Developing assays to address identity, potency, purity and safety: cell characterization in cell therapy process development

A major challenge to commercializing cell-based therapies is developing scalable manufacturing processes while maintaining the critical quality parameters (identity, potency, purity, safety) of the final live cell product. Process development activities such as extended passaging and serum reduction/elimination can facilitate the streamlining of cell manufacturing process as long as the biological functions of the product remain intact. Best practices in process development will be dependent on cell characterization; a thorough understanding of the cell-based product. Unique biological properties associated with different types of cell-based products are discussed. Cell characterization may be used as a tool for successful process development activities, which can promote a candidate cell therapy product through clinical development and ultimately to a commercialized product.

KEYWORDS: assay ■ cell-based therapy ■ cell characterization ■ cell therapy ■ cellular therapy ■ identity ■ potency ■ process development ■ regenerative medicine

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Ever since scientists began culturing cells in the laboratory they have been working on ways to characterize the cells they were maintaining *ex vivo*. Some early characterization methods were simply analyses of cell morphology and growth rates, but as biochemistry and molecular biology became more sophisticated, and understanding of protein and gene expression advanced, powerful analytical tools became available for characterization of phenotype and genotype of cell cultures. The increasingly quantitative nature of cell characterization enables cell and molecular biology to match the rigor of chemistry and biochemistry, and is critically important to the development of living cell-based therapies – the next generation of therapeutic drugs.

Creating therapeutic products composed of living cells presents many challenges, especially in today's highly regulated healthcare environment and considering that the regulatory framework was designed for chemical manufacturing in the last century [1]. Applying current GMPs to the manufacture of living biological drugs is hardly straightforward. Cell culture-based processes are inherently more complex and less well controlled than small molecule synthesis, and the products themselves, due to their living biologic nature, cannot be fully defined. These difficulties have given rise to a philosophy that 'the product is the process' in which manufacturers 'ensure product consistency, quality and purity by ensuring that the manufacturing process remains substantially the same over time' [101]. Cell therapy manufacturing processes inevitably change in the

course of clinical development, however, reflecting in part the challenges of increasing scale for a living biologic product. The critical quality attributes (CQAs), identity, purity, potency and safety, of cell-based drugs must be maintained as manufacturing processes are streamlined and scaled up or scaled out [2]. Manufacturing process development must be based on a foundation of characterization data, to ensure the continuity of quality, necessary for GMPs-compliant cell therapy manufacturing [3].

Cell characterization tools and technologies are critical to the successful development and scaling of therapeutic cell manufacturing processes [4,102]. The cell characterization tools available today for discovery research are now needed to support the product development and commercialization processes as well. Establishing which cell characteristics are the CQAs of cell-based drugs that must be maintained during manufacturing process development and scale-up is important so that the therapeutic efficacy seen in preclinical studies and early clinical development is maintained in later-stage trials and upon market introduction [5]. A variety of analytical tools and methods are available to help guide cell therapy product developers, although descriptions to guide their implementation have been limited. This review outlines product quality parameters in the context of cell therapies, and provides examples of how these tools may be used as part of effective process development and in product release. Readers are encouraged to consult the new glossary of terms used in regenerative

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medicine [6] for further clarification of any terms or ideas mentioned in this manuscript.

CQAs for therapeutic cells

Current GMPs, as applied to cell-based therapeutic products, require specifications for the following parameters:

- Identity: to confirm that product contains the intended cellular and noncellular components;

- Potency: to confirm that the product possesses the inherent or induced biological function(s) that is relevant to treating the intended clinical indication;

- Purity: to confirm that the product does not contain undesired components, such as contaminating cell types or residual process reagents;

- Safety: to confirm that the product is not contaminated with microbes or adventitious agents and, if appropriate, does not have tumorigenic potential.

This review focuses on the cellular characteristics of cell therapy products that shape the testing panel for these products. General product CQAs that apply to all biological products, including cell therapy drugs, which are independent of cell characterization, will not be covered thoroughly in this manuscript, but are addressed in a review by Rayment and Williams [7].

Identity

Identity assays verify the identity of the product in the master cell bank, working cell bank and final product and distinguish it from other products being manufactured in the same facility [8].

Identity testing is required to ensure that a cell therapy product contains the cell population(s) that the manufacturing process was expected to produce. This is essential to address the concern that the cells may have unexpectedly changed during the manufacturing process, or after modifications to the manufacturing process. Common process development activities such as serum elimination, extending culture duration or scaling up culture vessels can lead to phenotypic drift in the final cell product. A thorough knowledge of the identity of the cell product prior to undergoing these activities can help to assure that the cell product remains substantially similar as processes are changed during development activities. Having clearly defined identity tests for the cell therapy product supports the scale-up and streamlining of manufacturing processes, and provides documented proof that the appropriate product emerges from the process development campaign.

One measure of identity is that of morphology (also called morphometry) and is actually used as a release test for one regenerative medicine product, Carticel® (Genzyme®) [9]. However, best practices in establishing identity assays will be to use assays that provide a quantitative measure that is qualifiable and validatable [103]. Typically, identity testing of cell therapy products includes the use of flow cytometry because it is well established in the research community and it has translated well to GMPs manufacturing. Flow cytometry entails the staining of live (or fixed) cells with fluorescently labeled antibodies that recognize cell surface or, in some cases, intracellular expressed proteins [10]. Results can be obtained within hours and the assay can be validated using a number of antibody reagents that target distinct surface features of the cells and support identity assessment by defining their complex protein profiles. It is expected that each different cell population will express a sufficiently unique protein profile, thus facilitating its identification if the appropriate set of surface targets are identified. A typical identity specification may be that the product must be >80% for a particular cell surface marker (often actual data may be in the 90%+ range). Additional information regarding the remaining percentage of nonstaining cells will likely be required, as regulators may require information on the entire population of cells in the final cell product. For instance, does the remaining percentage represent the presence of other cell types that do not have this phenotype, or is it due to the analytical limit of detection? An understanding of analytical limits, linearity and dynamic range is necessary for the characterization package, but this rigorous approach is not always applied to flow cytometry.

Further challenges to creating GMPs assays for flow cytometry include establishing reference standards for each marker used as well as the subjective, operator-dependent nature of gating strategies. Automated gating has been shown to increase assay robustness, but is not yet a well-established quality control method [11]. Furthermore, flow cytometry analysis typically identifies the percentage of cells positive for a particular surface marker, although an additional measure of the mean fluorescent intensity of the positive cells may present a more clear representation of the level and uniformity of expression of a particular surface marker in the cell product.

