Intrapleural nano-immunotherapy promotes innate and adaptive immune responses to enhance anti-PD-L1 therapy for malignant pleural effusion

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Malignant pleural effusion (MPE) is indicative of terminal malignancy with a uniformly fatal prognosis. Often, two distinct compartments of tumour microenvironment, the effusion and disseminated pleural tumours, co-exist in the pleural cavity, presenting a major challenge for therapeutic interventions and drug delivery. Clinical evidence suggests that MPE comprises abundant tumour-associated myeloid cells with the tumour-promoting phenotype, impairing antitumour immunity. Here we developed a liposomal nanoparticle loaded with cyclic dinucleotide (LNP-CDN) for targeted activation of stimulators of interferon genes signalling in macrophages and dendritic cells and showed that, on intrapleural administration, they induce drastic changes in the transcriptional landscape in MPE, mitigating the immune cold MPE in both effusion and pleural tumours. Moreover, combination immunotherapy with blockade of programmed death ligand 1 potently reduced MPE volume and inhibited tumour growth not only in the pleural cavity but also in the lung parenchyma, conferring significantly prolonged survival of MPE-bearing mice. Furthermore, the LNP-CDN-induced immunological effects were also observed with clinical MPE samples, suggesting the potential of intrapleural LNP-CDN for clinical MPE immunotherapy.

Malignant pleural effusion (MPE) secondary to metastatic cancer represents an enormous challenge in clinical patient management. The appearance of MPE is an ominous prognostic sign for patients with cancer; the average survival of patients with MPE is 4–9 months. Moreover, accumulation of pleural effusion commonly causes dyspnoea that severely compromises quality of life. The current standard of care treatment for MPE includes catheter drainage or chemical/surgical pleurodesis but is largely palliative. MPE is a build-up of extra fluid in the space between the lungs and chest wall, comprising tumour cells and various types of immune cells. Accompanying MPE, disseminated and unresectable tumour foci (carcinomatosis) often develop on the pleural surface. Clinical immunopathological studies suggest that the tumour microenvironment (TME) of MPE is profoundly immunosuppressive with abundant tumour-promoting myeloid immune cells and high levels of immunosuppressive cytokines, which negatively affects antitumour immunity. Moreover, variable levels of programmed death ligand 1 (PD-L1) expression have been detected on tumour cells in the transcriptional landscape in MPE, mitigating the immune cold MPE in both effusion and pleural tumours. Moreover, combination immunotherapy with blockade of programmed death ligand 1 potently reduced MPE volume and inhibited tumour growth not only in the pleural cavity but also in the lung parenchyma, conferring significantly prolonged survival of MPE-bearing mice. Furthermore, the LNP-CDN-induced immunological effects were also observed with clinical MPE samples, suggesting the potential of intrapleural LNP-CDN for clinical MPE immunotherapy.

The stimulator of interferon genes (STING) pathway has recently been identified to play an important role in antitumour immunity. As a potent STING agonist, the cyclic dinucleotide (CDN), 2’3’-cGAMP, functions in the cytosol to ligate STING and activate the STING pathway and type I interferon (IFN) production. While recent preclinical studies involving intratumoral injection of CDN have demonstrated its ability to enhance antitumour immunity in solid tumours, the potential of intrapleural CDN has not been explored. However, there are some concerns with the use of free CDN in MPE. Built from labile phosphodiester bonds, the CDN is susceptible to degradation by ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1). Soluble ENPP1 exists in human and mouse serum and higher levels of ENPP1 have been reported in malignant effusion. More recent studies have shown...
that activation of the STING pathway within tumour-resident antigen-presenting cells (APCs) is necessary for the induction of antitumor CD8+ T cell immunity, whereas non-targeted, free CDN can induce T cell apoptosis or increase the resistance of tumor cells to immune checkpoint blockade (ICB).

To overcome the immune cold MPE, we synthesized CDN-loaded, phosphatidylserine (PS)-coated liposomes (LNP-CDN) that demonstrate favorable pharmacokinetic profiles in MPE and selective targeting of intrapleural phagocytes. Loading of CDN complexed with calcium phosphate (CaP) enables pH-responsive release of CDN from the endosome to the cytosol, where it ligates STING to initiate STING signaling. To gain insights into the immunological effects of intrapleural LNP-CDN on individual immune cell populations, we conducted single-cell RNA sequencing (scRNA-seq) of MPE in mouse models. We then investigated if LNP-CDN-induced pro-inflammatory MPE and upregulation of PD-L1 would set the
stage for response to anti-PD-L1 immunotherapy against MPE in mouse models. Furthermore, MPE samples of patients with non-small cell lung cancer (NSCLC) were obtained to demonstrate that the immunological effects induced by LNP-CDN in mice could be reproduced in humans.

**Intrapleurally injected LNP-CDN targets phagocytes in MPE.**

We synthesized a series of LNPs with variable surface compositions of different phospholipids with/without polyethylene glycol (PEG), to load CDN complexed with CaP (Supplementary Fig. 1). Among them, the PS-coated LNPs with/without DSPE-PEG2000 (10%; LNP-1 and LNP-2) exhibited excellent in vitro phagocytosis targeting specificity and PS-mediated cell uptake (Supplementary Fig. 1). The PEGylated LNP-2 was stable over time with little CDN release at pH 7.4, but destabilized rapidly to release CDN at an acidic pH (Supplementary Fig. 1). In vivo pharmacokinetic data revealed that LNP-2 achieved better MPE retention and less extrathoracic distribution than the non-PEGylated LNP-1 (Supplementary Fig. 2). Therefore, LNP-2-based LNP-CDN was chosen to use in this study.

Intrapleural LNP-CDN has an average diameter of ~120 nm and a negative surface charge of ~−15 mV (Fig. 1a). A mouse MPE model of Lewis lung cancer (LLC) developed both the fluid in the pleural cavity and multifocal tumours on the pleural surface, clearly seen by in vivo magnetic resonance imaging (MRI) and at autopsy (Fig. 1a). An in vivo imaging system (IVIS) at different times post-intrapleural injection of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine iodide (DiR)-labelled LNP clearly showed that the majority of signals remained in the chest region and were sustained for at least 48 h (Fig. 1b,c). Ex vivo IVIS detected DiR-LNP signals in pleural tumours, tumour-draining lymph nodes (TDLNs), lung and liver (Fig. 1d), which were quantitated by high-performance liquid chromatography (HPLC) (Supplementary Fig. 2). There was minimal DiR-LNP in the blood and other major organs. Immunochemistry of the cells collected from MPE after intrapleural DiR-LNP revealed predominant uptake of LNP-CDN by CD11c+ monocytes/macrophages and dendritic cells (DCs; Fig. 1e). Immunohistochemistry of pleural tumour tissues depicted well-dispersed LNPs that also colocalized well with CD11c+ monocytes/macrophages and DCs (Fig. 1f). Consistently, our flow data showed that DiR-LNPs were captured primarily by macrophages, CD103+ DCs and CD11b+ DCs, while minimal uptake was observed in tumour cells, T cells or natural killer (NK) cells in MPE, pleural tumours and TDLNs (Fig. 1g–i). CD103+ DCs are considered as the most competent APCs to prime-class CD8+ T cells[36–38]. Together, these data indicate that intrapleural LNP-CDN enables phagocyte-targeted delivery of CDN in both MPE and pleural tumours in the pleural cavity. Notably, LNP-CDN itself or carried by APCs can migrate from MPE to TDLNs, which probably potentiates APC-mediated cross-priming of cytotoxic T cells in TDLNs.

