The interplay between SARS-CoV-2 infected airway epithelium and immune cells modulates regulatory/inflammatory signals

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Highlights

HAE as a model to study the cross-talk between infected epithelium and immune cells

Immune cells failed to inhibit SARS-CoV-2 replication

Immune cells dampen the production of several signals induced by SARS-CoV-2 infection

Decrease of NK/γδ T and increase of CD8 T cells in SARS-CoV-2 infected HAE co-culture

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The interplay between SARS-CoV-2 infected airway epithelium and immune cells modulates regulatory/inflammatory signals

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SUMMARY
To assess the cross-talk between immune cells and respiratory tract during SARS-CoV-2 infection, we analyzed the relationships between the inflammatory response induced by SARS-CoV-2 replication and immune cells phenotype in a reconstituted organotypic human airway epithelium (HAE). The results indicated that immune cells failed to inhibit SARS-CoV-2 replication in the HAE model. In contrast, immune cells strongly affected the inflammatory profile induced by SARS-CoV-2 infection, dampening the production of several immunoregulatory/inflammatory signals (e.g., IL-35, IL-27, and IL-34). Moreover, these mediators were found inversely correlated with innate immune cell frequency (NK and γδ T cells) and directly with CD8 T cells. The enriched signals associated with NK and CD8 T cells highlighted the modulation of pathways induced by SARS-CoV-2 infected HAE. These findings are useful to depict the cell-cell communication mechanisms necessary to develop novel therapeutic strategies aimed to promote an effective immune response.

INTRODUCTION
The clinical presentation of SARS-CoV-2 infection ranges from asymptomatic cases to mild upper respiratory tract infection to bilateral pneumonia with acute respiratory distress syndrome (ARDS) and multiple organ failure (Guan et al., 2020; Huang et al., 2020; Zhou et al., 2020). Pathological examination indicates that SARS-CoV-2 targets primarily the airways tract and the lungs (Tian et al., 2020; Xu et al., 2020). COVID-19 infection is characterized by inflammatory infiltrates in the alveolar space and by a systemic inflammatory cytokine storm, suggesting the role of excessive immune response in damaging lung function (Huang et al., 2020; Liao et al., 2020; Song et al., 2020). The inflammatory storm is able to orchestrate pleiotropic activities, resulting in tissue damage, thrombotic events, lymphocytes apoptosis and finally affecting the generation of a balanced and protective immune response (Zhou et al., 2020; Mehta et al., 2020). Induction of interferons appears to be limited in the more severe clinical cases, suggesting an imbalance between protective antiviral and detrimental inflammatory cytokine responses (Blanco-Melo et al., 2020; Hadjadji et al., 2020).

The interplay between epithelial and immune cells during respiratory viral infections is critical in orchestrating and inducing antiviral responses rather than an excessive inflammation. Cytokines and chemokines produced by infected epithelial cells are able to recruit, activate, and coordinate innate and adaptive immune functions. Although a majority of studies focused on the interactions between respiratory epithelial and innate myeloid immune cells, few data are available on respiratory epithelium/lymphocytes cross talk. For example, RSV-infected epithelial cells promote a T cell-independent antibody response, which is important for protection against reinfection (McNamara et al., 2013). Furthermore, the strong interactions between epithelial and immune cells described in nasopharyngeal and bronchial samples from patients with severe COVID-19 are likely to contribute to epithelial cell death (Chua et al., 2020).

Organotypic cell culture of human airway epithelial cells (HAE) sustains SARS-CoV-2 replication and has been used to characterize SARS-CoV-2 cell tropism, replication, and pathogenesis (Zhou et al., 2020; Ravindra et al., 2021; Zhou et al., 2020; Hao et al., 2020; Pizzorno et al., 2020).
Here, to better understand the interplay between immune cells and the epithelial tissue during SARS-CoV-2 replication, we analyzed the impact of SARS-CoV-2 infection on inflammatory response and immune cells phenotype in an organotypic co-culture of human bronchial epithelium and immune cells model.

RESULTS
Co-culture of SARS-CoV-2 infected airway epithelium with immune cells: the experimental model
To investigate the cross talk between SARS-CoV-2-infected human airway epithelium (HAE) and the immune cells, we set up a co-culture model. Specifically, we cultured primary Human Bronchial Epithelial Cells at an air-liquid interface for 21 days and then challenged the apical surface of the epithelium with SARS-CoV-2. Soon after the removal of viral inoculum, the HAE was co-cultured with peripheral blood mononuclear cells (HAE-PBMC) in the lower chamber. Infected cultures were collected for gene expression analysis, flow cytometry, inflammatory/regulatory soluble factors profile at 6-days postinfection. Four independent experiments using immune cells isolated from four different healthy donors were performed in duplicate.

