Supplementary Methods

PEMC and PBMC isolation from LUAD patients and HD

MPEs were obtained by thoracentesis and collected aseptically in non heparinized bottles/tubes. After a centrifugation step (1,200 rpm for 5min at 4°C) supernatants were harvested and stored at -80°C. The cell fraction was collected, washed twice with phosphate-buffered saline (PBS; Sigma, St. Louis, MO) by centrifugation (1,200 rpm for 5min at 4°C), layered on OncoQuick gradient separation medium (Greiner Bio-One) and centrifuged (1,600 g, no brake, for 20 min at 4°C) to partially purify tumour cells, that are enriched at the interphase layer of the gradient, from erythrocytes and leucocytes that migrate into the lower phase through the bottom of the tube. To further separate erythrocytes from pleural effusion mononuclear cells (PEMC), cells derived from the lower phase were resuspended in PBS and further stratified on Lympholyte-H density gradient separation medium (Cedarlane, Ontario, CA, USA). After a centrifugation step (1,800 rpm, no brake, for 20 min at room temperature) PEMC fraction (ring) were collected, washed twice with PBS and frozen in 10% v/v DMSO, 90% v/v fetal bovine serum (FBS) solution (Sigma-Aldrich, St. Louis, MO) for subsequent RNA extraction.

Peripheral blood was collected from LUAD patients and healthy donors (HD) in anti-coagulant supplemented blood tubes and centrifuged (1,800 rpm for 10 min at 4°C) to separate cells from plasma. Plasma was then collected and stored at -80°C and cells were stratified on Lympholyte-H density gradient separation medium to isolate mononuclear cells (PBMC).

Bulk RNA Sequencing (RNA-Seq), data processing, DEGs identification and functional enrichment analysis

RNA libraries for sequencing were generated using the TruSeq RNA Exome kit (Illumina, San Diego, CA, USA), optimized for low input and low-quality RNA. The procedure consists of a general whole transcriptome strand specific library preparation followed by a specific exon targeting enrichment. The quality of the resulting libraries was assessed via Bioanalyzer (High Sensitivity DNA Kit). The intermediate library, before exon enrichment, was quantified by Qubit, the final library by qPCR. Samples were sequenced in paired-end mode, sequencing 76 bp from each side, with NextSeq 500 System (Illumina).

RNA-seq raw data were processed thanks to the nf-core/rnaseq pipeline (v 2.4)[1], which carries out the primary analysis of the mapping onto the reference genome (GRCh37) providing Quality Control metrics of the analysed samples, obtaining both the raw counts (FeatureCounts) and the TPM (Transcript Per Million) normalized pseudo counts. The DEseq2 Bioconductor package[2] was used to test for the differentially expressed genes (DEGs) between the groups using the negative binomial

distribution and Wald's test. For DEG identification, genes with an adjusted p-value < 0.05 were considered as statistically significant and an additional filter cut-off criterion of | log2FC|> 2 was applied to select the signature with the strongest induction among conditions. The biological function of DEGs was identified by Gene Ontology (GO) analysis using the R package "enrichR"[3]. GO visualizations were produced via the auto-GO/ARGO framework. Fisher's exact test was employed, and the occurrence of false positives was corrected by Benjamini-Hochberg (B-H) multiple test correction method. For the enrichment analysis, an adjusted p-value < 0.05 was set as the cut-off criterion.

MPEs Mφ signature: ImmGen interrogation and network analysis

As a starting point for the selection of the MPEs Mφ signature we used the upregulated genes in LUAD PEMC compared to matched PBMC (n=2363 Log2FC >2 and adjusted p-value < 0.05), exploiting the fact that the macrophagic component is present only at the tumour site (i.e. MPEs) and not in the circulation. Subsequently additional filter cut-off criteria, log2FC ≥ 3 and mean_PEMC_≥ 10 (where mean stands for mean of the normalized values with the variance stabilizing transformation, vsd () function, of DeSeq), were applied to select the genes with the strongest induction in PEMC. After filtering, 194 genes were selected. Finally in order to identify the most relevant genes related to macrophage phenotype we used the public ImmGen (Immunological Genome Project)[4,5] resources available online (http://www.immgen.org), obtaining a 37 gene list (MPEs Mφ signature, Table S2). My Gene Set browser was used to examine the expression pattern of the identified genes across different immune cell types, exploiting the ImmGen expression data. In detail, the ImmGen ULI RNA-seq dataset GSE127267, that encompasses primary RNAseq data from carefully sorted immunocyte populations, sequenced using ImmGen's SOP for 'ultra-low-input' population RNAseq (typically 500 to 1,000 cells) performed by Smartseq2, was used to test MPEs Mφ signature as well as TMR and MDM signatures[6] and to generate the reported graphs (Figure 5 and Supplementary Figure S6).

Gene expression data of 505 LUAD patients were downloaded from Xena Browser[7] as RSEM_TPM log2 (tpm+0.001) and transformed applying the (2^tpm)-0.001 formula to then calculate the geometric mean of the 37 genes expression (MPEs Mφ signature) across all patients, divided into different stage disease. Stage I (N=274), Stage II (N=122), Stage III (N=83), Stage IV (N=26).

The protein-protein interaction (PPI) networks for the 37 genes included in the MPEs Mφ signature was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING) online database[8] (http://string-db.org; version11.0). An interaction score > 0.4 was regarded as statistically significant. The molecular interaction network was visualized using Cytoscape[9]

software (v 3.8.2) and the Cytohubba plugin app[10] within Cytoscape was used to calculate degree of interaction between these 37 genes. The top 10 genes were defined as hub genes.