**Intrapleural LNP-CDN reprograms myeloid immune cells.**

To elucidate the immune landscape in MPE and its response to intrapleural LNP-CDN, we applied scRNA-seq without bias to characterize cell type-specific transcriptional profiles after intrapleural PBS, LNP-CDN, anti-PD-L1 antibody (Ab) or LNP-CDN+ anti-PD-L1 Ab. About 4,000 single cells from the pooled MPE of three LLC MPE-bearing mice per condition were subjected to scRNA-seq. As depicted in t-distributed stochastic neighbour embedding (t-SNE) projection, unsupervised clustering singled out 20 distinct cell clusters, comprising tumour cells and various immune cells of monocytes/macrophages, neutrophils, DCs, NKs, CD4+ T cells and CD8+ T and B cells (Fig. 2a,b). Clearly, MPE contained a large number of myeloid cells including macrophages (Cd68 and Adgre1; ~55%) and neutrophils (Ly6g; ~30%). In-depth scRNA-seq clustering of the macrophage population yielded six subclusters (Fig. 2c). Mac_3, Mac_4 and Mac_6 were exclusively induced after LNP-CDN alone or in combination with anti-PD-L1 Ab (Fig. 2c–e), which showed significantly upregulated M1-associated genes (Supplementary Fig. 3). By contrast, Mac_1 and Mac_2 that exhibited high expression of M2-associated genes were primarily found in the PBS control or anti-PD-L1 alone group, while Mac_5 was a mixture of cells from each treatment (Fig. 2c–e) and Supplementary Fig. 3).

To investigate the transcriptome dynamics during macrophage repolarization, we applied the Monocle2 method for trajectory analysis. Monocle determines and orders single cells along a trajectory of two alternative cellular fates, namely the M2 and M1 fates (Fig. 2f and Supplementary Fig. 4). Mac_1 and Mac_2 were located close to the M2 fate, while Mac_3 to Mac_6 were closer to the M1 fate (Fig. 2f). LNP-CDN alone or in combination clearly induced macrophage repolarization and gradual transition from the M2 to the M1 fate (Fig. 2g). Moreover, the trajectory heatmap revealed sequential gene expression changes (Fig. 2h). Clearly, gradually upregulated or downregulated expression of genes was shown during the switch from M2 to M1 (Fig. 2f) and Supplementary Fig. 4). Furthermore, we examined the enriched functions associated with these transitional genes by extracting functional gene ontology (GO) terms and biological pathways (Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways). We identified gene enrichment associated with antigen processing and presentation and the biological process related to inflammatory response during M2 to M1 repolarization (Fig. 2i).

To validate the scRNA-seq immunophenotyping, we conducted flow cytometry and found that LNP-CDN indeed skewed the M2 (F4/80+ arginase 1+) to M1-like macrophages (F4/80 + iNOS+) in both MPE and pleural tumours (Fig. 2k,l). On the contrary, intrapleural free CDN had no immunological effects, which probably resulted from rapid degradation of CDN by ENPP1, which was detected at a high concentration of 7,200 pg ml−1 in MPE (Supplementary Fig. 5). Intrapleural LNP-CDN plus anti-PD-L1 Ab was found to further enhance further the ratio of M1/M2 (Fig. 2k,l).

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**Fig. 2** | **Intrapleural LNP-CDN reprograms immunosuppressive myeloid cells towards pro-inflammatory phenotype and remodels the immune landscape in MPE.** a, Unsupervised scRNA-seq of pooled whole MPE cells (n = 3 mice per treatment) obtained 48 h later revealed 20 colour-coded cell clusters. b, t-SNE plot of all cells colour-coded by treatment. c, t-SNE subclustering of six colour-coded subsets of intrapleural macrophages combined from all four treatments. d, e, t-SNE plot, colour-coded by treatment (d) and bar plot (e) showing subpopulations of macrophages distinctly separated between LNP-CDN or combination treatment and PBS or anti-PD-L1 alone, of which Mac_3, Mac_4 and Mac_6 were exclusive to LNP-CDN alone or in combination with anti-PD-L1. f, Macrophage subclusters (Mac_1 to Mac_6) superimposed on pseudotime trajectory. g, Macrophage superimposed on the pseudotime trajectory colour-coded by treatment. h, Heatmap of differentially expressed genes ordered based on their common kinetics through pseudotime during M2 to M1 repolarization, Cells (columns) are ordered along the M2 to M1 path. i, Representative gene expression of transcriptional factors involved in M2 to M1 repolarization plotted as a function of pseudotime. Each dot in the scatter plot represents the gene expression of a single cell. j, Selected significantly enriched terms in the GO and KEGG analyses based on the gradually upregulated (red) and downregulated (green) genes in M2 to M1 repolarization. The full list can be seen in Supplementary Table 1. k, l, Representative flow plots (k) showing intrapleural LNP-CDN-induced right shift of iNOS expression (M1-like marker) while a left shift of arginase1 (M2-like marker) on macrophages in both MPE and pleural tumours, and increased ratios of M1:M2 (l). Data are shown as the mean ± s.d. of n = 6 biologically independent mice. One-way ANOVA with Tukey’s post-hoc test. m, ELISA of various cytokines and chemokines in pleural fluid under the indicated treatment. Data are shown as the mean ± s.d. of n = 6 biologically independent mice.
An enzyme-linked immunosorbent assay (ELISA) also detected increased levels of pro-inflammatory cytokines and chemokines such as type I IFNs, interferon-γ (IFN-γ), interleukin (IL)-2, IL-12 and the IL-15–IL-15R complex in MPE after intrapleural LNP-CDN (Fig. 2m). Together, these data clearly indicate that the TME in MPE is profoundly immunosuppressive with enrichment of M2/N2-like...
myeloid cells but intrapleural LNP-CDN effectively repolarizes these myeloid cells towards the pro-inflammatory phenotype.

