Article

The unique ORF8 protein from SARS-CoV-2 binds to human dendritic cells and induces a hyper-inflammatory cytokine storm

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The novel coronavirus pandemic, first reported in December 2019, was caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 infection leads to a strong immune response and activation of antigen-presenting cells, which can elicit acute respiratory distress syndrome (ARDS) characterized by the rapid onset of widespread inflammation, the so-called cytokine storm. In response to viral infections, monocytes are recruited into the lung and subsequently differentiate into dendritic cells (DCs). DCs are critical players in the development of acute lung inflammation that causes ARDS. Here, we focus on the interaction of a specific SARS-CoV-2 open reading frame protein, ORF8, with DCs. We show that ORF8 binds to DCs, causes pre-maturation of differentiating DCs, and induces the secretion of multiple proinflammatory cytokines by these cells. In addition, we identified DC-SIGN as a possible interaction partner of ORF8 on DCs. Blockade of ORF8 leads to reduced production of IL-1β, IL-6, IL-12p70, TNF-α, MCP-1 (also named CCL2), and IL-10 by DCs. Therefore, a neutralizing antibody blocking the ORF8-mediated cytokine and chemokine response could be an improved therapeutic strategy against SARS-CoV-2.

Keywords: COVID-19, SARS-CoV-2, ORF8, cytokine storm, dendritic cells
Introduction
The novel coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Kraemer et al., 2020; Rothan and Byrareddy, 2020; Zumla and Niederman, 2020). This virus has risen to a global pandemic with over 676 million infections and over 6 million deaths (https://coronavirus.jhu.edu/map.html). During acute infection, the cytokine release syndrome seems to be responsible for severe conditions and the development of SARS (Campana et al., 2020). The disease is divided into two phases. During the non-severe stage, the virus triggers innate and adaptive immune responses. Innate immunity is mainly mediated by phagocytic cells, i.e. professional antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages, and granulocytes that reside in the lung or infiltrate into the lung tissue after infection (Campana et al., 2020). Notably, the recruitment of monocytes into the lung and their differentiation into inflammatory CD1c⁺ DCs play a crucial role in viral infections, including infections of influenza and SARS-CoV-2 (GeurtsvanKessel et al., 2008; Sanchez-Cerrillo et al., 2020; Zheng et al., 2021). If the body fails to develop a protective immune response during the early phase, viruses propagate and cause massive destruction of the affected tissues in the severe stage (Shi et al., 2020). Severe tissue damage leads to a strong activation of APCs. This second innate immune response, characterized by the rapid onset of widespread inflammation (the so-called cytokine storm), can elicit acute respiratory distress syndrome (ARDS), which causes life-threatening respiratory disorders (Xu et al., 2020). Amongst others, DCs are important players in the development of acute lung inflammation that causes ARDS (Law et al., 2005; Li et al., 2019; Campana et al., 2020).

Recently, Wang et al. (2020) demonstrated that ORF8 is a secretory protein. Additionally, the study reported that patients with SARS-CoV-2 infection showed early seropositivity for anti-ORF8 IgM, IgG, and IgA and that ORF8 can be used as an early diagnostic marker. As DCs and macrophages act as APCs, the infection of these cells by SARS-CoV-2 or the interaction of these cells with viral proteins may impair the adaptive immune responses against the virus, which may trigger the initial process of the cytokine and chemokine storm (Jafarzadeh et al., 2020). In this study, we focus on the interaction of the secreted ORF8 protein with DCs and its contribution to the cytokine storm observed in COVID-19 patients (Young et al., 2020).

Results
ORF8 specifically binds to monocytes and DCs
ORF8 contains a short signal peptide sequence comparable to that of the spike protein of SARS-CoV-2. Recently, it has been shown that ORF8 is secreted from infected cells, indicating the functionality of the signal peptide sequence (Wang et al., 2020). To confirm this result, we transfected HEK293 cells with the wild-type ORF8 coding sequence, which includes the short 5’ untranslated region. After induction of protein expression by doxycycline for 24 h, secreted ORF8 was detectable in the supernatant by coomassie blue staining and immunoblotting (Supplementary Figure S1). Since CD14⁺ monocytes are able to recognize foreign proteins, we analyzed whether the ORF8 protein can interact with this cell type. Therefore, we purified the recombinant, secreted ORF8 protein, and labeled a fraction with Atto488 (Supplementary Figure S1B and C). Contamination of the purified ORF8 protein by endotoxin could be excluded (Supplementary Figure S1D). We showed that ORF8 was mainly bound to CD14⁺ monocytes within human peripheral blood mononuclear cells (PBMCs) (Figure 1A). To exclude the possibility that this was a random protein binding, we used bovine serum albumin (BSA)-Atto488 as a control. The binding abilities of ORF8 to monocytes and DCs were significantly higher compared to those of the control protein (Figure 1A; Supplementary Figure S2A). Monocytes are the origin of antigen-presenting DCs (Figure 1B). We demonstrated that both immature and mature DCs have the ability to interact with the ORF8 protein (Figure 1C). Again, BSA-Atto488 showed only weak interactions with both immature and mature DCs (Supplementary Figure S2A). To validate the binding specificity, we used a commercially available recombinant polyclonal rabbit anti-ORF8 antibody. The anti-ORF8 antibody reduced the binding of ORF8 to immature and mature DCs (Figure 1D), while the corresponding isotype control did not (Supplementary Figure S2B), confirming the specificity of the interaction between ORF8 and DCs.

