Med-X (2024) 2:3
https://doi.org/10.1007/s44258-024-00016-4

Med-X

RESEARCH ARTICLE

Combining multi-mode thermal therapy with IL-6 and IL-17A neutralization amplifies antitumor immunity to facilitate long-term survival in LLC1-bearing mice

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Received: 8 October 2023 / Revised: 22 January 2024 / Accepted: 30 January 2024

Abstract

Non-small cell lung cancer (NSCLC) is known for rapid development and chronic inflammation-induced immunosuppression. IL-6 and IL-17A are the essential cytokines that facilitate NSCLC progression and myeloid-derived suppressive cell (MDSC)-mediated evasion. IL-6 or IL-17A targeting, especially IL-6, shown outstanding efficacy in patient NSCLC controlling, but failed to completely eradicate tumor. The local tumor multi-mode thermal therapy developed in our prior research was demonstrated to stimulate systemic and durable tumor-specific immune response thereby promoting long-term tumor-free survival of mice and prolong the progression-free survival of patients, although the therapeutic efficacy was still affected by high-level preoperative MDSCs. To further improve the efficacy, in this study, IL-6 and IL-17A neutralization were combined with multi-mode thermal therapy in mouse LLC1 NSCLC model. Study revealed that combined with single cytokine neutralization only prolonged the survival time while triple combination therapy efficiently improved the survival rate. Additionally, triple combination therapy reduced the accumulation of MDSCs but promoted their maturation with strengthened activation and function of myeloid cells, thereby triggering a Th1-dominant-$CD4^+$ T cell-response and enhancing the malignant cell-killing capacity of immune cells. Our study highlights the extraordinary efficacy of combining multi-mode thermal therapy with IL-6 and IL-17A neutralization, revealing a new strategy for refractory NSCLC patients.

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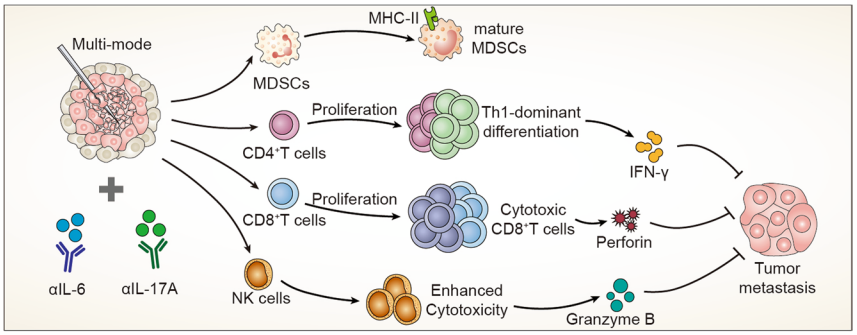
Published online: 03 April 2024

Graphical Abstract



Highlights

- Combining multi-mode thermal therapy with anti-IL-6&IL-17A promotes survival of NSCLC mice
- Triple combination therapy reduces the proportion of MDSCs and arouses $CD4^+$ Th1 immunity
- Triple combination therapy enhances T and NK cell-mediated tumor-killing

Keywords Multi-mode thermal therapy · Cytokine neutralization · Combination therapy · Antitumor immunity · Non-small cell lung cancer

Introduction

Highly metastatic non-small cell lung cancer (NSCLC) is the major diagnosed subtype of lung cancer, leading to high quantities of deaths worldwide [1–4]. The rapid development of molecular targeted therapies and immunotherapies over the past several decades has efficiently improved the prognosis [5, 6]. Unfortunately, drug resistance will develop rapidly which eventually results in the progression of metastatic NSCLC patients and leads to a median overall survival of less than 3 years [7]. Therefore, new therapeutic strategies are urgently required for the treatment of NSCLC patients.

Thermal ablation uses extreme temperatures to destroy primary tumor tissues and has been proven to enhance the tumor antigens releasing thus initiating the antitumor-immune cycle in various malignant tumor types [8]. However, the immunostimulatory effects of existing thermal therapies do not seem to be strong enough to control distant tumor metastasis, as evidenced by the comparable five-year survival of patients treated with thermal therapies versus surgical resection [9]. Our prior study has established the novel multi-mode thermal ablative strategy which can entirely destroy the tumor tissue via rapid temperature changes caused thermal stress. Multi-mode thermal therapy efficiently improves the long-term survival rate of melanoma and triple-negative breast cancer-bearing mice by eliciting a $CD4^+$ T-helpers (Th) 1-dominant immune response [10, 11]. However, a preliminary study showed that Lewis lung cancer 1 (LLC1)-bearing mice, which develop spontaneous NSCLC metastasis [12, 13], cannot be cured by individual multi-mode thermal therapy. Therefore, combination with immunotherapy was performed to amplify the multi-mode thermal therapy induced immune response, thereby achieving long-term survival in LLC1 mice model.

