The Journal of Immunology

RESEARCH ARTICLE | JUNE 02 2023

SARS-CoV-2 ORF8 Mediates Signals in Macrophages and Monocytes through MyD88 Independently of the IL-17 Receptor

Nicole O. Ponde; ... et. al

J Immunol (2023) 211 (2): 252–260.
https://doi.org/10.4049/jimmunol.2300110

SARS-CoV-2 ORF8 Mediates Signals in Macrophages and Monocytes through MyD88 Independently of the IL-17 Receptor

Nicole O. Ponde,* Karsen E. Shoger,† Mst Shamima Khatun,‡ Mrinal K. Sarkar,§ Ipsita Dey,* Tiffany C. Taylor,* Rylee N. Cisney,* Samyuktha P. Arunkumar,* Johann E. Gudjonsson,§ Jay K. Kolls,‡ Rachel A. Gottschalk,† and Sarah L. Gaffen*

SARS-CoV-2 has caused an estimated 7 million deaths worldwide to date. A secreted SARS-CoV-2 accessory protein, known as open reading frame 8 (ORF8), elicits inflammatory pulmonary cytokine responses and is associated with disease severity in COVID-19 patients. Recent reports proposed that ORF8 mediates downstream signals in macrophages and monocytes through the IL-17 receptor complex (IL-17RA, IL-17RC). However, generally IL-17 signals are found to be restricted to the nonhematopoietic compartment, thought to be due to rate-limiting expression of IL-17RC. Accordingly, we revisited the capacity of IL-17 and ORF8 to induce cytokine gene expression in mouse and human macrophages and monocytes. In SARS-CoV-2–infected human and mouse lungs, *IL17RC* mRNA was undetectable in monocyte/macrophage populations. In cultured mouse and human monocytes and macrophages, ORF8 but not IL-17 led to elevated expression of target cytokines. ORF8-induced signaling was fully preserved in the presence of anti–IL-17RA/RC neutralizing Abs and in *Il17ra*$^{-/-}$ cells. ORF8 signaling was also operative in *Il1r1*$^{-/-}$ bone marrow–derived macrophages. However, the TLR/IL-1R family adaptor MyD88, which is dispensable for IL-17R signaling, was required for ORF8 activity yet MyD88 is not required for IL-17 signaling. Thus, we conclude that ORF8 transduces inflammatory signaling in monocytes and macrophages via MyD88 independently of the IL-17R. *The Journal of Immunology*, 2023, 211: 252–260.

Severe acute respiratory syndrome coronavirus 2 surfaced as a novel virus responsible for the ongoing COVID-19 pandemic, infecting >750 million individuals to date according to the World Health Organization. Although the immune responses that govern disease outcome have not been completely elucidated, severe infection is often associated with a systemic "cytokine storm," characterized by hyperactivation of immune cells and overproduction of proinflammatory cytokines (1).

SARS-CoV-2 open reading frame 8 (*ORF8*) encodes a secreted multifunctional protein that is highly expressed in severe disease, and patients infected with *ORF8*-deleted viral mutants exhibit milder symptoms (2, 3). ORF8 has been described to play multiple roles in the course of infection, including immune modulation by regulation of MHC expression, acting as a histone mimic, and antagonizing a type I IFN response (3). Two recent studies reported unexpectedly that ORF8 activates downstream cytokine responses in murine macrophages and human monocytes through the IL-17 receptor, and thus it was concluded that ORF8 is a viral "mimic" of IL-17 (IL-17A) (4, 5). This observation carries important implications, both for understanding the pathogenesis of coronavirus-induced pulmonary pathology and for strategies that may be employed to mitigate pathogenic inflammation such as biologic intervention (6).

IL-17 is the founding member of a distinct subclass of cytokines and protects against an assortment of microbes, especially extracellular bacteria and fungi (7). Production of IL-17A and its closest homolog, IL-17F, is limited to type 17 cells, most famously CD4$^+$ Th17 cells, but also subsets of CD8$^+$ cells, γδ-T cells, some NK cells, and group 3 innate lymphoid cells (8). Whereas IL-17 is made by lymphocytes, most studies have demonstrated that IL-17–responsive cells are nonhematopoietic, typically epithelial and mesenchymal cell types. This conclusion has been drawn from diverse approaches and model systems, including cultured primary and immortalized cells, bone marrow chimeras, and conditional IL-17R–knockout mouse lines (9–14). Sensitivity to IL-17 appears to be rate limited by restricted expression of the IL-17RC subunit (15–19). In contrast, the IL-17RA subunit is widely expressed in both the hematopoietic and nonhematopoietic compartments and is a coreceptor for several members of the IL-17 ligand family, including IL-17C, IL-25 (IL-17E), and IL-17F (15, 16, 19–21). Although IL-17 can direct myeloid cell recruitment to tissue during inflammation, this is generally believed to be mediated indirectly through the induction of chemokines and cytokines in epithelial or mesenchymal cell types (7).

Given our longstanding interest in IL-17 signaling mechanisms, we were intrigued by the suggestion raised in these and other reports

*Division of Rheumatology and Clinical Immunology, Department of Medicine, University of Pittsburgh, Pittsburgh, PA; †Department of Immunology, University of Pittsburgh, Pittsburgh, PA; ‡Tulane University, New Orleans, LA; and §Department of Dermatology, University of Michigan, Ann Arbor, MI

ORCIDs: 0000-0003-2704-0451 (N.O.P.); 0000-0001-8505-4259 (M.K.S.); 0000-0002-4971-3728 (T.C.T.); 0000-0002-8206-2955 (R.N.C.); 0000-0002-1397-9399 (S.P.A.); 0000-0002-0080-0812 (J.E.G.); 0000-0001-5151-6304 (J.K.K.); 0000-0002-5248-8816 (R.A.G.); 0000-0001-8511-2041 (S.L.G.).

Received for publication February 14, 2023. Accepted for publication May 11, 2023.

This work was supported by the National Institute of Allergy and Infectious Diseases Grants AI162616 and AI147383 (to S.L.G.), AR075043 (to J.E.G. and J.K.K.), and AI089443 (to T.C.T.), as well as by National Heart, Lung, and Blood Institute Grant HL13993 (to J.K.K.) and National Institute of General Medical Sciences Grant GM146896 (to R.A.G.). J.K.K. and M.S.K. were supported by the Louisiana Board of Regents Endowed Chairs for Eminent Scholars Program. M.K.S. was supported by National Psoriasis Foundation Translational Research Grant 852098.

Address correspondence and reprint requests to Dr. Sarah L. Gaffen, University of Pittsburgh, S702 Biomedical Science Tower, 200 Lothrop Street, Pittsburgh, PA 15261. E-mail address: sarah.gaffen@pitt.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; KC, keratinocyte; ORF8, open reading frame 8; qPCR, quantitative real-time PCR; scRNA-seq, single-cell RNA sequencing; WT, wild-type.

