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Immune profiling after SARS-CoV-2 infection: Investigation of T cell reactivity in convalescent individuals and persistent immune abnormalities in post-COVID Patients

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Erstes Gutachten:	Priv. Doz. Dr. Julia Roider
Zweites Gutachten:	Priv. Doz. Dr. Christof Geldmacher
Drittes Gutachten:	Prof. Dr. Lutz Gürtler
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	25.02.2025

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Table of content

Affidavit **1**

Table of content **2**

List of abbreviations **3**

List of publications **4**

1. Contribution to the publications **5**

1.1 Contribution to Publication I 5

1.2 Contribution to Publication II 7

2. Introduction **8**

2.1 Background 8

2.2 SARS-CoV-2 and coronaviruses 10

2.3 Immune system and response to SARS-CoV-2 12

2.4 Durable immunogenic changes after COVID-19 16

3. Summary (English) **18**

4. Zusammenfassung (Deutsch) **20**

5. Publication I **23**

6. Publication II **39**

7. References **56**

Acknowledgments **60**

List of abbreviations

CCL19: Chemokine (C-C motif) ligand 19

CCL5: Chemokine (C–C motif) ligand 5

COVID-19: Coronavirus disease 2019

CXCL1: Chemokine (C–X–C motif) ligand 1

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EI-S1-IgG: Anti-SARS-CoV-2-ELISA (IgG)

ELISA: Enzyme-linked immuno-sorbent assay

FLT3: Fms-related tyrosine kinase 3

HCoV: Human coronavirus

ICS: Intracellular cytokine staining

IGRA: Interferon gamma release assay

IFNγ: Interferon gamma

IL-1RA: Interleukin-1 receptor antagonist

ILC: Innate lymphoid cell

ILC2: Group 2 innate lymphoid cells

ILCP: ILC precursors

KoCo19: Prospective COVID-19 cohort study

M: Membrane (protein)

NC: Nucleocapsid (protein)

NSP: Non-structural protein

ORF: Open reading frame

PBMC: Peripheral blood mononuclear cells

PCC: Post-COVID-Care

PCS: Post-COVID syndrome

PCR: Polymerase chain reaction

PDGF-AA: Platelet-derived growth factor A

RBD: Receptor binding domain

Ro-N-Ig: Elecsys® Anti-SARS-CoV-2

ROC: Receiver operating characteristic

SARS-CoV-2: Severe acute respiratory syndrome coronavirus type 2

SCT: Spike-C-terminus (protein)

SNT: Spike-N-terminus (protein)

List of publications

Publications that are part of this cumulative dissertation:

- Brand I, **Gilberg L**, Bruger J, Garí M, Wieser A, Eser TM, Frese J, Ahmed MIM, Rubio-Acero R, Guggenbuehl Noller JM, Castelletti N, Diekmannshemke J, Thiesbrummel S, Huynh D, Winter S, Kroidl I, Fuchs C, Hoelscher M, Roider J, Kobold S, Pritsch M, Geldmacher C. • *Broad T Cell Targeting of Structural Proteins After SARS-CoV-2 Infection: High Throughput Assessment of T Cell Reactivity Using an Automated Interferon Gamma Release Assay* • Frontiers in Immunology 12, 688436 (2021) • doi:10.3389/fimmu.2021.688436.

- Sbierski-Kind J, Schlickeiser S, Feldmann S, Ober V, Grüner E, Pleimelding C, **Gilberg L**, Brand I, Weigl N, Ahmed MIM, Ibarra G, Ruzicka M, Benesch C, Pernpruner A, Valdinoci E, Hoelscher M, Adorjan K, Stubbe HC, Pritsch M, Seybold U, Roider J. • *Persistent immune abnormalities discriminate post-COVID syndrome from convalescence* • Infection 52, 1087-1097 (2024) • doi:10.1007/s15010-023-02164-y.

Further publications:

- Olbrich L, Castelletti N, Schälte Y, Garí M, Pütz P, Bakuli A, Pritsch M, Kroidl I, Saathoff E, Guggenbuehl Noller JM, Fingerle V, Le Gleut R, **Gilberg L**, Brand I, Falk P, Markgraf A, Deák F, Riess F, Diefenbach M, Eser T, Weinauer F, Martin S, Quenzel EM, Becker M, Druner J, Girl P, Müller K, Radon K, Fuchs C, Wölfel R, Hasenauer J, Hoelscher M, Wieser A. • *Head-to-head evaluation of seven different seroassays including direct viral neutralisation in a representative cohort for SARS-CoV-2* • Journal of General Virology 102 (2021) • doi:10.1099/jgv.0.001653.
- **Gilberg L**, Teodorescu B, Maerkisch L, Baumgart A, Ramaesh R, Gomes Ataide EJ, Koç AM. • *Deep Learning Enhances Radiologists' Detection of Potential Spinal Malignancies in CT Scans* • Applied Sciences 13,8140 (2023) • doi:10.3390/app13148140.
- Paderno A, Ataide Gomes EJ, **Gilberg L**, Maerkisch L, Teodorescu B, Koç AM, Meyer M. • *Artificial intelligence-enhanced opportunistic screening of osteoporosis in CT scan: a scoping Review* • Osteoporosis international 35, 1681-1692 (2024) • doi:10.1007/s00198-024-07179-1.
- Teodorescu B, **Gilberg L**, Melton PW, Hehr RM, Guzel HE, Koç AM, Baumgart A, Maerkisch L, Ataide Gomes EJ. • *A systematic review of deep learning-based spinal bone lesion detection in medical images* • Acta Radiologica 65, 115-1125 (2024) • doi:10.1177/02841851241263066.
- Teodorescu B, **Gilberg L**, Koç AM, Goncharov A, Berclaz LM, Wiedemeyer C, Guzel HE, Ataide Gomes EJ. • *Advancements in opportunistic intracranial aneurysm screening: The impact of a deep learning algorithm on radiologists' analysis of T2-weighted cranial MRI* • Journal of Stroke and Cerebrovascular Diseases 33, 108014 (2024) • doi:10.1177/02841851241263066.

1. Contribution to the publications

1.1 Contribution to Publication I

The title of the first publication where I am shared co-author is: *Broad T Cell Targeting of Structural Proteins After SARS-CoV-2 Infection: High Throughput Assessment of T Cell Reactivity Using an Automated Interferon Gamma Release Assay*. It was published in the journal Frontiers in Immunology in May 2021.

The foundation for the research project and all resulting publications was the establishment of the population-based prospective COVID-19 – Index Cohort Study (KoCo19-Index). I personally contributed to build up this cohort by recruiting participants, collecting and processing samples, and subsequently analysing the data in a study involving a large cohort of human subjects (n=407) over a period of five months. My contributions were essential in various aspects of the study, including assay execution in the laboratory, writing, and preparation of the publication.

More specifically, I fulfilled administrative tasks, including time scheduling of participants, household visits, giving study information and taking the informed consent. Furthermore, I had to administer logistic tasks like management of material and supplies and transportation of participants' samples.

I processed approximately half of all participants' samples (n=203) in a laboratory. This includes the isolation of peripheral blood mononuclear cells (PBMC) from the full-blood samples and the separation of plasma samples for further analysis. Subsequently, I conducted the interferon gamma release assay (IGRA) (manufactured by Euroim-

mun, Lübeck, Germany), which is the main experiment of this publication and I performed flow cytometry analyses for the supplement experiments.

As part of the data analysis, I integrated laboratory and patient data and displayed cohort characteristics. I conducted the initial data analysis, including the comparison of subject groups and assay reactivity. As a first step, I performed the receiver operating characteristic (ROC) curve analysis to determine the reactivity cut-offs of the IGRA results. This analysis was the baseline for all further analysis and a crucial step in evaluating the immune response of infected subjects. I provided the details in the Supplementary Table S1. Based on this, I also performed the analysis regarding the breath of T cell recognition of structural proteins of the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) in subjects with convalescent infection, which is shown in Figure 3. I also provided the Supplementary Table S2 and the Supplementary Figure 3, which are derived from the flow cytometry experiments we conducted. These experiments aimed to demonstrate that CD4 positive T cells are the source of released interferon-gamma upon stimulation. Most of the remaining figures and final statistics were provided by Mercè Garí, a data specialist and co-author with the appropriate academic background in the field of bioinformatics.

For the manuscript preparation, my primary contributions involved drafting the methods and results sections, along with the abstract. I outlined the study design, execution, and technical specifics of laboratory assays. Within the results section, I provided a comprehensive description of the study population, IGRA results, and comparisons among different groups. Additionally, I contributed to crafting sections of the introduction and discussion.

1.2 Contribution to Publication II

The title of the second Publication is: *Persistent immune abnormalities discriminate post-COVID syndrome from convalescence*. It was published in the journal Infection in February 2024.

As described in Chapter 1.1, I contributed profoundly to the establishment and management of the KoCo19-Index Cohort study. Subjects and samples from this cohort were used as important control groups for the second publication that focused on investigating the immune profile of patients suffering from post-COVID syndrome by comparing them to convalescent subjects without persisting symptoms and healthy controls.

I was responsible for the recruitment of these control groups and the processing of their blood samples in the wet laboratory. In particular, for the healthy control group, I conducted in-home visits to personally recruit all 32 subjects and their household members. During these visits, I obtained informed consent, administered questionnaires to gather household and symptom-related data, and collected blood samples from each participant. Consequently, I contributed to the publication by characterizing and describing the respective cohorts and providing vital symptom and laboratory-related data.

In conclusion, I contributed substantially to the acquisition, analysis, and interpretation of the data underlying the second publication.

2. Introduction

2.1 Background

In early 2020, the first cases of infections caused by the novel SARS-CoV-2 were registered in Germany, as well as in other parts of Europe, following a trend that was observed worldwide (Spiteri et al. 2020). The resulting pandemic caused over 6.6 million deaths globally by the end of 2022 and led to many more hospitalizations and long-term effects in recovered patients (WHO 2023). Alongside public health and social measures, the development and roll-out of vaccines against COVID-19 played a key role in protection against the virus, with the latter resulting in an estimated excess mortality reduction of 63% for the first year of vaccination (Watson et al. 2022).

However, the general and long-term effectiveness of these vaccines is compromised by mutations of the targeted proteins (mainly the spike protein) and hence the continuing rise of new (immune escape) virus variants (Malik et al. 2022). Therefore, research for a comprehensive understanding of SARS-CoV-2 specific immunity remains crucial for the development of further treatments and future vaccines.

This work and resulting publications are part of a collaborative project of the Division of Infectious Diseases and Tropical Medicine, the Division of Clinical Pharmacology and the Department of Infectious Diseases of the LMU University Hospital. The goal of this project was to establish a local Munich cohort (KoCo19-Index) of SARS-CoV-2 convalescent subjects that were infected during the first wave of the pandemic and to study their immune system, focusing on virus-specific immune responses.

For this purpose, citizens of the city of Munich, Germany, who had tested positive for SARS-CoV-2 between March and April 2020 were

contacted and enrolled into the study after having consented to participate. The study design included questionnaires on general demographics, participants' household situations, and symptoms, alongside the collection of blood samples for serological testing and further analysis of SARS-CoV-2 specific immunity. All participants who donated blood samples of sufficient quantity to receive the subsequent immunoassays (in addition to pure serological testing) were summarized under the KoCo19-Shield study. The duration of immune memory, the effects of contact and isolation inside participants' households and the potential cross-immunity to endemic coronaviruses were especially considered as topics of investigation for this sub-cohort.

Ethics approval was granted by the Institutional Review Board of the Medical Faculty at the Ludwig-Maximilians-University Munich under project numbers 20-692 and 20-371. Oral and written informed consent was obtained from all study subjects.

The experimental investigation of the immune response to the virus was conducted using a fast and scalable Interferon Gamma Release Assay that was performed to display the immune response (measured by interferon gamma release) of infected, exposed but not positive tested household members, and unexposed controls. The results of this experimental analysis led to the first publication “*Broad T Cell Targeting of Structural Proteins After SARS-CoV-2 Infection: High Throughput Assessment of T Cell Reactivity Using an Automated Interferon Gamma Release Assay*” published in the journal “Frontiers in Immunology” in May 2021. It demonstrates that SARS-CoV-2 infection induces specific T cell immune responses that last more than 200

days and it showcases a novel and scalable immunoassay as a feasible method for the measurement of cellular immune response in infected individuals.

Further in-depth investigations into the immunological profiling of this cohort, including intracellular staining and serological testing for other coronaviruses, are still ongoing. Preliminary results reveal more complex dynamic of immune responses to SARS-CoV-2, considering the potential impact of cross-reactivity with other human coronaviruses (HCoVs).

For a subsequent project, convalescent and uninfected participants from the established KoCo19-Index cohort were used as control groups in the context of the Post-COVID-Care (PCC) study. This study aimed to investigate persistent immune alterations in patients with prolonged sequelae after acute Coronavirus Disease 2019 (COVID-19) also known as post-COVID syndrome. Ethics approval was granted by the Ethics Committee of the Medical Faculty at LMU Munich (No. 21-1165). The article "*Persistent immune abnormalities discriminate post-COVID syndrome from convalescence*", published in February 2024 in the journal Infection, presents the respective results.

2.2 SARS-CoV-2 and coronaviruses

SARS-CoV-2 is an enveloped, single-stranded RNA virus and the most recent addition to the Coronaviridae family. The virus was first identified in China in 2019 and is responsible for causing the disease known as coronavirus disease 2019 (COVID-19) (Zhu et al. 2020).

Today, seven known strains of coronaviruses are reported to infect humans:

- Human Coronavirus 229E (HCoV-229E) and Human Coronavirus NL63 (HCoV-NL63) from the genus alpha-coronaviruses.
- Human Coronavirus OC43 (HCoV-OC43), Human Coronavirus HKU1 (HCoV-HKU1), the Middle East respiratory syndrome (MERS) coronavirus, SARS-CoV-1 and SARS-CoV-2 from the genus beta-coronaviruses.

The severity of diseases caused by coronavirus infections ranges from mild, as seen with common cold coronaviruses, to severe and potentially lethal outcomes, as associated with SARS-CoV-1 (lethality ~9.5%) and MERS-CoV (lethality ~35%). The risk of a severe or fatal disease course is further influenced by cofactors such as advanced age, obesity, diabetes, and other pre-existing health conditions (Petersen et al. 2020; WHO 2022).

All HCoVs express the immunodominant structural proteins spike, envelope, membrane, and nucleocapsid. Additionally, they produce accessory proteins encoded by open reading frames (ORFs) and a large group of non-structural proteins (NSPs) (Yadav et al. 2021).

The coronavirus spike protein is of special interest because it plays a crucial role for the entrance of the virus into the human cell. It contains two subunits, S1 and S2. The S1 subunit, containing the receptor binding domain (RBD), facilitates interaction with the cellular receptor ACE2, while the S2 subunit is responsible for the fusion of viral and host cell membranes (Graham and Baric 2010). The spike protein has been proven to induce high levels of humoral and cellular immunity

and therefore most established and approved vaccines today are targeted against the spike protein (Du et al. 2009).

At time of recruitment of participants for the cohort underlying this work, the predominant variant in Germany was the original Wuhan strain (RKI 2021). In recent years, evolutionary pressure and immune selection gave rise to mutations – especially in the spike protein – and resulted in various SARS-CoV-2 variants with varying transmission rates and differing propensities for severe disease (Markov et al. 2023; Mistry et al. 2021). However, the investigation of the immune response to the original strain is crucial in order to gain a comprehensive understanding of the underlying mechanisms associated with the virus. Moreover, it paves the way for the development of more effective treatments and preventive measures in the future.

2.3 Immune system and response to SARS-CoV-2

Antiviral immunity

The immune system can be divided into two main parts: the innate immune system and the adaptive immune system.

The innate immune system serves as the first line of defence, effectively limiting the ability of pathogens to enter and disseminate within the human body. This defence mechanism involves the recruitment of immune cells, the liberation of cytokines and chemokines, and the facilitation of antigen presentation to activate the subsequent part of the immune response.

The second part is known as the adaptive immune system, which exhibits a slower response rate but offers greater specificity in its protective capabilities. It mainly comprises CD4 and CD8 T cells, B cells

responsible for antibody production, as well as antigen-presenting cells such as macrophages and dendritic cells.

For effective control and elimination of human pathogenic viruses, this adaptive part of the immune system is essential. It is the key to immune memory and vaccine functionality and therefore the understanding of SARS-CoV-2 specific adaptive immune responses will play a highly important role even in the future (Sette and Crotty 2021).

Typically, the adaptive immune responses require approximately 6-10 days after initial exposure to a virus to exhibit effective antiviral activity. This time is needed for the differentiation and proliferation of primarily naive immune cells to antigen specific effector cells (Moss 2022). A delay, for example because of an impaired innate immune response, reduced naive T cell pools, or viral immune evading strategies, is associated with severe or chronic disease and is also reported in the case of SARS-CoV-2 infections (Hope and Bradley 2021).

CD4, CD8 and B cells all play crucial roles in defence against SARS-CoV-2. From early on during the pandemic many studies have postulated that the B cell derived SARS-CoV-2 specific neutralizing antibodies, as the key effector of the humoral arm of the adaptive immune system, show protective capacities against infection (Robbiani et al. 2020; Khoury et al. 2021). They, however, wane over time and may lose effectiveness due to immune escape variants (Yaugel-Novoa, Bourlet, and Paul 2022). In contrast, cellular immunity might bear the potential for long-lasting protection, especially as variant cross-reactive T-cell responses have been reported in Sars-CoV-2 infection and might represent the correlate of protection against severe disease course (Lasrado et al. 2024).

SARS-CoV-2 specific T cell immunity

T cell immunity plays a vital role in the hosts' response to SARS-CoV-2 infection. CD4 positive T cells orchestrate the immune response by releasing cytokines, supporting the antibody production in B cells and aiding in the activation and expansion of CD8 T cells. A key mechanism in T cellular immunity is the formation of long-lasting memory cells that can persist in the body after infection. This immunologic memory is critical for a specific and protective immune response against future encounters with the virus, potentially reducing the severity of subsequent infections.

Multiple studies investigated the T cellular immune memory after SARS-CoV-2 infection. Jung et al. reported persistent memory T cells in COVID-19 convalescent patients 10 months after infection, including the development of stem cell-like memory T cells (Jung et al. 2021). Dan et al. found CD4 and CD8 cell memory up to 8 months with a half-life decline in immune responses of 3-5 months (Dan et al. 2021). Other studies estimate the half-life of virus-specific cellular immune responses between several months and more than one year (Dan et al. 2021; Zuo et al. 2021; Jung et al. 2021; Wragg et al. 2022), which is comparable with previous reporting’s of immune memory in SARS-CoV-1 (Le Bert et al. 2020; Ng et al. 2016).

Various factors can impact the magnitude and duration of cellular immune response. This variability is reflected in the wide range of clinical manifestations seen in COVID-19, spanning from asymptomatic cases to lethal outcomes, which in turn aligns with the diverse CD4 T cell responses observed in convalescent individuals.