Interleukin (IL)-6 and IL-17A are both the participants of NSCLC progression. IL-6 and IL-17A directly enhance the expansion and exploration of NSCLC cells [14, 15]. Meanwhile, myeloid-derived suppressive cells (MDSCs) induced by IL-6 and IL-17A maintain an immunosuppressive environment, and contribute to the formation of metastatic niches [16]. IL-17A can attenuate type I polarization of $CD4^+$ T-helpers [17]. Moreover, IL-6 leads to the synthesis of IL-17A [18, 19], which in turn accelerates IL-6 generation in LLC1 cells, resulting in an autofeedback loop in NSCLC [20]. In clinical lung cancer patients, the accumulation of these two cytokines also strongly correlated with poor prognosis [21, 22], which may be the potent therapeutic targets for NSCLC treatment [23].

In this study, hypothesize has been established that the unsatisfactory prognosis of multi-mode thermal therapy in LLC1-bearing mice was related to IL-6 and IL-17A. Thus, the highly metastatic LLC1 mouse model was used to explore the efficacy of multi-mode thermal therapy combining with IL-6 and IL-17A double neutralization. Our results showed that single multi-mode thermal therapy slightly downregulated the quantity of MDSCs but promoted the expansion of T cells and nature killer (NK) cells, but had little impact on their cytotoxic function. The combination therapy of multi-mode with single IL-6 or IL-17A signaling blockade partially stimulated the maturation of myeloid cells and promoted a $CD8^+$ T-cell response but was still not sufficient to induce $CD4^+$ Th1 polarization. Triple combination therapy further attenuated MDSC-induced suppression, promoted a $CD4^+$ Th1-dominant immune response, and enhanced the functionality of both

$CD8^+$ T and NK cells, which together led to improved survival rate of LLC1-bearing mice. This study provides an efficacious combination strategy for patients with refractory solid tumor.

Materials and methods

Cell cultivation and mouse tumor model establishing

The LLC1 cell line was donated by Professor Weiliang Xia, Shanghai Jiao Tong University. The conventional cultivation reagents including fundamental DMEM solution (Hyclone, UT, USA) plus 10% (volume fraction) fetal bovine serum (GEMENI, Calabasas, USA) and penicillin–streptomycin antibiotic union (100 U/mL and 100 μg/mL respectively, Hyclone, UT, USA) were used for cell culture. A humid 37℃ incubator with the 5% CO_2 environment is maintained.

A million of LLC1 cells were subcutaneously injected into the right back of female C57BL/6 mice (aged 6–8 weeks, Slaccas, Shanghai, China) to establish tumor model. The multi-mode ablation procedure was performed on day twelve after model establish. All animal experiments were approved and restrictively obey the guidelines of animal care ethics declared in the postscript statement.

The multi-mode thermal therapy and cytokines neutralization strategies

Randomly divided mice in different groups were anesthetized with chloral hydrate (Sinopharm, Shanghai, China) before treatment. Then multi-mode thermal therapy was performed by sequentially rapid LN_2 freezing at − 20℃ for 5 min and radiofrequency ablation at 50℃ for 10 min.

For IL-6 and IL-17A neutralization, anti-IL-6 mAb (MP5-20F3, 20 μg in 100 μL PBS, Bio X Cell, NH, USA) and anti-IL-17A mAb (17F3, 20 μg in 100 μL PBS, Bio X Cell, NH, USA) was administered i.p. on Day 1, 4, 7, 10, 13 and Day 5, 9, 13 after ablation respectively. Same dose of PBS was performed in single multi-mode group.

Detection of serum IL-6 and IL-17A

The serum was collected at Day 3,7 after multi-mode treatment or time-paired tumor-bearing control mice, and the concentration of cytokines was detected by corresponding enzyme-linked immunosorbent assay (ELISA) Kits (Boster, Wuhan, China).

Flow cytometry analysis

To prepare single-cell suspension, tissues were collected on Day 14 after multi-mode thermal therapy. The lungs were physically shredded into segments and enzymolyzed with Collagenase (type I), Hyaluronidase (Yeasen, Shanghai, China) and Dnase (Solarbio, Beijing, China) at 37℃ for 30 min. Then spleens and lungs were separated manually and red blood cells were depleted by erythrocyte-lysing.

For transcription factor or intercellular cytokines staining, cells were treated with the True-Nuclear Transcription Factor Buffer Set or Fixation and Permeabilization Buffer Set (Biolegend, CA, USA) respectively referring to the given protocol. For cytokines expressive ability detection, cells were pre-stimulated with Cell Activation Cocktail (Biolegend, CA, USA) for 4 h. Fluorescence-coupled antibodies for membrane molecules and cytokines were cultured for 20 min, and antibodies for transcriptional factors were stained for 45 min. All related antibodies were listed in Supplementary.

Data was collected by the BD/Aria II cytometer, and gating and counting using FlowJo (version 10.8.2). Gating strategies was shown in Figure S1.

