The C-C motif chemokine ligand 3 like 3, CCL3L3, is differentially expressed in the blood in cases of viral co-infection.

Shahan Mamoor, MS¹

¹shahanmamoor@gmail.com

East Islip, NY 11730

Co-infection is defined by the infection of a host or cell by more than one pathogen^{1,2}. Infections from the novel coronavirus severe acute respiratory syndrome coronavirus 2, SARS-CoV-2³, have resulted in the death of more than 200,000 Americans⁴ and over 1,000,000 people worldwide⁵ in less than one year. An upcoming influenza season raises the prospect of potential complications arising from co-infections involving SARS-CoV-2⁶. We mined published microarray data⁷ to discover transcriptional features of viral co-infection in the blood of human patients. We identified the C-C motif chemokine ligand 3 like 3, CCL3L3, as among the genes whose expression was most different in the blood of patients with a variety of viral co-infections, including co-infection of human coronavirus OC43 and influenza A. Transcriptional changes in CCL3L3 appeared to be specific to co-infection settings. CCL3L3 may be of relevance to fundamental biological processes underlying viral co-infections of human blood.

Keywords: viral co-infection, CCL3L3, SARS-CoV-2, coronavirus, influenza virus, systems biology of viral infection.

1 The most basic relationship between host and pathogen is a simple infection between one
2 pathogen and one host or cell. Re-infection, or a second infection of a host with the same
3 pathogen despite an initial immune response can occur⁸. Super-infection, a process by which a
4 bacterial infection complicates an existing viral infection, such as those found in the respiratory
5 tract, can occur^{9,10}. A third type of complex infection setting is known as co-infection, a process
6 by which a cell or organism already infected with a virus is then infected with a second, different
7 virus^{1,2}. The novel coronavirus SARS-CoV-2 has resulted in the death of over 200,000
8 Americans in less than one year^{3,4}. The upcoming influenza season raises the prospect of
9 complications resulting from influenza and SARS-CoV-2 co-infection. A recent study of patients
10 in Northern California found that of 116 patients testing positive for a SARS-CoV-2, 24 (20.7%)
11 possessed a co-infection⁶. Little is known about the transcriptional changes that accompany
12 coronavirus co-infection. We mined published microarray data⁷ to understand in an unbiased
13 fashion and at the systems-level the most significant gene expression changes in the blood of
14 patients with coronavirus co-infections, as well as in the blood of patients with other viral co-
15 infections not involving a human coronavirus. We observed significant, recurrent transcriptional
16 changes in the C-C motif chemokine ligand 3 like 3, CCL3L3, that were among the largest gene
17 expression changes transcriptome-wide in the blood of multiple patients with viral co-infection.
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25 **Methods**

26 We used dataset GSE68310⁷ for this differential gene expression analysis of the blood in
27 patients with viral co-infections. GSE68310 was generated using Illumina HumanHT-12 V4.0
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1 expression beadchip technology; analysis was performed using platform GPL10558. In the
2 study from which this microarray data was obtained, investigators collected peripheral blood
3 from patients at baseline, day 0 of infection, correlating with a visit to the clinic upon infection
4 with a respiratory illness; day 2, day 4, day 6, and day 21 of infection following the clinic visit,
5 as well as in the season following infection (“Spring”). We compared the blood of $n=1$ patient
6 with respiratory syncytial virus A (RSVA) to the blood of $n=1$ patient with co-infection of RSVA
7 and human rhinovirus. We compared the blood of $n=1$ patient with enterovirus (ID: JR0471) to
8 the blood of $n=1$ patient with co-infection of enterovirus and human rhinovirus (ID:JR0209). We
9 compared the blood of $n=1$ patient with Influenza A infection (ID: JR0021) with the blood of
10 $n=1$ patient with human coronavirus HCoV OC43 and Influenza A co-infection (ID: JR1829).
11 We compared the blood of $n=1$ patient with Influenza A infection (ID: JR0021) with the blood of
12 $n=1$ patient with human coronavirus HCoV 229E and Influenza A co-infection (ID: JR0173). We
13 compared the blood of $n=4$ patients with influenza A infection only at baseline, day 0, day 2, day
14 4, day 6, day 21, and Spring (IDs: JR0021, JR0023, JR0035 and JR0053). We compared the
15 blood of patients with $n=6$ patients with rhinovirus infection only (IDs: JR0148, JR0154,
16 JR0223, JR0228, JR0276, JR0287) at baseline, day 0, day 2, day 4, day 6, day 21 and Spring.
17 The Benjamini and Hochberg method of p -value adjustment was used for ranking of differential
18 expression but raw p -values were used for assessment of statistical significance of global
19 differential expression. Log-transformation of data was auto-detected, and the NCBI generated
20 category of platform annotation was used. A statistical test was used to evaluate the significance
21 of difference in CCL3L3 gene expression between the blood of a patient with a single infection
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and the blood of a patient with co-infection, using a two-tailed, unpaired t-test with Welch's correction. All statistical tests were performed with PRISM 8.4.0 (455).