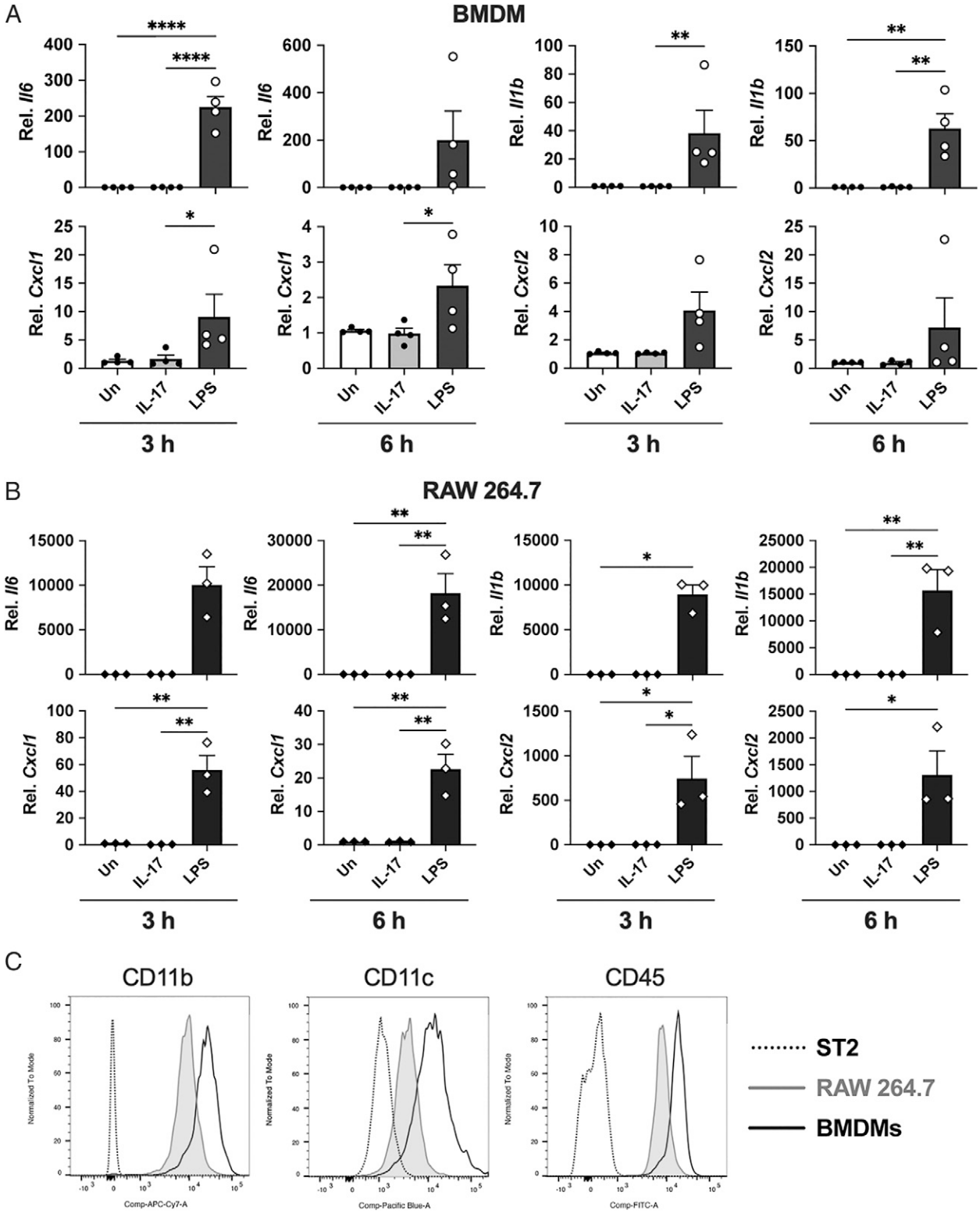

FIGURE 1. Mouse macrophages do not respond to IL-17. (**A** and **B**) BMDMs from (A) WT mice or (B) RAW 264.7 cells were treated with IL-17 (200 ng/ml) or *E. coli* LPS (1 μg/ml) for 3 or 6 h. Expression of the indicated genes was quantified by qPCR normalized to *Gapdh*. Data are presented as fold change relative to unstimulated ± SEM of three to four independent experiments. Each symbol indicates an individual mouse ($n = 4$) or biological replicates ($n = 3$). (**C**) Histograms of CD11b, CD11c, and CD45 staining on ST2, RAW 264.7, and BMDM cells is shown. Significance was assessed by one-way ANOVA with a Bonferroni's test or a Kruskal–Wallis test with Dunn's multiple comparison tests. Data are representative of two independent experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

that myeloid cells potentially have the capacity to respond to IL-17, and moreover that SARS-CoV-2 ORF8 may exploit the IL-17R pathway to promote pathologic inflammation in the lung (4, 5). In this study, we reproduce findings that ORF8 potently induces inflammatory cytokines in myeloid cells, including murine macrophages (RAW 264.7 cells, primary bone marrow–derived macrophages [BMDMs]) and in primary human monocytes. However, in our hands, IL-17 did not detectably induce these events in

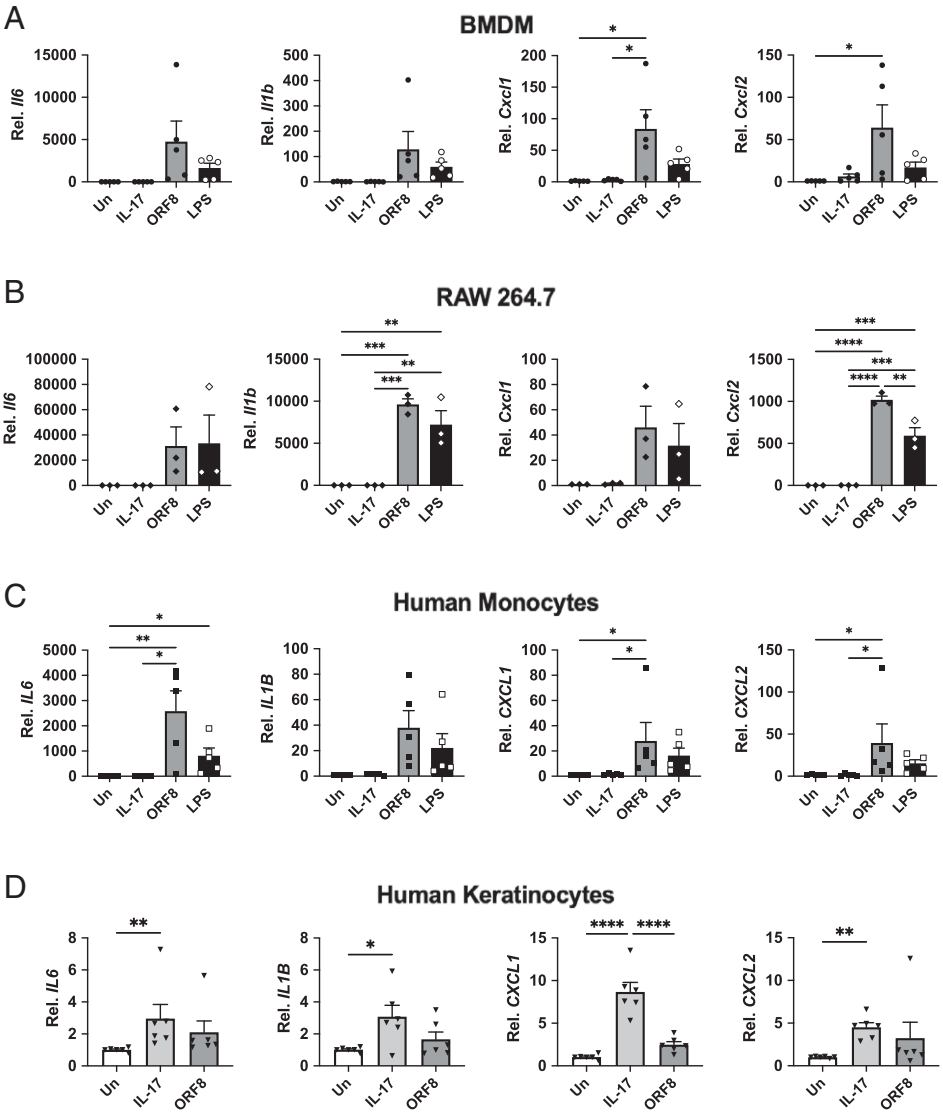

FIGURE 2. ORF8 but not IL-17 activates monocytes/macrophages. (**A**–**C**) Primary BMDMs derived from (A) C57BL/6 mice, (B) RAW 264.7 cells, or (C) human monocytes derived from PBMCs were stimulated with IL-17 (200 ng/ml), His-ORF8 (1 μg/ml), or LPS (1 μg/ml) for 3 h. Expression of the indicated genes was quantified by qPCR normalized to *Gapdh*. Data are presented as fold change relative to unstimulated ± SEM of three to five independent experiments. Each symbol indicates one mouse ($n = 5$), biological replicates ($n = 3$), or a human donor sample ($n = 5$). (**D**) Human KCs were treated with IL-17 (50–100 ng/ml) or ORF8 (1 μg/ml) for 3 h. Expression of indicated genes was quantified by qPCR normalized to *GAPDH* or *RPLP0*. Data are fold change relative to unstimulated ± SEM. Each symbol represents a biological replicate from two independent experiments. Significance was assessed by one-way ANOVA with a Bonferroni's test or a Kruskal–Wallis test with Dunn's multiple comparison tests. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

macrophage/monocyte cell types. We show that IL-17RA but not IL-17RC is expressed in macrophage/monocyte cells isolated from SARS-CoV-2–infected lungs isolated from humans with severe COVID-19 disease or from infected K18-hACE2 mice. Knockout of IL-17RA or Ab blockade of IL-17RA and IL-17RC did not impair ORF8 signaling. Rather, ORF8 relies on the TLR/IL-1 family adaptor MyD88, and these observed effects are not due to contaminating endotoxin. Thus, ORF8-induced monocyte and macrophage responses appear to occur independently of the IL-17R pathway.