Tumor cell killing assay

Pulmonary single-cell suspension was labelled with CD3-PE conjunct antibody, and separated using PE Positive Selection Kit. The NK cells were obtained by NK cell Isolation Kit. Both the isolation kits were purchased from StemCell Technologies (BC, Canada). Cells were then co-cultured with calcein-AM pre-labeled LLC1 cells at a ratio of 10:1 for 4 h. The released calcein was detected in the supernatant to reflect the killing capacity by the followed formula.

$$Killing\ ratio = \frac{experimental\ group - free\ release\ group}{positive\ control\ group - free\ release\ group} \times 100\%$$

Analysis of RNA-Seq

The whole lungs were obtained at the indicated time and groups. All the RNA extraction followed with reverse transcription to obtain cDNA, and library construction, HiSeq X Ten sequencing were all performed by OE Biotech (Shanghai, China).

H&E staining and immunohistochemistry

The lungs of mice from different treatment groups were harvested at the indicated time. All the fresh tissues were immediately fixed in 4% formaldehyde, and then embedded

by paraffin. For pathological examination, the speciemens were sliced into sections and stained with hematoxylin–eosin sequentially. Light microscope was used for pathological changes observation.

Statistical analysis

Statistics were calculated by one-way ANOVA and two-sided Student's t-test (only for two groups). Survival analysis of mice was performed using the K-M curves and log-rank tests. All the analyses were carried out by GraphPad Prism 7.0. Data were presented as mean ± SD. *P* values lower than 0.05 were presented by * in the figures and regarded as significant.

Results

Triple combination therapy improved the survival of LLC1 tumor-bearing mice

To investigate whether IL-6 and IL-17A affect the therapeutic effect of multi-mode thermal therapy (MTT), serum level of these two cytokines from tumor-bearing control (control) and MTT-treated mice were measured through ELISA. As showed, the concentration of IL-17A in serum was reduced on Day 3, but markedly increased to a higher level on Day 7 after MTT compared with that of the control (Fig. 1a). Furthermore, the content of IL-6 increased in the control mice from Day 3 to Day 7 (Fig. 1b). Higher level of IL-6 on Day 3 was observed in the MTT group, and the level was comparable in the two groups on Day 7 (Fig. 1b). These suggested that the serum IL-17A and IL-6 maintained high level after MTT and may achieve unsatisfactory therapeutic effects. Therefore, the therapeutic efficacy of combining multi-mode thermal therapy with IL-6 and IL-17A blockade was investigated. As shown in Fig. 1c, d, multi-mode thermal therapy alone failed to improve the survival rate. All mice died from in situ recurrence or lung metastasis. However, multi-mode thermal therapy combined with anti-IL-6 (MTT + αIL-6), or combined with anti-IL-17A (MTT + αIL-17A), or triple combination therapy (MTT + αIL-6&αIL-17A) all prolonged survival time as compared to the untreated group. Moreover, MTT + αIL-17A significantly prolonged the survival time compared to single MTT. Interestingly, MTT + αIL-6&αIL-17A notably increased the survival rate by over 30% in LLC1-model mice (Fig. 1d), suggested that triple combination therapy could achieve an obvious curative effect to improve the prognosis in LLC1 tumor model.

Triple combination therapy reduced MDSC accumulation and promoted myeloid cells maturity in the lungs

To understand the cellular mechanism of triple combination therapy, the phenotypes of innate and adaptive immune cells were detected two weeks after multi-mode ablation. Macrophages and dendritic cells (DCs) are critical for antigen presentation and processing, which activate T-cell

