

A Two-Cytokine Module Reconstructs *Acinetobacter baumannii*–Driven Immunopathology and Reveals IRF1 as a Targetable Node

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Abstract

Severe *Acinetobacter baumannii* infection is accompanied by widespread cytokine release, but the specific cytokine combinations that directly drive host injury remain poorly defined. We combined lung transcriptomic analysis and serum cytokine profiling with pairwise cytokine screening to identify pathogenic cytokine interactions. Among the combinations tested, IL-1 α or IL-1 β together with IFN- γ induced marked macrophage death, whereas each cytokine alone had little effect. In mice, administration of either IL-1–IFN- γ pair reproduced several features observed during *A. baumannii* infection, including rapid mortality, gastrointestinal and hepatic injury, and tissue-specific changes in immune-cell populations. The combined cytokines simultaneously activated pyroptotic, apoptotic, and necroptotic pathways and promoted colocalization of RIPK3, ASC, and caspase-8, consistent with coordinated inflammatory cell death. Transcriptomic comparison further showed that cytokine-treated and infected mice shared NF- κ B- and STAT1-associated inflammatory programs, with IRF1 emerging as a common regulatory node. Neutralizing IL-1 α/β and IFN- γ after challenge improved survival and reduced bacterial burdens. Inhibition of IRF1 also limited macrophage death, tissue injury, and mortality in both cytokine-challenge and infection models, including infections caused by clinical isolates. Together, these findings define IL-1–IFN- γ cooperation as a discrete pathogenic circuit in *A. baumannii* infection and support IRF1 as a potential target for host-directed intervention.

Introduction

Antimicrobial resistance is a major global health threat and was directly responsible for more than 1.2 million deaths in 2019¹. A major contributor to this crisis is not only unchecked bacterial growth, but also the ability of certain pathogens to hijack a dysregulated host immune response¹. *Acinetobacter baumannii* (*Ab*) is a WHO critical-priority Gram-negative pathogen that frequently causes hospital-acquired sepsis, with mortality driven not only by antimicrobial resistance but also by dysregulated host inflammation. Despite appropriate antibiotic therapy, mortality rates often exceed 40%, as the subsequent cytokine storm triggers rapid multiorgan failure^{1,2}.

Sepsis is fundamentally characterized by a dysregulated host response, marked by the concurrent occurrence of hyperinflammation and immunosuppression³. In sepsis, IL-1, IFN- γ , TNF, and IL-6 do not act in isolation; they converge on NF- κ B, JAK–STAT, and stress-kinase pathways to sustain inflammatory myeloid-cell activation^{4,5}. Excessive inflammatory signaling can lower the threshold for programmed inflammatory cell death. Under some inflammatory conditions, pyroptotic, apoptotic, and necroptotic pathways can become coordinately engaged through PANoptosome-associated signaling complexes, a process termed PANoptosis^{6,7}. While this coordinated cell death pathway aids in eliminating infected cells during typical infections, it becomes maladaptive during overwhelming inflammation: it releases damage-associated molecular patterns (DAMPs) that propagate tissue injury^{8,9}. Because inflammatory cell death can amplify tissue injury, upstream regulators of these pathways may represent potential host-directed intervention points. Interrupting upstream signal integration could break the vicious cycle of

hyperinflammation without inducing the profound immunosuppression associated with many existing therapies^{2,10}.

How cytokine combinations drive inflammatory cell death in sepsis remains poorly defined. Most mechanistic studies have focused on individual mediators or on TNF–IFN- γ synergy, leaving other cytokine pairs largely unexplored^{4,11}. This gap is especially important in *Ab* sepsis, where the inflammatory milieu is complex but the cytokine combinations that are sufficient to kill myeloid cells remain unclear. PANoptosis, which integrates pyroptotic, apoptotic, and necroptotic machinery into a coordinated inflammatory cell death program, offers a mechanistic framework for studying this process¹². However, whether defined cytokine inputs in *Ab* infection induce bona fide PANoptosis, and which upstream regulators couple these signals to cell death execution, remains unknown. Defining this pathway could identify host-directed strategies to limit tissue-damaging inflammation during severe infection.^{13,14}.

Results

Systematic cytokine-pair screening identifies cytotoxic IL-1–IFN- γ combinations

To identify cytokine interactions associated with *A. baumannii* sepsis, we reanalyzed lung RNA-seq data from infected mice and quantified circulating cytokines using a multiplex assay¹⁵. *Ab* ATCC 17978 was used as the reference strain for mechanistic discovery. To assess whether key host-directed intervention findings were applicable beyond this laboratory strain, two clinical *Ab* isolates, *Ab191* and *Ab2*^{16,17}, were further included for validation experiments (**Figure S1**). Transcriptional profiling revealed a marked upregulation of 13 inflammation-associated cytokine genes in infected mice compared to uninfected controls (**Figure 1A**). Among these, 6 genes, *Il1b*, *Il6*, *Il10*, *Tnf*, *Il1a*, and *Ifng*, exhibited the most significant differential expression (**Figure 1B**), suggesting that both proinflammatory mediators and the anti-inflammatory cytokine IL-10 characterize the acute response to *Ab*. We next quantified circulating cytokine concentrations using a multiplex Luminex assay. Consistent with the mRNA data, we observed elevated levels of IL-1 α , IL-1 β , IL-6, and IFN- γ in the serum of infected mice relative to controls (**Figure 1C**), alongside other mediators, indicating a broad systemic inflammatory response.

Given the established association between cytokine storm and tissue injury, we next sought to determine whether these elevated cytokines directly trigger cell death in myeloid cells, which are key effectors in sepsis pathogenesis¹⁸. We treated THP-1 cells and immortalized bone marrow-derived macrophages (iBMDMs) with each of the 13 cytokines individually. However, none of the individual cytokines induced detectable cytotoxicity (**Figure 1D**), suggesting that single-cytokine exposure was insufficient to trigger cell death. In contrast, a cocktail containing all 13 cytokines (All) triggered profound cell death (**Figure 1D, Figure S2**). To dissect the specific cytokine interactions responsible, we systematically screened all possible pairwise combinations. Only three pairs, IL-1 α +IFN- γ , IL-1 β +IFN- γ , and IL-6+IFN- γ , were sufficient to recapitulate the cytotoxicity observed with the full cocktail (**Figure 1E, 1F**). This synergistic effect was further visualized and quantified via propidium iodide (PI) staining in THP-1 cells (**Figure 1G**) and CCK-8

assays in iBMDMs, identifying these combinations as the dominant cytotoxic pairs under the conditions tested.

Combined IL-1 and IFN- γ treatment induces systemic inflammation and tissue injury in mice

To determine the *in vivo* pathological relevance of the synergistic cytokine pairs, we administered combinations of IL-1 α plus IFN- γ , IL-1 β plus IFN- γ , or IL-6 plus IFN- γ to mice by intraperitoneal injection. Co-administration of IL-1 α +IFN- γ resulted in 100% mortality within 18 h, whereas IL-1 β +IFN- γ and IL-6+IFN- γ caused only moderate mortality over 24 h (**Figure 2A**). Further investigation into the pathological consequences of IL-1-IFN- γ synergy revealed severe gastrointestinal injury in mice, showing diffuse erythema, marked luminal distension, and gas accumulation (**Figure 2B**). Histopathological analysis demonstrated profound tissue disruption, including moderate autolysis, villus atrophy, and extensive epithelial denudation exposing the lamina propria (**Figure 2C**). To further assess the extent of systemic injury, we measured tissue damage biomarkers. Mice administrated IL-1-IFN- γ combinations exhibited marked elevations in lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (**Figure 2D-F**), suggesting that IL-1 and IFN- γ act synergistically to drive systemic tissue injury and multiorgan dysfunction, which are hallmarks of cytokine storm syndromes^{10,19}.

