



Persistent immune abnormalities discriminate post-COVID syndrome from convalescence

Julia Sbierski-Kind^{1,2,3} · Stephan Schlickeiser^{4,5} · Svenja Feldmann⁶ · Veronica Ober⁶ · Eva Grüner⁶ · Claire Pleimelding⁶ · Leonard Gilberg⁶ · Isabel Brand⁷ · Nikolas Weigl⁸ · 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 Seybold⁶ · Julia Roeder^{6,7} on behalf of The Post COVID Care and KoCo19 study groups

Received: 13 November 2023 / Accepted: 19 December 2023
© The Author(s) 2024

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 Roeder
Julia.Roeder@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

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/arthritis, 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—IFN γ ; 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)
<i>n</i>	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/m²

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 $450 \times g$ for 10 min at room

temperature (RT). Plasma was then transferred to 1.8-ml polyethylene Cryotube™ vials (ThermoFisher), aliquoted, and stored at $-80\text{ }^{\circ}\text{C}$. 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 $450 \times g$ 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 $-180\text{ }^{\circ}\text{C}$ for cryopreservation.

Flow cytometry

Cryopreserved PBMCs were thawed in a $37\text{ }^{\circ}\text{C}$ 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 $4\text{ }^{\circ}\text{C}$, 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, CXCL1, 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 down-sampling 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 \pm 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/m²; mean BMI CC group 23.4 kg/m²; mean BMI HC group 25.2 kg/m²; 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