ORF8 overrules DC differentiation into pre-maturity
Next, we aimed to define if ORF8 has a functional effect on DCs (Figure 2A). Similar to BSA, we found that ORF8 alone did not act as an inducer of monocyte differentiation (Figure 2B). Monocytes treated with BSA or ORF8 remained positive for the monocyte marker CD14 and negative for the DC markers DC-SIGN and CD1c (Figure 2B).

Next, we differentiated monocytes into immature DCs with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) and then analyzed if the addition of ORF8 could influence the differentiation process (Figure 3A). We observed an ORF8 dose-dependent upregulation of different surface maturation markers. For CD40, CD80, and CD83, a first plateau phase was reached between 500 and 1000 ng/ml and had an additional small increase at 2000 ng/ml (Figure 3B). Based on that, we chose a concentration of 1000 ng/ml (1 μg/ml) for all subsequent experiments to elicit robust activation. DCs differentiated in the presence of ORF8 showed a significantly different stellate-like morphology compared to immature DCs (Supplementary Figure S3A). In line with the morphological changes, the expression levels of the maturation markers CD40, CD80, and CD83 were also markedly upregulated (Figure 3B). For comparison, the expression profiles of the surface markers of lipopolysaccharide (LPS)-induced maturation are shown in Supplementary Figure S3B. None of the controls, including BSA, the anti-ORF8 antibody alone, its isotype control, or a human Fc-antibody fragment, induced DC maturation (Supplementary Figure S3C).
Figure 1 ORF8 binds specifically to monocytes and DCs. (A) The binding ability of ORF8-Atto488 to CD14+ monocytes within PBMCs was analyzed by flow cytometry (unspecific control: cells stained for BSA-Atto488; negative control: unstained cells). (B) Experimental setup for the differentiation and maturation of DCs from monocytes. (C) The binding abilities of ORF8-Atto488 to immature and mature DCs were analyzed by flow cytometry. (D) The anti-ORF8 antibody reduced the binding of ORF8 to immature and mature DCs as analyzed by flow cytometry. The blue arrows indicate the shift of the peak. αORF8, anti-ORF8 antibody. The experiments were repeated three times. *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001. ns, not significant.
In addition, we analyzed the DC-specific marker DC-SIGN in this experimental setup. Interestingly, the cell surface expression of DC-SIGN was almost completely downregulated in DCs differentiated in the presence of ORF8 (Figure 3C and D). Neutralizing ORF8 with the anti-ORF8 antibody partially restored the cell surface expression of DC-SIGN (Figure 3D). By co-immunoprecipitation, we showed that ORF8 interacts with the extracellular part of the DC-SIGN receptor (Figure 3E, blot 1). Using the anti-ORF8 antibody to neutralize ORF8, the binding between DC-SIGN and ORF8 was reduced (Figure 3E, blot 2).

**ORF8 induces unique pro-inflammatory cytokine secretion during DC differentiation**

SARS-CoV-2 can induce a cytokine storm leading to ARDS (Xu et al., 2020). Therefore, we also analyzed the cytokine and chemokine expression profiles of monocyte-derived DCs exposed to ORF8 during differentiation. Strikingly, compared with that in untreated cells, the secretion of IP-10, IL-1β, TNF-α, IL-10, MCP-1 (also named CCL2), IL-6, and IL-12p70 was significantly elevated in ORF8-treated cells (Figure 4A). This cytokine profile was correlated with COVID-19 adverse outcome pathways as well as with SARS-CoV-2 innate immune evasion and cell-specific immune response pathways (Figure 5A). In an independent experiment, the elevated secretion of IP-10, IL-1β, TNF-α, IL-10, MCP-1 (also named CCL2), IL-6, and IL-12p70 in monocyte-derived DCs could be partly reversed by the simultaneous addition of the anti-ORF8 antibody during differentiation, verifying the ORF8-specific induction of the increased cytokine secretion (Figure 4B). Furthermore, ORF8 induced a unique DC activation profile (Supplementary Figure 5A). By RNA sequencing, 211 unique RNAs were identified by ORF8 induction in comparison to LPS stimulation (Supplementary Figure 5B and C). Using the metascape platform for enrichment and pathway analysis (Zhou et al., 2019), we showed that this cytokine profile was significantly associated with COVID-19 adverse outcome pathways as well as with SARS-CoV-2 innate immune evasion and cell-specific immune response pathways (Figure 5B).

