

Temporal Proteomic and Phosphoproteomic Profiling Reveals Rapid Immune Suppression Induced by Lung Adenocarcinoma Pleural Effusions

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Temporal Proteomic and Phosphoproteomic Profiling Reveals Rapid Immune Suppression Induced by Lung Adenocarcinoma Pleural Effusions

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Running Title

Tumor EVs role on the peripheral immune cells

ABSTRACT

Background: Malignant pleural effusion (MPE) constitutes a highly immunosuppressive tumor microenvironment, yet the temporal dynamics by which it rewires peripheral immune cells remain poorly understood.

Methods: Here, we established an *ex vivo* temporal model by exposing peripheral blood mononuclear cells (PBMCs) from healthy donors to transudative, benign exudative, or lung adenocarcinoma-derived malignant pleural effusions. To isolate the mechanistic mediators, PBMCs were additionally co-cultured with tumor-derived extracellular vesicles (TDEVs) and extracellular vesicle-depleted (EV-depleted) conditioned media. Cellular responses were evaluated using time-resolved mass spectrometry-based proteomic and phosphoproteomic profiling, multiparametric immunophenotyping, and kinase set enrichment analysis (KSEA).

Results:

MPE drove a dose- and time-dependent reduction in PBMC viability that significantly exceeded that of non-malignant effusions. At 24 h, immunophenotyping revealed marked changes in immune cell composition, including monocyte depletion and the enrichment of a T follicular helper-like (Tfh-like) CD4+ T-cell population expressing PD-1+ and CXCR5+. Time-resolved proteomic profiling revealed that while all effusions triggered a rapid initial response within 6 hours, a stark biological divergence occurred at 24 hours. Benign effusions elicited a robust, sustained immune rebound, whereas MPE exposure uniquely drove a profound, progressive downregulation of global immune pathways, alongside the enrichment of networks driving apoptosis, ferroptosis, and extracellular vesicle signaling. Crucially, EV-depleted conditioned media failed to induce severe cell death, establishing that TDEVs, rather than soluble factors alone, carry the primary cytotoxic payload. Subsequent KSEA of TDEV-treated PBMCs identified distinct, vesicle-size-dependent hijacking of critical intracellular networks, highlighted by, for example, the significant dysregulation of cyclin-dependent kinases.

Conclusions: Together, these findings demonstrate the rapid, time-dependent immune alterations induced by lung adenocarcinoma-associated pleural effusions and identify tumor-derived

extracellular vesicles as critical drivers of immune-cell exhaustion and cell death through the modulation of intracellular kinase signaling.

KEYWORDS: Malignant Pleural Effusion, Non-malignant Pleural Effusion, Lung Cancer, Peripheral Blood Mononuclear cells, Tumor Microenvironment, Immunosuppression, Extracellular Vesicles, Phosphorylation

INTRODUCTION

Malignant pleural effusion (MPE) affects up to 15% of patients with cancer, and its incidence and prevalence are increasing [1–4]. Most patients are symptomatic; with dyspnea being the predominant symptom due to lung atelectasis, impaired chest wall movement, and diaphragmatic dysfunction [1, 3–6]. MPE usually reflects advanced or metastatic disease, with a poor prognosis, and median survival ranging from 3-12 months depending on the primary cancer site and other prognostic factors [1, 7].

Historically, MPE was considered a passive mechanical complication of advanced disease driven by fluid accumulation [5, 8]. However, an emerging clinical paradigm now recognizes MPE as an actively pathogenic reservoir where dynamic tumor-host interactions drive disease progression [9–11]. Indeed, MPE is richer than matched plasma in tumor-derived factors, including soluble proteins, extracellular vesicles (EVs), and RNA [6]. Consequently, the MPE constitutes a unique, complex tumor microenvironment (TME) [12], serving as a unique clinical window to investigate local tumor-host interplay. Previous studies have explored the tumor-host interconnection via cytokine profiling of the pleural space [13, 14], a factor known to influence patient responses to immunotherapy [15]. They have also characterized the broad immune profiles of both malignant [16] and non-malignant [17] PE. However, the cellular composition of MPE does not entirely mirror the immune contexture of the primary tumor tissue [18]. It is well-established that the MPE microenvironment is highly immunosuppressive, impairing T cell cytotoxicity and enabling tumor cells to evade immune surveillance [19, 20].

Among the critical mediators of this immunosuppression, extracellular vesicles have emerged as potent regulators of the TME [21]. Tumor-derived EVs (TDEVs) contain functional molecules that dictate immune cell fate, heavily influencing lung cancer progression. Growing evidence demonstrates that differentially expressed proteins within MPE-derived EVs exert cytotoxic effects on immune populations [22], while TDEVs can rapidly alter immune cell function by mediating intracellular signaling and phosphorylation cascades across various cancers [23, 24]. Similarly, soluble molecules in lung cancer MPE have been shown to impact immune responses, including neutrophil viability and degranulation [25]. Despite these insights, the precise temporal dynamics and holistic proteomic rewiring of peripheral immune cells upon exposure to diverse PE microenvironments remain poorly understood.

Expanding our previous proteomics work on liquid biopsies, including bronchoalveolar lavage [26] and PE [27], the present study aims to elucidate the functional and proteomic aberrations specific to immune cells induced by MPE versus non-malignant PE. To achieve this, we established an *ex vivo* temporal model utilizing healthy donor (HD) peripheral blood mononuclear cells (PBMCs) exposed to MPE from patients with lung adenocarcinoma, benign exudative, and transudative effusions. Time-resolved mass spectrometry-based proteomics revealed profound temporal immune rewiring, with functional enrichment analysis highlighting the critical involvement of EV-related pathways. To mechanistically dissect these findings, we further investigated the influence of EVs secreted by lung cancer cell lines on HD PBMC function. Utilizing global phospho-proteomics, we comprehensively mapped the early phosphorylation events and kinase signaling networks triggered by TDEV treatment. Ultimately, this time-resolved multi-omics approach provides high-resolution insights into the rapid communication between tumor cells and the peripheral immune system, identifying novel mechanistic targets for future intrapleural immunotherapies.

MATERIAL AND METHODS

Clinical Characteristics of cohort participants

MPE and non-MPE samples were obtained from nine patients: three lung adenocarcinoma (LUAD) patients with exsudative MPE; three heart failure patients with transudative PE (TPE); and three pneumonia patients with benign parapneumonic effusion (BEPE), according to Light's criteria. Lung adenocarcinoma was cytologically confirmed, and MPE was defined by the presence of malignant cells on pleural fluid cytology.

The study protocol was approved by the NOVA Medical School Ethics Committee (Registry No. 85/2018/CEFCM) and the ethics committee of CUF Descobertas Hospital, Lisbon Portugal. The research adhered to the ethical guidelines of the Declaration of Helsinki, ensuring all patient information was anonymized, and written informed consent was obtained from all participants or their legal representatives.

Pleural fluid consisted of residual material obtained during clinically indicated diagnostic thoracentesis; no additional procedures were performed for research purposes. Exclusion criteria included patients under 18 years of age, individuals with severe cognitive impairment, and those with a critical performance status unable to provide informed consent. Pleural fluid samples were collected in sterile 50 mL tubes during thoracentesis. The samples underwent low-speed centrifugation ($800 \times g$) to pellet the cells, and both the cell pellet and supernatant were subsequently stored at -80°C until further analysis.

Isolation of PBMCs from HDs

Blood samples were collected in EDTA tubes. PBMCs were isolated by density gradient centrifugation (Ficoll, Merck Millipore, L-6715), and freshly isolated cells were used for all subsequent *ex vivo* experiments. Briefly, whole blood was diluted with phosphate-buffered saline (PBS, 1 $\times$) at a 1:1 ratio in a 50 mL Falcon tube and mixed thoroughly. The diluted blood was then carefully layered onto the Ficoll gradient using a Pasteur pipette at a 1:2 ratio (Ficoll: blood). Samples were centrifuged at $1200 \times g$ for 30 min at room temperature with the centrifuge brake off to prevent phase mixing. The mononuclear cell layer, visible as a whitish ring at the plasma-Ficoll interface, was carefully collected using a Pasteur pipette and transferred to a new tube. The cells were washed by adding 20 mL of PBS (1 $\times$) followed by centrifugation at $700 \times g$ for 5 min. The supernatant was discarded, and this washing step was repeated twice. Finally, the isolated PBMCs were counted using a Neubauer chamber with Trypan Blue exclusion and plated for subsequent co-culture analyses at 6, 24, and 48-h time points.

Cell culture

PBMCs isolated from healthy donors (HDs) were cultured in RPMI-1640 medium (Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, according to the manufacturer's instructions, prior to co-culture with pleural fluids. The A549 human lung adenocarcinoma cell line (ATCC, CRM-CCL-185) was cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, following the manufacturer's protocol. Cell lysis was performed using a RIPA buffer containing 2% SDS. The resulting lysates were sonicated using a Branson sonicator and cleared by centrifugation at $14,000 \times g$ for 20 min at 4°C .

***Ex vivo* co-culture of PBMCs with sEVs and IEVs**

To investigate immune regulation mediated by tumor-derived small EVs (sEVs) and large EVs (IEVs) *ex vivo*, freshly isolated PBMCs were seeded in 96-well plates at a density of 5×10^4 cells/well in 200 μL of RPMI-1640 medium supplemented with 10% EV-depleted FBS and 1% penicillin/streptomycin. To determine the optimal EV-to-cell ratio, PBMCs were treated with varying doses of EVs (10, 20, and 40 μg) and incubated for 24 h. Control conditions included PBMCs cultured in standard medium (RPMI-1640 with regular 10% FBS), conditioned medium, and EV-depleted medium. All 24-h conditions and dose-optimization experiments were assessed in three independent experiments, performed in triplicate. Separately, for the evaluation of rapid signaling dynamics via

phosphoproteomic and short-exposure immunoblotting analyses, PBMCs were incubated with EVs for 10 min, 30 min, and 2 h in a single exploratory experiment. Following this kinetic incubation, the PBMCs were washed twice with PBS (1×) and harvested.

***Ex vivo* viability and cytotoxicity assays of PBMCs**

Cell viability was assessed using the Trypan Blue exclusion method. PBMCs were seeded in 96-well culture plates at a density of 2.5×10^5 cells/mL. Following incubation with various types of PE (malignant and non-malignant) for 6, 24, and 48 h, 10 μ L aliquots of the cell suspension were mixed with an equal volume (10 μ L) of 0.4% Trypan Blue solution in PBS (1×) (Invitrogen, Italy). Viable (Trypan Blue-negative) cells were counted using a Neubauer chamber. All measurements were performed in triplicate for each time point.

The cytotoxic effects of PE and TDEVs on PBMCs were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay, which measures the ability of viable cells to reduce MTT into a spectrophotometrically detectable formazan dye. PBMCs were suspended at a density of 2.5×10^5 cells/mL and cultured in 96-well plates in a final volume of 200 μ L/well under the various PE conditions. After 24 and 48 h of incubation, 10 μ L of MTT solution was added to each well, and the plates were incubated for an additional 3 h at 37 °C in a humidified 5% CO₂ atmosphere. The resulting formazan crystals were solubilized by adding 150 μ L of MTT solvent (0.1 M HCl in isopropanol) to each well. Absorbance was measured at 590 nm, with a reference wavelength of 630 nm, using a Synergy HT microplate reader (BioTek). PBMCs cultured in standard complete medium served as the control for all conditions.

Flow Cytometry (FACS)

To evaluate immunophenotypic changes, PBMCs were collected 24 h post-exposure to the various pleural fluid conditions (MPE, TPE, and BEPE). After washing with cold PBS (1×), the samples were incubated for 15 min with specific monoclonal antibody (mAb) panels. For T cell evaluation, the eight-color DryFlowEx ACT T Screen kit (Cat. No. ED7705, EXBIO Praha, a.s., Vestec, Czech Republic) was utilized. This pre-formulated panel includes anti-CD45-Pacific Blue™ (clone 2D1), anti-CD3-PE-Cy™7 (clone SK7), anti-CD4-FITC (clone MEM-241), and anti-CD8-Pacific Orange™ (clone MEM-31), alongside activation and exhaustion markers anti-HLA-DR-PerCP-Cy™5.5 (clone L243), anti-CD38-APC-Cy™7 (clone HIT2), anti-PD-1-PE (clone EH12.2H7), and anti-CXCR5-APC (clone J252D4). For B cell evaluation, the seven-color DryFlowEx ASC Screen kit (Cat. No. ED7704, EXBIO Praha, a.s., Vestec, Czech Republic) was used following gating according to the manufacturer's instructions. This panel consists of anti-CD45-Pacific Blue™ (clone 2D1), anti-CD19-PE-Cy™7 (clone LT19), anti-IgD-FITC (clone IA6-2), anti-CD27-PE (clone LT27), anti-CD38-APC-Cy™7 (clone HIT2), anti-CD21-APC (clone LT21), and anti-CD24-PerCP-Cy™5.5 (clone SN3). Following staining, cells were processed using a lyse-wash protocol with BD FACS Lysing Solution (BD Biosciences, San Jose, CA, USA). Data acquisition was performed on a BD FACSCanto II cell analyzer utilizing FACSDiva Software v8.0.1 (BD Biosciences). Data analysis was conducted using FlowJo software v10 (FlowJo, LLC).

