


Deconvolution of malignant pleural effusions immune landscape unravels a novel macrophage signature associated with worse clinical outcome in lung adenocarcinoma patients

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ABSTRACT

Background Immune checkpoint inhibitors are still unable to provide clinical benefit to the large majority of non-small cell lung cancer (NSCLC) patients. A deeper characterization of the tumor immune microenvironment (TIME) is expected to shed light on the mechanisms of cancer immune evasion and resistance to immunotherapy. Here, we exploited malignant pleural effusions (MPEs) from lung adenocarcinoma (LUAD) patients as a model system to decipher TIME in metastatic NSCLC.

Methods Mononuclear cells from MPEs (PEMC) and peripheral blood (PBMC), cell free pleural fluid and/or plasma were collected from a total of 24 LUAD patients and 12 healthy donors. Bulk-RNA sequencing was performed on total RNA extracted from PEMC and matched PBMC. The DESeq2 Bioconductor package was used to perform differential expression analysis and CIBERSORTx for the regression-based immune deconvolution of bulk gene expression data. Cytokine analysis of cell-free pleural fluid and plasma samples was performed using a 48-Plex Assay panel. THP-1 monocytic cells were used to assess macrophage polarization. Survival analyses on NSCLC patients were performed using KM Plotter (LUAD, N=672; lung squamous cell carcinoma, N=271).

Results Transcriptomic analysis of immune cells and cytokine analysis of soluble factors in the pleural fluid depicted MPEs as a metastatic niche in which all the components required for an effective antitumor response are present, but conscripted in a wound-healing, proinflammatory and tumor-supportive mode. The bioinformatic deconvolution analysis revealed an immune landscape dominated by myeloid subsets with the prevalence of monocytes, protumoral macrophages and activated mast cells. Focusing on macrophages we identified an MPEs-distinctive signature associated with worse clinical outcome in LUAD patients.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Tumor immune microenvironment (TIME) is a key element in determining resistance to immunotherapy with immune checkpoint inhibitors. Malignant pleural effusions (MPEs), a pathological condition associated with 30% of non-small cell lung cancer cases, represent an easily accessible window onto metastatic disease.

WHAT THIS STUDY ADDS

⇒ By using MPEs and blood samples directly derived from lung adenocarcinoma (LUAD) patients and exploiting cytokine analysis and transcriptomic data combined with an immune deconvolution-based approach, we provide a deep characterization of cellular and soluble components of MPEs microenvironment. The main novelty of our findings relies in the identification in MPEs of a cytokine fuelling monocytes infiltration and protumoral Macrophage (Mφ) polarization and of a novel Mφ genes signature associated with poor prognosis in LUAD.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ This study highlights the involvement of the myeloid compartment in the context of LUAD TIME and provides a solid ground for the identification of new targets for the treatment of metastatic disease.

Conclusions Our study reports for the first time a wide characterization of MPEs LUAD microenvironment, highlighting the importance of specific components of the myeloid compartment and opens new perspectives for the rational design of new therapies for metastatic NSCLC.

INTRODUCTION

Immunotherapy is revolutionizing the clinical management of non-small cell lung cancer (NSCLC), mainly through the introduction of immune checkpoint inhibitors (ICIs).^{1,2} However, despite previously unanticipated long-term responses in advanced disease have been obtained in a subset of patients, the main hurdle remains the lack of efficacy in the vast majority of cases.^{3,4} Several resistance mechanisms to immunotherapy have been identified, involving tumor cell intrinsic as well as tumor cell-extrinsic factors.⁵ The latter can be mainly ascribed to components of the tumor microenvironment (TME).⁶ Although the main efforts have been devoted to T-cell characterizations with the development of ICI therapies, other immune cells of the innate and adaptive immune systems, including DCs, macrophages, NK cells, and B cells, have also been shown to contribute to tumor progression and response to immunotherapy.^{7,8} Hence, a comprehensive characterization of the tumor immune microenvironment (TIME) is essential.⁹ In this regard recently a detailed immune cell atlas of early-stage lung cancer has provided novel insights into the functional states and developmental lineages of heterogeneous myeloid subsets.¹⁰ An exemplary proof of myeloid complexity is given by the macrophage compartment. It is well known nowadays that tumor-associated macrophages (TAMs) are highly plastic cells and have a pivotal role in shaping the TME and response to therapy.¹¹ Moreover, high infiltration of TAMs is associated with poor overall survival in different tumor types,¹² making them an important target for cancer treatment.^{13,14} Nevertheless, the lack of complete understanding of molecular and functional diversity of the tumor macrophage compartment strongly limits therapeutic approaches. Very recently, focusing on how different macrophage lineages contribute to TME and cancer progression, tissue-resident macrophages have been proposed as target for prevention and treatment of early NSCLC.¹⁵ However, currently the majority of NSCLC cases are diagnosed at an advanced stage, and approximately 30% present the development of malignant pleural effusion (MPEs), a pathological condition mostly associated with poor prognosis.^{16,17} Even if in the last years several studies reported a thorough characterization of tumor heterogeneity and TME landscape of lung cancer from early to advanced stages,^{10,18–20} a real focus on metastatic sites was not yet provided.

MPEs, that arise when metastatic cancer cells infiltrate thoracic lymph nodes as well as the pleura, result in the accumulation of a fluid in which cancer and immune cells, stroma and their soluble factors interact promoting tumor proliferation, epithelial-mesenchymal transition and the emergence of most aggressive neoplastic cells.^{21–24} In this view MPEs, that are routinely therapeutically drained, represent an easily accessible and little invasive approach to investigate the types of interactions that occur in the TME of advanced lung cancer. In past years, our group has reported that MPEs from lung adenocarcinoma (LUAD) patients are a valuable source of primary

tumor cells, that show stem-like features when cultured in three-dimensional conditions.^{25–27} In this study, we exploited MPEs from LUAD patients as a model system to investigate the TME of metastatic NSCLC.

METHODS

Human specimens

MPEs and blood samples were obtained from 24 patients with histologically or cytologically confirmed diagnosis of LUAD complicated with MPEs. Patients were enrolled from three different medical centers in Rome, Istituti Fisoterapici Ospedalieri, Sant'Andrea Hospital and Policlinico Umberto I (Sapienza University of Rome) and named respectively as BBIRE, SA and PUC followed by a progressive patient number. Twenty-four patients consisted of 9 males and 15 females with a median of 66 years old. Blood samples from 12 healthy donors (HDs) were also collected (median age of 62 years old). Additional information regarding the clinical history of subjects involved in the study are reported in online supplemental table S1.

Mononuclear cell from pleural effusions and peripheral blood isolation from LUAD patients and HD

Mononuclear cell from pleural effusions (PEMC) and peripheral blood (PBMC) of LUAD patients and HD were isolated via OncoQuick (Greiner Bio-One) and Lympholyte-H (Cedarlane, Ontario, California, USA) gradient separation media. More details on samples processing are reported in online supplemental methods.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was performed as previously described.²⁸ TaqMan probes for CCL3, CCL20, CXCL2, IL6, VEGFA and GAPDH were purchased from Applied Biosystems. Primers used for individual genes were previously reported,²⁹ except for β -actin: Fw (5'–3')-GCCGGGACCTGACTGACT; Rv (5'–3')-TGGTGATGACCTGGCCGT. The mRNA levels were normalized using β -actin or GAPDH, as specified. The two housekeeping genes present similar expression levels among the various groups.

