An in silico analysis identifies drugs potentially modulating the cytokine storm triggered by SARS-CoV-2 infection

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The ongoing COVID-19 pandemic is one of the biggest health challenges of recent decades. Among the causes of mortality triggered by SARS-CoV-2 infection, the development of an inflammatory "cytokine storm" (CS) plays a determinant role. Here, we used transcriptomic data from the bronchoalveolar lavage fluid (BALF) of COVID-19 patients undergoing a CS to obtain gene-signatures associated to this pathology. Using these signatures, we interrogated the Connectivity Map (CMap) dataset that contains the effects of over 5000 small molecules on the transcriptome of human cell lines, and looked for molecules which effects on transcription mimic or oppose those of the CS. As expected, molecules that potentiate immune responses such as PKC activators are predicted to worsen the CS. In addition, we identified the negative regulation of female hormones among pathways potentially aggravating the CS, which helps to understand the gender-related differences in COVID-19 mortality. Regarding drugs potentially counteracting the CS, we identified glucocorticoids as a top hit, which validates our approach as this is the primary treatment for this pathology. Interestingly, our analysis also reveals a potential effect of MEK inhibitors in reverting the COVID-19 CS, which is supported by in vitro data that confirms the anti-inflammatory properties of these compounds.

Since the first patient was hospitalized in Wuhan on December 12th, 20191, the ongoing COVID-19 pandemic has caused 108.2 million confirmed infections and 5.3 million deaths by Dec 16th, 2021 (http://covid19.who.int). The disease-causing pathogen is SARS-CoV-2, a novel positive sense single-stranded RNA coronavirus belonging to the lineage B of the betacoronavirus genus2. Infections by different coronavirus strains have been historically present in humans, although they most often only cause a mild cold (e.g. infections by HKU, 229 and hCOV-OC43 coronaviruses). However, several new coronavirus strains have emerged in the last 2 decades that cause an Acute Respiratory Distress Syndrome (ARDS) with severe health consequences including patients’ death. These include SARS-CoV, which between 2002 and 2003 led to 8000 cases with a mortality of 9.5% and MERS-CoV, which in 2012 caused 2500 confirmed infections with a mortality rate of 36%4. As of today, and while respiratory failure associated to ARDS is the leading cause of mortality in COVID-19 patients, accumulating evidence shows that the lethality in a subgroup of the severe patients occurs due to the late onset of an inflammatory "cytokine storm" (CS)5–7. First defined in 1993 in the context of graft-versus-host disease11, the CS has been observed in a wide range of inflammatory diseases such as multiple sclerosis or pancreatitis, and in the context of viral infections including SARS-CoV-212,13. In COVID-19, the CS is caused by an overproduction of proinflammatory cytokines such as IL-6, IL-1β and TNFα by lung infiltrating alveolar macrophages, which triggers vascular hyperpermeability, multiorgan failure and ultimately cell death14. Potential treatments for the CS of COVID-19 patients included broad spectrum anti-inflammatory therapies such as corticosteroids or more targeted therapies like anti-IL-6 receptor antibodies.

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by "ATPase inhibitors", "Topoisomerase Inhibitors", "Insulin sensitizers" and "CDC inhibitors" (Fig. 1D,E).

Results

Biological pathways related to the hypercytokinemia found in COVID-19 patients. In order to define a transcriptional signature related to COVID-19, we used recently published transcriptomic data from bronchoalveolar lavage fluid (BALF) cells where COVID-19 patients (n = 8) were compared to healthy individuals (n = 20) and community-acquired pneumonia patients (n = 146)25. Consistent with clinical observations, this study found that COVID-19 patients present a distinct activation of an IFN-dependent cytokine response. We thus defined a transcriptional signature based on the cytokines and cytokine receptor genes that were differentially expressed in COVID-19 patients (COVIDCS) (signature available in Table S1). We then mined CMap using its clue.io tool (http://clue.io), in order to compare the COVIDCS with transcriptional signatures associated to specific perturbations (drug treatments, gene overexpression or knockdowns and biological pathways (CMap classes)). The output of these analyses are similarity scores, which are positive for those signatures that are similar to COVIDCS and negative for perturbations driving transcriptional signatures opposed to it (Fig. 1A).

