**Significance of the work**

The ongoing work is crucial for the advancement of antiviral drug development against a backdrop where rapid responses to emerging viral threats are increasingly necessary. By perfecting iPSC-derived models for high-throughput screening, we're paving the way for the discovery and optimization of antiviral compounds with greater efficiency and cost-effectiveness. This work directly addresses the urgent need for innovative approaches to antiviral therapy, particularly in the face of pandemics.

**Significant project-generated resources**

1. Enhanced iPSC-derived cell models optimized for antiviral drug screening.
2. Streamlined pneumocyte differentiation protocols that reduce cost and time.
3. RNA-seq and single-cell RNA-seq data sets characterizing cellular responses post-viral infection.
4. Adapted protocols for medium-throughput antiviral assays in 96-well formats.

**A. Specific Aims for the MP/DRP**

The Specific Aims have not been modified from the original, competing application.

**B. Studies and Results**

A critical objective of aim 1 was to optimize a cost-effective and medium-throughput antiviral assay using induced pluripotent stem cell (iPSC)-derived cells. A collaborative effort between the Zwaka and White laboratories has culminated in the development of a functional immunofluorescence (IF)-based antiviral assay BFIGUREtargeting SARS-CoV-2 in iPSC-derived type II pneumocytes. The assay employs staining for the viral antigen N at 488nM to enumerate infected cells, complemented by a DAPI counterstain for total cell quantification. Initial experiments were conducted using human pluripotent stem cells cultured in mTeSR. To induce alveolar differentiation, cells at 70-80% confluency were collected and seeded at high density on Vitronectin-coated tissue culture plates in mTeSR. The differentiation medium was IMDM supplemented with FBS, all-trans-retinoic acid, FGF-10, EGF, Wnt3a, KGF, and BMP-4. Viral infections were performed on day nine post-differentiation induction, with subsequent analysis two days post-infection. During pilot studies, attachment issues were observed when cells were transferred to the BSL-3 facility. To address this, various substrates including laminin, gelatin, fibronectin, and standard plastic were tested. The most efficient substrate was found to be standard 1% gelatin with a seeding density of 50,000 cells per well, which enhanced survival and plating efficiency.

Further optimization included evaluating the necessity of specific cytokines for infectibility. It was determined that Wnt3a, despite being the most costly component, was dispensable for infectibility. This finding presents a cost-saving opportunity in the differentiation protocol. This result allowed for a reduction in the protocol duration by four days, resulting in cost savings and increased robustness of the assay. Additionally, the optimal number of cells required for seeding and the precise timing for cell transfer to the White lab were determined, minimizing the risks of contamination and cell detachment. The differentiation and culture protocols were successfully adapted for high-throughput screening in a 96-well format. This adaptation facilitates medium-throughput antiviral assays, allowing for more efficient screening of potential antiviral compounds (see Figure 1 and 2).

A diagram of a plate with arrows

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**Figure1 Overview of the Optimized Differentiation Protocol for Type II Pneumocytes.** This panel illustrates the refined step-by-step procedure for the differentiation of iPSCs into type II pneumocytes. Starting with the iPSC culture, the process details the timeline and conditions for each differentiation stage, including the introduction of specific growth factors and the transition from a pluripotent state to a specialized pneumocyte phenotype. Key optimizations such as substrate variations (laminin, gelatin, fibronectin) and cytokine reduction steps are highlighted to emphasize improvements in efficiency and cost-effectiveness.

**A graph of infection with numbers and a chart

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**Figure 2: Efficacy of Rupintrivir Against EV-D68 Infection in iPSC-Derived Pneumocytes**

A. Infection and Cell Viability Assessment: The top graph presents the results from an infection assay of iPSC-derived pneumocytes with Enterovirus D68 (EV-D68), plotted against the multiplicity of infection (MOI). The blue line indicates the percentage of infected cells, while the red line represents the count of cell nuclei (DAPI count), serving as an indirect measure of cell viability. The data suggest an increase in infection rate with higher MOI, with a subsequent reduction in DAPI count, indicating cytotoxic effects associated with viral infection.

B. Antiviral Activity of Rupintrivir: The bottom graph displays the antiviral potency of Rupintrivir, an inhibitor of picornavirus 3C protease, against EV-D68 in the iPSC-derived pneumocytes. The percentage of infection and cell viability are plotted against the logarithmic concentration of Rupintrivir. The IC50 and IC90 values, indicating the concentration required to inhibit 50% and 90% of viral infection respectively, are shown in the inset. Both IC50 and IC90 values are marked as being less than 0.022 µM and 0.022 nM, respectively, demonstrating the high efficacy of Rupintrivir at very low concentrations.