Gene expression profiling also may be used to characterize cell therapy product identity [12]. For most of these assays, total mRNA is extracted from cells and tested to determine the profile of actively expressed gene transcripts. Analysis of mRNA may be performed by either quantitative PCR (qPCR; also called real-time PCR), by qPCR arrays, or by using microarrays (gene chips). With qPCR, individual genes or groups of genes can be screened. Most commonly, these assays are performed semiquantitatively, with the expression of the gene of interest from the test population of cells compared against the expression level from a well-characterized or reference population of cells. Absolute levels of mRNA transcripts within the cells sampled cannot be determined this way, although fold-changed differences in gene expression, relative to the reference population, can be attained. Quantitative assessment of gene expression levels can be determined when mRNA from cells of the test population is compared against a standard curve of the gene of interest. Focused PCR arrays that look at a matrix of relevant genes may be an economical and time efficient way of monitoring cell characteristics during process development activities.

Another promising genetic screen that may be useful for establishing cellular identity is miRNA expression analysis. These small RNA molecules control the expression of whole classes of genes and screening the mRNA profile for certain miRNAs in a particular cell may indicate the types of genes being expressed in that cell [13]. Similar to miRNA screening, epigenetic profiling also sheds light on the program of genes being expressed by a particular cell. The study of DNA methylation is referred to as 'epigenetics' meaning 'on top of genetics' because although the underlying DNA sequence is unchanged, the methylation state of the genomic DNA (gDNA) determines the gene expression pattern. For these assays, gDNA is isolated from cells and the methylation state of selected gene promoters can be used to characterize the identity of a cell. Hypermethylated promoters identify genes that are silenced while hypomethylated promoters suggest highly expressed genes. The profile of active promoters within the cell's genome dictates the genes expressed by the cell and thus provides an indication of cellular identity. The hypermethylation (silencing) of pluripotent genes, for instance, assures that through subsequent cellular divisions the cells remain differentiated and do not revert back to pluripotency. Likewise, a change in the methylation state of specific promoters can indicate if a cell is shifting its gene expression profile; or in other words, changing identity. Currently, these types of assays are under development, although they could play an important role in characterizing the identity of cell therapy products in the future, particularly those derived from pluripotent cells.

■ Identity consideration: pluripotent stem cells

It may be prudent to develop unique identity assays for cell therapy products derived from pluripotent cells. As the cells progress through stages of differentiation on their way to becoming a physiologically mature cell their identity changes [14]. The cells in these products implicitly change identity through the manufacturing process. Evidence of the pluripotent nature of the cells, in process, will likely need to be demonstrated while a lack of pluripotent cells in the final product will also be desirable. It is unlikely that flow cytometry alone will be sufficient to address both the pluripotent identity of these products in process as well as the differentiated identity of the final products. A combination of flow cytometry and molecular assays may more comprehensively characterize the cellular identity of products derived from pluripotent cells. The protein expression profile will reveal the current identity of the cells while the genetic analysis will give a better assurance of complete differentiation (silencing of pluripotent genes). For a product in which identity shifts through the manufacturing process, a more comprehensive approach to characterizing the identity of the cells will be helpful in order to allow for the appropriate cells to be present in the final product.

Potency

The US FDA describes potency tests as measures of appropriate biological activity [9].

Potency assays provide a measurable readout of a relevant biological function of the cell product with respect to the clinical indication. While identity assays indicate what a cell is, potency assays measure what a cell does. It is important to note that potency tests are not expected to be a direct measure of product efficacy *in vivo*, although they can be. The biological function measured by potency testing must simply be a relevant aspect of the product's function. It is certainly desirable for potency testing results to correlate with clinical outcome, for example, but this is not required, and in many cases may not be possible. Quantitative assays are preferable,

although qualitative assays may be acceptable as part of a potency assay matrix [103]. Relevant examples of each would be a quantitative measurement of bioactive cytokine levels secreted into the supernatant of cultured cells or a qualitative assay involving observations of cellular morphology. Potency testing, ideally, evaluates the product as administered, with all its components. For a cell/device combination product, for example, in which cells are delivered on a scaffold, potency testing development should seek to evaluate the cell/scaffold product, which could entail testing culture supernatant, or differential gene expression profiling of the cells alone as well as assessing the biological attributes of the cells on the scaffold.

Best practice in establishing potency testing is to establish a set of appropriate biologically functional assays that are relevant to the expected mechanism of action (MOA) of a drug for the specific indication. It is not uncommon for a cell therapy product to be considered for multiple clinical indications. Indeed, the same cells may be in different formats or at varying concentrations for a range of indications. It may be appropriate for indication-specific potency testing be developed. Even cell therapy products that are manufactured in the same way and at the same concentration but intended for different clinical usage may require indication-specific potency tests. For instance, the expression of anti-inflammatory cytokines may be part of the potency assay matrix for a cell therapy product indicated for graft-versus-host disease [15]. If this same cell product is also indicated for neovascularization in acute myocardial infarction the potency assay matrix for the acute myocardial infarction product might include relevant tests for angiogenic activity (e.g., VEGF secretion) [16]. It may be difficult to set an absolute productivity output (pg/cell/day) in an *in vitro* assay setting in the absence of firm evidence as to how much factor(s) is required for the cell product to achieve the indicated MOA. The possibility that a product may be useful in targeting more than one (unrelated) disease further underscores the need for case-by-case consideration of assay references.

The potency assay for a cell therapy product will likely be a matrix of several tests that adequately capture many of the key activities of the cell product [17]. When determining tests to create the potency matrix, several potential assays should be followed as 'for information only' (FIO) as the cell product moves through various stages of development. This accumulated data is beneficial to track over many manufactured lots of product, and the assays that provide the most reproducible data may be the best candidates for moving forward to validate. Problems may arise when validating a release test which would preclude the use of that test and it is beneficial to have back-up assays so that development does not need to start from scratch. In addition, the supply of key reagents used for the potency test may change, necessitating the development of a new test. Tracking the results of key measures of biological function as FIO measures throughout development will ensure that sufficient data are collected in the event that a new test of biological function can be qualified and validated as a release test when necessary.

■ Potency consideration: mesenchymal stromal cells

Biological functions relevant to potency of mesenchymal stromal cells (MSCs) from various tissues (including bone marrow, fat and umbilical cord) have been widely studied. It is hypothesized that MSC-based products tend to derive more therapeutic value from their secreted paracrine factors, rather than from their ability to differentiate into bone, cartilage or adipose cells [15]. Likewise, relevant measures of biological function for these products often involve screening for factors that they secrete in culture supernatants, most commonly by ELISA. In addition, the colony-forming unit (CFU) fibroblast test has also been classically used as a measure of MSC biological activity. The cytokines and factors secreted by MSC-based products typically link back to the indicated MOA thereby rendering them useful assays to develop as potential release tests for potency.