Results

We mined published microarray data⁷ to determine in an unbiased fashion and at the systems-level the most significant transcriptional changes in the blood of patients with viral co-infection.

CCL3L3 is differentially expressed and at increased levels in the blood of a patient with co-infection of RSVA and human rhinovirus.

We identified the gene encoding the C-C motif chemokine ligand 3 like 3, CCL3L3, as among the most differentially expressed in the blood of a patient with co-infection of RSVA and human rhinovirus when compared to the blood of a patient with infection of RSVA only (Table 1). When sorting each of the genes expressed in the blood during RSVA and human rhinovirus co-infection based on significance of change in expression as compared to RSVA infection only, CCL3L3 ranked 41 out of 47254 total transcripts (Table 1), equating to 99.9% differential expression. Differential expression of CCL3L3 in RSVA and human rhinovirus co-infection was statistically significant (Table 1; $p=1.31\text{E-}07$). CCL3L3 was expressed at significantly higher levels in the blood during co-infection of RSVA and human rhinovirus as compared to that of a patient with infection of RSVA only (Figure 1; $p<0.0001$). We calculated a mean fold change of 1.0308 ± 0.0080 in CCL3L3 levels in the blood during co-infection of RSVA and human rhinovirus (Table 1).

CCL3L3 is differentially expressed and at decreased levels in the blood of a patient with co-infection of enterovirus and human rhinovirus.

We also observed significant transcriptome-wide differential expression of CCL3L3 in the blood of a patient with co-infection of enterovirus and human rhinovirus when compared to the blood of a patient with infection of enterovirus only (Table 2). When sorting each of the genes expressed in the blood during enterovirus and human rhinovirus co-infection based on significance of change in expression as compared to enterovirus infection only, CCL3L3 ranked 7 out of 47254 total transcripts (Table 2), equating to 99.98% differential expression.

Differential expression of CCL3L3 in enterovirus and human rhinovirus co-infection was statistically significant (Table 2; $p=2.41E-11$). CCL3L3 was expressed at significantly lower levels in the blood during co-infection of human rhinovirus and enterovirus infection as compared to that of a patient with infection of enterovirus only (Figure 2; $p<0.0001$). We calculated a mean fold change of 0.8967 ± 0.0031 in CCL3L3 levels in the blood during co-infection of enterovirus and human rhinovirus (Table 1).

CCL3L3 is differentially expressed in the blood of a patient with co-infection of influenza A and human coronavirus OC43.

We asked whether differential expression of CCL3L3 could be observed in co-infections involving a human coronavirus. We again found that CCL3L3 was among the most differentially expressed genes in the blood during co-infection, here in the blood of a patient with co-infection of HCoV OC43 and influenza A when compared to the blood of a patient with infection of

1 influenza A only (Table 3). When sorting each of the genes expressed in the blood during HCoV
2 OC43 and influenza A co-infection based on significance of change in expression as compared to
3 influenza A infection only, CCL3L3 ranked 2848 out of 47254 total transcripts (Table 3),
4 equating to 94.0% differential expression. Differential expression of CCL3L3 in HCoV OC43
5 and influenza A co-infection was statistically significant (Table 3; $p=3.91E-02$).
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9 CCL3L3 is differentially expressed in the blood of a patient with co-infection of influenza A and
10 human coronavirus 229E.

11 Further analysis of patient blood during viral co-infection revealed that CCL3L3 was
12 among the most differentially expressed genes in the blood of a patient with a different human
13 coronavirus: here, in co-infection of influenza A and human coronavirus-229E when compared to
14 the blood of a patient with infection of influenza A only (Table 4). When sorting each of the
15 genes expressed in the blood during influenza A and human coronavirus 229E co-infection based
16 on significance of change in expression as compared to influenza A infection only, CCL3L3
17 ranked 485 out of 47254 total transcripts (Table 4), equating to 99.0% differential expression.
18 Differential expression of CCL3L3 in influenza A and HCoV-229E co-infection was statistically
19 significant (Table 4; $p=1.52E-03$).
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22 After finding that CCL3L3 was among the most differentially expressed genes in the
23 blood during four separate types of viral co-infections, we asked whether differential expression
24 of CCL3L3 was specific to viral co-infection or could be observed in patient blood during the
25 course of infection with a single virus.
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CCL3L3 is not differentially expressed in patient blood during infection with influenza A.

