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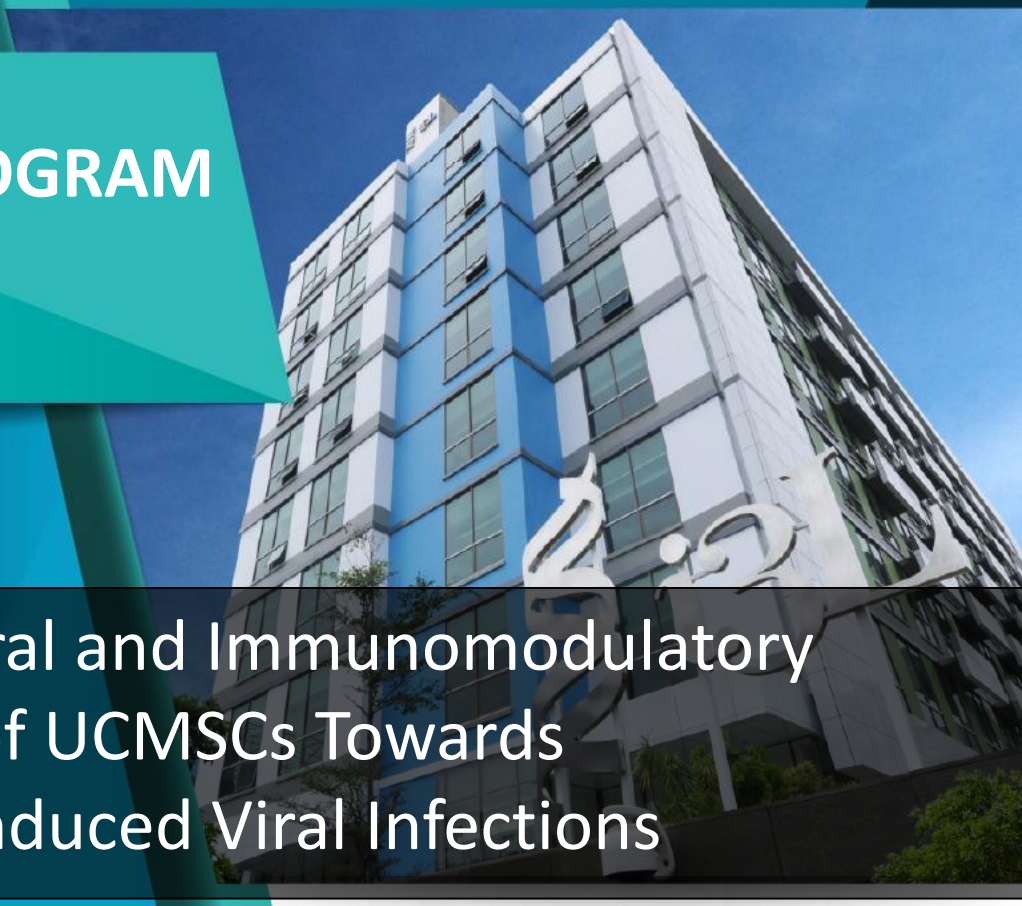

The Antiviral and Immunomodulatory Potential of UCMSCs Towards Poly(I:C)-Induced Viral Infections

STUDY PROGRAM
Biomedicine

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INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES (i3L)
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RESEARCH REPORT

The Antiviral and Immunomodulatory Potential of UCMSCs Towards Poly(I:C)-Induced Viral Infections

By

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Submitted to

i3L – Indonesia International Institute for Life Sciences

School of Life Sciences

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Biomedicine

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Jakarta, Indonesia

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Approval Page

I hereby submit the final draft of EP Report as a requirement to participate in EP Final Presentation.

EP Title : The Antiviral and Immunomodulatory Potential of UCMSCs Towards Poly(I:C)-Induced Viral Infections

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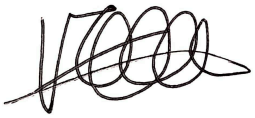

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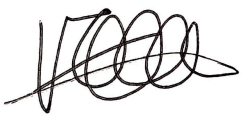

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ABSTRACT

Mesenchymal stem cells (MSCs) derived from the umbilical cord (UCMSC), bone marrow (BMMSC), and adipose tissue (ADMSC) show significant potential as regenerative medicine. Nevertheless, viral infections that can cause prolonged inflammatory responses often compromise their therapeutic effectiveness. To address this challenge, an experimental model can use the effect of polyinosinic-polycytidylic acid (poly I:C), a synthetic analog of double-stranded RNA, often used in research as a powerful mimic of viral infection, to stimulate immune responses. Furthermore, bioinformatics tools such as Metascape and Ingenuity Pathway Analysis (IPA) were used and validate that UCMSCs activate antiviral pathways, such as ISGylation and type I interferon signaling, more effectively than bone marrow-derived MSCs. Subsequently, in vitro MSC propagation through analysis of peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) provides insights into the immunomodulatory functions of MSCs in response to Poly(I:C). Following by UCMSCs' effects on immune regulation were assessed using flow cytometry to analyze reactive oxygen species (ROS) production, neutrophil apoptosis, and T-cell expression. The findings reveal that UCMSCs aid in the inflammation resolution and increase regulatory T cells (Tregs) critical for immune balance. These results suggest that UCMSCs can mitigate excessive immune responses while promoting viral clearance. Thus, this study highlights the therapeutic potential of UCMSCs in addressing viral infections and their complications. Further research is needed to validate these findings in vivo and develop clinical applications.

Keywords: *Mesenchymal stem cells, poly I:C, IFN-I, TLR3.*

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Praise and gratitude I express to the presence of God Almighty, who has bestowed His grace and gifts during the writing of this paper. Because without His mercy, this paper would not be finished. The report title I propose is "The Antiviral and Immunomodulatory Potential of UCMSCs Towards Poly(I:C)-Induced Viral Infections" This report fulfills the BM41932: Research Internship course as the part of the enrichment program and implements the knowledge of theoretical biomedicine, writing, and technical skills. It is undeniable that completing this paper took a lot of effort and patience.

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LIST OF ABBREVIATIONS

Abbreviation	Full Term
ADMSC	Adipose-Derived Mesenchymal Stem Cell
BMMSC	Bone Marrow Mesenchymal Stem Cell
CFSE	Carboxyfluorescein Succinimidyl Ester
DCFDA	2',7'-Dichlorodihydrofluorescein Diacetate
FITC	Fluorescein Isothiocyanate
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
IPA	Ingenuity Pathway Analysis
NaCl	Sodium Chloride
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
Poly (I:C)	Polyinosinic-polycytidylic Acid
PMN	Polymorphonuclear Neutrophils
UCMSC	Umbilical Cord Mesenchymal Stem Cell
Tregs	T Regulatory cells

I. INTRODUCTION

1.1 Background Information

Mesenchymal stem cells (MSCs) derived from various sources, such as the umbilical cord (UCMSCs), bone marrow (BMMSCs), and adipose tissue (ADMSCs), and have been explored for their immunomodulatory properties, making them potential therapeutic agents for various inflammatory and immune-mediated diseases (Műzes & Sipos, 2022). Among these, UCMSCs have emerged as particularly promising due to their superior immunomodulatory potential compared to BMMSCs. This has led to increased use in clinical trials, positioning UCMSCs as the second most utilized MSC type after BMMSCs, which have been in clinical use since 2003 (Yen et al., 2020). Furthermore, the umbilical cord offers a convenient and non-invasive source of mesenchymal stem cells (MSCs), unlike bone marrow extraction, which is both painful and carries risks for the donor. Being derived from neonatal tissues, UC-MSCs are biologically younger. They may have stronger regenerative potential than bone marrow-derived MSCs (BM-MSCs) from adult tissues, which are more likely to accumulate genetic mutations over time. Additionally, UC-MSCs exhibit lower immunogenicity than BM-MSCs, which reduces the risk of immune rejection in allogeneic recipients (Wang et al., 2021). Thus, this makes them more suitable for off-the-shelf therapies, such as to treat viral infections.