Potency tests can measure the activity of the cell product 'as is' or they may measure an induced activity. For example, following product delivery of MSCs to a site of inflammation, their response may be the secretion of anti-inflammatory and/or proangiogenic cytokines. Examples of induced activity with MSCs include one study in which MSCs were found to secrete elevated levels of the angiogenic cytokine VEGF when stimulated with TNF-α or IFN-γ [16]. The secretion of VEGF by MSCs is also relevant for angiogenic activity, which has also been attributed to these cells [18]. Another example of induced activity with MSCs is the treatment of MSCs with IFN-γ in order to stimulate the activity of indolamine-pyrrole 2,3-dioxygenase (IDO) [19]. The IDO enzyme converts L-tryptophan to *N*-formylkynurenine (kynurenine), an inhibitor

of cell proliferation including T cells (inflammation) as well as pathogens. Detection of kynurenine in the supernatant of IFN-γ stimulated MSCs is a measure of induced biological function (for more information on IDO activity see [20]). Mimicking these responses in an *in vitro* assay could be a useful surrogate of *in vivo* potency.

An additional set of potency testing that is common with MSC products is that of determining the trilineage potential of the cells. An implicit characteristic of MSCs is their ability to differentiate into osteocytes (bone), chondrocytes (cartilage) and adipocytes (fat) [21]. Traditional histochemical-based methods used to demonstrate trilineage potential were qualitative, but newer approaches are being developed to quantify the differentiation capacity of the cells. An alternate approach to histochemical testing, which could reduce test times and overall costs, is genetic analysis. Although MSCs have trilineage potential, most diseases for which MSCs are indicated do not involve the differentiation of the cells into bone, cartilage or adipose tissue. Therefore, unless the cells are differentiating into one of these cell types *in vivo*, demonstration of trilineage potentiality may not be the most relevant potency test for an anti-inflammatory or angiogenic indication for an MSC-based therapy. The potency assay matrix for an MSC product may better demonstrate MOA by consisting of more relevant tests, and product release would not be hindered by assays irrelevant to therapeutic benefit.

■ Potency consideration: patient-specific and/or autologous cells

Many autologous products are T cell- or dendritic cell (DC)-based, and will likely include a measurement of T-cell activity as a relevant assessment of biological activity since many of these products involve the direct (T cell-based) or indirect (DC-based) activation and expansion of T cells *in vivo*. This may be achieved either with monocyte or DC products, which activate the endogenous T cells, or alternatively, products may consist of purified and/or engineered T cells. Therefore, a good measure of biological function for these cell products is going to be *in vitro* assessments of T-cell activation. For monocyte or DC products this can be achieved by direct incubation of the manufactured cells with naive T cells or treatment of T cells with conditioned media from the manufactured product. For T-cell products, the product will be stimulated directly. Measurements of secreted cytokines that promote T-cell activation may also be good

markers of biological function for monocyte or DC products. An example of a potency test for an autologous cell therapy product is the measurement of IL-12p70 from postelectroporated CD40L-expressing DCs [22]. The authors have found that measurement of IL-12p70 secretion by their product is a surrogate measure of *in vivo* activation of T cells and have demonstrated the biological relevance of this molecule in the activity of their product.

Measures of biological function for hematopoietic cell-based products (either patient-specific or autologous) include the CFU-granulocyte macrophage test. For CFU tests, the product is stimulated with activating factors and then cultured for approximately 7 days before the number of colonies is determined via microscope imaging and normalized to the number of cells initially seeded [23,24]. If the cell therapy product is comprised of a mixed population of cells, such as Aastrom's tissue repair cells (also known as Ixmyelocel-T), then multiple CFU tests may be necessary to characterize the different populations in the final product [25]. There are newer approaches to the CFU test, which are based on bioluminescence readouts [26]. These assays reduce the subjectivity of visually observing the colonies and may be preferable as the results are readily quantifiable and can be qualified to a reference standard.

■ Potency consideration: pluripotent stem cells

Potential tests of potency for cell therapy products derived from pluripotent cells, such as human embryonic stem cells (hESCs) and induced pluripotent stem cells, will be very diverse as these products are typically differentiated into mature (or near-mature) cell types prior to administration to patients. Therefore, the measures of biological function for these products will be as varied as the types of diseases and conditions they are indicated to treat. One pluripotent-based cell therapy product currently in clinical trials is the Geron® hESC-derived oligodendrocyte progenitor cells indicated for patients with spinal cord injury [104]. Preclinical studies of these cells in rodents demonstrate that the cells secrete neurotrophic factors, which may be linked to the MOA and could possibly serve as a measure of biological function [27]. Another pluripotent cell product is ViaCyte®'s Pro-Islet™ hESC-derived product for the regeneration of pancreatic β-cells indicated for Type 1 diabetes mellitus [105]. When implanted into mice, the ViaCyte product differentiates into human islet

cells, with detectable levels of human insulin present in the blood of mice following a glucose challenge [28]. Advanced Cell Technology® has a retinal pigment epithelial program for the treatment of juvenile and age-related macular regeneration with hESC-derived cells intended for the regeneration of photoreceptor degeneration; a rescue of vision deterioration [106]. Studies of these cells *in vitro* demonstrate the capacity of the cells for phagocytosis and the expression of neuronal markers [29]. Cell therapy products derived from pluripotent cells may have the widest variety of potency measures, as each product-specific potency matrix will contain assays pertinent to the activity of the differentiated cells.

Purity

Purity tests ensure that cell therapy products are free from unwanted material, including unwanted cell types, endotoxin, residual proteins, peptides or other agents used in manufacturing such as animal serum [9].

Ideally, a cell therapy product will contain the specified therapeutic cell type(s) in a physiologic buffer suitable for human administration that 'meets generally accepted standards of purity and quality' [107]. Therefore, it is necessary to fully characterize the contents of a dose of formulated finished product in order to describe all cell types present (and in what percentage) as well as identify any extraneous compounds that may be present. Cell therapy products will need to be demonstrated to be free of endotoxin (also known as pyrogen, or more commonly, lipopolysaccharide) although further discussion of endotoxin testing can be found elsewhere [7]. Serum albumin levels, a surrogate measure of residual animal serum, will also likely need to be determined for any cell product cultured or processed in the presence of animal serum. Culturing of cells with animal serum (usually bovine) is still common for cell therapy product manufacturing [5]. The Code of Federal Regulations states that the animal serum levels must be reduced to below 1 ppm in the final formulation of the product [107]. Typically, most residual protein assays (such as the serum albumin test) are standard ELISA-based tests, which are relatively easy to qualify to a reference standard and validate. Harvest reagents such as trypsin should also be reduced in the cell product prior to final formulation; however, there are no stated guidance levels for trypsin. Currently, there are a few commercially available ELISA-based tests for detection of trypsin, rendering direct measurement of porcine-derived trypsin protein levels possible. There are, however,

fluorescent substrate-based trypsin activity assays that can be used to estimate the drop in residual trypsin activity during post-harvest processing. It may also be necessary to demonstrate that cytokines, growth factors, antibodies or other agents used during the manufacturing process are not present in the final product [8]. As standard release panels are established for cell therapy products, it is likely that multiplexed ELISA assays will be used to streamline and simplify the release testing process [30].

