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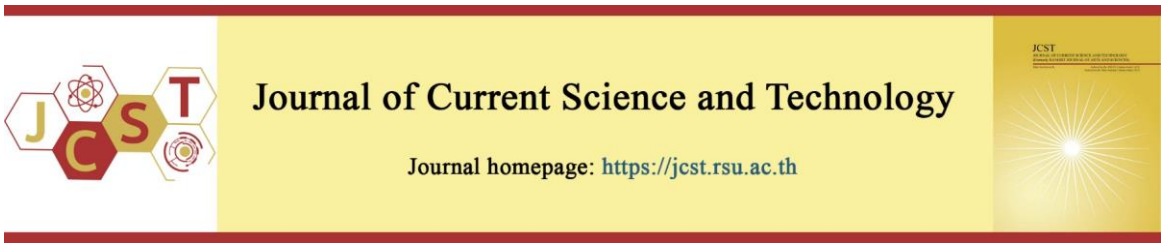

Practical applications of peripheral blood mononuclear cells (PBMCs) in immunotherapy preclinical research

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Abstract

In recent years, we have faced more and more new diseases caused by viruses and opportunistic infections. There are still no preclinical models that would improve the evaluation of the proposed therapies and the immune system's response to drugs and infections. That is why the models based on the peripheral blood mononuclear cells (PBMCs) have a great potential to evaluate the human body's cellular, cytotoxic and humoral responses to vaccines and drugs. At the same time, studying these models may be the next stage of preclinical research, allowing for the initial verification of the tested factors before starting animal testing. This review has compiled the key information on the isolation, culture, cryopreservation, and application potential of PBMCs in the preclinical research focusing on viral pathogenesis and therapy.

Keywords**:** *cryopreservation; microfluidics; PBMC isolation; peripheral blood mononuclear cells (PBMCs); preclinical research; viruses.*

1. Introduction

Over the last few years, immunotherapy has advanced immensely, developing breakthrough therapies such as immune checkpoint therapies (Constantinidou, Alifieris, & Trafalis, 2019; Sharma, & Allison, 2015), which modulate immune effector mechanisms (Durgeau, Virk, Corgnac, & Mami-Chouaib, 2018). However, research into new therapeutic options requires a deeper understanding of the human body's immune response to realise immunotherapy's full potential. In the face of the COVID-19 pandemic, developing a system that could enable rapid preclinical verification of vaccines and drugs appears crucial for the dynamically progressing mutations of SARS-CoV-2 (Krause et al., 2021). Peripheral blood mononuclear cell models could bridge the gap between animal experiments and clinical trial systems. They are useful for *in vitro* evaluation of memory T cell responses to vaccines (Tapia-Calle et al., 2019).

Peripheral blood mononuclear cells (PBMCs) consist of different blood cells characterised by a single, spherical cell nucleus. They include, among others, monocytes, lymphocytes and macrophages (Kleiveland, 2015). The composition of PBMCs may differ when we consider a few key factors, such as the donor's age (Table 1) (Autissier, Soulas, Burdo, & Williams, 2010). As PBMCs are proved to be a valuable immune response model, they are used in various *in vitro* and *ex vivo* tests that investigate the impact of

bacteria (Alvarez-Rueda et al., 2020; Deng et al., 2021; Peña-Cearra et al., 2021) and viruses (Sałkowska, Karwaciak, Karaś, Dastych, & Ratajewski, 2020; Z. Wang et al., 2021; Zhou et al., 2020) on different pathways.

In the following review, we focus on the possibilities of using PBMCs in virus immunotherapy research and on the critical steps of laboratory work involving these cells.

Table 1 Composition of human PBMCs. Data were obtained from nine healthy adult donors (age range 21-48 years) by an automated haematology blood analyser and FACS. Modified on the base of (Autissier et al., 2010).

Type of cells	Range
Total lymphocytes	41 – 60%
	Containing:
	49 – 77% T cells
	6 – 17% B cells
NK	7 – 35%
Monocytes	6 – 12%
Dendritic cells	~1%

2. Isolation

PBMCs can be isolated from fresh whole blood (Weiskopf et al., 2020; Zhou et al., 2020) or a buffy coat (Luukkainen et al., 2018). The isolation from fresh blood often needs protection against spontaneous clot formations complicating the separation of distinguished cell types (Hwai, Chen, & Tzeng, 2018). For this purpose, various substances are used, such as EDTA (Kofanova et al., 2019; Weiskopf et al., 2020), heparin (Ansell et al., 2019), and acid–citrate–dextrose (ACD) (Banka, & Eniola-Adefeso, 2021). EDTA works by binding calcium ions, thus blocking the coagulation cascade (Moore, Knight, & Blann, 2021). Heparin derivatives inhibit blood clotting due to the potentiation of the action of antithrombin III (AT III). The type of anticoagulant should be selected according to the planned tests; for example, heparin is the best anticoagulant for T cell studies (Mallone et al., 2011).

