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Vaccine- and infection-induced cellular immunity against SARS-CoV-2 in humans

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Tiivistelmä – Referat – Abstract

In this thesis, I studied T cell responses to SARS-CoV-2 structural proteins in subjects who had been both vaccinated and infected (n=30), who had only been infected (n=22), and as controls, in subjects who had been neither vaccinated nor infected (n=6). In addition, I compared cellular responses between groups of subjects who had been infected with either wild-type (WT) SARS-CoV-2, Alpha (B.1.1.7), or Beta (B.1.351) variants. Before analyzing the samples to be studied, I optimized the conditions for the cell stimulations.

Peripheral blood mononuclear cells (PBMCs) were collected from infected subjects six months after infection. PBMCs were stimulated with SARS-CoV-2 wild-type nucleoprotein, spike-, envelope-, and membrane protein peptide pools. I quantified cytokines and effector molecules characteristic of CD4+ and CD8+ cell responses; perforin, tumor necrosis factor alpha (TNF-a), granzyme B, interferon gamma (IFN-γ), interleukin 2 (IL-2) and interleukin 4 (IL-4) secreted by PBMCs were quantified.

In this study, I found that subjects with infection, or combination of infection and vaccination had higher cellular immune responses compared to uninfected controls. Infection induced higher granzyme B, IFN-γ, and IL-2 secretion, and the combination of infection and vaccination induced higher granzyme B, perforin, IFN-γ, IL-2 and IL-4 secretion. I found that subjects with hybrid immunity, defined as immunity acquired from combined vaccination and infection, had on average higher IL-4 responses compared to those who had been infected only. In this study, I found that nucleoprotein, spike-, and membrane proteins stimulated T cell responses whereas envelope protein did not stimulate T cell responses. I found that WT, Alpha or Beta infection produced equally good T cell responses to WT spike peptide.

In conclusion, I found that COVID-19 patients have long-lasting T cell responses. I found that T cells recognize different SARS-CoV-2 variants. Mutations present in the spike proteins of the different variants do not affect T-cell ability to recognize these antigens. Immunity based on T cells is not as susceptible to antigenic changes as the humoral immunity. T cells have a vital role in protection against variants, when new SARS-CoV-2 variants evaded antibody-based immunity.

Avainsanat – Nyckelord – Keywords

Cellular immunity, T cell, SARS-CoV-2, cytokine, effector molecule, vaccination, SARS-CoV-2 variant

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Rokotteen ja infektion tuottama soluvälitteinen immuniteetti SARS-CoV-2 virusta vastaan ihmisillä

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Tiivistelmä – Referat – Abstract

Tässä maisterintutkielmassa tutkittiin T-soluvasteita SARS-CoV-2:n rakenneproteiineille. T-solu vasteita tutkittiin rokotetuilla ja tartunnan saaneilla (n=30), vain tartunnan saaneilla (n=22) sekä henkilöillä, joilla ei ollut aiempaa tartuntaa eikä rokotteita (n = 6). Lisäksi vertailin T-solu vasteita ryhmien välillä, jotka olivat saaneet joko villityypin- (WT), Alfa- (B.1.1.7) tai Beta -infektion (B.1.351). Ennen tutkittavien näytteiden analysointia optimoin solustimulaatio olosuhteet.

Perifeerisen veren mononukleaarisolut (PBMC) kerättiin tartunnan henkilöiltä kuusi kuukautta tartunnan jälkeen. Mononukleaarisoluja stimuloitiin SARS-CoV-2 villityypin nukleo-, piikki-, vaippa- ja kalvoproteiini- peptidi pooleilla. Tässä työssä määritin mononukleaaristensolujen erittämät sytokiinit ja efektorimolekyylit, jotka olivat perforiini, tuumorinekroositekijä alfa (TNF-a), grantsyymi B, interferoni gamma (IFN-y), interleukiini 2 (IL-2) ja interleukiini 4 (IL-4). Nämä sytokiinit ja efektorimolekyylit kertovat CD4+- ja CD8+ solu vasteista.

Löysin että tutkittavilla, joilla oli pelkästään infektio tai infektion ja rokotuksen yhdistelmä, oli voimakkaampi soluvälitteinen immuunivaste verrattuna terveisiin kontrolli henkilöihin. Infektio sai aikaan suurempaa grantsyymi B:n, IFN-y:n ja IL-2:n eritystä. Infektion ja rokotuksen yhdistelmä sai aikaan suurempaa grantsyymi B:n, perforiinin, IFN-y:n, IL-2:n ja IL-4:n eritystä. Huomasin, että henkilöillä, joilla oli rokotteen sekä infektion tuottamaa hybridi-immuniteettiä SARS-CoV-2 vastaan, oli keskimäärin suurempi IL-4:n eritys verrattuna henkilöihin, jotka olivat saaneet vain tartunnan. Tässä tutkielmassa havaitsin, että nukleo-, piikki- ja kalvoproteiini stimuloivat T-soluvasteita, kun taas vaippaproteiini ei saanut aikaan T-soluvasteita. Löysin, että WT-, Alfa- tai Beeta-infektiot tuottivat yhtä hyviä T-soluvasteita villityypin piikkiproteiinille.

Löysin tässä tutkielmassa, että COVID-19-potilaille syntyy pitkäkestoinen T-soluvaste. Löysin myös, että T-solut tunnistavat erilaisia SARS-CoV-2-variantteja. Eri varianttien piikkiproteiineissa esiintyvät mutaatiot eivät vaikuta T-solujen kykyyn tunnistaa näitä antigeenejä. T-soluihin perustuva immuniteetti ei ole siis yhtä herkkä antigeeni muutoksille kuin vasta-aine välitteinen immuniteetti on. T-soluilla on tärkeä rooli uusia variantteja vastaan suojautumisessa, kun uudet SARS-CoV-2-variantit väistävät vasta-aine välitteistä immuniteettia.

Avainsanat – Nyckelord – Keywords

Soluvälitteinen immuniteetti, T-solu, SARS-CoV-2, sytokiini, efektorimolekyyli, rokotus

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List of Abbreviations

IFN-γ	**Interferon gamma**
IL-2	**Interleukin 2**
IL-4	**Interleukin 4**
TNF-α	**Tumor necrosis factor alpha**
SARS-CoV-2	**Severe acute respiratory syndrome coronavirus 2**
COVID-19	**Coronavirus disease 2019**
PBMC	**Peripheral blood mononuclear cell**
WT	**Wild-type**
WHO	**World Health Organization**
VOC	**The variant of consent**
FMIA	**Fluorescent multiplex immunoassay**
MNT	**Microneutralization test**
CO2	**Carbon dioxide**
S protein	**Spike glycoprotein**
N protein	**Nucleoprotein**
M protein	**Membrane protein**
E protein	**Envelope protein**
DMSO	**Dimethyl sulfoxide**
UK	**United Kingdom**
HCW	**Health care workers**
RBD	**protein receptor binding domain**

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes a viral respiratory disease, coronavirus disease 2019 (COVID-19). SARS-CoV-2 has been circulating all over the world since it emerged in late 2019. First, wild-type SARS-CoV-2 spread and eventually caused the COVID-19 pandemic. Over time SARS-CoV-2 virus has been changed and SARS-CoV-2 variants have been detected since late in 2020. Some of the changes are beneficial, such as causing more effective spreading or impaired neutralizing responses (Haveri *et al.*, 2021; Cheng *et al.*, 2022; Wang *et al.*, 2022). World Health Organization (WHO) is tracking SARS-CoV-2 variants and will be defining new variant to be variants of concern (VOCs) if it will be a risk to global public health at that moment (Tracking SARS-CoV-2 variants). Over time, VOCs have caused multiple waves of infections. Previously defined VOC, the Alpha variant (B.1.1.7) started circulating in Finland in December 2020. Also previously defined VOC, the Beta variant (B.1.351) started circulating in Finland right after the Alpha variant. The Alpha variant was the dominant variant in Finland in the two first quarters of 2021 (7.2 – 13.6.2021). The Beta variant (B.1.351) was the second dominant variant in the second quarter of 2021 (14.3 – 9.5.2021) ("SARS-CoV-2-koronaviruksen genomiseuranta - THL").

Immunity can be divided into innate immunity and adaptive immunity. Innate immunity reacts the same way to all pathogens and provides immediate and non-specific protection. Adaptive immunity is highly specific and can create long-lasting memory. Adaptive immunity requires time to develop after the first exposure to a new antigen. Adaptive immunity is based on two main types of immune cells, B cells and T cells, where B cells play a vital role in the humoral immune response, mediated by antibodies. This thesis focuses on cellular immunity which is mediated by T cells. More specifically, cytotoxic T cells (CD8+) directly recognize and eliminate virus-infected cells, whilst T helper cells (CD4+) coordinate and activate other immune cells. Signaling molecules, cytokines and effector molecules regulate and coordinate adaptive immunity, and are produced by immune cells of which T cells are one of the most significant producers (Castellino and Germain, 2006).

SARS-CoV-2 virus has four structural proteins spike (S), membrane (M), envelope (E), and nucleoprotein (N), which will be used for stimulations in this thesis. The most abundant protein, membrane protein is the main component of the shell. On the other hand, envelope protein is the smallest of the structural proteins. Nucleoprotein is found inside the virus particle and supports genome packing by binding to RNA (Yan *et al.*, 2022). Spike protein is a membrane glycoprotein that

mediates virus fusion and entry to the host cell, via the receptor-binding domain (RBD) of the spike protein binding to the host cell angiotensin-converting enzyme 2 (ACE2) receptors (Tai *et al.*, 2020; Zhou *et al.*, 2020).

Humoral immunity is primarily based on antibody binding to spike protein. Neutralizing antibodies prevent virus entry into the host cells by target the RBD region of the spike protein (Ju *et al.*, 2020). In contrast to antibodies, T cells recognize different SARS-CoV-2 protein regions when antigen-presenting cells break up and present these antigens to T cells. Different mutations have accumulated in SARS-CoV-2 variants, especially mutations that have accumulated in the RBD region in spike protein. RBD mutations in variants can affect neutralizing antibodies' ability to recognize mutated RBD regions when neutralizing antibodies targeted against RBD region in wild-type SARS-CoV-2 virus. Therefore is reported impaired neutralizing responses against SARS-CoV-2 variants (Haveri *et al.*, 2021; Cheng *et al.*, 2022; Wang *et al.*, 2022). For that reason, humoral immunity is vulnerable to spike protein mutations.

The COVID-19 pandemic caused rapid development of different types of COVID-19 vaccines. Different types of COVID-19 vaccines based on SARS-CoV-2 spike protein, like messenger RNA (mRNA) vaccines, such as Pfizer BioNTech vaccine BNT162b2 (Krammer, 2020; Lamb, 2021) and Moderna vaccine Spidevax mRNA-1273 (*COVID-19 Vaccine Moderna (mRNA-1273)*), or like adenovirus vector vaccines, such as AstraZeneca vaccine Vaxzevria ChadOx1-S (*AstraZeneca ChAdOx1-S/nCoV-19, COVID-19 vaccine*). After the large-scale introduction of COVID-19 vaccines, infections have also occurred in a growing part of the population, producing so-called hybrid immunity. Hybrid immunity has been found in studies to produce the most effective protection against severe disease (Lasrado and Barouch, 2023).

SARS-CoV-2-specific humoral immunity has been extensively studied over the past couple of years of the COVID-19 pandemic. At the same time, SARS-CoV-2-specific cellular immunity has received less attention partly due to time-consuming and expensive cell methods and protocols to study cellular immunity. Also, COVID-19 cellular immunity studies are not standardized and therefore laboratories have used various methods and protocols to study cellular immunity to SARS-CoV-2. Because of the above, all data from COVID-19 T cell studies are valuable. This thesis focuses on cellular immunity, showing cellular immune responses by measuring cytokines, such as Interferon gamma (IFN-γ), Interleukin 2 (IL-2), Interleukin- 4 (IL-4) and Tumor necrosis factor alpha (TNF-a) and effector molecules, such as perforin and Granzyme B (GrB). Cytokines and effector molecules were

measured from peripheral blood mononuclear cell (PBMC) samples, which include round nuclei immune cells, T cells, B cells, natural killer cells, monocytes, and dendritic cells.