A unique purity issue associated with cell therapy products is that of unwanted cell types. Purity analysis of a cell-based product should ensure that it contains no more than a defined and consistent percentage of nontarget cells. All cell therapy products (allogeneic, autologous and pluripotent) derive from a heterogeneous mixture of cells. For allogeneic and autologous products the desired cell is typically isolated from biological tissue or body fluids. Pluripotent products, however, derive from progenitor cells, meaning that the final product carries an implicit risk of containing undifferentiated or partially differentiated cells. Furthermore, in the cases of autologous or pluripotent cell products in which the manufacturing processes may include targeted differentiation or transformation, additional opportunities for unwanted cell types leading to product heterogeneity exist. Therefore, it is unlikely that complete product purity can be achieved with any type of cell therapy product. For these reasons most cell-based assays for purity and potency will not likely have a gold standard to be compared with but rather seek to demonstrate consistency (whatever that may look like) with early product batches that were deemed to be safe and efficacious. Efforts to quantitatively describe the complete cell content of the final product are expected by the FDA [8]. The use of multiple analyses (such as multitargeted FACS and gene arrays) may be the best strategy to achieve an acceptable level of purity characterization. Establishment of purity assays to succinctly address unwanted cell types is a work in progress; best practices are still being developed.

Safety

In-process testing as well as testing of final formulated product (prior to cryopreservation) and of thawed products is required in order to ensure the quality and consistency of the product. The testing shall include sterility (bacteria), mycoplasma, as well as testing for adventitious viral agents [9].

Characterizing cell therapy products for safety is given the highest priority in the FDA guidance documents. Sterility testing has been well characterized and is thoroughly described for cell therapy manufacturing elsewhere [7,31]. The most common risk of a sterility breach comes from cells grown in an open-system process. Cells grown in an open system are more susceptible to microbial and mycoplasma contamination. Transitioning the culturing protocol for cell therapy products from open-system to closed-system processing minimizes the contamination risk in the final product. Furthermore, process development activities that reduce or eliminate animal serum from the culture protocol improve the safety of the product by reducing the chances of mycoplasma contamination in the final product as mycoplasma often can be traced to products of animal origin [8]. Process development activities such as transitioning to closed-system processing and reducing serum can reduce the risk of manufacturing contaminated products [5].

Virus screening may be required for cell therapy products. The human genome contains numerous integrated viral genomes. Human cells can contain endogenous retroviruses (for further reading, see [32,33]) as well as the genomes of inactive viruses, such as those of the *Herpesviridae* family (e.g., *Cytomegalovirus*, Epstein–Barr virus, and the chickenpox virus; human HPV6), which have the potential to reactivate once cells are in culture. Screening of the master cell bank for these adventitious agents is standard for all biological pharmaceuticals, including cell therapy products, in order to demonstrate that the cells used to generate the product lots were free from such adventitious agents. Testing of the working cell bank and/or final product may be necessary as well, depending on the product [34]. If viral vectors were used to engineer the cellular product [35] then monitoring of cell banks and/or product doses for viral particles may need to be performed as well [36].

■ Safety consideration: autologous cells

The standard sterility panel of testing takes 14 days to complete [108]. Some patient-specific and autologous cell products are administered fresh (never cryopreserved); the processing time may be as little as 48 h. This means that the results from a standard sterility panel will not be confirmed prior to administration of the product to the patient. For example, Dendreon®'s sipuleucel-T (also known as Provenge®) autologous DC product for treating castration-resistant prostate cancer is administered to patients prior to the results of a full sterility panel of testing being received [37]. For Provenge, a 2-day Gram stain and endotoxin test are performed prior to administration. Patients sign a waiver acknowledging that the results of the full sterility test will be received several days after administration. If evidence of contamination is found, the results will be communicated back to the administering doctor, as well as information about the contaminating microorganisms (if determined) for treatment purposes. Until recently, products that must be administered prior to confirmation of sterility by current standard methods were intended to be administered 'at risk' with results from the standard sterility panel pending. However, in September 2011, the FDA proposed an amendment to 21 Code of Federal Regulations 600, 610 and 680 [109], which would allow for the acceptance of rapid sterility testing for some biological products that should help assuage some of the sterility considerations associated with these products.

■ Safety consideration: pluripotent stem cells

The proliferative nature of cells in culture contributes to the manufacturing of the product, but also presents some unique safety concerns. Tests for tumorigenicity, the propensity of the product to form tumors, are typically performed on master cell banks, although in some cases working cell banks are also tested. Typically, tumorigenicity tests are conducted by injecting tenfold more cells than the (body mass-adjusted) therapeutic dose into immune-compromised mice in order to test if the cells form tumors [38]. Pluripotent cells, with the potential to harbor a small percentage of undifferentiated cells, may have a propensity to form teratomas (tumors comprising of cells from all three germ layers), a unique and significant safety concern. A risk of cyst (small tumor) was evident in Geron's first investigational new drug for using a hESC-based product to treat spinal cord injury, resulting in delayed patient recruiting and establishment of new release testing for the product (summarized in [31,39,110]). Ideally, cell therapy products should also be tested for karyotypic abnormalities at a number of population doublings (PDLs) that exceeds what is typical of the product doses (at least one to three passages past the expected product dose PDL) in order to ensure that genetic abnormalities are absent. For pluripotent cells, this testing will likely be very important due

to the highly proliferative nature of these cells. Embryonic stem cells have the potential to harbor chromosomal abnormalities, or to acquire them through multiple passages, although it remains to be determined if those anomalies affect product differentiation capacity or even represent a real safety concern for patients [38,40]. For induced pluripotent stem cells, somatic cells reprogrammed into pluripotent cells via administration of a transcription factor cocktail, there exists a high propensity for acquisition of genetic anomalies [41]. Further studies need to be performed to better understand the true safety risk of these genetic challenges in pluripotent cell products. It is likely that tumor and karyotype testing will be an important component of safety testing for all product types, and especially pluripotent cell-based products as more of these products enter clinical trials.

Choosing assays & assigning their roles in support of a cell therapy product

The topic of greatest confusion and frustration for developers of new cellular therapies is that of identifying an adequate measure of biological function for their product. As a potential cell product evolves from an interesting finding in the laboratory to a therapeutic agent tested in animal models its developers identify unique attributes associated with the cells. In practice, measures of biological function for cell products typically derive from preclinical observations in animal models. Tests initially performed in animal models become distilled down to distinct assays that can be qualified to a reference standard and serve as a surrogate for the initial *in vivo* observation. These tests will likely become the foundation for the product-specific analytical toolbox that can be used to characterize the biological activity and safety of the cells as the cell product advances through clinical trials and, at the same time, the manufacturing process is scaled up and streamlined. This toolbox will consist of multiple, independent assays whose value and utility are under ongoing assessment.