Bulk RNA Sequencing

Total RNA was extracted from PEMC and matched PBMC of five LUAD patients and from PBMC of four HDs, using Qiazol (Qiagen, Hilden, Germany), purified from DNA contamination through a DNase I (Qiagen) digestion step, and further enriched by Qiagen miRNeasy columns profiling (Qiagen). Quantity and integrity of the extracted RNA were assessed by Nanodrop Spectrophotometer (Nanodrop Technologies, Thermofisher, Waltham, Massachusetts, USA) and by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA), respectively. RNA libraries preparation, sequencing and subsequent bioinformatic analyses are described in online supplemental methods. RNA-seq

derived visualizations were generated via the AUTO-go framework.³⁰

CIBERSORTx deconvolution analysis

To determine the proportion of 22 different immune cell types in each sample, the gene expression data, obtained by RNA-seq, were uploaded to the CIBERSORTx webtool³¹ (<https://cibersortx.stanford.edu/>). The algorithm was run using the LM22 signature matrix, that defines 22 infiltrating immune populations based on the expression of 547 different genes, for 1000 permutations. We used bulk-mode batch correction and the output was in absolute mode, that reflects the absolute proportion of each cell type in the mixture. Only cases with a CIBERSORTx output $p < 0.05$ were chosen for further analysis. Then, the estimated cell type abundances were used to realize a differential deconvolution analysis by applying a paired or unpaired Student's t-test between the immune cell populations for the groups of interest.

Statistical analysis

Statistical analyses were performed using GraphPad Prism V.8.0 software and R environment for statistical

computing (V.4.1.0). Groups were compared by Student's t-test or Wilcoxon signed-rank test as indicated and statistical significance is represented in figures as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

RESULTS

Transcriptional profiling of LUAD PEMC and PBMC reveals an immunosuppressive and tumor-promoting TIME in LUAD pleural effusions

An overview of the workflow followed in the study is schematically represented in [figure 1](#). First, we performed a bulk RNA Sequencing (RNA-seq) on immune mononuclear cells isolated from MPEs (PEMC) and peripheral blood (PBMC) of five LUAD patients and on PBMC from a group of four HDs, as control. More detailed information on patients' characteristics and HDs enrolled are reported in online supplemental table S1.

Principal component analysis (PCA) and hierarchical clustering clearly segregated the transcriptomic profiles of the three different groups ([figure 2A,B](#)). This suggests that not only PEMC and PBMC from the

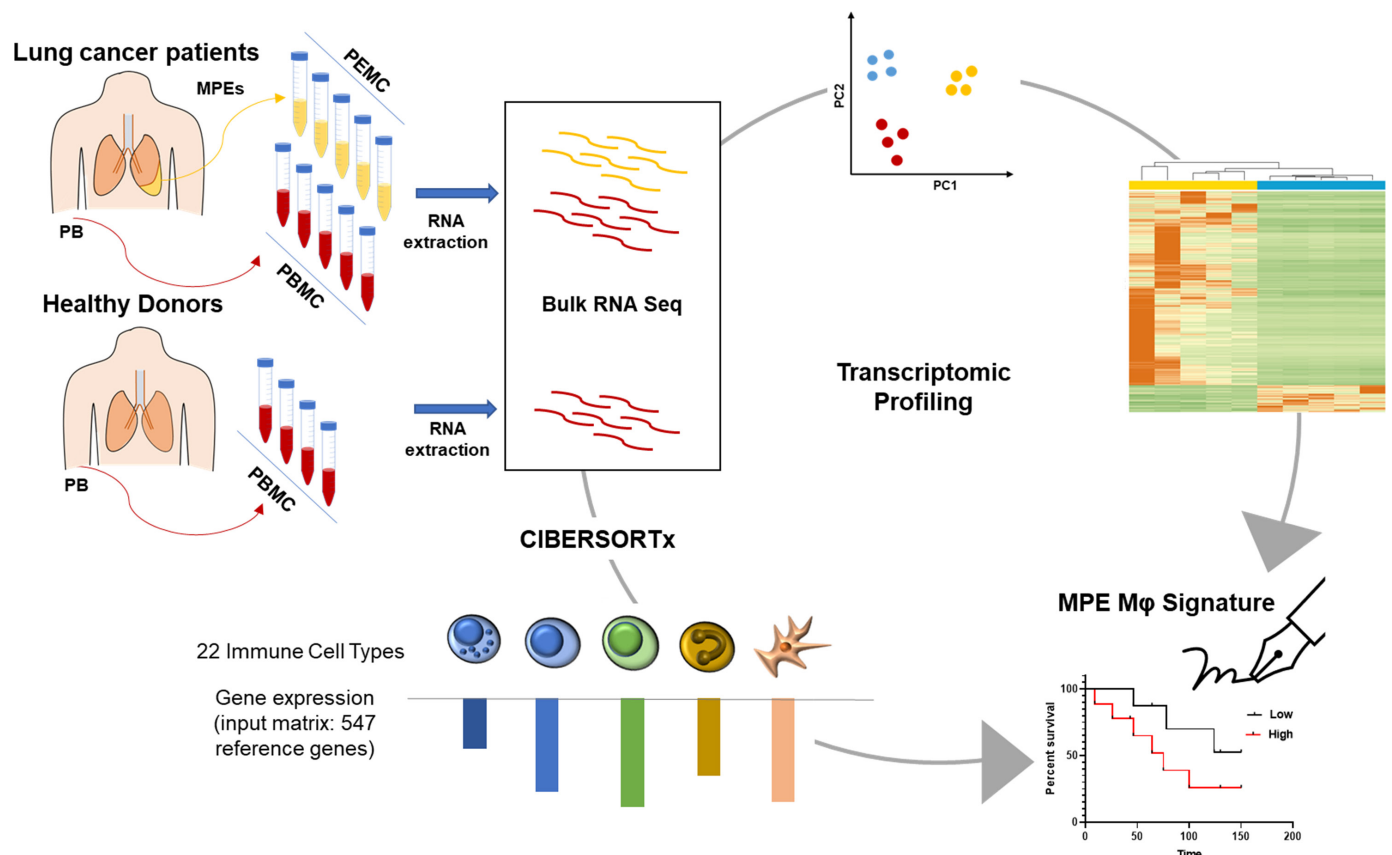
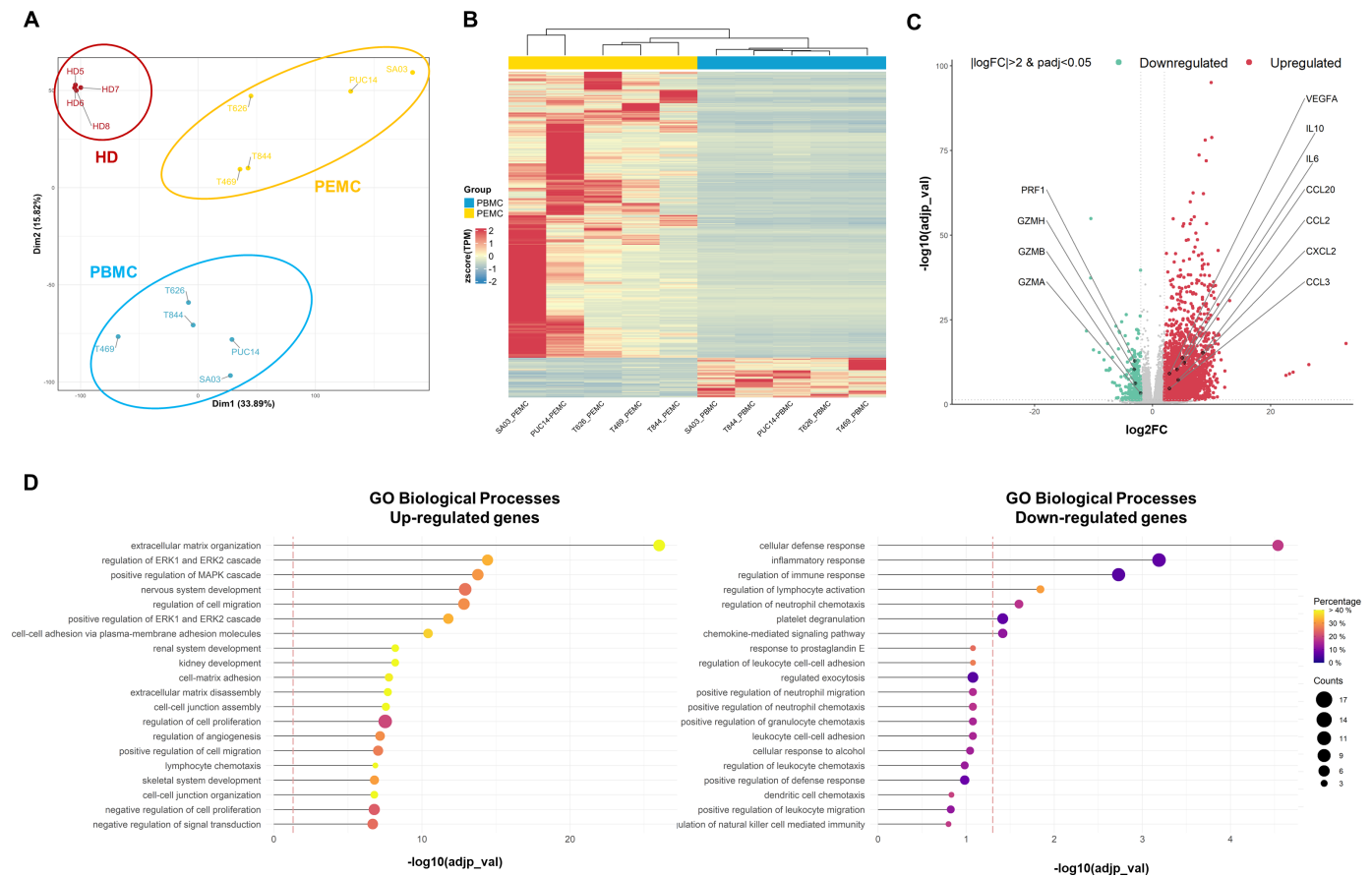