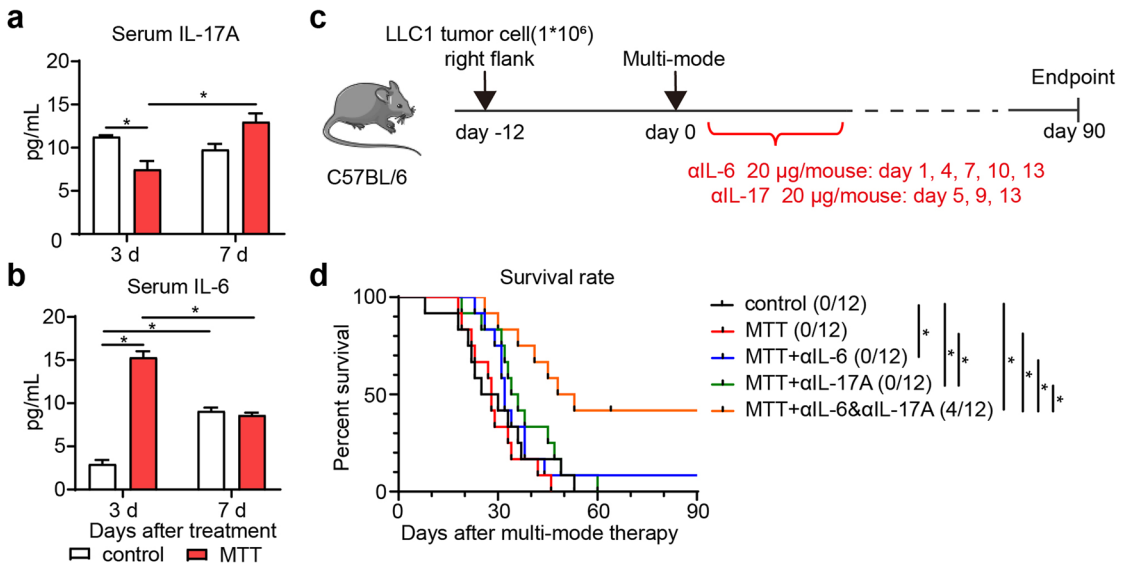


Fig. 1 Triple combination therapy promoted tumor-free survival in LLC1 model (**a**, **b**) The concentration of IL-17A (**a**), IL-6 (**b**) in serum from the control and MTT mice at different time points were analyzed by using ELISA. (**c**) Schematic of the combining strategy design. 20 μg of anti-IL-6 or IL-17A mAb were administered i.p.. (**d**) Kaplan–Meier survival plot of survival observation $n = 12$ for each group. Data in (**a**, **b**) was calculated by two-sided Student's t-test and data in (**d**) was analyzed using log-rank tests. *$p < 0.05$ was regarded as significant

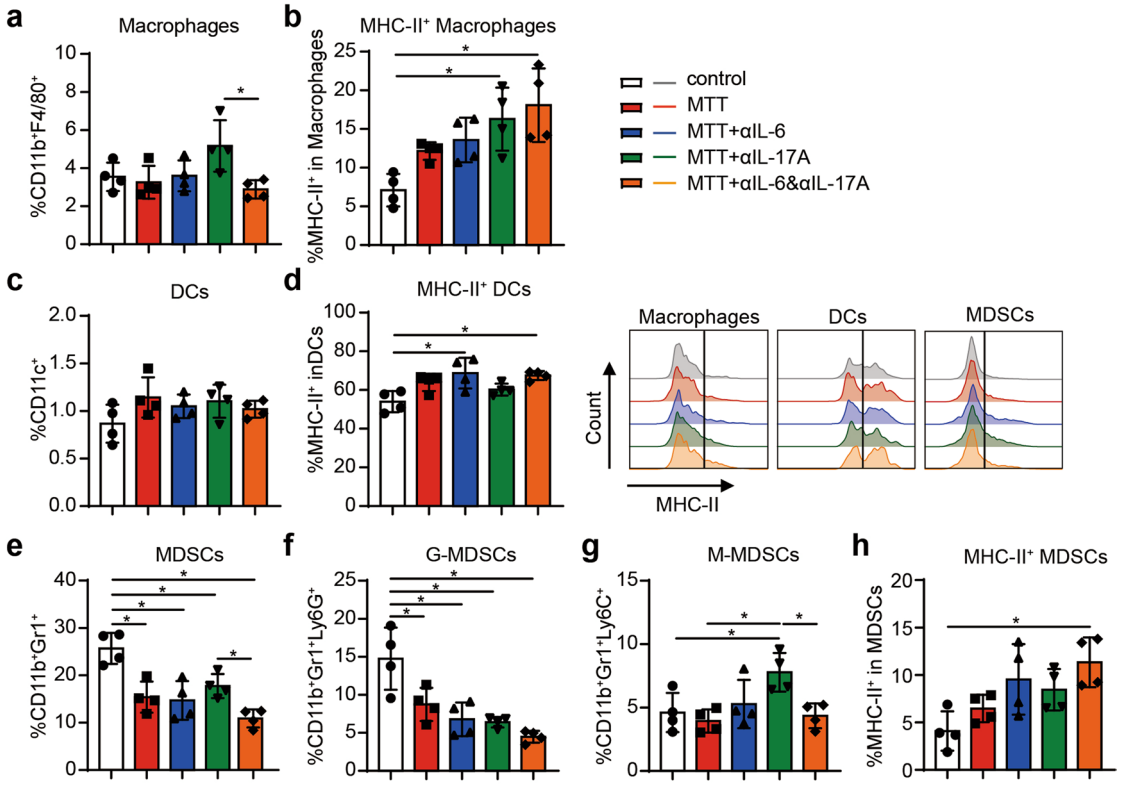


Fig. 2 Triple combination therapy reduced the accumulation of MDSCs and promoted the maturity of antigen-presenting cells in the lungs Proportion and phenotype of pulmonary macrophages (**a**, **b**), DCs (**c**, **d**), and MDSCs and granulocytic, monocytic subsets (**e**–**h**). $n=4$. One-way ANOVA was used for data analysis. *$p<0.05$ was regarded as significant

responses [24, 25]. The proportion of macrophages in the lungs was downregulated after MTT + αIL-6&αIL-17A compared to MTT + αIL-17A (Fig. 2a), but the proportion of major histocompatibility complex class II (MHC-II) expressing macrophages was significantly upregulated after MTT + αIL-6&αIL-17A and MTT + αIL-17A compared with that of the control (Fig. 2b). Additionally, the frequency of mature DCs with high-level of MHC-II was also significantly upregulated after MTT + αIL-6&αIL-17A and MTT + αIL-6, although the proportions of DCs were not obviously changed compared to those in the control (Fig. 2c, d). Similarly, the frequencies of mature macrophages and DCs in the spleens were also increased after single or triple combination therapy (Fig. S2a, c). These indicated that single neutralization combining or triple combination therapy could all effectively activate DCs and macrophages.

