NOVA SCHOOL OF SCIENCE & TECHNOLOGY

Department of Life Sciences

COVID-19 Vaccination

A study on red blood cells (RBCs) and their impact on the immune system

Beatriz de Matos Borrego Gil Ferreira

Bachelor's in Biology

Master's degree in Genetic and Molecular Biomedicine

NOVA University, Lisbon

2023, September

Department of Life Sciences

COVID-19 Vaccination

A study on red blood cells (RBCs) and their impact on the immune system

Beatriz de Matos Borrego Gil Ferreira

Bachelor's in Biology

Supervisor: Ph.D. Deborah Penque, from National Institute of Health Doctor Ricardo Jorge

Co-supervisor: Ph.D. Paula Videira, from NOVA School School of Science and Technology University

The jury was composed as follows:

President: Ph.D. Pedro Manuel Brôa Costa, Assistant Professor at the Faculty of Sciences and Technology of NOVA University of Lisbon.

Examiner: Ph.D. Hugo Miguel Baptista Carreira dos Santos, Assistant Researcher at the Faculty of Sciences and Technology of NOVA University of Lisbon.

Supervisor: Ph.D. Deborah Penque, Principal Researcher at the Dr. Ricardo Jorge National Institute of Health.

Master's degree in Genetic and Molecular Biomedicine

NOVA University, Lisbon

2023, September

Acknowledgments

I would like to express my heartfelt gratitude to all those who have contributed to the successful completion of this master's thesis.

First and foremost, I am deeply thankful to Dr. Deborah Penque for her invaluable guidance, unwavering support, and insightful mentorship throughout this journey. Her expertise and dedication have been instrumental in shaping this work. I am, also, immensely grateful to the rest of the team at the Laboratory of Proteomics at the National Institute of Health Dr. Ricardo Jorge, where a significant part of this thesis was conducted. The laboratory's collaborative spirit, camaraderie, and shared knowledge have greatly enriched my research experience. My sincere thanks to Fátima Vaz, Cristina Coelho, Sofia Neves, and Joana Saraiva, for their assistance, discussions, and camaraderie.

Equally, I extend my appreciation to Professor Paula Videira and the colleagues at the Glycoimmunology Laboratory at the Faculty of Sciences and Technology, Nova University of Lisbon. Their guidance, encouragement, and collaborative efforts have been invaluable in shaping part of this thesis. I would like to express my gratitude to Vanessa Luz, Rita Lourenço, Patricia Mexia, Joana Cunha, Mariana Barbosa, Beatriz Pereira, Salvador Sobral, Pedro Granjo, and Zélia Silva, for their support.

I extend my special thanks to Professor Marília Antunes from the Faculty of Sciences, University of Lisbon, for her expert assistance in statistical analysis. Her guidance has been pivotal in analyzing and interpreting the data effectively.

I also want to thank the 39 patients and donors who generously contributed their blood samples, without whom this project would not have been possible. Your contribution is deeply appreciated and has significant implications for advancing scientific knowledge.

Lastly, I would like to acknowledge my friends and family for their unwavering support, patience, and encouragement throughout this journey. Your belief in me has been a constant source of motivation.

In conclusion, this work stands as a testament to many individuals' collective efforts, support, and encouragement. Thank you all for being an integral part of this endeavor.

Abstract

Red blood cells (RBCs) are emerging as regulators of the innate immune response by interacting with inflammatory molecules, including cytokines/chemokines, nucleic acids, and pathogens, thereby modulating immune responses.

This thesis investigated how RBCs impact immune responses after COVID-19 vaccination, focusing on (a) cytokine profiles in RBCs and their conditioned media (RBC-CM) before and after vaccination; (b) RBC effects on peripheral blood mononuclear cells (PBMCs) activity, especially T-cell expansion.

The initial phase optimized the ELISA assay for TNF-α, establishing Lysis Method 2 and a 1000 µg total protein extract as optimal parameters. TNF-α presence was confirmed in both RBCs and RBC-CM.

IL-1β, IL-6, IL-12, IL-15, and TNF-α cytokines were, then, analyzed in RBCs and RBC-CM samples, from healthy subjects (n=8) across different time points (T0-T4) of COVID-19 vaccination (Biobank-COVID-19 vaccines). The results revealed that these proinflammatory cytokines are bound to or can be released from cultured RBCs in varying amounts in response to vaccination. Similar temporal modulation in plasma following COVID-19 vaccination has been described for these cytokines, suggesting an interaction between plasma and RBC might exist in response to vaccine-induced proinflammatory cytokine signaling.

The subsequent phase delves into RBC samples (T0-T4) and PBMC interactions in cell culture. ELISA assays measuring IL-12, IL-15, TNF-α, and IFN-γ in culture medium showed that RBCs presenting differential cytokine profiles in response to vaccination can in turn differentially modulate PBMC cytokines secretion *in vitro* with potential downstream consequences in the immune activity of these cells.

Preliminary flow cytometry results suggested a specific T-cell subpopulations expansion in response to vaccination in the presence of RBCs.

This study provides initial insights into cytokine changes in RBCs post-vaccination and their influence on PBMC cytokine response and T-cell subpopulation expansion. Further research confirming these findings will be necessary to better understand the complex interplay that might exist between RBCs and immune cells during vaccination-induced immunization.

Keywords: RBC, RBC-CM, COVID-19 Vaccination, Immune System, Modulation, and Cytokines.

Resumo

Os glóbulos vermelhos (GVs) têm surgido como reguladores da resposta imunitária inata, interagindo com moléculas inflamatórias, incluindo citocinas/quimiocinas, ácidos nucleicos e patógenos, influenciando as respostas imunológicas.

Neste estudo, investigámos o impacto dos GVs nas respostas imunitárias após a vacinação COVID-19, focando-nos: (a) nos perfis de citocinas dos GVs e dos seus meios condicionados (GV-CM) antes e após a vacinação; (b) nos efeitos dos GVs na atividade das células mononucleares do sangue periférico (CMSP), especialmente na expansão das células T.

Na primeira fase, otimizamos a técnica ELISA para TNF-α nos GVs e nos GV-CMs, estabelecendo parâmetros como o Método de Lise 2 e a quantidade de 1000 µg de proteína total nos GVs. Presença de TNF-α foi confirmada em ambas as amostras.

IL-1β, IL-6, IL-12, IL-15 e TNF-α foram analisadas por ELISA, nas amostras de GV e GV-CM de indivíduos saudáveis (n=8), em diferentes momentos (T0-T4) da vacinação COVID-19 (Biobanco de Vacinas-COVID-19). Estas citocinas pró-inflamatórias mostraram estar ligadas aos GVs ou a serem libertas em quantidades variáveis em resposta à vacinação. No plasma, esta modulação temporal após a vacinação COVID-19 já foi observada, sugerindo uma possível interação entre o mesmo e os GVs em resposta à sinalização pró-inflamatória induzida pela vacina.

Posteriormente, avaliaram-se as interações entre as amostras de GV (T0-T4) e as CMSP em cultura celular. A análise de IL-12, IL-15, TNF-α e IFN-γ no meio de cultura indicou que os GVs com diferentes perfis de citocinas em resposta à vacinação podem modular a secreção de citocinas pelas CMSP *in vitro*, afetando potencialmente a atividade imunitária dessas células.

Resultados preliminares da citometria de fluxo sugeriram uma expansão específica de subpopulações de células T em resposta à vacinação na presença de GV.

Este estudo fornece uma perspetiva inicial sobre as alterações das citocinas nos GV após a vacinação e o seu impacto na resposta das CMSP. No entanto, é necessário validar estas descobertas e compreender melhor a complexa interação entre os GV e as células do sistema imunitário durante a imunização induzida pela vacinação contra a COVID-19.

Palavras-chave: GV, GV-CM, vacina COVID-19, Sistema Imune, Modulação e Citocinas.

Contents

Acknowledgments ---------- **vii**

Abstract ---------- **ix**

Resumo ---------- **xi**

Contents ---------- **xiii**

List of Figures ---------- **xv**

List of Tables ---------- **xxi**

Abbreviations ---------- **xxiii**

Symbols ---------- **xxv**

I Introduction ---------- **1**

1. Immune System ---------- 1

1.1 Innate immune system ---------- 1

1.2 Adaptive/specific immunity ---------- 4

2. Red blood cells (RBCs) ---------- 8

2.1 RBCs and the immune system ---------- 8

II Aim ---------- **13**

III Methods and Materials ---------- **15**

1. Reagents, kits, and antibodies ---------- 15
2. Samples ---------- 16
3. Cell culture between PBMCs and RBCs lysates or RBC-CM ---------- 21
4. Sample Treatment after Cell Culture between PBMCs and RBC Lysates or RBC-CM- 23

4.1 PBMCs and Supernatant ---------- 23

4.2 PBMC Lysis ---------- 23

5. Quantification of the total protein concentration ---------- 24

5.1 For RBCs lysates ---------- 24

5.2 For PBMC lysates and RBC-CM ---------- 24

6. Protein detection – Cytokine profile analysis by Sandwich Enzyme-Linked Immunosorbent Assays (ELISAs) ---------- 25

6.1 ELISA Technique Overview ---------- 25

6.2 ELISA in RBC Lysates and RBC-CM ---------- 26

6.3 ELISA in the supernatant/medium from the cell culture between PBMC and RBCs lysates or RBC-CM ---------- 27

7. Flow cytometry in PBMCs resulted from the cell culture with the RBCs lysates – a Preliminary Optimization Study ---- 27
7.1 Technique introduction ---- 27
7.2 Viability and Population Study ---- 28
8. Statistical Analysis ---- 30
IV Results ---- 31
1. Cytokine profile analysis of red blood cells (RBCs) and RBC-conditioned media (RBC-CM) ---- 31
1.1 ELISA – optimization assay ---- 31
1.2 Assay - Cytokine profile modulation in RBCs and RBC-CM under effect of COVID-19 vaccination ---- 33
2. Immunomodulatory role of RBCs in the immune system, namely in peripheral blood mononuclear cells (PBMCs) ---- 37
2.1. Sandwich ELISAs - Analysis of the cell culture supernatant of PBMCs incubated with RBCs or RBC-CM ---- 37
2.2 Flow cytometry – Analysis of PBMC populations, in particular, T-cell subpopulations ---- 43
V Discussion ---- 47
Cytokine profiles bound or released by RBCs are modulated in response to the COVID-19 vaccine ---- 47
Immunomodulatory role of RBCs in the immune system ---- 54
VI Conclusions and Future Perspectives ---- 61
VII Bibliography ---- 63

List of Figures

Figure 1. Representation of an example of the interaction between a type of PAMP (pathogen-associated molecular pattern), in this case, LPS, and the surface receptor present in the macrophages, which can be described as a pattern recognition receptor (PRR). This results in the release of the cytokines Interleukin-8 (IL-8), Interleukin-1 (IL-1), and Tumor Necrosis Factor-α (TNF-α), which allows for phagocytic cells, like neutrophils, to enter from blood vessels into the affected tissue. (Source: [3]) 2

Figure 2. Missing-self recognition of target cells. The activating and inhibitory receptor signaling regulates the natural killer (NK) cells' activation. Cells undergoing stress such as tumor cells lose their MHC class I molecules, a ligand for inhibitory receptors on NK cells. At the same time, they acquire stress-associated molecules which act as ligands for activating receptors. Thus, the lack of inhibitory signaling coupled with the induction of activating signaling shifts the balance toward NK cell activation, leading to the secretion of cytokines and the killing of tumor cells. (Source: [5]) 4

Figure 3. Antigen-specific T-cell activation requires three distinct signals: signal 1 is antigen-specific signaling mediated by T-cell receptor (TCR) engagement with the major histocompatibility complex (MHC) molecules, which possesses the pathogenic peptides, signal 2 is costimulatory signaling, mainly mediated by the interaction of CD28 with one of the B7 molecules (CD80 and CD86); and, finally, signal 3 that is polarizing signaling mediated by various cytokines produced by dendritic cells, like IL-12. (Source: [7]) 5

Figure 4. Differentiation of $CD4^+$ T-cells into specialized Th1 or Th2 effector cells. Upon activation with a specific antigen, $CD4^+$ T-cells proliferate and differentiate into either the Th1 or the Th2 subset. Th1 cells promote cellular immunity and are involved in the development of autoimmune diseases, secreting major levels of proinflammatory cytokines (IFN-γ, Interleukin-2 (IL-2), and TNF- α (the same as LT)); Th2 cells mediate humoral immunity and are involved in allergic immune responses, producing more of anti-inflammatory cytokines (Interleukin-4 (IL-4) and Interleukin-5 (IL -5). (Source: [8]) 6

Figure 5. B-cells can, regardless of the presence of APCs, activate their function through direct contact with the pathogen, through their PAMPs. Thus, releasing specific antibodies against the pathogen in question. (Source: [12]) 7

Figure 6. Illustration of RBCs (Red Blood Cells) in circulation. It´s possible to see their cell surface receptors, responsible for their interaction with different inflammatory molecules, including cytokines/chemokines, nucleic acids, and pathogens. (Source: [16])..... 9

Figure 7. Isolation of RBCs and/or Peripheral mononuclear blood cells (PBMCs) from a buffy coat or a blood sample. The figure demonstrates the peripheral blood diluted with PBS (or DPBS) 1X is layered over the Ficoll-Paque (left side) and followed by centrifugation, the blood components are separated into plasma, peripheral blood mononuclear cells, and erythrocytes (right side). (Source: [41])..... 16

Figure 8. Scheme of the RBCs lysis selected method, using the lysis buffer based on sodium phosphate buffer and distilled water, in equal proportions to one another. 18

Figure 9. Scheme of the 24-well culture plaque, used for the optimization studies (n=1). Per condition, there were technical triplicates. In the A-line, the wells with the minus (-) signal represent a control, where only PBMCs were present, whereas the plus (+) signal represents another control, with PBMCs and the stimuli (PMA + Io). For the other lines (B-D), PBMCs and stimuli were present, together with RBCs' lysates (B and C lines) and with RBCs' conditioned media (D line): in the B-line, the proportion PBMC: RBC was 1:100; in the C-line, the proportion PBMC: RBC was 1:10; lastly, in the D-line RBC-CM was collocated, at a 15 µg of protein extract, per well..... 22

Figure 10. **Calibration curve for the total protein quantification (µg/mL) –** example**.** 24

Figure 11. Procedure for the Sandwich ELISA immunoassay used for the analysis of the cytokine profile observed in the different samples. The method goes as follows: 1 is the microplate coating, with the capture antibody (overnight); 2 represents the connection between the capture antibody and the specific antigen (in this case, the wanted cytokine); in 3 we observe the addition of the detection antibody biotinylated, which binds to the same antigen; 4 is when the Streptavidin-HRP; and, finally, 5 represents the addition of TMB (chromogenic substrate, sensitive for HRP) and the "colored product", which in this study was sulfuric acid, is considered also a stop solution. In the end, the microplate is read at a high-performance spectrophotometer (SpectraMax 190 Microplate Reader, Molecular Devices) at a wavelength of 450 nm. (Source: [43]) 25

Figure 12. Flow cytometry experiments involve multiple stages, including sample preparation, instrument setup, data acquisition, and data analysis. Sample preparation, often beginning with Ficoll gradient separation and staining with fluorescent antibody conjugates, can

introduce variability. Instrument setup, focusing on adjusting photomultiplier tube voltages for optimal sensitivity, is another potential source of variation. Data acquisition captures fluorescence emissions from bound antibodies, with instrument differences impacting variability. Finally, data analysis defines cell populations of interest and contributes significantly to overall variability. (Source: [44])..... 28

Figure 13. Flow cytometry – compensation phase. Eppendorf tubes containing, in each one, 1×105 non-stimulated PBMCs. (A) only unstained PBMCs; (B) PBMCs with the antibody CD8-PE; (C) PBMCs stained with only CD3-APC; (D) PBMCs with 7-AAD..... 29

Figure 14. Flow cytometry – optimization assay. Eppendorf tubes with 1×105 PBMCs each. In the blue ones (E, F, and G), only non-stimulated PBMCs were present, in contrast with the ones represented in red (H, I, and J) where only stimulated PBMCs. In both situations, there were 3 settings, where E and H only had unstained PBMCs present; in F and I, PBMCs had only 7AAd; and, in G and J, PBMCs were stained with all the antibodies (CD8-PE, CD3-APC, and 7AAd). Furthermore, the scheme represented by the red Eppendorf tubes was repeated for each one of these conditions: PBMCs only stimulated with PMA and Io; stimulated PBMCs that were in culture with RBCs' lysates from T0; and stimulated PBMCs that were in culture with RBCs' lysates from T1..... 29

Figure 15. Cytokine profiles in RBCs and RBC-CM in response to COVID-19 vaccination. (A, C, E, G, and I) RBCs and (B, D, F, H, and J) RBC-CM samples were analyzed by Sandwich ELISA for the following cytokines: (A and B) Interleukin-1β (IL-1β), (C and D) Interleukin-6 (IL-6), (E and F) Interleukin-12 (IL-12), (G and H) Interleukin-15 (IL-15), and (I and J), and Tumor Necrosis Factor-α (TNF-α). The longitudinal time points considered during COVID-19 vaccine immunization were T0: before vaccination, T1: 24h-72h after the first vaccine dose, T2: before the second vaccine dose, T3: after the second vaccine dose, and T4: after one month of the last vaccine dose. Data was analyzed by statistical Linear Mixed Model (LMM). Statistically significant differences between pre-COVID-19 vaccination (T0) and post-vaccination time points (T1, T2, T3, and T4) are indicated by: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$. Between T1 and T2 these differences are denoted by # $p<0.001$ and ## $p<0.0001$, and between T3 and T2 or T4 by ^ $p<0.001$ and ^^ $p<0.0001$..... 35

Figure 16. **PBMCs cytokines secretion under the effect of RBCs and RBC-CM from subjects undergoing COVID-19 vaccination –optimization study.** RBC (T0 and T1) and RBC-CM (T0 and T3) samples from one subject (n=1, Patient ID: 136) that underwent COVID-19 vaccination were selected from the Biobank, lysed by Method 2 and incubated in triplicate (technical triplicates) with PBMCs under stimulation of phorbol myristate acetate (PMA) and Ionomycin

(Io). Different ratios of PBMC: RBC were tested (1:10 and 1:100). The cell culture was conducted over a 5-day incubation period at 37°C, 5% CO2, and 99% humidity. After incubation, the cell culture medium was recovered and analyzed by sandwich ELISAs for (A) Interleukin-12 (IL-12); (B) Interferon-gamma (IFN-γ); and (C) Tumor Necrosis Factor-α (TNF-α). Non-stimulated and stimulated PBMCs without RBC/RBC-CM lysates were used as controls. Regarding the time points: T0: before COVID-19 vaccination; T1: 24h-72h after the first COVID-19 vaccine dose; T3: 24h-72h after the second COVID-19 vaccine dose. The statistical test employed for these findings was the Paired T-test. Statistically significant discrepancies between the PMA+Io condition and the other conditions are denoted as follows: ● $p<0.05$, ●● $p<0.01$, ●●● $p<0.001$, and ●●●● $p<0.0001$. Statistically significant differences between pre-COVID-19 vaccination (T0) and post-vaccination time points (T1, and/or T3) are indicated by: Δ $p<0.05$, ΔΔ $p<0.01$, ΔΔΔ$p<0.001$, and ΔΔΔΔ $p<0.0001$. Finally, significant differences between the proportions PBMC: RBC, 1:10 and/or 1:100, belonging to the same time point, are represented by: □ $p<0.05$, □□ $p<0.01$, □□□ $p<0.001$, and □□□□ $p<0.0001$..... 39

Figure 17. PBMC cytokines secretion resulted from the presence, in culture, of RBCs from subjects undergoing COVID-19 vaccination, in histogram form– main study. RBC samples collected from eight subjects (n=8) who received COVID-19 vaccination were chosen from the Biobank. These samples were subjected to lysis using method 2 and were then cultured with PBMCs stimulated with phorbol myristate acetate (PMA) and Ionomycin (Io). The time points correspond as follows: T0 represents the pre-vaccination period; T1 and T3 indicate time intervals of 24-72 hours after the first and second vaccine doses, respectively; T2 signifies one month following T1; and T4 corresponds to the time point 30 days after T3. The histograms (A-D) illustrate the PBMC-produced cytokines: (A) Interleukin-12 (IL-12); (B) Interleukin-15 (IL-15); (C) Tumor Necrosis Factor-α (TNF-α); and (D) Interferon-gamma (IFN-γ). The statistical test employed for these findings was the Paired T-test. Statistically significant discrepancies between the PMA+Io condition and the other conditions are denoted as follows: ● $p<0.05$, ●● $p<0.01$, ●●● $p<0.001$, and ●●●● $p<0.0001$..... 40

Figure 18. PBMCs cytokines secretion under the effect of RBCs from subjects undergoing COVID-19 vaccination– main study. RBC (T0-T4) samples of eight subjects (n=8) that underwent COVID-19 vaccination were selected from the Biobank, lysed by Method 2 and incubated with PBMCs under stimulation of phorbol myristate acetate (PMA) and Ionomycin (Io). T0 corresponds to the period before COVID-19 vaccination; T1 and T3 denote intervals of 24-72 hours after the first and second doses of the COVID-19 vaccine, respectively; T2 represents one month after the T1; and finally; and T4 corresponds to the time point 30 days after T3. (A-D) represents the production of these cytokines, by PBMCs: (A) Interleukin-12 (IL-12); (B)

Interleukin-15 (IL-15); (C) Tumor Necrosis Factor-α (TNF-α); and (D) Interferon-gamma (IFN-γ). Data was analyzed by statistical Linear Mixed Model (LMM). Statistically significant distinctions between the pre-COVID-19 vaccination phase (T0) and the post-vaccination time points (T1-T4) are indicated by: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$. Furthermore, significant disparities between T1 and T2 are represented by # $p<0.05$, ## $p<0.01$, ### $p<0.001$, and #### $p<0.0001$, and, between T3 and T2 or T4 by ^ $p<0.05$, ^^ $p<0.01$, ^^^ $p<0.001$, and ^^^^ $p<0.0001$. 41

List of Tables

Table 1. Representation of some of the main proinflammatory cytokines secreted during inflammation, and their functions. Note: NK cells stand for Natural killer cells, which are lymphocytes of the innate immune system. Based on the work of Chen et al. (2015) [4]. 3

Table 2. Representation of the equivalencies between the number of cells/RBCs and the total protein extract (µg) of the correspondent cell lysates, for n=3 (mean ± s.d. (n)). The selected values for the number of cells to analyze are from studies such as Karsten et al. (2020) and Arosa et al. (2011) [26, 34]. 18

Table 3. Demographic information about eight patients (n=8) whose blood samples were analyzed in this study. BMI stands for Body Mass Index; SMH for Smoking Habits; PIC19 for Previous Infection with COVID-19; and CMB for Comorbidities. 20

Table 4 TNF-α ELISA optimization assay for RBCs from three biological replicates (n=3), not included in the Biobank-COVID-19 vaccines..... 32

Table 5. TNF-α ELISA assay optimization for RBC-conditioned media (RBC-CM) from patients belonging to the Biobank-COVID-19 vaccines. 33

Table 6. Average quantification (n=8) of IL-1β, IL-6, IL-12, IL-15, and/or TNF-α cytokines (pg per one mg of total protein) in RBC lysates and/or RBC-Conditioned media (RBC-CM). The values are similar to the ones expressed in Figure 15. 35

Table 7. Averages (n=8) of cytokine quantifications (IL-12, IL-15, TNF-α, and/or IFN-γ) for the cell culture supernatant in this study (pg /mg of total protein). These values closely align with those depicted in Figure 18. 42

Table 8. Representation of the quantification of proinflammatory cytokines in pg/mL of whole blood. Comparison between the Karsten et al. (2018) [22] study and our study, for the RBC samples lysates from T0 time point (pre-vaccination). 51

Abbreviations

7-AAD - 7-Aminoactinomycin D (fluorescent dye)

APC – Allophycocyanin (fluorescent dye)

APCs - Antigen Presenting Cells

B-cells - Lymphocytes B

BSA - Bovine serum albumin

CD - Cluster of Differentiation

cf-mtDNA - Mitochondrial DNA

COVID-19 - Coronavirus disease 2019

DARC - Duffy antigen receptor for cytokines/chemokines

DCs - Dendritic Cells

DPBS - Dulbecco's phosphate-buffered saline

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

EVs - Extracellular vesicles

FBS - Fetal bovine serum

GYPA - Glycophorins A

HLA - Human leukocyte antigen

HRP - Horseradish peroxidase

ID: Identification

i.e.- “id est” in Latin, means "that is."