**LNP-CDN promotes cytotoxic effector CD8+ T cells and NK cells.** In addition to its effects on myeloid cells, intrapleural LNP-CDN led to marked expansion of MPE-infiltrating CD8+ T cells (Fig. 3a and Supplementary Figs. 6 and 7). scRNA-seq depicted five distinct subclusters of CD8+ T cells (Fig. 3b). CD8_3 and CD8_4 were predominantly induced after intrapleural LNP-CDN and to a lesser degree by anti-PD-L1 Ab (Fig. 3b,c). While both the subclusters exhibited upregulated expression of effector molecules (Ifng, Prf1, Gzmb and Klrg1) and costimulatory receptors (Cd28 and Icos), consistent with an activating effector phenotype, CD8_4 was found to have higher expression of cell cycle transcripts (Cdki, Mki67 and Pena) (Fig. 3d,e). CD8T_1, also largely expanded by LNP-CDN, presented a naive-like phenotype (Tcf7+, Ccr7+, Pdcd1+), but also expressed high levels of stem cell antigen 1 (Sca-1 or Ly6a), the haematopoietic stem cell engragment genes Hoxa1 and L pneum and memory markers such as Il2rb and Cxcr3 (Fig. 3d,e), probably associated with the stem-like memory CD8+ T phenotype40-44. Conversely, most T cells under PBS treatment were located in CD8_2 and CD8_5 (Fig. 3b,c), of which CD8_2 showed high expression of Ccr7, IIf7 and Tcf7, but low expression of Cd44, probably indicative of naive-like T cells, while CD8_5 expressed high levels of Tox, Pdcd1, Lag3 and Cila4 (Fig. 3d,e and Supplementary Fig. 8), commonly linked to T cell exhaustion45-47. We also applied the Monocle2 to CD8+ T cells and yielded trifurcating trajectories (Supplementary Fig. 9). Concurring with the t-SNE characterization, CD8T_1 and CD8T_2 were enriched in the memory/naive branch, CD8T_3 and CD8T_4 were largely located in the effector branch, while CD8T_5 was found primarily in the exhausted branch (Supplementary Fig. 9). In response to LNP-CDN or LNP-CDN+ anti-PD-L1, CD8+ T cells were found to accumulate on the effector trajectory with some on the memory/naive trajectory (Supplementary Fig 9). Similarly, bulk RNA-seq analysis showed that the combination immunotherapy enriched genes in multiple signalling pathways relevant to T cell proliferation and activation, differentiation and migration and T cell-mediated immunity and cytotoxicity (Supplementary Figs. 10 and 11).

Notably, LNP-CDN alone or combined with anti-PD-L1 Ab significantly increased the number of polyfunctional CD8+ T cells with both IFN-γ+ and granzyme B (Gzmb)+ in MPE and pleural tumours (Fig. 3f,g and Supplementary Fig. 7). Utilizing the LLC-ovalbumin (OVA) MPE-bearing mice, our data showed that there was a significant increase in the uptake of exogenous OVA-fluorescein isothiocyanate (FITC) by CD103+ DCs (Supplementary Fig. 12) and presentation of the SIINFEKL–MHC class I molecule Kb complex on CD103+ DCs (CD11c+, CD103+, CD11b+) DCs in MPE, pleural tumours and TDLNs. Data are shown as the mean±s.d. of n=6 biologically independent mice. One-way ANOVA with Tukey’s post-hoc test.

**Intrapleural LNP-CDN enhances anti-PD-L1 immunotherapy.** It is known that PD-L1 is inducible by IFNs to serve as a counter-regulatory mechanism57-60. LNP-CDN was found to initiate the expression of PD-L1 and MIC class I on tumour cells at the transcript and protein levels (Supplementary Fig. 19). Thus, combining anti-PD-L1 Ab to counteract overexpressed PD-L1 may be a rational strategy to augment anticancer immunity. Anti-PD-L1 Ab is commonly administered systemically. To explore an alternative administration, we compared intra-

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**Fig. 3 | Intrapleural LNP-CDN promotes polyfunctional CD8+ effector T cells, expands stem-like memory CD8+ T cells and generates tumour-specific cytotoxic T cells in MPE.** a. t-SNE plots of intrapleural CD8+ T cells colour-coded by treatment. b. t-SNE projection showing CD8α+ T cells colour-coded by subclusters. c. Bar plots showing the frequency of CD8α+ T cells within each cluster as a function of treatment. d. Gene expression patterns projected onto t-SNE plots of Ifng, Gzmb, Cd28, Mki67, Cd44 and Tcf7 (scale, log, fold change). e. Heatmap showing z-scores of differentially expressed individual genes in subcluster 1–5 shown in a. b–e Data are from one experiment with n=3 MPE samples pooled per treatment. The full differential expression gene list between subclusters is provided in Supplementary Table 4. f. Flow plots showing polyfunctional CD8+ T cells with double-positive staining for IFN-γ and Gzmb. g. Number of cells with double-positive staining quantitated. Data are shown as the mean±s.d. of n=6 biologically independent mice. One-way ANOVA with Tukey’s post-hoc test. h. Representative flow plots (h) and quantitative data (i) of the OVA peptide SIINFEKL-MHC class I molecule Kb complex on CD103+ (CD11c+, CD103+, CD11b+) DCs in MPE, pleural tumours and TDLNs. Data are shown as the mean±s.d. of n=6 biologically independent mice. One-way ANOVA with Tukey’s post-hoc test.
pleural versus intraperitoneal (i.p.) injection of anti-PD-L1 Ab in this study. Pharmacokinetic data showed that the intra-pleural approach achieved a tenfold and threefold higher pleural concentration of anti-PD-L1 Ab at 3 h and 24 h, respectively (Supplementary Fig. 18). Intrapleural anti-PD-L1 also led to significantly higher Ab concentrations in pleural tumours and TDLNs.
but much lower blood concentrations (Supplementary Fig. 18). Thus, we included intrapleural anti-PD-L1 Ab in our treatment study but with less than one-third of the i.p. dose (100 µg). Intrapleural LNP-CDN or anti-PD-L1 (30 µg) monotherapy delayed tumour growth and MPE progression; the combination treatment resulted in a further decrease in MPE volume and pleural tumour burden and significantly prolonged survival in wild-type but not STING−/− mice bearing LLC stably transfected with firefly luciferase (LLC-Luc) MPE (Fig. 5a–f and Supplementary Fig. 18). There was no significant difference in survival benefit between intrapleural and systemic anti-PD-L1 Ab (Fig. 5c). Similar antitumour immune responses and therapeutic efficacy of the combination immunotherapy were also observed in the CMT-167-Luc MPE model (Fig. 5g–j and Supplementary Figs. 20 and 21). Immunohistochemistry of pleural tumours revealed significantly more apoptotic cells induced by the combination treatment (Fig. 5kl). Intriguingly, in contrast to the enlarged and distorted angiogenic vessels observed in pleural tumours with PBS control, LNP-CDN alone or in combination resulted in ‘normalized’ blood vessels (Fig. 5m–p). Through bulk RNA-seq, the combination treatment was found to upregulate the expression of vascular normalization/stabilization genes and anti-angiogenic genes, while downregulating pro-angiogenic genes (Supplementary Figs. 10 and 11).