The gating strategy applied to characterize the major lymphocyte subsets is illustrated in **Fig. S1**. First, doublets were eliminated (FSC-A vs. FSC-H/W). Single cells were then gated based on forward scatter (FSC) and side scatter (SSC) properties to identify the lymphocyte population, followed by the selection of CD45⁺ cells. For T cell subtyping, CD3⁺ cells were selected and subsequently divided

into CD4⁺, CD8⁺, and double-negative (DN) T cell subsets. Within each of these specific T cell populations, the expression of activation (CD38⁺, HLA-DR⁺) and exhaustion (PD-1⁺, CXCR5⁺) markers was quantified. For B cell subtyping, total CD19⁺ cells were identified from the lymphocyte gate and further evaluated for the relevant subset markers.

Isolation of Extracellular Vesicles (EVs)

EVs were isolated from conditioned medium (CM) of A549 cells cultured in RPMI 1640 supplemented with 10% EV-depleted FBS (prepared by ultracentrifugation at 100000 $\times g$ for 18h, 4°C) for 72 h (yielding approximately 48–50 $\times 10^6$ cells). The culture medium was supplemented with 10% EV-depleted FBS (prepared via ultracentrifugation). To remove cells and cellular debris, the CM was initially centrifuged at 300 $\times g$ for 10 min at 4 °C, followed by a centrifugation at 3000 $\times g$ for 20 min at 4 °C. The clarified supernatant was transferred to a new tube and centrifuged at 12,000 $\times g$ for 60 min at 4 °C to pellet the large EVs (lEVs). The lEV pellet was resuspended in ice-cold PBS (1 $\times$). Subsequently, the supernatant was carefully transferred to new tubes and ultracentrifuged at 100,000 $\times g$ for 120 min at 4 °C using a Beckman Coulter Optima L-90K ultracentrifuge equipped with a Type SW 32.1 Ti rotor (k-factor: 229) to pellet the sEVs (sEVs). Following ultracentrifugation, the sEV pellet was resuspended in PBS (1 $\times$). The protein concentration of the EV suspensions was determined using a BCA colorimetric assay, and the isolated EVs were stored at –80 °C until further use.

Nanoparticle tracking analysis (NTA)

The size distribution and concentration of the isolated EVs were determined using a NanoSight NS300 instrument (Malvern Panalytical, Malvern, UK) equipped with a 488 nm laser and an sCMOS camera. EV samples were diluted in PBS (1 $\times$) to a final volume of 1 mL to achieve an optimal analysis concentration of 1 $\times 10^8$ to 2 $\times 10^9$ particles/mL. Samples were loaded into the sample chamber via a syringe pump operating at a continuous flow rate of 20 μ L/min. The camera focus was manually adjusted for each sample to achieve optimal particle visualization. For each measurement, five 60-second videos were captured under the following standardized settings: temperature, 25 °C; viscosity, 0.9 cP; and camera level, 13–14 in light scatter mode. Captured videos were analyzed using NanoSight NTA Software version 3.4 (Build 3.4.4) with the detection threshold set to 5.

PBMC co-culture model with different PE conditions

To determine the optimal ratio of pleural fluid to culture medium, freshly isolated PBMCs were seeded at a density of 2.5 $\times 10^5$ cells/mL in 96-well plates. Cells were cultured in a final volume of 200 μ L of RPMI-1640 medium (supplemented with 10% FBS and 1% penicillin/streptomycin) containing 25%, 50%, 75%, or 100% (v/v) pleural fluid. PE samples derived from patients with lung adenocarcinoma (MPE), heart failure (transudative PE), and pneumonia (exudative PE) were evaluated. The cells were incubated for 24 and 48 h at 37 °C in a 5% CO₂ atmosphere. Control wells contained PBMCs cultured in standard supplemented RPMI-1640 medium without pleural fluid. Based on these optimization steps, the final co-culture experiments with MPE and non-malignant PE were performed by plating Trypan Blue-counted PBMCs for exposure times of 6, 24, and 48 h.

Immunoblotting Analysis

For immunoblotting, PBMC were lysed in RIPA buffer supplemented with protease inhibitors. Following lysate clearance by centrifugation, protein concentrations were determined using the Pierce™ BCA Protein Assay Kit. Equal amounts of protein (20 μ g per sample) were mixed with Laemmli buffer, denatured by heating at 95 °C for 10 min, and separated by 10% SDS-PAGE. Proteins

were then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) and subsequently incubated overnight at 4 °C with the following primary antibodies: anti-CD63 (1:2000; SICGEN, Portugal), anti-Alix (1:2000; SICGEN, Portugal), anti-phospho-tyrosine (P-Tyr-102, #9416, Cell Signaling Technology), and anti- β -actin (MA5-15739, Thermo Fisher Scientific) as a loading control (1:10000). Following primary incubation, the membranes were washed and incubated with the appropriate HRP-conjugated secondary antibodies, donkey anti-goat (1:10000) or goat anti-mouse (1:10000), for 1 h at room temperature. Immunoreactive bands were visualized using a ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA). Band intensity was quantified using ImageJ software, and protein expression was normalized to either Ponceau S total protein staining or β -actin expression from the same sample.

Peptide preparation

PBMCs and extracellular vesicles preparation extracts, were processed using the filter-aided sample preparation (FASP) method [28]. Briefly, proteins were reduced with 0.1 M dithiothreitol (DTT) and loaded onto 30 kDa molecular weight cutoff spin filter columns (Millipore, Billerica, MA, USA). The samples were exhaustively washed with 8 M urea in 0.1 M HEPES buffer (pH 8.0), followed by alkylation with 50 mM iodoacetamide (IAA). After equilibrating the columns with ammonium bicarbonate buffer, protein digestion was performed overnight at 37°C using sequencing-grade trypsin (Promega, Madison, WI, USA).

Mass Spectrometry (MS) Analysis

Peptide samples (5 μ L) were analyzed using a Dionex RSLCnano 3000 system coupled to an Exploris 480 Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK), as previously described [29]. Briefly, samples were loaded onto a custom-made fused capillary precolumn (2 cm length, 360 μ m outer diameter [OD], 75 μ m inner diameter [ID]) packed with ReproSil-Pur C18 5.0 μ m resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) at a flow rate of 5 μ L/min for 6 min. Trapped peptides were subsequently separated on a custom-made fused capillary analytical column (25 cm length, 360 μ m OD, 75 μ m ID) packed with ReproSil-Pur C18 1.9 μ m resin (Dr. Maisch GmbH). Separation was achieved at a flow rate of 250 nL/min using a 56-min linear gradient from 13% to 32% solvent B (0.1% formic acid in 80% acetonitrile) against solvent A (0.1% formic acid in water).

Mass spectra were acquired in positive ion mode utilizing a data-dependent acquisition (DDA) strategy. The instrument automatically alternated between a full Orbitrap survey MS scan (mass range 350–1200 m/z) and higher-energy collision dissociation (HCD) fragmentation, followed by Orbitrap detection of fragment ions with a cycle time of 2 s between master scans. The maximum injection times for both MS and MS/MS were set to Auto. HCD fragmentation in the ion routing multipole was performed with a normalized collision energy of 30% and an ion selection threshold of 10,000 counts. Sequenced precursor ions were dynamically excluded for 30 s.

MS database search

The acquired LC-MS/MS raw data were processed and searched using VEMS [30] and MaxQuant [31] software platforms against the UniProt Human database (3AUP000005640). The search parameters allowed for a maximum of four missed tryptic cleavages. For the VEMS analysis, the mass tolerance was set to 5 ppm for precursor ions and 0.01 m/z for fragment ions. Carbamidomethylation of cysteine was set as a fixed modification. Variable modifications included methionine oxidation, N-terminal protein acetylation, lysine acetylation, di-glycine remnant

(ubiquitination tag), and phosphorylation of serine, threonine, and tyrosine residues. All other search parameters in MaxQuant were kept at their default settings. A false discovery rate (FDR) threshold of 1% was applied at both the peptide and protein levels.

Quantitative proteomics analysis

For the PBMC co-cultures treated with pleural effusion (PE), the cohort consisted of 36 biological samples (n=36), resulting in a total of 72 LC-MS measurements. For the remainder of the study, the sample size was n=12, yielding 24 LC-MS measurements. Intensity-based absolute quantification (iBAQ) values were estimated from total ion counts by dividing by the number of theoretical canonical tryptic peptides for each given protein (excluding theoretical peptides with missed cleavages). All quantitative data exported from MaxQuant were analyzed using the R statistical programming environment. Label-free protein expression values were preprocessed by filtering out common MS contaminants, followed by a $\log_2(x+1)$ transformation and quantile normalization. Statistical comparative analyses were performed using the *limma* R package [32]. Data visualization, including heatmaps, box plots, and line plots, was conducted using the *ggplot2* R package [33]. Principal component analysis (PCA) was performed using the *FactoMineR* R package [34], and volcano plots were generated using the *EnhancedVolcano* R package [35]. P-values were adjusted for multiple testing using the Benjamini-Hochberg (BH) false discovery rate (FDR) method [35].

Phosphopeptide enrichment

For phosphoproteomic analysis, precipitated protein extracts were reduced, alkylated with iodoacetamide, and digested with trypsin according to the manufacturer's protocol (Thermo Fisher Scientific). The resulting tryptic peptide mixtures (approximately 200 µg) were subjected to phosphopeptide enrichment using titanium dioxide (TiO₂) chromatography, essentially as previously described[36]. The purified phosphopeptide mixtures were subsequently analyzed by nano-LC-MS/MS virtually as described above with the following modification: The linear gradient (56 min) was from 10% to 28% solvent B (0.1% formic acid in 80% acetonitrile) against solvent A (0.1% formic acid in water).

Phosphoproteomics data processing

Peptide and phosphopeptide identifications and intensities were derived using the SAGE [37] search engine (version 0.14.6) against the human UniProt reference proteome (UP000005640). The search parameters included a precursor ion mass tolerance of 20 ppm and a fragment ion mass tolerance of 0.05 Da. Charge states ranging from 2+ to 4+ were considered. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation was set as a variable modification for all datasets. For the phosphoproteomic analysis, phosphorylation of serine, threonine, and tyrosine residues (S/T/Y) was included as an additional variable modification. Identifications were filtered to a strictly controlled false discovery rate (FDR) of 1% at the peptide-to-spectrum match (PSM) and protein levels. Signal intensities of the identified peptides and phosphopeptides were extracted and normalized across samples. Label-free quantitative values were preprocessed by filtering out common MS contaminants, followed by a $\log_2(x+1)$ transformation and quantile normalization. Statistical significance between experimental groups was evaluated using an unpaired, two-tailed Student's t-test, followed by Benjamini-Hochberg correction for multiple hypothesis testing to generate adjusted p-values.

Functional enrichment analysis (FEA) of proteomic and phosphoproteomic data

Functional enrichment analysis (FEA) was performed using a hypergeometric probability test, essentially as previously described [38]. Significantly up- and down-regulated proteins ($p < 0.05$) were subjected to enrichment analysis across multiple categories, including Gene Ontology (GO) Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) terms, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Kinase prediction and sequence motif analysis

To predict the upstream kinases responsible for each identified phosphosite, the PhosphoSitePlus database was utilized [39]. To generate kinase sequence logos for serine (S), threonine (T), and tyrosine (Y) phosphorylation events, 15-amino acid sequence windows surrounding the modified residues (denoted as pS, pT, or pY) were extracted. These site-specific sequences were then used as inputs for kinase-substrate prediction and motif generation via the Kinase Library tool [40] followed by enrichment analysis in R using the cumulative hypergeometric function. Additionally, Kinase-Substrate Enrichment Analysis (KSEA) was performed to estimate the relative changes in kinase activity across the experimental conditions [41]. KSEA derives a normalized kinase z-score by evaluating the quantitative fold-changes of the empirically mapped downstream substrates for each specific kinase network.

Statistical analysis of immunoblotting data

Statistical analysis and data visualization of immunoblotting results were performed using R software (R version, 4.1.2) within the RStudio environment (version 1.3.1073). All immunoblotting experiments were conducted in at least three independent biological replicates. Multiple pairwise comparisons between different clinical effusion phenotypes were evaluated using one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) post-hoc test.

Results

General overview of the study

A schematic overview of the experimental workflow is presented in **Fig. 1**. To elucidate how the PE microenvironment alters peripheral immune cell function, we established an *ex vivo* temporal exposure model using healthy donor (HD) PBMCs. These PBMCs were co-cultured with three distinct clinical PE fluid types: malignant exudate (MPE), benign parapneumonic effusion (BEPE), and transudate pleural effusion (TPE). To capture the temporal dynamics of immune rewiring, treated and untreated control PBMCs were analyzed at 6, 24, and 48 h post-exposure. Readouts for PE exposure included cell viability and cytotoxicity assays, comprehensive immunophenotyping via flow cytometry (FACS), and global MS-based proteomics.

Furthermore, to mechanistically dissect the specific role of tumor-derived factors in this immunosuppressive cascade, HD PBMCs were also exposed to small (sEVs) and large (lEVs) extracellular vesicles isolated from the A549 lung cancer cell line. These EV-treated PBMCs were subsequently analyzed for viability, global proteomic and phosphoproteomic alterations, and early kinase signaling events. Finally, orthogonal immunoblotting was performed to validate the rapid phosphotyrosine signaling alterations identified in the phosphoproteomic datasets.