By analyzing the RNA sequencing data of DCs that were differentiated in the presence or absence of ORF8, we found a total of 843 differentially expressed genes (P-value < 0.05 after Benjamini Hochberg correction; Supplementary Figure 5A, n = 4 for each group). Subsequent pathway and process enrichment analysis (metascape platform) of the differentially expressed genes in ORF8-activated DCs revealed a significant enrichment of inflammatory and immune-activating pathways (Supplementary Figure 5B). Using the Coronascape network (www.metascape.org/COVID; Zhou et al., 2019), we compared our data with single-cell mRNA sequencing data from monocytes (Zhang et al., 2020) and bronchoalveolar immune cells (Liao et al., 2020) in patients with COVID-19. These cell types are the most closely related cell populations available in the metascape databases. We found a strong overlap in the gene profiles between our study and the published studies (Figure 5C and D) and even a stronger overlap in genes that

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**Figure 2** ORF8 lacks the potential to differentiate monocytes into DCs. (A) Experimental setup: CD14+ monocytes were treated with ORF8 and analyzed for markers of immature DCs. (B) The expression levels of DC-SIGN, CD1c, and CD14 on monocytes after incubation with ORF8 or BSA were analyzed by flow cytometry. Positive control: monocytes differentiated into immature DCs by GM-CSF and IL-4 (orange line). Negative control: monocytes without any stimulation (black line). The experiments were repeated three times.
Figure 3 ORF8 overdrives DC differentiation into pre-maturity. (A–C) Monocytes were differentiated into immature DCs in the presence or absence of ORF8. (A) Schematic diagram of the differentiation procedure. (B) The relative expression levels of CD40, CD80, CD83, CD11c, CD86, and MHCII during differentiation were analyzed by flow cytometry. (C) The expression levels of DC-SIGN during differentiation were analyzed by flow cytometry. Red line: cells treated in the presence of ORF8; blue line: cells treated without ORF8; black line: unstained cells. (D) Quantification of DC-SIGN surface expression. αORF8, anti-ORF8 antibody; MFI, mean fluorescence intensity. (E) Co-immunoprecipitation of ORF8 and DC-SIGN. The immunoprecipitates were immunoblotted with an anti-DC-SIGN antibody. The experiments were repeated three times. *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001.
Figure 4 ORF8 induces a strong cytokine storm during DC differentiation. (A and B) Monocytes were differentiated into DCs in the presence of 1 μg/ml ORF8 or in the absence of ORF8 (control). (A) ORF8-induced cytokine and chemokine production was determined by the cytokine bead array. (B) The effect of the anti-ORF8 antibody on ORF8-induced cytokine and chemokine production was determined by the cytokine bead array. bAB, anti-ORF8 antibody; IgG, isotype control. The experiments were repeated six times. *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001.
Figure 5 ORF8 induces an inflammatory mRNA profile involved in SARS-CoV-2 infection. (A) The enriched pathways of the upregulated cytokines and chemokines in ORF8-induced DCs (Figure 4A) are presented in a bar graph. (B) By RNA sequencing, 211 unique RNAs were identified by ORF8 induction in comparison to LPS stimulation (Supplementary Figure S4B and C). The enriched pathways of the 211 unique RNAs are presented in a bar graph. (C and D) The datasets used in C and D are as follows: ORF8 induction (843 differentially expressed genes in DCs differentiated in the presence of ORF8 in this study); severe SARS-CoV-2 infection (analyzed in BALF) (Liao et al., 2020); severe and moderate SARS-CoV-2 infection (analyzed in monocytes) (Zhang et al., 2020). −log10(P) represents −log10(P-value). BALF, bronchoalveolar lavage fluid. (C) Cluster analysis of enriched terms across the three datasets, colored by P-values (https://metascape.org/; Zhou et al., 2019). (D) Overlaps of the three datasets. Purple curves link identical genes. Blue curves link genes that belong to the same enriched ontology term. The inner-circle represents gene lists, where hits are arranged along the arc. Genes that hit multiple lists are colored in dark orange, and genes unique to a list are shown in light orange. Calculation source: www.metascape.org/COVID (Zhou et al., 2019).
reflect the same pathways and functions (Supplementary Figure S5B). The overlapped genes in all three studies were mainly enriched in the regulation of cytokine production, myeloid leukocyte activation, and leukocyte migration (Figure 5C; Supplementary Figure S5B). Next, we compared the genes in ORF8-treated DCs with antiviral-related genes and cytokine genes in host cells after SARS-CoV-2 infection (Sun et al., 2020). We found an overlap of genes, including LAMP3, TRIM14, IL-1A, CXCL8, IL-15, IL-6, CCL5, EB13, LIF, and IL-1B. In comparison with the expression profile of blood APCs in patients with severe COVID-19 (Saichi et al., 2021), we found an overlap of genes, including CXCR4, TNAP3, KLF4, LCH, NR4A1, RETN, and IL-1B. All detected genes were related to severe COVID-19 (Supplementary Figure S5C).