As previously described in a review by Sette and Crotty, conflicting data has been reported to determine the cause for this heterogeneity (Sette and Crotty 2021). While some studies show a significant influence of factors like viral load, age, general health and cross-reactivity

to other coronaviruses (Rydyznski Moderbacher et al. 2020; Yu et al. 2021; Sagar et al. 2021), others reported highly heterogenic results even in homogeneous young and healthy subject cohorts (Le Bert et al. 2021).

Targets of cellular immunity

Although more than 1.400 potential epitopes across the SARS-CoV-2 virion have been identified (Grifoni et al. 2021; Quadeer, Ahmed, and McKay 2021), most studies focused on the T cell response to the structural proteins spike, membrane and nucleocapsid (Moss 2022). Even though epitopes from other regions can induce significant cellular responses, the main structural proteins were reported to be the immunodominant (Grifoni et al. 2020). The spike protein, particularly its receptor binding domain, was prioritized as an early target for vaccine development. Furthermore, these structural proteins exhibit the strongest evidence of cross-immunity to other types of coronaviruses, making them likely to play a pivotal role in potential future endeavours to develop a pan-coronavirus vaccine (Murray et al. 2022).

While recent studies focus more on vaccine derived or convalescent and vaccinated cross-immunity, the research included in this work aligns with the previously mentioned studies in detecting immune responses against structural viral proteins in an unvaccinated population, more than 200 days post infection. It adds to the mosaic of knowledge about cellular immune responses by providing evidence of durable T cell reactivity targeting multiple SARS-CoV-2 structural proteins.

2.4 Durable immunogenic changes after COVID-19

According to the World Health Organization's 2021 definition, post-COVID-19 syndrome (PCS) is a condition that arises after a confirmed or suspected SARS-CoV-2 infection, typically presenting >3 months after the initial onset of COVID-19, with symptoms persisting for a duration of two months or longer and not explained by alternative diagnoses (Soriano et al. 2022).

The often used term “long COVID” usually refers to all prolonged conditions after acute SARS-CoV-2 infection and includes the period from 4 to 12 weeks, as well as the post-COVID-19 syndrome.

Approximately 10% of all SARS-CoV-2 infected suffer from the post-COVID-19 syndrome (Ballering et al. 2022) with the actual prevalence much likely being higher, depending on factors such as age, comorbidities, disease severity and vaccination status (Nittas et al. 2022).

Over 200 symptoms, affecting multiple organ systems have been associated with the condition (Davis et al. 2021).

The syndrome, sometimes also referred to as SARS-CoV-2 related post-infection sequelae, aligns with a spectrum of other post-acute infection syndromes (PAISs), characterized by common clinical presentation, yet limited understanding of their underlying mechanisms. These syndromes – such as those following infections with respiratory syncytial virus and influenza viruses, among others – share the feature of enduring or newly emerging symptoms after infection without the associated pathogen remaining detectable in the body (Narasimhan et al. 2022).

Because of the large number of patients suffering from post-COVID syndrome and the resulting burden on public health systems, the need for immunological characterisation of the disease and the identification of specific biomarkers is crucial.

Clinical inflammation markers like C-reactive protein and lactate dehydrogenase can be elevated in post COVID-19 syndrome but remain unspecific (Yong et al. 2023).

Several more in-depth immunological phenotypes have been described in patients suffering from post-COVID syndrome. Reported trends include enriched memory T and B cells in lung tissue, dysregulation in specific CD8 positive T cell populations and elevated proinflammatory cytokines like interferon β, type III interferons and interleukin-6 (Cheon et al. 2021; Phetsouphanh et al. 2022).

Innate lymphoid cells (ILCs) can be understood as the innate counterpart to T cells. They play an important role in antiviral immune response through the regulation of both adaptive and innate immune cells and are particularly located in the mucosal surface of the gastrointestinal and respiratory tract (Panda and Colonna 2019).

A depletion of ILCs has been associated with acute COVID-19 severity but their role in post-COVID syndrome is still largely unknown (Silverstein et al. 2022).

Hence, the second publication included in this work aims to characterize patterns of circulating innate lymphoid cells and pro-inflammatory chemokines and cytokines in patients with post-COVID syndrome, comparing them to convalescent and healthy controls.

3. Summary (English)

The coronavirus disease 19 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), resulted in over 6.6 million deaths, and its aftereffects still weigh heavy on health systems around the globe. Clinical presentation of the infection varies from asymptomatic to severe COVID-19, with a significant fraction of patients suffering from lasting symptoms even months after infection, which is known as post COVID-19 syndrome. Biological explanations for these heterogeneous phenomena remain insufficient, highlighting the need for further characterization of patients' immune responses in the context of infection.

We established a local Munich cohort (KoCo19-Index) consisting of PCR-confirmed convalescent participants (n=177), their household members (n=145), and SARS-CoV-2 naïve controls (n=85). We studied their adaptive immune responses to SARS-CoV-2 structural proteins after their first infection in 2020. Using a high-throughput automated interferon gamma release assay (IGRA), we tested T cell responses against the nucleocapsid, membrane and spike proteins in 407 participants from 193 households, more than 200 days after infection. Broad T cell reactivity against these antigens was found in participants with confirmed SARS-CoV-2 infection, and the magnitude of interferon gamma response correlated with anti-nucleocapsid antibody titers. We found T cell responses also in PCR-confirmed infected, but seronegative individuals, suggesting cellular immune memory that outlived the presence of circulating antibodies. Both, serological positive and negative household contacts of the PCR confirmed index participants showed a trend towards increased T cell reactivity against the structural proteins compared to confirmed unexposed controls.

Taken together, the first study demonstrated broad T cell responses even after a prolonged period post-infection, showcasing the IGRA as

an effective method for large-scale assessment of SARS-CoV-2-specific cellular immune responses, with high accuracy against the three tested target proteins.

For a subsequent study, we utilized the serologically confirmed convalescent individuals and the serologically plus IGRA confirmed uninfected individuals as controls in an investigation of persistent immune abnormalities in patients suffering from post-COVID syndrome (PCS). PCS, a condition that can present with a variety of lasting symptoms such as fatigue, dyspnea, headaches, cognitive impairment, and others, occurs in approximately 10% of patients infected with SARS-CoV-2. To explore immunological alterations in PCS patients, we performed multicolor flow cytometry and multiplex cytokine assays on the plasma of these patients, as well as on our convalescent and healthy control groups. We quantified a total of 46 plasma cytokines and further analyzed circulating innate lymphoid cells (ILCs) and their subpopulations, which are known to play a role in prolonged infection and tissue repair. On the cellular level, it was observed that circulating ILC precursors (ILCPs) were expanded without showing clear signs of enhanced activation. Conversely, ILC2 levels were decreased, while ILC1 levels remained largely unchanged. On the cytokine level, PCS patients exhibited elevations in proinflammatory factors such as interleukin (IL)-1RA and IL-1a, as well as elevated chemokines associated with immune cell trafficking (CCL19/MIP-3b, FLT3-ligand) and endothelial inflammation and repair (CXCL1, EGF, RANTES, etc.). These findings characterize immunological profiles linked to PCS and may aid in identifying novel biomarkers and subsequent therapeutic strategies relevant not only to PCS but also to other post-acute infection syndromes.

4. Zusammenfassung (Deutsch)

Die Pandemie der Coronavirus-Krankheit-2019 (COVID-19), die durch das Schweres Akutes Respiratorisches Syndrom-Coronavirus-2 (SARS-CoV-2) ausgelöst wurde, führte weltweit zu über 6,6 Millionen Todesfällen, und ihre Nachwirkungen belasten die Gesundheitssysteme auf der ganzen Welt noch immer schwer. Das klinische Erscheinungsbild der Infektion ist vielfältig und reicht von asymptomatisch bis hin zu schwerem Covid-19. Ein erheblicher Teil der Patienten leidet noch Monate nach der Infektion unter anhaltenden Symptomen, die als Post-Covid-19-Syndrom bezeichnet werden. Die biologischen Erklärungen für diese heterogenen Phänomene sind nach wie vor unzureichend, was die Notwendigkeit einer weiteren Charakterisierung der Immunreaktionen der Patienten im Zusammenhang mit der Infektion unterstreicht.

Im Rahmen der vorliegenden Studie wurde eine lokale Münchner Kohorte (KoCo19-Index) rekonvaleszent infizierter Teilnehmer, ihren Haushaltsmitgliedern und SARS-CoV-2 naiven Kontrollen aufgebaut, um die adaptive Immunantwort auf SARS-CoV-2-Strukturproteine nach der Erstinfektion im Jahr 2020 zu untersuchen. Mittels eines automatisierten Hochdurchsatz-Assays zur Interferon-Gamma-Freisetzung (IGRA) wurden bei 407 Teilnehmenden aus 193 Haushalten mehr als 200 Tage nach der Infektion T-Zell-Reaktionen gegen das Nukleokapsid-, das Membran- und das Spike-Protein getestet. Bei Teilnehmenden mit bestätigter SARS-CoV-2-Infektion konnte eine breite T-Zell-Reaktivität gegen diese Antigene festgestellt werden. Das Ausmaß der Interferon-Gamma-Reaktion korrelierte mit den Anti-Nukleokapsid-Antikörpertitern. Auch bei PCR-bestätigten, seronegativen Personen, bei denen eine Infektion nachgewiesen wurde, konnten T-Zell-Antworten beobachtet werden. Dies deutet auf ein zelluläres Immungedächtnis hin, welches die Anwesenheit zirkulierender Antikörper überdauert. Sowohl serologisch positive als auch negative

Haushaltskontakte der PCR-bestätigten Index-Teilnehmer wiesen einen Trend zu einer erhöhten T-Zell-Reaktivität gegen die Strukturproteine im Vergleich zu bestätigten nicht exponierten Kontrollen auf.

Insgesamt konnte im Rahmen der ersten Studie eine breite T-Zell-Antwort auch nach einem längeren Zeitraum post infectionem nachgewiesen werden. Dies belegt die Eignung des IGRA als Methode für eine groß angelegte Auswertung der SARS-CoV-2-spezifischen zellulären Immunantwort mit hoher Genauigkeit gegen die drei getesteten Zielproteine.

In einer weiteren Studie wurden die serologisch bestätigten Rekonvaleszenten und die serologisch plus IGRA bestätigten Nichtinfizierten als Kontrolle verwendet, um anhaltende Immunanomalien bei Personen zu untersuchen, die am Post-COVID-Syndrom (PCS) leiden. PCS, eine Erkrankung, die mit einer Vielzahl von persistierenden Symptomen wie Müdigkeit, Atemnot, Kopfschmerzen, kognitiven Beeinträchtigungen etc. einhergehen kann, manifestiert sich bei etwa 10% der mit SARS-CoV-2 infizierten Betroffenen. Zur Untersuchung immunologischer Veränderungen bei PCS-Patienten führten wir Multicolor-Durchflusszytometrie und Multiplex-Zytokin-Assays mit Plasma dieser Personen sowie an unseren rekonvaleszenten und gesunden Kontrollgruppen durch. Im Rahmen der Studie wurden insgesamt 46 Zytokin- und Chemokin-Parameter in Plasma quantifiziert und darüber hinaus die zirkulierenden, angeborenen lymphatischen Zellen (ILCs) und ihre Subpopulationen analysiert. Letztere spielen bekanntermaßen eine Rolle bei anhaltenden Infektionen und der Gewebereparatur. Auf der Zytokinebene wiesen PCS-Patienten erhöhte proinflammatorische Faktoren wie Interleukin (IL)-1RA und IL-1a sowie erhöhte Chemokine auf, die mit dem Transport von Immunzellen (CCL19/MIP-3b, FLT3-Ligand) und der endothelialen Entzündung und Reparatur (CXCL1, EGF, RANTES u. a.) in Zusammenhang stehen. Die präsentierten Ergebnisse charakterisieren immunologische Profile, die mit dem PCS assoziiert sind. Sie leisten einen Beitrag zur

Identifizierung neuer Biomarker und folgender möglicher therapeutischer Ansätze, die nicht nur für PCS, sondern auch für andere postakute Infektionssyndrome relevant sind.

5. Publication I

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Broad T Cell Targeting of Structural Proteins After SARS-CoV-2 Infection: High Throughput Assessment of T Cell Reactivity Using an Automated Interferon Gamma Release Assay

Isabel Brand[1,2†], Leonard Gilberg[1,3†], Jan Bruger[1†], Mercè Garí[4], Andreas Wieser[1,5], Tabea M. Eser[1,5], Jonathan Frese[1], Mohamed I. M. Ahmed[1,5], Raquel Rubio-Acero[1], Jessica M. Guggenbuehl Noller[1], Noemi Castelletti[1], Jana Diekmannshemke[4,6], Sophie Thiesbrummel[4,6], Duc Huynh[2], Simon Winter[1], Inge Kroidl[1,5], Christiane Fuchs[4,6,7], Michael Hoelscher[1,5,8‡], Julia Roider[3,5‡], Sebastian Kobold[2,9,10‡], Michael Pritsch[1,5‡] and Christof Geldmacher[1,5*‡] on behalf of the KoCo19 study group*

[1] Division of Infectious Diseases and Tropical Medicine, University Hospital, Ludwig-Maximilians-Universität (LMU) Munich, Munich, Germany, [2] Division of Clinical Pharmacology, Department of Medicine IV, University Hospital, LMU Munich, Munich, Germany, [3] Department of Infectious Diseases, University Hospital, LMU Munich, Munich, Germany, [4] Institute of Computational Biology, Helmholtz Zentrum München – German Research Center for Environmental Health (HMGU), Neuherberg, Germany, [5] German Center for Infection Research (DZIF), Partner Site Munich, Munich, Germany, [6] Faculty of Business Administration and Economics, Bielefeld University, Bielefeld, Germany, [7] Center for Mathematics, Technische Universität München, Garching, Germany, [8] Center for International Health (CIH), University Hospital, LMU Munich, Munich, Germany, [9] German Center for Translational Cancer Research (DKTK), Partner Site Munich, Munich, Germany, [10] Unit for Clinical Pharmacology (EKLiP), Helmholtz Zentrum München – German Research Center for Environmental Health (HMGU), Neuherberg, Germany

Background: Adaptive immune responses to structural proteins of the virion play a crucial role in protection against coronavirus disease 2019 (COVID-19). We therefore studied T cell responses against multiple SARS-CoV-2 structural proteins in a large cohort using a simple, fast, and high-throughput approach.

Methods: An automated interferon gamma release assay (IGRA) for the Nucleocapsid (NC)-, Membrane (M)-, Spike-C-terminus (SCT)-, and N-terminus-protein (SNT)-specific T cell responses was performed using fresh whole blood from study subjects with convalescent, confirmed COVID-19 (n = 177, more than 200 days post infection), exposed household members (n = 145), and unexposed controls (n = 85). SARS-CoV-2-specific antibodies were assessed using Elecsys® Anti-SARS-CoV-2 (Ro-N-Ig) and Anti-SARS-CoV-2-ELISA (IgG) (EI-S1-IgG).

Results: 156 of 177 (88%) previously PCR confirmed cases were still positive by Ro-N-Ig more than 200 days after infection. In T cells, most frequently the M-protein was targeted by 88% seropositive, PCR confirmed cases, followed by SCT (85%), NC (82%), and SNT (73%), whereas each of these antigens was recognized by less than 14% of non-exposed control subjects. Broad targeting of these structural virion proteins was characteristic of convalescent SARS-CoV-2 infection; 68% of all seropositive individuals targeted all four tested antigens. Indeed, anti-NC antibody titer correlated loosely, but significantly

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***Correspondence:**
Christof Geldmacher
geldmacher@lrz.uni-muenchen.de
Michael Pritsch
pritsch@lrz.uni-muenchen.de

†These authors share first authorship

‡These authors share senior authorship

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with the magnitude and breadth of the SARS-CoV-2-specific T cell response. Age, sex, and body mass index were comparable between the different groups.

Conclusion: SARS-CoV-2 seropositivity correlates with broad T cell reactivity of the structural virus proteins at 200 days after infection and beyond. The SARS-CoV-2-IGRA can facilitate large scale determination of SARS-CoV-2-specific T cell responses with high accuracy against multiple targets.

Keywords: SARS-CoV-2, COVID-19, T cell response, interferon gamma release assay (IGRA), high through put

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), started in December 2019. More than one year later, SARS-CoV-2 is still a serious threat to global health and a significant cause of mortality, especially in the elderly. Vaccines, mostly targeting the Spike protein of SARS-CoV-2 have been developed and approved at an unprecedented pace in history based on evidence for high efficacy (1). Yet, at the same time, restricting the vaccine target to a single protein or parts thereof also poses a risk of failure to immunization due to variants arising from natural viral mutations within the single protein of interest. In fact, newly emerging viral variants, such as B1.335, P.1, or B1.617 carrying mutations in the Spike protein, which potentially enhances the infectiousness of the virus, currently raise concerns that existing vaccines could lose or diminish their efficacy against these strains (2, 3). It was recognized early on that SARS-CoV-2 mounts a specific antibody based response that can protect from reinfections (4). As fundamental immunology teaches that antibody responses cannot be generated without a (T) cellular helper response, unsurprisingly, specific T cell responses were found in convalescent patients (5). Along these lines, a growing body of evidence has also recognized the existence and importance of cellular responses to SARS-CoV-2 infection in the clearance and later protection from reinfections (6). By nature, such responses are less convenient to measure and unfortunately there are no high throughput methods available to quantify SARS-CoV-2 specific T cell responses in patients.

Adaptive SARS-CoV-2-specific T cell responses likely have the capacity to protect the host at least from severe courses of COVID-19 upon reinfection even with the aforementioned immune escape variants. Upon reinfection, T cell recognition should nonetheless attenuate COVID-19 in those infected individuals (7). A broad T cell recognition of virus structural proteins can contribute to immune control even of highly variable viruses, such as HIV (8, 9), which easily escapes immune pressure inflicted by individual epitope-specific T cell responses (10).

Besides their role in adaptive immunity, SARS-CoV-2-specific T cell responses may also have a diagnostic value, as it has been reported that antibody levels wane faster than T cells. For example, SARS-CoV-1-specific antibody responses were short-lived and dropped below the limit of detection within 2 to 3 years (11, 12). As for SARS-CoV-2, antigen-specific antibody responses are not even detectable in all individuals, particularly in those with milder forms of COVID-19 (13–15).

Here, we report on SARS-CoV-2 specific T cell and antibody responses in a large cohort of study subjects with convalescent, PCR confirmed COVID-19, which did not require hospitalization, and in their exposed household members, as well as in unexposed controls. Using an automated, easy-to-use whole blood interferon gamma release assay (IGRA), we demonstrate that most individuals with serological evidence of convalescent SARS-CoV-2 infection, T and B cell reactivity against multiple structural proteins can be detected in peripheral blood at 200 days after infection/exposure and beyond.