IL - Interleukin

IFN-γ - Interferon- γ

MVs - Microvesicles

NK cells - Natural Killer cells

NO - Nitric Oxide

PAMPs - Pathogen-associated Molecular Patterns

PBS - Phosphate-Buffered Saline

PE - Phycoerythrin (fluorescent dye)

PMA - Phorbol-12-myristate-13-acetate

PGE - Prostaglandin

RBCEVs - RBC-derived EVs

RBC - Red Blood Cells

RBC-CM - RBC- Conditioned Media

RNA - Double-stranded Ribonucleic Acid

RPMI - Roswell Park Memorial Institute

rpm - Revolutions Per Minute

s.d - Standard Deviation

TCR - T-cell receptor

Th or TH - T-helper type ($CD4^+$ cells)

TNF-α or β - Tumor Necrosis Factor- α or β

TLR9 - Toll-like receptor 9

T-cells – Lymphocytes T

TMB - 3,3′,5,5′-Tetramethylbenzidine

Symbols

°C – Celsius degrees

α - Alpha

β - Beta

γ - Gamma

I | Introduction

Having in mind our aim, it was decided that it was better to do a little introduction about the immune system, in the first place. Followed by an introduction to red blood cells (RBCs) and their, already-known, impact on the immune system.

1. Immune System

When an intruding pathogen is capable of passing the anatomic (skin and mucous membrane) and physiologic (temperature, low pH, and chemical mediators) defensive barriers, there is an initial immune response, that consists of three main reactions: the first one is the recognition of the pathogen by macrophages, which results in the activation of the innate immunity; the second one is based on the dendritic cells (DCs), which, in a simple manner of speaking, collect antigens from the pathogen and goes to the lymphatic system to track down lymphocytes (T- and B- cells), activating the specific/adaptative immune system; and the third one, which consists on the activation of the complement system, directly by via the lectin pathway or the alternative pathway.

In this chapter innate and adaptive immunity will be the main focus.

1.1 Innate immune system

The innate immune system activates within minutes or hours after aggression. This response can be divided into two situations, depending on the quantity of the intruding pathogens.

In both situations, normally, the macrophages are the first immune cells to respond to intruders. They recognize, through various types of surface receptors (including Toll-like receptors), the specific characteristics that occur in the pathogens and not in the “normal/healthy” cells. These characteristics are called pathogen-associated molecular patterns (PAMPs).

Examples of these include bacterial cell wall components such as lipopolysaccharides (LPS) and double-stranded ribonucleic acid (RNA) produced during viral infection (Figure 1) [(1)]. After this recognition, phagocytosis happens to destroy the pathogens.

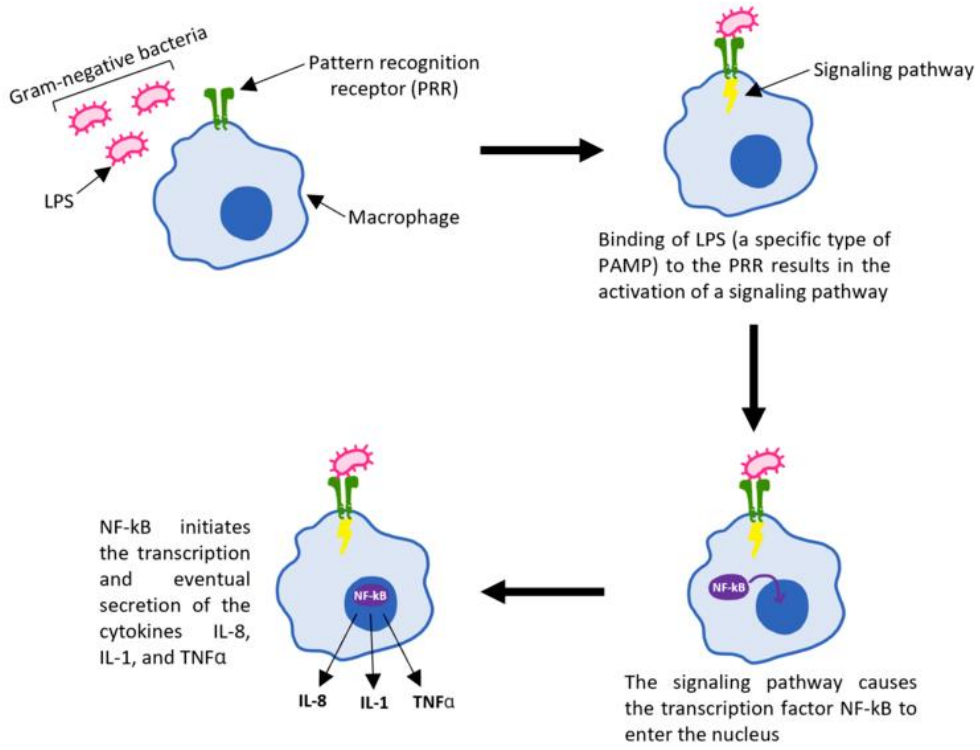

Figure 1. Representation of an example of the interaction between a type of PAMP (pathogen-associated molecular pattern), in this case, LPS, and the surface receptor present in the macrophages, which can be described as a pattern recognition receptor (PRR). This results in the release of the cytokines Interleukin-8 (IL-8), Interleukin-1 (IL-1), and Tumor Necrosis Factor-α (TNF-α), which allows for phagocytic cells, like neutrophils, to enter from blood vessels into the affected tissue. (Source: [3])

When there is only a single intruder, this method is sufficient for the protection of the organism. However, when there are multiple pathogens, macrophages need support. Because of that, they release cytokines and chemokines (small signaling proteins involved in cell–cell communication and recruitment), allowing the start of the inflammation (Figure 1). This consists of the recruitment of more phagocytes, which are subdivided into two main cell types: neutrophils and macrophages. Both cells share a function: to phagocytose microbes and kill them. Unlike neutrophils (which are short-lived cells), macrophages are long-lived cells [2].

In addition, the inflammatory response also involves other processes that help to contain and fight the infection, like vasodilation, increased vascular permeability, mast cell activation and degranulation (resulting in the release of more cytokines that further intensifies the inflammation process), activation of the clotting system and Kynan system.

Furthermore, inflammation stimulates the phagocytes to secrete cytokines, initiating the acute phase response, and leading to a more systemic inflammatory state. Some of the most common cytokines present in this phase are Interleukins such as, Interleukin-1 (IL-1), Interleukin-2 (IL-2), Interleukin -6 (IL-6), Interleukin-8 (IL-8), Interleukin-12 (IL-12), and Interleukin-15 (IL-15). Tumor Necrosis Factor-α (TNF-α), is another common cytokine released during inflammation. These are, thereby, considered proinflammatory cytokines (Table 1).

Table 1. Representation of some of the main proinflammatory cytokines secreted during inflammation, and their functions. Note: NK cells stand for Natural killer cells, which are lymphocytes of the innate immune system. Based on the work of Chen et al. (2015) [4].

Cytokine	Family	Main Sources	Function
IL-1β	IL-1	Macrophages, monocytes	Pro-inflammation, proliferation, apoptosis, differentiation
IL-6	IL-6	Macrophages, T-cells, adipocyte	Pro-inflammation, differentiation and cytokine production
IL-8	CXC	Macrophages, epithelial cells, endothelial cells	Pro-inflammation, chemotaxis, angiogenesis
IL-12	IL-12	Dendritic cells, macrophages, neutrophils	Pro-inflammation, and differentiation, activates NK cells
TNF-α	TNF	Macrophages, NK cells, CD4+ T-helper cells, adipocytes	Pro-inflammation, proliferation, apoptosis, cytokine production, and anti-infection
IL-2	**IL-2**	**Predominately secreted by CD4+ and CD8+ T-cells**	**Pro-inflammation, and T- cell proliferation**
IL-15	**IL-2**	**Macrophages, monocytes, dendritic cells, epithelial cells**	**Pro-inflammation, proliferation, activates T- and NK cells**

In addition to phagocytes, in the innate immune system, there are more acting immune cells important for the good response of this system [2]. One of them is the Natural killer cells (NK cells), which are considered cytotoxic lymphocytes, but instead of acting in the specific immune response, they act in the innate response [5]. They are non-specific cells and participate in early defense against foreign cells and autologous cells undergoing various forms of stress, such as microbial infection or tumor transformation, with a special taste for cells infected with a virus.

So, whereas phagocytes act by migrating to infected areas and by ingesting and killing microorganisms, NK cells induce apoptosis in “abnormal” cells, preventing viruses and cancer cells from spreading. Activated NK cells also release cytokines, summoning other white blood cells.

Their mechanism of action is like the $CD8^+$ cytotoxic T-cells. They interact directly with the infected cell, by verifying the surface receptors, mainly the major histocompatibility complex (MHC)-1. Since, if the cell is not healthy, MHC-1 will be different, activating the NK cells’ function (Figure 2).

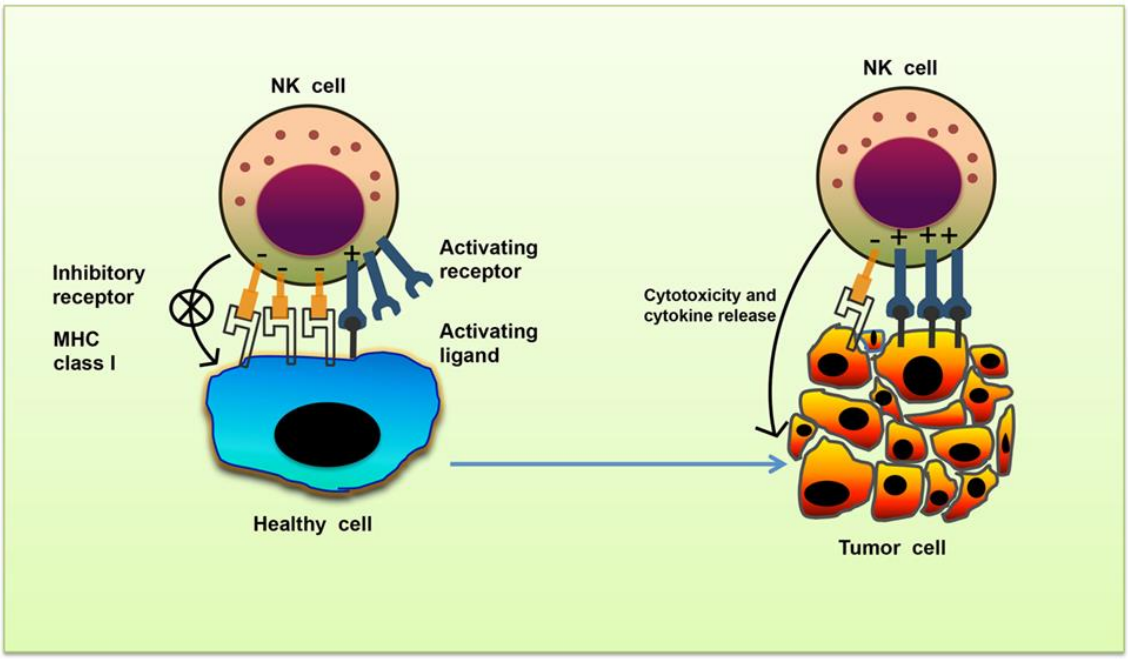

Figure 2. Missing-self recognition of target cells. The activating and inhibitory receptor signaling regulates the natural killer (NK) cells' activation. Cells undergoing stress such as tumor cells lose their MHC class I molecules, a ligand for inhibitory receptors on NK cells. At the same time, they acquire stress-associated molecules which act as ligands for activating receptors. Thus, the lack of inhibitory signaling coupled with the induction of activating signaling shifts the balance toward NK cell activation, leading to the secretion of cytokines and the killing of tumor cells. (Source: [5])

1.2 Adaptive/specific immunity

When innate immunity is ineffective in eliminating pathogenic agents, there is the development of adaptive/specific immunity [2]. Involves two types of lymphocytes, T-cells, and B-cells. These cells are free to be in the lymphatic system or blood circulation. But normally, they are situated in the lymph node and the mucosa-associated lymphoid tissue.

As said previously, each pathogen has a specific antigen profile on its membrane surface. On the other hand, each T-cell has surface receptors specific to a single type of antigen. B-cells also have specific antibodies on the surface that correspond to a specific single antigen. This results in the existence of millions of T- and B-cells specific for each antigen.

The fundamental difference between T-cells and B-cells is based on the fact that to recognize a specific antigen and to be activated, T-cells require the action of antigen-presenting cells (APCs), usually dendritic cells (DCs), but also macrophages, B-cells (sometimes), fibroblasts, and epithelial cells [2,6].

This happens because APCs, on their surface, express the major histocompatibility complex (MHC), normally the 2, where fragments of antigens are present when a cell comes in contact with the intruder (Figure 3) [7,8].

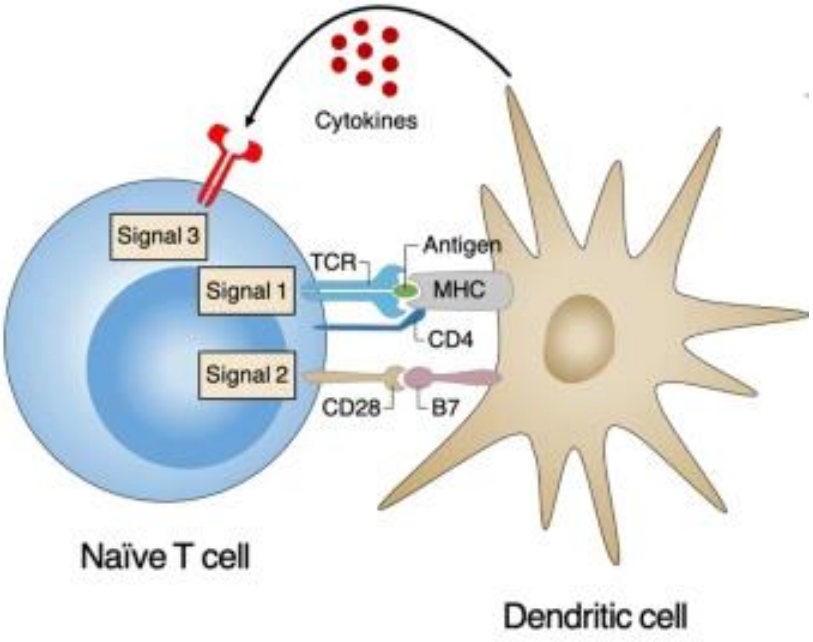

Figure 3. Antigen-specific T-cell activation requires three distinct signals: signal 1 is antigen-specific signaling mediated by T-cell receptor (TCR) engagement with the major histocompatibility complex (MHC) molecules, which possesses the pathogenic peptides, signal 2 is costimulatory signaling, mainly mediated by the interaction of CD28 with one of the B7 molecules (CD80 and CD86); and, finally, signal 3 that is polarizing signaling mediated by various cytokines produced by dendritic cells, like IL-12. (Source: [7])

The MHC-antigen complex activates the TCR (T-cell receptor), which results in the secretion of cytokines (for example: Interferon-γ (IFN-γ)) whose main role is to control the immune response. In addition, there are other two possible signals, beyond just the MHC presentation, capable of activating the T-cells, such as the costimulatory signaling, which is mainly mediated by the interaction of CD28 (Cluster of Differentiation 28) with one of the B7 molecules (CD80 and CD86); and/or the polarizing signaling mediated by various cytokines produced by dendritic cells (for example: IL-12) (Figure 3).

The antigen presentation process stimulates T-cell differentiation into either cytotoxic T-cells ($CD8^+$ cells) or T-helper (Th) cells ($CD4^+$ cells) [8].

1.2.1 T-cells (T-lymphocytes)

1.2.1.1 T-helper (Th) cells ($CD4^+$ cells)

The T-helper cells, after activated, can differentiate into two types of subsets called T-helper type 1 (TH1) and T-helper type 2 (TH2) cells, based on their production of certain cytokines.

TH1 cells secrete major levels of proinflammatory cytokines (resulting in greater recruitment of immune cells, such as macrophages), such as interferon-γ (IFN-γ), interleukin-2 (IL-2) and Tumor Necrosis Factor- α or -β (TNF-α/β).

They are essential for protection against a variety of intracellular infections. Whereas TH2 responses can be protective mostly against certain extracellular infections, producing predominantly cytokines with an anti-inflammatory effect, such as interleukin-4 (IL-4) and interleukin-5 (IL-5), recruiting lesser macrophages (Figure 4) [(10)].

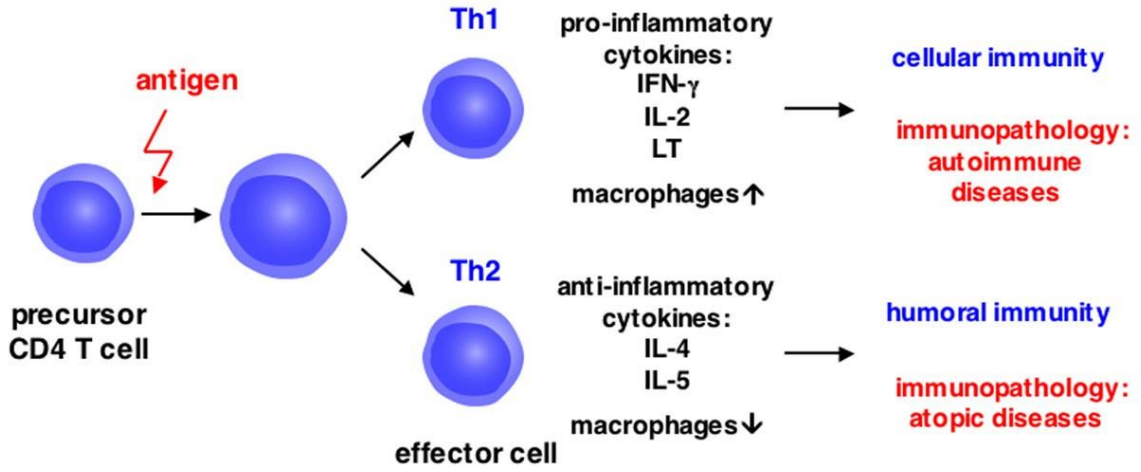

Figure 4. Differentiation of $CD4^+$ T-cells into specialized Th1 or Th2 effector cells. Upon activation with a specific antigen, $CD4^+$ T-cells proliferate and differentiate into either the Th1 or the Th2 subset. Th1 cells promote cellular immunity and are involved in the development of autoimmune diseases, secreting major levels of proinflammatory cytokines (IFN-γ, Intereleukin-2 (IL-2), and TNF- α (the same as LT)); Th2 cells mediate humoral immunity and are involved in allergic immune responses, producing more of anti-inflammatory cytokines (Intereleukin-4 (IL-4) and Intereleukin-5 (IL -5). (Source: [(8)])

In addition, T-helper cells, mainly TH1, play critical roles in controlling viral infections by promoting $CD8^+$ T-cell responses, since they secrete cytokines responsible for $CD8^+$ T-cell proliferation and differentiation into cytotoxic T-cells.

On the other hand, T-helper cells, mostly TH2, can also promote humoral immunity, by producing cytokines responsible for the B-cells' proliferation and differentiation into plasma cells, which can release large quantities of antibodies, and differentiation into memory B-cells, which are part of the immune memory to respond quickly to future infections for that specific pathogen (Figure 4).

These cells can also migrate to areas of infection and secrete cytokines that help to recruit and activate phagocytes in the infected tissue.

1.2.1.2 Cytotoxic T-cells ($CD8^+$ cells)

$CD8^+$ T-cells mediate their effector functions through the production of cytokines such as IFN-γ and TNF-α, and/or by cytolytic mechanisms.

Cytotoxic T-cells, after activated, are responsible for killing cells that have been infected by pathogens such as virally infected cells. To do that, they need to attach themselves to the HLA (Human leukocyte antigen) class 1 receptor, using the T-cell receptor, that expresses the antigen protein.

Once they are bound, they have two mechanisms of acting: the first is called granule exocytosis, where they "spray" the infected cell with perforin, which creates an opening in the target cell so the lymphocyte can insert granzymes, killing the cell; the second one is based on the activation of a fast molecule, causing the apoptosis of the infected cell [(11)].

1.2.2 B-Cells (B-lymphocytes)

B-cells, on the other hand, do not need the APCs to activate. The antigen-binding receptor of B-cells recognizes and binds to an antigen in its native form, initiating the production of antibodies, as represented in Figure 5.

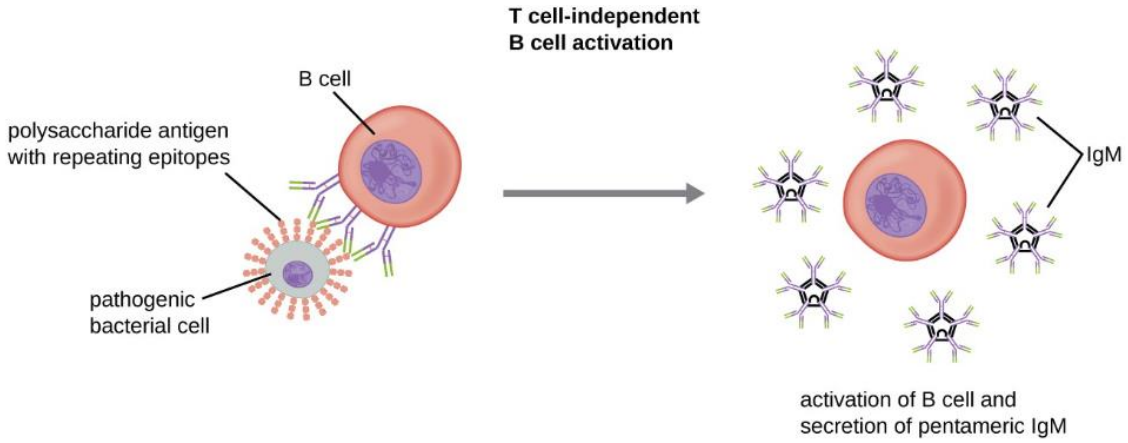

Figure 5. B-cells can, regardless of the presence of APCs, activate their function through direct contact with the pathogen, through their PAMPs. Thus, releasing specific antibodies against the pathogen in question. (Source: [(12)])

The secreted antibodies bind to antigens on the surface of pathogens, flagging them for destruction through complement activation, opsonin promotion of phagocytosis, and pathogen elimination by immune effector cells [(7)]; they can also attach to the released toxins (neutralizing their effect) and attach to the healthy cells' receptors (preventing the viral infection); in addition, the antibodies may clump together to slow down the spread of the pathogen (agglutination).

What was written before, in the last topics about B-cells and T-cells, can be related to the definition of humoral or antibody-mediated immunity and cell-mediated immunity, respectively.

The first one is the responsibility of B-cells, mostly. The last one is through the activation of antigen-specific cytotoxic T-cells, macrophages, and NK cells [7]. This type of immunity is most effective in eliminating virus-infected cells and cancer cells, but can also participate in defending against fungi, protozoa, cancers, and intracellular bacteria [12].

2. Red blood cells (RBCs)

Red blood cells (RBCs), or erythrocytes, are considered the most common type of cell in the blood, with each cubic millimeter of blood containing 4-6 million RBCs [13].

Every second, 2-3 million of these cells are produced in the bone marrow and released into circulation [13].

Erythropoiesis is the process that produces RBCs. After the final stage of this process, known as terminal erythropoiesis, the enucleated reticulocytes are then released into the bloodstream to complete their maturation process [13].

After a lifespan of approximately 120 days, RBCs are cleared by macrophages in the spleen and liver [14].

Normally, erythrocytes are mainly associated with the transportation of oxygen and carbon dioxide between the tissues and lungs, maintaining the acid-base balance of the organism too. In addition, they are also considered important modulators of nitric oxide (NO) metabolism and, via their concentration (hematocrit), are also seen as participating in the control of blood rheology, defining its viscosity [14].

2.1 RBCs and the immune system

However, RBCs are emerging as important modulators of the innate immune response. Depending on the conditions of the microenvironment, erythrocytes may either promote immune activation or maintain immune quiescence [15].

