

Supplemental Materials and Methods

Clinical and demographic data analysis. Continuous clinical and demographic variables, including fungal burden (*Pneumocystis mtLSU* and *nad4* copy numbers), viral loads (BALf and serum CMV), and physiological parameters (P/F ratio, LDH, β -D glucan), were compared between survivor and non-survivor cohorts using the nonparametric Wilcoxon rank-sum (Mann-Whitney U) test. To accommodate broad dynamic ranges, viral and fungal load measurements were log₁₀-transformed prior to graphical visualisation. Categorical clinical variables, such as sputum IFA and PCP classifier status, were compared using Fisher's exact test. To evaluate the continuous relationships between oxygenation impairment and microbiological severity, Spearman's rank correlation analysis was performed between the P/F ratio and specific pathogen burden metrics (BALf *mtLSU*, BALf *nad4*, β -D glucan, and BALf CMV viral load), yielding correlation coefficients (R) and corresponding statistical significance.

RNA extraction and RNA-sequencing. BALf samples (10mL) were centrifuged and cell pellets lysed in 1mL TRIzol LS (Invitrogen). Total RNA was extracted using the Zymo Direct-zol RNA mini-prep kit (Zymo Research). RNA-sequencing libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep kit (NEB), where mammalian rRNA was depleted with NEBNext rRNA Depletion kit (NEB). The final libraries were assessed using TapeStation 2000 System (Agilent) and sequenced on Illumina NextSeq instrument with paired-end 75 cycle reactions.

RNA-seq data analysis. The quality of the sequencing fastq files was analysed using FastQC (v0.11.5) and poor quality samples were excluded from further differential gene expression analysis. Sequence reads were adapter- and quality-trimmed using Trimmomatic (v0.38) before aligning to the human genome (Ensembl GRCh38 build 88) using STAR aligner (v2.7.11b). Gene expression was quantified using RSEM (v1.3.0) and differential gene expression analysis was performed using DESeq2 (v1.42.1). DESeq2 uses a generalised linear model to estimate log₂ fold change (log₂FC) between comparison groups. The Benjamini-Hochberg false discovery rate (FDR) was applied as multiple testing correction, resulting in an adjusted p-value (padj) for each gene per comparison. Gene Set Enrichment Analysis on the whole transcriptome was performed by pre-ranking all genes to the Wald statistic, referenced to the Reactome biological pathway database.

Weighted Gene Co-expression Network Analysis. To identify systems-level transcriptional networks associated with clinical phenotypes, co-expression network analysis was performed using the WGCNA package in R. Variance-stabilised transformed (VST) count data generated from DESeq2 were utilised as the input expression matrix. A signed network was constructed by calculating the Pearson correlation matrix for all interacting genes, which was subsequently transformed into a Topological Overlap Matrix (TOM) to minimise the effects of spurious correlations. Genes were hierarchically clustered based on TOM-based dissimilarity, and discrete co-expression modules were identified using the dynamic tree-cut algorithm. To determine the clinical relevance of these networks,

Module Eigengenes (MEs), defined as the first principal component of a given module, were correlated with continuous and categorical patient traits to generate module-trait association matrices. Functional annotation of phenotypically significant modules was conducted by mapping constituent genes to the Reactome biological pathway database, with enrichment visualised based on gene ratios and adjusted p-values. To extract highly connected regulatory targets, hub genes were defined with the following mathematical thresholds: an absolute Module Membership (MM; the correlation between an individual gene and its ME) ≥ 0.8 , and an absolute Gene Significance (GS; the correlation between the gene and the specific clinical trait) ≥ 0.5 , with a threshold of $p \leq 0.05$. For topological visualisation of hub gene trajectories, continuous clinical traits (e.g., P/F ratio, BALf CMV viral load, and fungal burden) were categorised into statistical tertiles (High, Medium, Low) based on percentile ranking. The mean Z-score standardised expression of the extracted hub genes was calculated for each clinical bin and visualised using the ComplexHeatmap package to demonstrate dose-dependent transcriptomic shifts.

