Lasting changes to circulating leukocytes in people with mild SARS-CoV-2 infections

Allison E. Kennedy, Laura Cook, Jessica A. Breznik, Braeden Cowbrough, Jessica G. Wallace, Angela Huynh, James W Smith, Kiho Son, Hannah Stacey, Jann Ang, Alison McGeer, Brenda L. Coleman, Maggie Larché, Mark Larché, Nathan Hambly, Parameswaran Nair, Kjetil Ask, Matthew S. Miller, Jonathan Bramson, Megan K. Levings, Ishac Nazy, Sarah Svenningsen, Manali Mukherjee, Dawn M. E. Bowdish

1McMaster Immunology Research Centre, McMaster University, Hamilton, Ontario, Canada
2Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario, Canada
3Department of Medicine, Michael G. DeGroote School of Medicine, McMaster University, Hamilton, Ontario, Canada
4Department of Microbiology and Immunology, University of Melbourne, Melbourne, Australia
5The Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Australia
6Firestone Institute of Respiratory Health, St Joseph’s Healthcare, Hamilton, Ontario, Canada
7Department of Biochemistry & Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada
8Sinai Health, Toronto, Ontario, Canada
9University of Toronto, Toronto, Ontario, Canada
10School of Biomedical Engineering, University of British Columbia, Vancouver, British Columbia, Canada
11Department of Surgery, University of British Columbia, Vancouver, British Columbia, Canada
12British Columbia Children’s Hospital Research Institute, Vancouver, British Columbia, Canada
13McMaster Centre for Transfusion Research, Hamilton, Ontario, Canada

¶AEK and LC are Joint Senior Authors

*Corresponding author. Email: bowdish@mcmaster.ca (DMEB)

Short Title: Immune changes after mild COVID-19

NOTE: This preprint report new research that has not been certified by peer review and should not be used to guide clinical practice.
Abstract

Survivors of severe SARS-CoV-2 infections frequently suffer from a range of post-infection sequelae. Whether survivors of mild or asymptomatic infections can expect any long-term health consequences is not yet known. Herein we investigated lasting changes to soluble inflammatory factors and cellular immune phenotype and function in individuals who had recovered from mild SARS-CoV-2 infections (n=22) compared to those that had recovered from other mild respiratory infections (n=11). Individuals who had mild SARS-CoV-2 infections had elevated levels of C-reactive protein 1-3 months after symptom onset, and changes in phenotype and function of circulating T cells that were not apparent in individuals 6-9 months post-symptom onset. Markers of monocyte activation and expression of adherence and chemokine receptors indicative of altered migratory capacity were also higher at 1-3 months post-infection in individuals who had mild SARS-CoV-2, but these were no longer elevated by 6-9 months post-infection. Perhaps most surprisingly, polyclonal activation of T cells was higher in individuals who had recently experienced a mild SARS-CoV-2 infection compared to individuals with other recent respiratory infections. These data are indicative of prolonged immune activation and systemic inflammation that persists for up to three months after mild or asymptomatic SARS-CoV-2 infections.
SARS-CoV-2 infection has unusual effects on circulating immune cells. In general, respiratory infections are associated with rapid and sustained release of neutrophils and monocytes from the bone marrow, with no immediate change in production or function of lymphocytes [1]. In contrast, SARS-CoV-2 infections are consistently associated with lymphopenia, which is a reliable predictor of mortality [2]. The loss of T cells may be due to apoptosis in the secondary lymphoid organs, or possibly inappropriate recruitment to the lungs and other organs [3, 4]. Severe cases of SARS-CoV-2 infection are also associated with increased granularity and changes in nuclear morphology in neutrophils and monocytes, which are generally due to premature egress from the bone marrow and stunted maturation [5]. Whether myelopoiesis and egress are affected in mild COVID is not yet known.

Hospitalization with any respiratory infection is associated with long-term health consequences [6]; however, the consequences of COVID-19 appear to be particularly broad and include “long COVID” (a constellation of symptoms that can include severe fatigue often exacerbated by exercise; pain, and respiratory complications) [7, 8], and a higher than expected risk of re-hospitalization for a variety of causes [9-11]. Although it has been documented that there are differences in acute immune responses in asymptomatic, mild, moderate, and severe cases of SARS-CoV-2 infection [12, 13], it is not clear whether there is any risk of long-term immune dysregulation or health consequences in survivors of asymptomatic or mild cases.

We investigated whether there are any changes in phenotype and frequency of circulating leukocytes in individuals who had mild COVID-19 (i.e. did not require medical attention) and compared them to those who had other mild respiratory infections. We found evidence of transient changes in circulating NK cells, T cells, and monocytes 1-3 months post mild COVID-19 but not after other mild respiratory infections. These changes generally resolved 6-9 months post-COVID-19 infection, indicating...
that there is an early period of immune dysregulation greater than that of other respiratory infections, which occurs even after mild SARS-CoV-2 infection.

Materials and Methods

Participant recruitment and blood collection

Research participants with symptoms consistent with COVID-19 (as summarized in Table 1) were recruited from the greater Hamilton area (Ontario, Canada) between February 2020 and January 2021. All protocols were approved by the Hamilton Research Ethics Board (#10757 and #11471). Venous blood was drawn in anti-coagulant free vacutainers for isolation of serum, and in heparin-coated vacutainers for experiments that required viable leukocytes. Serum and leukocytes were isolated as per standard protocols [14]. Immunophenotyping was performed on fresh blood within four hours of collection. None of the participants had been vaccinated against SARS-CoV-2 at the time of sample collection. Three donors who had a positive PCR test for SARS-CoV-2 were either asymptomatic or had mild symptoms (i.e. headache or tiredness that lasted for a day). The remaining donors had symptoms that they reported as consistent with a typical respiratory infection (e.g. cough, fever, chills) but these generally resolved within 2-4 weeks. The duration of symptoms and demographics of the 38 participants in this study (i.e. sex, age, body mass index, comorbid conditions, medications) are summarized in Table 1. Participants were characterized as having had COVID-19, ‘other respiratory infection’, or ‘indeterminate’, as detailed in S1 Table. Most participants did not consult a medical professional about their condition, consistent with the public health advice in the early months of the pandemic that individuals who did not need medical attention should stay home, and since none were hospitalized, we defined these as ‘mild’ infections. Donors recovered from COVID-19 gave blood either 1-3 months post-symptom onset or 6-9 months post-symptom onset, but no donor gave blood at both time points and therefore there were no repeat measures from the same donor.
**Table 1. Participant demographics**