Previous work showed reduced lymphocyte abundance and increased myeloid-cell infiltration in the lungs during *A. baumannii* infection¹⁵. We therefore compared immune-cell composition in the lungs and spleens of cytokine-treated mice. For the lymphocytes compartment, IL-1+IFN- γ treatment resulted in different alterations in lungs and spleens, with T cells, B cells, and NK cells increased in lungs (**Figure 2G-I, Figure S3A-C**) but decreased in spleen (**Figure 2J-L**), highlighting the organ-specific nature of immune remodeling during cytokine treatment. Flow cytometric analysis also revealed an increased frequencies of neutrophils in IL-1+IFN- γ treated lungs and spleens when compared to controls (**Figure 2M-P**). Although we observed the increased expression of CD86 mean fluorescence intensity (MFI) on the surface of macrophages, the total number of macrophages in lungs and spleens are declined (**Figure 2Q-T, Figure S3D-F**), suggesting either cell death or impaired recruitment under excessive cytokine signaling conditions.

IL-1 α/β and IFN- γ synergy induces PANoptosis

Macrophages play a pivotal role in maintaining the inflammatory microenvironment during *Ab* infection¹⁵, and cytokine storm-driven organ injury is increasingly recognized to result from inflammatory cell death¹⁵. We next asked whether combined cytokine treatment engages inflammatory cell-death pathways in macrophages. Lung transcriptomic analysis showed increased expression of genes associated with pyroptotic (*Casp1*, *Gsdmd*), apoptotic (*Casp7*, *Casp8*), and necroptotic (*Ripk1*, *Mkl1*) pathways following combined cytokine treatment. (**Figure 3A**). Given the crosstalk among these pathways, we propose that *Ab* triggers PANoptosis—a synergistic cell death process regulated by molecules such as caspase-3, caspase-8, and MLKL. Then we treated immortalized bone marrow-derived macrophages (iBMDMs) with PBS, cytokine alone, or with the combinations of IL-1 α +IFN- γ or IL-

1 β +IFN- γ for 48 h. Our results showed that only the combinatorial treatments induced the cleavage of Gasdermin D (GSDMD) and Gasdermin E (GSDME) (**Figure 3C**), suggesting that IL-1-IFN- γ synergy effectively activates the inflammasome pathway and leads to pyroptosis. Additionally, apoptosis was evidenced by cleavage of caspase-3, and -7 (**Figure 3D**), while phosphorylated MLKL indicated necroptosis (**Figure 3E**).

RNA-seq analysis revealed that ZBP1, RIPK3, and NLRP12, key molecules implicated in PANoptosome signaling, were significantly upregulated in the IL-1+IFN- γ -treated group compared with controls, suggesting that IL-1+IFN- γ primes the molecular machinery for PANoptosis (**Figure 3B**). Transcriptomic profiling of lung tissues from IL-1 β +IFN- γ -treated mice showed enrichment of inflammatory and programmed cell death pathways, including cytokine–cytokine receptor interaction, NOD-like receptor signaling, and TNF signaling (**Figure S4A**), supporting the systemic activation of innate immune responses. To determine whether this transcriptional activation was accompanied by assembly of the PANoptotic signaling complex, we performed confocal microscopy and observed colocalization of RIPK3, ASC, and caspase-8 (**Figure 3F, Figure S4B–E**). These results support the formation of a PANoptosome-like complex and are consistent with engagement of a PANoptotic cell-death program.

Neutralization of IL-1 α / β and IFN- γ synergy confers complete protection against cytokine storm-induced lethality and attenuates sepsis mortality

We next tested whether blockade of the cytokine combination could improve survival. Treatment with neutralizing antibodies against IL-1 α / β and IFN- γ prevented death completely in the cytokine-injection model (**Figure 4A, B**). We next tested whether post-challenge cytokine neutralization could improve survival during *A. baumannii* infection. Mice infected with ATCC 17978 received the same neutralizing antibodies 3 h after bacterial challenge. IL-1 α plus IFN- γ blockade reduced mortality from 100% to 20%, whereas IL-1 β plus IFN- γ blockade reduced mortality to 40% (**Figure 4C–E**). Cytokine-neutralized mice exhibited significantly lower bacterial burden in lungs, spleen, liver, and kidneys compared with control IgG-treated mice (**Figure 4F–I**). These findings show that IL-1–IFN- γ cooperation is sufficient to induce severe inflammatory pathology and contributes substantially to mortality during *A. baumannii* infection.

IRF1 inhibition limits cytokine- and infection-induced inflammatory cell death

To identify regulators shared by cytokine challenge and bacterial infection, we compared lung transcriptomes from mice treated with IL-1 α plus IFN- γ or IL-1 β plus IFN- γ with those from mice infected with *A. baumannii* ATCC17978. The three conditions showed enrichment of overlapping inflammatory pathways, including cytokine–cytokine receptor interaction, NOD-like receptor, TNF, Toll-like receptor, NF- κ B, and JAK–STAT signaling (**Figure 5A and Figure S5**).

A shared transcriptional signature included NF- κ B-associated genes (*Nfkb1a*, *Tnf*, *Il6*, and *Ccl2*), STAT1/interferon-responsive genes (*Stat1*, *Irf1*, *Cxcl9*, *Cxcl10*, *Isg15*, and *Gbp2*), and inflammatory cell-death-associated genes (*Zbp1*, *Ripk3*, and *Mkl1*) (**Figure 5B**). Among the candidate regulators, IRF1 was of particular interest because it can be induced downstream of both NF- κ B and STAT1 associated

signaling. These observations identified IRF1 as a candidate regulator shared by cytokine- and infection-induced inflammatory programs.

IL-1 receptor signaling engages MyD88-dependent NF- κ B and MAPK pathways, whereas IFN- γ activates JAK-STAT1 signaling and induces interferon-responsive genes. IFN- γ activates the JAK-STAT pathway, leading to STAT1 phosphorylation and induction of interferon-stimulated genes (ISGs)²⁰. IRF1 can be induced downstream of STAT1- and NF- κ B-associated signaling and has been implicated in inflammatory and cell-death programs^{13,14}. Because IRF1 is responsive to both NF- κ B and STAT1-associated signaling, we examined whether it contributes functionally to the inflammatory cell-death program identified here. In contrast, PKR, while an ISG primarily activated by IFN- γ -driven STAT1 signaling and further potentiated by IL-1-mediated stress signals, functions predominantly as a downstream effector kinase that enforces translational arrest and selective amplification of death pathways in IFN- γ -dominant contexts²¹.

RT-qPCR analysis of lung tissues from mice infected with Ab or challenged with IL-1 α/β + IFN- γ revealed significantly elevated transcription of IRF1 and PKR compared to uninfected controls (Figure 5C, Figure S6A). Consistently, pretreatment of immortalized bone marrow-derived macrophages (iBMDMs) with IRF1-IN-2 markedly attenuated IL-1 α/β +IFN- γ induced cytotoxicity relative to controls. Cell death rates were also substantially reduced during Ab infection (Figure 5D). These pharmacological results suggest that IRF1 contributes to inflammatory cell death under both sterile cytokine challenge and live bacterial infection, whereas the contribution of PKR is more context dependent (Figure S6B).

