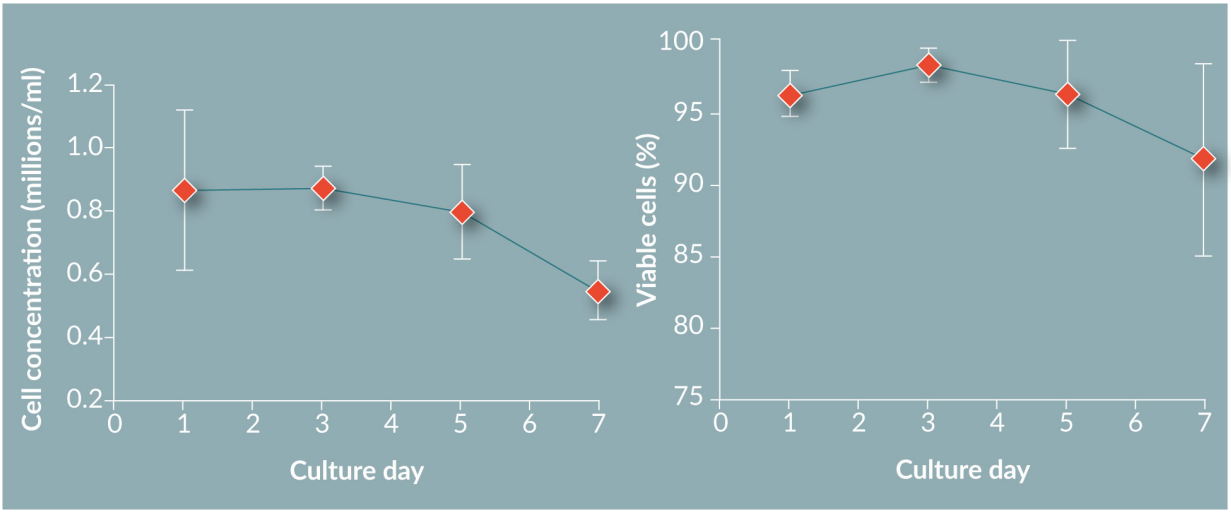
Chemistry analysis

The chemistry analysis highlights that the pH, pO2 and pCO2 levels all remained consistent during culture, maintaining equilibrium with the environment (Figure 7). The pH of dendritic medium was 7.2 and this remained throughout culture highlight the bag properties ensured a constant pH during culture. Both lactate and ammonium increased during culture, both are by-products of cell metabolism, so highlight active metabolism from cells. As there were no medium exchanges during culture, increasing levels of both of these metabolites would be expected. Lactate dehydrogenase, as a measure of cell death, remained consistent showing there were no detrimental effects during the culture that pertain to increased levels of cell death. Glucose levels decreased over the culture period, as an input to cell metabolism this would be expected to decrease as active cells consume this reagent. Glutamine is an amino acid essential for cell growth. The decline in glutamine – beyond the amount expected due to its inherent instability in culture medium – is in line with the declines in glucose and total protein, which all show active metabolism in cells.

Other chemistries (lactate, ammonium, glucose, glutamine, LDH and total protein) were within expected ranges for monocyte to DC culture (Figure 8).