|                               | Other respiratory infections (not COVID-19) | COVID-19 infections n=22 | Indeterminate n=5 | P value * |
|-------------------------------|-------------------------------------------|--------------------------|-------------------|-----------|
| **Age (mean ± STDEV)**        | 55 ± 16                                   | 55 ± 15                  | 57 ± 11           | 0.9249    |
| **Sex (% female)**            | 8 (73%)                                   | 11 (50%)                 | 3 (60%)           | 0.2783    |
| **BMI (kg/m²)**               | 24.0 ± 2.9                                | 25.0 ± 3.6               | 24.5 ± 2.3        | 0.4172    |
| **Health Conditions (frequency)** |                                 |                          |                   |           |
| Asthma                        | 1 (9%)                                    | 1 (5%)                   | 1 (20%)           | -         |
| COPD (including emphysema and chronic bronchitis) | 1 (9%)                                    | 0 (0%)                   | 0 (0%)           | -         |
| Other lung disease            | 0 (0%)                                    | 1 (5%)                   | 0 (0%)            | -         |
| Diabetes                      | 0 (0%)                                    | 3 (14%)                  | 0 (0%)            | -         |
| Hypertension                  | 1 (9%)                                    | 1 (5%)                   | 1 (20%)           | -         |
| Heart disease                 | 0 (0%)                                    | 2 (9%)                   | 0 (0%)            | -         |
| Cancer                        | 2 (18%)                                   | 2 (9%)                   | 0 (0%)            | -         |
| Autoimmune condition          | 1 (9%)                                    | 1 (5%)                   | 1 (20%)           | -         |
| **Medications**               |                                           |                          |                   |           |
| Number of medications (mean ± STDEV) | 1 ± 2                                     | 2 ± 2                    | 1 ± 1             | 0.2049    |
| **Symptoms (frequency)**      |                                           |                          |                   |           |
| Cough                         | 6 (55%)                                   | 13 (59%)                 | 2 (40%)           | -         |
| Shortness of breath           | 4 (36%)                                   | 9 (41%)                  | 1 (20%)           | -         |
| Chest pain                    | 3 (27%)                                   | 9 (41%)                  | 2 (40%)           | -         |
| Fever                         | 3 (27%)                                   | 12 (55%)                 | 1 (20%)           | -         |
| Feeling generally unwell      | 9 (82%)                                   | 21 (95%)                 | 2 (40%)           | -         |
| Abnormally tired              | 7 (64%)                                   | 20 (91%)                 | 2 (40%)           | -         |
| New confusion                 | 4 (36%)                                   | 1 (5%)                   | 1 (20%)           | -         |
| New generalized muscle aches and pains | 5 (45%)                                   | 16 (73%)                 | 2 (40%)           | -         |
| New joint pain                | 3 (27%)                                   | 5 (23%)                  | 2 (40%)           | -         |
| Earache/infection             | 0 (0%)                                    | 1 (5%)                   | 0 (0%)            | -         |
| Headache                      | 6 (55%)                                   | 14 (64%)                 | 1 (20%)           | -         |
| Runny/stuffy nose             | 7 (64%)                                   | 7 (32%)                  | 2 (40%)           | -         |
| Sinus pain                    | 2 (18%)                                   | 4 (18%)                  | 1 (20%)           | -         |
| Sore/scratchy throat          | 7 (64%)                                   | 10 (45%)                 | 2 (40%)           | -         |
| Loss of appetite              | 4 (36%)                                   | 8 (36%)                  | 2 (40%)           | -         |
| Loss of taste/smell           | 2 (18%)                                   | 11 (50%)                 | 1 (20%)           | -         |
| **Duration of Symptoms**      |                                           |                          |                   |           |
| No symptoms - 1 week          | 4 (36%)                                   | 3 (14%)                  | 1 (20%)           | -         |
| 2-4 weeks                     | 3 (27%)                                   | 11 (50%)                 | 4 (80%)           | -         |
| 4 or more weeks               | 4 (36%)                                   | 8 (36%)                  | 0 (0%)            | -         |
| **Diagnosis**                 |                                           |                          |                   |           |
| PCR test for SARS-CoV-2 performed | 0 (0%)                                   | 15 (68%)                 | 0 (0%)            | -         |
| Told by a health care professional that they had SARS-CoV-2/COVID-19 without a PCR test | 1 (8%)                                   | 5 (25%)                  | 0 (0%)            | -         |

*Statistical comparisons were made between ‘other respiratory infections’ and ‘COVID-19 infections’ groups only since the ‘indeterminate’ group was not used in subsequent analyses. Student’s unpaired parametric t-test was used to compare age and BMI, which were normally distributed. Sex distribution was measured by Fisher’s exact test to analyze a 2x2 contingency table. Differences in the number of medications, which was not normally distributed, was measured using an unpaired non-parametric test not assuming Gaussian distribution.
Measurements of anti-SARS-CoV-2 antibodies

Anti-SARS-CoV-2 full-length S protein and RBD IgG and IgA seropositivity were identified via validated serology ELISA as described by Huynh et al., 2021 [15]. Briefly, NUNC™ Maxisorp 384 well plates (ThermoScientific) were coated with S (5 μg/mL) or RBD (2 μg/mL) antigens in 50 mM carbonate buffer (pH 9.6) overnight at 4°C and blocked with 3% skim milk in PBS-0.05% Tween-20. After washing with PBS, plates were coated with diluted serum (1:100) for 1 hour at room temperature, washed, and incubated with 25 μL alkaline phosphatase conjugated antibodies goat anti-human IgG (1:2000) or goat anti-human IgA (1:500) (Jackson Immuno). Plates were washed and antibody levels were quantified by adding 50 μL of substrate buffer (0.27 μM p-nitrophenyl phosphate/diethanolamine buffer, 1M, pH 9.6) and reading optical density (OD) at 405 nm detection every 1 min, with 490 nm reference.

Assessment of T cell activation induced markers (AIM) for SARS-CoV-2 peptides

To detect antigen-specific T cell recall responses, 100 μL of venous blood was cultured with an equal amount of Iscove’s Modified Dulbecco’s Medium Glutamax (Invitrogen Life Technologies) and antigen for 44 hours in 96-well flat bottom plates at 37°C. Wells were stimulated using SARS-CoV-2 peptide pools (PepTivators from Miltenyi Biotec) containing overlapping peptides covering the complete sequence of the membrane glycoprotein (M; #130-126-702), the nucleocapsid phosphoprotein (N; #130-126-699) or the immunodominant sequence domains of the spike glycoprotein (S; #130-126-701); each used 1 μg/mL. Unstimulated wells served as negative controls, and polyclonal stimulation with CytoStim™ (0.5 μl/well, Miltenyi Biotec) was included as positive control. The fluorescently conjugated monoclonal antibody panel used for analysis is within S2 Table. Samples were run on a CytoFLEX LX (4 laser, Beckman Coulter). In this
AIM assay the antigen-specific T cells (AIM-positive) are defined by co-expression of CD25 and CD134 (OX40) for CD4+ T cells [16, 17] and by co-expression of CD69 and CD137 (4-1BB) for CD8+ T cells [18].