Aims of the study

The main aim of the study was to understand cellular immune responses to SARS-CoV-2. The first objective of this study was to determine the optimal stimulation time and quantity of cells to achieve maximal cytokine and effector molecule secretion. The second objective was to compare cellular immune responses between three study groups consisting of i) subjects who had been vaccinated and infected, ii) subjects who had been infected but not vaccinated, and iii) controls who were neither vaccinated nor infected. The third objective was to compare cellular immune responses between groups of subjects who had been infected with either WT, Alpha (B.1.1.7), or Beta (B.1.351) variants.

Materials and Methods

Participants

This thesis is part of a larger study cohort, serological population study of the coronavirus epidemic (*Koronaepidemian serologinen väestötutkimus - THL*). The study was approved by the ethical committee of the Hospital District of Helsinki and Uusimaa and registered under study protocol HUS/1137/2020. Written informed consent has been received from all participants.

Potential participants were identified in the National Infectious Disease Register maintained by the Finnish Institute for Health and Welfare. Participants with RT-PCR-confirmed SARS-CoV-2 infection were invited to participate in the study. PBMC samples analyzed in this thesis were collected six months after a laboratory-confirmed SARS-CoV-2 infection. Control group samples were collected from family members of the patients who had not tested positive for SARS-CoV-2 with PCR. The control samples were confirmed to be from non-infected individuals by measuring SARS-CoV-2 antibodies via in-house fluorescent multiplex immunoassay (FMIA) and microneutralization test (MNT) (Haveri *et al.*, 2021; Solastie *et al.*, 2021). The control samples were handled the same way as samples from SARS-CoV-2 infected patients. Blood sampling was performed at the Finnish Institute for Health and Welfare, Helsinki. COVID-19 vaccination information was verified from the

national vaccination register. Information on SARS-COV-2 infections was obtained from the National Infectious Diseases Register.

The study subjects included in this study had a prior RT-PCR confirmed infection with either wild-type (WT) (n=20), Alpha B.1.1.7 (n=20), and Beta B.1.351 (n=12). Samples were collected at six months after SARS-CoV-2 infection. Samples from subjects with WT infection were collected between May 31 to June 14, 2021; from subjects with Alpha variant infection were collected between August 16 to August 25, 2021, and from subjects with Beta variant infection were collected from August 17 to September 7, 2021. From each variant group, subjects who had received both the COVID-19 vaccine and SARS-CoV-2 infection and subjects who had only been infected but not vaccinated, were selected for this study.

The total sample size of vaccinated and infected subjects was 30 (WT n=10, Alpha n=14, and Beta n=6). The total sample size of only infected subjects was 22 (WT n=10, Alpha n=6, and Beta n=6). In addition to these SARS-CoV-2 infection samples, 6 unvaccinated uninfected subjects were selected for the control group. Accordingly, the total sample size in this study was 58 and these study participants were 21 – 72 years old (mean 47 years) and 62% were female (36/58). The vaccinated subjects had received a single dose of a COVID-19 vaccine after the SARS-CoV-2 infection. The time between vaccination and blood sampling was 5 – 76 days. The majority of these vaccinees had received Comirnaty (BNT162b2, BioNTech/Pfizer, n=28) only one vaccinee had received Vaxzevria (ChadOx1-S, AstraZeneca) and one vaccinee had received Spikevax (mRNA-1273, Moderna).

Division of labor

Coworker Liedes collected blood samples with the help of Vara, who took care of human peripheral blood mononuclear cells (PBMCs) isolation. Vara developed a new PBMC stimulation method based on the old cell stimulation method. Vara also performed PBMC stimulations and collected PBMC culture supernatants. Vara also performed cytokine and effector molecule detection in cell culture supernatants.

Cell isolation

Whole blood samples were collected into BD Vacutainer® CPT™ Cell Preparation Tubes (sodium citrate additive). PBMCs were isolated from CPT™ tubes by using a centrifuge to separate red blood cells and plasma. PBMCs formed a layer above a plasma layer. The PBMC layer was carefully collected and washed two times with Ficoll salt solution using density gradient centrifugation. PBMCs were counted with Scepter™ 2.0 (Sigma-Aldrich) cell counter. CryoStor® CS10 (Stem Cell Technologies) was added to the PBMC samples and PBMCs were stored in a concentration of $1{,}7x10^7$ cells per ml at ≤ 68 °C for 24h. Frozen PBMCs were transferred to vapor phase liquid nitrogen (under –135°C) until further analysis.

Optimization of PBMC stimulation

Optimal cell stimulation conditions, the number of cells per well, and stimulation time were tested before further analyses. Stimulation time was optimized by comparing the level of IFN-y production at two and four days. Cell concentration was optimized by testing 200 000 and 400 000 PBMCs per well. As a positive control sample, cells were stimulated with antibodies that bind to T-cell receptors, CD3 and CD28 antibodies (BioLegend; purified anti-human CD28 and CD3 Antibody). In addition, the effect of 0,4 % dimethyl sulfoxide (DMSO, Sigma-Aldrich) supplement was tested before further analyses, via stimulation with CD3 & CD28 antibodies with and without DMSO.

PBMCs stimulation

First, cryopreserved PBMCs were thawed quickly in a warm (37°C) water bath. PBMCs were counted with Countess automated cell counter (Thermo) and cell viability was determined with trypan blue dye (Invitrogen). 400 000 PBMCs were placed into 96 well-round base plates (Sarstedt) in 100 µl cell culture media (RPMI 1640 + 25mmol/l Hepes, Thermo) supplemented with 2mmol L-glutamine, 25 µg/ml gentamycin and 5% of heat-inactivated Human AB serum (Innovative Research, Novi, MI, USA). Thawed cryopreserved PBMCs were rested on the 96-well plate for one hour at 37°C, 5% CO^2. Following the resting period, PBMCs were stimulated for two days (48 to 50 hours) with SARS-CoV-2 protein peptide pools at a concentration of 0.5 µg/ml in culture media. Each peptide pool, spike

(S), nucleoprotein (N), envelope (E) and membrane (M) (PepMiX, JPT Peptide Technologies) contains peptides of 15 amino acids length covering the complete sequence of the SARS-CoV-2 protein with an 11 amino acid- overlap between peptides. 2 µg/ml of CD3 & CD28 antibody and 0,4 % DMSO in culture media was used as a positive control. 0,4 % DMSO in culture media was used as a negative control. PBMC stimulation conditions were maintained optimal at the carbon dioxide (CO^2) incubator (5% CO^2, 95% humidity, and 37°C). After two days of stimulation, the 96-well plates were centrifuged, and the supernatant was carefully collected from the top of the PBMCs. At last, supernatants were stored at ≤ 68 °C.

Cytokine and effector molecule detection in cell culture supernatants

MILLIPLEX® multiplex immunoassays based on Luminex® xMAP® multiplex assay technology, was used to determine cytokine and effector molecule concentrations via cytokine/effector molecule-specific magnetic microspheres and LED detection. The levels of six secreted cytokines and effector molecules (IFN-γ, IL-2, TNF-α, IL-4, Granzyme B, and Perforin) in the SARS-CoV-2 peptide pool stimulated PBMC supernatants were analyzed with a 96-well plate assay. Multi-well plate (96 well) was analyzed using a custom-made MILLIPLEX® MAP Kit (HCD8MAG-15K, Millipore™). The fluorescence of the samples was measured with Luminex MAGPIX magnetic bead analyzer (Luminex® Corporation, Austin, TX). Cytokines and effector molecules median fluorescent intensity were converted to concentration (pg/ml) by interpolation from seven diluted standards using a 5-parameter logistic regression. All PBMC supernatant samples that were in the linear range were given their measured concentration. Whereas samples below the lowest standard in the linear range were given half the value of the standard (2,4 pg/ml for IFN-γ, 3.6 pg/ml for IL-2, 5 pg/ml for IL-4, 2,4 pg/ml for TNF-α, 0,6 pg/ml for Granzyme B, and 97,7 pg/ml for Perforin). In the same way, samples over the highest standard in the linear range were given the highest value of the standard which were 5 000 pg/ml for IFN-γ, 7 500 pg/ml for IL-2, 10 000 pg/ml for IL-4, 2 000 pg/ml for TNF-α, 5 000 pg/ml for Granzyme B, and 50 000 pg/ml for Perforin. There were seven standards in the kit and each standard had a duplicate. Seven different four-fold dilutions of the standard were made for each plate, starting with the dilution of 1:4, in true duplicates. Standards with a coefficient of variation of less than 22% between duplicates were accepted. The kit included two control samples, with a low and a high concentration of cytokines and effector molecules, that I analyzed in true duplicates. Control samples with a coefficient of variation of less than 15% between duplicates were

accepted. According to the kit manufacturer, if there were less than 35 beads in the well could not be given a dependable concentration. For this reason, samples with under 35 beads in the well were rejected from the final analysis.

Statistical analyses

Data was collected in Excel 2016 (Microsoft 365) and analyzed and visualized with GraphPad Prism (GraphPad Software, San Diego, California, USA, version 9.0.0). Data was determined to be independent even though data samples have biological dependence. I used Mann-Whitney U-test to assess the effect of the DMSO supplement on stimulation, and to compare the IFN-y concentrations between stimulation with CD3 and CD28 antibodies and stimulation with CD3 and CD28 antibodies with 0,4% DMSO. I tested the effect of cell concentration with Mann-Whitney U-test by comparing spike-specific IFN-y concentrations between cells cultured in a concentration of 200 000 versus 400 000 cells per well. Similarly, I tested the effect of stimulation time with Mann-Whitney U-test by comparing spike-specific IFN-y concentrations between cells stimulated for two- versus four days. I compared the concentrations of cytokines and effector molecules between study groups (vaccinated and infected, only infected and control group) with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Likewise, I compared the concentrations of cytokines and effector molecules induced by stimulation with spike protein peptide pool between study groups (WT, Alpha, and Beta infected group) with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. The Kruskal-Wallis test was used to analyze the statistical significance between the groups and Dunn's multiple comparisons test to see which groups differed significantly from each other. P-values <0.05 were considered statistically significant. Statistical tests used in respective data analyses have been mentioned in the figure legends.

Results

Optimization of PBMC stimulation

Before analyzing the samples to be studied, I optimized the conditions for the cell stimulations. Six PBMC samples were selected for testing these PBMC stimulation conditions, the selected samples were from vaccinated and infected (n=2), only infected (n=2) and neither vaccinated nor infected subjects (n=2). Finding the optimal stimulation time and cell concentration for the cellular immunity studies was the first objective of the study. The optimal quantity of PBMCs was tested with a comparison between 200 000 and 400 000 cells in a well and stimulation time was tested by comparing stimulation for two and four days. For these comparisons, the cells were stimulated with a spike protein peptide pool. As a positive control, the cells were stimulated with antibodies that bind to T cell receptors, CD3 and CD28 antibodies. T cell stimulation with CD3 and CD28 antibodies mimics T cell activation. As a negative control, the cells were cultured in the cell culture media with 0,4 % DMSO, in the absence of any stimulation. In addition to testing the cell concentration and stimulation time, the effect of the presence of DMSO on stimulation was assessed by stimulating the cells with antibodies that bind to T cell receptors, anti-CD3 and anti-CD28. Therefore, positive control with the 0,4 % DMSO supplement was tested before study sample stimulations. The effect of 0,4 % DMSO supplement was tested by comparison of two different stimulation circumstances, stimulation with CD3 and CD28 antibodies and stimulation with CD3 and CD28 antibodies with 0,4% DMSO.

The results indicated that the IFN-γ concentrations measured from cells stimulated for two or four days were similar (Figure 1, panel A). There was a trend for slightly higher IFN-γ concentrations with the 4-day stimulation with spike. However, there was no statistically significant difference between two- and four-days stimulation with spike protein, when the cell concentration was 200 000. There was also no statistically significant difference between two- and four-days stimulation with spike protein, when the cell concentration was 400 000. Similarly, there was a trend for slightly higher IFN-γ concentrations with higher cell concentration (Figure 1, panel A). However, the differences between stimulation with 200 000 and 400 000 cells for 2- or 4 days were not statistically significant.