Materials and Methods

Mice

Wild-type (WT), *Myd88*$^{-/-}$ (no. 009088), *Il1r1*$^{-/-}$ (no. 003245), and *K18-hACE2* (no. 034860) mice (on the C57BL/6 background) were from The Jackson Laboratory (Bar Harbor, ME) or generated in-house. *Il17ra*$^{-/-}$ mice were from Amgen. Experiments were performed on males (6–10 wk of age) under Institutional Animal Care and Use Committee protocols approved by the University of Pittsburgh.

Cell culture and stimulations

BMDMs were generated by 6-d culture of femoral bone marrow in DMEM (10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 20 mM HEPES, 60 ng/ml M-CSF) and replated 1 d before stimulation. RAW 264.7 cells (American Type Culture Collection) were grown in IMDM (10% FBS [Life Technologies], sodium pyruvate, MEM nonessential amino acids, 1% penicillin/streptomycin). ST2 cells were cultured in MEM (10–12% FBS, 1% penicillin/streptomycin). Human monocytes were isolated with an EasySep human monocyte isolation kit (STEMCELL Technologies) and cultured in X-VIVO 15 media (Lonza). Human immortalized keratinocytes (KCs; N/TERT-2G [22]) were grown in KC SFM (serum-free medium; Thermo Fisher Scientific) with 30 μg/ml bovine pituitary extract, 0.2 ng/ml epidermal growth factor, and 0.3 mM $CaCl_2$. Murine or human IL-17A (PeproTech) was used at 50–200 ng/ml, *Escherichia coli* LPS (Sigma-Aldrich) at 1 μg/ml, and His-Tag ORF8 (Thermo Scientific) at 1 μg/ml. Rat anti–IL-17RA, rat IgG2A, goat anti–IL-17RC, goat IgG (Bio-Techne, 5 μg/ml), or polymyxin B (Sigma-Aldrich, 30 μg/ml) were applied 1 h prior to cytokine stimulation.

Quantitative Real-Time PCR

RNA was isolated with RNeasy mini kits (Qiagen), cDNA was synthesized with an iScript cDNA synthesis kit, and quantitative real-time PCR (qPCR) was performed with SYBR Green Supermix on a CFX Opus 96 (Bio-Rad). Primers were from QuantiTect (Qiagen).

Single-cell RNA sequencing

Raw fastq data were from public datasets of lungs of 4-d SARS-CoV-2–infected K18-hACE2 mice (accession no. GSE175996, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE175996) (23) and six severe COVID-19 patients at Shenzhen Third People's Hospital (accession no. GSE145926, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145926) (24). Raw 3′ single-cell RNA sequencing (scRNA-seq) data

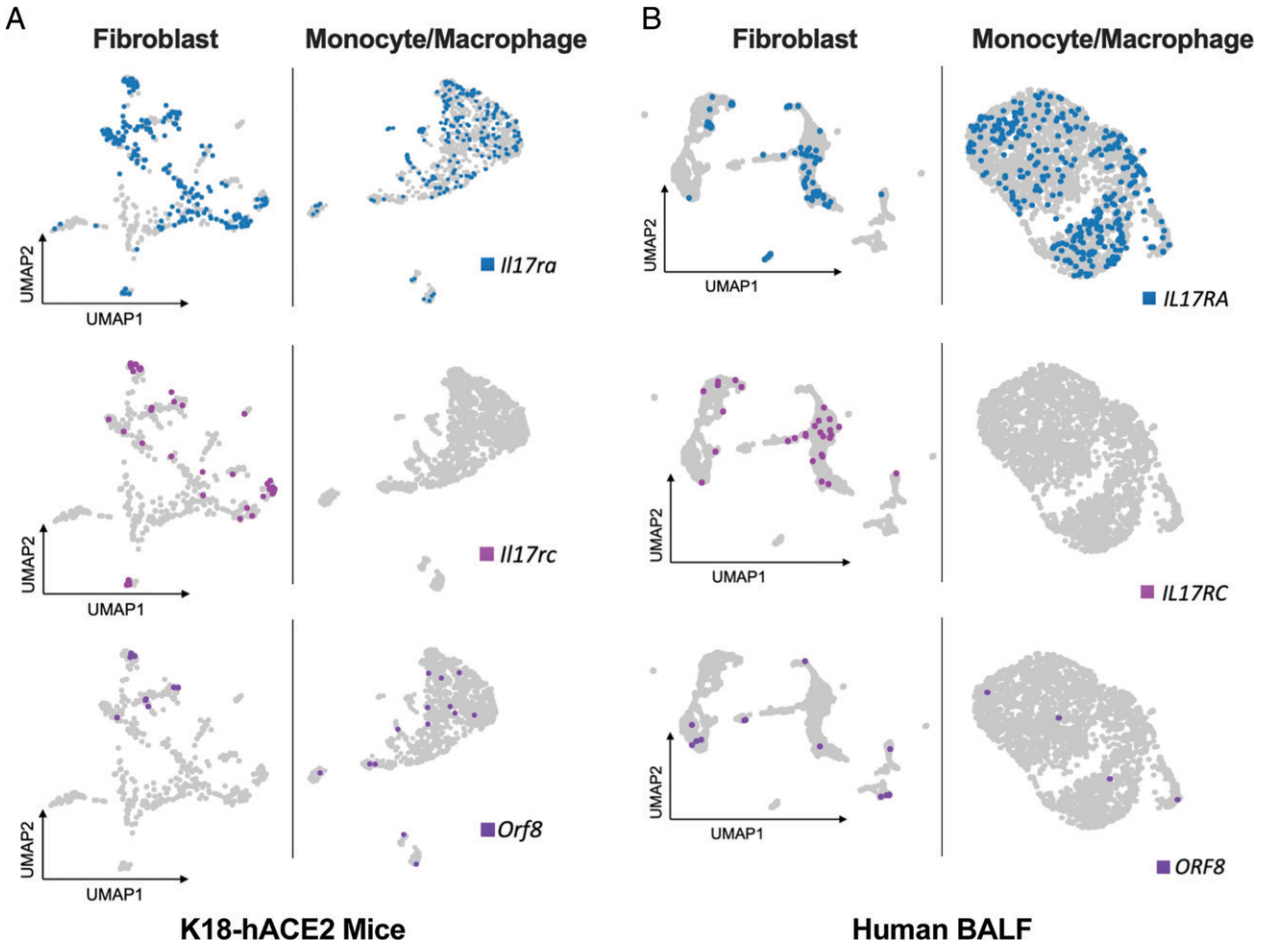

FIGURE 3. IL-17 receptor subunit expression in SARS-CoV-2–infected humans and mouse lung. (**A**) UMAP of scRNA-seq data from lungs of SARS-CoV-2–infected K18-hACE2 transgenic mice (USA-WA1/2020 strain, 4 d) showing murine *Il17ra*, *IL17rc*, and viral *Orf8* in monocyte/macrophage and fibroblast clusters. (**B**) Severe COVID-19 patient bronchoalveolar lavage fluid (BALF) *IL17RA*, *IL17RC*, and viral *ORF8* expression in monocyte/macrophage and fibroblast clusters. Each point represents an individual cell; colors are based on marker annotation. Gray indicates that the gene of interest was below the threshold in the marker set. The cutoff was $\log_2$norm > 1 (log-transformed and normalized). Data are from published datasets (see *Materials and Methods*).

were processed with Cell Ranger v6.1.2 (10x Genomics). Transcripts were aligned to a customized reference genome in which *ORF8* (MN985325.1, https://www.ncbi.nlm.nih.gov/nuccore/MN985325.1/) was added to human and mouse reference genomes GRCh38 and mm10-2020. An entry summarizing the ORF8 gene was appended to GRCh38 and mm10-2020 annotation gtf files and the genome was indexed using cellranger_mkref. Loupe Cell Browser (10x Genomics) was used to define clusters. Barcodes were filtered to exclude high counts of unique molecular identifiers, representing likely doublets. A threshold unique molecular identifier count of 50,000 and 60,000 per barcode on a linear scale was used for humans and mice, respectively. Seurat suite v4.0.6 was used for quality control and analysis.