Human airway epithelium-peripheral blood mononuclear cells co-culture sustains SARS-CoV-2 replication and induces the interferon response
The ability of airway epithelium to sustain SARS-CoV-2 replication and the impact of immune cells co-culture were analyzed. SARS-CoV-2 replication was measured at two and six days postinfection (p.i.) comparing viral RNA levels released by or associated with epithelial cells, as well as by measuring the infective titers of the viral particles shed to the apical side of the HAE culture model. In HAE, an increase

Figure 1. Experimental model and design
Human bronchial epithelial cells were seeded onto the permeable membrane of a cell culture insert and once confluence was reached, the cells were subjected to "airlift," where the medium was supplied only to the basal chamber, driving differentiation toward a mucociliary phenotype. After 21 days, the organotypic human airway epithelial (HAE) culture were infected (or not) with SARS-CoV-2 and co-cultured in the absence (HAE) or in the presence of healthy Peripheral Blood Mononuclear Cells (HAE-PBMC) in the lower chamber. Infected cultures were collected for gene expression analysis, flow cytometry, inflammatory/regulatory soluble factors profile at 6-days postinfection. Four independent experiments using immune cells isolated from four different healthy donors were performed in duplicate.
Immune cells modulate the inflammatory response induced by SARS-CoV-2 infection

To evaluate the impact of immune cells on the inflammatory profile induced by SARS-CoV-2 infection, we quantified 36 key biomarkers, belonging to the TNF superfamily proteins, IFN family proteins, regulatory cytokines, and Matrix metalloproteinases (MMPs) in SARS-CoV-2 infected HAE-PBMC. PCA analysis was, therefore, performed in order to identify the major trends inherent to the inflammatory profile. Unsupervised PCA of 36 soluble mediators seemed more efficient at segregating HAE cultures on the basis of the presence of immune cells than on the basis of infection. As showed in Figure 3A: not infected and SARS-CoV-2 infected HAE cultures (blue and red points, respectively) are grouped on the third and fourth quadrants of score plot, while not infected and infected HAE-PBMC are grouped on the opposite (first and second) quadrants (purple and green points), suggesting that the immune cells strongly shape the inflammatory response. The quantification of all the mediators in SARS-CoV-2 infected HAE and in HAE-PBMC are shown in Table 1. In the absence of PBMC, 33/36 cytokines screened showed a slight increase, although they did not reach the statistical significance (Figure 2C). Furthermore, SARS-CoV-2 infection did not change the gene expression levels of the two main viral entry factors (Figure 2C), namely ACE2 and TMPRSS2 (Hoffmann et al., 2020).

Correlation matrix analysis showed a coordinate IFNs response in HAE culture where viral RNA directly correlates with ISG15, IFN-α, and IFN-β; ISG15 correlated with IFNs and ISG56 with IFN-β (Figure 2D), suggesting the activation of IFN signaling by viral replication. In the presence of PBMC, these correlations seemed to be even stronger and new ones appeared: ISG15 correlated with ISG56 and ACE2, ISG56 correlated with viral RNA and, finally, ACE2 correlated with IFN-β. These results suggested that viral replication in the HAE model induced a Type I-IFN signaling independently from the presence of immune cells.

SARS-CoV-2 infection of human airway epithelium-peripheral blood mononuclear cell co-culture modulates immune cell frequency

To verify if SARS-CoV-2 infected HAE may modulate PBMC phenotype we performed a multiparametric flow cytometry analysis of co-cultured PBMCs, focusing on NK cells (CD56 dim, bright, and negative
subsets), γδ T cells, monocytes, and adaptive CD4 and CD8 T cells. The results showed a significant decrease of innate cells (CD56+ bright and γδ T cells) and a weak increase of CD8+ T cells between not infected and SARS-CoV-2 infected HAE-PBMC. Of note, the frequency of CD8 T cells was positively correlated with SARS-CoV-2 RNA (r = 0.78, p = 0.02). No significant differences were observed when looking at CD16+ and CD16 negative monocytes and CD4 T cells (Figure 4).

SARS-CoV-2 infection modulates the soluble mediator’s network associated with immune cell subsets in human airway epithelium-peripheral blood mononuclear cell co-cultures

To define possible associations among the immunological parameters and the inflammatory profile in not infected and SARS-CoV-2 infected HAE-PBMC, we performed a correlation matrix among the (36 + 8) continuous variables and build a protein-protein network using Cytoscape (STRING App).