Figure 1 Schematic overview of the experimental workflow followed in the study. First, RNA from PEMC and matched PBMC of five LUAD patients and PBMC of four HD was extracted to perform a bulk RNA-Seq. PCA and DEA were performed to define the transcriptomic profile of LUAD immune cells and highlight differences between samples. Afterwards, to infer the immune cell proportions in each sample, RNA Seq data were subjected to CIBERSORTx deconvolution analysis. The tool permits to quantify at once the abundance of 22 immune cell types, based on a 547 genes signature matrix (LM22). Finally, this combined approach also allowed us to identify an MPEs macrophage (MPEs Mφ) signature related to patients' clinical outcome. DEA, differential expression analysis; HD, healthy donors; LUAD, lung adenocarcinoma; PCA, principal component analysis; PBMC, peripheral blood mononuclear cells; PEMC, pleural effusion mononuclear cells



same patients are transcriptionally distinct, as in part expected given their different origin, but also that PBMC from LUAD patients are substantially transcriptionally different from those of HDs (online supplemental files 3; 4).

Differential expression analysis (DEA) revealed 2694 differentially expressed genes (DEGs) in PEMC compared with their matched PBMC (2363 upregulated and 331 downregulated; $|\log_2\text{Fold Change}| > 2$ and adjusted $p < 0.05$, online supplemental table S2A) (figure 2C) and 1275 DEGs in LUAD vs HD PBMC (834 upregulated and 441 downregulated; $|\log_2\text{Fold Change}| > 2$ and adjusted $p < 0.05$, online supplemental table S2B and figure S1B).

The functional gene ontology (GO) enrichment analysis performed on the DEGs resulting from LUAD versus HD PBMC comparison, revealed, with respect to biological processes (BP), a set of enriched terms such as cellular response to cytokine stimulus, inflammatory response, positive regulation of cell migration and

angiogenesis for the downregulated genes in patients' PBMCs and no significant enriched term for the upregulated ones (online supplemental figure S1C and table S3A,B). This could suggest a certain level of dormancy of circulating immune cells in LUAD patients with respect to HDs.

Most importantly, the GO BP enrichment analysis performed on DEGs in PEMC with respect to their matched PBMC revealed a number of enriched terms related to tumor promotion for the upregulated genes, such as cell migration, extracellular matrix organization, angiogenesis, cellular proliferation, and a set of terms related to immune response for the downregulated genes (figure 2D and online supplemental table S3C,D).

Based on this analysis, we selected a set of DEGs more representative of this tumor-promoting and immune suppressive phenotype and split them into three main clusters: growth factors and metastatic/angiogenic promoters, inflammatory chemokines, and cytokines/

enzymes related to regulation of immune response (online supplemental figure S2A). qRT-PCR analysis performed on the RNA-Seq cohort and on nine additional LUAD patients (online supplemental table S1) confirmed a significantly increased expression of some of these proinflammatory cytokines and chemokines genes such as CXCL2, CCL20, IL6 and VEGFA in PEMC compared with matched LUAD PBMC (online supplemental figure S2B). The same validation performed on LUAD and HD PBMC confirmed the downregulation of CXCL2, CCL20 and VEGFA in LUAD patients respect to HDs (online supplemental figure S1D). Overall, these data suggest that in the microenvironment of MPEs immune cells acquire, with respect to their circulating counterpart (ie,PBMC), an immunosuppressive and tumor-promoting profile.