However, the sample size was small, which must be taken into account when drawing conclusions. Therefore, I looked more at geometric means when drawing conclusions than the results of

statistical tests. Statistically significant difference was not observed between two- and four-day stimulation, based on these results, and also taking into consideration the feasibility of the stimulation, two-day stimulation time was selected for study sample stimulations. There was also no statistically significant difference between 200 000 and 400 000 cells. However, a slightly higher IFN-y concentration was observed with 400 000 cells. Therefore, a higher cell concentration, 400 000 cells was selected for study sample stimulations. Additionally, 400 000 cell is more suitable for further research questions.

Because DMSO is present in the SARS-CoV-2 peptide pools in a concentration of 0,4 %, it would be desirable for both the positive and negative control samples to contain DMSO. There was no significant difference in levels of IFN-y between positive control with DMSO and without DMSO (p= 0,6447) (Figure 1, panel B). The IFN-y concentrations were high, as a large part of the samples were given the highest value of the standard which was 5000 pg/ml, these samples are shown in the figure as overlapping dots (Figure 1, panel B). Based on these findings, CD3 and CD28 antibodies with 0,4% DMSO as a positive control was selected for study sample stimulations. In conclusion, for optimal stimulation conditions, two-day stimulation, 400 000 cells in well and positive control with DMSO was selected for study sample stimulations.

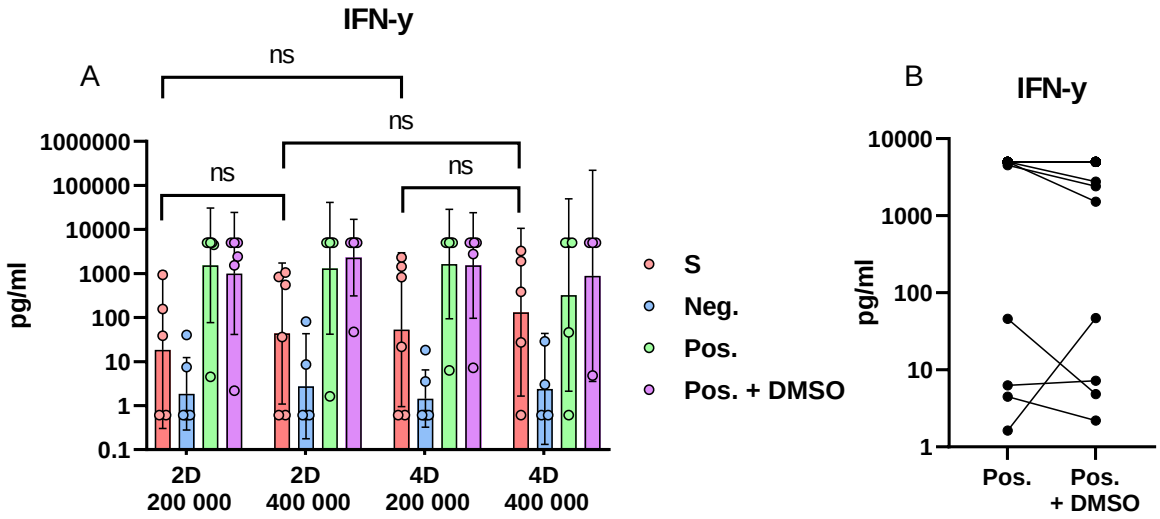

Figure 1 - Optimization of PBMC stimulation. The y-axis represents the concentration (pg/ml) of IFN-y. **Panel A** shows IFN-y secretion from six PBMC supernatants. **Panel A** presents a comparison of two different PBMC concentrations (200 000 and 400 000 cells) and two different stimulation times (two and four days). Stimulants are presented in different colors, spike peptide pool (with 0,4 % DMSO), negative control (culture media and 0,4 % DMSO), positive control (CD28 & CD3 antibodies) and positive control with 0,4 % DMSO (CD28 & CD3 antibodies and DMSO). Statistical analysis was performed with the Mann-Whitney test for comparison of 200 000 and 400 000 cells, two- and four-day stimulation with spike protein. The difference between 200 000 and 400 000 cells was statistically not significant (p= 0,7619 and p= 0,6537). The difference

between two and four days was statistically not significant (p=0,6277 and p=0,6537). The geometric means and 95% confidence intervals are shown in the figure. **Panel B** shows IFN-γ secretion from 22 PBMC supernatants, stimulated with CD28 & CD3 antibodies and CD28 & CD3 antibodies with DMSO supplement. Lines connected the same supernatant samples. Statistical analysis was performed with the Mann-Whitney test for comparison of positive control without DMSO and positive control with DMSO. The difference between positive control and positive control with DMSO was statistically not significant (p= 0,6447). S., SARS-CoV-2 spike protein peptide pool; Neg., Negative control (culture media and DMSO); Pos., Positive control (CD28 & CD3 Antibody); Pos. + DMSO, Positive control (CD28 & CD3 Antibody and DMSO)

Cellular immune responses in three study groups, hybrid immunity, only vaccinated and uninfected controls

The second objective of this thesis was to compare cellular immune responses between three study groups. Cellular immune responses were compared between subjects who had been vaccinated and infected (n=30), subjects who had only been infected (n=22), and subjects who had been neither vaccinated nor infected (n=6). Cells were stimulated with spike, nucleoprotein, membrane or envelope peptide pools for two days. In addition, each sample was stimulated with negative (culture media and DMSO) and positive control (CD28 & CD3 antibody and DMSO). Supernatants were collected and concentrations of cytokines and effector molecules were analyzed via MILLIPLEX® multiplex immunoassays based on Luminex® xMAP® multiplex assay technology.

High levels of cytokines and effector molecules granzyme B, TNF-a, perforin and IFN-γ and lower levels of cytokines IL-2 and IL-4 were measured following stimulation with the SARS-CoV-2 S, N and M peptide pools, indicating that the antigens are able to stimulate cellular responses in the selected stimulation conditions (Figure 2). Also, high levels of cytokine and effector were measured stimulation with CD28 & CD3 antibodies which indicates that stimulation of PBMCs has succeeded when T-cells have been activated via CD28 & CD3 antibodies. In addition, stimulation with only culture media and DMSO induces low responses which shows that cell media or DMSO do not induce PBMC activation. Consequently, background response is weak, and therefore negative control is also functional.

At the beginning of the comparison between groups, infection- and hybrid immunity-induced T-cell responses were compared to subjects who had been neither vaccinated nor infected. First, focus on comparison between the hybrid immunity group and uninfected control group. Higher concentrations of cytokines and effector molecules were measured in subjects who had been infected and vaccinated compared to uninfected controls (Figures 3-6). There was statistically

significant difference in levels of granzyme B ($p<0.001$), perforin ($p<0.5$), IFN-γ ($p<0.0001$), IL-2 ($p<0.001$) and IL-4 ($p<0.001$) between infected and vaccinated subjects and uninfected controls stimulation with spike protein (Figure 3). Statistically significant difference was observed also in levels of granzyme B ($p<0.05$), IFN-γ ($p<0.01$) and IL-2 ($p<0.001$) between infected and vaccinated subjects and uninfected controls stimulation with membrane protein (Figure 4). Similarly, statistically significant difference was observed in levels of granzyme B ($p<0.01$), IFN-γ ($p<0.01$) and IL-2 ($p<0.001$) between infected and vaccinated subjects and uninfected controls stimulation with nucleoprotein (Figure 5). On the other hand, envelope-specific hybrid immunity-induced T-cell responses were not observed (Figure 6). In conclusion, subjects who had been infected and vaccinated had higher cellular immune responses compared to uninfected controls.

Next, focus on comparison between subjects who had been infected and uninfected controls. Similarly, as seen in hybrid group subjects, higher concentrations of cytokines and effector molecules were measured in subjects who had been infected compared to uninfected controls (Figures 3-6). There was statistically significant difference in levels of granzyme B ($p<0.05$), IFN-γ ($p<0.05$) and IL-2 ($p<0.001$) between infected subjects and uninfected controls stimulation with spike protein (Figure 3). Statistically significant difference was observed in levels of the same cytokine and effector molecules, granzyme B ($p<0.05$), IFN-γ ($p<0.05$) and IL-2 ($p<0.001$) between infected subjects and uninfected controls stimulation with membrane protein (Figure 4). Similarly, statistically significant difference was observed in levels of the same cytokine and effector molecules, granzyme B ($p<0.01$), IFN-γ ($p<0.01$) and IL-2 ($p<0.001$) between infected subjects and uninfected controls stimulation with nucleoprotein (Figure 5). However, envelope-specific infection activated cellular immune responses were not observed (Figure 6). Based on the findings, subjects who have been only infected have higher cellular immune responses compared to uninfected controls.

Next, I assessed, whether subjects with hybrid immunity have cellular immune responses that differ from subjects who have been infected but not vaccinated. The concentrations of granzyme B, TNF-a, perforin, IFN- γ or IL-2 did not differ between the two groups, but those with hybrid immunity had on average higher IL-4 responses compared to those who had been infected only ($p < 0.0001$, Figure 7).

T cell responses against SARS-CoV-2 nucleoprotein, spike -, envelope - and membrane proteins

In this thesis, I also compared T cell responses to different structural proteins of SARS-CoV-2. I assess similarities or differences between PBMC stimulations with nucleoprotein, spike -, membrane - and envelope protein. Spike protein induced strong secretion of cytokines and effector molecules from PBMCs. Generally, it can be observed that stimulation with spike protein induces the highest levels of cytokine and effector molecules compared to other SARS-CoV-2 proteins (Figure 2). Spike protein also stimulates the widest range of cytokines and effector molecules compared to other SARS-CoV-2 proteins. Spike protein stimulated secretion of granzyme B, perforin, IFN-y, IL-2 and IL-4 (Figure 3). TNF-a was the only cytokine whose secretion was not activated via spike protein stimulation (Figure 3, panel B). As well, nucleoprotein and membrane protein stimulate observable secretions of cytokines and effector molecules (Figure 2). Stimulation with nucleoprotein and membrane protein induced similar T-cell responses. Stimulation with nucleoprotein or membrane protein increases levels of the same cytokines and effector molecules, granzyme B, IFN-y and IL-2 (Figures 4 and 5). On the other hand, stimulation with envelope protein did not stimulate T-cell responses. After envelope peptide pool stimulation, levels of cytokines and effector molecules are at the same levels as stimulation with culture media and DMSO. This indicates that envelope protein does not stimulate any significant cellular responses (Figure 1). All cytokines and effect molecule concentrations are at very low levels stimulation with envelope peptide pool (Figure 6).