The frequency of MDSCs in the lungs was significantly decreased in all treated groups compared to the control mice, and was also prominently decreased after MTT + αIL-6&αIL-17A compared with MTT + αIL-17A (Fig. 2e). Consistently, the proportion of granulocytic MDSCs (G-MDSCs), one of the major subsets of MDSCs [26], was obviously decreased in the lungs in all treated groups (Fig. 2f). The proportion of monocytic MDSCs (M-MDSCs), the other subset of MDSCs, was slightly increased after MTT + αIL-17A and was comparable in other groups (Fig. 2g). In addition, the proportion of pulmonary MHC-II$^+$ MDSCs, representative of mature MDSCs [27], was obviously increased after MTT + αIL-6&αIL-17A (Fig. 2h). At the same time, the proportions of splenic MDSCs and M-MDSCs were also decreased after MTT + αIL-6&αIL-17A, although their maturation was not further promoted (Fig. S3e-h). These suggested that multi-mode or single neutralization combination therapy prevented the accumulation of MDSCs in the lungs, while triple combination therapy not only markedly reduced the proportion of MDSCs in both the lung and spleen but also promoted the maturation of pulmonary MDSCs.

Triple combination therapy promoted the CD4$^+$Th1 cell response

Multi-mode thermal therapy has been demonstrated to trigger a strong CD4$^+$ Th1-dominant response, which mediates systematic antitumor immunity [10]. The T-cell responses were then analyzed. Compared to the untreated group, the proportion of pulmonary CD4$^+$ T cells was notably

