

1 **Distinct lung-homing receptor expression and activation profiles**
2 **on NK cell and T cell subsets in COVID-19 and influenza**

3

4 **Short title: Lung homing receptor expression in respiratory viral infections**

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6 Demi Brownlie¹, Inga Rødahl¹, Renata Varnaite¹, Hilmir Asgeirsson^{2,3}, Hedvig Glans^{2,4}, Sara
7 Falck-Jones⁵, Sindhu Vangeti⁵, Marcus Buggert¹, Hans-Gustaf Ljunggren¹, Jakob
8 Michaëlsson¹, Sara Gredmark-Russ^{1,2}, Anna Smed-Sörensen⁵, Nicole Marquardt¹

9

10 ¹Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet,
11 Stockholm, Sweden

12 ²Department of Infectious Diseases, Karolinska University Hospital, Stockholm, Sweden

13 ³Division of Infectious Diseases, Department of Medicine Huddinge, Karolinska Institutet,
14 Stockholm, Sweden

15 ⁴Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden

16 ⁵Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet,
17 Karolinska University Hospital, Stockholm, Sweden

18

19 **Corresponding author:** Nicole Marquardt, Center for Infectious Medicine, Department of
20 Medicine, Karolinska Institutet, Stockholm, Sweden. Phone: 0046(0)8 524 843 22

21 E-mail: Nicole.Marquardt@ki.se, ORCID: <https://orcid.org/0000-0003-3186-4752>

22 **Abstract**

23 Respiratory viral infections with SARS-CoV-2 or influenza viruses commonly induce a strong
24 infiltration of immune cells into the lung, with potential detrimental effects on the integrity of
25 the lung tissue. Despite comprising the largest fractions of circulating lymphocytes in the lung,
26 little is known about how blood natural killer (NK) cells and T cell subsets are equipped for
27 lung-homing in COVID-19 and influenza. Using 28-colour flow cytometry and re-analysis of
28 published RNA-seq datasets, we provide a detailed comparative analysis of NK cells and T
29 cells in peripheral blood from moderately sick COVID-19 and influenza patients, focusing on
30 the expression of chemokine receptors known to be involved in leukocyte recruitment to the
31 lung. The results reveal a predominant role for CXCR3, CXCR6, and CCR5 in COVID-19 and
32 influenza patients, mirrored by scRNA-seq signatures in peripheral blood and bronchoalveolar
33 lavage from publicly available datasets. NK cells and T cells expressing lung-homing receptors
34 displayed stronger phenotypic signs of activation as compared to cells lacking lung-homing
35 receptors, and activation was overall stronger in influenza as compared to COVID-19.
36 Together, our results indicate migration of functionally competent CXCR3⁺, CXCR6⁺, and/or
37 CCR5⁺ NK cells and T cells to the lungs in moderate COVID-19 and influenza patients,
38 identifying potential common targets for future therapeutic interventions in respiratory viral
39 infections.

40 **Author summary**

41 The composition of in particular CXCR3⁺ and/or CXCR6⁺ NK cells and T cells is altered in
42 peripheral blood upon infection with SARS-CoV-2 or influenza virus in patients with moderate
43 disease. Lung-homing receptor-expression is biased towards phenotypically activated NK cells
44 and T cells, suggesting a functional role for these cells co-expressing in particular CXCR3
45 and/or CXCR6 upon homing towards the lung.

46

47 **Key words**

48 NK cells, T cells, chemokine receptors, lung, homing, SARS-CoV-2, COVID-19, influenza
49 virus

50 **Introduction**

51 The ongoing pandemic of coronavirus disease 19 (COVID-19), caused by the novel severe
52 acute respiratory syndrome coronavirus 2 (SARS-CoV-2), highlights the need for a better
53 understanding of respiratory viral infections which have the potential to cause recurrent
54 epidemics or pandemics. In addition to SARS-CoV-2, this also includes influenza virus,
55 respiratory syncytial virus (RSV), SARS-CoV, and the Middle East Respiratory Syndrome
56 (MERS)-CoV. Future disease outbreaks with novel viruses affecting the airways are to be
57 expected and prepared for.

58 During acute infections with respiratory viruses such as with SARS-CoV-2 or influenza,
59 specific chemokines mediating leukocyte recruitment are increased in the lung and
60 bronchoalveolar lavage (BAL) fluid. These chemokines include CCL2, CCL3, CCL20,
61 CXCL1, CXCL3, CXCL10, and IL8, attracting cells expressing chemokine receptors such as
62 CCR2, CCR5, CXCR3, and CXCR6 (1-3). In patients suffering from severe COVID-19, recent
63 reports suggest exacerbated lung tissue damage and epithelial cell death resulting from
64 hyperactivated immune cells, such as inflammatory macrophages (1), natural killer (NK) cells
65 (4) and/or T cells (1,5). While moderate disease in COVID-19 and in influenza patients is per
66 definition not fatal, patients may still require hospitalization and/or experience persistent long-
67 term symptoms such as fatigue, respiratory problems, loss of taste or smell, headache, and
68 diarrhea. Since lung-homing cytotoxic lymphocytes are likely involved in lung pathology
69 during acute infection, a better understanding of their major homing mechanisms will help in
70 developing and improving treatment strategies in COVID-19 and other respiratory viral
71 infections.