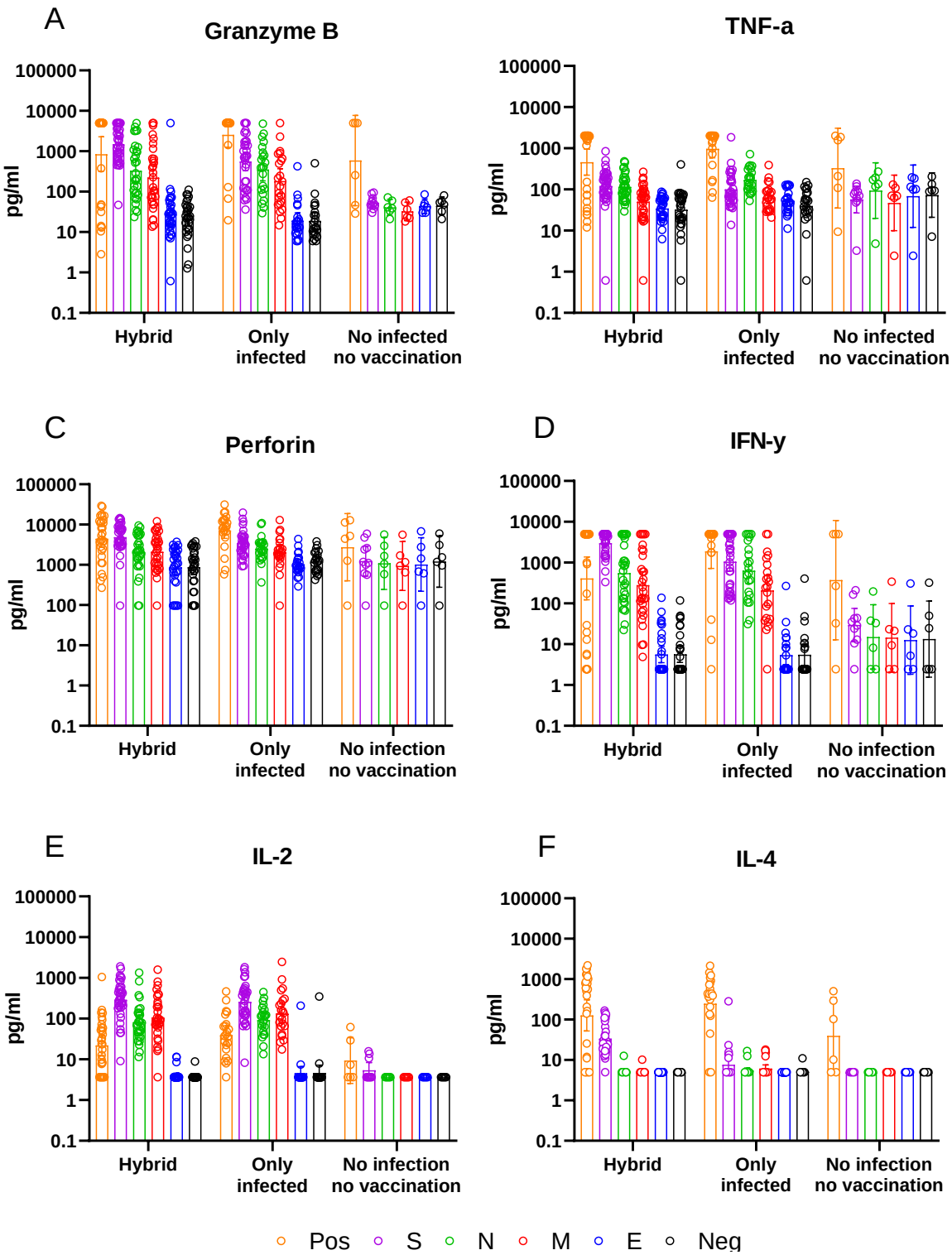

Figure 2 – Showing the entire data of the thesis. Cytokine and effector molecule (granzyme B, TNF-a, perforin, IFN-γ, IL-2 and IL-4) concentrations in stimulated PBMC supernatants. The y axis represents concentration (pg/ml) of cytokine or effector molecule. PBMCs stimulated with CD28 & CD3 antibodies (positive control), SARS-CoV-2 peptide pools (spike protein, nucleoprotein, membrane protein and envelope protein) and DMSO (negative control). PBMCs were collected from vaccinated and infected subjects (n=30), only infected subjects (n=22) and neither vaccinated nor infected subjects (n=6). The geometric means and 95% confidence intervals are shown in the figures. Pos, Positive control; S, Spike protein; N Nucleoprotein; M, Membrane protein; E, Envelope protein; Neg, Negative control

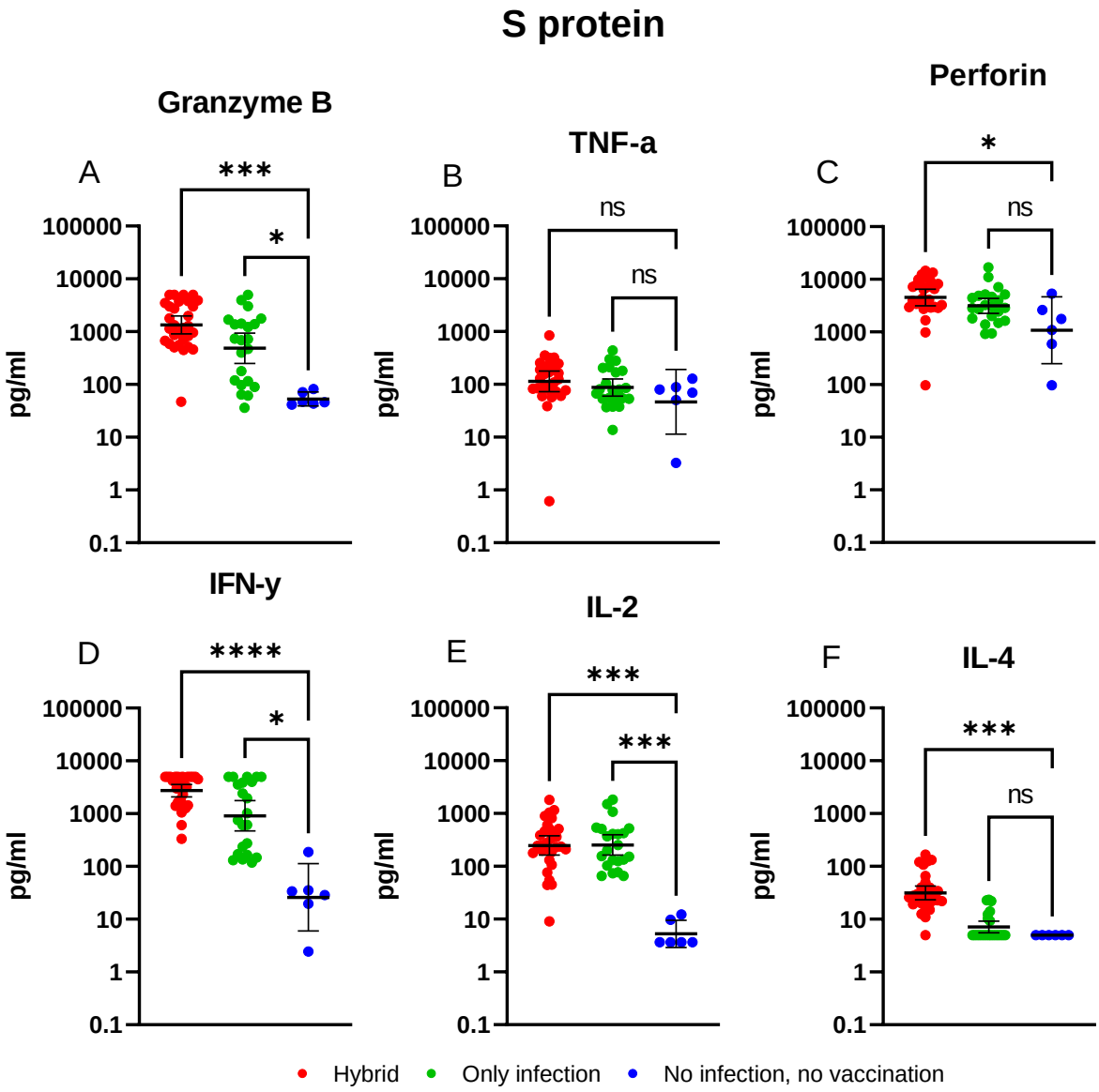

Figure 3 – Cytokine and effector molecule (granzyme B, TNF-a, perforin, IFN- γ, IL-2 and IL-4) concentrations in spike peptide pool stimulated PBMC supernatants. The y-axis represents the concentration (pg/ml) of cytokine or effector molecule. Data is divided into three groups, hybrid group which includes vaccinated and infected (n=30), only infected (n=22) and neither infection nor infected (n=6). The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. *p < 0.05; ***p < 0.001; ****p < 0.0001

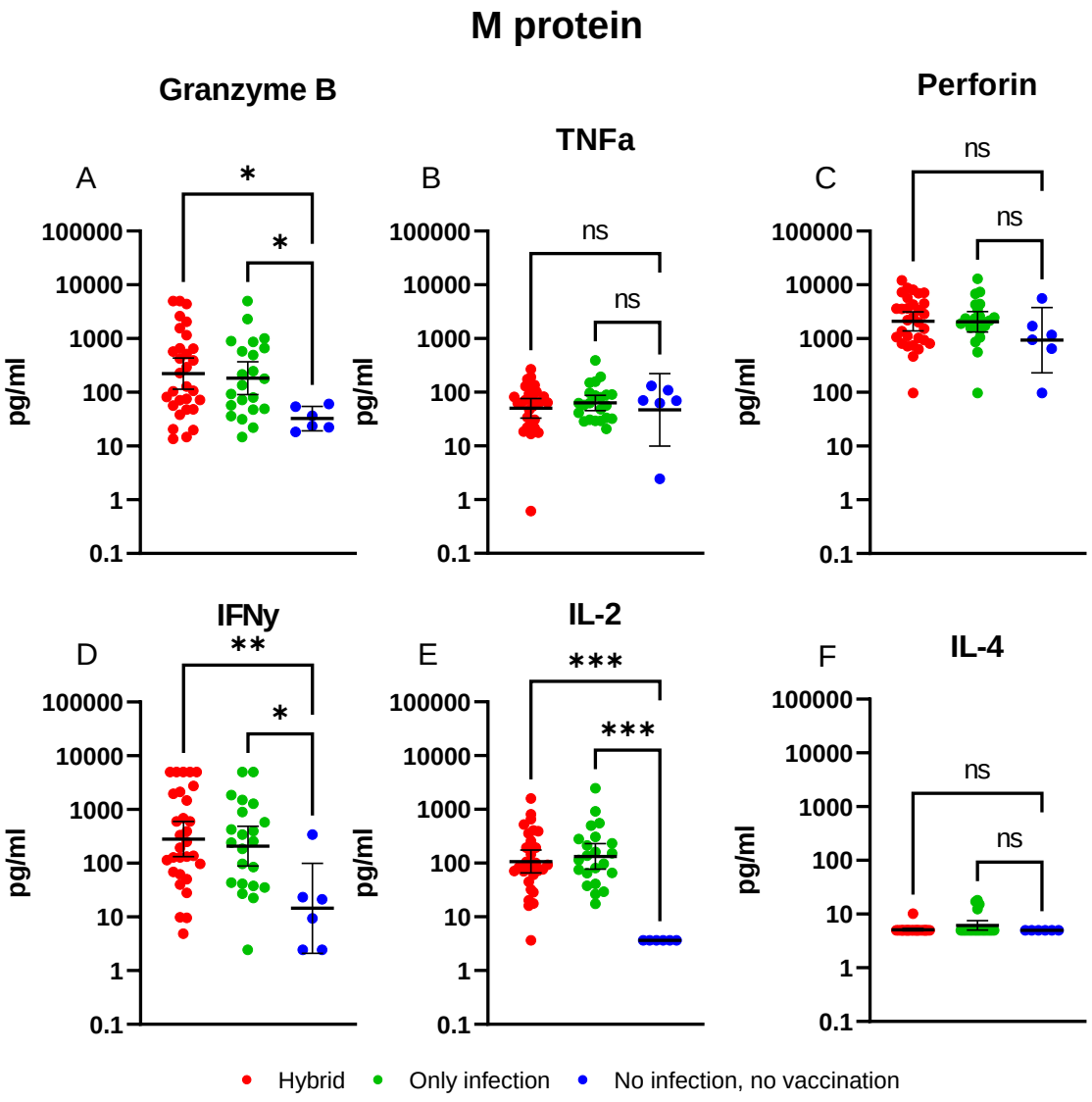

Figure 4 – Cytokine and effector molecule (granzyme B, TNF-a, perforin, IFN- γ, IL-2 and IL-4) concentrations in membrane peptide pool stimulated PBMC supernatants. The y-axis represents the concentration (pg/ml) of cytokine or effector molecule. Data is divided into three groups, hybrid group which includes vaccinated and infected (n=30), only infected (n=22) and neither infection nor infected (n=6). The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. *$p < 0.05$. **$p < 0.01$; ***$p < 0.001$