The correlation matrix highlighted that SARS-CoV-2 infection of HAE-PBMC deeply changed the association between soluble factors and lymphomonocytes subsets. The four hierarchical clusters identified in HAE-PBMC were modified by SARS-CoV-2 infection (Figure 5A), where a more consistent group of inflammatory/regulatory mediators was observed (Figure 5A). In not infected co-cultures, the soluble mediators were negatively correlated to NK cells (mainly CD56dim subset) and γδ T cells (Figure 5A green boxes and Figure 5B blue circles), and positively to CD8 T cells (Figure 5A red box, and Figure 5B blue circles). Differently, in SARS-CoV-2 infected co-cultures, the negative associations with NK cells were strongly reduced (Figure 5A green box and Figure 5B, orange circle) while the positive associations with CD8 T cells increased (Figure 5A red box and Figure 5B, orange circles). Furthermore, during the interplay with SARS-CoV-2 infected epithelium, several inflammatory mediators underwent new associations with immune cells (Figures 5A and 5B). Specifically: (i) IFN-α2, IFN-λ1, IL-12p40, IL-20, IL-26, IL-35, and osteocalcin are positively associated with CD8 T cells; (ii) IL-10, IL-27 and IL-34 are negatively related to NK CD56bright during infection; and (iii) IFN-α2, IL-35, and MMP3 are negatively associated with γδ T cells. Moreover, PTX3 during infection is negatively correlated to CD4 T cells frequency.

Collectively, these data showed that in SARS-CoV-2 infected HAE-PBMC co-cultures, five of 10 soluble mediators that were modulated by the presence of immune cells, resulted correlated to CD8 T cells, NK-bright and γδ T cells in these ways: IFN-λ, as well as IL-35, were directly correlated with CD8 T cells frequency; MMP3 as well as IL-35 inversely correlated with γδ T cells frequency; IL-27 as well as IL-34 inversely correlated with NK CD56 bright frequency.

In order to explore the enrichment of biological pathways resulting from inflammatory profile and NK cells subset association, we performed STRING Enrichment analysis, grouping soluble factors based on their association with specific lymphomonocytes subset, in the presence or absence of SARS-CoV-2 infection. In SARS-CoV-2 infected HAE-PBMC, the pathways associated with NK-dim cells subsets were profoundly affected, losing most of the associations found in the uninfected conditions (Figure 5C). Interestingly, during SARS-CoV-2 infection, the CD8 T cells subset was associated with the RIG-I pathway.

DISCUSSION

Epithelial cells that span the length of the respiratory tract respond to viral infection by producing soluble mediators that communicate with immune cells to activate and regulate the antiviral response. Organotypic cell culture of human airway epithelial cells (HAE) has been successfully used to isolate SARS-CoV-2 and developed to widely characterize SARS-CoV-2 cell tropism, replication, and pathogenesis (Zhu et al., 2020a; Ravindra et al., 2021; Zhu et al., 2020b; Hao et al., 2020; Pizzorno et al., 2020). However, the cross-talk between immune cells and the infected HAE during SARS-CoV-2 infection has not yet been
| Soluble mediators | Not infected [median pg/mL (IQR)] | SARS-CoV-2 [median pg/mL (IQR)] |
|------------------|----------------------------------|----------------------------------|
| TNFSF13          | 77483 (45696–109333)             | 127025 (62742–172721)            |
| TNFSF13B         | 3112 (2901–3735)                 | 3989 (3380–4914)                 |
| TNFRSF58         | 75.3 (62.4–112.5)                | 78.1 (67.6–121.6)                |
| sCD163           | 3112 (2901–3735)                 | 3989 (3380–4914)                 |
| Chitinase 3-like 1| 1671 (1165–2445)                | 1728 (1391–4669)                 |
| gp130            | 2408 (1362–4180)                 | 403.2 (49.4–2484)                |
| IFNa2            | 47.6 (38.7–76.9)                 | 44.9 (31.1–52.5)                 |
| IFNb             | 9.2 (7.2–14.3)                   | 9.3 (6.4–10.4)                   |
| IFNg             | 37.2 (24.8–61.3)                 | 30.4 (24.8–49.5)                 |
| IL-2             | 24.8 (18.1–38.8)                 | 27.6 (24.3–56.7)                 |
| sIL-6Ra          | 44 (20–262.1)                    | 20 (20–168.2)                    |
| IL-8             | 11724 (4993–25370)               | 11724 (11724–30956)              |
| IL-10            | 1.9 (1.9–26.9)                   | 1.9 (1.9–17.6)                   |
| IL-11            | 755.9 (503.4–1772)               | 501.4 (189.5–840.4)              |
| IL-12p40         | 66.8 (51.3–121.9)                | 62.7 (41.9–78.4)                 |
| IL-12p70         | 0.7 (0.5–1.4)                    | 0.5 (0.5–1)                      |
| IL-19            | 172.1 (111.8–254.1)              | 151.6 (93–189.2)                 |
| IL-20            | 112.1 (46.8–182.3)               | 93.9 (65.6–117.5)                |
| IL-22            | 150.3 (135.6–236.1)              | 184.8 (159.9–239.2)              |
| IL-26            | 942.2 (753.9–1390)               | 1331 (728.3–1462)                |
| IL-27            | 150.2 (121.6–200.9)              | 156.6 (110.3–199.2)              |
| IFN-L2           | 58.8 (40.7–90.7)                 | 56.3 (38.9–66.8)                 |
| IFN-L1           | 128.6 (106.6–196.1)              | 114.7 (78.3–133.6)               |
| IL-32            | 37.2 (31.5–49.5)                 | 28.7 (12.8–37.7)                 |
| IL-34            | 6008 (4228–8936)                 | 6712 (4229–8540)                 |
| IL-35            | 179.3 (130.2–290.6)              | 172.1 (110.8–186.9)              |
| TNFSF14          | 33 (26–53.6)                     | 42.9 (33.6–60.2)                 |
| MMP-1            | 2084 (1103–3683)                 | 1922 (1167–2728)                 |
| MMP-2            | 23857 (6142–50637)               | 8230 (4656–13600)                |
| MMP-3            | 9303 (8148–10293)                | 9388 (6995–9973)                 |
| BGLAP            | 621.3 (424–967.9)                | 577.4 (396.7–674.9)              |
| OPN              | 46.2 (34.9–70.8)                 | 628.5 (52.8–3813)                |
| Pentraxin-3      | 1087 (567.4–2569)                | 3311 (2008–4393)                 |
| sTNF-R1          | 3604 (1990–8959)                 | 3278 (1931–6536)                 |
| sTNF-R2          | 86.8 (70.7–116.6)                | 251.6 (134.6–577)                |
| TNFSF12          | 4.2 (3–31.7)                     | 40.1 (14.6–78.5)                 |