The blood counts are separated from each other and the plasma by centrifugation in a sugar gradient, e.g. Ficoll, at a density of 1.077 g/mL (Cui, Schoenfelt, Becker, & Becker, 2021). In the research literature, there are comparisons of various isolation techniques, e.g. with the use of unique test tubes such as Cell Preparation Tubes™ (CPT, BD Biosciences) and Lymphoprep™ Tubes (Axis-Shield) (Chen et al., 2020; Grievink, Luisman, Kluft, Moerland, & Malone, 2016). Depending on the chosen isolation technique, PBMCs will vary in viability, the number of individual populations, and their ability to produce pro-inflammatory cytokines. However, the technician's experience performing the test is one of the main factors influencing the divergence of the results.

It needs to be highlighted that the temperature during the isolation process, starting with the buffy coat/blood temperature itself, is crucial for further results of the conducted experiments.

The storage of the material at a reduced temperature (4°C) harms the process of cell isolation and the subsequent performance of PBMCs (Jerram et al., 2021) as it increases the contamination of PBMCs with granulocytes significantly. At the same time, the long-term storage (> 8h) causes a similar phenomenon (Mallone et al., 2011). The low temperature during isolation causes the isolate contamination with red blood cells and platelets, resulting in sticking them to leukocytes (Burlingham et al., 2005). It is highly undesirable, especially if Real-Time PCR is planned (Banas, Kost, Hillebrand, & Goebel, 2004). This problem can be solved by extending the centrifugation time and carrying out the isolation at room temperature (Merck, n.d.). As an additional step, ACK buffer, ammonium chloride, or commercial solutions lysis can be applied to remove red blood cells from the suspension (Betsou, Gaignaux, Ammerlaan, Norris, & Stone, 2019).

Storing blood at room temperature increases the number of low-density neutrophils in the final suspension. However, there is no detectable effect on the content of other cells relevant to immune response studies, such as monocytes, CD4+, CD8+, CD8+CD45RA, NK, and B cells (Jerram et al., 2021).

Following the gradient centrifugation, the PBMCs must be rinsed with the culture medium

RPMI-1630, Opti-MEM (Akbaba, Akkaya-Ulum, Demir, Ozen, & Balci-Peynircioglu, 2021) or PBS (Chen, Cheung, Shi, Zhou, & Lu, 2018). Adding the human serum at this step improves the isolation yield with the upward trend of the yield. The content of the serum should be adapted to further planned applications (Mallone et al., 2011).

3. Culturing

After the isolation, the cells can be either maintained as a culture or cryopreserved in the samples; however, the proliferation process of PMBCs is minimal since the isolated cells are in the mature form. Primary lines isolated from blood or buffy coat can be grown for one passage and will not proliferate without stimulation (De Fries, & Mitsuhashi, 1995; Panda, & Ravindran, 2013).

The induction of proliferation can take place using factors of various origins. Lipopolysaccharide (LPS) of bacterial origin, phytohaemagglutinin (PHA) and other mitogens have been successfully used for this purpose (Dawes et al., 2008; Janský, Reymanová, & Kopecký, 2003; Shin, Ye, Kim, & Kang, 2007). Depending on the planned tests, the appropriate stimulant should be selected, bearing in mind that it may induce a specific immune response to a given reagent and thus change the result of further tests.

PBMCs can be cultured for about 7-10 days in 24 or 96 well plates (Schroers et al., 2000; Tapia-Calle et al., 2019). The use of U-shaped culture wells (Suni et al., 2003) may be advisable as PBMCs are composed of different cells with different specificities and adhesion to the substrate. As the standard, the cells are seeded at approximately one million per 1 mL of culture medium. The RPMI-1640 is the most often used medium (Loughran et al., 2018; Luukkainen et al., 2018; Naseem, Manzoor, Javed, & Abbas, 2018; Z. Wang et al., 2021) but then the use of Opti-MEM is an excellent solution, especially for protein-sensitive tests as it allows the reduction of serum level (Majumdar, Ratho, Chawla, & Singh, 2014; Somamoto, Nakanishi, & Nakao, 2013). The cells are grown under the standard conditions and incubated at 37°C in a humidified, 5% CO_2 atmosphere.