Flow cytometry

Cell suspensions were blocked with Fc receptor Abs (BioLegend) in 5% goat serum (Jackson ImmunoResearch) and stained with CD11c-BV421, CD11b-allophycocyanin/Cy7 (BioLegend) and CD45-FITC (Invitrogen). Data were collected on an Aurora (Cytek) and analyzed with FlowJo.

Statistical analysis

Datasets were tested for normality using the Shapiro–Wilk normality test. Data were analyzed by 1- or 2-way ANOVA with Bonferroni's multiple comparisons test (parametric data) or Kruskal-Wallis tests with Dunn's multiple comparisons test (nonparametric data) in GraphPad Prism. A p value <0.05 was considered significant, and values are expressed as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Results

ORF8 but not IL-17 activates cytokines in human and mouse monocytes/macrophages

A paradigm in the IL-17 field is that IL-17–mediated signaling is limited to nonhematopoietic cells. Given reports that ORF8 may bind to and activate the IL-17R complex in macrophages and monocytes (4, 5), we felt compelled to re-examine the capacity of IL-17 to induce inflammatory signals in myeloid cells. Accordingly, we treated BMDMs from C57BL/6 WT mice and RAW 264.7 macrophage cells with LPS (1 μg/ml) or recombinant murine IL-17 (200 ng/ml) for 3 and 6 h (Fig. 1). Cytokine genes ascribed to IL-17 and/or ORF8 signaling were assessed by qPCR (*Il6, Ilb, Cxcl1, Cxcl2*) normalized to *Gapdh*. These early time points were used based on prior experience showing that IL-17–sensitive cells (e.g., fibroblasts, epithelial cells, lymph node stromal cells) reliably upregulate target genes within this time frame (25–29). IL-17 did not detectably upregulate these cytokine transcripts in BMDMs, whereas LPS potently induced their expression (Fig. 1A). Similarly, LPS but not IL-17 enhanced target gene expression in RAW 264.7 cells (Fig. 1B).

Cell line contamination (e.g., HeLa cells) is a widespread but often unacknowledged problem in cell culture studies (30). Therefore, we authenticated cells used in the current study by staining for

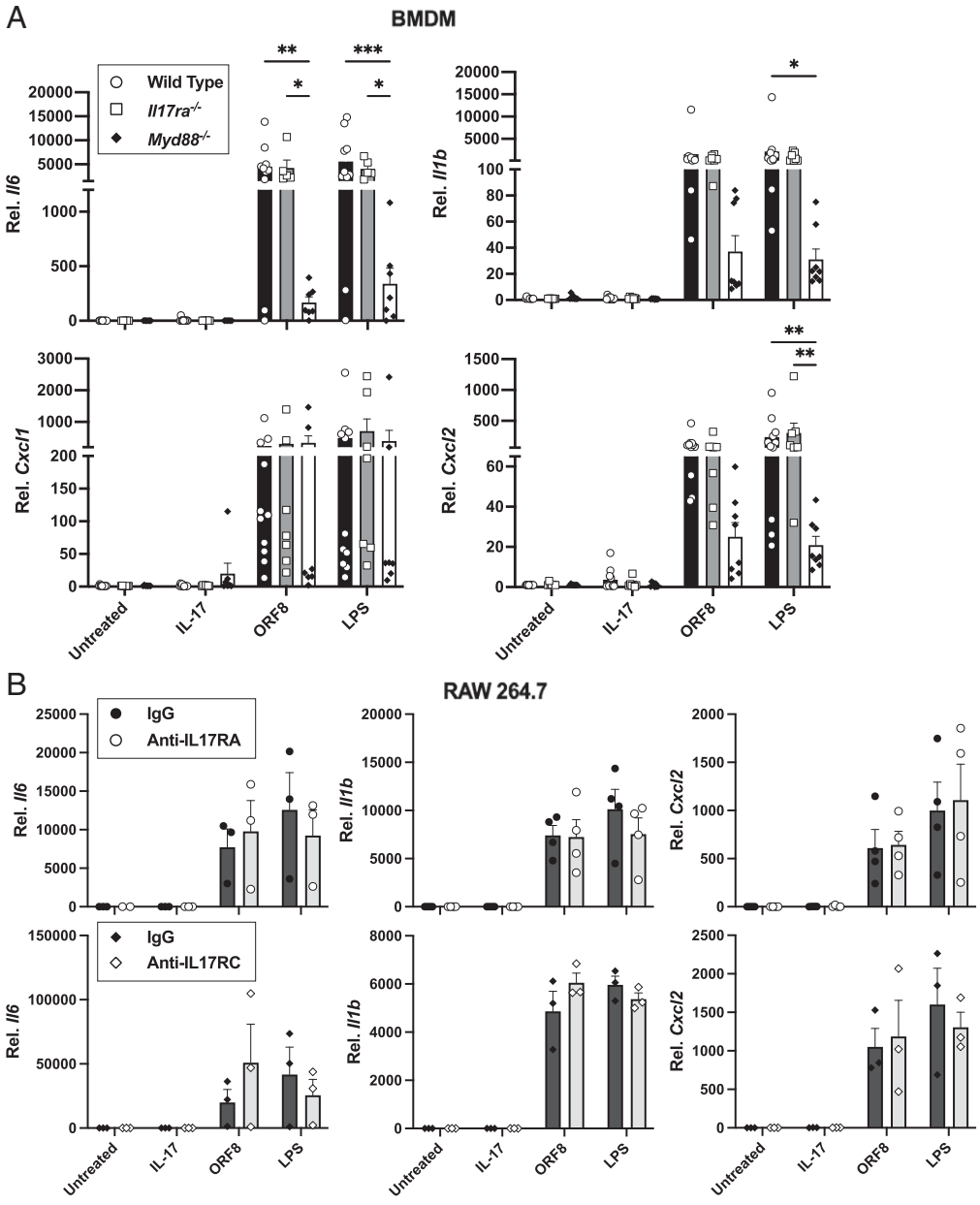

FIGURE 4. ORF8 signals are MyD88-dependent. (**A**) BMDMs from the indicated mice were treated with IL-17 (200 ng/ml), ORF8 (1 μg/ml), or LPS (1 μg/ml) for 3 h. Expression of the indicated genes was quantified by qPCR normalized to *Gapdh*. Data are presented as fold change relative to unstimulated ± SEM. Each symbol indicates one mouse (n = 5–9), and data are from four independent experiments. (**B**) RAW 264.7 cells were pretreated with control IgG, IL-17RA, or IL-17RC Abs at 5 μg/ml for 1 h and stimulated with IL-17 (50 ng/ml), ORF8 (1 μg/ml), or LPS (1 μg/ml) for 3 h. Expression of indicated genes was quantified by qPCR normalized to *Gapdh*. Data are shown as fold change relative to untreated, and show mean ± SEM. Each symbol represents a biological replicate (n = 3–4), and data are from four independent experiments. Significance was assessed by two-way ANOVA with a Bonferroni's test. $*p < 0.05$, $**p < 0.01$.

the hematopoietic marker CD45 and monocyte and macrophage markers CD11b and CD11c. ST2 stromal cells, an IL-17–responsive line routinely used to evaluate IL-17 signaling (29), served as a control (Fig. 1C). The high expression levels of CD45, CD11b, and CD11c on BMDMs and RAW 264.7 cells used in these studies were consistent with bona fide macrophage identity.

