HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2024 July 15.

Published in final edited form as:

J Immunol. 2023 July 15; 211(2): 252–260. doi:10.4049/jimmunol.2300110.

SARS-CoV-2 ORF8 mediates signals in macrophages and monocytes through MyD88 independently of the IL-17 Receptor

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Abstract

SARS-CoV-2 has caused an estimated 7 million deaths worldwide. 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 non-hematopoietic 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 antibodies and in *Il17ra*$^{-/-}$ cells. ORF8 signaling was also operative in *Il1r1*$^{-/-}$ BMDMs. However, the TLR/IL-1R family adaptor MyD88, which is dispensable for IL-17R signaling, was required for ORF8 activity. Thus, we conclude that ORF8 transduces inflammatory signaling in monocytes and macrophages via MyD88 but independently of the IL-17R.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) surfaced as a novel virus responsible for the ongoing COVID-19 pandemic, infecting more than 750 million individuals to date according to the World Health Organization. Though the immune responses that govern disease outcome have not been completely elucidated, severe infection

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The authors declare no conflicts of interest

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 interferon response (3). Two recent studies reported unexpectedly that ORF8 activates its 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 homologue, IL-17F, is limited to Type 17 cells, most famously $CD4^+$Th17 cells, but also subsets of $CD8^+$ cells, γ-T cells, some NK cells and ILC3s (8). Whereas IL-17 is made by lymphocytes, the majority of studies have demonstrated that IL-17-responsive cells are non-hematopoietic, 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 non-hematopoietic compartments and is a co-receptor 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 some other reports that myeloid cells potentially have the capacity to respond to IL-17, and moreover that SARS-CoV2 ORF8 may exploit the IL-17R pathway to promote pathologic inflammation in the lung (4, 5). Here, 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 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-17R subunits or antibody blockade of IL-17RA and IL-17RC did not impair ORF8 signaling. Rather, ORF8 relies on the TLR/IL-1-family adaptor MyD88, and its effects are not due to contaminating endotoxin. Thus ORF8-induced monocyte and macrophage responses occur independently of the IL-17R pathway.

Methods

Mice

WT, *Myd88*$^{-/-}$ (#009088), *Il1r1*$^{-/-}$ (#003245) and *K18-hACE2* (#034860) mice (on the C57BL/6background) were from JAX (Bar Habor, ME) or generated in-house. *Il17ra*$^{-/-}$ mice were from Amgen. Experiments were performed on males (6–10 weeks) under IACUC protocols approved by the University of Pittsburgh.

Cell Culture and stimulations

BMDMs were generated by 6-day culture of femoral BM in DMEM (10% FBS, 1% pen/strep, 2 mM L-Glutamine, 20 mM HEPES, 60 ng/ml M-CSF), and re-plated 1 day before stimulation. RAW264.7 cells (ATCC) were grown in IMDM (10% FBS, Gibco, Na pyruvate, MEM non-essential amino acids, 1% pen/strep). ST2 cells were cultured in MEM (10–12% FBS, 1% pen/strep). Human monocytes were isolated with EasySep™ Human Monocyte Isolation Kit (Stem Cell Technologies) and cultured in X-vivo 15 media (Lonza). Human immortalized KC cells (N/TERT-2G (22)) were grown in Keratinocyte-SFM medium (ThermoFisher) with 30 μg/ml bovine pituitary extract, 0.2 ng/mL EGF, 0.3 mM $CaCl_2$. Murine or human IL-17A (Peprotech) was used at 50–200 ng/mL, *E. 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, 30 μg/mL) were applied 1 h prior to stimulation.

qPCR

RNA was isolated with RNeasy Mini Kits (Qiagen), cDNA was synthesized with iScript cDNA Synthesis Kit, and qPCR performed with SYBR Green Supermix on a CFX Opus 96 (Bio-Rad). Primers were from QuantiTect (Qiagen).