Leaving the PBMCs for an overnight rest is usually preferable, as incubation for 18-20h prior to the scheduled testing allows for getting rid of apoptotic cells (Kierstead et al., 2007). It can also be helpful to increase the sensitivity of functional T-cell assays (Kutscher et al., 2013), but the cell with and without the rest day should not be compared in the analysis.

4. Cryopreservation

Due to the frequent need to freeze blood cells for further analysis, many research teams have focused on checking whether this process affects the content of individual populations and the immune response of the PBMCs. Cryopreservation enables to conduct of the research according to a predetermined plan and reduces the divergence of results caused by other experimental conditions.

The traditional freezing medium contains fetal bovine serum (FBS) and dimethylsulfoxide (DMSO). Other ingredients such as the mixtures of osmolytes composed of sugars, sugar alcohols and amino acids were also tested. However, they showed a more than 10% decrease in viability in both T helper and T cytotoxic cells (Pi, Hornberger, Dosa, & Hubel, 2020). The composition of the freezing medium, e.g. the concentration of the main components (DMSO, FBS), significantly impacts the recovery of cells after freezing.

DMSO reduces the number of ice crystals formed during freezing, thus protecting cells from damage. The research shows that the optimal concentration is between 10 and 15%. Despite the possible toxic effect of DMSO on cells, the thawed suspension can be kept in the freezing medium for up to 30 minutes without observing a loss in viability. It is beneficial when considering batch thawing of samples for research. Typically, the freezing process uses a medium containing 90% FBS. However, the studies show that this amount can be reduced to 40% while keeping the cells in good condition after thawing (Nazarpour et al., 2012).

PBMCs are at their best after cryopreservation if the cooling rate is $-1°C\ min^{-1}$ or slower in a controlled manner (Baboo et al., 2019). It is worth emphasising that Ramachandran with the team reported that adding the cold medium to the cold sample reduces the viability and recovery of the PBMCs population (Ramachandran et al., 2012). Adding the warm culture medium to PBMCs heated in the water bath gives significantly better results.

However, there is no substantial effect of cryopreservation on the content of CD4+, CD8+ and CD25+ cells in the suspension (Tompa, Nilsson-Bowers, & Faresjö, 2018) so freezing the preparations is not ideal for all possible tests.

Cryopreservation can reduce the number of naive fractions and central memory T cells, decrease CCR-5 expression, and increase CD8 + effector (Betsou et al., 2019; Costantini et al., 2003). It also affects CD4+ apoptosis, which may cause a reduction in response. CD8+ responses to

immunogenic peptides and peptide pools are well preserved.

Long-term cryopreservation (over a year) may reduce the CD4+ response and CD4+ markers (Owen et al., 2007). There is also a clear decrease in cell proliferation in response to antigen. For example, the lymphocytes from whole blood can be successfully transformed with Epstein-Barr virus; however, they are characterised by decreased viability and increased cell content in apoptosis (Betsou et al., 2019).

Despite the possible decrease in cell viability during the freezing process, recovery is still sufficient for most analyses, as presented in Figure1. Therefore thawed PBMCs can be used successfully in immunophenotyping (Y. Wang et al., 2021) and proliferation assays of lymphocytes (Olajide et al., 2021), host cell reactivity (HCR) tests (Athas, Hedayati, Matanoski, Farmer, & Grossman, 1991), lymphoblastoid cell lines (LCLs) determination (Tremblay, & Khandjian, 1998), and a mutagen susceptibility test to assess DNA repair capacity (Cheng et al., 2001).

Special attention should be paid to whether the freezing process will significantly impact the tested parameters, as it can, for example, impact the levels of many clinically significant T cell markers, such as PD-1, CTLA4, KLRG1, CD25, CD122, CD127, CCR7 can occur (Capelle et al., 2021).

Notably, there is a particular need to compare the studies on PBMC's response with the results from the same way of preparation, as cryopreservation can impact many parameters, e.g. cytokine production, the number of individual populations or cell viability (Martikainen, & Roponen, 2020).