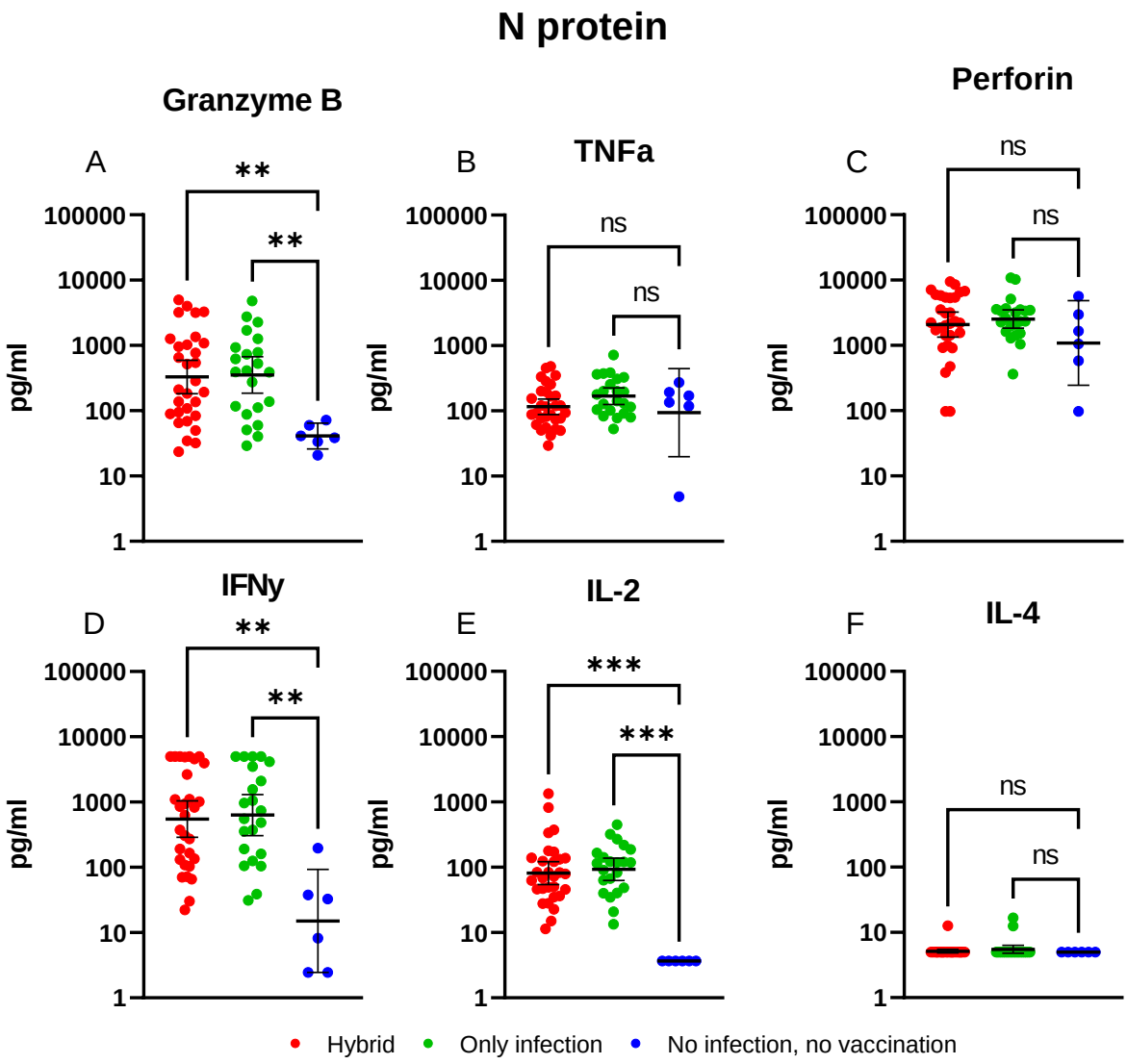

Figure 5 – Cytokine and effector molecule (granzyme B, TNF-a, perforin, IFN- γ, IL-2 and IL-4) concentrations in nucleoprotein peptide pool stimulated PBMC supernatants. The y-axis represents the concentration (pg/ml) of cytokine or effector molecule. Data is divided into three groups, hybrid group which includes vaccinated and infected (n=30), only infected (n=22) and neither infection nor infected (n=6). The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. **p < 0.01; ***p < 0.001

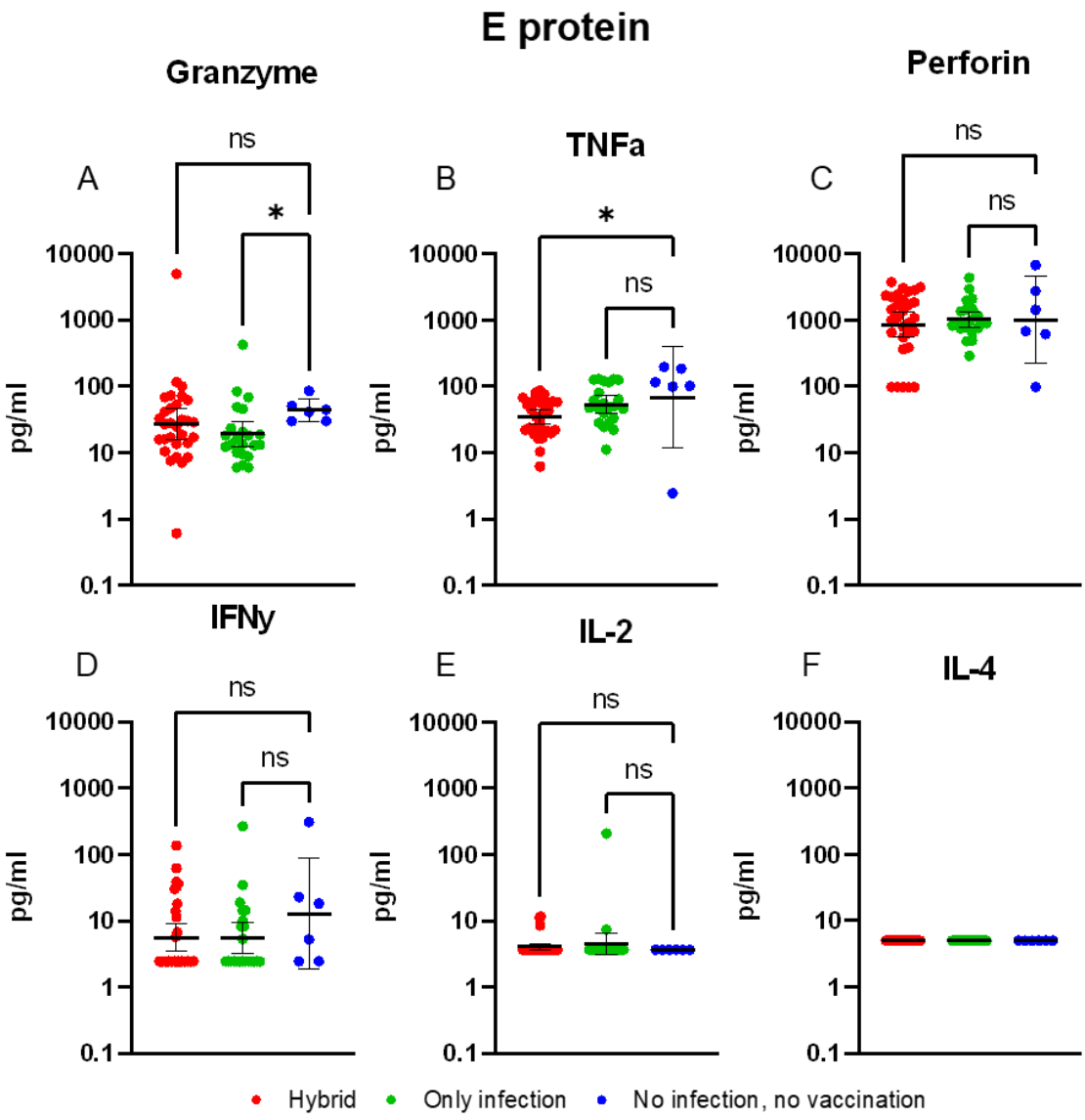

Figure 6 – Cytokine and effector molecule (granzyme B, TNF-a, perforin, IFN- γ, IL-2 and IL-4) concentrations in envelope peptide pool stimulated PBMC supernatants. The y axis represents concentration (pg/ml) of cytokine or effector molecule. Data is divided into three groups, hybrid group which includes vaccinated and infected (n=30), only infected (n=22) and neither infection nor infected (n=6). The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. **p < 0.01

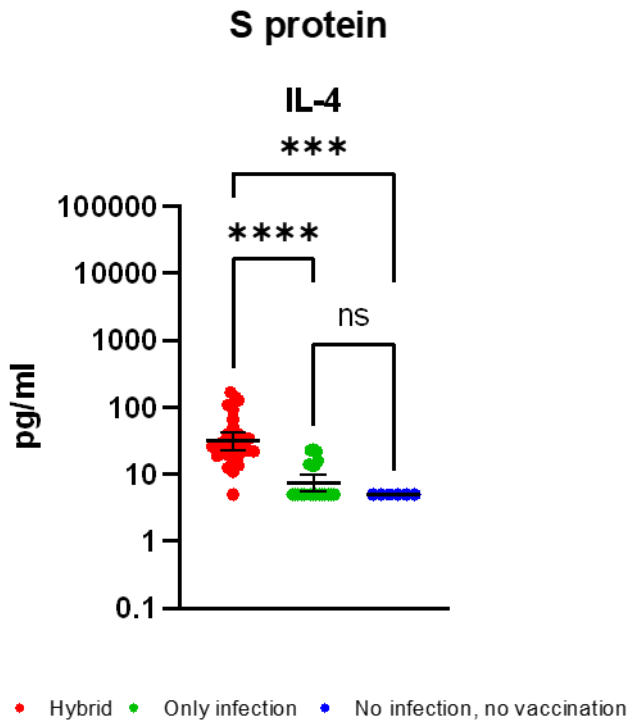

Figure 7 – Hybrid-immunity T-cell responses against SARS-CoV-2 IL-4 concentrations in nucleoprotein peptide pool stimulated PBMC supernatants. The y-axis represents the concentration (pg/ml) of IL-4. Data is divided into three groups, hybrid group which includes vaccinated and infected (n=30), only infected (n=22) and neither infection nor infected (n=6). The geometric means and 95% confidence intervals are shown in the figure. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. ***p < 0.001; ****p < 0.0001

T-cell responses against wild-type, Alpha, and Beta SARS-CoV-2 strains

The third objective of this thesis was to assess whether the cellular immune responses are similar or different depending on which SARS-CoV-2 variant caused the infection. To assess this, cellular responses were compared between groups of subjects who had been infected with either WT, Alpha (B.1.1.7), or Beta (B.1.351) variants. Since some of the subjects had also received the COVID-19 vaccine, each group was divided into subjects who had immunity produced only by the infection or by both the infection and the vaccine. Comparison between variants was performed only with spike protein stimulation because this is the antigen that is also the target of COVID-19 vaccines and mutations have concentrated in the RBD region of the spike protein. PBMC stimulations were performed only with WT derived spike peptide pool.

Subjects who had immunity produced only by the Alpha or Beta infection had T cells that had been initially activated with antigens expressed by these variants. Subjects who had immunity produced only by WT infection had T cells that had been activated with WT antigens. Therefore, it was interesting to see if T cells that had been activated with Alpha or Beta spike proteins would also be recognized and be activated by stimulation with WT spike peptides. This was assessed by stimulating samples of unvaccinated subjects infected with WT, Alpha or Beta variant with WT spike peptide pool (Figures 7-12 panel E). There was no statistically significant difference between unvaccinated Alpha and WT infected subjects (Figures 7-12 panel E). There was also no statistically significant difference in levels of granzyme B, TNF-a and IL-4 between Beta and WT infected subjects (Figure 7E, 8E and 12E). Statistically significant difference was observed in levels of perforin (Figure 9E, $p<0.05$), IFN-y (Figure 10E, $p<0.05$) and IL-2 (Figure 11E, $p<0.01$) between Beta and WT infected subjects. Generally, these results show that WT, Alpha or Beta infected subjects present equally good T cell responses after stimulation with WT spike peptide pool. Importantly, these results also show that T cells that have been activated with Beta or Alpha variant infection, will also recognize WT spike peptides. Similar results were seen in stimulations with N, M and E peptide pools (Figures 7-12 panels B, C, D, F, G and H). T cells by Alpha or Beta infection recognized and were stimulated with WT N, M and E peptide pools. As these antigens are not included in the COVID-19 vaccines, in this comparison it would not matter whether the subjects were vaccinated or not.