ScRNASeq

Raw fastq data was from public datasets of lungs of 4-day SARS-CoV-2-infected K18-hACE2 mice (accession GSE175996, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE175996) (23) and 6 six severe COVID-19 patients at Shenzhen Third People's Hospital (GSE145926, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145926) (24). Raw 3′ scRNA-seq data were processed with CellRanger *V*6.1.2 (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 UMIs (Unique Molecular Identifiers), representing likely doublets. A threshold UMI count of 50,000 and 60,000 per barcode on a linear scale was used for human and mouse, 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-APC/Cy7 (Biolegend) and CD45-FITC (Invitrogen). Data were collected on an Aurora (Cytek) and analyzed with FlowJo.

Statistics

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 (parameteric data) or Kruskal-Wallis tests with Dunn's multiple comparisons test (nonparametric data) in GraphPad Prism. *P* values < 0.05 were considered significant. **P* < 0.05, ** < 0.01, *** <0.001, ****<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 non-hematopoietic 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 bone marrow-derived macrophages (BMDMs) from C57BL/6 wild-type (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 hours (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, LN 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 here by staining for 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 of CD45, CD11b and CD11c on BMDMs and RAW 264.7 cells used in these studies are 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, B, C). Accordingly, in

keeping with most prior observations, macrophages/monocytes are sensitive to LPS but do not respond to IL-17.

IL-17 but not ORF8 activates signaling in keratinocytes

Unlike myeloid cells, keratinocytes (KC) respond robustly to IL-17 (28, 32). We reasoned that if ORF8 can 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-dependent 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 scRNASeq 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 BAL fluid from patients with severe COVID-19, *IL17RA* was found in both fibroblasts and monocyte/macrophages whereas *IL17RC* was not 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, B). Hence, given the absence of IL-17RC, ORF8 is unlikely to be able to activate the IL-17R pathway in monocyte/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 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). 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, 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 and the predicted structural similarities between ORF8 and IL-1 (37), we evaluted the role of IL-1 signaling in ORF8-induced inflammatory responses. To that 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 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 pre-treated 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 activites mediated by ORF8 are unlikely to be a result of endotoxin contamination.

Discussion

Many viruses encode cytokine mimics, particularly in the interferon 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, though a few studies implicate IL-17 in antiviral immunity (25, 42, 43). The 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 over 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 felt it was important to explore this issue further.

As noted, most reports using BM chimeras or conditional IL-17R deletions have not observed roles for IL-17 signaling in the hematopoietic system, though 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 co-stimulation 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 pathogencity (25, 33). In natural killer (NK) cells, it was reported that IL-17RA-deficiency causes cells to be hyporesponsive to microbial stimuli (45), and in pre-osteoclasts (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 COVID19 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 gDNA and mtDNA released by SARS-CoV-2-infected epithelial cells (50) and trigger inflammasome signaling. The resulting release of mature IL-1β from co-cultured 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 if ORF8 can act directly on lung epithelial cell types, though in human keratinocytes we saw no activation of a cytokine gene panel in response to ORF8 treatment.

Here, we reproduce observations that ORF8 induces inflammatory cytokines in monocytes and macropahges (4, 5). While IL-17 effectively activated gene expression in stromal cells and immortalized KCs, as expected, we saw no IL-17-induced cytokine induction in BMDMs, RAW264.7 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 vs. IL-17, a widespread challenge is cell line misidentification, especially cross-contamination of HeLa cells (30). HeLa are cervical epithelial cells that are very responsive to IL-17 (53, 54). It will be valuable to determine if 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 co-IP and proximity ligation assays (PLA) (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). Co-IP and PLA 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 ER/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 followup report indicates that ORF8 is secreted in both glycosylated and non-glycosylated forms, but only non-glycosylated ORF8 can co-IP 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 monocyte/macrophages. Additionally, this new study (56) uses NF-κB activation as a readout of IL-17 activity, and only non-glycosylated ORF8 was found to activate this transcription factor. However, NF-κB was assessed at an inappropriately late time point (12 hours), 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-17 receptor in mediating these activities is open to question.

Canonical IL-17 signaling requires IL-17RA and IL-17RC (15, 16, 19), though other configurations of the receptor have been proposed, e.g., 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 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. While our studies using *Il1r1*$^{-/-}$ BMDMs suggest 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 COVID19. 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-CoV2 severe disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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

NIH supported SLG (AI162616, AI147383), JEG (AR075043), JKK (AR075043, HL13993), TCT (AI089443) and RAG (GM146896). JKK and MSK were supported by the Louisiana Board of Regents Endowed Chairs for Eminent Scholars Program. MKS was supported by a National Psoriasis Foundation Translational Research grant (852098).