**Quantification of peripheral immunophenotype**

Circulating neutrophils, monocytes, T, B and NK cells were quantitated by multi-colour flow cytometry as previously described [14, 19]. Direct application of monoclonal antibodies (specificities outlined in S2 Table) to 100 µL of whole blood was performed for 30 minutes at room temperature.

Following staining, samples were incubated with either 1 x Fix/Lyse Buffer (eBioscience) for 10 minutes or following standard protocols for the FOXP3 Transcription Factor Staining Kit (eBioscience), washed with PBS, and resuspended in FACS Wash (5 mM EDTA, 0.5% BSA in PBS) for analysis with a CytoFLEX LX (4 laser, Beckman Coulter). Absolute counts for circulating immune populations were determined using CountBright™ absolute counting beads (Invitrogen). Gating strategies and representative FACS dot plots to determine circulating immune populations are shown in S1 Fig.

**Measurements of cytokines and C-reactive protein**

Serum cytokines IL-6 and TNF and C-reactive protein (CRP) were measured using the Ella™ Automated Immunoassay System (Biotecne). Serum was diluted 1:2 as per the manufacturer’s protocol.

The lower limits of quantification for CRP, IL-6, and TNF are 32 pg/mL, 0.28 pg/mL, 0.3 pg/mL, respectively.

**Statistical analysis**

Data and statistical analyses were done in FlowJo version 10.7.1, GraphPad Prism version 9, and R. Multiple group comparisons were tested using Welch’s One-Way ANOVA and Games-Howell post-hoc test. As data were non-normally distributed, correlations were tested using Spearman correlation. Outliers were removed using Grubbs’ test ($\alpha =0.05$).
Results

Demographics and symptoms of individuals who had SARS-CoV-2 and other respiratory infections

During the early phase of the COVID-19 pandemic (February – June 2020) diagnostic PCR testing in Ontario was generally limited to individuals who were hospitalized, had a confirmed exposure with an infected person, or had travelled to an area with active infections. At the time, public health advice was that people who did not need medical attention should self-isolate at home without being tested. Serologic studies have been used to estimate an infection rate of 0.5-1.5% during the March-June ‘first wave’ [20]. Compounding the lack of testing were the high rates of influenza and unusually high rates of respiratory syncytial virus (RSV) infections [21]. Consequently, some people with respiratory infections in this early period were told by a health care professional that they had COVID-19 without a nasopharyngeal swab PCR test, and not all of our study participants with symptoms of mild respiratory illness had COVID-19 (Table 1). Participants were classified as having had COVID-19 if they received a positive diagnostic PCR test and/or had anti-SARS-CoV-2 antibodies (S1 Table). There were no statistically significant differences in age, sex, BMI, co-morbidities, medications, or duration of symptoms between individuals that had mild COVID-19 (n=22) and individuals that had other mild respiratory infections (n = 11, Table 1).

Survivors of mild SARS-CoV-2 infections have reduced T cell responses to SARS-CoV-2 peptides 6-9 months post symptom onset

Lymphopenia is commonly reported in severe SARS-CoV-2 infections; however, it is not clear whether mild infections impact T cell numbers or function. We measured T cell responses to the SARS-CoV-2 structural membrane protein (M), nucleocapsid protein (N), and immunodominant regions of the spike (S) protein in all participants. After 44 hours incubation with antigen this assay identifies antigen-specific
CD4⁺ T cells by induced co-expression of CD25 and CD134 (OX40) and antigen-specific CD8⁺ T cells by co-expression of CD69 and CD137 (4-1BB) (Fig 1A) [22, 23]. A positive assay result was defined as > 3 standard deviations above an unstimulated sample (negative control well) and consisting of at least 20 events. We identified 5 individuals who had mild respiratory symptoms, did not have a positive diagnostic PCR test, and were seronegative for SARS-CoV-2 antibodies but had detectable levels of SARS-CoV-2 reactive T cells to some, but not all, of the SARS-CoV-2 M, N, and S peptides (S1 Table). Although it is possible these individuals had been infected with SARS-CoV-2 and had antibodies at some point, these data are also consistent with reports of pre-existing cross-reactive T cells to SARS-CoV-2 antigens in as many as 30% of individuals [24]. Consequently, we classified these 5 individuals as “indeterminate” and did not include them in subsequent analyses (their demographic data is summarized in Table 1).

**Fig 1.** CD4⁺ and CD8⁺ T cell responses to the M, N, and S peptide pools after mild SARS-CoV-2 infection. A) The number of SARS-CoV-2 specific T cells is measured as a percent of CD4⁺ T cells expressing both CD25 and OX40 or CD8⁺ T cells expressing both CD69 and CD137 after activation with the S, M or N peptide pools 1-3 months and 6-9 months after infection. The polyclonal activator Cytostim is used as a positive control. B) All COVID-19 seropositive donors had an increase in CD25⁺OX40⁺CD4⁺ T cells in response to at least one of the M, N or S antigens 1-3 months after mild COVID-19 infection compared to seronegative individuals recovered from other mild respiratory infections. Each participant is indicated by a single data point: other respiratory infection n=11; 1-3 months post COVID-19 infection n=11; 6-9 months post COVID-19 infection n=8. Multiple group comparisons were tested using Welch’s One-Way ANOVA and Games-Howell post-hoc test; bars represent the mean ± standard deviation. *P<0.05; **P<0.01; ***P<0.001

For analysis of the T cell responses, we split our cohort into those without antibody or T cell responses to SARS-CoV-2 (other respiratory infection; n=11) and those with antibody responses to SARS-
CoV-2 (COVID-19; n=22), the majority of whom also had detectable T cell responses to SARS-CoV-2 M, N, and S antigens (90.9%; n=20/22) (Fig 1; S1 Fig). Of the 22 participants who had COVID-19 infections, 14 provided samples 1-3 months post-symptom onset and 8 provided samples 6-9 months post-symptom onset. Consistent with other reports of lasting memory T cell responses to SARS-CoV-2 infection [13], we observed that all COVID-19 participants that provided samples at 1-3 months or 6-9 months post-symptom onset had generated T cell memory to at least one of the M, N and S peptides pools (Fig 1B, S3 Table). These data are consistent with previous observations that survivors of mild infections have lasting memory responses [13], however, there appear to be long-term differences in T cell functionality in COVID-19 survivors.