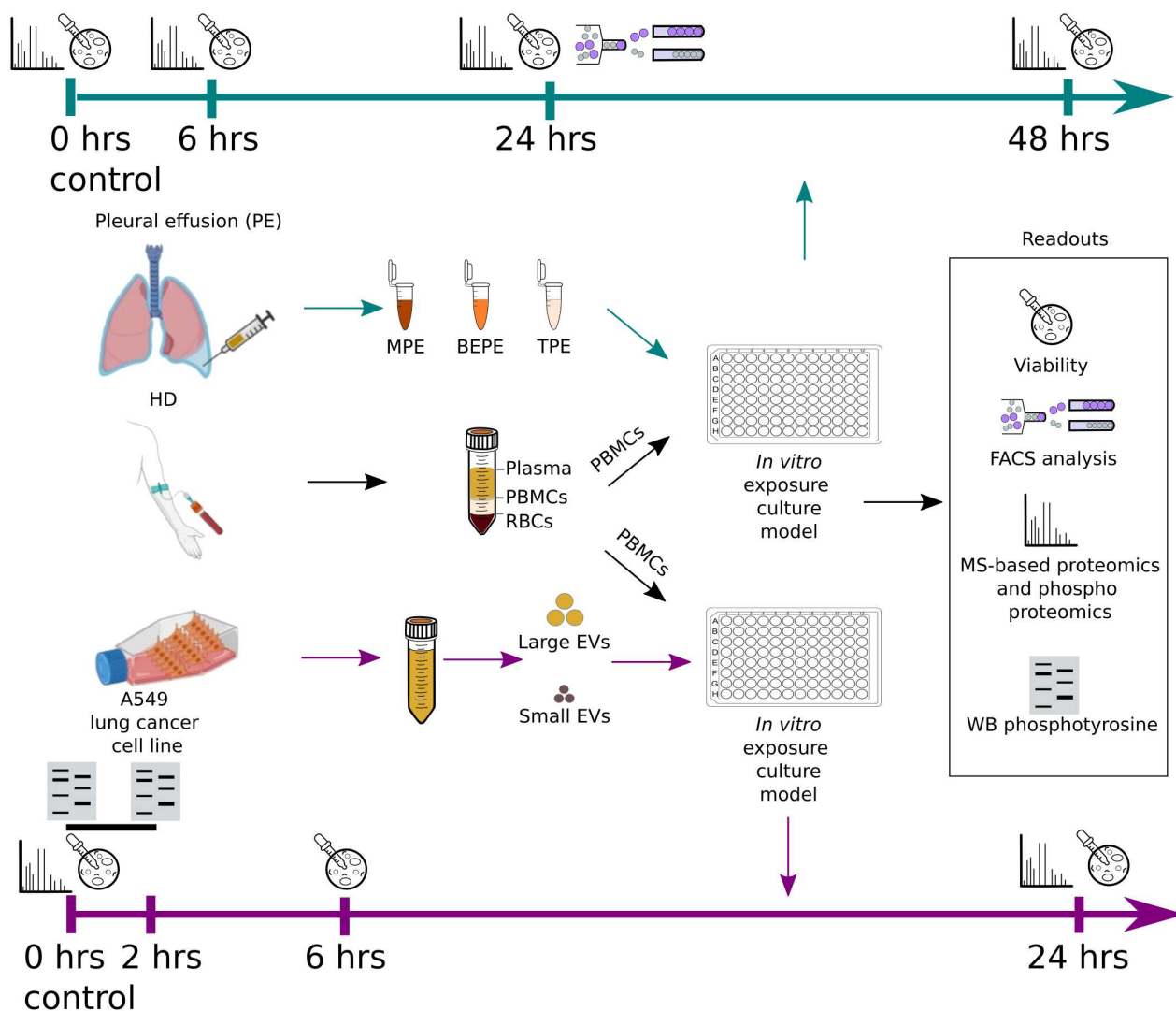


Fig. 1. Schematic overview of the three temporal multi-omics experimental workflows. Freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy donors (HD) were utilized in two distinct ex vivo co-culture models. Top (Clinical Model): PBMCs were exposed to three types of clinical pleural effusions (MPE, BEPE, and TPE) to simulate the tumor microenvironment. Phenotypic and multi-omics data were collected at 0, 6, 24, and 48 hours (hrs) post-exposure. Bottom (Mechanistic Model): To isolate the specific effects of tumor-derived factors, PBMCs were co-cultured with Large and Small extracellular vesicles (EVs) isolated from the A549 lung adenocarcinoma cell line, with early signaling and phenotypic readouts collected at 0, 2, 6, and 24 hrs. Right: The comprehensive readouts key denotes the specific assays (cell viability, flow cytometry: FACS, mass spectrometry (MS)-based proteomics, and western blot: WB phosphotyrosine validation) performed at the indicated time points along each experimental timeline. Black timeline (bottom left) from 0 to 2 hrs is for WB phosphoproteomics experiments.

Patient demographics

A statistical summary of the patients' clinical characteristics is outlined in **Table 1**. In total, nine patients were included for the current study, comprising three patients with stage IV lung adenocarcinoma-associated MPE, three with BEPE, and three with transudative pleural effusion (TPE). BEPE samples were collected from patients with pneumonia-associated pleural effusion, whereas TPE samples were obtained from patients with heart failure.

Table 1. Clinical characteristics of patients. *N* indicates the total number of patients in each subgroup. Continuous variables are presented as Mean (SD); categorical variables are presented as *n* (%).

Characteristic	Transudate PE (Heart Failure)	Benign Exudate PE (Pneumonia)	Malignant Exudate PE (LUAD)
Total numbers (N = 9)	3	3	3
Age (Years)	81.6 (8.7)	73.6 (7.5)	61.0 (12.2)
Gender			
Male	1 (33.3%)	2 (66.7%)	1 (33.3%)
Female	2 (66.7%)	1 (33.3%)	2 (66.7%)
Stage of tumor			
Stage IV	N/A	N/A	3 (100%)
Pleural fluid analysis			
Pleural proteins (g/dL)	2.0 (0.7)	3.86 (0.8)	4.2 (0.8)
Pleural LDH (U/L)	78.6 (5.9)	430.6 (264.6)	337.6 (120.5)
Ethnicity			
Black	0 (0%)	0 (0%)	1 (33.3%)
Caucasian	3 (100%)	3 (100%)	2 (66.7%)
Smoking status			
Smoker	1 (33.3%)	0 (0%)	1 (33.3%)
Ex-smoker	1 (33.3%)	2 (66.7%)	1 (33.3%)
Nonsmoker	1 (33.3%)	1 (33.3%)	1 (33.3%)
Cytology			
Positive	0 (0%)	0 (0%)	3 (100%)
Negative	3 (100%)	3 (100%)	0 (0%)
Status			
Alive	3 (100%)	3 (100%)	0 (0%)
Dead	0 (0%)	0 (0%)	3 (100%)

MPE reduces PBMC viability and metabolic activity in a concentration- and time-dependent manner

To determine the optimal exposure ratio of PE to culture media, PBMCs from HDs were cultured with varying concentrations (25%, 50%, 75%, and 100%) of MPE. Cell viability was evaluated at 24 and 48 hours post-incubation and compared to time-matched control groups grown in media alone. As demonstrated by direct cell counts (**Fig. 2A**) and MTT reduction assays (**Fig. 2B**), PBMC viability decreased significantly in a dose-dependent manner. Compared to the time-matched media controls, cell viability was significantly reduced across all MPE concentrations at both 24 hours ($p < 0.01$) and 48 hours ($p < 0.05$). Notably, at a 50% MPE concentration (a 1:1 mixture of PE and RPMI medium), PBMC viability was significantly impacted ($p < 0.001$) but remained robust enough (above 50%) at both early and late time points to allow for downstream omics analyses. Consequently, this 50% ratio was selected for all subsequent comparative exposure experiments.

Next, we evaluated the temporal effects of different clinical PE phenotypes (MPE, BEPE, and TPE) on immune cell viability using the established 50% exposure model. Following a brief 6-hour exposure, neither direct cell counts nor MTT assays revealed any significant changes in PBMC

viability across the different PE groups compared to the time-matched media control (**Fig. 2C, D**). However, prolonged exposure resulted in marked cytotoxicity. By 24 and 48 hours, exposure to all three PE types significantly reduced PBMC viability and metabolic activity compared to the untreated, time-matched controls. Crucially, MPE exerted the most profound cytotoxic effect; direct pairwise comparisons confirmed that MPE exposure resulted in significantly lower PBMC viability compared to both the BEPE ($p < 0.01$) and TPE ($p < 0.001$) groups at both the 24- and 48-hour marks.

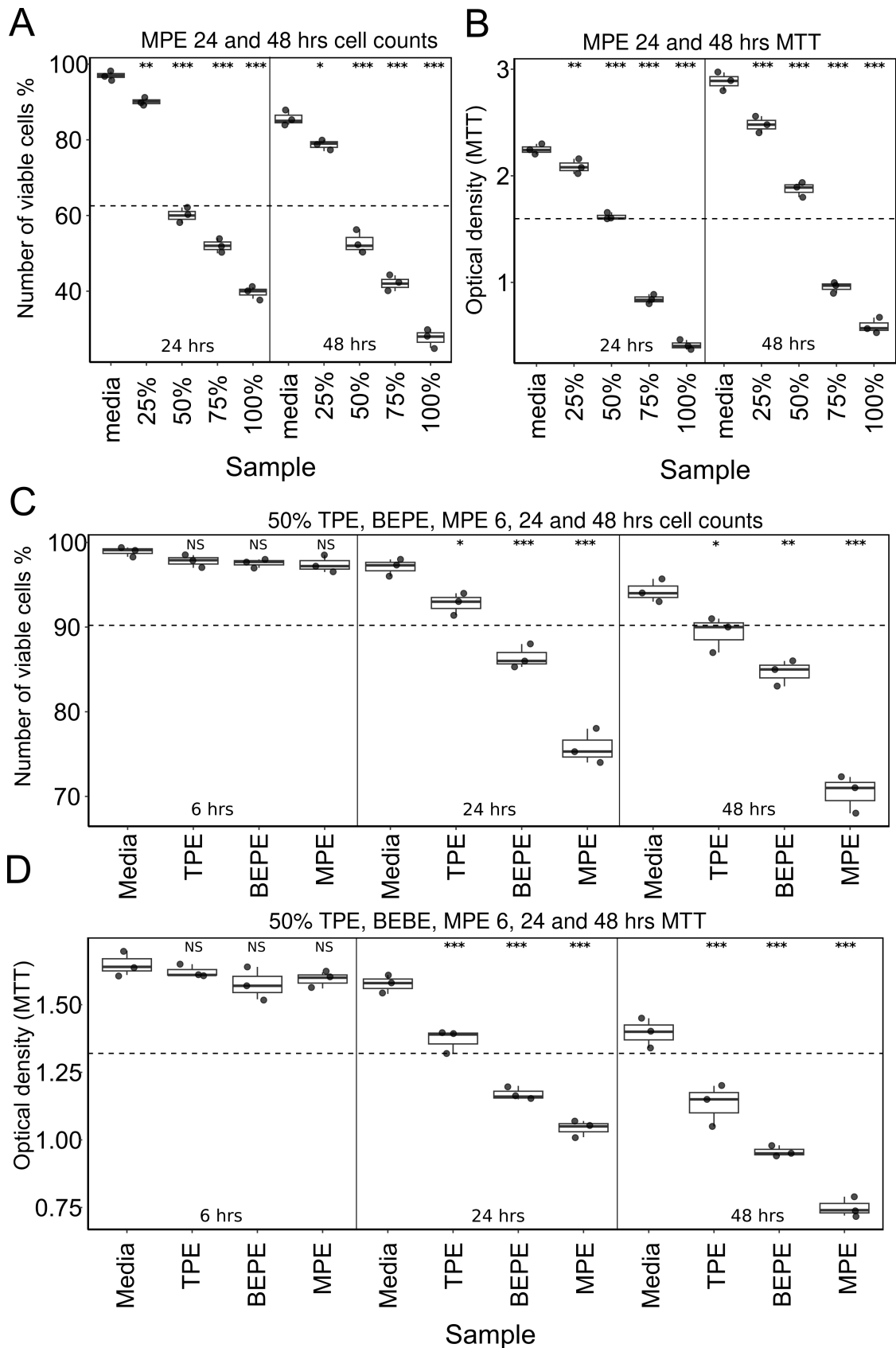


Fig. 2.

PBMC viability and metabolic activity decrease following exposure to pleural effusions. (A) Cell viability determined by direct cell counts and (B) metabolic activity determined by MTT reduction assay of PBMCs exposed to varying concentrations (25%, 50%, 75%, and 100%) of malignant pleural effusion (MPE) for 24 and 48 h. (C) Cell counts and (D) MTT reduction assays of PBMCs exposed to a 50% concentration of different clinical pleural effusion phenotypes (TPE, BEPE, and MPE) at 6, 24, and 48 hours (hrs) time points. For all experiments, treatments were compared to

their respective time-matched media controls to account for baseline temporal variations. The dashed line represents the mean of the corresponding control group. Statistical significance was calculated using a one-way ANOVA followed by Dunnett's multiple comparisons test ($p < 0.05$, $p < 0.01$, *** $p < 0.001$, NS = not significant).*

Immunophenotyping of PBMCs reveals changes in immune contexture after treatment with various types of PE

Lymphocytes are the main type of immune cells in MPE, and its microenvironment is described to modulate the immune transcriptome. To systematically evaluate the impact of PE exposure on peripheral mononuclear immune cells, multiparametric flow cytometry was performed on healthy donor PBMCs cultured for 24 hours in media or challenged with 50% MPE, BEPE, or TPE. A multicolor panel enabled immunophenotyping of activated T lymphocyte subsets including specific populations of circulating follicular T helper cells, activated CD4⁺ and CD8⁺ T cells (**Fig. S1E–L**; **Fig. S2B–C**). Additionally, gating of lymphocytes CD45⁺CD3[−] cells were evaluated and ascribed to NK cells or B cells (**Fig. S1H**). On the other hand, a population distinct from lymphocytes, (SSC-A low FSC-A low), SSC-A high FSC-A high cells were ascribed to monocytes/macrophages (**Fig. S1**, Mono-MAC Gating A). The comprehensive gating strategy and high-dimensional t-SNE landscapes illustrating global population distributions across the different PE conditions are provided in **Supplementary Figs. S1 and S2**.