5. Applications

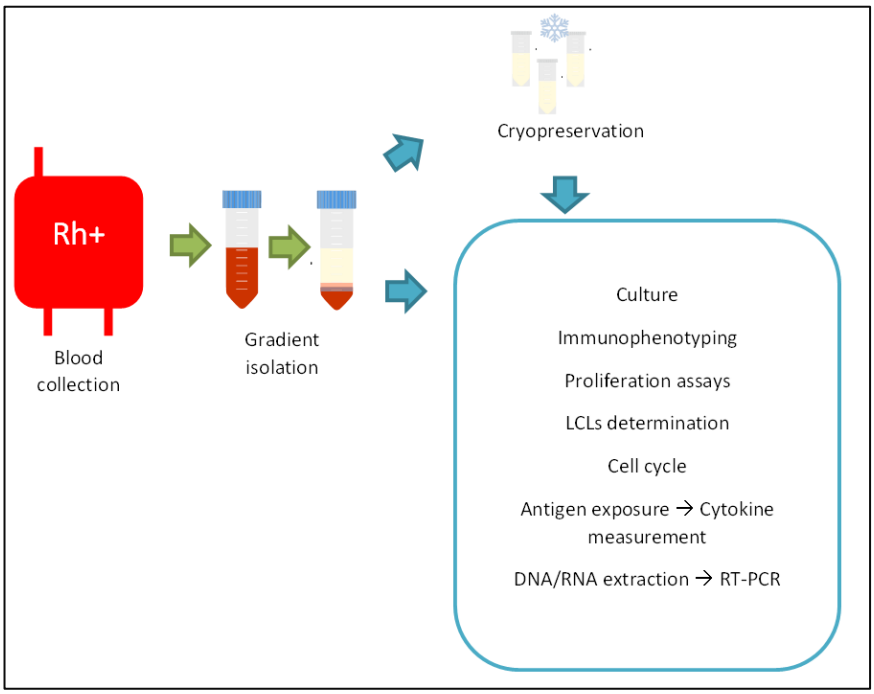

Figure 1 Possible workflows using PBMCs. *LCLs - lymphoblastoid cell lines.*

The isolated PBMCs can have many applications, as shown in Figure 1. By exposing PBMCs to various antigens, we can create an effective model of the immune response for preclinical research. It is also worth noting that these cells can be activated globally on all cell phenotypes and individually on a specific cell type (due to the sorting process).

Sałkowska with the team investigated the impact of SARS-CoV-2 proteins on the differentiation of CD4+ lymphocytes (Sałkowska et al., 2020). The cells were isolated from healthy donors and then incubated in the presence of the S (spike) and N (nucleocapsid) proteins. Differentiation of Th1 and Th17 cells under the influence of these factors was checked using RT-

PCR and ELISA. The studies showed that CD4+ cells isolated from healthy donors, which differentiated towards the Th1 lineage in the presence of the tested immunogenic factors, led to the induction of IFNG (Interferon Gamma). The researchers suggested that the response to the proteins of SARS-CoV-2 is primarily related to these lymphocytes and may depend on the prior infection with other types of coronavirus or vaccination that induce a non-specific cellular response. The transcriptomic analysis of PBMC data from COVID-19 patients enabled the identification of biomarkers based on abnormal mRNA and lncRNA expression (Shaath, & Alajez, 2021). The immune response is clearly suppressed in COVID-19 patients who enter the ICU. Understanding the role of PBMC biomarkers could help identify therapeutic options for COVID-19 patients. It was also found that patients suffering from the severe disease showed significantly increased expression of TNF, IFN-γ, antiviral,

HLA-DQA1 and HLA-F genes (Sadanandam et al., 2020).

Another SARS-CoV-2 research (Weiskopf et al., 2020) used the *ex vivo* PBMCs model for checking the cytokine profiles after stimulation with MegaPool (MPs; peptide pools), such as MP_S, MP_CD4_R (R=reminder) and MD_CD8. The specific cell activation was measured by flow cytometry via the expression of CD69, CD137, CD45RA and CCR7. The research provided information on the virus-specific immune responses in COVID-19 ARDS patients, including the lymphocyte phenotype and the kinetics of the post-infection changes. Wang et al. isolated PBMCs from COVID-19 patients and healthy people in close contact with them and then stimulated PBMCs with a peptide pool containing 446 15-mer peptides and 110 18-mer peptides for ten days. It was observed that both tested populations developed the virus-specific CD4+ and CD8+ responses. Moreover, the memory T cell pool size in COVID-19 patients was more significant and better than in the close contact population. The research suggests that relative donors may acquire cellular immunity to SARS-CoV-2 despite no detectable infection and have similar levels of memory lymphocytes to the patients with symptoms of the disease.