Deconvolution of bulk gene expression data depicts the Immune Landscape of LUAD MPEs and underpins the importance of myeloid compartment

In order to define the immune cell landscape of MPEs, the next step was to perform a deconvolution analysis of the gene expression data obtained from the RNA-Seq,

described in the previous paragraph, exploiting CIBERSORTx, a tool which uses predefined immune cells specific gene signature matrices for the in silico quantification of 22 different immune types.³¹ Algorithm execution allowed us to infer the immune composition of our samples (online supplemental table S1) reported both as absolute score (see heatmap figure 3A and online supplemental table S4A), and as estimated relative fractions (see bar plot figure 3B). These data clearly show how samples belonging to each group cluster together, demonstrating that there are strong differences in the immune cell composition among the three groups. Moreover, samples belonging to each group show a similar immune landscape, even if, similar to the observations in other studies,¹⁸⁻²⁰ a certain variability among patients' samples is appreciable and most pronounced in the PEMC group. Of note, the four HD PBMC samples show a very homogeneous and reproducible immune pattern, thus confirming the robustness of the deconvolution analysis. The analysis revealed that out of the detectable 22 immune cell types, each deconvoluted group contains a variety of them ranging from 11 in HD PBMC to 15 in LUAD PBMC and

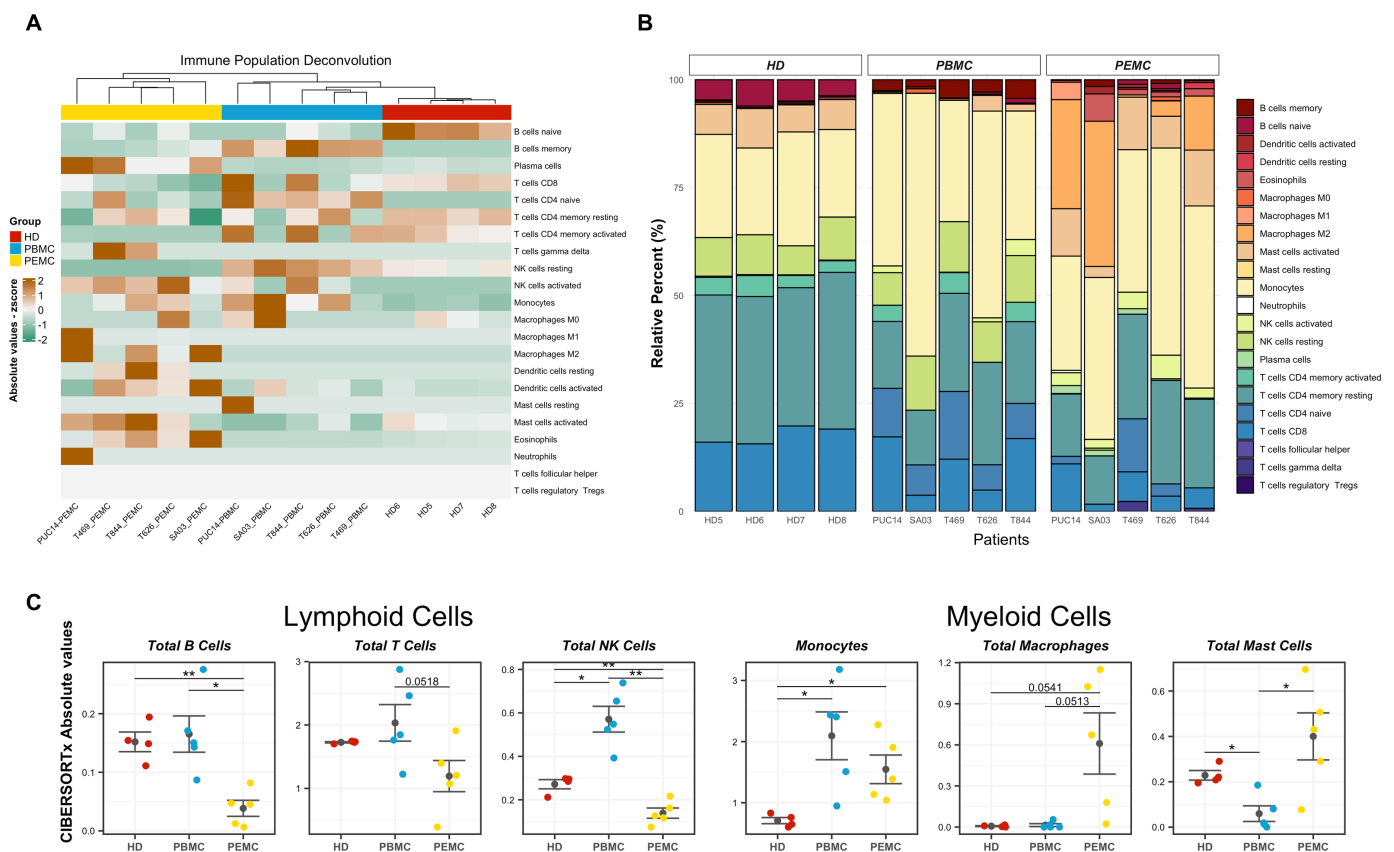


Figure 3 CIBERSORTx deconvolution analysis depicts the Immune Landscape of LUAD MPEs. (A) Heatmap showing the absolute abundances of 22 immune cell types in each sample. Hierarchical clustering shows a clear segregation of the samples according to their originating group, LUAD PEMC (yellow), LUAD PBMC (blue) and HD PBMC (red). Absolute values are Z score transformed. (B) Stacked bar plots reporting the estimated relative fractions of each immune cell type in a given mixture sample. (C) Dot plots showing the comparisons of total lymphoid and myeloid immune cells between the three sample groups. Each dot represents one patient. Mean values and SD for each cell subset were calculated for each patient group and compared using paired (LUAD PEMC vs PBMC) or unpaired (LUAD PBMC vs HD) two-tailed Student's t-test. *P<0.05; **p<0.01. HD, healthy donor; LUAD, lung adenocarcinoma; PBMC, peripheral blood mononuclear cells; PEMC, pleural effusion mononuclear cells.

19 in LUAD PEMC. Only follicular helper T cells and regulatory T cells (Tregs) were not found in any group. Instead, both eosinophils and neutrophils were detected in a very low proportions and the latter only in one PEMC sample (figure 3A,B and online supplemental table S4A). Regarding the immune cell distribution in HD PBMC the most abundant populations are represented by CD4 T cells (mainly CD4 memory resting), followed by monocytes and CD8 T cells (roughly in a 2:1 ratio with CD4 T cells) with a smaller fractions of NK cells resting, activated mast cells, naïve B cells and a very small fraction of activated dendritic cells (DCs) (figure 3B,C, online supplemental figure S3). These results are completely in line with the well-known relative abundancies of immune cell populations found in normal human blood samples,³² underpinning the reliability of this *in silico* approach.

Looking at the immune composition of LUAD PBMC, even if the overall proportion between immune cell types is conserved with respect to that of HD PBMC, some differences can be identified, as shown by the differential deconvolution analysis (online supplemental table S4B). T CD4 cells remain the most abundant lymphocytes. However, respect to HD it is possible to detect not only CD4 memory resting and activated ones, but also naïve CD4 T cells. The total proportion of B cells is also conserved with a switch from naïve B cells to memory ones. Instead, an increased fraction of NK cells is observed with the appearance also of activated NK, even if in a 1:10 ratio with NK resting cells. Furthermore, a strong decrease in activated mast cells is appreciable. Most importantly, in LUAD PBMC the most abundant cell type is represented by monocytes and not anymore by CD4 T cells (figure 3B,C, online supplemental figure S3). Of note, this finding is in line with the upregulation in LUAD versus HD PBMC of the most relevant receptors involved in monocytes migration and macrophage differentiation,^{33,34} such as CCR2, CX3CR1 and CSF1R, as shown by DEA performed on RNA-seq data (online supplemental figure S1B).