We saw similar frequencies of CD4+ T cell responses to all SARS-CoV-2 antigens and these were highest in COVID-19 patients 1-3 months post symptom onset (means of ~3-4% of CD4+ T cells), being significantly higher than in individuals with other respiratory infections (Fig 1B). Compared to COVID-19 patients 1-3 months post symptom onset, responses were reduced in COVID-19 patients 6-9 months post symptom onset and were not significantly different to those in individuals recovered from other respiratory infections. Similar results were found for CD8+ T cell responses, being highest in COVID-19 patients 1-3 months post symptom onset (means of ~0.4-0.5% of CD8+ T cells) although there were no significant differences between groups.

**Survivors of mild SARS-CoV-2 infections have evidence of sustained inflammation 1-3 months post symptom onset**

Prolonged immune activation can occur after recovery from severe infections and is thought to contribute to malaise and other symptoms in survivors [25]. One of the unusual features of COVID-19 is that a significant number of patients with mild to moderate illness report symptoms weeks to months after infection, despite having cleared the virus [26]. Although the participants in our study generally had
symptoms resolve within 1 month post infection (Table 1), we have nonetheless found evidence of prolonged inflammation. Seropositive individuals had higher levels of CRP, TNF, and IL-6 in circulation 1-3 months post-symptom onset; but in samples from individuals 6-9 months post-symptom onset, levels were equivalent to those individuals who were seronegative and were recovered from other respiratory infections (Fig 2A, B & C).

Figure 2. Transient increases in soluble mediators of inflammation occur after mild SARS-CoV-2 infection. A) CRP levels in serum were higher 1-3 months after mild COVID-19 infection, and there was a trend towards remaining elevated 6-9 months after mild COVID-19 infection, compared to levels observed in seronegative individuals after other respiratory infections. Serum TNF levels (B) and IL-6 levels (C) were higher 1-3 months after COVID-19 infection but returned to levels seen in individuals who had other respiratory infections by 6-9 months. Each participant is indicated by a single data point: other respiratory infection n=11; 1-3 months post-COVID n=9-12; 6-9 months post-COVID n=8. Multiple group comparisons were tested using Welch’s One-Way ANOVA and Games-Howell post-hoc test; bars are presented as mean ± standard deviation. *P<0.05; **P<0.01.

Surprisingly, there were differences in the degree of epitope independent polyclonal activation of T cells between the COVID-19 and non-COVID-19 groups. When whole blood was stimulated with Cytostim, which acts like a superantigen by crosslinking TCR (regardless of Vβ usage) and MHC, there was a statistically significant difference in the percent of CD25⁺OX40⁺CD4⁺ T cells and CD69⁺CD137⁺CD8⁺ T cells in COVID-19 patients 1-3 months post-symptom onset (Fig 3A&B). Whether this superantigen-like crosslinking activation contributes to, or is a result of, systemic inflammation is not clear, but CRP levels correlate with the percent of Cytostim-activated CD4⁺ T cells (Fig 3C). The Cytostim-activated T cells in the COVID-19 group also had higher expression levels of OX40, CCR6, CCR4 and CD69 than the other respiratory infection
group, implying a stronger activation (Fig 3D). Although these levels of OX40 and CD69 were reduced in samples collected 6-9 months post-symptom onset compared to samples collected 1-3 months post-symptom onset, levels of CCR4 and CCR6 remained similar. Collectively these data imply that there may be subtle changes in inducible T cell activation in convalescent SARS-CoV-2 patients that do not occur in response to other respiratory infections.

Figure 3. Evidence of prolonged T cell activation after mild SARS-CoV-2 infection.

The polyclonal activator Cytostim was used to measure T cell responses. COVID-19 seropositive individuals had a higher proportion of CD4⁺ (A) and CD8⁺ (B) activated T cells 1 to 3 months after infection compared to seronegative individuals after other respiratory infections. C) The number of CD4⁺ T cells that responded to polyclonal stimulation correlated with CRP in COVID-19 seropositive individuals. D) Activation markers on the Cytostim-responsive CD4⁺ T cells OX40, CCR6, CCR4, and CD69 were higher 1-3 months after COVID-19 infection compared to after other respiratory infections. Each participant is indicated by a single data point: other respiratory infection n=7-11; 1-3 months post COVID-19 infection n=11-12; 6-9 months post COVID-19 infection n=5-8. Multiple group comparisons in A-B and D were tested using Welch’s One-Way ANOVA and Games-Howell post-hoc test; bars are presented as mean ± standard deviation. Data in C was assessed by Spearman’s rank correlation. *P<0.05; **P<0.01.

Survivors of mild SARS-CoV-2 infections have changes in circulating immunophenotype 1-3 months post-symptom onset

The total numbers of circulating CD45⁺ cells, CD4⁺ T cells and CD8⁺ T cells were not different between individuals who had other respiratory infections and those who had COVID-19 at either 1-3 months or 6-9 months post symptom onset (Fig 4A-C). Compared to participants who were recovered from
other respiratory infections, there was an expansion of Tregs (CD45+CD3+CD4+CD25+CD127lowFOXP3+) and a
decrease in NK cells (CD45+CD56+/NKp46+) in COVID-19 patients at 1-3 months post-symptom onset that
returned by 6-9 months post-symptom onset to levels observed in individuals with non-COVID-19
respiratory infections (Fig 4D,G). We also measured the proportions of circulating naïve (CD45RA⁺CCR7⁻),
central memory (CD45RA⁺CCR7), effector memory (CD45RA⁺CCR7), terminally differentiated effector
memory cells re-expressing CD45RA (TEMRA; CD45RA⁺CCR7⁻), and terminally differentiated (CD45RA⁺CCR7⁻
CD57⁺CD28⁻) CD4⁺ and CD8⁺ T cells. Compared to patients with non-COVID-19 respiratory infections, there
was an expansion of central memory CD4⁺ cells and terminally differentiated CD8⁺ T cells in COVID-19
patients at 1-3 months post-symptom onset that returned to levels observed in patients with other
respiratory infections by 6-9 months post-symptom onset (Fig 4E,F). There were no significant differences
between participants who had other respiratory infections and those who had COVID-19 for any other
measured CD4⁺ and CD8⁺ T cell subsets.

Figure 4. Transient changes in circulating lymphocytes occur 1-3 months after COVID-19 infection.

Absolute numbers of circulating CD45⁺ cells (A), CD4⁺ T cells (B), and CD8⁺ T cells (C) were not different
after 1-3 months or 6-9 months post-symptom presentation in seropositive individuals recovered from mild
COVID-19 infection compared to seronegative individuals recovered from other respiratory infections;
however, NK cell numbers (D) were lower in the 1-3 months post-COVID-19 infection group. At 1-3 months
post recovery from COVID-19 there was an increase in CD45RA⁺CCR7⁺ central memory CD4⁺ cells (E) and an
increase in CD45RA⁺CCR7⁺CD57⁻CD28⁻ terminally differentiated CD8⁺ T cells (F) compared to individuals
recovered from other respiratory infections, but these differences were not apparent in individuals who
had recovered from COVID-19 6-9 months prior. (G) Levels of circulating regulatory T cells (measured as a
% of CD4⁺ T cells) were higher in individuals 1-3 months post COVID-19 infection. Each participant is
indicated by a single data point: other respiratory infection n=11; 1-3 months post COVID-19 infection
n=11-13; 6-9 months post COVID-19 infection n=8. Multiple group comparisons were tested using Welch’s One-Way ANOVA and Games-Howell post-hoc test; bars are presented as mean ± standard deviation. *P<0.05.