Detection of ORF8 and anti-ORF8 antibodies in COVID-19 patients

Since monocytes circulate in the blood stream and migrate into the lung tissue upon inflammation to differentiate into DCs (GeurtsvanKessel et al., 2008; Li et al., 2019), we analyzed whether ORF8 is present in these compartments of COVID-19 patients. In hospitalized patients, ORF8 was detected in a small amount of plasma at different time points after infection (Figure 6A). In addition, high levels of ORF8 were observed in the lung tissue of severe cases of infection. Locotypical cells, such as vascular endothelial and smooth muscle cells, as well as the respiratory epithelium of bronchi, stained positive for ORF8 (Figure 6B).

Next, we were interested in whether patients infected with SARS-CoV-2 produce antibodies against ORF8. Therefore, we used the enzyme-linked immunosorbent assay (ELISA) to detect anti-ORF8 antibodies in convalescent plasma. First, we analyzed plasma collected from 64 COVID-19 patients at 0–90 days post diagnosis (Figure 6C) and found eight patients highly positive for anti-ORF8 IgG antibodies. Anti-ORF8 antibody was still detectable in the plasma of COVID-19 patients over 90 days post diagnosis (Figure 6D; n = 104). Similarly, we also performed an ELISA for detecting spike antibodies (Supplementary Figure S6) to compare the immunogenicity of the two viral proteins. We found that seven out of the eight anti-ORF8 antibody-positive samples also had a very strong reaction toward the SARS-CoV-2 spike protein. An overview of all routinely performed assays using patient sera can be found in Supplementary Table S1.

Lack of anti-ORF8 neutralizing antibodies in COVID-19 patients

Having shown that patients develop antibodies against ORF8, we next analyzed if these antibodies have the potential to neutralize the effect of ORF8 on DCs. Therefore, the eight identified sera with high anti-ORF8 antibody titer from the patient cohort at late time points (Figure 6D) were pre-incubated with the ORF8 protein overnight to ensure the binding of the anti-ORF8 antibodies to ORF8. Subsequently, monocytes were incubated with the ORF8/sera mixture, sera alone, or ORF8 alone during differentiation. Interestingly, anti-ORF8 antibody-containing sera could not reduce the binding of ORF8 to DCs (Figure 7A). On the contrary, we showed that ORF8 bound even better to DCs in combination with the sera. This effect could be reversed by blocking the Fc receptor on DCs (Figure 7B). By analyzing the surface expression of DC-specific markers and activation markers, we showed that anti-ORF8 antibody-positive sera were not able to reduce ORF8-induced DC activation (Figure 7C). Furthermore, most of the sera did not reduce ORF8-triggered cytokine or chemokine production (Figure 7D). The loop region of ORF8, which is recognized by antibodies, has high plasticity (Supplementary Figure S7). This may justify the missing inhibitory effect of patient-derived anti-ORF8 antibodies. This region may also be responsible for the interaction with the cell surface of DCs.

Discussion

Recently, it was shown that ORF8 is a glycosylated homodimer secreted by SARS-CoV-2-infected cells and could be detected in the sera of COVID-19 patients (Wang et al., 2020; Matsuoka et al., 2022). Furthermore, it was reported that a mutant SARS-CoV-2 strain originating in Singapore displayed a deletion of 382 nucleotides in the region of ORF8. This deletion was associated with decreased pathogenicity (Su et al., 2020; Young et al., 2020). These findings underline the importance of ORF8 as a virulence factor in the pathogenicity of this infectious disease. Notably, all the main strains of SARS-CoV-2, including the Omicron variants, contain the ORF8 protein (https://covariants.org/).

In our study, we used for the first time ORF8 expressed and purified from a mammalian system, whereas previous studies used bacterial-derived proteins (Flower et al., 2021). Previous reports showed that recombinant SARS-CoV-2 proteins can be contaminated by endotoxins, including LPS. In our study, we could exclude the possibility that protein contamination (Supplementary Figure S1C) or endotoxin contamination (Supplementary Figure S1D) are responsible for any effects on immune cells. Schwarz et al. (2014) postulated the minimum contamination level of endotoxins needed to drive the immune response and cytokine production in monocytes and DCs. In our protein samples, the amount of endotoxins (Supplementary Figure S1D) remained below this limit. Furthermore, the values were significantly lower than those for contaminated spike protein samples described elsewhere (0.038 EU/mL vs. 3 EU/mL, respectively) (Ouyang et al., 2021; Cinquegrani et al., 2022).