Furthermore, viruses pose significant threats to global health due to their capacity to trigger severe immune responses (de Reuver & Maelfait, 2024). For instance, double dsRNA is synthesized as an intermediate in the viral life cycle of Sars-CoV-2 and many other viruses, which activates the Toll-like receptor 3 (TLR3), a key component of the innate immune response (Manik & Singh, 2021). However, in contrast to the pathogenic effects of viral dsRNA, polyinosinic-polycytidylic acid, or poly (I:C), serves as a synthetic analog that mimics viral dsRNA and is used in research to activate TLR3 in a controlled manner (Fujiwara et al., 2024). Moreover, the immune response to viral infections involves several key components, such as TLR3, TLR8, and TLR9, which detect different pathogen-associated molecular patterns and initiate immune defenses (Fernandes-Santos & Azeredo, 2022). The interaction between these immune cells and TLR3 activation is essential to the body's defense against viral infections. This research, using flow cytometry to measure the expression and activation of T cells marked by CD3, CD4, and CD8, aims to shed light on how mesenchymal stem cells (MSCs) influence the immune response towards poly (I:C) and explore potential therapeutic strategies for managing viral infections like cytokine storm during Sars-CoV-2 infection.

1.2 Aim

This research aims to evaluate the immunomodulatory and antiviral potential of umbilical cord mesenchymal stem cells in the viral infection defense system through the utilization of poly(I:C) as a model to simulate viral infection.

1.3 Hypothesis

H0 = The addition of UCMSC does not show both antiviral capabilities and a significant effect on immune response under Poly(I:C) stimulation

H1 = The addition of UCMSC will show antiviral capabilities and regulate both neutrophil expression and T cell function under Poly(I:C) stimulation

II. LITERATURE REVIEW

2.1 Mesenchymal stem cell

Mesenchymal stem cells (MSCs) are multipotent stem cells with the ability to differentiate into various cell types and play a critical role in regenerative medicine and immunomodulation. According to Robert et al. (2020), they can differentiate into mesodermal lineage cells, such as osteoblasts (bone cells), adipocytes (fat cells), and chondrocytes (cartilage cells). MSC can maintain the capacity for self-renewal through replication and preservation of their undifferentiated state over multiple passages, typically exhibiting fibroblast-like morphology. MSCs express surface markers such as CD73, CD90, and CD105 while lacking hematopoietic markers like CD34 and CD45 (Shi et al., 2023). These versatile cells can be derived from various tissues, including bone marrow (BMMSCs), which, while possessing high differentiation potential, require invasive extraction; adipose tissue (ADMSCs), an abundant and easily accessible source though potentially variable in immunomodulatory potency; and umbilical cord (UCMSCs), a non-invasive source characterized by a high proliferation rate and strong immunosuppressive properties. Biologically, MSCs contribute significantly to tissue regeneration by differentiating into cell types essential for repairing and regenerating damaged tissues (Sagaradze et al., 2020).

2.1.1 Bone Marrow MSC

Bone marrow-derived mesenchymal stem cells (BMMSCs) are among the most studied and clinically utilized MSCs, renowned for their robust differentiation potential and potent immunomodulatory properties (Huang et al., 2022). Typically harvested from the bone marrow, a rich yet limited reservoir of MSCs, these cells are obtained through a bone marrow aspiration procedure, often from the iliac crest under anesthesia or sedation. However, this process is invasive, associated with patient discomfort, and carries risks such as pain, infection, and bleeding. Additionally, the yield and quality of BMMSCs decline with donor age, making bone marrow a less practical source for older individuals (Česnik & Švajger, 2024). These limitations have driven the exploration of alternative MSC sources, such as adipose tissue and umbilical cord, which offer less invasive collection methods, higher proliferation rates, and potentially enhanced immunosuppressive capabilities. Despite these drawbacks, BMMSCs remain a cornerstone of regenerative medicine and cell-based therapies, valued for their well-characterized biological properties and extensive history of clinical application (Mudasir Bashir Gugjoo, 2022).

2.1.2 Adipose Tissue MSC

Adipose-derived mesenchymal stem cells (ADMSCs) are isolated from adipose tissue, a highly abundant and easily accessible source obtained through minimally invasive procedures like liposuction. This makes ADMSCs a convenient and relatively low-risk option for stem cell therapy, with fewer complications and lower patient discomfort compared to other sources such as bone marrow (Czerwiec et al., 2023). ADMSCs can differentiate into a variety of cell types, including adipocytes, osteocytes, and chondrocytes, and they exhibit immunomodulatory properties. However, according to Huang et al. (2023), despite their abundance and ease of extraction, ADMSCs generally display lower functionality than other mesenchymal stem cells, particularly umbilical cord-derived mesenchymal stem cells (UCMSCs). Notably, ADMSCs tend to have a lower proliferation rate and reduced differentiation potential, which can limit their effectiveness in therapeutic contexts. Additionally, ADMSCs are more prone to senescence, leading to decreased functionality over time, and their characteristics can vary significantly among individuals due to factors like age and metabolic conditions (Weng et al., 2022). Thus, making UCMSCs a more favorable choice in certain clinical applications, particularly for immunotherapy and regenerative medicine.

2.1.3 Umbilical Cord MSC

Umbilical cord mesenchymal stem cells (UCMSCs) are multipotent stem cells derived from the umbilical cord, particularly from Wharton's jelly and perivascular areas (Walker et al., 2020). They are recognized for their high self-renewal capacity, multi-directional differentiation potential, and low immunogenicity. UCMSCs exhibit significant immunomodulatory capabilities, influencing both innate and adaptive immune systems through mechanisms such as cytokine secretion, which include IL-6, IL-10, and TGF-β (Li et al., 2022). UCMSC also plays a role in direct cell-to-cell interactions that promote regulatory T-cell or Tregs differentiation. This unique ability to modulate immune responses without triggering significant allogeneic reactions makes UCMSCs attractive for therapeutic use in conditions characterized by excessive inflammation or autoimmunity (Dabrowska et al., 2021). Additionally, their capacity to secrete various bioactive molecules plays a crucial role in controlling inflammation and enhancing antiviral immunity. Consequently, UCMSCs are not only valuable in tissue regeneration but also represent a promising candidate for antiviral treatments, especially in cases of chronic viral infections or viral-induced immune dysregulation.

2.2 Viral Infection

Viral infections occur when viruses invade a host organism, replicate within its cells, and cause disease, as they require a living host for reproduction due to their lack of cellular machinery (Lozach, 2020). These infections can lead to various health issues, ranging from mild symptoms like the common cold to severe diseases that may require hospitalization or even result in death. Therefore, when a virus infects the body, it triggers an immune response aimed at eliminating the pathogen. However, in severe cases, this response can escalate into a cytokine storm, which is a phenomenon characterized by an overproduction of cytokines that leads to excessive inflammation (Murdaca et al., 2021). This inflammation can damage tissues and organs, resulting in severe complications such as acute respiratory distress syndrome (ARDS), organ failure, or even death (Polidoro et al., 2020). Thus, understanding viral infections and their potential to induce cytokine storms is crucial for managing their impact on health and society.

