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Microfluidics for cell therapy and manufacturing in oncology and regenerative medicine

Josie L. Duncan, † Julio P. Arroyo † and Rafael V. Davalos *

Microfluidics has offered invaluable insight into diagnostics and point-of-care applications due to its small footprint, low costs, and minimal power requirements. As cellular manufacturing has shown significant promise for treating previously insurmountable diseases, microfluidics has expanded its reach into immunotherapy and regenerative medicine with a clinical perspective. Conventional methods to reprogram a target cell to improve prognosis, while innovative on their own, face challenges that miniaturized systems are poised to address. Here, we provide an overview of microfluidic-based technology that highlights significant strides within the field of cell manufacturing to treat cancer and degenerative diseases. We highlight commonly used mechanisms to isolate, transfect, and expand target cells in microfluidic devices. We discuss specific innovative microfluidic-based approaches that demonstrate comparable or exceptional performance compared to traditional methods.

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1 Introduction

Cell therapy refers to the collection and reintroduction of cells to a patient to treat previously insurmountable conditions such as cancer, hereditary disorders, and severe degenerative diseases.[1,2] Some cell therapy techniques require engineering the cell *via* genetic modification or editing where genetic material is introduced into the cell, termed as gene therapy. Cell therapies and the process set in place for manufacturing these potent cells have a hopeful outlook for treating various diseases and have been estimated to support the health of roughly 350 000 patients by 2030.[3] Though promising, cell-based therapeutic approaches still require refinement in the manufacturing of highly specific cells to improve efficacy and safety.

The process of generating these cells for cell therapy generally requires three main steps: isolation of the cells of interest from a patient sample, transfection or transduction of target cells with desired cargo (DNA, mRNA, and other genetic or proteinous material), and expansion of the modified cell to achieve an efficacious dose prior to reintroduction into the patient. Due to the cell concentration needed for reintroducing these cells into the patient, conventional bulk methods for isolation, transfection or transduction, and expansion are used. For isolation, common approaches such as centrifugation or the use of specific antibody-bound labels to identify and enrich for target cells are often used.[4,5] Once the desired population is secluded, the process for intracellular delivery of cargo involves using viral or non-viral vectors to either enact carrier-mediated trafficking or physically disrupt the membrane to insert cargo directly into the cell.[6,7] Post-transfection, current systems for cell expansion range in scale and complexity from 2D culture vessels (flasks, well plates, *etc.*) to 3D suspension cultures in bag-based systems or bioreactors. Traditional methods for harvesting, manufacturing, and administering the target cells suffer from low isolation specificity, transfection/transduction efficiency, and expansion viability.[8–11]

Conventionally, a variety of bulk methods exist in both academic and commercial sectors to achieve each of the steps required to manufacture therapeutic approaches. These methods, however, come at high manufacturing and transportation costs, thus decreasing accessibility and often resulting in low-quality yields.[12,13] Additionally, bulk methods may have method-specific limitations such as cargo size for viral vectors,[14,15] low transfection/transduction efficiencies, and high-stress conditions that greatly reduce cell viability.[5,16,17] Utilizing alternative technologies, particularly those that shift from bulk techniques, can alleviate one or more of these pitfalls during the cell manufacturing process.

One well-established and viable approach involves the use of microfluidic devices to capitalize on cell-sized fluid dynamics, which introduces an entirely new scale of physics and unique advantages of cellular engineering not yet implemented in the current process. Microfluidic technologies have a long history of sequestering desired cells from mixed populations and

Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology-Emory University, Atlanta, GA, USA.
E-mail: rafael.davalos@bme.gatech.edu
† Authors contributed equally to the work.

permeabilizing their membranes to introduce cargo as a means of increasing the efficiency of transfection/transduction for numerous novel disease initiatives.[18,19]

Not always under the guise of cell therapy-specific applications, advances in microfluidic technologies can provide a solution more effectual yet less recognized than conventional methods. By scaling down the environment to a magnitude relevant to a single cell at which these steps take place (*e.g.*, bulk processing to microfluidic devices), the overall cost of the process can drastically decrease while also producing comparable or even higher yields regarding purity and transfection efficiency. Further, these miniaturized devices provide an environment that enables gentle handling of cell populations, increasing cell viability.[20–23]

Moreover, these benefits attributed to microfluidic systems promote their assistance in developing potential clinical applications for novel oncological drugs, immunotherapies, and regenerative medicine. Due to their ease in handling and reproducibility for studying intracellular delivery, various cancer lines have been investigated for the separation of metastatic-causing circulating tumor cells (CTCs) and the delivery of harmful therapeutic agents into bulk tumors, resulting in cell death.[24–27] Additionally, microfluidics can be used to develop and expand the potency behind immunotherapies for specialized cancer targeting.[28,29] By assisting with the process for adoptive cell therapy, minute innate and adaptive immune populations can be properly isolated, genetically engineered for anti-tumor responses, and expanded at a clinical scale. Beyond cancer therapeutics, microfluidics offers an efficacious avenue for advancing regenerative medicine for targeting debilitating diseases through stem cell therapy by delicately handling these cells in order to direct differentiation.[30–32]

Over the years, several excellent reviews have been curated to provide an overview of the impact microfluidics has previously had and may eventually have on the field of cell therapy for oncology[33–35] and regenerative medicine.[36–39] These reviews provide a detailed look at niches within the field of microfluidics for oncological or regenerative medicine applications. Here, we investigate state-of-the-art microfluidic technologies for the three steps imperative to manufacturing cells for cell therapy toward disease treatment: isolation, transfection/transduction, and expansion (Fig. 1). We first introduce and discuss the microfluidic mechanisms commonly implemented to achieve a cell manufacturing workflow. We subsequently discuss specific applications and works that highlight the benefits of microfluidics in engineering cells for cell therapy. We then discuss the opportunities for microfluidics to be integrated into this workflow and the unique advantages and limitations associated with microscale devices. We provide an overview of common mechanisms used to isolate target cells, promote cell transfection through permeabilization, and expand cells of interest towards cell therapy applications. This review is structured such that the reader may familiarize themselves with an overview of common microfluidics mechanisms and tools employed in each of the three steps of cell manufacturing and then explore the numerous studies that applied these mechanisms to advance microfluidics in the fields of oncology and regenerative medicine.

2 Microfluidic mechanisms for cellular engineering for cell therapy applications

The process of cellular manufacturing for therapeutic applications in the clinic involves isolation of the desired cell type from a heterogeneous sample, permeabilization of the isolated cells to introduce genetic material, and expansion of the transformed cells for eventual reintroduction into the patient. Microfluidics, with its innate ability to apply cell-

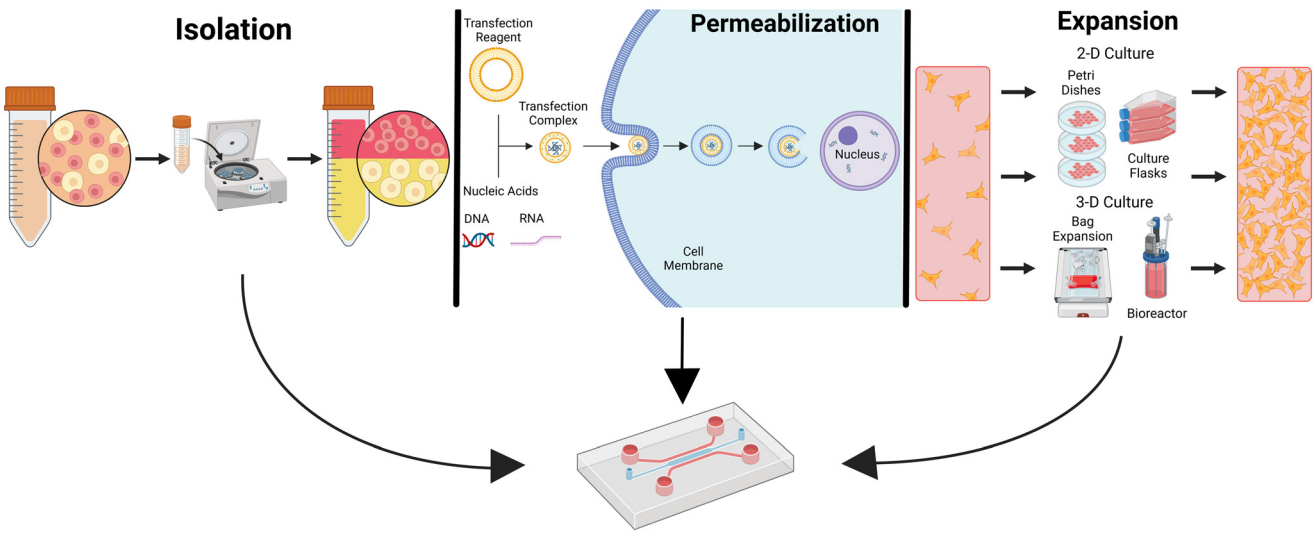

Fig. 1 Isolation, permeabilization, and expansion are the three essential steps for the development of cell therapy manufacturing. Microfluidics has the capabilities to complete each of these processes with increased sensitivity, efficiency, and specificity. Created with **https://BioRender.com**.

scale forces at the single-cell level, provides innovative approaches to each of these cell manufacturing stages that may overcome the viability and transfection efficiency limitations of conventional methods.

Microfluidic techniques for manipulating fluid flow and particle/cell behavior are often described as either active or passive. The distinction given to each technology depends on whether the function of the device is performed with or without an external force.[40] Passive devices, for example, may manipulate flow streams *via* channel geometries, channel obstructions, flow rate variations, or fluid dynamic phenomena such as capillary action. Active methods, on the other hand, implement external forces, such as electrical, magnetic, or acoustic fields, to manipulate fluid flow and particle behavior within the microfluidic device.[41] Here, we give an overview of commonly used microfluidic techniques, both active and passive, for cell and gene therapy applications.

2.1 Isolation

For clinical applications of cell therapy, patient or donor cells are collected, modified, and enriched *ex vivo*, often derived from tissue samples and complex biological fluids such as blood or aspirates. Within these specimens, the cells of interest (*i.e.*, immune and stem cell populations) are heterogeneously lost among the other constituents, necessitating sensitive technologies for isolation. Conventional approaches to isolation rely on centrifugation to isolate cells of certain density or labeling such as fluorescent- and magnetic-activated cell sorting (FACS and MACS). The use of labels and high centrifugation forces may decrease viability, increase cell stress, modify the morphology of the cells of interest, lack sensitivity, and require identifiable and specific biomarkers that may not be available for rare cell types.[42,43] Microfluidic techniques offer alternative solutions to cell isolation that can mitigate the shortcomings of conventional methods, particularly circumventing the loss of viable cells and the need for labels.

2.1.1 Mechanical. For isolation purposes, the field of microfluidics abounds in techniques to separate individual cells from a heterogeneous sample with high sensitivity by exploiting fluid dynamic principles. Notably, passive techniques such as hydrodynamic-, inertia-, and filtration-based microfluidic devices provide an approach to isolating cells that differ in size by capitalizing on geometric features and fluid dynamics on-chip.