MATERIALS AND METHODS

Study Design, Study Subjects, and Specimen Collection

To establish a solid data basis for this study, we included study subjects in whose household at least one person has had a PCR confirmed SARS-CoV-2 infection. In May and June 2020, all households of Munich with at least one registered positive PCR for SARS-CoV-2 to date (more than 6000 households) were contacted by the responsible official authorities (City of Munich Health Department) and were provided information about COVID-19 related studies as well as contact details of the study center at the Division of Infectious Diseases and Tropical Medicine, University Hospital, LMU Munich, where upon more than 1000 households declared their interest in participating. Chronological enrollment took place from September 29, 2020 until January 27, 2021 of 177 PCR-positive individuals starting with the earliest registered PCR-positives and 145 of their household members. Furthermore, we randomly selected 40 households from a previously described population-based cohort study (KoCo 19) as controls (12, 16, 17) without any seropositive members on baseline as well as during follow up. A total of 36 of those households comprising 85 eligible members agreed to participate and were recruited during January 6-27, 2021. To investigate serology, cellular immune response and transmission, the study subjects of both groups were asked to provide a venous blood sample. Enrollment as well as specimen collection took place during household visits or at a central testing facility depending on study subjects' preferences.

Personal Information

Personal data of the study subjects was collected as described previously (18–20). In short, the mobile data collection tool OpenDataKit (ODK) was used to capture data during study visits by field workers on Android smartphones. Consecutively, study subjects completed household as well as personal questionnaires using a web-based application. Non responders were reminded first by email, and in case of continued non-response with a telephone reminder. Telephone interviews were offered to those who felt unable to complete the questionnaires online.

Serologic Testing Methods

We determined antibody reactivity in plasma derived from ethylenediaminetetraacetic acid (EDTA)-coated blood tubes using Elecsys® Anti-SARS-CoV-2 (Roche, Mannheim, Germany) hereafter Ro-N-Ig and Anti-SARS-CoV-2-ELISA (IgG) (Euroimmun, Lübeck, Germany), hereafter EI-S1-IgG. Testing was conducted in accordance with the manufacturers` recommendations. An optimized cutoff of 0.422 (instead of 1.0) for Ro-N-Ig was used to determine seropositivity in our study subjects, as described previously (18). An optimized cutoff of 1.015 (instead of 1.100) for EI-S1-IgG was only used in supplemental **Figure S1** as an additional marker for seropositivity in one subgroup.

SARS-CoV-2 Interferon Gamma Release Assay (IGRA)

0.5 ml of fresh heparinized whole blood was added to each "Euroimmun" stimulator tube coated with SARS-CoV-2 specific antigens (Nucleocapsid protein, Spike-C-Terminus, Spike-N-Terminus and Membrane protein) and to negative and positive control tubes according to manufacturer instructions (Euroimmun, Lübeck, Germany). Tubes were inverted six times. After 16 to 20 hours of incubation at 37°C, 5% CO_2 the samples were centrifuged at 12000 rcf for 10 minutes. The plasma supernatant was then transferred into a cryotube and stored at –80°C until testing. Interferon gamma (IFNγ) was detected automatically in the supernatants by an enzyme-linked immuno-sorbent assay (ELISA, Euroimmun, Lübeck, Germany) using the Euroimmun Analyzer I according to the manufacturer's instructions. Using a standard curve, the IFNγ concentration was calculated. Background subtraction was carried out. Negative calculated values after background subtraction were set to 0 mIU/ml. All, but 3 out of 55 (5%) from the group exposed seropositive, 3 out of 90 (3%) from the group exposed seronegative and 5 out of 85 (6%) from the unexposed controls were stimulated with 3 antigens (NC, M and SCT). Study subjects, who were not stimulated with all antigenic regions provided too little blood. A subset of 232 (57%) study subjects was also stimulated with the SNT. All antigens were pools of synthetic 15mer peptides with 11 amino acid overlap (JPT Peptide Technologies), were based on the SARS-CoV-2 WUHAN isolate and were used at a final concentration of 5µg per stimulation. The Spike-N-terminal (PM-WCPV-S-2: P0DTC2) pool consisted of 158 peptides and the Spike-C-terminus (PM-WCPV-S-2: P0DTC2) of 157 peptides. The Nucleocapsid protein (PM-WCPV-NCAP: P0DTC9) contained 102 and the Membrane protein (PM-WCPV-VME: P0DTC5) 53 peptides. The utilized sequences for the peptides were previously used and described by others (21).

Data Analysis

Data analysis and graphics were performed using the statistical software R (R Development Core Team, 2021) and the ggplot package (Wickham, 2016), as well as GraphPad Prism version 8 (GraphPad Software Inc.). Concentrations of IFNγ (mIU/ml) were log2 transformed for visual representation. The receiver operating characteristics (ROC) curve was used to define an optimized cutoff of IFNγ of 40 mIU/ml (**Tables S1A**, **B**). Differences in the IFNγ concentrations between the response to the antigenic regions were tested for significance using the Wilcoxon signed rank test and for differences in the IFNγ between EI-S1-IgG seropositive and seronegative study subjects the unpaired Wilcoxon test was used. Resulting p-values were adjusted for multiple testing using the Bonferroni correction. Spearman's correlation coefficient (rho) was used to assess the correlation between Ro-N-Ig and the number of antigens detected. The flowchart was designed using (diagrams.net).

RESULTS

Description of Study Population

A total of 182 households with 322 household members were recruited into this study (**Figure 1**). At least one resident of each household had been infected with SARS-CoV-2 and was diagnosed by a positive PCR result between March and April 2020 and registered by the City of Munich Health Department. These 322 study subjects were then tested using the IGRA, including individuals with PCR confirmed, convalescent SARS-CoV-2 infection. Only 11 of these study subjects had visited hospital outpatient facilities due to COVID-19 related symptoms, but none was hospitalized. All other COVID-19 cases in this study showed a mild course or did not report any symptoms at all.

In addition, 85 study subjects from 36 non-exposed households participating in the COVID-19 cohort Munich (KoCo19) were recruited as a control group and were also tested using the IGRA (**Figure 1**) (20, 22). These study subjects did not report contact to SARS-CoV-2 infected individuals and were previously tested twice for SARS-CoV-2 antibodies, being seronegative both times. At the time of blood collection, these individuals were tested again and remained seronegative. T cell responses against three SARS-CoV-2 structural antigens (Nucleocapsid (NC), Membrane protein (M), and Spike-C terminal region (SCT)) were tested for a total of 407 subjects (including the 85 controls) between October 2020 to January 2021 using a high throughput fresh whole blood IGRA. Within a subset of 232 study subjects (including 42 controls), reactivity to a fourth antigen (Spike-N terminal region (SNT)) was tested additionally. **Table 1** summarizes basic characteristics of these 407 individuals and shows that sex, age, and BMI were comparable between the groups with an overall median age of 41 years, a sex proportion of 51% females, and a median BMI of 23.9 kg/m^2. The median time between PCR testing and sample measurement was 243 (IQR 228.5 - 259.3) days in PCR-positive

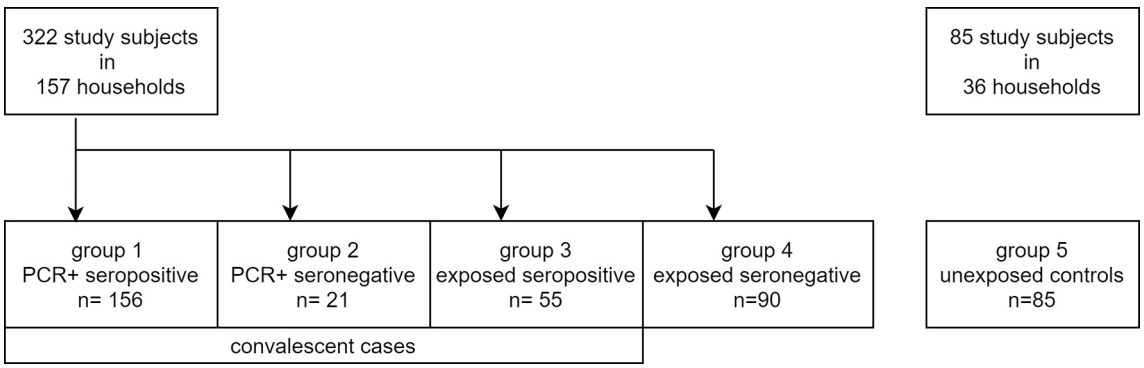

FIGURE 1 | Flowchart of study subject groups.

seropositive study subjects and 233 (IQR 223.0 - 244.5) days in PCR-positive seronegative ones.

T Cell Reactivity to Structural SARS-CoV-2 Proteins in PCR-Positive Convalescent Cases and Unexposed Controls

To define SARS-CoV-2-specific T cell reactivity with high sensitivity and specificity, we determined a single optimized cutoff for the concentration of IFNγ in stimulated whole blood supernatants for each of the tested SARS-CoV-2 antigenic regions NC, M, SCT, and SNT. To this end, we used PCR-positive seropositive cases as cases and unexposed individuals as controls (**Figure 2A**). ROC analysis confirmed an optimized cutoff at 40 mIU/ml IFNγ to define positive T cell responses against these antigenic regions. This resulted in a sensitivity of 82% and a specificity of 91% for T cell responses targeting the NC, a sensitivity of 88% and specificity of 94% for those targeting M protein, a sensitivity of 85% and specificity of 85% for those targeting SCT, and a sensitivity of 73% and specificity of 97% for those targeting SNT. As shown in **Figures 2A** and **S2**, for each of the antigenic regions most PCR-positive seropositive cases had IFNγ values of 40 mIU/ml or above upon *in vitro* stimulation with each of the antigens, whereas few non-exposed individuals had mounted such responses (10% to NC, 7% to M, 14% to SCT, 4% to SNT). SARS-CoV-2-specific T cells are most likely source of IFNγ production in this IGRA. We hence analyzed 10 convalescent SARS-CoV-2 patients (175 - 210 days post infection) using standard intracellular cytokine staining techniques after *in vitro*

TABLE 1 | Overview on basic characteristics of the 407 study subjects.

	PCR-positive seropositive	PCR-positive seronegative	Exposed seropositive	Exposed seronegative	Unexposed controls	All study subjects
n	156	21	55	90	85	407
Sex						
Male	71 (45.5%)	15 (71.4%)	28 (50.9%)	43 (47.8%)	44 (51.8%)	201 (49.4%)
Female	85 (54.5%)	6 (28.6%)	27 (49.1%)	47 (52.2%)	41 (48.2%)	206 (50.6%)
Age (years)						
14-19	1(0.60%)	0 (0.00%)	6 (10.1%)	7 (7.80%)	11 (12.9%)	25 (6.10%)
20-34	32 (20.5%)	8 (38.1%)	20 (36.3%)	30 (33.3%)	15 (17.6%)	105 (25.8%)
35-49	68 (43.6%)	8 (38.1%)	13 (23.6%)	31 (34.4%)	36 (42.4%)	156 (38.3%)
50-64	44 (28.2%)	3 (14.3%)	11 (20.0%)	18 (20.0%)	16 (18.8%)	92 (22.6%)
65-79	11 (7.10%)	2 (9.50%)	5 (9.10%)	4 (4.40%)	3 (3.50%)	25 (6.10%)
80+	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	4 (4.70%)	4 (1.00%)
Median	44	39	35	40	43	41
Body Mass Index (kg/m²)						
< 18,5	2 (1.30%)	0 (0.00%)	2 (3.60%)	3 (3.30%)	2 (2.40%)	9 (2.20%)
18,5-25	89 (57.1%)	16 (76.2%)	32 (58.2%)	54 (60.0%)	47 (55.3%)	238 (58.5%)
25-30	54 (34.6%)	5 (23.8%)	11 (20.0%)	29 (32.2%)	24 (28.2%)	123 (30.2%)
> 30	11 (7.10%)	0 (0.00%)	9 (16.4%)	3 (3.30%)	11 (12.9%)	34 (8.40%)
NA	0 (0.00%)	0 (0.00%)	1 (1.80%)	1 (1.10%)	1 (1.20%)	3 (0.70%)
Median	24.2	24.2	23.7	23.3	24.1	23.9
Time from PCR to visit						
median (in days)	243	233				
IQR (in days)	228.5 - 259.3	223.0 - 244.5				

Sex, age and body mass index were comparable between the individual groups with an overall median age of 41 years, 51% females and BMI of 23.9 kg/m².

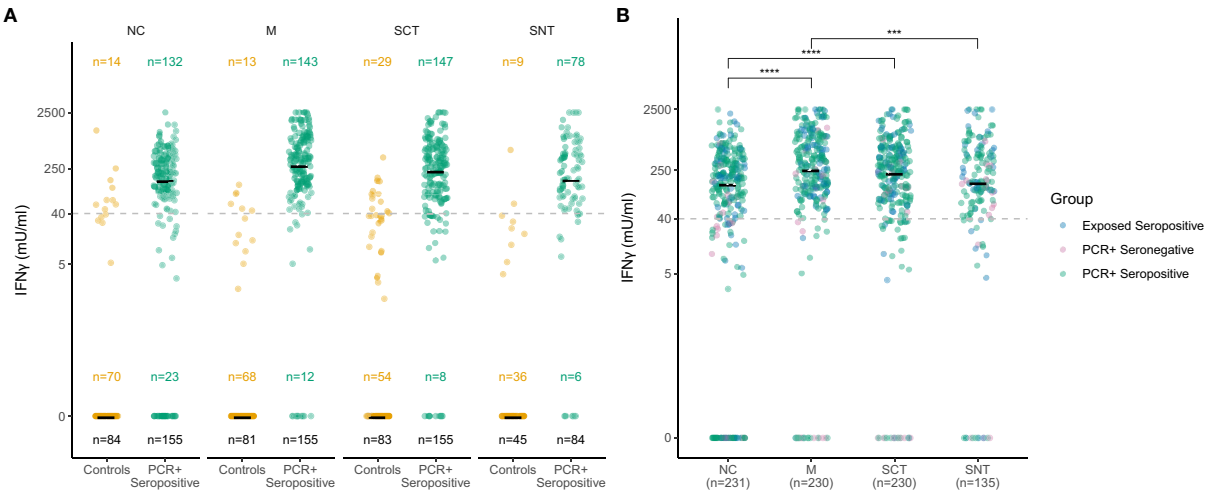

FIGURE 2 | Sensitive and specific detection of T cell responses to four SARS-CoV-2 antigenic regions. Concentration of IFNγ in stimulated whole blood supernatants (y-axis) is shown as mIU/ml for the Nucleocapsid (NC), Membrane protein (M), Spike-C-Terminus (SCT) and Spike-N-Terminus (SNT). The numbers of subjects tested are indicated for each antigenic region and group. The black number at the bottom indicates overall number of study subjects in each group, numbers in the middle and the top show the number of subjects with IFNγ concentration of or above 0 mIU/ml, respectively. Cutoff of 40 mIU/ml IFNγ for T cell reactivity to an antigenic region is indicated as dashed line. Thick black lines mark median values. Each dot represents one study subject. Due to low blood volume, not all participants underwent the same analysis regarding the stimulation with the main three tested antigenic regions (NC, SCT and M). Therefore, sample sizes at each group between Antigens differ (see black sample size n below). 232 study subjects were also stimulated with SNT. **(A)** Ro-N-Ig seropositive subjects with PCR confirmed convalescent COVID-19 (green dots) were compared to negative controls from unexposed households (orange dots). **(B)** T cell recognition to the four tested structural antigens was compared for subjects with serological and/or PCR confirmed convalescent COVID-19. The p-values were calculated using Wilcoxon signed rank test. ***$p \leq 0.001$, ****$p \leq 0.0001$.

restimulation of fresh PBMCs with a Spike-specific or NC-specific peptide pool (**Figure S3** and **Table S2**). The source of antigen specific IFNγ production were mostly CD4 and sometimes CD8 T cells, which is consistent with previous reports (5, 23, 24). CD3 negative cells did not produce antigen specific IFNγ.

Next, we determined the concentration of IFNγ in stimulated supernatants for all study subjects with evidence of convalescent SARS-CoV-2 infection. All subjects were tested for NC, M and SCT antigens. 135 convalescent cases were stimulated with a fourth antigenic region, the SNT (**Figure 2B**). The median IFNγ concentration in stimulated supernatants for all study subjects with evidence of convalescent SARS-CoV-2 infection was 151 mIU/ml, 258 mIU/ml, 231 mIU/ml, and 162 mIU/ml for NC, M, SCT and SNT proteins, respectively (**Figure 2B**). The magnitude of the memory response towards the small M-protein was the strongest observed and significantly increased when compared to NC ($p < 0.0001$) and SNT ($p < 0.001$). There was also a significant increase in IFNγ production measured after stimulation with SCT when compared to NC ($p < 0.0001$). **Figure S2** shows IFNγ concentrations in mIU/ml against all four tested antigenic regions in all five groups (unexposed controls, exposed seronegatives, exposed seropositives, PCR-positive seronegatives and PCR-positives seropositives). We also highlight, that exposed seronegatives did not differ from unexposed controls with very narrow or non-existent SARS-CoV-2 T cell recognition. Overall, these results show that almost all individuals with evidence of convalescent SARS-CoV-2 infection mount memory T cell responses against structural proteins of the SARS-CoV-2 virion with the highest median IFNγ response magnitude determined for the small M protein.