Building upon our extensive experience in cell preservation, as documented instance in our paper by Leitner and Zwaka, we have made significant strides in the batch freezing of primary iPSC-derived cells. This expertise has been successfully translated to the preservation of iPSC-derived type II pneumocytes. The ability to freeze these cells is an important advancement, as it greatly enhances the scalability and flexibility of our antiviral assays. The implementation of batch freezing protocols for pneumocytes is advantageous for several reasons. Firstly, it allows for the creation of a cell bank, ensuring a consistent supply of cells that are readily available for experimentation. This is useful for large-scale drug screening efforts, where the demand for a reliable and uniform cell population is high. Moreover, batch freezing facilitates the simultaneous testing of multiple drug candidates. This is because cells can be thawed and cultured in parallel, allowing for high-throughput screening of antiviral compounds. This approach not only saves time but resources, as it minimizes the need for continuous cell culturing, which can be labor-intensive and costly. The protocol we have applied to the lung cells has been optimized to preserve cell integrity and function upon thawing. This ensures that the post-thaw cells retain their differentiation status and functionality, which is essential for their use in antiviral assays. (Figure 3).

A graph with lines and a red line

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**Figure 3: Post-Thaw Recovery and Growth Kinetics of iPSC-Derived Pneumocytes**

This graph illustrates the growth kinetics of iPSC-derived pneumocytes after thawing from cryopreservation compared to a non-frozen control over a period of three days. The black line represents the control group of continuously cultured cells, while the red line indicates the cells that were frozen and subsequently thawed before plating. The Y-axis measures a parameter indicative of cell survival and proliferation (e.g., cell count or confluency), and the X-axis denotes the days after plating. Error bars represent the standard deviation of the measurements.

Both lines show similar trends in growth, indicating that the process of cryopreservation and thawing does not adversely affect the survival and proliferative capacity of the cells. On day 2, both control and frozen cells demonstrate a peak in the parameter measured, with the frozen cells exhibiting a comparable trend to the control cells. This suggests that the cryopreservation protocol is effective in preserving cell viability and does not impede the subsequent growth of iPSC-derived pneumocytes.

**Expansion of Cell Line Repertoire to TZ14**

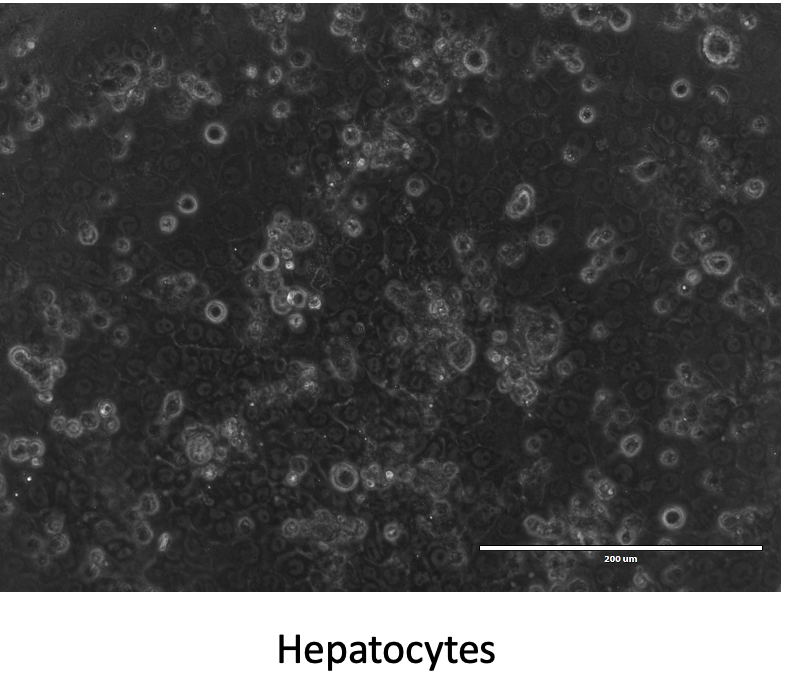
We have expanded our cell line repertoire to include TZ14, a line we have previously published on. Through differentiation studies, we have established that TZ14 cells can reproduce the differentiation phenotype and infectibility characteristic of pneumocytes. This expansion is useful as it allows us to leverage the genetic heterogeneity inherent in different cell lines to enhance the robustness and applicability of our research findings. The heterogeneous cell lines allow for the assessment of drug efficacy across a spectrum of genetic backgrounds, which is crucial and utilizing a variety of cell lines can help validate results, ensuring that findings are not specific to a single cell line The differentiation studies conducted on TZ14 have shown that this cell line can mimic the behavior of primary pneumocytes, both in terms of differentiation and susceptibility to infection.

