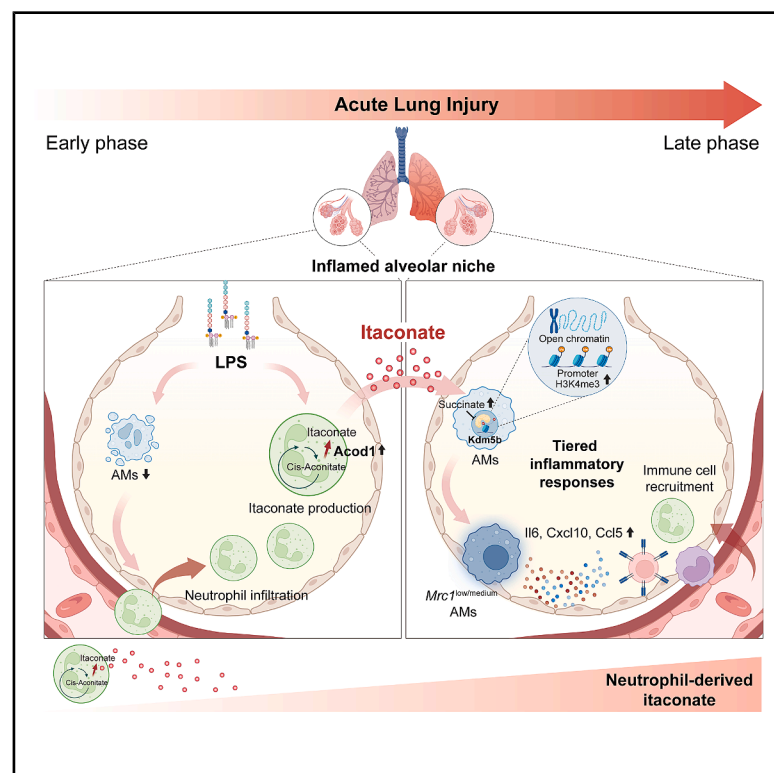


Neutrophil-derived itaconate facilitates tiered pulmonary inflammation via Kdm5b-associated epigenetic remodeling in alveolar macrophages

Graphical abstract



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In brief

Lim et al. identify neutrophil-derived itaconate as a key environmental factor shaping tiered inflammatory responses in ALI. Itaconate metabolically promotes KDM5B-associated epigenetic activation of proinflammatory genes in alveolar macrophages, coordinating later-stage immune cell infiltration, including monocytes and T cells.

Highlights

- Neutrophils are a major source of extracellular itaconate in BALF during ALI
- KDM5B inhibition by itaconate leads to epigenetic shifts in alveolar macrophages
- Neutrophil-derived itaconate coordinates tiered lung inflammatory responses in ALI



Report

Neutrophil-derived itaconate facilitates tiered pulmonary inflammation via Kdm5b-associated epigenetic remodeling in alveolar macrophages

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SUMMARY

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), present a substantial clinical burden, magnified by conditions such as COVID-19. While these conditions provide a model for exploring complex inflammatory processes, the environmental factors coordinating these responses remain poorly understood. Here, we employ comprehensive multi-omics and biochemical analyses and identify neutrophil-derived itaconate as an extracellular factor associated with sequential immune cell infiltration, including neutrophils, T cells, and monocytes. Mechanistically, extracellular itaconate metabolically facilitates Kdm5b-associated epigenetic changes at the *Il6*, *Ccl5*, and *Cxcl10* gene promoters in alveolar macrophages, which are important for immune cell recruitment. Consistent with this, *Mrp8-Cre Acod1^{fl/fl}* mice lacking neutrophil-derived itaconate are significantly protected from ALI-induced inflammation, with similar patterns observed in an *Acinetobacter baumannii* infection model. Collectively, these findings identify neutrophil-derived itaconate as an environmental factor that epigenetically shapes tiered inflammatory responses in the lung.

INTRODUCTION

The intermediate tricarboxylic acid (TCA) cycle metabolite itaconate is synthesized by aconitate decarboxylase-1 (*Acod1*), which was found in 2013 to rapidly accumulate within lipopolysaccharide (LPS)-stimulated macrophages.¹ This accumulation is associated with profound antimicrobial function. Initial work

showed that this was due to the direct binding of itaconate to bacterial isocitrate lyase, which inhibited the glyoxylate-shunt pathway in intracellularly vacuolized bacteria.^{2–5} However, more recently, itaconate was also found to alter the immune responses of macrophages.^{6,7} The initial investigations suggested that itaconate derivatives promote anti-inflammatory responses by macrophages.^{8–11} Proposed mechanisms include the



regulation of KEAP1, a central mediator of the antioxidant response; Mills et al. demonstrated that 4-octyl itaconate, a cell-permeable itaconate derivative, alkylates multiple cysteine residues on KEAP1, thereby releasing the cytosolic master transcription factor Nrf2. This enables Nrf2 to translocate into the nucleus and induce anti-inflammatory gene expression,⁸ although these effects may largely reflect the electrophilic properties of the modified itaconate rather than those of endogenous itaconate itself. Another mechanism involves competitive inhibition of itaconate with the TCA cycle metabolite succinate for binding to the active site of succinate dehydrogenase (SDH). The resulting accumulation of succinate and/or loss of succinate oxidation blocks SDH-mediated induction of pro-inflammatory genes such as *IL-6*.^{7,12} However, subsequent research suggested that itaconate may also promote pro-inflammatory macrophage responses albeit with a limited *in vivo* relevance due to the lack of genetic model: for example, alternatively activated macrophages can take up itaconate and 4-octyl itaconate, which alkylates the receptor kinase JAK1, thus preventing it from activating the STAT transcription factors. This blunts the ability of the macrophages to express the anti-inflammatory cytokines *IL-13* and interferon- β .¹³ Also, itaconate can shape inflammatory responses by enhancing type I interferon responses via inhibition of peroxiredoxin 5.^{14,15} Thus, itaconate may encourage the development of not only anti-inflammatory but also pro-inflammatory macrophages.^{13,16} The disparate outcomes of these studies suggest that the microenvironmental context dictates the direction of macrophage polarization.

The above studies on the immunomodulatory mechanisms of itaconate were mainly based on *in vitro* analyses of macrophages. However, several studies showed that itaconate is not only abundantly expressed in the lungs of mice infected with various bacteria, itaconate-resistant bacterial strains associate with metabolic reprogramming in local and circulating myeloid cells that induces an anti-inflammatory phenotype.^{17–19} Thus, the *in vitro* ability of itaconate to promote anti-inflammatory macrophages has also been observed *in vivo*. Interestingly, the study by Tomlinson et al. on a murine pneumonia model showed recently that neutrophils may synthesize itaconate during lung inflammation, which hampers neutrophil-mediated bacterial killing.²⁰ Thus, neutrophils can also produce itaconate in inflammation. Moreover, a line of investigation shows that the tumor microenvironment can promote the secretion of itaconate by macrophages, which impairs anti-tumor activities of CD8⁺ T cells.^{21–23} The findings suggest that itaconate can be secreted and could potentially regulate immune cell functions *in vivo* by metabolite-related epigenetic mechanisms.²¹ However, the physiological and functional implications of itaconate secreted in various microenvironments remain poorly understood, partly due to the absence of genetic models that permit cell-type-specific depletion of itaconate.

Acute lung injury (ALI) and its more severe manifestation, acute respiratory distress syndrome (ARDS), represent critical clinical syndromes characterized by acute respiratory failure, severe morbidity, and high mortality rates.^{24–26} Notably, the COVID-19 pandemic has significantly amplified the global burden of ALI/ARDS, with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection frequently leading to diffuse alveolar

damage and refractory hypoxemia in affected patients.^{27–29} This underscores the urgent need for a deeper understanding of ALI/ARDS pathogenesis and the development of effective therapeutic strategies.

Beyond its profound clinical burden, ALI serves as a crucial biological paradigm for understanding the intricate and dynamic interplay of immune cells within a multi-tiered inflammatory process. The pathogenesis of ALI involves a complex cascade of inflammatory cytokines and chemokines released by various cell types, leading to extensive damage to both the pulmonary vascular endothelium and alveolar epithelium.²⁶ The immune response in ALI consists of distinct yet interconnected phases. The early phase is characterized by the rapid activation and necrosis of alveolar macrophages (AMs), which release pro-inflammatory mediators like pro-interleukin-1 α (pro-IL-1 α) via CD14 and P2X7 receptor signaling, thereby initiating vascular leakage and the crucial early recruitment of neutrophils into the lung parenchyma.³⁰ The neutrophils, while essential for pathogen control, contribute significantly to acute lung damage through the release of cytotoxic substances and neutrophil extracellular traps.^{31,32} As the disease progresses into its later stages, the landscape of immune infiltration shifts into the lung, with T lymphocytes, including regulatory T cells, playing a vital role in modulating immune responses and facilitating the resolution of injury.^{33–36} Concurrently, circulating monocytes are recruited to the lung, where they differentiate into monocyte-derived macrophages crucial for inflammation resolution and tissue repair.^{37–39} Throughout these dynamic transitions, AMs remain orchestrators, exhibiting remarkable functional plasticity to coordinate both the initiation and resolution of inflammation. Their secretion of key mediators such as IL-6; CXCL1, 2, 9, and 10; and CCL5 significantly influences the progression and outcome of ALI by modulating tiered inflammatory cascades, from neutrophils to T cells, and monocytes.^{26,31,40,41}

While the cellular and cytokine dynamics of ALI are increasingly understood, the precise mechanisms by which broader environmental factors govern these complex, multi-stage immune response in ALI, from initial injury to resolution, remain largely unexplored. In particular, the impact of immunomodulatory metabolites, which have emerged as critical environmental factors of immune cell function in other contexts,^{42–46} has not been comprehensively investigated in the context of the pulmonary inflammation. Therefore, this study aims to identify the key environmental factors, particularly immunomodulatory metabolites, that orchestrate the multi-tiered immune processes in ALI.

RESULTS

ALI-stimulated lung-infiltrating neutrophils represent a major source of extracellular itaconate in BALF

To identify the metabolites functioning as environmental factors that orchestrate the tiered inflammatory responses, we induced ALI in mice with a pulmonary injection of LPS, isolated the cell component-removed bronchoalveolar lavage fluid (BALF) 24 h later, and subjected the BALF to metabolomics with gas chromatography-tandem mass spectrometry (GC-MS/MS) (Figure 1A). Eighty metabolites were detected, of which itaconate was the most enriched. Longitudinal BALF sampling also

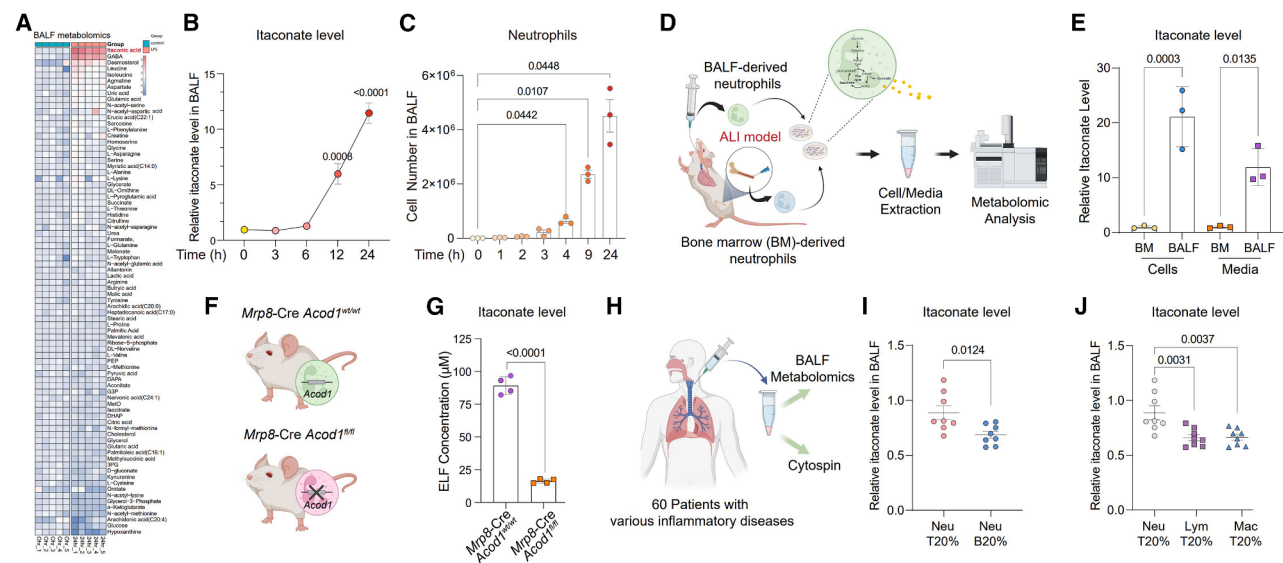


Figure 1. Neutrophils serve as the predominant source of extracellular itaconate in the BALF during ALI