Broad T Cell Recognition of Structural SARS-CoV-2 Proteins at More Than 200 Days in Individuals With Convalescent SARS-CoV-2 Infection

The cutoff 40 mIU/ml was applied to define the breadth of SARS-CoV-2-specific T cell targeting of structural proteins in all groups stratified by serostatus, confirmatory PCR diagnoses and history of SARS-CoV-2 exposure. **Figure 3A** shows that, when tested against NC, M and SCT, most study subjects (70%) in the group PCR-positive seropositive targeted all tested SARS-CoV-2 specific antigenic regions and above 85% reacted to two of the three tested antigens. A similar pattern was detected for exposed seropositive study subjects, who were not confirmed by a positive PCR. A reduced breadth of SARS-CoV-2-specific T cell recognition was observed for the PCR-positive seronegative individuals. By contrast, above 70% of unexposed controls reacted to none of the tested antigens and the remaining ones typically reacted to only one of the tested SARS-CoV-2 antigenic regions. More than 70% of exposed seronegative study subjects also did not target any of the tested structural proteins, however the proportion of responders recognizing two or more antigens was increased, although not statistically significant ($p = 0.055$), when compared to unexposed controls. In individuals with four

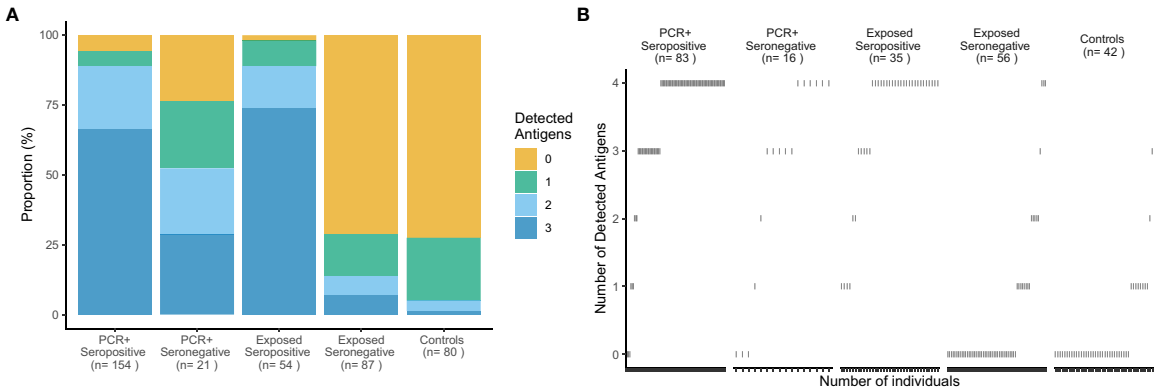

FIGURE 3 | Broad T cell recognition of structural SARS-CoV-2 proteins in subjects with convalescent infection. Percentages of subjects (y-axis) who responded to 0, 1, 2 or 3 of 3 tested antigenic regions are shown in **(A)** for 5 groups delineated by PCR, Ro-N-Ig serostatus and SARS-CoV-2 exposure status. The number of study subjects in each group and the breadth of T cell recognition from 0 to 3 structural proteins are indicated for each group. A subgroup of 208 study subjects was tested with a fourth antigen - the Spike-N-Terminus **(B)**. The breadth of T cell recognition of the four tested antigens is shown for each of the 5 groups. Every dot represents an individual. Reactivity to Nucleocapsid Protein (NC), Spike-C-Terminus (SCT), and Membrane protein (M) was tested in stimulated whole blood supernatants.

tested antigens (NC, M, SCT and SNT) a similar pattern was observed (**Figure 3B**); most subjects with evidence of convalescent SARS-CoV-2 infection reacted against all four tested antigens. Of note, we observed T cell reactivity to multiple antigenic regions in 75% (12 of 16 tested with four antigens) of study subjects who had been diagnosed by PCR but were seronegative at the time point of study inclusion. EI-S1-IgG against the SARS-CoV-2 S1 region was additionally measured in 17 PCR-positive, Ro-N-Ig-seronegative study subjects. 35% of these (6 of 17) had Spike-specific IgG antibody responses. Comparison of T cell reactivity to the M protein, but not the other 3 antigenic regions, differed significantly between these EI-S1-IgG-positive and EI-S1-IgG-negative study subjects (**Figure S1**). These findings suggest that many of these PCR-positive, seronegative study subjects are true convalescent COVID-19 cases and were not falsely diagnosed with COVID-19 in the past. However, we cannot exclude false positivity for a some of the subjects, who also did not have detectable Spike-specific IgG antibodies, nor a broader SARS-CoV-2 T cell response. Anti-NC

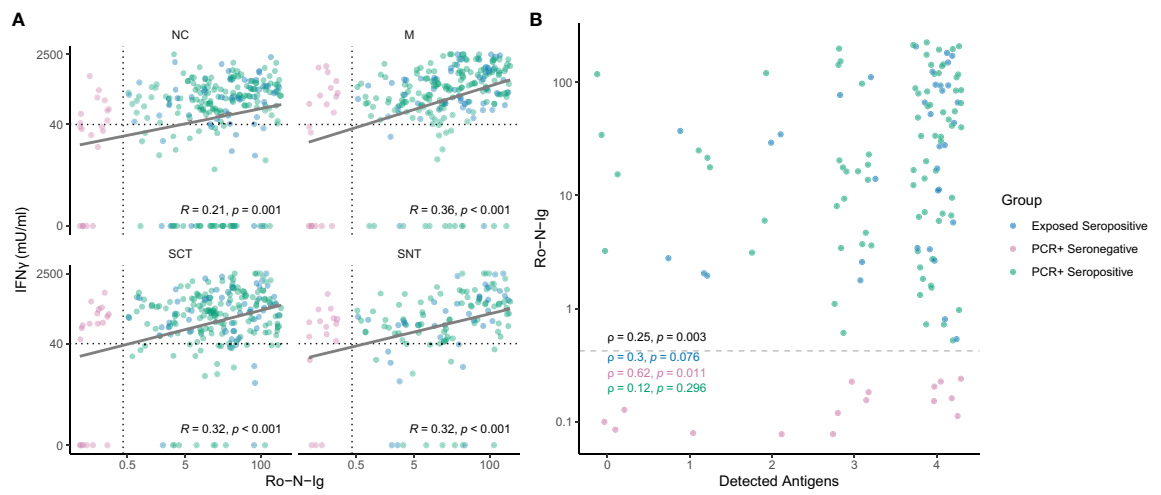

FIGURE 4 | **(A)** Correlation of SARS-CoV-2-specific T cell reactivity to different antigenic regions and Roche-N-Ig titer. Shown are individuals of the convalescent group in different colors for each subgroup. Strong humoral immune response correlates with cellular reactivity to SARS-CoV-2 specific antigens measured in IFNγ (y-axis). Cutoffs for seropositivity and T cell reactivity to an antigenic region are indicated as dashed lines. Each dot represents one study subject. **(B)** Correlation of Roche-N-Ig and breadth reactivity of detected antigens. The plot shows Roche-N-Ig values for each individual recognizing 0, 1, 2, 3 or 4 antigenic regions. Only individuals which were tested for all four antigenic regions (Nucleocapsid NC, Spike-C-Terminus SCT, Spike-N-Terminus SNT and Membrane M) are shown. Cutoff for seropositivity is indicated by a dashed line. The p- value of non-zero correlation from all groups combined is shown in black. A low p-value means that the correlation is unlikely to be non-zero due to chance. Each dot represents one study subject.

antibody correlated loosely, but significantly with the magnitude and breadth of the SARS-CoV-2-specific T cell response (**Figure 4**). In summary, these results demonstrate broad T cell targeting of structural SARS-CoV-2 proteins long after convalescent infection in subjects with moderate, mild, or even asymptomatic SARS-CoV-2 infection. The high throughput interferon gamma release assay detected these responses with high sensitivity and specificity even in likely asymptomatic cases or in seronegative individuals.

DISCUSSION

Our study included study subjects of households, in which at least one member had a PCR confirmed SARS-CoV-2 infection between March and April 2020, including a subgroup of cases who had been infected, but had reported mild or no COVID-19-specific symptoms. Using a simple IGRA approach, we show that whole blood stimulation with different SARS-CoV-2 antigens can detect a broad cellular immune response to different structural proteins in convalescent individuals after moderate, mild, or completely asymptomatic COVID-19 at least 200 days after infection. In addition, the used approach provides high sensitivity and specificity. IFNγ production upon *in vitro* restimulation typically derives from CD4 and CD8 T cells and the tested structural antigens belong to the most immunodominant in acute SARS-CoV-2 infection (23). To prove this aspect, we used flow cytometry in 10 convalescent subjects more than 175 days after their reported infection, that also have been stimulated with structural SARS-CoV-2 proteins.

NC, M and S peptide pools were chosen for stimulation, because these represent structural proteins of the SARS-CoV-2 virion and were previously shown to induce high magnitude T cell responses (23). Because a previous study showed that T cell reactivity to the SNT peptide pool has high SARS-CoV-2 specificity (21), whereas the SCT peptide pool identified more non-SARS-CoV-2-specific T cell responses, the Spike protein was split into these two pools for the purpose of this study (23). Previous research has shown that CD4+ T cell responses are often stronger than corresponding CD8+ T cell responses, at least when using cryopreserved PBMC (5, 23, 24). Analyses of fresh, whole blood are the most direct way to assess antigen-specific cell function and avoid potential losses associated with PBMC cryopreservation. We therefore consider our approach to detect SARS-CoV-2 specific T cell responses as highly sensitive.

While most of exposed anti-nucleocapsid-seronegative study subjects did not mount T cell responses, there was a trend towards increased T cell recognition of multiple antigenic regions compared to the unexposed controls. This suggests that some formerly infected, now seronegative subjects retained SARS-CoV-2-specific T cell reactivity. Hence, assessment of a broad SARS-CoV-2-specific T cell response besides antibody responses increased detection of past SARS-CoV-2 transmission events in our study, which has been reported previously (16).

Another observation made was that most of the exposed seronegative study subjects did not differ from unexposed controls with very narrow or non-existent SARS-CoV-2 T cell recognition, probably because either no transmission event took place or narrow positive T cell responses to a single SARS-CoV-2 antigen could be the result of cross reactivity to other common cold coronaviruses (17, 21, 25). Indeed, 28% of unexposed controls had some narrow reactivity to SARS-CoV-2 structural proteins. Thereof, our results suggest that the current approach could be suited for identifying individuals with pre-existing cross-reactive T cell responses. This could facilitate studies on the potentially protective role of those T cells in SARS-CoV-2 infection.

Subjects with convalescent SARS-CoV-2 infection are well protected from reinfection, which correlates not only with anti-Spike antibodies, but also with anti-NC-antibodies (26). Our data show that anti-NC-seropositivity is also indicative of a broad T cell response against structural SARS-CoV-2 proteins in most seropositive individuals, including those individuals who did not report any COVID-19 specific symptoms. From the data it might also be concluded that in a certain fraction of subjects, specific T-cells are detected longer after the initial infection than antibodies tested with serological assays such as the one used here. A broad T cell recognition of virus structural proteins can contribute to immune control of variable viruses, such as HIV (8, 9). We therefore speculate that such broad virus-specific T cell immunity could contribute to reduce peak viral loads, to accelerate virus clearance and hence also reduce transmission risk and attenuate COVID-19 in case of reinfection with viral variants of concern, such as B1.335, P.1, or B1.617. Virus neutralization by antibodies is decreased for these variants (27), but to the best of our knowledge these have not escaped from T cell mediated immune pressure. Next generation polyvalent SARS-CoV-2 vaccines should therefore incorporate the comparatively small and immunogenic proteins M and NC to broaden vaccine-induced T cell recognition.

One limitation of this study is that we only included mild or asymptomatic COVID-19 convalescent cases and no severe ones. Another limitation is that the final antigen concentration differed between the different peptide pools on a per peptide level. We can therefore not exclude some effect of per peptide concentration on cellular responsiveness to the individual peptide pools. Nevertheless, this should not affect the overall results and interpretation of our study.

One strength of this study is that inclusion of SARS-CoV-2 exposed seropositive study subjects, who did not receive a PCR confirmed diagnosis, should have enriched for formerly infected subjects who had minimal or no COVID-19 specific symptoms and therefor did not get PCR tested. Unfortunately, due to a recall-bias, disease symptom reporting may have been incomplete after more than 200 days and hence we cannot conclude on differences in SARS-CoV-2-specific T cell memory between subjects with truly asymptomatic, very mild, or mild to moderate disease. It would be interesting to learn whether such individuals differ in their SARS-CoV-2-specific T cell memory and immunoreactivity in this assay. The major strength of this study relies on the combination of high throughput IGRA and automated serology platforms, that allowed us to be capable of investigating SARS-CoV-2-specific T and B cell responses for a large cohort in a limited amount of time. In addition, this also enabled us to analyze cellular responses to multiple structural virion proteins with high accuracy and in a diverse subset of

individuals such as those with PCR or serologically confirmed convalescent COVID-19 as well as seronegative, exposed household members and unexposed controls. In conclusion, our results show that most subjects have broad T cell and B cell immunity at least 200 days after SARS-CoV-2 infection and beyond regardless of disease severity.

KOCO19 STUDY GROUP MEMBERS

Emad Alamoudi, Jared Anderson, Abhishek Bakuli, Marc Becker, Franziska Bednarzki, Olimbek Bemirayev, Jessica Beyerl, Patrick Bitzer, Rebecca Boehnlein, Friedrich Caroli, Lorenzo Contento, Alina Czwienzek, Flora Deák, Maximilian N. Diefenbach, Gerhard Dobler, Jürgen Durner, Judith Eckstein, Philine Falk, Volker Fingerle, Felix Forster, Turid Frahnow, Guenter Froeschl, Otto Geisenberger, Kristina Gillig, Philipp Girl, Pablo Gutierrez, Anselm Haderer, Marlene Hannes, Jan Hasenauer, Tim Haselwarter, Alejandra Hernandes, Matthias Herrmann, Leah Hillari, Christian Hinske, Tim Hofberger, Sacha Horn, Kristina Huber, Christian Janke, Ursula Kappl, Antonia Kessler, Zohaib N. Khan, Johanna Kresin, Arne Kroidl, Magdalena Lang, Silvan Lange, Michael Laxy, Ronan Le Gleut, Reiner Leidl, Leopold Liedl, Xhovana Lucaj, Petra Mang, Alisa Markgraf, Rebecca Mayrhofer, Dafni Metaxa, Hannah Mueller, Katharina Mueller, Laura Olbrich, Ivana Paunovic, Claire Pleimelding, Michel Pletschette, Stephan Prueckner, Kerstin Puchinger, Peter Puetz, Katja Radon, Elba Raimundéz, Jakob Reich, Friedrich Riess, Camilla Rothe, Viktoria Ruci, Nicole Schaefer, Yannik Schaelte, Benedikt Schluse, Elmar Saathoff, Lara Schneider, Mirjam Schunk, Lars Schwettmann, Peter Sothmann, Kathrin Strobl, Jeni Tang, Fabian Theis, Verena Thiel, Jonathan von Lovenberg, Julia Waibel, Claudia Wallrauch, Roman Woelfel, Julia Wolff, Tobias Wuerfel, Sabine Zange, Eleftheria Zeggini, Anna Zielke

DATA AVAILABILITY STATEMENT

Data at individual level is not available due to protection of data privacy of our study subjects. However, data are accessible subject to data protection regulations upon reasonable request to the corresponding authors. Requests will be scientifically reviewed including the respective institutional review board if necessary and an appropriate data transfer agreement will have to be signed if approved. To facilitate reproducibility and reuse, the code used to perform the analyses and generate the figures has been made available on GitHub (https://github.com/koco19/IGRA).

ETHICS STATEMENT

The study protocol was reviewed and approved by the Institutional Review Board of the Medical Faculty at Ludwig-Maximilians-University Munich, Germany under the project number 20-692 (vote of approval dated Sept. 21st, 2020) and 20-371 (vote of approval dated May 15th, 2020. Oral and written informed consent was obtained from all study subjects. For youths (ages 14 to 17) age-appropriate versions of the information and consent forms were used.

AUTHOR CONTRIBUTIONS

MH is the principal investigator and obtained most funds. MH, MP, CG, SK, and JR designed the study with help from JG and JF. SK and JR also obtained funds. Sample collection was led by JG, MP, JF, and IK. IB, LG, TE, MA, and DC performed the interferon gamma release assay. TE performed PBMC isolation and flow cytometry. JB generated study questionnaires. MG, JD, ST, JB, IB, LG, SW, NC, AW, and CF performed data cleaning and statistical analysis. LG, IB, and TE analyzed flow cytometry data. Data management and visualization was prepared by MG. High throughput serological testing was conducted by AW and RR-A. IB, LG, MP, JB, JF, JR, SK, MG, CF, and CG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.688436/full#supplementary-material

Supplementary Table 1A | Cutoff determination using ROC-Analysis. Shown are Sensitivity and Specificity for different amounts of interferon gamma for Nucleocapsid (NC) and Spike-C-terminus (SCT). Determined cutoff is indicated as a line. To define a general cutoff for a positive T cell response, we compared sensitivity and specificity of NC, SCT, SNT and M. 40mlU/ml showed the best combination of sensitivity and specificity when defining one single cutoff for all four antigenic regions.

Supplementary Table 1B | Cutoff determination using ROC-Analysis. Shown are Sensitivity and Specificity for different amounts of interferon gamma for Spike-N-Terminus (SNT) and Membrane protein (M). Determined cutoff is indicated as a line. To define a general cutoff for a positive T cell response, we compared sensitivity and specificity of NC, SCT, SNT and M. 40mlU/ml showed the best combination of sensitivity and specificity when defining one single cutoff for all four antigenic regions.

Supplementary Table 2 | Phenotypic characterization of IFNγ-positive cells responding to Spike- and Nucleocapsid peptide pools using intracellular cytokine staining. Shown are the percentages of parent cell populations for 10 study subjects. Columns from left to right according to the gating strategy. In total, 10 study subjects were tested after overnight *in vitro* restimulation of fresh PBMC at 180 days after SARS-CoV-2 symptom onset. A summary of these results and materials and methods are provided in **Supplementary Figure 3**.

Supplementary Figure 1 | Comparison of IFNγ in PCR-positive, EI-S1-IgG seropositive, but Roche-N-Ig seronegative study subjects. Shown are individuals of the PCR-positive Ro-N-Ig seronegative group. The figure shows the concentration of IFNγ in stimulated whole blood supernatants (y-axis) in mIU/ml in 17 PCR-positive, EI-S1-IgG seropositive, but Ro-N-Ig seronegative study subjects. Six out of 17 (35%) Ro-N-Ig negative study subjects are EI-S1-IgG positive, arguing for being serologic Nucleocapsid non-responders. Comparison of T cell reactivity to the M protein, but not the other 3 antigenic regions, differed significantly between these EI-S1-IgG-positive and EI-S1-IgG-negative study subjects. Cutoff for EI-S1-IgG is indicated by a dashed line. Each dot represents one study subject. Wilcox Test p-values were calculated. In four study subjects EI-S1-IgG was not measured and only 12 out of 17 were tested with SNT. *$p \leq 0.05$.

Supplementary Figure 2 | Comparison of reactivity to the four structural antigenic regions in all study subjects delineated by exposure, PCR result and serostatus. Concentration of IFNγ in stimulated whole blood supernatants (y-axis) is shown as mIU/ml for the Nucleocapsid (NC), Membrane protein (M), Spike-C-Terminus (SCT) and Spike-N-Terminus (SNT). The numbers of subjects tested are indicated for each antigenic region and group. The black number at the bottom indicates overall number of study subjects in each group, numbers in the middle and the top show the number of subjects with IFNγ concentration of or above 0 mIU/ml, respectively. Cutoff of 40 mIU/ml IFNγ for T cell reactivity to an antigenic region is indicated as dashed line. Thick black lines mark median values. Each dot represents one study subject. Due to low blood volume, not all participants underwent the same analysis regarding the stimulation with the main three tested antigenic regions (NC, SCT and M). Therefore, sample sizes at each group between Antigens differ (see black sample size n below). 232 study subjects were also stimulated with SNT.

