Hypothesis

Production of SARS-CoV-2 Specific IFN-γ/IL-10 Co-producing CD4 T Cells from Convalescent Donors to Treat COVID-19: A Hypothesis

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has alarmed people worldwide as an emerging coronavirus, which to date remains a major global public health challenge. Patients are being treated with different therapies; however, no evidence of a single therapy has been found to improve the clinical outcomes significantly; therefore, there is currently no single effective treatment against COVID-19. The research related to virus-specific T cell therapy has provided positive results when treating Epstein-Barr virus (EBV) and cytomegalovirus (CMV). Therefore, when facing a new virus, it is necessary to continue innovating the therapeutic strategies that have worked to treat viral infections previously, adapting to the pathogenesis of this new disease, to treat patients infected with SARS-CoV-2 effectively and safely. This proposal presents the research idea of creating SARS-CoV-2 specific interferon-gamma (IFN-γ) and interleukin-10 (IL-10) co-producing CD4 T cells to determine the cytokine secretion and viability of their production for virus-specific T cell therapy.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),

which began in Wuhan, a central city in China, in December 2019, has alarmed people worldwide as an emerging coronavirus, which to date remains a major global public health challenge.[1] In the first few days, patients that are infected with SARS-CoV-2 might develop the disease in several stages starting from an asymptomatic state. In the following days, the virus travels through the upper airways, where it spreads and migrates along the conductive airways, and a more robust immune and innate response is triggered, in approximately 80% of patients with COVID-19 the disease will be mild and mainly restricted to the upper and conductive airways.[2,3] Approximately 20% of infected patients will progress to the next stage and develop pulmonary infiltrates, and some of them will develop severe diseases, such as hypoxia and progression to acute respiratory distress syndrome (ARDS). The pathological result of severe acute respiratory syndrome (SARS) and COVID-19 is diffuse alveolar damage with fibrin-rich hyaline membranes and some giant multinucleated cells, and aberrant wound healing could lead to scarring and fibrosis that is more severe than other forms of SARS.[2,4,5] Other typical symptoms of COVID-19 include fever, dry cough, and shortness of breath; gastrointestinal symptoms, such as nausea, vomiting, abdominal pain, and diarrhea have been reported, and neurologically related symptoms, in particular, anosmia, hyposmia, and dysgeusia.[6]

Currently, there is no single effective treatment against COVID-19.[7] Hydroxychloroquine does not have any benefit, and thera-

pies, such as cytokine storm inhibitors and monoclonal antibodies are ineffective and require further investigation and convalescent plasma therapy (CPT) and interferon provide some clinical benefits, such as faster recovery time and reduced mortality; however, these effects were not clinically significant.[7] Therefore, is necessary to consider therapies that have worked when treating infected patients with other diseases that are caused by viruses as a potential therapeutic basis for treating COVID-19. A promising therapeutic strategy that has been used to treat viral infections is the adaptive therapy with virus-specific T cells, as in with Epstein-Barr virus (EBV).[8] Other studies obtained positive antiviral responses from T cells, without inducing graft-versus-host disease (GvHD), or the acute side effects in specific T cell adoptive transfer therapy to treat cytomegalovirus (CMV).[9] The targets of T cell-mediated immunity to SARS-CoV-2 have been identified,[10] which indicates how to develop peripheral blood mononuclear cell (PBMC)-derived products from COVID-19 recovered patients, which is currently controversial and has initiated multinational research for this approach.[11–14]

Hypothesis: SARS-CoV-2 specific IFN-γ/IL-10 co-producing CD4 T cells from convalescent donors to treat COVID-19

Hypercytokinaemic immune dysregulation in COVID-19, which is known as cytokine storm syndrome, has been described as immune deregulation that is characterized by the continuous activation of immune cells, which are secreters of large amounts of cytokines that lead to overwhelming systemic inflammation and high-mortality multiorgan insufficiency.[15,16] This could be mediated by interleukin-10 (IL-10), a potent immunomodulator. IL-10 inhibits the production of pro-inflammatory cytokines, such as interferon (IFN), tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) in various cell types; and it prevents the maturation of dendritic cells by blocking IL-12.[16] IL-10 levels increase in the second week after the onset of symptoms; although they have not associated with the immune drawback, it is an indication of latent immune efforts to control the cytokine storm.[17,18]