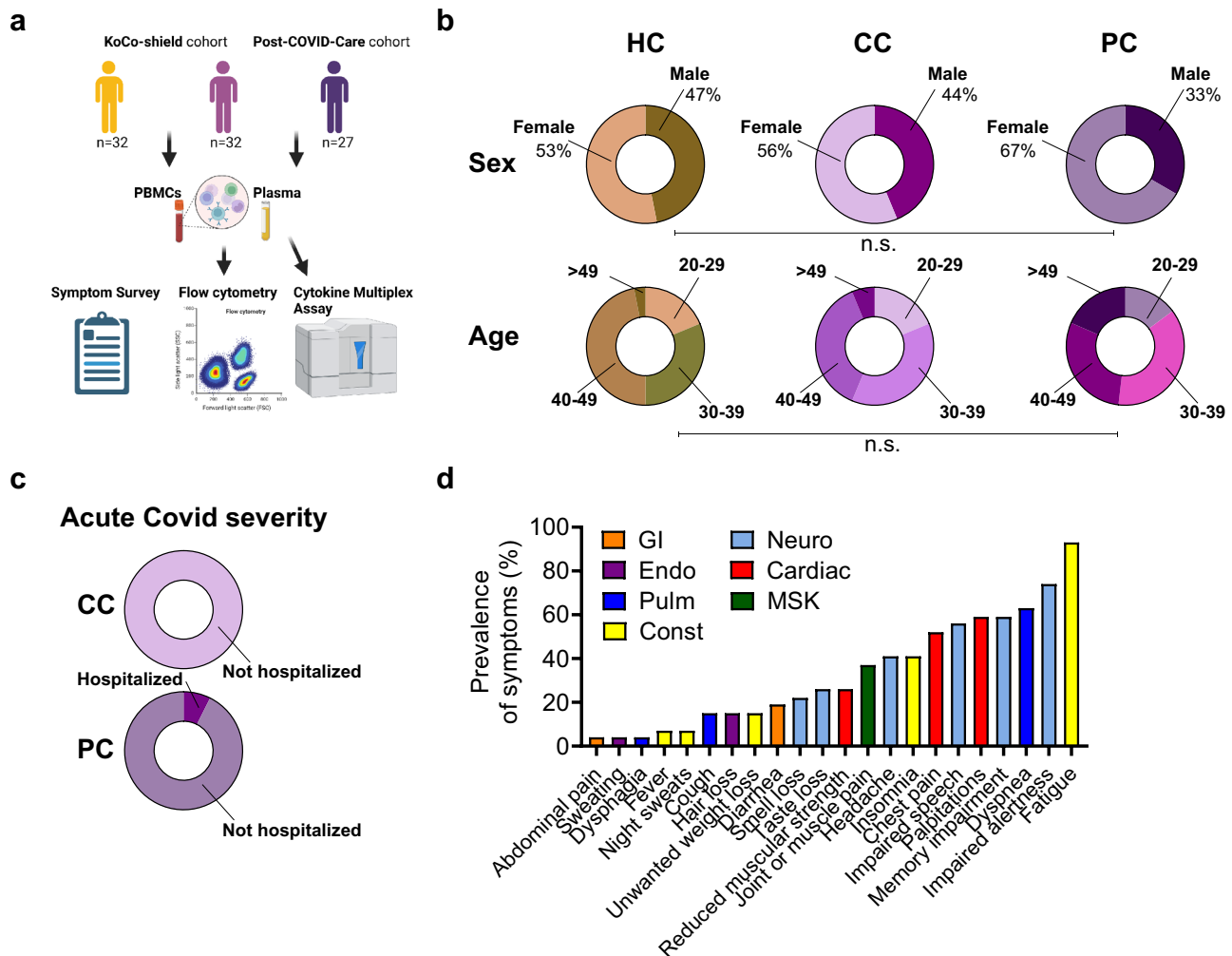


Fig. 1 Clinical characteristics of study cohorts. **a** Overview of study cohorts and methods. The figure is partly created with BioRender.com. **b** Demographic data for healthy, uninfected controls (HC), convalescent SARS-CoV-2 participants without persisting symptoms (CC) and convalescent SARS-CoV-2 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