We demonstrated that the extracellular ORF8 protein binds specifically to monocytes as well as immature and mature DCs. The specificity of the binding was confirmed by blocking ORF8 with an anti-ORF8 antibody (Figure 1D). Nevertheless, ORF8 was not able to directly induce the differentiation of monocytes into DCs, as previously shown for several other viruses, e.g. HIV-1 (Majumder et al., 2005). Instead, we showed that ORF8 induced dose-dependent pre-maturity of DCs differentiated in the presence of IL-4 and GM-CSF.

Pre-maturity was characterized by a mature cell morphology (Xing et al., 2011) as well as upregulation of the maturation markers CD40, CD80, and CD83. Surprisingly, the DC marker
Figure 6 Detection of anti-ORF8 antibody titers in COVID-19 patients. (A) Immunoblot detection of ORF8 in the sera of hospitalized patients ($n = 5$). (B) Immunohistochemical detection of ORF8 in the lung tissue at different severity stages of SARS-CoV-2 infection (severity stage: patient 1 > patient 2 > patient 3). ORF8 is expressed in the hyaline membranes (red asterisk), pneumocytes (red arrow heads), histiocytes (green arrows), and fibroblastic cells in the fibroblastic foci of the organizing pneumonia (blue arrows). ORF8 is negative in lymphocytes in all cases, independent of the severity of the infection. Control: the lung tissue of a non-COVID-19 patient. (C) Anti-ORF8 antibody levels were detected by ELISA in SARS-CoV-2-positive ($n = 64$) and -negative patients ($n = 55$) during 0–90 days post diagnosis. Positive control: ORF8 positive plasma from a COVID-19 patient; negative control: plasma of a healthy volunteer (SARS-CoV-2-negative). OD values above 0.4: high titers of anti-ORF8 antibodies; OD values below 0.4: low titers of anti-ORF8 antibodies or negative for anti-ORF8 antibodies. (D) Anti-ORF8 antibody levels were detected by ELISA in SARS-CoV-2-positive ($n = 104$) and -negative patients ($n = 100$) at >90 days post diagnosis. OD values above 1.0: high titers of anti-ORF8 antibodies; OD values below 1.0: low titers of anti-ORF8 antibodies or negative for anti-ORF8 antibodies. σORF8, anti-ORF8 antibody; dpd, days post diagnosis; OD, optical density.
Figure 7 Failure of patient-derived anti-ORF8 antibodies to neutralize ORF8. (A–D) The sera of eight COVID-19 patients were highly positive for anti-ORF8 IgG antibodies. Monocytes were incubated with the ORF8/sera mixture, sera alone, or ORF8 alone during differentiation into immature DCs. iDCs, immature DCs. *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001. (A and B) The binding capacity of ORF8 to immature DCs was determined by FACS and quantitative analysis. (C) Effect of anti-ORF8 antibody-containing sera on ORF8-induced maturation of immature DCs. Samples were normalized to the geometrical mean of immature DCs. (D) Effect of anti-ORF8 antibody-containing sera on ORF8-triggered cytokine and chemokine production in immature DCs. Values of immature DCs were set to one, and expression changes were calculated accordingly and plotted in a heat map.
DC-SIGN was entirely downregulated during DC differentiation in the presence of ORF8 (Figure 3C). DC-SIGN was shown to be a receptor for SARS-CoV-2 in lung and kidney epithelial and endothelial cells (Amarael et al., 2021). Even more interesting, DC-SIGN was also shown to be key for SARS-CoV-2 infection of DCs (Yang et al., 2004). During HIV infection, DC-SIGN is known to form an infectious synapse between infected DCs and T cells to facilitate HIV infection (McDonald et al., 2003). Furthermore, it was shown that DC-SIGN mediates the internalization of intact HIV into a low-pH nonlysosomal compartment in DCs. This internalization is needed for the trans-activation of T cells by DCs (Kwon et al., 2002). In this study, we showed that blockade of ORF8 partly rescued DC-SIGN expression on the surface of immature DCs (Figure 3D), suggesting that ORF8 interacts with DC-SIGN. We further showed that ORF8 interacted directly with DC-SIGN in a co-immunoprecipitation assay and that this interaction was reduced by an anti-ORF8 neutralizing antibody (Figure 3E). Hence, DC-SIGN is a potential interaction partner of ORF8 and might be internalized upon binding to ORF8. DC-SIGN is known for its immune regulatory role in DCs (Gringuéhuis et al., 2014) and macrophages (Lugo-Villarino et al., 2018). Downregulation of DC-SIGN on the surface of DCs might lead to altered cytokine expression. It was shown that DC-SIGN ligation on DCs results in ERK and PI3K activation and modulates IL-10 secretion (Caparasos et al., 2006).