The reason for this lies in the fact that, despite their deletion of organelles during erythropoiesis and subsequent inability to carry out transcription and translation, mammalian erythrocytes retain the ability to bind and interact with a variety of inflammatory molecules — including cytokines/chemokines, nucleic acids, and pathogens — thereby regulating and modulating immune responses (Figure 6).

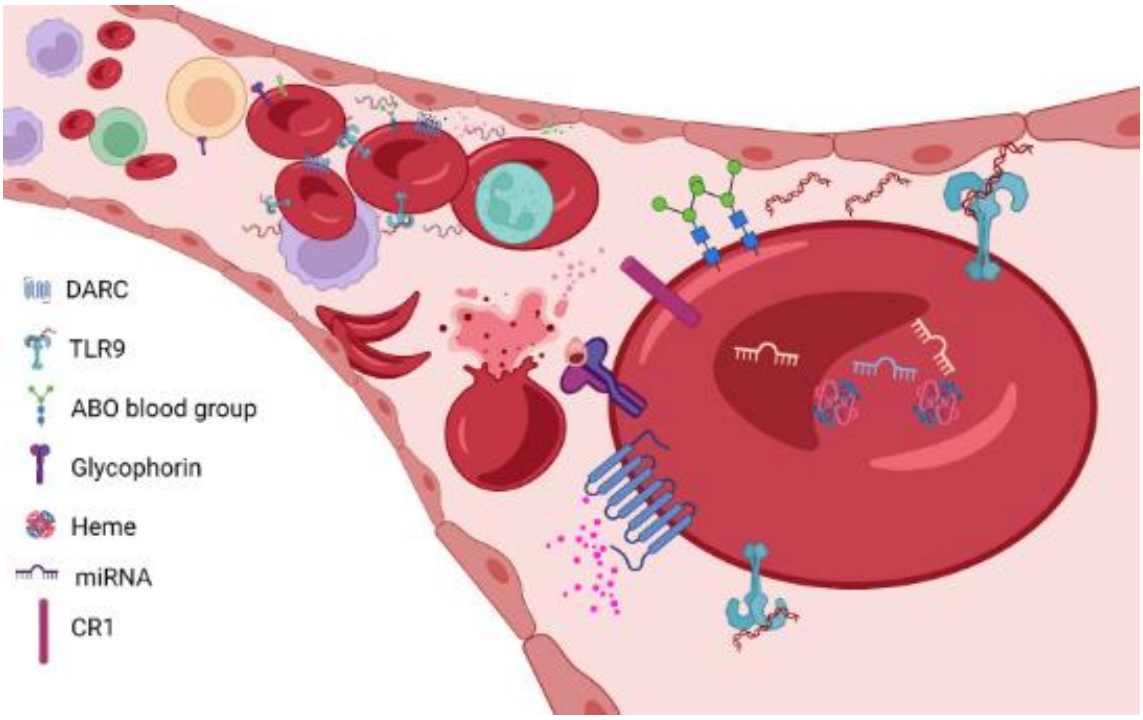

Figure 6. Illustration of RBCs (Red Blood Cells) in circulation. It´s possible to see their cell surface receptors, responsible for their interaction with different inflammatory molecules, including cytokines/chemokines, nucleic acids, and pathogens. (Source: **(16))**

This is possible because these RBCs express a larger number of cell surface receptors, compared to the non-mammalian vertebrates' RBCs, since in these animals the RBCs are nucleated, allowing the production of inflammatory mediators, which possibilities the regulation of the immune response [(16)].

2.1.1 RBCs and DNA molecules

In a 2018 study, Mangalmurti's team found that RBCs harbor a type of molecular sensor, known as Toll-like receptor 9 (TLR9), which recognizes and scavenges DNA molecules containing multiple pairs of the nucleotide bases cytosine and guanine, or CpG motifs, including mitochondrial DNA (cf-mtDNA) [(17, 18)].

This type of DNA can be released by damaged human cells and is characteristic of bacteria and other pathogens too, like malaria. If this DNA binds to the RBCs in the bloodstream, it leads to their phagocytosis by macrophages and to innate immune activation in pathological settings [(18)].

In addition, these processes can lead to the development of anemia and induction of both local and systemic inflammation, with the constant secretion of proinflammatory cytokines, like interleukin-6 (IL-6), as demonstrated in a study about critically ill sepsis patients [(19)]. This study

also described the same premises for critically ill coronavirus disease 2019 (COVID-19) patients with pneumonia, where the levels of cf-mtDNA-bound erythrocytes are directly associated with anemia and disease severity [19].

2.1.2 RBCs and cytokines/chemokines

Another important form of the immune mechanism of the erythrocytes is the proven existence of the Duffy antigen receptor for cytokines/chemokines (DARC) on the membrane of the RBC, recognized first by Darbonne and colleagues in 1991[20]. The same study also concluded that cytokine/chemokine binding is superficial and easily reversible.

At present, there are two models as to why RBCs regulate blood cytokine/chemokine levels: the sink model (consists of the fact that, for example, these cells bind to cytokines/chemokines to diminish immune-activating signals, to dampen immune response via attenuation of neutrophil signaling) and the "reservoir" hypothesis (where is dictated that RBCs' binding prevents cytokine/chemokine clearance, thereby prolonging its half-life in the blood) [20].

While these models appear contradictory at first, they may not be mutually exclusive: Fukuma et al. in 2003, suggested that erythrocytes do scavenge cytokines/chemokines from sites of inflammation, but eventually release them in response to decreases of their concentration in the plasma, effectively maintaining homeostasis [21].

Additionally, in a 2018 study by Karsten *et al*., the list of signaling molecules identified was increased to 42 cytokines present in RBCs' lysates at concentrations ≥4-fold higher than plasma per volume of whole blood [22].

2.1.3 RBCs and pathogens

In addition to the RBCs' affinity for endogenous molecules like DNA molecules and cytokines/chemokines, these cells also bind to pathogens.

The genus *Plasmodium*, a group of protists responsible for causing malaria, is a known example of a pathogen that binds to human erythrocytes [17]. This happens because the Glycophorins A (GYPA) (one of the major sialoglycoproteins of the human erythrocyte membrane) may act as a "decoy receptor," chaperoning pathogens away from important tissues and into the spleen to facilitate their destruction by macrophages [23].

Furthermore, it is also known that in erythrocytes, hemoglobin and heme trigger the production of reactive oxygen species (ROS) to destroy and eliminate hemolytic pathogens. These components of RBCs also promote inflammation and auto-immune responses.

2.2 RBCs and induction of vascular dysfunction – acute inflammation

In a recent study about COVID-19, another mechanism associated with RBC immune function was identified, which is the induction of vascular dysfunction [24].

In this study, the authors conclude that RBCs may be carriers of IFN-γ during acute inflammatory states that may drive vascular dysfunction, resulting in endothelial-dependent and endothelial-independent relaxation of tissues.

As stated by the authors, these findings deserve further investigation since IFN-γ is a well-known inducer of endothelial cytotoxicity.

2.3. RBCs and the regulation of the immune cells

The RBCs in the immune system are increasingly recognized, as it was shown that RBCs from healthy individuals regulate T-cell activity, in particular its proliferation and apoptosis, through cytokine interaction modulation [25].

Karsten et al. (2020) demonstrated that RBCs exposed to a cancer line in culture (modified RBCs) had a cytokine profile different from the RBCs not exposed to this cancer cell line. The incubation of healthy RBCs pre-exposed to cancer cells or RBCs obtained from cancer patients with healthy T-cells resulted in differences in the proliferation/ survival of the T-cells (Jurkat cells and from peripheral blood mononucleated cells (PBMCs)) but also in their cytokines expression profile [26].

In this study, RBCs were also shown to stimulate the release of proinflammatory cytokines by T-cells in a dose-dependent manner [26].

In another research, it was verified that RBCs isolated from patients with carotid atherosclerosis differ from RBCs isolated from healthy individuals in that they have a more attenuated capacity to protect T-cells from apoptosis [27].

Besides, in different studies it was also demonstrated that RBCs may influence the maturation of monocytes, especially into DCs, which are APCs, having a significant influence on the T-cells' activation **[28,29].**

2.3.1 RBCs' released vesicles and soluble factors – impact on the immune response

RBCs can release extracellular vesicles (EVs) including both endosome-derived exosomes and plasma-membrane-derived microvesicles (MVs). RBC-derived EVs (RBCEVs) are secreted during erythropoiesis, physiological cellular aging, disease conditions, and in response to environmental stressors [30].

The immunomodulatory effect of RBCEVs was analyzed by *in vitro* studies, which suggested that mixing RBCEVs with PBMCs, for example, causes the secretion of proinflammatory chemokines and cytokines, and increases the survival of unstimulated PBMCs [31]. In addition, it was also proven that these vesicles amplified the replication of mitogen-induced $CD4^+$ and $CD8^+$ T-lymphocytes in an antigen-presenting cell-dependent manner.

Furthermore, another study by Fisher et al. (2017) also demonstrated the existence of an increased interaction of platelets with neutrophils and monocytes upon incubation with RBCEVs [32,33].

Altogether these studies suggested that RBCEVs have an important immunomodulatory role in the immune system.

In contrast, Arosa et al. (2011), demonstrated that RBC-derived soluble factors rather than RBCEVs hold bioactivities capable of modulating T-cell growth, proliferation, and survival [34]. Other studies are also in agreement with Arosa's group observation [25,34,35,36].

Some evidence indicates that RBC contains inactive molecules that under stimulus are ready for rapid release in active forms. For example, Oonishi et al. (1998), showed that after mechanical stress, Prostaglandin E1 (PGE1) and E2 (PGE2) were detectable in RBC cytosols [37].

Interestingly, PGE2 mediates inflammation whereas PGE1 acts as an anti-inflammatory factor. Reinforcing the fact that there are soluble molecules released by RBCs, that can influence the immune response.

Further studies are thus needed to better clarify the different impacts of RBCEVs and RBC-released soluble molecules on the immune response.

2.4. RBCs and COVID-19 infection

Under COVID-19 infection the RBC proteome, metabolome, and lipidome can be significantly modified, which, eventually, has an impact on the immune function. It will be important to analyze, in future studies, whether these alterations are generalizable to other infectious and inflammatory conditions [38,39,40].

II Aim

Although the role of RBCs in the immune system is well documented, the putative impact of RBCs on the immunization process triggered by vaccines remains to be investigated. [40,34].

Taking as a model the emergent COVID-19 vaccines, the main aim of this project is to investigate whether vaccines induce modifications in the RBCs' cytokine profile capable of affecting the immune cells activity, T-cells (present in the peripheral blood mononucleated cells (PBMCs) isolated from a buffy coat), as a mechanist test for vaccine immunization regulated by RBCs.

III | Methods and Materials

1. Reagents, kits, and antibodies

RPMI-1640 base medium (Gibco, New York, United States of America (USA)), Fetal bovine serum (FBS) (Sigma Aldrich, Missouri, USA / Gibco), L-glutamine or GlutaMAX (Invitrogen, California, USA/Gibco), Non-essential amino acids (NEAA) (Gibco / Invitrogen), Sodium pyruvate (Gibco / Invitrogen), Penicillin/streptomycin (Gibco / Invitrogen). Phosphate-buffered saline, a buffer solution (PBS), and Dulbecco's phosphate-buffered saline (DPBS). Bovine serum albumin (BSA) (Invitrogen), Ficoll-Hypaque (composition: 9,1% w/v Sodium Diatrizoate, 5.7% w/v Polysaccharide, water. Density of 1.077 g/ml (Stemcell Technologies, Oslo, Norway). Distilled water (dH2O), RBC's lysis buffer (sodium phosphate buffer with dH2O, pH=7.4), PierceTM IP lysis buffer (Thermo Fisher Scientific, Massachusetts, USA), IP (proteases' inhibitor), buffy coat, Ionomycin (Io, from Streptomyces conglobatus, Sigma Aldrich), PMA (phorbol-12-myristate-13-acetate, Sigma Aldrich). ELISA kits for Interleukin -6, Interleukin -12, Interleukin-15 and Interferon-γ (ImmunoTools, Friesoythe, Germany). ELISA kits for Interleukin -1β and Tumor necrosis factor- α(Invitrogen). DMSO (Dimethyl sulfoxide, Sigma Aldrich). Antibodies CD8-PE (Immunotools), CD3-APC (Immunotools), and 7-AAD (7-Aminoactinomycin D, Sigma Aldrich). Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), Pierce™ 660nm Protein Assay Reagent (Thermo Fisher Scientific), TMB ELISA Substrate Solution (Thermo Fisher Scientific), Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set (Thermo Fisher Scientific), Tween 20, 10% (Thermo Fisher Scientific). Sulfuric acid 2 mM. Complete RPMI-1640 medium, which is constituted by: RPMI-1640 base medium, 10% FBS, 2mM L-glutamine (or GlutaMAX), 1% NEEA, 1% sodium pyruvate, and 100 µg/mL penicillin/streptomycin.

2. Samples

2.1 Red Blood Cells (RBCs)

2.1.1 Optimization Assay

In the optimization assay, three distinct blood samples (n=3) were utilized, sourced from female donors, with an age range between 30 and 65 years, who had been inoculated with COVID-19 vaccines, more than six months before sample collection.

These samples were used to establish optimal parameters, including the total protein extract concentration (µg) equivalent to a specific number of RBCs, and to determine the optimal laboratory procedure for RBC lysis.

2.1.1.1 RBC isolation from peripheral blood

The isolation of RBCs from whole blood involved standard Ficoll-Paque density gradient separation.

Briefly, 5 mL of whole blood in a 15 mL Falcon tube was mixed with an equal volume of Dulbecco's phosphate-buffered saline (DPBS) 1X. The diluted blood was gently transferred to a tube containing 4 mL of Ficoll, followed by centrifugation for 25 minutes at 800xg and 18°C (without brake) (see Figure 7).

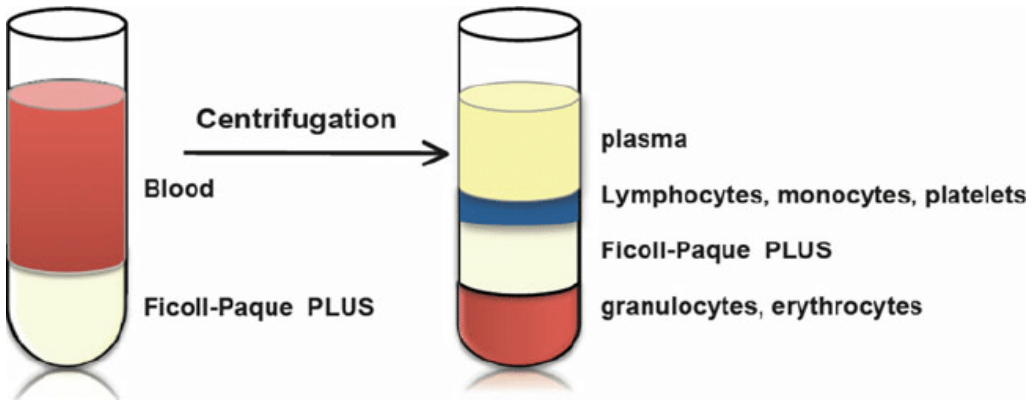

Figure 7. Isolation of RBCs and/or Peripheral mononuclear blood cells (PBMCs) from a buffy coat or a blood sample. The figure demonstrates the peripheral blood diluted with PBS (or DPBS) 1X is layered over the Ficoll-Paque (left side) and followed by centrifugation, the blood components are separated into plasma, peripheral blood mononuclear cells, and erythrocytes (right side). (Source: [41])

RBCs were then transferred to a new Falcon tube, and washed with DPBS 1X, added until final volume of 14 mL. Next, the samples were centrifuged at 250xg and 4°C, for 10 minutes, and the supernatant was discarded. This was repeated one more time, for a total of two washes.

Afterward, the supernatant was discarded, and a complete RPMI-1640 medium was added to a final volume of 10 mL, and the RBCs were counted using Neubauer's chamber.

For the optimization assay, the cell counting had the aim of making three defined cell numbers, through dilutions, derived from the literature [26,34] (see point 2.1.1.3, Table 2).

Following cell counting, the samples were centrifugated at 13,000 rpm for 5 minutes at 4°C. The supernatant was discarded, and the RBCs were frozen at -80°C.

2.1.1.2 RBC lysis

The RBC lysis procedure involved the evaluation of two distinct methods to determine the one yielding optimal results with ELISA (see point 6.1.1).

- Method 1

In this method, the lysis buffer consisted of a 2:1 ratio of sodium phosphate buffer (5 mM, pH 7.4) and distilled water. Frozen RBC samples stored at -80°C were thawed on ice, and 50 µL of these samples was added to the lysis buffer, at a proportion of 1:10. After pipetting (1x) and vortexing, the samples underwent sonication for 1 minute. Subsequently, they were centrifuged at 16,000xg for 30 minutes at 4°C, and the supernatant was collected and stored at -20°C.

- Method 2

In comparison to the above, in this method, the lysis buffer consisted of a 1:1 ratio of sodium phosphate buffer (5mM, pH 7.4) and distilled water, as depicted in Figure 8.

Following pipetting (10x) and intermittent vortexing, the samples were centrifuged at 2,000xg for 5 minutes at 4°C. The supernatant was collected and stored at -20°C.

Lysis Method 2 was selected as it yielded superior results, as detailed in the "Results" chapter (Figure 8).

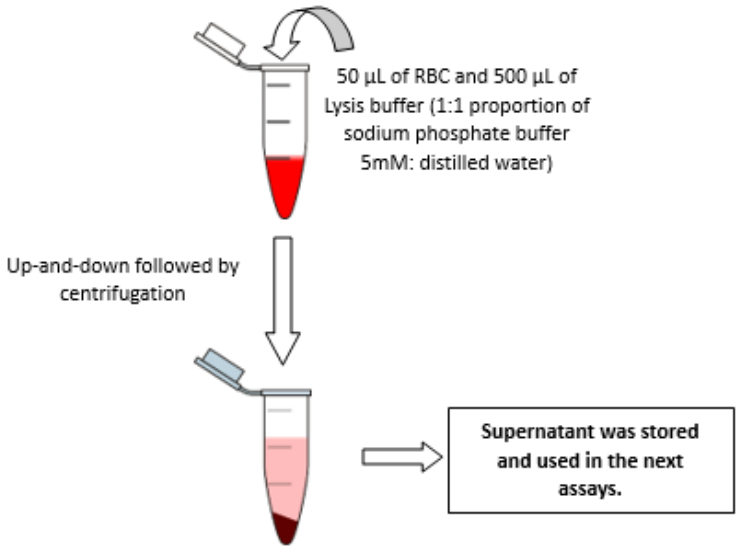

Figure 8. Scheme of the RBCs lysis selected method, using the lysis buffer based on sodium phosphate buffer and distilled water, in equal proportions to one another.

2.1.1.3 Equivalence Between Cell Number and Protein Extract

To establish a predetermined equivalence between the number of cells/RBCs and the total protein extract (µg), cell counting was performed following RBC isolation for the optimization study.

The total protein content in their lysate was quantified, and the results are presented in Table 2.

Table 2. Representation of the equivalencies between the number of cells/RBCs and the total protein extract (µg) of the correspondent cell lysates, for n=3 (mean ± s.d. (n)). The selected values for the number of cells to analyze are from studies such as Karsten et al. (2020) and Arosa et al. (2011) [26, 34].

Number of cells/RBCs	Total protein extract (µg) #
5×10^5 RBCs	11,63 µg ± 1,85 (3)
1×10^6 RBCs	25,04 µg ± 2.04 (3)
5×10^6 RBCs	133,63 µg ± 2.72 (3)

 # represents the average (mean ± s.d. (n=3)) of the total protein extract (µg) for a specific number of RBCs.

The final total protein extract (µg) for 1×10^6 RBCs was determined by averaging the values for total protein corresponding to 5×10^5 RBCs, 1×10^6 RBCs, and 5×10^6 RBCs, as shown in Table 2. This calculation resulted in an average total protein extract of 25.01 µg ± 2.12431 for 1×10^6 RBCs.

2.1.2 Assays

2.1.2.1 Study Population and Biobank

After establishing optimal parameters, subsequent studies focused on analyzing samples from vaccinated patients, which were obtained from a pre-existing Biobank (Biobank-COVID-19 vaccines) affiliated with the proteomics laboratory at the National Institute of Health Ricardo Jorge (INSA), as part of the COVID-19 Vaccination approved project by the Etic Commission on 13 of April of 2021: "Molecular impact of COVID-19 vaccines: a proteomic and metabolomic profiling of immune response regulated by erythrocytes " (Ref INSA: 2021DGH2016).

The Biobank was created by longitudinally collecting blood samples from 39 subjects who received two doses of COVID-19 vaccination between April and September 2021. In this study, only eight distinct biological samples were examined.

Longitudinal collection encompassed five time points: T0, T1, T2, T3, and T4. T0 and T2 represented pre-vaccination time points, while T1 and T3 corresponded to 24-72 hours post the first (1^{st}) and second (2^{nd}) vaccine doses, respectively. T4 indicated the time point 30 days after the last vaccination.

Demographic information for the eight patients (n=8) analyzed in this study is detailed in Table 3. All these patients received the Pfizer mRNA-based vaccine.

Table 3. Demographic information about eight patients (n=8) whose blood samples were analyzed in this study. BMI stands for Body Mass Index; SMH for Smoking Habits; PIC19 for Previous Infection with COVID-19; and CMB for Comorbidities.

Patient ID	Age	Sex	BMI	SMH	PIC19	CMB
118	44	F	23,795	0	0	0
122	41	F	25,970	1	0	0
123	39	F	20,077	0	0	0
124	34	F	23,634	0	0	0
126	41	F	24,435	0	0	0
127	43	F	30,110	0	0	0
134	33	F	22,313	0	0	0
136	31	F	18,750	0	0	0

2.1.2.2. RBC isolation and lysis

The procedure for isolating RBCs from blood samples obtained from COVID-19 vaccinated patients followed the same protocol as outlined in the optimization assays (see point 2.1.1.1).

Furthermore, the selected method for lysing these cells was Method 2, as previously described (point 2.1.1.2).

2.1.2.3 RBC-Conditioned media (RBC-CM)

To obtain RBC-CM, 500 µL of freshly isolated RBCs were directly placed into Petri dishes containing 30 mL of complete RPMI-1640 medium. The resuspension was made using a pipette. Subsequently, the Petri dishes were incubated at 37°C, 5% CO2, and 99% humidity for 48 hours to 72 hours. RBC-CM was collected after two successive centrifugations at 1,700g for 10 minutes to remove red blood cells. The resulting RBC-CM was then frozen at -80°C in 1.5 mL Eppendorf tubes.

For the assays, these samples were also lysed using Method 2, above mentioned (point 2.1.1.2).

2.2 Peripheral mononuclear blood cells (PBMCs)

2.2.1 PBMCs isolation from a buffy coat

PBMCs were isolated from buffy coat samples, which are concentrated leukocyte fractions obtained from healthy volunteers, provided and ethically approved by the Portuguese Blood Institute (*Instituto Português do Sangue e da Transplantação, IPST*).

The procedure closely followed Diana Sousa master's thesis [42] method, with some modifications.

Key differences were as follows, reduced time of the second centrifugation step to 20 minutes after mixing samples with Phosphate-buffered saline (PBS) 1X and Ficoll. PBMCs were collected, transferred to a new tube, and washed once at 600xg for 10 minutes at 24°C. After discarding the supernatant, the PBMC pellet was resuspended in 10 mL of PBS 1X, and cell counting was performed using an automated cell counter (EVE™) with trypan blue solution. Finally, the samples were centrifuged at 400xg for 5 minutes at 24°C and cryopreserved.

2.2.2 Cryopreservation of PBMCs

After the last centrifugation in the isolation of PBMCs, the supernatant was removed, and the pellet was resuspended in RPMI GlutaMAX medium supplemented with 20% FBS and 10% DMSO. The cell suspension was aliquoted into 2.0 mL cryovials, placed in a "Mr. Frosty" container, and stored at -80°C.

2.2.3 Thawing and counting of the PBMCs

To thaw cryopreserved PBMCs, multiple vials were defrosted simultaneously, with each vial receiving 500 µL of pre-warmed complete RPMI-1640 medium. After gentle pipetting, all PBMCs from individual vials were pooled in a 15 mL Falcon tube and topped up with medium to a total volume of 10 mL Followed by centrifugation at 400xg for 5 minutes at 24°C. The supernatant was discarded, and an additional 10 mL of medium was added to the PBMC pellet. Cell counting was done in the same way as previously mentioned (point 2.2.1).