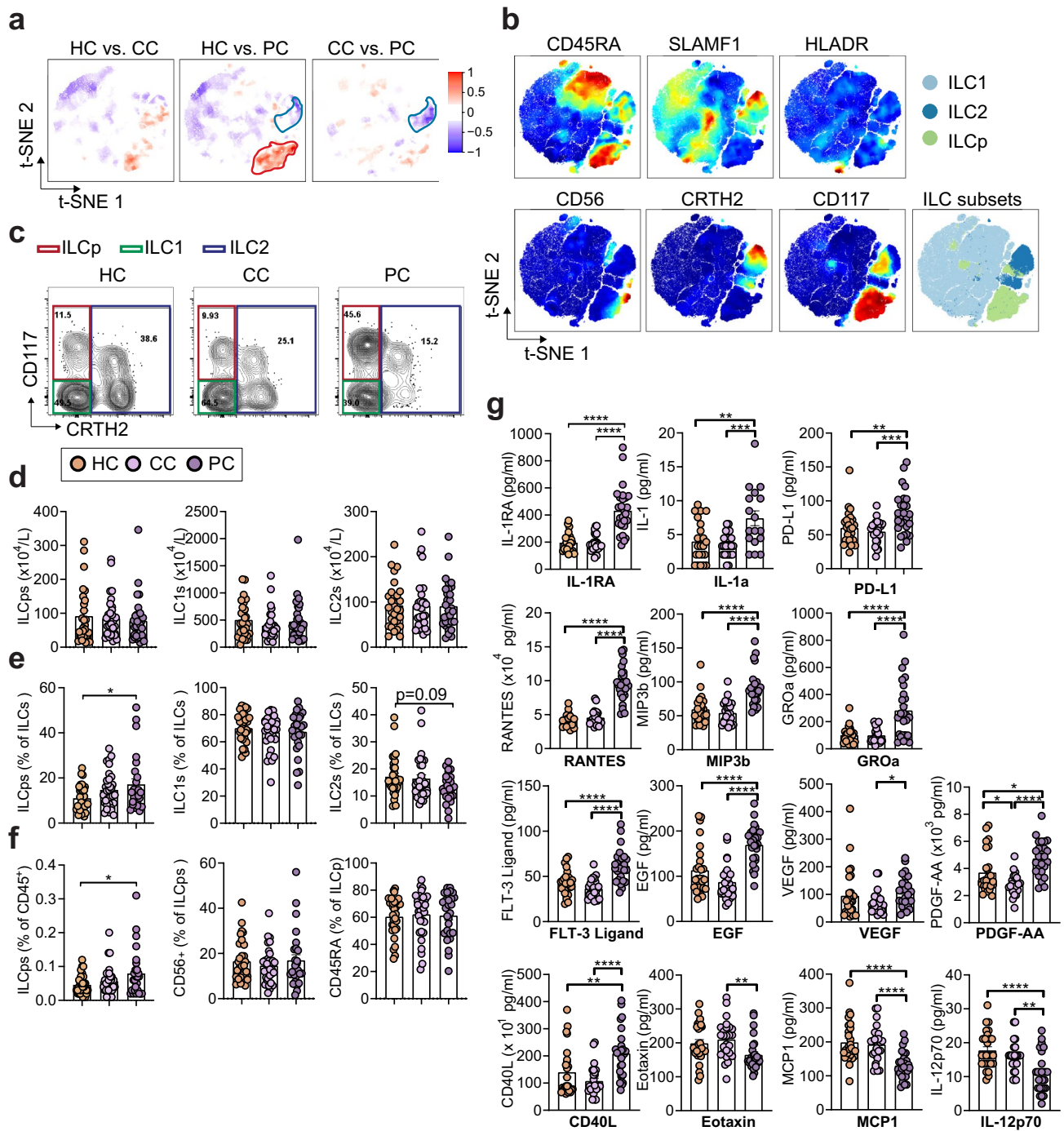


Fig. 2 Post-COVID participants show altered cytokine expression and levels of innate lymphoid cells. **a** High-dimensionality reduction analysis of innate lymphoid cells (ILCs, gated as lymphocytes, singlets, and $CD45^+CD3^-Lin^-CD127^+$ cells as shown in Suppl. Fig. 1) from peripheral blood mononuclear 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 (\pm SE), $n = 27\text{--}32$ 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- κ B*) 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-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 (TNF α), IFN- γ and IL-1 β (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 TNF α , 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.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s15010-023-02164-y>.

Acknowledgements We thank Renate Stirner and Gabriele Reiling for excellent technical assistance. The Post-COVID^{LMU} research project is financially supported by the Bavarian State Ministry for Health and Care and the Bavarian State Office for Health and Food Safety (LGL). There is a close link to the nationwide research project “Network University Medicine” (NUM), funded by the Federal Ministry

of Education and Research (BMBF) (funding code: 01KX2021) and the NUM-associated research projects. JR is supported by the German Center for Infection Research (DZIF) and Else Kröner-Fresenius-Stiftung (EKFS). JSK received funding from the German Research Foundation (DFG, Deutsche Forschungsgemeinschaft), the German Diabetes Society (DDG, Deutsche Diabetes Gesellschaft), and FoE-FoLe, LMU Munich. JSK is also supported by the German Society of Internal Medicine (DGIM, Deutsche Gesellschaft für Innere Medizin, Clinician Scientist Program).

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.

Funding Open Access funding enabled and organized by Projekt DEAL.