We first looked into CMap classes with transcriptional signatures that correlate positively with the COVIDCS. Consistent with their key roles in inflammation26, these analyses identified activation of NFκB and PKC signaling as the biological pathways with associated signatures that are most significantly similar to the COVIDCS (Table S2). Of note, PKC activation triggers NFκB and TNFa-dependent inflammatory responses in human bronchial epithelial cells, suggesting that PKC could act early on in triggering the inflammatory response27. Regarding specific genes our analysis identified IL1 (IL1R1) or TNFα receptors (TNFRS10A and TNFRSF1A) and IFN-response genes (IRF2 and IFR5) as the factors whose overexpression triggers a transcriptional signature that most significantly mimics the COVIDCS (Table S3). Thus, as expected, the transcriptional signature of the hypercytokinemia triggered by SARS-CoV-2 infection resembles that of activated inflammatory pathways regulated by PKC, NFκB, TNFa and IL1.

In what regards to genes that, when downregulated, trigger a signature similar to the COVIDCS, the list was not as restricted to immune factors as the one from overexpressed genes, although it included anti-inflammatory factors such as NFκB inhibitor alpha (NFKBIA) (Table S4). Interestingly, Gene Ontology (GO) analyses of genes whose downregulation triggers a transcriptional signature mimicking that of the COVIDCS detected an enrichment in pathways related to female sexual hormones (Fig. 1B). We find this of particular interest since even if male and females are equally infected with SARS-CoV-2 the fatality rate is significantly higher in men, which could relate to the well-established gender-related differences in the intensity of inflammatory responses28. In fact, several studies have indicated that estrogen levels influence the severity of diseases linked to inflammation, including cancer, due the modulation of NFκB signaling29,32. Moreover, gender differences in COVID-19 mortality rates are biggest in younger patients and decline progressively, further supporting a connection with female hormone levels. Noteworthy, estrogen treatments are already been explored in clinical trials for reducing the severity of COVID-19 in men and women 55 and older (as estrogen levels decline after menopause)33.

Drugs with transcriptional signatures that mimic the COVIDCS. Next, we interrogated CMap for the identification of compounds triggering a similar effect on the transcriptome to the one observed in COVID-19 patients, as these compounds could in principle potentiates the severity of the hypercytokinemia. Consistent with the analysis of biological pathways, three of the top compounds were PKC activators: prostratin, phorbol-12-myristate-13-acetate (PMA) and ingenol (Table S5). Besides specific compounds, we aimed to identify compound classes with signatures mimicking the COVIDCS, for which we adapted Gene Set Enrichment Analyses (GSEA) to conduct a "drug GSEA"34,35. PKC activating drugs were the most significantly enriched class, followed by "ATPase inhibitors", "Topoisomerase Inhibitors", "Insulin sensitizers" and "CDC inhibitors" (Fig. 1D,E). Regarding insulin, a recent study has found that the triglyceride and glucose index (TyG) correlates with the severity and mortality of COVID-19 patients, which builds upon epidemiological observations identifying obesity as a risk factor among COVID-19-related fatalities36. If we restrict our analysis to medically approved drugs, only Topoisomerase inhibitors showed a significant enrichment as drugs mimicking the COVIDCS (Fig. 1E and Table S6), although the absence of other compound classes from this list can be influenced by the low number of
clinically available drugs in other pathways. As for the potential aggravating effects of topoisomerase inhibitors on the COVID-19 CS, we believe that this is most likely related to Topoisomerase-II. In fact, Topoisomerase-I activity has been found to mediate the expression of inflammatory genes. In contrast, Topoisomerase-II inhibition triggers the expression of inflammatory cytokines through activation of cGAS-STING innate immune sensing of cytoplasmic DNA.