3. Cell culture between PBMCs and RBCs lysates or RBC-CM

For the cell culture, in a 24-well culture plate, each well contained, except for the control wells (+ and -), 1×10^6 PBMCs combined with the chosen stimulants, PMA (Phorbol 12-

myristate 13-acetate) and Io (Ionomycin). In addition, in each of these wells RBC lysates and/or RBC-CM were added, following the specific assay conditions. The PBMC to RBC ratio varied based on the assay type.

As mentioned before, two control groups were included: one with only PBMCs (-) and one with PBMCs and stimuli (+).

a) Optimization Assays

These assays involved a single patient (n=1, Patient ID: 136). Time points T0 and T1 were selected for RBC lysates, while T0 and T3 were chosen for RBC-CM. Technical triplicates were used for optimization. Two PBMC to RBC ratios were tested: 1:10 and 1:100. Considering that each well contained 1×10^6 PBMCs, these ratios corresponded to 250 µg and 2500 µg of protein extract, respectively, with the knowledge that 1×10^6 RBCs represented an average of 25 µg of total protein extract (Table 2). Figure 9 represents the culture plaque scheme.

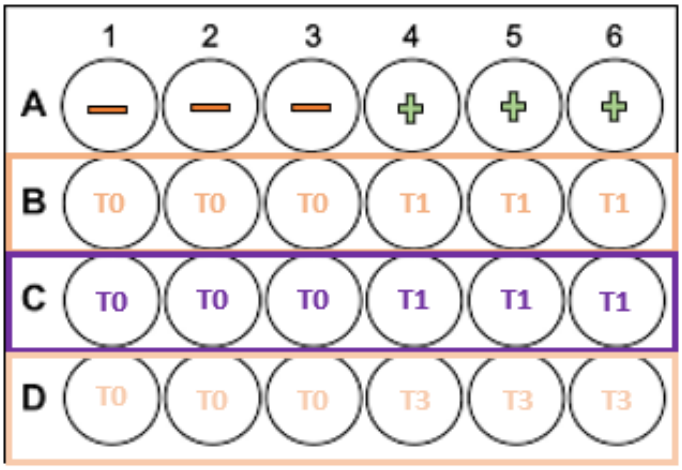

Figure 9. Scheme of the 24-well culture plaque, used for the optimization studies (n=1). Per condition, there were technical triplicates. In the A-line, the wells with the minus (-) signal represent a control, where only PBMCs were present, whereas the plus (+) signal represents another control, with PBMCs and the stimuli (PMA + Io). For the other lines (B-D), PBMCs and stimuli were present, together with RBCs' lysates (B and C lines) and with RBCs' conditioned media (D line): in the B-line, the proportion PBMC: RBC was 1:100; in the C-line, the proportion PBMC: RBC was 1:10; lastly, in the D-line RBC-CM was collocated, at a 15 µg of protein extract, per well.

b) Main Assay

Building on the optimization studies, the culture between PBMCs and RBC lysates was conducted at a 1 PBMC:10 RBC ratio. For this assay, all eight patients (n=8) were analyzed at five time points (T0-T4). As in (a) included, two control groups were integrated: one with only PBMCs (-) and one with PBMCs and stimuli (+).

For both assay types (a) and (b), incubation conditions remained uniform: a five-day duration at 37°C, 5% CO2, and 99% humidity. This protocol was established based on procedures outlined by Karsten et al. (2020) and Arosa et al. (2011) [26, 34].

4. Sample Treatment after Cell Culture between PBMCs and RBC Lysates or RBC-CM

4.1 PBMCs and Supernatant

Following a five-day incubation, the samples underwent preparation for subsequent analysis. They were transferred from the culture wells to 1.5 mL Eppendorf tubes and centrifuged at 300xg for 5 minutes at 24°C. The supernatant was separated from the pellet and subjected to a second centrifugation at 17,000xg for 5 minutes at 24°C.

The resulting supernatant was stored at -20°C for cytokine profile analysis using Enzyme-Linked Immunosorbent Assays (ELISA).

The pellet was treated with 1 mL of PBS 1X and subjected to a similar centrifugation step as the initial one. The supernatant was discarded, and the pellet was stored at -80°C.

4.2 PBMC Lysis

In 1.5 mL Eppendorf tubes, PBMCs from the aforementioned cell culture were processed. For every 5×10^5 PBMCs, 20 µL of a lysis buffer (PierceTM) combined with a 1:10 ratio of protease inhibitor (IP) was added. This was followed by vortexing at alternating temperature intervals (the samples would be on the ice for 5 minutes followed by a vortex action and put at room temperature (24°C) for 45 minutes.

The samples were, then, centrifuged at 17,000xg for 10 minutes at 4°C. The supernatant was collected and utilized for the total protein quantification assay, as a normalization method for the ELISA results.

5. Quantification of the total protein concentration

5.1 For RBCs lysates

The quantification of the total protein in the prepared RBC lysates was performed using the *Thermo Scientific Pierce 660nm Protein Assay*. This assay involves a working reagent and pre-diluted standards (bovine serum albumin - BSA), in a 96-well microplate. Absorbance measurements were carried out at a wavelength of 660 nm using a spectrophotometer (*Spectrostar Nano – BMG LABTECH*). Through a calibration curve (Absorbance/[Protein]), the protein concentration of each sample was calculated based on the curve equation. Figure 10 represents an example of a calibration curve. Figure 10 represents an example of a calibration curve.

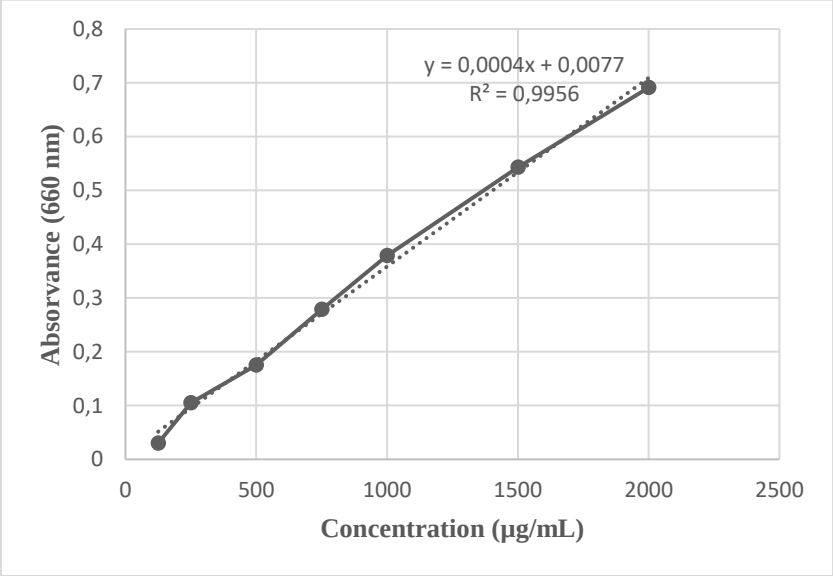

Figure 10. **Calibration curve for the total protein quantification (µg/mL) –** example**.**

5.2 For PBMC lysates and RBC-CM

For quantifying total protein in PBMC lysates and RBC-CM, *The Pierce BCA Protein Assay Kit* was employed. This assay differs from the previous one in that the standards are not pre-diluted, and the working reagent is prepared by mixing two different solutions, A and B, in a 50:1 ratio, respectively. In addition, this method requires incubating the microplate at 37°C for 30 minutes and reading the absorbance at 562 nm using a high-performance spectrophotometer (*SpectraMax 190 Microplate Reader, Molecular Devices*)

6. Protein detection – Cytokine profile analysis by Sandwich Enzyme-Linked Immunosorbent Assays (ELISAs)

6.1 ELISA Technique Overview

Sandwich Enzyme-Linked Immunosorbent Assays (ELISAs) are a widely employed technique in immunology for the precise detection and quantification of specific proteins, such as cytokines, within biological samples. This method relies on a "sandwich" principle, where the target protein is captured between two specific antibodies, facilitating highly sensitive and selective measurements (Figure 11).

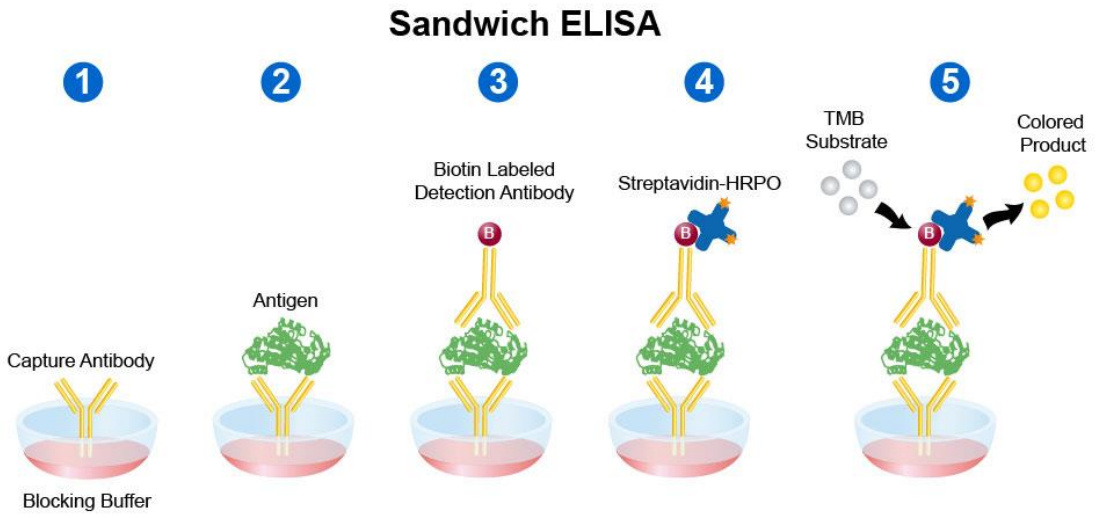

Figure 11. Procedure for the Sandwich ELISA immunoassay used for the analysis of the cytokine profile observed in the different samples. The method goes as follows: 1 is the microplate coating, with the capture antibody (overnight); 2 represents the connection between the capture antibody and the specific antigen (in this case, the wanted cytokine); in 3 we observe the addition of the detection antibody biotinylated, which binds to the same antigen; 4 is when the Streptavidin-HRP; and, finally, 5 represents the addition of TMB (chromogenic substrate, sensitive for HRP) and the "colored product", which in this study was sulfuric acid, is considered also a stop solution. In the end, the microplate is read at a high-performance spectrophotometer (*SpectraMax 190 Microplate Reader, Molecular Devices*) at a wavelength of 450 nm. (Source: [43])

6.2 ELISA in RBC Lysates and RBC-CM

6.2.1 Optimization study

The optimization study aimed to refine the ELISA protocol for the detection of anti-human TNF-α (Tumor Necrosis Factor-α) within RBC lysates and RBC-CM samples. It encompassed three primary objectives:

- Benchmark Protein Extract: The study sought to establish a baseline total protein extract (µg) for RBC lysates. To achieve this, three distinct total protein values (250 µg, 500 µg, and 1000 µg) were analyzed in triplicates (n=3). These concentrations were determined based on the methodology described in the study by Karsten et al. (2018) [22].

- Presence of TNF-α in RBC-CM: The second objective was to confirm the presence of TNF-α in RBC-CM, as suggested by existing literature [20]. The confirmation of TNF-α in RBC-CM samples facilitated the continued analysis of this sample type.

- Selection of Optimal RBC Lysis Method**:** The final objective was to determine the RBC samples lysis method, denoted as Method 1 or Method 2.

TNF-α was chosen as the target cytokine for this study due to its significant presence in RBCs from healthy individuals, as reported in studies such as Karsten et al. (2018) [22].

6.2.2 Assay - Cytokine profiles analysis of RBCs and RBC-CM from patients vaccinated against COVID-19

With the assay parameters established, the cytokine profiles within RBC lysates and RBC-CM obtained from COVID-19 vaccinated patients were assessed. The following cytokines were analyzed: Interleukin-6 (IL-6), Interleukin-12 (IL-12), Interleukin-1β (IL-1β), Interleukin-15 (IL-15), and TNF-α.

Protocols for IL-6, IL-12, and IL-15 ELISAs adhered to the procedures outlined by *Immunotools*. In contrast, protocols for IL-1β and TNF-α ELISAs were under *Invitrogen's* guidelines.

6.3 ELISA in the supernatant/medium from the cell culture between PBMC and RBCs lysates or RBC-CM

6.3.1 Optimization studies

The optimization study focused on the analysis of supernatants derived from the preliminary culture between PBMCs and either RBC lysates or RBC-CM, as previously outlined in section 3a. The assay was for the quantification of IL-12, Interferon-γ (IFN-γ), and TNF-α, by ELISA.

6.3.2 Assay - Cytokine profile analysis of supernatants from cell culture between PBMCs and RBCs from patients vaccinated against COVID-19

In this assay, the analysis occurred on the supernatants collected during the main study, as described in section 3b. The evaluation encompassed the same cytokines mentioned above, with the addition of IL-15. The detection was achieved using Sandwich ELISA immunoassays.

The protocols employed in both these assays were the same as delineated before in point 6.2.2.

7. Flow cytometry in PBMCs resulted from the cell culture with the RBCs lysates – a Preliminary Optimization Study

7.1 Technique introduction

Flow cytometry is a valuable analytical technique used in immunology to assess cellular characteristics, including viability and population distribution. This method relies on the detection of fluorescence signals emitted by labeled antibodies or markers, enabling precise cell analysis. A general representation of this technique is observed in Figure 12.

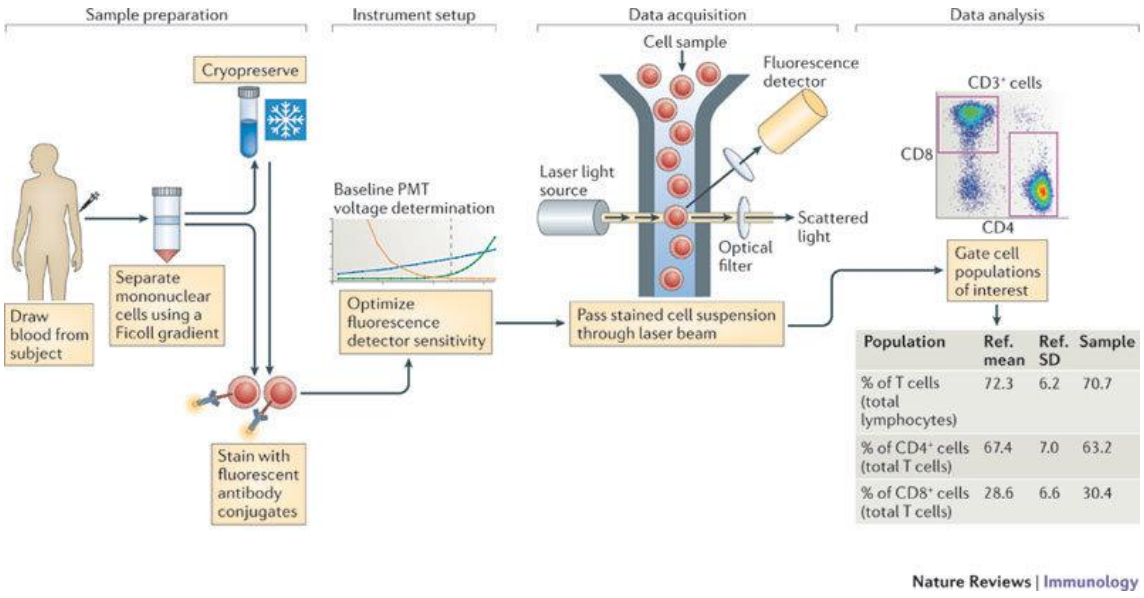

Figure 12. Flow cytometry experiments involve multiple stages, including sample preparation, instrument setup, data acquisition, and data analysis. Sample preparation, often beginning with Ficoll gradient separation and staining with fluorescent antibody conjugates, can introduce variability. Instrument setup, focusing on adjusting photomultiplier tube voltages for optimal sensitivity, is another potential source of variation. Data acquisition captures fluorescence emissions from bound antibodies, with instrument differences impacting variability. Finally, data analysis defines cell populations of interest and contributes significantly to overall variability. (Source: [44])

In this study, the fresh PBMCs treated as described above (point 4.1), were followed by antibody staining.

7.2 Viability and Population Study

This assay exclusively focused on PBMCs derived from the first cell culture (point 6, section 3a) between PBMCs and RBC lysates, involving a single biological sample (n=1, Patient ID: 136).

7.2.1 Antibody Staining

Freshly isolated PBMCs, treated as previously described in section 4.1, underwent antibody staining. The cells were divided into multiple Eppendorf tubes (1×10^5 PBMCs per tube).

The markers used in this study were: CD3-APC, CD8-PE, and 7-AAD. CD3-APC stains T-cells, as CD8-PE marks the T-cell subpopulation $CD8^+$ T-cells, and 7-AAD was used as a viability marker.

Four compensation tubes were prepared using non-stimulated PBMCs (Figure 13).

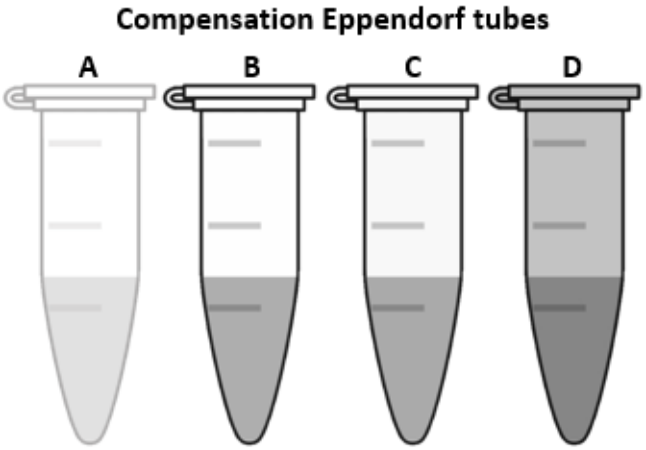

Figure 13. Flow cytometry – compensation phase. Eppendorf tubes containing, in each one, 1×10^5 non-stimulated PBMCs. (A) only unstained PBMCs; (B) PBMCs with the antibody CD8-PE; (C) PBMCs stained with only CD3-APC; (D) PBMCs with 7-AAD.

Additionally, three Eppendorf tubes for each condition, consisting of non-stimulated or stimulated PBMCs, were assembled (Figure 14).

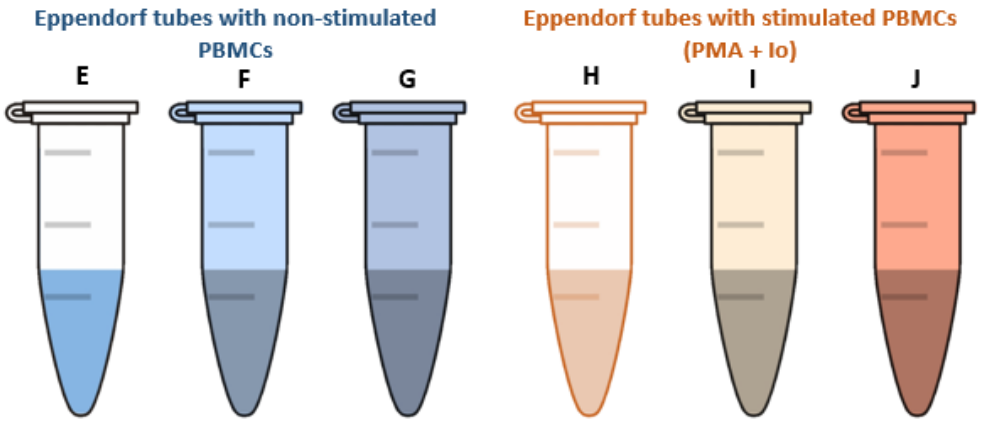

Figure 14. Flow cytometry – optimization assay. Eppendorf tubes with 1×10^5 PBMCs each. In the blue ones (E, F, and G), only non-stimulated PBMCs were present, in contrast with the ones represented in red (H, I, and J) where only stimulated PBMCs. In both situations, there were 3 settings, where E and H only had unstained PBMCs present; in F and I, PBMCs had only 7AAd; and, in G and J, PBMCs were stained with all the antibodies (CD8-PE, CD3-APC, and 7AAd). Furthermore, the scheme represented by the red Eppendorf tubes was repeated for each one of these conditions: PBMCs only stimulated with PMA and Io; stimulated PBMCs that were in culture with RBCs' lysates from T0; and stimulated PBMCs that were in culture with RBCs' lysates from T1.

7.2.1.1 Incubation and Analysis

After antibody staining, a 30-minute incubation at 4 °C was conducted. The cells were washed with 1 mL of PBS (1X), followed by centrifugation at 300xg for 4 minutes at 4 °C. The supernatant was discarded, and the PBMCs were kept on ice until analysis using the *Attune™ CytPix™ Flow Cytometer* by *Invitrogen*.

8. Statistical Analysis

Flow cytometry data analysis was carried out using *FlowJo software version 10.0.5* (TreeStar, San Carlos, CA, USA).

For statistical analysis of cytokine data, *'R' version 4.3.0* (2023-06-01, R: A Language and Environment for Statistical Computing) was the main tool. But, for the Paired T tests realized, when comparing only two conditions at time, mainly represented in the histograms, *GraphPadTM Prism 5 software* was also employed together with *'R'* program to have a more statistical certainty.

Linear Mixed Models (LMM) were utilized to examine the relationship between cytokine concentrations and various time points (T0-T4) during COVID-19 vaccination, and this model was only made with the *'R'* program.

The graphical representations were created using *Excel* and *GraphPadTM Prism 5 software*.

IV | Results

1. Cytokine profile analysis of red blood cells (RBCs) and RBC-conditioned media (RBC-CM)

To evaluate the cytokine profile of RBCs in response to COVID-19 vaccine immunization, RBCs and RBC-CM samples, from the Biobank-COVID-19 vaccines, were analyzed for Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-12 (IL-12), Interleukin-15 (IL-15), and Tumor Necrosis Factor- α (TNF-α) by using a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) method, as described in the Material and Methods chapter. Before proceeding with the cytokine analysis using ELISA, several optimization tests were conducted to assess critical parameters within the laboratory procedure.

1.1 ELISA – optimization assay

Due to financial constraints, ELISA optimization assays were performed only for TNF-α, following the manufacturer's recommended protocol.

Two distinct approaches (Lysis Method 1 and Lysis Method 2) were tested to identify the optimal total protein extract (µg) for cytokine detection in RBC lysates using ELISA. It is worth noting that RBC-CM contains soluble proteins and released vesicles containing proteins, which like RBCs, must be solubilized in a suitable lysis buffer before any protein assessment method, including ELISA.

The key differences between these two approaches are the composition of the lysis buffer, variations in the proportion of sodium phosphate buffer to distilled water, differences in the centrifugation process, and the inclusion of a sonication step, being this latter exclusively employed in Method 1.

For the optimization assays, newly collected RBC samples from three female subjects and biobanked RBC-CM samples from three subjects (Patient ID.: 118, 122, and 123) collected at time point T0 (before COVID-19 vaccination) were utilized as biological replicates. The tests were performed in triplicates to minimize potential technical variability and three different total protein extracts—250 µg, 500 µg, and 1000 µg— were evaluated. These values were calculated and established using the Karsten et al. (2020) study [26].

The results of these experiments in RBCs are summarized in Table 4.

Table 4 TNF-α ELISA optimization assay for RBCs from three biological replicates (n=3), not included in the Biobank-COVID-19 vaccines.

	Concentrations of TNF-α (pg/mL) #	
Total protein extract (µg)	**RBC Lysis** Method 1	**RBC Lysis** Method 2
250 µg	6.54 ± 0.1216 (3)	8.36 ± 0.093 (3)
500 µg	7.33 ± 0.0194 (3)	8.78± 0.0625 (3)
1000 µg	8.0 ± 0.0237 (3)	17.04 ± 0.041 (3)

 # represents the average (mean ± s.d. (n)) concentrations of TNF-α (pg/mL of sample) between biological triplicates of RBC lysates samples. Two different methods of cell lysis (Method 1 and Method 2) were compared for optimal protein solubilization/extraction and concentration for cytokine detection and quantification by ELISA assay.