The selection of assays that populate the analytical toolbox will begin with fundamental biological properties of the cells and lead to qualified assays that can be used to characterize the cells. For most products, the product cells may cause an effect in a target cell population, which can be linked back to either the expression of a unique surface marker on the product cells and/or the secretion of a unique cytokine or growth factor that elicits the desired response. Screening the cell product for these unique cell surface markers and the secretion of defined proteins will likely be the foundations for the identity and potency (release) assays for such a cell product. Literature searches can also shed light on typical tests performed to characterize the product cell type by identifying other secreted proteins or regulated gene pathways that can be followed with protein or genetic array screening products. This may also be necessary in order to identify unique, indication-specific biological activities that can be attributed to the product. Once identified as product (and indication) relevant assays, they can be tracked as FIO until they can be used in comparability studies and potentially as release testing. Assays to be used for release testing must be first qualified and validated during later-phase clinical study [42], an endeavor involving a significant effort. Even early in the lifetime of a cell therapy product, consideration should be given to whether a perceived release assay can actually be validated based on the underlying technology and reference materials available. It should be noted that characterization assays not used for product release (e.g., assays tracked for comparability and/or FIO) need only be qualified.

One important practical consideration related to product release assays is the importance of developing assays to test the final product, rather than a manufacturing intermediate (e.g., cells at harvest prior to downstream processing and formulation). After final harvest, cells often go through several hours of processing to concentrate, wash, formulate, fill and preserve the product, as well as cryopreservation, prior to administration. During transfer from research to development, assays that are typically performed on cells directly after harvest will need to be established at the appropriate place in the manufacturing process in order to avoid a misinterpretation of the safety, identity or potency of the intended product. Thus, developing assays and release criteria for identity and potency of the final product should involve measuring the characteristics of the cells in a typical formulated dose, and not an in-process intermediate. Building on this concept, it is also best practice to perform 'worst case scenario' studies in which cell viability and biological function are impaired by stressing some aspect of processing (e.g., holding time and/or temperature). Defining the limits of processing parameters helps to establish a processing space that accommodates a viable product.

Comparability is a tool recommended by the FDA in order to ensure that a product maintains its CQAs through manufacturing process changes [43]. During process development, assays are used to demonstrate consistency or similarity between products manufactured before and after process improvement in order to maintain safety and efficacy parameters. As the process is developing, assays used to characterize the product are evolving as well. An assay development plan might start with a broad list of targets which is then edited down to a more meaningful subset. An ideal strategy would be to establish a larger number of candidate assay targets, then generate data from available cell materials (e.g., end product originating from different donors or produced before and after process improvements) and identify trends and patterns in these results. Often the final assay choice will be a balance of assay relevance and performance. Test results that are most reproducible over time and batch-to-batch will emerge as candidate assays. Test candidates may also be selected due to an advantage associated with specificity or availability of a particular reagent. Once candidate assays are identified, test specifications will need to be set. Product release test specifications and acceptance ranges generally start out being rather broad and then narrow as the overall process is more refined and becomes more consistent based upon data accumulated over time. Assays used (or tracked) during process development for comparability, are the most likely tests to become qualified and validated release tests for commercially manufactured product.

In addition to facilitating product release, qualified assays are used to determine the stability (safety and efficacy) of the cell therapy products. The same strategy for determining commercial product shelf-life and expiration dating for drugs can also be applied for cell therapies. Product stability testing is required at all stages of clinical testing in order to ensure that (at a minimum) the cell therapy product is stable for the duration of the clinical trial [8]. Products intended for use within 1 year should be tested at increments of 1, 2, 3, 6 and 9 months to establish a pattern of product stability and then tested at 12 months and at least one subsequent point (15 or 18 months) to confirm stability is maintained at least slightly beyond the intended expiry claim. Products targeting a shelf life of 2 or more years should also be tested at 18, 24 and 30 months and annually after year 2 [44]. Stability testing is also essential at key process operations (although in a much shorter time frame, perhaps hours or days) to establish the time frame at which cells can be held under specific conditions (time and temperature). A recommended method for outlining stability testing of a cell therapy product is to create a table highlighting the parameters to be tested, especially the CQAs, versus time (for an example stability matrix see TABLE 1). Not all parameters will need to be tested at all time points, although basic tests such as sterility, mycoplasma and endotoxin as well as viable cell recovery, identity markers and key measures of biological function should be tested at each time point. Performing stability testing on all product lots manufactured for clinical trials will ensure a sufficient database to identify limitations to the longer-term stability performance necessary for commercial-scale production.

While implementation of relevant assays at appropriate times during process development is important, statistical design of experiments and analysis of the results are important considerations not to overlook. A 'process quality engineering' approach [45] can be taken to begin to understand how critical process parameters drive process variability in the CQAs. Identifying the sources of process variability will facilitate the implementation of what is known as a Quality by Design approach to process development. As ranges for critical process parameters are established and their impact on the CQAs understood over many manufacturing runs over many donors, six sigma statistical process control can be implemented for continual improvement of the manufacturing process [46].

Cell characterization assays as tools for process development

The manufacturing process for therapeutic cells is expected to change dramatically throughout clinical development [47]. During early clinical development there should be a focused hypothesis-driven research effort to understand how the cell characteristics impact the safety and therapeutic efficacy of the intended product. This product focus is necessary to direct experiments to answer questions on what specific cells and biologic functions are central to addressing the disease of interest. As more information is gathered regarding the intended cell product, the process development program can be established to answer questions related to the efficient and robust manufacturing of the intended therapeutic cells with prespecified quality attributes. Process development experiments can be focused on establishing culture parameters that

Table 1. Sample stability matrix.

Category	Test	Specification	Lot release T = 0	3 months	6 months	9 months	12 months	18 months	24 months	Optional 36 months	48 months
Viability	Cell counting	≥70%	X	X	X	X	X	X	X	X	X
Cell count	Viable cell recovery	≥75% targeted dose	X		X	X	X		X		X
Safety	Sterility USP <71>	Negative	X	X		X	X	X	X		X
Safety	Mycoplasma USP <63>	Negative	X	X		X	X	X	X		X
Purity	Endotoxin USP <85>	≥5 EU/ml	X				X	X	X		X
Identity	Flow marker #1	≥90%	X	X	X	X	X	X	X	X	X
Identity	Flow marker #2	≥90%	X	X	X	X	X	X	X	X	X
Identity/purity	Flow marker #3	≥5%	X	X	X	X	X	X	X	X	X
Potency	Cytokine #1 ELISA	50–100 pg/cell/day	X	X	X	X	X	X	X	X	X
Potency	Cytokine #2 ELISA	25–75 pg/cell/day	X	X	X	X	X	X	X	X	X
Potency	Elicited cytokine #3 ELISA	200–300 pg/cell/day	X	X	X	X	X	X	X	X	X

The simplest mechanism for identifying tests to be performed for stability testing is to form a stability matrix. Here, we have indicated the stability tests recommended by the International Conference on Harmonisation and US FDA [8,44]. We have assigned generic identity, potency and purity tests along with example specifications in order to demonstrate the degree to which these assays need to be developed prior to stability testing for a cell therapy product manufactured for all phases of a clinical trial.
USP: United States Pharmacopeia.

impact the CQAs of the therapeutic cells that can then be utilized to design larger-scale and more efficient production processes.