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Key Points

- SARS-Cov-2 ORF8 but not IL-17 upregulates cytokines in monocytes/macrophages
- Monocytes and macrophages infected with SARS-Cov-2 do not express IL-17RC
- ORF8 signals through MyD88

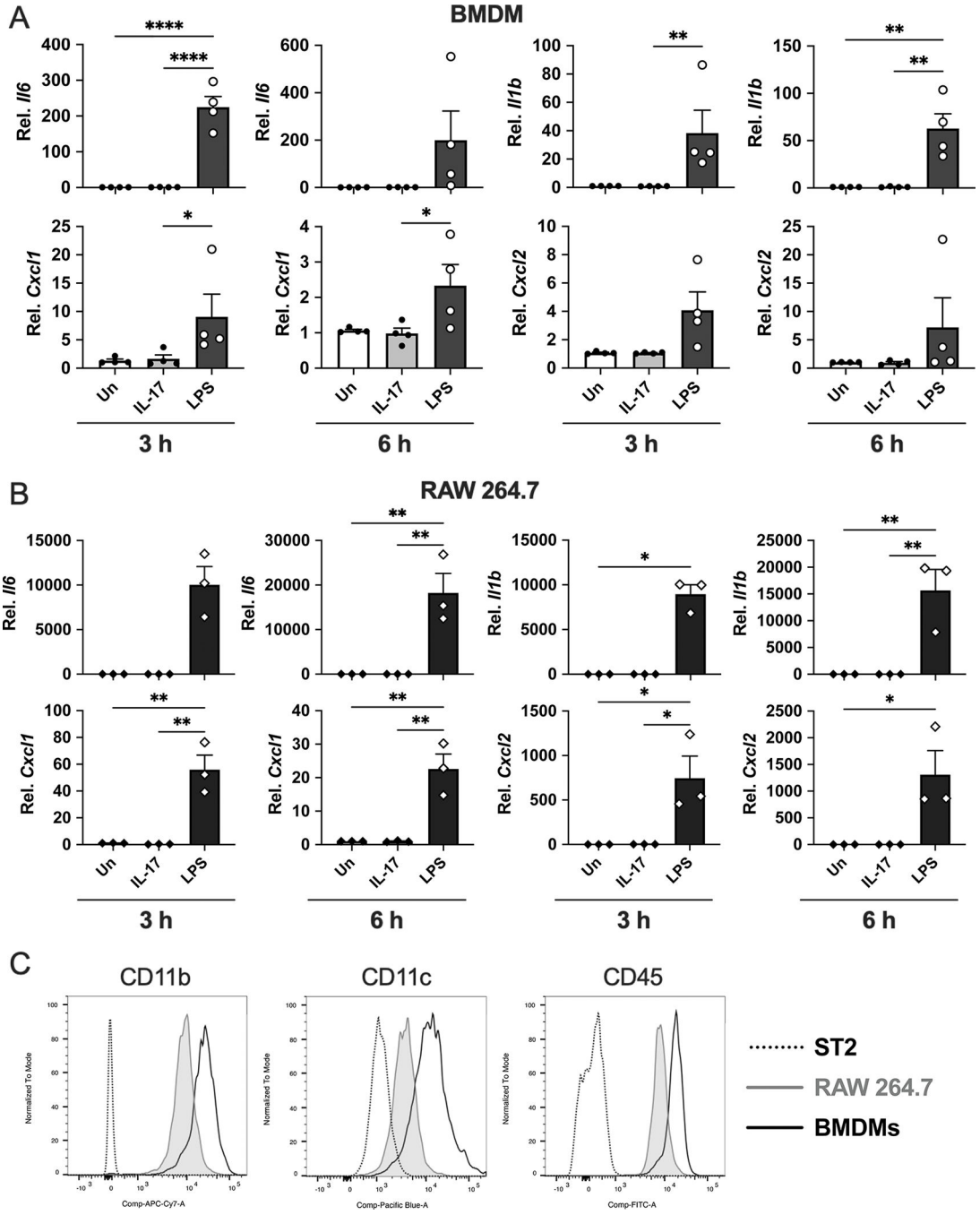

Figure 1. Mouse macrophages do not respond to IL-17.