Interferon-gamma (IFN-γ) is a type II interferon that is produced by various cells, such as CD4 T cells. IFN-γ participates in numerous immunological and adaptive functions, promotes macrophage activation and antigen presentation, and is highly involved in antibacterial and antiviral immunity and the transduction of signals.[16,19] The inflammatory response to COVID-19 results in the formation of fibrosis in patients; it has been suggested that early intervention of antiviral infection using IFN-γ could be substantial in inhibiting fibrosis for a better functional recovery.[19]

Effector memory T cells that co-produce IFN-γ and IL-10 perform an essential role in regulating pulmonary inflammatory development and fatal injury in acute infections that are caused by respiratory viruses.[20] Effector memory CD4 T cells that co-produce IFN-γ/IL-10 accumulate in peripheral tissues in areas where the infection is persistent to prevent local damage; however, despite its IL-10 production, these cells express especially high levels of IFN-γ.[21] In addition, when these cells are cultivated with macrophages that have been infected with Toxoplasma gondii, the pathogen is efficiently eradicated, which indicates that their simultaneous production of IL-10 does not compromise the effector function of these T-helper type 1 (Th1) cells.[21,22]

By focusing on the capabilities and therapeutic potential of these Th1 cells, it is essential to consider their use to combat the disease that is caused by SARS-CoV-2 (Fig. 1). In addition, it is necessary to determine the viability of its production and analyze the IFN-γ and IL-10 secretion of these cells. Therefore, the meth-

ods that support the study design of this research hypothesis are explained in detail in the following section.

Proposed methods for the hypothesis

Donor eligibility

Donor eligibility must be performed under all criteria set by PAHO/WHO. People between the ages of 21 and 65 years, with registered COVID-19 positive documentation of reverse-transcription-polymerase chain reaction (RT-PCR) nasopharyngeal samples from when they had the disease; however, at the time of this study, they must show negative results of the same RT-PCR tests. People using immunosuppressive treatments and that have diseases, such as human immunodeficiency virus (HIV), Hepatitis C virus (HCV), and Hepatitis B Virus (HBV) infection do not qualify as donors. In addition, informed consent will be required for the use of blood components from the donor and approval from the institutional bioethics committees for the study's development must be obtained.

Cell processing to obtain SARS-CoV-2 specific IFN-γ T cells

Following extraction of PBMCs, the cells will undergo a fully automated process that uses the CliniMACS Prodigy IFN-γ Cytokine Capture System (CCS) (Miltenyi Biotec, Germany). The cells will be stimulated with PepTivator Prot-S SARS-CoV-2 that contains the amino acids sequence domains 304-338, 421-475, 492-519, 683-707, 741-770, 785-802, and 885-1273; PepTivator Prot-M and Prot-N SARS-COV-2, each comprises a set of peptides, which consist mainly of 15-mer sequences with 11 amino acids overlap that covers the complete sequence of the nucleocapsid phosphoprotein (N) and the complete sequence of the SARS-CoV-2 membrane glycoprotein (M) respectively. (GenBank MN908947.3, Protein QHD43416.1, Protein QHD43419.1, Protein QHD43423.2) (Miltenyi Biotec, Germany). Then, the cells will be labeled with the Catchmatrix reagent (Miltenyi Biotec, Germany) that contains biospecific antibodies for CD45 and specific for IFN-γ, which will be secreted by the target cells on stimulation. Briefly, IFN-γ bound to the cell surface will be guided by the CliniMACS enrichment reagent; this IFN-γ specific antibody is conjugated to MACS Micro-Beads, which leads to cell separation. Finally, the cells that secrete IFN-γ labeled with the microbeads will be isolated by the built-in magnetic column of the CliniMACS Prodigy; however, the unlabeled cells pass through this and the microbead-labeled cells are retained in the magnetic field, and then diluted in the target cell bag.[12]

Isolation of IFN-γ-secreting CD4 T cells

Effector memory CD4 T cells will be isolated from the previous procedure's target cell bag, using the effector memory CD4 T cell isolation kit (Miltenyi Biotec, Germany), with the autoMACS Pro Separator (Miltenyi Biotec, Germany) following the manufacturer's protocols.