**Hepatocyte differentiation and infection**

The differentiation of induced pluripotent stem cells (iPSCs) into hepatocytes is a multi-step process that mimics liver development in vivo. This process involves the sequential induction of iPSCs into definitive endoderm (DE), hepatic endoderm (HE), and finally mature hepatocyte-like cells (HLCs). We have adopted and optimized a protocol for the differentiation of iPSCs into hepatocytes. The first step in the differentiation process is the induction of iPSCs into DE cells. This is achieved by culturing iPSCs in a medium supplemented with factors that promote endoderm formation. We established a protocol that involves the use of Activin A and CHIR99021, a GSK3β inhibitor, which synergistically induce the formation of DE cells. The basal medium consists of RPMI-1640 supplemented with B27, penicillin, and streptomycin. The DE medium was further supplemented with 100 ng/ml Activin A and 3 µM CHIR99021. The iPSCs are treated with this medium for approximately four days, during which they underwent morphological changes and begin to express DE markers.

Following the establishment of DE cells, the next step was to direct these cells towards a hepatic fate. This was achieved by treating the DE cells with a medium that promotes the formation of hepatic endoderm. The HE medium contained basal medium supplemented with growth factors such as basic fibroblast growth factor (bFGF) and bone morphogenic protein 4 (BMP4), along with 0.5% DMSO to enhance differentiation. The concentration of bFGF and BMP4 was 5 ng/ml and 20 ng/ml, respectively. The cells were cultured in HE medium for five days, during which they begin to express markers indicative of hepatic lineage.

The final stage of differentiation involves the maturation of HE cells into functional hepatocyte-like cells. This is achieved by culturing the cells in a medium that supports hepatocyte maturation. The mature hepatocyte (MH) medium was composed of hepatocyte basal medium supplemented with the growth factors hepatocyte growth factor (HGF), oncostatin M, and dexamethasone, along with 0.5% DMSO. The concentrations of HGF and oncostatin M optimized at 20 ng/ml, and dexamethasone was used at 100 nM. The cells were treated with MH medium for 10-12 days, after which they exhibited characteristics of mature hepatocytes, including the ability to store glycogen, uptake and release indocyanine green (ICG), and secrete albumin. Throughout the differentiation process, we monitored the expression of stage-specific markers using techniques such as real-time RT-PCR, immunofluorescence staining, and flow cytometry. For example, the expression of DE markers such as CXCR4 and SOX17 is assessed at the end of the DE induction stage, while hepatic markers such as ALB and AFP are evaluated during the HE and MH stages. The differentiation of iPSCs into hepatocytes is a complex process that requires careful manipulation of the cellular microenvironment to mimic developmental cues. The protocol developed by us has been optimized to produce hepatocyte-like cells with functional characteristics similar to primary hepatocytes (Figure 4).



**Figure 4: Morphological Characteristics of iPSC-Derived Hepatocytes**

This micrograph of a dense culture of iPSC-derived hepatocytes, displaying key morphological characteristics indicative of mature liver cells. The hepatocytes exhibit a polygonal shape with distinct nuclei and prominent nucleoli, resembling the in vivo architecture of liver tissue. The cytoplasm appears granular, which may indicate the presence of organelles such as mitochondria and endoplasmic reticulum, essential for the liver functions, including metabolism and detoxification processes. Binucleation, a common feature of primary hepatocytes, can also be observed in some cells, further confirming the mature state of the iPSC-derived hepatocytes.

**Differentiation of iPSCs into Macrophages and infection**

The differentiation of iPSCs into macrophages is a process that recapitulates aspects of hematopoiesis and myeloid cell lineage commitment. Our laboratory has successfully developed a protocol to generate functional macrophages from iPSCs. We began the differentiation process by inducing iPSCs to form embryoid bodies (EBs), which are three-dimensional aggregates that can give rise to various cell types. To do this, we cultured iPSCs in a medium that supports EB formation, consisting of DMEM/F12 supplemented with 20% fetal bovine serum (FBS), non-essential amino acids, L-glutamine, and β-mercaptoethanol. After several days, we observed the formation of EBs, indicating the initiation of differentiation.