Best practices in manufacturing process development will involve focusing on scaling-up in order to meet clinical and eventual commercial demand. For example, a single indication requiring 100,000 (10^5) patient doses per year of 108 cells would demand approximately 10 trillion (10^{13}) cells per year to be manufactured to meet market demand, a scale currently not possible in therapeutic cell processing. Additional focus on 'process streamlining' will be necessary to minimize costs and position the cell product for commercial success. During this period of process development, there are three major areas that are addressed:

- The production and processing of cells is moved to a sterile, closed system to minimize risks of contamination, and to scalable processing platforms that will enable commercial lot sizes;

- Animal-derived components are reduced or completely eliminated to minimize risk of disease transmission, as well as to reduce supply chain risks [48];

- The manufacturing process must be scaled up to increase the lot size and streamlined to minimize processing steps (reducing risk of contamination or failure) and reduce the overall costs of these inherently expensive cell-based products.

All of the above process development activities have the potential to change the phenotype, function or safety profile of the cell product [47]. Quick and efficient characterization of the cells that come out of the development experiments is important to ensure that the CQAs are not changing to the point that the drug developer now has a different product, and most importantly that the unique biological functions that are relevant to the MOA of the therapeutic are retained at appropriate levels. The variety of cell characterization tools and technologies discussed are central to achieving success during these development activities. We will now illustrate how identity and potency assays can be used to help understand common process development activities. Unfortunately, there is an 'identity crisis' in the field of cell therapy. There is a tendency to rely on little more than flow markers (identity) as a characterization panel for cells undergoing process development. To demonstrate the danger in this minimalistic approach,

we hypothesized that the flow cytometry profile of a cell product could be uncoupled from potential potency measures of biological functionality (such as cytokine secretion). To address this, we used research-grade MSCs (Lonza®, Basel, Switzerland) in two experimental systems common in translational process development; extended culture passaging, and serum-containing versus serum-free media expansion. In these two experimental examples, both reveal that while the identity assays for the cells are not changing, the biological functions of the cells do change in some instances, and this information should all be taken into account when making decisions on accepting manufacturing process changes.

■ Extended passage

Extended passage studies are a useful tool for determining the population doubling limit for a cell therapy product. Determining the allowable population doubling range for the final cell product allows for maximization of the final lot size boosting the number of doses that can be generated in each lot. It is critical that the cells maintain not just their identity during process changes, but also their important biologic functions. We have performed an experiment in which human MSCs (Lonza) from a single donor were grown in ten-layer cell factories over ten passages, or 21 PDLs. The cells were characterized by flow cytometric analysis for the markers CD45, CD105 and CD166 as example identity parameters, which are a common subset of markers used to identify human MSCs [21]. As MSCs are known to secrete a wealth of cytokines that are believed to contribute to *in vivo* potency, supernatants from cells subcultured from PDLs three, eight, 15 and 21 were assayed for secretion of IL-6, IL-8 and VEGF. Evidence of IDO enzyme activity was measured by probing culture supernatants for kynurenine, which is generated by IFN-γ-stimulated cells that use IDO to transform tryptophan. During the ten passages (21 PDLs), the cells maintained characteristic flow cytometry markers and were >95% positive for CD90, CD105 and CD166, and <5% for CD45 (FIGURE 1A). However, there were differences found in the levels of cytokine secretion with each population doubling studied (FIGURE 1B–D). In some instances IL-6 and IL-8 levels were higher at PDL three, while VEGF levels were lower in the higher PDL cultures. Measurement of IDO activity via kynurenine accumulation in the supernatant from IFN-γ-stimulated cells revealed a steady decline with increasing PDL,

indicating that this component of their biological function was faltering (FIGURE 1E).

This type of experiment demonstrates that it is critical to not only evaluate flow markers of identity, but also biological functions that may contribute to the *in vivo* MOA of the cell product. Extended passage experiments should ideally be repeated with 3–5 donors prior to making process decisions, in order to account for donor-to-donor variability. Once executed, data from these experiments will facilitate the identification of process parameters that maintain biological functions. In addition, specifications can be set with regard to determining at which PDL the minimal levels of relevant biological function are maintained for the indication of interest. If PDLs are extended without appropriate cell characterization testing, it is possible for the product to lose biological functions related to potency that could lead to clinical data inconsistent with earlier-stage trials. Additional PDLs can also compromise the genetic stability of MSC products, especially in the presence of FBS [49,50]; an important safety concern associated with extended passaging that should be minimized.