72 In this study, we investigated the composition of NK cell and T cell subsets which are
73 equipped with lung-homing properties in the peripheral blood of patients suffering from
74 moderate COVID-19 or influenza, and in healthy controls. In addition to analyses by 28-colour
75 flow cytometry, we assessed transcript expression in NK cells and T cells using three publicly

76 available single cell (sc)RNA-seq datasets of cells from peripheral blood and bronchoalveolar
77 lavage, respectively (6-8). Our data indicate a universal role for CXCR3-mediated lung-homing
78 of NK cells and T cells in COVID-19 and influenza and an additional role for recruitment via
79 CXCR6 and CCR5.

80 Together, we provide an extensive characterization of the lung-homing potential of
81 functional NK cells and T cells in homeostasis and acute respiratory viral infections such as
82 COVID-19 and influenza. The present results are of relevance for the understanding of the
83 disease progression and for identifying target molecules to improve future therapeutic treatment
84 strategies.

85 **Results**

86 **Altered frequencies of chemokine receptor-positive NK cell and T cell subsets in** 87 **peripheral blood in COVID-19 and influenza patients**

88 Chemokine receptors relevant for lung-homing such as CXCR3, CXCR6, CCR2, and CCR5,
89 could be identified in all detectable NK and T cell subsets in peripheral blood both from healthy
90 donors and COVID-19 patients (Fig. 1a, b; see Fig. S1a for gating strategies). The frequency
91 of chemokine receptor-positive NK cell and T cell subsets was decreased in COVID-19 patients
92 (Fig. 1a, b). Unbiased analysis of single chemokine receptors revealed a loss of CXCR2⁺,
93 CXCR3⁺, CXCR6⁺, and CCR2⁺ cells despite an increase in the frequency of CCR5⁺ NK cells
94 and T cells, respectively (Fig. 1c). When compared to influenza patients, we observed only
95 minor and non-significant differences for chemokine receptor expression on NK cells in both
96 diseases (Fig. 1d, e). Notably, a strong trend towards a loss of CXCR3⁺ NK cells in COVID-19
97 and influenza patients was observed (Fig. 1d, e). Lower frequencies of CXCR6⁺ NK cells were
98 accompanied with an increase in CCR5⁺CD56^{bright}CD16⁻ NK cells in influenza but not in
99 COVID-19 patients (Fig. 1d, e). In contrast to NK cells, in particular CD8⁺ T cells were
100 significantly affected both in COVID-19 and influenza patients (Fig. 1f, g). Similar to NK cells,
101 CXCR3⁺ T cells were markedly reduced most, both in COVID-19 and influenza. Additionally,
102 CXCR6⁺CD8⁺ T cells were reduced in influenza (Fig. 1f, g). Changes in chemokine receptor
103 expression were observed both in naïve and non-naïve CD4⁺ and CD8⁺ T cells (Fig. S2a, b;
104 gating strategy in Fig. S1a) and likewise when CD8⁺ TCM, TEM, and TEMRA cells were
105 compared (Fig. S2c). Unbiased principal component analysis (PCA) revealed segregation
106 between healthy controls and influenza patients for NK cells as well as between COVID-19
107 and influenza patients for T cells, respectively (Fig. 1h-j). The differences were largely driven
108 by CXCR3 and CCR5 for NK cells and by CCR5 on CD4⁺ T cells as well as by CCR2, CXCR3,
109 and CXCR6 on CD8⁺ T cells (Fig. 1i). Together, despite differences between NK cells and
110 CD8⁺ T cells, both lymphocyte subsets displayed a similar pattern in terms of CXCR3 and

111 CXCR6 expression in COVID-19 and influenza patients, with T cells being more strongly
112 affected.

113 In order to compare the present phenotypic results, with those of other data collections,
114 we analyzed two publicly available scRNA-seq datasets from peripheral blood from a different
115 cohort of SARS-CoV-2-infected patients with moderate COVID-19 disease (6) (Fig. 1k) and
116 from patients infected with either SARS-CoV-2 or influenza A virus (IAV) (7) (Fig. 1l),
117 respectively. In order to allow a fair comparison of the RNA-seq data to the present dataset, we
118 selectively analyzed data from patients with similar clinical characteristics. This analysis
119 revealed differences of chemokine receptor expression between NK cells and T cells at the
120 mRNA level in peripheral blood. While *CXCR2* was largely confined to NK cells, *CCR2* and
121 *CCR5* dominated in T cells, both in healthy controls and in COVID-19 patients (Fig. 1k) (6).
122 Lower average expression of *CXCR3* was found in all three lymphocyte subsets (NK cells,
123 CD4⁺ T cells, CD8⁺ T cells) in COVID-19 patients as compared to healthy controls (Fig. 1k).
124 However, in contrast to protein data, *CXCR6*, *CCR2*, and *CCR5* were strongly increased both
125 in frequency and in mean intensity in T cells in COVID-19 patients as compared to healthy
126 controls (Fig. 1k), suggesting post-transcriptional regulation of these chemokine receptors.
127 Finally, direct comparative analysis of transcript expression of *CXCR2*, *CXCR3*, *CXCR6*, and
128 *CCR5* revealed stronger loss of *CXCR2*, *CXCR3* and *CXCR6* in NK cells from influenza
129 patients as compared to COVID-19 patients as well as a trend towards upregulation of *CCR5*
130 (Fig. 1l) (7), in line with the similarly observed trend at the protein level (Fig. 1d, e) and
131 suggesting a role for these chemokine receptors particularly in influenza infection. While
132 *CXCR3*, *CXCR6*, *CCR2*, and *CCR5* are predominantly expressed on CD56^{bright}CD16⁻ NK cells
133 in peripheral blood from healthy controls and patients with COVID-19 or influenza,
134 respectively, *CXCR2* is strongly expressed on CD56^{dim}CD16⁺ blood NK cells (Fig. S2d, e). In
135 COVID-19 patients, *CXCR2*⁺CD56^{dim}CD16⁺ NK cells were mainly lost within the least
136 differentiated NKG2A⁺CD57⁻ subset (Fig. S2f). On T cells, *CXCR2* expression was overall