Survival analyses

Survival analyses were performed using the KM-plotter database (http://kmplot.com)[11]. Kaplan–Meier curves stratify 672 LUAD and 271 LUSC patients by high and low MPEs Mφ signature expression. The upper quartile value was used as cut-off criterion. $p < 0.05$ was considered as statistically significant (log rank test).

The analyses were performed on expression data from tissue biopsies of 672 independent patient samples from seven LUAD cohorts: GSE19188, GSE29013, GSE30219, GSE31210, GSE3141, GSE37745, GSE50081; and on 271 independent patient samples from six LUSC cohorts: GSE19188, GSE29013, GSE30219, GSE3141, GSE37745, GSE50081. The main clinical characteristics of those patients are reported in the table below:

Dataset	Cancer Type	Sex	Median Age	Disease Stage
GSE19188	LUAD (N=40)	M (N=25);F(N=15)	N/A	I-II
	LUSC (N=24)	M (N=22); F(N=2)	N/A	I-II
GSE29013	LUAD (N=30)	M (N=20);F(N=10)	66	I(N=16)-II(N=6)-III(N=8)
	LUSC (N=25)	M (N=18); F(N=7)	65	I(N=8)-II(N=8)-III(N=9)
GSE30219	LUAD (N=85)	M (N=67);F(N=18)	60	N/A
	LUSC (N=61)	M (N=57); F(N=4)	63	N/A
GSE3141	LUAD (N=58)	N/A	N/A	N/A
	LUSC (N=53)	N/A	N/A	N/A
GSE37745	LUAD (N=106)	M (N=46);F(N=60)	64	I(N=70)-II(N=19)-III(N=13)-IV(=4)
	LUSC (N=66)	M (N=46);F(N=20)	67	I(N=40)-II(N=15)-III(N=11)
GSE50081	LUAD (N=127)	M (N=65);F(N=62)	70	I(N=92)-II(N=35)
	LUSC (N=42)	M (N=24);F(N=18)	70	I(N=26)-II(N=16)
GSE31210	LUAD (N=226)	M (N=105); F(N=121)	61	I(N=168)-II(N=58)

THP1-Monocytes differentiation and MPEs stimulation

Human THP-1 monocytes were maintained in culture medium RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% of inactivated FBS and incubated at 37°C in a 5% v/v CO2 atmosphere. For monocyte-macrophage differentiation, cells were seeded at a density of $1x10^5$ cells/ml in 6-well plates and macrophage differentiation was initiated by exposing the cells to 100ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) in 10% FBS culture medium for 24 hours. Subsequently, THP-1 derived macrophages were cultured for 48 hours with serum free medium (M0 macrophages) or medium supplemented with 10% of cell-free pleural fluids from LUAD patients. M1/M2 polarization was then assessed by qRT-PCR. Experiments were performed

independently at least three times and the data were expressed as average ± SE of the mean (SEM). Differences between groups were analysed with a two-tailed Student's t-test and were considered statistically significant for p-value <0.05

Cytokine Array

Levels of 48 different cytokines, including chemokines, interleukins and growth factors, were evaluated at the same time in cell-free pleural fluids and matched plasma of 15 LUAD patients and in plasma of 4 HDs, using Pro Human Cytokine Screening 48-Plex Assays panel (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions.

Immediately prior to the analysis, the frozen samples were thawed and clarified by centrifugation (15 min at 1,000 x g) in a cold room environment (4°C). Pleural fluid samples were also filtered through a 0.2 µm filter prior to the analysis. Cytokines were quantified on the Luminex platform using the Bio-Plex MagPix instrument (Bio-Rad, Hercules, CA, USA) and the Bio-Plex Manager MP software was used for data acquisition and analysis. All the samples were run in duplicate and ten-point standard curve was run for each cytokine. Determinations that were designated "Out of Range Below" (i.e. below the limit of quantification) by the analytical software were arbitrarily filled with a zero value. Cytokines fold change, calculated as the ratio between cytokine levels in pleural fluid samples with respect to those in matched plasma samples of LUAD patients, were considered statistically significant if p-value < 0.05 (two-tailed Student's t-test). For the complete list of 48 analysed cytokines see Supplementary Table S5.

Wound healing assays

Wound healing assays were performed on two MPEs-derived primary cell lines (i.e BBIRE-T248 and PUC36) isolated and characterized as previously reported[12]. Primary cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS and incubated at 37°C in a 5% v/v CO2 atmosphere. For wound healing assay 9 x 10^4 cells were plated into a 2 well culture-insert (Ibidi GmbH, Martinsried, Germany). When cells reached complete confluence, the culture-insert was removed, cells were washed with PBS, and cultured for 24 hours with serum free medium (CTRL) or medium supplemented with 10% of matched cell-free pleural fluids. Images were captured immediately after insert removal (T0) and after 24 hours of treatment (T24), with an Axiocam 208 color (Zeiss, Oberkochen, Germany), coupled with a ZEISS Primovert Photo optical microscope (Zeiss, Oberkochen, Germany). The cell-free space (open residual area) was measured by using the ImageJ software (NIH, Rockville, USA). Experiments were performed independently at least three times and the data were expressed as average ± SE of the mean (SEM). Differences between groups

were analysed with paired Student's t-test and were considered statistically significant for p-value <0.05.

List of the Online Tools Used

http://string-db.org

http://www.immgen.org

http://kmplot.com

https://cibersortx.stanford.edu

Supplementary References

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