(A–G) Mice were challenged oropharyngeally with LPS (1 mg/kg) to induce an ALI model. BALF was sampled at 0, 3, 6, 12, and/or 24 h after challenge. (A) Heatmap showing the GC-MS/MS-based targeted metabolomics of the WT BALFs at 24 h ($n = 5$ mice per group). The heatmap depicts the log2 fold change in the relative abundance of the indicated metabolites. (B) Changes in itaconate levels in the WT BALF over time ($n = 3$ mice per group). (C) Quantification of neutrophil number (gated on single live cells, CD11b⁺ Gr1⁺) in BALF of ALI mice at indicated time points (0, 1, 2, 3, 4, 9, and 24 h) post-LPS challenge ($n = 3$ mice per group). (D) Schematic depiction showing the analytical workflow used to measure intracellular and extracellular itaconate levels in neutrophils derived from BALF and BM. (E) Relative itaconate level in the cell extract (Cell) and culture medium (Media) of neutrophils derived from BALF or BM ($n = 3$ mice per group). (F) Schematic diagram illustrating the generation of the *Mrp8-Cre Acod1^{fl/fl}* mice in which *Acod1* is specifically deleted in neutrophils. (G) Itaconate concentration of the ELFs in *Mrp8-Cre Acod1^{wt/wt}* and *Mrp8-Cre Acod1^{fl/fl}* mice ($n = 4$ mice per group). (H–J) BALF samples from 60 patients with inflammatory lung diseases were subjected to metabolomics analysis of itaconate levels and neutrophil, lymphocyte, and macrophage cell counting. (H) Schematic depiction of patient-derived BALF metabolomics. (I) Comparison of the 20% of patients with the highest neutrophil numbers to the 20% of patients with the lowest neutrophil numbers in terms of relative itaconate levels in the BALF (8 patients per group). (J) Comparison of the 20% of patients with the highest neutrophil numbers, the 20% of patients with the highest lymphocyte numbers, and the 20% of patients with the highest macrophage numbers in terms of relative itaconate levels in the BALF (8 patients per group). The data are shown as mean $\pm$ SEM and statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test (B, C, E, and J) or two-tailed Student's *t* test (G and I).

showed that itaconate levels in BALF sharply increased over the 24 h after LPS injection (Figure 1B). Considering the concurrent infiltration of neutrophils observed within 24 h post-LPS injection, alongside a unique self-repopulation pattern of AMs^{47–50} and a negligible presence of monocyte-derived macrophages (Figures 1C and S1A–S1C), we hypothesized that neutrophils synthesize and release itaconate into the BALF during lung inflammation.

To this end, we isolated the neutrophils from bone marrow (BM) and BALF 24 h after LPS exposure, obtained their mRNAs, and examined *Acod1* expression, which encodes the itaconate-synthesizing enzyme. Indeed, LPS exposure not only increased neutrophil numbers in the lung but also initiated strong *Acod1* expression by the neutrophils (Figure S1D). Although Tomlinson et al. suggested that lung neutrophils might synthesize itaconate upon infection,²⁰ whether neutrophils are responsible for extracellular itaconate in BALF was not definitively examined. To address this, we measured itaconate levels in matched BALF- and BM-derived neutrophils isolated from the same ALI mice,

as well as in their corresponding culture media (Figure 1D). While acknowledging that BM-derived neutrophils may represent a distinct baseline state, our analysis suggests that BALF-infiltrating neutrophils possess a robust capacity for *de novo* itaconate synthesis and subsequent extracellular release (Figure 1E). Thus, the enriched itaconate in the BALF of ALI mice is not solely due to the intracellular accumulation of this metabolite within neutrophils; a significant portion is also released into the extracellular environment.

To determine whether neutrophils are a physiologically relevant source of BALF itaconate, we generated *Mrp8-Cre Acod1^{fl/fl}* mice (Figure 1F). The specific ablation of *Acod1* expression in neutrophils, but not in AMs (Figures S1E and S1F), in these mice validates this model for studying the functional relevance of neutrophil-derived itaconate. Notably, this knockout largely eliminated the high levels of itaconate observed in the epithelial lining fluid (ELF)—a physiologically relevant lung fluid—isolated from ALI mice, where concentrations typically reach approximately 100 μ M (Figures 1G and S1G). This

indicates that neutrophils are the primary source of the highly enriched BALF itaconate during ALI.

Next, we investigated the human relevance of neutrophil-derived itaconate in pulmonary diseases. To this end, we collected BALF from 60 patients suffering from various inflammatory lung conditions, including pneumonia, tuberculosis, and bronchiectasis; determined BALF neutrophil, lymphocyte, and macrophage counts; and measured itaconate levels in the BALF (Figure 1H). Patients with the highest BALF neutrophil numbers exhibited significantly higher itaconate levels in their BALF compared to those with the lowest neutrophil numbers (Figure 1I). Furthermore, patients with the highest BALF neutrophil counts also had significantly more BALF itaconate than patients with high BALF lymphocyte or macrophage counts (Figure 1J). These findings suggest an association between neutrophil abundance and elevated BALF itaconate levels in human pulmonary diseases.

Taken together, these data establish neutrophils as the predominant source of BALF itaconate during pulmonary inflammation, highlighting their release into the lung local environment.

Neutrophil-derived itaconate ablation upon ALI results in the induction of *Mrc1^{high}* macrophage

Recent studies have demonstrated that macrophages secrete itaconate into the tumor microenvironment, thereby exhausting the infiltrating CD8⁺ T cells and aggravating tumor growth.^{21,22} This suggests that itaconate might serve as a cellular signal that modulates the activities of other cells. Given these findings, coupled with our observation regarding neutrophil-driven BALF itaconate enrichment, we asked whether the neutrophil-derived itaconate in ALI lungs affected other local cell populations.

To this end, we induced ALI in *Mrp8-Cre Acod-1^{fl/fl}* mice and their WT littermates for 24 h; the BALF cells were then collected and subjected to single-cell RNA sequencing (scRNA-seq) and clustering analysis (Figures 2A and S2A–S2D). Our analysis surprisingly revealed that ablation of neutrophil itaconate production had negligible effects on the intrinsic transcriptional features, including apoptosis (Figure S2E). Furthermore, it did not significantly change the proportion of most cell types, such as neutrophils, natural killer cells, monocytes, dendritic cells (DCs), and macrophages, although epithelial and T cell numbers were notably less enriched in the knockout mice (Figures 2B and S2F).

Interestingly, when we used the FindSubCluster function of Seurat, we observed that the total macrophage population (i.e., AMs) in the knockout mice largely split into an *Mrc1^{high}* macrophage subcluster and the remaining *Mrc1^{low/medium}* macrophage subcluster (Figure 2C): the former subcluster was almost completely absent in the wild-type (WT) littermates. Specifically, in the WT littermates, almost all macrophages were *Mrc1^{low/medium}* cells, whereas 70.8% and 29.2% of the macrophages from knockout mice were *Mrc1^{low/medium}* and *Mrc1^{high}* macrophages, respectively (Figures 2C and S2F–S2H). *Mrc1* (also known as *CD206*) is well known to be upregulated by anti-inflammatory cytokine including IL-4 and is considered to be a marker of anti-inflammatory tissue-resident macrophages.⁵² Consistent with this, the *Mrc1^{high}* macrophages barely express pro-inflammatory mediators such as *Il6*, *Mmp12*, *Cxcl1*, *Cxcl2*,

Cxcl9, and *Cxcl10*; by contrast, the remaining *Mrc1^{low/medium}* macrophages expressed these mediators at higher levels (Figure 2D). Moreover, pathway enrichment analysis showed that while the *Mrc1^{low/medium}* macrophages expressed high levels of pro-inflammatory pathways and genes (Figure 2E), the *Mrc1^{high}* macrophage transcriptome was dominated by distinct pathways (Figure S2I).

Finally, cell-cell interaction analyses showed that the *Mrc1^{low/medium}* macrophages in WT littermate BALF interacted strongly with most other immune cells, while the *Mrc1^{high}* macrophage population was tiny and did not interact with the other cells. By contrast, the large *Mrc1^{high}* macrophage population in the knockout mice interacted strongly with most other immune cells in the lung, including the neutrophils and *Mrc1^{low}* macrophages (Figure S2J). Given that itaconate can modulate pro-inflammatory macrophage responses *in vitro*,^{13,16} we speculate that neutrophil-derived itaconate in ALI may lead to a substantial population of pro-inflammatory *Mrc1^{low}* macrophages that recruits and activates other immune cells in the lung.

To examine whether neutrophil-derived itaconate effects on AMs are also relevant in human contexts, we revisited the publicly available scRNA-seq data of 35 BALF or pleural-fluid samples from patients with varying degrees of COVID-19 severity⁵¹ (Figure 2F). Clustering analysis of the whole cohort of 49,014 cells showed the presence of multiple immune and other cell types (Figures S2K–S2M). First, patients were categorized based on the presence of neutrophils expressing *ACOD1* (i.e., *ACOD1^{pos}* vs. *ACOD1^{neg}*) (Figure S2K). Notably, *ACOD1* was significantly higher in patients with severe/critical COVID-19 symptoms than in patients with mild/moderate symptoms (Figure 2G). Of note, *ACOD1* expression was more significantly enriched in neutrophils than macrophages (Figure 2H). Next, we assessed AMs from both groups for their expression of *MRC1* (Figures S2N and S2O). This showed that there were significantly fewer *MRC1*-expressing AMs in patients with *ACOD1^{pos}* neutrophils than in patients without these neutrophils (Figure 2I). Moreover, the AMs from patients with *ACOD1^{pos}* neutrophils expressed various inflammatory genes such as *Il6* at higher levels than the AMs from the other patients (Figures 2J and 2K). Importantly, this is consistent with a strong correlation between the AMs and COVID-19 severity (Figures 2L and 2M). These observations suggest that dysregulated BALF itaconate levels may be associated with the severity of human inflammatory pulmonary diseases, including COVID-19, presumably by promoting excessive inflammatory responses.

Taken together, these findings revealed that neutrophil-derived extracellular itaconate serves as an environmental factor that directly promotes the pro-inflammatory phenotype of AMs.

Extracellular itaconate dictates AMs' phenotypic plasticity via epigenetic reprogramming

The emergence of *Mrc1^{high}* macrophages upon genetic ablation of BALF itaconate suggested that neutrophil-derived itaconate may influence AM plasticity in ALI mice. To test this *in vivo*, we first analyzed the spatial relationship between these two critical immune cell types in the lung tissue of ALI-induced mice (Figures S3A and S3B). Immunostaining of lung tissue sections