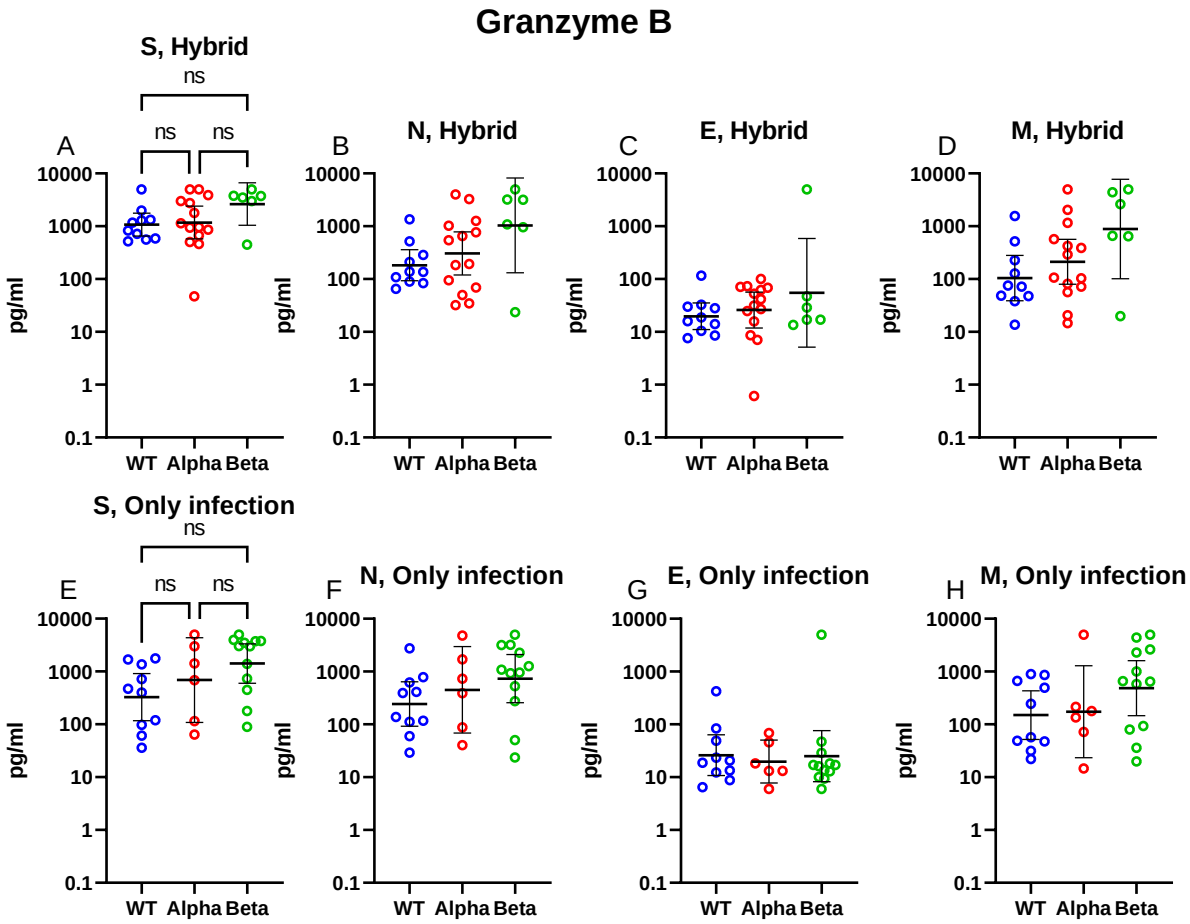

Figure 7 – Granzyme B secretion from stimulated PBMC supernatants. Data is divided into three groups based on which SARS-CoV-2 strain (WT n=20, Alpha n=20 and Beta n=12) has caused COVID-19. **Panels A-H** shows granzyme B secretion with spike, nucleoprotein, membrane or envelope peptide pool stimulations. **Panels A-D** present hybrid immunity (vaccinated and infected) data and **panels E-H** present only infected data. The y-axis represents the concentration (pg/ml) of granzyme B. The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Comparison between variants was performed only with spike protein stimulation because spike protein is the target of COVID-19 vaccines and mutations have concentrated in the RBD region of the spike protein.

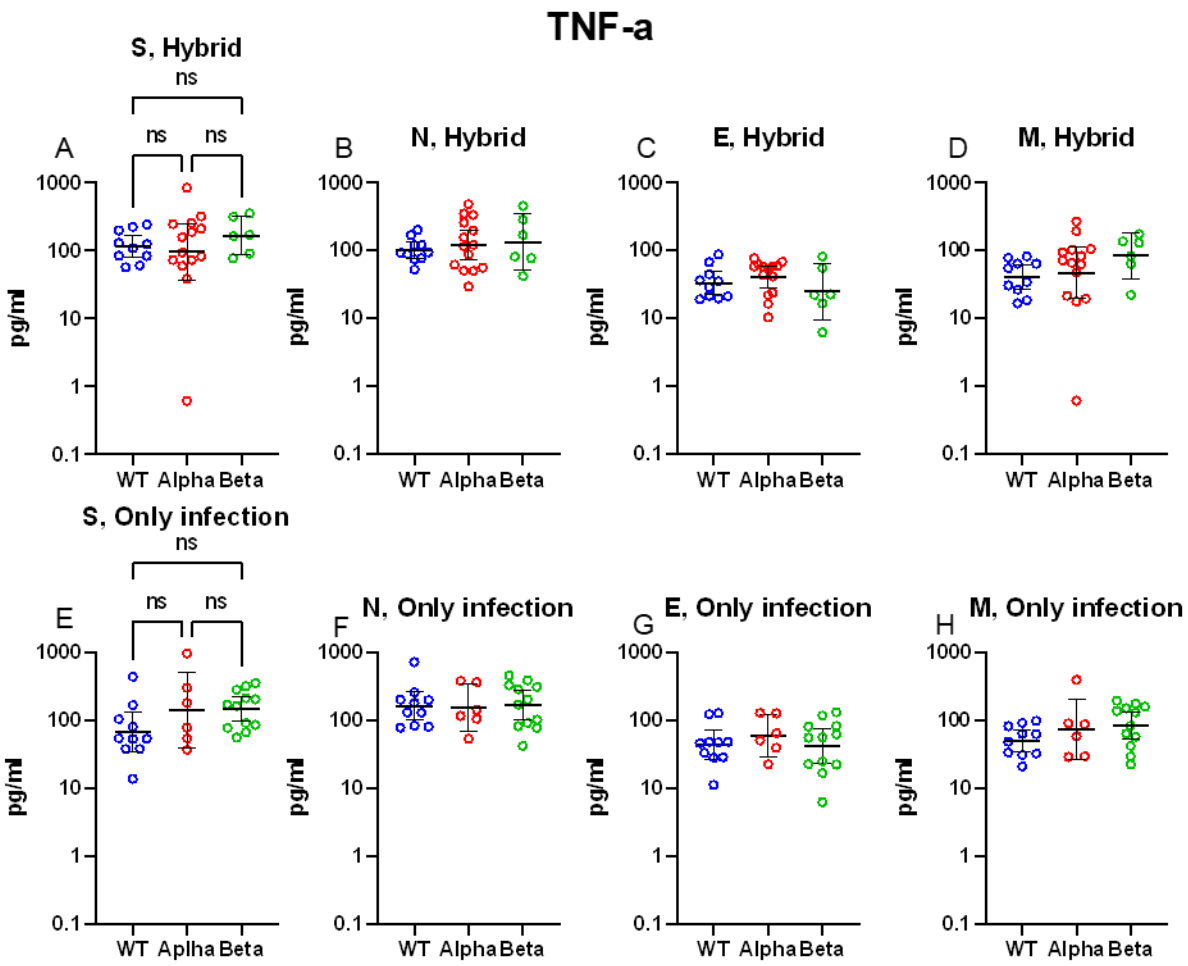

Figure 8 – TNF-a secretion from stimulated PBMC supernatants. Data is divided into three groups based on which SARS-CoV-2 strain (WT n=20, Alpha n=20 and Beta n=12) has caused COVID-19. **Panels A-H** show TNF-a secretion with spike, nucleoprotein, membrane or envelope peptide pool stimulations. **Panels A-D** presents hybrid immunity (vaccinated and infected) data and **panels E-H** present only infected data. The y-axis represents the concentration (pg/ml) of granzyme B. The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Comparison between variants was performed only with spike protein stimulation because spike protein is the target of COVID-19 vaccines and mutations have concentrated in the RBD region of the spike protein.

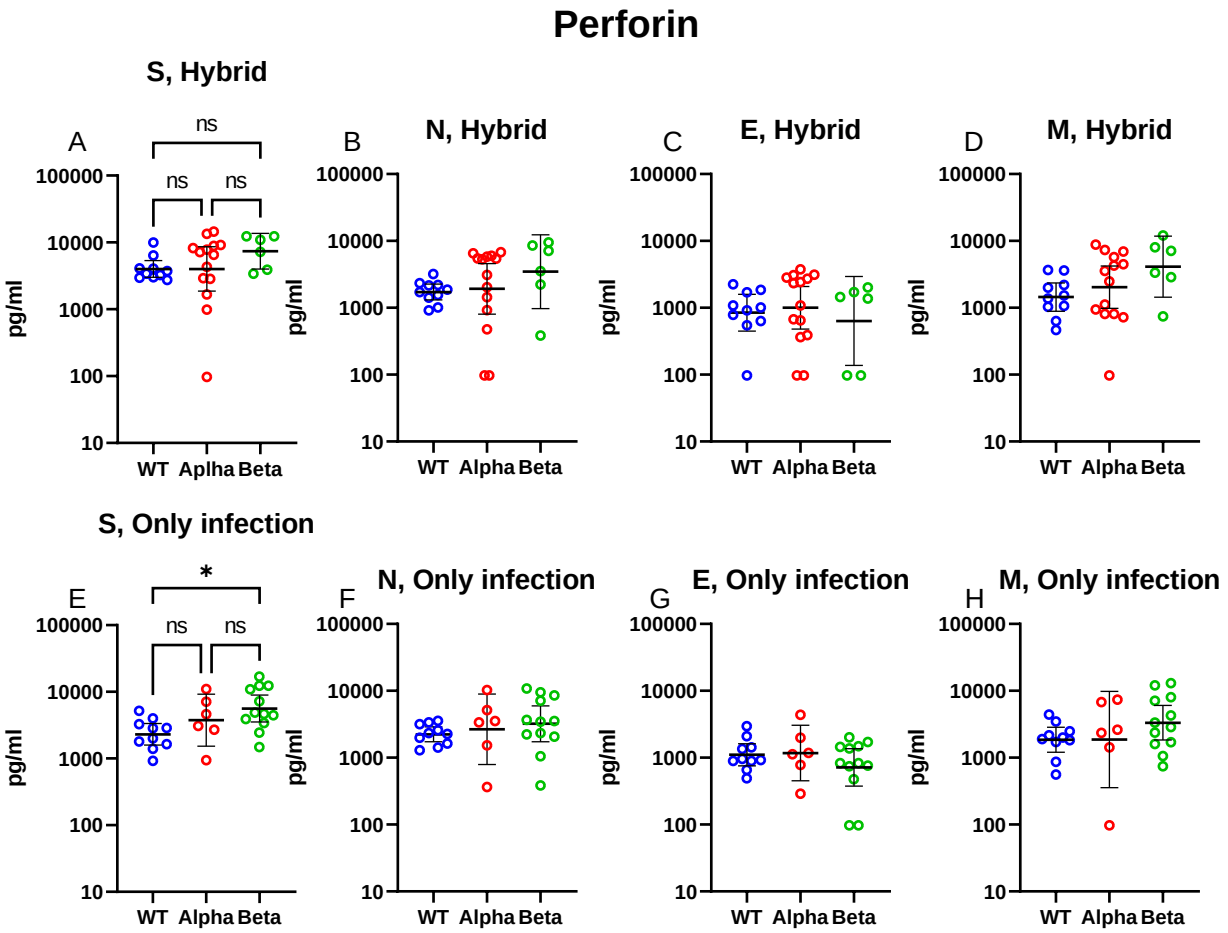

Figure 9 –Perforin secretion from stimulated PBMC supernatants. Data is divided into three groups based on which SARS-CoV-2 strain (WT n=20, Alpha n=20 and Beta n=12) has caused COVID-19. **Panels A-H** show perforin secretion with spike, nucleoprotein, membrane or envelope peptide pool stimulations. **Panels A-D** present hybrid immunity (vaccinated and infected) data and **panels E-H** present only infected data. The y-axis represents the concentration (pg/ml) of granzyme B. The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Comparison between variants was performed only with spike protein stimulation because spike protein is the target of COVID-19 vaccines and mutations have concentrated in the RBD region of the spike protein. *$p < 0.05$.