SomaScan proteomic assay. Patient BALf samples were profiled using the aptamer-based SomaScan 7k proteomics assay at SomaLogic, Inc (Boulder, CO, USA). Prior to analysis, the total protein concentration of the BAL fluid samples was quantified using PierceTM BCA assay (ThermoFisher Scientific), and all samples were normalised to an input concentration of 4 μ g/mL to control for dilution variance. To control for dilution variance, particularly for samples presenting below the standard 4 μ g/mL input threshold, raw relative fluorescence intensity (RFU) values were adjusted by their respective sample dilution factors. RFU of each protein analyte for each sample was first independently normalised to hybridisation control, followed by median signal and interplate calibration normalisation, and scale factor was determined. The RFUs were log₂-transformed for statistical comparison. Differential protein abundance between non-survivors and survivors was assessed using linear models with empirical Bayes moderation as implemented in the limma package (v3.58.1) in R (v4.3.3). To control for multiple comparisons, p-values were adjusted using the Benjamini-Hochberg FDR method.

Multi-trait Lasso regression analysis. To identify predictive proteomic biomarker panels associated with distinct clinical and microbiological phenotypes, Least Absolute Shrinkage and Selection Operator (Lasso) regression was performed using the glmnet package in R. Log₂-transformed RFU matrices from the targeted proteomics assay were utilised as the input predictor variables, subset to synchronise with patient-matched clinical metadata. To accommodate the diverse statistical nature of the clinical endpoints, regression models were tailored prior to fitting: binomial logistic regression was applied for binary categorical outcomes (e.g. mortality, sex, PCP classifier status), whereas Gaussian linear regression was used for continuous physiological and microbiological parameters (e.g. P/F ratio, BALf and serum CMV viral loads, *Pneumocystis mtLSU* and *nad4* copy numbers, β -D-glucan). To penalise model complexity, mitigate multicollinearity among highly correlated proteins, and prevent overfitting, internal k-fold cross-validation was conducted to calculate the optimal tuning parameter (λ). Lasso regression was specifically selected because it applies an L1 penalty that shrinks non-

informative feature coefficients to exactly zero. This algorithm is highly efficient for feature selection in high-dimensional omics datasets and explicitly constrains the number of variables to mitigate the risk of model overfitting inherent in studies with a small sample size. The penalty threshold yielding the minimum mean cross-validated error (λ min) was selected for final model fitting, enforcing an elastic net mixing parameter of $\alpha=1$. Proteins retaining non-zero coefficients at this threshold were extracted as a predictive biomarker signature for each respective trait.

Untargeted metabolomics of BAL fluid. Polar metabolites were extracted from BAL fluid using chloroform:water:methanol (3:2:4 v/v) and the polar fraction was concentrated by SpeedVac. The dried samples were resuspended in a mixture of 30% methanol and 2% acetonitrile and analysed on an Agilent 1290 Infinity II UHPLC system interfaced with an Agilent 6546 LC/QTOF mass spectrometer. Chromatographic separation was performed on a Poroshell 120 HILIC-Z column (100 $\times$ 2.1 mm, 2.1 μ m; Agilent). A 2 μ L aliquot was injected, and the column was maintained at 15 $^{\circ}$ C. The LC was operated at 400 μ L/min using a gradient elution with mobile phase A consisting of 20 mM ammonium acetate supplemented with 5 μ M medronic acid (pH adjusted to 9.3) and mobile phase B consisting of acetonitrile. The gradient was programmed as follows: 0-1 min, 85% B; 1-8 min, 75% B; 8-12 min, 60% B; 12-19.1 min, 10% B; followed by re-equilibration at 85% B until 24 min. The QTOF source settings were: gas temperature, 225 $^{\circ}$ C; drying gas, 9 L/min; nebuliser, 30 psi; sheath gas temperature, 375 $^{\circ}$ C; capillary voltage, 3,000 V; nozzle voltage, 500 V; fragmentor, 100 V; skimmer, 45 V; octupole RF amplitude, 750 V (Vpp). Acquisition was performed in positive and negative ionisation modes with detection up to 1700 m/z in the low-mass range. To ensure accurate identification, authentic standards from an internal compound library were run to confirm retention times and MS/MS fragmentation signatures under identical acquisition conditions.