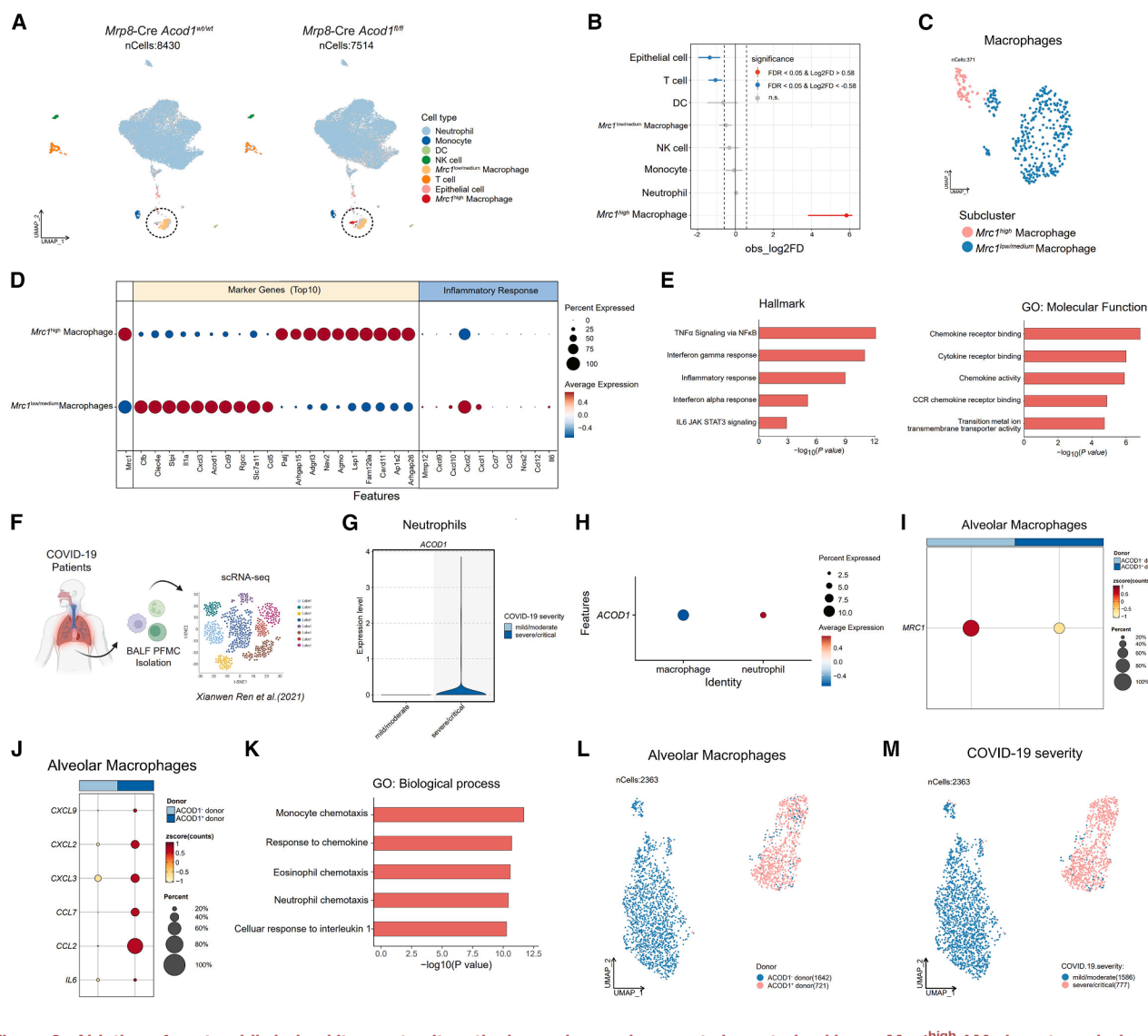
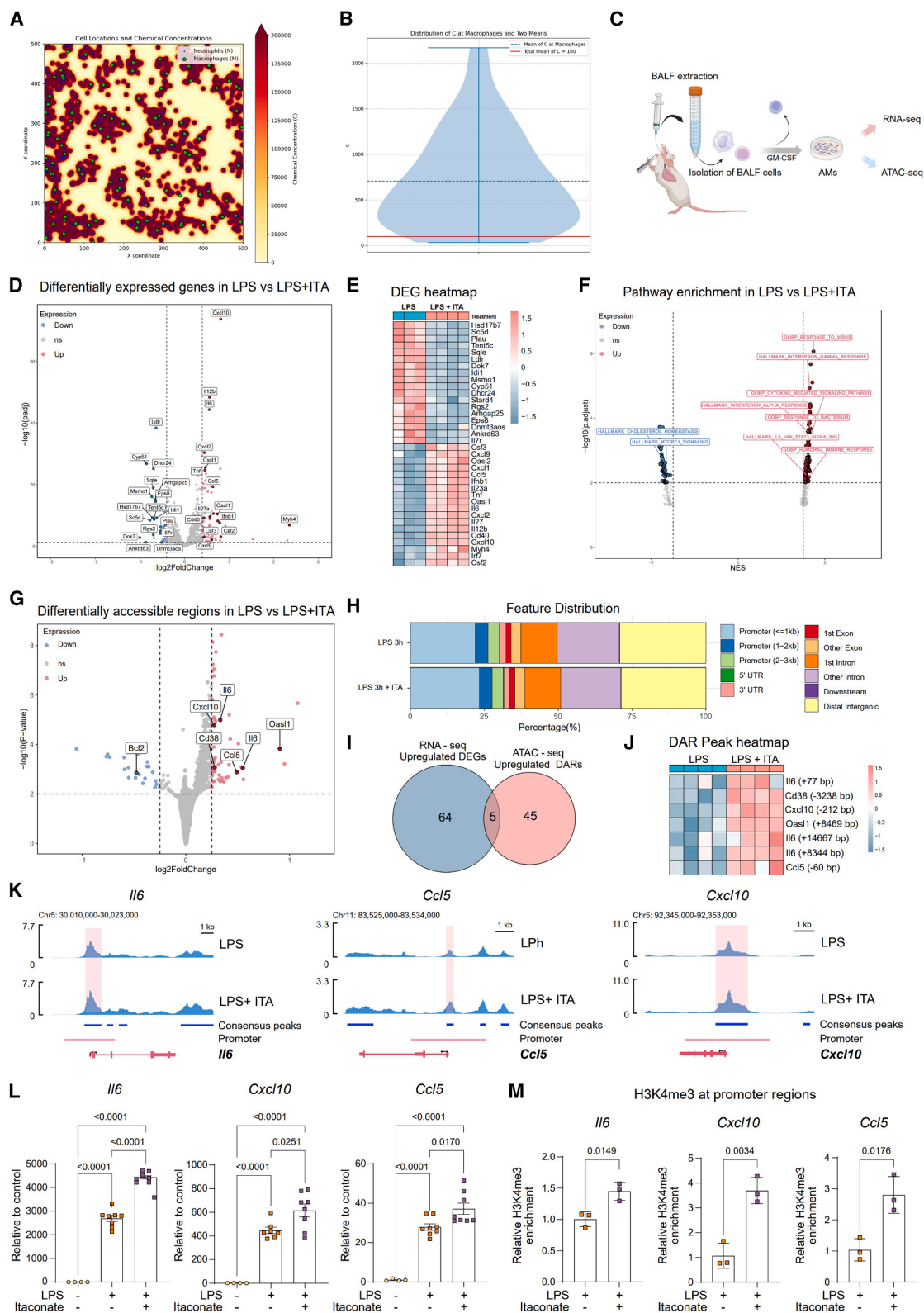


Figure 2. Ablation of neutrophil-derived itaconate alters the lung microenvironment characterized by an *Mrc1^{high}* AM phenotype during ALI (A–E) BALF from *Mrp8-Cre Acod1^{wt/wt}* and *Mrp8-Cre Acod1^{fl/fl}* mice was sampled 24 h after ALI induction (1 mg/kg LPS) and subjected to scRNA-seq and annotation and clustering according to the parameters shown in Figures S2A–S2D. BALF samples were prepared by pooling from three mice per group. (A) Uniform manifold approximation and projection (UMAP) representation of all cell clusters in BALF samples from *Mrp8-Cre Acod1^{wt/wt}* and *Mrp8-Cre Acod1^{fl/fl}* mice. (B) Single-cell proportion analysis reveals differences in the distribution of cell types between *Mrp8-Cre Acod1^{wt/wt}* and *Mrp8-Cre Acod1^{fl/fl}* BALF samples. (C) UMAP visualization of macrophage subclusters. (D) Dot plot showing the top 10 marker genes and inflammatory response-related genes expressed in macrophage subclusters. (E) Over-representation analysis of *Mrc1^{low/medium}* macrophages (left, hallmark pathways; right, Gene Ontology: molecular function). (F) Schematic diagram depicting the analysis of public scRNA-seq dataset from BALF/PFMC samples of COVID-19 patients.⁵¹ (G) *ACOD1* expression levels in neutrophils from COVID-19 patients with mild/moderate or severe/critical disease. (H) *ACOD1* expression levels in macrophages and neutrophils from BALF samples of COVID-19 patients. (I) Dot plot of *MRC1* expression in alveolar macrophages from neutrophil-specific *ACOD1*-negative and *ACOD1*-positive donors. (J) Dot plot showing expression of inflammatory response genes in alveolar macrophages from neutrophil-specific *ACOD1*-negative and *ACOD1*-positive donors. (K) Over-representation analysis of marker genes of alveolar macrophages from *ACOD1*-positive donors. (L) UMAP plot of alveolar macrophages from *ACOD1*-negative and *ACOD1*-positive donors. (M) UMAP plot showing alveolar macrophages stratified by COVID-19 disease severity.

from ALI mice revealed that AMs and neutrophils were positioned in close proximity within the inflamed pulmonary microenvironment (Figure S3B). Quantitative analysis of the distance be-

tween AMs and neutrophils (~10.6 μ m on average) further confirmed that these cells were significantly closer under ALI conditions. Importantly, this characteristic distance was not



(legend on next page)

only observed histologically but also recapitulated in our computational simulation, where neutrophil-macrophage radial positioning was modeled from a truncated normal distribution (Figures 3A and S3C). By scaling the reaction-diffusion model to the experimentally determined concentration of $\sim 100 \mu\text{M}$ (Figure 1), the simulation further demonstrated that such proximity yielded a mean steady-state concentration of C around $\sim 685 \pm 50 \mu\text{M}$ at macrophage surfaces (Figures 3B and S3D). This quantitative agreement between spatial distance and model-derived chemical accumulation provides a potential mechanistic explanation for how the anatomical closeness of AMs and neutrophils may establish a permissive microenvironment for paracrine crosstalk.

To further evaluate whether itaconate exerts direct effects on AMs, we established an *in vitro* culture system where primary AMs were treated with LPS as an inflammatory stimulus or a combination of LPS and itaconate (Figure 3C). Subsequently, we employed a multiomics approach combining RNA-seq for analyzing global gene expression changes and ATAC sequencing (ATAC-seq) for profiling chromatin accessibility, thereby gaining a comprehensive view of itaconate-mediated transcriptional and epigenetic regulation.

RNA-seq analysis of AMs treated with LPS alone or a combination of LPS and itaconate revealed significant changes in gene expression profiles (Figures 3D, S3E, and S3F). Specifically, compared to LPS alone, numerous pro-inflammatory genes were robustly upregulated, and a distinct pattern of further modulation was notably observed upon LPS/itaconate cotreatment (Figures 3D–3F and S3G–S3I). This underscores the role of itaconate in modulating the inflammatory transcriptional program of AMs.

Complementary ATAC-seq analysis revealed dynamic changes in chromatin accessibility in response to a combination of LPS and itaconate (Figures 3G and S3J–S3N). Notably, a significant opening of promoter regions for specific genes was observed, indicating increased chromatin accessibility at these regulatory elements (Figures 3H, S3L, and S3M). This finding

suggests that the influence of itaconate on gene expression may extend to the epigenetic level, potentially impacting the accessibility of DNA to the transcriptional machinery. The concomitant observation of both changes in gene expression and alterations in chromatin accessibility at promoter regions provides potential evidence that the immunomodulatory effects of itaconate on gene expression may be, at least in part, mediated by epigenetic remodeling.

To identify targets of itaconate, an integrated analysis was performed by overlapping differentially expressed genes (DEGs) upregulated in RNA-seq with differentially accessible regions (DARs) upregulated in ATAC-seq. This integrated analysis identified 5 key genes that were not only transcriptionally upregulated but also exhibited increased chromatin accessibility in their regulatory regions (Figure 3I). Among these 5 genes, three major pro-inflammatory genes (*Il6*, *Ccl5*, and *Cxcl10*) with significantly opened promoters and upregulated expression were specifically identified (Figure 3K). These genes represent core mediators of the inflammatory response in AMs,^{53–57} underscoring the regulatory effects of itaconate.

Further detailed examination of the ATAC-seq data confirmed that the promoter regions of these three identified pro-inflammatory genes exhibited distinct open chromatin peaks in response to LPS/itaconate cotreatment, directly correlating with their increased expression (Figures 3J and 3K). The effects of itaconate at physiologically relevant concentration on three identified pro-inflammatory genes (*Il6*, *Cxcl10*, and *Ccl5*) were confirmed at the relevant concentrations using quantitative PCR (qPCR), aligning with the trends observed in the RNA-seq data (Figures 3L and S3O).

Epigenetic remodeling through specific histone modifications increases chromatin accessibility at regulatory sites, thereby regulating gene expression.⁵⁸ In particular, H3K4me3 is often found at the transcription start sites (TSSs) of active genes, and its levels generally correlate with transcriptional output, promoting active transcription when located near the TSSs.⁵⁹ Finally, chromatin immunoprecipitation (ChIP)-qPCR of the three

Figure 3. Extracellular itaconate drives AM phenotypic plasticity via coordinated transcriptomic and epigenetic remodeling

(A and B) Mice were challenged oropharyngeally with LPS (1 mg/kg) to induce an ALI model, and the lung tissues were sampled 24 h after challenge and then subjected to Opal multiplex immunohistochemistry staining analysis. Computational simulations based on spatial tissue staining data were used to analyze neutrophil-macrophage radial positioning and concentration gradients of itaconate released from neutrophils.

(A) Representative images from Monte Carlo simulation illustrating the spatial distribution of neutrophils and macrophages and chemical concentrations.

(B) Violin plot showing the concentration distribution of itaconate released from neutrophils.

(C) Schematic diagram of AM isolation and treatment for subsequent multi-omics analysis. Cultured AMs were treated with or without 1 mM itaconate for 3 h, followed by stimulation with 10 ng/mL LPS for an additional 3 h, and then subjected to transcriptomic and epigenomic profiling ($n = 4$ mice per group).

(D and E) Volcano plot (D) and heatmap (E) showing the significantly DEGs (adjusted $p < 0.05$, $|\log_2 \text{fold change}| \geq 0.4$, baseMean > 10).

(F) Volcano plot showing $\log_2$ fold changes of significantly enriched pathways identified by GSEA ($p < 0.01$, NES cutoff = 1.5).

(G) Volcano plot showing $\log_2$ fold changes of significantly enriched DARs between LPS and LPS + itaconate conditions ($p < 0.01$, $|\log_2 \text{fold change}| \geq 1.2$).

(H) Bar plot illustrating the feature distribution between LPS and LPS + itaconate conditions.

(I) Venn diagram showing the overlap of upregulated DEGs and DARs between LPS and LPS + itaconate.

(J) Heatmap showing the intersecting genes and their associated DAR peaks, including *Il6*, *Cd38*, *Cxcl10*, *Oasl1*, and *Ccl5* (DAR peaks: $p < 0.01$).

(K) Genomic tracks showing chromatin accessibility at the promoter regions of *Il6*, *Ccl5*, and *Cxcl10* based on the ATAC-seq profiling (promoter region defined as ± 2 kb from the transcription start site based on RefSeq mm10 database).

(L) Quantitative reverse-transcription PCR (RT-qPCR) analysis of *Il6*, *Cxcl10*, and *Ccl5* expression in mouse AMs under the indicated conditions ($n = 3$ per group). Data are representative of three independent experiments.