2.2.1 dsRNA in viral life cycle

Double-stranded RNA (dsRNA) is integral to the replication cycle of many viruses (Chen & Hur, 2021). It serves either as the viral genome in dsRNA viruses, which comprise approximately 5-6% of all known viruses, or as an intermediate in RNA viruses, which account for around 60-70% of all viruses. In dsRNA viruses, the genome consists of two complementary RNA strands released into the host cell during infection, facilitating direct replication and transcription to produce viral mRNAs (Liu et al., 2023). Conversely, RNA viruses, including positive-sense ssRNA viruses, contribute to about 40–45% of all viruses. In addition to negative-sense ssRNA viruses of around 10-12% that generate dsRNA during genome replication. The presence of dsRNA is recognized by nearly all organisms as a signal of viral infection, activating immune responses through pattern recognition receptors (Chen & Hur, 2021). This recognition triggers both innate immune responses, such as the interferon response, and adaptive responses involving T-cells that target infected cells. Therefore, dsRNA plays a dual role in that they are essential for viral replication across a diverse range of viruses and serves as a critical trigger for antiviral immune defenses.

2.2.2 Poly (I:C)

Polyinosinic-polycytidylic acid, or poly(I:C), is a synthetic analog of double-stranded RNA (dsRNA) that mimics the molecular patterns associated with viral infections, making it an effective agonist for toll-like receptor 3 or TLR3 (Jain et al., 2022). TLR3, located in endosomes and on the cell surface, recognizes poly(I:C) through its leucine-rich repeat (LRR) domain. This binding triggers TLR3 dimerization, leading to the recruitment of the adapter protein TRIF (TIR-domain-containing

adapter-inducing interferon-β) and the activation of downstream signaling pathways. These include the IRF3 pathway, which drives the production of type I interferons like IFN-β, and the NF-κB pathway, which induces pro-inflammatory cytokines such as TNF-α, IL-6, and IL-12 (Ramasamy & Subbian, 2021). The resulting cytokine production enhances antiviral defenses by upregulating interferon-stimulated genes (ISGs) and activating immune cells, closely mimicking the innate immune response to viral dsRNA. As a synthetic molecule, poly(I:C) offers precise control over TLR3 activation levels, enabling consistent and reproducible studies of TLR3-mediated immune responses. It is widely used in immunological research to simulate viral infection in a safe and controlled manner, serving as a valuable tool to investigate pathways involved in antiviral defense and immunomodulation without the risks associated with live viruses or natural dsRNA (McGarry et al., 2021).

2.3 Immune Response

The immune response to viral infections relies heavily on the interplay between TLR3, Type I interferons, neutrophils, and T cells. Furthermore, TLR3, a key pattern recognition receptor, detects double-stranded RNA (dsRNA) generated during viral replication, triggering a signaling cascade through the TRIF pathway that leads to the production of Type I interferons including IFN-α and IFN-β (Chen et al., 2021). These interferons establish an antiviral state by inhibiting viral replication and enhancing antigen presentation. Type I interferons also recruit neutrophils, which, though traditionally associated with bacterial infections, play an essential antiviral role by phagocytosing infected cells (Stegelmeier et al., 2021). Meanwhile, T cells are crucial for adaptive immunity, with CD8+ cytotoxic T cells recognizing and killing virus-infected cells, and CD4+ helper T cells coordinating the broader immune response by secreting cytokines that enhance both T cell activity and antibody production (Swain et al., 2012). This coordinated response ensures effective viral clearance, with TLR3 initiating the cascade, Type I interferons amplifying the signal, neutrophils providing rapid containment, and T cells delivering specificity and long-term immunity. However, dysregulation of these components can lead to excessive inflammation, underscoring the importance of balance in the immune response.

2.3.1 Innate Immune response

The innate immune response is the first line of defense against viral infections, acting swiftly and nonspecifically to limit viral replication and spread. Neutrophils, a key component of this response, are recruited to infection sites by signaling molecules such as IFNs. Upon activation, neutrophils execute various effector functions, including phagocytosis of viral particles or infected cells (Johansson & Kirsebom, 2021). Additionally, neutrophils produce reactive oxygen species (ROS)

via the NADPH oxidase complex, generating molecules like superoxide, hydrogen peroxide, and hypochlorous acid, which damage viral particles from infected cells and promote inflammatory cytokine production. However, the rapid apoptotic rate of neutrophils is further accelerated by ROS-induced oxidative stress and proapoptotic signals which is critical to preventing prolonged inflammation and tissue damage (Chen & Hur, 2021). In contrast, some viruses, such as influenza and SARS-CoV-2, delay neutrophil apoptosis, exacerbating inflammation and tissue injury (Flerlage et al., 2021). Therefore, maintaining a balance in neutrophil activation, ROS production, and apoptosis is vital, as insufficient ROS leads to ineffective viral clearance, while excessive ROS or delayed apoptosis causes tissue damage, as observed in severe infections like ARDS and COVID-19.

2.3.2 Adaptive Immune response

The adaptive immune response to viral infections is complex and involves various immune cells, with T cells playing a central role. T cell activation begins when T cells recognize viral antigens presented by MHC molecules on antigen-presenting cells (APCs), such as dendritic cells and macrophages (Fasano et al., 2022). CD8+ cytotoxic T cells (CTLs) recognize antigens in MHC class I, while CD4+ T helper cells (Th cells) recognize those in MHC class II. Activation requires co-stimulatory signals, such as CD28 on T cells interacting with CD80/CD86 on APCs, which prevent anergy or apoptosis. T regulatory cells (Tregs), a subset of CD4+ T cells, are crucial for maintaining immune balance and preventing autoimmunity by suppressing excessive immune responses (Grover et al., 2021). They suppress immune responses through several mechanisms, including the secretion of immunosuppressive cytokines, such as TGF-β and IL-10. During viral infections, Th1 cells promote viral clearance by activating cytotoxic T cells and enhancing macrophage responses, while Tregs help modulate the immune response to prevent excessive inflammation and tissue damage. The interplay between T helper cells and Tregs is critical for clearing viruses without causing excessive immune damage (Fisicaro et al., 2020).

III. MATERIALS & METHODS

3.1 Metascape Analysis

The GSE file was downloaded and sorted using filters for FC⩾2, 1.5, and 1.2. Then, a .csv file containing only the gene symbols was uploaded to Metascape for analysis. Human sapiens was selected as the species, followed by initiating a custom analysis. For pathway analysis, GO pathways were exclusively selected for GO analysis, while KEGG pathways were chosen when analyzing KEGG. Enrichment analysis was performed, and upon completion, the "Analysis Report Page" was accessed to download the "All-in-One Zip file." Within the Enrichment_GO folder, the _FINAL_GO file was opened, and relevant pathway descriptions were identified based on their suitability to the research topic. To generate a graphical representation, the SR Plot website was accessed. The horizontal bar plot option was selected, and data, including the GO number, Logp values, and color codes, were prepared in Excel. The label number option was set to "No," and the figure was saved before being further analysed.

3.2 Ingenuity Pathways Analysis

The dataset to be processed was copied and accessed using a remote desktop connection to log in to the main computer for Ingenuity Pathways Analysis (IPA). The dataset was pasted and processed within the IPA platform. The appropriate pathway was selected, and the dataset was overlaid onto it. Fold change data were displayed, and the resulting figure was saved for further analysis or presentation.

3.3 MSC Seeding

Non-consumable materials include a T75 flask, trypsin, PBS, cDMEM, trypan blue, a hemocytometer, 5 and 10 mL serological pipettes, P1000 and P10 micropipettes with tips, a 15 mL falcon tube, and a 96-well plate. The old media was removed using a suction system, and 7 mL of PBS was added to wash the cells before being discarded. Next, 1 mL of trypsin was added to the flask. The flask was gently tapped to detach the cells, followed by the addition of 10 mL of cDMEM to deactivate trypsin activity. The cell suspension was transferred to a 15-mL falcon tube and centrifuged at 1500 rpm at 25°C for 5 minutes. The remaining media was aspirated, and the cell pellet was broken by gently flicking the tube. The pellet was resuspended in 1 mL of PBS, and 10 µL of the cell suspension was mixed with 10 µL of trypan blue in a 96-well plate. 10 µL was aspirated onto a hemocytometer for cell counting. The total cell number was calculated. After determining the cell number, a master mix was prepared by dividing the total cells by the required number of cells and adjusting the suspension volume to 1 mL for seeding 5000 cells into the well plate.