Because ORF8 was suggested to serve as an IL-17R agonist (4, 5, 31), we next attempted to reproduce findings that ORF8 upregulates expression of cytokine genes in BMDMs, RAW 264.7 cells, and primary human monocytes isolated from PBMCs of healthy donors. Cells were treated with recombinant ORF8 (1 μg/ml), IL-17 (200 ng/ml), or LPS (1 μg/ml) for 3 h. ORF8 induced inflammatory transcripts in all cell types tested at a magnitude similar to LPS, whereas IL-17 did not enhance gene expression (Fig. 2A–C). Accordingly, in keeping with most prior observations, macrophages/monocytes are sensitive to LPS but do not detectably respond to IL-17.

IL-17 but not ORF8 activates signaling in KCs

Unlike myeloid cells, KCs respond robustly to IL-17 (28, 32). We reasoned that if ORF8 can indeed activate the IL-17R, KCs would respond to ORF8 in a manner similar to IL-17. Accordingly, we treated immortalized human KCs (22) with IL-17 or ORF8 for 3 h. As expected, KCs showed elevated levels of *IL6*, *IL1B*, *CXCL1*, and *CXCL2* in response to IL-17, but ORF8 did not significantly activate these genes (Fig. 2D), further arguing against an IL-17R–mediated mechanism of action for this viral protein.

SARS-CoV-2–infected lung monocytes and macrophages do not express IL-17RC

Although the predominance of literature suggests that IL-17RC expression is limited to hematopoietic cells, there are reported exceptions (18, 33–36). To visualize IL-17RC and IL-17RA in the setting of SARS-CoV-2 infection, we interrogated public scRNA-seq datasets from lung of K18-hACE2 transgenic mice (37) infected with SARS-CoV-2 or in humans with severe COVID-19 disease (23). In mice, *Il17ra* was ubiquitously expressed, whereas *Il17rc* mRNA was highly expressed in fibroblasts but undetectable in monocyte/macrophage clusters (Fig. 3A). Likewise, in human bronchoalveolar lavage fluid from patients with severe COVID-19, *IL17RA* was found in both fibroblasts and monocyte/macrophages

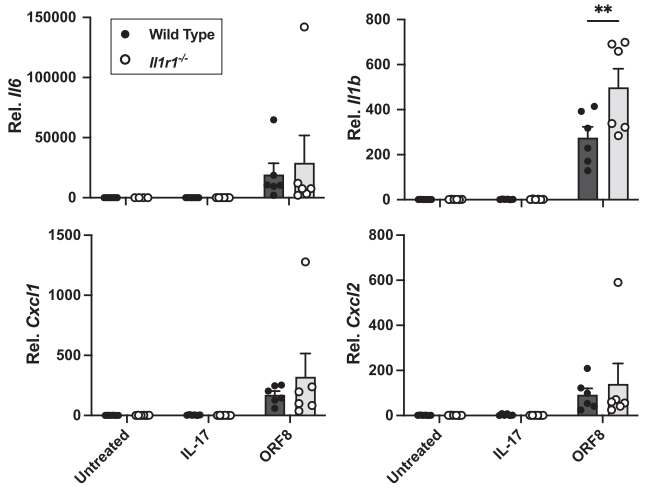

FIGURE 5. ORF8 signals are MyD88-dependent, but do not require IL-1R1. BMDMs from the indicated mice were treated with IL-17 (200 ng/ml) or ORF8 (1 μg/ml) for 3 h. Expression of the indicated genes was quantified by qPCR normalized to *Gapdh*. Data are presented as fold change relative to unstimulated ± SEM. Each symbol indicates experimental replicates from two mice performed once. Significance was assessed by two-way ANOVA with a Bonferroni's test. $^{**}p < 0.01$.

whereas *IL17RC* was not observed in the myeloid compartment (Fig. 3B). *ORF8* mRNA was detectable in both cell types in mice and humans, confirming that samples were virally infected (Fig. 3A, 3B). Hence, given the absence of IL-17RC, ORF8 is unlikely to be able to activate the IL-17R pathway in monocytes/macrophages.

ORF8-induced signaling is mediated by MyD88, not the IL-17R

To determine whether the IL-17R is required for ORF8-driven responses, *Il17ra*$^{-/-}$ or WT BMDMs were treated with IL-17 and ORF8 and cytokines were evaluated by qPCR. The response to ORF8 was fully preserved in *Il17ra*$^{-/-}$ BMDMs but markedly impaired in *Myd88*$^{-/-}$ cells (Fig. 4A). We also evaluated the capacity of blocking Abs against IL-17RA or IL-17RC to inhibit ORF8 signaling. In ST2 stromal cells, these Abs efficiently blocked IL-17 induction of *Il6*, a canonical IL-17–induced target gene (Supplemental Fig. 1A). However, anti–IL-17RA/C Abs did not diminish ORF8-induced signals in RAW 264.7 macrophages (Fig. 4B). Taken together, these data support a model in which ORF8 signals independently of IL-17RA and IL-17RC.

In the same BMDM experiments, we evaluated the ability of ORF8 to signal in the absence of MyD88, given reported structural similarities of ORF8 to IL-1 (38). Notably, MyD88 is dispensable for IL-17 signaling (27). In contrast to both WT and *Il17ra*$^{-/-}$ BMDMs, the response to ORF8 was markedly impaired in *Myd88*$^{-/-}$ cells. As expected, LPS responses were largely mitigated in the absence of MyD88 but not IL-17RA (Fig. 4A). The modest signals in *Myd88*$^{-/-}$ BMDMs upon LPS treatment are likely mediated through TRIF (Toll/IL-1R domain-containing adapter inducing IFN-β), a MyD88-independent adaptor activated by TLR4 but also not required for IL-17 signaling (27).

Given that the IL-1 receptor is upstream of MyD88 as well as the predicted structural similarities between ORF8 and IL-1 (37), we evaluated the role of IL-1 signaling in ORF8-induced inflammatory responses. To this end, *Il1r1*$^{-/-}$ or WT BMDMs were treated with IL-17 and ORF8, and cytokines were evaluated by qPCR. The response to ORF8 was preserved in the absence of IL-1R (Fig. 5), indicating that ORF8-induced responses are not reliant on the IL-1R in monocytes/macrophages.

Based on similarities between ORF8 and LPS signaling responses, we questioned whether residual LPS might contaminate the commercial ORF8 preparation (made in *E. coli*) and whether trace levels of endotoxin capable of eliciting signaling might account for cytokine induction induced by this protein (39), especially given the exquisite sensitivity of monocytes/macrophages to bacterial LPS. To rule this out, we pretreated RAW 264.7 cells with polymyxin B, which inactivates endotoxin and inhibits LPS-induced MyD88 activation (40), and then treated cells with LPS or ORF8. As shown, ORF8 induced *Il6*, *Il1b*, *Cxcl1*, or *Cxcl2* regardless of polymyxin B, but polymyxin B effectively blocked LPS signaling (Fig. 6). Thus, the activities mediated by ORF8 are unlikely to be a result of endotoxin contamination.

Discussion

Many viruses encode cytokine mimics, particularly in the IFN and TNF pathways (41). In fact, mammalian IL-17 was initially recognized based on homology to an ORF in *Herpesvirus saimiri*. The advantage to a virus in encoding an IL-17 agonist is unclear, although a few studies implicate IL-17 in antiviral immunity (25, 42, 43). The

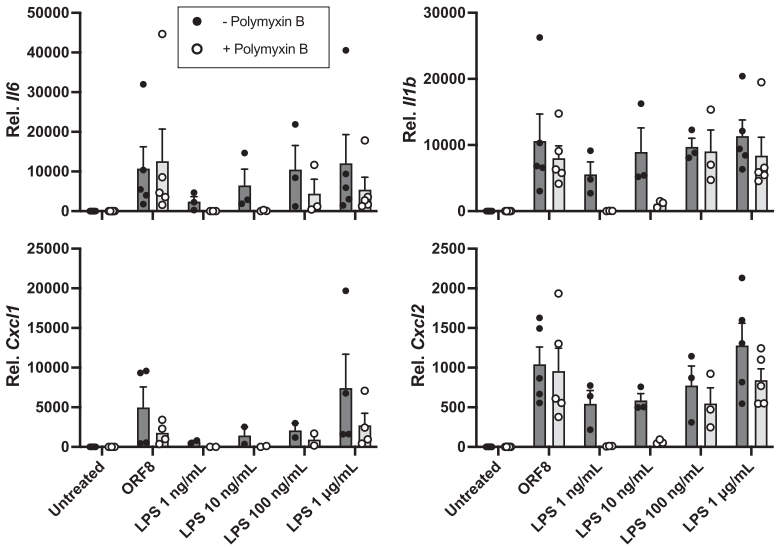

FIGURE 6. ORF8-induced gene expression is not a result of endotoxin contamination. RAW 264.7 cells were pretreated with polymyxin B (30 μg/ml) or vehicle for 1 h and then stimulated with ORF8 (1 μg/ml) or LPS at the indicated concentrations for 3 h. Expression of the indicated genes was quantified by qPCR normalized to *Gapdh*. Data are presented as fold change relative to unstimulated, and show mean ± SEM. Each symbol represents one biological replicate from 3–4 independent experiments. Significance was assessed by two-way ANOVA with a Bonferroni's test.

potential for SARS-CoV-2 ORF8 to be a viral agonist of the IL-17R was intriguing.