137 low, without significant differences between healthy controls and COVID-19 patients (Fig.
138 S2g, h).

139 Together, the present phenotypic analyses indicate that CXCR3 is a common lung-
140 homing receptor for both NK cells and T cell subsets during acute infection with COVID-19 or
141 influenza, despite major differences for *CXCR6*, *CCR2*, and *CCR5* between the two diseases at
142 transcriptional level. Furthermore, CXCR6⁺ NK and T cells were strongly affected in influenza
143 but not in COVID-19 patients, indicating potential differences in homing capacities in the two
144 diseases.

145

146 **Activation profiles in chemokine-receptor positive NK cells differ in COVID-19 and** 147 **influenza**

148 We and others previously demonstrated an activated phenotype in peripheral blood NK
149 cells and T cells in COVID-19 and influenza, respectively (4,9,10). Here, we aimed at
150 identifying NK and T cell activation markers in relation to expression of lung-homing receptors
151 as identified by boolean gating, combining cells expressing either CXCR3, CXCR6, CCR2, or
152 CCR5 (Fig. S1b, c). Expression of activation markers such as CD69, CD38, Ki67, and NKG2D
153 was elevated on NK cells in moderate COVID-19 patients (Fig. 2a, b), with strongest increases
154 detected for CD69 and Ki67 (Fig. 2b, c). In detail, induction of CD69 expression was highest
155 on CD56^{bright}CD16⁻ NK cells co-expressing lung-homing receptors, while Ki67 induction was
156 highest in corresponding CD56^{dim}CD16⁺ NK cells, particularly in those co-expressing lung-
157 homing receptors (Fig. 2c). Due to very low numbers of CD56^{bright}CD16⁻ NK cells lacking any
158 relevant chemokine receptor in healthy controls, no comparisons could be performed for this
159 subset. In contrast to NK cells from COVID-19 patients, upregulation of CD69 was highest in
160 CD56^{dim}CD16⁺ NK cells in influenza patients, irrespective of co-expression of lung-homing
161 receptors (Fig. 2d, e, f). Expression and upregulation of CD38 was similar in COVID-19 and
162 influenza patients (Fig 2c, f). While these data indicate fundamental differences in activation

163 patterns for NK cells in COVID-19 and influenza patients, other phenotypic characteristics
164 remained stable between the two diseases (Fig. S3a, b), despite induction of granzymes and
165 perforin observed in particular in chemokine receptor-positive CD56^{bright}CD16⁻ NK cells (Fig.
166 S3c-f). In the latter subset, upregulation of perforin expression was three times higher in
167 influenza patients as compared to COVID-19 patients (Fig. S3e, f), indicating stronger
168 activation of blood NK cells in influenza.

169 As indicated by transcript level, CXCR2 might have an additional role in lung-homing
170 for NK cells. Since activation affected both CD56^{dim} and CD56^{bright} NK cells in COVID-19
171 patients, we next sought to determine changes in CXCR2⁺ expression (Fig. 2g, h). In this regard,
172 expression of CXCR2 was higher on CD56^{dim}CD16⁺ NK cells lacking other lung-homing
173 receptors (Fig. 2g). The frequency of this CXCR2⁺CD56^{dim}CD16⁺ NK cell subset was
174 significantly reduced in COVID-19 patients as compared to healthy controls (Fig. 2h),
175 indicating an alternative migration mechanism for CXCR2⁺CD56^{dim}CD16⁺ NK cells to the lung
176 in COVID-19 patients.

177 Together, our data show that the activation patterns differ for NK cell subsets in
178 COVID-19 and influenza patients, respectively, with generally stronger activation of blood NK
179 cells in influenza as compared to moderate COVID-19 patients. Furthermore, the data suggest
180 that CXCR2 might act as an alternative lung-homing receptor for CD56^{dim}CD16⁺ NK cells
181 lacking other lung-homing receptors.