Deterministic lateral displacement (DLD) is a well-regarded technique that employs a micropillar array with each subsequent row of pillars slightly offset from the previous along the width of the channel by a designated amount relevant to a set quantity termed the critical diameter. The critical diameter is essentially a particle size threshold that determines the trajectories of particles/cells with diameters above and below that threshold. Because of low Reynolds numbers, DLD devices have highly predictable streamlines of fluid flow through the micropillar array which allows the user to predict particle behavior. Under flow conditions ($Re < 1$), when encountering a post in the array, cells with diameters above the desired critical diameter will be displaced from their current streamline into an adjacent streamline (Fig. 2A). Larger cells move to the adjacent streamline due to their centroid being displaced by its encounter with the post, forcing the larger cell to move laterally across the channel. Smaller cells, on the other hand, have a centroid closer to the post, allowing the cell to behave rather undisturbed and continue straight along the length of the channel. These distinct behaviors of cells below and above the critical diameter create differentiable streamlines and thus, separation based on cell size is achieved. This technique has been shown to have a resolution of 10 nm and allows for continuous separation, a covetable feature for high-throughput applications.[44–46] In fact, for separation of circulating tumor cells from blood, flowrates of up to 10 mL min^{-1} have been used, greatly increasing the ability of DLD devices to be integrated into higher throughput workflows despite their microscale volumes and features.[47] The relevant parameters to achieve desired separation of particles are well reviewed by McGrath *et al.*, highlighting a multitude of device configurations, post geometries, and physics to consider for specific applications.[48]

Inertial methods use fluid dynamic principles that rely on carrier or sheath fluid velocity and device geometry to capitalize on cell-scale forces capable of separation based on cell size (Fig. 2B). Inertial microfluidics typically operate at higher Reynolds numbers ($1 < Re < 100$) than other microfluidic approaches ($Re \ll 1$), where fluid viscosity becomes negligible and inertial forces become dominant contributors for cell manipulation.[49] Inertial devices, commonly curved or spiral in nature, use particle inertia within a fluid to displace particles into adjacent streamlines, achieving separation or concentration based on size and deformability. Phenomena such as Dean flow, inertial lift, or elastic forces may also be exploited *via* channel design to manipulate particle position within the channel and facilitate separation. Forces acting on the particles or cells can be modified by tuning channel geometry and altering the operating flow rate (100 μL/min–24 mL/min).[50] In addition to their use as cell sorters, inertial microfluidic devices excel in cell focusing, which is often a desirable feature for integration with downstream on-chip transfection devices.[51] Inertial devices fit well within the cellular manufacturing workflow due to their continuous, high-throughput nature. Similar microfluidic devices generally have simplistic design, fabrication, and potential scale-up opportunities due to their constant cross-section channels and planar geometries, where both are often compatible with mass production manufacturing processes.

Other passive microfluidic filtration techniques, such as microsieving or cross-flow microfiltration, sort particles based on size by passing them over gaps or membranes to filter certain particle sizes. These devices often struggle with clogging at high cell numbers and lack specificity due to cells similar in size or high in elasticity, though offering a simple solution to cell sorting.[52–55]

2.1.2 Electrical. Though mechanical separation techniques have high sensitivity for size-based sorts, electrical methods

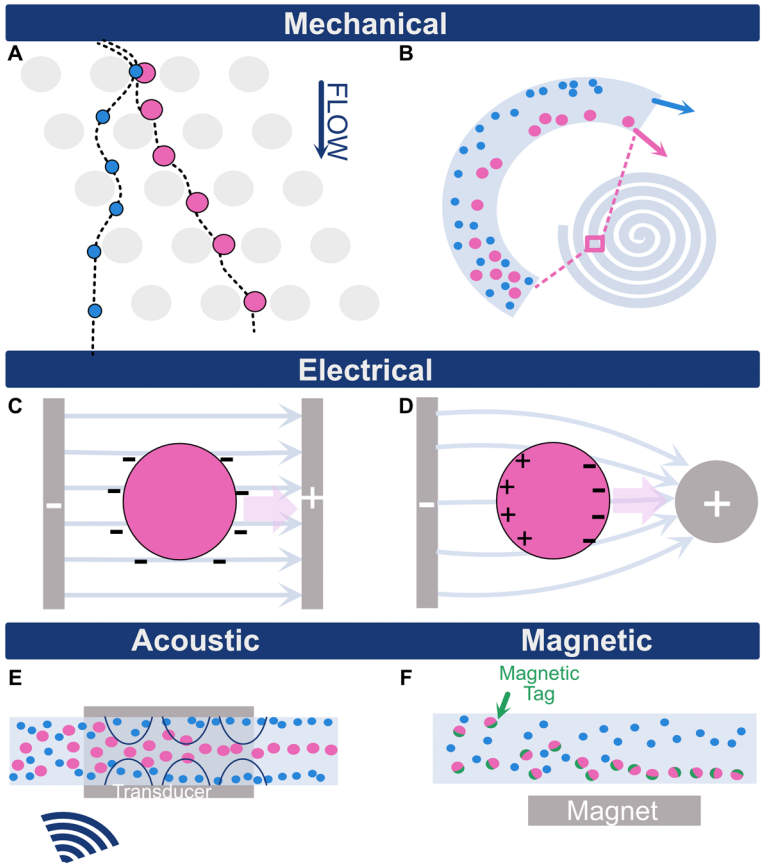

Fig. 2 Passive and active microfluidic isolation techniques towards the advancement of cell and gene therapy for oncology and regenerative medicine. A) Deterministic lateral displacement, B) inertial, C) electrophoresis, D) dielectrophoresis, E) acoustophoresis, F) magnetophoresis.

offer an additional layer of specificity by capitalizing on the electrical properties of cells to positively or negatively select cells of interest. Electrokinetic techniques, such as electrophoresis or dielectrophoresis (DEP), manipulate cells based on their electrical charge or polarizability, respectively. These techniques have been shown to be gentle on sensitive cells, highly specific, and can be used label-free. For cell-based therapy applications, label-free techniques are highly beneficial for applications involving stem cells, as there are few biomarkers for targeting these cells.

Electrophoresis, or the induced movement of a particle in the presence of an electric field, manipulates cells based on their zeta potential. The zeta potential refers to the organization of charges near the surface of the cell. Electrophoresis commonly employs DC or low-frequency AC fields as a means of applying a force to the organization of charges close to the cell surface (Fig. 2C). In the presence of this force, the cell moves through its suspending medium at a characteristic velocity reflective of biophysical differences evidenced by small changes in zeta potential. Optimizing electrophoresis in microfluidic devices often involves modulating the applied voltage and device geometry as a means to generate sufficient electric field.[56]

Dielectrophoresis, a similar technique, uses electric fields to induce the movement of particles in the presence of a large field gradient induced by an AC field. When exposed to a high field gradient, cells suspended in a low conductivity medium will experience an induced dipole. According to the force acting on the dipole, the field will attract or repel the cell to or from the area of the highest electric field gradient (Fig. 2D). The response of the cell depends on intrinsic electrical properties resulting from its biophysical makeup, such as size, membrane capacitance, and cytoplasmic conductivity, and how these characteristics interact with their surrounding medium. To capitalize on the sensitivity of DEP, parameters such as voltage, flow rate of the suspending medium, and device geometry can be tuned appropriately while applied frequency is typically used as a characteristic property unique to the cell in that environment.[57,58]

2.1.3 Acoustic. Similar to the electrical techniques mentioned previously, ultrasonic acoustic forces can be used to separate cells based on size, density, and compressibility, harboring innate advantages over passive techniques such as DLD, which primarily relies on size alone. Acoustofluidics, or the use of acoustics in tandem with flow profiles of microfluidics to manipulate cells or particles of interest, is a label-free technique where a frequency-dependent acoustic field is applied *via* a transducer to isolate cells of interest. The frequency of the transducer is a tunable parameter for achieving specific sorts and preserving cell integrity and phenotypic

identity. Acoustofluidics uses acoustic streaming and radiation forces dispersed across the cell surface to manipulate particles based on size and mechanical properties.[59] The difference between the acoustic properties of the particle and its medium is what allows the movement of the particle through the medium towards pressure nodes or antinodes in response to the acoustic field gradient (Fig. 2E).[59,60] Uniquely, by transmitting waves to exert force on the cell/particle of interest, acoustofluidics (or acoustophoresis, referring to the induced movement of a particle in response to sound waves) is essentially contactless, significantly reducing stress or damage to the desired cells. Acoustophoresis can achieve isolation of specific cell types, but it is also a viable method for continuous and high-throughput cell focusing for easy integration.[61]

2.1.4 Magnetic. Magnetofluidics uses electromagnetic fields and fluid dynamics in microchannels to induce movement of particles or cells (magnetophoresis) (Fig. 2F). Magnetophoresis capitalizes on the relationship of the particles and their suspending medium by either manipulating diamagnetic particles in a magnetic fluid or magnetic particles in a diamagnetic fluid, termed negative and positive magnetophoresis, respectively.[62]

In this context, cells (with the exclusion of red blood cells) tend to be diamagnetic, thus requiring either a magnetic tag to be attached to the cell or the sample to be resuspended in a magnetic medium.[62] Unlike the previously described active methods of microfluidic separation, magnetophoresis is not always label-free for biological samples. If the cell itself is not magnetic and a biocompatible medium must be maintained, magnetic beads to tag the population are necessary for manipulation. The reliance on a tag is beneficial as it increases the sensitivity and purity of the resulting sample. The use of a label also enables flexible functionality and an antigen-specific surface for sorting.[63] Label-free techniques primarily capitalize on holistic manifestations of phenotypic deviances; for example, dielectrophoresis does not detect phenotypic changes directly, but rather the manifestation of those changes in the cells' bioelectric signature. The use of a label allows the user to modify the surface of the cell intentionally and predictably to improve specific sorts using magnetophoresis. On the other hand, the use of a tag is more expensive, and removal of the tag involves cell lysis or centrifugation, which decreases the concentration of viable cells for subsequent processes.[64,65] To implement negative magnetophoresis, which uses magnetic fields to repel cells to the field's area of lowest gradient, the cells must be suspended in a paramagnetic medium that hinders visualization of the movement of the cells. This technique requires a fluorescent label for tracking cell movement through the magnetic fluid in response to a magnetic field. Additionally, it is possible to use this technique for negative isolation of target cells by tagging undesired cells for removal, leaving the desired cells untagged and undamaged.[66] In this way, magnetofluidics can be useful for removing red blood cells from biological samples label-free and, when used for negative selection of cells, can be a gentle method that does not induce harmful thermal fluctuations within the microchannel. To modify magnetophoresis in a microfluidic device, the user can use a variety of conjugated beads for tagging (~nm–μm), tune operational parameters such as the AC frequency and amplitude for electromagnets, and adjust magnet position and geometry to alter the magnetophoretic response.

There are a plethora of configurations, orientations, and energies that can be applied in microfluidic devices to manipulate cells that may prove useful for cell and gene therapy applications. Passive methods generally offer simplistic set-ups, easy integration, and are ideal for size-based sorting. Active methods use external forces to balance fluidic dynamic forces within the device to induce particle movement. Active methods of microfluidic isolation include a wide variety of applied fields that yield more sensitive sorts and expand the cell properties by which to sort. These methods also tend to be more complicated to fabricate, involve complex setups external to the chip, and may induce damage to the cells of interest by way of high forces or heating within the device.