Six months after SARS-CoV-2 infection there was a statistical trend towards a lower ratio of myeloid to lymphoid cells, though this was due to a non-significant decrease in neutrophil numbers rather than changes in numbers of monocytes (Fig 5A-C). However, as circulating monocytes are a sensitive marker of chronic inflammation, we assessed monocyte subsets as well as their expression of migratory and activation markers (Fig 5D, S4 and S5 Tables). Classical monocytes (CD14^+CD16^-) expressing CCR2 are the first to leave the bone marrow. They have a half-life in the circulation of less than 24 hours since they are either recruited to sites of acute inflammation in response to CCL2/MCP or differentiate into CX3CR1-expressing intermediate (CD14^+CD16^-) monocytes [27, 28]. Classical monocytes increase in the circulation during acute infection so, as expected, there was no difference in the number of circulating monocytes after COVID-19 infection had resolved (Fig 5C). Surprisingly, at 1-3 months after infection levels of CCR2 were lower on intermediate and non-classical monocyte populations (Fig 5D), implying that cells with the highest levels of CCR2 emigrated from the circulation. In general, the chemokine receptor CX3CR1 is expressed more highly on intermediate and non-classical (CD14^hiCD16^-) monocytes and is associated with recruitment to the tissues or vasculature and repair of damage in response to CX3CL1/fractalkine [29, 30]. A transient decrease in CX3CR1-expressing classical monocytes was found 1-3 months post COVID-19 infection, which likely indicates that monocytes with the highest levels of CX3CR1 (and therefore the most responsive to CX3CL1) had emigrated from the circulation. Expression of the integrin CD11b was also transiently increased on monocytes at 1-3 months post-symptom onset, but not neutrophils (data not shown), providing further evidence of transient but systemic immune activation and possible changes in the migratory capacity of monocytes after mild SARS-CoV-2 infection.
Figure 5. Evidence of sustained cellular inflammation after mild SARS-CoV-2 infection.

A) There was a trend towards a decreasing ratio of myeloid to lymphoid cells after SARS-CoV-2 infection, compared to individuals recovered from other infections, which was driven by a decrease in circulating neutrophils (B). Although total monocyte numbers did not change after infection (C), surface expression of the migratory markers CX3CR1 and CCR2 decreased transiently (D). Concurrent increases in surface expression of the migration and activation marker CD11b implies that the monocytes were activated. E) Correlation analysis (Spearman’s correlation) of SARS-CoV-2 specific CD4+ and CD8+ T cell responses with measures of monocyte activation and migratory potential. Multiple group comparisons in A-D were tested using Welch’s One-Way ANOVA and Games-Howell post-hoc test; in A-C bars are presented as mean ± standard deviation and each dot indicates a participant. The spread of expression of monocyte surface markers in D was visualized by concatenating uncompensated CD45+CD19-CD3-CD56-CD11b+HLADR+CD14+ events in FlowJo for each infection group prior to overlaying geometric mean fluorescence intensity expression data from all donors onto the same histogram plot. Other respiratory infection (grey) n=11, 1-3 months after COVID-19 infection (red) n=14, 6-9 months from COVID-19 infection (pink) n=8. Data in E were assessed with the rcorr function in the Hmisc package in R and only statistically significant associations are shown. *P<0.05.

Changes in monocyte activation markers could be due to the increase in basal inflammation or could be proportionate to the SARS-CoV-2 specific immune response. We found that decreasing CX3CR1 expression was most strongly associated with the percentage of S-antigen specific CD4+ T cells (p=0.0389) and that there was a strong association between M-antigen specific CD8+ T cells and HLA-DR expression on non-classical/patrolling monocytes (p=0.0198) (Fig 5E). Importantly, there was no relationship between polyclonal T cell activation (i.e. Cytostim) and myeloid activation, implying that these changes are not due
to the general increase in T cell activation observed in convalescent patients, but rather SARS-CoV-2 specific responses.

Discussion

Infections severe enough to require hospitalization can have long-term health consequences, but one of the unusual features of COVID-19 is that a significant proportion of mild infections have lasting cardiovascular, respiratory, and neurologic consequences [26, 31]. The range of long-term health consequences and the multi-organ immune pathology observed post-SARS-CoV-2 infection implies that there may be a greater degree of immune dysregulation than that commonly observed after infection with other respiratory pathogens. Importantly, the participants in this study did not have “long-COVID” in that they reported that their symptoms were mild and had mostly resolved within 2-4 weeks (Table 1); however, participants with even mild COVID-19 symptoms experience elevated inflammation and immune activation that endures at least 1-3 month after their infection had resolved. The fact that these participants had increased measures of systemic inflammation (e.g. CRP), and lasting phenotypic and functional changes to both monocytes and T cells, implies that inflammatory responses to mild COVID-19 infections are more protracted than expected.

The proportions of memory CD4+ and CD8+ T cells specific for SARS-CoV-2 S, M, and N proteins we observed were consistent with those reported for convalescent patients who had had more severe infections, implying that disease severity is not proportionate to SARS-CoV-2 memory T cell responses [24, 32]. Previous studies have reported differences in the expression of activation markers on SARS-CoV-2 specific memory CD4+ T cells between exposed but seronegative individuals compared to those who had either mild or severe infections [13]; however, to our knowledge, this is the first report of elevated activation of non-SARS-CoV-2 specific T cells (i.e. Cytostim treated) in convalescent COVID-19 patients.
Cytostim-activated CD4+ T cells in participants with mild COVID-19 1-3 months post-symptom onset,
compared to those in participants recovered from other respiratory infections, had higher expression of
the activation markers OX40, CCR4, CD69 and CCR6, and OX40 and CD69 expression remained elevated
after 6-9 months post-symptom onset. There were no differences between participant groups in the
expression of activation markers on circulating unstimulated cells (data not shown), demonstrating that
these differences are restricted to polyclonally activated cells. There are several possible explanations for
this result. CD4+ T cells from 1-3 months post-symptom onset of COVID-19 may be primed to respond
quicker and stronger to activation signals. Alternatively, since the number of activated CD4+ T cells in this
participant group correlated strongly with rising CRP levels, it is also possible that there may be circulating
cytokines (e.g. IL-6) or other factors that lower the threshold for T cell activation.