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 3–4 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 1-way

ANOVA with Bonferroni's or Kruskal-Wallis with Dunn's multiple comparisons tests. Data is representative of 2 independent experiments.

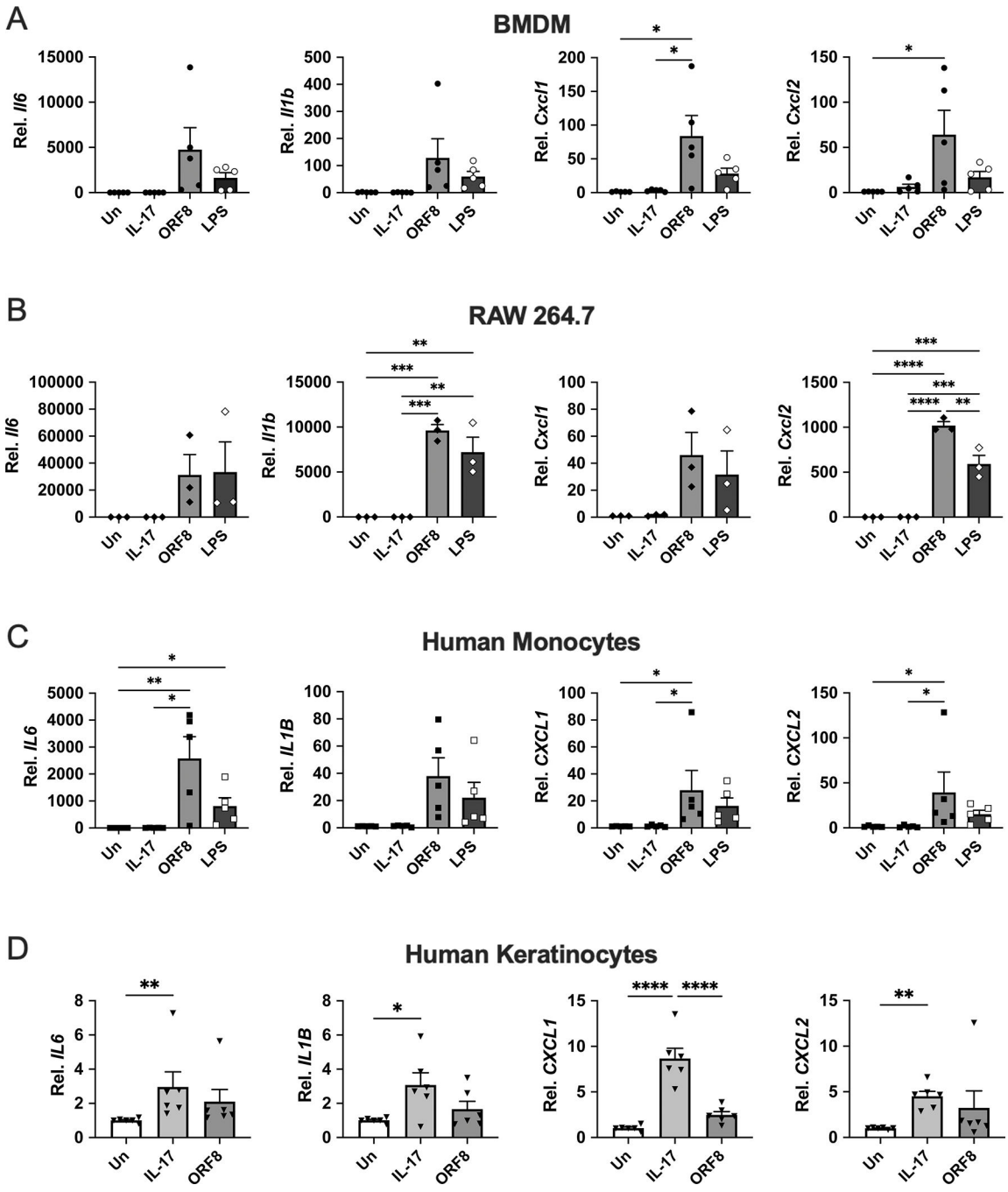

Figure 2. ORF8 but not IL-17 activates monocytes/macrophages.