Fig. 3 depicts the overall distribution of peripheral blood immune cells challenged by TPE, BEPE, and MPE. MPE exposure was associated with changes across several immune-cell populations, most notably within the non-T cell lymphocyte compartment (CD3[−] lymphocytes). This population experienced a significant reduction in frequency within the MPE microenvironment compared to TPE ($p = 0.035$), alongside a marginally significant decrease in BEPE ($p = 0.089$) (**Fig. 3A**). Concurrently, the activation status of these CD3[−] cells (CD38⁺ Activation) exhibited a downward trend in MPE compared to TPE ($p = 0.079$) (**Fig. 3B**). Further profiling of this non-T cell compartment revealed a distinct phenotypic shift, while TPE-challenged cells maintained a dominant CD38-bright population, exposure to BEPE and MPE trended toward a depletion of this highly active state in favor of CD38-dim and CD38-negative phenotypes (**Fig. S3B**).

While general T-cell activation markers (CD38⁺/HLA-DR⁺) remained largely unaltered across the conditions (**Fig. S3A**), the T-cell compartment showed distinct shifts toward specific antigen-experienced and exhausted phenotypes. The proportion of the antigen-experienced cTfh-like CD4⁺ subpopulation (PD-1⁺/CXCR5⁺) displayed a clear stepwise increase from TPE to BEPE, peaking in the malignant MPE microenvironment with a marginally significant enrichment compared to TPE ($p = 0.094$) (**Fig. 3C**). Meanwhile, exhausted CD8⁺ T cells followed a similarly elevated distribution, showing a marginal increase in BEPE relative to TPE ($p = 0.065$) (**Fig. 3D**). Despite these distinct population shifts, the overall CD4⁺/CD8⁺ ratio remained stable across the effusion types (**Fig. 3E**).

Furthermore, monocytes experienced a minor numerical reduction in frequency within the MPE microenvironment compared to benign counterparts (**Fig. 3F**). However, evaluation of the surviving monocytes revealed that their activation status (CD38⁺) remained robust and broadly comparable across all effusion conditions (**Fig. S3C**). Taken together, these data indicate that MPE establishes an immunosuppressive microenvironment characterized by a decrease in non-T cells (CD3[−], mainly B and NK cells) and a minor decreasing trend in monocytes, alongside an increase in CD4⁺ and CD8⁺ T-cell populations.

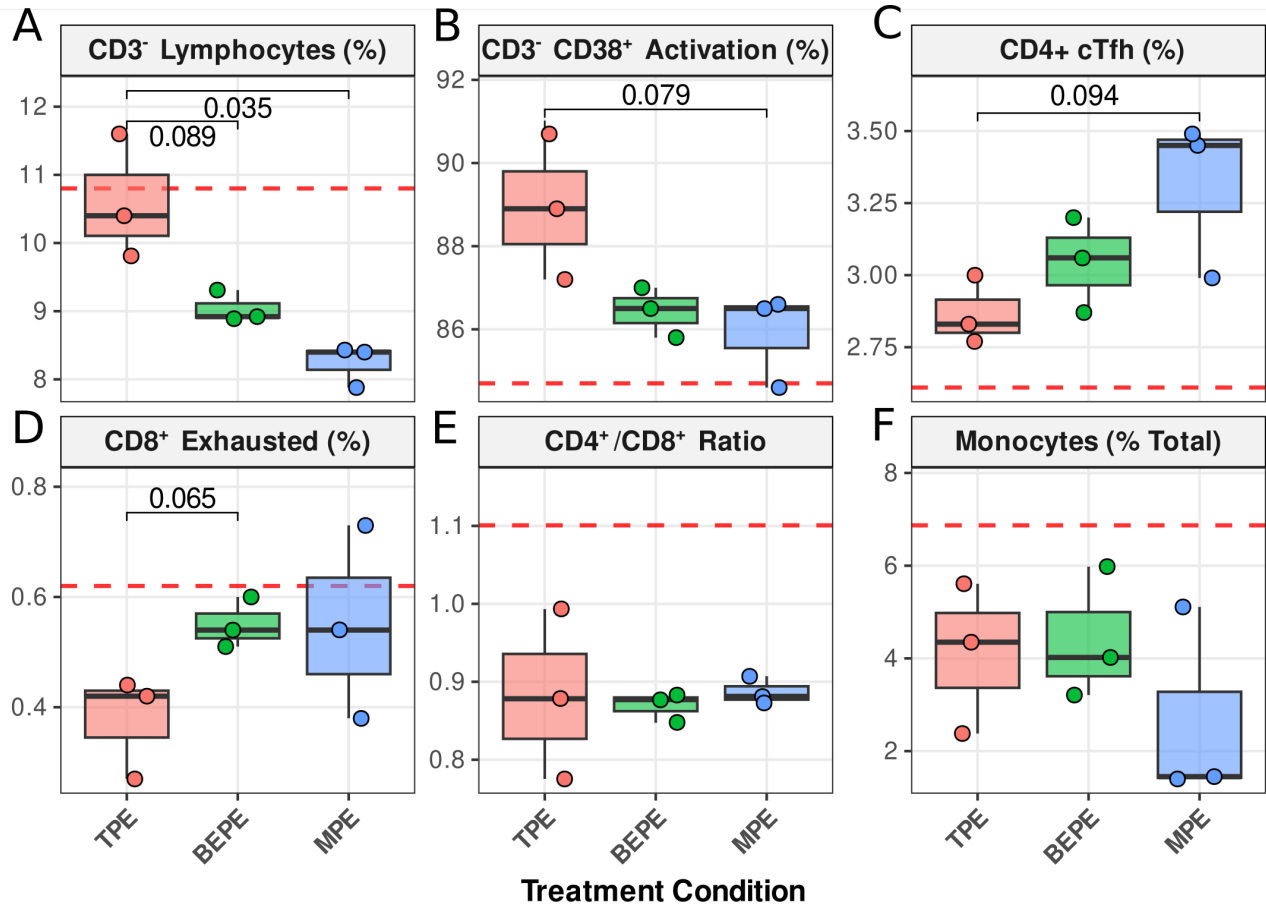


Fig. 3. Distribution of major immune populations and exhausted T-cell subsets across pleural effusion types. Boxplots show the relative abundance of core immune subsets across healthy donor PBMCs challenged with acellular pleural effusion collected from patients with TPE (transudative PE), BEPE (benign parapneumonic effusion), and MPE (malignant pleural effusion). (A) CD3⁺ lymphocyte frequency expressed as a percentage of total lymphocytes. (B) CD38⁺ activation expressed as a percentage of CD3⁺ lymphocytes. (C) Circulating Tfollicular helper (cTfh) CD4⁺ T cell subpopulation expressed as a percentage of CD4⁺ T cells. (D) Exhausted CD8⁺ T cell subpopulation expressed as a percentage of CD8⁺ T cells. (E) Ratio of CD4⁺ to CD8⁺ T cells. (F) Monocyte frequency expressed as a percentage of total events. Individual dots represent biological replicates (n=3 per experimental condition), and boxes indicate the interquartile range with the median shown as the center line. The horizontal dashed red line represents the baseline value for the unchallenged media control (n=1). Significant and marginally significant pairwise comparisons are denoted by adjusted p-values above the corresponding brackets.

Global proteomic profiling reveals rapid and distinct immune sensing of pleural effusions

To elucidate the temporal proteomic dynamics of immune cells exposed to the tumor and inflammatory microenvironments, we established an ex vivo model analyzing PBMC lysates at 0, 6, 24, and 48 hours post-exposure to TPE, BEPE, and MPE pleural effusions (**Tables S1-9**). In total, 72 LC-MS runs were performed across biological and technical replicates. Principal component analysis

(PCA) of the global quantified proteome immediately revealed a remarkable and distinct separation of PBMCs treated with all three effusion types compared to the media control (**Fig. 4A**). This early segregation indicates that immune cells rapidly sense and profoundly alter their intracellular protein landscapes in response to specific effusion microenvironments.

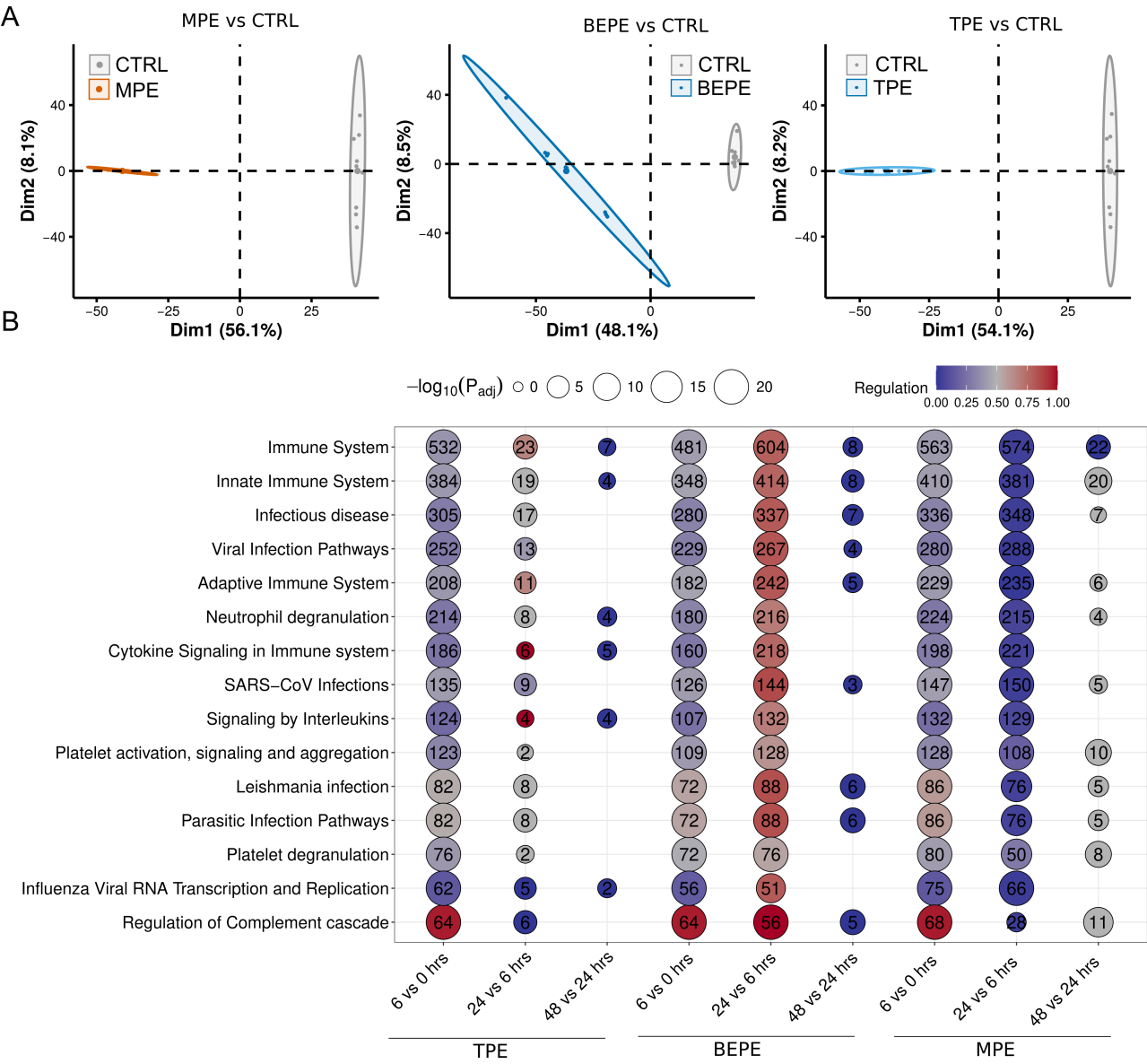


Fig. 4. Global and temporal proteomic dynamics of PBMCs exposed to distinct pleural effusions. (A) Principal component analysis (PCA) plots demonstrating the global proteomic separation of PBMCs treated with malignant pleural effusion (MPE), benign parapneumonic effusion (BEPE), and transudative pleural effusion (TPE) relative to media controls (CTRL). (B) Functional enrichment analysis (Reactome) mapping the temporal trajectory of immune system pathways following exposure to TPE, BEPE, and MPE. The x-axis represents the sequential temporal comparisons for each effusion type. Bubble size reflects the $-\log_{10}(\text{adjusted } p\text{-value})$ for enrichment, capped at a maximum of 20. The color gradient indicates the fraction of regulated proteins that are up-regulated (red = 1.0) versus down-regulated (blue = 0.0), with grey indicating an equal distribution (0.5). Text labels within the bubbles denote the total number of significantly regulated proteins mapping to that specific pathway.

Temporal dynamics expose MPE-induced immunosuppression

Limma contrasts across timepoints (6 vs 0 h, 24 vs 6 h, and 48 vs 24 h) were established to map the functional trajectories of these immune responses. Reactome pathway enrichment revealed distinct, time-profiled patterns highly dependent on the effusion phenotype (**Fig. 4B**). Transudate exposure induced a moderate immune response that peaked primarily within the first 6 hours, featuring complement activation, before stabilizing (**Fig. 4B**, left).