We probed microarray data from the blood of four patients with influenza A infection only, comparing total gene expression at baseline, day 0, day 2, day 4, day 6, day 21 and the following Spring, to determine if expression of CCL3L3 was significantly modulated during the course of influenza A infection in the absence of co-infection. CCL3L3 was not differentially expressed in human blood during influenza A infection (Table 5; $p=5.21E-01$). When sorting each of the genes expressed in the blood during the course of influenza A infection based on significance of change in expression as compared to baseline, CCL3L3 ranked 29625 out of 47254 total transcripts (Table 5), equating to 37.3% differential expression.

CCL3L3 is not differentially expressed in patient blood during infection with human rhinovirus.

We also probed microarray data from the blood of six patients with infection of human rhinovirus only, comparing total gene expression at baseline, day 0, day 2, day 4, day 6, day 21 and the following Spring, to determine if expression of CCL3L3 was significantly modulated during the course of human rhinovirus infection in the absence of co-infection. CCL3L3 was not differentially expressed in human blood during human rhinovirus infection (Table 6; $p=9E-01$). When sorting each of the genes expressed in the blood during the course of human rhinovirus infection based on significance of change in expression as compared to baseline, CCL3L3 ranked 43288 out of 47254 total transcripts (Table 6), equating to 8.4% differential expression.

1 Together, the data indicated that CCL3L3 was differentially expressed in the blood of
2 patients with four different types of viral co-infections, including co-infections involving
3 influenza A and human rhinovirus, but not during the course of influenza A or human rhinovirus
4 infections in the absence of co-infection. Though it is difficult to draw definite conclusions from
5 comparison of patient blood due to inherent variability in gene expression from patient to patient,
6 together with data demonstrating that CCL3L3 was not differentially expressed in patient blood
7 when studying whole blood transcriptome across time during the course of patients with single
8 infections these data argue that differential expression of CCL3L3 was specific to viral co-
9 infections in the blood.
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13 Thus, together, we conclude that CCL3L3 is among the genes whose expression is most
14 most significantly different transcriptome-wide in the blood of patients with viral co-infections,
15 including co-infections with human coronavirus OC43 and influenza A, human coronavirus 229E
16 and influenza A, RSVA and human rhinovirus, as well as enterovirus and human rhinovirus.
17 Differential expression of CCL3L3 appears to be specific to viral co-infection settings, as it does
18 not occur, across the course of infection in the blood of patients with single viral infection of
19 influenza A or human rhinovirus.
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23 **Discussion**

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25 CCL3L3 is a paralog of CCL3, also known as MIP1 α , a chemokine of the CC
26 subfamily¹¹. CCL3 was identified in 1988 as part of a protein doublet from mouse macrophages
27 stimulated with lipopolysaccharide (LPS)^{12,13}, and later distinguished as MIP1 α and MIP1 β ^{12,14}.
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1 CCL3L3 are both alternatively referred to as LD78β¹⁵. LD78β is a potent chemokine agonist of
2 CCR5, its cleavage by by CD26/dipeptidyl peptidase results in its conversion to a CCR1
3 agonist¹⁶. LD78β can function as a chemoattractant of lymphocytes and monocytes^{15,16}. While
4 MIP1α/LD78β functions to specifically recruit CD8+ T-lymphocytes, MIP1β functions to
5 specifically recruit CD4+ T-lymphocytes¹⁷. Importantly in this context, LD78β through its
6 action at the CCR5 HIV-1 co-receptor can function to inhibit HIV-1 infection¹⁸. In HL-60 cells
7 and in neutrophils, CCL3L3 is up-regulated following infection with Anaplasma
8 phagocytophilum¹⁹. In Ukrainian children, copy numbers of CCL3L genes has a significant
9 effect on HIV-1 transmission: if a mother and her offspring possessed a gene dose lower than
10 two, the risk of virus transmission increased 10-13 fold¹⁹. Moreover, the rate of progression to
11 AIDS in HIV-positive children with CCL3 copy number lower than two was nearly two times
12 faster¹⁹. Thus, in at least one important example of viral infection, CCL3 genes are important for
13 the process of viral infection and for viral pathogenesis in humans.