Re-stimulation of IFN-γ Th1 cells for IL-10 secretion

In this step, 10^7 cells/mL will be restimulated with the SARS-CoV-2 in a peptide pool previously described at a concentration of 5 µg/mL, in TexMACS medium (Miltenyi Biotec, Germany), with
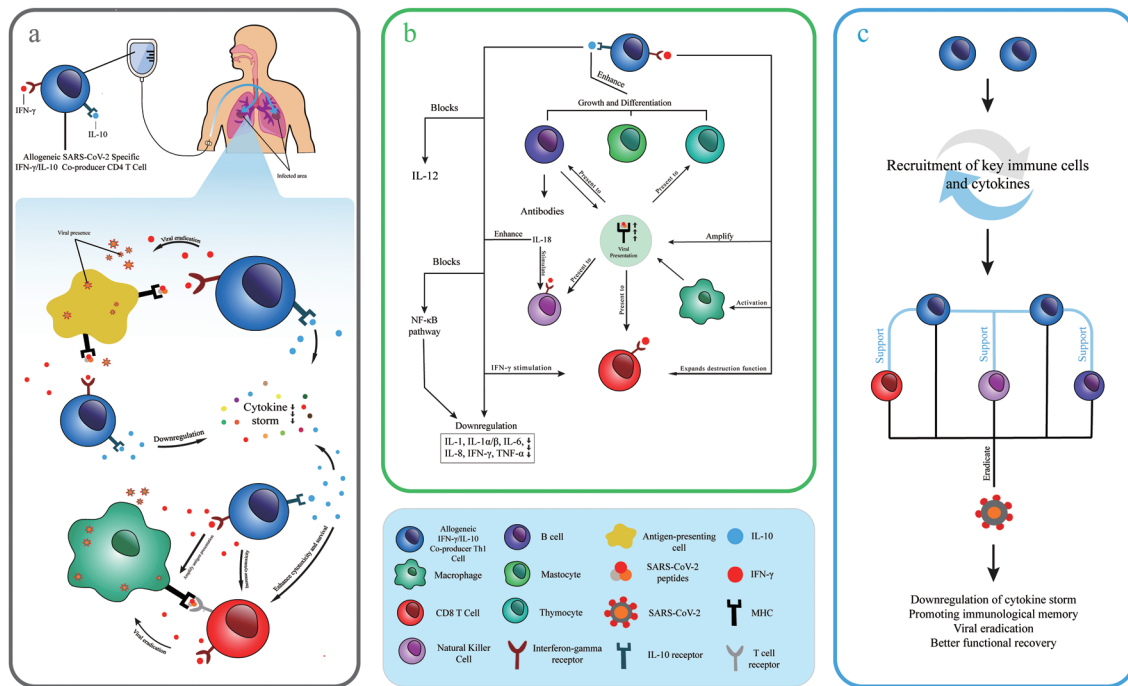
Fig. 1. SARS-CoV-2 Specific IFN-γ/IL-10 coproducing CD4 T cells; Hypothesis and possible function mechanism: (a) perfusion of virus-specific T cells, where the allogenic IFN-γ/IL-10 co-producing CD4 T cells travel to the areas infected by SARS-CoV-2. These cells will begin to produce IFN-γ when recognizing the viral presence and IL-10 to downregulate the cytokine storm along with their synergistic function; (b) allogeneic Th1 cells, when secreting IFN-γ/IL-10, will trigger a series of stimulations, functions, and regulations characterized by their secreted cytokines. IL-10 will serve to block the NF-κB pathway and downregulate interleukin-1 (IL-1), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), IFN-γ, and blocks interleukin-12 (IL-12); in this manner, it will downregulate pro-inflammatory cytokines within the cytokine storm. IL-10, when combined with Interleukin-18 (IL-18), can increase the proliferation, cytotoxicity, and production of IFN-γ of NK cells and play a role in stimulating IFN-γ production of CD8 T cells. IL-10 is an influential factor for the growth and differentiation of mastocytes cells, thymocytes, and B cells. On the other hand, IFN-γ, an essential antiviral cytokine in the immune response to viral infections, would expand CD8 T cells' cytotoxicity function and promote macrophage activation and amplify antigen presentation through APCs; and (c) These Th1 cells, with their potential, could recruit critical immune cells and cytokines to orchestrate a prepared defense to confront the infection caused by SARS-CoV-2. APC, antigen-presenting cell; IFN-γ, interferon-gamma; NF-κB, nuclear factor-Kappa B; NK, natural killer cell; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