In stark contrast, exposure to BEPE elicited a robust, prolonged immune activation. By 24 hours, BEPE exposure triggered a massive shift toward global up-regulation of immune system proteins, persisting into the 48-hour mark (**Fig. 4B**, middle). While MPE exposure triggered a similarly strong initial response at 6 hours, its trajectory sharply reversed; by 24 and 48 hours, MPE exposure drove a predominant down-regulation of immune-related proteins (**Fig. 4B**, right).

Malignant and benign exudates drive divergent apoptotic and immune trajectories

Given the clinical challenge of distinguishing benign from malignant exudates, we directly compared their temporal proteomic profiles (**Fig. 5A**, **Tables S10-12**). The quantitative analysis revealed a striking biphasic dynamic: the most profound differences occurred at 6 and 24 hours, characterized by a massive, hyperactive up-regulation of proteins in MPE-exposed PBMCs relative to BEPE (2,023 and 2,338 up-regulated proteins, respectively) (**Fig. 5B**). By 48 h, the proteomic divergence between MPE- and BEPE-exposed PBMCs had markedly collapsed, yielding a drastically reduced number of differentially expressed proteins.

PCA biplots mapped these distinct temporal trajectories, identifying specific protein drivers responsible for the early divergence between exudate types. Notably, ACTBL2 and DEFA3 are strongly associated with the BEPE response, whereas IGLV1-51 and PRAME are consistently clustered with MPE exposure (**Fig. 5C**).

Functional enrichment analysis further contextualized this early divergence, revealing completely opposed survival trajectories. At 6 and 24 hours, MPE exposure is massively and uniquely enriched for potent drivers of programmed cell death, including apoptosis, ferroptosis, and PANoptosis, appearing as up-regulated networks relative to BEPE (**Fig. 5D**). Furthermore, comparative enrichment of biological processes (**Fig. 5E**) revealed a distinct mechanistic divergence driving this early stress response. The direct comparison showed that relative to the benign response, MPE-exposed cells exhibit a disproportionate up-regulation of specific metabolic and translational pathways, including the respiratory electron transport chain, translational initiation, and the continuous cross-presentation of exogenous antigens via MHC class I. These networks are intensely up-regulated in the short term compared to BEPE, before undergoing a profound regulatory collapse at 48 hours, characterized by the near-total loss of pathway enrichment and diminished protein counts. Ultimately, this intense, divergent activation proved transient, with the differences between both conditions collapsing as the cells reached functional exhaustion by 48 hours. The true magnitude of this proposed collapse becomes even more evident in the subsequent section, where the cellular response is compared directly to the untreated control baseline at each time point.

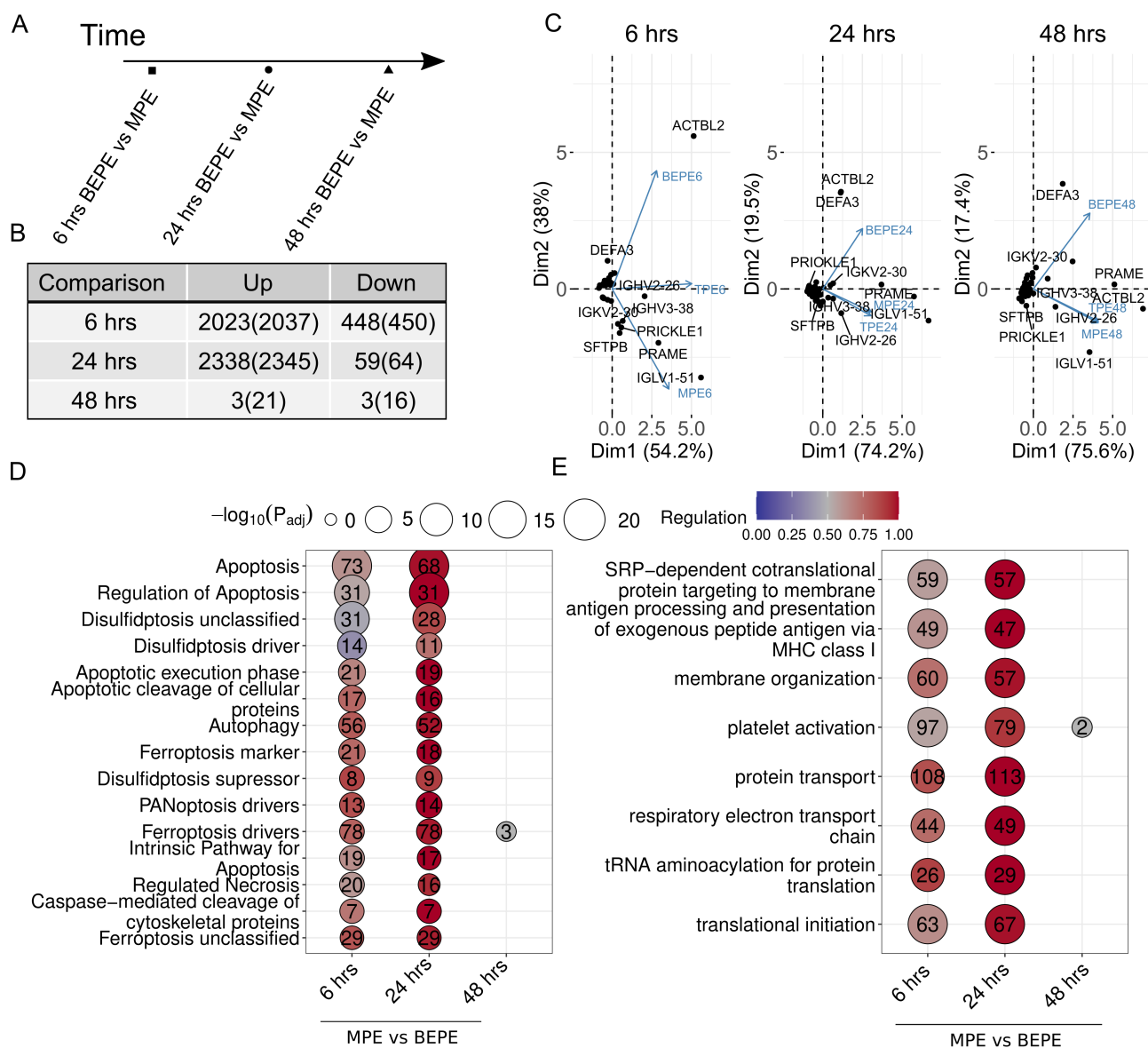


Fig. 5. Direct proteomic comparison of malignant versus benign exudative effusions. (A) Experimental timeline comparing BEPE to MPE exposures. (B) Table detailing the number of significantly up- and down-regulated proteins at 6, 24, and 48 h (unadjusted p -values in parentheses). (C) PCA biplots (Dim1 vs Dim2) identifying specific protein drivers (e.g., ACTBL2, PRAME) separating BEPE and MPE populations across time points. (D) Functional enrichment analysis highlighting the induction of specific cell death pathways (apoptosis, ferroptosis) in MPE compared to BEPE. (E) Comparative enrichment of biological processes highlighting metabolic hyperactivation, translational machinery, and antigen cross-presentation in MPE. For panels D and E, the x-axis represents the temporal comparisons of MPE versus BEPE. Bubble size reflects the $-\log_{10}(\text{adjusted } p\text{-value})$ for enrichment, capped at a maximum of 20. The color gradient indicates the fraction of regulated proteins that are up-regulated (red = 1.0) versus down-regulated (blue = 0.0) in MPE relative to BEPE, with grey indicating an equal distribution (0.5). Text labels within the bubbles denote the total number of significantly regulated proteins mapping to that specific pathway.

MPE exposure is associated with sustained alterations in vesicle-, metabolic-, and immune-related pathways

While longitudinal profiling revealed a highly dynamic, biphasic response that largely collapsed by 48 hours within the MPE-treated population (**Fig. 4**), cross-sectional comparisons against baseline controls demonstrated a profoundly sustained state of pathological dysregulation (**Fig. 6, Table S13-21**). Even as their active response rate slowed, MPE-treated PBMCs did not return to a healthy baseline; instead, they maintained a distinctly suppressed proteomic signature across the entire 48-hour observation window when compared to resting cells.

To define the mechanistic drivers of this MPE-specific response, we analyzed the overall net direction of protein regulation across Cellular Component (CC), Molecular Function (MF), and Biological Process (BP) pathways. This multi-dimensional analysis revealed a structural and metabolic shutdown, evidenced by the deep suppression of the 'mitochondrion' and 'respiratory electron transport chain' nodes (CC and BP), alongside a down-regulation of 'ribosome' and 'translation' machinery (CC, MF, and BP). This systemic shutdown was punctuated by a striking divergence in vesicular dynamics. Specifically, MPE exposure drove a massive net down-regulation of endogenous vesicle machinery, with 'extracellular vesicular exosome' (CC) and 'vesicle-mediated transport' (BP) heavily suppressed. This loss of exosome-related proteins contrasted with the distinct up-regulation of 'blood microparticle' and 'blood coagulation' (BP) signatures, occurring simultaneously with the breakdown of the 'structural constituent of cytoskeleton' (MF) and 'focal adhesion' (CC) networks. Furthermore, critical immune-related functions, including Toll-like receptor 4 signaling and degranulation pathways, exhibited suppression, alongside the sustained dysregulation of apoptotic processes.

Taken together, these data suggest that whole MPE establishes a highly toxic microenvironment that overwhelms PBMCs, driving them into a sustained state of structural and metabolic exhaustion within the observed 48-hour window. Given the profound and specific disruption of the PBMCs' own vesicular machinery, we hypothesized that TDEVs within the complex MPE mixture might be the primary mediators of this systemic collapse. To isolate this variable and determine if cancer EVs alone are sufficient to replicate these immunosuppressive and toxic effects, we next evaluated the response of healthy PBMCs to purified EVs derived from cancer cell lines.

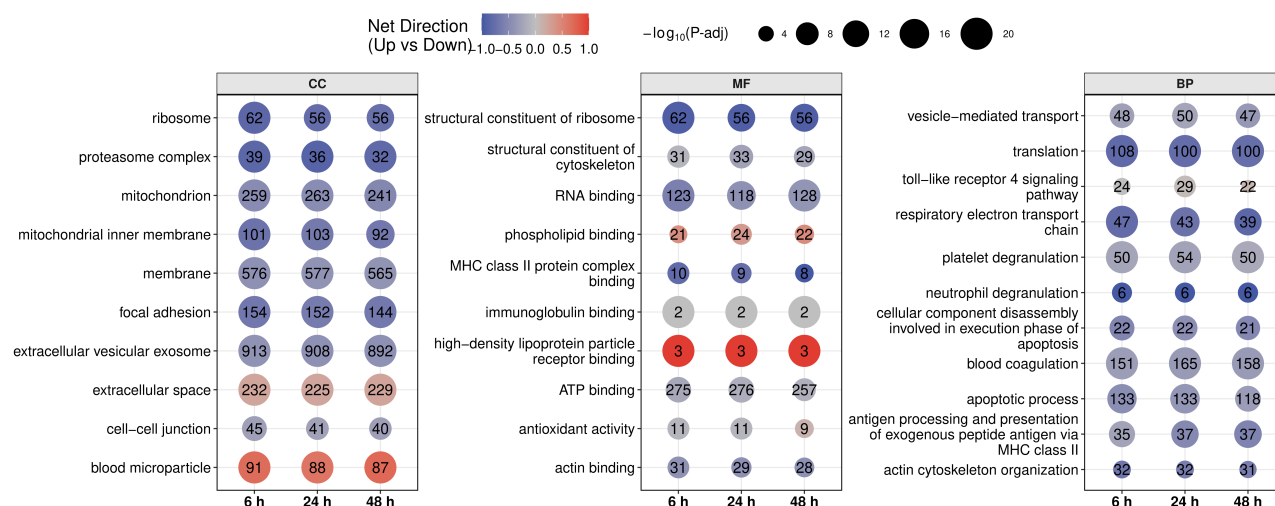


Fig. 6. MPE exposure suppresses endogenous exosome and mitochondrial networks while driving microparticle shedding. Multi-panel dot plot detailing Gene Ontology (GO) functional enrichment analysis across Cellular Component (CC), Molecular Function (MF), and Biological Process (BP) categories. The analysis compares MPE-exposed PBMCs to unexposed baseline controls at 6, 24, and 48 hours. The color gradient represents the Net Direction of protein regulation within each pathway (calculated as the ratio of up-regulated to down-regulated proteins), with red indicating net up-regulation, blue indicating net down-regulation, and grey representing a balanced response. Bubble size corresponds to statistical significance ($-\log_{10}(P\text{-adj})$). The number annotated inside each bubble indicates the total count of differentially expressed proteins (DEPs) associated with that specific GO term. Across all time points, MPE induces a sustained suppression of endogenous exosome machinery, translation, and mitochondrial metabolism, contrasting sharply with the distinct induction of pro-coagulant microparticle shedding and apoptotic processes.