2.2 Transfection/transduction

Intracellular delivery is dependent on both the method of cell permeabilization and the type of cargo or carrier being transported to the target cell for the alteration of cell behavior or function (Fig. 3). Carrier-mediated intracellular delivery incorporates a variety of biomolecules and protective containers to penetrate beyond the cellular membrane using different cell surface interactions to ultimately relinquish their cargo for achieving transduction or transfection. Packaging of the genetic material used for gene therapy is divided between viral and non-viral vectors. Transient membrane disruption-mediated intracellular transport relies on physical methods to open the cell membrane to allow both select carriers described above or free-floating cargo, including small molecules and DNA/RNA, directly into the cell to perform transfection. This allows for the evasion of a carrying capacity that traditionally limits carrier-mediated delivery.[67,68] Clinically, gene delivery has evolved to be the pinnacle for engineering new cell-based immunotherapies, editing genes within targeted diseased cells, and promoting advancements in regenerative medicine.[69] However, existing methods face challenges in balancing a high transfection/transduction efficiency with the number of cells successfully permeabilized/accessed to deliver desired cargo, as well as, ensuring the sufficient availability of cargo for large cell populations to match the quantity of cells required for therapeutic success. Implementation of these methods within microfluidics can prove to be fruitful when achieving both a high transfection efficiency and a high throughput of a largely viable population through cost-effective means.[70]

2.2.1 Carrier-mediated delivery. This delivery mode includes viral and non-viral approaches for efficiently loading genetic cargo into target cells. Viral delivery systems involve the insertion of cargo using a virus (*e.g.*, gamma retrovirus, lentivirus, adenovirus, adeno-associated virus (AAV)) to interact

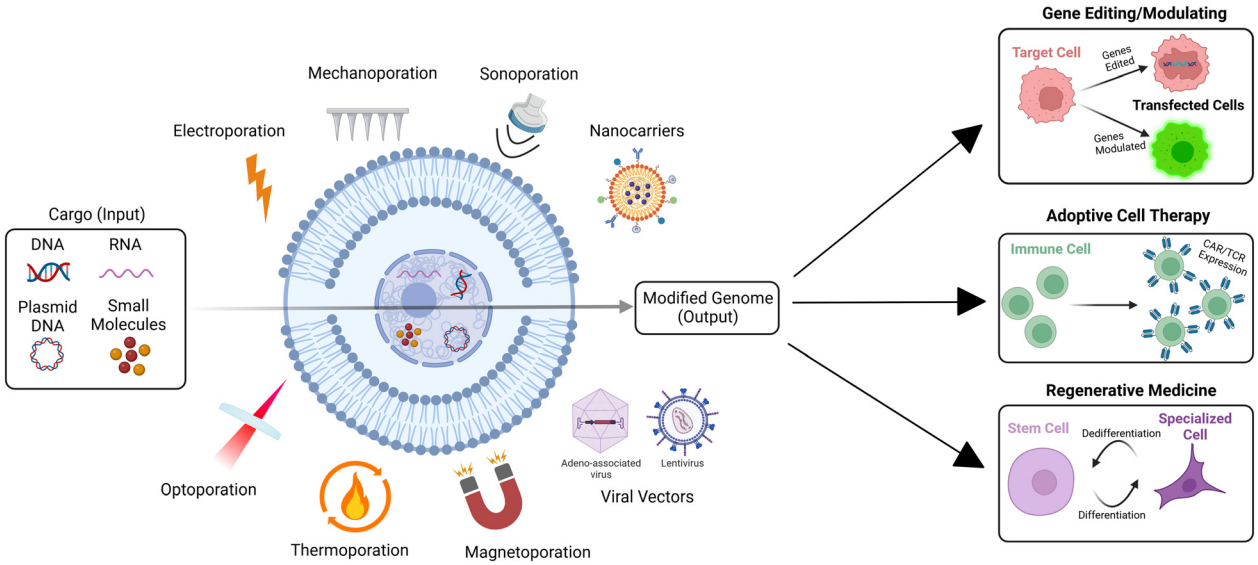

Fig. 3 Viral and non-viral techniques used for intracellular delivery of cargo for the purpose of cell and gene therapy which span gene editing/modulating, adoptive cell therapy, and regenerative medicine.

with a cell's surface to either become fused or endocytosed with the host cell. Each category of viral vector offers advantages behind the amount and type of genetic material that can be stored along with their tenacity within a system. These viruses offer both a high efficacy in regards to transduction efficiency, specificity, and persistence of cell modification to evade gene degradation; however, when administered clinically, viruses have been shown to activate an adverse immune response.[71,72] Non-viral delivery methods span over chemical vehicles (*e.g.*, exosomes and nanoparticles) which use similar interactions as the viral methods to penetrate into the cell. While chemical vehicles circumvent the disadvantages of viral vectors, evidence of cytotoxicity, low transfection/transduction efficiency, and unstable expression of induced genes remain to be the shortcomings of this approach.[73] Despite current novelties in microfluidics for viral nucleic acid purification[74,75] and post-transduction gene sequencing,[76] carrier-mediated delivery using microfluidics can be advanced by achieving closer proximity of cargo-bearing vehicles to target cells gently and at lower working volumes, resulting in lower volumes of costly genetic materials needed to promote both transduction/transfection efficiency and viability.[77,78]

2.2.2 Mechanical. Mechanoporation, documented in 1911 to be one of the first physical methods for intracellular delivery, is induced when mechanical forces rapidly applied to a cell lead to the development of transient defects along the cell membrane for delivery of cargo.[79] These defects arise under the application of compressive, tensile, or shear stresses enacted through perforation or constriction *via* microinjection, nanoneedles, or a microfluidic device. This technique has evolved in parallel with microfluidic design where alterations in chip architecture and flow gradients will influence the cell permeability for intracellular delivery. Further, these adjustments can produce high yields of transfection efficiency, purity, and viability for the target cells.[80] While these devices can be more expensive to fabricate due to the intricate components and less effective for suspension cell types, the incorporation of confinement and hydrodynamics within microfluidic-based mechanoporation displays increases in both transfection efficiency and viability. However, both suffer from clogging within the devices which make productivity drop.[81]

2.2.3 Electrical. The biophysical phenomenon of electroporation occurs when a cell membrane is disrupted with the formation of nanoscale defects due to an applied external electric field. This process is triggered when the induced electric field results in an increase of the cell's transmembrane potential (TMP) beyond a critical threshold of ~0.2–0.5 V. Promoted by this event, the cell membrane's phospholipid bilayer opens, creating reversible hydrophobic pores.[82–84] Deemed as reversible electroporation, cells in this state achieve increased permeability allowing for various cargo to be trafficked within. Other terms for this application of delivering foreign substances through these transient pores are electrotransfection or electropermeabilization.[85–88] Adopting electroporation for microfluidics may include adjusting the electrode geometry, configuration, and composition along with the applied voltage to achieve an amplified local electric field. The downsizing of components used to induce electroporation along with the constant flow-through produced in microfluidic devices can mitigate heat generation dramatically within the devices which can enhance transfection efficiency and throughput comparatively to bulk electroporation.[89,90]

2.2.4 Acoustic. In parallel to acoustophoresis, cells are subjected to acoustic waves at varying frequencies (MHz–GHz) delivered from an ultrasonic transducer, which causes cell membrane permeabilization, permitting biomolecules to enter through transient membrane pores. With its discovery in the 1980s, sonoporation initially manipulated acoustic

pressure to induce bubble formation to deliver cargo through the penetrated cell membrane. Bubble formation based sonoporation performance is reliant on the properties of the fluid medium, the bubbles causing cavitation, the frequency of the acoustic waves, and the transducer. Progression in the field has led to the use of non-bubble-based sonoporation, which relies on the acoustic radiation force and shear force to promote cargo delivery through cell permeabilization without accidental cell death by cavitation. Delivery of sonoporation within the context of microfluidics allows for ease in uniformity and lower power within the confined space, allowing for potentially higher cell viability and throughput.[91,92]

2.2.5 Magnetic. Initially, magnetoporation was believed to guide the delivery of cargo to a cell's membrane and stimulate absorption through endocytosis when subjected to a magnetic field. However, it has been likened to electroporation due to the applied magnetic field inducing a local electric field, causing cell membrane disruption and allowing magnetic-sensitive vectors, composed of the desired intracellular cargo and magnetic reagents, into the cell. Alterations to the applied magnetic field and material composition of the magnetic reagents lead to variations in transfection efficiency and viability. Challenges with magnetoporation arise from the unsuccessful delivery of magnetic vectors due to alterations in the field leading to clustering of the vectors, which often result in a low transfection efficiency; however, these obstacles provide opportunities for implementation within microfluidics.[93–96]

2.2.6 Optical. The permeabilization technique of optoporation is known to leverage the exposure and wavelength of light concentrated onto a cell's surface with and without the assistance of nanoparticles to form transient pores for the purpose of intracellular delivery.[97,98] Optimal delivery is derived from the duration of light exposure and wavelength applied to the system. Alterations to the duration of the focused light beam span from continuous application or through short pulses lasting on the scale of nanoseconds, picoseconds, or femtoseconds. Additionally, modulating the irradiation applied, such as the energy density or power density of the focused light, can create the necessary wavelength for inducing the physical effects needed to cause membrane permeation.[99,100] The high cost of optical equipment and potential for light diffraction can be limiting for transfection efficiency; however, microfluidics can leverage these focused light beams to be maintained uniformly within the channels where diffraction no longer becomes a limiting factor.[96]

2.2.7 Thermal. Thermoporation causes disruption of the cell membrane through induced fluctuations in temperature using laser-heating instruments. Thermal delivery methods of either rapid cycles of heating–cooling or continuous application of above physiological temperatures to the desired system allow for cell permeabilization.[101] Optimization of thermoporation is centered on tuning the duration and area heated by the laser system.[102] This physical approach can present harmful consequences beyond the intended cell populations through a lack of proper constraint on induced temperature, which can lead to severely damaging broader cell populations.[103,104] While this method remains infrequently integrated with microfluidics, benefits can arise from understanding thermoporation on a single-cell level, such as identifying the effects of thermal stress fluctuations for heat shock response and potential drug delivery.[102]

2.3 Expansion

Expansion after transfection is an integral part of cellular manufacturing for cell therapy applications. To achieve efficacious therapies, the modified cells must be expanded into a high number of cells for reintroduction back into the patient. Generally, higher cell numbers can yield more effective treatments, though they may also increase the risk of off-target effects and cytotoxicity. Some cell therapies, such as CAR-T cell therapy, require the expansion of up to 10^8–10^{10} cells occurring over 7–10 days. Likewise, some stem cell therapies require up to 10^8 cells for more efficacious outcomes when reintroduced into the patient.[105,106] While high cell quantities are necessary for these innovative therapies to bring about their promises, expansion is a tedious and time-consuming part of the cell engineering process.