In bold are indicated the soluble mediators significantly modulated by PBMC during SARS-CoV-2 infection.
investigated. The strength of this article is, therefore, the description of the epithelial-immune cell cross-talk during SARS-CoV-2 infection using an airway epithelial and immune cell co-culture system. Results showed that the immune cells modulate the production of immunoregulatory/inflammatory signals (e.g., IL-35, IL-27, and IL-34) from SARS-CoV-2 infected HAE. These mediators were found inversely correlated with innate cells (NK and $\gamma$-$\delta$ T cells) and directly with CD8 T cells, highlighting the strict interplay between immune cells and infected epithelium.

Figure 4. SARS-CoV-2 infection of HAE-PBMC co-cultures modulates immune cell frequency
Representative flow cytometry plots of NK cells, $\gamma$-$\delta$ T cells, CD16$^+$ and CD16$^-$ monocytes, CD4 and CD8 T cells frequency after 6 days of co-cultures with SARS-CoV-2 infected HAE (n = 4). Results are shown as Box and Whiskers Graphs. Wilcoxon matched-pairs signed-rank test was used for groups comparison. p values are indicated on the graphs.
SARS-CoV-2 replicates efficiently in HAE cultured in an airway-liquid interface culture inducing the IFN response, as shown by the direct correlation between viral RNA and ISGs; moreover, the presence of immune cells did not affect viral replication nor antiviral response. Differently from other observations (Chua et al., 2020; Nawijn and Timens, 2020), we did not observe a significant change in the ACE2 and TMPRSS expression probably owing to the high variability of their expression in a pluristratified airway epithelium. Nevertheless, the correlation between ISG15, ISG56, and ACE2 observed only in HAE-PBMC may suggest a possible enhancement in ISG coordination. Interestingly, a recent work demonstrates a crucial role of ISG15 in the MDAS-mediated antiviral response, representing a key immune evasion mechanism of SARS-CoV-2 (Chua et al., 2020).

Cytokines are crucial in the cell-cell communication processes and in shaping the host responses to SARS-CoV-2 infection. Principal component analysis revealed that the presence of immune cells discriminates the inflammatory/regulatory profile in SARS-CoV-2-infected HAE. SARS-CoV-2 infection of HAE induces the release of a wide profile of inflammatory/regulatory soluble mediators. Intriguingly, immune cells significantly reduced the levels of 10 SARS-CoV-2 induced cytokines. Specifically, in SARS-CoV-2 infected HAE-PBMC, the immune cells production of IFNs, MMPs (able to influence the inflammation processes), immunoregulatory cytokines (IL-27, IL-34, IL-35, and sTNFR1) and the inflammatory cytokines (sCD30/TNFRSF8, gp130/sIL6RB, IL32) decreased. The mechanisms responsible for immune cells-induced lowering of soluble mediators produced in response to SARS-CoV-2 infection need further analysis.