Tumor-derived extracellular vesicles reduce PBMC viability

To functionally validate whether the profound immune alterations observed in clinical MPEs are directly mediated by extracellular vesicles, we established an *ex vivo* co-culture model using TDEVs isolated from the A549 lung cancer cell line. Crucially, our experimental design utilized three distinct non-vesicular controls to isolate the specific effects of the EVs: fresh culture media (ctrl), complete conditioned media (CM) containing all tumor secretomes, and EV-depleted conditioned media. TDEVs were fractionated from the A549 CM via differential centrifugation into small (sEV) and large (IEV) populations. Successful isolation was biochemically validated via immunoblotting, which confirmed the robust enrichment of canonical small EV markers (Alix and CD63) specifically within the sEV fraction compared to the cell lysate, media, and IEV controls (**Fig. 7A**). Nanoparticle Tracking Analysis (NTA) confirmed the expected size distributions, with sEVs peaking sharply between 100 to 150 nm and IEVs exhibiting a broader profile extending up to 400 nm (**Fig. 7B**).

To evaluate the direct cytotoxic impact of these isolated TDEVs, healthy donor PBMCs were co-cultured with the sEVs, IEVs, or the respective controls. After 6 hours of co-culture, PBMC viability remained largely stable across most conditions, though a minor but statistically significant reduction was observed in the sEV-treated group ($p < 0.05$). However, following 24 hours of exposure, a striking divergence emerged (**Fig. 7C**). PBMCs treated with complete CM, isolated sEVs, and isolated IEVs all exhibited severe, highly significant reductions in cell viability compared to the fresh media control ($p < 0.01$ to $p < 0.001$). Importantly, the EV-depleted media did not induce significant cytotoxicity,

remaining comparable to the fresh media control and supporting a viability markedly higher than the complete CM or the isolated EV fractions. The preservation of PBMC viability following exposure to EV-depleted conditioned medium, compared with complete conditioned medium and isolated EV fractions, supports a contribution of tumor-derived EVs to the reduction in PBMC viability observed in this experimental model.

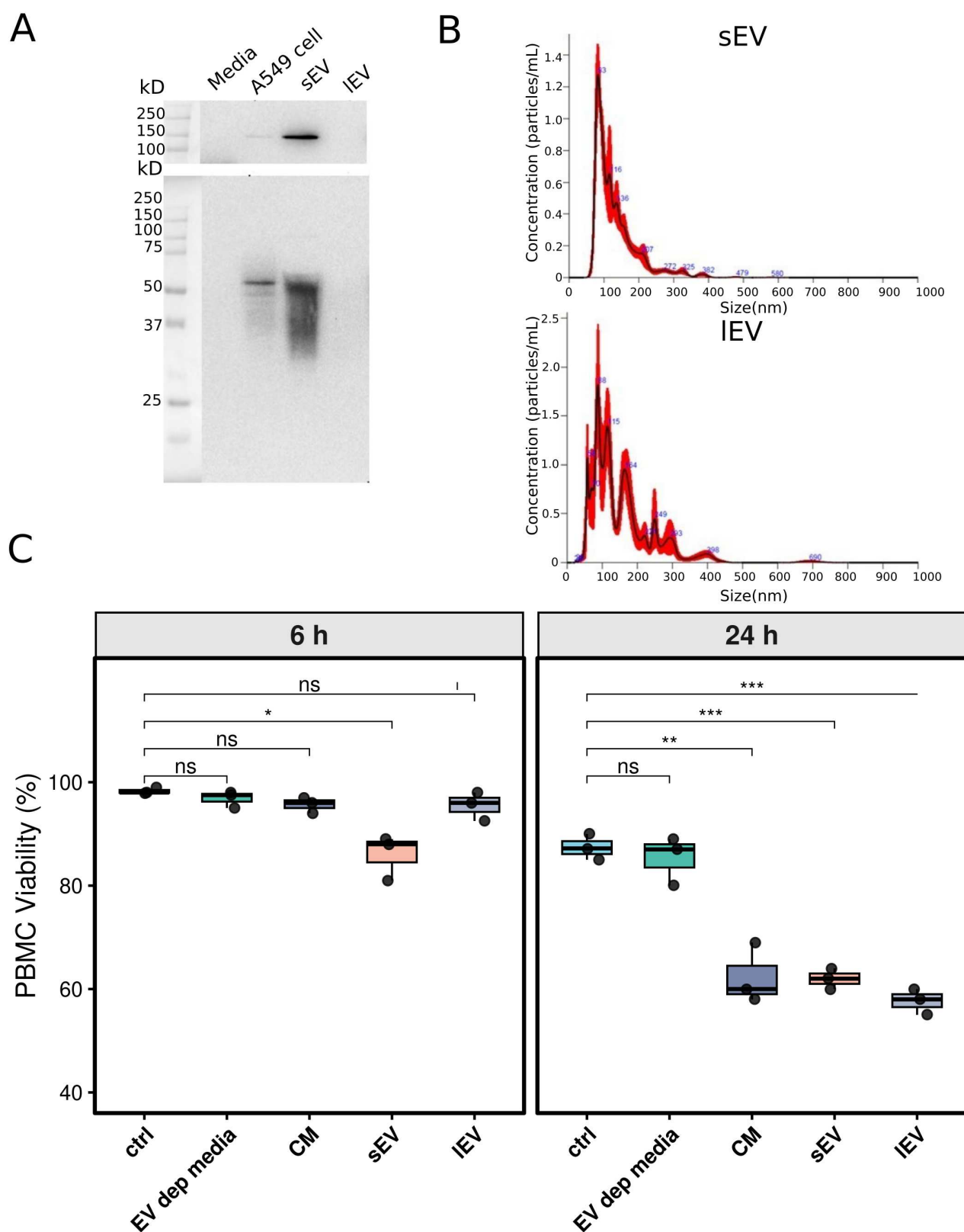


Fig. 7. Characterization and cytotoxic validation of tumor-derived extracellular vesicles (TDEVs). (A) Western blot analysis biochemically validating the EV isolation, confirming the strong enrichment of canonical markers Alix and CD63 in the sEV fraction relative to the A549 cell lysate, media, and IEV fraction. (B) Representative Nanoparticle Tracking Analysis (NTA) size profiles for the isolated sEV (top) and IEV (bottom) fractions. (C) Cell viability assay of healthy donor PBMCs following 6 h and 24 h ex vivo co-culture. Treatments included fresh media (ctrl), EV-depleted media, complete conditioned media (CM), small EVs (sEV), and large EVs (IEV). Statistical significance was

determined via ANOVA (*ns* = not significant, * $p < 0.05$, $p < 0.01$, *** $p < 0.001$ relative to the ctrl group at the respective time point).

Lung cancer-derived extracellular vesicles distinctly reprogram the PBMC proteome and kinome

To elucidate the specific intracellular mechanisms driving the response to TDEVs, global MS-based proteomic and phosphoproteomic profiling was conducted on PBMCs. Principal Component Analysis (PCA) of the global proteome revealed distinct regulatory shifts induced by the different secretome fractions compared to controls (**Fig. 8A-C, Table S22-24**). While EV-depleted conditioned media (dCM) and IEVs induced noticeable proteomic alterations, sEV treatment drove the most profound and distinct separation from the Ctrl group, indicating a highly potent, targeted reprogramming effect by this specific vesicular fraction. This strong size-dependent divergence was further amplified at the post-translational level; PCA of the phosphoproteome demonstrated that sEVs induce a highly distinct reorganization of intracellular phosphorylation states compared to both dCM and IEV treatments (**Fig. 8D-F, Table S25-27**).

To understand the biological relevance of these broad proteomic shifts, functional enrichment analysis was performed across the treatment groups (**Fig. 8G**). This analysis revealed significant regulation of proteins associated with the ribosome, mitochondria (including the inner membrane), focal adhesions, extracellular vesicular exosomes, and blood microparticles across all secretome fractions. However, while the magnitude of protein enrichment was comparable across the different treatments, the specific directionality of regulation induced by sEVs was striking. Notably, the regulatory pattern of blood microparticle-associated proteins driven by sEVs closely mirrors the functional profiles typically observed in MPEs. This shared directionality suggests that small lung cancer-derived EVs partly phenocopy key regulatory and metabolic aspects of the primary tumor microenvironment within recipient immune cells.

To pinpoint the upstream signaling master-regulators driving these downstream phosphoproteomic shifts, we implemented a highly rigorous, site-specific Kinase-Substrate Enrichment Analysis (KSEA). This targeted approach bypassed standard generalized mapping to ensure high-confidence evaluation of activation states. The analysis revealed a distinct signature of kinase suppression upon treatment, characterized by the significant inhibition ($Z\text{-score} < -1.0$) of key signaling nodes including PKG1, PKACA, PAK1, CDK2, CDK1, and Akt1 (**Fig. 8, Heatmap**). The attenuation of these specific pathways, particularly the Akt1 survival axis and CDK-mediated cell cycle regulation, highlights how TDEVs hijack and suppress specific upstream kinase networks, ultimately driving the functional reprogramming observed in the treated PBMCs. In the last step of the work, to validate the phosphorylation pattern changes of HD PBMCs after culturing with TDEVs, we performed immunoblotting of HD PBMCs exposed with media, CM, EV depleted media, sEVs, and IEVs (**Fig. S4A**) in a short time incubation period of 10 min, 30 min, and 2 hours. The results indicated an elevation of tyrosine phosphorylation in HD PBMCs after a short time treatment (30 min) with sEVs more than IEVs (**Fig. S4B**). Additionally, PBMCs treated with TDEVs depicted higher expression than media and CM which consists of the soluble factors.

In addition to the KSEA approach, we performed an orthogonal enrichment analysis based on the direct empirical mapping of identified phosphorylated substrates to their corresponding upstream kinases (**Fig. S5**). This independent methodology robustly validated our initial KSEA findings,

demonstrating a strong consensus in the hyperactivation of central kinase networks, such as PRKACA and AKT1, across the experimental conditions. Crucially, this direct mapping approach provided higher resolution into the distinct signaling topologies driven by the individual exudate fractions. The dCM induced the most expansive global phosphosignaling response, characterized by the broad and highly significant activation of acute signaling cascades, including the distinct enrichment of MAPK1. In contrast, sEVs exhibited a narrower overall phosphosignaling footprint, which aligns with their primary role in driving global protein abundance shifts via bulk cargo delivery. Despite this narrower footprint, sEVs mediated highly targeted, vesicle-specific signaling nodes. Specifically, sEV treatment uniquely drove the co-enrichment of CDK5 and CSNK2A1 activity, kinases critically associated with cytoskeletal remodeling, vesicular trafficking, and cellular survival under stress. This functional divergence suggests that while soluble factors within the dCM drive broad, acute receptor-mediated kinase cascades, cancer-derived sEVs execute highly specialized, targeted signaling networks to structurally and metabolically reprogram recipient immune cells.

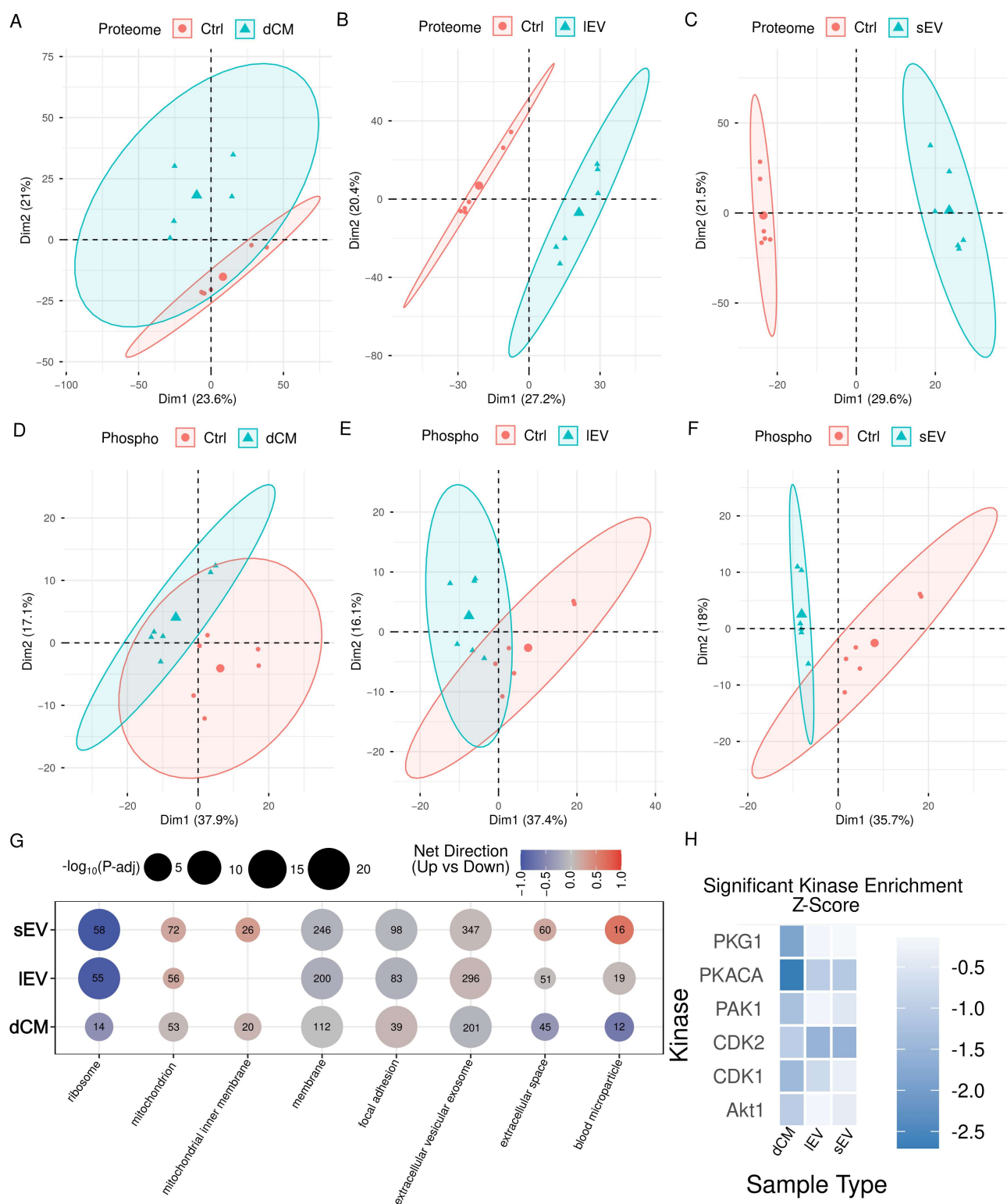


Fig. 8. Lung cancer-derived EVs profoundly reprogram the PBMC proteome and drive distinct upstream kinase signaling shifts. (A-C) Principal Component Analysis (PCA) of the global proteome in PBMCs treated with (A) EV-depleted conditioned media (dCM), (B) large EVs (lEVs), and (C) small EVs (sEVs) versus untreated controls (Ctrl). sEV treatment drives the most distinct separation from the control group. (D-F) PCA of the global phosphoproteome across (D) dCM, (E) lEV, and (F) sEV treatments versus controls, revealing pronounced phosphorylation state shifts primarily mediated by sEVs. (G) Bubble plot depicting functional enrichment across cellular components. Bubble size and color intensity correspond to statistical significance ($-\log_{10}(P\text{-adj})$) and net direction