We next tested whether pharmacological inhibition of IRF1 could improve outcomes *in vivo*. Administration of IRF1-IN-2 3 h after cytokine challenge improved survival following IL-1 α/β plus IFN- γ administration (**Figure 5E**). Post-challenge IRF1-IN-2 treatment also delayed mortality and improved survival after *A. baumannii* ATCC 17978 infection. By contrast, PKR inhibition did not improve survival during bacterial infection (**Figure S6F**). Mechanistically, IRF1 inhibition suppressed activation of inflammatory death execution pathways, as shown by reduced cleavage of caspase-3, caspase-7, and caspase-9, together with diminished GSDMD processing in macrophages challenged with either ATCC 17978 or IL-1 α/β +IFN- γ (**Figure 5F**). These findings are consistent with IRF1 acting upstream of the apoptotic and pyroptotic arms of the integrated inflammatory cell death program.

Consistent with the survival benefit, IRF1-IN-2 markedly attenuated lung pathology in both infection and cytokine-driven models. Vehicle-treated mice showed severe inflammatory infiltration, alveolar destruction, and tissue consolidation, whereas IRF1-IN-2-treated mice displayed improved preservation of alveolar architecture and reduced inflammatory damage (**Figure 5G**). Systemic tissue injury was also reduced, as reflected by lower serum LDH, ALT, and AST levels in IRF1-IN-2-treated groups compared with vehicle-treated controls (**Figure 5H–J**). To evaluate whether IRF1 inhibition affected bacterial clearance, Ab-infected mice were treated with IRF1-IN-2. The treatment significantly reduced bacterial load in lungs, spleen, liver, and kidneys compared with vehicle controls (**Figure S6G–J**). IRF1-IN-2 treatment was also associated with reduced bacterial burdens in the lungs, spleen, liver, and kidneys.

The mechanism underlying this reduction remains to be determined. Paralleling *in vitro* observations, PKR inhibition exerted effects solely in the cytokine synergy model, with no discernible impact during bacterial infection (**Figure S6B-F**). Consistent with these findings, IRF1 inhibition also restored macrophage viability following infection with the clinical isolates *Ab191* and *Ab2*, and treatment with either cytokine-neutralizing antibodies or IRF1-IN-2 improved survival in mice challenged with these clinical isolates (**Figure S1**). Together, these results identify IRF1 as a shared upstream node that couples IL-1/IFN- γ synergy and *Ab* infection to macrophage inflammatory death, lung injury, and lethal systemic inflammation. Targeting IRF1 may therefore provide a host-directed strategy to restrain pathogenic cytokine amplification during severe *Ab* sepsis.

Together, these pharmacological findings support IRF1 as a candidate regulator shared by cytokine- and infection-induced inflammatory cell death and warrant further evaluation of IRF1-directed host intervention.

Discussion

In this study, we identify IL-1 α/β and IFN- γ cooperation as an important contributor to inflammatory macrophage death during *A. baumannii* infection and identify IRF1-associated signaling as a candidate regulatory mechanism. These findings shift the focus from pathogen burden alone to a host-derived cytokine circuit that connects dysregulated innate immunity with multiorgan failure and identifies IRF1 as a potential therapeutic target in antibiotic-refractory infection.

A notable feature of our data is the strong dependence of macrophage cytotoxicity on combined cytokine exposure. Even at high concentrations, individual cytokines such as IL-1 β or IFN- γ were insufficient to trigger substantial macrophage death, whereas their combination rapidly induced a PANoptotic phenotype. This result argues against a purely dose-dependent view of inflammatory injury and instead supports a threshold model in which distinct signals perform nonredundant functions. One plausible interpretation, consistent with prior work on inflammatory cell death pathways, is that IFN- γ provides a transcriptional priming signal through JAK-STAT signaling, increasing the availability of inflammasome- and PANoptosis-related components, whereas IL-1 supplies a complementary activation signal that amplifies execution through NF- κ B-associated inflammatory programs^{7,12}. Within this framework, the simultaneous activation of GSDMD/E, p-MLKL, caspase-3/7/8, and PANoptosome assembly is not simply parallel pathway engagement; rather, it suggests that pyroptotic, necroptotic, and apoptotic machinery becomes functionally integrated once this dual-signal threshold is crossed. Caspase-8 is likely part of this integration step, given its established role in linking apoptosis and necroptosis.

Importantly, this dual-signal requirement was further validated *in vivo*: administration of neutralizing antibodies against IL-1 α/β or IFN- γ significantly attenuated macrophage PANoptosis and organ injury, both in live *Ab* infection and in sterile cytokine co-injection models. These interventions support a substantial contribution of IL-1-IFN- γ cooperation to tissue injury and systemic inflammation. Building

on this concept, targeting IRF1 provides an upstream approach that integrates inputs from both IL-1 and IFN- γ signaling, potentially offering a broader and more robust protective effect, even in the presence of multiple redundant inflammatory triggers^{8,14}.

The *in vivo* data further underscore that cell death potential observed *in vitro* does not translate uniformly into organismal lethality. Although IL-6 plus IFN- γ was sufficient to drive macrophage cytotoxicity in culture, it caused limited mortality and little gastrointestinal injury in mice. This divergence is informative. In tissues, IL-6 is not only a mediator of systemic inflammation but also an important regulator of STAT3-dependent epithelial repair and barrier maintenance¹⁰. By contrast, IL-1–IFN- γ treatment was associated with more severe gastrointestinal injury, raising the possibility that barrier disruption contributes to systemic pathology^{22,23}. Seen in this light, the higher lethality associated with IL-1 family cytokines probably reflects not only their direct inflammatory capacity but also their ability to destabilize tissue integrity and thereby sustain a feedforward loop of damage. The organ-level transcriptomic patterns are also consistent with this interpretation: the coexistence of inflammatory activation in the lung and lymphoid collapse in the spleen mirrors the clinical paradox recognized in sepsis, where hyperinflammation and immunosuppression develop in parallel rather than sequentially^{3,24}. The increase in IL-10 expression fits with a compensatory anti-inflammatory response, although whether this directly drives activation-induced lymphocyte loss will require more focused validation^{4,18}.

From a therapeutic perspective, our results indicate that the level at which this network is interrupted matters. Both PKR and IRF1 were activated across models, yet their pharmacological inhibition yielded very different outcomes. PKR inhibition protected mice in the sterile cytokine-shock setting but lost efficacy during live *Ab* infection, one possibility is that live bacterial infection activates additional pathways that are not engaged by sterile cytokine challenge^{25,26}. IRF1 behaved differently. Because it integrates inputs from IFN- γ -associated STAT1 signaling and IL-1/NF- κ B-linked inflammatory transcription, IRF1 may influence a broader transcriptional program than PKR under the conditions examined here. This likely explains why pharmacological inhibition of IRF1 remained protective even in the presence of ongoing infection^{13,27}. The implication is not that IRF1 is the sole regulator of sepsis lethality, but that it represents a mechanistically attractive node where convergent cytokine signals can be restrained before they are translated into a broad pro-death transcriptional program. In the context of antimicrobial resistance, this is especially relevant, as host-directed interventions could complement rather than replace antibacterial therapy.

These findings also refine current understanding of PANoptosis in severe infection. Much of the recent literature has emphasized PANoptosis as a common endpoint of inflammatory syndromes¹⁰, but the upstream cues that organize this response appear to vary across pathogens and tissue contexts. Our data place IL-1 α/β and IFN- γ upstream of this process in *Ab* sepsis and suggest that cytokine composition, rather than inflammatory magnitude alone, influences whether cell death remains localized or becomes systemically destructive. This point has conceptual value because it helps explain why

broad cytokine elevation does not always predict the same pathological outcome. It also provides a more precise framework for intervention: rather than indiscriminately suppressing inflammation, it may be more effective to target the specific cytokine interactions and transcriptional hubs that organize lethal immune collapse.