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 3–5 independent experiments. Each symbol indicates one mouse (n=5), biological replicates (n= 3) or 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 1-way ANOVA with Bonferroni's or Kruskal-Wallis with Dunn's multiple comparisons tests.

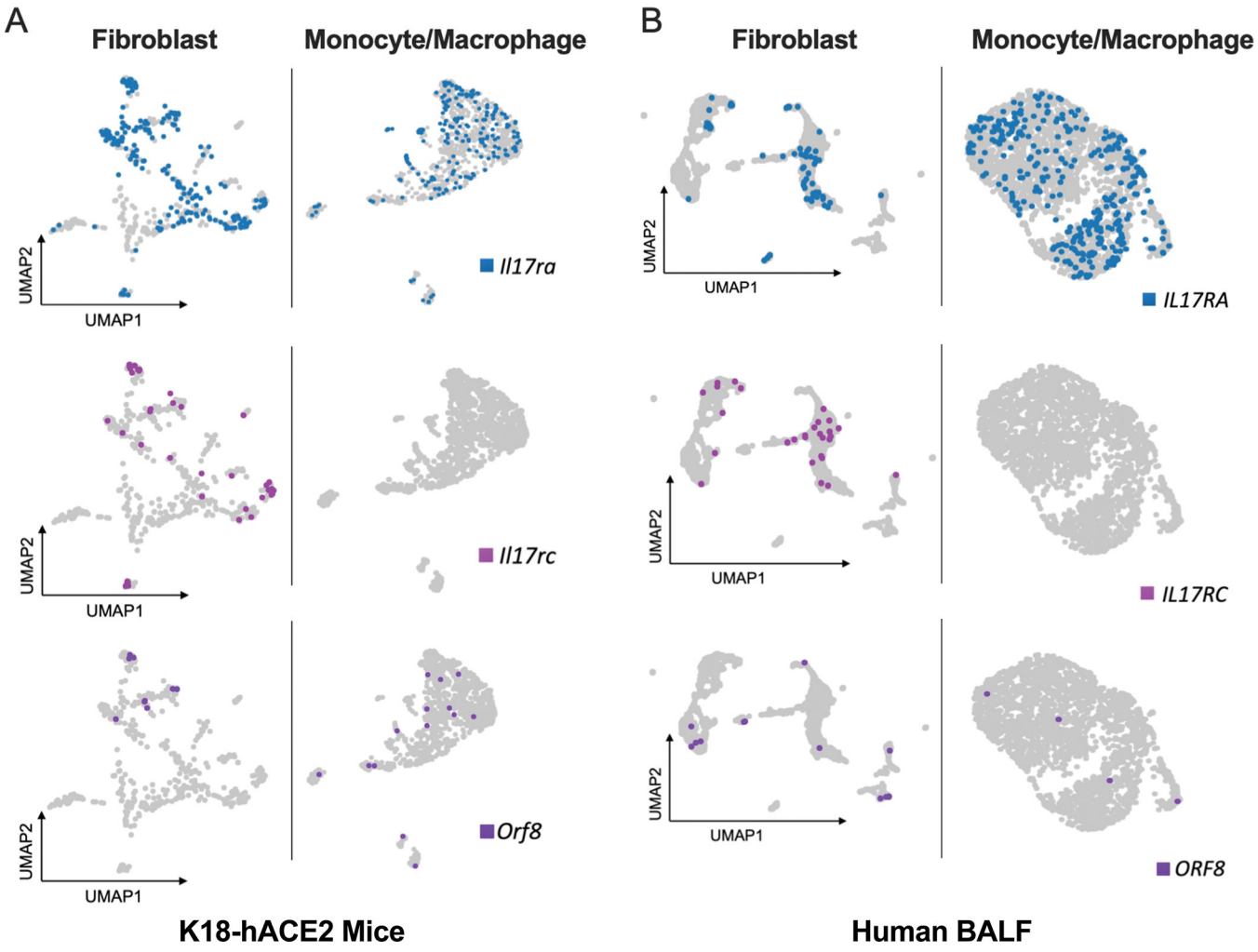

Figure 3. IL-17 receptor subunit expression in SARS-CoV-2-infected humans and mouse lung.