■ Serum elimination

Experiments that aim to reduce or eliminate animal-based products in the manufacture of cell therapy products will decrease both product safety risk and supply chain risk of cell therapy processes. Therefore, we performed the following experiment using cultured human MSCs from one donor in T-flasks over three passages using standard growth media containing 10% serum and compared cell growth and phenotype to two different serum-free media. After the final harvest (cumulative passage six) the cells were analyzed by flow cytometry for the flow markers CD45, CD105 and CD166 and in all conditions the cells were found to be greater than 95% positive for CD105 and CD166 and less than 5% positive for CD45 (FIGURE 2A). As in the extended passage study, IL-6, IL-8 and VEGF levels were measured from the supernatants of cells after the final harvest (FIGURE 2B–D). We found that cells grown in both chemically defined media failed to secrete as much IL-6 as the cells grown in serum-containing media. The levels of IL-8 were higher than serum-containing media with one type of chemically defined media, while lower with the other. For VEGF, the levels observed with the serum-containing media were comparable to that of one chemically defined media, but not the other. Finally, measurements of kynurenine levels, as an indication of IDO activity,
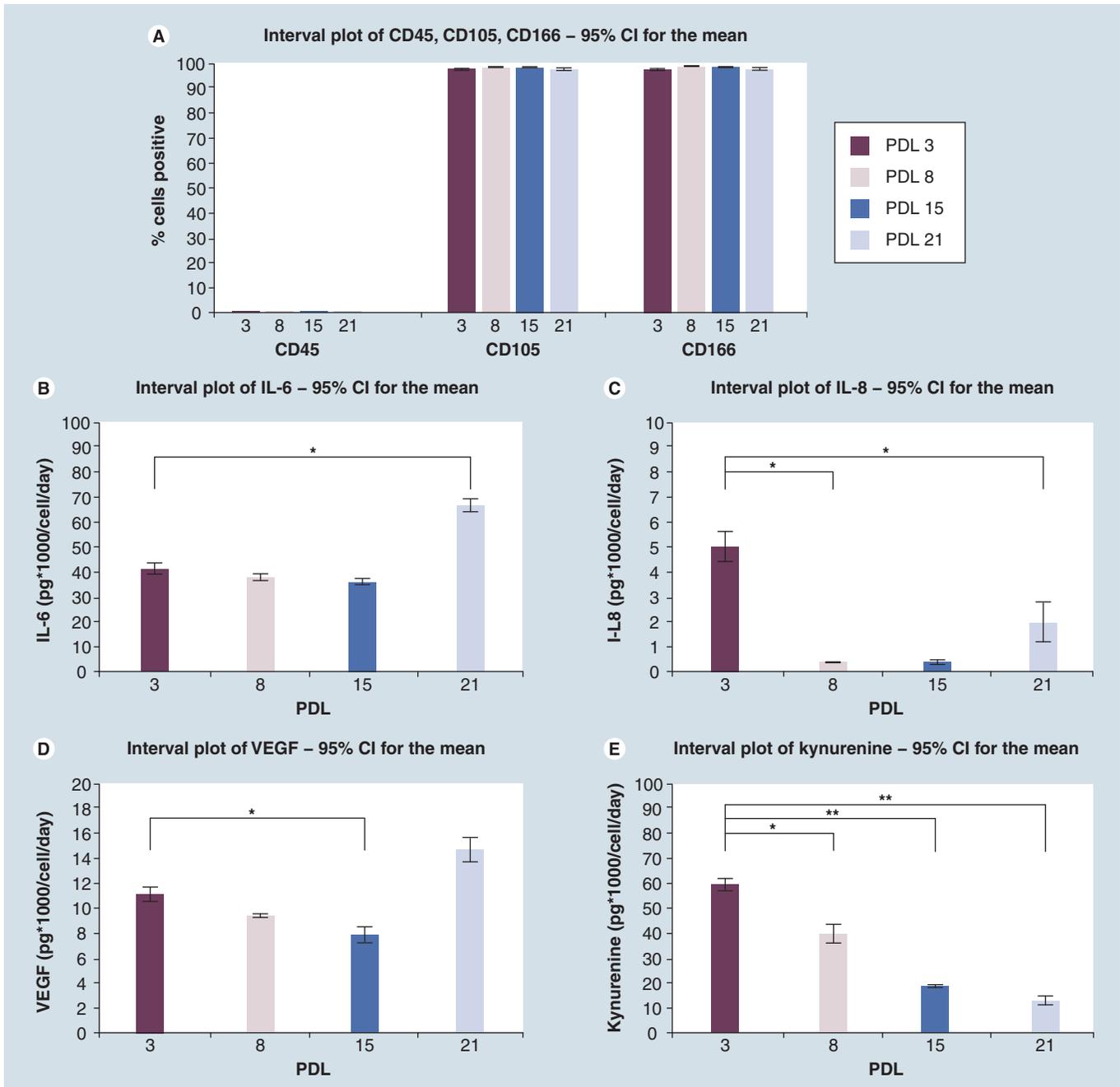
Figure 1. Extended passage study. Human mesenchymal stromal cells from one donor were grown through ten passages in three, ten-layer cell factories using DMEM supplemented with 10% fetal bovine serum (FBS; Lonza®, Basel, Switzerland) at each passage. **(A)** At passages one, three, six and ten (corresponding to PDLs 3, 8, 15 and 21) cells were thawed and stained for the surface markers CD45, CD105 and CD166 and analyzed using the FACSCalibur™ (BD Biosciences). At all passages/PDLs, the cells were consistently less than 5% positive for CD45 and greater than 95% positive for CD105 and CD166 ($p > 0.05$, three replicates from one donor). **(B–D)** Cells were plated at $7.6e^4$ cells/well in a 12-well plate and grown in DMEM + 10% FBS for 24 h and supernatants preserved at -80°C for ELISA analysis of the cytokines IL-6, IL-8 and VEGF (R&D Systems). Fluctuations in each of the cytokine expression profiles indicate that while the identity measures (FACS) are consistent, biological activity is changing in these cells through multiple PDLs (*$p < 0.05$, three replicates from one donor). The relevance of these changes can only be determined when measured against expected specifications for these cells. For instance, the significance of an elevation in IL-6 at PDL 21, or a drop in IL-8 levels at PDL 8 can only be determined in relation to the expected levels of the cytokines in a given cell therapy product. **(E)** Cells were also plated at a density of $5.6e^4$ cells/well in a 24-well plate and grown for 24 h in DMEM + 10% FBS. After 24 h half of the wells were stimulated wth IFN-γ in DMEM + 10% FBS while the other half were supplemented with fresh media. Supernatants were clarified and frozen at -80°C for measurement of kynurenine, an indicator of indolamine-pyrrole 2,3-dioxygenase activity (for more information please see the section on 'Potency'). As with the cytokine results, the stepwise reduction in kynurenine levels, corresponding to reduced indolamine-pyrrole 2,3-dioxygenase activity, can best be interpreted in the context of specifications for sample performance if this were a cell therapy product (*$p < 0.05$, **$p < 0.01$, three replicates from one donor).
PDL: Population doubling.
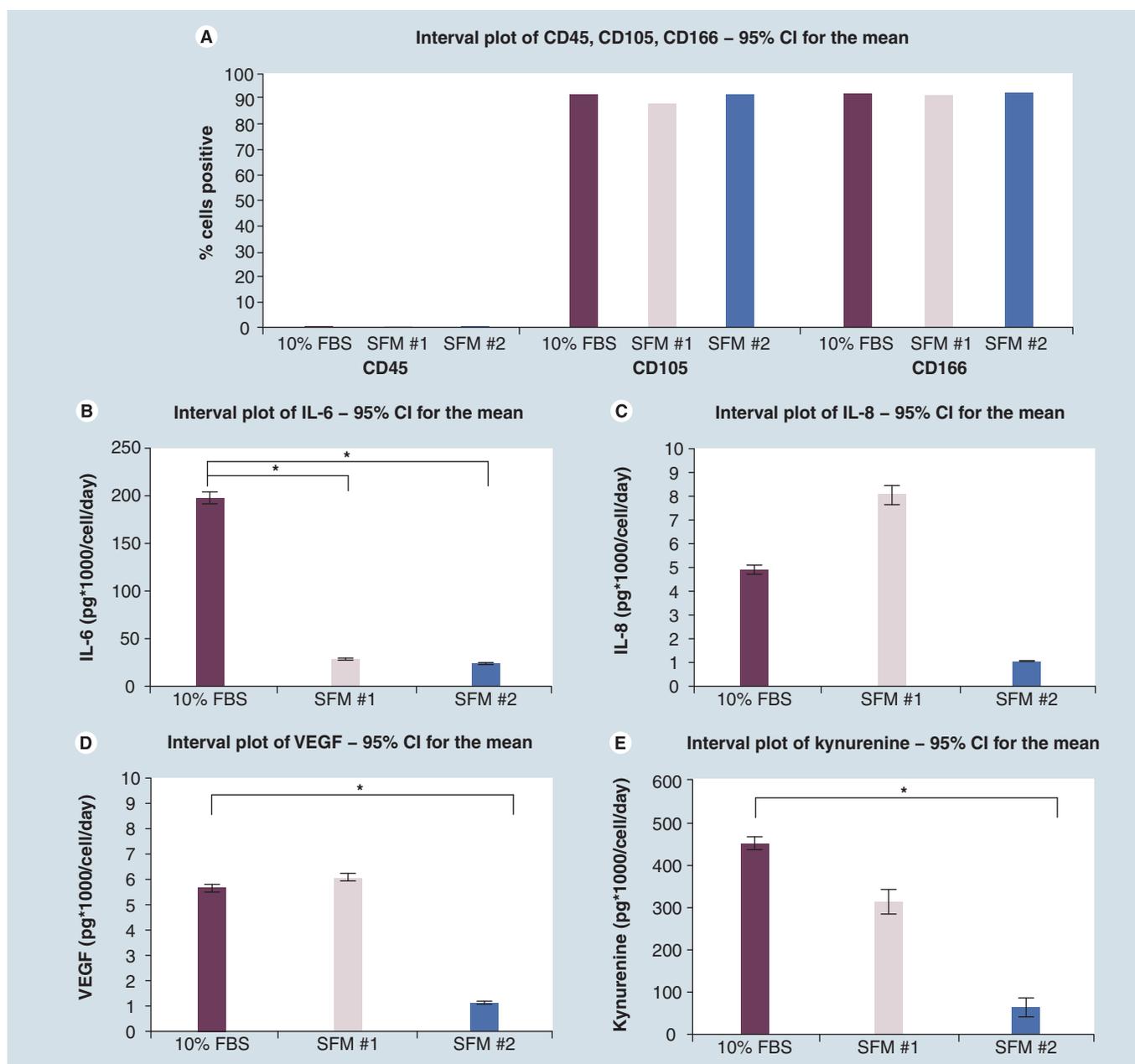
Figure 2. Serum elimination study. Human mesenchymal stromal cells from one donor were grown in either 10% FBS media or two different serum-free media identified as SFM #1 and SFM #2. **(A)** Cells from each media condition were thawed and stained for the surface markers CD45, CD105 and CD166 and analyzed using the FACSCalibur™ (BD Biosciences). In all media conditions, the cells were consistently less than 5% positive for CD45 and greater than 95% positive for CD105 and CD166. **(B–D)** Cells were plated at 7.6e⁴ cells/well in a 12-well plate and grown in DMEM + 10% FBS for 24 h and supernatants preserved at -80°C for ELISA analysis of the cytokines IL-6, IL-8 and VEGF (R&D Systems). Fluctuations in each of the cytokine expression profiles indicate that while the identity measures (FACS) are consistent, biological activity is changing in these cells grown in different medias (*p < 0.01, three replicates from one donor). As described for **FIGURE 1**, the relevance of these changes can only be determined when measured against expected specifications for these cells. **(E)** Cells were also plated at a density of 5.6e⁴ cells/well in a 24-well plate and grown for 24 h in DMEM + 10% FBS. After 24 h half of the wells were stimulated with IFN-γ in DMEM + 10% FBS while the other half were supplemented with fresh media. Supernatants were clarified and frozen at -80°C for measurement of kynurenine, an indicator of indolamine-pyrrole 2,3-dioxygenase activity (for more information please see the section on 'Potency'). As with the cytokine results, the reduced kynurenine levels in SFM #2 relative to the serum-containing media, corresponding to reduced indolamine-pyrrole 2,3-dioxygenase activity, can best be interpreted in the context of specifications for sample performance if this were a cell therapy product (*p < 0.01, three replicates from one donor).
FBS: Fetal bovine serum; SFM: Serum-free medium.