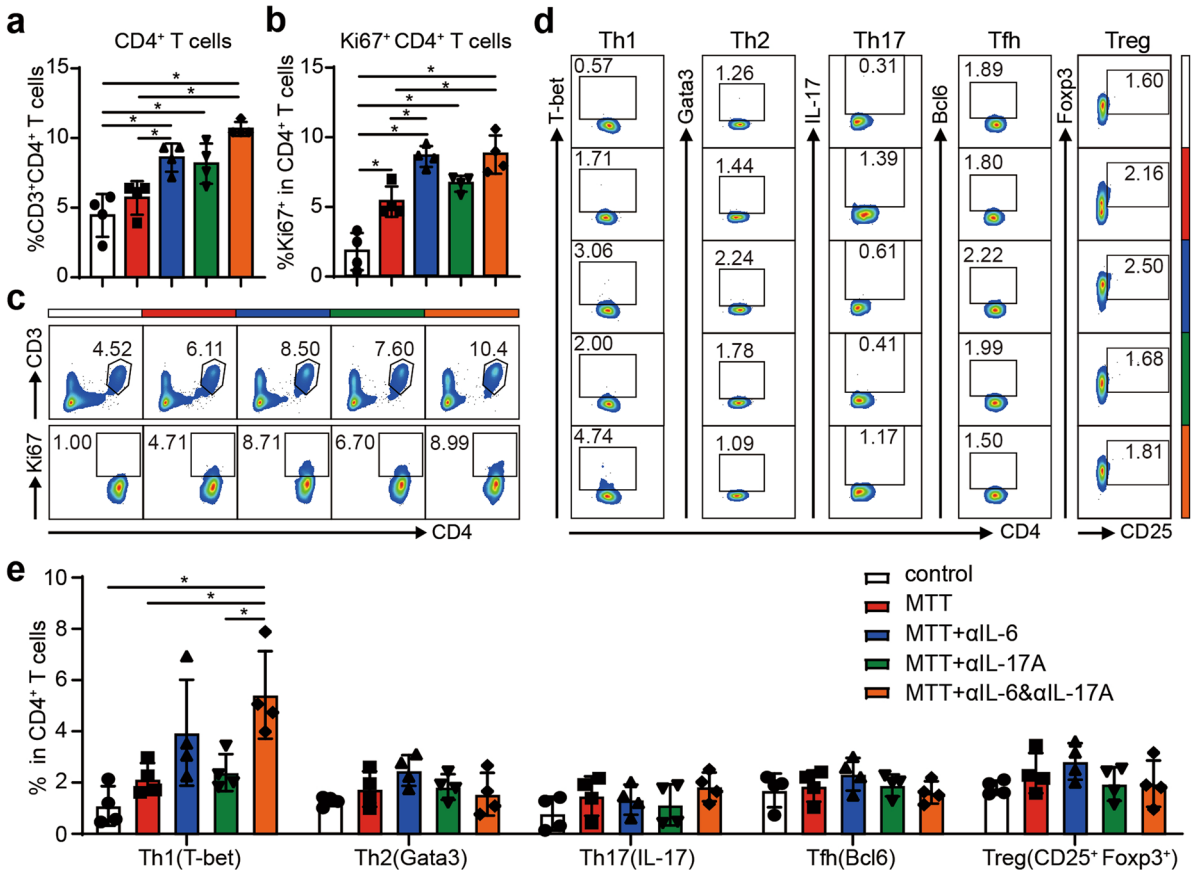


Fig. 3 Triple combination therapy promoted $CD4^+$ Th1 response in the lungs. (**a**-**c**) The proportion of $CD4^+$ T cells (**a**), $Ki67^+CD4^+$ T cells (**b**) in the lungs. (**c**) Representative flow cytometry graphics. (**d**, **e**) Subgroups of pulmonary $CD4^+$ T cells. $n=4$. One-way ANOVA was used for data analysis. $^*p<0.05$ was regarded as significant

increased after all combination strategies, especially after MTT + αIL-6 and triple combination therapy, and the level of $CD4^+$ T cells was much higher than that after MTT (Fig. 3a, c). Consistently, the proliferation capacity of $CD4^+$ T cells, represented by $Ki67^+$ cells [28], was significantly increased in all treated groups, and a higher frequency of $Ki67^+CD4^+$ T cells was observed after MTT + αIL-6 and MTT + αIL-6&αIL-17A than MTT alone (Fig. 3b, c). The proliferation capacity of splenic $CD4^+$ T cells was also significantly increased after MTT + αIL-6&αIL-17A, although shown comparable proportion with tumor-bearing control (Fig. S3a,b). Interestingly, the Th1 proportion ($T\text{-}bet^+$) was increased in both the lungs and spleen only after MTT + αIL-6&αIL-17A, but the proportions of other subset in all groups were not obviously changed (Fig. 3d, e, S3d). Collectively, these indicated that triple combination therapy could promote the expansion of $CD4^+$ T cells and uniquely induce Th1-dominant differentiation in the lungs and spleen.

Triple combination therapy enhanced the proliferation and cytotoxicity of $CD8^+$ T cells and NK cells in the lungs

$CD8^+$ T and NK cells are the major tumor killer cells [25, 29, 30], which characterized by interferon (IFN)-γ [31], perforin and granzyme B (GrzmB) expression [32, 33]. Compared with the control and MTT groups, the percentage of $CD8^+$ T cells after single or triple combination therapy was increased in the lungs (Fig. 4a, c). Consistently, a higher percentage of $Ki67^+CD8^+$ T cells was observed (Fig. 4b, c). In the spleen, an increased percentage of $CD8^+$ T cells was found after MTT and IL-6 or IL-17A single neutralization combination therapy, and the proportion of $Ki67^+CD8^+$ T cells was additionally increased in the MTT + αIL-6 and triple combination therapy groups (Fig. S5a-c). Moreover, compared with that in the MTT alone group, the frequency of $perforin^+CD8^+$ T cells in the lungs was significantly increased in all combination therapy groups, and the proportion of $GrzmB^+CD8^+$ T cells in the lungs was also increased after MTT + αIL-6,