Conventional methods of expansion after transfection in the context of cell therapies include both 2D and 3D approaches. Bulk expansion methods enable the use of a lower cell count for the preceding steps because large cell quantities can be achieved *via* cell culture flasks, closed-system bags, and bioreactors. After transfection, cells can be seeded onto a cell culturing platform such as well plates or traditional cell culture flasks with nutrient-rich growth-promoting (*e.g.*, dosed with cytokines, antibodies, *etc.*) media. Media constituents that may encourage proliferation, as well as the length of time over which expansion occurs, could decrease the proportion of potent cells as a result of unwanted differentiation, a common pitfall of bulk expansion for CAR-T cell manufacturing.[107,108] Bioreactors are another method of cell expansion for cell therapy applications. Bioreactors vary based on cell type but can be loosely described as environmentally-controlled vessels to promote cell growth *via* stimulation (*e.g.*, mechanical, electrical) and often host suspension-dependent cell types such as leukocytes for CAR-T cell therapies. Clinically relevant bioreactors take on forms such as disposable bags, stirred tanks, or perfusion vessels to handle up to 1000 L of cell media for expansion.[109] Other cell therapy treatments may require the expansion of adherent-cell types, such as mesenchymal or induced pluripotent stem cells (iPSCs), for regenerative medicine. Adherent cell culture conditions are spatially limited to adherent surface areas such as the floor of cell flasks or well plates and are subsequently limited by the storage of these culture platforms in environmentally controlled incubators. Workarounds of this limitation for the compatibility of large-volume bioreactors have yielded innovative solutions, such as the adherence of stem cells to microspheres for bulk expansion in a bioreactor.[109]

The opportunity of microfluidics to bring value to the expansion step of cellular engineering for cell therapy may seem daunting on the surface as the micro-scale nature of these technologies is not apparently compatible with the large volumes and cell quantities idealized for reimplantation back to the patient. While there may be some inherent challenges, the literature highlights distinctive and relevant features of microfluidic technologies that may aid in higher-quality cell expansions. For example, several microfluidic devices have entered the cell line development space and the expansion of rare cells for drug screening techniques which can be extrapolated to cell therapy. Wang *et al.* used a hydrophilic nano array to expand rare renal cancer stem cells. Each well hosted a single cell which proliferated into a spheroid over 7 days while maintaining its stemness for the purposes of drug screening.[110] Microfluidic devices enable highly controlled microenvironments that promote cell growth in a gentle and low-cost manner. In general, microscale technologies have revolutionized the ability to work with low cell quantities and rare cell types by creating platforms that are designed for single-cell expansion and tissue engineering, both potentially advantageous to the applications discussed here.

A large sect of microfluidics known as organ-on-a-chip (OOC) calls to mind another opportunity for microfluidics in cell manufacturing. Microfluidic organ-on-a-chip platforms offer an alternative approach to identifying effectiveness, cell–cell interactions, off-target complications, and potential new therapeutic approaches. Organ-on-a-chip devices often consist of an organoid or spheroid under dynamic culture conditions to study morphological changes undergoing various exposures.[111,112] As of late, cell therapy research is often limited to animal models which offer holistic insight, but lack the ability to glean answers to fundamental questions. Further, the need for an animal model makes researching new therapeutics using engineered cells costly. Microfluidics, heralded for being low-cost, offers the field of cell therapy an innovative technology *via* bioreactors and OOC platforms to shed light on the answers to the fundamental questions, the mechanisms of diseases, and treatments to better identify the limitations of such therapies prior to *in vivo* investigation.[112] Additionally, the implementation of microfluidics to characterize the limitations of cell therapies could greatly position the field of microscale devices towards highly translational research.[113]

3 Applications

In the United States, there are currently four adoptive cell therapy and three regenerative medicine clinical trials being conducted. These ongoing studies represent the advancements that modern medicine has for cell-based therapies to initiate anti-tumor responses or facilitate tissue reconstructive environments. Prolonged identification and expansion of select immune cells impede proper oncological treatment with this type of immunotherapy due to cell exhaustion, decreased proliferation, and loss of stemness.[114–116] Likewise, limited availability, directed differentiation, and immune rejection are severe constraints to the use of stem cells within regenerative medicine.[117,118] Since 2020, the studies discussed below have implemented a variety of microfluidic systems, highlighting both novelty and ingenuity to mitigate the challenges faced in their respective fields.

3.1 Adoptive cell therapy & oncology

3.1.1 Natural killer cells. One approach to effective immunotherapy is the use of natural killer (NK) cells, the main lymphocyte within the innate immune system, to target diseased cells in the body. NK cells have a wide bandwidth of targets since activation of these immune cells is based solely on the major histocompatibility complex (MHC) class I molecule presentation on normal healthy cells and stress-associated molecules on virus-infected/tumor cells. Due to these signaling molecules, NK cells do not require sensitization from antigen-presenting cells (APCs), unlike their antigen-specific counterparts (B and T cells), and can remain low in circulation until needed for targeting abnormal cells actively with cytotoxic granules or indirectly with pro-inflammatory cytokines.[119] NK cells may, likewise, be modified outside the body to enhance specificity, but their affinity towards distressed cells is a key advantage, not to mention the favorable safety results compared to T-cell therapies.[120] The implementation of microfluidics in natural killer cell-based therapy may be a solution for handling delicate cells found to be in low abundance within the blood.

Due to NK cells existing in much lower concentrations within the blood, the isolation of these cells has to be both specific and effective at maintaining their viability. To this end, Yang *et al.* designed a "minimal-stress" inertial device that uses on-chip centrifugation to isolate NK cells from whole donor blood. This allowed for a magnitude lower centrifugal force at half the time to obtain an 87% NK cell recovery rate when compared to 60% obtained by conventional centrifugation. Further, the microfluidic device yielded more reproducible results from donor samples with an average coefficient of variance of 6.9% compared to 30.3% conventionally (Fig. 4D).[121]

To actively separate NK cells from whole blood efficiently, a self-powered integrated magneto-microfluidic cell separation (SMS) chip employs an embedded lattice and buffer washing to form a phase gradient along with ferromagnetic beads to conduct a negative selection of the NK cells. Conventionally, magnetic-activated cell sorting (MACS) requires a sufficient amount of blood (5 mL) for the isolation of target cells using magnetic beads conjugated with antibodies for binding and removal of cells through an applied magnetic force. In comparison, the SMS device uses one drop of blood (8 μL) to achieve a purity of isolated NK cells at 80.4% and NK cytotoxicity was found to rival MACS-isolated NK populations. Additionally, the microfluidic chip's isolated NK cell populations from healthy and cancer

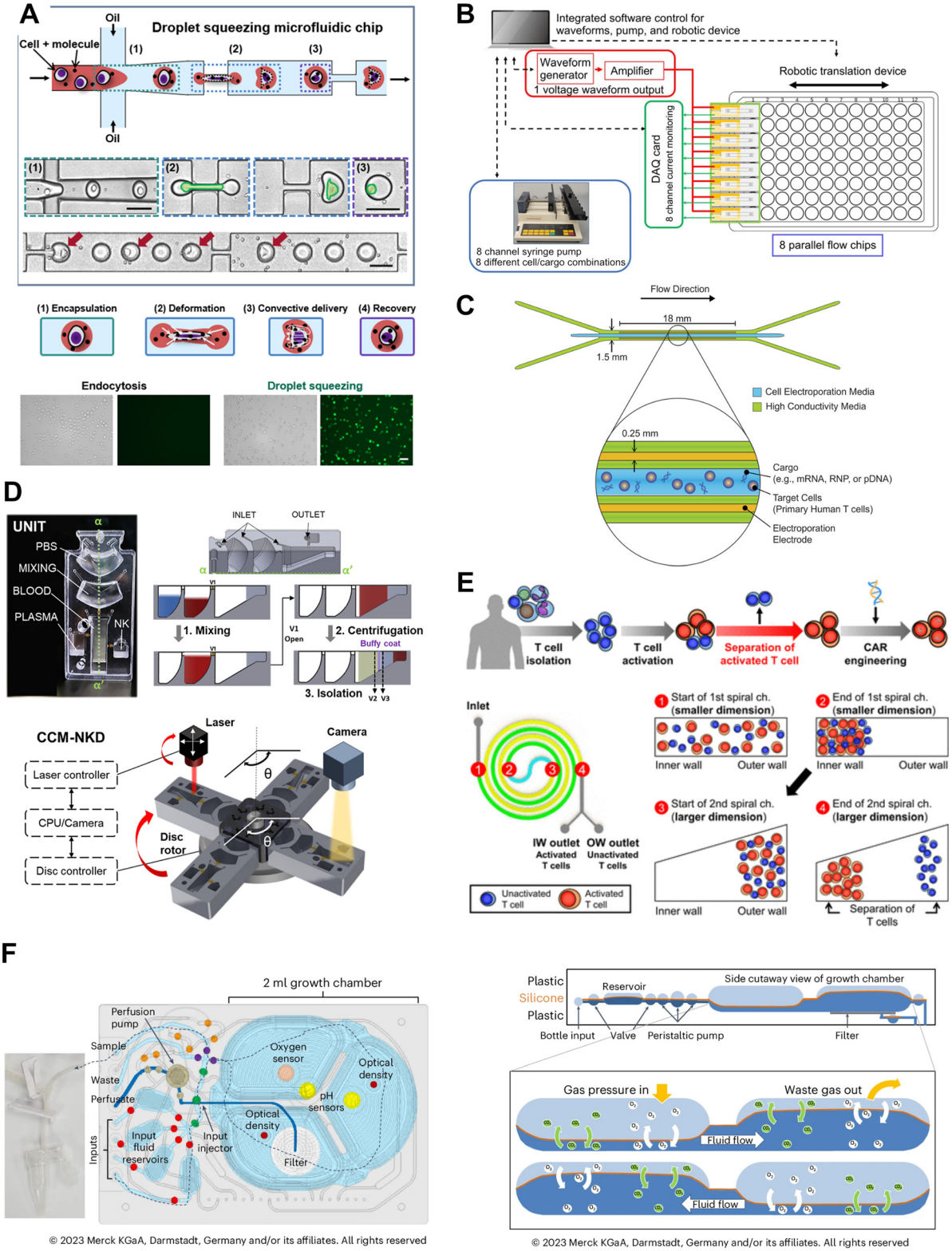

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Fig. 4 Microfluidics devices and platforms to improve cell manufacturing for oncology applications. A) Droplet-based mechanoporation for high-throughput gene transfection of human primary T-cells. Reprinted with permission from ref. 125 copyright 2021 American Chemical Society. B) Parallel plate electroporation in a microfluidic device integrated with a well plate and liquid handling robot for high-throughput transfection and expansion. Reprinted from ref. 128 copyright 2024 Springer Nature BV. C) A simple microfluidic channel with electroporation electrodes and high-conductivity sheath flow for >90% transfection efficiency at high-throughput volumes of primary T cells. Reprinted from ref. 130 copyright 2023 John Wiley and Sons. D) An inertial microfluidic device with on-chip centrifugation that isolates NK cells from whole blood samples. Reprinted from ref. 135 copyright 2023 American Chemical Society. E) Multi-dimensional double spiral for the separation of activated T-cells using inertial microfluidics for CAR-T cell manufacturing. Reprinted from ref. 131 copyright 2024 American Chemical Society. F) A perfused microfluidic bioreactor curates the microenvironment for clinically relevant CAR-T cell expansion. Reprinted from ref. 126 copyright 2024 Springer Nature.

patients were discovered to exhibit variations in cytotoxicity, promoting this device's functionality towards immune population monitoring.[122]

Other research groups have pursued efforts toward quantifying NK cells using microfluidics and machine learning software for prognostic and dosing applications.[123] Though relevant, the technology cited in these works emphasizes quantification rather than separation, transfection/transduction, and expansion and is thus outside the scope of this review. These studies do, however, highlight the accessibility of microfluidic technology and the ability to integrate these techniques in rapid and rural settings.