Both RBC lysis methods and the three protein total extracts tested demonstrated that the cytokine TNF-α was detectable in RBC cells. However, with Method 1, no significant variability was observed in the amount of this cytokine between the different increasing total protein extracts of tested samples. The difference between using 250 µg or 1000 µg of sample, (1 or 5 times more sample), was only a 1.25-fold-increase in the amount of cytokine detected, increasing from 6.54 pg/mL to 8.00 pg/mL, respectively (Table 4).

With Method 2, even using a small amount of sample, 250 µg, a detection of 8.36 pg/mL of TNF-α was obtained, i.e., almost the same as that obtained with 1000 µg of sample with Method 1. On the other hand, a 2-fold increase in cytokine detection was observed when 1000 µg of sample was used, i.e., 17.04 pg/mL on average (Table 4). In conclusion, Method 2 for RBC lysis and the total protein extract of 1000 µg of RBC lysate sample was shown to be the most effective in detecting cytokine in RBC cells (Table 4).

The same lysis Method was applied to the RBC-CM samples. However, contrary to the establishment of a benchmark for the total protein of RBC lysates, in RBC-CM samples this value, throughout the study, in general, varied between 4-10 µg.

The next aim was to confirm the presence of cytokines, in this case TNF-α, on RBC-CM samples. As documented in other studies, like Karsten et al. (2018) [22]. RBC-CM samples from three subjects collected at T0 from the Biobank-COVID-19 vaccines were selected and tested. In this particular case, the total protein extract (µg) of these samples varied between 4-4.5 µg, depending on the patient.

The obtained results, summarized in Table 5, confirmed that TNF-α is not only detectable in RBCs but also in the culture medium of these cells after 48h-72h of cultivation. Around 13.8 pg/mL, on average, of TNF-α was detected in these samples, where only RBCs collected at T0

(time point defined as the period before any COVID-19 vaccination) were cultivated, validating the use of this type of sample in the following investigations (Table 5).

Table 5. TNF-α ELISA assay optimization for RBC-conditioned media (RBC-CM) from patients belonging to the Biobank-COVID-19 vaccines.

		Concentrations of TNF-α (pg/mL) #
Patient ID	**Time point**	**RBC-CM**
118	T0	13.30 ± 0,05
122	T0	14.61 ± 0,1
123	T0	13.74 ± 0,03

 # represents the average (mean ± s.d.) concentrations of TNF-α (pg/mL of sample) between technical triplicates of RBC-CM T0 samples.

Altogether, the optimization assays indicated that the RBC lysis Method 2 and the total protein extract of, approximately, 1000 µg were the best choice for further ELISA studies to evaluate cytokines profile in RBC lysates samples under the effect of COVID-19 vaccination. The use of the correspondent RBC-CM samples was also validated for these studies.

1.2 Assay - Cytokine profile modulation in RBCs and RBC-CM under effect of COVID-19 vaccination

RBC and RBC-CM samples from healthy subjects (n=8) that underwent COVID-19 vaccination with Pfizer vaccine (T0-T4 time points) were selected from the Biobank for ELISA analysis targeting the following cytokines: Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-12 (IL-12), Interleukin-15 (IL-15), and Tumor Necrosis Factor-α (TNF-α), as demonstrated in Figure 15.

Both types of RBC samples were prepared by using RBC lysis Method 2. In addition, RBC lysates had approximately 1000 µg of total protein extract, as in RBC-CM this value varied between 4 -8 µg. Both RBC lysates and RBC-CM samples were analyzed in duplicate.

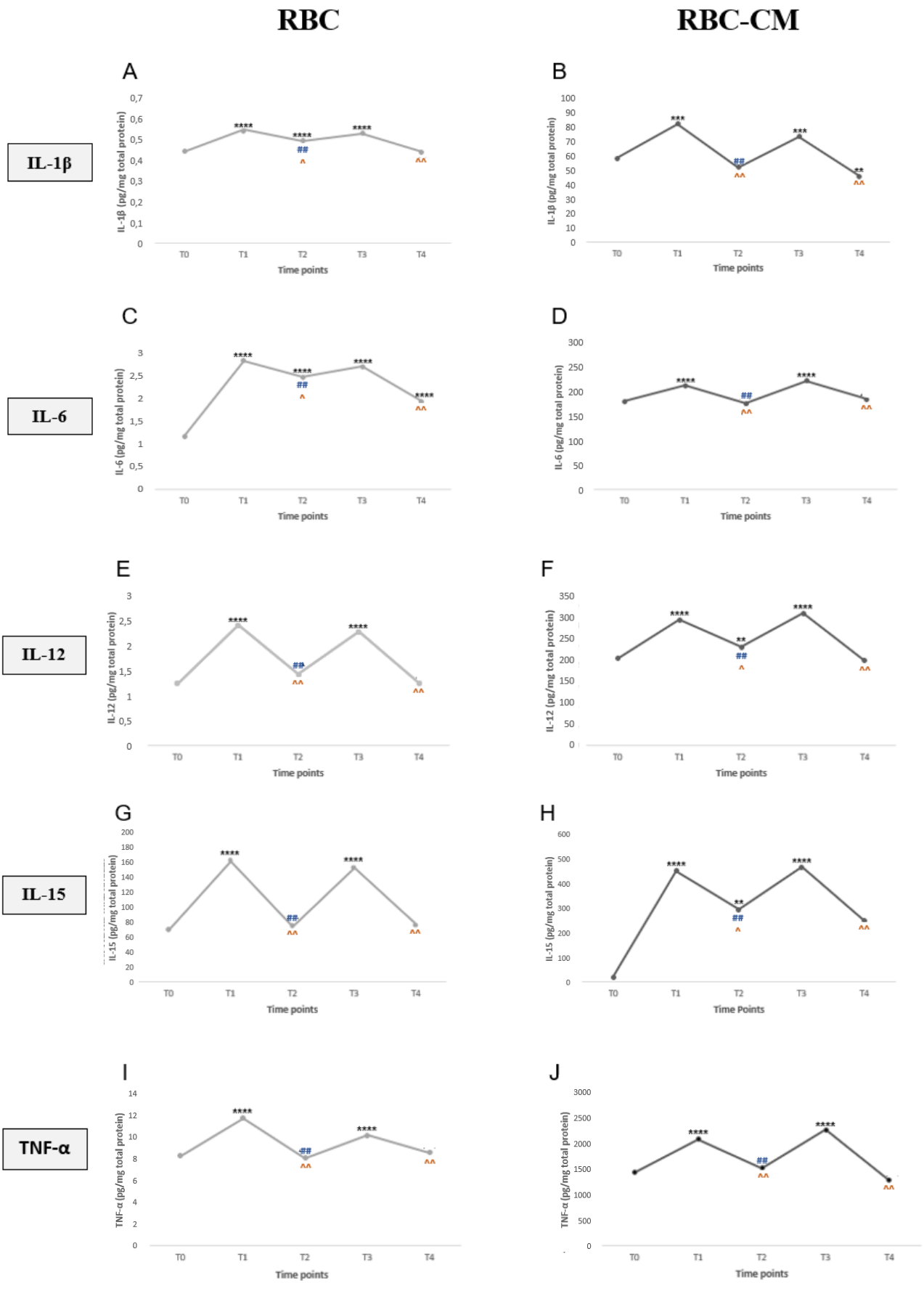
RBC
RBC-CM
IL-1β
IL-6
IL-12
IL-15
TNF-α
A
B
C
D
E
F
G
H
I
J
IL-1β (pg/mg total protein)
IL-6 (pg/mg total protein)
IL-12 (pg/mg total protein)
IL-15 (pg/mg total protein)
TNF-α (pg/mg total protein)
T0
T1
T2
T3
T4
Time points
Time Points

Figure 15. Cytokine profiles in RBCs and RBC-CM in response to COVID-19 vaccination. (A, C, E, G, and I) RBCs and (B, D, F, H, and J) RBC-CM samples were analyzed by Sandwich ELISA for the following cytokines: (A and B) Interleukin-1β (IL-1β), (C and D) Interleukin-6 (IL-6), (E and F) Interleukin-12 (IL-12), (G and H) Interleukin-15 (IL-15), and (I and J), and Tumor Necrosis Factor-α (TNF-α). The longitudinal time points considered during COVID-19 vaccine immunization were T0: before vaccination, T1: 24h-72h after the first vaccine dose, T2: before the second vaccine dose, T3: after the second vaccine dose, and T4: after one month of the last vaccine dose. Data was analyzed by statistical Linear Mixed Model (LMM). Statistically significant differences between pre-COVID-19 vaccination (T0) and post-vaccination time points (T1, T2, T3, and T4) are indicated by: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$. Between T1 and T2 these differences are denoted by # $p<0.001$ and ## $p<0.0001$, and between T3 and T2 or T4 by ^ $p<0.001$ and ^^ $p<0.0001$.

Table 6 shows the same results represented in Figure 15, reporting the average level of these cytokines in RBCs and RBC-CM of each time point under study along the COVID-19 immunization process.

Table 6. Average quantification (n=8) of IL-1β, IL-6, IL-12, IL-15, and/or TNF-α cytokines (pg per one mg of total protein) in RBC lysates and/or RBC-Conditioned media (RBC-CM). The values are similar to the ones expressed in Figure 15.

		RBC lysates	**RBC-CM**
Cytokine	**Time Point**	pg/mg of total protein #	pg/mg of total protein #
IL-1β	T0	0.47 ± 0.024	61.25 ± 6.05
	T1	0.59 ± 0.004	86.44 ± 7.03
	T2	0.53 ± 0.09	54.56 ± 4.29
	T3	0.57 ± 0.03	77.10 ± 6.58
	T4	0.47 ± 0.05	47.98 ± 5.08
IL-6	T0	1.17 ± 0.04	194.51 ± 10.22
	T1	2.83 ± 0.13	229.35 ± 10.81
	T2	2.49 ± 0.09	189.57 ± 13.72
	T3	2.70 ± 0.15	240.00 ± 11.20
	T4	1.94 ± 0.13	199.01 ± 14.04
IL-12	T0	1.33 ± 0.05	217.68 ± 20.67
	T1	2.58 ± 0.14	313.72 ± 21.29
	T2	1.53 ± 0.21	245.49 ± 8.26
	T3	2.43 ± 0.29	329.63 ± 9.27
	T4	1.34 ± 0.13	211.73 ± 13.68
IL-15	T0	74.59 ± 6.12	20.48 ± 1.02
	T1	172.39 ± 18.81	475.45 ± 22.77
	T2	79.10 ± 9.05	309.59 ± 6.82
	T3	162.92 ± 16.38	491.78 ± 7.07
	T4	81.46 ± 8.20	260.79 ± 8.27
TNF-α	T0	8.79 ± 0.88	1536.97 ± 17.2
	T1	12.48 ± 1.27	2240.61 ± 24.6
	T2	8.58 ± 0.86	1662.643 ± 11.4
	T3	10.84 ± 1.09	2435.77 ± 12.9
	T4	9.12 ± 0.91	1375.39 ± 11.7

 # represents the average (mean ± s.d. (n=8)) concentrations of the proinflammatory cytokines per mg of total protein of the RBC samples (pg/mg).

After a comprehensive analysis of the ELISA results for IL-1β, IL-6, IL-12, IL-15, and TNF-α, it can be concluded that statistically significant differences exist between T0 and T1, T0

and T3, and T2 and T3 for all these cytokines in both sample types, RBCs or RBC-CM (Figure 15).

Notably, there is an increase in these cytokine concentrations in both RBCs and RBC-CM samples collected at the time points T1 or T3, i.e., just 24h-72h after the first (1^{st}) or second (2^{nd}) dose of the vaccine, respectively. This leads to the conclusion that there was an alteration in the cytokine profile, both in the RBCs and in RBC-CM (whether soluble or present in vesicles) in response to COVID-19 vaccination.

Furthermore, between T1 and T2, and T3 and T4, there were statistically significant decreases of all these cytokines (Figure 15), indicating that although there was an increase in these cytokines after 24h-72h of taking the 1^{st} or 2^{nd} vaccine dose (T1 and T3), approximately, one month later the levels of these cytokines tended to return to the levels observed before vaccination (T2 and T4 compared with T0).

Although there was a significant decrease in T2 after an increase in T1, the levels of IL-1β and IL-6 in the RBCs (Figures 15B and 15D) and the levels of IL-12 and IL-15 in the RBC-CM (Figures 15F and 15H) remained significantly elevated after one month of the 1^{st} dose (T2 compared with T0).

By comparing T0 and T4, statistical differences were observed only for IL-6 in RBC lysates (Figure 15C), and IL-1β in RBC-CM (Figure 15B), suggesting that the vaccine-induced levels of these cytokines at T1 or T3 were still significantly higher, for both, although with a tendency to decrease after one month of taking the last vaccine dose for IL-1β, and with a tendency to maintain high levels in IL-6.

It is important to highlight that for all cytokines except IL-15, the concentrations in RBC-CM were notably higher, ranging from 100 to 1000 times higher when compared to RBC lysates. In the case of IL-15, a similar trend was observed, with generally higher quantities in RBC-CM, except for the T0 time point (Table 6).

2. Immunomodulatory role of RBCs in the immune system, namely in peripheral blood mononuclear cells (PBMCs)

In the previous assay, we demonstrated that the COVID-19 vaccine induces significant modulation in some proinflammatory cytokines present in RBCs and RBC-CM.

Given that these investigated cytokines are recognized proinflammatory cytokines able to influence the activity of immune cells, in this following study we investigated the impact of RBCs and/or RBC-CM from COVID-19 vaccinated subjects on the activity of PBMCs.

PBMCs are a heterogeneous population of immune cells, including lymphocytes such as T-cells, B-cells, NK cells, as well as monocytes, with functions encompassing immune surveillance, memory response, cytokine production, and immune regulation.

To address this question, cell culture assays were carried out by incubating freshly prepared PBMCs with Phorbol-12-myristate-13-acetate (PMA) and Ionomycin (Io) stimulators with and without lysates of RBCs or RBC-CM collected at different time points from the Biobank-COVID-19 vaccines.

After five days of incubation, the cell culture medium was recovered and analyzed for those cytokines under study by Sandwich ELISA. The collected PBMCs were analyzed by flow cytometry to assess possible differences in the T-cell subpopulations expansion in response to RBCs or RBC-CM from before and after COVID-19 vaccination.

2.1. Sandwich ELISAs - Analysis of the cell culture supernatant of PBMCs incubated with RBCs or RBC-CM

2.1.1 Optimization assay

Optimization assays were carried out to develop and implement the cell culture assay envisaged in this study.

RBC lysate (T0 and T1) and RBC-CM (T0 and T3) samples from only one subject (n=1, Patient ID.: 136) that underwent COVID-19 vaccination were selected from the Biobank, lysed by Method 2 and incubated in triplicates with PMBCs by testing 1:10 and 1:100 PBMC: RBC ratios, under PMA and Io stimulation. After five days of incubation, the cell culture medium and PBMCs were analyzed by ELISA and flow cytometry (see point 2.2), respectively.

To establish these PBMC: RBC ratios, as mentioned in the previous chapter, an equivalence between the number of RBCs and the corresponding total protein extract (µg) was determined during the first optimization assay. In summary, it was ascertained that approximately

1x 10^6 RBCs corresponded to 25 µg of total protein from the RBC lysate samples. Knowing this, the previously mentioned cell ratios (PBMCs: RBCs) were successfully implemented in the culture plate.

Additionally, taking this information into account, it was possible to normalize the final cytokine quantification values (in pg) per µg of total protein in the supernatant samples. For all cytokines studied in this interaction study between RBCs and PBMCs, except for Interferon-γ (IFN-γ), it was feasible to subtract the quantity of the respective cytokine originating from RBC lysates or RBC-CM from the total concentration of the same cytokine observed in the cell culture medium resulting from the PBMC and RBC and/or RBC-CM culture. This step ensured that the final cytokine amount in the culture supernatant originated solely from PBMCs and not from external sources.

However, for IFN-γ, this normalization was not possible since there was no quantification of it in RBCs and/or RBC-CM. Nevertheless, considering that these normalizations did not result in significant changes for the other cytokines, from a theoretical standpoint, we can infer that the results obtained, even without this normalization, are reliable. To further address this issue, we conducted normalization to T0 using the RBC values provided by Karsten et al. (2018) for IFN-γ, and the outcome remained largely unchanged, as expected [22].

With this consideration in mind, even if a similar normalization were applied to IFN-γ, the conclusions would probably persist unchanged, mirroring the outcomes observed for the other cytokines.

After five days of incubation, the cell culture medium and PBMCs were analyzed by ELISA and flow cytometry, respectively.

Figure 16 shows the results of the cell culture medium analysis by ELISA for IL-12, TNF-α, and IFN-γ cytokines.

All cytokines, except IL-12, showed to be increased in the PBMC cell culture medium after cell stimulation with PMA and Io alone. Non-stimulated PBMCs generated IL-12 (Figure 16A) but not TNF-α and IFN-γ (Figures 16B and 16C).

IL-12 was detected to significantly increase in PBMC cell culture supernatant after cell stimulation with PMA and Io alone in the presence of RBC lysates from both time points (T0 and T1). The ratio with better results was 1PBMC:10RBCs (Figure 16A).

The secretion of IFN-γ significantly increased when PBMCs were stimulated in the presence of RBC lysates or RBC-CM at any time point tested, being significantly higher in the presence of RBCs from T1 or RBC-CM from T3, compared with their respective T0 (Figure 16B). It is also observed that the 1:100, in RBC lysates, results in a major secretion of this cytokine, when compared to the 1:10 ratio.

The secretion of TNF-α was also significantly increased when cells were stimulated in the presence of RBC T0 or T1 lysates (1:10) but not with RBC T0 or T1 lysates (1:100), which in contrast seemed to cause a decrease in this cytokine secretion. A significantly higher increase of TNF-α was also observed in stimulated cells in the presence of RBC-CM T0 or T3 (1:100) (Figure 16C).

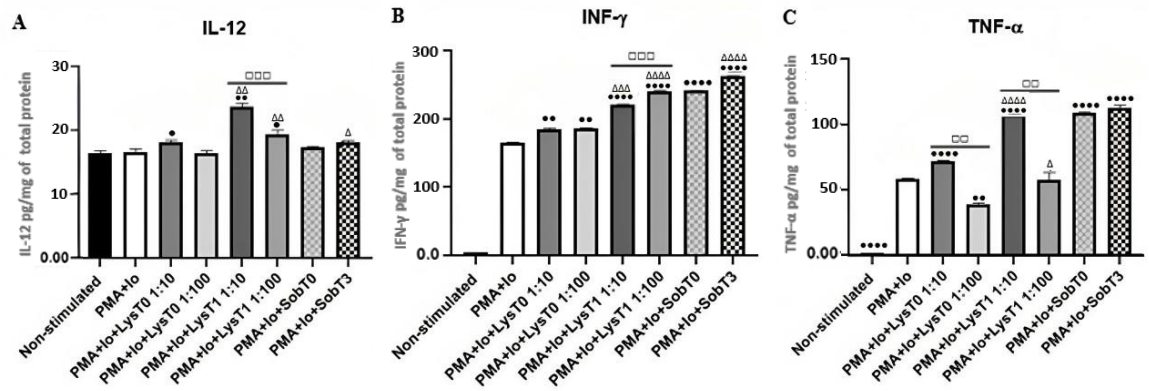

Figure 16. **PBMCs cytokines secretion under the effect of RBCs and RBC-CM from subjects undergoing COVID-19 vaccination –optimization study.** RBC (T0 and T1) and RBC-CM (T0 and T3) samples from one subject (n=1, Patient ID: 136) that underwent COVID-19 vaccination were selected from the Biobank, lysed by Method 2 and incubated in triplicate (technical triplicates) with PBMCs under stimulation of phorbol myristate acetate (PMA) and Ionomycin (Io). Different ratios of PBMC: RBC were tested (1:10 and 1:100). The cell culture was conducted over a 5-day incubation period at 37°C, 5% CO2, and 99% humidity. After incubation, the cell culture medium was recovered and analyzed by sandwich ELISAs for (A) Interleukin-12 (IL-12); (B) Interferon-gamma (IFN-γ); and (C) Tumor Necrosis Factor-α (TNF-α). Non-stimulated and stimulated PBMCs without RBC/RBC-CM lysates were used as controls. Regarding the time points: T0: before COVID-19 vaccination; T1: 24h-72h after the first COVID-19 vaccine dose; T3: 24h-72h after the second COVID-19 vaccine dose. The statistical test employed for these findings was the Paired T-test. Statistically significant discrepancies between the PMA+Io condition and the other conditions are denoted as follows: ● $p<0.05$, ●● $p<0.01$, ●●● $p<0.001$, and ●●●● $p<0.0001$. Statistically significant differences between pre-COVID-19 vaccination (T0) and post-vaccination time points (T1, and/or T3) are indicated by: Δ $p<0.05$, ΔΔ $p<0.01$, ΔΔΔ$p<0.001$, and ΔΔΔΔ $p<0.0001$. Finally, significant differences between the proportions PBMC: RBC, 1:10 and/or 1:100, belonging to the same time point, are represented by: □ $p<0.05$, □□ $p<0.01$, □□□ $p<0.001$, and □□□□ $p<0.0001$.

2.1.2 PBMCs cytokine secretion when in culture with RBC lysates, from COVID-19 vaccinated patients

Following optimization assays described in 2.1.1, sandwich ELISAs were conducted for the cytokines, Interleukin-12 (IL-12), Interleukin-15 (IL-15), Tumor Necrosis Factor-α (TNF-α), and Interferon-γ (IFN-γ) on the supernatants resulting from PBMC cell cultures stimulated with PMA/Io in the presence of RBC lysates from the eight patients (n=8), collected at the five time points under study (T0-T4).

Figure 17 illustrates the cytokine secretion patterns in PBMCs under various conditions.

Non-stimulated PBMCs exhibited minimal cytokine secretion, except for IL-12 and a low amount of IL-15 (Figures 17A and 17B). Upon stimulation with PMA and Io, significant secretion of IL-15, TNF-α, and IFN-γ was observed (Figure 17B-D), while IL-12 secretion remained comparable to non-stimulated cells (Figure 17A).

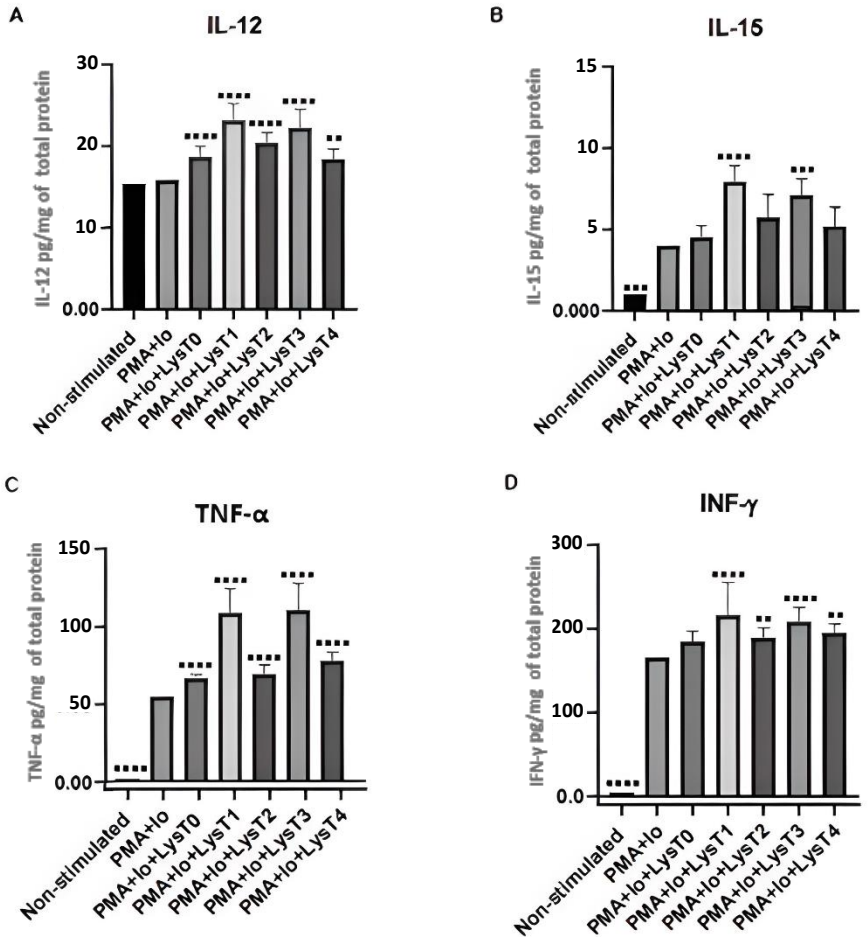

Figure 17. PBMC cytokines secretion resulted from the presence, in culture, of RBCs from subjects undergoing COVID-19 vaccination, in histogram form– main study. RBC samples collected from eight subjects (n=8) who received COVID-19 vaccination were chosen from the Biobank. These samples were subjected to lysis using method 2 and were then cultured with PBMCs stimulated with phorbol myristate acetate (PMA) and Ionomycin (Io). The time points correspond as follows: T0 represents the pre-vaccination period; T1 and T3 indicate time intervals of 24-72 hours after the first and second vaccine doses, respectively; T2 signifies one month following T1; and T4 corresponds to the time point 30 days after T3. The histograms (A-D) illustrate the PBMC-produced cytokines: (A) Interleukin-12 (IL-12); (B) Interleukin-15 (IL-15); (C) Tumor Necrosis Factor-α (TNF-α); and (D) Interferon-gamma (IFN-γ). The statistical test employed for these findings was the Paired T-test. Statistically significant discrepancies between the PMA+Io condition and the other conditions are denoted as follows: ● $p<0.05$, ●● $p<0.01$, ●●● $p<0.001$, and ●●●● $p<0.0001$.