3.4 Treatment using Poly(I:C)

The non-consumable materials used included one vial of Poly (I:C) LMW 25 mg, sterile water, a 1.5 mL microcentrifuge tube, P10 and P1000 micropipette, and tips. A stock solution of Poly (I:C) was prepared according to protocol instructions by adding 1.25 mL of endotoxin-free physiological water to the 25 mg vial, resulting in a stock concentration of 20 mg/mL. This stock solution was then diluted to prepare a working solution of 10 µg/mL. To achieve the desired concentration in each well, where 100 µL of media was already present after seeding, 100 µL of the 20 µg/mL solution was added, creating a final concentration of 10 µg/mL per well. Finally, 100 µL of the working solution was transferred, ensuring that each well contained 200 µL of media with 10 µg/mL of Poly (I:C).

3.5 PMN and PBMC Isolation

The non-consumable materials used included Ficoll-Paque™ PLUS, whole blood from a healthy donor, 50 mL and 15 mL centrifuge tubes, and PBS. Between 50 to 80 mL of blood was drawn, depending on the experiment, and PBS was added until the volume reached the 50 mL tick mark. For density gradient separation, 20 mL of Ficoll was added to a 50 mL tube, and 5 mL was added to a 15 mL tube. Blood was then gently layered onto the Ficoll in a 1:1 ratio, with 20 mL of blood added to the 50 mL tube and 5 mL added to the 15 mL tube. The tubes were centrifuged at 2000 rpm for 20 minutes with the brake set to zero. For PBMC isolation, the plasma (yellow layer) was discarded, and the second, whitish layer containing PBMCs was carefully collected and transferred to a new 50 mL centrifuge tube. For PMN isolation, the Ficoll layer (translucent layer) was discarded, and the PMNs were directly placed on ice to minimize apoptosis, with all further steps performed using the same tube.

3.6 PBMC and PMN Seeding

For PBMC seeding, the materials utilized included PBS, CFSE, a microcentrifuge tube, RPMI, and beads. Isolated PBMCs were transferred to a new 50-mL tube, and PBS was added to the 50-mL mark. The cells were centrifuged at 1500 rpm for 10 minutes, and the supernatant was removed. The cells were washed three times with 50 mL of PBS, centrifuging at 1500 rpm for 10 minutes each time. After removing the supernatant, the cells were resuspended in 10 mL of PBS for counting. A cell suspension containing 2×10^7 cells/mL of PBS was prepared in a 1.5 mL microcentrifuge tube, and CFSE was added to achieve a final concentration of 10 µM. The tube was incubated at 37℃ for 10 minutes. Following incubation, 1 mL of RPMI was added, and the cells were centrifuged at 1500 rpm for 3 minutes. The supernatant was removed, and the PBMCs stained with CFSE were seeded into a well plate with 100 µL of RPMI. Anti-CD3/CD28 beads were added in 50 µL of RPMI to activate T cells, bringing the total volume in each well to 150 µL.

Furthermore, for PMN seeding, the materials utilized included 0.2% NaCl, 1.6% NaCl, and RPMI. A 50-mL tube containing PMNs and RBCs was added with 0.2% NaCl up to the 25th tick mark. The tube was gently inverted for 30 seconds before adding 1.6% NaCl to the 40th tick mark. The mixture was centrifuged at 1200 rpm for 10 minutes at 4℃, and the supernatant was removed. Next, 0.2% NaCl was added to the 20th tick mark, and the tube was inverted for 30 seconds. 1.6% NaCl was then added to the 40th tick mark, and the solution was centrifuged again under the same conditions. The washing step using 0.2% NaCl was repeated twice more to ensure thorough purification. The cells were resuspended in 10 mL of PBS for counting. Finally, after removing the supernatant from the well plate, 100 µL of RPMI containing the prepared PMNs was added to each well.

3.7 Neutrophil and Surface Staining

For neutrophil staining, the materials utilized included a 96-well plate, a 96 V-shaped well plate, Annexin V, DCFDA, a multichannel pipette, and P100 tips. After 16 hours of co-culture with PMNs, 1 µL of either DCFDA or Annexin V was added to each well of the 96-well plate, and the plate was incubated in the dark for 30 minutes. The cells were then resuspended and transferred to a V-shaped well plate for both the Annexin V and DCFDA groups. The plate was centrifuged at 1200 rpm for 2 minutes, and the supernatant was removed. The cells were washed with 200 µL of staining buffer and centrifuged again. Stains were prepared as follows: for Annexin V-FITC, CD11b-PE, and DCFDA was prepared. For single stains, 50 µL was added per well. The plate was incubated for 20 minutes in the dark, covered with aluminum foil. Following incubation, the cells were washed three times with 200 µL of staining buffer and then fixed using a fixative buffer to complete the process.

For surface staining, the materials utilized included a 96-well plate, a 96 V-shaped well plate, antibodies, CFSE, a multichannel pipette, and P100 tips. After 5 days of incubation, the cells were transferred from the 96-well plate to a V-shaped plate and centrifuged at 1200 rpm for 2 minutes. The cells were washed with 200 µL of staining buffer, centrifuged again at 1200 rpm for 2 minutes, and the supernatant was removed. Antibodies were added for CD4-BV421, CD8-PerCP, CD25-APC, and CFSE-FITC and prepared with 50 µL per well. Additionally, 50 µL of staining buffer was added to the NS group. The cells were incubated for 20 minutes in the dark, and covered with aluminum foil. After incubation, the cells were centrifuged at 1200 rpm for 2 minutes, and the supernatant was removed. The cells were washed three times with 200 µL of staining buffer, centrifuging after each wash. Following the washes, the cells were fixed with 50 µL/well of Fluoro Fix™ Buffer, and pipetted evenly. The cells were centrifuged again at 1200 rpm for 2 minutes, and the cells were resuspended in 100-150 µL of staining buffer. Finally, the resuspended cells were transferred to a 1.2 mL library tube in an adaptor, sealed with parafilm, and stored at 4 °C until flow cytometry analysis.

3.8 Data analysis

Data analysis involved running flow cytometry to collect data, followed by gating for single cells and the respective desired cell populations using the FlowJo 10.8.1. application. The resulting data were processed and visualized using GraphPad Prism 10.2.3. Graphs were plotted, and statistical analysis was performed using one-way ANOVA. A p-value was considered significant if it was less than 0.05 ($P < 0.05$).