Supplementary Figure 3 | Phenotypic characterization of IFNg+ cells responding to Spike- and Nucleocapsid peptide pools using intracellular cytokine staining. Shown are results for representative 5 study subjects (different columns). **(A)** shows CD3 versus IFNγ staining for cells of the lymphocyte gate. CD3+ T cells were then further delineated into CD4 **(B)** and CD8 **(C)** T cells. The stimulation antigen is indicated on the left. In total, 10 study subjects were tested 180 days after SARS-CoV-2 symptom onset. The associated data is provided in **Table S2**. In summary, out of the 10 subjects, 60% showed IFNγ+CD4+ T cells and 10% showed IFNγ+ CD8+ T cells upon *in vitro* restimulation with the Spike peptide pool. 90% and 50% had detectable IFNγ+ CD4+ and CD8 T cell responses to the Nucleocapsid peptide pool, respectively. 60% had IFNγ+CD4+ T cell reactivity to the Spike and Nucleocapsid peptide pools. A positive response is defined by a minimum of 15 cells/gate, a minimum of 0.01% IFNγ+ cells of CD4+ cells or CD8+ cells and a minimum of the double percentage of the negative control. The source of interferon gamma was determined by standard ICS procedures as follows; for the PBMC isolation, CPDA blood was centrifuged at 1285g for 10 minutes. After adding PBS, the suspension was filled into Leucosept tubes (Greiner) with Ficoll-Paque and centrifuged at 800g. PBMCs were harvested and directly prepared for flow cytometric analysis. PBMCs were incubated in complete medium at 37°C in 5% CO2 for 16h in the presence of either Nucleocapsid (NC), Spike-peptide pools (S), staphylococcus enterotoxin B (SEB) as positive control or nothing as negative control, and with a stimulation master mix containing costimulatory antibodies CD28, CD49d (Becton Dickinson, clones L293 and L25, respectively) and Brefeldin A (SIGMA). The PBMCs were then washed and stained with a surface antibody mix containing CD4-ECD (Beckman Coulter, clone SFCI12T4D1) and CD8–APC A750 (Beckman Coulter, clone B9.11) and incubated for 20 minutes in the dark. As the assays were performed on freshly isolated PBMC, no life/dead stain was added. After another washing step, the cells were permeabilized using pre-diluted FoxP3 Perm fixation buffer (ebioscience) and incubated of 25 minutes before adding diluted permeabilization buffer (ebioscience). PBMCs were then stained intracellularly using CD3–APC A700 (Beckman Coulter clone, UCHT1) and IFNγ-FITC (Biolegend, clone B27) for 30 minutes. Permeabilization buffer was added, and cells were acquired using a Cytoflex flow cytometry instrument (Beckman Coulter). Flow cytometry data analyses performed using FlowJo 10.7.2.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Table S1A. Cutoff determination using ROC-Analysis. Shown are Sensitivity and Specificity for different amounts of interferon gamma for Nucleocapsid (NC) and Spike-C-terminus (SCT). Determined cutoff is indicated as a line. To define a general cutoff for a positive T cell response, we compared sensitivity and specificity of NC, SCT, SNT and M. 40mlU/ml showed the best combination of sensitivity and specificity when defining one single cutoff for all four antigenic regions.

NC			SCT		
	Sensitivity (%)	Specificity (%)		Sensitivity (%)	Specificity (%)
> 2.410	89.13	83.08	> 1.190	95.65	63.08
> 5.070	88.04	83.08	> 2.635	95.65	64.62
> 5.915	88.04	84.62	> 3.040	95.65	66.15
> 10.70	86.96	84.62	> 4.895	95.65	67.69
> 16.14	85.87	84.62	> 7.365	94.57	67.69
> 18.47	84.78	84.62	> 8.415	94.57	69.23
> 21.59	83.7	84.62	> 9.240	94.57	70.77
> 25.50	82.61	84.62	> 10.00	93.48	70.77
> 28.69	82.61	86.15	> 11.12	93.48	72.31
> 32.22	82.61	87.69	> 13.79	92.39	72.31
> 35.32	82.61	89.23	> 16.95	92.39	73.85
> 37.37	81.52	89.23	> 18.71	92.39	75.38
> 40.58	81.52	90.77	> 19.32	91.3	75.38
> 45.13	80.43	90.77	> 24.78	90.22	75.38
> 48.11	79.35	90.77	> 30.47	90.22	76.92
> 52.80	78.26	90.77	> 31.61	89.13	76.92
> 57.24	77.17	90.77	> 34.25	89.13	78.46
> 57.79	77.17	92.31	> 36.21	86.96	78.46
> 58.59	77.17	93.85	> 36.26	86.96	80
> 63.92	76.09	93.85	> 36.84	86.96	83.08
> 68.93	75	93.85	> 37.71	86.96	84.62
> 70.61	75	95.38	> 39.19	85.87	84.62
> 74.43	73.91	95.38	> 40.38	84.78	84.62
> 78.57	72.83	95.38	> 42.26	84.78	86.15
> 80.83	70.65	95.38	> 46.27	83.7	86.15
> 85.73	69.57	95.38	> 53.19	83.7	87.69
> 90.61	68.48	95.38	> 58.80	82.61	87.69
> 92.04	67.39	95.38	> 62.28	81.52	87.69
> 98.30	66.3	95.38	> 65.01	81.52	89.23
> 103.6	65.22	95.38	> 68.25	80.43	89.23
			> 71.60	79.35	89.23
			> 72.47	78.26	89.23
			> 73.55	77.17	89.23
			> 75.36	77.17	90.77
			> 77.41	76.09	90.77
			> 78.51	75	90.77
			> 81.58	73.91	90.77
			> 85.57	72.83	90.77
			> 87.62	71.74	90.77
			> 88.80	71.74	92.31
			> 92.89	71.74	93.85
			> 97.85	70.65	93.85
			> 102.4	69.57	93.85

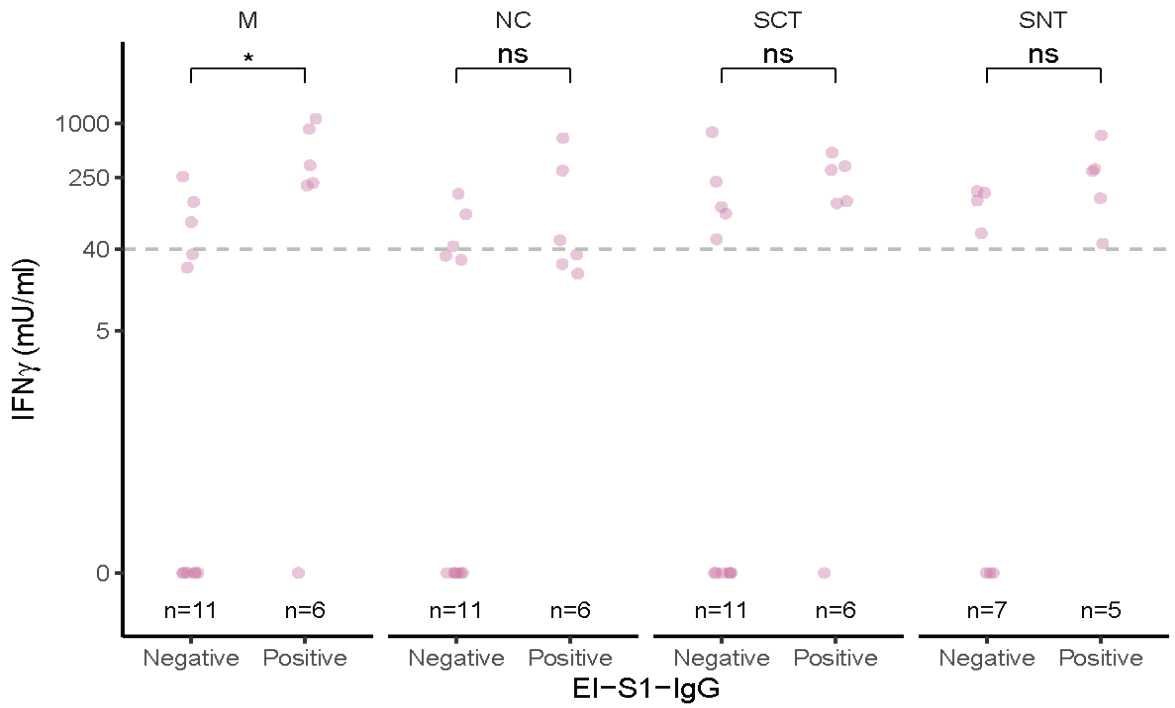
Table S1B. Cutoff determination using ROC-Analysis. Shown are Sensitivity and Specificity for different amounts of interferon gamma for Spike-N-Terminus (SNT) and Membrane protein (M). Determined cutoff is indicated as a line. To define a general cutoff for a positive T cell response, we compared sensitivity and specificity of NC, SCT, SNT and M. 40mlU/ml showed the best combination of sensitivity and specificity when defining one single cutoff for all four antigenic regions.

SNT			M		
	Sensitivity (%)	Specificity (%)		Sensitivity (%)	Specificity (%)
> 1.665	81.16	86.49	> 2.555	93.48	84.62
> 5.090	81.16	89.19	> 6.810	92.39	86.15
> 8.980	79.71	89.19	> 8.855	92.39	87.69
> 12.53	79.71	91.89	> 10.55	91.3	87.69
> 14.69	78.26	91.89	> 12.62	91.3	89.23
> 16.41	76.81	91.89	> 15.00	91.3	90.77
> 19.59	76.81	94.59	> 18.80	91.3	92.31
> 25.15	76.81	97.3	> 22.81	90.22	92.31
> 33.14	75.36	97.3	> 26.23	89.13	92.31
> 38.49	73.91	97.3	> 30.19	88.04	92.31
> 42.36	72.46	97.3	> 39.84	88.04	93.85
> 47.27	71.01	97.3	> 47.45	86.96	93.85
> 49.09	69.57	97.3	> 48.36	85.87	93.85
> 50.49	68.12	97.3	> 50.80	85.87	95.38
> 53.02	66.67	97.3	> 55.87	84.78	95.38
> 57.16	65.22	97.3	> 59.20	83.7	95.38
> 66.49	62.32	97.3	> 60.86	83.7	96.92
> 73.28	60.87	97.3	> 64.15	82.61	96.92
> 74.56	59.42	97.3	> 72.46	81.52	96.92
> 78.07	57.97	97.3	> 80.17	80.43	96.92
> 81.01	56.52	97.3	> 82.61	79.35	96.92
> 88.34	55.07	97.3	> 88.37	78.26	96.92
> 102.9	53.62	97.3	> 92.99	78.26	98.46
			> 93.29	77.17	98.46
			> 103.4	76.09	98.46

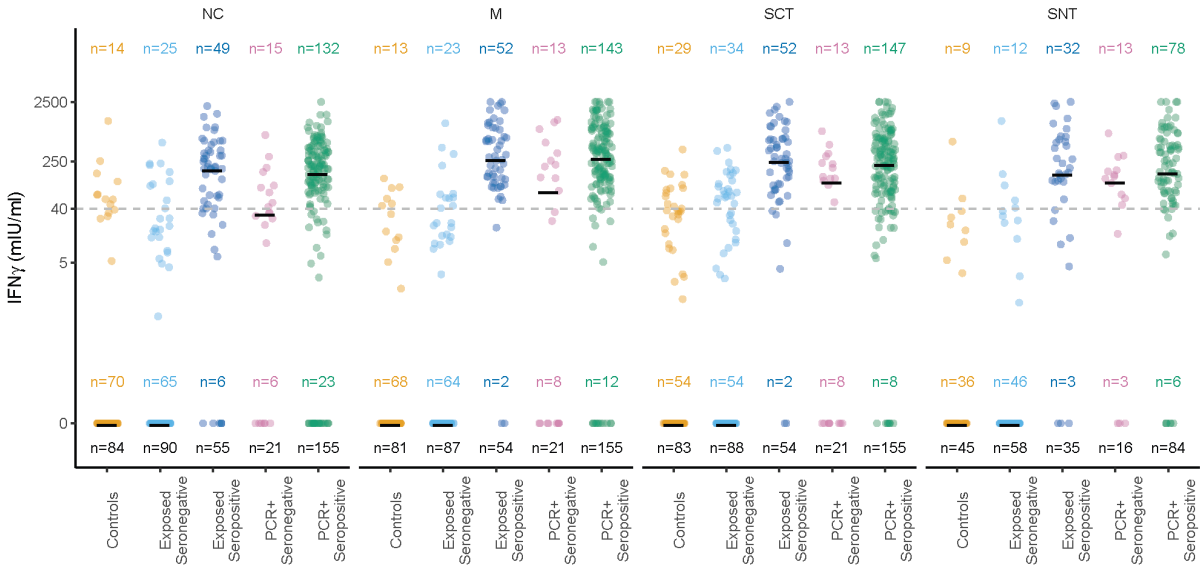
Table S2. Phenotypic characterization of IFNγ-positive cells responding to Spike- and Nucleocapsid peptide pools using intracellular cytokine staining. Shown are the percentages of parent cell populations for 10 study subjects. Columns from left to right according to the gating strategy. In total, 10 study subjects were tested after overnight in vitro restimulation of fresh PBMC at 180 days after SARS-CoV-2 symptom onset. A summary of these results and materials and methods are provided in supplementary figure 3.

	Lymphocytes	Singlets	CD3+	CD3+CD4+	CD4+IFNg+	CD3+CD8+	CD8+IFNg+
Study subject 1							
Nucleocapsid	38.200%	89.900%	75.200%	67.900%	0.034%	23.900%	0.140%
Spike	40.200%	85.500%	77.500%	66.300%	0.014%	25.900%	0.009%
positive control	47.900%	94.100%	74.500%	64.200%	4.740%	25.800%	2.580%
negative control	41.200%	78.600%	74.200%	66.900%	0.000%	25.600%	0.000%
Study subject 2							
Nucleocapsid	65.700%	93.400%	80.300%	57.400%	0.032%	28.400%	0.008%
Spike	66.500%	92.700%	81.200%	55.300%	0.016%	29.200%	0.011%
positive control	61.900%	79.900%	78.500%	56.300%	2.360%	27.400%	1.440%
negative control	66.600%	90.700%	78.300%	57.800%	0.002%	28.300%	0.000%
Study subject 3							
Nucleocapsid	62.200%	92.000%	80.800%	51.200%	0.028%	38.900%	0.015%
Spike	68.300%	95.400%	79.500%	46.300%	0.026%	43.300%	0.001%
positive control	66.300%	95.000%	79.300%	40.800%	2.240%	33.500%	2.230%
negative control	68.100%	95.300%	78.700%	51.200%	0.002%	41.100%	0.002%
Study subject 4							
Nucleocapsid	48.100%	89.500%	79.100%	50.300%	0.060%	41.700%	0.027%
Spike	60.700%	83.500%	76.500%	50.100%	0.007%	42.000%	0.001%
positive control	62.700%	81.500%	70.900%	49.400%	4.670%	40.100%	4.680%
negative control	61.300%	78.500%	73.500%	49.900%	0.003%	42.000%	0.002%
Study subject 5							
Nucleocapsid	68.100%	87.900%	68.200%	70.600%	0.094%	21.400%	0.005%
Spike	69.300%	88.900%	70.300%	69.100%	0.021%	22.600%	0.002%
positive control	66.300%	86.700%	69.500%	69.200%	2.320%	22.300%	7.740%
negative control	67.700%	86.400%	69.200%	69.500%	0.107%	22.600%	0.000%
Study subject 6							
Nucleocapsid	74.800%	90.600%	79.400%	75.100%	0.018%	16.400%	0.003%
Spike	71.200%	94.400%	77.200%	72.500%	0.008%	17.200%	0.002%
positive control	73.000%	92.300%	82.400%	73.300%	0.810%	17.000%	0.670%
negative control	75.300%	95.000%	76.300%	72.900%	0.000%	17.600%	0.001%
Study subject 7							
Nucleocapsid	60.400%	87.000%	83.400%	49.300%	0.026%	41.300%	0.020%
Spike	65.300%	88.600%	82.700%	50.200%	0.059%	40.600%	0.004%
positive control	65.100%	88.400%	86.600%	50.800%	2.490%	39.300%	4.830%
negative control	65.700%	86.800%	82.500%	50.200%	0.012%	41.000%	0.013%
Study subject 8							
Nucleocapsid	48.500%	88.400%	66.500%	79.500%	0.031%	14.900%	0.200%
Spike	53.200%	91.400%	69.000%	79.000%	0.010%	15.700%	0.007%
positive control	49.900%	84.500%	68.300%	75.800%	2.590%	14.500%	6.030%
negative control	46.000%	84.500%	66.000%	80.000%	0.008%	13.900%	0.000%
Study subject 9							
Nucleocapsid	61.500%	94.300%	67.800%	64.800%	0.027%	29.100%	0.022%
Spike	60.900%	92.300%	67.700%	64.700%	0.008%	28.500%	0.002%
positive control	60.100%	89.900%	69.500%	67.300%	1.330%	26.300%	2.220%
negative control	64.200%	94.400%	65.000%	65.600%	0.001%	27.600%	0.001%
Study subject 10							
Nucleocapsid	59.500%	89.100%	73.300%	71.900%	0.017%	24.100%	0.005%
Spike	63.300%	87.400%	71.300%	71.600%	0.020%	24.600%	0.007%
positive control	62.400%	68.800%	70.000%	75.400%	2.570%	20.400%	2.630%
negative control	64.000%	87.600%	69.400%	73.600%	0.003%	22.600%	0.003%

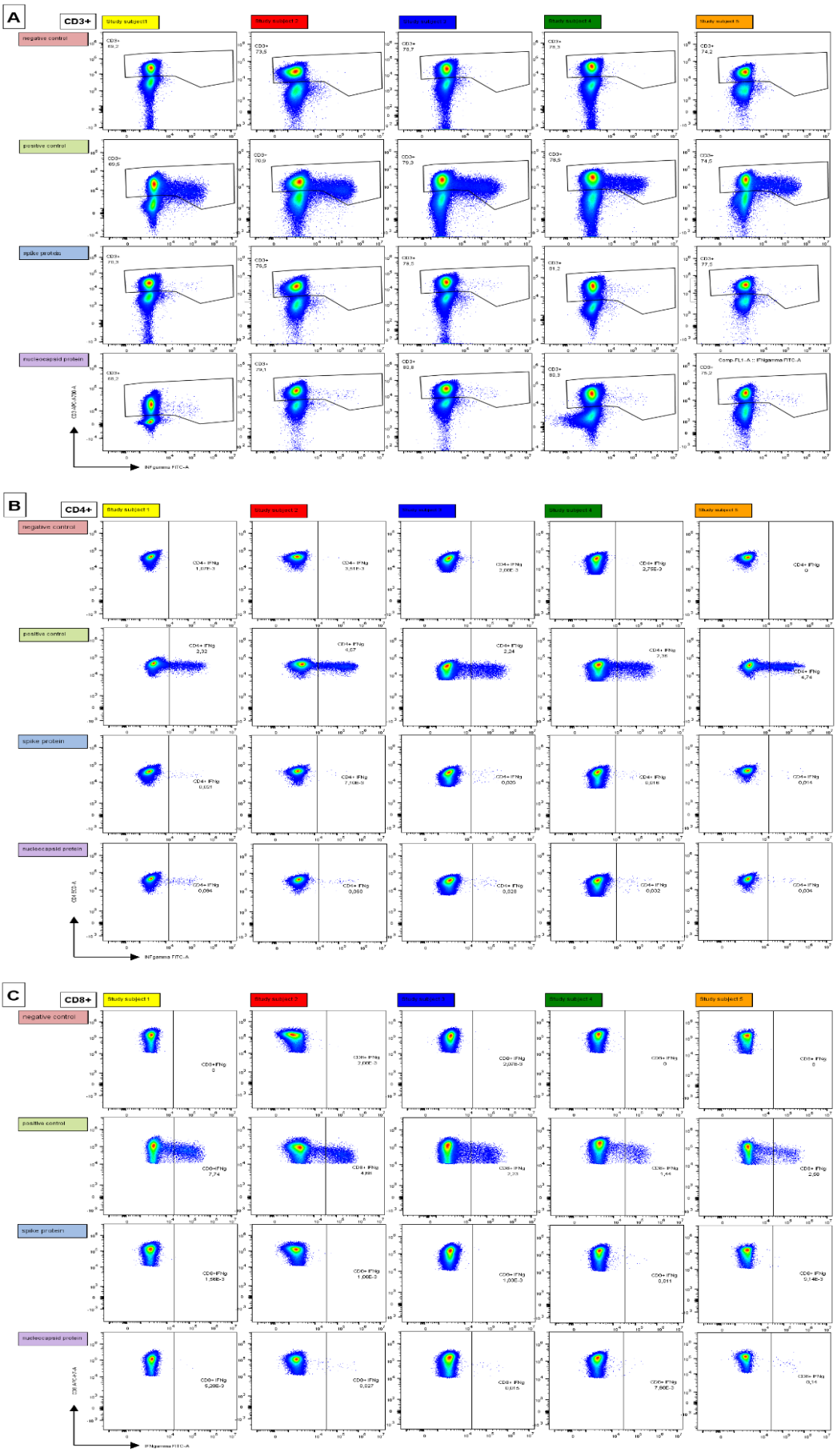
Supplementary Figure 1:



Supplementary Figure 2



Supplementary Figure 3:



6. Publication II

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RESEARCH

Persistent immune abnormalities discriminate post-COVID syndrome from convalescence

Julia Sbierski-Kind[1,2,3] · Stephan Schlickeiser[4,5] · Svenja Feldmann[6] · Veronica Ober[6] · Eva Grüner[6] · Claire Pleimelding[6] · Leonard Gilberg[6] · Isabel Brand[7] · Nikolas Weigl[8] · Mohamed I. M. Ahmed[7,9] · Gerardo Ibarra[3,10] · Michael Ruzicka[10,11] · Christopher Benesch[10,12] · Anna Pernpruner[10,12] · Elisabeth Valdinoci[10,12] · Michael Hoelscher[7,9] · Kristina Adorjan[10,13] · Hans Christian Stubbe[7,10,12] · Michael Pritsch[7,9] · Ulrich Seybold[6] · Julia Roider[6,7] on behalf of The Post COVID Care and KoCo19 study groups

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Abstract

Background Innate lymphoid cells (ILCs) are key organizers of tissue immune responses and regulate tissue development, repair, and pathology. Persistent clinical sequelae beyond 12 weeks following acute COVID-19 disease, named post-COVID syndrome (PCS), are increasingly recognized in convalescent individuals. ILCs have been associated with the severity of COVID-19 symptoms but their role in the development of PCS remains poorly defined.

Methods and results Here, we used multiparametric immune phenotyping, finding expanded circulating ILC precursors (ILCPs) and concurrent decreased group 2 innate lymphoid cells (ILC2s) in PCS patients compared to well-matched convalescent control groups at > 3 months after infection or healthy controls. Patients with PCS showed elevated expression of chemokines and cytokines associated with trafficking of immune cells (CCL19/MIP-3b, FLT3-ligand), endothelial inflammation and repair (CXCL1, EGF, RANTES, IL-1RA, PDGF-AA).

Conclusion These results define immunological parameters associated with PCS and might help find biomarkers and disease-relevant therapeutic strategies.

Keywords Innate lymphoid cells · COVID-19 · Post-COVID-19-syndrome · Immune activation · Tissue immunology

✉ Julia Roider
Julia.Roider@med.uni-muenchen.de

1 Department of Medicine IV, University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany

2 Department of Internal Medicine IV, Division of Diabetology, Endocrinology and Nephrology, University Hospital, Eberhard-Karls-Universität Tübingen, Tübingen, Germany

3 The M3 Research Center, University Clinic Tübingen (UKT), Medical Faculty, Otfried-Müllerstr. 37, Tübingen, Germany

4 Charité, Universitätsmedizin Berlin, Freie Universität Berlin and Humboldt- Universität Zu Berlin, Institute of Medical Immunology, Augustenburger Platz 1, 13353 Berlin, Germany

5 Berlin Institute of Health (BIH) at Charité, Universitätsmedizin Berlin, BIH Center for Regenerative Therapies (BCRT), Charitéplatz 1, 10117 Berlin, Germany

6 Department of Infectious Diseases, Department of Medicine IV, University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany

7 German Center for Infection Research (DZIF), Partner Site Munich, Munich, Germany

8 Department of Medicine IV, Division of Clinical Pharmacology, University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany

9 Division of Infectious Diseases and Tropical Medicine, University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany

10 COVID-19 Registry of the LMU Munich (CORKUM), University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany

11 Department of Medicine III, LMU University Hospital, LMU Munich, Munich, Germany

12 Department of Medicine II, University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany

13 Department of Psychiatry and Psychotherapy, University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany

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Introduction

Viral infections can result in chronic symptoms that persist in previously healthy convalescent individuals across a wide range of viral families, including Ebola virus, influenza, Epstein–Barr virus, and dengue fever [1, 2]. The main symptoms are fatigue, exertion intolerance, sleep disturbances, neurocognitive and sensory impairment, flu-like symptoms, myalgia/arthralgia, and a plethora of nonspecific symptoms [3]. These post-acute infection syndromes (PAIS) are associated with autoimmunity and endothelial dysfunction, affecting both large and small vessels [3, 4]; however, risk factors and the underlying pathophysiology remain largely unknown.

The COVID-19 pandemic, caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to an increasing prevalence of convalescent patients with prolonged and persistent sequelae following acute SARS-CoV-2 infection—known as 'long COVID' or 'post-COVID syndrome' (PCS) [5]. The estimated prevalence of PCS ranges from 5 to 50% [6], thus presenting an enormous global health burden, and can affect both patients with mild or severe forms of acute COVID-19 disease [7]. Clinical symptoms include fatigue, malaise, depression, cognitive impairment, persistent cough, dyspnea, palpitations, and headaches [8]. While the acute phase of COVID-19 has been extensively studied, providing health care professionals with efficient treatment options, the pathogenesis of PCS remains unclear, with current hypotheses including autoimmunity, latent virus reactivation, tissue, and endothelial damage [9].

The extreme respiratory distress in patients with acute COVID-19 is mediated primarily by immunopathology and systemic inflammation. Pathological immune signatures suggestive of T cell exhaustion, delayed bystander $CD8^+$ T cell activation, and higher plasma Granulocyte–macrophage colony-stimulating factor (GM-CSF) and C–X–C motif chemokine ligand 10 (CXCL10) levels are associated with severity of the disease [10–12]. Survivors of severe COVID-19 show persistent immune abnormalities, including elevated levels of pro-inflammatory cytokines [13]. In addition to systemic inflammation, SARS-CoV-2 infects endothelial cells, causing virus-mediated apoptosis and consecutive endotheliitis and, thus, may promote endothelial damage and increased recruitment of activated immune cells into the endothelium and surrounding tissue [14].

Dysregulated respiratory $CD8^+$ T cell responses may contribute to impaired tissue conditions and development of pulmonary sequelae [15]. Recent work identified persistent immunological dysfunction in patients with post-acute sequelae of COVID-19, including highly activated innate immune cells and marked differences in specific circulating myeloid and lymphocyte populations [16, 17].

Innate lymphoid cells (ILCs) are tissue-resident effector immune cells with crucial roles in normal tissue development and remodeling [18, 19]. ILCs can be grouped into type 1, type 2, and type 3/17 *flavors* with associated cytokines (type 1—IFNγ; type 2—IL-4, IL-5, IL-9, IL-13; type 3/17—IL-17A/F, IL-22) that coordinate discrete spatial and temporal aspects of anti-microbial immune responses as well as organ development, homeostasis, and repair [20].

These cells also participate in both protective and pathologic immune responses during lung tissue perturbation [21, 22]. Several studies detected a reduction in total circulating ILCs in severe COVID-19 patients, while relative group 2 innate lymphoid cells (ILC2) levels, particularly $NKGD^+$ ILC2s, were increased [23, 24]. Although ILCs appear central to lung infection and repair, their role in PCS remains critically unexplored.

Here, we used multicolor flow cytometry and multiplex cytokine assays on plasma from (1) healthy, uninfected controls ($n = 32$, 'HC'); (2) previously SARS-CoV-2-infected probands in the convalescent phase without persisting symptoms ($n = 32$, convalescent controls, 'CC'); and (3) patients with persisting symptoms following acute COVID-19 ($n = 27$, post-COVID, 'PC') to identify specific immunological alterations, including ILCs, in PCS. Most participants were non-hospitalized during acute SARS-CoV-2 infection and CC and PC individuals had persisting symptoms for more than 12 weeks from the initial infection. We found expanded circulating ILC precursors (ILCPs) in PC individuals while ILC2s were decreased. Patients with persisting symptoms also displayed elevated pro-inflammatory cytokines (interleukin (IL)-1RA, IL-1a), chemokines associated with trafficking of immune cells (Chemokine (C–C motif) ligand 19 (CCL19/MIP-3b), Fms-related tyrosine kinase 3 ligand (FLT3-Ligand)), and endothelial inflammation and repair (chemokine (C–X–C motif) ligand 1 (CXCL1), epidermal growth factor (EGF), Chemokine (C–C motif) ligand 5 (CCL5/RANTES), platelet-derived growth factor A (PDGF-AA)).

Materials and methods

Study design

post-covid-care study

The Post-COVID-Care (PCC) study is an ongoing prospective single-center study comprised of patients with persisting symptoms following acute COVID-19. Participants with COVID-19 sequelae were recruited from the post-COVID outpatient clinic at the Ludwig-Maximilian-University

(LMU) University Hospital in Munich. Samples were collected from participants enrolled between April and July 2022. Peripheral blood mononuclear cells (PBMCs) isolated from blood samples were analyzed from 27 age- and sex-matched patients with persisting symptoms for more than 12 weeks following acute SARS-CoV-2 infection (PC group). Inclusion criteria were age ≥ 18 years; persisting symptoms > 12 weeks within 6 months following initial COVID-19 infection. None of the participants reported co-infections (e.g., bacterial superinfections) during acute SARS-CoV-2 infection. Pre-specified exclusion criteria were other explanations for the symptom onset or complete resolution of symptoms. All participants were scheduled for follow-up for at least 6 months and up to 24 months if symptoms persisted. At baseline and during the routine follow-up visits, blood samples were obtained and each patient completed progressive web app (PWA)-based questionnaires (LCARS-C, LMU Munich, https://github.com/hcstubbe/lcarsc). Patients who did not undergo any follow-up on site were asked to fill out the follow-up surveys using the PWA-based questionnaire at home using a computer, smartphone or tablet. Informed consent was obtained from all participants before inclusion into the study. Clinical characteristics of study participants are reported in Table 1. The study was approved by the Ethics Committee of the Medical Faculty at LMU Munich (No. 21-1165) and registered to the German Clinical Trials Register (DRKS-ID: DRKS00030974).

Table 1 Clinical and demographic characteristics of study cohorts

		Healthy controls (HC)	Convalescent controls (CC)	Post-COVID (PC)
n	Number	32	32	27
Sex				
Male	Number (%)	15 (46.9%)	14 (43.8%)	9 (33.3%)
Female	Number (%)	17 (53.1%)	18 (56.2%)	18 (66.7%)
Age (years)				
20–29	Number (%)	6 (18.8%)	6 (18.8%)	4 (14.8%)
30–39	Number (%)	10 (31.2%)	12 (37.5%)	10 (37.1%)
40–49	Number (%)	15 (46.9%)	12 (37.5%)	8 (29.6%)
> 49	Number (%)	1 (3.1%)	2 (6.2%)	5 (18.5%)
	Mean	36	36	37
BMI (kg/m^2)				
< 18.5	Number (%)	3 (9.4%)	0 (0%)	0 (0%)
18.5–25	Number (%)	14 (43.8%)	22 (68.8%)	15 (65.2%)
25–30	Number (%)	10 (31.2%)	9 (28.1%)	5 (21.7%)
> 30	Number (%)	5 (15.6%)	1 (3.1%)	3 (13.1%)
	Mean	25.2	23.4	24*
Time from PCR to visit median (in days)			273 (min: 125; max: 318)	113 (min: 89; max: 292)
Disease severity				
Emergency hospitalization number (%)		n.a	1(3.1)	2 (7.4)
Severity score (0–5)	Median	n.a	3.0	n.a
Comorbidities				
No comorbidity	Number (%)	25 (78.1)	23 (71.5)	16 (59.3)
Coronary heart disease	Number (%)	1 (3.1)	0 (0)	2 (7.4)
Diabetes mellitus	Number (%)	0 (0)	0 (0)	1 (3.7)
Obesity	Number (%)	1 (3.1)	1 (3.1)	3 (11.1)
COPD/asthma	Number (%)	5 (15.6)	6 (18.8)	3 (11.1)
(Ex) smoker	Number (%)	6 (18.8)	12 (32.5)	5 (18.5)

Data are given as numbers (percentages). *BMI* body mass index. Sex, age and BMI were comparable between groups with an overall mean age of 36 years, 58% females and BMI of 24.2 kg/m^2

There were no significant differences in the proportion of male or female participants between groups ($p = 0.5530$ [Chi-square: 1.185, $d.f. = 2$]). Participants were well matched in age (Kruskal–Wallis post hoc $p = 0.9276$) and BMI (Kruskal–Wallis post hoc $p = 0.3315$)

*BMI was unknown for 4 individuals from the PC group

KoCo19-Shield study for control samples

For this project, two different control groups were used: (1) "CC-group": seropositive SARS-CoV-2-convalescent patients without persisting symptoms ($n=32$) and (2) "HC group": seronegative individuals without any previous contact to SARS-CoV-2 ($n=32$). Samples for these controls were derived from previously established cohorts and selection was performed to achieve optimal age and sex match with the PC group.

The KoCo19-Shield study cohort was originally established within a previously described population-based SARS-CoV-2 cohort study (KoCo19) [25, 26] to study SARS-CoV-2-specific immune responses in convalescent individuals > 3 months post-infection. Individuals from households with at least one person who had a PCR confirmed SARS-CoV-2 infection were contacted by the responsible official authorities (City of Munich Health department) in May and June 2020 and were recruited as previously described [27]. Individuals who expressed interest in participating were enrolled between September 29, 2020, and January 27, 2021. Furthermore, randomly selected 40 households from the KoCo19 study were selected as controls. In total, 36 households comprising 85 eligible members agreed to participate and were recruited during January 6–27, 2021. Participants of the control group did not show any seropositive tests for SARS-CoV-2 at baseline or during follow-up. PBMCs isolated from blood samples were analyzed from 32 age- and sex-matched seropositive convalescent patients without persisting symptoms (CC group) and from 32 controls without previous contact to SARS-CoV-2 (HC group). Personal data of the study participants were collected as previously described [25]. Participants with SARS-CoV-2 infection were asked to report date of symptom onset and acute disease severity, SARS-CoV-2 polymerase chain reaction (PCR) diagnostic testing results, and antibody testing results. All participants were also asked to provide SARS-CoV-2 vaccination status. Clinical demographics of study participants are reported in Table 1. The study was approved by the Ethics Committee of the Medical Faculty at LMU Munich (20–275 V) and the protocol is available online (www.koco19.de) [27]. Informed consent was obtained from all enrolled participants. The study is registered to the German Clinical Trials Register (DRKS-ID: DRKS00022155).

Blood sample processing

Peripheral blood samples from all participants were collected in four potassium-EDTA-coated blood collection tubes (Sarstedt) and were immediately processed at University Hospital, LMU, Munich, Germany. Whole blood was centrifuged at 450 × g for 10 min at room temperature (RT). Plasma was then transferred to 1.8-ml polyethylene Cryotube™ vials (ThermoFisher), aliquoted, and stored at − 80 °C. For isolation of PBMCs, two tubes each of the remaining whole blood sample were pooled and filled up to a total volume of 32.5 ml with Hank's Balanced Salts Solution (Capricorn or Sigma). 13.5 ml Histopaque®-1077 (Sigma) was added at the bottom of each tube and samples were centrifuged at 450 × g for 30 min at RT without break. PBMC layer on top of the Histopaque® layer was collected and washed twice in Hank's balanced salts solution. Isolated cells were counted using a CASY cell counter and analyzer (Schärfe System GmbH) before storage in liquid nitrogen at − 180 °C for cryopreservation.

Flow cytometry

Cryopreserved PBMCs were thawed in a 37 °C water bath, pipetted into Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FCS medium, and washed by centrifugation. Three to six million cells per sample were incubated with antibodies to surface antigens (Table S1) for 30 min at 4 °C, washed with FACS buffer (1XDPBS, 3% FCS, 0.05% NaN3), fixed with 2% paraformaldehyde for 10 min, washed again with FACS buffer, and resuspended in FACS buffer. Samples were acquired on a BD LSRFortessa X-20. Fluorochrome compensation was performed with single-stained UltraComp eBeads (Invitrogen, Cat# 01–2222-42). To exclude debris, FSC-A/SSC-A gating was used, followed by FSC-H/FSC-A gating to select single cells and Zombie NIR fixable to exclude dead cells. Innate lymphoid cells were identified as lineage negative ($CD1a^-$, $CD14^-$, $CD19^-$, $CD34^-$, $CD94^-$, $CD123^-$, $FcER1a^-$, $TCRab^-$, $TCRgd^-$, $BDCA2^-$), $CD45^+$, $CD161^+$, $CD127^+$, as indicated. The full gating strategy is shown in Fig. S1 and was adapted from previous work [28]. Data were analyzed using FlowJo version 10.7 software (TreeStar, USA) and compiled using Prism (GraphPad Software). T-distributed stochastic neighbor embedding (t-SNE) visualization of flow cytometry data was performed using Cytobank.

Quantification of plasma cytokine levels

Forty-six plasma cytokines (G-CSF, PDGF-AA, EGF, PDGF-AB/BB, VEGF, GM-CSF, FGF, GRZB, IL-1A, IL-1RA, IL-2, IL-27, IL-4, IL-6, IL-10, IL-13, TNF, IL-17C, IL-11, IL-18, IL-23, IL-6RA, IL-19, IFN-B, IL-3, IL-5, IL-7, IL-12p70, IL-15, IL-33, TGF-B, IFN-G, IL-1B, IL-17, IL-17E, CCL3, CCL11, CCL20, CXCCL1, CXCL2, CCL5, CCL2, CCL4, CCL19, CXCL1, CXCL10, PD-L1,

FLT3, TACI, FAS, LEPTIN R, APRIL, OPN, BAFF, LEPTIN, BMP4, CD40 LIGAND, FAS LIGAND, BMP7, BMP2, and TRAIL) were analyzed using a Luminex platform (Human Cytokine Discovery, R&D System, Minneapolis, MN) according to the manufacturer's instruction.