(M) Relative H3K4me3 enrichment in mouse AMs as determined by ChIP-qPCR at the promoter regions of the indicated genes under the conditions described above ($n = 3$ per group). Data are representative of four independent experiments.

The data are shown as mean $\pm$ SEM, and statistical significance was determined by one-way ANOVA with Turkey's multiple comparison test (L) and two-tailed Student's *t* test (M).

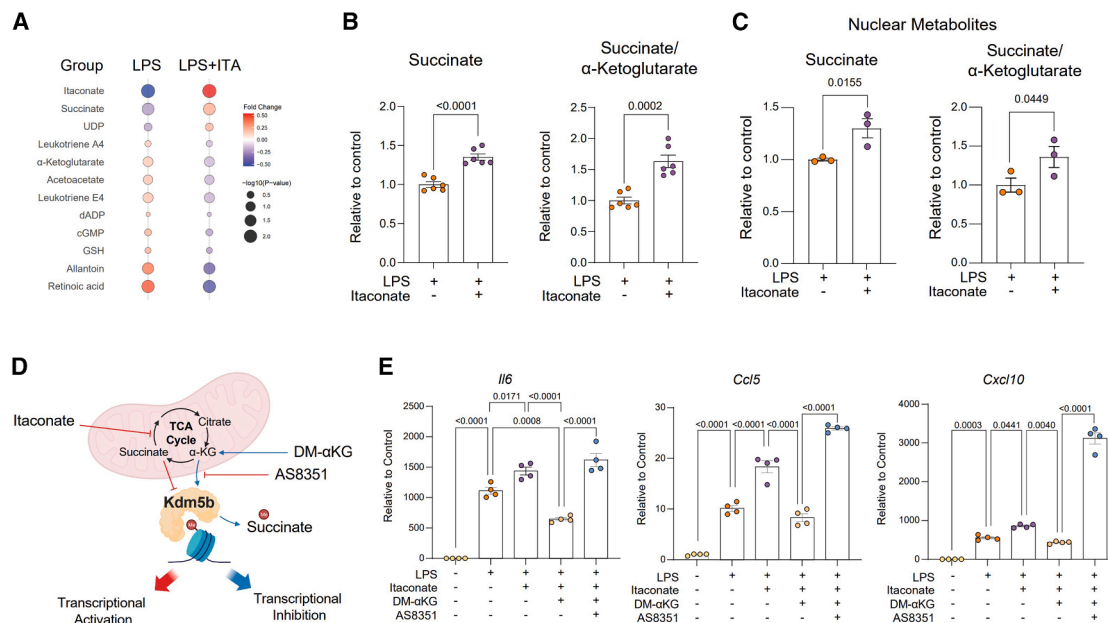


Figure 4. Extracellular itaconate orchestrates AM phenotypic plasticity through metabolic-epigenetic reprogramming

(A and B) LC-MS-based metabolomics analysis in AMs treated with or without 1 mM itaconate for 3 h, followed by stimulation with 10 ng/mL LPS for an additional 3 h ($n = 6$ per group). Data are representative of four independent experiments.

(A) Bubble plot showing the top 12 significantly altered metabolites identified by the bubble depicts the log2 fold change in the relative abundance and statistical significance of the indicated metabolites.

(B) Relative total succinate abundance and succinate/α-KG ratio in AMs.

(C) Relative nuclear succinate abundance and succinate/α-KG ratio in AMs.

(D) Schematic illustration of the proposed mechanisms underlying itaconate-mediated epigenetic regulation and the validation experiments. Itaconate modulates TCA cycle metabolism, leading to increased intracellular succinate and decreased α-KG levels, leading to suppression of the activity of the α-KG-dependent histone demethylase Kdm5b. AS-8351, a selective inhibitor of Kdm5b, mimicked the effect of itaconate and promoted transcriptional activation of pro-inflammatory genes. Conversely, supplementation with DM-αKG, which reverses the succinate/α-KG ratio, restored Kdm5b activity and consequently suppressed inflammatory gene expression.

(E) RT-qPCR analysis of *Il6*, *Ccl5*, and *Cxcl10* expression in AMs treated with 1 mM itaconate, 20 μM AS-8351, and/or 1 mM DM-αKG for 3 h, followed by stimulation with 10 ng/mL LPS for an additional 3 h ($n = 4$ per group).

Data are representative of three independent experiments. The data are shown as mean ± SEM, and statistical significance was determined by two-tailed Student's *t* test (B) or one-tailed Student's *t* test (C) and one-way ANOVA with Tukey's multiple comparison test (E).

target genes promoter in the LPS/itaconate-cotreated AMs showed the effects of itaconate on H3K4me3 levels at promoter regions of the three genes (Figure 3M).

Thus, itaconate can act as a potential environmental signal to regulate chromatin accessibility in AMs, thereby shaping AM phenotypic plasticity.

Kdm5b histone demethylase links itaconate-driven metabolic reprogramming to the epigenetic changes in AMs

When immune cells are activated, they often undergo metabolic reprogramming, which in turn induces epigenetic changes: this has been observed for T cells, monocytes, and macrophages.^{21,60–62} These findings, coupled with the itaconate effects on H3K4me3 levels at target gene promoter regions, led us to examine whether itaconate induces the epigenetic remodeling in macrophages by altering their cellular metabolism. Thus, primary AMs treated with LPS alone or LPS/itaconate cotreatment were subjected to liquid chromatography (LC)-MS-based metabolomics (Figures 4A and S4A). This showed that apart

from itaconate, the TCA cycle metabolite succinate was the most enriched of the metabolites (Figures 4A, 4B, and S4A). This is consistent with the fact that itaconate competes with succinate for binding to SDH, which blocks SDH activity and causes succinate to accumulate.^{7,12}

The accumulation of succinate significantly impacts epigenetic changes by inhibiting several epigenetic modifiers, including the KDM5 histone demethylase family proteins.⁶³ This inhibition alters the methylation status of specific genome regions, consequently changing target gene expression. Mechanistically, succinate suppresses these enzymes by competing with α-ketoglutarate (α-KG), a TCA cycle metabolite and an essential co-substrate for these demethylases.⁶³ Therefore, any changes in cellular metabolism that alter the intracellular succinate:α-KG ratio can drive epigenetic remodeling.^{21,64,65} Indeed, we observed that itaconate treatment of LPS-stimulated AMs markedly increased both total and nuclear succinate:α-KG ratios (Figures 4B and 4C).

The KDM5 histone demethylase family comprises four isoforms (KDM5A/B/C/D), each playing distinct roles in various

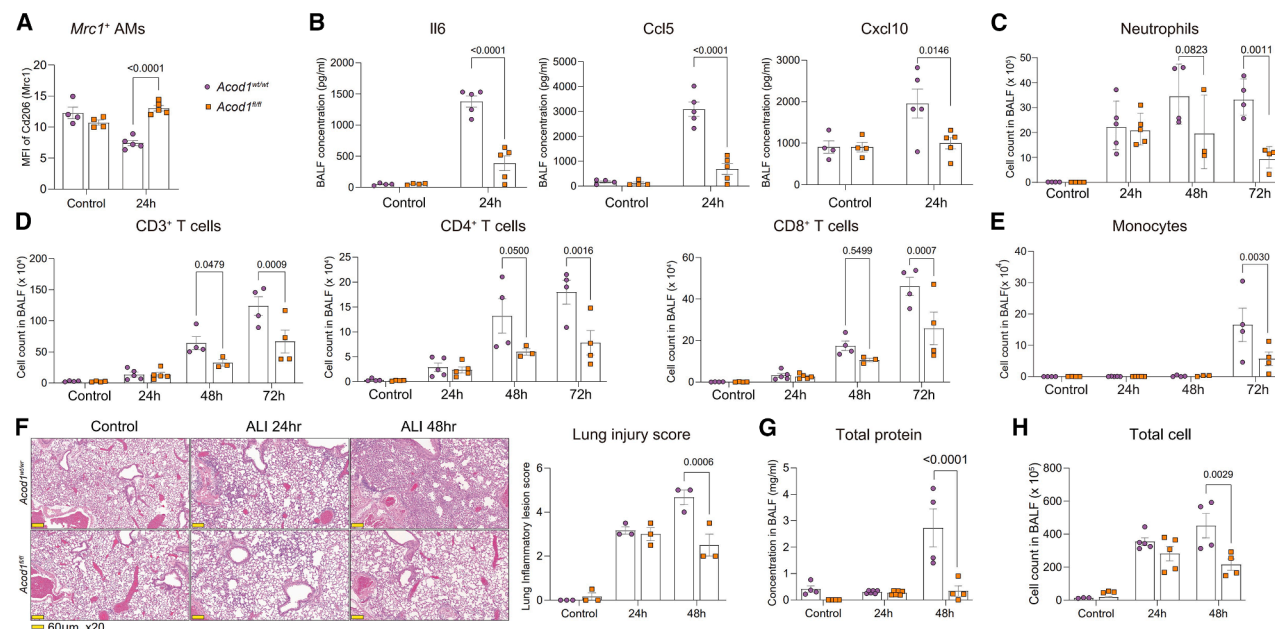


Figure 5. Depletion of neutrophil-derived itaconate ameliorates tiered inflammatory responses and alleviates ALI pathology

Mrp8-Cre Acod1^{wt/wt} and *Mrp8-Cre Acod1*^{fl/fl} mice (8 weeks old) were challenged with 1 mg/kg LPS to induce an ALI model and subjected to subsequent analyses shown in panels.

(A) Quantification of *Mrc1*⁺ cell counts in BALF from the *Mrp8-Cre Acod1*^{wt/wt} and *Mrp8-Cre Acod1*^{fl/fl} mice challenged with LPS at 0 and 24 h ($n = 4-5$ mice per group).

(B) Cytokine (Il6 and Ccl5) and chemokine (Cxcl10) levels measured by ELISA in BALF challenged with LPS from the *Mrp8-Cre Acod1*^{wt/wt} and *Mrp8-Cre Acod1*^{fl/fl} mice at 0 and 24 h ($n = 4-5$ mice per group).

(C) Neutrophil cell counts in BALF from the *Mrp8-Cre Acod1*^{wt/wt} and *Mrp8-Cre Acod1*^{fl/fl} mice at indicated time points (0, 24, 48, and/or 72 h) post-LPS challenge ($n = 4-5$ mice per group).

(D) Quantification of infiltrating T cell subsets (CD3⁺, CD4⁺, and CD8⁺) in BALF post-LPS challenge at 0, 24, 48, and 72 h ($n = 4-5$ mice per group).

(E) Quantification of CD115⁺ monocytes in BALF at indicated time points (0, 24, 48, and/or 72 h) post-LPS challenge ($n = 4-5$ mice per group).

(F) Representative H&E-stained lung sections harvested at indicated time (0, 24, and 48 h) after LPS challenge and quantified lung injury score compared between *Mrp8-Cre Acod1*^{wt/wt} and *Mrp8-Cre Acod1*^{fl/fl} mice ($n = 3$ mice per group).

(G) Total protein concentration measured in BALF from *Mrp8-Cre Acod1*^{wt/wt} and *Mrp8-Cre Acod1*^{fl/fl} mice at 0 and 24 h post-LPS challenge ($n = 4-5$ mice per group).

(H) Total cell counts in BALF from *Mrp8-Cre Acod1*^{wt/wt} and *Mrp8-Cre Acod1*^{fl/fl} mice at 0 and 24 h post-LPS challenge ($n = 4-5$ mice per group).

Data are presented as mean $\pm$ SEM. The number denotes biological replicates. Statistical significance was determined by two-way ANOVA.

physiological contexts, including inflammatory responses.⁶⁶⁻⁷¹

Among these, KDM5B is particularly known for its regulatory functions in promoter regions of pro-inflammatory genes including IL6, especially in macrophages.^{67,68} Notably, analysis of Kdm5b ChIP-qPCR analysis in LPS-stimulated MH-S cells, an AM cell line, showed that Kdm5b is enriched at the promoter regions of itaconate target genes, including *Il6*, *Cxcl10*, and *Ccl5* (Figure S4D).⁷¹ Therefore, we hypothesized that itaconate may induce epigenetic remodeling by suppressing Kdm5b (Figure 4D). To test this, AMs were stimulated with LPS in the presence or absence of itaconate or AS-8351, which is a specific inhibitor of Kdm5b in macrophages.^{72,73} AS-8351 mimicked itaconate in terms of the effect on the target gene expression in response to LPS (Figure S4E). The rescue experiment was then conducted with dimethyl α -KG (DM- α KG), a cell-permeable form of α -KG: when AMs were co-treated with itaconate and DM- α KG, the high succinate/ α -KG ratio generated by itaconate was recovered (Figure S4F), along with its ability to recover the target gene expression (Figure 4E). The itaconate-mimicking

and -suppressing effects of AS-8351 and DM- α KG are depicted in Figure 4D. Thus, Kdm5b may link itaconate-mediated metabolic changes in AMs to the epigenetic remodeling that dictates AM phenotypic plasticity.

Depletion of neutrophil-derived itaconate alleviates pulmonary inflammation through attenuated tiered inflammatory responses

We sought to determine the physiological relevance of our argument regarding itaconate as an environmental factor. To this end, we first analyzed the proportion of *Mrc1*-expressing AMs, which were found to be more enriched in *Mrp8-Cre Acod1*^{fl/fl} at 24 h post-LPS challenge (Figures 5A and S5A). This observation is consistent with our previous scRNA-seq findings (Figure 2).