Metabolomics data pre-processing, quality control and differential analysis. Raw LC-MS data were processed for peak picking, alignment, and gap filling. To correct for batch effects and technical variation, signal intensity drift was normalised using the BatchCorrMetabolomics R package. Features with >50% missing values in quality control (QC) samples or with a coefficient of variation (CV) >30% in QC samples were removed. The remaining missing values were imputed using the k-nearest neighbour algorithm. Following imputation, the data matrix underwent Probabilistic Quotient Normalisation to account for differences in sample dilution and was subsequently log₂-transformed to stabilise variance. To restrict downstream analysis to biologically interpretable features, unknown metabolites were removed from the dataset prior to differential comparison. Multivariate analysis was performed using principal component analysis (PCA) to visualise sample clustering and detect outliers. To identify differentially abundant metabolites between survivors and non-survivors, linear models were employed using the limma package in R. Empirical Bayes moderation was applied to standard errors to improve statistical power. To account for multiple comparisons, p-values were adjusted using the Benjamini-Hochberg FDR method, with statistical significance defined as an FDR-adjusted $p \leq 0.05$. Finally, to evaluate the relationship between specific metabolic sub-networks and

continuous clinical variables, composite pathway activity scores were derived using patient-level mean Z-scores and assessed via Spearman's rank-order correlation.

Metabolic pathway enrichment and topology analysis. Annotated metabolites were subjected to Pathway Enrichment Analysis using the MetaboAnalyst platform and the KEGG Pathway Database. Enrichment significance was assessed using the hypergeometric test. Pathway topology analysis was simultaneously performed to calculate the Pathway Impact scores based on the centrality of the differentially expressed metabolites within a given network. Pathways with FDR-adjusted $p \leq 0.05$ were considered significantly enriched.

Multi-omics data integration and biomarker discovery. To integrate disparate multi-omics datasets and identify a predictive biomarker signature associated with patient mortality, DIABLO (Data Integration Analysis for Biomarker discovery using Latent cOmponents) was performed using the mixOmics package (v6.26.0) in R. Input predictor matrices comprised variance-stabilised mRNA counts, log2-transformed protein relative fluorescence units, and log2-transformed annotated metabolite abundances, subset to synchronise with patient-matched metadata. For each platform, differential expression/abundance was tested using linear models adjusted for age, sex, CD4 count, PCP classification, and relevant technical covariates. We applied Benjamini-Hochberg FDR and defined significance as adjusted $p \leq 0.05$. Only FDR-significant features were carried forward into downstream pathway analysis. A design matrix was established with a linkage weight of 0.5 across all omics blocks to balance phenotypic discrimination and interblock correlation. To mitigate model complexity and prevent overfitting, hyperparameter tuning was conducted via repeated cross-validation. The optimal number of predictive features to retain per omics layer and latent component was determined by selecting the tuning threshold that yielded the minimum balanced classification error rate. Sparse partial least squares discriminant analysis was subsequently applied, incorporating an L1 penalisation step to shrink non-informative feature coefficients to zero. Features retaining non-zero coefficients were extracted as the final predictive multi-omics signature. Patient-level clustering and categorical class separation were visualised across individual omics blocks using sample projection scatter plots. To quantify patient-level molecular disease severity, individual sample coordinates (latent variates) corresponding to the first predictive component of the fitted DIABLO model were extracted across the constituent omics blocks. A composite multi-omics severity score was derived for each patient by calculating the mean of the block-specific latent variates. To evaluate the association between these DIABLO-derived component scores and clinical outcome, categorical class distributions comparing survivors and non-survivors were evaluated for statistical significance using the non-parametric Wilcoxon rank-sum test.