1% penicillin/streptomycin (Thermo Fisher Scientific, USA), 5% AB serum (Thermo Fisher Scientific), 60 ng/mL IL-12 (Miltenyi Biotec, Germany) at 37°C, 5% CO_2 for 4–6 h. To study the secretion of IL-10, a parallel sample cultured without peptides and IL-12 as control must be performed. After stimulation, the cells will be labeled with the IL-10 Secretion Assay-Detection Kit Capture Reagent, human (Miltenyi Biotec, Germany) composed from antibodies specific for IL-10 secreted by the target cells. Therefore, after the secretion phase, the IL-10 bound to the cell surface could be attacked using the IL-10 PE antibody included in the kit for their respective magnetic separation; therefore, obtaining the target cells.

Cell irradiation

Harvested effector memory IFN-γ/IL-10 co-producing CD4 T Cells will be irradiated by the RS 1800 Q Biological Irradiator (Rad Source Technologies Quastar, USA) at 7.5 Gy, for the inhibition of cell proliferation. A dose of 0 Gy will be used as a control sample.

IFN-γ and IL-10 secretion assay

IFN-γ/IL-10 co-producing Th1 cells will be simultaneously analyzed for the production of two different cytokines by combining two different cytokine secretion assays, using the IFN-γ Secretion Assay Detection Kit (PE) (Miltenyi Biotec, Germany) and the IL-10 Secretion Assay Detection Kit (PE) (Miltenyi Biotec, Germany), following the manufacturer's protocol (Two-color Cytokine Secretion Assays, Miltenyi Biotec, Germany).

Cell proliferation assay

The CellTrace CFSE Cell Proliferation Kit (Invitrogen) (Thermo Fisher Scientific) will be used for flow cytometry, following the manufacturer's specifications to analyze cell proliferation.

TCRVβ spectratyping

For evaluation of T cell receptor (TCR) spectra, the complementary-determining region 3 (CDR3) coding region of the T cell receptor variable (TCRV) gene will be expanded using 24 specific primers of the TCRV subfamily and a primer conjugated per carboxyfluorescein region (FAM);[12] The PCR products will be denatured with Hi-Di formamide (Applied Biosystems, Carlsbad, CA) and electrophoresed along with the standard size Gene Scan-600 LIZ (Applied Biosystems) on a SeqStudio Genetic Analyzer (Applied Biosystems).[12]

Flow cytometry panels for immune cell purity and composition

To analyze the cell composition, two antibody panels must be performed with MACSQuant Analyzer (Miltenyi Biotec, Germany) using the express mode. The first panel, CCS_Immune_Cell_Composition_h_01, (Panel A) will be used to determine immune cell composition and target cell number with CD45-VioBlue, CD4-VioGreen, CD3-FITC, CD16/CD56-PE, CD19-PEVio770, CD14-APC, CD8-APCVio 770, and 7-AAD; the second panel, CCS_Purity_h_01, (Panel B) will be used to determine phenotype and frequency of target cells with CD45-VioBlue, CD3-FITC, IFN-γ-PE, CD45RO-PEVio770, CD62L-APC, and CD8-APCVio770.[12]

Evaluation of the hypothesis and the proposed methods

COVID-19 is a disease that remains a major global public health challenge.[1] Vaccine development and implementation is accelerating and the number of vaccines that are entering phase IV clinical trials is increasing;[23] however, there is currently no single effective treatment against COVID-19.[7] One of the strategies used to treat patients with COVID-19 has been CPT, which is composed of neutralizing antibodies specific for SARS-CoV-2.[7,24] The Food and Drug Administration (FDA) currently approves CPT for use in patients with COVID-19 for research purposes; observational studies have shown treated patients have significantly improved oxygen saturation, CRP, 30-day survival rate, and reduced need for mechanical ventilation.[7] However, CPT requires active compatible donors for urgent transfusion, which for some people could be difficult to obtain if a donor is not available at the right time.