Because MPE is often associated with NSCLC, we created a mouse model with concomitant lung cancer and MPE and treated mice with intrapleural combination immunotherapy. Assessment of the treatment effects by IVIS and ex vivo examinations of lung tumour foci showed that combination immunotherapy significantly inhibited lung tumour growth (Supplementary Fig. 22), indicating that antitumour immune responses are not confined to the pleural cavity but extended to the lung parenchyma. Moreover, long-term surviving MPE mice resisted secondary LLC tumour but not B6 melanoma challenge, suggesting that combination treatment triggers tumour-specific antitumour memory (Supplementary Fig. 23).

To determine if the observed antitumour immune responses were derived from the integrative efforts of both innate and adaptive immune cells, we conducted depletion studies and analysed the effects of loss of individual immune cells. Depletion of macrophages/DCs in MPE by LNP-clodronate (LNP-Clod) completely abrogated the treatment-induced cytotoxicity observed in both in vivo apoptosis assay and Kaplan–Meier survival study (Supplementary Fig. 18). Sequential depletion studies revealed that depleting CD8+ T cells or NK cells starting 1 day before treatment or depleting CD8+ T cells 7 days after treatment largely abolished the antitumour effects of combination immunotherapy (Supplementary Fig. 18). However, loss of NK cells at the latter time had little impact.
Fig. 5 | Intrapleural LNP-CDN in combination with anti-PD-L1 Ab suppresses pleural tumour growth, reduces MPE volume and prolongs survival of MPE mice. a. Intrapleural tumour burden monitored with BLI of mice with the indicated treatment on days 4 and 14. b. Quantitative photon counts within the chest area. c. Kaplan–Meier survival assay of LLC MPE mice with the indicated treatment. n = 10 in the LNP-CDN + PD-L1 group, n = 8 in the other groups. P = 0.0012 in LNP-CDN versus PBS and P = 0.0056 in PD-L1 versus PBS, respectively; two-sided log-rank test. d–f, MPE volume (d), non-haematological cell (CD45−) counts in MPE by flow cytometry (e) and pleural tumour weight (f) were measured on day 14 in the LLC-Luc MPE mice (n = 6 in the PBS and CDN groups, n = 7 in the other group). Data are shown as the mean ± s.d.; one-way ANOVA with Tukey’s post-hoc test. g–j, Kaplan–Meier survival assay of CMT167-Luc MPE mice with the indicated treatment (g). MPE volume (h), non-haematological cell (CD45−) counts in MPE (i) and pleural tumour weight (j) on day 14 in the CMT167-Luc MPE mice. n = 10 in the LNP-CDN + PD-L1 group, n = 8 in the other groups for the survival assay; P = 0.011 in LNP-CDN versus PBS; two-sided log-rank test. n = 6 per group for the MPE evaluation in h–j. Data are shown as the mean ± s.d. Two-sided log-rank test for the survival assay, one-way ANOVA with Tukey’s post-hoc test for the others. k–p, Immunohistochemical staining (k) and quantification (l) of active caspase3+ apoptotic cells, immunohistochemical staining (m) and quantification (n) of CD31+ cells and immunofluorescence staining (o) and quantification (p) of CD31+ and NG2+ cells in pleural tumours of n = 3 biologically independent mice with LLC-Luc MPE under the indicated treatment. Data are shown as the mean ± s.d. Scale bars, 20 μm (k) or 50 μm (m and o). One-way ANOVA with Tukey’s post-hoc test.
**Fig. 6 | LNP-CDN reprograms tumour-associated macrophages, activates cytotoxic effector NK cells and CD8\(^+\) T cells and enhances the cytotoxic activity of NK cells in the MPE of patients with NSCLC.**

**a**, Schemes of treating patients’ MPE with LNP-CDN. **b**, Cellular compositions of MPE freshly obtained from patients with NSCLC (\(n=5\)) determined by flow cytometry. Each spot represents an individual MPE sample. Tumor and CD45\(^+\) cells were the percentage of live cells; other immune cells were the percentage of CD45\(^+\) cells. Data are shown as the mean ± s.d. **c**, Pairwise comparison of gene expression profiles showing LNP-CDN induced M1-associated genes while suppressing M2-associated genes in macrophages. The heatmap data are shown as the mean log2 fold change normalized to normal human PBMCs. **d**, Specific uptake of DiR-LNP by macrophages and DCs in patients' MPE (\(n=5\)) by flow cytometry. The percentage of DiR\(^+\) cells in their own population are presented. Data are shown as the mean ± s.d. **e**, Immunofluorescence imaging of the uptake of DiR-LNP (red) by macrophages (green) isolated from representative patients' MPE. Scale bars, 20 \(\mu\)m. Representative images of three independent experiments. **f**, Flow plots showing increased ratios of M1 (CD80)/M2 (CD206) after LNP-CDN treatment of individual MPE samples (\(n=5\)). **g,h**, LNP-CDN activated NK cells in patients' MPE (\(n=5\)). Sorted NK cells from patient MPEs were cocultured with supernatant from LNP-CDN-treated macrophages for 18h; the cell surface expression of NKG2D (g) and intracellular expression of IFN-\(\gamma\) (h) were determined by flow cytometry. **i**, LNP-CDN activated CD8\(^+\) T cells in patients' MPE (\(n=5\)). **j**, NK cell cytotoxicity and PD-L1 Ab-mediated ADCC enhanced by LNP-CDN. Data are shown as the mean ± s.d. of \(n=5\) patient samples. One-way ANOVA with Tukey's post-hoc test. **k**, Heterogeneous expression of PD-L1 on tumour cells observed among the samples of five individual patients. Data are shown as the mean ± s.d. of \(n=3\) biologically independent experiments.
on animal survival. These data suggested the critical role of NK cells during the early phase of antitumour immunity. Together, these data indicate that the observed anticancer immunity is indeed macrophage/DC-initiated/mediated and collaborative efforts of the innate and adaptive effector lymphocytes are required to execute the antitumour activity.