IV. RESULTS AND DISCUSSION

4.1 UCMSC as a candidate for treating viral infection

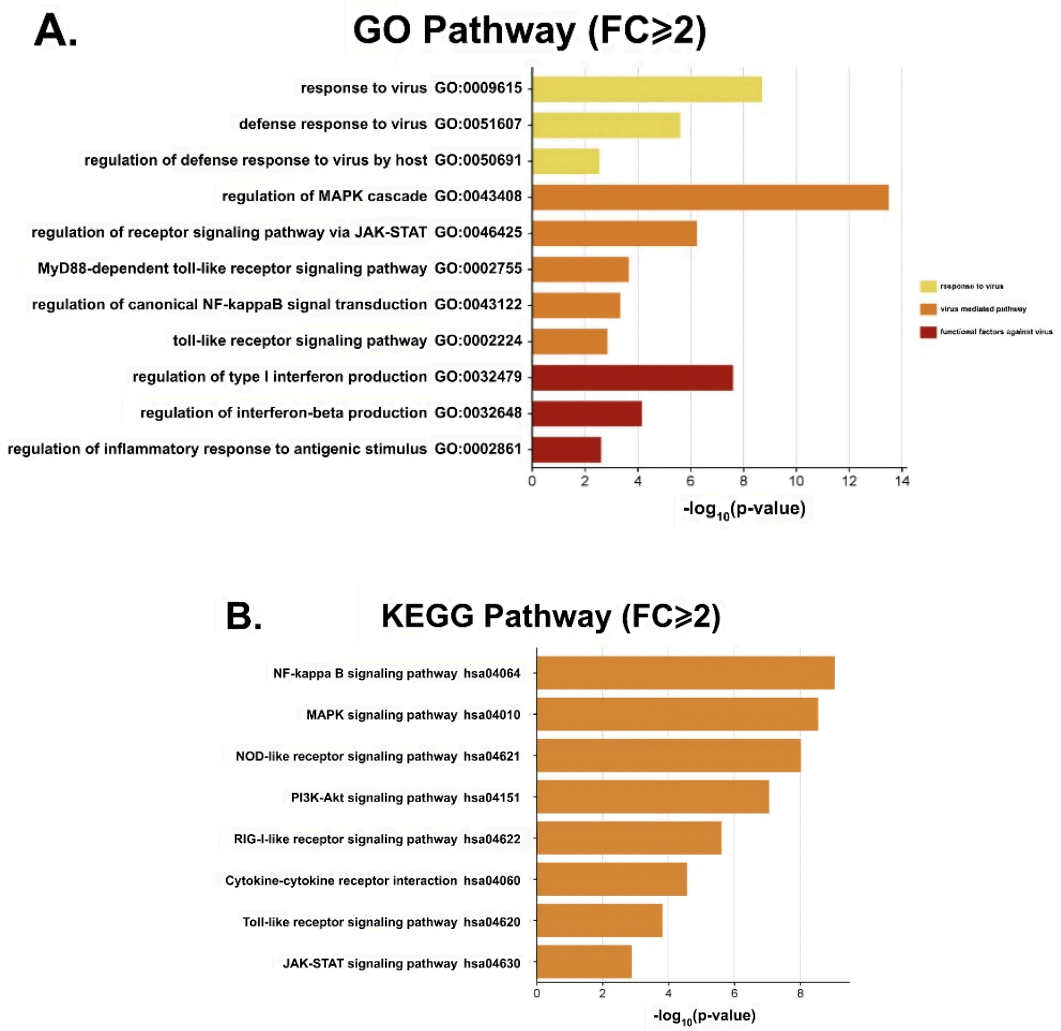

Figure 1. Metascape result from GSE77685 comparing from UCMSC and BMMSC with the fold change Greater than or equal to 2. A)GO pathway. B)KEGG pathway

Figure 1 displays analysis through Metascape, a powerful tool for functional enrichment analysis, integrating data from various biological databases, including KEGG and GO, to provide insights into the biological functions of specific gene sets, by analyzing fold-change data, such as from UCMSC versus BMMSC comparisons. These pathways shed light on the immune mechanisms activated in response to viral infections, offering insights into the differential immunomodulatory properties of UCMSCs and BMMSCs, especially in Figure 1A. The GO analysis pathway represents a coordinated host immune response to viral infections. The pathways in the figure can be grouped into three key categories that reflect distinct roles in antiviral defense. The yellow pathways indicate

the response to the virus, including the defense response to the virus, and represent the broad activation of the UCMSC to help the immune system upon detecting viral components, such as RNA, triggering innate immune responses to combat infections (Dabrowska et al., 2021). The orange pathways represent virus-mediated pathways, such as regulation of MAPK cascade, toll-like receptor signaling pathway, and canonical NF-kappaB signal transduction, which highlight critical signaling mechanisms that amplify immune responses, drive inflammation, and regulate cell survival during viral infections (Battagello et al., 2020). Lastly, the Red pathways display functional factors against viruses, including regulation of type I interferon production, interferon beta production, and regulation of inflammatory responses, which emphasize the production of interferons and inflammatory cytokines, which are essential for inhibiting viral replication and recruiting immune cells to clear infections (Jung et al., 2023). Together, these pathways illustrate that UCMSCs demonstrate superior regulation of key immune pathways compared to BMSCs, particularly in the context of antiviral defense.

Furthermore, from Figure 1B, KEGG pathways identified in the analysis collectively highlight critical mechanisms involved in viral defense. The NF-kappa B signaling pathway regulates immune responses and inflammation, producing pro-inflammatory cytokines and interferons essential for viral clearance (Hariharan et al., 2020). Similarly, the MAPK signaling pathway modulates cellular stress responses and survival. The NOD-like receptor (NLR) signaling pathway activates inflammasomes and triggers IL-1β and IL-18 production, enhancing immune responses against viral pathogens. The RIG-I-like receptor signaling pathway detects viral double-stranded RNA (dsRNA) and induces type I interferons (IFN-α, IFN-β), establishing an antiviral state (Batool et al., 2021). Cytokine-cytokine receptor interactions are crucial for immune signaling, particularly through type I interferons. The Toll-like receptor (TLR) signaling pathway further detects viral RNA and DNA through receptors like TLR3, TLR7/8, and TLR9, initiating cytokine production and interferon signaling, which viruses sometimes subvert to avoid immune activation. Lastly, the JAK-STAT signaling pathway transduces interferon signals to upregulate interferon-stimulated genes (ISGs) that inhibit viral replication (Ezeonwumelu et al., 2021). Together, these pathways underline key immune and inflammatory responses to viral infections, with their differential enrichment between UCMSC and BMMSC suggesting distinct immunomodulatory properties that may influence their effectiveness in viral defense. Therefore, the differential enrichment of pathways between UCMSCs and BMMSCs underscores the unique immunomodulatory potential of UCMSCs. Thus, by activating critical immune processes, UCMSCs not only recognize viral components but also initiate a coordinated response to suppress viral replication and restore immune homeostasis.

4.2 UCMSC as viral inhibitor through ISGylation pathway

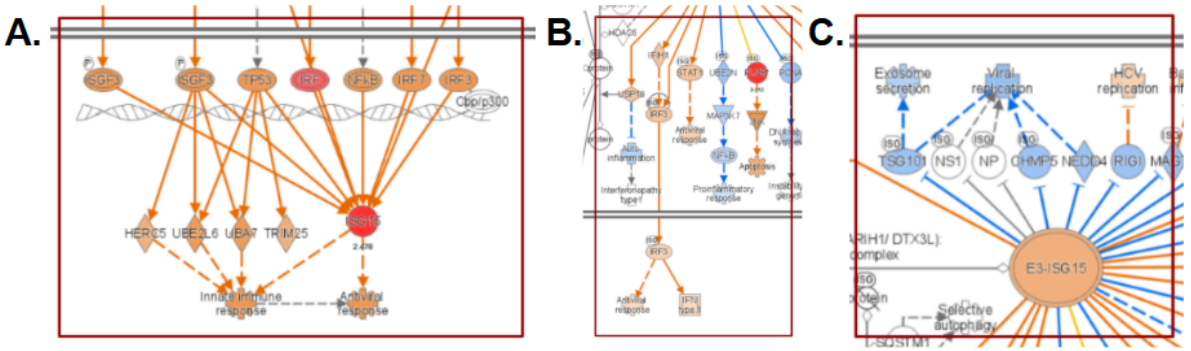

Figure 2. IPA analysis of ISGylation signaling pathway that overlaid from the GSE48022 dataset comparing UCMSC and BMMSC with the fold change bigger than 2.