We used these results as a baseline of the immune status in LUAD patients to unravel the specific features of the MPEs immune landscape. When we looked at immune cell composition of LUAD PEMC we found that, as in LUAD PBMC, the two most abundant immune cell types are represented by monocytes and CD4 T cells. However, the differential deconvolution analysis (online supplemental table S4B) showed a decreasing trend in the overall content of all effector cells, such as total T and B lymphocytes and NK cells in PEMC compared with PBMC (figure 3C). With respect to CD4 T cells the main difference was found in CD4 T activated memory cells. The same trend was observed in CD8 T cells, even if statistical significance was not reached. There is also an overall decrease in B cells content with a significant switch from memory B cells to plasma cells. Of note, even if a small fraction of NK activated cells is detectable there is a clear decrease in the overall content of NK cells (figure 3C and online supplemental figure S3). As in PBMC a very small fraction of activated DC cells is detectable, with the appearance of

a comparable fraction of resting DC in three out of five patients (figure 3A and online supplemental figure S3). Most importantly, in MPE compartment the high proportion of monocytes is supported by a prominent increase of protumoral M2 polarized macrophages and activated mast cells (figure 3C and online supplemental figure S3). This is in line with the immunosuppressive and tumor promoting profiling of PEMC that we defined by DEA (figure 2C,D). Overall, these results depict a TIME in which both adaptative and innate immune cells are still present but dominated by monocytes/protumoral M2 macrophages, that in concert with activated mast cells, could sculpt the TIME toward an immunosuppressive phenotype.

Since the enrichment of M2 macrophages in LUAD PEMC respect to PBMC is very pronounced, but borderline for statistical significance ($p=0.0528$), to validate these results, we assessed by qRT-PCR the mRNA expression levels of M2 (ie, CD163, IL10, CD206 and CCL22) and M1 (ie, COX-2, IL1B and IL12) specific macrophage markers in PEMC and matched PBMC of the RNA-Seq cohort and nine additional LUAD patients. The analysis confirmed a significantly upregulation of the M2 markers in PEMC. Instead, no significant variations were detected for the M1 markers (figure 4A). The same analysis performed on LUAD and HD PBMC revealed no clear polarization toward M1 or M2 phenotypes (figure 4B).

Finally, to further strengthen the concept that, in the microenvironment of MPEs, monocytes are induced to acquire a protumoral M2 phenotype, THP1-derived macrophages were stimulated for 48 hours with serum free medium (M0 macrophages) or medium supplemented with cell-free MPE material from LUAD patients (figure 4C). qRT-PCR evaluation of M1/M2 markers clearly showed that MPEs from three different patients, included in the RNA-seq experiment, induced a marked and significant increase of three M2 genes (CD163, IL10 and CD206) and a parallel decrease of two M1 genes (IL1B and IL12) when compared with the baseline mRNA levels of M0 macrophages (figure 4D), thus confirming the M2-polarizing activity of MPEs.

Cytokine analysis of LUAD MPEs confirms an immunosuppressive, wound healing and tumor promoting environment

The bioinformatic analysis described in previous paragraphs revealed a proinflammatory, immunosuppressive and tumor promoting profile of the cellular component in the TIME of MPEs. Since soluble factors are known to play a pivotal role in shaping the TME, the levels of 48 different cytokines and growth factors were evaluated in MPEs-derived cell free supernatant and plasma of 15 different LUAD patients and of 4 HDs, as control (online supplemental table S5A). PCA and hierarchical clustering show that MPEs and plasma of LUAD patients are characterized by distinctive cytokine patterns that could, in part, explain the differential immune cell composition in these two compartments. Instead, a similar cytokine pattern