(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 COVID19 patient BALF *IL17RA, IL17RC* and viral *ORF8* in monocyte/macrophage and fibroblast clusters. Each point is an individual cell; colors are based on marker annotation. Gray indicates that gene of interest was below threshold in the marker set. Cutoff: Log2Norm > 1 (log- transformed and normalized). Data are from published datasets, see Methods.

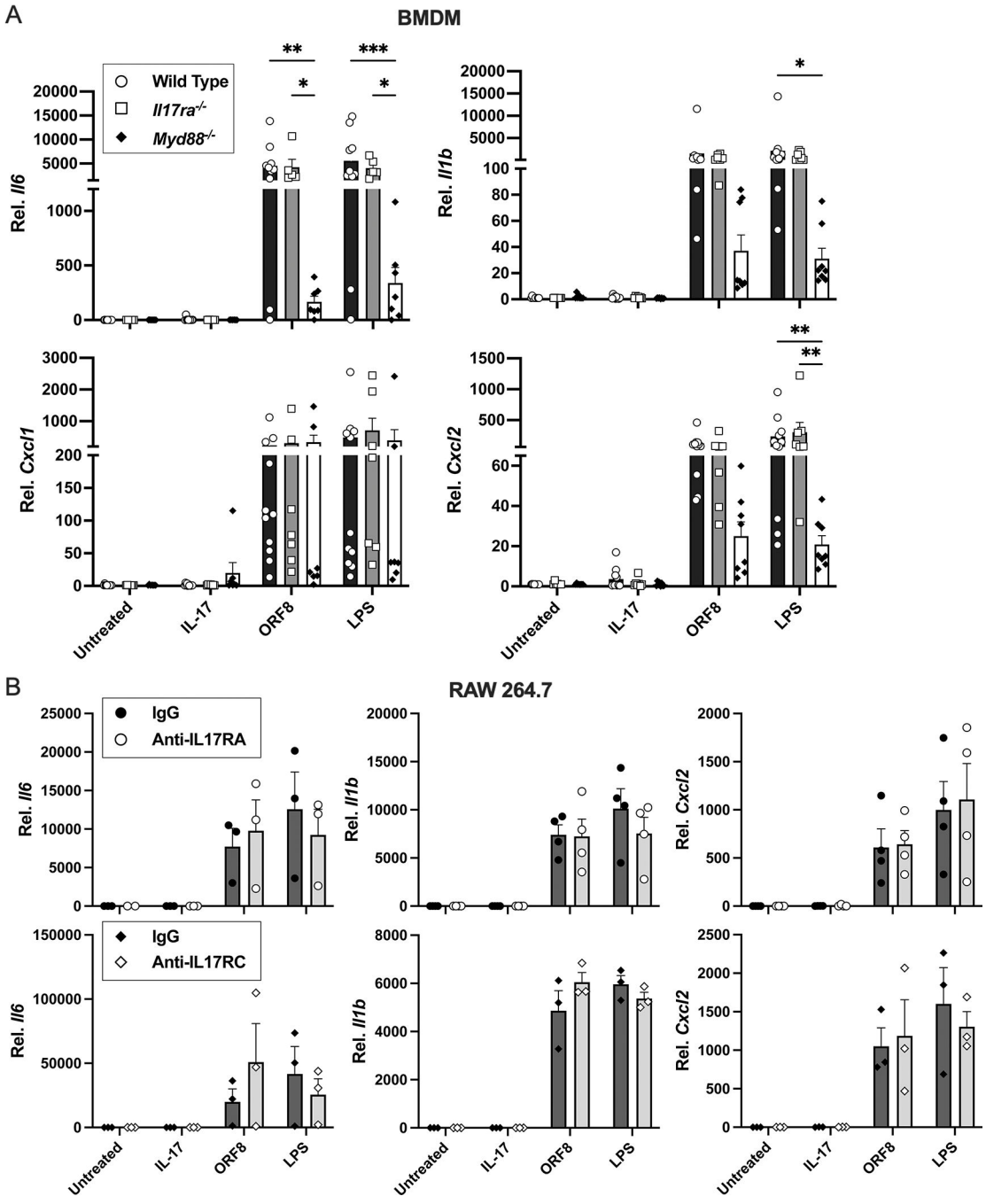

Figure 4. ORF8 signals are MyD88-dependent.