Following EB formation, we induced the differentiation of these structures into hematopoietic progenitor cells. We transferred the EBs into a medium designed to promote hematopoiesis, which included cytokines such as stem cell factor (SCF), interleukin-3 (IL-3), and interleukin-6 (IL-6). The presence of these cytokines is crucial as they mimic the hematopoietic niche of the bone marrow and drive the differentiation towards the myeloid lineage. After approximately 14 days of culture, we harvested the cells and analyzed them for the expression of hematopoietic markers such as CD34 and CD43, confirming the presence of myeloid progenitors.

To further differentiate the myeloid progenitors into macrophages, we cultured the cells in a medium containing macrophage colony-stimulating factor (M-CSF), which is essential for macrophage differentiation and survival. The medium was composed of RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, and 50 ng/ml M-CSF. We maintained the cells in this medium for an additional 10 to 14 days, during which they acquired morphological characteristics of macrophages, such as an irregular shape and the presence of pseudopodia.

Upon completion of the differentiation protocol, we characterized the iPSC-derived macrophages by assessing the expression of macrophage-specific markers, including CD14, CD68, and CD80, using flow cytometry and immunocytochemistry. We evaluated the functional capabilities of the derived macrophages, such as phagocytosis, cytokine production, and the ability to mount an immune response upon stimulation with lipopolysaccharide (LPS). The differentiation of iPSCs into macrophages was a methodical process that involved the careful orchestration of developmental cues to guide iPSCs through stages of hematopoietic and myeloid lineage commitment.

Following the differentiation of iPSCs into macrophages, our laboratory embarked on a series of experiments to investigate the infectivity SARS-CoV-2 and Enterovirus (EV) in these iPSC-derived immune cells. The study aimed to assess the susceptibility of the macrophages to viral infection at various multiplicities of infection (MOIs) and to measure the resultant cell death. We initiated the infection experiments by exposing the iPSC-derived macrophages to SARS-CoV-2 and EV at different MOIs, ranging from low to high, to evaluate the dose-dependent effects of viral infection. The chosen MOIs were designed to reflect a spectrum of viral loads that macrophages might encounter during an infection. After inoculating the macrophages with the viruses, we incubated the cultures under optimal conditions to allow for viral entry and replication.

To assess the infectivity of SARS-CoV-2 and EV in the iPSC-derived macrophages, we measured the viral RNA levels in the cells at various time points post-infection using quantitative reverse transcription PCR (qRT-PCR). This technique enabled us to quantify the replication kinetics of the viruses within the macrophages, providing a clear picture of viral infectivity. Simultaneously, we evaluated cell death resulting from viral infection through several assays, including DAPI to measure cell viability and to detect apoptosis and necrosis. These assays allowed us to determine the cytotoxic effects of SARS-CoV-2 and EV on the macrophages at different MOIs.

Our experiments revealed that iPSC-derived macrophages are susceptible to infection by both SARS-CoV-2 and EV across the range of MOIs tested. We observed a dose-dependent increase in viral RNA levels within the macrophages, indicating successful viral replication. Notably, the extent of cell death increased with higher MOIs, reflecting the cytotoxic impact of the viral infections.

Despite the observed cytotoxicity, the iPSC-derived macrophages demonstrated a remarkable capacity to support viral replication, underscoring their utility as a model system for studying viral infections. The ability to modulate the MOI allowed us to mimic various stages of infection, from mild to severe, providing a comprehensive understanding of how macrophages respond to these viruses. Our work not only confirms the feasibility of using iPSC-derived macrophages as a model for viral infection but highlights the importance of MOI in studying the dynamics of viral infectivity and immune response.

A close-up of a microscope

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**Figure 5: Morphology of iPSC-Derived Macrophages**

Displayed is a field of iPSC-derived macrophages, characterized by their distinct morphology. These cells exhibit an irregular shape with a ruffled membrane, a hallmark of macrophages that facilitates their role in phagocytosis. The heterogeneity in cell size and shape reflects the diverse states of activation and function within the population. Several cells show the formation of pseudopodia, the protrusions of the cell membrane which are critical for cell movement and the engulfment of pathogens. The granular appearance within the cytoplasm suggests the presence of phagosomes and lysosomes, essential for the degradation of ingested material. Notably, the cells maintain a consistent morphology indicative of a well-differentiated and functional macrophage phenotype.

**Differentiation of iPSCs into Neurons**

Our laboratory has meticulously executed a protocol for neuron differentiation, adhering to a series of well-defined stages that mimic neural development in vivo. This section provides a detailed account of the steps we developed to generate functional neurons from iPSCs.