Intrapleural LNP-CDN and anti-PD-L1 Ab were well tolerated by the MPE mice. Safety studies showed no abnormality in blood liver enzyme levels or morphological changes in major organs from the short-term treated or the long-term surviving mice (Supplementary Fig. 24), supporting the notion that intrapleural LNP-CDN alone or in combination with anti-PD-L1 Ab is safe.

**Immunological effects of LNP-CDN are confirmed in human MPE.** To assess the translational potential of intrapleural LNP-CDN, we obtained clinical MPE samples (n = 5 patients) during thoracotomies of patients with NSCLC with confirmed MPE cytology (Fig. 6a). Flow cytometry of MPE identified tumour cells (~30%) and diverse immune cells (~60%) with a wide range of variation between individual patients (Fig. 6b). Among the immune cells, CD3+ T cells, monocytes/macrophages and neutrophils were more prevalent. Despite the high percentage of T lymphocytes, the fraction of CD8+ T cells was much lower than that of CD4+ T cells and a considerable number of CD4+ regulatory T cells were present (Fig. 6b). Monocytes/macrophages isolated from each patient’s MPE exhibited homogeneously upregulated expression of M2-associated genes (Fig. 6c). ELISA detected low levels of IFNs and other pro-inflammatory cytokines/chemokines in MPE (Supplementary Fig. 25). These data are in good agreement with previous clinical observations, suggesting the immune cold MPE.

To study the utility of LNP-CDN in human MPE, we first evaluated its targeting specificity by incubating DiR-LNP with fresh MPE or isolated macrophages from MPE (Supplementary Fig. 26 and 27). Flow cytometry and immunofluorescence microscopy revealed the predominant uptake of DiR-LNP by monocytes/macrophages and DCs (Fig. 6d,e). We next investigated the ex vivo effects of LNP-CDN on various immune cells in MPE. Incubation of LNP-CDN with isolated macrophages induced increased expression of M1-associated genes in all five patient samples (Fig. 6c,f). We also isolated NK cells from individual samples and treated NK cells with the supernatant from the above LNP-CDN-treated macrophages (Supplementary Fig. 27). Flow cytometry detected a marked increase in expression of the activating receptor, NKG2D and production of intracellular IFN-γ (Fig. 6g,h). Moreover, incubation of LNP-CDN with fresh MPE led to a drastic increase in IFN-γ+ CD8+ T cells in all five samples (Fig. 6i). Furthermore, the LNP-CDN-activated NK cells demonstrated enhanced cytotoxicity of autologous tumour cells (Fig. 6i). As variable levels of PD-L1 were expressed on pleural tumour cells in all samples (Fig. 6k), combining avelumab, the clinically approved IgG1 anti-PD-L1 monoclonal Ab, further increased NK cell cytotoxicity (Fig. 6i). Together, these data support the potential use of intrapleural LNP-CDN in human MPE.

**Conclusions**

Consistent with previous reports, our data showed that MPE in mice and humans was profoundly immunosuppressive with abundant tumour-promoting myeloid cells. Despite being a potent STING agonist, 23’-cGAMP CDN has a short biological half-life in MPE because MPE contains a high level of ENPP1 that degrades CDN. We developed the CDN-loaded liposomal nanoparticle LNP-CDN, which protected CDN from enzymatic degradation in MPE and exhibited its specific targeting of macrophages and DCs to activate STING signalling. Through scRNA-seq, intrapleural LNP-CDN was found to induce drastic changes in the transcriptional landscape in MPE by reprogramming myeloid cells, activating DCs for cross-presentation of TA, promoting polyfunctional NK cells and CD8+ T cells and expanding stem-like memory CD8+ T cells. We further showed that LNP-CDN-induced remodelling of the MPE TME set a stage for response to anti-PD-L1 ICB.

In the context of clinical practice, LNP-CDN can be administered serially via indwelling pleural catheters. Clinically, the presence of MPE often precludes surgical intervention and many patients with MPE are not fit for chemotherapy due to their extremely poor condition. Thus, successful management of MPE may renew opportunities for combining with other treatment options to maximize therapeutic efficacy.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41565-021-01032-w.

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Methods

Preparation and characterization of LNP-CDN. The liposome LNP-CDN with CaP core complexed with CDN (LNP) was prepared in two steps using the water-in-oil reverse microemulsion method57,58. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[lysine rhodamine B sulfonyl] (Rhod-b)) were purchased from Avanti Polar Lipids. The CaP core with single-layer anionic lipid coating (PS:DSPC:cholesterol=5:4:1) was prepared and 4 lipid mixtures (molar ratios) for the outer coating of LNP were prepared: (1) LNP-1, brain-PS:DSPC:cholesterol = 5:4:1; (2) LNP-2, brain-P:DSPE:cholesterol:DSPE-PEG2000 =5:4:1:1; (3) LNP-3, DSPA:DSPC:cholesterol = 5:4:1; (4) DOTAP:PS:DSPC:cholesterol = 1:5:4:1. LNP-CaP nanoparticles with bilayer lipid coating were formed by adding 1 mL of rehydrating solution against release medium at different pHs (pH 7.4, 6.5 and 5.0) with 10% fetal bovine serum (FBS) (v/v) at 37 °C for 12 h to study the CDN, half of the desired CDN content was mixed with each of the CaCl2 and Na2HPO4 solutions, to fluorescently label NPs, 18.1 Liss Rhod PE (Rhod-b) was added to the second lipid mixture with a molar ratio of 1%; alternatively, DiR was used for labelling the NPs by adding DiR directly to the second lipid mixture at a molar ratio of 1%. Loading of CDN without the use of CaP in liposomes (lipid mixture, 20 mM, brain-PS:DSPC:cholesterol:DSPE-PEG2000 =5:4:1:1) was also prepared for the water-in-oil thin-film hydration method.

The size, size distribution and zeta potential of LNP-CDN in aqueous solution were measured in a Malvern Zetasizer Nano ZS90 and analysed by Zetasizer (7.12). Transmission electron microscopy (TEM) measurements were performed on an FEI Tecnai Bio TWIN transmission electron microscope.