ARDS is the main cause of death in patients infected with MERS-CoV, SARS-CoV, and SARS-CoV-2 (Lew et al., 2003; Drosten et al., 2013). ARDS is the final outcome of a cytokine storm reflected by key pro-inflammatory cytokines such as IL-6, IL-8, IL-1β, GM-CSF and chemokines such as CCL2, CCL-5, IP-10 (CXCL10), and CCL3 (Jiang et al., 2005; Reghunathan et al., 2005; Cameron et al., 2008). Furthermore, high expression levels of IFN-γ, IP-10, IL-1β, MCP-1 (also named CCL2), and TNF-α have been detected in patients with SARS-CoV-2 infection (Costela-Ruiz et al., 2020). These inflammatory cytokines may activate T helper cells (e.g. Th1). Th1 activation is a key event in the activation of adaptive immunity. However, unlike patients with SARS, patients with SARS-CoV-2 also present elevated levels of Th2-secreted anti-inflammatory cytokines, including IL-4 and IL-10 (Lew et al., 2003; Huang et al., 2020; Marchingo et al., 2020). Recent publications showed an alternative pro-inflammatory role of IL-10 in SARS-CoV-2 infection (Islam et al., 2021; Hasanwand, 2022). In line with this, we found a significant upregulation of pro-inflammatory cytokines and chemokines, including IL-10. The observed ORF8-induced cytokine profile showed close similarities with COVID-19 adverse outcome pathways as well as SARS-CoV-2 innate immune evasion and cell-specific immune response pathways (Figure 5A). The binding of ORF8 to monocytes was recently reported by other groups in preprinted articles (Nisha Kriplani, 2021; Wu et al., 2022). Their observations back up and support our findings of a unique function of ORF8 in the context of inflammation.

In our two cohorts of convalescent plasma, about 10% of the patients infected with SARS-CoV-2 contained anti-ORF8 IgG antibodies. In contrast, Hachim et al. (2020) showed that anti-ORF8 antibodies could be detected in almost all patients infected with SARS-CoV-2. Since the conclusion of the study by Hachim et al. (2020) needs to be validated by a conventional ELISA, further studies are required to explain the different findings.

Nevertheless, the identified anti-ORF8 IgG+ sera had almost no neutralizing effect on ORF8-induced cytokine secretion. Instead, pre-incubation of ORF8 with anti-ORF8 IgG+ sera led to enhanced binding of ORF8 to DCs (Figure 7A). This antibody-enhanced binding of different viral proteins to DCs is well documented for several viruses, including influenza (Skowronska et al., 2010), dengue (Ambuel et al., 2014), and HIV-1 (Willey et al., 2011). This phenomenon has also been intensively discussed for SARS-CoV-2 infection, with many studies demonstrating that antibodies against SARS-CoV-2 are key to enhancing the infection rate and are a critical challenge for developing future vaccines (Eroshenko et al., 2020; Lee et al., 2020; Rieke, 2021). This enhancement seems to be mediated by the Fc-receptors on the DCs since it could be blocked by conventional Fc-block (Figure 7B). Fu et al. (2020) discussed therapeutical tools to reduce SARS-CoV-2-induced inflammatory responses, including multiple methods to block FcR activation.

The detection of anti-ORF8 antibodies in patient serum underscores the importance of our finding that ORF8 is an important factor in the development of COVID-19. The missing neutralizing capacity of the serum antibodies shows that there is an unmet need to develop a neutralizing anti-ORF8 antibody to block, amongst others, the proinflammatory effect of ORF8 on DCs. Screening of a larger cohort of patient sera has the potential to identify a potent ORF8-blocking antibody, as was identified for the spike protein (Vanshylla et al., 2022).

We used here SARS-CoV-2 ORF8 purified from HEK293 cells, which includes all protein modifications, e.g. glycosylation. When determining the structure of this protein at a resolution of 2.6 Å, we observed that the overall structure of ORF8 was consistent with published structures of ORF8 isolated from E. coli (Flower et al., 2021). Interestingly, we noted a considerable degree of flexibility between monomers within the ORF8 dimer compared to reported structures, despite the presence of an identical dimer interface. To date, ORF8 has been implicated in indirectly binding several cell surface receptors, including MHC-I (Zhang et al., 2021) and IL17RA (Lin et al., 2021). How the structural flexibility of ORF8 involves in the binding and downregulation of multiple cell receptors to facilitate any immunomodulatory functions needs to be analyzed in further studies. Similarly, ORF8 has been postulated to form large assemblies mediated by SARS-CoV-2-specific sequence motifs (Flower et al., 2021). Whether ORF8 oligomerization is relevant to DC-SIGN binding and DC cell differentiation remains to be determined.