3.1.2 T cells. The manufacturing of CAR-T cells is a flourishing area of research, offering one of the most promising alternative treatments for cancer in the clinic. Though a brilliant direction of research, the burden of the manufacturing process is often characterized by high costs of labor and reagents, lengthy time scales, transfection efficiency, and quality assurance of transfected cells. The benefits of microfluidics for addressing drawbacks and bottlenecks of the CAR-T manufacturing process have been largely investigated. A review of the role of microfluidics in CAR-T cell manufacturing from Kim *et al.* provides a highly comprehensive synthesis of efforts to this end.[124] Here, we highlight select studies that rely on the unique advantages of microfluidic phenomena to address a burden in CAR-T cell manufacturing.

Joo *et al.* combined mechanoporation with the benefits of droplet microfluidics, namely localized concentration of cargo and single-cell sensitivity, to achieve high transfection efficiency of primary T-cells as a means for manufacturing CAR-T cells relevant to clinical applications (Fig. 4A).[125] By encasing both the cell of interest and the cargo within a single droplet, constriction of the droplet results in membrane permeabilization and subsequent media exchange across the membrane. This method achieves 90% mRNA transfection efficiency and 82% cell viability while reducing cargo waste and decreasing the overall cost.

Sin *et al.* approach CAR-T cell manufacturing from activation to expansion with a single microfluidic bioreactor.[126] The researchers compared their bioreactor to a gas-permeable well plate using Dynabeads for activation, lentiviral vectors for transduction, and perfusion for high-density expansion of these cells. The microfluidic bioreactor maintained comparable viability while outperforming the gas-permeable well plate in viable CAR-T cells, required vector load, and fold expansion (Fig. 4F). This research highlights the benefits of cell-scaled technology for cell and gene therapy. The data presented by Sin *et al.* suggests that microfluidics may cut cost, time, and labor appealingly compared to conventional bulk methods.

Mechanoporation devices based on tiny constrictions may result in clogging and severely decrease the throughput of the device, making it difficult to view microfluidic mechanoporation as a viable option for clinical application. Sevenler *et al.* uses fluidic forces within a constriction to permeabilize the cell using viscoelastic mechanoporation, demonstrating the ability to process 250 million cells/min.[127] By designing two spiral inlets that converge before the constriction, the fluid applies pressure to the cell and elongates it through the narrowing channel without contacting the channel walls, significantly reducing chances of clogging and achieving high throughput. Sevenler *et al.* demonstrates the potential for this device to be used to deliver various cargo such as RNA and CRISPR into numerous cell types, including primary T-cells for cell-therapy applications.

Microfluidics allows for the use of low voltages for transfection *via* electroporation, affording the engineers the ability to use more complex waveforms compared to high-voltage DC pulses commonly used in conventional electroporation. VanderBurgh *et al.* capitalized on this principle (27 V) and the plug-like fluid dynamics in microfluidic devices to electroporate cells with a time varying waveform as they pass through a region of the channel with parallel plate electrodes. Upon exiting the device, the platform is integrated with a liquid-handling robot and well plate system for high-throughput screening (Fig. 4B). By combining an array of these devices in parallel, the user has the flexibility to choose waveforms and liquid chemistries to be tested simultaneously, offering a tried-and-true gateway to higher-throughput microfluidics. After determining the optimal waveform, Jurkat cells were processed at 6.4×10^7 cells/min and maintained at an efficiency of 81% and a viability of 91%.[128] Similarly, Welch *et al.* utilized high-conductivity sheath flow along electroporation electrodes through a straight microfluidic channel to transduce primary T cells for high-throughput cell therapy applications (Fig. 4C). Building upon the work of Lissandrello *et al.*,[129] the cells would be exposed to the electric field generated from applying 100 V across the electrodes for 250 μs at 10 Hz through the device, allowing for an extended period for either mRNA, CRISPR/Cas9 single guide RNA ribonucleoprotein complexes (RNP), or (pDNA) to enter the cells for viable transfection. The conductivity of the cell medium informed the optimized electric field magnitude parameters and resulted in high mRNA transfection viability of 99% of the 10 million cells/mL flown through the device. High-throughput of this device was evaluated by increasing the cell concentration to 100 million cells/mL at a flow rate of 1.6 mL/min within the device which proceeded to produce high transfection efficiency rates of $<$93% for mRNA and $<$96% for RNP within the cells along with high post-transfection recovery rates. At the speed and level of high transfection rates, T cells could be modified for immunotherapy at a rate of 9.6 billion cells/hour.[130]

Separation of T cell subpopulations is vital for CAR-T cell manufacturing due to the success behind cell surface CAR expression relying primarily on the collected T cell being activated prior to genetic modification. This can be facilitated utilizing inertial separation at varying flow rates through a multidimensional double spiral (MDDS) design for activated and inactivated primary T cells isolated from patient whole blood (Fig. 4E). Research utilizing this microfluidic device found

benefits in both activating and transducing the T cells to express CAR prior to separation through MMDS to enhance viable CAR-expressing T cells in an isolated population. With the MMDS not requiring labeling of cells, low manufacturing cost, and proven to mitigate activation of sensitive cells, the MMDS shows a high appeal for implementation within the current CAR-T cell manufacturing process.[131]

3.1.3 Dendritic cells. An alternative approach within immunotherapy aims to leverage the capabilities of the dendritic cell, which acts as the primary APC responsible for capturing antigens and priming various T cells within the lymph tissue for adaptive immunity. Implementing dendritic cells as an effective immunotherapy comes with several limitations ranging from difficulties with conventional techniques of isolation and expansion, infrastructural barriers requiring highly skilled individuals, and successfully supplementing antigens to dendritic cells for T cell activation.[132] These challenges provide a unique opportunity for microfluidics to offer solutions to ameliorate such problems.

Caffiyar *et al.* utilized a canonical spiral microfluidic device to induce passive forces for the isolation of human dendritic cells from a patient blood sample for the downstream development of dendritic cell vaccines against various tumor types. The optimized spiral channel shape expands in size from its initial single inlet (200 μm) throughout each of its 7 turns to achieve optimal separation of varying cell sizes amongst 4 outlets (600 μm) using constant flow rates. 100–200× dilution of blood samples seemed to yield the best results for the inertial lift and Dean forces to more evenly separate based on the different cell sizes appropriately amongst the outlets without large cell densities affecting intended results. This research successfully supplied the outlets with sufficient dendritic cell populations for enrichment towards point-of-care immunotherapeutic treatment, while demonstrating a microfluidic capability for a potential replacement for expensive equipment requiring highly skilled users to conduct the same process.[133]

Resident CD8+ T cells often encounter barriers to effective tumor-specific antigens priming due to limited cross-presentation pathways and immunosuppressive elements within the tumor microenvironment (TME), such as regulatory T cells and checkpoint pathways, which hinder robust anti-tumor responses. To remedy this, Booty *et al.* subjected dendritic cells to a microfluidic device containing a 10–30 μm long constriction channel which caused cell squeezing, resulting in cell membrane permeabilization for the desired antigen to diffuse into the cytosol. This process identified a lower concentration of antigen was required for dendritic cells to activate CD8+ T cells through MHC I presentation. This process was found to be successfully replicated by squeezing any white blood cells found in the spleen. *In vivo* evaluation of the squeezed cells targeting various tumor-bearing models proved to yield superior survival rates, specifically when utilizing an additional T cell immunostimulant such as CpG. This microfluidic process promotes the potential for a substantial production of APCs rapidly for swift expansion and clinical implementation.[134]

3.1.4 Cancer cells. Investigating cancer for intracellular therapy has become a gold standard within microfluidics due to the disease's significance and history, along with the ease of handling and reproducibility when using neoplastic cells. Over 2 million people were diagnosed with cancer in the US during 2024 and joined millions of other patients afflicted with the same disease.[136] As accurate diagnostic tools and novel therapeutics become more vital, implementing microfluidics can improve on difficulties within this process where conventional methods struggle to find success. To capitalize using passive inertial forces through a continuous-flow, single spiral microchannel with a rectangular cross-section, Shiriny *et al.* demonstrated the capability to isolate two different-sized circulating tumor cells (CTCs), HeLa and MCF-7 cells, and healthy blood cells into three different outlets.[137] Flow rates were varied to produce an optimized Reynolds number to alter the Dean's drag force and inertial lift force for efficient cell-sized separation. A Reynolds number between 90–110 was found to accomplish a 100% separation efficiency of the cells, demonstrating an alternative for cancer diagnostics of subpopulations.

Expanding this methodology to a novel application, Tsou *et al.* used a spiral microfluidic device to separate cells obtained from a patient's pleural effusion, fluid buildup within the lung, to provide rapid diagnostic screening for cancer development. Out of 15 patient samples, accurate detection of malignancy was found in the patient population diagnosed with cancer. The separation efficiency and purity discovered experimentally were 98% and 90%, respectively, with a throughput of 65 mL/h. From these studies, the inertial spiral devices offer high throughput and purity at low costs, demonstrating their capabilities for further downstream analyses of label-free populations such as tracking cancer metastasis evolution and identifying various drug or immunotherapy options optimal for personalized cancer treatments.[138]