182

183 **Biased activation of T cells co-expressing lung-homing receptors in COVID-19 and** 184 **influenza**

185 Since NK cell subset activation in COVID-19 and influenza is associated with
186 chemokine receptor expression, we next determined whether T cells expressing lung-homing
187 receptors displayed an equivalent phenotypic bias towards stronger activation of chemokine
188 receptor-positive T cells (Fig. 3). Similar to NK cells, a larger proportion of CD4⁺ and CD8⁺ T

189 cells expressed CD69, both in COVID-19 (Fig. 3a, c) and influenza (Fig. 3b, c) compared to
190 healthy controls. While in COVID-19 patients CD69 upregulation was biased towards CD8⁺ T
191 cells co-expressing lung-homing receptors (Fig. 3a, c), upregulation was overall higher and
192 more uniform between the T cell subsets in influenza (Fig. 3b, c). Furthermore, CD38 was
193 strongly upregulated on CD8⁺ T cells in influenza but not COVID-19 patients (Fig. 3c). Finally,
194 expression of Ki67 was largely confined to chemokine receptor-positive T cells, both in healthy
195 controls and in COVID-19 patients (Fig. 3a). Upregulation of Ki67 was strongest in chemokine
196 receptor-positive CD8⁺ T cells (Fig. 3c), which is in line with strongest upregulation in
197 CD56^{dim}CD16⁺ chemokine receptor-positive NK cells (Fig. 2c), indicating a particular
198 activation of cytotoxic lymphocytes expressing lung-homing receptors in COVID-19 patients.

199 In comparison to NK cells where upregulation of granzymes and perforin was more
200 uniform between COVID-19 and influenza patients (Fig. S3), differences were more distinct
201 for T cells (Fig. 3d-f). As to be expected, expression of cytotoxic effector molecules was to a
202 large extent contained to cytotoxic CD8⁺ T cells (Fig. 3d, f), although some expression was also
203 observed in CD4⁺ T cells both in COVID-19 and influenza patients, respectively, with a
204 particular bias towards chemokine receptor-negative CD4⁺ T cells (Fig. 3e, f). Expression of
205 granzyme B and perforin was highest in chemokine receptor-negative CD8⁺ T cells in healthy
206 controls as well as in COVID-19 and influenza patients (Fig. 3d, e). Importantly, however,
207 lung-homing receptor-positive T cells displayed the highest increase compared to the same
208 subset in healthy controls (Fig. 3f).

209 Together, these data indicate an overall stronger *in vivo* priming of T cells and NK cells
210 in influenza patients as compared to moderate COVID-19 patients and also suggest a specific
211 role for cytotoxic lymphocytes co-expressing lung-homing receptors.

212

213 **Distinct CXCR3- and CXCR6-mediated accumulation of phenotypically primed NK cells**
214 **and T cells in BAL fluid of COVID-19 patients**

215 Activation of cytotoxic NK cells and CD8⁺ T cells in COVID-19 and influenza patients
216 was associated with high expression of effector molecules (Fig. S3, Fig. 3). Stratification of
217 cells based on expression of chemokine receptors, granzyme A, granzyme B, and perforin
218 revealed distinct co-expression patterns between CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺ NK cells
219 and CD8⁺ T cells as well as between COVID-19 and influenza (Fig. 4a). Shared between the
220 different subsets and between COVID-19 and influenza was an increase of all effector
221 molecules particularly on chemokine receptor-positive cells. Increased expression of effector
222 molecules could also be confirmed at transcriptional level both for NK cells (Fig. 4b) and CD8⁺
223 T cells (Fig. 4c) in peripheral blood of COVID-19 patients as compared to healthy controls (6).
224 Interestingly, levels of effector molecules gene transcripts were nearly identical between
225 ventilated (severe) and non-ventilated (moderate) COVID-19 patients, both in NK cells (Fig.
226 4b) and CD8⁺ T cells (Fig. 4c), indicating that similar NK and T cell activation is similar in
227 patients with moderate and severe disease.

228 The loss of NK cells and T cells expressing lung-homing receptors in the peripheral
229 blood of COVID-19 patients suggests migration of the respective cells to the infected lung
230 tissue. In order to identify characteristics of NK cells and T cells in the lung, we next used a
231 publicly available scRNAseq dataset from BAL fluid cells from COVID-19 patients (8) and
232 analyzed expression of transcripts of relevant chemokines in total BAL cells (Fig. 4d) as well
233 as chemokine receptor expression on NK cells and CD8⁺ T cells (Fig. 4e). High transcript levels
234 of a large number of chemokines were found in patients with severe disease, while moderate
235 patients were mainly distinguished from healthy patients by a significantly upregulated
236 expression of *CXCL10* and *CCL5*, in addition to increased levels of *CXCL11* and *CXCL16* (Fig.
237 4d), highlighting the role for CXCR3, CXCR6, and CCR5 in moderate COVID-19. In line with
238 these results, transcripts for *CXCR3*, *CXCR6*, and *CCR5* were highly enriched in NK cells as
239 well as CD4⁺ and CD8⁺ T cells in COVID-19 patients with moderate disease (Fig. 4e).
240 Although it is possible that some of the cells in BAL fluid are comprised of tissue-resident NK

241 cells and memory T cells which express high levels of CXCR3 and CXCR6 at the
242 transcriptional and protein levels (11,12), our data strongly suggest infiltration of NK cells and
243 T cells from peripheral blood into the lung in COVID-19 patients. Since NK cells and CD8⁺ T
244 cells from peripheral blood displayed upregulated levels of effector molecules, these cells might
245 have important cytotoxic implications upon infiltration into the lung. Indeed, NK cells and
246 CD8⁺ T cells in BAL fluid from COVID-19 patients displayed increased expression levels of
247 *GZMA*, *GZMB*, and *PRF1* in patients with moderate and severe disease (Fig. 4e, f). We
248 previously demonstrated that exposure to IAV-infected cells functionally primes human blood
249 and lung NK cells *in vitro* towards increased target cell-responsiveness (9). However, despite
250 phenotypic activation and upregulation of effector molecules both at RNA and protein levels,
251 blood NK cells from patients with moderate COVID-19 showed no elevated responses to K562
252 target cells as compared to healthy controls (Fig. 4h, i). Instead, NK cell responses were slightly
253 reduced with time after symptom onset (Fig. 4j). While our data suggest a low state of NK cell
254 activation in peripheral blood of patients with moderate COVID-19, it remains to be determined
255 whether NK cells migrating towards the lungs remain functional and contribute to lysis of virus-
256 infected cells and possibly even to tissue pathology in patients with moderate or severe COVID-
257 19.