The release of CDN from LNP was assessed by the digestion of LNP-CDN solution against release medium at different pHs (pH 7.4, 6.5 and 5.0) at various times by Agilent 1100 HPLC. The LNP's were incubated in PBS (pH 7.4, 6.5 and 5.0:0.01 M) with 10% fetal bovine serum (FBS) (v/v) at 37 °C for another 2 min at 70 °C. The resulting nanoparticles were further filtered with a 0.45 µm membrane to remove the free lipid aggregates and stored at 4 °C. To load the CDN, 30 mL of the desired CDN content was mixed with each of the CaCl2 and Na2HPO4 solutions, to fluorescently label NPs, 18.1 Liss Rhod PE (Rhod-b) was added to the second lipid mixture with a molar ratio of 1%; alternatively, DiR was used for labelling the NPs by adding DiR directly to the second lipid mixture at a molar ratio of 5%. Loading of CDN without the use of CaP in liposomes (lipid mixture, 20 mM, brain-PS:DSPC:cholesterol:DSPE-PEG2000 =5:4:1:1) was also prepared for the water-in-oil thin-film hydration method.

Cell lines. The mouse NSCLC LLC (ATCC), CMT-167 (clone CMTC6), Sigma-Aldrich), OVA-transfected LLC (LLC-OVA) and B16 melanoma (provided by Y. Lu; Wake Forest) cells, LLC-Luc cells (stably transfected with firefly luciferase (CMT-Luc) were cultured in DMEM, supplemented with 10% FBS, 100 U ml−1 of penicillin and 100 µg ml−1 of streptomycin and maintained in a humidified atmosphere containing 5% carbon dioxide at 37 °C. Cell lines were maintained in a 12–12 light–dark cycle with a temperature of 20–23 °C and humidity of 48–52%.

Flow cytometry. LLC or CMT MPE mice were randomly grouped and treated on day 11 with intrapleural PBS, free CDN (1 µg), LNP-CDN (1 µg), anti-PD-L1 Ab (30 µg) or LNP-CDN plus anti-PD-L1 Ab. Mice were killed on day 13 and pleural fluid was gently aspirated using a 1 ml syringe through the diaphragm and its volume was measured with a 1 ml pipette. Solid tumours on the pleural surface and mediastinal lymph nodes were also collected and processed for flow cytometry. Flow cytometry was performed on a BD Canto II flow cytometer; data were collected with the FACSDiva software v6.1.3 and analysed using the FlowJo software v10.1 (FlowJo LLC). A list of antibodies used is summarized in Supplementary Table 1. All antibodies for mouse flow cytometry were used at a 1:100 dilution. Doublets and dead cells were defined by their differential expression across cells. Flow cytometry was performed on a BD Canto II flow cytometer; data were collected with the FACSDiva software v6.1.3 and analysed using the FlowJo software v10.1 (FlowJo LLC). A list of antibodies used is summarized in Supplementary Table 1. All antibodies for mouse flow cytometry were used at a 1:100 dilution. Doublets and dead cells were defined by their differential expression across cells. Flow cytometry was performed on a BD Canto II flow cytometer; data were collected with the FACSDiva software v6.1.3 and analysed using the FlowJo software v10.1 (FlowJo LLC). A list of antibodies used is summarized in Supplementary Table 1. All antibodies for mouse flow cytometry were used at a 1:100 dilution. Doublets and dead cells were defined by their differential expression across cells. Flow cytometry was performed on a BD Canto II flow cytometer; data were collected with the FACSDiva software v6.1.3 and analysed using the FlowJo software v10.1 (FlowJo LLC). A list of antibodies used is summarized in Supplementary Table 1. All antibodies for mouse flow cytometry were used at a 1:100 dilution. Doublets and dead cells were defined by their differential expression across cells. Flow cytometry was performed on a BD Canto II flow cytometer; data were collected with the FACSDiva software v6.1.3 and analysed using the FlowJo software v10.1 (FlowJo LLC). A list of antibodies used is summarized in Supplementary Table 1. All antibodies for mouse flow cytometry were used at a 1:100 dilution. Doublets and dead cells were defined by their differential expression across cells.
PD-L1, apoptosis or vasculature staining, anti-mouse PD-L1 antibody (1:2,000 dilution; B7-H1; Bio X Cell), anti-mouse cleaved caspase 3 antibody (1:400 dilution; Cell Signaling Technology) or anti-mouse CD31 antibody (1:200 dilution; BD Biosciences), followed by horseradish peroxidase-conjugated goat anti-rabbit (1:500 dilution; Jackson ImmunoResearch) or goat anti-rat secondary antibody (1:500 dilution; Jackson ImmunoResearch) were applied, respectively. Sections were then developed with 3,3′-diaminobenzidine kits (Vector Laboratories) and counterstained with haematoxylin.

In vivo experimental treatment in MPE mouse models. LLC-Luc MPE or CMT-Luc MPE mice, after confirming tumour formation by IVIS, were randomly grouped (n = 8–10 per group) and treated on day 4 and repeated on days 6 and 8 as follows: (1) PBS (intrapleural injection); (2) LNP-CDN (1 µg x 3, intrapleural injection); (3) free CDN (1 µg x 3, intrapleural injection); (4) anti-human PD-L1 Ab (1:500 dilution; Cell Signaling Technology); or LNP-CDN (intrapleural injection) + anti-PD-L1 (intrapleural injection); (5) LNP-CDN (intrapleural injection) + anti-PD-L1 (intrapleural injection, 100 µg x 3). For depletion of macrophages and DCs in pleural fluid, mice were injected i.p. with 200 µg of LNP-Clod 1 day before treatment and followed by one or three doses of intrapleural LNP-Clod. To study the role of the STING pathway, STING knockout mice were also treated with LNP-CDN + PD-L1. To deplete C8 T cells or NK cells throughout the treatment, 400 µg of anti-mouse NK1.1 (clone PK136; Bio X Cell) or anti-mouse CD8a antibody (clone 2.43; Bio X Cell) were injected i.p. 1 day before the treatment and 100 µg of anti-mouse NK1.1 or anti-mouse CD8a were injected intravenously with LNP-CDN at the time of treatment, followed by twice per week i.p. injection of 100 µg of anti-mouse NK1.1 or anti-mouse CD8a throughout treatment. For different groups of mice, C8 T cells or NK cells were depleted starting 7 days after the initial treatment by i.p. injection of the Abs twice per week throughout treatment. The development of pleural tumours and MPE were monitored longitudinally by BLI. MPE volume and solid tumour weight were measured on day 14. Tumour cell numbers in the fluid and memory CD8 T cells were evaluated by flow cytometry. Mice survival (n = 8–10 per group) was followed up to 80 days.

Human MPE sample acquisition. In this study, malignant pleural effusion samples (100 ml per patient) from five patients diagnosed with NSCLC were collected during thoracocentesis and distributed by tumour tissue and pathology shared resource (TTPSR) of the Wake Forest Baptist Medical Center Comprehensive Cancer Center. Acquisition of de-identified MPE samples from TTPSR for research use was in accordance with the institutional review board of Wake Forest University (protocol no. IRB00004151). All patients provided written informed consent.