Several limitations should be considered when interpreting these results. First, the mechanistic role of IRF1 was supported primarily by a small-molecule inhibitor rather than a genetic loss-of-function model. Although this approach has translational advantages and better reflects the timing of clinical intervention, off-target effects cannot be fully excluded. Second, although the mechanistic framework was primarily established using the reference strain ATCC 17978, key intervention findings were further validated using two clinical *Ab* isolates, *Ab191* and *Ab2*. These data support the broader relevance of the IL-1–IFN- γ –IRF1 axis beyond a single laboratory strain. Nevertheless, additional multidrug-resistant and geographically diverse clinical isolates should be examined in future studies to define potential strain-dependent differences in inflammatory kinetics and therapeutic responsiveness. More virulent or carbapenem-resistant isolates may engage this pathway with different kinetics or intensity²⁸. Third, our experiments focused mainly on macrophage death, whereas endothelial cells, epithelial cells, and other myeloid populations may also contribute substantially to barrier failure and organ dysfunction during sepsis²⁹. Finally, although IRF1 inhibition improved survival in our models, the balance between limiting immunopathology and preserving antimicrobial defense remains a key question for therapeutic development. These considerations define the boundaries of the present study, but they do not diminish the central conclusion that an IL-1/IFN- γ –IRF1 circuit is a major determinant of pathogenic host injury in this setting.

In summary, our study identifies cytokine synergy between IL-1 α/β and IFN- γ as a principal trigger of macrophage PANoptosis and positions IRF1 as an upstream regulator linking this response to fatal sepsis progression. Further work should test this axis across diverse clinical isolates, clarify its cell type-specific effects in target organs, and determine how IRF1-directed intervention can be combined with standard antimicrobial therapy to improve outcomes in drug-resistant *Ab* infection^{13,30}.

Materials and Methods

Bacterial strains and culture conditions

Ab strain ATCC 17978, obtained from the American Type Culture Collection (VA, USA), was routinely cultured in LB broth at 37°C with shaking at 250 rpm. Two clinical *Ab* isolates, *Ab191* and *Ab2*, were included for validation experiments, which were used as the references.

Mice

Age- and sex-matched C57BL/6 mice (6-8 weeks old) were used for experiments. Mice were provided and bred in-house at the Laboratory Animal Research Unit of the City University of Hong Kong under controlled conditions (21°C, 12:12 h light/dark cycle) with ad libitum access to food and water. The

Laboratory Animal Research Unit of the Hong Kong Polytechnic University approved all animal procedures.

Cell Culture

Immortalized Bone marrow-derived macrophages (iBMDMs) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 µg/mL penicillin-streptomycin at 37 °C in a humidified incubator of 5% CO₂ and sub-cultured every 2-3 days by using a cell scraper.

Mouse sepsis infection model

Mice were randomly assigned to several experimental groups (five animals per group) and subsequently infected with 7.5×10^7 CFU of *Ab* test strain via intraperitoneal injection. To assess survival, mortality rates were monitored at 12-hour intervals over 120 hours. At 12 h post-infection (hpi), mice were euthanized, and organs were harvested for downstream analysis. Tissue samples were acquired for metabolomics analysis, RNA sequencing (RNA-seq), and quantitative real-time PCR (qRT-PCR), bacterial burden measurement, histological analysis, and flow cytometry. Serum samples were collected.

Flow cytometric analysis

Single lung and spleen cells isolated from the test mice were incubated with different combinations of fluorochrome-conjugated antibodies against mice: CD45 (Cat# 103106), CD11b (Cat# 101235), Ly6G (Cat# 127615), F4/80 (Cat# 123113), CD206 (Cat# 141703), CD86 (Cat# 105029), CD3 (Cat# 100235), CD19 (Cat# 115529), CD4 (Cat# 100437), CD8 (Cat# 100751), NK1.1 (Cat# 156513), ; appropriate isotype controls were acquired from Biolegend. Propidium iodide (Sigma-Aldrich, Cat#P4170-1G) was used to exclude dead cells. Flow cytometric analyses were carried out by using a FACSCelesta™ flow cytometer (BD Biosciences, CA, USA); data analysis was conducted by FlowJo software (Version 10.8.1, Treestar, CA, USA).

RNA sequencing

Total RNA from mice lung samples was sequenced by BGI Technology (Shen Zhen, China). RNA was qualified and quantified using the Bioanalyzer 2100 (Agilent Technologies, CA, USA), and cDNA libraries were constructed with the Optimal Dual-mode mRNA Library Prep Kit (BGI, China). Libraries were then amplified by phi29 and rolling circle amplification (RCA) to make DNA nanoball (DNB), which were loaded into the patterned nanoarray for sequencing on the BGI G400 platform, generating PE150 reads.

HISAT2³¹ was used for sequence alignment, and gene fragments were counted using Feature Counts v2.0.2³². Differential expression analysis was conducted with DESeq2³³, identifying differentially expressed genes (DEGs) with $P_{\text{adjust}} < 0.05$ and $|\text{Log}_2\text{Fold Change}| > 1$. Principal component analysis (PCA) was conducted on the full set of processed detected genes to view the distributional differences. The data visualization was also achieved through volcano plots and heatmaps, and the pathway enrichment analysis was conducted using the KEGG database.

Intestines histopathology

Histopathological analysis was carried out by Servicebio (Shenzhen, China). The intestine samples of the test mice were fixed overnight in 10% formalin at 4°C, embedded in paraffin wax, and stained with hematoxylin and Eosin (H&E). Then, the stained samples were sectioned (4 µm thick), scanned, and imaged at a magnification of 100× or 400×.

Cell infection

In the groups infected with ATCC 17978, THP-1 or iBMDMs were infected under specific conditions depending on the assay. For caspase immunoblot analysis, iBMDMs were infected with ATCC 17978 for 3 h, after which 250 µg/mL gentamicin was added to the culture medium, and incubation continued for an additional 36 h. For immunofluorescence microscopy, immunoblot analysis of signaling pathways and PANoptosome sensors, as well as an immunoprecipitation assay, iBMDMs were infected for 6 h. For the cell death assay, cells were infected for 12 h.

Cell stimulation

iBMDMs were stimulated with the following concentrations of cytokines, where indicated, unless otherwise noted: 50 ng/mL of IFN-γ, 20 ng/mL of others. For the inhibition of NO, cells were co-treated with 1 mM of L-NAME hydrochloride.

Serum collection

Mice blood was acquired from mice via orbital bleeding and left at room temperature for one hour to clot. Clotted blood was centrifuged at 10,000 g for 20 min at 4°C. Serum was immediately stored at -80°C until use for subsequent analysis.

In vivo IL- 1α/β + IFN-γ–induced inflammatory shock

Age- and gender-matched 6- to 8-week-old mice of the indicated genotypes were injected intraperitoneally with 20 µg IL- 1α/β alone, 20 µg IFN-γ alone, or IL- 1α/β + IFN-γ diluted in DPBS. Animals were under permanent observation, and survival was assessed every 30 min. Blood was collected 5 h after cytokine injection. Blood composition was analyzed using an automated hematology analyzer (Serum LDH, ALT, and AST).

For neutralizing antibody treatment, age- and gender-matched 6- to 8-week-old WT mice were administered 500 µg of neutralizing antibody against IL-1α/β plus 500 µg of neutralizing antibody against IFN-γ (n = 5) 3 h after *i.p.* injection of IL-1α/β and IFN-γ. Mice were monitored for survival.