258 Altogether, our data suggest distinct recruitment of CXCR3⁺ and CXCR6⁺ NK cells and
259 CD8⁺ T cells to the lung in patients with moderate COVID-19 and influenza, indicating
260 overlapping recruitment mechanisms in these two respiratory viral infections. Hence, a better
261 understanding of lung-homing of innate and adaptive cytotoxic lymphocytes in COVID-19 and
262 influenza patients might reveal universal concepts of disease progression in these two, and
263 possibly other, respiratory viral infections.

264 **Discussion**

265 The COVID-19 pandemic has raised the awareness about the need for a better understanding
266 of the course of respiratory viral infections. So far, only a few studies have compared immune
267 responses in COVID-19 and other respiratory viral infections side by side (13) (14,15). Here,
268 we compared COVID-19 with influenza. Both viruses are to different degrees transmitted by
269 contact, droplets and fomites. They cause respiratory disease with similar disease presentation
270 ranging from asymptomatic or mild to severe disease and death. Here, we demonstrate that NK
271 cells and T cells, in particular CD8⁺ T cells, largely overlap in their chemokine receptor
272 expression profile in the blood of COVID-19 and influenza, indicating similar lung-homing
273 mechanisms for cytotoxic lymphocytes in both infections. We identified a stronger loss of
274 CXCR3⁺ and CXCR6⁺ CD8⁺ T cells, overall stronger activation of NK cells and T cells, as well
275 as reduced transcript level expression of lung-homing receptors in the blood of influenza
276 patients as compared to COVID-19 patients with moderate disease, in line with the recent
277 observation of a lower inflammatory profile in COVID-19 patients as compared to influenza
278 patients (14). The activated profile of NK cells and CD8⁺ T cells, including elevated expression
279 of CD69 and Ki67, and induction of perforin, was biased towards subsets co-expressing one or
280 more of the lung-homing receptors CXCR2, CXCR3, CXCR6, CCR2, or CCR5. Levels of
281 corresponding leukocyte-recruiting chemokines such as IL-8, CCL5, CXCL1, CXCL2,
282 CXCL5, and CXCL10 (IP-10), are elevated in the BAL fluid of patients infected with SARS-
283 CoV-2 (16,17). Monocytes producing ligands for CXCR3 have been shown to be expanded in
284 the lungs of COVID-19 patients (8). Data regarding chemokine expression in the lung in
285 influenza are limited to patients with fatal outcome (2). However, in particular CXCL10 has
286 been shown to be upregulated in serum of influenza patients (18,19) and in SARS-CoV-1
287 patients with ARDS (20), in *in vitro* influenza virus-infected human macrophages (21), in
288 human lung tissue explants infected with SARS-CoV-2 (16), and in the lungs of mice infected
289 with influenza virus (3,22,23), suggesting a predominant role for the CXCR3: CXCL10 axis for

290 lung-homing upon respiratory viral infection and, interestingly, also in non-viral lung tissue
291 injury (24). Elevated CXCL10 levels in BAL was associated with longer duration of mechanical
292 ventilation in COVID-19 patients (17). In mice, CXCR3-deficiency rescued CCR5-deficient
293 mice from IAV-induced mortality (25). Other murine IAV infection models demonstrated a
294 role for CXCR3 and CCR5 for NK cell lung-homing and showed NK cell accumulation in the
295 lung was not due to proliferation or apoptosis (23). For CD8⁺ T cells, murine models
296 demonstrated that virus-specific T cells express CXCR3 and migrate to CXCR3 ligands *in vitro*
297 (26). Furthermore, CCR5 is required for recruitment of memory CD8⁺ T cells to IAV-infected
298 epithelium and is rapidly upregulated on the surface of memory CD8⁺ T cells upon viral
299 challenge (26). Interestingly, these mouse models also revealed that CCR5 is required for
300 circulating CD8⁺ memory T cells to migrate to respiratory airways but not lung parenchyma
301 during virus challenge (26), indicating potential distinct migration patterns depending on
302 chemokine receptor expression. In addition to CCR5 and CXCR3, CXCR6 has been suggested
303 to be of importance for recruitment of resident memory T cells to the airways both in mice (12)
304 and in moderate COVID-19 (8).