Kundu's team study showed the example of incorporating *in silico* and further *ex vivo* approaches in evaluating the indication of immune pressure or capsid and non-capsid regions coxsackie B viruses (Kundu, Knight, Dunga, & Peakman, 2018). PBMCs were stimulated with synthetic peptides based on component-restricted sequences, which led to IFNγ responses measured by the ELISpot assay. The results showed that anti-CBV CD8 T cell immunity is connected with multiple effector memory responses, which might help design preventive measures against SARS-CoV-2.

Another team investigated whether IL-6 would protect lymphocytes from apoptosis and functional exhaustion in chronic HCV infection using PBMCs (Naseem et al., 2018). The cells isolated from HCV patients and healthy donors were incubated with or without adding IL-6. Then the cells were stained with Annexin V for flow cytometry, or RNA extraction was performed. The expression of genes related to the apoptosis process was tested – interferon-gamma, TIM-3, BIM, MCL-1, BCL-2 and caspase 3. The study confirmed the positive role of IL-6 in inhibiting lymphocyte apoptosis, but its use as the only factor is insufficient to maintain acquired immunity. Based on these conclusions, IL-6 can be tested further as one component of combination therapy for the adoptive treatment of chronic HCV infection.

The *ex vivo* system using PBMCs can also study the course of the disease and verify the factors determining its severity. The examples include the studies in which the role of IL-10 and regulatory B cells in the pathogenesis of hepatitis B (HBV) was verified using such a model (Das et al., 2012). The role of B lymphocytes and changes in their populations were studied using different stimulants, such as CpG and HBV Ags. Over time the correlation between the level of B lymphocytes and symptoms of disease exacerbation was observed in the patients and *ex vivo* studies. It was proved that the levels of these cells in patients are adequately reflected in *ex vivo* models. PBMC models can be an excellent way to study various therapeutic possibilities and immunogenic factors in preclinical tests.

HIV infections are among the most studied using PBMC models (Jamshidi et al., 2020; O'Connell, Zheng, Amalfitano, & Aldhamen et al., 2019). Investigating the pathogenesis of viral infection is critical in designing a therapy for this disease. O'Connell's team proposed the protocol to study the role of myeloid cells with a particular emphasis on monocytes, which are an essential

mediator of chronic inflammation in HIV-1 infected patients (Merino, Allers, Didier, & Kuroda, 2017; O'Connell et al., 2019). The proposed method with FACS enables the analysis of monocyte infection without the need for intracellular p24 staining.

Another virus studied just as often using PBMC models is the *dengue virus* (DV) (Blackley et al., 2007; Kou et al., 2008; Weiskopf et al., 2013). It is so because this study requires the accurate identification of permissive DV target cells. The infectivity of the virus towards specific cells of the immune system can be performed by the examination of the presence of viral RNA by flow cytometry with DV E antigen (anti-E) as a target (Blackley et al., 2007; Kou et al., 2008). Imaging of monocyte infections was also performed using microscopic techniques. Thanks to electron microscopy, it is possible to present the attachment of virus particles to the cell surface (Espina, Valero, Hernández, & Mosquera, 2003) while using confocal microscopy active DV replication in human B cells (Lin et al., 2002). An important discovery in research on the DV was that TNF alpha does not affect its replication in monocyte-derived macrophages. At the same time, DV infection changes the response to stimulation with this factor (Wati, Li, Burrell, & Carr, 2007).

These examples prove that the PBMC model is extremely useful for studying viral infections. It is worth emphasising that co-infections are one of the biggest and most present problems in modern medicine are co-infections. Viruses can modulate the host's response, and potential drug interactions often make them difficult to treat (McArdle, Turkova, & Cunnington, 2018).

Influenza A is a well-known case often associated with a severe course of *Streptococcus pneumonia* infection (Loughran et al., 2018). The mechanism of this dependency is not fully understood. It is known that a competent Th17 response is a critical element of protection against pneumococcal lung infection. In the 2018 study, a model based on healthy donors' PBMCs was used to check how the immune response of cells was affected by influenza A. It was confirmed that the infection with the Influenza A virus inhibits innate immune responses in human antigen-presenting cells and T helper cells, such as the production of cytokines. This virus was also tested in the studies of Al-Ghazal's and Bardelli's teams (Bardelli et al., 2013; Al-Ghazal, Ismail, Al-Umary, Al-Khuzie, & Assaf, 2016).