Immunoblot analysis

For caspase analysis, cell supernatant was collected and precipitated by TCA at the ratio of 9:1, washed with cold acetone, and then solubilized in sample buffer with (4× SDS loading buffer, 1×protease

inhibitors, and 1×phosphatase inhibitors). For signaling analysis, the cell supernatants were removed, and the cells were lysed with RIPA buffer with protease and phosphatase inhibitors. Protein concentration was measured using BCA protein assay (Thermo Fisher Scientific, USA). Cell lysates were loaded onto 12% SDS-PAGE gel and then electroblotted to PVDF membranes (Millipore, MA, USA) by a Trans-Blot Turbo Transfer System (Bio-rad, CA, USA). Non-specific binding was blocked by 5% skim milk in TBST, and then membranes were incubated overnight at 4 °C with primary antibodies against: IL-1 β (Cell Signaling Technology [CST], Cat#12507, 1:1000), cleaved IL-1 β (CST, Cat#63124, 1:1000), caspase-1 (CST, Cat#24232, 1:1000), cleaved caspase-1 (CST, Cat#89322, 1:1000), gasdermin D (CST, Cat#39754, 1:1000), cleaved gasdermin D (CST, Cat#10137, 1:1000), caspase 3 (CST, Cat#14220, 1:1000), cleaved caspase 3 (CST, Cat#9664, 1:1000), caspase 7 (CST, Cat#9492, 1:1000), cleaved caspase 7 (CST, Cat#9491, 1:1000), caspase 9 (CST, Cat#9508, 1:1000), cleaved caspase 9 (CST, Cat#9507, 1:1000), MLKL (CST, Cat#37705, 1:1000), phospho-MLKL (CST, Cat#37333, 1:1000), beta-actin (Bioss, Cat#bs-0061R). Membranes were washed with TBST and incubated with HRP-linked secondary antibodies (CST, Cat#7074, 1:2000). Immunoreactive bands were visualized by Western HRP Substrate (Advansta, USA).

Cell death assay

Lytic cell death was measured by propidium iodide (PI) incorporation as previously described. Briefly, PI (2 μ g/mL) solutions were added into the culture medium and incubated at room temperature in the dark for 10 min. The stained cells were immediately observed using the Nikon Eclipse Ti2-E Live-cell Fluorescence Imaging System (Nikon, Japan). The cell death rate was calculated as the percentage of PI-positive (dead) cells among total cells. The mortality rate of cells was also assessed by measuring lactate dehydrogenase (LDH) release using the CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen, USA) according to the manufacturer's instructions.

Statistical analysis

Sample size and data analysis methods are detailed in the respective figure legends. Mean $\pm$ S.D. was utilized for data presentation. The statistical analysis was conducted using unpaired Student's t-test between two groups, and one-way ANOVA with Tukey correction for pairwise comparisons among three or more groups. The Log-rank (Mantel-Cox) test was applied to compare Kaplan-Meier survival curves in animal studies. Statistical analyses were performed using GraphPad Prism 9 (GraphPad software). $P < 0.05$ was considered indicative of statistical significance where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Heatmaps and dumbbell plots in Figure 1A and 1B were created using R version 4.5.1 and the ggplot library.

Declarations

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Data Availability declaration

Data available on request from the authors.

Ethics and Consent to Participate declarations

Female C57BL/6J mice (6-8 weeks old, ~20 g) were purchased from Zhuhai BesTest Bio-Tech Co., Ltd. (Zhuhai, China) and housed under specific pathogen-free conditions. All animal experiments were approved by the Animal Subjects Ethics Sub-committee (ASESC) of The Hong Kong Polytechnic University (approval no. 23-24/821-FSN-R-TRS). All efforts were made to minimize animal suffering.

Funding Declaration in the manuscript

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Author contributions

W.Z. performed the experiments and drafted the manuscript; H.W., Q.X., and S.C. designed the project; X.Y., W. Z., and H.W. helped with the animal experiments; H.W. helped with flow cytometry analysis; S.C. supervised the project and wrote the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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Figures

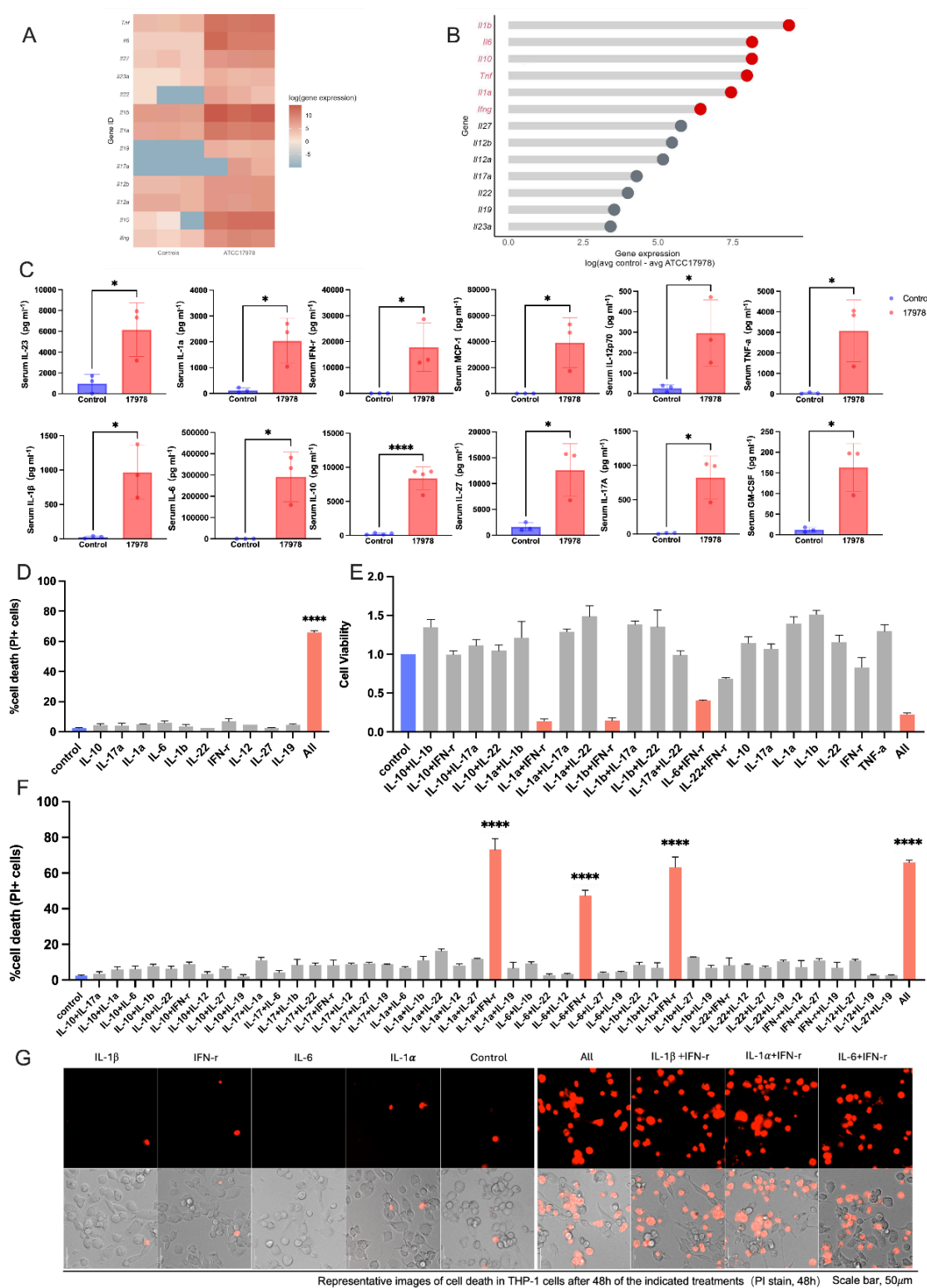


Figure 1

Inflammatory Cytokines in Ab Sepsis, and Synergy and Their Synergistic Effects on Cell Death.