The protocol from iPSCs to neurons begins with the initiation of neural induction, a process aimed at directing the pluripotent cells towards a neural lineage. We achieved this by culturing iPSCs in a neural induction medium (NIM), which consists of DMEM/F12 supplemented with N2 supplement, non-essential amino acids, L-glutamine, and a dual SMAD inhibition cocktail of SB431542 and LDN193189. These inhibitors block the TGF-β and BMP signaling pathways, respectively, which are essential for neural induction. The iPSC cultures were maintained in NIM for approximately 7 to 10 days, during which they formed neural rosettes, a hallmark of early neural differentiation. Following neural induction, we manually selected and expanded the neural rosettes to enrich the culture with neural progenitor cells (NPCs). The NPCs were then dissociated and plated onto poly-ornithine and laminin-coated dishes to promote adhesion and expansion. During this phase, we maintained the cells in a medium designed to support NPC growth, consisting of DMEM/F12 supplemented with N2, B27 without vitamin A, FGF2, and EGF. This expansion phase lasted for approximately 2 weeks, allowing for the proliferation of NPCs while maintaining their undifferentiated state.

To initiate the differentiation of NPCs into mature neurons, we switched the culture medium to one that supports neuronal differentiation. This medium includes Neurobasal medium supplemented with B27 with vitamin A, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and ascorbic acid. The addition of these factors promotes the maturation of NPCs into various neuronal subtypes, including excitatory and inhibitory neurons. We cultured the cells in this differentiation medium for an additional 3 to 4 weeks, during which the NPCs underwent morphological changes characteristic of maturing neurons, such as the extension of neurites and the formation of synaptic connections. Upon completion of the differentiation protocol, we characterized the iPSC-derived neurons by assessing the expression of neuronal markers using immunocytochemistry and quantitative PCR. Markers such as MAP2, NeuN, and synapsin were used to confirm neuronal identity and maturity. Additionally, we performed functional assays, including calcium imaging and patch-clamp recordings, to evaluate the electrophysiological properties of the neurons, confirming their functionality.

We observed reasonable levels of infection in iPSC-derived neurons when exposed to various viruses. These infection levels coincided with a reduction in DAPI count, indicative of cell death, which is a common outcome of viral infection. The correlation between infection efficiency and cell viability is an expected phenomenon, as viral replication leads to cellular damage and apoptosis. While we have successfully processed data for several viruses, we are in the process of analyzing the results for additional viral pathogens.

The infection of neurons with low efficiency aligns with what is expected for this cell type. Neurons are post-mitotic cells, meaning they do not divide, which can limit the spread of certain viruses that rely on cell division to propagate. Despite the lower infection rates, the ability to infect iPSC-derived neurons is significant. It provides a model to study neurotropic viruses and their interactions with the nervous system, which is a critical aspect of the AViDD program's objectives (Figure 5).

A close-up of a grey surface

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**Figure 6: Morphological Presentation of iPSC-Derived Neurons**

Captured in this image is a culture of iPSC-derived neurons, characterized by their complex morphology. These cells display long, intricate neurites that form networks essential for neuronal communication. The neuronal somas (cell bodies) are of varying sizes, and the extensions (axons and dendrites) can be seen reaching out to connect with neighboring cells, indicative of synapse formation. The contrast in the image highlights the typical clear, rounded cell bodies from which multiple neurites emerge. This complex neuritic architecture is characteristic of healthy, mature neurons and suggests functional neural networks

**Antiviral Efficacy and Resistance Core Viral infection of iPSC Models**

The AER core has also been tasked with the development of high-throughput primary cell models to meet the needs of all ASAP programs. The AER core now has coronavirus, enterovirus, and flavivirus programs entering our live-virus antiviral assays, and primary cell models will be needed for comprehensive potency analysis of each program as leads approach proof-of-concept animal studies. Furthermore, the AER core has a AViDD Developmental Award in collaboration with Thomas Zwaka from Mt Sinai to develop multiple primary-like iPSC models, representing the many important cell types infected by these target viral families, in 96-well format for medium-throughput antiviral screening (See the Zwaka Developmental Award Report for more details on the generation of these models). The Zwaka laboratory regularly produces iPSC-derived pneumocytes for SARS-CoV-2 antiviral screening in the AER core, which has been well-established over years of collaboration. Over the first year of the developmental award, the Zwaka laboratory has produced iPSC-derived hepatocytes, macrophages, and neurons in 96-well format for the establishment of IF-based antiviral assays. The AER core has established IF-based assays for coronavirus **(Fig. 7A)** and enterovirus **(Fig. 7B)** in these 3 iPSC cell types. We generally detected infection of hepatocytes and macrophages ranging from 9-24% infection, which are appropriate for robust medium-throughput assays. EV-D68 was able to infect the iPSC-derived neurons at up to 4%, which is on the borderline of robustness for an effective medium-throughput antiviral assay. SARS-CoV-2 did not show detectable infection of the neurons, which is in line with the 0.1% infection observed in the literature. We are now expanding these observations to flaviviruses to ensure we have assays prepared for all ASAP programs. The AER has also established EV-D68 infection of iPSC-derived pneumocytes and validated our rupintrivir control in this assay **(Fig. 7C)**.A collage of graphs and charts