IL-27 has been proposed as an early biomarker of COVID-19 severity (Tamayo-Velasco et al., 2021). The ability of immune cells to dampen IL-27 production may suggest a possible role in reducing lung damage. On the other way, IL-27, as well as IL-34 levels, may affect the innate immune response, as shown by the negative correlation with NK-bright cells. Accordingly, in a previous work in patients with COVID-19, we demonstrated that the increase of inflammatory mediators was correlated with a reduction of innate and adaptive cytotoxic antiviral function (Bordoni et al., 2020).

The wide immunoprofiling analysis performed in this study highlights the pivotal role of immune-regulatory mediators in shaping the immune response. IL-27 and IL-35 (together with TGFβ and IL-10) are the most extensively studied cytokines in tissues such as the lung (Branchett and Lloyd, 2019). IL-35 can inhibit T cell proliferation and cytotoxicity and reduce T cell activation. Moreover, IL-35 was found to increase in both lungs of mice and serum of humans following influenza infection, and its expression was further enhanced in influenza-infected mice following secondary pneumococcal infection (Chen et al., 2016). The main role of IL-27 in respiratory infection has been proposed. In influenza infection, the delivery of recombinant IL-27 limited the recruitment of inflammatory monocytes, neutrophils, and NK cells into the lung, without affecting the T cell response, suggesting that IL-27 can control the immune response to influenza (Liu et al., 2014). IL-34 plays diverse roles in diseases, owing to its inflammatory and immunosuppressive properties. Elevated IL-34 expression has been observed in lung cancers and pulmonary infections, although its role is not well defined (Baghdadi et al., 2018; Zhou et al., 2018). γδ T cells frequency was reduced in SARS-CoV-2-infected HAE co-cultures and negatively correlated with IL-35. Accordingly, a decrease of γδ T cell has been observed in patients with severe COVID-19 (Carissimo et al., 2020; Lei et al., 2020), and a possible protective role has also been proposed in SARS-CoV infection (Poccia et al., 2006).

Interestingly the analysis of enrichment pathway resulting from 36 soluble mediators associated with different immune cell subsets, revealed that the RIG-I pathway (a key activator of type I interferon (IFN)}
antiviral signaling) was enriched in infected HAE-PBMC and correlated to CD8 T cells. Notably, a high concentration of RIG-I is found around tight junctions, suggesting that they could act as apical foci for antiviral sensors (Mukherjee et al., 2009).

Altogether, we described a strict interplay between immunoregulatory/inflammatory signals (e.g., IL-35, IL-27, and IL-34) induced by SARS-CoV-2 infection and innate and adaptive immune cells that can be useful to develop novel therapeutic strategies aimed at promoting effective immune responses against respiratory viruses.

Limitations of the study
A major limitation of this work, as for all the in vitro model of infection, is that the airway epithelial and immune cell co-culture system described in this study likely does not reflect the dynamic interaction of infiltrating immune cells within the airway at different stages of SARS-CoV-2 infection. Nevertheless, this experimental approach may allow to dissect epithelial-immune cell cross-talk during SARS-CoV-2 infection, and to identify possible key pathogenic/protective processes that should be subsequently validated in an in vivo animal model.

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.103854.