(A) Heatmap depicting the levels of pro-inflammatory cytokines from RNA sequencing on lung tissues from Ab-infected mice and healthy mice. (B) The most highly upregulated cytokines are among all the pro-inflammatory cytokines in terms of expression levels. (C) Pro-inflammatory cytokines in the serum of mice infected with Ab (ATCC17978) and healthy mice. (E) Cell viability of immortalized bone marrow-derived macrophages (iBMDMs) after 48 h of the indicated treatments. (D, F) Percent of THP-1 cells that are dead 48 h after treatment with the indicated cytokines. (G) Representative images of cell death in THP-1 cells after 48 h of the indicated treatments. Scale bar, 50 μ m. Statistical analyses were conducted by unpaired t-test (b-f). The data are representative of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

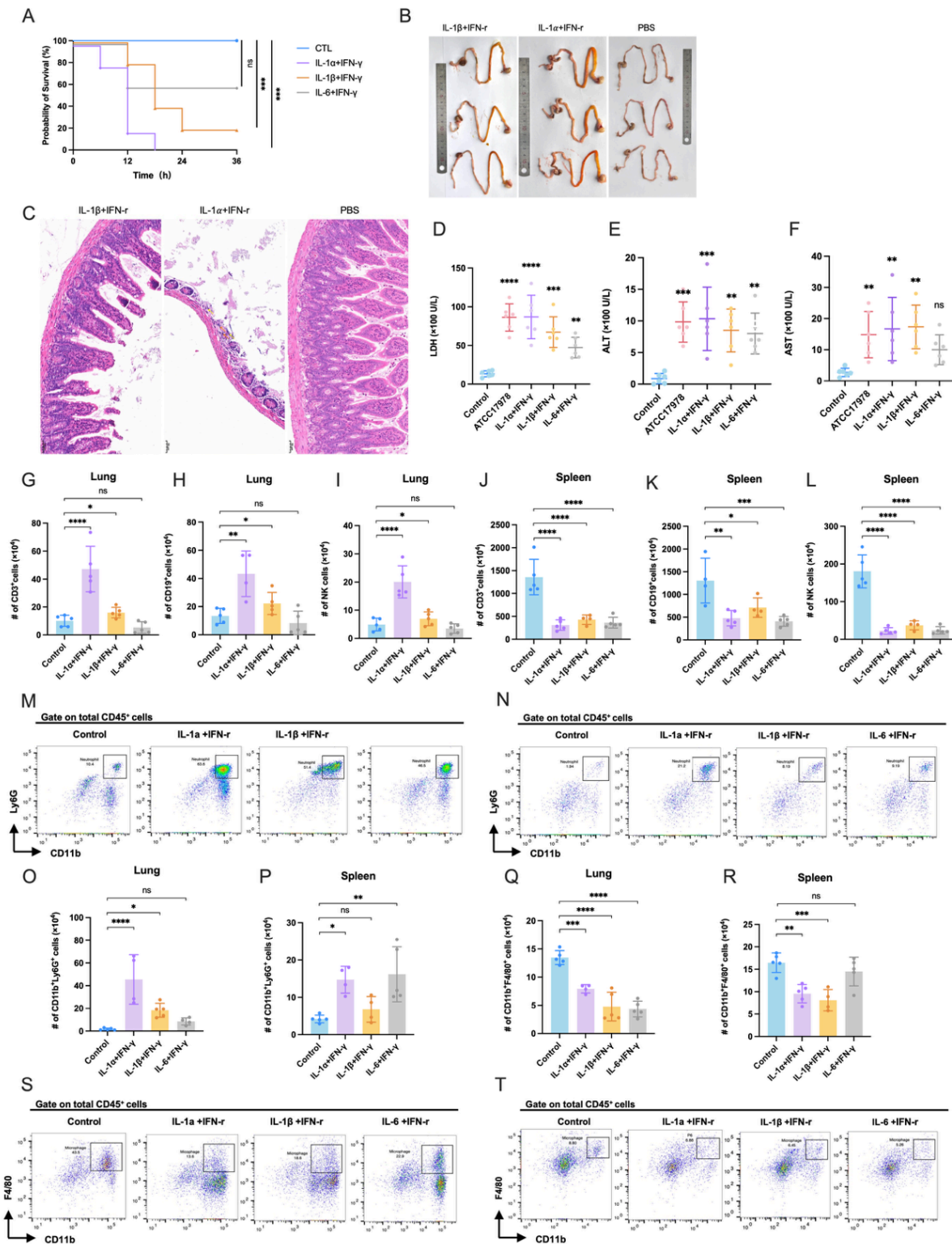


Figure 2

Synergistic IL-1α/β and IFN-γ Signaling Induces Lethal Systemic Inflammation and Multiorgan Damage *In Vivo*. (A) Survival of 6- to 8-week-old WT mice after intraperitoneal (*i.p.*) injection of PBS (n = 5), IL-1α+IFN-γ (n = 5) or IL-1β+IFN-γ (n = 5). (B) Representative image of colon samples from mice injected with PBS or IL-1α+IFN-γ (n = 5) or IL-1β+IFN-γ (n = 5) after 5 h. (C) H/E staining of colon samples from mice injected with PBS or IL-1α+IFN-γ (n = 5) or IL-1β+IFN-γ (n = 5) after 5 h. (D, E, F) Analysis of (C)

serum levels of LDH, ALT, and AST. (G, H, I) Quantification of total CD3+, CD19+, NK1.1+ cells, lymphocytes in the lung cells (n=5). (J, K, L) Quantification of total CD3+, CD19+, NK1.1+ cells, lymphocytes in the spleen cells (n=5). (Q, S) Quantification of total F4/80+ macrophages and Ly6G+ neutrophils in lung cells analyzed by flow cytometry. (R, T) Quantification of total F4/80+ macrophages and Ly6G+ neutrophils in spleen cells analyzed by flow cytometry.

Statistical analyses were conducted by log-rank test (A) and ANOVA (D-L, Q-R). Data are representative of at least three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant.