305 The relevance of the CXCR3: CXCL10 axis for lung tissue-homing of cytotoxic immune
306 cells such as NK cells and CD8⁺ T cells might be of interest for future approaches of
307 intervention. Antibody-mediated targeting of CXCL10 improved survival of H1N1-infected
308 mice (27), revealing a novel approach for immunotherapy in patients with severe respiratory
309 viral infections. A thorough review recently summarized relevant known factors and cells
310 involved in lung-homing during infection with SARS-CoV-2 and influenza, emphasizing the
311 role of circulating NK cells not only in terms of their cytotoxic potential but also in potentially
312 facilitating the recruitment of other cell types such as neutrophils (28). The potential
313 immunoregulatory roles of NK cells in the human lung in health and disease however remains
314 to be studied further.

315 Despite the parallels between SARS-CoV-2 and influenza virus infection, both diseases
316 differ at an immunological level including that SARS-CoV-2 does not infect NK cells or other
317 mononuclear blood cells due to the lack of ACE2 expression (29), while influenza virus can
318 infect NK cells (30). In contrast to influenza virus, SARS-CoV-2 can spread to other organs if
319 not cleared efficiently from the respiratory tract (31), and the viruses induce different antiviral
320 responses in lung epithelial cells (32).

321 Here, we demonstrate common lung-homing potential of circulating NK cells and CD8⁺
322 T cells in SARS-CoV-2 and influenza patients. A shortcoming of our study is a low number of
323 patients, impeding a detailed stratification by clinical or other parameters. However, the present
324 cohort was limited to clinically well-defined patients with moderate disease. Future studies will
325 assess how acute respiratory viral infection with SARS-CoV-2, influenza viruses, and other
326 viruses affects the landscape of activated NK cells and T cells in the lung.

327 Together, our results strongly implicate an importance of CXCR3 as a lung-homing
328 receptor in respiratory viral infections such as SARS-CoV-2 and influenza virus in humans.
329 The results also reveal a role for other receptors such as CXCR6, CCR5 on CD56^{bright}CD16⁻
330 NK cells, as well as CXCR2 on CD56^{dim}CD16⁺ NK cells, as potential alternative receptors of
331 importance. A better understanding of how these chemokine receptors are affecting disease
332 progression might help to develop future immunotherapeutic interventions in patients that
333 developed disease in current or future epidemics or pandemics with respiratory viral infections.

334 **Material and Methods**

335 **Patients and processing of peripheral blood**

336 We enrolled a total of 10 hospitalized patients (four females and six males; age range 24-70;
337 average age 55.3) who were diagnosed with COVID-19 by RT-qPCR for SARS-CoV-2 in
338 respiratory samples. Patients were sampled on average 11 days (range 6-16) after symptom
339 onset. All of the COVID-19 patients were considered ‘moderate’ based on the guidelines for
340 diagnosis and treatment of COVID-19 (Version 7) released by the National Health Commission
341 and State Administration of Traditional Chinese Medicine (33). Furthermore, we enrolled 18
342 patients who tested positive for IAV (n=12) or IBV (n=6) (nine females and nine males; age
343 range 21-84; median age 45) by RT-qPCR who were recruited during the four months
344 immediately preceding the outbreak of COVID-19 in the Stockholm region. Nine of the 18
345 influenza patients were hospitalized.

346 None of the COVID-19 patients and only one of the influenza patients received
347 immunosuppressive treatment. Diagnostics for all patients were performed at the diagnostic
348 laboratory at the Karolinska University Hospital, Stockholm, Sweden. Patients were sampled
349 on average 5 days (range 1-11) after symptom onset. Mononuclear cells from peripheral blood
350 were isolated by density gradient centrifugation (Lymphoprep). For each of the two separate
351 cohorts, blood was collected from healthy blood donors and processed in parallel with patient
352 samples.

353 The study was approved by the Regional Ethical Review Board in Stockholm, Sweden,
354 and by the Swedish Ethical Review Authority. All donors provided informed written consent
355 prior to blood sampling.

356

357 **Transcriptome analysis**

358 Preprocessed and annotated scRNA-seq datasets of cells from peripheral blood and BAL fluid
359 from healthy controls, COVID patients, and influenza patients were obtained in RDS formats

360 from published data (6-8). The data was read and analyzed using Seurat (v3.2.2). Quality
361 filtering and cell and cluster annotations were retained from the respective publications. The
362 datasets were scaled and transformed using the SCTransform function with mitochondrial gene
363 expression regressed out, this was repeated after excluding populations and groups.
364 Differentially expressed genes present in at least 25% of the cells were determined in a pairwise
365 manner comparing patient groups using the sctransform outcome and the FindMarkers function
366 implemented in Seurat. NK cells and T cell subsets were selected separately and analyzed based
367 on patient group for transcript expression of chemokine receptors and effector molecules. CD4⁺
368 T cells and CD8⁺ T cells were identified from the BAL T cell subset based on expression of
369 CD4 and CD8. NK cells and T cell populations were further studied in only moderate and non-
370 ventilated COVID patients in the datasets from peripheral blood and BAL fluid, respectively.

371

372 **Flow cytometry**

373 Antibodies and clones used for phenotyping are listed in supplementary table 1. Secondary
374 staining was performed with streptavidin BUV630 (BD Biosciences) and Live/Dead Aqua
375 (Invitrogen). After surface staining, peripheral blood mononuclear cells (PBMC) were fixed
376 and permeabilized using FoxP3/Transcription Factor staining kit (eBioscience).