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AUTHOR CONTRIBUTIONS
Conceptualization, C.A.; Data curation, V.B., G.M., D.M., M.A.; Formal analysis, V.B., G.M., D.M., M.A, G.M.F. and C.A.; Funding acquisition, G.I. and C.A.; Investigation, V.B., G.M., D.M., A.S., C.C., and C.A.; Methodology, E.C., E.T., R.C., G.G., S.T.; Supervision, G.M.F., M.R.C. and C.A.; Writing—original draft, V.B., G.M. D.M., M.A.; Writing—review and editing, C.A. All authors have read and agreed to the published version of the article.
The authors declare no competing interests.
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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse anti-human CD3 PerCP-Cy5.5 (Clone SP34-2) | BD Biosciences | Cat.#552852, RRID: AB_394493 |
| Mouse Anti-Human CD56 APC (Clone B159) | BD Biosciences | Cat.#555518, RRID: AB_398601 |
| Mouse Anti-Human CD16 PE-Cy7 (Clone 3G8) | BD Biosciences | Cat.#557744, RRID: AB_396850 |
| Mouse Anti-Human CD4 FITC (Clone RPA-T4) | BD Biosciences | Cat.#555346, RRID: AB_395751 |
| Mouse Anti-Human CD8 PE (Clone RPA-T8) | BD Biosciences | Cat.#555367, RRID: AB_395770 |
| Mouse Anti-Human CD14 APC-H7 (Clone M4P9) | BD Biosciences | Cat.#641394;RRID:AB_1645725 |
| Mouse Anti-Human CD16 V450 (Clone 3G8) | BD Biosciences | Cat.#560475, RRID: AB_1645561 |
| Mouse Anti-Human CD45 Krome Orange (Clone J33) | Beckman Coulter | Cat.#B36294;RRID:AB_2833027 |
| Mouse Anti-Human V62 Brilliant Violet 605 (Clone B6) | Biolegend | Cat.#331430;RRID:AB_2783212 |
| Rabbit Polyclonal Antibody anti-ZO-1 | Thermo Fisher Scientific | Cat.#40-2200, RRID:AB_2533456 |
| Mouse monoclonal Antibody anti-SARS-CoV-2 spike antibody (Clone 1A9) | GeneTex | Cat.#GTX632604, RRID:AB_2864418 |
| goat Anti-Mouse IgG1 (γ1), CF 488A | Sigma Aldrich | Cat.#SA84600238 |
| goat Anti-Rabbit IgG, CF 647 | Sigma Aldrich | Cat.#SA84600352 |
| **Bacterial and virus strains** | | |
| Human 2019-nCoV strain 2019-nCoV/Italy-INMI1, clade V | EVAg Portal | Ref-SKU: 008V-03893 |
| **Biological samples** | | |
| Peripheral blood Mononuclear cells from healthy donors | Recruited at INMI L Spallanzani (Ethical approval: 70/2018) | N/A |
| **Critical commercial assays** | | |
| Bio-Plex Pro Human Inflammation Panel 1, 37-Plex | Bio-Rad | Cat.#171AL001M |
| Direct-zol RNA MicroPrep KIT | Zymo Research | Cat.#R1051 |
| AMV-reverse transcriptase | Promega | Cat.#M5108 |
| Qiaprep viral RNA kit | Qiagen | Cat.#52904 |
| RealStar SARS-CoV-2 RT-PCR Kit | Altona Diagnostics | Cat.#821005 |
| FastStart Essential DNA Green Master | Roche | Cat.#06402712001 |
| **Experimental models: Cell lines** | | |
| Human adult Bronchial Epithelial Cells (HBEpC) | Cell Applications | Cat.#502–05a |
| Cercopithecus aethiops Vero E6 Cells | ATCC | CRL-1586 |
| **Oligonucleotides** | | |
| See Table S1: Oligonucleotides | This paper | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for reagents may be directed to and will be fulfilled by the lead contact, Chiara Agrati (chiara.agrati@inmi.it).

Materials availability
This Study did not generate new unique reagents.

Data and code availability
- The data presented in this study are openly available at http://rawdata.inmi.it.
- This paper does not report original code. All code utilized in the paper is available online and listed in the key resources table.
- Any additional information required to re-analyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects
The immune cells from residual samples obtained for diagnostic purpose of healthy donors were isolated in compliance with the principles included in the Declaration of Helsinki. The Ethics Committee of the INMI L Spallanzani approved the study protocol (Ethical approvement: 70/2018). PBMCs from four different healthy donors (age: 46, 51, 53, 36; negative for SARS-CoV-2 molecular and serological tests) were isolated from whole blood by density gradient centrifugation (Lympholyte-H; Cedarlane Labs). PBMCs were suspended in RPMI 1640 (Corning Incorporated) supplemented with 2 mM L-glutamine, 50 U/mL penicillin, and 50 \mu g/mL streptomycin.

Human primary bronchial epithelial cells
Human primary adult Bronchial Epithelial Cells (lot 2463, HBEpCs, Cell Applications) were thawed in Bronchial/Tracheal Epithelial growth medium (Cell Applications), expanded and frozen in single aliquots for further experiments. After 3–5 days, cells were detached using the Animal Component-Free (ACF) Cell Dissociation Kit (STEMCELL Technologies) and seeded in 12 mm transwell inserts (STEMCELL Technologies) in PneumaCult Ex Plus Medium (STEMCELL, Technologies), supplemented with 2 mM L-glutamine (Corning Incorporated, Corning, New York, USA), 50 U/mL penicillin, and 50 \mu g/mL streptomycin (Corning Incorporated), at the density of 1.1 \times 10^5 cells/mL in a humidified atmosphere (5% CO2) at 37°C. At the confluence, the medium was cleared from both apical and basal sides and PneumaCult-ALI-S Medium (STEMCELL Technologies) was only added in the lower chamber, leaving the apical chamber in contact with the air and inducing the differentiation for 21 days. The medium was changed every two days until complete differentiation, observable from mucus production, which can be removed using 0.5 mL of Phosphate buffered saline 1 × (PBS). The well differentiated human airway epithelium (HAE) was therefore used for successive experiments. The cell viability was verified overtime checking the structural epithelial architecture by immunofluorescence analysis.
Vero E6 cell line
Vero E6 cells (ATCC® CRL-1586) were culture in Eagle’s Minimum Essential Medium (MEM, Sigma-Aldrich St. Louis, Missouri, USA) supplemented with 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin, and 10% Foetal Bovine Se-rum (FBS, GIBCO).