We were motivated to pursue the present analyses because our extensive experience with IL-17 signaling has shown that its functions are limited to mesenchymal and epithelial cell types (13, 14), and not to myeloid cells as described in the studies of ORF8 and the IL-17R. Only minimal studies during the past few decades have observed IL-17 to act on monocytes or macrophages, and in some cases alternative explanations for such phenomena, such as HeLa contamination or indirect effects, were not ruled out rigorously. Also, often in these settings only investigation of IL-17RA was performed, which is a shared subunit for other cytokines in the IL-17 ligand family (44). Given the global significance of the COVID-19 pandemic, we thought it was important to explore this issue further.

As noted, most reports using bone marrow chimeras or conditional IL-17R deletions have not observed roles for IL-17 signaling in the hematopoietic system, although some studies credibly describe IL-17 activity on immune cells. For example, in activated T cells, one of the initial studies of IL-17 showed it could mediate costimulation of T cell proliferation, in a similar manner to *Herpesvirus saimiri* gene 13 (HVS13), a viral homolog of this cytokine. IL-17 was shown to act in an autocrine manner in Th17 cells to limit pathogenicity (25, 33). In NK cells, it was reported that IL-17RA deficiency causes cells to be hyporesponsive to microbial stimuli (45), and in preosteoclasts (which are from a hematopoietic lineage), IL-17RC deficiency abolishes IL-17F–induced osteoclast differentiation (18).

Understanding regulation of inflammatory cytokines in the context of severe COVID-19 is clinically important, given the mortality arising from the cytokine storm. For example, blockade of IL-1 and IL-6 improves disease outcomes in severe infection settings (46–49). A recent study revealed an intricate relationship between airway epithelial cells and myeloid cells, in which bystander myeloid cells sense extracellular genomic DNA and mitochondrial DNA released by SARS-CoV-2–infected epithelial cells (50) and trigger inflammasome signaling. The resulting release of mature IL-1β from cocultured epithelial cells and PBMCs in turn promotes IL-6 release, amplifying inflammation (50). IL-17 is also thought to participate in the COVID cytokine storm (51). In the lung, cellular targets of IL-17 signaling are airway epithelial cells (13); in this regard, it is not known whether ORF8 can act directly on lung epithelial cell types, although in human KCs we saw no activation of a cytokine gene panel in response to ORF8 treatment.

In this study, we reproduce observations that ORF8 induces inflammatory cytokines in monocytes and macrophages (4, 5). Although IL-17 effectively activated gene expression in stromal cells and immortalized KCs, as expected, we saw no IL-17–induced cytokine induction in BMDMs, RAW 264.7 cells or primary human monocytes, in keeping with another recent report (52). The cytokine gene targets tested were selected based on prior publications, but we did not do more extensive transcriptomic analyses that might have uncovered other potential target genes. Although we are not certain why our findings differ from prior reports with respect to ORF8 versus IL-17, a widespread challenge is cell line misidentification, especially cross-contamination of HeLa cells (30). HeLa cells are cervical epithelial cells that are very responsive to IL-17 (53, 54). It will be valuable to determine whether discordant results at least in some cases could be explained by HeLa contaminants or other technical considerations.

ORF8 was concluded to bind to IL-17RA based on coimmunoprecipitation and proximity ligation assays (4, 5, 31), but there are important caveats in interpreting results based on the systems used (e.g., overexpression in HEK293 cells, NIH-3T3 fibroblasts). Coimmunoprecipitation and proximity ligation assay approaches do not distinguish well between protein–protein interactions occurring at the cell surface versus intracellularly, and excess IL-17RA upon transfection accumulates at high levels within the endoplasmic reticulum/Golgi, potentially resulting in nonspecific protein aggregation (55). No studies to our knowledge have directly demonstrated cell surface interactions of ORF8 with IL-17RA (or IL-17RC), which are needed to support a bona fide ligand–receptor relationship. A recent follow-up report indicates that ORF8 is secreted in both glycosylated and nonglycosylated forms, but only nonglycosylated ORF8 can coimmunoprecipitate with IL-17RA (56). However, our studies use *E. coli*–derived ORF8, which is not glycosylated, and we still observed no induction of NF-κB–dependent cytokines in human or murine monocytes/macrophages. Additionally, this new study (56) uses NF-κB activation as a readout of IL-17 activity, and only nonglycosylated ORF8 was found to activate this transcription factor. However, NF-κB was assessed at an inappropriately late time point (12 h), and more specific markers of IL-17 signaling activation such as Act1 phosphorylation or proximal NF-κB/IκBξ or C/EBPβ/δ induction (57) were not tested. Thus, although ORF8 is clearly secreted and capable of activating monocytes and macrophages, the role of the IL-17R in mediating these activities is open to question.

Canonical IL-17 signaling requires IL-17RA and IL-17RC (15, 16, 19), although other configurations of the receptor have been proposed, for example, IL-17RA/RD heterodimers and IL-17RC homodimers (58–60). Our studies with *Il17ra*$^{-/-}$ BMDMs and IL-17RA/RC blocking Abs suggested that ORF8 responses are not mediated through the IL-17R complex, but instead they occur through a MyD88-dependent receptor. It is not evident that ORF8 has three-dimensional homology with IL-17. Rather, its structural properties bear resemblance to IL-1β and IL-1RA agonists (38), in keeping with its dependence on MyD88. Although our studies using *Il1r1*$^{-/-}$ BMDMs suggest that this does not occur via IL-1R, the nature of which receptor engaged by ORF8 remains unknown. We speculate that this may be a member of the TLR family, but further studies are warranted in this regard.

In summary, we reproduce findings that the multifunctional SARS-CoV-2 ORF8-secreted protein provokes cytokine secretion in monocytes and macrophages, which could contribute to the deadly cytokine storm seen in severe manifestations of COVID-19. However, our data argue against ORF8 mediating effects through the IL-17R. We suggest that secreted ORF8 is more likely to act on TLR family receptors that signal through MyD88. Defining the function of ORF8 and its ability to modulate cytokine expression in key immune cells is important for probing effective ways to combat the consequences of SARS-CoV-2 severe disease.

Acknowledgments

We thank Amgen for *Il17ra*$^{-/-}$ mice, and B.M. Coleman and S. Sarkar for valuable input.

Disclosures

The authors have no financial conflicts of interest.

References

1. Moore, J. B., and C. H. June. 2020. Cytokine release syndrome in severe COVID-19. *Science* 368: 473–474.
2. Young, B. E., S. W. Fong, Y. H. Chan, T. M. Mak, L. W. Ang, D. E. Anderson, C. Y. Lee, S. N. Amrun, B. Lee, Y. S. Goh, et al. 2020. Effects of a major deletion in the SARS-CoV-2 genome on the severity of infection and the inflammatory response: an observational cohort study. *Lancet* 396: 603–611.