377 Samples were analyzed on a BD LSR Fortessa equipped with four lasers (BD
378 Biosciences), and data were analyzed using FlowJo version 9.5.2 and version 10.7.1 (Tree Star
379 Inc). UMAPs were constructed in FlowJo 10.7.1 using the UMAP plugin. For calculation of
380 the UMAP, the following parameters were included: CXCR3, CCR7, GzmA, GzmB, Perforin,
381 FcεR1g, NKG2C, Ki67, CD8, CD16, CD56, CD38, CXCR2, CD3, CXCR6, CD4, CD57,
382 CCR5, CCR2, CD69, CD45RA, NKG2A, NKG2D, pan-KIR, NKp80.

383

384 **Degranulation assay**

385 Cells were co-cultured in R10 medium alone or in presence of K562 cells for 6 hours in the
386 presence of anti-human CD107a (H4A3, FITC, BD Biosciences). Golgi plug and Golgi stop
387 (BD Biosciences) were added for the last 5 hours of the assay. The PBMC were subsequently
388 stained as described above.

389

390 **Principal component analysis**

391 Analyses were performed using GraphPad Prism 9 (GraphPad Software). PCs with eigenvalues
392 greater than 1.0 (“Kaiser rule”) were used for component selection.

393

394 **Statistical analyses**

395 GraphPad Prism 8 and 9 (GraphPad Software) was used for statistical analyses. The statistical
396 method used is indicated in each figure legend.

397

398 **Author contributions**

399 Conceptualization/study design: D.B., M.B., S.G.R., A.S.S., N.M.; Investigation: D.B., I.R.,
400 R.V., S.F.J., S.V., J.M., N.M.; Resources: H.A., H.G., S.F.J., S.V., S.G.R., A.S.S.; Writing –
401 original draft: D.B., N.M.; Writing – review and editing: D.B, I.R., R.V., H.A., H.G., S.F.J.,
402 S.V., M.B., H.G.L., J.M., S.G.M., A.S.S., N.M.

403

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408

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516

517 **Figure legends**

518 **Figure 1: Altered composition of NK cells and T cells expressing different lung-homing**
519 **receptors in peripheral blood in acute viral infection. (a)** Uniform manifold approximation
520 and projection (UMAP) analysis of NK and T cell subsets in healthy controls (HC) and COVID-
521 19 patients. 15 healthy controls and 10 COVID-19 patients were included in the analysis with
522 3,000 cells of each donor. Concatanated HC data were downsampled to 30,000 events. **(b)**
523 Chemokine receptor-positive cells (blue) in HC (left plot) or COVID-19 patients (right plot)
524 were defined by applying a Boolean algorithm ($CXCR2^+$ OR $CXCR3^+$ OR $CXCR6^+$ OR $CCR2^+$
525 OR $CCR5^+$) on data from (a). **(c)** Receptor-positive lymphocytes in HC (upper panel) and
526 COVID-19 patients (lower panel) are indicated in blue, using the dataset from (a). **(d, f)**
527 Representative overlays and **(e, g)** summary of data of chemokine receptor expression on **(d, e)**
528 $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ blood NK cells in HC and COVID-19 patients (upper
529 panel) or influenza patients (lower panel). ($CXCR3$: n = 14; $CXCR6$: n = 12; $CCR2$: n = 18;
530 $CCR5$: n = 18. HC: n = 12), or on **(f, g)** $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells in peripheral blood
531 of HC and COVID-19 patients (n = 10) (upper panel) or influenza patients (lower panel;
532 $CXCR3$: n = 18; $CXCR6$: n = 13; $CCR2$: n = 18; $CCR5$: n = 18. HC: n = 12). **(h)** PCA plots
533 showing the distribution and segregation of healthy controls and COVID-19 and influenza
534 patients, respectively based on expression of chemokine receptors ($CXCR3$, $CXCR6$, $CCR2$,
535 $CCR5$) on $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ NK cells (left) and $CD4^+$ and $CD8^+$ T cells
536 (right), respectively. Each dot represents one donor. **(i)** PCA plots showing corresponding
537 trajectories of key receptors on NK and T cell subsets that influenced the group-defined
538 segregation. **(j)** Dot plot showing the distribution of donors in PC2 for NK cells (left) and T
539 cells (right). **(k)** Transcript expression of chemokine receptors in peripheral blood of HC (n =
540 3) and patients with moderate COVID-19 (n = 13) and influenza (n = 4) in NK cells (left), $CD4^+$
541 T cells (middle), and $CD8^+$ T cells (right). **(l)** Dot plots showing the average transcript
542 expression of chemokine receptors on NK cells from healthy controls and COVID-19 and

543 influenza patients. (k, l) Dataset derived from a publicly available scRNA-seq dataset (7). (e, g,
544 j) Kruskal-Wallis rank-sum test with Dunn's post hoc test for multiple comparisons. * $p < 0.05$,
545 ** $p < 0.005$, **** $p < 0.0001$.