Our observations of transient increases in central memory CD4+ T cells and terminally
differentiated CD8+ T cells are consistent with T cell responses to acute infection; however, a pronounced
increase in regulatory T cells is generally associated with minimizing pathology during the acute, not the
convalescent, phase of respiratory viral infections. Severe SARS-CoV-2 infections are associated
with the development of autoantibodies that contribute to severity. Mild SARS-CoV-2 infections are
associated with autoimmune inflammatory syndromes (e.g. arthritis, vasculitis) after the primary infection
has resolved. Whether this expansion of Treg protects from autoimmune sequelae is not clear but
this has been reported to occur after other zoonotic infections. Appropriate regulation of Treg may be
especially important in SARS-CoV-2 infections, since pathology of these infections are caused in part by
dysregulation of TGF-β production, a major cytokine produced by Treg that is required for their
appropriate differentiation.

Severe COVID-19 infections are associated with a dysregulation of myelopoiesis that is so extreme
that monocytes and neutrophils are unrecognizable by blood smear and have dramatic changes in
granularity, size and surface marker expression. Both mild and severe COVID-19 are associated with
changes in number and migratory potential of dendritic cells for at least 7 months post infection [43]. We do not know if the transient changes in expression of CCR2 and CX3CR1 on circulating monocytes 1-3 months after infection are due to emigration of cells with the highest expression of those markers to inflamed or damaged tissues, however, CCL2 has been implicated in recruitment of monocyte to the lungs during infection [44]. The observation that decreasing CX3CR1 is associated with the level of SARS-CoV-2 specific T cells may imply that there is a relationship between immune responsiveness and innate immune activation. These observations in combination with the elevated expression of monocyte CD11b, which increases during acute and chronic inflammation and alters monocyte migration and adherence to the vasculature[45], imply even though the symptoms of mild COVID-19 infection may resolve in weeks, immune activation persists for at least 1-3 months.

Collectively, these data provide evidence that mild, and in some individuals even asymptomatic SARS-CoV-2 infections, can lead to sustained immune activation after resolution of symptoms, which is not observed in response to other mild respiratory infections. Whether this immune activation is more pronounced in patients with long-COVID, or more serious infections, remains to be seen.

Acknowledgements

The authors would like to thank Dr. Brian Dixon, Dr. Marc Aucoin, Dr. Mark Bruder and Dr. Aaron Frenette (University of Waterloo) for the recombinant antigens used in the serology assays, and Carmen Venegas for assistance with participant recruitment and data collection.
References

1. Alon R, Sportiello M, Kožlović S, Kumar A, Reilly EC, Zarbock A, et al. Leukocyte trafficking to the lungs and beyond: lessons from influenza for COVID-19. Nature Reviews Immunology. 2021;21(1):49-64. doi: 10.1038/s41577-020-00470-2.

2. Huang I, Pranata R. Lymphopenia in severe coronavirus disease-2019 (COVID-19): systematic review and meta-analysis. Journal of Intensive Care. 2020;8(1):36. doi: 10.1186/s40560-020-00453-4.

3. Chen Z, John Wherry E. T cell responses in patients with COVID-19. Nature Reviews Immunology. 2020;20(9):529-36. doi: 10.1038/s41577-020-0402-6.

4. de Candia P, Prattichizzo F, Garavelli S, Matrese G. T Cells: Warriors of SARS-CoV-2 Infection. Trends Immunol. 2021;42(1):18-30. Epub 2020/12/06. doi: 10.1016/j.it.2020.11.002. PubMed PMID: 33277181; PubMed Central PMCID: PMCPMC7664351.

5. Zhang D, Guo R, Lei L, Liu H, Wang Y, Wang Y, et al. COVID-19 infection induces readily detectable morphologic and inflammation-related phenotypic changes in peripheral blood monocytes. Journal of leucocyte biology. 2020.

6. Prescott HC, Girard TD. Recovery From Severe COVID-19: Leveraging the Lessons of Survival From Sepsis. JAMA. 2020;324(8):739-40. doi: 10.1001/jama.2020.14103.

7. Greenhalgh T, Knight M, A'Court C, Buxton M, Husain L. Management of post-acute covid-19 in primary care. BMJ. 2020;370:m3026. doi: 10.1136/bmj.m3026. PubMed PMID: 33325566; PubMed Central PMCID: PMCPMC7707210.

8. Ayoubkhani D, Khunti K, Nafilyan V, Maddox T, Humberstone B, Diamond I, et al. Post-covid syndrome in individuals admitted to hospital with covid-19: retrospective cohort study. BMJ. 2021;372:n693. doi: 10.1136/bmj.n693.

9. Chopra V, Flanders SA, O’Malley M, Malani AN, Prescott HC. Sixty-Day Outcomes Among Patients Hospitalized With COVID-19. Ann Intern Med. 2020. Epub 2020/11/12. doi: 10.7326/m20-5661. PubMed PMID: 33175566; PubMed Central PMCID: PMCPMC7707210.

10. Donnelly JP, Wang XQ, Iwashyna TJ, Prescott HC. Readmission and Death After Initial Hospital Discharge Among Patients With COVID-19 in a Large Multihospital System. JAMA. 2020. doi: 10.1001/jama.2020.21465.

11. Ayoubkhani D, Khunti K, Nafilyan V, Maddox T, Humberstone B, Diamond SI, et al. Epidemiology of post-COVID syndrome following hospitalisation with coronavirus: a retrospective cohort study. medRxiv. 2021;2021.01.15.21249885. doi: 10.1101/2021.01.15.21249885.

12. Sekine T, Perez-Potti A, Rivera-Ballesteros O, Strålin K, Gorin JB, Olsson A, et al. Robust T Cell Immunity in Convalescent Individuals with Asymptomatic or Mild COVID-19. Cell. 2020;183(1):58-68 e14. Epub 2020/09/28. doi: 10.1016/j.cell.2020.08.017. PubMed PMID: 32979941; PubMed Central PMCID: PMCPMC7427556.

13. Sekine T, Perez-Potti A, Rivera-Ballesteros O, Strålin K, Gorin J-B, Olsson A, et al. Robust T Cell Immunity in Convalescent Individuals with Asymptomatic or Mild COVID-19. Cell. 2020;183(1):58-68.e14. doi: https://doi.org/10.1016/j.cell.2020.08.017.

14. Verschoor CP, Kohli V, Balion C. A comprehensive assessment of immunophenotyping performed in cryopreserved peripheral whole blood. Cytometry B Clin Cytom. 2018;94(5):662-70. Epub 2017/04/06. doi: 10.1002/cyto.b.21526. PubMed PMID: 28378896.