When PBMCs were stimulated in the presence of RBCs collected at different time points (T0-T4) following vaccination, noteworthy changes in cytokine secretion were observed.

Specifically, IL-12 and TNF-α secretion significantly increased in stimulated PBMCs when exposed to RBCs from any vaccination time point (Figures 17A and 17C).

Conversely, IL-15 showed a significant increase in stimulated PBMCs only when exposed to RBCs from T1 or T3, corresponding to RBCs collected immediately after the 1st and 2nd vaccine doses.

IFN-γ secretion was elevated in stimulated cells exposed to RBCs from T1-T4 but not from T0 (Figure 17D).

In summary, these findings suggest that RBCs, influenced by the COVID-19 vaccines, differentially modulate and enhance the secretion of IL-12, IL-15, TNF-α, and TNF-γ by PBMCs in an *in vitro* setting.

Figure 18 provides a comprehensive overview of the statistical results derived from a Linear Mixed Model (LMM) analysis, focusing on the impact of RBCs collected at various time points during COVID-19 vaccination on cytokine secretion by PBMCs.

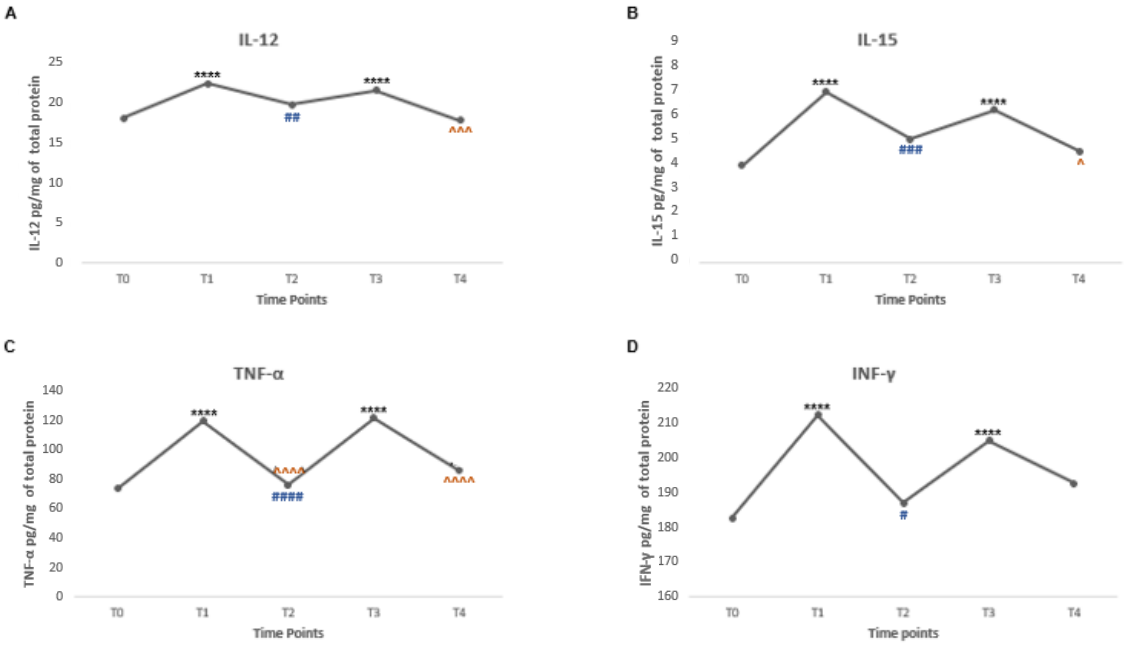

Figure 18. PBMCs cytokines secretion under the effect of RBCs from subjects undergoing COVID-19 vaccination– main study. RBC (T0-T4) samples of eight subjects (n=8) that underwent COVID-19 vaccination were selected from the Biobank, lysed by Method 2 and incubated with PBMCs under stimulation of phorbol myristate acetate (PMA) and Ionomycin (Io). T0 corresponds to the period before COVID-19 vaccination; T1 and T3 denote intervals of 24-72 hours after the first and second doses of the COVID-19 vaccine, respectively; T2 represents one month after the T1; and finally; and T4 corresponds to the time point 30 days after T3. (A-D) represents the production of these cytokines, by PBMCs: (A) Interleukin-12 (IL-12); (B) Interleukin-15 (IL-15); (C) Tumor Necrosis Factor-α (TNF-α); and (D) Interferon-gamma (IFN-γ). Data was analyzed by statistical Linear Mixed Model (LMM). Statistically significant distinctions between the pre-COVID-19 vaccination phase (T0) and the post-vaccination time points (T1-T4) are indicated by: * p<0.05, ** p<0.01, *** p<0.001, and **** p<0.0001. Furthermore, significant disparities between T1 and T2 are represented by # p<0.05, ## p<0.01, ### p<0.001, and #### p<0.0001, and, between T3 and T2 or T4 by ^ p<0.05, ^^ p<0.01, ^^^ p<0.001, and ^^^^ p<0.0001.

As detailed in Figure 18, it was observed that PBMCs exhibited a significant increase in the secretion of all cytokines, including IL-12, IL-15, TNF-α, and IFN-γ, when stimulated with lysates from RBC T1 or RBC T3 (collected immediately after the 1st and 2nd vaccine doses, respectively), in comparison to RBC T0 (pre-vaccination state).

However, notable changes in cytokine secretion were observed 28-30 days after the 1st vaccine dose (T2), where the levels of all these cytokines exhibited a significant decrease (Figure 18A-D). A similar trend was noted 28-30 days after the 2nd vaccine dose (T4), with IL-12, IL-15, and TNF-α showing a significant decrease compared to T3. Although IFN-γ demonstrated, also, a decrease, that did not reach statistical significance when compared to T3 (Figure 18A-C).

Table 7 summarizes the results by showing the average levels of each of these cytokines measured in the culture medium of PBMCs stimulated in the presence of RBCs from different time points, (T0-T4) throughout the COVID-19 vaccination process.

Table 7. **Averages (n=8) of cytokine quantifications (IL-12, IL-15, TNF-α, and/or IFN-γ) for the cell culture supernatant in this study (pg /mg of total protein)**. These values closely align with those depicted in Figure 18.

Cytokine	**Time Point**	**Cell culture medium** pg/mg of total protein #
IL-12	T0	185.60 ± 11.98
	T1	227.75 ± 19.23
	T2	202.81 ± 10.3
	T3	222.21 ± 17.2
	T4	183.57 ± 10.5
IL-15	T0	4.55 ± 0.42
	T1	8.49 ± 0.68
	T2	5.78 ± 1.2
	T3	7.43 ± 0.83
	T4	5.76 ± 1.13
TNF-α	T0	66.26 ± 24.5
	T1	113.20 ± 38.3
	T2	68.96 ± 62.0
	T3	116.06 ± 61.0
	T4	78.08 ± 48.9
IFN-γ	T0	184.13 ± 13.0
	T1	215.99 ± 38.9
	T2	188.9 ± 12.0
	T3	208.1 ± 17.25
	T4	194.78 ± 11.27

 # represents the average (mean ± s.d. (n=8)) concentrations of the proinflammatory cytokines per mg of total protein of the PBMCs culture medium (pg/mg).

Observing Table 7 is possible to affirm that, TNF-α and IFN-γ are the most secreted cytokines by PBMCs, in culture with RBC samples.

2.2 Flow cytometry – Analysis of PBMC populations, in particular, T-cell subpopulations

As outlined in section 2.1.1, in addition to analyzing the cytokine profile in cell culture supernatants between PBMCs and RBC samples, a preliminary flow cytometry analysis was conducted on the PBMCs resulting from the culture. The culture conditions and implied samples are identical to those described above (point 2.1.1). Specifically, this experiment was conducted using only RBC T0 and RBC T1 samples obtained from the Biobank-COVID-19 vaccines (n=1 subject ID: 136).

The primary objective of this assay was to assess the PBMC population, with a particular focus on the expansion of the T-cell population in the presence or absence of RBC lysates collected either before vaccination (T0) or between 24-72 hours after the administration of the 1st vaccine dose (T1).

Three flow cytometry markers were employed for this study: CD3-APC to label T-cells within the PBMCs, CD8-PE to specifically mark cells expressing the CD8 receptor on their surface, and a cell viability marker, 7-AAD, which enabled the selection of viable cells for the study.

Regarding the flow cytometry results presented in Figure 19, beginning with the $CD3^+$ population, it is apparent, based on the Mean Fluorescence Intensity (MFI), that this population exhibits a significantly larger size when stimulated with RBC lysate from T1 and at a PBMC to RBC ratio of 1:10 (Figure 19C).

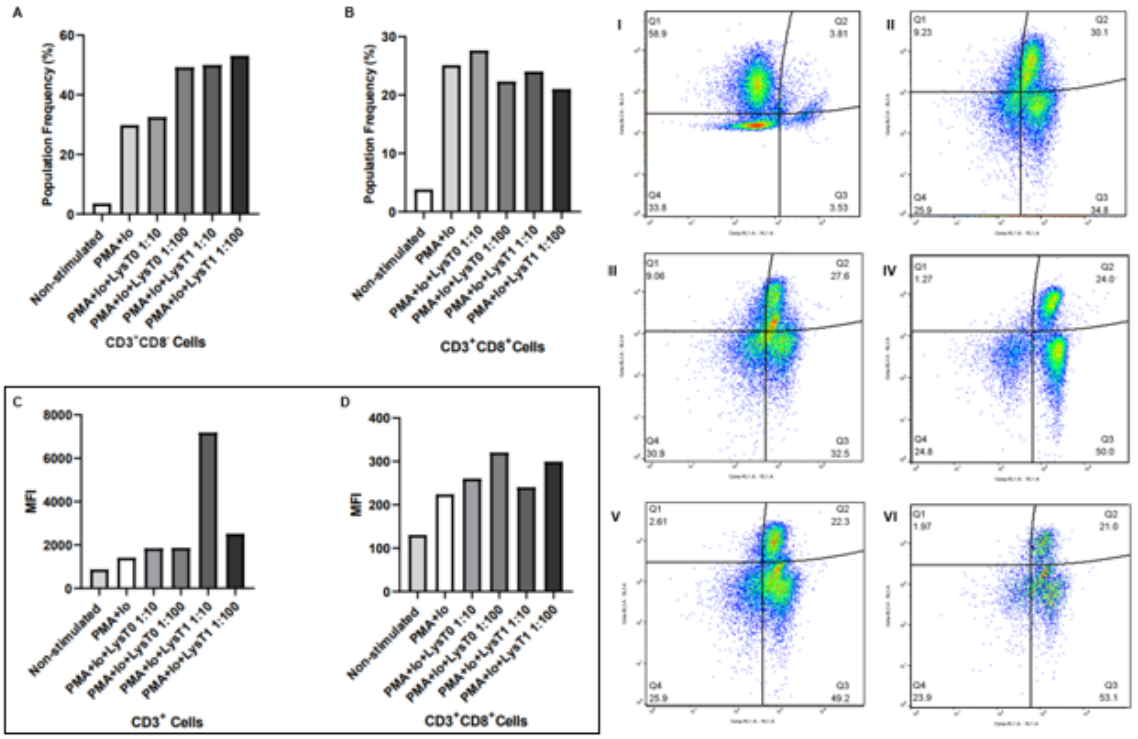

Figure 19. **Flow cytometry of peripheral mononuclear cells (PBMCs) stimulated under the effect of RBCs before (T1) and after (T1) COVID-19 vaccination – an optimization study.** RBC (T0 and T1) samples from one subject (n=1, Subject ID: 136) that underwent COVID-19 vaccination were selected from the Biobank, lysed by Method 2, and incubated with PBMCs under stimulation of phorbol myristate acetate (PMA) and Ionomycin (Io). (A and B) Represent population frequencies of PBMCs in histogram form. (A) corresponds to the $CD3^+CD8^-$ population and (B) pertains to population $CD3^+CD8^+$ population. (I-VI) demonstrates, also, population frequencies, but in quadrants. The X-axis denotes the RL1-A channel associated with the CD3-APC marker, while the Y-axis represents the BL2-A channel linked to the CD8-PE antibody labeling. (C and D) Mean Fluorescence Intensity (MFI) for $CD3^+$ and $CD3^+CD8^+$ cell populations, respectively. MFI and the population frequencies resulted from the statistics provided by the *Flowjo software*.

Concerning the frequencies of T-cell subpopulations, the $CD3^+CD8^-$ subpopulation, generally indicative of $CD4^+$ T helper cells, showed an increased presence when PBMCs were stimulated in the presence of RBC lysates, regardless of whether they were collected at time point T0 or T1, compared to stimulated cells in the absence of these samples (Figure 19A). In this instance, the 1:10 ratio demonstrates a more pronounced alteration in the T0 to T1 subpopulation increase when compared to the 1:100 ratio, despite having slightly inferior secretion values.

The $CD3^+CD8^+$ subpopulation ($CD8^+$ T-cells) exhibited slightly higher expansion levels when PBMCs were stimulated in the presence of "non-vaccinated" RBC T0 for both ratios, compared to the other conditions. There was even a reduction in the size of the population in the presence of T1 lysates compared to T0 (Figure 19B). The same trend is observed for the MFI values shown in Figure 19D for this T-cell subpopulation. This trend is consistent for both ratios,

with the 1:100 ratio generally displaying higher values, but 1:10 demonstrates a more significant decrease.

In summary, the conclusions from this assay are as follows: there is an expansion in the T-cell population when exposed to RBC lysates from COVID-19 vaccinated patients. Within this cell population, $CD4^+$ T-cells are more prominently present in greater quantities compared to $CD8^+$ T-cells following the administration of the 1^{st} dose of the COVID-19 vaccine. Additionally, there is even a decrease from T0 to T1 for the latter subpopulation (Figure 19).

Given these results, it would be valuable to conduct a similar study with a larger sample size of biological replicates from RBC lysate and RBC-CM samples collected at all five time points (at least n=8) and employing additional cytometry markers to gain a more comprehensive understanding of this cellular dynamic in response to COVID-19 vaccination.

V | Discussion

Red blood cells (RBCs) have been recognized as carriers of cytokines, potentially influencing immune responses [22]. Diseases and infections can modify the RBC proteome, including the cytokines profile bound or released by RBCs. These modifications can affect the immune system, such as the T-cell response [25,26,27]. Despite this knowledge, the RBC cytokine profile in response to immunization by vaccination has not yet been investigated nor the effect it may have on immune cell activity.

To address these questions, the main objectives of this preliminary research were: i) to evaluate changes in the RBC cytokine profile due to COVID-19 vaccination and ii) to investigate any resulting downstream effects those modified RBCs may have on peripheral mononuclear blood cells (PBMCs) cytokines secretion and T-cell expansion *in vitro*. For that, ELISAs were performed to evaluate cytokines in RBCs and RBC-conditioned media (RBC-CM) samples at different time points (T0-T4), derived from healthy subjects that underwent COVID-19 vaccination (Biobank- COVID-19 vaccines). PBMCs recovered from disposable buffy coats from blood donation were cultured and stimulated in the presence/absence of those RBC or RBC-CM lysates, for ELISA cytokine analysis in culture medium and cell flow cytometry for evaluation of T-cell subpopulations expansion.

Cytokine profiles bound or released by RBCs are modulated in response to the COVID-19 vaccine

The following cytokines were selected to be ELISA evaluated: Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-12 (IL-12), Interleukin-15 (IL-15), and Tumor Necrosis Factor-α (TNF-α). The rationale behind selecting these cytokines stemmed from their pivotal roles in the immune response, particularly their proinflammatory functions, which encompass the activation of the immune system in response to vaccination by reacting to the vaccine antigen.

Recent studies have revealed a direct correlation between the levels of proinflammatory cytokines and the severity of adverse reactions following COVID-19 vaccination, as well as the disease itself. Individuals with heightened proinflammatory cytokine levels at the time of vaccination were more prone to experiencing more severe post-vaccination side effects [45,46, 47].

Additionally, research by Bergamaschi et al. (2021) has shed light on the significance of transient increases in IL-15 levels shortly after booster vaccinations [48]. These increases were found to correlate with elevated Spike antibody levels, underscoring their potential as a valuable

biomarker for assessing the development of effective immunity in response to COVID-19 vaccination. Also, it has already been described that IL-15 is related to antibody development against COVID-19 [49].

In summary:

- IL-1β, as extensively studied by Dinarello in 2018 [50], plays a crucial role in propagating inflammation and facilitating the recruitment of leukocytes to infection sites.

- IL-6, as elucidated by Tanaka et al. (2014) [51], is essential for orchestrating the acute-phase response and promoting the development of adaptive immune reactions.

- Both IL-12 and IL-15, as highlighted in various studies [52, 53], are involved in regulating T-cell responses. IL-12 enhances cytotoxic activity in natural killer (NK) cells and induces Th1 immune responses, as explained by Vignali et al. (2012) [54]. On the other hand, IL-15 contributes to the viability and proliferation of memory $CD8^+$ T-cells and NK cells, as demonstrated by Dubois et al. (2002) and Bergamaschi et al. (2021) [55, 56].

- TNF-α exerts a significant influence on various immune cells. Its functions encompass the initiation of inflammation, activation of immune cells such as macrophages, and regulation of immune responses [57].

Although these cytokines have been extensively investigated mainly in plasma or immune cells, few studies have been carried out in RBCs. Therefore, before proceeding with the assessment of the cytokine profile in both RBCs and RBC-CM, there was a need to optimize the ELISA cytokine assay for these types of samples under our laboratory conditions.

To optimize the ELISA assay for RBC samples, two critical parameters were considered: the selection of the best lysis method, between Method 1 and Method 2, and the determination of the optimal total protein extract (μg) that would yield the highest cytokine detection signal in these particular samples.

For optimization experiments, we selected the ELISA for TNF-α based on prior findings reported by Karsten et al. (2018) [22]. In their study, the average quantification of this cytokine (pg/mL of whole blood) was observed to be substantially elevated when compared to values of other proinflammatory cytokines, in RBC lysates. In addition, its presence in RBC-CM was also demonstrated [22]. The comparison between their results and ours, for RBC samples from T0, will be discussed below.

Fresh RBC samples from three healthy subjects, collected at least six months after their COVID-19 vaccination, and RBC-CM samples from three healthy subjects, from the Biobank-COVID-19 vaccine dataset (Patient ID: 118, 122, 123) at time point T0, corresponding to the pre-COVID-19 vaccination period, were used for ELISA optimization purposes.

The results obtained from both the RBC and RBC-CM samples not only validated our approach but also established an optimal benchmark for subsequent ELISA assays.

Lysis Method 2 and the total protein extract of 1000 µg (for RBC lysate sample) were determined to be the best parameters to be used in further ELISA assay using RBC samples.

One potential explanation for the superior performance of Method 2 compared to Method 1 lay in the absence of sonication of the sample. The use of sonication in Method 1, although described to better fragment and dissolve cell membranes, can inadvertently lead to protein degradation due to the increasing temperature within the sample. This effect can persist even when the sample is maintained in ice during the process. Furthermore, Method 2 involved shorter and less intense centrifugation of the lysates compared to Method 1, which may have contributed to minimizing protein degradation in these samples and increasing the protein content in the final supernatant used for cytokines analysis.

In conclusion, the optimization tests for ELISA cytokine assay applied to RBC samples assay, evaluating TNF-α, not only identified the best total protein extract for a reliable detection signal but also confirmed the presence of this cytokine not only in RBC lysates but also in RBC-CM samples.

For the subsequent ELISA assays in RBC lysates and RBC-CM in response to COVID-19 vaccines, samples from a Biobank-COVID-19 were selected, corresponding to healthy subjects (n=8), which samples were collected before $1^{st}/2^{nd}$ dose vaccination (T0 and T2, respectively), after 24h-72h post- $1^{st}/2^{nd}$ dose (T1 and T3, respectively), and 28-30 days after the 2^{nd} vaccine dose (T4).

The ELISA assay results confirmed that all cytokines, IL-1β, IL-6, IL-12, IL-15, and TNF-α are present in both types of samples, RBC lysates and RBC-CM, albeit at varying concentrations.

Notably all cytokines, except IL-15, exhibited concentrations approximately 100 to 1000 times higher in RBC-CM compared to RBC lysates. The same result was demonstrated for TNF-α and IL-6 in Karsten et al. (2018) work [(22)].

IL-15 demonstrated a similar pattern of response except for the T0 time point, in which concentration was shown similar between RBC lysates and RBC-CM.

One plausible technical explanation for the observed differences between RBC lysate and RBC-CM samples could be attributed to the lysis method employed, which may not be optimally suited for solubilizing cytokines bound to RBCs. In future investigations, alternative lysis methods should be explored to enhance the solubilization of cytokines from intact RBCs, thereby facilitating more accurate cytokine analysis. An example of an alternative approach is the freeze-thaw cycle method applied to RBCs, as described [22].

Furthermore, from a biological perspective, RBC-CM samples may exhibit higher cytokine enrichment compared to RBC lysates, translating into a higher concentration per microgram of total protein in the sample. This phenomenon may be attributed to the enhanced solubilization of cytokines in RBC-CM, as opposed to cytokines present within the RBC membranes. It is plausible that cytokines may have been bound to chaperones or formed complexes when lysate from intact cells was analyzed but were subsequently released when the cells were incubated for 48h-72 hours [22,26].

Additionally, there is evidence suggesting that certain molecules are contained in RBCs in an inactive form, becoming detectable only after experiencing mechanical stress. Oonishi et al. (1998) [37] showed that molecules such as PGE1 and PGE2 were detectable in RBC cytosols only after mechanical stress, implying that enzymes may convert these molecules into an active and detectable form.

In response to COVID-19 vaccination, we observed a significant increase in all those cytokines in both RBC samples—whether bound (RBC lysates) or (*in vitro*) released (RBC-CM), collected after 48h-72hours of the 1st or 2nd vaccine dose (T1 and T3, respectively).

In RBC lysates, after 28 to 30 days following either dose (T2 and T4, respectively), the levels of all these cytokines significantly decreased with a tendency to return to pre-vaccination levels (T0).

While IL-1β demonstrated a notable decline 28 to 30 days after the 1st vaccine dose (T2), following an initial increase just 24-72hours post-vaccination (T1), it continued to be significantly elevated when compared to the pre-vaccination baseline (T0).

Il-6, a proinflammatory cytokine associated with acute inflammation and the adaptive immune response [49], although it showed a tendency to recover 28-30 days after an increase in response to both 1st and 2nd vaccine dose, the level of this cytokine remained significantly increased throughout the immunization process (T1-T4) compared to the period before any contact with the vaccine (T0). This result was also demonstrated by Hartog et al. (2023) [58].