Network construction and biomarker validation. Multi-omics correlation networks generated by DIABLO were exported to Cytoscape (v3.10.4) for topological visualisation. Network nodes represented individual omics features, and edges denoted correlation directionality ($r \geq 0.50$), with continuous colour mapping applied to distinguish positive and negative associations. To identify

structural master regulators acting as interblock biological bridges, topological network analysis was conducted. Node size was mapped to the betweenness centrality metric, and features exhibiting the highest centrality scores were extracted as candidate prognostic biomarkers. The prognostic capacity of these selected biomarkers to classify patient mortality was evaluated via Receiver Operating Characteristic (ROC) curve analysis. To address sparse outcome events and mitigate model overfitting, an internal validation framework was established using the caret (v7.0.1) and pROC (v1.19.01.1) packages in R. Standard logistic regression models were constructed to evaluate each master regulator individually, alongside an unpenalised combined multi-omics panel integrating all four features. Out-of-sample predictive performance was assessed using repeated k-fold cross-validation. A 2-fold cross-validation split was specified to ensure the equitable distribution of mortality events across training and testing partitions. This procedure was repeated 100 times with random patient reshuffling to stabilise variance and generate smoothed out-of-fold probabilities. Final predictive accuracy was reported as the cross-validated area under the curve (CV AUC) derived from these hold-out predictions.

***Pneumocystis murina* antigen preparation and Western blot screening.** To generate *Pneumocystis* protein antigens for validation, *P. murina* were isolated from the lung tissue of Rag2^{-/-} Il2rγ^{-/-} mice (C57BL/6 background) that had been previously inoculated with *P. murina* for 8 weeks. Following collection, the fungal organisms were purified from the lung homogenates via differential centrifugation. To confirm the presence and purity of the *P. murina* organisms, aliquots of the resuspended pellets in PBS were evaluated microscopically using a modified Giemsa stain, alongside standard Gram staining to exclude any concurrent bacterial contamination. The purified *P. murina* pellets were subsequently subjected to sonication for 5 minutes to release intracellular and cell-surface protein antigens. The total protein concentration of the resulting sonicate was quantified using a BCA assay to ensure standardised loading. To confirm that the IVIG preparation possessed sufficient cross-reactive antibodies against the fungus, aliquots of the *P. murina* antigen (5µg, 10µg, and 20µg) were resolved by SDS-PAGE alongside uninfected lung homogenates from both Rag2^{-/-} Il2rγ^{-/-} and wild-type C57BL/6 mice (5µg and 25µg) to serve as negative host-tissue controls. The resolved proteins were transferred to a membrane and blocked to prevent non-specific binding. The membrane was subsequently probed with the selected IVIG preparation (Octagam 10%, Lot no: M451c8541), followed by incubation with an appropriate secondary antibody (anti-human IgG) conjugated for detection.

***Ex Vivo* Precision Cut Lung Slice infection and opsonisation Assay.** Precision-cut lung slices (PCLS) were generated from immunodeficient Rag2^{-/-} Il2rγ^{-/-} mice. Lungs were perfused with PBS, inflated with either 1.5% low-melting agarose or 15% gelatin, and sectioned into 250 µm slices using a vibratome (Leica VT1200s). Slices were cultured in DMEM supplemented with 10% FBS. Bone marrow-derived macrophages (BMDMs) were generated from the femurs of C57BL/6 mice and differentiated for 6 days in M-CSF (20 ng/mL). To assess macrophage-mediated killing, PCLS were infected with *Pneumocystis murina* (2x10⁵ asci/well) and co-cultured with or without BMDMs (1x10⁶

cells/well) under three conditions: (1) Incubation in media alone; (2) Incubation with polyclonal mouse anti-*Pneumocystis* serum (1:300) for 1 h prior to infection; and (3) Incubation with 2 mg/mL intravenous immunoglobulin (Octagam 10%, Lot number: M451c8541).

RT-qPCR. Fungal burden was quantified 24 h post-infection by RT-qPCR targeting the *P. murina* single-copy gene serine protease (*Sp*). Expression was normalised to the housekeeping gene *Gapdh* and calculated as fold change ($2^{-\Delta\Delta C_t}$). For the *ex vivo* inhibitory experiments, fungal burden data were log10-transformed to normalise distribution prior to analysis. Differences between treatment groups were assessed using two-tailed Student's t-tests, followed by Benjamini-Hochberg FDR correction for multiple comparisons. Statistical significance was defined as an adjusted $p \leq 0.05$.