T cell therapy might be a promising strategy when treating patients infected by SARS-CoV-2 based on the evidence and positive results of this therapy previously,[8,9,25,26] and could give us the possibility to create a repertory of cells samples, because they can be cultured and stored in a biobank.[27] Therefore, haploidentical patients might obtain a living drug to be treated quickly and efficiently, an idea that has initiated multinational research into this approach.[11–14] The recommended doses are approximately >2.5×10⁴/kg of the recipient's body weight for perfusion of virus-specific T cells in incompatible/haploidentical donors.[28] The use of third-party virus-specific T cells in biobanks is a feasible and safe method to treat severe viral infections rapidly; previous studies reflect the efficacy of virus-specific cells, even when combined with a single relevant human leukocyte antigen (HLA) allele with the receptor, as long as this allele presents antigens derived from viruses.[27]

The acute hyperinflammatory response, the phenomenon that has been implicated in critically ill patients that are infected with SARS-CoV-2,[15,16,29,30] could be regulated by IL-10; a cytokine that these proposed effector cells secrete. IL-10 is a mediator cytokine of the immune response; this inhibits the secretion of other pro-inflammatory cytokines, such as IL-1, IL-1α, IL-1β, IL-6, IL-8, IFN-γ, and TNF-α, and blocks IL-12.[16,31,32] In addition, the modulatory effect of IL-10 in the inflammatory responses is associated with the inhibition of the nuclear factor-kappa B (NF-κB) pathway.[33–36] The NF-κB coordinates the activation of numerous genes in response to pathogens and pro-inflammatory cytokines and is; therefore, critical in the development of acute and chronic inflammatory diseases.[34,36] In COVID-19, the NF-κB activation in various cells, such as macrophages of the lung, liver, kidney, central nervous system, gastrointestinal system,

and cardiovascular system leads to pro-inflammatory cytokines and chemokines production.[37] Various authors proposed that the inhibition of the NF-κB pathway as a potential target for therapeutic strategies to treat severe COVID-19.[37–39] IL-10 blocks NF-κB activity at two levels; through the suppression of I kappa B kinase (IKK) activity and through the inhibition of NF-κB DNA binding activity.[34]

These proposed cells secrete IFN-γ, which is an essential antiviral cytokine in the immune response to viral infections and eradicating pathogens.[16,19] In addition to its antiviral activity, IFN-γ has antifibrotic properties.[19] IFN-γ increases the destruction function of CD8 T cells by inducing major histocompatibility complex (MHC) class I; in addition, it improves the immune response by stimulating the MHC class II receptors.[40] IFN-γ amplifies antigen presentation through antigen-presenting cells (APCs) by enhancing antigen recognition via cognate T cell interaction.[40,41] It has been suggested that early intervention in antiviral infection using IFN-γ could be substantial in inhibiting fibrosis for better functional recovery;[19] and a study with results that were limited to a small number of patients obtained data on the positive effect of IFN-γ on the rate of clinical stabilization and recovery of patients with moderate COVID-19.[42]

IFN-γ/IL-10 coproducing CD4 T cells express T-box transcription factor (TBX21), which is known as T-box expressed on T cells (T-bet).[21,22,43] T-bet plays a critical role in defending against Vaccinia virus (VV) infection in mice; it is required to control IFN-γ production in CD4 T cells and natural killer (NK) cells.[43] NK cells play a central role in maintaining immune homeostasis, which is a critical requirement when facing the challenge of a novel pathogen; SARS-CoV-2 infection impedes NK cell function, and therefore, disrupts this vital balance.[42] IL-10 has previously been associated with the inhibition of NK cell production of IFN-γ due to its ability to suppress accessory cell production of pro-inflammatory cytokines.[44–46] However, IL-10 increases NK cell production of IFN-γ when combined with interleukin-18 (IL-18),[46] which is a cytokine presented in the cytokine storm in COVID-19.[47,48] In addition, IL-10 enhanced the ability of IL-18 to stimulate NK cell cytotoxicity and proliferation,[46] and plays a role on the stimulation of IFN-γ production by CD8 T cells.[16] IL-10 is an influential factor for the growth and differentiation of mastocytes, thymocytes, and B cells;[16] IL-10 supports the differentiation of B cells in the complete absence of IL-2.[49]