MPE preparation from human samples. MPE from patients was centrifuged at 800 × g for 10 min at 4 °C; supernatants were collected and stored at −80 °C. The cell pellets containing red blood cells were treated with 2 ml of ACK lysing buffer at 4 °C for 10 min followed by adding 10 ml of PBS to stop the lysis. Cell suspensions were further centrifuged at 500 × g for 5 min. Cell pellets were resuspended with PBS buffer to single-cell solutions for the following analyses.

Flow cytometry of cellular compositions of human MPE. As described above, flow cytometry was performed on a BD Canto II flow cytometer and analysis using the FlowJo software. A list of the anti-human antibodies used is summarized in Supplementary Table 2. All antibodies for human flow cytometry were used at a 1:100 dilution. For the T cell activation study, fresh MPE was incubated with LNP-CDN (100 nM) overnight. MPE cells were treated with a phorbol 12-myristate 13-acetate ionomycin cocktail according to the manufacturer’s specification (BioLegend) before intracellular IFN-γ staining.

Isolation and culture of macrophages and NK cells from patients’ MPE samples. Monocytes/macrophages from MPE were isolated by magnetic-activated cell sorting (MACS) using magnetic beads conjugated with human CCR2 antibodies and analyzed with flow cytometry. MonoMACs were collected from MPE by fluorescence-activated cell sorting (MACS) using magnetic beads conjugated with human CCR2 antibodies and analyzed with flow cytometry. MonoMACs were collected from MPE by fluorescence-activated cell sorting (MACS) using magnetic beads conjugated with human CCR2 antibodies and analyzed with flow cytometry.

Ex vivo treatment of macrophages from the patient sample with LNP-CDN. Sorted macrophages (10^5 in 60 mm culture dishes) were treated with PBS or LNP-CDN (100 nM) for 18 h and the culture medium was collected for ELISA assay of cytokines and further coculture with NK cells. Macrophages after LNP-CDN treatment were stained with M1 macrophage marker (anti-human iNOS, anti-human human leukocyte antigen–DR isotype and anti-CD80 monoclonal Abs) and M2 macrophage marker (anti-human CD206 and anti-human CD163 monoclonal Abs) for flow analysis.

Real-time PCR of M1- and M2-like gene profiles. The total RNAs of macrophages after treatment were extracted for real-time PCR analysis with M1 signature genes (such as CD86, IL12, IL15, IL6, IL18, IFN18, and so on) and M2 signature genes (MRC1, CD163, IL10, TGFB1, and so on); details are listed in Supplementary Table 3. Individual gene expression was normalized with the housekeeping gene GAPDH and compared with gene expression level from healthy human peripheral blood mononuclear cells (PBMCs).

Ex vivo NK cell activation mediated by LNP-CDN-induced activation of macrophages. One million (10^6) NK cells were cultured with 4 ml of supernatants collected from autologous macrophages previously treated with PBS or LNP-CDN for 18 h in 60 mm culture dishes. NK cells were collected by centrifuging at 500 × g for 5 min at 4 °C. After the addition of autologous tumour cells (E:T = 20:1) for another 16 h, NK cells were collected from the supernatants for ELISA assay of IFN-γ and GzmB. Anti-human NKG2D, anti-human Nkp46 and anti-human FcγRIII monoclonal Abs were stained as surface markers of NK activation and the intracellular expression of IFN-γ was determined by flow cytometry after ex vivo stimulation with phorbol 12-myristate 13-acetate-ionomycin cocktail.

The cytoxicity and ADCC effects of NK cells were assessed in the presence of human IgG1 isotype, anti-human PD-L1, avelumab (10 µg ml⁻¹) or avelumab (F(ab)²). The effector to target cell ratios were titrated and a ratio of 20:1 was used throughout the experiment.

Statistics and reproducibility. Statistical analysis was performed using Microsoft Excel (2016) and Prism 9.0 (GraphPad Software). Data are presented as the mean ± s.d. P < 0.05 was considered statistically significant. Unpaired one-tailed Student’s t-tests were conducted for comparisons between two groups. One-way analysis of variance (ANOVA) with Tukey’s post-hoc test was performed to compare more than two groups. The survival assay was analysed using a log-rank test. No statistical methods were used to predetermine sample size. The experiments were repeated three times independently with similar results.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The scRNA-seq data are available in the NCBI Gene Expression Omnibus (GEO) under accession no. GSE164487. The bulk messenger RNA-seq data are available in the GEO under accession no. GSE179783. Source data are provided with this paper. The authors declare that other data supporting the findings of this study are available within this article and its supplementary information; all additional data are available from the corresponding author upon reasonable request.

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Author contributions
Y. Liu and D.Z. conceptualized the study. Y. Liu, L.W., W.N.C., D.Z., Q.S. and Y. Lu were responsible for the methodology. Q.S., G.A.H., W.Z., L.D.M., W.N.C., L.W. and Y. Liu were responsible for the software. Y. Liu, L.W., W.N.C. and Q.S. carried out the formal
Articles

Y. Liu, L.W., Q.S., M.A., W.J.P and D.Z. carried out the investigation. M.A., G.L.K., S.S., G.A.H., L.D.M., M.A., K.W.T and C.R.B. were responsible for resources. Y. Liu, G.A.H. and L.D.M. were responsible for data curation. Y. Liu, L.W. and D.Z. wrote the original draft. Y. Liu, M.A., Q.S., W.N.C., Y. Lu, C.R.B., W.Z., A.A.H., W.J.P and D.Z. reviewed and edited the manuscript. C.R.B., W.J.P and D.Z. supervised the study. Y. Liu, L.W., Q.S., W.N.C. and D.Z. were responsible for data visualization.

Competing interests

The authors declare no competing interests.

Additional information

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Software and code

Policy information about: availability of computer code

Data collection
- Flow cytometry data was collected using FACSDiva software (6.1.3); IVIS; Living Image software (4.5); DLS/zeta (Zetasizer 7.12);

Data analysis
- GraphPad Prism (9.0) was used for statistical analysis; FlowJo (10.1) was used for flow cytometry data analysis;
- Living Image software (v4.5) for IVIS images;
- Zetasizer (7.12) for DLS data;
- Cell Ranger (3.0.2), R software (3.6), Monocle 2 (2.20.0), and Loupe Browser (5.0.0) were used for scRNA-seq analysis;
- fastp (1.2.0), STAR (2.7.5c), DESeq2 (1.32.0), and clusterProfiler (4.0.5) softwares packages were used for bulk-RNA seq data analysis.