revealed comparable levels between the serum-containing media and one chemically defined media, but not the other (**FIGURE 2E**).

In this study, as with the extended culture study, cellular identity (surface phenotype) remains unchanged while some measures of

biological function vary. Therefore, comparability tests should not rely on identity data alone, but rather should include measures of biological function in order to ensure maintenance of product integrity through process development. We also demonstrate that not all serum-free media are equivalent in performance. It is best to test several conditions in a single experiment, ideally repeated with multiple donors, in order to determine the biological activity of a cell product in different media formulations. Understanding the important cell parameters of a given cell therapy product will facilitate the optimization of new media formulations for maintenance of the quality parameters of the product.

Future perspective

Nearly 1900 cell therapy product candidates have been, or are, in clinical development today [111] [MASON C. PERS. COMM.]. In the coming years, the cell therapy landscape will evolve and there will be multiple products on the market from which to learn. Best practices in cell characterization for process development and product release will be developed and refined, leading to multiple paradigms to apply to next-generation cell therapies. Molecular mechanisms of both safety and efficacy/potency of cell therapy drugs will become much better defined and then incorporated into drug development activities. Current assays will likely be simplified and multiplexed (for multiparametric analysis) leading to an overall reduction in cost (as well as turnaround time) and complexity regarding cell characterization as it applies to process development, comparability and release testing. A paradigm shift to more quantitative assays will be better able to support process improvements in cell therapy manufacturing. These assays will likely be based on the identification of new characteristics related to identity, potency and safety and will help establish a more comprehensive set of quality parameters for cell therapy products. In the next 5 years analytical advances may well outpace process improvements in the cell therapy field and should play a leading role in superior, faster development of new products as well as contributing to derisking clinical development using new surrogate measures of MOA.

Financial & competing interests disclosure

Executive summary

Critical quality attributes for therapeutic cells

- Analytical definition of a product must target four critical quality attributes for allogeneic, autologous and pluripotent cell therapy products.
  - Identity: to confirm that product contains the intended cellular and noncellular components.
  - Potency: to confirm that the product possesses the inherent or induced biological function(s) that is relevant to treating the intended clinical indication.
  - Purity: to confirm that the product does not contain undesired components, such as contaminating cell types or residual process reagents.
  - Safety: to confirm that the product is not contaminated with microbes or adventitious agents and, if appropriate, does not have tumorigenic potential.

Choosing assays & assigning their roles in support of a cell therapy product

- Identifying the tests that will characterize a product is often challenging, although in practice these tests derive from preclinical observations that can be focused into qualified and validated assays.
- Cell characterization assays are most often used to demonstrate comparability through process changes and a subset of this collection typically evolves into release testing of final product lots.

Cell characterization assays as tools for process development

- Product/process comparability testing provides the analytical basis to define what process improvements can be implemented to improve process scale-up and economics while minimizing risk to product quality.
- Extended passage: maximizing cell expansions to increase lot size is often needed to meet projected clinical demand but requires preserving all key attributes of the product.
- Serum reduction/elimination: minimizing the use of animal-derived serum in cell manufacturing processes can reduce the risk of adventitious agent contamination as well as supply-chain (e.g., animal serum) limitations as long as the product maintains biological functions.
- Cell characterization involves the measures of the biological function of a cell therapy product, as well as measures of cellular identity. Flow cytometry analysis alone is not sufficient to determine comparability through process development activities.

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