In conclusion, our findings show that ORF8 contributes to the cytokine and chemokine storm in patients with SARS-CoV-2 infection (Coperchini et al., 2020; Costela-Ruiz et al., 2020; Noroozi et al., 2020; Tang et al., 2020) through interaction with DCs. Furthermore, our findings shed light on the function of ORF8 and its role in the development of ARDS.
Materials and methods

Differentiation of monocytes into DCs

Human PBMCs were isolated from leukocyte concentrate using a density gradient (Lymphoprep, Stemcell). Ethical clearance was obtained from the ethical review committee of the University Hospital Cologne (N\(^{11}\)-339). The harvested PBMCs were washed with PBS and resuspended in X-Vivo 15 media (Biozym). To enrich monocytes, we used the adherence strategy as described previously (Thurner et al., 1999). In each well of a 6-well plate, we seeded 2 \( \times 10^7 \) PBMCs in 2 ml of X-Vivo 15 media and incubated them for 75 min under 5% CO\(_2\) and 90% humidity at 37°C. The non-adherent fraction was removed. To differentiate monocytes into immature DCs, X-Vivo 15 media were supplemented with 100 ng/ml human GM-CSF and 20 ng/ml human IL-4 (both from PeproTech). The cells were cultured under 5% CO\(_2\) and 90% humidity at 37°C for 5 days. To analyze the function of ORF8 with monocytes and DCs, cells were pre-incubated with the ORF8-Atto488 protein before staining for all other cell surface markers. To exclude random protein binding, we used BSA-Atto488 as a control. CD14, DC-SIGN, and CD1c (BioLegend) were used for the staining of monocytes. MHCII, CD11c, CD40, CD80, CD83, CD86, and DC-SIGN (BioLegend) were used for the staining of immature DCs. Data acquisitions were performed on a FACSCanto II Flow Cytometer (BD Biosciences). The data were analyzed with FlowJo v10 software (BD).

Blocking of ORF8

To block ORF8, cells were pre-incubated with a polyclonal rabbit anti-ORF8 antibody (ABIN6992307, antibodies-online GmbH) for 30 min on ice. To avoid cell damage caused by sodium azide in the antibody solution, we used no more than 2 \( \mu \)g of anti-ORF8 antibody in the experiment. As an isotype control, an unspecific polyclonal rabbit antibody (NEB) was used. To block ORF8 during differentiation, monocytes were pre-incubated with the polyclonal rabbit anti-ORF8 antibody or the isotype control overnight, then GM-CSF and IL-4 were added to induce monocyte differentiation.

Co-immunoprecipitation of ORF8 and DC-SIGN

For co-immunoprecipitation, 25 \( \mu \)l of streptavidin-coated magnetic beads (NEB) was pre-incubated at 4°C for 1 h with agitation. Samples included an empty control (no protein), ORF8 protein with a Strep II tag, and 2 \( \mu \)g of ORF8 protein plus 2 \( \mu \)g of anti-ORF8 antibody (ABIN6992307, antibodies-online GmbH). Unbound protein was washed from the beads with 500 \( \mu \)l of PBS, spun down, and resuspended in 50 \( \mu \)l of PBS. For analyzing protein–protein interaction, pre-incubated beads were incubated a second time with a recombinant human DC-SIGN/Fc tag protein [2 \( \mu \)g; N-terminally truncated extracellular domain (Lys62–Ala404) of human DC-SIGN; Sino Biological] for 1 h at 4°C with agitation. The beads were washed three times with 500 \( \mu \)l of PBS before denaturation and separation by a polyacrylamide gel (4%–12% gradient). The gels were blotted onto a nitrocellulose membrane and blocked with 5% milk in TBST buffer. Then, a direct horseradish peroxidase (HRP)-conjugated anti-human Fc (Invitrogen) antibody was used, and bands were detected with an imager (Bio-Rad).

Multiplex analysis of cytokines and chemokines

We collected supernatant from differentiated DCs of several donors in the presence or absence of the ORF8 protein. The supernatant was cleared twice by centrifugation to exclude cells and cellular fragments. For the LegendPlex assay, we followed the manufacturer’s instructions of the LEGENDplexTM HU Essential Immune Response Panel (BioLegend). The acquisitions of the beads were performed on a FACSComp II Flow Cytometer (BD Biosciences). The data were analyzed using LEGENDplex v8 software (BioLegend) to determine the standard curves and current concentrations of the samples. The data were transferred to GraphPad Prism (version 9) for visualization and statistical analysis (one-way ANOVA).