A study from 2016 showed the changes in the mRNA expression in leukocyte genes responsible for inflammatory responses. The research material was isolated from the buffy coats of the infected patients. Influenza A caused several alterations in the gene expression of cells, including genes encoding interleukins 1B, 8, 10 and 13, and many C-C Motif Chemokine Ligands. The analysis helped find the correlation with complications in patients with asthma.

The research from 2013 focuses on the *ex vivo* analysis of human B lymphocytes, which are specific for A and B influenza hemagglutinin. The presented method using PBMCs as a model allowed the quantitative and functional analysis of lymphocytes by staining the B-cells with CD20 and CD27 antibodies mixed with recombinant H1 bait or human serum albumin (conjugated with the Alexa-488 fluorochrome) to identify cells engaged in BCR-specific interactions. The flow cytometry analysis showed the contribution of pre-existing human memory B-cells produced after the infection or vaccination in antibody responses against novel IA viruses**.**

The opportunistic infections are also a significant risk in the *measles virus* (MV) infection (Laksono et al., 2018). The infection of the virus is due to the CD150 receptor in the lymphocytes, dendritic cells, macrophages and thymocytes. Laksono's team studies checked the susceptibility and permissiveness of naïve and memory cells from the human peripheral blood and tonsils. The research based on immunophenotyping showed that both naive and memory B cells are essential to target cells of MV infection. It was concluded that the depletion of these cells contributed to disease suppression.

The studies of co-cultures in batch culture or microfluidic systems are another possibility of using the PBMCs. These models enable immune response analysis in conditions similar to selected organs *in vivo*. At the same time, they can contribute to the development of systemic models and search for targeted immunomodulatory therapies. Using models using microfluidic cultures enables the study of synergistic effects, e.g. between the epithelium and endothelium and the immune system, the study of cytokine secretion, or even the identification of new biomarkers crucial for the pathogenesis of the disease. An example is research

on culture inserts that allow the differentiation of epithelial cells, e.g., nasal epithelium cells or lung cells.

In the studies from 2018, an *in vitro* co-culture model was established that included H3N2 infection of human nasal epithelium and PBMCs (Luukkainen et al., 2018). The study aimed to test crosstalk during the infection. The cells were infected with the virus for 24h before establishing co-culture. Subsequently, cultivation was performed for 24 or 48 hours and the PBMCs were taken for further tests such as immunophenotyping using flow cytometry. The content of cytokines and chemokines such as INF-γ, INF-α, and numerous interleukins in the basal media supernatant and the content of viral RNA in it were also measured. It was proven that infection with the H3N2 virus of nasal epithelium causes an increase in interferons, pro-inflammatory cytokines, and chemokines as early as 24 hours. It indicates the activation of monocytes, NK cells and innate T-cells.

Another model includes the co-culture of epithelial NCI-H441 and endothelial HUVEC cells with the macrophages isolated from PMBCs (Deinhardt-Emmer et al., 2020). The researchers established the system resembling the human alveolus architecture and functions used for co-infection studies of influenza virus and *Staphylococcus aureus*. The model allowed for novel studies showing the crosstalk between the pathogen and the host. The analysis of the response of macrophages isolated from PBMCs to infection showed their effect on preserving the integrity of biological membranes during infection. This integrity is closely related to the formation of inflammation leading to a dynamically developing organism disease. Benams and Huh's research teams showed similar lung-on-chip models which can be incorporated into toxicology immune response research (Benam et al., 2016; Huh, 2015).

Although many indirect disease biomarkers are in the blood, their detection is problematic due to the non-specific background. Circulating lymphocytes can be the indicators of various diseases because of changes due to immunogenic factors such as pathogens. In the studies from 2021, a label-free microfluidic impedance assay was used to activate lymphocyte profiling based on native and antigen-specific T-lymphocyte responses (Petchakup et al., 2021). Apparent biophysical differences were observed in the size of healthy cells, activated and dead lymphocytes. Based on these results, a model was constructed to directly quantify activated lymphocyte impedance directly. The presented results can serve as the basis for developing a multiplex screening test to detect antigen-specific T cell responses, e.g. for diagnosing infectious diseases, including COVID-19.

In conclusion, we proved the key importance of PBMC models for the study of preclinical viral infections. Working with these models, however, requires paying much attention to the selection of individual parameters such as the isolation temperature and the composition of the cryopreservation medium. The method of thawing cryopreserved samples also needs to be considered carefully.

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