15. Huynh A, Arnold DM, Smith JW, Moore JC, Zhang A, Chagla Z, et al. Characteristics of Anti-SARS-CoV-2 Antibodies in Recovered COVID-19 Subjects. Viruses. 2021;13(4):697. PubMed PMID: doi:10.3390/v13040697.
16. Zaunders JJ, Munier ML, Seddiki N, Pett S, Ip S, Bailey M, et al. High Levels of Human Antigen-Specific CD4+ T Cells in Peripheral Blood Revealed by Stimulated Coexpression of CD25 and CD134 (OX40). The Journal of Immunology. 2009;183(4):2827-36. doi: 10.4049/jimmunol.0803548.

17. Seddiki N, Cook L, Hsu DC, Phetsouphanh C, Brown K, Xu Y, et al. Human antigen-specific CD4(+) CD25(+) CD134(+) T cells are enriched for regulatory T cells and comprise a substantial proportion of recall responses. Eur J Immunol. 2014;44(6):1644-61. Epub 2014/04/23. doi: 10.1002/eji.20134102. PubMed PMID: 24752698.

18. Wolfl M, Kuball J, Ho WY, Nguyen H, Manley TJ, Bleakley M, et al. Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. Blood. 2007;110(1):201-10. Epub 2007/03/21. doi: 10.1182/blood-2006-11-056168. PubMed PMID: 17371945; PubMed Central PMCID: PMCPMC1896114.

19. Loukov D, Karampatos S, Maly MR, Bowdish DME. Monocyte activation is elevated in women with knee-osteoarthritis and associated with inflammation, BMI and pain. Osteoarthritis Cartilage. 2018;26(2):255-63. Epub 2017/11/13. doi: 10.1016/j.joca.2017.10.018. PubMed PMID: 29128509.

20. Ontario. OAfHPaPPH. COVID-19 Sero surveillance Summary - Seroprevalence in Ontario: March 27, 2020 to June 30, 2020. In: Ontario PH, editor. Toronto: Queen's Printer for Ontario; 2020.

21. Canada Go. Respiratory Virus Report, week 34 - ending August 22, 2020 2020. Available from: https://www.canada.ca/en/public-health/services/surveillance/respiratory-virus-detections-canada/2019-2020/week-34-ending-august-22-2020.html.

22. Sadler R, Bateman EA, Heath V, Patel SY, Schwingshakl PP, Cullinane AC, et al. Establishment of a healthy human range for the whole blood "OX40" assay for the detection of antigen-specific CD4+ T cells by flow cytometry. Cytometry B Clin Cytom. 2014;86(5):350-61. Epub 2014/05/16. doi: 10.1002/cyto.b.21165. PubMed PMID: 24827553.

23. Zaunders JJ, Munier ML, Seddiki N, Pett S, Ip S, Bailey M, et al. High levels of human antigen-specific CD4+ T cells in peripheral blood revealed by stimulated coexpression of CD25 and CD134 (OX40). J Immunol. 2009;183(4):2827-36. Epub 2009/07/29. doi: 10.4049/jimmunol.0803548. PubMed PMID: 19635903.

24. Mateus J, Grifoni A, Tarke A, Sidney J, Ramirez SI, Dan JM, et al. Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans. Science. 2020;370(6512):89-94.

25. Honigsbaum M, Krishnan L. Taking pandemic sequelae seriously: from the Russian influenza to COVID-19 long-haulers. Lancet. 2020;396(10260):1389-91. Epub 2020/10/16. doi: 10.1016/s0140-6736(20)32134-6. PubMed PMID: 33058777; PubMed Central PMCID: PMCPMC7550169.

26. Maheb E. Covid-19: What do we know about "long covid"? Bmj. 2020;370:m2815. Epub 2020/07/16. doi: 10.1136/bmj.m2815. PubMed PMID: 32665317.

27. Kratofil RM, Kubes P, Deniset JF. Monocyte Conversion During Inflammation and Injury. Arteriosclerosis, Thrombosis, and Vascular Biology. 2015;35(6):1306-16. doi: 10.1161/ATVBAHA.114.304650.

28. Al-Aly Z, Xie Y, Bowe B. High-dimensional characterization of post-acute sequelae of COVID-19. Nature. 2021. doi: 10.1038/s41586-021-03553-9.
32. Rydzynski Moderbacher C, Ramirez SI, Dan JM, Grifoni A, Hastie KM, Weiskopf D, et al. Antigen-Specific Adaptive Immunity to SARS-CoV-2 in Acute COVID-19 and Associations with Age and Disease Severity. Cell. 2020;183(4):996-1012.e19. Epub 2020/10/05. doi: 10.1016/j.cell.2020.09.038. PubMed PMID: 33010815; PubMed Central PMCID: PMCPMC7494270.

33. Sanchez AM, Zhu J, Huang X, Yang Y. The Development and Function of Memory Regulatory T Cells after Acute Viral Infections. The Journal of Immunology. 2012;189(6):2805-14. doi: 10.4049/jimmunol.1200645.

34. Betts RJ, Prabhu N, Ho AWS, Lew FC, Hutchinson PE, Rotzschke O, et al. Influenza A Virus Infection Results in a Robust, Antigen-Responsive, and Widely Disseminated Foxp3+ Regulatory T Cell Response. Journal of Virology. 2012;86(5):2817-25. doi: 10.1128/jvi.05685-11.

35. Bastard P, Rosen LB, Zhang Q, Michailidis E, Hoffmann HH, Zhang Y, et al. Autoantibodies against type I IFNs in patients with life-threatening COVID-19. Science. 2020;370(6515). Epub 2020/09/26. doi: 10.1126/science.abd4585. PubMed PMID: 32972996; PubMed Central PMCID: PMCPMC7857397.

36. Verdoni L, Mazza A, Gervasoni A, Martelli L, Ruggeri M, Ciuffreda M, et al. An outbreak of severe Kawasaki-like disease at the Italian epicentre of the SARS-CoV-2 epidemic: an observational cohort study. Lancet. 2020;395(10239):1771-8. Epub 2020/05/16. doi: 10.1016/s0140-6736 (20) 31103-x. PubMed PMID: 32410760; PubMed Central PMCID: PMCPMC7220177.

37. Ramos-Casals M, Brito-Zeron P, Mariette X. Systemic and organ-specific immune-related manifestations of COVID-19. Nature Reviews Rheumatology. 2021. doi: 10.1038/s41584-021-00608-z.

38. Lanteri MC, O’Brien KM, Purtha WE, Cameron MJ, Lund JM, Owen RE, et al. Tregs control the development of symptomatic West Nile virus infection in humans and mice. The Journal of Clinical Investigation. 2009;119(10):2866-77. doi: 10.1172/JCI39387.