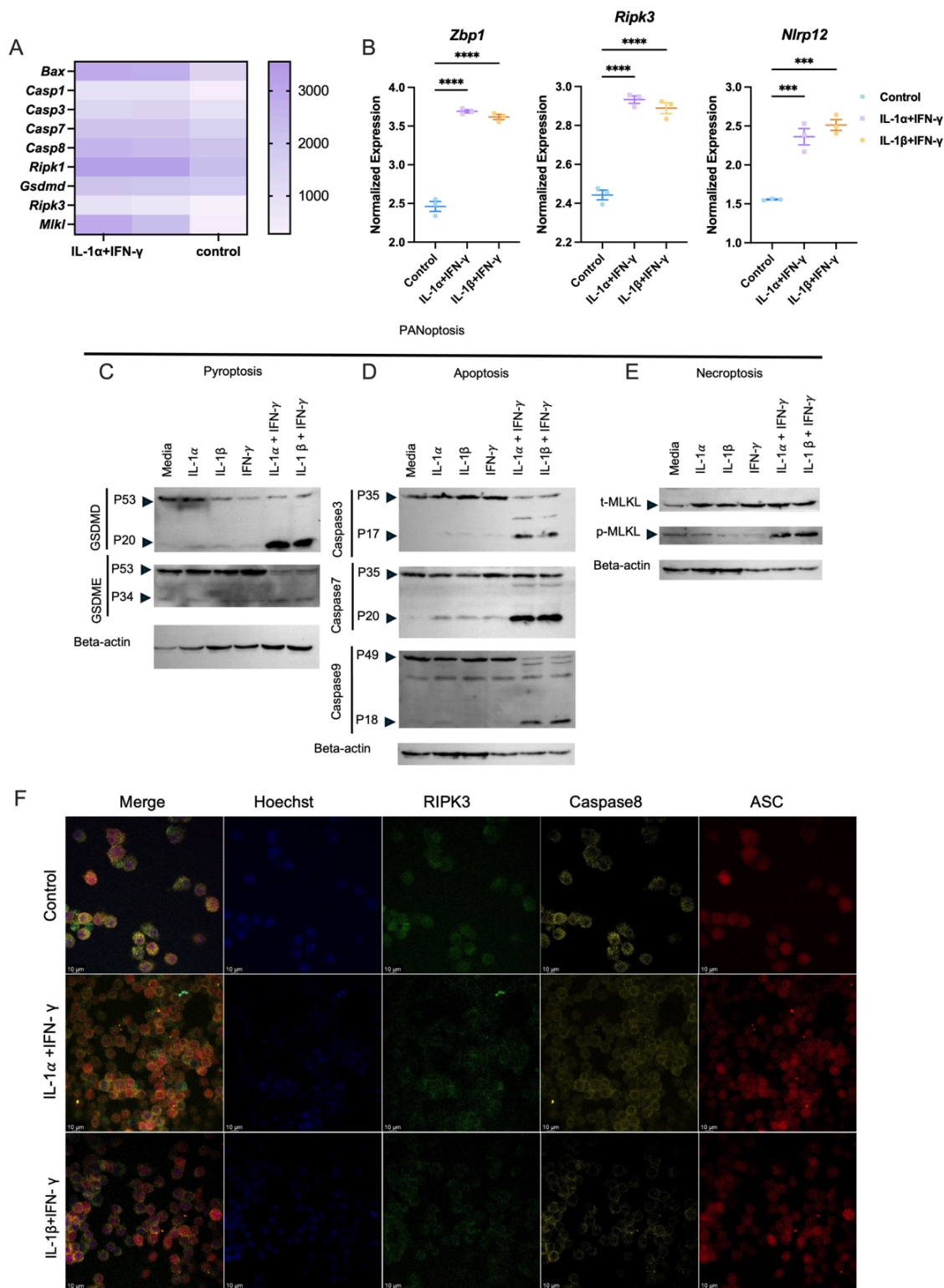


Figure 3

IL-1 α / β and IFN- γ Synergy Induces PANoptosis through Concurrent Activation of Pyroptotic, Necroptotic, and Apoptotic Pathways

(A) Heatmap showing the relative expression of genes PANoptosis-related in the indicated groups. (B) Normalized RNA-seq expression of Zbp1, Ripk3, and Nlrp12 in control (PBS), IL-1 α + IFN- γ and IL-1 β + IFN-

γ treated samples. Each dot represents one biological replicate. Data are presented as mean ± SEM. (C-E) Immunoblot analysis of (C) pro- (P53), activated (P30), and inactivated (P20) GSDMD, pro(P53) and activated (P34) GSDME; (D) pro- (P35) and cleaved (P19 and P17) CASP3, pro- (P35) and cleaved (P20) CASP7, and pro- (P49) and cleaved (P18) CASP9; and (E) phosphorylated MLKL (pMLKL), total MLKL (tMLKL) in iBMDMs treated with IL-1α, IL-1β, IFN-γ, IL-1α+ IFN-γ or IL-1β+ IFN-γ. (F) iBMDMs treated with PBS, Control, IL-1α + IFN-γ or IL-1β + IFN-γ for 4 h, immunofluorescence microscopy was adopted to reveal the distribution of RIPK3, CASP8, and ASC.

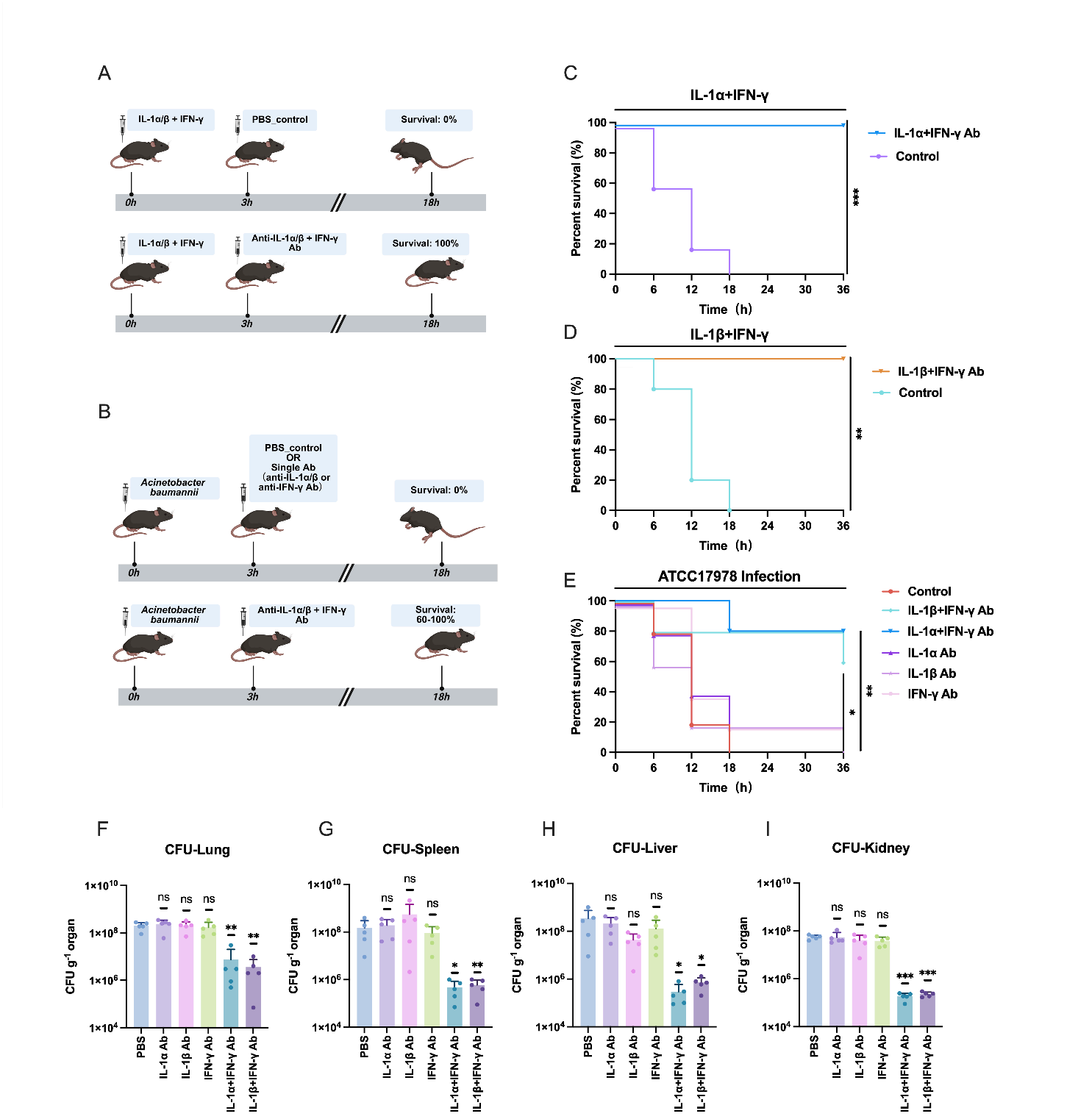


Figure 4

Neutralization of IL-1 α / β and IFN- γ Synergy Confers Complete Protection Against Cytokine Storm–Induced Lethality and Attenuates Sepsis Mortality