FIGURE 6

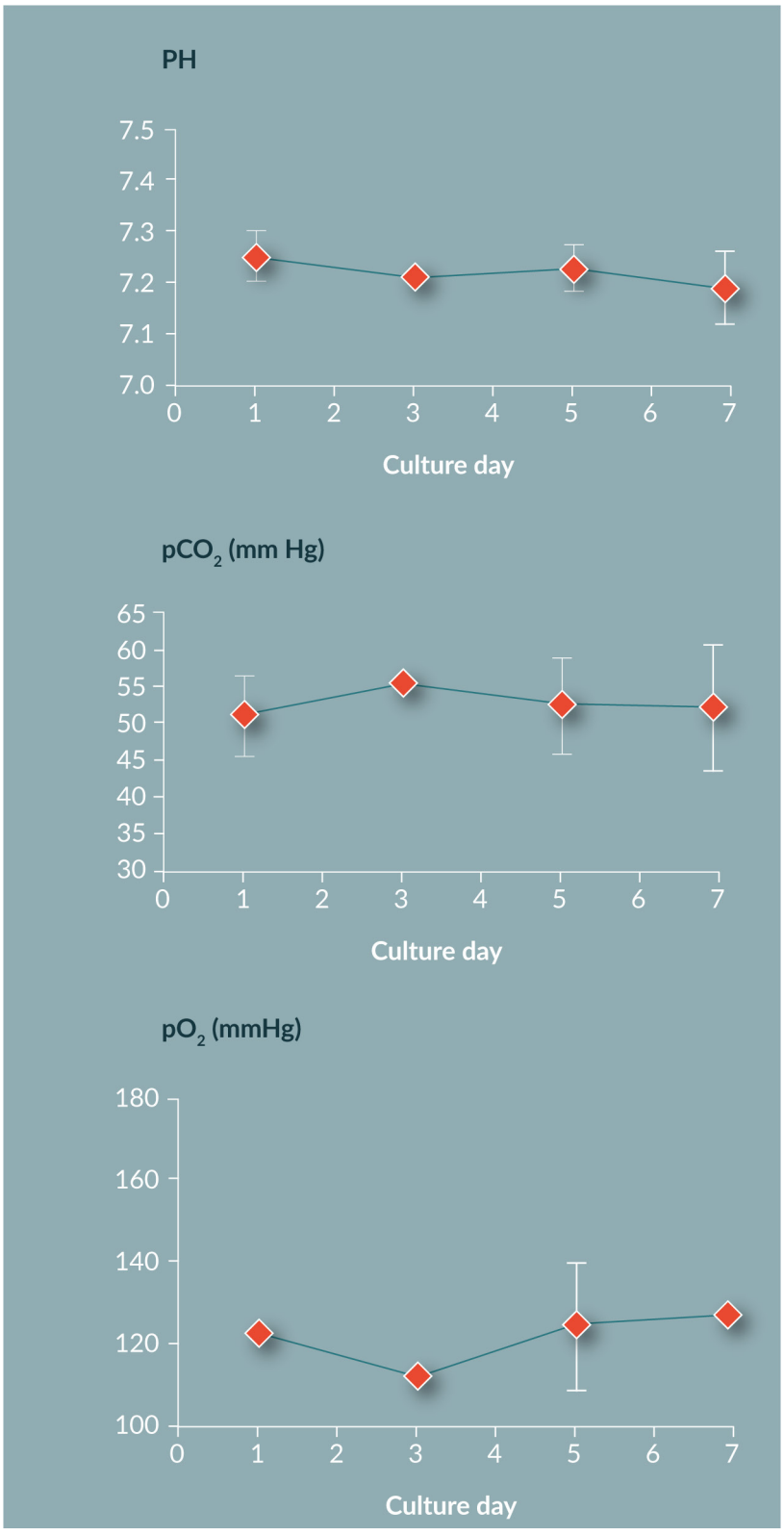
Cell yield and cell viability for dendritic cell differentiation experiments using VueLife culture bags.



N=3. All values are mean (red diamonds) ± standard deviation (error bars). All bags had on average a 60% yield of mature DCs on Day 7 compared to the number of monocytes plated on Day 0. Cell viability was good at 92% on average.

FIGURE 7

pH, pO_2 and pCO_2 measurements for dendritic cell differentiation experiments.



N=3 Measurements were performed on fresh samples using a Nova pHOx bionanalyzer.

Phenotype analysis

Phenotyping by flow cytometry showed an increase in cell size and complexity (increase in forward and side scatter) during differentiation (Figure 9). The loss of CD14 expression was essentially complete by Day 7. Increases in expression of markers associated with DC differentiation and maturation were seen over the course of the culture period. Of these markers, CD86 and HLA-DR expression were constitutively high in the starting monocytes on Day 0, but increased still further during the differentiation process. CD40, CD1a, CD80 and CD83 were essentially absent on Day 0 and increased substantially by the end of the differentiation process, with CD1a and CD80 showing the greatest variability.

Summary

The work in this paper has shown that the use of FEP bags for the culture and differentiation of monocytes to DCs produces results in line with values from other vessels. The yield (60%) and viability (92%) are comparable to previous reported values. Chemistry analysis suggests that FEP bag properties suitably control the cell environment by providing great gas exchange as measured by pO_2, pCO_2 and pH. The metabolite data suggests active metabolism of cells, suggesting again the cells are in a healthy state within the FEP bag. Phenotyping of the cells shows a change over time from a large monocyte population above 75% (by CD14 marker) on day 1 to an immature DC population by day 7 (loss of CD14+ to less than 10%