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**Summary**

Our investigations into the infectivity of various viruses in iPSC-derived cell types, including neurons, hepatocytes, and macrophages, have yielded valuable data. We observed infection levels that were consistent with expectations, given the nature of the cell types and the viruses involved. Notably, the infection of iPSC-derived neurons occurred with lower efficiency, which is typical due to their post-mitotic state, yet it remains a significant finding for understanding virus-neuron interactions. The correlation between viral infection and cell death, as evidenced by reduced DAPI counts, further corroborates the typical cellular response to viral replication. The results of these studies contribute to the broader objectives of the AViDD program, providing a proof of principle for the use of iPSC-derived cells as models for viral infection. As we continue to process data for additional viruses, these models stand to offer further insights into viral pathogenesis and host responses.

**Challenges and Innovations**:

We faced challenges in ensuring model fidelity and assay scalability. These were addressed by validating our models against primary tissues and conducting rigorous optimization and standardization for high-throughput formats.

**C. Significance**

The significance of our work in differentiating induced pluripotent stem cells (iPSCs) into various cell types and subsequently infecting them with a range of viruses extends beyond the immediate findings of infectivity and cell death. This research provides a versatile and dynamic platform for modeling human diseases, particularly those caused by viral infections. By utilizing iPSC-derived cells, we are able to replicate the complex interactions between viruses and their host cells in a controlled environment. This approach not only enhances our understanding of viral mechanisms of infection and pathogenesis but also offers a valuable tool for the development of antiviral therapies. The ability to study virus-cell interactions across different cell types, including neurons, hepatocytes, and macrophages, is particularly crucial for diseases that affect multiple organ systems or have neurotropic characteristics.

Furthermore, the application of iPSC technology in virology research represents a significant advancement in the field of regenerative medicine and personalized therapy. The generation of patient-specific iPSCs and their differentiation into relevant cell types can facilitate the study of individual responses to viral infections, paving the way for personalized treatment strategies. Additionally, the insights gained from these studies contribute to the broader scientific understanding of how viruses can influence cell differentiation and development, potentially revealing novel targets for therapeutic intervention. As we continue to explore the interactions between viruses and iPSC-derived cells, the implications of this research are poised to impact not only the field of infectious diseases but also the development of regenerative therapies and personalized medicine.

**D. Plans**

In the forthcoming year, our research into viral infection studies and iPSC technology is set to embark on exciting new paths. We aim to delve deeper into the realm of viral pathogenesis and host interactions. Our plan includes the expansion of viral infection studies to assess infectivity and viral replication across a wider array of iPSC-derived cell lines. This endeavor will not only broaden our understanding of virus-cell dynamics but also illuminate the diverse ways in which different viruses interact with various cell types. By casting a wider net over the spectrum of viruses, we anticipate uncovering novel insights into viral mechanisms and identifying potential vulnerabilities that could be targeted by future therapies.

In parallel, we will dedicate efforts to characterizing new iPSC lines, analyzing their expression patterns and assessing their capabilities to support viral replication. This will involve a deep dive into the genetic and epigenetic landscapes of these cells, unraveling the complexities that govern their susceptibility to infection. By understanding the nuances of each cell line, we can tailor our approaches to studying specific viruses, thereby enhancing the precision and relevance of our models. Furthermore, we plan to revolutionize our experimental workflows by optimizing protocols for high-throughput screening.

By refining our techniques for both 96- and 384-well formats, we aim to establish a robust platform capable of evaluating a vast array of antiviral compounds efficiently. This high-throughput approach will not only accelerate the pace of discovery but also enable us to screen for antiviral drugs on an unprecedented scale, opening new avenues for therapeutic intervention.