of regulation, respectively. Numbers within the bubbles denote the total count of differentially expressed proteins mapping to that term. While enrichment magnitudes are comparable across fractions, the specific directionality of regulation induced by sEVs (e.g., within the blood microparticle and focal adhesion categories) is functionally distinct and mirrors the profile of malignant pleural effusions (MPEs). (H) Heatmap visualizing Kinase-Substrate Enrichment Analysis (KSEA) Z-scores of significant kinase inhibition (blue tiles) upon treatment. The analysis revealed targeted suppression of key signaling nodes including *PKG1*, *PKACA*, *PAK1*, *CDK2*, *CDK1*, and *Akt1*. Enrichment Z-scores were calculated based on the mean fold change of site-specific kinase-substrate phosphosites, normalized by the square root of the number of substrates ($n \geq 3$ per kinase).

Discussion

The immune system is considered to play a major role in the pathogenesis and biology of lung cancer, operating not only locally around the tumor site but also systemically [42]. This systemic immune engagement underscores why some patients, particularly those exhibiting high PD-L1 expression, can successfully respond to systemic immunotherapy drugs. Historically, MPE was drained via thoracentesis primarily as a palliative measure to improve symptoms such as dyspnea. However, a current hypothesis recognizes that MPE is more than a passive byproduct of advanced cancer. Rather, it is sustained by a vicious loop of tumor host interactions involving inflammatory, vascular, and immune pathways that actively support tumor progression. Consequently, therapeutic thoracentesis may theoretically play a role in diminishing the overall tumor burden by removing this immunosuppressive reservoir. By physically draining the pleural space, clinicians could potentially reduce systemic immunosuppression, circulating oncogenic signaling, and tumor-promoting EV exposure, indicating that pleural intervention may offer profound benefits beyond simple symptom palliation. Highlighting the active and aggressive nature of these secondary sites, recent landmark evidence in post-mortem patients reveal that lung cancer metastases themselves act as primary factors of disease progression and dissemination [43].

MPE, because of its proximity to the tumor microenvironment (TME), has gained significant attention. Its soluble products can hinder circulating PBMCs, such as T cell activity, through various mechanisms including the secretion of soluble factors [44] and tumor-derived extracellular vesicles (TDEVs) [45]. Tumor cells and PBMCs are engaged in a dynamic interaction that deeply influences the progression of lung cancer [46]. The proteome profile of lung cancer-associated MPE originates from diverse cell types, including tumor cells, epithelial cells, immune cells and soluble factors. Previous findings have demonstrated the proteomics landscape of MPE, distinguishing it from non-malignant pleural effusions such as TPE and BEPE [47]. Likewise, our prior work identified differentially expressed proteins (DEPs) among various PE types, pinpointing the involvement of immune-related, extracellular region, and vesicle-related proteins when comparing the proteomes of MPE with TPE cases [27].

In the present study, we systematically investigated the impact of the soluble fractions of diverse PEs on healthy donor (HD) PBMC viability, immunophenotyping, and temporal proteome alterations. Furthermore, we isolated TDEVs to directly interrogate their role as mechanistic drivers of these alterations in a controlled *ex vivo* co-culture system. Crucially, our data demonstrates that MPE exerts a profoundly more cytotoxic effect on PBMCs than benign effusions. The viability of PBMCs decreased significantly after 24 and 48 hours of incubation due to the effects of MPE, an effect that was statistically far more pronounced than in PBMCs cultured with BEPE or TPE. Interestingly, this reduction was not significantly visible after a short-term (6 h) incubation. This temporal dynamic was

consistently validated by our global proteomics findings; PBMCs cultured with MPE exhibited a time-dependent upregulation of potent cell death-related proteins (such as CLU, GSN, and CASP8) and unique apoptotic trajectories that distinctly separated malignant from non-malignant PEs.

The proteomic analysis of PBMCs treated with MPE, BEPE, and TPE provides a high-resolution map of immune-tumor crosstalk. GO functional analysis of the unique DEPs in MPE-exposed PBMCs overwhelmingly underlined the importance of membrane-bounded organelles and vesicle clusters, fundamentally differentiating the MPE response from non-malignant conditions. Conversely, while early BEPE treatment induced the enrichment of innate immune responses and some EV-related pathways, it failed to trigger the catastrophic apoptotic collapse seen in MPE. Our functional analysis revealed a striking dichotomy in the vesicular dynamics of MPE-exposed PBMCs, characterized by the profound suppression of endogenous exosome pathways alongside the up-regulation of blood microparticle and coagulation signatures. In the context of PBMCs, particularly the monocyte fraction, we hypothesize that this reflects a severe pathological shift. As healthy, regulated exosome secretion shuts down, likely a consequence of the broader metabolic collapse, the cells instead undergo stress-induced microparticle shedding. Because we observed this occurring simultaneously with the breakdown of the actin cytoskeleton and focal adhesions, it suggests that this pro-coagulant shedding is driven by the structural dismantling of the cells under MPE-induced toxicity.

It has been established that MPE contains immunosuppressive soluble factors capable of suppressing macrophage activation *ex vivo* [20]. In human NSCLC tumors, T cells remain the dominant immune population [48]. In the current work, our flow cytometry data revealed a subpopulation of activated mono-MAC ablation alongside a shift in the T cell compartment. The frequency of CD4⁺ T follicular helper (Tfh) cells expressing PD-1 was notably higher in PBMCs treated with MPE compared to the control and TPE treatments, highlighting the immune modulation effect of the microenvironment driven by the tumor (**Fig. 3**). The PD-1/PD-L1 axis has a critical immunosuppressive function in lung cancer TME. Recent evidence indicates that CD4⁺ CXCR5⁺ Tfh cells are recruited by exhausted CD8⁺ T cells expressing high levels of CXCL13, the primary ligand for CXCR5 [49]. Alternatively, this Tfh expansion may represent an attempted anti-tumor humoral response, specifically aimed at facilitating B-cell activation and antibody class switching, that is ultimately insufficient to overcome the broader immunosuppressive milieu. Previous reports indicate that blood samples from NSCLC patients exhibit higher PD-1 expression in CXCR5⁻ and CXCR5⁺ CD4⁺ T cells compared to healthy subjects[50]. Similarly, we observed high expression of PD-1⁺ CXCR5⁺ CD4⁺ cells in HD PBMCs treated with MPE. High expression of PD-1⁺ CD4⁺ T cells has been linked to extensive-stage disease and poor prognosis in lung cancer patients, with overexpression noted more prominently in bronchoalveolar lavage fluid (BALF) than in peripheral blood[51]. Clinically, the marked PD-1⁺/CXCR5⁺ phenotype, coupled with the activated monocyte depletion observed in our model, strongly suggests that the unique immune architecture of MPE could dictate patient responses to immune checkpoint inhibitors. Profiling the cellular and fluid compartments of pleural effusions may therefore serve as a vital stratification tool to predict immunotherapy responsiveness or resistance.

Regarding the cytotoxic compartment, we found an overexpression of global CD8⁺ T cells in MPE-treated PBMCs compared to untreated controls. Interestingly, previous work revealed that CXCR5⁺ CD4⁺ T cells can mediate effects on CD8⁺ T cell function and promote their proliferation [50]. This finding likely explains the elevated CD8⁺ populations detected in our MPE-treated cohort, as we concurrently observed a higher proportion of CD4⁺ CXCR5⁺ cells. Moreover, Tfh-like cells characterized by PD-1⁺ CXCR5⁺ CD4⁺ expression were elevated in MPE-treated PBMCs, mirroring

findings previously reported in the blood and tissue of lung cancer patients compared to healthy controls [52]. This population indicates the skewed activation of adaptive immune cells in lung cancer, similarly to activated CD8⁺ T cells implicated in the development of peripheral immune responses [53]. Ultimately, higher infiltration of PD1-TILs, loss of HLA class I, and overexpression of PD-L1 on immune cells are strongly correlated with poor prognosis in MPE patients [54]. In our model, the non-T cell compartment (CD45⁺CD3⁻ lymphocytes) was predominantly represented by natural killer (NK) cells, given the scarcity of B cells (**Fig. S6**) in the healthy donor PBMCs. Profiling this population revealed that CD38 expression on NK cells is modulated by the specific type of pleural effusion. Notably, MPE induced a decrease in the CD38^{bright} fraction, accompanied by a reciprocal increase in the CD38^{dim} and CD38⁻ populations. Because the CD38^{bright} NK cell fraction is clinically associated with an activated, highly cytotoxic, and cytokine-responsive phenotype [55], its selective depletion suggests that the MPE microenvironment actively suppresses or remodels this critical innate immune subset. This remodeling likely drives NK cell exhaustion and limits survival, a hypothesis corroborated by the significant upregulation of cell death pathways observed in our proteomic analysis specifically following 24 hours of MPE exposure. This immunosuppressive signature contrasts with highly inflammatory states, such as autoimmune diseases, where a high percentage of CD38⁺ NK cells persists in both peripheral blood and synovial fluid [56]. Conversely, clinical studies demonstrate that the overall frequency of circulating NK cells is significantly diminished in MPE patients compared to healthy donors [16], underscoring a systemic immune escape mechanism. This active suppression is likely mediated by the well-documented abundance of immunosuppressive soluble factors in MPE, such as IL-10 and TGF-beta, which directly impair NK cell cytotoxicity, cytokine production, and the expression of activating receptors [11].

Functional enrichment analysis of PBMCs cultured with MPE persistently highlighted pathways related to extracellular vesicles and the regulation of protein autophosphorylation. These clinical proteomic signatures motivated our hypothesis that communication within the lung TME is actively mediated by TDEVs regulating circulating PBMCs. Using an *ex vivo* model, we isolated sEVs and lEVs to mimic this interaction. TDEVs play a critical role in intercellular communication with various biological functions on immune cells [57]. Strikingly, our viability assays confirmed that dCM was significantly less toxic than complete CM or isolated EV fractions, demonstrating that TDEVs are the primary carriers of the cytotoxic payload. Co-culture with TDEVs induced massive proteome changes in PBMCs. Functional enrichment analysis depicted that vesicular treatment altered pathways involving the ribosome, mitochondria, focal adhesions, and extracellular vesicular exosomes. Crucially, while enrichment magnitude was comparable across fractions, the specific directionality of regulation induced by sEVs, particularly concerning blood microparticle-associated proteins, closely mirrored the distinct functional profile of MPEs. This indicates that small lung cancer-derived EVs uniquely phenocopy the immunosuppressive and metabolic aspects of the primary tumor microenvironment within recipient immune cells.

Immunosuppressive roles of TDEVs in innate and adaptive immunity have been widely reported. In breast cancer, TDEV-induced CD8⁺ T cell exhaustion leads to the failure of immunotherapy by diminishing host anti-tumor activity [58]. Similarly, in lung cancer, TDEV cargo mediates tumor progression primarily by suppressing antitumor immunity [59]. We detected the massive upregulation of MUC5AC in the proteome of sEV-treated PBMCs, a known poor prognostic factor in lung cancer [60]. STAT1, a master regulator of the immune system in cancers, was also among the top regulated proteins [61]. *Ex vivo* STAT1 silencing has been shown to mediate MHC class II suppression, leading to immune escape [62]. TDEV treatment directly induced cell death-associated proteins, including

APP, GSR, HMGB2, and ETF1 in sEV-cultured PBMCs. GSR is an extracellular protein associated with ROS signaling that modulates immune cell proliferation [63]. Similarly, lEV exposure regulated DNM1L and PRF1; DNM1L acts as a regulator of necroptosis in lung cancer [64], is associated with necrosis in patients, and mediates immune cell infiltration [65].