Next, we measured the concentrations of AM-derived key inflammatory mediators regulated by itaconate (i.e., Il6, Ccl5, and Cxcl10) in the BALF. At 24 h post-LPS challenge, the levels of Il6, Ccl5, and Cxcl10 were significantly reduced in the BALF of

Mrp8-Cre Acod1^{fl/fl} compared to WT littermates (Figure 5B). Given the established role of IL-6 in promoting neutrophil survival in the lung,⁷⁴ the reduced Il6 levels observed at 24 h in *Mrp8-Cre Acod1^{fl/fl}* mice may contribute to impaired neutrophil maintenance within the pulmonary environment at 48 h and beyond (Figures 5C and S5B). Moreover, observations were further corroborated by adoptive transfer experiments using CD45.1 isotype mice, where neutrophils isolated from *Mrp8-Cre Acod1^{fl/fl}* animals induced lower chemokine expression compared to those from WT littermates (Figures S5C and S5D). While the precise mechanisms underlying this temporal effect remain to be fully elucidated, these findings suggest that the itaconate-Il6 axis influences neutrophil longevity during the progression of lung injury. Importantly, the extracellular itaconate-mediated suppression of chemokine/cytokine expression was also recapitulated in a bacterial infection model (*Acinetobacter baumannii*), indicating that these mechanisms may broadly be relevant to pulmonary inflammation (Figure S5E).

Given the well-established functional relevance of Ccl5 and Cxcl10 in infiltration of T cells and monocytes, we investigated the infiltration of these subsequent immune cell populations. T cells (CD3⁺, CD4⁺, and CD8⁺), which typically begin to appear in the microenvironment from 48 h in ALI, showed a significant reduction in their numbers in the BALF of *Mrp8-Cre Acod1^{fl/fl}* at both 48 and 72 h (Figures 5D and S5F). Similarly, monocytes, which are typically observed from 72 h in ALI, also exhibited a decreased presence in the BALF of *Mrp8-Cre Acod1^{fl/fl}* (Figures 5E and S5G). These findings collectively suggest that the depletion of neutrophil-derived itaconate leads to an attenuated recruitment of various immune cell types.

Finally, to assess the overall impact on ALI pathology, we performed lung histology analysis. While a mild pathological phenotype was observed at 24 h in both groups, by 48 h post-LPS challenge, the lung histology of *Mrp8-Cre Acod1^{fl/fl}* showed a marked amelioration of ALI pathology compared to WT littermates (Figure 5F). This amelioration was further supported by reduced total protein levels in both BALF (Figure 5G) and total cells (Figure 5H) at 48 h in *Mrp8-Cre Acod1^{fl/fl}*, indicating decreased vascular leakage and tissue damage.

In conclusion, these results suggest that neutrophil-derived itaconate may influence ALI pathology by modulating AM-governed tiered inflammatory processes, including the sequential recruitment of neutrophils, T cells, and monocytes, ultimately influencing the overall severity of lung injury.

DISCUSSION

ALI is a substantial clinical burden, particularly when coupled with other infectious diseases such as COVID-19, necessitating urgent clinical care. Beyond its clinical significance, ALI serves as an invaluable biological model for exploring the delicate coordination of acute and chronic inflammatory responses. Unlike many other bacterial infections, ALI is characterized by a massive and dominant neutrophil infiltration, which profoundly determines the extent of its pathophysiology.^{31,75,76} Subsequently, T cells and monocytes sequentially infiltrate the lung, further contributing to the disease progression.^{35,36} Of note, the intratracheal injection of LPS is a highly relevant and well-es-

tablished model that accurately reflects *in vivo* ALI pathology, providing a robust platform for studying environmental factors that systemically shape these tiered processes and their subsequent pathological outcomes.^{77–80} Recent studies utilizing the lung injury model have begun to uncover such environmental factors.^{81–84} However, despite the long-standing recognition of cell-derived metabolites as dynamic and rapid environmental cues in contexts such as cancer, their role as lung environmental factors in inflammation has remained largely unexplored. In this study, using the LPS-induced ALI model, we propose neutrophil-derived itaconate as a potential environmental factor associated with these complex inflammatory cascades.

The immunomodulatory roles of itaconate have gained significant attention, with a predominant focus on its anti-inflammatory functions within macrophages, particularly in the context of BM-derived macrophages (BMDMs).^{7–9} However, the physiological relevance of these findings, especially within tissue-specific contexts, remains incompletely understood, which is largely due to the reliance on *in vitro* studies using BMDMs, which may not fully recapitulate the complex microenvironmental cues experienced by tissue-resident macrophages. Furthermore, many studies have employed itaconate derivatives, such as dimethyl itaconate and 4-octyl itaconate, which display distinct physicochemical properties and do not precisely mimic the function of native itaconate.⁸⁵ Our study addresses these limitations by employing a comprehensive multi-omics approach, including scRNA-seq, bulk RNA-seq, ATAC-seq, and metabolomics, especially in AMs, the tissue-resident macrophages in the lung, and identifies a key role for neutrophil-derived itaconate as a potential environmental factor that may shape the plasticity of AMs, thereby influencing pro-inflammatory responses, a function opposite to the established anti-inflammatory roles observed in BMDMs.

While previous research has primarily focused on the intrinsic functions of itaconate within macrophages, recent studies have recognized the roles of macrophage-derived extracellular itaconate. For instance, extracellular itaconate secreted by macrophages has been shown to promote hepatocellular carcinoma progression by epigenetically inducing CD8⁺ T cell exhaustion and to suppress cytotoxic CD8⁺ T cells, thereby promoting tumor growth.²¹ In the context of pulmonary inflammation, Tomlinson et al. recently demonstrated that neutrophils possess the capacity for itaconate synthesis during *Staphylococcus aureus* pneumonia, where it suppresses the oxidative burst and limits bacterial clearance.²⁰ Our study significantly expands these observations by showing that neutrophils may serve as a primary source of BALF itaconate and release it into the lung microenvironment. Through detailed analysis of *Mrp8-Cre Acod1^{fl/fl}* mice, which lack neutrophil-derived itaconate, we uncover its potential role as an environmental factor associated with tiered inflammatory responses. While some reports indicate potential macrophage expression leakage in *Mrp8-Cre* models, (1) our biochemical validation using LPS-stimulated primary AMs from *Mrp8-Cre Acod1^{fl/fl}* mice showing intact *Acod1* expression in AMs (Figure S1F); (2) previous literature supporting the utility of *Mrp8-Cre* as a model for studying neutrophil-myeloid cell interactions⁸⁶; (3) Zhao et al. showing *Mrp8-Cre Acod1^{fl/fl}*, but not *Ly6g-Cre Acod1^{fl/fl}*, as an efficient model for studying the effects

of neutrophil-specific *Acod1* deletion⁸⁷; and (4) adoptive transfer experiments using CD45.1 isotype mice further support our findings regarding neutrophil-derived itaconate as a key environmental factor and its physiological relevance in ALI. Of note, our findings are particularly complementary to the very recent study by Shan et al., who demonstrated a pro-inflammatory function of itaconate intrinsically synthesized in AMs. While Shan et al.¹⁶ focused on the intracellular effects of itaconate produced by AMs themselves, our study highlights the crucial role of extrinsic, neutrophil-derived itaconate. Given that neutrophil-derived itaconate targets AMs from the extracellular environment, and considering that LPS-induced *Acod1* expression in AMs requires a significant time interval (Figure S1H), we propose a possible temporally coordinated mechanism: neutrophil-derived itaconate may serve as an initial trigger, or “first hit,” for stimulating self-repopulated AMs^{47–50} into a pro-inflammatory state. This initial pro-inflammatory signal can then be further amplified by the intrinsic itaconate synthesized by AMs themselves as their *Acod1* expression upregulates. This potential interplay between extrinsic and intrinsic itaconate, in which neutrophil-derived itaconate initiates and shapes the AM response, may provide a more physiologically relevant understanding of ALI pathogenesis. Our study, therefore, offers a distinct and crucial perspective on the initiation and propagation of inflammatory responses in the lung, emphasizing the dynamic role of intercellular metabolic crosstalk in determining disease outcomes.

While our human analyses are correlative in nature, the observed associations between BALF itaconate levels, neutrophil abundance, ACOD1 expression, and AM inflammatory states closely parallel those identified in our murine model. These data do not establish causality or definitively define the cellular origin of itaconate in the human lung, but they support the relevance of neutrophil-associated itaconate signaling in human pulmonary inflammation. Further studies using isolated human neutrophils or advanced *ex vivo* lung models will be required to directly test causal relationships.

Our investigations into epigenetic regulation highlight the potential role of KDM5B. The accumulation of succinate, a consequence of itaconate-mediated SDH inhibition, significantly impacts epigenetics by competing with α -KG, an essential co-substrate for various epigenetic modifiers, including the Kdm5 histone demethylase family.⁶³ Our findings, coupled with previous literature, suggest that Kdm5b may play a significant role in these mechanisms. Specifically, a previous study has indicated that Kdm5b can regulate inflammatory responses, for instance, by modulating H3K4me3 levels at gene promoters such as *Il6*.⁶⁸ Moreover, our ChIP-qPCR analysis of itaconate target genes in AM cells further supports the notion that KDM5B may occupy these promoter regions, suggesting its potential involvement in the observed epigenetic changes.⁸⁸ While our study suggests that itaconate-driven metabolic reprogramming may lead to the suppression of KDM5B activity, thereby promoting pro-inflammatory gene expression, further research using established genetic KO models will be required to elucidate the precise mechanisms by which itaconate specifically regulates KDM5B over other KDM5 proteins. Moreover, we cannot rule out the involvement of other α -KG-regulated en-

zymes in this intricate process, which likely contribute to the broader epigenetic landscape shaped by itaconate. Furthermore, it is important to acknowledge that potential itaconate receptor signaling, such as OXGR1, and SDH inhibition followed by succinate accumulation likely regulate multiple signaling pathways in AMs simultaneously. For instance, Shan et al. recently demonstrated that itaconate activates the NLRP3 inflammasome via SDH inhibition, thereby driving the production of pro-inflammatory cytokines such as IL-1 β and IL-6.¹⁶ These collective observations suggest that itaconate-induced metabolic shifts may promote AM activation through diverse nodes, including but not limited to KDM5B. Notably, these established mechanisms and our current findings may not be mutually exclusive; rather, they likely operate in a complementary or synergistic manner to orchestrate itaconate-driven inflammatory responses in the lung. Finally, it may also be of interest to identify the relevant itaconate transporter and its functional contribution to epigenetic regulation. To fully dissect itaconate's precise regulatory mechanisms, various biochemical approaches are essential. Future work should specifically aim to identify how itaconate selectively impacts Kdm5b compared to other Kdm5 family members and to comprehensively characterize its interplay with succinate, α -KG, and other α -KG-dependent epigenetic enzymes. In this context, *in situ* detection of subtle shifts in nuclear α -KG and succinate using efficient but non-toxic probes could provide invaluable insights. These combined analyses will provide a clearer picture of the intricate metabolic and epigenetic crosstalk governing inflammatory responses.

Limitations of the study

While our targeted ChIP-qPCR analysis in an AM cell line supports KDM5B enrichment at the promoter regions of itaconate target genes, an unbiased genome-wide ChIP sequencing approach would provide a more comprehensive view of KDM5B's contribution to itaconate-mediated regulation.

Furthermore, although our pharmacological perturbation suggests that KDM5B may serve as a key target of itaconate-induced metabolic changes, genetic perturbation studies are required to rigorously evaluate the specific contribution of KDM5B, as well as other α -KG-dependent dioxygenases (e.g., KDM5A, KDM5C, and KDM5D).

Due to the technical challenges of acute genetic manipulation, including RNA interference, in primary AMs—potentially related to macrophage-specific immune surveillance and phagolysosomal degradation—future studies using genetic KDM5B-knockout AMs will be essential to define the associated inflammatory phenotype and epigenetic landscape and to further clarify the role of KDM5B in AMs.

Although our study mainly focused on the role of extracellular itaconate in promoting inflammatory responses through intracellular metabolic and epigenetic regulation in AMs, additional mechanisms may also contribute to this process. For example, loss-of-function experiments targeting itaconate transporters (e.g., SLC13A3 and MCT1/4) would not only further support our findings but also help identify the key transporters responsible for mediating extracellular itaconate uptake in AMs. Moreover, in addition to itaconate uptake and its intracellular mechanisms involving metabolic and epigenetic regulation, it will also

be important to investigate the potential role of extracellular receptors such as OXGR1, which may function as a ligand-sensing receptor for itaconate. Clarifying the contribution of OXGR1 in our experimental context may, therefore, further expand our findings and help guide future studies.