Unsupervised data analysis

Cytobank [29] was used for initial manual gating of Lineage-negative cells and ILC subsets group 1 innate lymphoid cell (ILC1), ILC2, and ILCP, using the same gating strategy as described above. Lineage-negative cells were subjected to dimensionality reduction using Cytobank opt-SNE with default hyperparameters and following embedding markers with normalized scales Cytobank arcsinh transformation: CD117, CD127, CD161, CD45RA, CD56, CRTH2, HLA-DR, and SLAMF1. All pre-gated events were used without prior downsampling from 91 samples. To perform downstream statistical analyses in R (http://www.r-project.org/) and visualize t-SNE maps across the 91 samples, events within ILC subsets were exported from Cytobank as tab-separated values containing compensated and transformed marker expression levels as well as t-SNE coordinates and metacluster assignment. T-SNE plots were generated after subsampling each sample to contain a maximum of 2500 events. High-resolution group differences were visualized by calculating Cohen's D for a given comparison across the t-SNE map. We used the probability binning algorithm available through the R *flowFP* package [30] and generated adaptive 2D histograms. A single binning model was created on collapsed data from all samples, by recursively splitting the events at the median values along the two t-SNE dimensions. We chose a grid of 256 bins to have on average, at least eight cells per bin in each sample for statistical accuracy. Since there was a significant difference between cellular frequency distributions between the six measurement days, the batch effect was first regressed out by fitting a linear model to each bin after applying the arcsine-square-root transformation for proportions. The group-difference effect sizes were then calculated for each bin using the cohen.d function of the *effsize* package. To get a smoothed representation of the effect size map, adaptive binning was performed on a series of rotated coordinates and per cell-averaged effect size values were used to color-encode each cell throughout the t-SNE map. All analyses were performed using R version 4.1.1, available free online at https://www.r-project.org.

Statistical analysis

The sample size was not pre-determined through formal power analysis. Data were analyzed using Prism version 8 (GraphPad Software, La Jolla, CA). All column graphs are presented as means ± standard error of the mean (SEM) unless otherwise noted with $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$, $**** = p < 0.0001$. For comparisons between three groups, one-way ANOVA with Tukey´s multiple comparisons test was used. For comparison of age and BMI between study groups, one-way analysis of variance with Kruskal–Wallis and Dunn's correction for multiple comparisons were performed (see Table 1). For comparison of age between study groups, a Chi-squared test was used (see Table 1). Correlation analyses were performed using Pearson's correlation coefficient. Each symbol reflects individuals for flow analysis or plasma cytokine levels.

Results

Clinical characteristics of study participants

Patients, enrolled in the Post-COVID-Care study at the LMU University Hospital Munich, presented with persisting symptoms for more than 12 weeks following acute SARS-CoV-2 infection (PC group; $n = 27$) and were compared to convalescent patients without persisting symptoms (CC group; $n = 32$) and 'healthy controls' without previous contact to SARS-CoV-2 (HC group; $n = 32$), enrolled in the KoCo19-Shield sub study (Fig. 1a). Clinical demographics of both study cohorts are reported in Table 1.

The PCS, convalescent, and 'healthy control' groups were well matched in sex (67% female PC; 56% female CC; 53% female HC; $p = 0.5530$ [Chi-square: 1.185, $d.f. = 2$]), age (mean 37.15 years old PC; mean 36.09 years old CC; mean 35.91 years old HC; Kruskal–Wallis post hoc $p = 0.9276$), and BMI (mean BMI PC group 24.0 kg/m^2; mean BMI CC group 23.4 kg/m^2; mean BMI HC group 25.2 kg/m^2; Kruskal–Wallis post hoc $p = 0.3315$) (Fig. 1b and Table 1). Only two patients with COVID-19 sequelae were hospitalized during acute infection, whereas none of the convalescent study participants were hospitalized (Fig. 1c), reflecting that some patients experience long-term health-consequences after acute COVID-19, regardless of disease severity. Consistent with numerous previous reports of PCS, the most common reported symptoms included constitutional symptoms, such as fatigue (93%) and insomnia (41%), and neurological symptoms, such as impaired alertness (74%), memory impairment (59%), and impaired speech (56%). Cardiac symptoms, including palpitations (59%), chest pain (52%), and reduced muscular strength (26%) were also a common complaint (Fig. 1d).

Circulating ILCPs are elevated in PC patients with concurrent decrease in ILC2s

To investigate circulating ILC levels via flow cytometry in PCS, convalescent and 'healthy controls', we used a well-established gating strategy [28] (Suppl. Figure 1). Lin$^-$CD127$^+$ ILC subsets were defined as

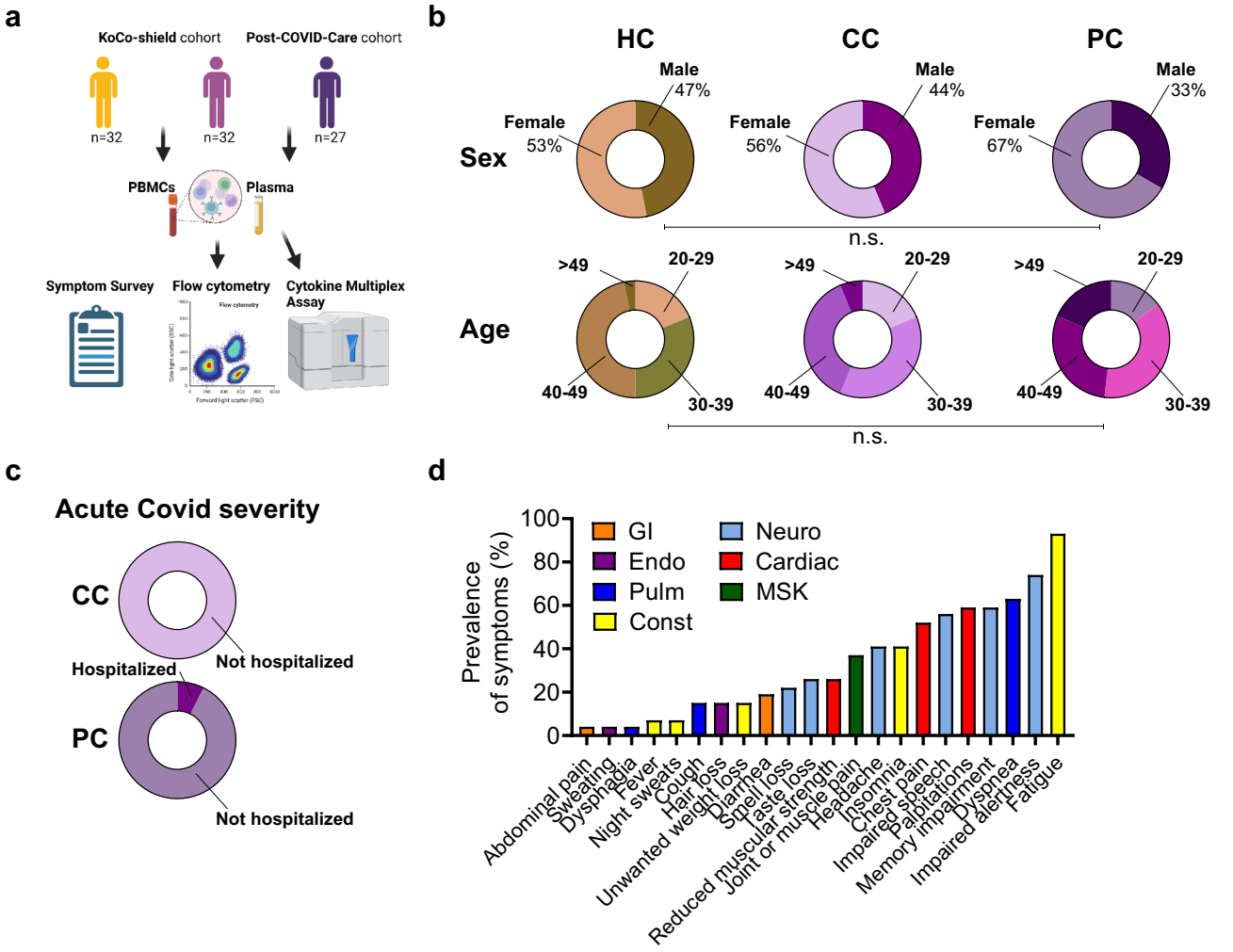

Fig. 1 Clinical characteristics of study cohorts. **a** Overview of study cohorts and methods. The figure is partly created with BioRender.com. **b** Demographic data for healthy, uninfected controls (HC), convalescent SARS-CoV-2 participants without persisting symptoms (CC) and convalescent SARS-CoV-2 participants with persisting symptoms (PC) displayed as ring charts. Statistical significance is shown by capped lines as Chi-square tests for 'Sex' and post hoc comparisons for 'Age'. Further characteristics are detailed in Table 1. **c** Percentage of hospitalization during acute COVID infection for CC and PC participants displayed as ring charts. **d** Prevalence of top 22 self-reported symptoms in PC participants (least prevalent (left) to most prevalent (right)). Symptoms are colored according to physiological systems. Gastrointestinal (GI), endocrine (Endo), pulmonary (Pulm), constitutional (Const), neurological (Neuro), cardiac, and musculoskeletal (MSK)

$CD117^{-}CRTH2^{-}$ ILC1s, $CD117^{+}$ ILC progenitors (ILCP) [31], and $CRTH2^{+}$ ILC2s. We used CD56 as a marker of activated or ILC3/NK cell-committed ILCP and CD45RA for naïve ILCP [28]. Recent work discovered $CD45RA^{+}$ naïve-like ILCs, lacking proliferative activity, indicative of cellular quiescence [32]. To visualize multiple dimensions in simple two-dimensional plots and compare flow cytometry data between groups, we used stochastic neighbor embedding analysis (Fig. 2a, b). We found increased expression of the ILCP marker CD117 in PC compared to HC groups, while CRTH2 (marker for ILC2s) was decreased in PC compared to both CC and HC groups (Fig. 2a, b). However, the expression of proteins associated with ILC activation, CD56 (also defining NK cells with intermediate or high expression levels) and HLA-DR, was not different between groups (Fig. 2a, b). Next, we evaluated total numbers and frequencies of circulating ILCs and NK cells in patients with persisting symptoms after COVID-19 infection as compared to convalescent patients and healthy controls. We did not observe significant changes in total ILCs and subsequent ILC subsets (ILC2s, ILC1s, ILCPs) in PC compared to CC and HC groups (Fig. 2c, d, Suppl. Figure 2a). However, PC patients had significantly expanded levels of ILCPs with concurrent decreased ILC2 frequencies, while ILC1 levels remained unchanged (Fig. 2e). The role of Bar

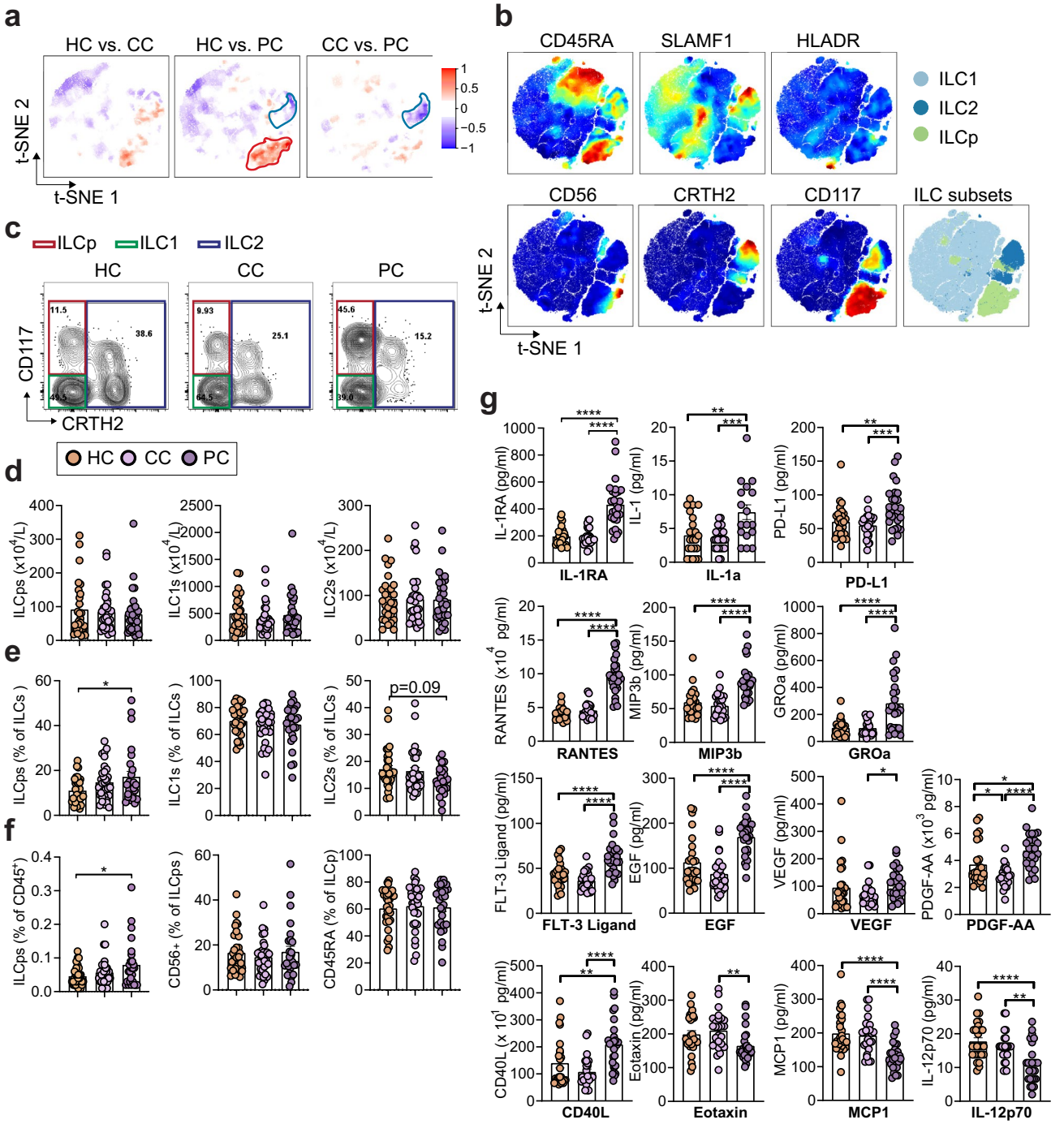

Fig. 2 Post-COVID participants show altered cytokine expression and levels of innate lymphoid cells. **a** High-dimensionality reduction analysis of innate lymphoid cells (ILCs, gated as lymphocytes, singlets, and $CD45^{+}CD3^{-}Lin^{-}CD127^{+}$ cells as shown in Suppl. Fig. 1) from peripheral blood mononuclear cells (PBMCs) of HC, CC, and PC groups. High-resolution group differences were visualized by calculating Cohen's D for a given comparison across the t-SNE map. Residual plot showing differences between maps. Phenotypes within red circles were confirmed to be statistically more common in PC samples, and phenotypes within blue circles were less common in PC samples. Analysis is based on flow cytometry data from 32 HC, 32, CC, and 27 PC samples. **b** Relative expression intensities (combined HC, CC, and PC samples) of parameters used in the t-SNE analysis. **c–f** Representative flow cytometry plots (**c**) and quantification (**d–f**), showing total numbers (**d**) and percent (**e**, **f**) innate lymphoid cell populations in HC, CC, and PC groups at 3–10 months after acute COVID infection. **g** Multiplex assay quantification showing plasma levels of IL-1RA, IL-1a, PDL-1, RANTES, MIP-3b, Groa, FLT3 Ligand, EGF, VEGF, PDGF-AA, CD40L, Eotaxin, MCP1, and IL-12p70 in healthy controls with no prior SARS-CoV-2 infection (HC), convalescent SARS-CoV-2 participants without persisting symptoms (CC), and convalescent SARS-CoV-2 participants with persisting symptoms (PC) at 3–10 months after acute COVID infection

graphs indicate mean (± SE), $n = 27–32$ individuals per group, one-way ANOVA with Tukey's multiple comparisons test (D, E, F), $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$. See also Suppl. Fig. 2 and Suppl. Fig. 3.

ILC2s in viral-induced lung pathogenesis remains controversial. Although increased levels of IL-18, IL-13, and IL-6 have been reported along with accumulation of ILC2s during acute COVID-19, increased circulating ILC2s in moderate but not severe COVID-19 patients were found in other studies [33], consistent with their attrition by interferon (IFN)-γ in type 1 (viral-induced) inflammation [21]. Thus, ILC2s might have important roles in tissue repair during viral-induced epithelial cell damage, perhaps through crosstalk with other ILC subsets.

Recent work suggested that human ILCPs can interact with endothelial cells, fostering the adhesion of other innate and adaptive immune cells by stimulating pro-inflammatory cytokine expression of adhesion molecules. This activation occurs through the tumor necrosis factor receptor- and RANK-dependent engagement of Nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB)* pathway [34]. ILCP levels as percentage of all $CD45^+$ leukocytes were also increased in PC patients compared to the HC group (Fig. 2f). Nevertheless, PC patients did not show significant changes in $CD45RA^+$ ILCPs, although $CD56^+$ ILCPs were trending upwards, suggesting a circulating ILCP expansion without overt altered activation (Fig. 2f). Surprisingly, the expression of CD45RA was increased in ILC1 subsets in the PC group compared to HC and CC groups, while $CD45RA^+$ ILC2 subsets remained unchanged (Suppl. Figure 2b), suggesting the increase of a quiescent local reservoir for the generation of differentiated ILCs [32]. Frequencies of $HLA\text{-}DR^+$ ILC1s, percentages of $CD117^+$ ILC2s, and the transcriptional expression of Signaling lymphocytic activation molecule 1 (SLAMF1) within the ILC2 compartment were similar between PC, CC, and HC groups (Suppl. Figure 2b). We could not find significant differences in NK cell frequencies between patients with PCS, convalescent, and healthy controls (Suppl. Figure 2c). We also did not find significant differences between frequencies of $CD4^+$ or $CD8^+$ T cells or regulatory T cells (data not shown). Together, these data indicate that ILCPs expand in patients with COVID-19 sequelae, without alteration of their activation state.