Patients and sample collection

Patients positive for SARS-CoV-2 by reverse transcription–quantitative polymerase chain reaction (RT–qPCR) after admission to the University Hospital Cologne were included in this study. Our study enrolled 268 adult patients who tested positive for SARS-CoV-2 between 0 and 90 days after the first diagnosis to cover the early onset of the infection. Ethical clearance for the use of human material was obtained from the ethical review committee of the University Hospital Cologne. For 10 patients, information about age and sex, as well as hospitalization, was not available. The ages of the study cohort ranged from 24 to 80 years, with an average of 41 years. The study included 37.5% males, 46.8% females, and 15.6% undefined individuals. Twelve patients were hospitalized during follow-up, and neutralizing antibodies against the virus were detected in 76% of the patients (DKRS number: DRKS00021468 EIKOS).

ELISA

For detecting anti-ORF8 antibodies, 96-well Maxisorp Nunc Immuno plates (Thermo Fisher) were coated at 4°C overnight with 0.5 \( \mu \)g of ORF8. After washing with TBS, unspecific binding was blocked with 3% BSA/TBS blocking buffer for 1 h at room temperature. Plasma samples were diluted 1:100 with blocking buffer. After 90 minutes of incubation at room temperature, the plates were washed three times with TBS buffer. Next, a rabbit anti-human HRP antibody (1:3000; Dako) in blocking solution was added and incubated for 1 h at room temperature, and the plates were washed three times. HRP activity was detected with 50 \( \mu \)l of 1-Step Ultra TMB ELISA substrate solution (Thermo Scientific).
Fisher). Then, the reaction was stopped with 50 µl of 10% H₂SO₄, and the absorbance was measured at 450 nm. For detecting spike antibodies, samples were measured as described previously (Meinberger et al., 2021). The manufacturer provides positive and negative controls and a cut-off sample to facilitate the evaluation of the patients. Due to the fact that in the negative group (n = 100, PCR negative), some of the plasma from patients with SARS-CoV-2 infection showed low reactivity towards ORF8, the cut-off had to be set at OD = 1.1. Samples were measured in duplicate. The data were plotted and analyzed with GraphPad Prism (version 9).

Immunoblotting and immunohistochemistry

The circulation of ORF8 was analyzed in the sera of five hospitalized patients collected at two timepoints during infection [day of diagnosis (t1) and 4 to 5 days during follow-up (t2)]. Sera (2 µl) were boiled in 22 µl of denaturing laemmli loading buffer and separated by a 4%–12% NuPAGE SDS gel, running with MES buffer. As a control, healthy donor sera were spiked with bacteria-expressed ORF8 (RP-87666; Invitrogen) at the indicated amount. The separated protein was transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk for 1 h, followed by incubation with the primary polyclonal rabbit anti-ORF8 antibody (1:1000, ABIN6992307, antibodies-online GmbH) overnight. F(ab)² anti-rabbit HRP (1:7000, NA9340, Amersham) was used as a secondary antibody. The development was performed with West Pico PLUS Chemiluminescent Substrate (Thermo) and an imaging system from Bio-Rad.

For immunohistochemistry, ORF8 was detected in formalin-fixed tissues (3 µg) of SARS-CoV-2-infected patients. The epitope was retrieved by citrate treatment (pH 8). ORF8 was stained with an anti-ORF8 monoclonal antibody (Clone #1041422, R&D) in a BOND immunostainer (dilution of 1:500). After blocking, incubation with H₂O₂ for 5 min, and chemical enhancement for 10 min, the antibody was developed using poly-HRP-anti mouse/rabbit IgG (Bright Vision, Medac-diagnostics) and the DAB away kit (Biocare Medical). In addition, the morphology of the formalin-fixed paraffin-embedded tissues was examined using routine H&E staining.

Screening for ORF8 neutralizing antibodies in patients with high anti-ORF8 antibody titers

The ORF8 protein was incubated with 5 µl of patient serum (n = 8) overnight to get maximum binding of the antibodies. Monocytes were incubated with the ORF8/sera mixture, sera alone, or ORF8 alone for 5 days during differentiation. The differentiated DCs were analyzed for the specific upregulation of dendritic and activation markers by FACS analysis. The supernatant was used for multiplex analysis of cytokines and chemokines.

Statistical analysis

The ‘n’ represents the number of biological replicates, with a minimum of n = 3. For the statistical analysis, the software GraphPad Prism (version 9) was used, and a one- and two-way ANOVA were performed.

Data availability

For sequencing data and LegenPlex data, please contact Felix Bock at felix.bock@uk-koeln.de.

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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