Ingenuity Pathway Analysis (IPA) is a web-based bioinformatics application designed to analyze and interpret high-throughput biological data by mapping experimental data, such as gene expression changes, onto known signaling pathways and networks. This helps identify key molecules, including transcription factors or cytokines, that drive observed changes in cellular processes. The IPA results for the ISGylation signaling pathway indicate that umbilical cord-derived mesenchymal stem cells (UCMSCs) are more effective than other cell sources in inhibiting viral replication and autoinflammatory responses. The analysis reveals that UCMSCs significantly modulate genes involved in ISGylation, a process critical for antiviral defense, leading to reduced viral replication (Zhang et al., 2021). Additionally, UCMSCs are predicted to activate Type I Interferon (IFN) signaling, an essential pathway in the host's antiviral response, which enhances the expression of interferon-stimulated genes (ISGs) (Lukhele et al., 2019). This activation of Type I IFN strengthens the immune system's antiviral mechanisms, promoting a more effective and rapid clearance of viral infections (Fritsch & Weichhart, 2016). Together, these findings suggest that UCMSCs not only suppress viral replication but also potentiate the host's immune response, underscoring their potential therapeutic advantage in managing viral infections and autoinflammatory conditions.

4.3 UCMSC enhances controlled oxidative reaction

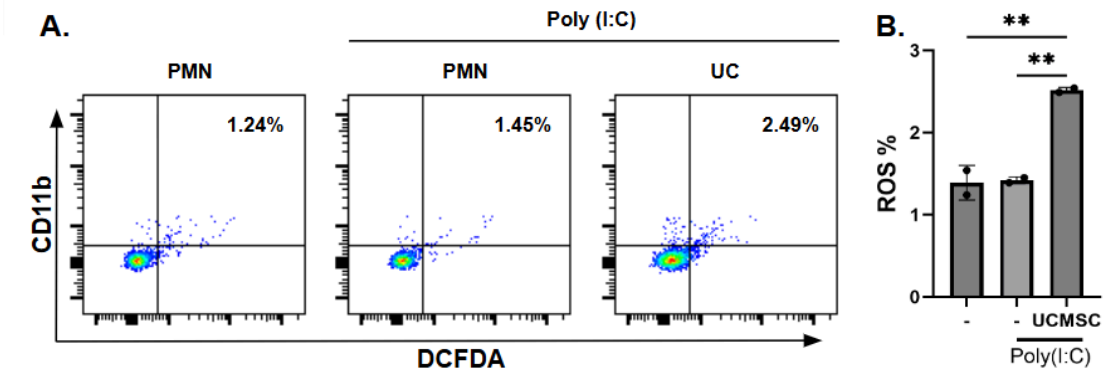

Figure 3. DCFDA staining that detects the ROS production of neutrophils. A) Flow cytometry result. B) Percentage of ROS production between PMN, PMN, and UCMSC after stimulation using Poly(I:C).

Figure 3 demonstrates the role of UCMSCs in modulating reactive oxygen species (ROS) production by neutrophils (PMNs) following stimulation with Poly(I:C), as detected by DCFDA staining using flow cytometry. The ROS level measurement uses the H_2DCFDA probe, a non-fluorescent, cell-permeable reagent that is deacetylated by cellular esterases into non-fluorescent H_2DCF. This compound is then oxidized by ROS into highly fluorescent 2',7'-dichlorofluorescein (DCF). Figure 3A shows that unstimulated PMNs exhibit a baseline ROS production of 1.24%, which slightly increases to 1.45% after Poly(I:C) stimulation. Co-culture with UCMSCs, however, significantly enhances ROS production to 2.49%, as quantitatively shown in Figure 3B, where the ROS increase is statistically significant compared to Poly(I:C)-stimulated PMNs alone. The result suggests that UCMSCs enhance controlled oxidative reactions, crucial for pathogen clearance and signaling pathways (Wu et al., 2024). ROS, while essential for pathogen defense, can cause oxidative damage to cellular components, disrupting normal cellular functions and contributing to tissue injury and chronic inflammation. As such, this result also aligns with a previous study that UCMSC can regulate ROS production in a controlled manner in order to avoid excessive oxidative damage (Lin et al., 2023). However, the experiment faced a limitation in the number of isolated PMNs, which could impact the robustness and reproducibility of the findings. As such, insufficient PMNs may result in variability in ROS production and hinder statistical significance in smaller sample sizes. Nonetheless, ROS production is often associated with the apoptotic rate, as ROS can act as intracellular mediators, activating apoptosis pathways through mitochondrial dysfunction and caspase activation (Brillo et al., 2021). Therefore, by promoting controlled ROS production, UCMSCs help balance oxidative stress, enabling effective pathogen clearance and inflammation regulation. This highlights the therapeutic potential of UCMSCs in modulating immune responses.

4.4. UCMCS increased the rate of neutrophil apoptosis

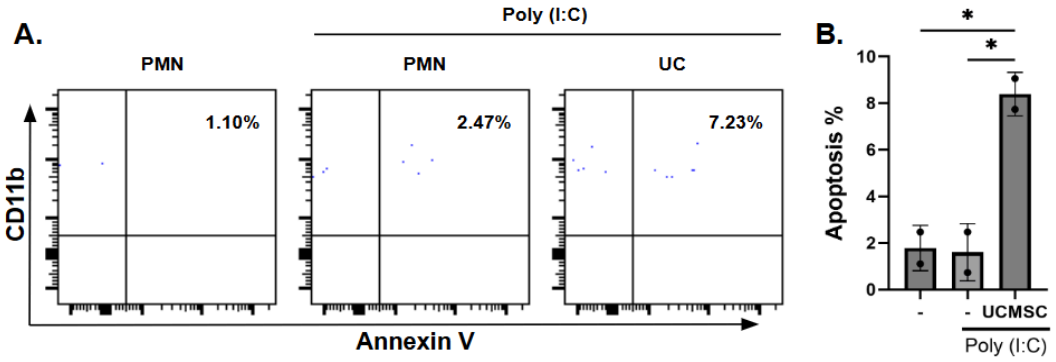

Figure 4. Annexin V staining that detects the apoptosis rate of neutrophils. A) Flow cytometry result. B) Percentage of apoptosis rate between PMN, PMN, and UCMSC after stimulation using Poly(I:C).

This figure 4 illustrates the role of UCMSCs in modulating neutrophil apoptosis during inflammation, as assessed by Annexin V staining and flow cytometry. Annexin V staining, which binds to phosphatidylserine (PS) exposed on the outer surface of apoptotic cells, provides a sensitive assay for detecting apoptosis. PS is normally located on the cytoplasmic side of the plasma membrane in healthy cells, but during apoptosis, structural changes occur, translocating PS to the outer surface, where annexin V can bind and be visualized using fluorescent conjugates. Therefore, the utilization of this protocol highlights the therapeutic potential of UCMSCs in regulating the apoptotic rate of neutrophils, thus managing inflammation and promoting tissue repair (Zhidu et al., 2024). Furthermore, CD11b, a surface integrin specific to neutrophils, was used to ensure that the apoptosis data accurately reflect the functional neutrophils involved in the inflammatory response. In Figure 4A, without stimulation, neutrophils (PMNs) exhibit a low apoptosis rate (1.10%), which modestly increases upon Poly(I:C) stimulation (2.47%), indicating a pro-apoptotic effect. However, co-culture with UCMSCs significantly enhances apoptosis to 7.23%, as confirmed quantitatively in Figure 4B where UCMSCs show a statistically significant increase in apoptosis compared to Poly(I:C)-stimulated PMNs alone. This elevated apoptosis rate is likely due to increased ROS production induced by UCMSCs, suggesting that UCMSCs promote neutrophil apoptosis, aiding in the resolution of inflammation by reducing neutrophil lifespan and limiting their pro-inflammatory activity, thereby preventing prolonged inflammation and tissue damage (Feng et al., 2023). Mechanistically, UCMSCs likely achieve this through the secretion of immunomodulatory factors such as PGE2, TGF-β, or IL-10, which enhance neutrophil apoptosis and support immune homeostasis.