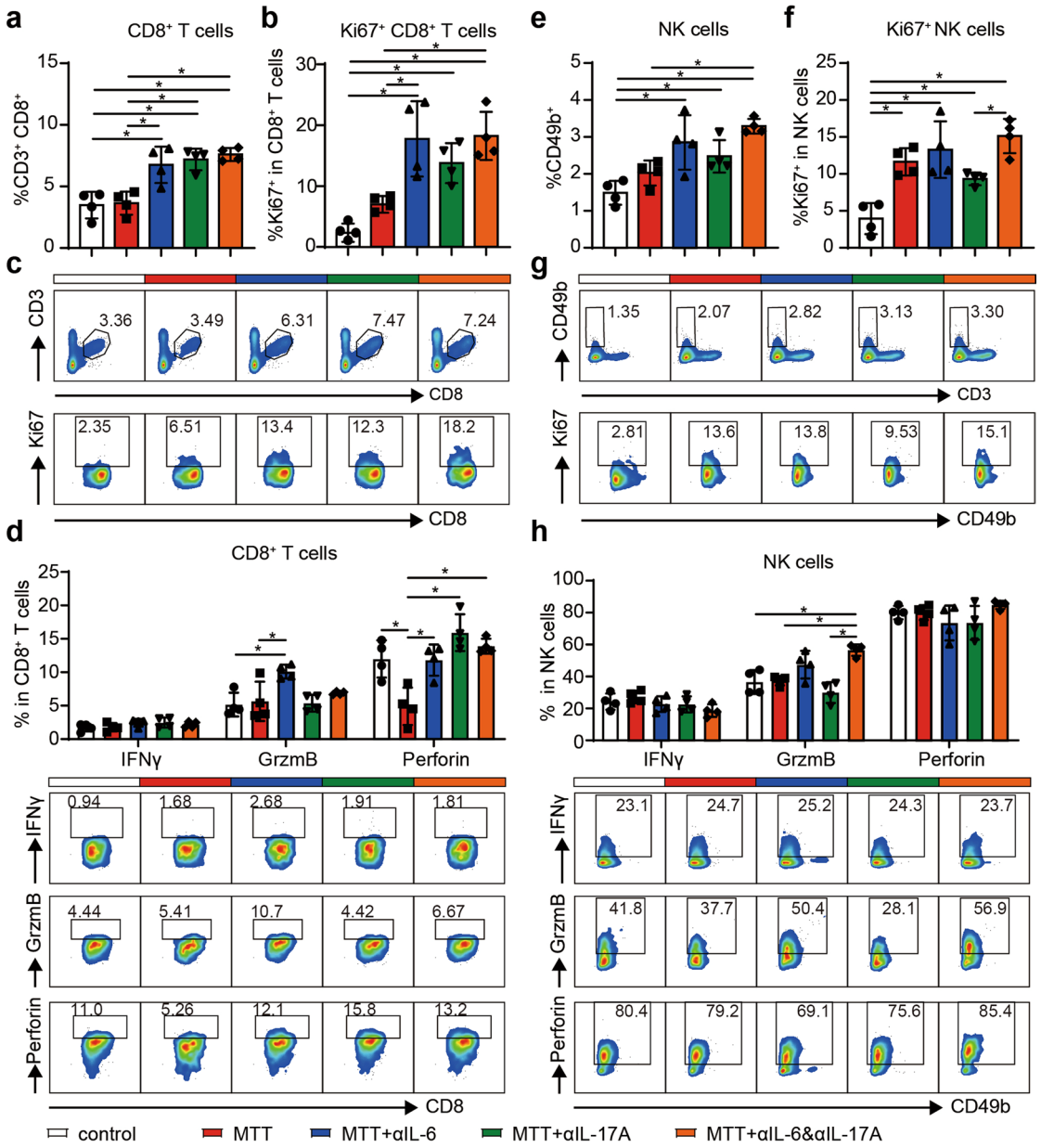


Fig. 4 Triple combination therapy promoted the proliferation and cytotoxicity of $CD8^+$ T cells and NK cells in the lungs (**a**-**d**) The proportion (**a**), proliferation level (**b**) and cytotoxicity molecules expression (**d**) of pulmonary $CD8^+$ T cells. (**e**–**h**) The proportion (**e**), proliferation level (**f**) and cytotoxicity molecules expression (**h**) of pulmonary NK cells. $n=4$. One-way ANOVA was used for data analysis. $^*p<0.05$ was regarded as significant

although a smaller difference was found in the spleen (Fig. 4d, S5d). These suggested that multi-mode thermal therapy combined with single or double cytokine neutralization effectively increased the proliferation and cytotoxicity of $CD8^+$ T cells in the lungs, while multi-mode thermal therapy combined with anti-IL-6 or double neutralization additionally promoted the proliferation of $CD8^+$ T cells in the spleen.

At the same time, increased proliferation of pulmonary NK cells was noticed after all treated groups, while the proportions were increased after all combining therapies compared with those in the untreated control group (Fig. 4e-g). In the spleen, the proportion of NK cells was not affected (Fig. S5e, g), despite an increased proportion of $Ki67^+$ NK cells in the MTT and both double combination therapy groups (Fig. S6f, g). At the same time, a higher percentage of $GrzmB^+$ NK cells in the lungs was detected in the triple combination therapy group (Fig. 4h), but the levels of cytotoxic molecules in splenic NK cells were downregulated after MTT + αIL-6&αIL-17A (Fig. S6h). These suggested that only triple combination therapy promoted the expansion and cytotoxicity of NK cells in the lungs.