3. Arduini, A., F. Laprise, and C. Liang. 2023. SARS-CoV-2 ORF8: a rapidly evolving immune and viral modulator in COVID-19. *Viruses* 15: 871.
4. Lin, X., B. Fu, S. Yin, Z. Li, H. Liu, H. Zhang, N. Xing, Y. Wang, W. Xue, Y. Xiong, et al. 2021. ORF8 contributes to cytokine storm during SARS-CoV-2 infection by activating IL-17 pathway. *iScience* 24: 102293.
5. Wu, X., T. Xia, W. J. Shin, K. M. Yu, W. Jung, A. Herrmann, S. S. Foo, W. Chen, P. Zhang, J. S. Lee, et al. 2022. Viral mimicry of interleukin-17A by SARS-CoV-2 ORF8. *MBio* 13: e0040222.
6. Orlov, M., P. L. Wander, E. D. Morrell, C. Mikacenic, and M. M. Wurfel. 2020. A case for targeting Th17 cells and IL-17A in SARS-CoV-2 infections. *J. Immunol.* 205: 892–898.
7. Li, X., R. Bechara, J. Zhao, M. J. McGeachy, and S. L. Gaffen. 2019. IL-17 receptor-based signaling and implications for disease. *Nat. Immunol.* 20: 1594–1602.
8. Chung, S. H., X. Q. Ye, and Y. Iwakura. 2021. Interleukin-17 family members in health and disease. *Int. Immunol.* 33: 723–729.
9. Miossec, P., and J. K. Kolls. 2012. Targeting IL-17 and T_H17 cells in chronic inflammation. *Nat. Rev. Drug Discov.* 11: 763–776.
10. Lubberts, E., P. Schwarzenberger, W. Huang, J. R. Schurr, J. J. Peschon, W. B. van den Berg, and J. K. Kolls. 2005. Requirement of IL-17 receptor signaling in radiation-resistant cells in the joint for full progression of destructive synovitis. *J. Immunol.* 175: 3360–3368.
11. Lin, X., S. J. Gaudino, K. K. Jang, T. Bahadur, A. Singh, A. Banerjee, M. Beaupre, T. Chu, H. T. Wong, C. K. Kim, et al. 2022. IL-17RA-signaling in $Lgr5^+$ intestinal stem cells induces expression of transcription factor ATOH1 to promote secretory cell lineage commitment. *Immunity* 55: 237–253.e8.
12. Ramani, K., C. V. Jawale, A. H. Verma, B. M. Coleman, J. K. Kolls, and P. S. Biswas. 2018. Unexpected kidney-restricted role for IL-17 receptor signaling in defense against systemic *Candida albicans* infection. *JCI Insight* 3: e98241.
13. Chen, K., T. Eddens, G. Trevejo-Nunez, E. E. Way, W. Elsegeiny, D. M. Ricks, A. V. Garg, C. J. Erb, M. Bo, T. Wang, et al. 2016. IL-17 receptor signaling in the lung epithelium is required for mucosal chemokine gradients and pulmonary host defense against *K. pneumoniae*. *Cell Host Microbe* 20: 596–605.
14. Conti, H. R., V. M. Bruno, E. E. Childs, S. Daugherty, J. P. Hunter, B. G. Mengesha, D. L. Saevig, M. R. Hendricks, B. M. Coleman, L. Brane, et al. 2016. IL-17 receptor signaling in oral epithelial cells is critical for protection against oropharyngeal candidiasis. *Cell Host Microbe* 20: 606–617.
15. Kuestner, R. E., D. W. Taft, A. Haran, C. S. Brandt, T. Brender, K. Lum, B. Harder, S. Okada, C. D. Ostrander, J. L. Kreindler, et al. 2007. Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. *J. Immunol.* 179: 5462–5473.
16. Wright, J. F., F. Bennett, B. Li, J. Brooks, D. P. Luxenberg, M. J. Whitters, K. N. Tomkinson, L. J. Fitz, N. M. Wolfman, M. Collins, et al. 2008. The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex. *J. Immunol.* 181: 2799–2805.
17. Ho, A. W., F. Shen, H. R. Conti, N. Patel, E. E. Childs, A. C. Peterson, N. Hernández-Santos, J. K. Kolls, L. P. Kane, W. Ouyang, and S. L. Gaffen. 2010. IL-17RC is required for immune signaling via an extended SEF/IL-17R signaling domain in the cytoplasmic tail. *J. Immunol.* 185: 1063–1070.
18. Hu, Y., N. Ota, I. Peng, C. J. Refino, D. M. Danilenko, P. Caplazi, and W. Ouyang. 2010. IL-17RC is required for IL-17A- and IL-17F-dependent signaling and the pathogenesis of experimental autoimmune encephalomyelitis. *J. Immunol.* 184: 4307–4316.
19. Toy, D., D. Kugler, M. Wolfson, T. Vanden Bos, J. Gurgel, J. Derry, J. Tocker, and J. Peschon. 2006. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J. Immunol.* 177: 36–39.
20. Ishigame, H., S. Kakuta, T. Nagai, M. Kadoki, A. Nambu, Y. Komiyama, N. Fujikado, Y. Tanahashi, A. Akitsu, H. Kotaki, et al. 2009. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity* 30: 108–119.
21. Zrioual, S., M. L. Toh, A. Tournadre, Y. Zhou, M. A. Cazalis, A. Pachot, V. Miossec, and P. Miossec. 2008. IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR^+ CXC chemokine expression in synoviocytes and are overexpressed in rheumatoid blood. *J. Immunol.* 180: 655–663.
22. Dickson, M. A., W. C. Hahn, Y. Ino, V. Ronfard, J. Y. Wu, R. A. Weinberg, D. N. Louis, F. P. Li, and J. G. Rheinwald. 2000. Human keratinocytes that express hTERT and also bypass a $p16^{INK4a}$-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol. Cell. Biol.* 20: 1436–1447.
23. Qin, Z., F. Liu, R. Blair, C. Wang, H. Yang, J. Mudd, J. M. Currey, N. Iwanaga, J. He, R. Mi, et al. 2021. Endothelial cell infection and dysfunction, immune activation in severe COVID-19. *Theranostics* 11: 8076–8091.
24. Liao, M., Y. Liu, J. Yuan, Y. Wen, G. Xu, J. Zhao, L. Cheng, J. Li, X. Wang, F. Wang, et al. 2020. Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nat. Med.* 26: 842–844.
25. Yao, Z., W. C. Fanslow, M. F. Seldin, A.-M. Rousseau, S. L. Painter, M. R. Comeau, J. I. Cohen, and M. K. Spriggs. 1995. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 3: 811–821.
26. Qian, Y., C. Liu, J. Hartupee, C. Z. Altuntas, M. F. Gulen, D. Jane-Wit, J. Xiao, Y. Lu, N. Giltiay, J. Liu, et al. 2007. The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nat. Immunol.* 8: 247–256.
27. Maitra, A., F. Shen, W. Hanel, K. Mossman, J. Tocker, D. Swart, and S. L. Gaffen. 2007. Distinct functional motifs within the IL-17 receptor regulate signal transduction and target gene expression. *Proc. Natl. Acad. Sci. USA* 104: 7506–7511.
28. Swindell, W. R., M. K. Sarkar, Y. Liang, X. Xing, J. Baliwag, J. T. Elder, A. Johnston, N. L. Ward, and J. E. Gudjonsson. 2017. RNA-seq identifies a diminished differentiation gene signature in primary monolayer keratinocytes grown from lesional and uninvolved psoriatic skin. *Sci. Rep.* 7: 18045.
29. Shen, F., Z. Hu, J. Goswami, and S. L. Gaffen. 2006. Identification of common transcriptional regulatory elements in interleukin-17 target genes. *J. Biol. Chem.* 281: 24138–24148.
30. Horbach, S. P. J. M., and W. Halffman. 2017. The ghosts of HeLa: how cell line misidentification contaminates the scientific literature. *PLoS One* 12: e0186281.
31. Gordon, D. E., G. M. Jang, M. Bouhaddou, J. Xu, K. Obernier, K. M. White, M. J. O'Meara, V. V. Rezelj, J. Z. Guo, D. L. Swaney, et al. 2020. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 583: 459–468.
32. Konieczny, P., Y. Xing, I. Sidhu, I. Subudhi, K. P. Mansfield, B. Hsieh, D. E. Biancur, S. B. Larsen, M. Cammer, D. Li, et al. 2022. Interleukin-17 governs hypoxic adaptation of injured epithelium. *Science* 377: eabg9302.
33. Chong, W. P., M. J. Mattapallil, K. Raychaudhuri, S. J. Bing, S. Wu, Y. Zhong, W. Wang, Z. Chen, P. B. Silver, Y. Jittayasothorn, et al. 2020. The cytokine IL-17A limits Th17 pathogenicity via a negative feedback loop driven by autocrine induction of IL-24. *Immunity* 53: 384–397.e5.
34. Schmidt, T., J. Luebbe, C. Kilian, J. H. Riedel, S. Hiekmann, N. Asada, P. Ginsberg, L. Robben, N. Song, A. Kaffke, et al. 2021. IL-17 receptor C signaling controls $CD4^+$ T_H17 immune responses and tissue injury in immune-mediated kidney diseases. *J. Am. Soc. Nephrol.* 32: 3081–3098.
35. Nesmond, S., C. Muller, R. Le Naour, M. Viguier, P. Bernard, F. Antonicelli, and S. Le Jan. 2019. Characteristic pattern of IL-17RA, IL-17RB, and IL-17RC in monocytes/macrophages and mast cells from patients with bullous pemphigoid. *Front. Immunol.* 10: 2107.
36. Liu, L., D. Ge, L. Ma, J. Mei, S. Liu, Q. Zhang, F. Ren, H. Liao, Q. Pu, T. Wang, and Z. You. 2012. Interleukin-17 and prostaglandin E2 are involved in formation of an M2 macrophage-dominant microenvironment in lung cancer. *J. Thorac. Oncol.* 7: 1091–1100.
37. McCray, P. B., Jr., L. Pewe, C. Wohlford-Lenane, M. Hickey, L. Manzel, L. Shi, J. Netland, H. P. Jia, C. Halabi, C. D. Sigmund, et al. 2007. Lethal infection of K18-*hACE2* mice infected with severe acute respiratory syndrome coronavirus. *J. Virol.* 81: 813–821.
38. Valcarcel, A., A. Bensussen, E. R. Álvarez-Buylla, and J. Díaz. 2021. Structural analysis of SARS-CoV-2 ORF8 protein: pathogenic and therapeutic implications. *Front. Genet.* 12: 693227.
39. Schwarz, H., M. Schmittner, A. Duschl, and J. Horejs-Hoeck. 2014. Residual endotoxin contaminations in recombinant proteins are sufficient to activate human $CD1c^+$ dendritic cells. *PLoS One* 9: e113840.
40. Cardoso, L. S., M. I. Araujo, A. M. Góes, L. G. Pacífico, R. R. Oliveira, and S. C. Oliveira. 2007. Polymyxin B as inhibitor of LPS contamination of *Schistosoma mansoni* recombinant proteins in human cytokine analysis. *Microb. Cell Fact.* 6: 1.
41. Hernaez, B., and A. Alcamí. 2020. Virus-encoded cytokine and chemokine decoy receptors. *Curr. Opin. Immunol.* 66: 50–56.
42. Gaffen, S. L. 2011. Life before seventeen: cloning of the IL-17 receptor. *J. Immunol.* 187: 4389–4391.
43. Yao, Z., S. L. Painter, W. C. Fanslow, D. Ulrich, B. M. Macduff, M. K. Spriggs, and R. J. Armitage. 1995. Human IL-17: a novel cytokine derived from T cells. *J. Immunol.* 155: 5483–5486.
44. Iwakura, Y., H. Ishigame, S. Saijo, and S. Nakae. 2011. Functional specialization of interleukin-17 family members. *Immunity* 34: 149–162.
45. Bär, E., P. G. Whitney, K. Moor, C. Reis e Sousa, and S. LeibundGut-Landmann. 2014. IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells. *Immunity* 40: 117–127.
46. Kyriazopoulou, E., T. Huet, G. Cavalli, A. Gori, M. Kyprianou, P. Pickkers, J. Eugen-Olsen, M. Clerici, F. Veas, G. Chatellier, et al.; International Collaborative Group for Anakinra in COVID-19. 2021. Effect of anakinra on mortality in patients with COVID-19: a systematic review and patient-level meta-analysis. *Lancet Rheumatol.* 3: e690–e697.
47. Kyriazopoulou, E., P. Panagopoulos, S. Metallidis, G. N. Dalekos, G. Poulakou, N. Gatselis, E. Karakike, M. Saridaki, G. Loli, A. Stefos, et al. 2021. An open label trial of anakinra to prevent respiratory failure in COVID-19. *eLife* 10: e66125.
48. Choy, E. H., F. De Benedetti, T. Takeuchi, M. Hashizume, M. R. John, and T. Kishimoto. 2020. Translating IL-6 biology into effective treatments. *Nat. Rev. Rheumatol.* 16: 335–345.
49. Fu, B., X. Xu, and H. Wei. 2020. Why tocilizumab could be an effective treatment for severe COVID-19? *J. Transl. Med.* 18: 164.
50. Barnett, K. C., Y. Xie, T. Asakura, D. Song, K. Liang, S. A. Taft-Benz, H. Guo, S. Yang, K. Okuda, R. C. Gilmore, et al. 2023. An epithelial-immune circuit amplifies inflammasome and IL-6 responses to SARS-CoV-2. *Cell Host Microbe* 31: 243–259.e6.
51. Pacha, O., M. A. Sallman, and S. E. Evans. 2020. COVID-19: a case for inhibiting IL-17? *Nat. Rev. Immunol.* 20: 345–346.
52. Kohyama, M., T. Suzuki, W. Nakai, C. Ono, S. Matsuoka, K. Iwatani, Y. Liu, Y. Sakai, A. Nakagawa, K. Tomii, et al. 2023. SARS-CoV-2 ORF8 is a viral cytokine regulating immune responses. *Int. Immunol.* 35: 43–52.
53. Tartour, E., F. Fossiez, I. Joyeux, A. Galinha, A. Gey, E. Claret, X. Sastre-Garau, J. Couturier, V. Mosseri, V. Vives, et al. 1999. Interleukin 17, a T-cell-derived cytokine, promotes tumorigenicity of human cervical tumors in nude mice. *Cancer Res.* 59: 3698–3704.
54. Chen, X., G. Cai, C. Liu, J. Zhao, C. Gu, L. Wu, T. A. Hamilton, C. J. Zhang, J. Ko, L. Zhu, et al. 2019. IL-17R-EGFR axis links wound healing to tumorigenesis in $Lrig1^+$ stem cells. *J. Exp. Med.* 216: 195–214.