In the proposed process (Fig. 2), PBMCs samples will be extracted from convalescent patients (Fig. 2.1). Recovered COVID-19 patients consistently generated a substantial CD4 T cell response against SARS-CoV-2.[10] After extraction of the PBMCs, lymphocytes will be automatically processed with specific peptides of the SARS-COV-2 for IFN-γ secretion (Fig. 2.2), this process takes approximately 12 h, is fully automatic, and it requires limited human input for its development.[12] In brief, the cells will be isolated to obtain the effector memory CD4 T cells that secrets IFN-γ (Fig. 2.3) along with an automated magnetic cell separation with the autoMACS Pro Separator for fast and gentle isolation; therefore, the target cells will be separated without requiring density gradient centrifugation. In the re-stimulation process (Fig. 2.5), the cells will be re-stimulated with SARS-COV-2 peptides and IL-12; IFN-γ/IL-10 co-producing Th1 cells require a high dose induced phosphorylation of antigen and IL-12 induced signal transducer and activator of transcription 4 (STAT-4) activation.[50] After re-stimulation (Fig. 2.4), the harvest cells will be irradiated to ensure perfusion and attenuate the alloreactivity to avoid GvHD. 7.5 Gy was a safe dose used in previous studies to inhibit cell proliferation in treatments as donor lymphocyte infusion (DLI), 7.5 Gy likewise preserves the cells for their secretion
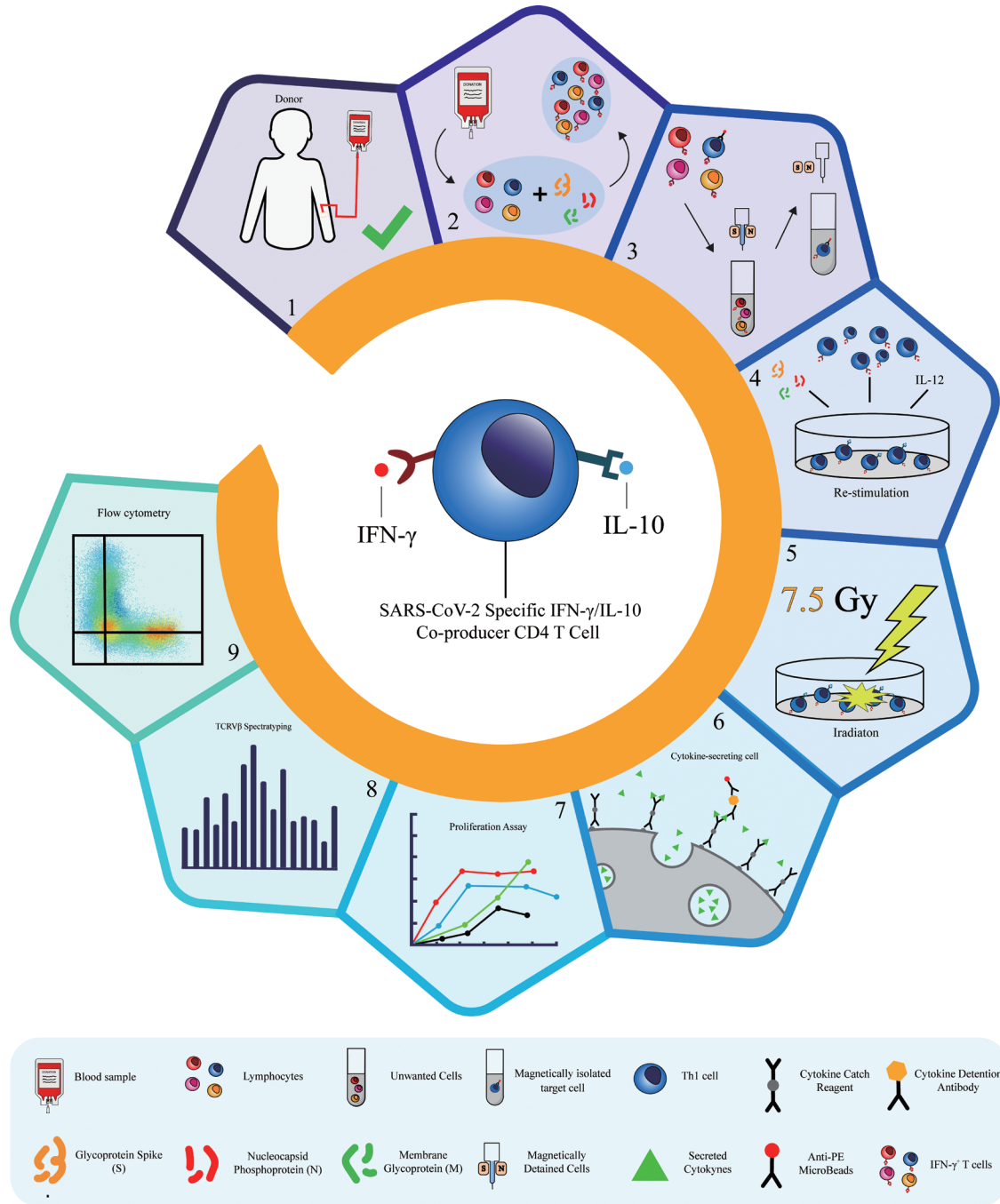
Fig. 2. Order of the study design methods, which consists of the following: (1) donor eligibility, where people who qualify for the study can provide blood samples; (2) automated cell processing to obtain SARS-CoV-2 specific IFN-γ T cells; (3) magnetic cell isolation to separate effector memory CD4 T cells; (4) re-stimulation of IFN-γ CD4 T cells with IL-12 and SAR-CoV-2 peptides for IL-10 secretion; (5) cell irradiation at 7.5 Gy; (6) IFN-γ and IL-10 secretion assay; (7) cell proliferation assay; (8) TCRVβ spectrometry analysis; and (9) flow cytometry panels to analyze the purity and composition of immune cells. IFN-γ, interferon-gamma; IL, interleukin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TCRV, T cell receptor variable.