Concerning the cytokine levels at T0, in RBCs, our results can be compared to the findings of Karsten et al. (2018) [22], which previously studied the cytokine profile of RBCs in healthy individuals (n=10). Table 8 succinctly presents these values, in both studies.

Table 8. Representation of the quantification of proinflammatory cytokines in pg/mL of whole blood. Comparison between the Karsten et al. (2018) [22] study and our study, for the RBC samples lysates from T0 time point (pre-vaccination).

		Cytokine concentration (pg/mL)#	
Cytokine	**Time Point**	Karsten et al. (2018) [22]	Our study
IL-1β	T0	13.1 ± 4,2	45.7 ± 1.5
IL-6	T0	0	113.1 ± 3.59
IL-12 (p40)	T0	1603.7 ± 629.7	104.03 ± 3.4
IL-15	T0	77.2 ± 54.8	7193.4 ± 223.0
TNF-α	T0	113.0 ± 79.2	84.63 ± 2.8

 # represents the average (mean ± s.d. (n=10 or n=8)) concentrations of the proinflammatory cytokines per mL of whole blood (pg/mL).

The observed discrepancies can be attributed to several factors, beginning with the protocols employed for the detection and quantification of specific cytokines. Notably, Karsten et al. (2018) [22] used the Multiplex Luminex technique, which, in comparison to the ELISAs employed in our study, possesses greater sensitivity, enabling the detection of lower levels of cytokines, such as TNF-α and IL-12. Consequently, this leads to higher measurements in samples with relatively low concentrations of this protein. Furthermore, in the case of IL-12, the substantial elevated results in the Karsten et al. (2018) [22] study, when compared to our results, can be associated to the fact that while both Luminex and ELISA methods can be influenced by IL-12 (p40), Luminex, due to its multiplex nature, might face challenges distinguishing between IL-12 and interleukin-23 (IL-23), as both these cytokines share the subunit p40, impacting specificity. ELISAs, being more targeted, can offer greater specificity in detecting IL-12, minimizing interference from other related molecules, resulting in more correct values of the quantification.

As mentioned before, although there is a bigger sensitivity, the specificity is more significant in the ELISA technique. This characteristic can be crucial to ensure the detection of some cytokines, in this case, IL-6, for example.

The sample treatment, specifically the RBC lysis, was also performed differently between the two studies, potentially leading to differences in the solubilization of cytokines in the supernatants, thus affecting the quantification of these molecules. For example, the last centrifugation was minor in the lysis protocol of Karsten et al. (2018) [22], and in their work, they

did not use a lysis buffer, but three freeze-thaw cycles to ensure complete cellular lysis. In addition, the final protein concentration of the RBC lysates, used in our study was superior to the one employed by Karsten et al. (2018) [22], about approximately 2-times, which could be an additional reason for the superior values of concentration of some cytokines (Table 8).

Another important factor is the existing biological variation between these studies. Different patients from diverse backgrounds were tested, and the number of biologically distinct samples used in both studies was not notably high, impacting the statistical power.

As a final point, is evident from the values in Table 8, those from Karsten et al. (2018) [22] exhibit a higher standard deviation, in general, indicating greater data variability, which suggests less consistency and greater data dispersion when compared to the values from our study.

Regarding RBC-CM, the quantitative patterns of these cytokines at all measured time points closely mirrored those observed in RBC lysates, which exhibited a return to baseline levels around T0, occurring 28 to 30 days after the last vaccine, except for IL-1β. Moreover, the levels of IL-12 and IL-15, while showing a significant decrease after 28 to 30 days following an initial increase post-1st vaccine dose, remained significantly elevated compared to the pre-vaccination period (T0).

These observations, in both RBC and RBC-CM, suggest that the composition of these cytokines, whether bound to RBCs or released as soluble factors or within vesicles, underwent modifications in response to the COVID-19 vaccine. These alterations in the quantitative cytokine profile appeared to occur within a few hours or days following vaccine administration. However, this response appeared to be temporary, as 28 to 30 days later, there was a return of cytokine levels to values similar to those observed before vaccination.

The observed increase in these proinflammatory cytokines immediately after vaccine administration may be associated with either transient inflammatory side effects or the immune response cascade induced by vaccination.

The inflammatory response has been described as an event directly correlated with the increased immunogenicity of the vaccine [59]. Nakayama (2016) reported that the production of inflammatory cytokines occurs approximately three hours after vaccination, both systemically and locally, on average [60]. Considering this, the elevated production of these proinflammatory cytokines (among others), upon vaccination, is expected.

Regarding the COVID-19 vaccine, high concentrations of IL-1β, IL-6, IL-12, and TNF-α in plasma/serum shortly after vaccination have already been demonstrated by Heo et al. (2022), Wang et al. (2022) and Hartog et al. (2023) [58, 61,62]. The same has been observed for IL-15 [48, 63].

Nakayama et al. (2022) further described that the production of inflammatory cytokines following the administration of messenger RNA vaccines BNT162b2 and mRNA-1273 typically occurs within the first 24 hours after a single dose, regardless of the specific vaccine [64]. Additionally, Murata et al. (2022) studied four cases where a cytokine storm occurred after COVID-19 vaccination, resulting in significantly elevated levels of proinflammatory cytokines in the plasma and fatalities within 1-10 days post-vaccination [65].

It has been suggested that to maintain homeostasis, erythrocytes gather proinflammatory cytokines from areas of inflammation, where their concentration is elevated, and release them in plasma [21]. RBCs have Duffy antigen receptors (DARC) [20] on their membranes, allowing them to bind to cytokines from the circulating plasma, and release them. Recently, Karsten et al. (2018), demonstrated that the level of cytokines in RBCs is 1000-fold higher than in plasma [66], suggesting that RBC has a relevant role in the regulation of cytokine signaling.

Our findings show a modulation in the RBC proinflammatory cytokines similar to that, which has been described following COVID-19 vaccination. Thus, it is plausible to propose that a crosstalk between plasma and RBC might also exist in response to vaccine-induced proinflammatory cytokine signaling involved in the immunization process.

The tendency for all cytokines to restore baseline values from an increase just after vaccination could be attributed to the body's counterbalancing efforts to avoid an exacerbated immune reaction, that might lead to unwanted side effects or systemic inflammation [67]. This modulation could also reflect the coordinated effort between the innate and adaptive immune responses triggered by the vaccine.

In summary, the comprehensive analysis of cytokine RBC profiles, bound or released to the extracellular microenvironment demonstrated a significant alteration induced by the COVID-19 vaccine. The augmentation of proinflammatory cytokines in RBCs or RBC-CM suggests an erythrocyte-mediated inflammatory process in response to vaccination, maybe involving a complex interplay between plasma, RBCs, and the immune system.

In addition, temporal variations in cytokine concentrations emphasize the dynamic nature of this immune response, providing valuable insights into the intricate interactions between RBCs and the immune system during vaccination.

These findings also align with other recent scientific research on immune responses to vaccinations and cytokine dynamics [66,68,69,70].

Immunomodulatory role of RBCs in the immune system

PBMCs are considered an immune cell population that consists of both lymphocytes and monocytes [40]. In this study, we examined how RBCs under COVID-19 vaccination affect PBMCs *in vitro,* specifically the profile of cytokine release by these cells or the expansion of T-cell subpopulations in activated PBMCs. For this, optimization experiments for cell culture were carried out.

PBMC culture assays were based on a combination of stimulation factors with/without RBCs lysates or RBC-CM, all collected before COVID-19 vaccination (T0) or just after the 1st or 2nd vaccine dose (T1 and T3, respectively), from a single patient (n=1, Patient:136). The choice of these time points was based on our previous findings showing the higher concentration of cytokine in RBC or RBC-derived samples at those time points.

Overall, these initial studies aimed to confirm whether there was indeed a potential modulation of PBMCs' response in the presence of the RBC samples, before extending our investigation to a larger number of patients (n=8). Moreover, the appropriate PBMC: RBC ratio was also determined, by testing 1 PBMC:10 RBC and 1 PBMC:100 RBC ratio as previously reported in the literature [26,34].

The results of this experimental setup allowed us to measure cytokine levels in PBMC cell culture medium using Sandwich ELISAs and assess changes in PBMCs, particularly T-cell subpopulations, using flow cytometry.

These proinflammatory cytokines are known to be secreted by PBMCs [4]:

- IL-12, primarily produced by dendritic cells, macrophages, and some lymphocytes such as B-cells and natural killer (NK) cells. Its primary function involves the differentiation of helper T cells (Th) into type 1 helper T cells (Th1) [52,54]. Furthermore, it induces the production of IFN-γ, primarily by T-cells and NK cells.

- IL-15, known for promoting the survival and proliferation of lymphocytes, particularly NK cells and memory $CD8^+$ T-cells. This cytokine aids in sustaining these lymphocytes and increasing their numbers as needed [53, 55]. It is produced by macrophages, dendritic cells, antigen-presenting cells, and lymphocytes (T-cells and NK cells) [71].

- IFN-γ has various functions, including activating macrophages and NK cells to combat virus-infected cells by promoting the Th1 response and MHC (Major Histocompatibility Complex) activation, leading to an increase in active T-cells in circulation. Elevated IFN-

γ levels in plasma/serum samples have been correlated with heightened Spike antibody levels resulting from COVID-19 vaccination [48].

- TNF-α was also selected due to its well-documented functions in the immune system and its primary production by macrophages and active Th1 cells [57]. It can also be produced by dendritic cells and NK cells.

The laboratory procedure was initially conducted as an optimization test and afterward, as a statistically relevant assay using a larger cohort of samples. As previously mentioned, the key differences between the two studies lay in the number of biological replicates used and the number of time points analyzed per RBC sample. Additionally, in the optimization test, the two tested PBMC: RBC ratios were analyzed, along with RBC-CM.

Concerning the optimization test by ELISA for IL-12, IFN-γ, and TNF-α in stimulated PBMCs culture medium, it was observed that the secretion of these cytokines is significantly modulated in the presence of RBC lysates either collected before (T0) or after vaccination (T1).

Regarding the PBMC: RBC ratios evaluated during this assay, it was observed that the secretion of IFN-γ appears to thrive under a larger dose of RBCs, resulting in higher production at the 1:100 ratio. Conversely, for TNF-α and IL-12, the 1:10 ratio yielded more favorable outcomes. This effect was especially pronounced for TNF-α, where the 1:100 ratio resulted in diminished cytokine production compared to PBMCs solely stimulated with PMA and Io.

These distinct observations suggest that varying amounts of RBC lysates or RBC type in relation to the time point may differentially influence the secretion of these cytokines by stimulated PBMCs.

Regarding the observation made for TNF-α, a negative feedback response may be occurring, where the high total TNF-α concentration in the 1PBMC:100 RBC proportion triggers PBMCs to reduce the secretion of this cytokine into the medium. This could be a regulatory mechanism, reflecting what happens *in vivo* to prevent a cytokine storm or a prolonged inflammation state [72]. A regulatory mechanism can be the activation of intracellular pathways that suppress further production of TNF-α, for example [73]. This action is primarily mediated by specific cell surface receptors like cytokine receptors and Toll-like receptors (TLRs), present in immune cells, that control the cytokine concentration in the medium [7].

Conversely, the opposite may be happening for IFN-γ, where the presence of high concentrations of IL-12 in RBC lysate samples may favor IFN-γ secretion by PBMCs, possibly initiating a cellular immune response against the given stimulus.

Additionally, the results from the same optimization assay by ELISA demonstrated a heightened production of IFN-γ and TNF-α in response to RBC-CM, indicating the potent cytokine-modulating potential. This could be attributed to a higher concentration of proinflammatory cytokines in the extracellular medium of RBC-CM compared to the RBC sample itself. This observation aligns with research conducted by Wang et al. (2019 and 2021) [74, 75], demonstrating the impact of exosomes derived from RBC units on immune cell activation and the release of proinflammatory cytokines, ultimately enhancing T-cell responses and similar effects observed in monocytes.

Following these findings, a final culture study was made using a larger cohort of RBC lysate samples from subjects (n=8), collected at the different time points (T0-T4) of COVID-19 vaccination. The PBMC: RBC ratio selected for the final assay was 1:10, which is also consistent with the study by Arosa et al. (2011) [34]. In addition to the three cytokines analyzed before, IL-15 was also studied in this final assay.

PBMCs were shown to significantly secrete IL-15, IFN-γ, and TNF-α only after stimulation. As for IFN-γ, and TNF-α, its' secretion was negative without any stimulation, as also demonstrated in Karsten et al. (2020) [27] and Stopinšek et al. (2010) [76]. In contrast, the secretion of IL-12 by PBMCs was always evident even in the absence of any stimulus and the addition of PMA and Io stimulants showed no significant effect on its secretion. This effect may likely occur because IL-12 is primarily produced by monocytes, including macrophages and dendritic cells, rather than T-cells [77,78]. Therefore, the PMA and Io stimulus, which mainly target T-cell activation and proliferation, might not significantly influence IL-12 secretion compared to the pronounced influence of RBCs on monocyte-driven secretion as previously described in the literature [78]. These studies have suggested that RBCs may influence the maturation of monocytes, particularly into dendritic cells (DCs), which are the primary producers of IL-12 [28,29].

In the presence of RBC lysates before vaccination (T0), stimulated PBMCs significantly increased the secretion of IL-12 and TNF-α. No significant effect was observed for IL-15 and IFN-γ. However, in the presence of RBC lysates after vaccination (time points T1 and T3) the secretion of all those proinflammatory cytokines in stimulated PBMCs was significantly modulated in a similar pattern as those observed in RBC themselves for these cytokines either bound or released, in response to vaccination.

Incubation with RBC lysates at 24h-72h after the 1st or 2nd vaccine dose (T1 and T3) induced a higher increase of those cytokines' secretion by stimulated PBMCs, which significantly decreased with the incubation of RBC lysates collected 28 to 30 days later after taking these doses

(T2 and T4), with a tendency to return to the levels observed with RBC T0 incubation (before vaccination).

These results are in line with Karsten et al. (2020) [26] and Profumo et al. (2011) [27]. These authors showed that the cytokine profile secreted by stimulated PBMCs in the presence of healthy RBCs or diseased RBCs was significantly different compared to PBMCs with stimulants alone, and between the former two, the cytokine profile was also different. However, our work showed for the first time in our knowledge that the cytokines profile secreted by stimulated PBMCs can be differentially modulated in the presence of RBCs from healthy subjects that underwent COVID-19 vaccination. This observation suggests that RBCs presenting differential cytokine profiles in response to vaccination as we confirmed in this study can in turn differentially modulate PBMC cytokines secretion *in vitro* with potential downstream consequences in the immune activity of these cells *in vivo*.

Vacaflores et al. (2016 and 2017) studies showed that $CD4^+$ and $CD8^+$ T-cells treated with IL-12 induced IFN-γ and TNF-α production [79,80]. IL-12 is the primary cytokine known to induce IFN-γ production by T-cells [80]. Although RBCs before vaccination (T0) showed some level of cytokines such as IL-12, this seemed to be not enough to significantly induce IFN-γ in stimulated PBMCs. Conversely, the presence of high concentrations of IL-12 in RBCs after vaccination, in particular just after 24h-72hrs of 1st or 2nd dose, may favor induction of INF-γ and TNF-α (demonstrates a significant increase, when compared with the pre-vaccination state (T0)) secretion by stimulated PBMCs, possibly initiating a cellular immune response activity in response to the given stimulus. A dose-response effect associated with the IL-12 impact on IFN-γ and TNF-α secretion by PBMCs can be considered. The same reason can be applied to the increase verified in the secretion by PBMCs of IL-15, only in the presence of vaccinated RBCs, as this cytokine can indirectly be associated with the concentrations of IL-12. However, further studies are necessary to confirm this effect.

To analyze T-cell subpopulations expansion in PBMCs under the effect of RBCs from vaccinated subjects, a preliminary flow cytometry optimization assay was carried out. For that, cells were marked with CD3-APC, to label the entire T-cell population, and with CD8-PE, to identify the $CD8^+$ T-cell subpopulation. In addition, the viability marker 7-AAD was utilized to mark the DNA of dead cells, excluding them from the viable cell population through statistical analysis.

An examination of Mean Fluorescence Intensity (MFI) within the $CD3^+$ cell population, which encompasses all T-cells, revealed higher MFI values in PBMCs stimulated with RBC lysate at 24h-72h after the 1st vaccine dose (T1), particularly at the PBMC: RBC ratio of 1:10.

This observation is in accordance with the enhanced expansion of activation of T-cells following COVID-19 vaccination [81]. A study by Moss et al. (2022) also demonstrated that all COVID-19 vaccines induce T-cell responses in addition to antibody production [82]. Moreover, as previously established, T-cells are activated by the presence of proinflammatory cytokines, such as TNF-α, IL-12, and IL-15, which were present in elevated concentrations in RBC samples collected 24-72 hours after vaccination (T1 and T3). This may account for the observed increase in the T-cell population expansion in the presence of these RBC lysates.

The $CD3^+CD8^-$ T-cell subpopulation includes T helper (Th) cells, memory $CD4^+$ T-cells, regulatory $CD4^+$ T-cells, and distinct Th subsets such as Th1, Th2, and Th17 cells. These cells contribute to immune modulation, memory recall, and fine-tuning of immune responses [82, 83, 84].

In this very preliminary study, this specific T-cell subpopulation showed a similar increased expansion in stimulated PBMCs in the presence of RBC lysates, regardless of the time point of vaccination (T0 or T1), suggesting a potential regulatory role of RBCs independently of vaccination condition. This increase was more evident/significant with the incubation of a 1:10 PBMC: RBC ratio.

$CD3^+CD8^+$ T-cell subpopulation plays roles in immune surveillance, memory formation, immune regulation, and direct elimination of infected or abnormal cells [84]. $CD3^+CD8^+$ subset T-cell population, comprises cytotoxic T-cells (CTLs), memory $CD8^+$ T-cells, regulatory $CD8^+$ T-cells, and effector $CD8^+$ T-cells [83,84].

In contrast to the $CD3^+CD8^-$ T-cell subpopulation, the expansion $CD3^+CD8^+$ T-cell subpopulation showed be increase in stimulated PMBCs in the presence of RBC T0 lysates but not in the presence of RBC lysates 24-72 hours after 1st vaccine dose, which cause instead a reduction in this cell expansion. This observation was substantial for the 1:10 PBMC: RBC ratio.

A similar trend is evident in the MFI values for this T-cell subpopulation. It is worth noting that when considering the different ratios, there is a distinction between MFI and population frequency for the $CD3^+CD8^+$ subset. The choice between these two metrics largely depends on the specific context of the analysis.

MFI serves as a metric that assesses the average fluorescence emitted by all cells expressing a particular marker. However, it is crucial to emphasize that even among cells with identical marker expression, variations in observed fluorescence intensity can arise. These variations in MFI may be attributed to diverse factors, including variations in antibody quality, differences in the instrumentation employed during the experiment, or disparities in sample preparation protocols. Given MFI's sensitivity to fluctuations in fluorescence intensity, the results presented in terms of population frequency for this subpopulation are more appropriate in this case. Population frequency considers the percentage of cells positive for the marker, irrespective

of the intensity of the fluorescence signal. This metric provides a more robust representation of the data and is less susceptible to the aforementioned variations in fluorescence intensity.

Despite the slight differences observed depending on the method used, all together the results obtained with cell culture, although very preliminary, appear to mirror the immunocellular events occurring in response to the COVID-19 vaccine.

After vaccination, $CD8^+$ T-cells are activated and migrate to the site of the simulated infection, where they multiply and differentiate into effector cells to combat the antigen present in the vaccine. This can lead to a temporary decrease in circulating $CD8^+$ cells just after vaccination [77,84]. As the immune response resolves, some of these cells differentiate into memory immune cells, which remain in the body to provide long-term protection, resulting in a gradual recovery of circulating $CD8^+$ cells.

Conversely, after vaccination against COVID-19, it is common to observe a temporary increase in $CD4^+$ cells (helper T-cells) in the body [80]. This occurs because $CD4^+$ cells play a fundamental role in coordinating the immune response. They assist in antibody production, recruit other immune cells, and help regulate the inflammatory response. This increase in $CD4^+$ cells is a normal response of the immune system to the vaccine, aimed at creating immunological memory and more effective protection against the COVID-19 virus in the future [81, 85]. These changes in cell populations are temporary and tend to normalize as the immune response stabilizes.

In summary, our results align with those demonstrated by other studies on the COVID-19 vaccine, where it is described that this vaccine induces more prominent $CD4^+$ T-cell responses than $CD8^+$ T-cell responses, characterized by a predominant Th1 and weak Th17/Th2 helper response [80, 82, 86]. This can also be explained by the previous ELISA results, where $CD4^+$ Th1 cytokines, such as IFN-γ and TNF-α, were shown to be elevated in stimulated PBMCs treated with RBCs lysates at 24h-72h after the 1st vaccine dose (T1) [87]. These observations are also supported by Ewer et al. (2020) [68] and Zhu et al. (2020) [88] in plasma/serum.

In addition, the observed effects of different PBMC: RBC ratios on cytokine secretion or T-cell expansion by PBMCs leads us to suggest that specific PBMC: RBC ratios, which are correlated to the concentration of proinflammatory cytokines or other factors present in RBC, may induce different degrees of immune cell activity in PBMCs [89].

Although preliminary, limitations in this study should be addressed. Sample size and exclusion of male RBC samples limit the data generalization.

In conclusion, the findings of this study reveal the intricate interactions between RBCs and PBMCs and their possible implications for the immunization process. The multifaceted nature of these discoveries underscores the delicate balance within the immune system that could be harnessed to enhance vaccination strategies. However, a deeper understanding of the mechanisms driving these interactions is essential to fully leverage this potential. As we go deeper into these complex interactions between RBCs and immune cells, further research is warranted to uncover.

VI | Conclusions and Future Perspectives

In summary, the discussions presented in this thesis encompass two distinct yet interconnected lines of research that shed light on the interactions between RBCs and the immune system within the context of COVID-19 vaccination.

The first part of the thesis, focusing on the analysis of cytokine profiles bound to or *in vitro* released by RBCs has provided compelling insights into the dynamic immune responses triggered by COVID-19 vaccination. Through an examination of cytokine concentrations, it became evident that the vaccination process induced significant alterations in the immune response.

The heightened levels of proinflammatory cytokines, such as Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-12 (IL-12), Interleukin-15 (IL-15), and Tumor Necrosis Factor-α (TNF-α), pointed towards a cascade of immune reactions initiated by the vaccine. The diverse functional roles of these cytokines in immune regulation further illuminated the intricate orchestration of immune signaling involving RBCs.

Notably, the temporal variations in cytokine concentrations across different time points underscored the nature of immune activation, initiation, and resolution in response to the vaccination.

The second part of the thesis delved into the immunomodulatory role of RBCs, particularly their effects on immune cells activity such as PBMCs. By combining RBC lysates and RBC-CM with PBMCs, a rich interplay was revealed, showcasing the potential influence of RBC effects on cytokine release (ELISA) and, possibly, T-cell subpopulations (verified only in the preliminary study, without statistical proof).

The cytokine dynamics and shifts in T-cell subpopulations expansion presented a complex landscape, indicative of RBCs' potential to modulate immune responses. This observation is in line with a growing body of research highlighting RBCs' role beyond their traditional function of oxygen transport, extending into the realm of immune regulation.

In conclusion, this study advances the understanding of the multifaceted interaction between RBCs and the immune system in response to COVID-19 vaccination. It highlights the potential of erythrocytes as pivotal mediators of immune responses and underscores the significance of their interactions in shaping effective vaccination strategies. As the field continues to uncover the intricate mechanisms governing these interactions, the implications for vaccine development and immune modulation remain profound and promising.

Looking to the future, these findings open several avenues for research. Optimizing lysis techniques to enhance cytokine extraction from intact RBCs could improve cytokine analysis precision. The clinical potential of RBC-associated cytokines as diagnostic biomarkers for various health and disease conditions holds considerable promise.

Further research into the immunomodulatory roles of RBCs, including their therapeutic applications in immune regulation, represents novel and impactful directions. Investigating how specific RBC-associated cytokine profiles influence vaccine efficacy may lead to more personalized and potent vaccination strategies.

Continued research into RBC interactions with immune cells, with a larger sample size, can validate and refine observations on cytokine dynamics and shifts within T-cell populations. In addition, the exploration of genetic and environmental factors influencing interindividual variations in RBC cytokine concentrations may be a helping tool for personalized medicine approaches.