Drugs triggering transcriptional signatures opposed to the COVID CS. As mentioned in the introduction, one of the initial ideas behind the development of CMap was to identify drugs that trigger transcriptional responses that negatively correlate with those associated to a given disease, in order to identify potential new therapies through drug repurposing. Based on this concept, we next interrogated CMap for drugs that trigger signatures opposed to the COVID CS. Remarkably, all compound classes that have been already proposed for the treatment of the COVID-19 CS including corticosteroids and JAK or HMGCR inhibitors had transcriptional signatures that negatively correlated with the CS (Similarity score > 95). The panel represents those with a False Discovery Rate (FDR) < 0.05 and a fold enrichment > 22.4. Pathways related to female hormone signaling are highlighted in yellow. (C) Drug GSEA analysis of compound classes triggering a transcriptional signature that positively correlates to the COVID CS. Compounds were classified by their mechanism of action. (D) Similarity scores of specific compounds with a COVID CS similarity score bigger than 95. Compounds belonging to the classes defined in (C) are highlighted in different colors: PKC activators (purple), CDC inhibitors (pink), Insulin sensitizers (yellow), Topoisomerase inhibitors (black) and ATPase inhibitors (green). (E) Enrichment plot of Topoisomerase inhibitors resulting from the Drug GSEA analysis on medically approved compounds mimicking the COVID CS signature.
Ticoid dexamethasone is currently the most widely used treatment for the CS in COVID-19 patients (Fig. 2B,C). Besides glucocorticoids, this analysis identified Vitamin K antagonists and MEK inhibitors as potentially countering the COVIDCS, although the analysis of Vitamin K antagonists was based on very few compounds (Fig. 2B,D). An independent analysis of CMap classes confirmed glucocorticoid receptor agonists (C) and MEK inhibitors (D) resulting from the Drug GSEA analysis mentioned in (B) illustrating the overall negative correlation between the transcriptional signatures triggered by these compounds and the COVIDCS. (E) Similarity scores of the top CMap Classes with an associated transcriptional signature that negatively correlates to the COVIDCS. (F) Similarity scores of specific compounds with a COVIDCS similarity score lower than −95. MEK inhibitors (blue), RAF/EGFR/MAPK inhibitors (orange) and glucocorticoid receptor agonists (red) are highlighted by the indicated colors.

Figure 2. Compounds triggering transcriptional signatures opposed to the COVIDCS. (A) COVIDCS similarity scores for drug types (CMap Classes) already under study for the treatment of the COVID-19 CS. (B) Drug GSEA analysis of compound classes with a transcriptional signature that negatively correlates to the COVIDCS. Enriched pathways with padj value < 0.05 are shown, as well as their gene ranks, Normalized Enrichment Scores (NES), p-value (pval) and adjusted p-value (padj). (C, D) Enrichment plots of Glucocorticoid receptor agonists (C) and MEK inhibitors (D) resulting from the Drug GSEA analysis mentioned in (B) illustrating the overall negative correlation between the transcriptional signatures triggered by these compounds and the COVIDCS. (E) Similarity scores of the top CMap Classes with an associated transcriptional signature that negatively correlates to the COVIDCS. (F) Similarity scores of specific compounds with a COVIDCS similarity score lower than −95. MEK inhibitors (blue), RAF/EGFR/MAPK inhibitors (orange) and glucocorticoid receptor agonists (red) are highlighted by the indicated colors.
a mouse cytokine-storm model of SARS-CoV-2 infection\(^{32}\). Altogether, these evidences support that the validity of our approach to identify inhibitors that could potentially reduce the severity of COVID\(^{32}\). Among these different types of compounds, the potential effects of MEK inhibitors in counteracting the cytokine response seem of particular interest for several reasons. First, because even if MEK inhibitors have been mainly developed as antineoplastic agents, numerous studies support that targeting MEK limits cytokine responses in various contexts like graft-vs-host disease, cerebral ischemia, activation of T cells by superantigens or influenza A infection\(^{33-38}\). Second, because MEK inhibition has been previously shown to inhibit replication of other coronaviruses such as the mouse hepatitis virus (MHV)\(^{39}\). Third, because besides MEK, several compounds with the highest scores of opposing signatures to the COVID\(^{32}\) are inhibitors of other factors from the same signaling route as MEK (RAF, EGFR and MAPK inhibitors) (Fig. 2F and Table S8). Finally, and most interestingly, there is actually an ongoing clinical trial exploring the efficacy of a MEK inhibitor (ATR-002) for the treatment of COVID-19 and its associated CS\(^{30}\).