Finally, although we observed an association between immune cells and itaconate in human patient-derived BALF, the potential effects of additional factors, such as gender, should be addressed in future studies.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Dong Wook Choi (dongwook_choi@korea.ac.kr).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

- Targeted metabolomics data generated by GC-MS/MS are provided in [Table S2](#). Raw RNA-seq/ATAC-seq/single-cell RNA-seq data have been deposited at BioProject: PRJNA1449024. Untargeted metabolomics data generated using LC-QTOF have been deposited in the Metabolomics Workbench: ST004653. This study also analyzed publicly available data from GEO: GSE158055.
- This paper does not report original code. All analyses were performed using publicly available software, packages, and standard pipelines as listed in the [key resources table](#) and described in the [STAR Methods](#).
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.L., D.P., H.K., S.Y., C.K., and D.W.C.; methodology, G.L., D.P., J.K., J.H.L., Y.J.R., S.B., J.P., H.S., H.Y., P.S., S.E.L., Y.E.K., H.-s.Y., D.H.K., H.K., C.K., S.-j.Y., and D.W.C.; validation and investigation, G.L., D.P., J.K., and J.H.L.; formal analysis, G.L., D.P., J.K., J.H.L., and C.K.; writing – original draft, G.L. and D.W.C.; writing – review and editing, G.L., J.K., Y.J.R., Y.P., D.H.K., H.K., C.K., S.-j.Y., and D.W.C.; funding acquisition, C.K., S.-j.Y., and D.W.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD11b	Cell Signaling Technology	Cat#93169; RRID:AB_3731290
Anti-F4/80	Cell Signaling Technology	Cat#70076; RRID:AB_2799771
Anti-Ly6G	Cell Signaling Technology	Cat#87048; RRID:AB_2909808
Anti-CD11c	Cell Signaling Technology	Cat#97585; RRID:AB_2800282
Anti-H3K4me3	Abcam	Cat#ab8580; RRID:AB_306649
Anti-IgG	Abcam	Cat#ab37415; RRID:AB_2631996
APC/Cyanine7 anti-mouse CD11c	BioLegend	Cat#117323; RRID:AB_830646
APC anti-mouse CD64 (FcγRI)	BioLegend	Cat#139305; RRID:AB_11219205
PerCP anti-mouse Ly-6G/Ly-6C (Gr-1)	BioLegend	Cat#108425; RRID:AB_893560
Alexa Fluor 488 anti-mouse/human CD11b	BioLegend	Cat#101219; RRID:AB_493545
Anti-Ly6G	BioXcell	Cat#BE0075-1; RRID:AB_1107721
F4/80 antibody	BioLegend	Cat#123106; RRID:AB_893501
Anti-Acod1	Abcam	Cat#ab222411; RRID:AB_2868510
Anti-SDHA	Cell Signaling Technology	Cat#11998; RRID:AB_2750900
Anti-β-Actin	Santa Cruz Biotechnology	Cat#sc-47778; RRID:AB_626632
Anti-rabbit-HRP	Jackson ImmunoResearch Labs	Cat#111-035-003; RRID:AB_2313567
Anti-mouse-HRP	Jackson ImmunoResearch Labs	Cat#115-035-003; RRID:AB_10015289
Bacterial and virus strains		
<i>A. baumannii</i>	ATCC	Cat#19606
Chemicals, peptides, and recombinant proteins		
LPS	Sigma-Aldrich	Cat#L2630
RPMI-1640	Gibco	Cat#11875-093
FBS	HyClone	Cat#SH30919.03
RBC Lysis Buffer	BioLegend	Cat#420301
Penicillin-streptomycin	Corning	Cat#30-002-CI
Sodium pyruvate	Gibco	Cat#11360070
GM-CSF	PeproTech	Cat#AF-315-02
Itaconate	Sigma-Aldrich	Cat#I29204
DM-αKG	Sigma-Aldrich	Cat#349631
AS8351	Sigma-Aldrich	Cat#SML1692
Methanol	Fisher Chemical	Cat#A456
DL-norvaline	Sigma-Aldrich	Cat#N7502
Methoxyamine hydrochloride	Sigma-Aldrich	Cat#226904
Anhydrous pyridine	Sigma-Aldrich	Cat#270970
MTBSTFA(<i>N</i> - <i>tert</i> -Butyldimethylsilyl)- <i>N</i> -methyltrifluoroacetamide)	Sigma-Aldrich	Cat#394882
NP-40	Thermo Scientific	Cat#85124
BSA	Sigma-Aldrich	Cat#A-421-500
Trizol reagent	Invitrogen	Cat#15596026
Formaldehyde solution (37%)	Sigma-Aldrich	Cat#818708
Glycine	SAMCHUN	Cat#G0288
EDTA	Thermo Scientific	Cat#15575020
Triton X-100	Thermo Scientific	Cat#A16046

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Proteinase K	Invitrogen	Cat#25530049
Accutase	Sigma	Cat#A6964
Digitonin	Promega	Cat#G9441
Transposase	Illumina	Cat#FC-121-1030
Hematoxylin	Thermo Scientific	Cat#H345
Eosin	Thermo Scientific	Cat#40943
Xylene	Thermo Scientific	Cat#A11358
Paraformaldehyde	Thermo Scientific	Cat#AAJ19943K2
MojoSort buffer	BioLegend	Cat#480017
Tween 20	Sigma-Aldrich	Cat#P9416
Protease/phosphatase inhibitors	Thermo Scientific	Cat#78446
β -mercaptoethanol	Bio-Rad	Cat#1610710
Ficoll	Sigma-Aldrich	Cat#F5415

Critical commercial assays

Opal Polaris 7-Color Manual IHC Kit	AKOYA Biosciences	Cat#NEL861001KT
Neutrophil isolation kit	Miltenyi Biotec	Cat#130-097-658
Chromium Single Cell 3' Reagent Kit v3.1	10X Genomics	N/A
Quant-IT RiboGreen	Invitrogen	Cat#R11490
Illumina TruSeq Stranded Total RNA Library Prep Gold Kit	Illumina	Cat#20020599
KAPA Library Quantification kits	KAPA BIOSYSTEMS	Cat#KK4854
PCR purification kit	QIAGEN	Cat#28106
Qubit dsDNA high sensitivity assay kit	Invitrogen	Cat#Q32851
Zymo DNA Clean and Concentrator	Zymo	Cat#D4014
Nextera DNA Library Preparation Kit	Illumina	Cat#FC-121
mouse IL-6 DuoSet ELISA	R&D Systems	Cat#DY406
mouse/rat CCL5/RANTES Quantikine ELISA Kit	R&D Systems	Cat#MMR00
mouse CXCL10/IP-10/CRG-2 DuoSet ELISA	R&D Systems	Cat#DY466
QIAGEN RNeasy Puls Mini Kit	QIAGEN	Cat#74136
cDNA synthesis kit	Takara	Cat#6110B
KAPA SYBR FAST qPCR Master Mix (2X)	KAPA BIOSYSTEMS	Cat#KK4602
CellAmp Direct TB Green RT-qPCR Kit	TaKaRa	Cat#3735S
BCA assay kit	Thermo Scientific	Cat#23228, 1859078

Deposited data

GSE158055 dataset	GEO	https://cellxgene.cziscience.com/collections/0a839c4b-10d0-4d64-9272-684c49a2c8ba
mouse MsigDB (category: C3-TFT-TFT_Legacy)	N/A	http://www.gsea-msigdb.org/gsea/msigdb/
Agilent METLIN PCDL library	Agilent	N/A
ImmGenData database in the celldex (version 1.12.0)	celldex (Bioconductor)	N/A
Single cell RNA-seq, RNA-seq, ATAC-seq datasets	NCBI Bio project	BioProject:PRJNA1449024
LC-qTOF-based untargeted metabolomics dataset	Metabolomics Workbench	MetabolomicsWorkbench:ST004653, and GEO:GSE158055

Experimental models: Cell lines

MH-S	ATCC	Cat#CRL-2019
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Experimental models: Organisms/strains

WT C57BL/6J mice	KRIBB	N/A
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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Mrp8</i> -Cre C57BL/6J mice	Jackson Laboratory	021614
<i>Acod1</i> ^{fl/fl} C57BL/6J mice	Cyagen Biosciences	CKOCMP-16365-Acod1-B6J-VA
<i>Mrp8</i> -Cre <i>Acod1</i> ^{fl/fl} C57BL/6J mice	KRIBB	N/A
<i>Mrp8</i> -Cre <i>Acod1</i> ^{wt/wt} C57BL/6J mice	KRIBB	N/A
Oligonucleotides		
Primers for qPCR, see Table S1	This paper	N/A
Software and algorithms		
Monte Carlo simulation	MGH	RRID:SCR_002588
MassHunter Quantitative Analysis	Agilent	RRID:SCR_015040
MassHunter Profinder software	Agilent	RRID:SCR_017026
CellRanger (version 7.1.0)	10x Genomics	RRID:SCR_017344; https://www.10xgenomics.com/support/software/cell-ranger/latest
Seurat package (version 4.3.0.1)	10x Genomics	RRID:SCR_016341; https://satijalab.org/seurat/get_started.html
R (version 4.3.0)	R Core Team	https://www.r-project.org/
Harmony (version 1.2.0)	Harmony	RRID:SCR_022206; https://github.com/immunogenomics/harmony
SCP (version 0.4.8)	Zhang H. (GitHub: zhanghao-njmu)	https://github.com/zhanghao-njmu/SCP
SingleR (version 2.4.0)	Aran et al. ⁸⁹	RRID:SCR_023120; https://www.bioconductor.org/packages/release/bioc/html/SingleR.html
genekitr (version 1.2.5)	GitHub	https://github.com/GangLiLab/genekitr
ggplot2 (version 3.4.2)	Wickham et al. ⁹⁰	RRID:SCR_014601; https://cran.r-project.org/web/packages/ggplot2/index.html
scProportionTest (version 0.0.0.9000)	Miller et al. ⁹¹	https://github.com/rpolicaastro/scProportionTest
CellChat (version 2.1.1)	Jin et al. ⁹²	RRID:SCR_021946; https://github.com/sqjin/CellChat
fastp (version 0.23.2)	GitHub	RRID:SCR_016962; https://github.com/OpenGene/fastp
STAR aligner (version 2.7.10a)	Dobin et al. ⁹³	RRID:SCR_004463; https://github.com/alexdobin/STAR
FeatureCounts (version 2.0.1)	Liao et al. ⁹⁴	RRID:SCR_012919; https://github.com/ShiLab-Bioinformatics/subread
DESeq2 (version 1.40.0)	Love et al. ⁹⁵	RRID:SCR_015687; https://bioconductor.org/packages/release/bioc/html/DESeq2.html
msigdb (version 7.5.1)	CRAN	RRID:SCR_022870; https://cran.r-project.org/package=msigdb
nf-core ATAC-seq pipeline (version 2.0)	nf-core community	https://github.com/nf-core/atacseq
ChIPseeker (version 1.38.0)	Yu et al. ⁹⁶	RRID:SCR_021322; https://bioconductor.org/packages/ChIPseeker/
Spark (version 2.6.2)	Apache Software Foundation	RRID:SCR_006207; https://github.com/apache/spark
FlowJo (v10.10.1)	BD Biosciences	RRID:SCR_008520
GraphPad Prism	GraphPad Software	RRID:SCR_002798; http://www.graphpad.com/
Adobe Illustrator	Adobe	RRID:SCR_010279; http://www.adobe.com/products/illustrator.html
Inform software	Akoya Biosciences	https://www.quanterix.com/software-data-analysis-solutions/inform-software/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
SuperScript II reverse transcriptase	Invitrogen	Cat#18064014
Streptavidin-conjugated Nanobeads	BioLegend	Cat#480016
4–20% Mini-PROTEIN TGX Precast Gels	Bio-Rad	Cat#456-1095
Polyvinylidene difluoride membrane	Millipore	Cat#IPVH-00010

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

The Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (KRIBB) approved all animal studies. C57BL/6J mice from the KRIBB were housed in a pathogen-free animal facility under a 12 h light-dark cycle with standard rodent chow and water provided *ad libitum*. The experimental groups were all age- and sex-matched (male, 18–23 g, 8-week-old and 20-month-old). Hemizygous *Mrp8*-Cre TG mice were purchased from The Jackson Laboratory. *Acod1*-floxed mice were generated from Cyagen Biosciences Inc. by co-injecting the donor vector containing *loxP* sites flanking exon 1 of the *Acod1* gene and Cas9 mRNA into fertilized mouse eggs. To generate mice lacking *Acod1* expression and therefore itaconate production in neutrophils, hemizygous *Acod1*-floxed mice were bred to generate homozygous offspring, which were then crossed with hemizygous *Mrp8*-Cre mice to produce *Mrp8*-Cre *Acod1*^{fl/fl} mice. Homozygous *Acod1*-floxed *Mrp8*-Cre-negative littermates served as a control.

In vivo ALI model and BALF-cell preparation

The murine ALI model was generated by depositing 1 mg/kg LPS into the oropharynx. BALF samples were obtained by bronchoalveolar lavage 24 h after LPS stimulation. Briefly, a cannula was inserted into the trachea and repeated 1 mL washes of PBS were infused in and out of the airways. The BALF from each mouse was separately centrifuged and the cells were resuspended in complete RPMI containing 10% FBS.