METHOD DETAILS
SARS-CoV-2 infection of HAE
HAE cells were infected with SARS-CoV-2, (Human 2019-nCoV strain 2019-nCoV/Italy-INM1, clade V; Ref-SKU: 008V-03893 European Virus Archive – GLOB-AL, GiSAID: BetaCoV/Italy/INM1—isl/2020: EPI_ISL_410545) in MEM at a multiplicity of infection (M.O.I.) of 0.1. Viral inoculum or medium only (not infected) was applied on the apical side and cells were incubated for 1 h and 30’ at 37°C, 5% CO2. Viral inoculum was then removed and cells washed with 0.3 mL of 1×PBS two times. Next, 1 mL of RPMI 1640 with 5% Human Serum (Sigma Aldrich) was added to the basal chamber of each well and cells were cultured at 37°C, 5% CO2. In co-culture experiments (HAE-PBMC), two million of PBMCs were plated in the basal chamber. Soon after inoculum removal and at two- and six-days post infection, we collected HAE cells, PBMCs, Viral quantification and titration
SARS-CoV-2 infection of HAE
Viral quantification and titration

Flow cytometry staining and analysis
NK, T and Monocyte cell surface antigens were analysed by flow cytometry. Briefly, two million PBMCs were stained with a surface antibody’s cocktail: anti-CD3 PerCP-Cy 5.5, anti-CD56 APC and anti-CD16 PE-CyTM7 from BD Biosciences, anti-CD45 Krome Orange from Beckman Coulter and anti-V450 from BD Biosciences and anti-CD45 Krome Orange (Mix Monocytes), in 1× PBS, 1% Bovine Serum Albumin (BSA) (from Sigma Aldrich) and 0.1% Sodium azide (NaN3) (from Serva) solution for 15 min at 4°C. Afterwards, PBMCs were washed with 0.3 mL of 1× PBS, 1% BSA and 0.1% NaN3 solution and centrifuged at 1600 rpm for 5 min. Next, PBMCs were fixed with 1% Paraformaldehyde (PFA) (Bio-Rad, Hercules, California, USA) in 1× PBS, 1% BSA and 0.1% NaN3 solution for 15 min at room temperature in the dark, washed as before and centrifuged at 1600 rpm for 5 min. Cells were acquired using a FACSLyric (BD Biosciences) and analysed by BD FACSuite software (BD Biosciences).

Real time RT-PCR
Total RNA was extracted from HAE culture using Direct-zol RNA MicroPrep KIT (Zymo Research Corp.) according to the manufacturer’s instructions. For RT-PCR analyses, single-stranded cDNA was obtained by reverse transcription of 1 μg of total RNA using AMV-reverse transcriptase (Promega Corporation). For cellular gene expression analysis, ISG15, ISG56, INFα, INFβ, ACE2, truncated ACE2 and TMPRSS2 mRNA were analysed by quantitative PCR using the following primers purchased from Sigma-Aldrich: ISG15 forward: 5'-GCCAGATCCCAAGAGATCG-3', reverse: 5'-GGGCCCTTGGATTTCCTCAAC-3'; ISG56 forward: 5'-CTCTTGTGAGTGGAGGAGA-3'; reverse: 5'-CCAGGCGATAGGCAGAGA-3'; INFα forward: 5'-TGGGCTGTGACTCTGCTCAAAC-3'; reverse: 5'-CAGCCTTTTGGAACCTGTTGCC-3'; INFβ forward: 5'-CTTGCATCTACAAGAGGACGC-3'; reverse: 5'-TCCCTTTGGAACCTGCTGCA-3'; ACE2 forward: 5'-GGGCCGACCTAGGATCTTAT-3'; reverse: 5'-GGATATGCGCATCTCATGAGT-3'; truncated dACE2 forward: 5'-GGAAGCACGGGCGGAGGACAA-3'; reverse: 5'-AGCTGTGAGGAGTGGCTCATT-3'; TMPRSS2 forward: 5'-GTCCCACTGTCTCGAGGT-3'; reverse: 5'-CAGACGACGGGTGGGAAA-3'. qPCR was performed with FastStart Essential DNA Green Master (Roche) according to the manufacturer’s instructions. The expression levels were normalized to GAPDH level (GAPDH forward: 5'-GGTG TCTCCTGACTCTCAACA-3'; reverse: 5'-GTGGTCTGAGGAGGCAATG-3') using the equation “2−ΔΔCt”. All qPCR reactions were performed in a Corbett 212 Rotor-gene6000 Real-Time PCR System.