**Validation of CMap analyses in two additional COVID-19 signatures.** In order to complement our analyses, we defined another two transcriptional signatures associated to the COVID CS. First, we defined a transcriptional signature specifically related to severe patients undergoing a CS, for which we used data from a manuscript where the BALF transcriptome of a COVID-19 patient suffering from a CS was compared to that of healthy individuals or a milder case of the disease (signature available in Table S1; CS\(^{[sc]}\)). Second, we used a study where Liao et al. reported single-cell RNA-seq data from BALF samples obtained from healthy individuals and from COVID-19 patients with either mild disease or CS symptoms\(^{40}\). Using transcriptomic data from the lung-infiltrating macrophages analyzed in this study (6 patients), we defined a new transcriptional signature associated to the COVID-19 CS (signature available in Table S1; CS\(^{[sc]}\)). Consistent with our previous analyses, NFκB and PKC signaling were the CMap classes most significantly similar to the CS\(^{[sc]}\) and to CS\(^{[sc]}\) (Fig. S1A,B and Table S2). Furthermore, TNFα receptors and IFN-response genes were once again found among the top list of factors that, when overexpressed, trigger a transcriptional signature similar to the CS\(^{[sc]}\) (Fig. S1A,B and Table S2). Drug GSEA analysis also revealed MEK inhibitors together with ”Bacterial cell wall synthesis inhibitors” and PI3K inhibitors as the most significantly enriched classes of compounds with a negatively correlating transcriptional signature to the CS\(^{[sc]}\) (Fig. S2C). Likewise, the analysis of CMap classes negatively correlating to the CS\(^{[sc]}\) had MEK inhibitors at the top of the list, together with loss of function of aminoacyl-tRNA synthetases and inhibitors of the reverse transcriptase, bacterial 30S ribosome, PKA and glycogen synthase kinase (Fig. S2D and Table S7).

**Anti-inflammatory properties of MEK and ERK inhibitors.** As the efficacy of glucocorticoid receptor agonists for the treatment of the COVID-19 CS is well established, we decided to complete our study by investigating the potential anti-inflammatory properties of inhibitors of the MAPK/MEK/RAF route, which as mentioned have been primarily developed as anticancer agents. First, in vitro, we differentiated cells from a human monocyte cell line (THP-1) into macrophages, and triggered cytokine production by exposing them to lipopolysaccharide (LPS), an endotoxin found in the outer membrane of Gram-negative bacteria. Differentiated THP-1 cells were exposed to LPS together with two independent inhibitors (trametinib and selumetinib), one ERK inhibitor (SCH-772984) and two glucocorticoids (dexamethasone and hydrocortisone). The treatment with LPS promoted the activation of an inflammatory program, exemplified by an increased expression of cytokines such as IL-1β, TNF-α and IL6 (Fig. 3A). As expected, cytokine expression was reduced upon treatment with glucocorticoids. Strikingly, MEK and ERK inhibitors not only did they reduce LPS-induced cytokine production, but they actually had a bigger effect than glucocorticoids on IL-1β and TNF-α (Fig. 3A). Of note, we also tested the effect of inhibitors of RAF (verumarfenib) or EGFR (gefitinib and erlotinib), which act upstream of MEK and ERK in growth factor signaling. However, these compounds only showed partial effects on IL-6 and did not affect the expression of IL-1β or TNF-α (Fig. S3).