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.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Clark DV, Kibuuka H, Millard M, Wakabi S, Lukwago L, Taylor A, et al. Long-term sequelae after Ebola virus disease in Bundibugyo, Uganda: a retrospective cohort study. *Lancet Infect Dis.* 2015;15:905–12. [https://doi.org/10.1016/S1473-3099\(15\)70152-0](https://doi.org/10.1016/S1473-3099(15)70152-0).
- A Post-Graduate Lecture ON THE NERVOUS SEQUELÆ OF INFLUENZA. *Lancet* 1893;142:73–6. [https://doi.org/10.1016/S0140-6736\(00\)65088-2](https://doi.org/10.1016/S0140-6736(00)65088-2).
- Fluge Ø, Tronstad KJ, Mella O. Pathomechanisms and possible interventions in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). *J Clin Invest* 2021. <https://doi.org/10.1172/JCI150377>.
- Newton DJ, Kennedy G, Chan KKF, Lang CC, Belch JF, Khan F. Large and small artery endothelial dysfunction in chronic fatigue syndrome. *Int J Cardiol.* 2012;154:335–6. <https://doi.org/10.1016/j.ijcard.2011.10.030>.
- Datta SD, Talwar A, Lee JT. A proposed framework and timeline of the spectrum of disease due to SARS-CoV-2 infection: illness beyond acute infection and public health implications. *JAMA.* 2020;324:2251–2. <https://doi.org/10.1001/jama.2020.22717>.
- Sivan M, Taylor S. NICE guideline on long covid. *BMJ.* 2020;371:m4938. <https://doi.org/10.1136/bmj.m4938>.
- Crook H, Raza S, Nowell J, Young M, Edison P. Long covid—mechanisms, risk factors, and management. *BMJ.* 2021;374:n1648. <https://doi.org/10.1136/bmj.n1648>.
- Margalit I, Yelin D, Sagi M, Rahat MM, Sheena L, Mizrahi N, et al. Risk factors and multidimensional assessment of long coronavirus disease fatigue: a nested case-control study. *Clin Infect Dis.* 2022;75:1688–97. <https://doi.org/10.1093/cid/ciac283>.
- Choutka J, Jansari V, Hornig M, Iwasaki A. Unexplained post-acute infection syndromes. *Nat Med.* 2022;28:911–23. <https://doi.org/10.1038/s41591-022-01810-6>.
- Kreutmair S, Unger S, Núñez NG, Ingelfinger F, Alberti C, De Feo D, et al. Distinct immunological signatures discriminate severe COVID-19 from non-SARS-CoV-2-driven critical pneumonia. *Immunity.* 2021;54:1578–1593.e5. <https://doi.org/10.1016/j.immuni.2021.05.002>.
- Bergamaschi L, Mescia F, Turner L, Hanson AL, Kotagiri P, Dunmore BJ, et al. Longitudinal analysis reveals that delayed bystander CD8+ T cell activation and early immune pathology distinguish severe COVID-19 from mild disease. *Immunity.* 2021;54:1257–1275.e8. <https://doi.org/10.1016/j.immuni.2021.05.010>.
- Blot M, Bour J-B, Quenot JP, Bourredjem A, Nguyen M, Guy J, et al. The dysregulated innate immune response in severe COVID-19 pneumonia that could drive poorer outcome. *J Transl Med.* 2020;18:457. <https://doi.org/10.1186/s12967-020-02646-9>.
- Lim J, Puan KJ, Wang LW, Teng KWW, Loh CY, Tan KP, et al. Data-driven analysis of COVID-19 reveals persistent immune abnormalities in convalescent severe individuals. *Front Immunol* 2021;12.
- Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, et al. Endothelial cell infection and endothelitis in COVID-19. *The Lancet.* 2020;395:1417–8. [https://doi.org/10.1016/S0140-6736\(20\)30937-5](https://doi.org/10.1016/S0140-6736(20)30937-5).
- Cheon IS, Li C, Son YM, Goplen NP, Wu Y, Cassmann T, et al. Immune signatures underlying post-acute COVID-19 lung sequelae. *Sci Immunol.* 2022;6:1741. <https://doi.org/10.1126/sciimmunol.abk1741>.
- Klein J, Wood J, Jaycox J, Lu P, Dhodapkar RM, Gehlhausen JR, et al. Distinguishing features of Long COVID identified through immune profiling. *MedRxiv.* 2022. <https://doi.org/10.1101/2022.08.09.22278592>.
- Phetsouphanh C, Darley DR, Wilson DB, Howe A, Munier CML, Patel SK, et al. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. *Nat Immunol.* 2022;23:210–6. <https://doi.org/10.1038/s41590-021-01113-x>.
- Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of TH2 cytokines by adipose tissue-associated c-Kit+Sca-1+ lymphoid cells. *Nature.* 2010;463:540–4. <https://doi.org/10.1038/nature08636>.
- Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell.* 2018;174:1054–66. <https://doi.org/10.1016/j.cell.2018.07.017>.
- Annunziato F, Romagnani C, Romagnani S. The 3 major types of innate and adaptive cell-mediated effector immunity. *J Allergy Clin Immunol.* 2015;135:626–35. <https://doi.org/10.1016/j.jaci.2014.11.001>.
- Cautivo KM, Matatia PR, Lizama CO, Mroz NM, Dahlgren MW, Yu X, et al. Interferon gamma constrains type 2 lymphocyte niche boundaries during mixed inflammation. *Immunity.* 2022;55:254–271.e7. <https://doi.org/10.1016/j.immuni.2021.12.014>.
- Dahlgren MW, Molofsky AB. All along the watchtower: group 2 innate lymphoid cells in allergic responses. *Curr Opin Immunol.* 2018;54:13–9.
- Gomez-Cadena A, Spehner L, Kroemer M, Khelil MB, Bouiller K, Verdeil G, et al. Severe COVID-19 patients exhibit an ILC2 NKG2D+ population in their impaired ILC compartment. *Cell Mol Immunol.* 2021;18:484–6. <https://doi.org/10.1038/s41423-020-00596-2>.
- Silverstein NJ, Wang Y, Manickas-Hill Z, Carbone C, Dauphin A, Boribong BP, et al. Innate lymphoid cells and COVID-19 severity in SARS-CoV-2 infection. *Elife.* 2022;11:e74681. <https://doi.org/10.7554/eLife.74681>.
- Pritsch M, Radon K, Bakuli A, Le Gleut R, Olbrich L, Guggenbühl Noller JM, et al. Prevalence and risk factors of infection in the representative COVID-19 cohort Munich. *Int J Environ Res Public Health.* 2021. <https://doi.org/10.3390/ijerph18073572>.
- Radon K, Saathoff E, Pritsch M, Guggenbühl Noller JM, Kroidl I, Olbrich L, et al. Protocol of a population-based prospective COVID-19 cohort study Munich, Germany (KoCo19). *BMC Public Health.* 2020;20:1036. <https://doi.org/10.1186/s12889-020-09164-9>.
- Brand I, Gilbert L, Bruger J, Garí M, Wieser A, Eser TM, et al. 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. *Front Immunol* 2021;12.
- Mazzurana L, Bonfiglio F, Forkel M, D'Amato M, Halfvarson J, Mjösberg J. Crohn's disease is associated with activation of circulating innate lymphoid cells. *Inflamm Bowel Dis.* 2021;27:1128–38. <https://doi.org/10.1093/ibd/izaa316>.
- Kotecha N, Krutzik PO, Irish JM. Web-based analysis and publication of flow cytometry experiments. *Curr Protoc Cytom* 2010;Chapter 10:Unit10.17-Unit10.17. <https://doi.org/10.1002/0471142956.cy1017s53>.
- Rogers WT, Holyst HA. FlowFP: a bioconductor package for fingerprinting flow cytometric data. *Adv Bioinform.* 2009;2009:193947. <https://doi.org/10.1155/2009/193947>.
- Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, et al. Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell.* 2017;168:1086–1100.e10. <https://doi.org/10.1016/j.cell.2017.02.021>.
- Kokkinou E, Pandey RV, Mazzurana L, Gutierrez-Perez I, Tibbitt CA, Weigel W, et al. CD45RA+CD62L– ILCs in human tissues represent a quiescent local reservoir for the generation of