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- MRI-based neuroimaging

Antibodies

Antibodies used in the current study have been listed as Supplementary Table S1 and Table S2. Dilution of each antibody used for FACS analysis is 1:100. Dilutions for ex vivo staining of MPE cells were: anti-mouse CD11c-FITC (1:100) and anti-luciferase (1:800), and cy3-anti-rabbit secondary antibody (1:100; Jackson Immuno). Dilutions for antibodies used for immunohistochemistry as following: For immunofluorescence staining of T cells, cryosections (10 μm) of LLC-Luc lung MPE-bearing tumor tissues obtained from the above treatment group were immunostained with anti-mouse CD8α-cy3 (1:200; BioLegend), or anti-mouse FoxP3-Alexa Fluoro 647 antibody (1:250; BioLegend). For immunofluorescence staining of vasculature, anti-mouse CD31 (1:200; BD Biosciences) and anti-mouse NG2 (1:100, Abcam), followed by goat anti-rat Cy3 (1:400, Jackson Immuno) and goat anti-rabbit Alexa Fluor 488 (1:100,
For PD-L1, apoptosis or vasculature staining, anti-mouse PD-L1 antibody (1:2000; B7-H1; BioXCell), anti-mouse cleaved active caspase 3 antibody (1:400; Cell Signaling, Danvers, MA) or anti-mouse CD31 antibody (1:200; BD Biosciences), followed by HRP-conjugated goat anti-rabbit (1:500; Jackson Immuno) or goat anti-rat secondary antibody (1:500; Jackson Immuno) were applied respectively.

**Validation**

All antibodies were well-recognized in the field and have their validation statement on their manufacturers' websites: https://www.biolegend.com, https://www.thermofisher.com, https://www.cellsignal.com, https://bxcell.com, https://www.bdbiosciences.com/en-us, https://www.sinobiological.com, or http://www.abmole.com. These antibodies are further validated for species and application, and are routinely used in our lab.

**Eukaryotic cell lines**

| Policy information about | cell lines |
|-------------------------|------------|
| Cell line source(s)     | LLC-L1/2, CRL-1642, ATCC, LLC-Luc, LLC-RFP, and CMT-167-Luc were generated in our lab by stable transfection with lenti-virus. |
| Authentication           | None of the cells were authenticated in the lab. |
| Mycoplasma contamination | The cells lines were tested negative for mycoplasma every two week. |
| Commonly misidentified lines (See iCLAC register) | No commonly misidentified cell lines were used. |

**Animals and other organisms**

| Policy information about | studies involving animals; ARIVE guidelines recommended for reporting animal research |
|-------------------------|---------------------------------------------------------------------------------|
| Laboratory animals      | C57BL/6 mice (6–8 weeks, female: male at 1:1) were purchased from Charles River Laboratories; STING knockout mice ((B6(Cg)-Sting1tm1.2Camb/J, 6–8 weeks, female: male=1:1) were purchased from the Jackson Laboratory. |
| Wild animals            | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight        | Institutional Animal Care and Use Committee at the Wake Forest University School of Medicine |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

**Human research participants**

| Policy information about | studies involving human research participants |
|-------------------------|------------------------------------------------|
| Population characteristics | Malignant pleural effusion samples (100 ml/patient) from 5 patients diagnosed with non-small cell lung cancer (NSCLC) were collected during thoracentesis and distributed by Tumor Tissue and Pathology Shared Resource (TTPSR) of the Wake Forest Baptist Medical Center Comprehensive Cancer Center (WFBMC-CCC). Acquisition of de-identified MPE samples from TTPSR for research use was in accordance with Institutional Review Board of Wake Forest University (IRB protocol # IRB00040151). All patients provided written informed consent. |
| Recruitment              | Eligible patients were invited to participate by one of the investigators or a designee of one of the investigators. If they are willing to participate, the investigator or their designee reviewed the consent form and obtained informed consent. All patients with non-small cell lung cancer (NSCLC) with planned thoracentesis were consented to MPE collection protocol. Patients were approached in clinic by study personnel without relation to age, race, gender, disease stage, or prior therapies. Patients with MPE collected had no significant biases appeared to be present. |
| Ethics oversight         | Institutional Review Board of Wake Forest University (IRB protocol # IRB00040151) |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

**Flow Cytometry**

**Plots**

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.
Methodology

Sample preparation

The pleural fluids were centrifuged at 500 g for 10 min, and the supernatants were collected for ELISA assay. The cell pellet was further treated with 2 mL ACK Lysing Buffer (Thermo Fisher) to remove red blood cells for 10 min at 4 °C in 15-mL conical centrifuge tube. 10 mL cold PBS was added to stop the lysis, and the cells were centrifuged at 500 g for 5 min. The cell pellets were re-suspended for further surface and cytosolic Abs staining for flow analysis. For solid tumors and TDLNs, tissues were cut into small pieces in PBS on ice and further processed in 1 x tumor digestion buffer (1 mg/mL Collagenase IV, 0.1 mg/mL Hyaluronidase, and 20 U/mL DNAse) at 37 °C for 1 h under rotation. The cell suspension was gathered by filtering through cell strainer. After centrifuge at 500 g for 5 min, the cell pellet was washed once by 5 mL PBS followed by centrifuge at 500 g for 5 min. The pellet was treated with ACK lysing buffer to remove red blood cells as mentioned above. The cells were re-suspend for sub-sequenced Abs staining for flow analysis.

Flow cytometry was performed on a BD Canto II flow cytometer and analyzed using the FlowJo software (BD Biosciences). A list of antibodies used here was summarized in Table S1. For intracellular staining of IFN-γ, fresh isolated cells are treated with phorbol 12-myristate 13-acetate/ionomycin cocktail according to the manufacturer’s specification (BioLegend). The cells were then washed, stained with antibodies against CD3, CD4, and CD8α, fixed with fixation buffer and subsequently stained intracellularly with antibodies against IFN-γ and granzyme B (GZMB) in Intracellular Staining Permeabilization Wash Buffer (BioLegend). Doublets and debris of dead cells were excluded before various gating strategies were applied. Gates and quadrants were set based on isotype control staining, and the mean fluorescence intensity (MFI) values are calculated by subtracting the MFI of isotype control antibodies.

Instrument

BD Canto II flow cytometer

Software

Flow cytometry data were collected by FACSDiva software (6.1.3) and analyzed by FlowJo (10.1)

Cell population abundance

No sorting was performed by flow cytometry.

Gating strategy

Gates and quadrants were set based on isotype control staining.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.