Finally, we explored if the effects of MEK inhibition in counteracting cytokine expression are also observed in cell types that are involved in the inflammatory responses triggered by COVID-19 infection. To this end, we took advantage of publicly available GEO data from human bronquial cells (GSE63229) and human monocytes (GSE123574) treated with either DMSO or trametinib (MEK inhibitor) to perform differential expression
followed by Gene Set Enrichment Analyses (GSEA). Consistent with our THP-1 data, GSEA from human bronchial cells revealed that the pathways most significantly downregulated in trametinib-treated cells were "TNF-α signaling via NFκB" and "inflammatory response", together with the expected downregulation of Ras signaling ("KRAS signaling up") and "MYC targets" (Fig. 3B,C). Likewise, GSEA in human monocytes showed these two pathways, plus "allograft rejection", "IL6-JAK-STAT3 signaling" and "interferon-γ response" as the ones being most repressed by trametinib (Fig. 3D,E). Collectively, these experiments provide support to the concept of...
using inhibitors of MEK and ERK kinases to alleviate pathologies linked to hypercytokinemia such as the CS that evolves in severe cases of COVID-19.

**Discussion**

We here present our results using large-scale public repositories of transcriptional signatures and bioinformatics tools to identify compounds and genetic perturbations that trigger a transcriptional response similar or opposite to that observed in severe patients of COVID-19 undergoing a CS. We are aware of the caveats of the limited data available to perform these studies at this point, which should get stronger as more transcriptional data from these patients becomes available. We find nevertheless remarkable the large degree of overlap that we found in our analyses, despite using three different datasets and two different models (whole tissue vs single-cell) to define our transcriptional signatures. Besides identifying drugs that are already being used (e.g. glucocorticoid receptor antagonists) or are being tested in clinical trials (e.g. JAK inhibitors) as COVID-19 therapies, our study supports the potential for drug repositioning of compounds like MEK or ERK inhibitors for treating the COVID-19-associated CS, which in the case of MEK is already being tested in clinical trials. It is interesting to note that the effects of MEK inhibitors in suppressing hypercytokinemia seem independent from their well-established antiproliferative roles that relate to cancer therapy, as the anti-inflammatory effects we observed in THP-1 cells were made in differentiated non-growing cells. We must again emphasize that, in any case, any therapy based on anti-inflammatory drugs should be restricted to the late and severe stages of COVID-19, as their use could limit the efficacy of the immune system in fighting the infection at earlier stages.

Besides identifying compounds that could potentially be of use for treating severe cases of COVID-19, we raise awareness on the possible adverse effects that some drugs like Topoisomerase inhibitors might have in potentiating the CS. In this regard, our manuscript supports that the gender-related differences on the severity of COVID-19 might be related to the anti-inflammatory properties of female hormones. We want to end this manuscript with a clear statement in that by no means we are proposing novel clinical indications for any of these agents. We simply wanted to contribute in the context of the current health crisis and provide a resource that others might find of use in the selection of drugs that could be tested for efficacy in experimental models of CS.

**Materials and methods**

**Calculation of similarity scores.** Three independent transcriptional signatures defined from COVID-19 patients (COVID92, CS9 and CS(sc)93) were used as inputs to query CMap using its clue.io tool (https://clue.io), in order to obtain similarity scores for the signatures associated to all the perturbagens (compounds, CMap classes and the over-expression or knock-down of genes) available at CMap94. The Query CMap tool was used, employing Gene expression (L1000), Touchstone and individual query as query parameters. Similarity scores were downloaded, and results sorted based on their scores and the type of perturbagen. For restricting the analysis to medically approved drugs, we used drug information available at CMap.

**Gene ontology (GO).** For GO analyses, a list of genes in which their perturbation (overexpression or knockdown) led to a similarity score > 95 with COVID-19-associated signatures was used as input in "The Gene Ontology Resource", release 2020-04-23 (http://geneontology.org/)63–65.

**Drug “Gene Set Enrichment Analysis” (GSEA).** We adapted Gene Set Enrichment Analysis (GSEA) to enable enrichment analyses of "drug classes" based in their mechanism of action (MOA), for which we used the GSEA method implemented in the R package fgsea34, similarly as done by Sinha et al.24. In brief, similarity scores from the signatures associated to all compounds were ranked and the GSEA method applied to the ranked list with "gene sets" for GSEA analysis, being "gene sets" the sets of drugs within each class. Drug classes were defined based on their mechanisms of action (MOA); for which annotation data was included in CMap analysis as "description". R version 3.6.3 (2020-02-29) was used for computational analyses and a sample code can be obtained at https://github.com/Genomic-Instability-Lab/An-in-silico-analysis-of-drugs-potentially-modulating-the-cytokine-storm-triggered-by-SARS-CoV-2-inf.