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19 We found that CCL3L3 was among the genes most differentially expressed when
20 comparing the blood of patients with viral co-infections, in four separate types of co-infection,
21 including co-infections involving a human coronavirus: influenza A and HCoV OC43, influenza
22 A and HCoV-229E, respiratory syncytial virus A and human rhinovirus, as well as in enterovirus
23 and human rhinovirus co-infection. CCL3L3 has important roles in immunity functioning as a
24 chemoattractant to cells of the immune system, including cytotoxic CD8+ T-lymphocytes, and
25 can function to inhibit HIV-1 infection. CCL3L3 may have specific roles in the context of viral
26 co-infection of cells of the hematopoietic system.

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Rank	ID	p-value	t	B	FC	Gene	Gene name
41	ILMN_2105573	1.31E-07	9.87	7.94273	1.0308 ± 0.0080	CCL3L3	C-C motif chemokine ligand 3 like 3

Table 1: Differential expression of CCL3L3 in the blood of a patient with co-infection of RSVA and human rhinovirus as compared to that of a patient with RSVA infection.

The rank of differential expression relative to all transcripts measured, probe ID, *p*-value with respect to global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the patients compared, fold change of CCL3L3 expression in the blood of a patient with RSVA and human rhinovirus co-infection relative to that of a patient with infection of RSVA only, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
7	ILMN_2105573	2.41E-11	-19.1290146	15.376548	0.8967 ± 0.0031	CCL3L3	C-C motif chemokine ligand 3 like 3

Table 2: Differential expression of CCL3L3 in the blood of a patient with co-infection of enterovirus and human rhinovirus as compared to that of a patient with enterovirusinfection.

The rank of differential expression relative to all transcripts measured, probe ID, *p*-value with respect to global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the patients compared, fold change of CCL3L3 expression in the blood of a patient with influenza A and enterovirus co-infection relative to that of a patient with infection of enterovirus only, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
2848	ILMN_2105573	3.91E-02	2.2852781	-4.2302	CCL3L3	C-C motif chemokine ligand 3 like 3

Table 3: Differential expression of CCL3L3 in the blood of a patient with co-infection of influenza A and human coronavirus OC43 as compared to that of a patient with influenza A infection.

The rank of differential expression relative to all transcripts measured, probe ID, *p*-value with respect to global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the patients compared, fold change of CCL3L3 expression in the blood of a patient with influenza A and HCoV OC43 co-infection relative to that of a patient with infection of influenza A only, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
485	ILMN_2105573	1.52E-03	-3.9557088	-1.17294	CCL3L3	C-C motif chemokine ligand 3 like 3

Table 4: Differential expression of CCL3L3 in the blood of a patient with co-infection of influenza A and human coronavirus 229E as compared to that of a patient with influenza A infection.

The rank of differential expression relative to all transcripts measured, probe ID, *p*-value with respect to global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the patients compared, fold change of CCL3L3 expression in the blood of a patient with influenza A and HCoV 229E co-infection relative to that of a patient with infection of influenza A only, gene and gene name are listed in this chart.

Rank	ID	p-value	F	Gene	Gene name
29625	ILMN_2105573	5.21E-01	0.8865	CCL3L3	C-C motif chemokine ligand 3 like 3

Table 5: CCL3L3 is not differentially expressed during the course of infection in the blood of patients with infection of influenza A.

The rank of differential expression relative to all transcripts measured, probe ID, *p*-value with respect to global differential expression, F statistic, gene and gene name are listed in this chart.

Rank	ID	p-value	F	Gene	Gene name
43288	ILMN_2105573	9E-01	0.35815	CCL3L3	C-C motif chemokine ligand 3 like 3

Table 6: CCL3L3 is not differentially expressed during the course of infection in the blood of patients with infection of human rhinovirus.

The rank of differential expression relative to all transcripts measured, probe ID, *p*-value with respect to global differential expression, F statistic, gene and gene name are listed in this chart.

CCL3L3 Respiratory Syncytial Virus A (RSVA) vs. RSVA and Rhinovirus

Figure 1: CCL3L3 is expressed at significantly higher levels in the blood of a patient with RSVA and human rhinovirus infection as compared to that of a patient with RSVA infection only.

Messenger RNA levels of CCL3L3 are graphically depicted in the blood of a patient with RSVA infection (left) and in the blood of a patient with co-infection of RSVA and human rhinovirus (right), with the result of a statistical test evaluating significance of difference in CCL3L3 expression, a *p*-value, listed above.

Figure 2: CCL3L3 is expressed at significantly lower levels in the blood of a patient with enterovirus and human rhinovirus infection as compared to that of a patient with enterovirus infection only.

Messenger RNA levels of CCL3L3 are graphically depicted in the blood of a patient with enterovirus infection (left) and in the blood of a patient with co-infection of enterovirus and human rhinovirus co-infection (right), with the result of a statistical test evaluating significance of difference in CCL3L3 expression, a *p*-value, listed above.