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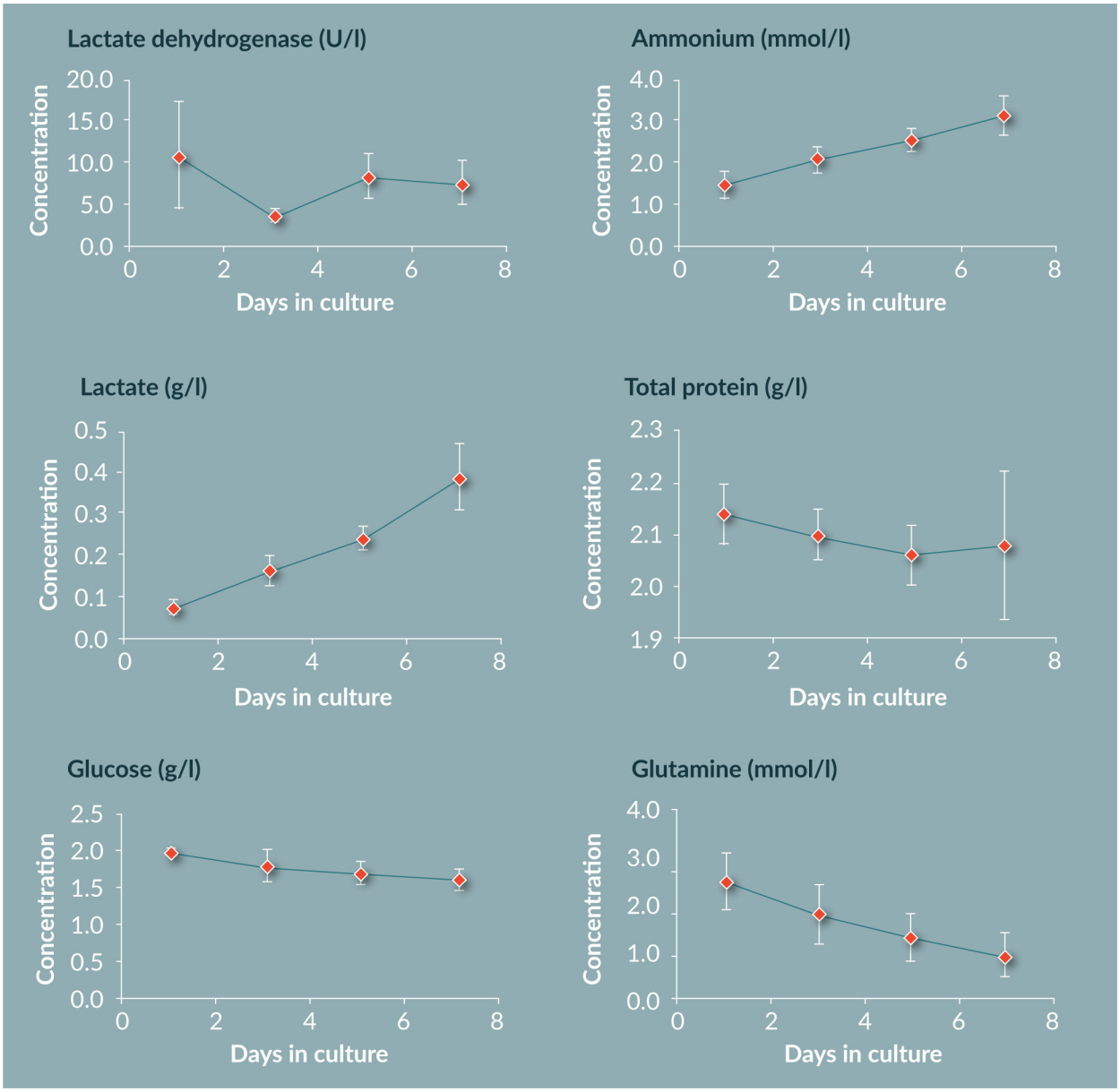
FIGURE 8

Lactate, glucose, ammonium, glutamine, LDH and total protein measurements for dendritic cell differentiation experiments.



N=3 Measurements were performed on frozen samples using a Roche Cedex bioanalyzer.

and greater than 75% for CD40+, CD83+, CD86+ and HLA-DR+).