Pro-inflammatory cytokines and growth factors are elevated in PCS

In COVID-19 patients with severe disease, cytokine storm and uncontrolled inflammatory responses, including endothelial inflammation and associated tissue damage, are recognized as one of the driving immunopathological features that can lead to death [10]. To uncover the immunological dysregulation in PCS, we quantified 46 molecular analytes in the plasma of patients from the CC and PC groups > 3 months after acute SARS-CoV-2 infection using a multiplex cytokine assay and compared them to healthy controls. Four key pro-inflammatory cytokines (IL-8, IL-6, interleukin-1 receptor antagonist (IL-1Ra) and IL-1a) were elevated in the PC group compared to the CC group; IL-1RA and IL-1a levels were also significantly higher in the PC group compared to healthy controls (Fig. 2g, Suppl. Figure 3a), while no difference was observed in transforming growth factor alpha (TGF-α), IL-7, IL-5, IL-4, IL-13, tumor necrosis factor (TNFα), IFN-γ and IL-1β (Suppl. Figure 3b). IL-10 was also elevated in the PC group compared to the CC group (Suppl. Figure 3c). IL-8 has been previously associated with a prothrombotic neutrophil phenotype in severe COVID-19 and blocking IL-8 signaling reduced SARS-CoV-2 spike protein-induced, human Angiotensin-converting enzyme 2 (ACE2)-dependent pulmonary microthrombosis in mice [35]. Surprisingly, levels of IL-8 were lower in CC compared to HCs, whereas other pro-inflammatory cytokines were not different between these groups (Suppl. Figure 3a, b). IL-1Ra was 2.16-fold higher in the PC group compared to the HC group and 2.22-fold higher compared to the CC group; other pro-inflammatory cytokines were only slightly increased (Fig. 2g, Suppl. Figure 3b). Importantly, programmed death-ligand 1 (PD-L1) was increased in the persisting symptom group compared to both convalescent and healthy control groups, consistent with previous reports, highlighting the prognostic role of sPD-L1 in COVID-19 patients [36] (Fig. 2g). Several chemokines (RANTES, MIP-3b, CXCL1) and growth factors (FLT3 Ligand, EGF, vascular endothelial growth factor (VEGF), PDGF-AA), that could be associated with trafficking of immune cells (MIP-3b, FLT3-Ligand) and endothelial inflammation (CXCL1, EGF, RANTES, PDGF-AA), and CD40L were also elevated in PC participants compared to both CC and HC groups (Fig. 2g). Interestingly, Eotaxin (CCL11), monocyte chemoattractant protein 1 (MCP1), and IL-12p70 were decreased in PC patients compared to both convalescent and healthy controls (Fig. 2g); some of these chemokines were associated with severe cases of acute COVID-19 [37]. The frequencies of plasma TNFα, FLT3-Ligand and CXCL1 (Groa) were positively correlated with levels of ILCPs (Suppl. Figure 4a–c), whereas PDGF-AA was negatively correlated with levels of naïve $CD45RA^+$ ILCPs (Suppl. Figure 4d), indicating a strong coregulation of pro-inflammatory markers with activated ILCPs. Together, these data suggest persisting immune abnormalities in patients suffering from post-acute sequelae of COVID-19.

Discussion

Persistent sequelae following acute COVID-19 are increasingly recognized in convalescent individuals. Our exploratory analyses identified immunological differences in patients with PCS as compared to well-matched convalescent and HC individuals at > 3 months

post-infection. We found significant changes in circulating ILC subsets, including increased ILCPs and concurrent decreased ILC2 levels. In addition, pro-inflammatory cytokines (IL-1RA, IL-1a), chemokines associated with trafficking of immune cells (CCL19/MIP-3b, FLT3-Ligand) and endothelial inflammation and -repair (CXCL1, EGF, RANTES, PDGF-AA) were elevated in PC participants. We also observed an association between frequencies of circulating ILCPs and plasma markers associated with (endothelial)—inflammation and tissue repair. A limitation of our study is that for PC and CC groups, elapsed days since initial SARS-CoV-2 infection were different from acute disease (113 days for PC group vs. 273 days for CC group, data not shown); however, initial enrollment and collection of blood for immunophenotyping took place more than 3 months after onset of COVID-19 and none of the convalescent participants reported persisting symptoms after acute disease. Several studies have shown that pro-inflammatory cytokines remained significantly elevated in PC patients at month 8 after acute infection [17]. Acute SARS-CoV-2 infections within the PC group occurred in the period when the Omicron BA.2 variants were dominant (between January and March 2022), whereas participants of the convalescent group were confirmed to be infected with SARS-CoV-2 between March and April 2020, when parental strains drove the majority of new cases. While several risk factors, including comorbidities and virus variants, have been identified for the development of PCS [38], clinical symptoms are similar for different SARS-CoV-2 strains, with the exception of musculoskeletal pain, where chronic burden may be lower for Omicron compared to Delta variants [39]. Our work does not dissect how ILCPs or other activated innate and adaptive immune cells, contribute mechanistically to endothelial dysfunction in PCS. However, ILCP expansion along with elevated markers for endothelial inflammation in PC supports their interaction with endothelial cells; thereby facilitating enhanced inflammatory responses and endotheliitis in several organs. These findings may not only be interesting for long-term sequelae of COVID-19, but also for other viral infections that can result in PAIS in convalescent individuals. Further exploration of immunological alterations in PCS may delineate mechanisms of ILC-endothelial cell crosstalk and lead to disease-relevant targeted therapies.

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Author contributions Conception and design: JR and JSK. Cohort initiation, study follow-up, data management and sample processing KoCo19-Shield: MH, MP, CP, IB, LG, NW. Cohort initiation, study follow-up, data management and sample processing PCC: HS, KA, US, GI, EG, MR, CB, AP, EV. Acquisition of data: JSK, SF, VO, HS, MA. Analysis and interpretation of data: JSK and SS. Writing of the manuscript: JSK and JR. Critical reagents and manuscript editing: SF, VO, SS, and MA. Funding acquisition: MH, HS, KA, JSK, JR.

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Data availability statement Further information and requests for resources and reagents should be delivered to and will be fulfilled by the Lead Contact, Julia Sbierski-Kind (Julia.Sbierski-Kind@med.uni-tuebingen.de).

Declarations

Conflict of interest The authors declare no commercial or financial conflicts of interest.

Ethics approval statement for human studies KoCo19-Shield: The study protocol was reviewed and approved by the Institutional Review Board of the Medical Faculty at Ludwig-Maximilians-University Munich, Germany under the project number 20-692 (vote of approval dated Sept. 21st, 2020) and 20-371 (vote of approval dated May 15th, 2020). Oral and written informed consent was obtained from all study subjects. PCC: The study protocol was reviewed and approved by the Institutional Review Board of the Medical Faculty at Ludwig-Maximilians-University Munich, Germany under the project number 21-1165 (vote of approval dated Feb 15, 2021, amendment approved Aug. 11, 2021). Oral and written informed consent was obtained from all study subjects.

Patient consent statement The patients/participants provided their written informed consent to participate in this study.

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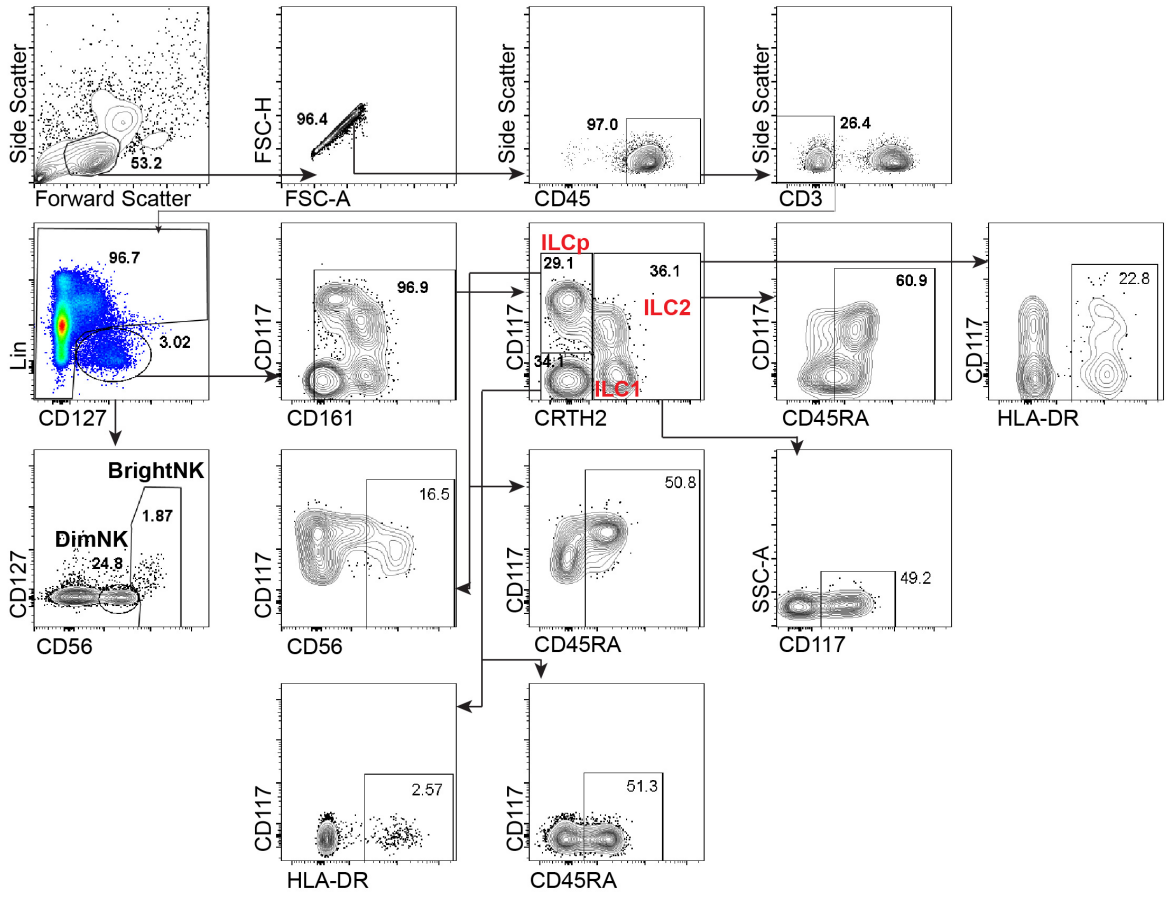
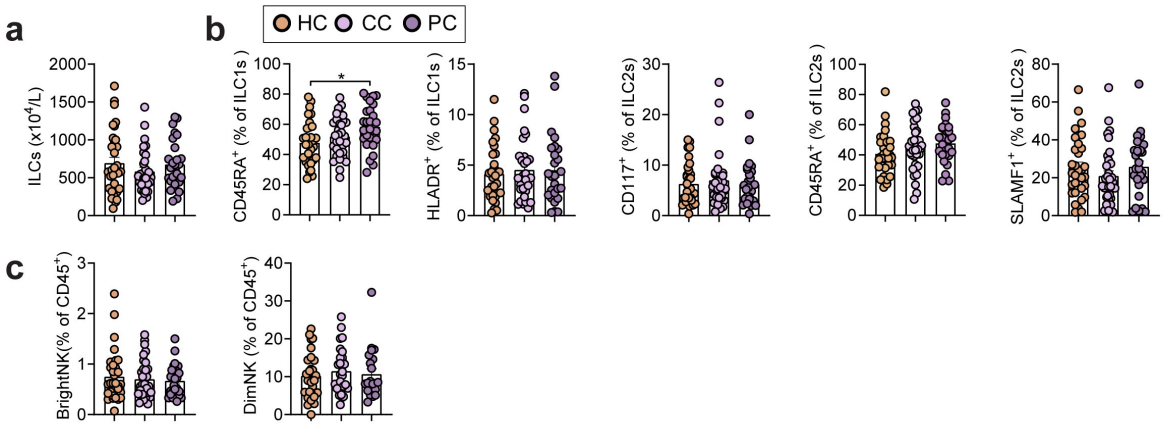
Supplementary Material

Supplementary Figure 1



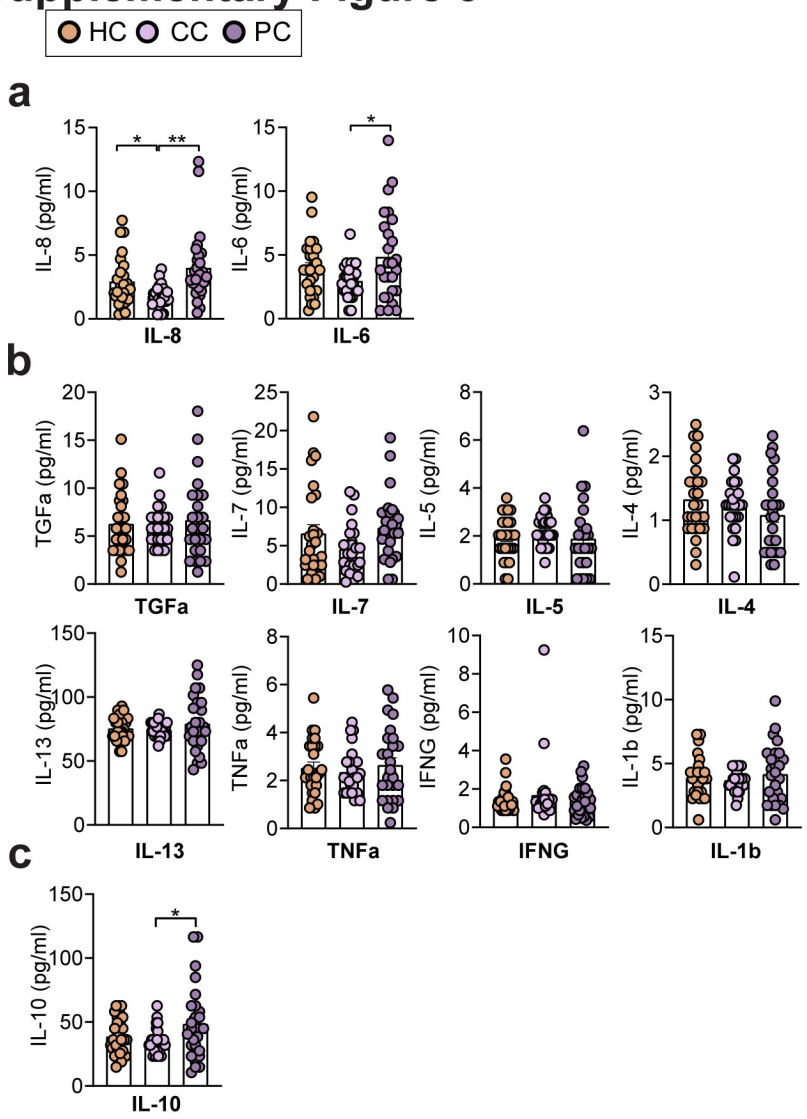
Supplementary Figure 1: Flow cytometry gating scheme.
Representative flow cytometry gating scheme for analysis of innate lymphoid cells (ILCs) in peripheral blood mononuclear cells (PBMCs). Numbers represent percentages of cells in respective gates. Lineage markers contained CD1a, CD14, CD19, CD123, BDCA2, FceR1, CD34, CD94, TCRαβ, TCRγδ, FceR1, and dead cell marker.

Supplementary Figure 2



Supplementary Figure 2: Levels of innate lymphoid cells among study groups.
(a-c) Flow cytometry quantification, showing total numbers (a) and percent (b, c) of innate lymphoid cells (ILCs) and NK cells in peripheral blood mononuclear cells (PBMCs) of healthy controls with no prior SARS-CoV-2 infection (HC), convalescent SARS-CoV-2 participants without persisting symptoms (CC) and convalescent SARS-CoV-2 participants with persisting symptoms (PC) at 3-10 months after acute COVID infection.
Bar graphs indicate mean (±SE), one-way ANOVA with Tukey´s multiple comparisons test (A-C), *p ≤ 0.05.

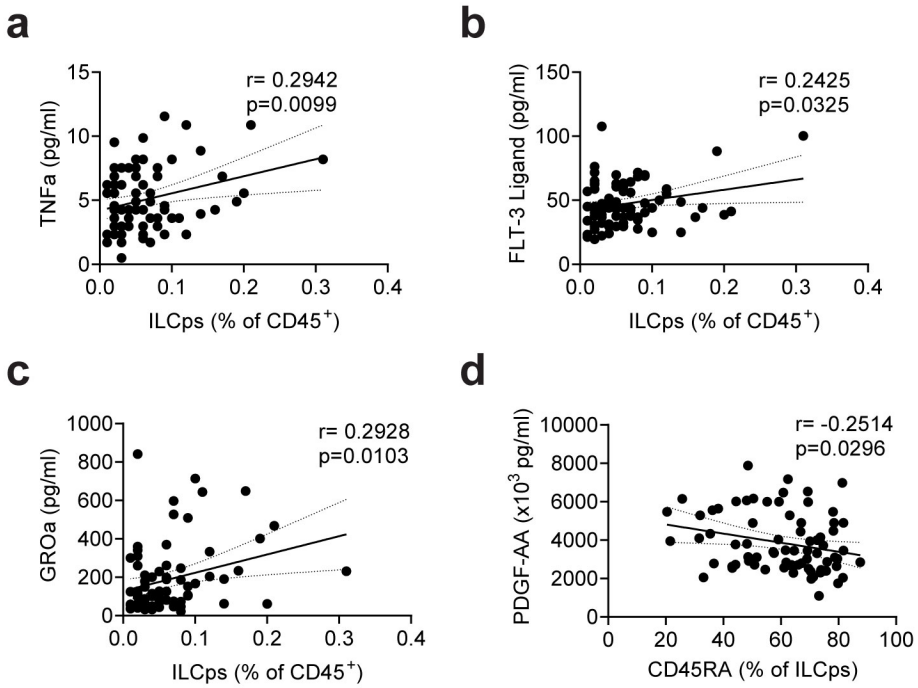
Supplementary Figure 3



Supplementary Figure 3: Post-COVID participants display altered plasma cytokine expression levels.

(a-c) Multiplex assay quantification showing levels of IL-8, IL-6 (a), TGFa, IL-7, IL-5, IL-4, IL-13, TNFa, IFNG, IL-1b (b), and IL-10 (c) in plasma of healthy controls with no prior SARS-CoV-2 infection (HC), n=32, convalescent SARS-CoV-2 participants without persisting symptoms (CC), n=32, and convalescent SARS-CoV-2 participants with persisting symptoms (PC), n=27 at 3-10 months after acute COVID infection.
Bar graphs indicate mean (±SE), one-way ANOVA with Tukey's multiple comparisons test, *p ≤ 0.05, **p ≤ 0.01.

Supplementary Figure 4



Supplementary Figure 4: The percentage of circulating ILCps is associated with plasma cytokines involved in immune cell trafficking and proliferation.

(a-d) Correlation of percentages of ILCps of leukocytes (a-c) or percentages of CD45RA$^+$ ILCps (d) with levels of TNFa (a), FLT3-Ligand (b), CXCL1 (Groa) (c), and PDGF-AA (d) in plasma of healthy controls with no prior SARS-CoV-2 infection (HC), n=32, convalescent SARS-CoV-2 participants without persisting symptoms (CC), n=32, and convalescent SARS-CoV-2 participants with persisting symptoms (PC), n=27 at 3-10 months after acute COVID infection. The *r* indicates the Pearson correlation coefficient.

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