546

547 **Figure 2: Lung-homing receptor-positive NK cells display an activated phenotype in**
548 **peripheral blood of COVID-19 and influenza.** Chemokine receptor-positive cells in COVID-
549 19 and influenza patients were identified using the Boolean gate “CXCR3⁺ OR CXCR6⁺ OR
550 CCR2⁺ OR CCR5⁺” (see representative gates in Figure S1b). Cells lacking all of these receptors
551 were identified as chemokine receptor-negative. (a) Representative overlays and (b) summary
552 of data showing CD69 and CD38 on chemokine receptor-positive/negative CD56^{dim}CD16⁺
553 and CD56^{bright}CD16⁻ blood NK cells from COVID-19 patients (upper panel; n = 5-10) and
554 healthy controls (lower panel; n = 20). (c, f) Heatmaps displaying the ratio of mean expression
555 of (c) CD69, CD38, Ki67, and NKG2D between COVID-19 patients and healthy controls or (f)
556 of CD69 and CD38 between influenza patients and healthy controls in CR⁻ and CR⁺
557 CD56^{dim}CD16⁺ and CR⁺ CD56^{bright}CD16⁻ NK cells. Baseline value = 1 (white). (d)
558 Representative overlays and (e) summary of data of CD69 and CD38 expression on CR⁺ and
559 CR⁻ CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻ blood NK cells from influenza patients (upper panel;
560 n = 4-12) and healthy controls (lower panel; n = 12), respectively. (g) Representative overlays
561 and (h) summary of data of CXCR2 expression on CR⁻ and CR⁺ CD56^{dim}CD16⁺ and
562 CD56^{bright}CD16⁻ NK cells from COVID-19 patients (orange; n = 5) and healthy controls (grey;
563 n = 16), respectively. Box and Whiskers, min to max, mean shown as '+'. Kruskal-Wallis rank-
564 sum test with Dunn's post hoc test for multiple comparisons. ** $p < 0.005$.

565

566 **Figure 3: Circulating lung-homing receptor-positive T cells are phenotypically activated**
567 **in COVID-19 and influenza.** (a, b) Expression of (a) CD69, CD38, and Ki67 on CR⁻ and CR⁺
568 CD4⁺ and CD8⁺ T cells from COVID-19 patients (upper panel; n = 10) and healthy controls

569 (lower panel; n = 21) or **(b)** of CD69, and CD38 on CR⁻ and CR⁺ CD4⁺ and CD8⁺ T cells from
570 influenza patients (upper panel; n = 12) and healthy controls (lower panel; n = 12). **(c)** Heatmaps
571 displaying the ratio of mean expression of CD69, CD38, and Ki67 between COVID-19 patients
572 and healthy controls (top) or of CD69 and CD38 between influenza patients and healthy
573 controls (bottom) in CR⁻ and CR⁺ CD4⁺ and CD8⁺ T cells. Baseline value = 1 (white). **(d, e)**
574 Expression of effector molecules in CR⁻ and CR⁺ CD4⁺ and CD8⁺ T cells from **(d)** COVID-19
575 patients (upper panel; n = 10) and healthy controls (lower panel; n = 21), or **(e)** from influenza
576 patients (upper panel; n = 8 (GzmA/B), n = 11 (perforin)) and healthy controls (lower panel; n
577 = 12). **(f)** Heatmaps displaying the ratio of mean expression of effector molecules between
578 COVID-19 patients (top) or influenza patients (bottom) and healthy controls in CR⁻ and CR⁺
579 CD4⁺ and CD8⁺ T cells. Baseline value = 1 (white). (a, b, d, e) Box and whiskers, min to max,
580 mean is indicated as '+'. Kruskal-Wallis rank-sum test with Dunn's post hoc test for multiple
581 comparisons. *p<0.05, ****p<0.0001.

582

583 **Figure 4: Upregulation of cytotoxic profile in circulating NK cells and CD8⁺ T cells in**
584 **COVID-19. (a)** SPICE analysis of CD56^{bright}CD16⁻ NK cells (left), CD56^{dim}CD16⁺ NK cells
585 (middle), and CD8⁺ T cells (right) in COVID-19 patients (upper panel) and influenza patients
586 (lower panel) and the respective healthy controls, displaying co-expression of effector
587 molecules in CR⁻ and CR⁺ cells. n = 10/21 (COVID/healthy), n= 6/12 (influenza/healthy) **(b)**
588 Violin plots of indicated genes encoding granzyme A, granzyme B, and perforin in NK cells
589 and **(c)** CD8⁺ T cells from peripheral blood of healthy controls and non-ventilated and ventilated
590 COVID-19 patients (6). **(d)** Dot plots of indicated genes in total BAL fluid cells, and **(e)** NK
591 cells as well as CD4⁺ and CD8⁺ T cells from healthy controls and moderate and severe COVID-
592 19 patients. **(f)** Violin plots of indicated genes in BAL fluid NK cells and **(g)** in CD8⁺ T cells
593 in healthy controls and moderate and severe COVID-19 patients. **(h)** Representative dot plots
594 showing CD107a and Ki67 expression in bulk NK cells from peripheral blood of a healthy

595 control (HC) and a COVID-19 patient following stimulation with K562 cells. **(i)** Frequencies
596 of CD107a expression in CD56^{bright} and CD56^{dim} NK cells of healthy controls (n = 6) or
597 COVID-19 patients (n = 7) following 6 hour-incubation with or without K562 target cells. **(j)**
598 Correlation analysis of CD107a expression frequencies in CD56^{dim} and CD56^{bright} NK cells (n
599 = 7) versus time since symptom onset in COVID-19 patients. (d-g) RNAseq dataset derived
600 from Liao *et al.* (8).

601

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