4.5 UCMSC regulates the expression of T cells

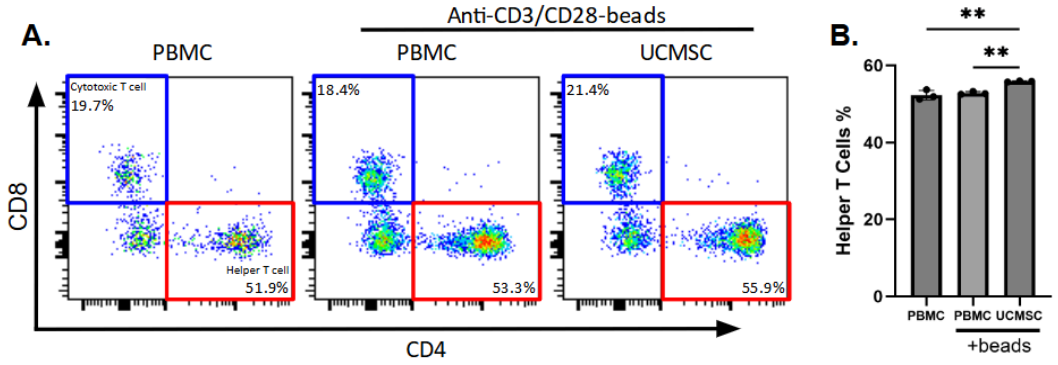

Figure 5. Surface staining that detects the expression ratio of T cells. A) Flow cytometry result. B) The CD4 expression percentage of PBMC, PBMC, and UCMSC after activation using Anti-CD3/CD28-beads under the stimulation of Poly(I:C)

From Figure 5, it can be seen that surface staining detects the expression of T cell subsets, specifically helper T cells (CD4+) and cytotoxic T cells (CD8+), in peripheral blood mononuclear cells (PBMCs) with and without UCMSC co-culture, following activation with anti-CD3/CD28 beads under Poly(I:C) stimulation. Figure 5A shows that unstimulated PBMCs have a helper T cell population of 51.9%, which increases slightly to 53.3% after activation. Furthermore, co-culture with UCMSCs significantly enhances the helper T cell population to 55.9%, while the CD8+ cytotoxic T cell population slightly increases to 21.4% from 18.4%, as quantified in Figure 5B. This suggests that UCMSC enhances CD4+ T cell expression, particularly regulatory T cells while maintaining balanced regulation of cytotoxic T cells (Ma et al., 2023). However, a limitation of this study is that the failure of activation beads to sufficiently stimulate T cells in the experimental setups made the use of CFSE dye unsuitable for tracking cell proliferation, as its fluorescence remains unchanged without cellular division. Mechanistically, UCMSCs express MHC class II molecules, which present antigens to CD4+ T cells, driving their activation and proliferation. Additionally, UCMSCs secrete cytokines such as IL-10 and TGF-β, which promote CD4+ T cell differentiation into regulatory T cells, and suppress overactive immune responses. The slight increase in CD8+ cytotoxic T cells indicates that UCMSCs may also support their activity to sustain an effective antiviral defense (Li et al., 2022). This dual regulation by UCMSCs highlights their ability to regulate immune responses by enhancing CD4+ T cell-mediated regulation while ensuring cytotoxic T cell functionality for robust antiviral immunity.

4.6 UCMSC promotes the induction of Tregs

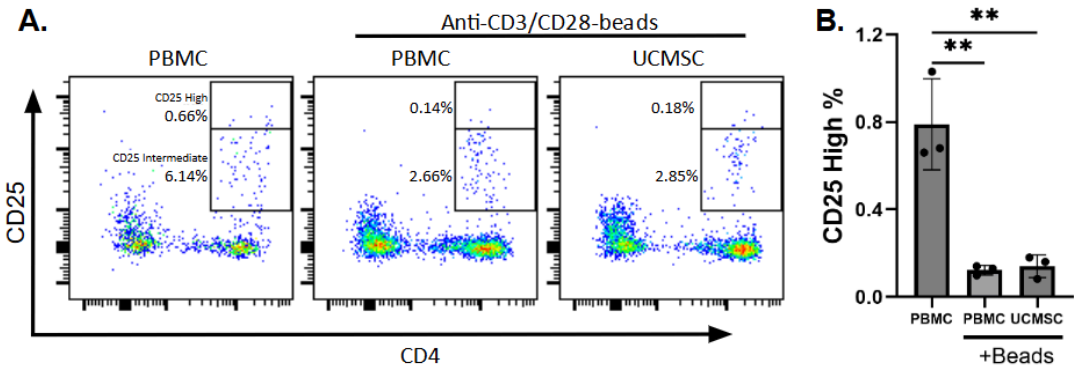

Figure 6. Surface staining that detects the expression of activated T cells. A) Flow cytometry result. B) The Treg expression percentage of PBMC, PBMC, and UCMSC after activation using Anti-CD3/CD28-beads under the stimulation of Poly(I:C)

From Figure 6, it is evident that UCMSCs play a role in modulating T cell activation, as indicated by the expression of CD25, a marker of activated T cells. Figure 6A shows that while anti-CD3/CD28 beads stimulate T cells in PBMC, the proportion of CD25-high regulatory T cells (Tregs) remains low at 0.14%. In contrast, the addition of UCMSC to bead-stimulated PBMC slightly increases CD25-high Tregs to 0.18%, as quantified in Figure 6B. Tregs, characterized by their high CD25 expression, play a crucial role in regulating inflammation during viral infections by suppressing excessive immune responses, minimizing tissue damage, and preventing immune overactivation (Wei et al., 2023). The UCMSC-mediated secretion of IL-2, a cytokine critical for Treg proliferation and maintenance, likely drives this effect (Han et al., 2022). Thus, by promoting Treg differentiation and activation through IL-2-dependent mechanisms, UCMSCs help balance immune responses, aiding inflammation resolution and antiviral defense (Ye et al., 2018). However, a limitation of this experiment is the suboptimal activation of Tregs using anti-CD3/CD28 beads alone, which could be due to inadequate bead-to-cell ratios. Therefore, to address this, troubleshooting approaches may include optimizing the bead-to-cell ratio or supplementing the culture with exogenous IL-2 to support Treg activation. These adjustments could improve the activation and differentiation of Tregs, providing clearer insights into the immunomodulatory effects of UCMSCs.

V. SELF REFLECTION

As for the self-reflection, I gained numerous valuable skills that will benefit my future career. Notably, I honed my proficiency in flow cytometry, an essential technique in biomedical research. Additionally, I improved my time management skills by balancing multiple tasks efficiently and developed public speaking skills, particularly in conducting public communication in a new and foreign environment, thus preparing me for future roles that require clear and effective communication. These experiences enhanced my analytical abilities and collaborative skills, though I identified weaknesses in time management and adapting to setbacks, such as equipment failures, which I aim to improve. The values instilled at i3L played a crucial role in my success, enabling me to persevere through challenging experiments. Through this journey, I identified my strengths, such as adaptability and teamwork, alongside areas for improvement, including overcoming hesitation in initiating discussions. This experience has not only prepared me for future employment but also reinforced my aspirations as a future researcher in the biomedical field.