39. Stukalov A, Girault V, Grass V, Karayol O, Bergant V, Urban C, et al. Multilevel proteomics reveals host perturbations by SARS-CoV-2 and SARS-CoV. Nature. 2021. Epub 2021/04/13. doi: 10.1038/s41586-021-03493-4. PubMed PMID: 33845483.

40. Sun X, Cui Y, Feng H, Liu H, Liu X. TGF-β signaling controls Foxp3 methylation and T reg cell differentiation by modulating Uhrf1 activity. Journal of Experimental Medicine. 2019;216(12):2819-37. doi: 10.1084/jem.20190550.

41. Lüke F, Orsó E, Kirsten J, Poock H, Grube M, Wolff D, et al. Coronavirus disease 2019 induces multilineage, morphologic changes in peripheral blood cells. ElHaem. 2020. Epub 2020/08/25. doi: 10.1002/jha2.44. PubMed PMID: 32838398; PubMed Central PMCID: PMCPMC7361732.

42. Schulte-Schrepping J, Reusch N, Paclik D, Baßler K, Schlickreiser S, Zhang B, et al. Severe COVID-19 Is Marked by a Dysregulated Myeloid Cell Compartment. Cell. 2020;182(6):1419-40.e23. Epub 2020/08/19. doi: 10.1016/j.cell.2020.08.001. PubMed PMID: 32810438; PubMed Central PMCID: PMCPMC7405822.

43. Pérez-Gómez A, Vitallé J, Gasca-Capoté C, Gutierrez-Valencia A, Trujillo-Rodriguez M, Serna-Gallego A, et al. Dendritic cell deficiencies persist seven months after SARS-CoV-2 infection. Cellular & Molecular Immunology. 2021. doi: 10.1038/s41423-021-00728-2.

44. McKechnie JL, Blish CA. The Innate Immune System: Fighting on the Front Lines or Fanning the Flames of COVID-19? Cell Host Microbe. 2020;27(6):863-9. Epub 2020/05/29. doi: 10.1016/j.chom.2020.05.009. PubMed PMID: 32464098; PubMed Central PMCID: PMCPMC7237895.

45. Miller LJ, Bainton DF, Borregaard N, Springer TA. Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. The Journal of clinical investigation. 1987;80(2):535-44. doi: 10.1172/JCI113102. PubMed PMID: 3038962.
Supporting information

Supplementary Figure 1. Gating strategies for flow cytometry data analysis
Supplementary Table 1. Criteria and identification of individuals with SARS-CoV-2 and other respiratory infections
Supplementary Table 2. Fluorophore-conjugated antibodies used for flow cytometry
Supplementary Table 3. Summary of SARS-CoV-2 peptivator AIMS T cell activation observations
Supplementary Table 4. All immune parameter data analysis
Supplementary Table 5. Monocyte surface receptor expression as Mean Fluorescence Intensity (MFI)
Figure 1

A)  

CD4+ T cells:  
- Unstimulated: 0.77% (M), 0% (N), 2.88% (S)  
- Positive control/Cytostim: 27.0% (M), 28.8% (N), 6.57% (S)  
- M peptivator pool: 5.92% (M), 0.54% (N), 0.06% (S)  
- N peptivator pool: 6.06% (M), 1.23% (N), 0.06% (S)  
- S peptivator pool: 5.82% (M), 0.06% (N), 0.06% (S)  

CD8+ T cells:  
- Unstimulated: 0.01% (M), 11.0% (N), 0.26% (S)  
- Positive control/Cytostim: 37.6% (M), 6.57% (N), 0.22% (S)  
- M peptivator pool: 92.6% (M), 0.94% (N), 0.12% (S)  
- N peptivator pool: 91.1% (M), 1.64% (N), 0.54% (S)  
- S peptivator pool: 93.1% (M), 1.23% (N), 0.01% (S)  

B)  

% of CD4+ T cells:  
- M: 11.0% (1-3 months), 0% (6-9 months)  
- N: 0.26% (1-3 months), 0.06% (6-9 months)  
- S: 0.12% (1-3 months), 0.01% (6-9 months)  

% of CD8+ T cells:  
- M: 0% (1-3 months), 0.06% (6-9 months)  
- N: 0% (1-3 months), 0.06% (6-9 months)  
- S: 0% (1-3 months), 0.06% (6-9 months)  

Other respiratory infection:

- 1-3 months post-COVID infection  
- 6-9 months post-COVID infection
Figure 2

A) CRP (ng/mL)

B) TNF (pg/mL)

C) IL-6 (pg/mL)
**Figure 4**

A) CD45+ Absolute Count (x10^6) /mL

B) CD4+ Absolute Count (x10^5) /mL

C) CD8+ Absolute Count (x10^6) /mL

D) NK Cells

E) CD4+

- Other Respiratory Infection
- 1-3 months Post-COVID
- 6-9 months Post-COVID

- Naive (CD45RA+ CCR7+)
- Central Memory (CD45RA- CCR7+)
- Effector Memory (CD45RA- CCR7-)
- EMRA (CD45RA+ CCR7-)
- TD (CD45RA+ CCR7- CD57+ CD28-)

p = 0.017

F) CD8+

- Other Respiratory Infection
- 1-3 months Post-COVID
- 6-9 months Post-COVID

- Naive (CD45RA+ CCR7+)
- Central Memory (CD45RA- CCR7+)
- Effector Memory (CD45RA- CCR7-)
- EMRA (CD45RA+ CCR7-)
- TD (CD45RA+ CCR7- CD57+ CD28-)

p = 0.048

G) Regulatory T cells

- % CD4+ T cells
Figure 5

A) Myeloid/Lymphoid

| Months Post-COVID | Other | 1-3 | 6-9 |
|-------------------|-------|-----|-----|
| 0                 |       |     |     |
| Absolute Count (10^6) /mL |       |     |     |

B) Neutrophils

| Months Post-COVID | Other | 1-3 | 6-9 |
|-------------------|-------|-----|-----|
| 0                 |       |     |     |
| Absolute Count (10^6) /mL |       |     |     |

C) Monocytes

| Months Post-COVID | Other | 1-3 | 6-9 |
|-------------------|-------|-----|-----|
| 0                 |       |     |     |
| Absolute Count (10^6) /mL |       |     |     |

D) CX3CR1

- All Monocytes
- Classical Monocytes (CD14+CD16-)
- Intermediate Monocytes (CD14+CD16+)
- Non-classical Monocytes (CD14lowCD16+)
- Neutrophils

E) Cytostim

- M Peptivator
- N Peptivator
- S Peptivator

CD8+ Activation

- (CD69+CD137+)

CD4+ Activation

- (CD25+OX40+)

A) Myeloid/Lymphoid

B) Neutrophils

C) Monocytes