(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 are from 2 independent experiments. (B) RAW 264.7 cells were pre-treated 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 as fold-change relative to untreated, and show mean (± SEM). Each symbol represents a biological replicate (n=4) and data are from 2 independent experiments. Significance was assessed by 2-way ANOVA with Bonferroni's test.

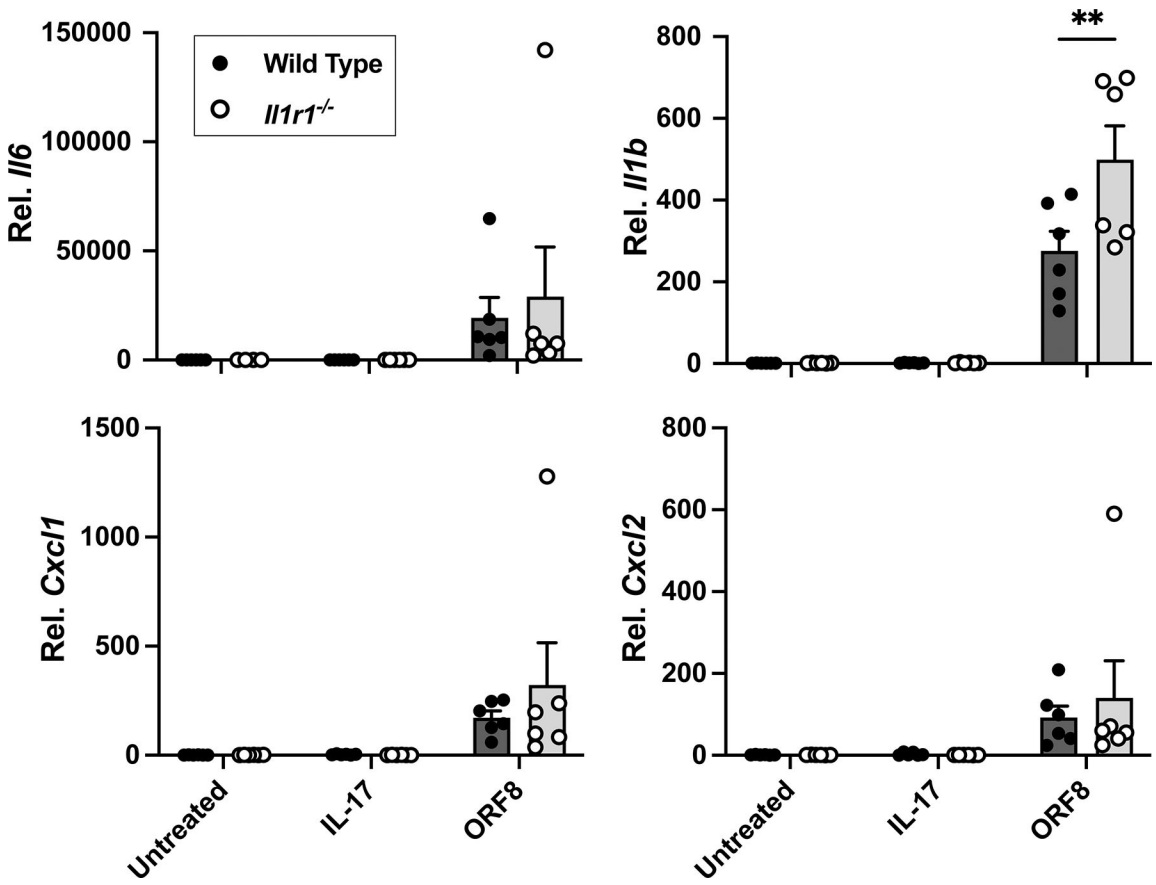

Figure 5. ORF8 signals are MyD88-dependent, but do not require IL-1R1.