Analysis of BALF samples from humans with inflammatory lung diseases

This study was conducted according to the principles of the Declaration of Helsinki and approved by the institutional review board of Chungnam National University Hospital (2018-01-059). All study samples were obtained following acquisition of the study participants' written informed consent. Peripheral blood was collected from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll (GE Healthcare) density-gradient centrifugation. BALF was collected from patients with infectious lung diseases. Bronchoscopy was performed using Olympus (Tokyo, Japan) flexible bronchoscopes. BALF was obtained conforming to official recommendations⁹⁷ by instillation of isotonic saline solution in total 100 mL into the wedged pulmonary segment that showed the most prominent finding in the most recent chest CT. Through gentle suction of injected solution, BALF was yielded and pooled in a collection tube. For processing of the study samples, BALF was filtered through a 100µm cell strainer to remove clumps and debris and centrifuged at 2,000 rpm for 10 min at room temperature. After separating cell-pellets, supernatant was aliquoted and stored at –80°C. Metabolite extraction from the supernatant was prepared in the same manner as for mice BALF described below.

Isolation and culture of primary murine AMs

Isolation of AMs was performed as previously described.⁹⁸ Briefly, trachea of euthanized mice was surgically exposed, and 18-gauge Intravascular catheter attached to a 1 mL syringe containing 1 mL pre-warmed PBS supplied with 2 mM EDTA and 0.5% heat-inactivated FBS was inserted. The PBS buffer was carefully infused into the lung and then slowly withdrawn into the syringe followed by put into a 5 mL tube. This infusion-withdrawal step was repeated 3 times per mouse. The collected BALF was centrifuged at 300 g for 5 min at 4°C, and the cell pellet was resuspended in 0.5 mL RBC lysis buffer and incubated for 3 min at 37°C. The RBC lysis was then stopped by adding 4.5 mL PBS, followed by centrifugation at 300g for 5 min at 4°C. The pellet was resuspended in AM culture medium (RPMI 1640 medium containing 10% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate and 10 ng/mL GM-CSF, filtered through a cell strainer. The resuspended cells were pooled and seeded onto 6-well culture plate at a density of 4×10^5 cells per well. Cultured AMs were incubated at 37°C in a humidified 5% CO₂ incubator. After 24 h, the culture medium was removed, and the cells were washed twice with PBS before being replenished with fresh AM culture medium. The remaining AMs were pretreated with 1 mM itaconate for 3 h, followed by stimulation with 10 ng/mL LPS for additional 3 h. To modulate Kdm5b activity, AMs were treated with 1 mM DM-αKG and/or 20 µM AS8351 for 3 h prior to additional 3 h of LPS stimulation, respectively.

Pulmonary neutrophil transplantation (PNT)

Bone marrow-derived neutrophils were administered directly into the lungs of 10-week-old CD45.1 male mice using a previously modified method.⁹⁹ Briefly, using anti-Ly6G antibody induced depletion of CD45.1 neutrophil during 24h. Next, mice received anesthesia by isoflurane inhalation and then challenged with LPS (1mg/kg) at three hours. Neutrophils were harvested from 7-8-week-old *Mrp8-Cre Acod^{wtl/wt}* or *Mrp8-Cre Acod1^{fl/fl}* mice femurs according to the Neutrophil Isolation Kit. Neutrophils containing 2×10^6 cells in PBS (50 μ L) were administered into neutrophil-depleted CD45.1 mice at 21 h. PNT mice were used for the analysis of cytokines and chemokines.

Bacterial infection model

A. baumannii was cultured in LB broth at 37°C as previously described.¹⁰⁰ Bacteria were harvested at an OD600 of 1.0, washed, and diluted to 5×10^7 CFU in 50 μ L PBS. Eight-week-old male *Mrp8-Cre Acod^{wtl/wt}* or *Mrp8-Cre Acod1^{fl/fl}* mice were infected via intra-tracheal instillation with the inoculum. BALF was collected 24 h post-infection and analyzed for cytokine levels.

Culture of murine alveolar macrophage cell line

The murine alveolar macrophage cell line MH-S was obtained from ATCC and maintained according to the provider's instructions. Cell line authentication was not performed. Cells were routinely tested for mycoplasma contamination and confirmed to be negative. MH-S cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin. For ChIP-qPCR analysis, MH-S cells were stimulated with LPS (10 ng/mL) for 3 h and subsequently harvested for downstream experiments.

METHOD DETAILS

Neutrophil isolation and preparation

Neutrophils were isolated from BM-derived cells and BALF using a magnetic-activated cell sorting (MACS)-based neutrophil isolation kit, according to the manufacturer's instructions. Briefly, cell pellets from both sources were resuspended in MACS buffer and labeled with anti-Ly6G microbeads for positive selection. The labeled cells were passed through MACS separation columns placed in a magnetic field to enrich neutrophils. Purified neutrophils were resuspended in RPMI 1640 medium supplemented with 10% FBS and used for subsequent experiments.

Multiplex fluorescent immunohistochemistry staining

Multiplex fluorescent immunohistochemistry staining was performed on lung tissue sections using Opal Polaris 7-Color Manual IHC Kit according to the manufacturer's instructions. Briefly, lung sections from mice 24 h after ALI challenge were sequentially stained with primary antibodies targeting CD11b, F4/80, Ly6G, CD11c. Subsequently, the sections were incubated with the corresponding Opal fluorophore-conjugated secondary antibodies. Images of stained slides were acquired using a Phenomager Fusion (AKOYA Biosciences Inc.) and analyzed using Inform software (AKOYA Biosciences Inc.) to quantify mean fluorescence expression levels and nearest neighbor distances among cell populations.

Modeling of Neutrophil-AM spatial distribution and itaconate concentrations

The simulation has been conducted in a two-dimensional domain with periodic boundary conditions. A population of 100 macrophages was distributed uniformly at random. To model the observed cellular clustering, 10 neutrophils were positioned around each macrophage. Based on the previous macrophage-neutrophil distance data, the radial distance of each neutrophil from its associated macrophage was sampled from a truncated normal distribution ($\mu = 10.6$, $\sigma = 15$, range = [0, 100]), while its angle was chosen from a uniform distribution. The steady-state concentration of chemical *C*, governed by a 2d reaction-diffusion model,

$$0 = D\Delta C - \beta C + \alpha \sum_i \delta(x - x_i),$$

has been calculated by summing the contribution from each neutrophil source. Here, $\delta(x - x_i)$ denotes the Dirac delta source at x_i (neutrophils), and the parameters $D = 1$, $\alpha = 1$ and $\beta = 0.1$ denote the diffusion coefficient, production rate and degradation rate of *C*, respectively.

To quantify the concentration of itaconate released from neutrophils, Monte Carlo simulations were conducted to model extracellular diffusion dynamics ($n = 100$). In each run, the spatial distribution of cells was generated, and the resulting chemical concentrations were calculated based on the reaction-diffusion model.

Metabolite extraction

Metabolite extraction and metabolomics were performed as previously described with slight modifications.^{101,102} Briefly, to extract the metabolites from BALF, 400 μ L MS-grade methanol containing 100 μ M DL-norvaline as an internal standard (ISTD) was added to each sample, followed by vortexing for 10 s. To extract the metabolites from BALF neutrophils or primary AMs, the cells were washed twice with ice-cold 0.9% NaCl and prepared by the addition of 350 μ L of 80% methanol(v/v) that also contained 100 μ M DL-norvaline as the ISTD. The cells were fully lysed by sonication at high intensity with a 30 s on/off cycle for 10 min at 4°C by using a BioRuptor

(Diagenode). The extracted samples were centrifuged at 12,000 rpm for 30 min at 4°C, after which 400 µL of the BALF supernatants or 300 µL of the cell-lysate samples were transferred to the 1.5 mL microtubes. The supernatants were completely dried in a vacuum centrifuge (LABCONCO) for 4 h at 4°C.

Quantification of itaconate in epithelial lining fluids

ELF volume was estimated according to the previously described methods.¹⁰³ ELF was sampled by BALF collection from mice, and blood was obtained by cardiac puncture for serum preparation. Serum was separated from whole blood by centrifugation and used for metabolite extraction. For serum metabolite extraction, 20 µL of serum was mixed with 80 µL of ice-cold methanol containing 100 µM norvaline as an internal standard, briefly vortexed, and centrifuged at 12,000 rpm for 20 min at 4°C. Following centrifugation, 80 µL of the supernatant was transferred to a new tube and dried in a vacuum centrifuge at 4°C for 4 h. Metabolites were extracted from mouse BALF using the same protocol as described for serum.

Dried serum and BALF extracts were analyzed by GC-MS. Urea and itaconate in each sample were quantified using external calibration curves generated from authentic standards. ELF itaconate concentrations were then calculated using the urea dilution method with the following equation: $V_{\text{ELF}} = V_{\text{BALF}} \times ([\text{Urea}]_{\text{BALF}} / [\text{Urea}]_{\text{serum}})$, where V_{ELF} is the epithelial lining fluid volume and V_{BALF} is the recovered BALF volume.¹⁰³

Metabolomics with GC-MS/MS and GC-MS

The dried samples were derivatized by two steps. First, 30 µL MOX solution (10 mg methoxyamine in 1 mL anhydrous pyridine) was added to each sample, vortexed for 1 min, and incubated at 37°C for 30 min. Second, 70 µL MTBSTFA (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide) was added to each sample and incubated at 70°C for 1 h. GC-MS/MS or GC-MS analysis was performed using the Agilent 7890/7000 GC triple quadrupole mass spectrometer system or Agilent 8890/5977B GC MSD, respectively. Briefly, 1 µL of each derivatized sample was injected in the splitless mode using the auto sampler Agilent 7683A or 7693A. Gas chromatography was equipped with the Agilent J&W HP-5ms capillary column. Helium served as the carrier gas with a constant column flow rate of 1.500 mL/min. The inlet temperature was 280°C and the oven temperature was initiated at 60°C and increased to 320°C at a rate of 10°C/min. Mass detection was performed in the electron ionization mode at 70 eV, the dynamic multiple reaction monitoring (dMRM) mode, or the 50–550 m/z scan mode. The software Agilent MassHunter Data Acquisition Software was used for data acquisition and the MassHunter Workstation Software for Quantitative Analysis was used for peak quantification. The data table was exported and normalized to the ISTD.

Metabolite profiling using LC-QTOF

Dried AM samples were reconstituted in 30 µL of 50% methanol(v/v), followed by transfer to glass inserts placed in glass vials for subsequent LC-QTOF analysis.

Metabolite profiling was performed using an Agilent 1290 infinity II LC system coupled to Agilent Poroshell 120 HILIC-Z (3 × 150 mm, 2.7 µm) and Agilent 6530 quadrupole time of flight mass spectrometer using Dual Agilent Jet Stream Electrospray Ionization. Injection volume of sample was 2 µL. LC separation was achieved on Agilent Poroshell 120 HILIC-Z column (3 × 150 mm, 2.7 µm) using a mobile phase A (10 mM ammonium acetate in water, pH 9.0) and mobile phase B (10 mM ammonium acetate water/acetonitrile 10:90 (v/v) pH 9.0). Flow rate was 0.300 mL/min. The LC gradient was 0 min 90% B, 3.5 min 82.5% B, 6 min 70% B, 7.5–8.5 min 65% B, 9 min 90% B, 12 min 90% B. Electrospray ionization parameters included gas temperature of 280°C, drying gas flow of 8 L/min, nebulizer pressure of 45 psi, sheath gas temperature of 350°C, sheath gas flow of 10 L/min, capillary voltage of 3500 V, fragmentor of 120 V, skimmer of 65 V, Oct 1 RF vpp of 750 V. Data acquisition was carried out using electrospray ionization in the negative mode using full-scan analysis (MS) over a mass range of m/z 60 to 1100 in 2 GHz extended dynamic range mode.

Peak area integration was carried out using MassHunter Profinder software (Agilent). Metabolite identification was based on matching the accurate mass-to-charge ratio (m/z) to the Agilent PCDL library.

Nuclear isolation and fractionation

Nuclear isolation was carried out as previously described with slight modifications.¹⁰⁴ Briefly, 3×10^6 AMs were washed twice with ice-cold PBS, and 0.5 mL ice-cold lysis buffer (250 mM sucrose, 15 mM Tris-HCl (pH 7.6), 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, and 0.1% [v/v] NP-40 adjusted to pH 7.6) was added to each dish. AMs were scraped into the buffer, cell clumps were broken up by pipetting against the dish five times. The suspension was collected into 1.5 mL microcentrifuge tubes and centrifuged at 600 × g for 5 min at 4°C. The supernatant (non-nuclear fraction) was transferred to a fresh 1.5 mL tube. The nuclear pellet was washed twice with ice-cold PBS and re-centrifuged at 600 × g for 5 min at 4°C. For downstream metabolite and protein analyses, the nuclear pellet and the non-nuclear fraction were each quenched by adding ice-cold 80% (v/v) methanol containing 100 µM DL-norvaline, followed by sonication to achieve homogenization. The resulting nuclear and non-nuclear homogenates were used for metabolite profiling or analyzed by immunoblotting to verify compartment-specific protein markers.

Single-cell RNA sequencing library

Cells were suspended in PBS containing 0.04% BSA to generate a cell concentration of 1000 cells/ μ L and then loaded into Chromium Controller (10X Genomics, CA, USA). RNA-seq libraries were prepared by using Chromium Single Cell 3' Reagent Kit v3.1 according to the manufacturer's instructions (10X Genomics, CA, USA). The recovery target was 10000 cells per sample. The libraries were sequenced on an Illumina Novaseq6000 (Illumina).