Finally, TDEVs profoundly perturbed the intracellular kinase networks of recipient PBMCs. Previous studies have shown that TDEVs alter Smad signaling to inhibit NK cells [66], modulate p-STAT3 in CD14⁺ monocytes, and alter phosphorylated p70S6 kinase and Erk1/2 [67]. Colorectal cancer-derived EVs suppress T cell proliferation via MAPK, AKT, and TGF- β /Smad perturbation [68], while TEV-stimulated STAT5 phosphorylation inhibits Th1/Th17 differentiation and promotes Treg expansion [69]. Given the importance of phosphorylation signaling in the lung cancer TME, our rigorous site-specific Kinase-Substrate Enrichment Analysis (KSEA) highlighted a distinct signature of targeted kinase suppression. Specifically, we identified the significant inhibition of key signaling nodes including PKG1, PKACA, PAK1, CDK2, CDK1, and Akt1 following treatment. The specific attenuation of the Akt1 survival axis and CDK-mediated cell cycle regulation demonstrates how tumor-derived EVs hijack and suppress upstream kinase networks to force immune cell reprogramming. Furthermore, the analysis underscored the importance of protein kinase A catalytic subunit alpha (PKACA/PRKACA) and PKG1 inhibition. The role of PKACA in protein modifications of NSCLC has been studied previously [70]. In our model, the targeted suppression of these nodes, alongside PAK1, suggests a coordinated EV-mediated mechanism to disable normal immune cell activation and survival pathways. To dissect the distinct signaling topologies driving the observed proteomic shifts, we complemented our directional KSEA with a broader, direct kinase enrichment mapping across the isolated fractions derived from lung cancer cell conditioned media. This direct substrate-to-kinase mapping validates and expands upon the KSEA findings, confirming the broad dysregulation of central nodes like PRKACA and AKT1 while providing higher resolution into fraction-specific responses. Notably, the dCM induced the most expansive and highly significant phosphosignaling response, characterized by the profound activation of the PRKACA, AKT1, and MAPK1 axes. This contrasts with our global proteomic PCA data, which demonstrated that sEVs and lEVs primarily drive total protein abundance changes. Despite the narrower global phosphosignaling footprint of sEVs, kinase mapping revealed highly specific vesicular signaling nodes, notably the unique co-enrichment of CDK5 and CSNK2A1 activity in sEV-treated cells compared to all other fractions. This divergence suggests that while soluble factors in the conditioned media drive broad acute kinase cascades (e.g., MAPK1), sEVs mediate highly targeted signaling networks alongside their bulk cargo delivery.

Furthermore, this dual-analysis framework highlighted a remarkable consistency in the metabolic reprogramming observed across our clinical and *ex vivo* models. Specifically, the anomalous stress and hyperactivation of the mTOR signaling axis observed in the early temporal clinical comparisons of MPE versus BEPE were directly phenocopied in the sEV and CM fractions derived from the lung cancer cell line. This agreement demonstrates that both the vesicular and soluble components of the tumor cell secretome actively collaborate to force a state of metabolic burnout within immune cells. In general, pathological phosphorylation signaling mediates profound immunosuppression [71] and aggressively induces immune cell death in cancer [72].

Clinically, the identification of this specific EV-mediated kinase rewiring suggests immediate therapeutic opportunities. Disrupting these networks, whether through the pharmacological inhibition of EV biogenesis and cellular uptake, localized intrapleural immunotherapy, or the deployment of

systemic kinase-targeted therapies, could restore anti-tumor immunity and effectively neutralize the MPE microenvironment.

Within our current findings, we demonstrated that MPE radically alters the viability and immunophenotype of HD PBMCs by 24 hours, but its impact on the intracellular proteome begins much earlier, following a distinct ~6-hour lag phase. The uniquely toxic soluble factors and TDEVs present in MPE drive completely divergent proteomic trajectories compared to non-malignant PEs. This emphasizes that MPE actively forces the phenotype and proteome of T cells, B cells, and monocytes into an exhausted, suppressed state. Because MPE reflects these active tumor–immune interactions in real time, it represents a highly dynamic diagnostic compartment. Future clinical research should evaluate serial pleural fluid analyses to monitor tumor evolution, shifting immune exhaustion profiles, and emerging therapeutic resistance.

While this study provides a high-resolution map of immune-tumor crosstalk, we acknowledge certain limitations. Primarily, the clinical sample size of patient effusions utilized in the *ex vivo* models is relatively small. However, this study was explicitly designed to prioritize extensive temporal and multi-omics depth over broad cohort screening. By meticulously profiling multiple distinct biological conditions (MPE, BEPE, TPE, sEVs, IEVs, and EV-depleted conditioned media) across highly resolved temporal kinetics (from 10 minutes to 48 hours) using combined global proteomic, exploratory phosphoproteomic, and immunophenotyping platforms, we generated an exhaustive quantitative dataset comprising 27 supplemental data tables. This rigorous, high-dimensional approach was necessary to capture the complex, time-dependent mechanistic shifts in intracellular pathways and kinase networks. Ultimately, this deep exploratory framework identifies specific EV-associated pathways and temporal dynamics that must now be validated across larger, independent patient cohorts.

Given the high resolution, scale, and distinct condition-based separation achieved by the proteomic and phosphoproteomic profiling in this study, a central priority for future research must be the targeted clinical validation of these EV-driven molecular signatures across larger patient cohorts. Complementing this MS-based validation, deep FACS analysis should be utilized to further sub-phenotype the exhausted CD4⁺ and CD8⁺ T cell populations following MPE exposure, while reciprocal studies on how PBMC-derived EVs alter lung cancer cells will clarify the bidirectional nature of this crosstalk. Ultimately, our molecular data strongly supports an emerging clinical paradigm: rather than being a mere passive byproduct of advanced cancer, MPE acts as an actively pathogenic reservoir that drives disease progression [9–11]. Future clinical trials must proactively explore this hypothesis. Moving beyond standard symptom palliation, therapeutic drainage should be evaluated as a direct, active strategy to mitigate systemic immune exhaustion and improve patient outcomes.

Conclusion

In conclusion, our study demonstrates that MPE establishes a profoundly immunosuppressive microenvironment characterized by rapid monocyte depletion and T cell exhaustion. By employing an *ex vivo* temporal model and multi-omics profiling, we reveal that these immunological shifts are driven primarily by tumor-derived extracellular vesicles (TDEVs) rather than soluble factors alone. TDEVs actively hijack highly targeted intracellular kinase networks (such as CDK5 and CSNK2A1), while soluble factors drive broad acute cascades, collaboratively rewiring the PBMC proteome and

precipitating structural and metabolic collapse. These findings provide critical mechanistic insights into early immune evasion within the lung cancer microenvironment and highlight vesicle-mediated signaling pathways as potential therapeutic vulnerabilities for restoring anti-tumor immunity in patients with malignant effusions.

Abbreviation List

BALF: Bronchoalveolar lavage fluid

BEPE: Benign parapneumonic effusion

BP: Biological Process

CC: Cellular Component

CM: Conditioned medium

dCM: EV depleted condition medium

cTfh: Circulating T follicular helper

DDA: Data-dependent acquisition

DEP: Differentially expressed protein

DTT: Dithiothreitol

EVs: Extracellular vesicles

FACS: Flow cytometry (Fluorescence-activated cell sorting)

FASP: Filter-aided sample preparation

FBS: Fetal bovine serum

FDR: False discovery rate

FEA: Functional enrichment analysis

FSC: Forward scatter

GO: Gene Ontology

HCD: Higher-energy collision dissociation

HD: Healthy donor

IAA: Iodoacetamide

iBAQ: Intensity-based absolute quantification

KEGG: Kyoto Encyclopedia of Genes and Genomes

KSEA: Kinase set enrichment analysis

IEVs: Large extracellular vesicles

LUAD: Lung adenocarcinoma

MF: Molecular Function

MPE: Malignant pleural effusion

MS: Mass spectrometry
NTA: Nanoparticle tracking analysis
PBMCs: Peripheral blood mononuclear cells
PBS: Phosphate-buffered saline
PCA: Principal component analysis
PE: Pleural effusion
PVDF: Polyvinylidene fluoride
sEVs: Small extracellular vesicles
SSC: Side scatter
TDEVs: Tumor-derived extracellular vesicles
Tfh: T follicular helper
TME: Tumor microenvironment
TPE: Transudative pleural effusion

Data availability

The mass spectrometry proteomics data that support the findings of this study have been deposited in ProteomeXchange Consortium [73] via the PRIDE [74] partner with the accession codes PXD082520 (10.6019/PXD082520) and PXD082543. Proteomics of PBMCs exposed to pleural effusion: Data access during review phase (to be deleted upon publication where the data will be made public). Project Name: Human PBMCs exposed to pleural effusions, Project accession: PXD082520, Project DOI: 10.6019/PXD082520, Reviewer account details: Project accession: PXD082520 Password: eJW4CNWerUiH. Project Name: Phosphoproteomic Profiling of PBMCs exposed to Lung Adenocarcinoma EVs, Project accession: PXD082543, Reviewer account details: Project accession: PXD082543 Password: 6fKsdeKXvG80.

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Supplementary Information

Supplementary Tables S1–S9 for Figure 4. Proteomic dynamics of PBMCs exposed to pleural effusions. These tables contain the quantitative data and *limma* statistical results for the pairwise comparisons of PBMCs exposed to TPE, BEPE, and MPE at 0, 6, 24, and 48 hours. Each table includes additional sheets detailing the functional enrichment of all significantly regulated proteins (p-value < 0.05 and $|\log_2(\text{fold change})| > 1$), strictly down-regulated proteins (p-value < 0.05 and $\log_2(\text{fold change}) < -1$), and strictly up-regulated proteins (p-value < 0.05 and $\log_2(\text{fold change}) > 1$). Table S1 is for TPE 6 hrs vs untreated. Table S2 TPE 24 hrs vs TPE 6 hrs. Table S3 TPE 48 hrs vs TPE 24 hrs. Table S4 BEPE 6 hrs vs untreated. Table S5 BEPE 24 hrs vs BEPE 6 hrs. Table S6 BEPE 48 hrs vs BEPE 24 hrs. Table S7 MPE 6 hrs vs untreated. Table S8 MPE 24 hrs vs MPE 6 hrs. Table S9 MPE 48 hrs vs MPE 24 hrs.

Supplementary Tables S10–S12 for Figure 5. Proteomic dynamics of PBMCs exposed to pleural effusions. These tables contain the quantitative data and *limma* statistical results for the pairwise comparisons of PBMCs exposed to BEPE, and MPE at 6, 24, and 48 hours. Each table includes additional sheets detailing the functional enrichment of all significantly regulated proteins (p-value < 0.05 and $|\log_2(\text{fold change})| > 1$), strictly down-regulated proteins (p-value < 0.05 and $\log_2(\text{fold change}) < -1$), and strictly up-regulated proteins (p-value < 0.05 and $\log_2(\text{fold change}) > 1$). Table S10 is for MPE 6 hrs vs BEPE 6 hrs. Table S11 is for MPE 24 hrs vs BEPE 24 hrs. Table S12 is for MPE 48 hrs vs BEPE 48 hrs.

Supplementary Tables S13–S21 for Figure 6. Proteomic dynamics of PBMCs exposed to pleural effusions. These tables contain the quantitative data and *limma* statistical results for the pairwise comparisons of PBMCs exposed to untreated, TPE, BEPE, and MPE at 6, 24, and 48 hours. Each table includes additional sheets detailing the functional enrichment of all significantly regulated proteins (p-value < 0.05 and $|\log_2(\text{fold change})| > 1$), strictly down-regulated proteins (p-value < 0.05 and $\log_2(\text{fold change}) < -1$), and strictly up-regulated proteins (p-value < 0.05 and $\log_2(\text{fold change}) > 1$). Table S13 is for TPE 6 hrs vs untreated 6 hrs. Table S14 is for TPE 24 hrs vs untreated 24 hrs. Table S15 is for TPE 48 hrs vs untreated 48 hrs. Table S16 is for BEPE 6 hrs vs untreated 6 hrs. Table S17 is for BEPE 24 hrs vs untreated 24 hrs. Table S18 is for BEPE 48 hrs vs untreated 48 hrs. Table S19 is for MPE 6 hrs vs untreated 6 hrs. Table S20 is for MPE 24 hrs vs untreated 24 hrs. Table S21 is for MPE 48 hrs vs untreated 48 hrs.

Supplementary Tables S22–S27 for Figure 8. Protein and phosphopeptide dynamics of PBMCs exposed to lung adenocarcinoma EV-depleted conditioned media (dCM), IEVs, and sEV. These tables contain the quantitative data and *limma* statistical results for the pairwise comparisons of PBMCs exposed to dCM, IEV and sEV. Protein table includes additional sheets detailing the functional enrichment of all significantly regulated proteins (p-value < 0.05 and $|\log_2(\text{fold change})| > 1$), strictly down-regulated proteins (p-value < 0.05 and $\log_2(\text{fold change}) < -1$), and strictly up-regulated proteins (p-value < 0.05 and $\log_2(\text{fold change}) > 1$). Protein comparisons: Table S22 is for dCM vs ctrl (media), Table S23 is for IEV vs ctrl (media), Table S24 is for sEV vs ctrl (media). Phosphopeptide: Table S25 is for dCM vs ctrl (media), Table S26 is for IEV vs ctrl (media), Table S27 is for sEV vs ctrl (media).

Competing Interests/Declaration of Interest Statement

The authors declare no conflict of interest

Author contributions

S.Z.: Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft, review, and editing. M.E.: investigation; methodology; and writing—review and editing. H.C.B.: investigation, methodology and writing—review and editing. C.M. methodology and writing—review and editing. A.L.: methodology and writing—review and editing. P.B.: methodology and writing—review and editing. A.B.: Conceptualization; data curation; methodology; writing—original draft, review, and editing. A.S.C. Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft, review, and editing. R.M. Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft, review, and editing. All authors worked on the manuscript at the stage of reviewing and editing.

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