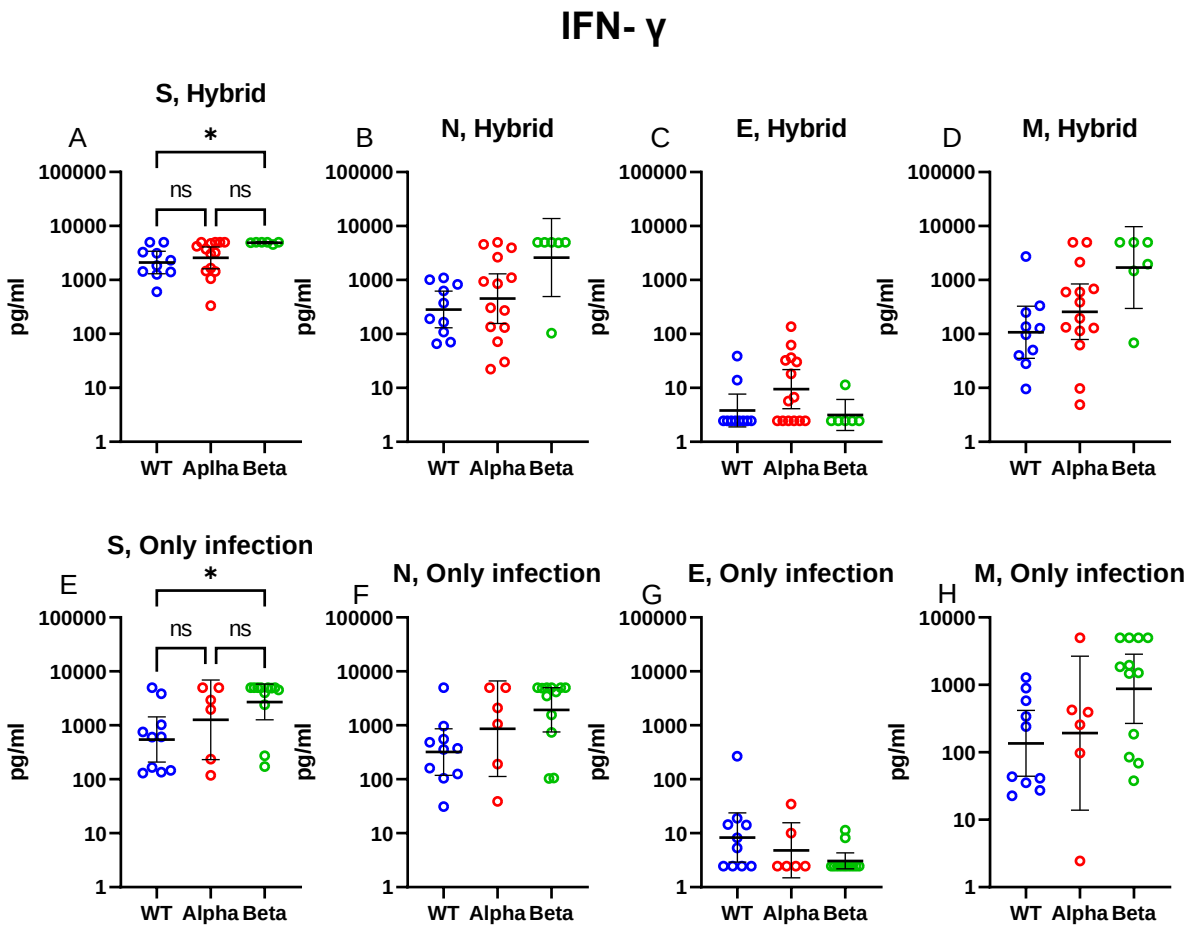

Figure 10 – IFN-y secretion from stimulated PBMC supernatants. Data is divided into three groups based on which SARS-CoV-2 strain (WT n=20, Alpha n=20 and Beta n=12) has caused COVID-19. **Panels A-H** show IFN-y secretion with spike, nucleoprotein, membrane or envelope peptide pool stimulations. **Panels A-D** present hybrid immunity (vaccinated and infected) data and **panels E-H** present only infected data. The y-axis represents the concentration (pg/ml) of granzyme B. The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Comparison between variants was performed only with spike protein stimulation because spike protein is the target of COVID-19 vaccines and mutations have concentrated in the RBD region of the spike protein. *$p < 0.05$.

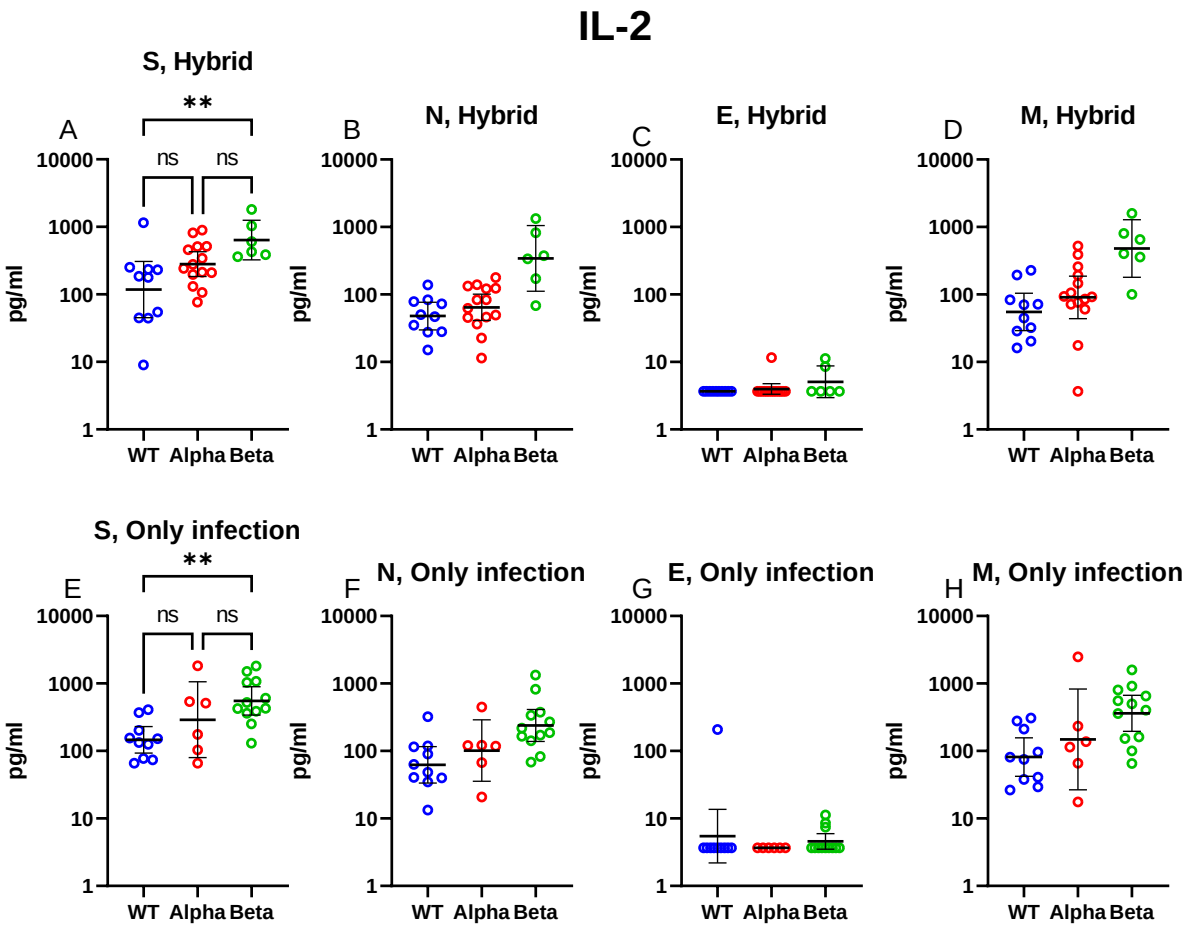

Figure 11 – IL-2 secretion from stimulated PBMC supernatants. Data is divided into three groups based on which SARS-CoV-2 strain (WT n=20, Alpha n=20 and Beta n=12) has caused COVID-19. **Panels A-H** show IL-2 secretion with spike, nucleoprotein, membrane or envelope peptide pool stimulations. **Panels A-D** present hybrid immunity (vaccinated and infected) data and **panels E-H** present only infected data. The y-axis represents the concentration (pg/ml) of granzyme B. The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Comparison between variants was performed only with spike protein stimulation because spike protein is the target of COVID-19 vaccines and mutations have concentrated in the RBD region of the spike protein. **$p < 0.01$

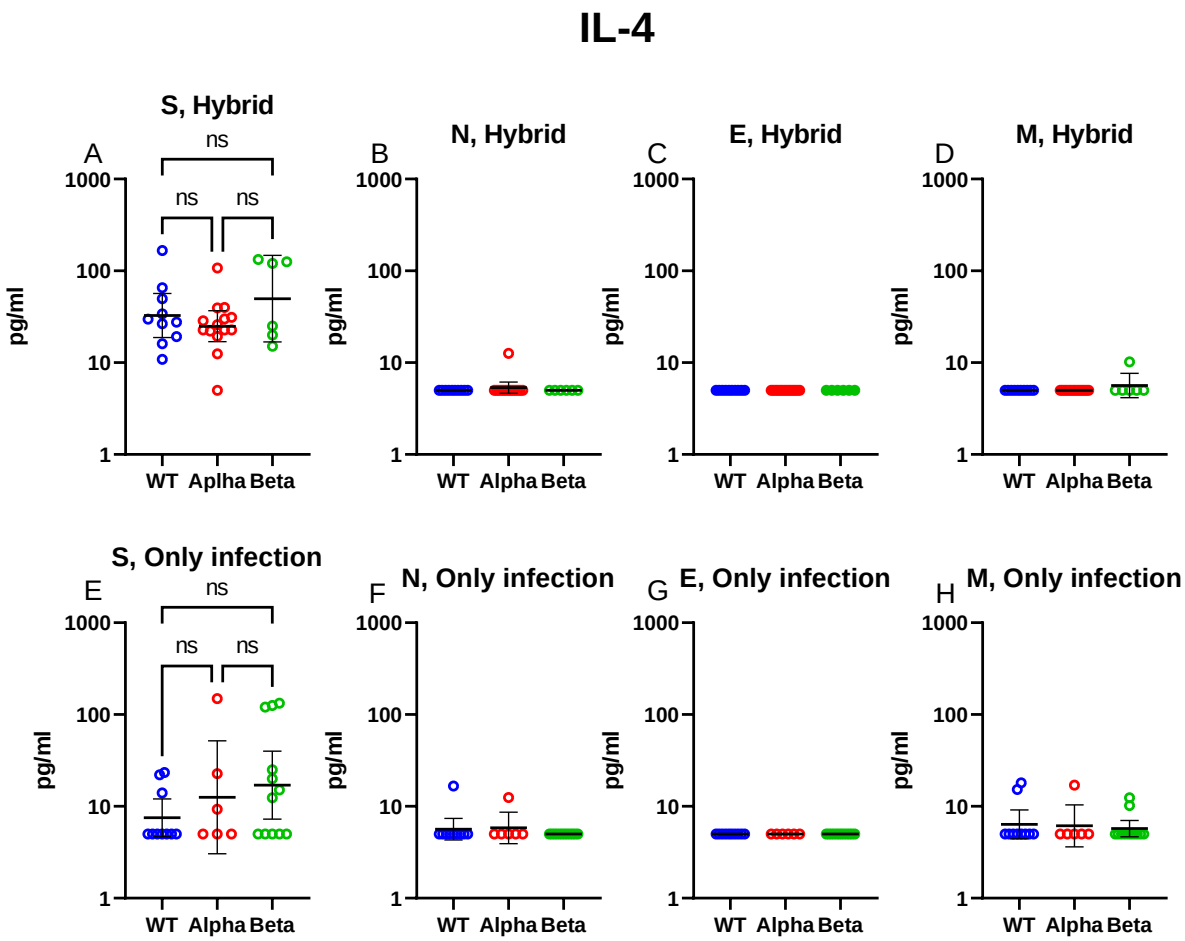

Figure 12 – IL-4 secretion from stimulated PBMC supernatants. Data is divided into three groups based on which SARS-CoV-2 strain (WT n=20, Alpha n=20 and Beta n=12) has caused COVID-19. **Panels A-H** shows IL-4 secretion with spike, nucleoprotein, membrane or envelope peptide pool stimulations. **Panels A-D** present hybrid immunity (vaccinated and infected) data and **panels E-H** present only infected data. The y-axis represents the concentration (pg/ml) of granzyme B. The geometric means and 95% confidence intervals are shown in the figures. Statistical significance was determined with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Comparison between variants was performed only with spike protein stimulation because spike protein is the target of COVID-19 vaccines and mutations have concentrated in the RBD region of the spike protein.