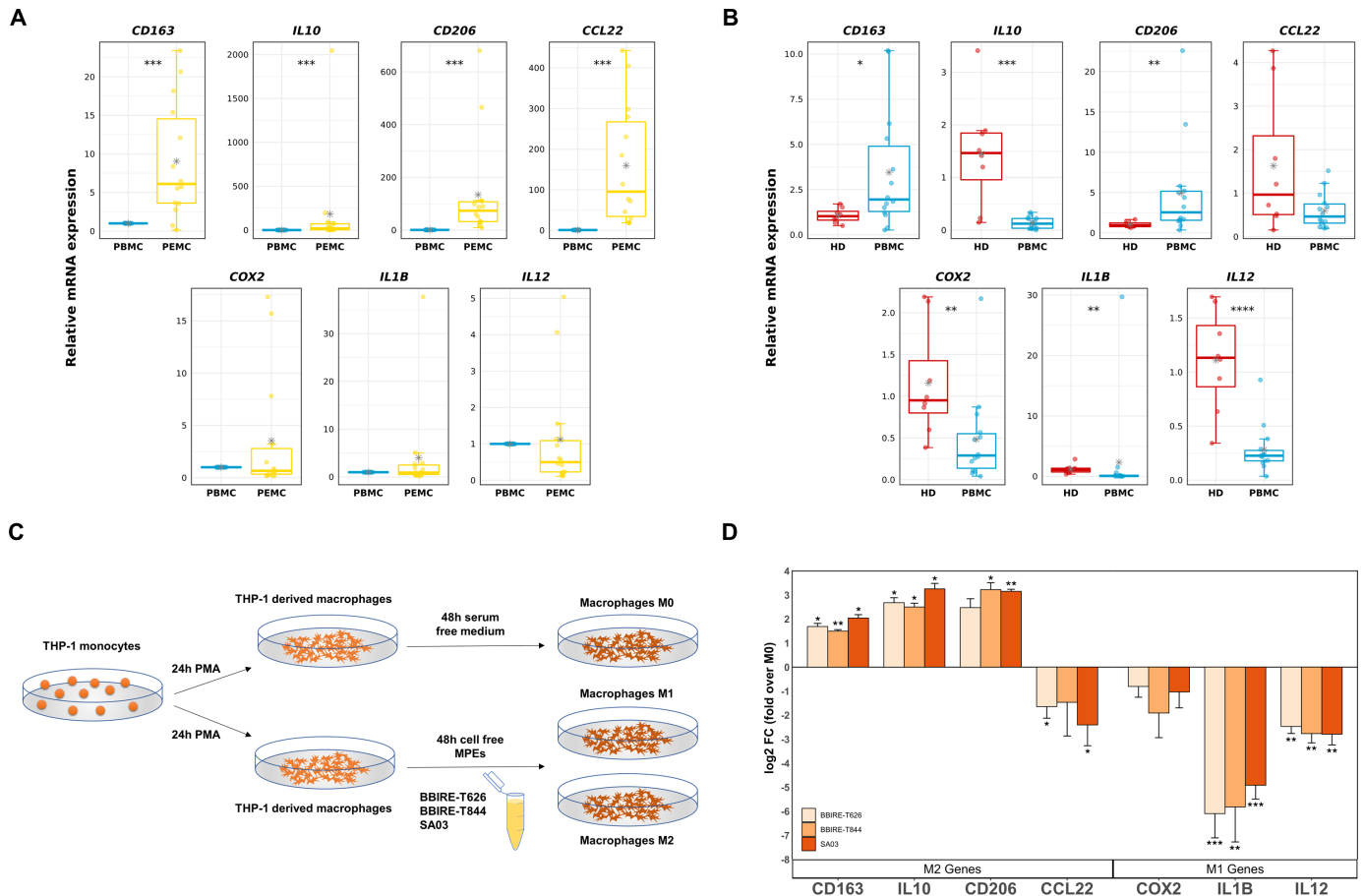


Figure 4 In the immune contexture of MPEs macrophages acquired a protumoral M2 phenotype. Quantitative real-time PCR (qRT-PCR) analyses showing the mRNA levels of M2 (CD163, IL10, CD206, CCL22) and M1 (COX2, IL1B, IL12) specific marker genes in LUAD PEMC compared with matched PBMC (A) and in LUAD PBMC compared with HD PBMC (B). β -actin was used as housekeeping gene. The box plot represents minimum and maximum values (whiskers), median values (center lines), mean values (center asterisk) and 25th and 75th percentiles (box edges), with all data points plotted. Each dot represents one patient (healthy: $n=8$; patients: $n=14$). * $P<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (Wilcoxon signed-rank test). (C) Schematic representation of THP-1 in vitro differentiation and macrophage polarization. THP1 monocytes were differentiated into macrophages in the presence of PMA (phorbol-12-myristate-13-acetate) and then stimulated with serum free medium (M0 macrophages) or with medium supplemented with 10% of cell-free pleural effusion material from three different LUAD patients (BBIRE-T626, BBIRE-T844 and SA03). After 48 hours, macrophage polarization was assessed by qRT-PCR evaluation of the mRNA levels of M1/M2-specific marker genes. (D) qRT-PCR analysis of M2 and M1 marker genes in THP-1 cells after 48 hours exposure to serum free medium (M0 macrophages) or medium supplemented with MPEs supernatant from LUAD patients. Data are reported as \log_2FC of the mRNA levels in treated THP1 over M0 macrophages (β -actin was used as housekeeping gene). The mean \pm SEM of three independent experiments is reported. * $P<0.05$, ** $p<0.01$, *** $p<0.001$ (two-tailed Student's t-test). HD, healthy donor; LUAD, lung adenocarcinoma; MPEs, malignant pleural effusions; PBMC, peripheral blood mononuclear cells; PEMC, pleural effusion mononuclear cells.

was observed in LUAD and HD plasma (figure 5A,B), suggesting that the main differences between patients and HDs do not reside in the soluble component. Based on this result, we focused our attention on LUAD patients and sought to define the characteristic cytokine profile of MPEs. To this purpose we (1) clustered the cytokines based on their concentration, ranging from <5 pg/mL up to 80 ng/mL (figure 5C); (2) calculated the fold increase or decrease of MPEs cytokines with respect to their systemic levels (ie, plasma of matched LUAD patients). As reported in table 1, 29 out of the 48 cytokines analyzed resulted statistically different between the two groups of samples. Fewer differences were observed for LUAD/

HDs comparison with increased levels of some cytokines, such as IL-6, IL-8, VEGFA and IL-2ra, in patients' plasma respect to that of HDs, as also previously reported³⁵ (online supplemental figure S4 and table S5B).

Focusing on LUAD plasma/MPEs comparison, even if SCGF-b represents the most concentrated cytokine in MPEs (83,287.1 \pm 8944 pg/mL in LUAD MPEs vs 25,082.5 \pm 3814 pg/mL in LUAD plasma), IL-6 shows a very high discrepancy between pleural fluid and plasma of LUAD patients (fold=413.9). Based on both parameters, it was possible to highlight different classes of cytokines that shape the MPEs microenvironment: (1) proinflammatory cytokines (ie, IL6, IL8, and CCL2); (2)