Viral quantification and titration
RNA from 140 μL of HAE culture supernatant was extracted using the Qiamp viral RNA kit (Qiagen), following manufacturers instruction and eluted in 50 μL of elution buffer.
For viral replication determination, Real time RT-PCR was performed on 10 µL of RNA extracted from supernatant, or 40 ng of cell-associated RNA using the RealStar® SARS-CoV-2 RT-PCR Kit RUO (Altona Diagnostics), which amplifies the E- and S- viral genes.

To estimate the production of infectious SARS-CoV-2, serial dilution of HAE cell supernatants were put in contact with sub-confluent VeroE6 cells seeded in 96-well plates in MEM containing 2% FBS. At day 5 after infection, cells were observed for cytopathic effect CPE and TCID50/mL was measured and analysed by Reed-Muench method.

**Multiplex microbead-based immunoassay**

The inflammation factor levels were analysed using a Bio-Plex Pro™ Human Inflammation Panel 1, 37-Plex (#171AL001M, Bio-Rad) according to the manufacturer’s instructions. 0.05 mL of each collected supernatant, at six days after infection, were used for the assay. The lecture of the plate was performed with BioPlex® MAGPIX Multiplex Reader.

**Immunofluorescence**

Transwells after six days from the infection were fixed in PAF 4% diluted in PBS 1× for 20 min at RT. The transwells were cut with a sterile scalpel and laid down in a new 24 cell culture well plate with the side cells located face-up. Then, transwells were washed with PBS 1× three times for five min each and blocked with Blocking Buffer (2% Goat Serum from Invitrogen; 1% BSA from Sigma Aldrich; 0.1% Fish Gelatin Blocking Agent from Biotium; 0.1% Triton X-100 and 0.05% Tween20 from Sigma Aldrich in 1×PBS) for 1 hour at RT. Later, blocking buffer was removed, transwells were washed as before, 0.45 mL of primary antibodies (anti-ZO1 from Invitrogen; anti-SARS-CoV-2 spike antibody from GeneTex; in Primary Antibody Buffer (1% BSA and 0.1% Fish Gelatin Blocking Agent in 1×PBS) were added to each transwell and incubated overnight at 4°C in the dark. The following day primary antibodies were removed, transwells were washed as before, 0.45 mL of secondary antibodies (anti-Mouse IgG1 AF488 and anti-Rabbit IgG Alexa-647 from Sigma Aldrich) diluted 1:1000 in Secondary Antibody Buffer (1× PBS) were added to each transwell and incubated for 2 h at 37°C. Afterward, secondary antibodies were removed, transwells were washed as before and membrane cell was mounted side up on slide using one drop of SlowFade Gold Antifade Mountant with DAPI (from Invitrogen). Therefore the images were acquired using a Leica THUNDER 3D Live Cell Imaging system (Leica Application Software (LAS) X 3.7.2; Leica Microsystems) using THUNDER Computational Clearing Settings at 63X magnification.

**Protein network and pathways analysis**

Cytoscape software (3.8.2 version) and STRING App were used to build protein-protein network using a 0.4 as confidence cutoff. STRING Enrichment analysis was performed to retrieve human KEGG pathways associated with different lymphomonocytes subset; redundant terms were filtered with 0.5 cutoff. RStudio software was used to build a bubble plot using ggplot2-based visualization.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Principal component analysis (PCA) was performed in order to identify the relevant information and visualize major trends inherent to the immunological profile. Data were analysed using RStudio software from [http://www.rstudio.org](http://www.rstudio.org) with the libraries FactoMineR (for the analysis) and factoextra (for ggplot2-based visualization). To visualize a correlation matrix in R we used the corplot function and generate a Heatmap object using correlation coefficients (computed using the Spearman) as input to the Heatmap. The heatmap was produced with the R package heatmap3. Quantitative variables were compared with nonparametric Wilcoxon test. Friedman along with Dunn’s multiple comparisons test evaluated the statistical differences between more than two conditions. A p value lower than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism v8.0 (GraphPad Software, Inc). More details of the statistical analysis are included in Figure legends.