(A) Survival of 6- to 8-week-old WT mice injected with PBS (n = 5) or neutralizing antibodies against IL-1 α and IFN- γ (n = 5) 3 h after i.p. injection of IL-1 α and IFN- γ . (B) Survival of 6- to 8-week-old WT mice injected with PBS (n = 5) or neutralizing antibodies against IL-1 β and IFN- γ (n = 5) 3 h after i.p. injection of IL-1 β and IFN- γ . (C) Survival of 6- to 8-week-old WT mice injected with PBS (n = 5) or neutralizing antibodies against IL-1 α , IL-1 β , IL-1 α and IFN- γ , or IL-1 β and IFN- γ (n = 5) 3 h after *i.p.* injection of Ab(ATCC17978)

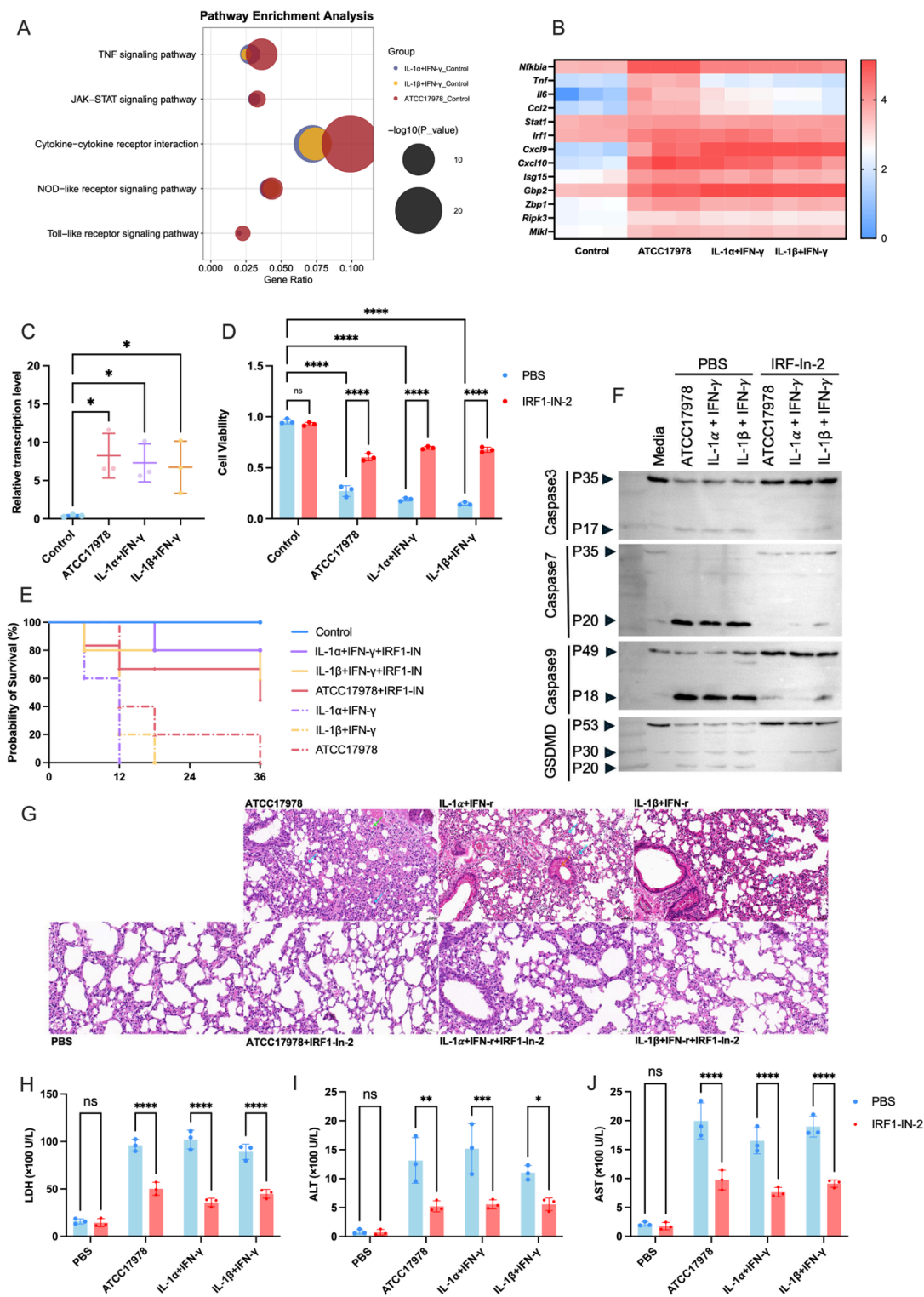


Figure 5

IRF1 Serves as a Critical Upstream Node Amplifying Pathogenic Inflammation in Cytokine Synergy and Ab Infection Models

(A) KEGG pathway enrichment analysis of differentially expressed genes in lung tissues from mice challenged with IL-1 α +IFN- γ , IL-1 β +IFN- γ , or Ab ATCC 17978, compared with control mice. Bubble size

indicates $-\log_{10}(\text{P value})$, and the x-axis indicates gene ratio. (B) Heatmap showing normalized expression of representative inflammatory, interferon-response, and PANoptosis-associated genes in control, ATCC 17978-infected, IL-1 α +IFN- γ -treated, and IL-1 β +IFN- γ -treated groups. (C) Quantitative PCR analysis of IRF1 mRNA expression in iBMDM cells 6 h after being infected with Ab(ATCC17978, 5×10^7 CFU) or treated with IL-1 α/β + IFN- γ , compared to uninfected PBS controls. (D) Cell death in iBMDMs pretreated with IRF1-IN-2 (20 μM) or vehicle (PBS) for 12 hours, followed by stimulation with IL-1 α/β + IFN- γ for 48 h or infection with Ab (MOI 10) for 6 h. Cell death was assessed by LDH release assay (percentage of maximum release). Data are mean $\pm$ SEM from three independent experiments (**** $p < 0.0001$ by two-way ANOVA). (E) Kaplan–Meier survival curves of 6–8-week-old C57BL/6 mice ($n = 5$ per group) treated with IRF1-IN-2 (100 $\mu\text{g}/\text{d}$, i.p.) or vehicle (PBS) 3 h after challenge with IL-1 α/β + IFN- γ or Ab infection (7.5×10^7 CFU). (F) Immunoblot analysis of caspase-3, caspase-7, caspase-9, and GSDMD processing in iBMDMs treated with vehicle or IRF1-IN-2 before ATCC 17978 infection or IL-1 α/β +IFN- γ stimulation. (G) Representative H&E-stained lung sections from mice treated as in (E). Scale bars, 50 μm . Images show preservation of alveolar architecture, reduced interstitial edema, myeloid infiltration, and epithelial damage in IRF1-IN-2–treated groups. (H–J) Serum LDH (H), ALT (I), and AST (J) levels in mice treated as in (E). Data are shown as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with appropriate multiple-comparison correction or log-rank test for survival analysis. $p < 0.05$; $p < 0.01$; $p < 0.001$; $p < 0.0001$; ns, not significant.

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