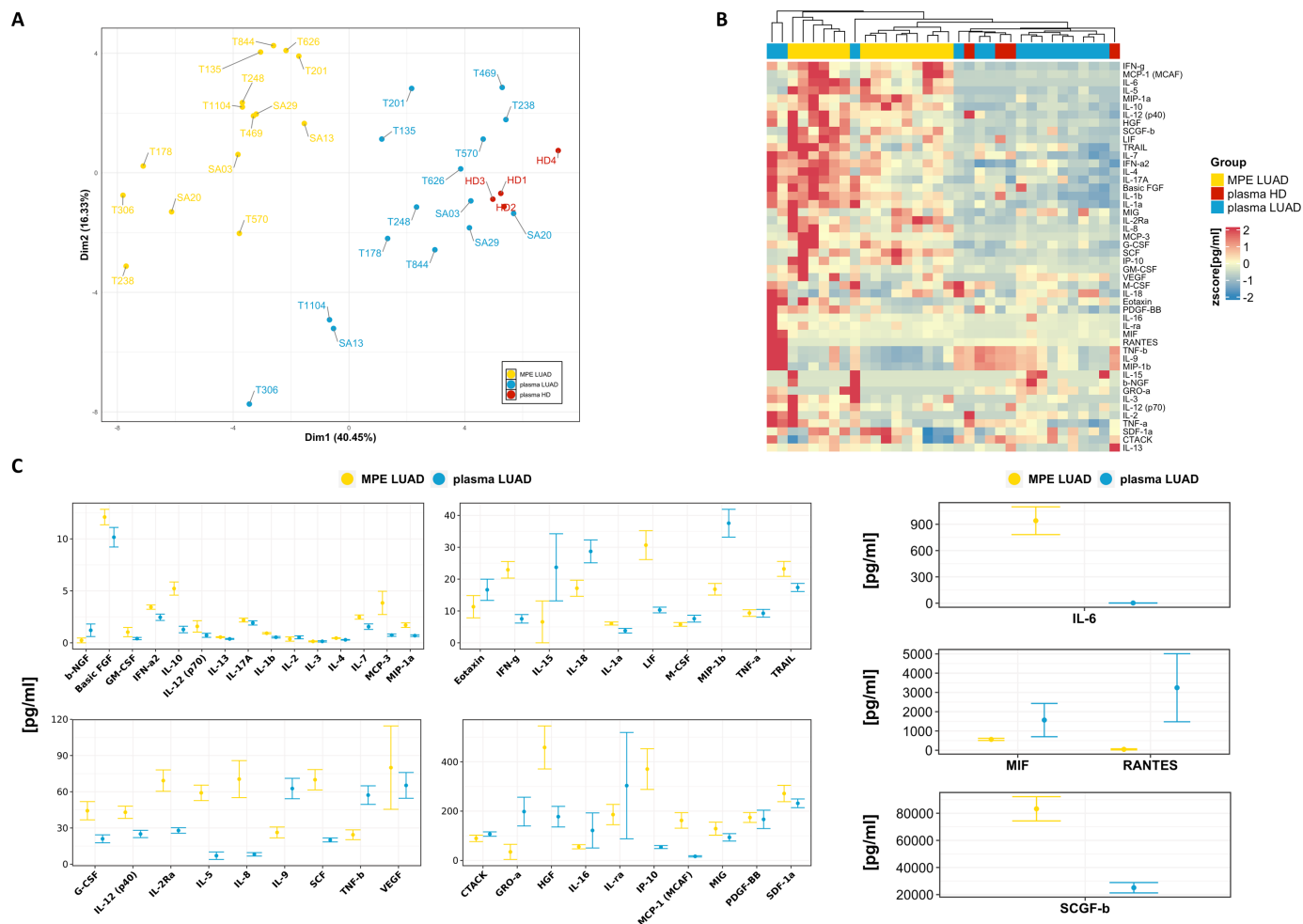


Figure 5 Cytokinome analysis of LUAD MPEs confirms an immunosuppressive, wound healing and tumor promoting environment. Principal component analysis (A) and hierarchical clustering (B) of 48 different cytokines levels evaluated in cell free pleural fluid and plasma of 15 LUAD patients (yellow and blue, respectively) and plasma of 4 HD (red) using a Bio-Plex sandwich immunoassay (see online supplemental methods). (C) Scatter plots showing the mean values \pm SEM (n=15) of cytokines concentration (pg/mL), evaluated in MPE and plasma of 15 LUAD patients. HD, healthy donor; LUAD, lung adenocarcinoma; MPE, malignant pleural effusion.

cytokines and growth factors linked to the maintenance of a stem like tumor phenotype (ie, IL6, IL8, SCF, SCF-b, LIF, and HGF)^{36,37}; (3) cytokines that promote monocytes infiltration and Th2/M2 polarization (ie, IL6, CCL2, IL5, IL10, and MCP-3),^{34,38,39} even if the lasts with medium and low concentration (table 1), but with high fold increase respect to plasma, suggesting a specific role in MPEs microenvironment. Furthermore a nascent but thwarted effector response in LUAD MPEs is witnessed by (1) the high concentration of CXCL10, a chemokine well known to play a role in T cell trafficking and activation,⁴⁰ in line with the high proportion of CD4 T cells in LUAD PEMC, as shown by our deconvolution analysis; (2) the presence of effector cytokines (ie, INF α 2, MIP1 α , INF γ , and TNF β) in a relatively low levels, but significantly higher with respect to plasma (excepted for TNF β). These results suggest that soluble factors identified in the microenvironment of MPEs contribute to the creation of a proinflammatory and immunosuppressive environment that probably shape the immune cellular component from

an effector toward an inactive mode. Moreover, wound healing assays, performed on two MPEs-derived primary cell lines stimulated for 24 hours with their matched cell free MPE supernatant, clearly show how these soluble components also impact onto tumor migratory capacity in the MPEs microenvironment (online supplemental figure S5).

A novel MPE-associated gene signature correlates with poor prognosis in LUAD patients

All our previous observations suggested the involvement of macrophages in the immune contexture of LUAD MPEs, where they acquire a protumoral M2 phenotype.

Two ontologically different macrophage types are known to populate adult lungs: tissue resident macrophages (TRMs) and monocytes derived macrophages (MDMs). To analyze the components that populate MPEs we tested the expression of a TRM and an MDM signature, identified in early-stage human NSCLC lesions,¹⁵ in our RNA Seq data from LUAD PEMC and PBMC.

Table 1 List of 29 cytokines differentially expressed between cell free pleural fluid and plasma of LUAD patients

	MPEs concentration (pg/mL)	Plasma concentration (pg/mL)	Fold MPEs/plasma	P value
SCGF-b	83 287.1 (±8944)	25 082.5 (±3814)	3.3	<0.0001
IL-6	940.1 (±158.3)	2.3 (±0.9)	413.9	<0.0001
HGF	458.1 (±87.1)	177.5 (±41.8)	2.6	0.0205
IP-10 (CXCL10)	370.5 (±82.6)	54.3 (±6.1)	6.8	0.0018
MCP-1 (CCL2)	162.3 (±31.4)	16.6 (±2.7)	9.8	0.0005
IL-8	70.5 (±15.3)	8.2 (±3.1)	8.6	0.0008
SCF	70.0 (±8.4)	20.1 (±1.7)	3.5	<0.0001
IL-2Ra	69.2 (±8.9)	27.9 (±2.3)	2.5	0.0001
IL-5	59.1 (±6.4)	7.0 (±3.1)	8.4	<0.0001
G-CSF	44.3 (±7.6)	21.0 (±3.3)	2.1	0.0021
IL-12 (p40)	43.0 (±5.1)	25.0 (±3.0)	1.7	0.0200
GRO-a (CXCL1)	34.7 (±30.7)	198.1 (±58.4)	0.2	0.0355
LIF	30.7 (±4.5)	10.3 (±0.9)	3.0	0.0008
IL-9	26.3 (±4.5)	62.7 (±8.5)	0.4	0.0022
TNF-b	24.4 (±4.1)	57.2 (±7.7)	0.4	0.0023
TRAIL	23.2 (±2.3)	17.4 (±1.3)	1.3	0.0363
IFN-g	22.9 (±2.6)	7.5 (±1.3)	3.0	<0.0001
IL-18	17.1 (±2.5)	28.7 (±3.6)	0.6	0.0282
MIP-1b (CCL4)	16.8 (±1.8)	37.6 (±4.4)	0.4	0.0008
IL-1a	6.1 (±0.5)	3.8 (±0.7)	1.6	0.0278
M-CSF	5.8 (±0.6)	7.6 (±1.1)	0.8	0.0274
IL-10	5.2 (±0.6)	1.3 (±0.3)	4.1	0.0002
MCP-3 (CCL7)	3.8 (±1.1)	0.7 (±0.1)	5.2	0.0132
IFN-a2	3.4 (±0.2)	2.5 (±0.3)	1.4	0.0284
IL-7	2.5 (±0.2)	1.6 (±0.3)	1.6	0.0008
MIP-1a (CCL3)	1.7 (±0.2)	0.7 (±0.1)	2.5	0.0001
IL-1b	0.9 (±0.1)	0.5 (±0.1)	1.7	0.0015
IL-13	0.5 (±0.04)	0.4 (±0.05)	1.5	0.0026
IL-4	0.5 (±0.03)	0.3 (±0.03)	1.6	0.0006

Cytokines are listed based on the higher concentration in MPEs. Mean values of concentration \pm SEM (n=15) are reported for each cytokine in each group. Fold is considered statistically significant if $p < 0.05$ (two-tailed Student's t-test).

LUAD, lung adenocarcinoma; MPEs, malignant pleural effusions.