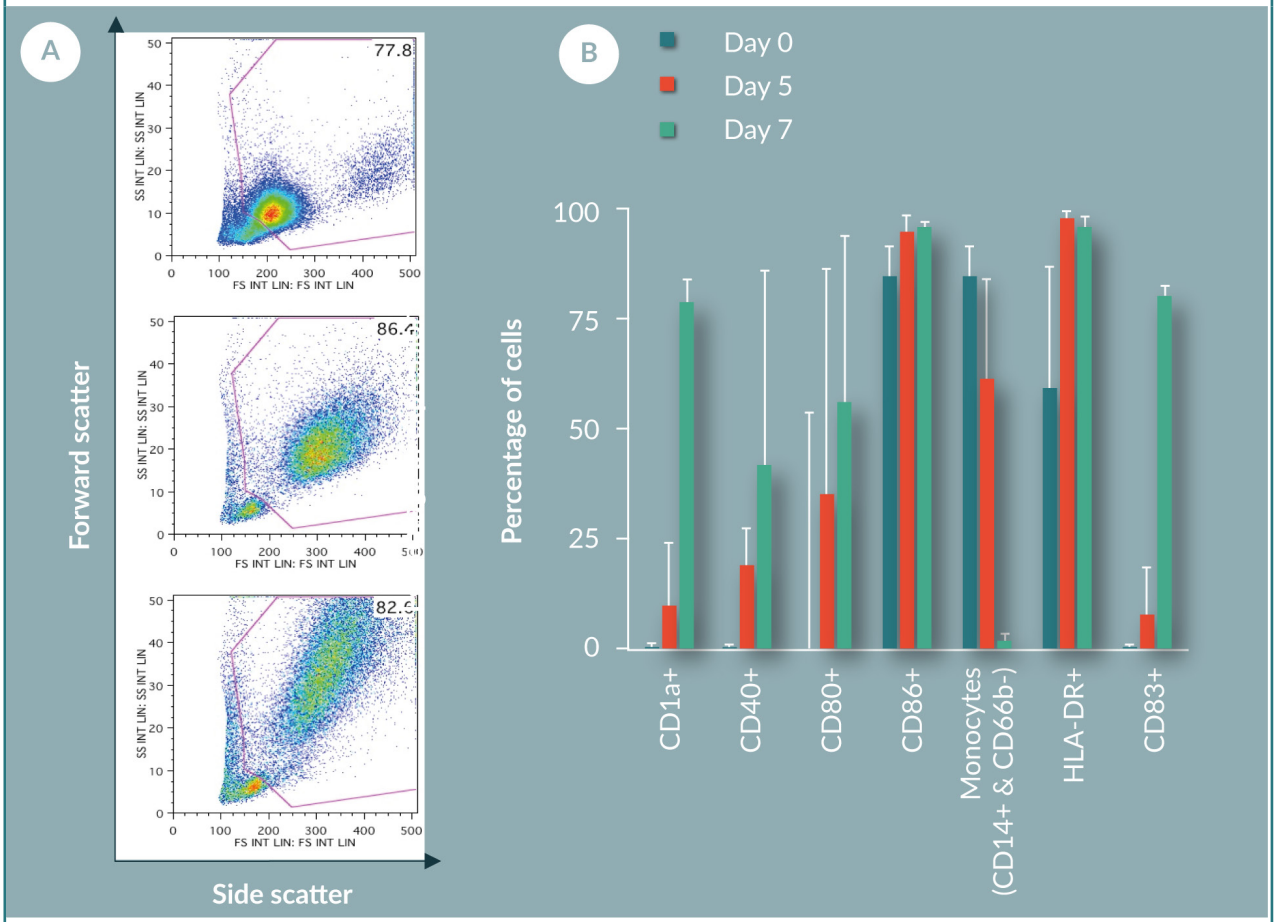
CONCLUSION

When choosing a container for cell culture, there are many elements to consider beyond traditional 'biocompatibility'. Some material properties to look for, and which have been outlined in this article include:

- Gas & water vapor permeability
- Transparency

FIGURE 9

Phenotype of cells during the transition from monocytes to dendritic cells in the 7 days of culture.



A) Forward against side scatter with increase in size and complexity over the culture period from Day 0 top, Day 5 middle, and Day 7 bottom, the starting product had 12% granulocyte, data not shown.
B) Increased expression in CD40, CD1a, CD80, and CD83, whilst maintaining CD86 and HLA-DR expression. The population loses expression of CD14+CD66b- over time. Data is the average from 3 donors.

- Extractables profile

Furthermore, there are a multitude of aspects to consider when selecting a finished culture container. These can include things such as:

- Is the container considered a close system?
- What size and shape configurations are available?
- What are the tubing, port, and connector options?
- How is the product sterilized?
- What is the product shelf life?

Based on critical material properties, VueLife®, made from FEP, was highlighted as a suitable option for the culture and differentiation of monocytes. However, given the diversity of manufacturing and cell types in the cell therapy market, there is rarely a one-size-fits-all solution for cell culture processes. Discussions with suppliers early on are critical to ensuring that the correct material is selected and design features are implemented to suit specific manufacturing needs.

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