Once CTC isolation is complete, further manipulation of the cells is required to effectively evaluate cancer therapies on this select population. Microfluidics offers direct solutions for investigating novel antitumor therapeutics on specialized cancer cell types. One study incorporated both human lung cancer cells (A549) and human umbilical vascular endothelial cells (HUVECs) within a microvascular network-forming microfluidic device to mimic the non-small cell lung cancer (NSCLC) TME. Utilizing this platform, exosome-loaded microRNA (miR-497) was delivered into the system to target the lung cancer cells, revealing a successful reduction of lung tumor development. This was seen by a stark decrease in tumor migration by 76.7% and a lack of vessel formation compared to evaluated controls. This therapeutic approach assessed the delivery of exosome-loaded miR-497 into the TME, similar microfluidic systems aim to recapitulate the *in vivo*-like states at a low cost to efficiently examine an assortment of targeted cancer therapeutics.[139] Furthermore, a study by Sun *et al.* aimed to introduce a gene knockout through the CRIPSR/Cas9 system to eliminate TRIM72, a gene within a large family of RING-finger E3 ubiquitin ligases,[140] that can lead to tumorigenicity and

migration in select cancer cell lines. Within this study, a single-cell microdroplet platform was used to conduct transfection through close proximity of an isolated single cell with sufficient TRIM72 gene knockout plasmid and transfection reagent. The microfluidic system implemented three inlets: the first and second to supply cells, plasmid DNA, and transfection reagent to achieve uniform mixing before meeting the oil supplied from the third outlet. The oil was introduced from two directions to form the emulsion. Transfection within the microdroplets was optimized to balance cell survival with the transfection, concluding with an ~80% efficiency for the ~60% surviving population. TRIM72 knockout was conducted in liver cancer (HepG2) cells and glioma (U251) cells, revealing lowered expression of F-actin and N-cadherin, respectively. Further, migration of the TRIM72 knockout U251 cells was evaluated with an *in vitro* scratch test and *in vivo* zebrafish inoculation, displaying a reduced rate of wound healing over 48 hours and an almost 4-fold decrease in tumor size compared to the wild-type tumor inoculation, respectively.[141] Finally, Quach *et al.* introduced novel digital microfluidic methodology for the development and operation of a multistep platform focused on incorporating the steps behind lentiviral generation, packaging, and transduction. Known as LENGEN, this self-automated technique utilizes several designated sites to facilitate viral production through lentiviral producing "packaging" cell lines for continuous production of the desired viral particles. From the production site, 15 kb in size of lentiviral DNA plasmids at a functional viral titer of 1×10^7 transducing unit (TU)/cell are transported *via* droplets to target cell culture sites for achieving transduction, where they can be easily retrieved for downstream expansion and analysis. Evaluation of this system involved the modification of oncogenes in breast cancer cell lines (MCF-7 and T47DKB-Luc) at 48 and 72 hours. Average viability reported for the MCF-7 was 83.7% and 85.7% and T47DKB-Luc was 98.4% and 88.6% for 48 and 72 hours, respectively, with heightened knockdown of the estrogen receptor 1 (ESR1) amongst the transfected cells. With this automated transduction method available, extraneous intermediate steps are eliminated which greatly increases the availability of transduced cells for various applications while limiting excessive handling of sensitive cell types.[77]

3.2 Regenerative medicine & stem cell therapy

3.2.1 Neural stem cells. Transplantation of neuronal stem cells is an attractive avenue for treating diseases and damaged tissue in the central nervous system. While these stem cells are conventionally obtained through patient samples or derived from pluripotent stem cells, the heterogeneity of their subpopulations, though known, is poorly characterized. Depending on the application, the efficiency of derivation for cell therapy may benefit from determining the fate of the neuronal stem cells prior to differentiation. Using dielectrophoresis as an isolation technique, Jiang *et al.* enriched astrocyte- and neuron-fated neuronal stem cells without a physical biomarker or label in a well-characterized microfluidic device. Rather, by using hydrodynamic-manipulating structures in a microchannel flanked by chevron-shaped interdigitated electrodes, cells with a higher membrane capacitance moved towards the center of the flow channel (Fig. 5A).[142] In correspondence with previous findings, higher membrane capacitance correlates with greater astrocyte potential.[143–145] Label-free techniques, whether electrical or hydrodynamic, are direct advantages of miniaturized systems suggesting their unique use in stem cell therapy.

Neurological diseases are excellent candidates for stem cell therapy. By directing the differentiation of stem cells, degenerative diseases and potential therapeutics can be studied. Following the discovery of the ability to reprogram human somatic cells to pluripotency *via* transfection, Gagliano *et al.* examined the benefits of microfluidics for reprogramming somatic cells using mRNA. Interestingly, it was the simple micron-scale dimensions of the device that yielded results as significant as a 50-fold increase in transfection. In comparison to a conventional well plate, the microfluidic device consisting of several straight channels in parallel, maintains the mRNA closer to the somatic cells and promotes more extrinsic endogenous factors for higher reprogramming efficiency (Fig. 5B). By implementing microfluidics, fewer cells are required and only 5 μl of daily reagent are required for each day of the 15-day reprogramming, decreasing the cost of the process >100-fold.[146] Due to these significant benefits of a miniaturized system, the work was expanded by Tolomeo *et al.* to reprogram human-induced pluripotent stem cells to neuronal stem cells with mRNA for *in vitro* studies on disease, degeneration, and therapies. The research shows that the implementation of this device yielded 60.9% GFP-positive cells after just 7 days in a microfluidic device without a permeabilization technique.[147] The scale of microfluidic devices, though often discounted as "low-throughput", may be the same characteristic that reduces the cost of cellular engineering, thus making these revolutionary treatments more accessible.

3.2.2 Cardiac. In addition to bone, tissue, and nerve regeneration, cell therapy has been shown to be a promising approach for addressing cardiac abnormalities and diseases such as myocardium infarction. Unlike other tissues and anatomical structures, the function of the heart, the movement it requires, and its electrical nature introduces complexities not resolved with mere cell growth. Cell orientation and electrical conduction, in particular, are key parameters for effective cardiac cell therapy. Microfluidics research has provided unique approaches to tune and manipulate these parameters to ensure appropriate cell function in the heart.

In an effort to design a rapid and effective method for manufacturing engineered patches for treating myocardial infarction, Jia *et al.* capitalize on the predictable flow dynamics in microfluidic devices by using hydrodynamic focusing to manipulate cell orientation to achieve the anatomically relevant anisotropy found in native myocardium. The flow-focusing portion is flanked by ion-photocrosslinking and parallel packing. Here, the device flows silver nanowires in an alginate-gel precursor in the main channel. Along the main channel, two

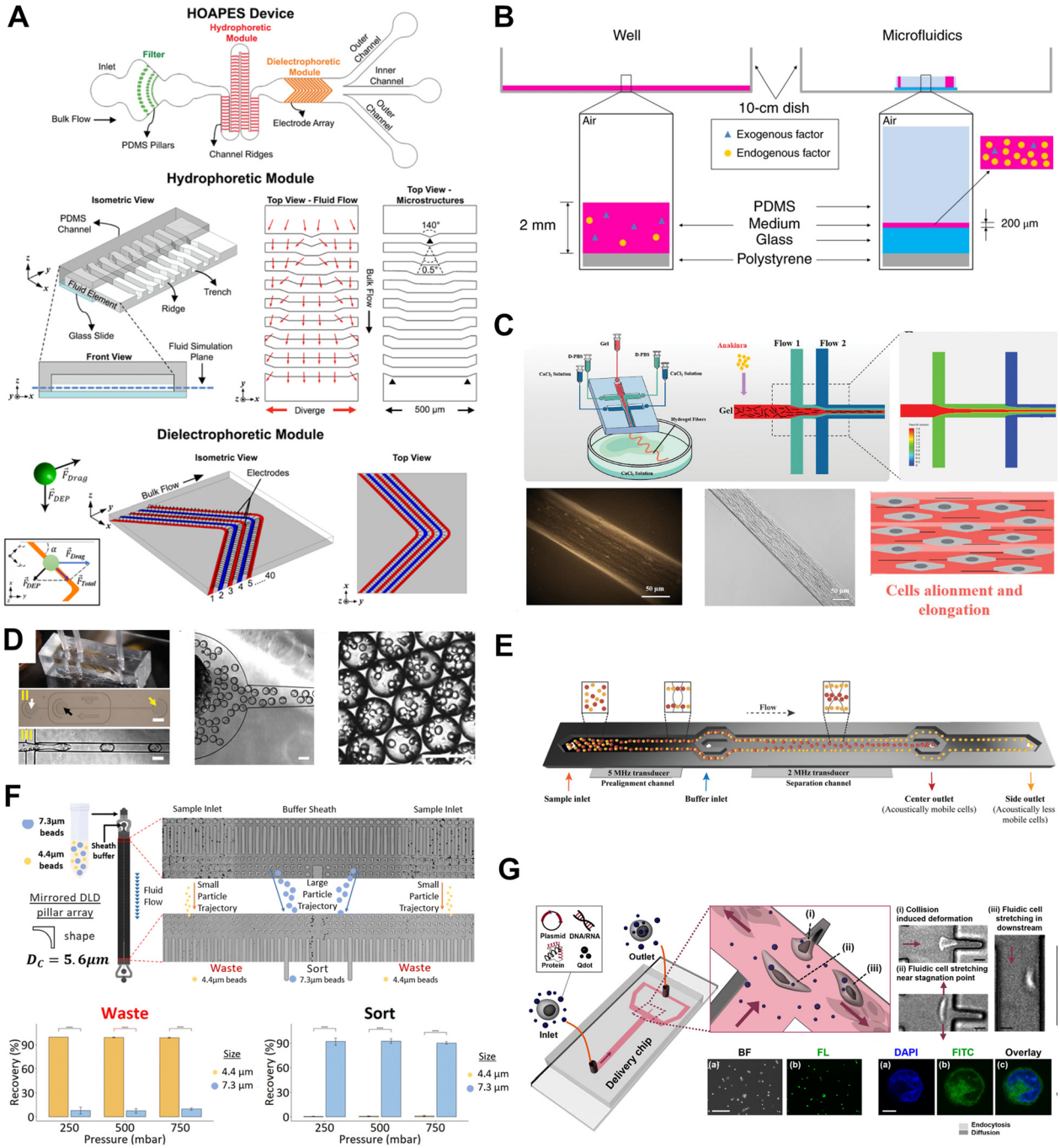

Fig. 5 Microfluidic devices towards the advancement of cell and gene therapy for regenerative medicine. A) Hydrodynamic and dielectrophoretic device for sorting neural pluripotent stem cells based on fate-bias. Reprinted from ref. 142 (CC BY). B) A microfluidic device for reprogramming human somatic cells to pluripotency that yields a 50-fold increase in transfection efficiency compared to a standard well plate.[146] C) Sheath flow for the high-throughput alignment of nanowires in a hydrogel for fabrication of cardiac patches.[148] D) Droplet microfluidics of cell-laden injectable hydrogels for treating myocardium infarction.[149] E) Acoustophoretic separation to enrich bone marrow MSCs for enhanced proliferation.[154] F) Deterministic lateral displacement for high-throughput isolation of MSCs from bone marrow aspirations. Adapted from[151] with permission from the Royal Society of Chemistry. G) Mechanoporation of bone-derived hMSCs by implementing i) an alcove at the end of a T-junction to permeabilize the membrane and induce cell stretching at the ii) stagnation point and iii) downstream. Reprinted with permission from ref. 155. Copyright 2020 American Chemical Society.

T-junctions are implemented to obtain hydrodynamic focusing *via* sheath flow to align the nanowires achieving the best alignment after the second T-junction (Fig. 5C). Not only did the cross channels provide forces to obtain 75% desired alignment, diffusion of calcium from the second T-junction medium diffused into the gel precursor promoting gel crosslinking. These hydrogels were removed from the device, fully cured in a calcium bath, and stacked to obtain a usable

patch. As the flow rates from the focusing flows increased, so did the percentage of aligned nanowires. The larger hydrodynamic forces, however, decreased the size of the extruded hydrogel exiting the device, which may decrease mechanical stability and be more inclined to breakage. These patches also showed significant antibacterial properties ranging from 65–100% sterility depending on the bacteria. In all, the patches manufactured through shear force alignment in a microfluidic device exhibited positive results regarding regeneration of electrical conduction, overall cardiac function recovery after myocardial infarction, and inhibition of fibrosis and ventricular remodeling.[148] Further, the use of microfluidics to establish anisotropy allows for the inclusion of biological macromolecules for functional hydrogels, which is difficult to achieve *via* high-voltage methods such as electrospinning.