**Gene Set Enrichment Analysis (GSEA) for GEO databases.** GEO data from human monocytes (GSE123574) and bronquial human cells (GSE63229) treated with DMSO or the MEK inhibitor trametinib was used to perform Gene Set Enrichment Analyses (GSEA). Differential expression data was obtained from the original paper in the case of the human monocytes66. For bronquial cells differential expression analysis was performed using the GEO2R GEO interface. The GSEA method implemented in the R package fgsea34 was used. Specifically, the gene expression log fold change values were ranked, and the GSEA method was applied to the ranked list with the Hallmark Signatures obtained from the GSEA database (https://www.gsea-msigdb.org/gsea/index.jsp). R version 3.6.3 (2020-02-29) was used for computational analysis and a sample code can be obtained at https://github.com/Genomic-Instability-Lab/An-in-silico-analysis-of-drugs-potentially-modulating-the-cytokine-storm-triggered-by-SARS-CoV-2-inf.

**In vitro assessment of LPS-induced inflammatory signaling.** THP-1 human monocyte cells were maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin, and grown at 37 °C in a humidified air atmosphere with 5% CO2. Cells in logarithmic growth phase were seeded as 2 x 106 cells in 10 cm dishes and differentiated into macrophages after incubation with 100 ng/mL PMA for 48 h. Differentiated cells were subsequently washed with PBS and grown in fresh RPMI 1640 medium for another 24 h. Cells were then pre-treated for 1 h with either DMSO (control) or 10 μM of each drug, followed by a 6 h treatment again
with either DMSO or 10 μM drugs in the absence or presence of 500 ng/mL LPS. Cell supernatants and pellets were obtained for ELISA and q-RT-PCR analyses, respectively. The following compounds were purchased from Selleckchem: Phorbol 12-myristate 13-acetate (PMA) (S7791), trametinib (S2673), selumetinib (S1008), SCH-772984 (S7101), vemurafenib (S1267), gefitinib (S1025) and erlotinib (S7786). LPS (L2630), dexamethasone (D4902) and hydrocortisone (3867) were purchased from Sigma.

ELISA. Cell supernatants were centrifuged at 10,000g for 1 min at 4 °C and analysed with IL-6 and IL-1β immunoassay kits (Invitrogen) according to the protocols provided by the manufacturer. LPS-treated supernatants were to be diluted prior to analysis: 1:5 for IL-6 detection, and 1:50 for IL-1β. Non-LPS-treated samples were not diluted for IL-6 detection and a 1:10 dilution was used in the case of IL-1β immunoassay. The absorbance of each well at 450 nm was measured and analysed using a PerkinElmer VICTOR Nivo Multimode Plate Reader and the MyAssays Desktop Explorer Software respectively.

Quantitative RT-PCR. Total RNA was extracted using the Agilent Absolutely RNA Miniprep Kit following manufacturer’s instructions. mRNA levels were measured by real-time quantitative PCR after reverse transcription of RNA, using for both the Invitrogen SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit with ROX. Quantitative RT-PCR was performed on an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System. We used the following primers sequences: GAPDH (GGA CTC ATG ACC ACA GTC CAT GCC, TCA GGG CTGA. The levels of GAPDH mRNA were used as control to normalize expression values.

Graphical representation and statistical analysis. “Drug” GSEA and GSEA graphs were obtained with R as explained in their corresponding section. Venn diagrams were represented using BioVenn®25, and the statistical significance for the overlap was calculated using the superexact test68. For the rest of the graph representations and the statistical analysis we used GraphPad Prism version 7.04.

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Most experiments, data analyses, and preparation of the figures: L.S.-B. Supervision of all the bioinformatic analysis: G.G.-L. and F.A.-S. Study conception, supervision and manuscript writing: O.F.-C. All authors reviewed the manuscript.

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