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 2 mice performed once. Significance was assessed by 2-way ANOVA with Bonferroni's test.

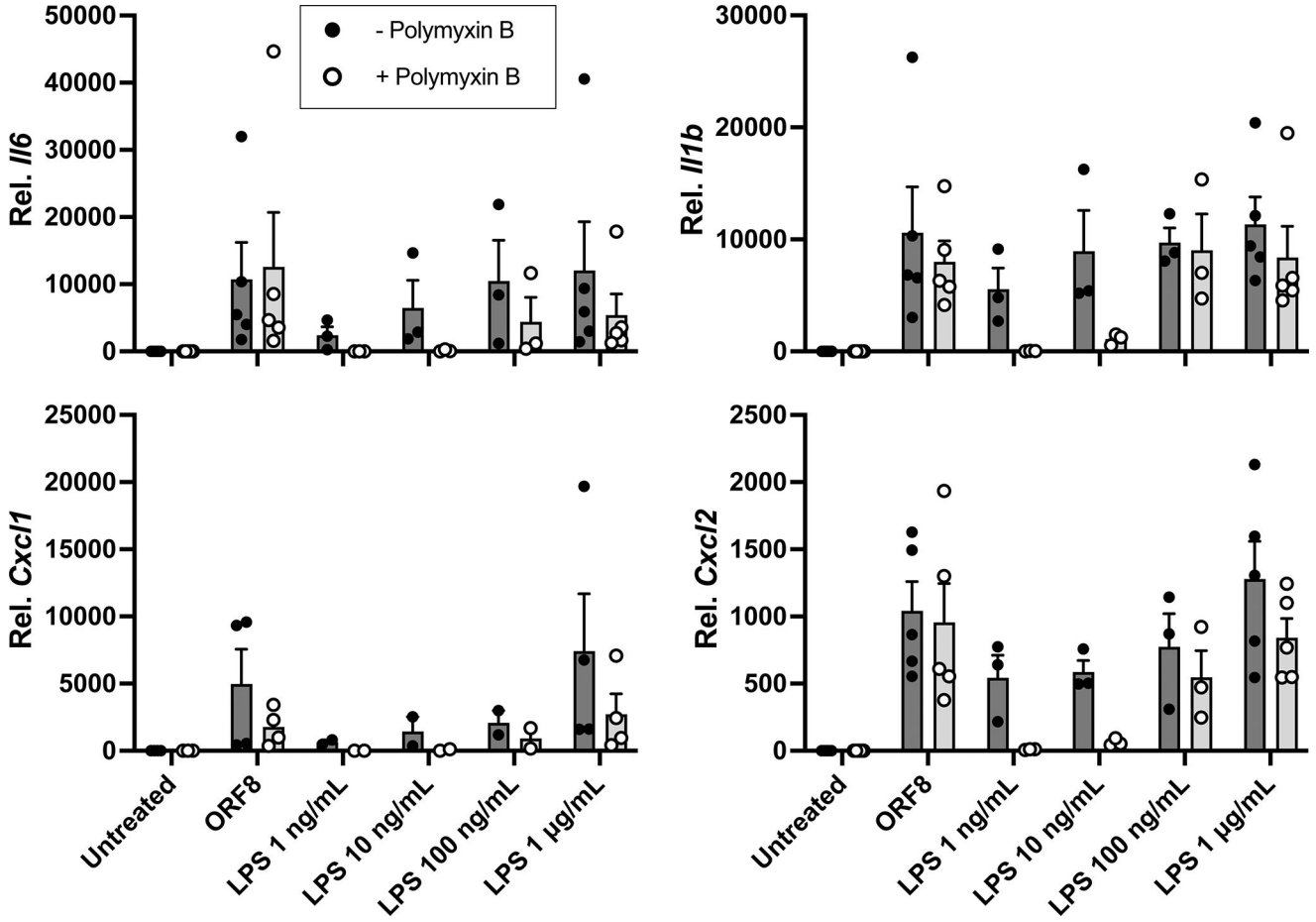

Figure 6. ORF8-induced gene expression is not a result of endotoxin contamination.

RAW 264.7 cells were pre-treated 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 2 independent experiments. Significance was assessed by 2-way ANOVA with Bonferroni's test.