of cytokines.[51]

Future directions

No data or results exist for the proposed idea; therefore, its de-velopment is required to determine its viability and initiate further research to study its therapeutic efficacy. This research idea implementation and therapy's direct practice will require an interdisciplinary approach, which should include but not be limited to biotechnologists, immunologists, geneticists, hematologists, oncologists, and other biomedical professionals.

Due to the global transmission of the virus, a large number of SARS-CoV-2 variants have appeared, especially the emerging strains that have been discovered in the UK (variant 20I/501Y.V1, lineage B.1.1.7), South Africa (variant 20H/501Y.V2, lineage B.1.351), Brazil (variant 20J/501Y.V3, lineage P.1),[52] California (variant 20C, lineage B.1.427/B.1.429),[53] India (variant G/452R.V3 or VUI-21APR-01, lineage B.1.617.1) (Kappa), and India (variant G/452R.V3 or VUI-21APR-02, lineage B.1.617.2) (Delta).[54] In addition to the peptides shown in the methods section of the research idea (PepTivator SARS-CoV-2 Prot_S B.1.1.7, Prot_N B.1.1.7, Prot_S B.1.351, Prot_S P.1, Prot_S B.1.427/B.1.429, Prot_S B.1.525, Prot_S B.1.617.1 and Prot_S B.1.617.2) (Miltenyi Biotec, Germany) could stimulate the target cells to broaden their specificity.

Conclusions

When facing a new virus, it is necessary to continue innovating the therapeutic strategies that have worked to treat previous viral infections, adapting to the pathogenesis of this new disease, to treat patients infected with SARS-CoV-2 effectively and safely. This proposal presents the research idea of creating SARS-CoV-2 specific IFN-γ and IL-10 co-producing CD4 T cells to determine its viability and cytokine secretion of the target cells. Target cell samples could be stored in a biobank; therefore, haploidentical patients might obtain the living drug to be treated quickly and efficiently. IFN-γ/IL-10 co-producing CD4 T cells have a synergistic function, and the effector role of this function is not affected by their IL-10 production,[21,22] which might amplify its feasibility to treat severe COVID-19 patients, because, they might eradicate the infection caused by SARS-CoV-2 and lead to the regulation of the immune system that has been altered by the cytokine storm. Therefore, it is essential to consider performing this study to provide new data that could result in a better strategy to treat COVID-19.

Acknowledgments

Funding

Conflict of interest

ANN registered the invention for a patent.

Author contributions

ANN was the only author.

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