55. Kramer, J. M., L. Yi, F. Shen, A. Maitra, X. Jiao, T. Jin, and S. L. Gaffen. 2006. Cutting edge: evidence for ligand-independent multimerization of the IL-17 receptor. *J. Immunol.* 176: 711–715.
56. Lin, X., B. Fu, Y. Xiong, N. Xing, W. Xue, D. Guo, M. Zaky, K. Pavani, D. Kunec, J. Trimpert, and H. Wu. 2023. Unconventional secretion of unglycosylated ORF8 is critical for the cytokine storm during SARS-CoV-2 infection. *PLoS Pathog.* 19: e1011128.
57. Amatya, N., A. V. Garg, and S. L. Gaffen. 2017. IL-17 signaling: the yin and the yang. *Trends Immunol.* 38: 310–322.
58. Goepfert, A., C. Barske, S. Lehmann, E. Wirth, J. Willemsen, J. E. Gudjonsson, N. L. Ward, M. K. Sarkar, R. Hemmig, F. Kolbinger, and J. M. Rondeau. 2022. IL-17-induced dimerization of IL-17RA drives the formation of the IL-17 signalosome to potentiate signaling. *Cell Rep.* 41: 111489.
59. Goepfert, A., S. Lehmann, J. Blank, F. Kolbinger, and J. M. Rondeau. 2020. Structural analysis reveals that the cytokine IL-17F forms a homodimeric complex with receptor IL-17RC to drive IL-17RA-independent signaling. *Immunity* 52: 499–512.e5.
60. Su, Y., J. Huang, X. Zhao, H. Lu, W. Wang, X. O. Yang, Y. Shi, X. Wang, Y. Lai, and C. Dong. 2019. Interleukin-17 receptor D constitutes an alternative receptor for interleukin-17A important in psoriasis-like skin inflammation. *Sci. Immunol.* 4: eaau9657.