Sequencing read alignment, quality control, and cell clustering

The sequencing data was aligned to the mouse genome reference in 10x Genomics by using Cell Ranger (version 7.1.0).¹⁰⁵ The Seurat package (version 4.3.0.1)¹⁰⁶ in R (version 4.3.0) was used for data pre-processing via a standard workflow. Quality control of the gene-cell matrix was conducted by filtering out the cells whose mitochondrial-gene counts were <10% and including the cells with between 200 and 7000 features. The two scRNA-seq datasets were normalized individually by using NormalizeData function and then merged. After merging, the FindVariableFeatures, ScaleData, and RunPCA functions were used according to the standard Seurat procedure. Harmony (version 1.2.0)¹⁰⁷ served as the batch effect-removal method. After batch-effect correction, the first 30 components and resolution 0.5 were used for UMAP embedding and cell clustering. The R package used for data analysis was SCP (version 0.4.8), which can be obtained at: <https://github.com/zhanghao-njmu/SCP>.

Marker-gene identification and cluster annotation

Marker genes were extracted by using the FindAllMarkers function and the top 30 with the greatest average log₂ fold change were determined. To annotate each cell cluster, SingleR (version 2.4.0)⁸⁹ were used with the immGenData database in the cellDex (version 1.12.0). Canonical markers were also used to score each cluster according to their normalized expression of each marker. The clusters (and their defining marker genes) were Immune cells (*Cd45*), T cells (*Cd3d*), NK cells (*Klrl1*), Macrophages (*Esr1*), Neutrophils (*Retnlg*, *Cxcr2*, *S100a9*), Epithelial cells (*Krt18*), Monocytes (*F13a1*), Dendritic cells (*Siglech*). Cluster 6 was separated into the *Mrc1*^{low/medium} macrophage and *Mrc1*^{high} macrophage subclusters by using the FindSubCluster function. The *Mrc1*^{low/medium} and *Mrc1*^{high} macrophage marker genes were extracted by using the FindAllMarkers function. Gene-set over-representation analysis (GSEA) was conducted by using genekitr (version 1.2.5)¹⁰⁸ and visualized with ggplot2 (version 3.4.2).⁹⁰ Only pathways that had an FDR-adjusted *p* value of <0.05 were considered. Cell proportion was plotted by using CellstatPlot function in SCP (version 0.4.8). To compare the cell populations in terms of cell-proportional differences, scProportionTest (version 0.0.0.9000)⁹¹ was used. Cell-cell interaction analysis was performed with CellChat (version 2.1.1).¹⁰⁹

Gene-set over-representation analysis

GSEA was conducted by using genekitr (version 1.2.5)¹⁰⁸ and visualized with ggplot2 (version 3.4.2).⁹⁰ Transcription-factor target analysis was performed by using genekitr (version 1.2.5) with mouse MsigDB¹¹⁰ (category: C3-TFT-TFT_Legacy). Only pathways that had an FDR-adjusted *p* value of <0.05 were considered.

Cell proportion, inflammatory-gene scoring, and cell-cell interaction analysis

Cell proportion was plotted by using CellstatPlot function in SCP (version 0.4.8). To compare the cell populations in terms of cell-proportional differences, scProportionTest (version 0.0.0.9000)⁹¹ was used. Inflammatory-gene scoring was conducted by using the AddModuleScore function with the following inflammation-related genes: *Mmp12*, *Cxcl9*, *Cxcl10*, *Cxcl2*, *Cxcl1*, *Ccl7*, *Ccl2*, *Nos2*, and *Ccl12*. Cell-cell interaction analysis was performed with CellChat (version 2.1.1).¹⁰⁹

Bulk RNA sequencing

AMs were pretreated with 1 mM itaconate for 3 h before being stimulated with 10 ng/mL LPS for 3 h followed by total RNA isolation using Trizol reagent. 500 ng of RNA was used for RNA sequencing. The total RNA concentration was calculated by using Quant-IT RiboGreen. To assess the integrity of the total RNA, samples were run on the TapeStation RNA screentape. Only high-quality RNA samples whose RIN was >7.0 were used for RNA library construction. Separate libraries were prepared from 0.5 μ g total RNA of each sample by using Illumina TruSeq Stranded Total RNA Library Prep Gold Kit. Thus, after removing the rRNA, the remaining mRNA was fragmented by using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first-strand cDNA by using SuperScript II reverse transcriptase and random primers. Second-strand cDNA synthesis was then conducted with DNA Polymerase I, RNase H, and dUTP. The resulting cDNA fragments were subjected to an end-repair process, the addition of a single 'A' base, and then ligation of the adapters. The products were purified and enriched with PCR to create the final cDNA library. The libraries were quantified by using KAPA Library Quantification kits for Illumina Sequencing platforms according to the qPCR Quantification Protocol Guide and their quality was determined with the TapeStation D1000 ScreenTape. The indexed libraries were then submitted to an Illumina NovaSeq (Illumina, Inc., San Diego, CA, USA) and the paired-end (2 $\times$ 100 bp) sequencing was performed by Macrogen Inc. Raw sequencing reads were subjected to quality control and adapter trimming using fastp (version 0.23.2). High-quality reads were then aligned to the mouse reference genome GRCm38 using the STAR aligner (version 2.7.10a). The resulting BAM files were used to quantify gene expression levels using the FeatureCounts (version 2.0.1), which generated a gene-level count matrix. Differentially gene expression analysis was performed using the DESeq2 (version 1.40.0) in R. Genes with an adjusted *p* value <0.05 and |log₂ fold change| $\geq$ 0.4 were considered significantly differentially expressed. To investigate the biological pathways

associated with the DEGs, GSEA was conducted. For this, gene sets were obtained from the MSigDB (Molecular Signatures Database), including Hallmark gene sets and C1–C8 collections using the msigdb (version 7.5.1). GSEA was performed using the ranked gene list based on the adjusted *p* value and log₂ fold change value.

ChIP-qPCR

To obtain cross-linked chromatin, 1×10^6 AMs were seeded onto 60-mm dishes, pretreated with 1 mM itaconate for 3 h, followed by 10 ng/mL LPS stimulation for additional 3 h. For assessment of Kdm5b occupancy at promoter regions, 1×10^7 MH-S were stimulated with LPS 10 ng/mL for 3 hr, and subsequently crosslinked with DSG 2 mM for 20 min. The AMs and MH-S cells were then incubated on a shaker for 10 min with 1% formaldehyde at room temperature, after which 0.125 M glycine was added and the cells incubated for another 5 min. After two washes with ice-cold PBS, the cells were incubated with 300 μ L lysis buffer (50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA and 1% Triton X-100, 1x PI) for 10 min on ice, followed by nuclei collection. The chromatin was sheared in a water-bath sonicator Bioruptor (Diagenode) until the fragments between 500 and 1000 bp were observed on electrophoretic analysis. The chromatin was then centrifuged at 8000 g for 3 min, after which the supernatant was collected and subjected to immunoprecipitation on a back-to-back rotator O/N at 4°C with the specific antibodies listed in the supplementary table. The protein-DNA complexes were eluted and the proteins were digested with 0.04 mg proteinase K for 1 h at 60°C. The extracts were centrifuged at 2000 g for 1 min and the DNA was recovered using a PCR purification kit. The DNA concentration was measured with Qubit dsDNA high sensitivity assay kit. The precipitated DNA was analyzed by RT-qPCR. The primers used for amplification of promoters of chemokine are listed in the [Table S1](#).

ATAC sequencing

ATAC sampling was performed as described previously.¹¹¹ AMs were dissociated with Accutase and 75,000 viable cells were centrifuged at 500 g for 5 min at 4°C. The cell membranes were lysed by incubation on ice in cold ATAC-seq lysis buffer (0.1% NP40, 0.01% Digitonin, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl₂) for 8 min. The nuclei were pelleted by centrifugation at 500g for 10 min at 4°C and resuspended in transposition mixture (25 μ L 2 x TD buffer, 2.5 μ L transposase [100 nM final], 16.5 μ L PBS, 0.5 μ L 1% Digitonin, 0.5 μ L 10% Tween 20, 5 μ L water). The transposition reactions were performed for 30 min at 37°C in a thermomixer with 1000 rpm mixing. Directly after transposition, the samples were purified by using Zymo DNA Clean and Concentrator. The libraries were amplified by using Nextera DNA Library Preparation Kit and quantified by using KAPA Library Quantification kit. ATAC-seq data analysis was performed by using the nf-core ATAC-seq pipeline (version 2.0).¹¹² This pipeline incorporates key steps such as read trimming, alignment, peak calling and peak annotation. DARs were identified using DESeq2 (version 1.40.0). Distribution of features and TF binding sites relative to TSSs was visualized using ChIPseeker (version 1.38.0). Visualization of peak tracks was achieved by using Spark (version 2.6.2).¹¹³

H&E staining

Lung tissues were fixed in formalin and embedded in paraffin prior to sectioning. The sections were then counterstained with H&E (hematoxylin and eosin), deparaffinized for 5 min in xylene, rehydrated by immersion in a graded ethanol series, and fixed for 15 min in 4% paraformaldehyde in PBS. Tissue sections were permeabilized by incubation for 20 min at room temperature in 100 μ L proteinase K solution (20 g/mL). All steps were performed according to the manufacturer's instructions.

Flow cytometry

BALF cells from individual mice were incubated with APC/Cyanine7 anti-CD11c and APC anti-mouse CD64 to identify the alveolar macrophage. PerCp anti-mouse Gr1 and Alexa Fluor 488 anti-mouse/human CD11b Antibodies were used for neutrophil staining. All antibodies were purchased from BioLegend. The cells were immediately analyzed by using a NovoCyte flow cytometer (Agilent) and the FlowJo software.

Enzyme-linked immunosorbent assay (ELISA) for BALF fluid

Levels of cytokines and chemokines in BALF were quantified using ELISA. Specifically, mouse IL-6 DuoSet ELISA, mouse/rat CCL5/RANTES Quantikine ELISA Kit, and mouse CXCL10/IP-10/CRG-2 DuoSet ELISA were according to the manufacturer's instructions. Optical density (OD) values were measured at 405 nm using a microplate reader. Cytokine and chemokine concentrations were calculated by interpolating OD values from corresponding standard curves.

RNA extraction and RT-qPCR

Total RNA was extracted using the QIAGEN RNeasy Puls Mini Kit and reverse-transcribed using the cDNA synthesis kit. For quantitative PCR analysis, cDNA was mixed with the KAPA SYBR FAST qPCR Master Mix (2X) kit and the primer pairs listed in Supplementary Table. The relative mRNA expression of each gene was determined by quantitative PCR using QuantStudio 1 Real-Time PCR instrument and normalized to β -Actin (for AM). Levels of *Acod1* mRNA in neutrophils and AMs isolated from BALF of mice post-LPS challenge were quantified using the CellAmp Direct TB Green RT-qPCR Kit, according to the manufacturer's instructions. Briefly, AMs were isolated from BALF by incubation with Biotin anti-mouse F4/80 antibody, followed by Streptavidin-conjugated Nanobeads diluted in MojoSort buffer. Macrophages were then magnetically separated and harvested using a magnetic separator.

Neutrophils were subsequently isolated from the macrophage-depleted fraction using the MojoSort Mouse Neutrophil Isolation Kit according to the manufacturer's protocol. The cells were centrifuged at 100 x g for 10 min, and then the pellets were lysed using the CellAmp Direct TB Green RT-qPCR Kit, containing buffer.

Western-blot analysis

Protein samples from cultured cells were prepared using lysis buffer (20 mM Tris, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, protease/phosphatase inhibitors). The protein concentration was measured by using the BCA assay kit. Lysates were treated with 200 mM β -mercaptoethanol and incubated at 90°C for 5 min. Protein samples were separated by 4–20% Mini-PROTEIN TGX Precast Gels and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 4% skim milk in TBST buffer (Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature and then incubated with a primary antibodies specific for ACOD1 or SDHA, Actin followed by incubation with anti-rabbit- or anti-mouse-HRP. The Immunoblots were imaged using a ChemiDoc XRS+ System (BioRad).

Analysis of a scRNA-seq database of COVID-19-patient samples

The GSE158055 dataset (<https://cellxgene.cziscience.com/collections/0a839c4b-10d0-4d64-9272-684c49a2c8ba>) displays the scRNA-seq profiles of 35 lung samples from COVID-19 patients.⁵¹ The dataset was used to analyze the *ACOD1* expression in COVID-19 patients. The donor IDs of the metadata were sorted according to whether the neutrophils had *ACOD1* expression >0 (which indicates a positive donor) or <0 (which indicates a negative donor). After subsetting macrophages from the whole dataset, AM clusters were identified based on the expression of known AM marker genes, such as *FABP4*, *MARCO* and *MRC1*.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data is presented as the mean $\pm$ SEM, as indicated. Unpaired Student's *t* test (two-tailed or one-tailed) was used to test the differences between two groups, based on the assessment of variance of the data. One-way or two-way ANOVA with multiple comparisons test was used to test the differences between three or more groups. Details of the *n* number are indicated in the appropriate figure legend. *p* values of less than 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism software (version 9) and R (v 4.2.2).