Discussion

The first objective of this thesis was to set up a PBMC stimulation assay based on a previously used protocol (Vuorela *et al.*, 2021). When I optimized PBMC stimulation conditions, I selected 400 000 cells in a well and four-day simulation time for study sample stimulations. In addition to testing optimal stimulation time and cell concentration, I tested the effect of DMSO on stimulation, to see if the DMSO supplement would influence CD3 and CD28 antibodies' ability to bind to T cell receptors, which was used as a positive control for stimulation. I found that there was no effect of DMSO on stimulation when there was no significant difference between CD3 and CD28 antibodies with and without DMSO. This was an important step to test to see if DMSO can also be added to positive control when all SARS-CoV-2 peptide pools are dissolved in DMSO. Due to all SARS-CoV-2 peptide pools containing DMSO, positive control also requires DMSO to show accurate stimulation. Therefore, positive control gives more accurate results, when DMSO is added.

The major aim of the study was to understand cellular immune responses to SARS-CoV-2, this aim was achieved in many ways. I compared cellular immune responses between different types of subjects. The second objective was to compare cellular immune responses between subjects who had been vaccinated and infected, subjects who had been only infected and controls who were neither vaccinated nor infected. First, I compared the concentration of cytokines and effector molecules between vaccinated and infected subjects and uninfected controls. I found that subjects who had a vaccine and infection have higher granzyme B, perforin, IFN-y, IL-2 and IL-4 secretion after spike protein stimulation and higher granzyme B, IFN-y, and IL-2 secretion after nucleoprotein and membrane protein stimulation. Consequently, I found that subjects with hybrid immunity have higher cellular immune responses compared to uninfected controls. These findings indicate that a combination of vaccination and infection produces effective cellular immune response, via activation of nucleoprotein-, spike-, and membrane-specific CD4+ and CD8+ cells. In a previous study, United Kingdom (UK) healthcare workers (HCWs) had similar vaccination and infection backgrounds as have been seen in this thesis. The study compared HCWs who had received one vaccine dose (BNT162b2) with previous SARS-CoV-2 infection to HCWs who had received one vaccine dose (BNT162b2) without previous SARS-CoV-2 infection. HCWs with previous infection show improved T cell responses against the Alpha variant (B.1.1.7) compared to HCWs without

previous SARS-CoV-2 infection. Even some of these HCWs without previous infection have so weak cellular immune responses, that these could not be measured (Reynolds *et al.*, 2021).

Next, I measured that subjects with infection have higher cellular immune responses compared to uninfected controls. I found that subjects with SARS-CoV-2 infection have higher Granzyme B, IFN-y, and IL-2 secretion after nucleoprotein, spike, and membrane peptide pool stimulation. These findings indicate that infection activates nucleoprotein-, spike-, and membrane-specific CD4+ and CD8+ cells. I found that infected subjects have higher cellular immune responses compared to uninfected controls. This is in line with previous COVID-19 research which detected that unvaccinated COVID-19 patients have high levels of IFN-y and IL-2 and have strong activation of CD4+ and CD8+ cells after spike peptide pool stimulation (Hurme *et al.*, 2022). Likewise, previous research reported that unvaccinated COVID-19 patients show strong IL-2 secretion and CD4+ activation after stimulation with SARS-CoV-2 structural peptide pools (Zuo *et al.*, 2021). Also, SARS-CoV-2 infected subjects were reported to have high IFN-y concentrations after SARS-CoV-2 structural protein stimulations compared to exposed or unexposed control subjects (Brand *et al.*, 2021). In addition, patients with severe or mild SARS-CoV-2 infection show increased IFN-y and IL-2 secretion after spike peptide pool stimulation (Sherina *et al.*, 2021). In conclusion, there is also strong evidence that subjects with only SARS-CoV-2 infection have strong cellular immune responses.

However, I found that subjects with infection and vaccination had significantly higher spike-specific IL-4 secretion compared to only infected subjects. This suggests that hybrid immunity produces enhanced CD4+ and CD8+ responses. T cell responses are likely to play a role in improved protection against severe COVID-19 disease in those with hybrid immunity. In contrast to this thesis's findings, a study about the Omicron variant (B.1.1.529) reported no observed differences in T cell responses. The Omicron variant study measured IFN-y with enzyme-linked immunosorbent spot (ELISpot) assay between only vaccinated subjects and hybrid immunity subjects. Both groups show similar T cell responses against Omicron and 21% of individuals from both groups have declined T cell reactivity to Omicron-derived spike protein (Naranbhai *et al.*, 2022). Also, another study reported that activation of CD4+ and CD8+ and secretion of IFN-y against the Omicron variant spike protein were weak (De Marco *et al.*, 2022). They found that overall there was no impact of previous infections or vaccination on measured T cell responses, only subjects who had received vaccination before SARS-

CoV-2 infection showed a slower decline in CD8+ response against omicron spike protein (De Marco *et al.*, 2022).

I observed that nucleoprotein, spike-, and membrane proteins stimulated T cell responses. I found that spike protein induced the strongest T cell responses seen by the widest range and strongest concentrations of secreted cytokines and effector molecules. I also found that nucleoprotein and membrane protein stimulated T cell responses. On the contrary, I observed that envelope protein did not stimulate T cell responses. In consideration of the small size of the envelope protein, there are fewer envelope peptide antigens to antigen-presenting cells to present T cells. Previous research about COVID-19 patients shows that S, M, and N proteins stimulated T cell responses, where they measured that M protein stimulates the highest IFN-y concentrations (Brand *et al.*, 2021). Similarly, another study reported that N and M peptides stimulated T cell responses when they measured IFN-y secretion and activation of CD4+ cells. They found also that E peptide pool stimulation did not activate high CD4+ T cell responses (Heide *et al.*, 2021).

The third objective of the thesis was to compare cellular immune responses between subjects who had been infected with WT, Alpha or Beta variants. I found that subjects with WT, Alpha or Beta infection had equally good T cell responses after stimulation with the WT spike peptide pool. I also found that T cells that have been activated with Beta or Alpha variant spike peptides will also recognize WT spike peptides. This suggests that mutations do not affect T-cell ability to recognize antigens. Therefore, immunity based on T cells is not as susceptible to antigenic changes in SARS-CoV-2 variants as the humoral immunity. Mutations accumulated in the spike protein have helped new variants evade antibody-based immunity and cause infections (Haveri *et al.*, 2021; Cheng *et al.*, 2022; Wang *et al.*, 2022). T cells recognize several short peptide fragments from the whole spike protein, whereas neutralizing antibodies recognize and bind to only the RBD region where most mutations have accumulated (Tai *et al.*, 2020).

One of the limitations of this thesis was that WT infected subjects' samples were not stimulated with a spike peptide pool derived from the Alpha and Beta variants. It would have been interesting to see if the T cells of subjects with different infections would recognize Alpha and Beta peptide pools equally compared to the WT peptide pool. Previous studies have reported results from T cell studies where cells were stimulated with spike peptide pools of different SARS-CoV-2 variants. For example, one previous study reported that stimulation with WT, Alpha, Beta, Gamma, and Delta variant spike peptide pools induces the same level of T cell responses (Hurme *et al.*, 2022). They

observed statistically significant differences only between WT and Gamma variants six weeks after vaccination and between WT and Beta variants six months after vaccination. Another study where the T cell immunity of subjects vaccinated with three doses of COVID-19 mRNA vaccines was assessed, it was demonstrated that T cells recognize both BA.1 and BA.2 spike peptide pools in the same way as the WT spike peptide pool (Belik *et al.*, 2023). In a study of common variable immunodeficiency (CVID) patients, it was reported that the study subjects had equal T cell responses to WT, Delta and Omicron BA.2 variants (Hurme *et al.*, 2023). Similarly, equal T cell responses, between WT, Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) have been reported (GeurtsvanKessel *et al.*, 2022). These findings show that T cells that have been activated with WT spike peptides will also recognize variant spike peptides. These different studies have reported equal or almost equal T cell responses to different SARS-CoV-2 variants. In conclusion, T cells can cross-recognize different SARS-CoV-2 variants spike peptides and mutations present in the spike proteins of the different variants do not affect T-cell ability to recognize these antigens.

Lastly, I found T cell responses last at least six months in subjects who had been infected with SARS-CoV-2. These long-lasting T cell responses were measured from samples collected six months after the SARS-CoV-2 infection. Other studies have also reported that T cell responses persist for six months (Sherina *et al.*, 2021; Zuo *et al.*, 2021; GeurtsvanKessel *et al.*, 2022; Hurme *et al.*, 2022). As well, T cell responses have been represented to be retained at three months after the third COVID-19 vaccination (Belik *et al.*, 2023). Similarly, the study of CVID patients reported that T cell responses last three months after the third vaccination (Hurme *et al.*, 2023). Even more long-lasting cellular immune responses have been measured, as long-lasting SARS-CoV-2 memory up to 10 months (Jung *et al.*, 2021). On the contrary previous study with COVID-19 patients presented declined T cell responses after 1 month (Dan *et al.*, 2021). They observed that 30 % of COVID-19 patients have declined CD8+ response against SARS-CoV-2.

This thesis has some limitations, the number of participants was rather low, 30 vaccinated and infected subjects, 22 only infected subjects, and 6 unvaccinated control subjects. However, there was all four SARS-CoV-2 protein stimulations and secretion of six cytokines and effector molecules were measured. Additionally, cell work is time-consuming and expensive, these cell work characteristics restrict sample size. Second, the study focused almost exclusively on analyzing responses to the BNT162b2 vaccine, while analysis of other COVID-19 vaccines was minimal. Third, vaccinated subjects had a variable interval between vaccination and blood sampling (5-76 days).

However, all participants have SARS-CoV-2 infection before vaccination. Therefore, memory T cells will be re-activated quickly after vaccination. T cell reactivation will take less than five days (Koutsakos *et al.*, 2023).

There were also some limitations in PBMC stimulation. PBMC stimulation protocol was optimized using a maximum of four-day stimulation. Over four-day stimulation was not tested since the maximal concentration of most cytokines is measured after two or five days (Surenaud *et al.*, 2016). I used cryopreserved PBMCs, which can affect total cell viability or the ratio of immune cell populations. Different studies have reported contradictory findings about whether cryopreservation will affect PBMCs. Some studies have reported that cryopreservation does not affect immune cell populations (Anderson *et al.*, 2019; Panch *et al.*, 2019) and reported that CD4+ and CD8+ ratio are remained the same after freezing (Panch *et al.*, 2019). Whereas the third study showed increased cytokine secretions after PBMCs were cryopreserved (Anderson *et al.*, 2019). However, in this thesis, effect of the cryopreservation on cell viability was tested by counting cells before and after cryopreservation. I found that cell viability was still high after freezing (over 80 %). Furthermore, because PBMC samples were cryopreserved, all samples could be stimulated and analyzed at the same time with the same conditions and reagents. Fourthly in this thesis, the real composition of the immune cell populations cannot be known. Still, PBMC samples usually have the same ratios of the immune cells. Moreover, there are plans to measure cell populations with flow cytometry using fluorescence-activated cell sorting (FACS) in future works.

This thesis was the first pilot study of cellular immune responses to SARS-CoV-2. In the future, more PBMC stimulations and cell work with flow cytometry are planned. In this thesis, I found that subjects who had been infected with SARS-CoV-2 have effective and long-lasting T cell responses and subjects who have been vaccinated and infected have improved spike-specific IL-4 secretion. I found that T cells recognize different SARS-CoV-2 variants. When mutations in the spike proteins of the different variants do not affect T-cell ability to recognize these antigens. Therefore, immunity based on T cells is not as susceptible to antigenic changes as the humoral immunity. T cells have a vital role in protection against new SARS-CoV-2 variants when these new variants evade antibody-based immunity. The vital role of T cells affects also future COVID-19 vaccine development when there is a new vaccine is being developed that contains segments of nucleoprotein and membrane protein. This new vaccine is developed to boost T cell response (Arieta *et al.*, 2023).

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