Finally, these future perspectives not only advance understanding but also hold the potential to impact clinical diagnostics, therapeutic interventions, and overall comprehension of immune regulation.

VII | Bibliography

1. Wegiel, B., Hauser, C. J., & Otterbein, L. E. (2015). Heme is a dangerous molecule in pathogen recognition. *Free Radical Biology and Medicine, 89*, 651-661. https://doi.org/10.1016/j.freeradbiomed.2015.08.020

2. Turvey, S. E., & Broide, D. H. (2010). Innate immunity. *Journal of Allergy and Clinical Immunology,* 125(Suppl 2), S24–S32. https://doi.org/10.1016/j.jaci.2009.11.014

3. Wikimedia Commons. (2020, March 8). PAMPs and PRRs in the Innate Immune System [Image]. Retrieved on 2023, July 15 from https://commons.wikimedia.org/wiki/File:PAMPs_and_PRRs_in_the_Innate_Immune_System.png

4. Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2015). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget, 9*. https://doi.org/10.18632/oncotarget.23208

5. Paul, S., & Lal, G. (2017). The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. *Frontiers in Immunology,* 8, 1124. https://doi.org/10.3389/fimmu.2017.01124

6. Stone, K. D., Prussin, C., & Metcalfe, D. D. (2010). IgE, mast cells, basophils, and eosinophils. *Journal of Allergy and Clinical Immunology,* 125(Suppl 2), S73–S80. https://doi.org/10.1016/j.jaci.2009.11.017

7. Lee, H. G., Cho, M. J., & Choi, J. M. (2020). Bystander CD4+ T cells: crossroads between innate and adaptive immunity. *Experimental & Molecular Medicine, 52*, 1255–1263. https://doi.org/10.1038/s12276-020-00486-7

8. Bonilla, F. A., & Oettgen, H. C. (2010). Adaptive immunity. *Journal of Allergy and Clinical Immunology,* 125(Suppl 2), S33–S40. https://doi.org/10.1016/j.jaci.2009.08.047

9. Skapenko, A., Leipe, J., Lipsky, P. E., *et al*. (2005). The role of the T cell in autoimmune inflammation. *Arthritis Research & Therapy,* 7(Suppl 2), S4. https://doi.org/10.1186/ar1703

10. O'Garra, A. (1998). Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity,* 8, 275–283. https://doi.org/10.1016/S1074-7613(00)80528-2

11. Marshall, J. S., Warrington, R., Watson, W., *et al*. (2018). An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology,* 14(2), 49. https://doi.org/10.1186/s13223-018-0278-1

12. Lumen Learning. (n.d.). B-Lymphocytes and Humoral Immunity. Retrieved on 2023, July 15, from https://courses.lumenlearning.com/suny-microbiology/chapter/b-lymphocytes-and-humoral-immunity/

13. Moras, M., Lefevre, S. D., & Ostuni, M. A. (2017). From Erythroblasts to Mature Red Blood Cells: Organelle Clearance in Mammals. *Frontiers in Physiology,* 8, 1076. https://doi.org/10.3389/fphys.2017.01076

14. de Back, D. Z., Kostova, E. B., van Kraaij, M., van den Berg, T. K., & van Bruggen, R. (2014). Of macrophages and red blood cells; a complex love story. Frontiers in Physiology, 5, 9. https://doi.org/10.3389/fphys.2014.00009

15. Anderson, H. L., Brodsky, I. E., & Mangalmurti, N. S. (2018). The Evolving Erythrocyte: Red Blood Cells as Modulators of Innate Immunity. *Journal of Immunology,* 201, 1343-1351. https://doi.org/10.4049/jimmunol.1800565

16. Dobkin, J., Wu, L., & Mangalmurti, N. S. (2023). The ultimate tradeoff: how red cell adaptations to malaria alter the host response during critical illness. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 324(2), L169-L178. https://doi.org/10.1152/ajplung.00139.2022

17. Hemmi H, Takeuchi O, Kawai T, *et al*. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature*, 408, 740–745. https://doi.org/10.1038/35047123

18. Hotz, MJ., Qing, D., Shashaty, MGS., *et al*. (2018). Red blood cells homeostatically bind mitochondrial DNA through TLR9 to maintain quiescence and prevent lung injury. *American Journal of Respiratory and Critical Care Medicine*, 197, 470–480. https://doi.org/10.4049/jimmunol.1800565

19. Lam, LKM., Murphy, S., Kokkinaki, D., *et al*. (2021). DNA binding to TLR9 expressed by red blood cells promotes innate immune activation and anemia. *Science Translational Medicine*, 13, eabj1008. https://doi.org/10.1016/s0006-291x(03)00293-6

20. Darbonne, WC., Rice, GC., Mohler, MA., *et al*. (1991). Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *Journal of Clinical Investigation*, 88, 1362–1369. https://doi.org/10.1172/JCI115438

21. Fukuma, N., Akimitsu, N., Hamamoto, H., *et al*. (2003). A role of the Duffy antigen for the maintenance of plasma chemokine concentrations. *Biochemical and Biophysical Research Communications*, 303, 137–139. https://doi.org/10.1016/s0006-291x(03)00330-0

22. Karsten, E., Breen, E., & Herbert, BR. (2018). Red blood cells are dynamic reservoirs of cytokines. *Scientific Reports*, 8, 3101. https://doi.org/10.1038/s41598-018-21387-w

23. Pruenster, M., Mudde, L., Bombosi, P., *et al*. (2009). The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nature Immunol*, 10, 101–108. https://doi.org/10.1038/ni.1675

24. Mahdi, A., Collado, A., Tengbom, J., *et al*. (2022). Erythrocytes induce vascular dysfunction in COVID-19. *JACC to Translational Science*, 7, 193–204. https://doi.org/10.1016/j.jacbts.2021.12.003

25. Antunes, RF., Brandão, C., Carvalho, G., *et al*. (2009). Red blood cells carry out T cell growth and survival bioactivities that are sensitive to cyclosporine A. *Cellular and Molecular Life Sciences*, 66, 3387–3398. https://doi.org/10.1007/s00018-009-0119-y

26. Karsten E, Breen E, McCracken SA, Clarke S, Herbert BR. (2020). Red blood cells exposed to cancer cells in culture have altered cytokine profiles and immune function. *Scientific Reports*, 10(1):7727. https://doi.org/10.1038/s41598-020-64319-3

27. Profumo, E., *et al*. (2011). Redox imbalance of red blood cells impacts T lymphocyte homeostasis: implication in carotid atherosclerosis. *Thrombosis and Haemostasis*, 106, 1117–1126. https://doi.org/10.1160/TH11-02-0110

28. Bettiol, E., Carapau, D., Galan-Rodriguez, C., *et al*. (2010). Dual effect of Plasmodium-infected erythrocytes on dendritic cell maturation. *Malaria Journal*, 9, 64. https://doi.org/10.1186/1475-2875-9-64

29. Urban, BC., Ferguson, DJ., Pain, A., *et al*. (1999). Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. *Nature*, 400, 73–77. https://doi.org/10.1038/21900

30. Thangaraju, K., Neerukonda, SN., Katneni, U., & Buehler, PW. (2020). Extracellular Vesicles from Red Blood Cells and Their Evolving Roles in Health, Coagulopathy and Therapy. *International Journal of Molecular Sciences*, 22(1), 153. https://doi.org/10.3390/ijms22010153

31. Danesh, A., Inglis, H.C., Jackman, R.P., *et al*. (2014). Exosomes from red blood cell units bind to monocytes and induce proinflammatory cytokines, boosting T-cell responses *in vitro*. *Blood*, 123, 687–696. https://doi.org/10.1182/blood-2013-10-530469

32. Straat, M., van Hezel, M.E., Böing, A., *et al*. (2016). Monocyte-mediated activation of endothelial cells occurs only after binding to extracellular vesicles from red blood cell products, a process mediated by β-integrin. *Transfusion*, 56, 3012–3020. https://doi.org/10.1111/trf.13851

33. Fischer, D., Büssow, J., Meybohm, P., *et al*. (2017). Microparticles from stored red blood cells enhance procoagulant and proinflammatory activity. *Transfusion*, 57, 2701–2711. https://doi.org/10.1111/trf.14268

34. Antunes, R.F., Brandão, C., Maia, M., & Arosa, F.A. (2011). Red blood cells release factors with growth and survival bioactivities for normal and leukemic T cells. *Immunology and Cell Biology*, 89, 111–121. https://doi.org/10.1038/icb.2010.61

35. Fonseca, A.M., Porto, G., Uchida, K., & Arosa, FA. (2001). Red blood cells inhibit activation-induced cell death and oxidative stress in human peripheral blood T lymphocytes. *Blood*, 97, 3152–3160. https://doi.org/10.1182/blood.V97.10.3152

36. Fonseca, A.M., Pereira, C.F., Porto, G., & Arosa, F.A. (2003). Red blood cells promote survival and cell cycle progression of human peripheral blood T cells independently of CD58/LFA-3 and heme compounds. *Cellular Immunology*, 224, 17–28. https://doi.org/10.1016/S0008-8749(03)00150-2

37. Oonishi, T., *et al*. (1998). Production of prostaglandins E1 and E2 by adult human red blood cells. *Prostaglandins & Other Lipid Mediators*, 56, 89–101. https://doi.org/10.1016/S0090-6980(98)00027-4

38. Thomas, T., Stefanoni, D., Dzieciatkowska, M., *et al*. (2020). Evidence of structural protein damage and membrane lipid remodeling in red blood cells from COVID-19 patients. *Journal of Proteome Research*, 19, 4455–4469. https://doi.org/10.1021/acs.jproteome.0c00476

39. Kuba´nkova´, M., Hohberger, B., Hoffmanns, J., *et al*. (2021). Physical phenotype of blood cells is altered in COVID-19. *Biophysical Journal*, 120, 2838–2847. https://doi.org/10.1016/j.bpj.2021.05.025

40. Morera, D., Roher, N., Ribas, L., *et al*. (2011). RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PLoS One*, 6(10), e26998. https://doi.org/10.1371/journal.pone.0026998

41. Bharadwaj, M., Mifsud, N., & McCluskey, J. (2012). Detection and Characterisation of Alloreactive T Cells. *Methods in Molecular Biology*, 882, 309-37. https://doi.org/10.1007/978-1-61779-842-9_18

42. Sousa, D. I. P. (2016). Improving the anti-tumor immune responses against cancer cells (Master's thesis). FCT/UNL, Lisbon, Portugal. URL: https://run.unl.pt/bitstream/10362/19550/1/Sousa_2016.pdf

43. Leinco Technologies. (n.d.). Sandwich ELISA Protocol. Retrieved August 1, 2023, from https://www.leinco.com/sandwich-elisa-protocol/

44. Maecker HT, McCoy JP, Nussenblatt R.(2012). Standardizing immunophenotyping for the Human Immunology Project. *Nature Reviews Immunology*, 12(3),191-200. https://doi.org/ 10.1038/nri315

45. Sahin, U., Muik, A., Derhovanessian, E., *et al.* (2020). COVID-19 vaccines: immune response, side effects, and safety. *Nature Reviews Immunology*, 20(8), 457-464. https://doi.org/10.1038/s41577-020-00404-4

46. Hawerkamp, H. C., Dyer, A. H., Patil, N. D., McElheron, M., O'Dowd, N., O'Doherty, L., Mhaonaigh, A. U., George, A. M., O'Halloran, A. M., Reddy, C., Kenny, R. A., Little, M. A., Martin-Loeches, I., Bergin, C., Kennelly, S. P., Donnelly, S. C., Bourke, N. M., Long, A., Sui, J., Doherty, D. G., Conlon, N., Cheallaigh, C. N., & Fallon, P. G. (2023). Characterization of the proinflammatory cytokine signature in severe COVID-19. *Frontiers in Immunology*, 14, 1170012. https://doi.org/10.3389/fimmu.2023.1170012

47. Hasanvand, A. (2022). "COVID-19 and the role of cytokines in this disease. *Inflammopharmacol*, 30, 789–798, https:// 10.1007/s10787-022-00992-2

48. Bergamaschi C, Terpos E, Rosati M, Angel M, Bear J, Stellas D, Karaliota S, Apostolakou F, Bagratuni T, Patseas D, Gumeni S, Trougakos IP, Dimopoulos MA, Felber BK, Pavlakis GN. (2021). "Systemic IL-15, IFN-γ, and IP-10/CXCL10 signature associated with effective immune response to SARS-CoV-2 in BNT162b2 mRNA vaccine recipients." *Cell Rep*, 36(6), 109504, https:// 10.1016/j.celrep.2021.109504.

49. Kandikattu, H. K., Venkateshaiah, S. U., Kumar, S., & Mishra, A. (2020). IL-15 immunotherapy is a viable strategy for COVID-19. *Cytokine Growth Factor Rev*, 54, 24-31. https:// 10.1016/j.cytogfr.2020.06.008.

50. Dinarello, CA. (2018). Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunological Reviews*, 281(1), 8-27. https://doi.org/10.1111/imr.12621

51. Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). IL-6 in inflammation, immunity, and disease. *Cold Spring Harbor Perspectives in Biology*, 6(10), a016295. https://doi.org/10.1101/cshperspect.a016295

52. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, Presky DH. (1998). The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annual Review of Immunology,* 16, 495-521. https://doi.org/10.1146/annurev.immunol.16.1.495

53. Wang X, Zhao XY. (2021). Transcription Factors Associated With IL-15 Cytokine Signaling During NK Cell Development. *Frontiers in Immunology,* 12, 610789. https://doi.org/10.3389/fimmu.2021.610789

54. Vignali DA, Kuchroo VK. (2012) IL-12 family cytokines: immunological playmakers. *Nature Immunology,* 13(8), 722-728. https://doi.org/10.1038/ni.2366

55. Dubois S, Mariner J, Waldmann TA, Tagaya Y. (2002). IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity*., 17(5), 537-47. https://doi.org/10.1016/s1074-7613(02)00429-6

56. Bergamaschi C, Stravokefalou V, Stellas D, Karaliota S, Felber BK, Pavlakis GN. (2021). Heterodimeric IL-15 in Cancer Immunotherapy. *Cancers (Basel)*., 13(4), 837. https://doi.org/10.3390/cancers13040837

57. Aggarwal, B.B. (2003). Signaling pathways of the TNF superfamily: a double-edged sword. *Nature Reviews Immunology*, 3(9), 745-756. https://doi.org/10.1038/nri1184

58. den Hartog, Y., Malahe, S.R.K., Rietdijk, W.J.R. *et al*. (2023). Th1-dominant cytokine responses in kidney patients after COVID-19 vaccination are associated with poor humoral responses. *npj Vaccines* 8, 70. https://doi.org/10.1038/s41541-023-00664-4

59. Alpatova, N. A., Avdeeva, Z. I., Nikitina, T. N., *et al*. (2020). Adjuvant Properties of Cytokines in Vaccination (Review). *Pharmaceutical Chemistry Journal,* 53, 991–996. https://doi.org/10.1007/s11094-020-02111-3

60. Nakayama T. (2016). An inflammatory response is essential for the development of adaptive immunity-immunogenicity and immunotoxicity. *Vaccine*. 11, 34(47), 5815-5818. https://doi.org/10.1016/j.vaccine.2016.08.051

61. Kumari, M., Lu, RM., Li, MC. *et al.* (2022). COVID-19 vaccine type-dependent differences in immunogenicity and inflammatory response: BNT162b2 and ChAdOx1 nCoV-19. *Frontiers in Immunology*. 13, 975363. https://10.3389/fimmu.2022.975363

62. Wang, C., Yang, S., Duan, L. *et al.* (2022). Adaptive immune responses and cytokine immune profiles in humans following prime and boost vaccination with the SARS-CoV-2 CoronaVac vaccine. *Virol J,* 19, 223. https://doi.org/10.1186/s12985-022-01957-1.

63. Hasanvand, A. (2022). "COVID-19 and the role of cytokines in this disease." *Inflammopharmacol*, 30, 789–798, https://doi.org/10.1007/s10787-022-00992-2

64. Nakayama T, Sawada A, Ito T. (2022) Comparison of cytokine production in mice inoculated with messenger RNA vaccines BNT162b2 and mRNA-1273. *Microbiol Immunol*, 67(3), 120-128. https://doi.org/10.1111/1348-0421.13043

65. Murata K, Nakao N, Ishiuchi N, Fukui T, Katsuya N, Fukumoto W, Oka H, Yoshikawa N, Nagao T, Namera A, Kakimoto N, Oue N, Awai K, Yoshimoto K, Nagao M. (2022). Four cases of cytokine storm after COVID-19 vaccination: Case report. *Frontiers Immunology*, 13:967226. https://doi.org/10.3389/fimmu.2022.967226.

66. Karsten, E., Hill, C., & Herbert, B. R. (2018). Red blood cells: the primary reservoir of macrophage migration inhibitory factor in whole blood. *Cytokine*, 102, 34-40. https://doi.org/10.1016/j.cyto.2017.12.005

67. Kany S, Vollrath JT, Relja B (2019). Cytokines in Inflammatory Disease. *International Journal of Molecular Sciences*. 20(23), 6008. https://doi.org/10.3390/ijms20236008

68. Ewer, K.J., Barrett, J.R., Belij-Rammerstorfer, S. *et al.* (2021). T cell and antibody responses induced by a single dose of ChAdOx1 nCoV-19 (AZD1222) vaccine in a phase 1/2 clinical trial. *Nature Medicine,* **27**, 270–278. https://doi.org/10.1038/s41591-020-01194-5

69. Dahlke C, Kasonta R, Lunemann S, Krähling V, Zinser ME, Biedenkopf N, Fehling SK, Ly ML, Rechtien A, Stubbe HC, Olearo F, Borregaard S, Jambrecina A, Stahl F, Strecker T, Eickmann M, Lütgehetmann M, Spohn M, Schmiedel S, Lohse AW, Becker S, Addo MM; VEBCON Consortium. (2017). Dose-dependent T-cell Dynamics and Cytokine Cascade Following rVSV-ZEBOV Immunization. *EBioMedicine*, 19, 107-118. https://doi.org/10.1016/j.ebiom.2017.03.045.

70. Karaba AH, Zhu X, Benner SE, Akinde O, Eby Y, Wang KH, Saraf S, Garonzik-Wang JM, Klein SL, Bailey JR, Cox AL, Blankson JN, Durand CM, Segev DL, Werbel WA, Tobian AAR. (2022). Higher Proinflammatory Cytokines Are Associated With Increased Antibody Titer After a Third Dose of SARS-CoV-2 Vaccine in Solid Organ Transplant Recipients. *Transplantation*, 106(4), 835-841. https://doi.org/10.1097/TP.0000000000004057.

71. Waldmann, T. A. (2014). The biology of interleukin-15: implications for cancer therapy and the treatment of autoimmune disorders. *Journal of Experimental Medicine*, 209(5), 961-964. https://doi.org/10.1084/jem.20131240.

72. Maini RN, Elliott MJ, Brennan FM, Feldmann M. (1995). Beneficial effects of tumour necrosis factor-alpha (TNF-alpha) blockade in rheumatoid arthritis (RA). *Clinical & Experimental Immunology*. 101(2), 207–212. https://doi.org/0.1111/j.1365-2249.1995.tb08340.x

73. Parameswaran N, Patial S. (2010). Tumor necrosis factor-α signaling in macrophages. *Critical Revisiews in Eukaryotic Gene Expression*, 20(2), 87-103. https://doi.org/10.1615/critreveukargeneexpr.v20.i2.10

74. Wang, Y., Jia, L., & Mao, X. (2019). Exosomes from RBC units bind to monocytes and induce proinflammatory cytokines release, boosting T cell responses *in vitro*. *Frontiers in Immunology*, 10, 1317. https://doi.org/10.3389/fimmu.2019.01317.

75. Wang, Y., Jia, L., Shen, J., Wang, Y., Cederbaum, A., & Mao, X. (2021). Exosomes From Red Blood Cell Units Bind to Monocytes and Induce Proinflammatory Cytokine Release, Increasing Expression of CD16, CD86 and CD36. *Frontiers in Immunology*, 12, 686776. https://doi.org/10.3389/fimmu.2021.686776

76. Stopinšek, S., Terčelj, M., Salobir, B., Wraber, B., Ihan, A., Rylander, R., & Simčič, S. (2010). Effects of fungal cell wall polysaccharides and lipopolysaccharide on *in vitro* tumor necrosis factor alpha production by peripheral blood mononuclear cells of sarcoidosis patients. *ZdravVestn*, 79(10). https://doi.org/10.2478/v10151-010-0017-9

77. D'Alessandro, A., D'Amici, G. M., Vaglio, S., & Zolla, L. (2019). Time-course investigation of SAGM-stored leukocyte-filtered erythrocyte concentrates: from metabolism to proteomics. *Haematologica*, 104(6), 1194-1206. https://doi.org/10.3324/haematol.2018.199802

78. Effenberger-Neidnicht, K., Schumacher, D., Gorges, J., & Heim, M. U. (2020). Erythrocytes modulate the immune response to antigen by induction of regulatory T cells. *International Journal of Molecular Sciences*, 21(18), 6806. https://doi.org/10.3390/ijms21186806.

79. Vacaflores A, Chapman NM, Harty JT, Richer MJ, Houtman JC. (2016). Exposure of human CD4 T cells to IL-12 results in enhanced TCR-induced cytokine production, altered TCR signaling, and increased oxidative metabolism. *PLoS One*, 11(6), e0157175. https://doi.org/ 10.1371/journal.pone.0157175

80. Vacaflores A, Freedman SN, Chapman NM, Houtman JC. (2017) Pretreatment of activated human CD8 T cells with IL-12 leads to enhanced TCR-induced signaling and cytokine production. *Molecular Immunol*ogy, 81, 1–15. https://doi.org/ 10.1016/j.molimm.2016.11.008

81. Takayuki Matsumura, *et al*. (2023). Immune responses related to the immunogenicity and reactogenicity of COVID-19 mRNA vaccines, *International Immunology*, 35(5), 213–220. https://doi.org/10.1093/intimm/dxac064. 80

82. Moss, P. (2022). The T cell immune response against SARS-CoV-2. *Nature Immunology,* 23, 186–193. https://doi.org/10.1038/s41590-021-01122-w

83. Josefowicz, S. Z., Lu, L. F., & Rudensky, A. Y. (2012). Regulatory T cells: mechanisms of differentiation and function. *Annual Review of Immunology*, 30, 531-564. https://doi.org/10.1146/annurev-immunol-020711-075036

84. Harty, J. T., & Badovinac, V. P. (2008). Shaping and reshaping CD8+ T-cell memory. *Nature Reviews Immunology*, 8(2), 107-119. https://doi.org/10.1038/nri2253

85. Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., ... & Sakaguchi, S. (1998). Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *International Immunology*, 10(12), 1969-1980. https://doi.org/10.1093/intimm/10.12.1969

86. DiPiazza, A. T., Graham, B. S., & Ruckwardt, T. J. (2021). T cell immunity to SARS-CoV-2 following natural infection and vaccination. *Biochem Biophys Res Commun*., 538, 211-217. https://doi.org/10.1016/j.bbrc.2020.10.060.

87. Guo, Y., Hu, K., Li, Y., *et al*. (2022). Targeting TNF-α for COVID-19: Recent Advanced and Controversies. *Front Public Health*, 10, 833967. https://doi.org/10.3389/fpubh.2022.833967.86

88. Zhu F.C., Li Y.H., Guan X.H., Hou L.H., Wang W.J., Li J.X., Wu S.P., Wang B.S., Wang Z., Wang L., Jia S.Y., Jiang H.D., Wang L., Jiang T., Hu Y., Gou J.B., Xu S.B., Xu J.J., Wang X.W., Wang W., Chen W. (2020). Safety, tolerability, and immunogenicity of a recombinant adenovirus type-5 vectored COVID-19 vaccine: a dose-escalation, open-label, non-randomised, first-in-human trial. *The Lancet.*, 395, 1845–1854. https://10.1016/S0140-6736(20)31208-3. 87

89. Barberis, M., Helikar, T., & Verbruggen, P. (2018). Simulation of Stimulation: Cytokine Dosage and Cell Cycle Crosstalk Driving Timing-Dependent T Cell

Differentiation. *Frontiers in Physiology*, 9, 00879. https://doi.org/10.3389/fphys.2018.00879