As an alternative approach to oriented patches for treating myocardial infarction, injectable cell therapy is also garnering attention. Current injectable cell therapy treatments for this condition have a decreased potency due to the slow or complete inability of the cells to form anticipated cell–cell interactions or develop a sustainable matrix. By encapsulation of a cell in a hydrogel droplet, the scaffold and its makeup offered by the hydrogel promote proliferation and differentiation. Further, encapsulation of these cells increases their viability and, as a byproduct, their efficacy by isolating the cells from harmful environments *in vivo*, such as hypoxia. Gal *et al.* show these benefits by generating encapsulated iPSCs in hydrogels using a single T-junction droplet microfluidic device (Fig. 5D).[149] The data displays the formation of reproducible drops, proliferative behaviors *in vitro* and *in vivo*, and the ability to inject the encapsulated droplets without rupturing the droplets. The authors comment on the ability of a similar infrastructure to be effective in the differentiation of stem cells citing the ability to reproducibly control the microenvironment of the droplet.

3.2.3 Bone, cartilage, and adipose. As the most commonly used stem cell within regenerative medicine, mesenchymal stem cells (MSCs) have become a central pillar for advancing the field forward due to their potential variety of stem cell lineages that can be achieved, their resilience to be grown in culture conditions prior to reimplantation, and their availability amongst several tissues for isolation.[117,150] Patient samples can be collected from bone marrow, adipose tissue, umbilical cord, or peripheral blood. These samples contain other constituents, debris, and cell types, often requiring a multi-step process to isolate MSCs for cell therapy applications. Conventional bulk isolation methods such as centrifugation often yield low recovery of the cells due to osmotic imbalance and large forces acting on the cells. To address the challenges of isolating MSCs without the harmful effects of the conventional workflow, Tan Kwan Zen *et al.* used DLD as a gentler approach to isolating desirable MSC from bone marrow aspiration samples (Fig. 5F). By implementing arrays of L-shaped pillars in their DLD device and employing multiple devices in parallel, the researchers were able to isolate MSCs from 2.5 mL of bone marrow aspirate in ~20 min with a cell recovery (~41%) twice that of conventional centrifugation (~13%).[151] Not only does this microfluidic approach outperform bulk centrifugation, but it is also entirely label-free, reducing the cost and time of isolation significantly. To the same end, several groups have implemented other passive microfluidic techniques for the isolation of adipose-derived stem cell[152] and chondrogenic MSC populations.[153] In a related effort, Olm *et al.* demonstrated a proof-of-concept microfluidic device that separated subpopulations of bone-marrow MSCs using acoustophoresis (Fig. 5E). Here, the data indicates acoustophoretic separation provides a label-free alternative for enriching MSC subpopulations that are 20% more likely to proliferate, suggesting more stem-like properties.[154] The applications of this work extend beyond the isolation of desirable cells from a highly heterogeneous sample but also suggest the potential to enhance the efficiency of gene and cell therapy by enriching a subpopulation using microfluidic technologies.

Towards the development of high-throughput, low-cost microfluidics for stem cell engineering, Hur *et al.* designed a device with a T-junction following a straight channel and an alcove at the junction to induce deformation of a cell (Fig. 5G). As the cell in a carrying medium flows through the straight channel towards the junction, the alcove induces intrinsic flows that reproducibly cause cell squeezing thus inducing permeabilization of the membrane. The researchers demonstrated plasma DNA transfection of adipose- and bone-derived human mesenchymal stem cells, which are notoriously difficult to transfect. The implemented device yielded a transfection efficiency of 50–60% for the adipose- and bone-derived human MSCs, a significant improvement over both the Electroporator and Lipofectamine 3000 bulk methods. Further, the researchers show the ability to use the device to deliver large cargo (300 nm) without a vector to primary cells. This microfluidic-based transfection approach provides promising evidence for the future of microfluidics in cellular engineering, by yielding desirable transfection metrics for stem cell therapy in devices that cost <$1 to produce and achieving a generous reduction in reagents consumed.[155] Moreover, ensuring the desired stem cell fate has been achieved *in vivo* has proven to be a limitation for efficacious tissue regeneration results. Nanoparticles have previously shown success when paired in tandem with several imaging modalities such as MRI or PET imaging; however, the lack of definitive labeling and long-term tracking have detracted from monitoring the differentiation of a stem cell implant. Suryadevara *et al.* employed a microfluidic system with a ridged internal channel to conduct mechanoporation on infused bone marrow-derived MSCs with MegaPro nanoparticles coated with polyethylene glycol. Results of the MegaPro nanoparticle transfection were highly successful with a survival rate of 94.21% ± 1.99%. Labeled cells were implanted within cartilage defects found in *in vivo* pig models along with chondrogenic pellets to promote cartilage cell development. Tracking the cellular expansion over 12 weeks with MRI displayed no significant difference in cartilage repair compared to measured controls while maintaining a higher intracellular iron content, demonstrating a safe and effective

visualization approach for extended tracking of implanted cells.[156] A similar study used analogous methodology by utilizing mechanoporation to insert 68Gn-labeled and lipid coated mesoporous nanoparticles (MSNs) within adipose-derived stem cells (ADSCs) for radiolabeling. Approximately 87% of transfected cells emitted a stronger fluorescence signal compared to unlabeled cells and discovered no significant decreases in viability post-transfection. Radiolabeled cells were evaluated similarly within an *in vivo* pig knee defect, revealing the radioactivity was readily identifiable post-transplantation using both MRI and PET imaging.[157]

To ensure effective MSC transplantation, appropriate supporting content (composition of cell carrier, growth factors, *etc.*) must be carefully selected to ensure the differentiation of the stem cell to the intended cell type. Through the implementation of microfluidics, the development of both complex and efficacious carrier systems can be designed to support MSC proliferation and expansion at the target site to optimize extended tissue remodeling.[158] In a similar study, Wu *et al.* used a microcarrier consisting of fibronectin (FN) and hyaluronic methacryloyl (HAMA) covalently bonded together to form a unique ADSC niche for targeting diabetic wounds by promoting cytocompatibility and mechanical signaling for stem cell adhesion and differentiation. To synthesize the FN–HAMA microcarriers, a microfluidic device was introduced which used a square glass, two circular capillaries, and a glass slide. Initially, water-in-oil emulsions were created through the device by introducing the FN–HAMA pregel and ADSCs (liquid phase) to 5% span 80 (oil phase) to form emulsion droplets. The droplets were declared microcarriers once solidified through UV light. When evaluated using *in vivo* skin punches on diabetic mice, the FN–HAMA ADSC-encapsulated microcarriers were shown to significantly increase angiogenesis, skin and hair thickness, and collagen deposition, leading to 90% of the area to be closed due to wound healing after nine days.[159] In a similar study, An *et al.* designed a droplet microfluidics platform that allows for the encapsulation and de-emulsification of MSCs in hydrogels to enhance viability and preserve regenerative function for stem cell therapy applications. The data suggests that MSCs remained over 90% viable when encapsulated cells were kept at 4 °C. The researchers used this device to preserve MSCs and then injected them into a rat medullary cavity after 7 days of differentiation. Remarkably, the cells encapsulated in hydrogel droplets showed a significant increase in osteogenesis and new bone tissue volume after 2 and 4 weeks when compared to an empty medullary cavity and a medullary cavity injected with naked MSCs cultured in 2D and empty microgels mixed together. The authors attribute this significant finding to the use of single-cell microgels as encapsulation in a hydrogel more closely mimics the 3D tissue scaffold found *in vivo* minimizing biological function inhibition.[160] Several related *in vivo* studies implemented fabricated carriers to target specific disease states utilizing stem cell therapy such as Wang *et al.* incorporated both ADSCs and fibroblast growth factor-19 into gelatin methacrylate microspheres for ischemic limb regeneration[161] and Song *et al.* loaded both dental pulp stem cells and chondrocyte-inducing agent, kartogenin, for remodeling of osteoarthritic diseased cartilage.[162]

Microfluidics can help overcome challenges associated with stem cell therapy from directing differentiation to quickening the manufacturing of injectable cell-laden hydrogels to fabricating tissue scaffolds to treat diseased tissue. Still, microfluidics in stem cell therapy encounters limitations concerning throughput and material selection.[30] For studies that pursue microfluidics for the production of hydrogel matrices, their limitations lie primarily in the application of stem cell therapy. Though offering a scaffold to promote growth and migration, additives to the hydrogel for crosslinking may result in toxicity to the cell or the recipient. The organ-on-a-chip sector of microfluidics, whose impact on the field is discussed later on, yields a multitude of chip-based models for studying tissue regeneration, toxicity and drug-screening, and cell therapy.[163–165]

3.2.4 Extended applications. Many of the aforementioned technologies and approaches can be expanded to other applications, such as infectious or hereditary diseases. CAR-T cells, for example, are being studied for their potential therapeutic effects for viruses such as HIV and hepatitis B.[166,167] Further, degenerative ocular diseases targeting the structures of the eye (retina, cornea, optical nerve, *etc.*) can easily disrupt this delicate system, causing visual impairment. Implementation of stem cell therapy can offer a route to circumvent these hindrances; however, this approach has been greatly subsided. One study by Jung *et al.* uses a "wine-shaped" channel design to capture human pluripotent stem cell-derived photoreceptor cell bodies and orient basal axon extensions to create uniform scaffolds for treating disorders that affect the outer retina.[168] Additionally, in all these applications and more, the higher viability and transfection efficiencies offered by microfluidic technology may be revolutionary in how we perceive treatment options for patients. A bulk of the microfluidic-related studies for these diseases are primarily for detection; this is surprising given the plethora of devices discussed in this review that have the potential to aid in developing new therapeutics for these diseases.

Additionally, some of these same techniques have been utilized in tandem with a single microfluidic device to produce novel avenues within adoptive cell therapy. One example of these combinatorial designs is used to promote the fusing of multiple cells' plasma membranes into one hybrid cell. A study by Pendharkar *et al.* implements an insulator-based dielectrophoretic (iDEP) lab chip to trap both colon carcinoma cells, CT26, and bone marrow-derived dendritic cells (BMDCs) within microwells with DEP as each of the cell types flow through the device. Once both cell types are in close proximity to each other, a DC electroporation square pulse bonds the cells physically. Using this approach, the device achieved 70% fusion efficiency with a 60% cell viability from this fused population. These electrofused cells can be implemented as a dendritic cell-tumor fusion vaccine method for allowing consistent antigen presentation to the

dendritic cells from the cancer to stimulate a more aggressive adaptive immune response.[169]

4 Discussion

4.1 Integration of microfluidics in cellular engineering for cell therapy

Though an innovative solution to treating disease, cell manufacturing for cell-based therapy applications also has limitations that must be resolved prior to widespread adoption. Currently, cell manufacturing is burdened by several limitations which result from protracted delays when completing each of the cumbersome steps for cell engineering, the requirement for highly-trained personnel to conduct each process, and inaccessible costs associated with expensive equipment and required personnel. Cell engineering for cell therapy also has its clinical limitations upon transplantation into the patient, thoroughly discussed elsewhere.[170] For cell therapies to truly be effective and safe in the clinic, improvements to each characteristic step (*i.e.*, isolation, transfection, and expansion) of cell manufacturing can lend a hand towards scale-up, cost reduction, and time optimization. Microfluidics offers an innovative approach to the challenges faced by conventional methods by significantly reducing footprint and cost.