VI. CONCLUSION

This study highlights the significant potential of umbilical cord mesenchymal stem cells (UCMSCs) in managing viral infections through their robust immunomodulatory and antiviral properties. The in silico findings demonstrate that UCMSCs display as promising candidates for antivirals through the GO, KEGG, and IPA analysis of the ISGylation pathway. Furthermore, in vitro results display that they might enhance controlled oxidative reactions to resolve inflammation. Subsequently, they can regulate T cell expression, including the induction of regulatory T cells (Tregs), to balance immune responses. These results lead to the rejection of the null hypothesis (H_0), which stated that the addition of UCMSC does not show both antiviral capabilities and a significant effect on immune response under Poly(I:C) stimulation, and support the acceptance of the alternative hypothesis (H_1), which proposed that UCMSCs aid as an antiviral agent that also regulate both neutrophil expression and T cell function. Despite these promising outcomes, the study is limited by variability in the number of isolated neutrophils, suboptimal activation of Tregs, and the lack of in vivo validation, which may affect the generalizability of the results. Future research should address these limitations by optimizing experimental conditions and conducting in vivo and clinical studies to confirm the therapeutic efficacy and scalability of UCMSCs. Overall, this study provides a strong foundation for leveraging UCMSCs in regenerative medicine and antiviral therapy, paving the way for innovative approaches to combat chronic viral diseases.

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APPENDICES

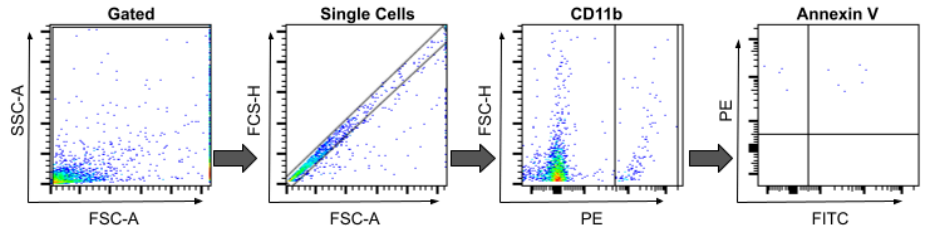
Appendix 1. Gene ontology data from metascape

Description	GO	-log10(P)
response to virus	GO:0009615	8.698937190
defense response to virus	GO:0051607	5.605181949
regulation of defense response to virus by host	GO:0050691	2.540244457
regulation of MAPK cascade	GO:0043408	13.492196680
regulation of receptor signaling pathway via JAK-STAT	GO:0046425	6.230268896
MyD88-dependent toll-like receptor signaling pathway	GO:0002755	3.649266910
regulation of canonical NF-kappaB signal transduction	GO:0043122	3.325564618
toll-like receptor signaling pathway	GO:0002224	2.842142692
regulation of type I interferon production	GO:0032479	7.603588406
regulation of interferon-beta production	GO:0032648	4.148495368
regulation of inflammatory response to antigenic stimulus	GO:0002861	2.600696648

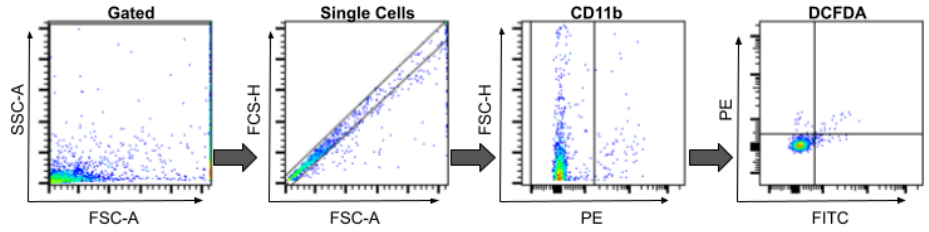
Appendix 2. Kyoto Encyclopedia of Genes and Genomes data from metascape

Description	GO	-log10(P)
NF-kappa B signaling pathway	hsa04064	9.035638482
MAPK signaling pathway	hsa04010	8.538019951
NOD-like receptor signaling pathway	hsa04621	8.015551920
PI3K-Akt signaling pathway	hsa04151	7.045921830
RIG-I-like receptor signaling pathway	hsa04622	5.609287351
Cytokine-cytokine receptor interaction	hsa04060	4.553136851
Toll-like receptor signaling pathway	hsa04620	3.812530127
JAK-STAT signaling pathway	hsa04630	2.880730183

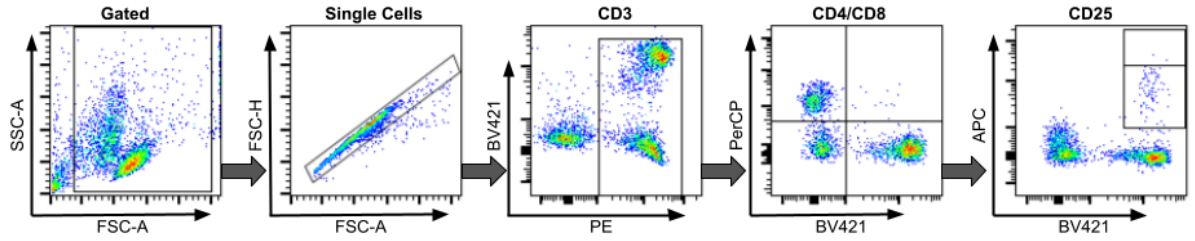
Appendix 3. Gating Strategy for PMN with Annexin V staining



Appendix 4. Gating Strategy for PMN with DCFDA staining



Appendix 5. Gating Strategy for PBMC with Surface Staining



Appendix 6. Geometric data of PMN from flow cytometry

Sample Name	Apoptosis %	ROS %
PMN-1	2,47 %	1,24 %
PMN-2	1,10 %	1,54 %
BM-1	3,85%	2,02 %
BM-2	4,19 %	1,57 %
UC-1	12,40%	2,28 %
UC-2	14,7 %	2,71 %
AD-1	3,50%	1,71 %
AD-2	7,65 %	1,35 %

DM-Ad-1	7,48%	2,00 %
DM-Ad-2	5,78 %	2,00 %
PMN-Poly-1	2,47 %	1,45 %
PMN-Poly-2	0,73 %	1,39 %
BM-Poly-1	4,46 %	2,71 %
BM-Poly-2	4,97 %	2,08 %
UC-Poly-1	9,05 %	2,49 %
UC-Poly-2	7,73 %	2,54 %
AD-Poly-1	7,23 %	1,31 %
AD-Poly-2	15,8 %	1,85 %
DM-Ad-Poly-1	8,21 %	1,40 %
DM-Ad-Poly-2	6,06 %	1,60 %

Appendix 7. Geometric data of CD4 and Tregs expression from flow cytometry

Sample Name	CD4	Tregs
AD-1	51,1 %	0,89 %
AD-2	50,7 %	0,53 %
AD-3	52,8 %	0,75 %
BM-1	47,6 %	0,74 %
BM-2	50,0 %	0,87 %
BM-3	50,9 %	0,78 %
DM-1	49,9 %	0,74 %
DM-2	50,9 %	0,51 %
DM-3	51,8 %	0,51 %
PBMC-1	51,9 %	0,66 %
PBMC-2	51,3 %	0,68 %
PBMC-3	53,7 %	1,03 %

UC-1	50,3 %	0,85 %
UC-2	50,8 %	0,84 %
UC-3	51,4 %	0,67 %
AD-beads-1	51,9 %	0,12 %
AD-beads-2	51,2 %	0,062 %
AD-beads-3	54,7 %	0,13 %
BM-beads-1	49,7 %	0,13 %
BM-beads-2	49,7 %	0,14 %
BM-beads-3	48,2 %	0,11 %
DM-beads-1	51,8 %	0,054 %
DM-beads-2	52,5 %	0,067 %
DM-beads-3	51,4 %	0,097 %
PBMC-beads-1	53,3 %	0,098 %
PBMC-beads-2	52,7 %	0,14 %
PBMC-beads-3	52,5 %	0,13 %
UC-beads-1	55,9 %	0,18 %
UC-beads-2	55,7 %	0,16 %
UC-beads-3	56,0 %	0,082 %

Appendix 8. Full figure of the IPA analysis result