Only 9 out of 22 genes of TRMs resulted significantly up regulated in PEMC *vs* matched PBMC compared with 28 out of 37 MDMs genes (|Log₂Fold Change|>2 and adjusted $p < 0.05$) (figure 6A,B). This result suggests that TAMs in MPEs may originate from MDMs, in line with the concept that TRMs accumulate at an early phase of tumor formation and that afterwards, during tumor propagation, TME becomes dominated by MDMs.¹⁵ Even if, for long time TAMs have been considered as tumor recruited, generally, protumoral population of differentiated monocytes that have an M2-like phenotype, it is accepted nowadays that in the TME macrophages exist on a continuum of phenotypes between classically (M1) and alternatively activated (M2).⁴¹ Indeed, when we looked at the most up regulated genes in PEMC we identified a set

of 37 genes (called MPEs M ϕ signature, see online supplemental methods and online supplemental table S2C), that do neither match with TRM nor with MDM signature (figure 6A,B) but are still related to macrophage, as shown by the analysis performed on ImmGen expression data⁴² (figure 6C). The same analysis on the MDM signature gave results comparable to MPEs M ϕ signature (online supplemental figure S6A); however, with respect to the TRM signature the results clearly show that TRMs genes are specifically enriched in the alveolar macrophage populations with respect to all the other immune cell types explored (online supplemental figure S6B), thus strengthening the findings obtained on MPEs signature. The protein–protein interaction network analysis

(figure 6E), indicating an histotype specificity for the identified signature. Similar but less impressive results were obtained by testing the MDM signature (online supplemental figure S6C) and no significant results were obtained from the TRM signature (online supplemental figure S6D), suggesting that the newly MPEs M ϕ signature identified in this study better represents myeloid targets for the treatment of metastatic LUAD.

DISCUSSION

The implication of TIME as a key element determining resistance to immunotherapy with ICIs is now widely appreciated.⁴⁴ Furthermore the metastatic involvement of pleural and peritoneal cavities was found to be associated with worse ICIs outcome in cancer patients.⁴⁵ In this context, the past few years have witnessed a series of studies reporting a complete characterization of tumor immune landscape in NSCLC from early to advanced disease.^{7 10 15 18–20} Here we performed a deep characterization of immune cells and soluble factors from MPEs and peripheral blood of LUAD patients to investigate the TIME of advanced NSCLC in a metastatic setting more easily accessible than others and, to our knowledge, still little explored.

All our observations concur to define an MPEs-specific microenvironment potentially prone to an effective immune-response, but conscripted in a wound-healing, proinflammatory and tumor-supportive mode. This is witnessed first by upregulation in PEMC, respect to the circulating PBMC counterpart, of genes related to (1) tumor promotion, spanning from angiogenesis and extracellular matrix organization (such as VEGFA, SERPINE1, FN1 and members of matrix metalloproteinase family) to cellular proliferation (such as LIF, FGF and WNT growth factors, also linked to the maintenance of tumor stem cells properties); (2) activation of chronic inflammation and immune suppression (such as IL6, IL1A, CCL3, IL10 and IDO1); (3) recruitment of TAMs, T Regs and MDSCs (such as CCL2, CCL7, CXCL1, CXCL2, CCL20); and parallel downregulation of genes (such as PRF1 and granzymes) related to the activation of effector cytotoxic cells (ie, CTL and NK cells). In accordance *in silico* immune deconvolution revealed a decreasing trend in the overall content of all effector cells (T and B lymphocytes and NK cells) in MPEs. On the other hand, the high proportion of monocytes, M2 polarized macrophages and activated mast cells suggests that these three immune cell types, well known to be involved in inflammatory, proangiogenic, metastatic and immune suppressive pathways,^{11 46 47} could represent the major drivers in shaping an immunosuppressive MPEs microenvironment. Indeed, the presence, even if in a relatively low levels, of potent effector cytokines such as INF α 2, MIP1 α , INF γ and TNF β , is a further hint of a present but thwarted immune response in the TME of MPEs. Instead, the most represented and characteristic soluble factors in MPEs are related to inflammatory processes, tumor propagation and stem like phenotype

maintenance and Th2/M2 polarization. Of note SCF is also known to mediated mast cells recruitment and activation, via c-kit receptor, in tumor microenvironment.⁴⁸

These findings are also coherent with the ability of cell free MPEs supernatant to induce *in vitro* M2 phenotype polarization. Our data are in line with previous findings that defined TAMs as the dominant immunosuppressive cell type in LUAD MPEs, leading to T cells disfunction via TGF- β .²¹ Given the correlation between high macrophage infiltration and poor prognosis in several human cancers, including NSCLC,^{12 13 49} these cells represent a promising target for anticancer therapies. Strategies, that aim at limiting macrophage recruitment or at reprogramming their phenotype toward an antitumor one, are currently under investigation.⁵⁰

IL6 and CCL2 that we found at high concentration and with a large difference between intrapleural and systemic levels, suggesting that they are locally secreted and carry out a pivotal role in that specific TME, are inflammatory factors correlated to MPEs and monocytes/macrophages recruitment.²⁴ In particular, CCL2 was shown to be a major player in the recruitment of monocytes and M2 macrophage differentiation in MPEs of mesothelioma patients.²² Furthermore, very recently it has been shown that the blockade of CCL2 expression could overcome the intrinsic PD-L1/PD-1 resistance in triple negative breast cancer.⁵¹ These evidences support our hypothesis that monocytes of LUAD patients, the most abundant circulating immune cell type, are recruited via CCR2/CCL2 axis into MPEs where they switch toward a proinflammatory and protumoral phenotype. This concept is supported by several findings: (1) the upregulation of CCR2 receptor gene expression in LUAD PBMC with respect to HD PBMC; (2) higher concentration of CCL2 in MPEs with respect to plasma; (3) high amounts of M2 macrophages in MPEs, as shown by CIBERSORTx deconvolution, and finally (4) the ability of MPEs to induce macrophage M2 polarization *in vitro*. Even if these observations require further *in vitro* investigations, they suggest CCL2 as a potential key target for therapy in this clinical setting. Of note, antibodies that selectively target CCL2 or its receptor (CCR2) have completed phase I and II clinical trials showing promising results in advanced prostate and pancreatic cancers.⁵⁰ Likewise, clinical trials testing the activity of antibodies anti-IL6/IL6R α are currently under investigations.⁵⁰ In this regard IL-6 resulted not only as one of the most represented cytokines in pleural effusions, but also as central gene in the identified MPEs M ϕ signature, along with FN1, highlighting a protumoral as well as a profibrotic phenotype, that could in turn promote the mobility of cancer cells in this metastatic site. Further investigations of this immune subtype may reveal novel tumor-immune interactions that are at the basis of metastatic process.

Although T cells have been the primary target of cancer immunotherapy, myeloid cells exhibit specific phenotypes and functions that could impact on cancer progression and immunotherapy response.¹ Noteworthy the 37-gene

MPEs M ϕ signature, identified in this study, resulted to be associated with poor clinical outcome specifically in LUAD patients. Hence approaches aiming to couple the specific targeting or reprogramming of immunosuppressive myeloid cells with immunotherapies directed to the reactivation of T cells could be a successful strategy to overcome drug resistance. In this regard a recent study showed how in cholangiocarcinoma the dual inhibition of TAM and G-MDSC potentiates ICI therapy, underpinning the importance of this type of combinatorial approach.⁵²

Overall, our study reported for the first time a comprehensive and wide characterization of MPEs microenvironment in LUAD patients. Furthermore, given the potentiality to collect from MPEs both tumor and immune cells, it represents a patient-derived system that could be used to establish *ex vivo* co-culture models as a reliable *in vitro* testbed to evaluate the efficacy of new therapeutics.

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