One of the key advantages of microfluidics is the ability to control the microenvironment near the cell of interest. By decreasing the length scales of a benchtop system to a lab-on-a-chip platform, the total volume of expensive reagents is significantly reduced to tens or hundreds of microliters rather than milliliters. The cargo is maintained closer to the cell for transfection, potentially improving the efficiency.[146] Further, the physics of chip-based platforms are more predictable as a result of operating at a low Reynolds number characteristic of laminar flow. Thus, intentional mixing or discrete separations of populations can be achieved in carefully designed microchannels.[171,172] Additionally, miniaturized systems may offer gentler handling, higher viability, and preserved biological functions *via* the application of forces relevant to the magnitude of the size of the cell.[173]

In addition to where microfluidics may comparably or outperform conventional methods, microfluidics technologies provide opportunities to refine the process in ways that might not be easily performed otherwise. Clinical approval and regulation of cell therapies is a whole hurdle in and of itself for innovative therapeutic approaches. To be used as therapeutic agents, manufactured cells must meet rigorous specifications to achieve regulatory clearance. The challenges associated with regulatory approval are often the result of out-of-specification (OOS) cells (with incidence rates up to 28% for CAR-T cells[174]) that do not meet these standards which pertain primarily to viability, abundance, and contamination.[175] This challenge may be addressed with the implementation of microfluidic techniques that offer enhanced control of the manufacturing process of cells prior to reimplantation into the patient, particularly those that prioritize viability and enrichment of desired cells.[176] Without necessary quality control screenings, the negative side effects cannot be properly predicted or mitigated and the safety and efficacy of such treatments cannot be evaluated accurately. Currently, the fields of cellular engineering and gene therapy lack a consistent method for quality control (QC) throughout the entire workflow. QC is a process that should be performed after each step of the cell manufacturing process (*e.g.*, isolation, transfection/transduction, expansion). Means for label-free techniques to perform QC at different points in this process may improve steps such as isolation of the most desirable T cells or stem cells, detecting and enriching for activated T-cells, and evaluating transfection efficiency are both necessary and made possible through the use of microfluidics.[177] By utilizing microfluidic systems to establish QC systems, the cell manufacturing process will be more repeatable, less costly, and potentially more efficacious if the most potent cells can be screened and isolated prior to expansion and reimplantation. Label-free microfluidic techniques such as mechanical deformation (*i.e.*, cell-squeezing) and dielectrophoresis offer sensitive and continuous methods to achieve this goal but have yet to be entirely explored.[178] For example, Ashby *et al.* achieved 100% purity of potent T cells for the purposes of T cell receptor therapy by implementing shear stress forces to determine cell avidity in a high-throughput microfluidic device.[176] Kiryo *et al.* used dielectrophoresis to separate embryonic stem cells based on pluripotency with 90% efficacy.[179] In industry, companies like Berkeley Lights and their recent acquiree, Isoplexis, have implemented optofluidics in their commercially available instrument to visualize and recover T cell phenotypes at the single-cell level for cellular engineering applications. By incorporating microfluidics throughout the cell-manufacturing process for cell-therapy applications, the regulatory challenges may subside or at the very least become better defined. Further, the overall performance of these treatments may significantly improve allowing these therapies to achieve the true innovation for which they are after.

Another avenue that positions the field of microfluidics well to enhance the field of cellular engineering for therapeutics is the ability to evaluate new therapeutics and toxicants for their potency and understand their mechanisms prior to *in vivo* investigation. Organ-on-a-chip (OOC) devices are microfluidic devices designed to mimic *in vivo* organ structures and relative disease states for testing therapeutic agents, stimulants, and toxicants without the cost or collateral of *in vivo* experiments for early-stage research. These on-chip 3D tissue structures cultivate a tailored biomimetic microenvironment and offer physiologically relevant responses to drug exposures for almost any organ in the body.[111,112,180] OOC devices enable an in-depth study of disease or malignant tissues by decoupling the multiplex of factors that may be contributing to the formation of the disease *in vivo*.[181] Several studies have utilized OOC platforms to study tumor responses to adjuvant therapies such as anti-PD1[182] and more recently, others have advanced OOC platforms to explore their contribution to immunotherapies. Ma *et al.* developed an immunocompetent microfluidic device to study the effectiveness of anti-CD19 CAR-T cells for treating leukemia. The

study yielded the elimination of B-cell acute lymphoid leukemia, induced inflammation, and relevant clinical responses. This work also sheds light on the potential failure modes of anti-CD19 CAR-T cells for treating leukemia, made possible by studying the therapy on-chip.[183,184] Others have utilized microfluidic-based devices to model immunological responses to CAR-T[185–187] and NK cell[188,189] therapies which provide information not readily available when explored *in vivo*. In regards to regenerative medicine, Achberger *et al.* developed a retina-on-a-chip (ROC) using iPSCs to examine the efficacy of various AAVs for ocular disease treatment. The ROC incorporates several retinal organoids enclosed in their respective reservoir that provides access to perfused media. With the benefit of the ROC recapitulating retinal tissue microstructure, select AAVs were screened to develop an improved second generation of AAVs and were found with prolonged application to produce stable expression of GFP. These results inform potential AAV delivery kinetics, such as time and dosage required to attain desired effects.[190] In addition, a joint-on-a-chip model, composed of hyaluronic acid and osteoarthritic synovial fluid, induces a diseased state model for implanted patient-specific chondrocytes and fibroblasts. This osteoarthritic OOC allows for studying personalized cellular responses leading to inflammation and cartilage deterioration.[191] OOC devices are positioned as a logical link between *in vitro* fundamental research to translation in the clinic by lending itself to valuable insight into disease formation, treatment, and mechanism. OOCs can bring edifying insight for developing and optimizing these therapies that might be instrumental in improving *in vivo* experimentation and clinical implementation. OOC devices also offer the ability to investigate a cell-based therapy's efficacy on individuals. Replicating or implementing *ex vivo* patient tissue into an OOC device, exposing it to therapeutics, and observing the response may yield translational insight for precision medicine applications.[192]

Revisiting the perhaps daunting task of using a micro-scale device for cell expansion towards 10^8 cells, microfluidics may have a significant contribution to the expansion step of cellular engineering. The most common solution to "scale-up" microfluidics to meet such a need is to recommend several devices operating in parallel in order to maintain the highly-tailored microenvironment engineered in the device while achieving adequate throughput. In addition to this solution, the authors posit that some of the greatest strengths can be gleaned from microfluidic technologies, not always by replacing conventional bulk methodologies and techniques, but by working in tandem with the tried-and-true methods to improve the process overall. Because high cell quantity and quality directly impact the success of the treatment, the ability to collect lower sample volumes from the patient, be highly selective throughout the cell manufacturing process, and expand the most desirable cells even if there exists a low quantity after transfection could revolutionize the process. Microfluidic devices make reliable single-cell expansion and architectural tissue scaffolds possible.[193–195] Introducing microfluidic technologies at any point in the cell manufacturing process may yield higher quality results due to their gentle, cell-scaled nature which is a resolution not achievable using conventional methods.[12]

While many cell manufacturing processes are conventionally done in bulk, these processes require highly skilled personnel to perform each step, which can be difficult to maintain a qualified staff. Development of automated processes that achieve comparable or higher yields is possible with microfluidics technologies. Microfluidic systems have been incorporated into automated infrastructures for high-throughput diagnostics and screening, making the extension of this technology feasible for cell manufacturing.[196,197] Further, microfluidic technologies can often be formed in parallel to increase throughput, reduce the number of expensive reagents and overall required volume, and require low power sources ultimately decreasing the cost of cell manufacturing. As areas such as automation and throughput continue to develop, microfluidics certainly provides opportunity the cell manufacturing process to become reliable, repeatable, cheaper, and more efficacious.

4.2 Challenges of microfluidic adoption in the cellular engineering workflow

Though microfluidics may excel in many areas relevant to cell manufacturing, these miniature approaches have limitations to consider. Microfluidics research produces significant and alluring results that are hopeful to revolutionize healthcare. Unfortunately, though this might be the aim, several efforts remain in the "proof-of-concept" era and optimization is seldom performed.[59,198] Further, the small feature size in devices with complex geometries (*e.g.*, high-aspect ratios, multilayer alignment, *etc.*) are extremely difficult to scale up using commonplace manufacturing methods such as injection molding, forcing scalable technologies to forego geometry-induced manipulations. Without being able to scale up lab-on-chip devices to meet the demand of the healthcare sector, microfluidics is unlikely to be a viable solution in the clinic.[199]

Another challenge inhibiting the full adoption of microfluidics is the upstream and downstream processes that require large volumes or minimum cell concentrations to be operational and accurate (*e.g.*, FACS, bulk transfection, expansion in large well plates, *etc.*). It is perhaps beneficial that new studies highlighting the performance and advantages of microfluidic devices also discuss the context in which the device is intended to exist.[200] For example, though microfluidic mechanoporation might result in high transfection efficiencies compared to bulk methods, it is important to discuss if the inlet cell concentration and volume are relevant to what might outflow from sorting *via* FACS. Likewise, the volume and cell concentration post-transfection in a microfluidic device should be discussed relevant to the expansion process to follow. Of course, the ideal scenario is to develop a multiplexed platform with fully integrated microfluidic devices for each step to eliminate this discrepancy and overcome such limitations.

5 Conclusion

Cell therapy is an extensive subject of interest in both the academic and industrial sectors. Since its onset, the field has been explored for applications to previously insurmountable diseases. By modifying the genome as a means to reprogram the cell to encourage the health of the host rather than cause detriment is a significant advancement for precision medicine. The challenges associated with cell manufacturing for therapeutic applications are in need of new solutions to decrease the cost, time, and labor required. Microfluidic technologies have been studied and validated to perform comparably or better than conventional methods throughout the entirety of the reprogramming process. Microfluidic techniques, both passive and active, can isolate populations of interest with unique specificity, increase transfection efficiency, and increase viability while preserving native function as the samples are prepared for reimplantation. Here, we have reviewed the recent past of innovative microfluidic technologies that address challenges associated with conventional tactics. Microfluidics, though limited in some ways, offers a pathway to improve current cell-manufacturing efforts that will increase efficiency and viability while reducing cost, footprint, and time.

Author contributions

JLD, JPA, RVD contributed to conceptualization. JLD and JPA contributed to synthesizing literature, writing, and editing of the manuscript.

Conflicts of interest

R. V. D. holds patents in electroporation and dielectrophoresis. There are no other conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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