- differentiated ILCs. *Sci Immunol.* 2023;7:eabj8301. <https://doi.org/10.1126/sciimmunol.abj8301>.
33. Fonseca W, Lukacs NW, Elesela S, Malinczak C-A. Role of ILC2 in Viral-Induced Lung Pathogenesis. *Front Immunol* 2021;12.
 34. Vanoni G, Ercolano G, Candiani S, Rutigliani M, Lanata M, Derré L, et al. Human primed ILCPs support endothelial activation through NF- κ B signaling. *Elife.* 2021;10:e58838. <https://doi.org/10.7554/eLife.58838>.
 35. Kaiser R, Leunig A, Pekayvaz K, Popp O, Joppich M, Polewka V, et al. Self-sustaining IL-8 loops drive a prothrombotic neutrophil phenotype in severe COVID-19. *JCI Insight.* 2021. <https://doi.org/10.1172/jci.insight.150862>.
 36. Sabbatino F, Conti V, Franci G, Sellitto C, Manzo V, Pagliano P, et al. PD-L1 dysregulation in COVID-19 patients. *Front Immunol* 2021;12.
 37. Zhang Z, Ai G, Chen L, Liu S, Gong C, Zhu X, et al. Associations of immunological features with COVID-19 severity: a systematic review and meta-analysis. *BMC Infect Dis.* 2021;21:738. <https://doi.org/10.1186/s12879-021-06457-1>.
 38. Antonelli M, Penfold RS, Merino J, Sudre CH, Molteni E, Berry S, et al. Risk factors and disease profile of post-vaccination SARS-CoV-2 infection in UK users of the COVID Symptom Study app: a prospective, community-based, nested, case-control study. *Lancet Infect Dis.* 2022;22:43–55. [https://doi.org/10.1016/S1473-3099\(21\)00460-6](https://doi.org/10.1016/S1473-3099(21)00460-6).
 39. Magnusson K, Kristoffersen DT, Dell’Isola A, Kiadaliri A, Turkiewicz A, Runhaar J, et al. Post-covid medical complaints following infection with SARS-CoV-2 Omicron vs Delta variants. *Nat Commun.* 2022;13:7363. <https://doi.org/10.1038/s41467-022-35240-2>.