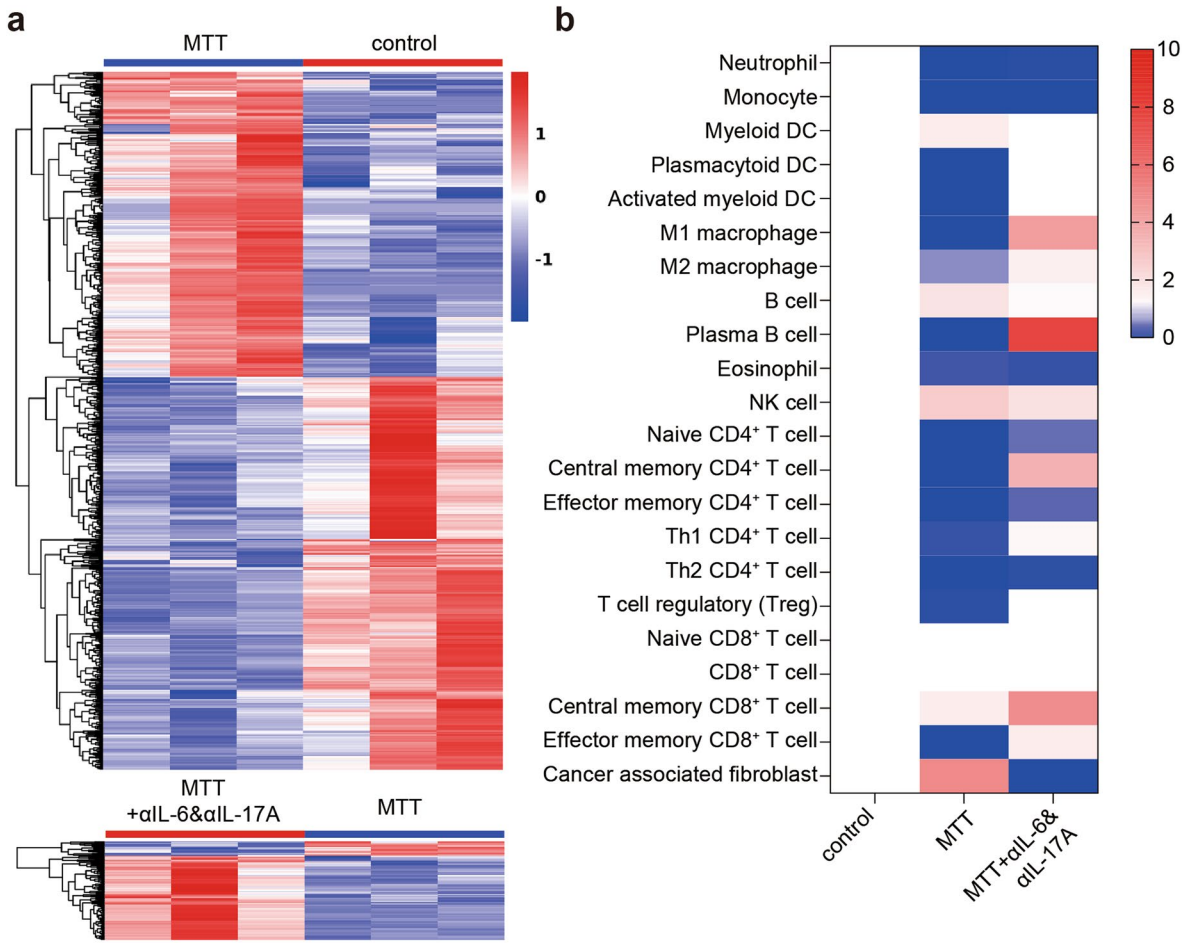


Fig. 5 Triple combination therapy upregulated the population of mature APCs and memory T cells and promoted $CD4^+$ Th1 immune response (**a**) Heatmap of differential genes, encompassing gene clustering. (**b**) Relative levels of cells in each group were calculated by TIMER2.0. DCs, Dendritic cells; NK cells, natural killers. $n = 3$

Triple combination therapy activated inflammation-related pathways and facilitated tumor killing of immune cells in the lungs

The above results highlighted that triple combination therapy could induce immune-stimulating responses in LLC1-bearing mice, especially in the lungs. To comprehensively understand the immune responses induced by triple combination therapy, RNA-sequencing was performed on Day 14 after treatment. Figure 5a showed that the lung tissues from the MTT group experienced robust transcriptional changes compared to the control group. There were 119 differentially expressed genes between the MTT + αIL-6&αIL-17A and MTT groups. The relative levels of cell subsets in each group calculated by TIMER 2.0 database represented that most myeloid-derived cells, $CD4^+$ T subsets, and effector memory $CD8^+$ T cells in the lungs were decreased after multi-mode thermal therapy, with increased frequency of myeloid DCs, B cells, NK cells, and cancer associated fibroblasts (CAFs) compared to tumor-bearing mice (Fig. 5b). However, the level of M1 macrophages and plasma B cells was significantly increased, accompanied by an increased level of the Th1 subset, but the quantity of CAFs was downregulated after MTT + αIL-6&αIL-17A compared to the multi-mode thermal therapy group (Fig. 5b). These indicated that triple combination therapy effectively mobilized the response of antigen-presenting cells (APCs), T cells and NK cells, but diminished the immunosuppressive effect by decreasing the proportion of CAFs, resulting in re-education and the creation of a tumor microenvironment to immunologically active state.

The GO and gene set enrichment analysis (GSEA) showed top 20 enrichment biological processes, where pathways including immune system processes, cellular response to IFN-β and IFN-γ, and peptide antigen binding were extensively activated after MTT + αIL-6&αIL-17A compared to MTT (Fig. 6a, b). Ingenuity Pathway Analysis (IPA) revealed downregulation of immunosuppressive

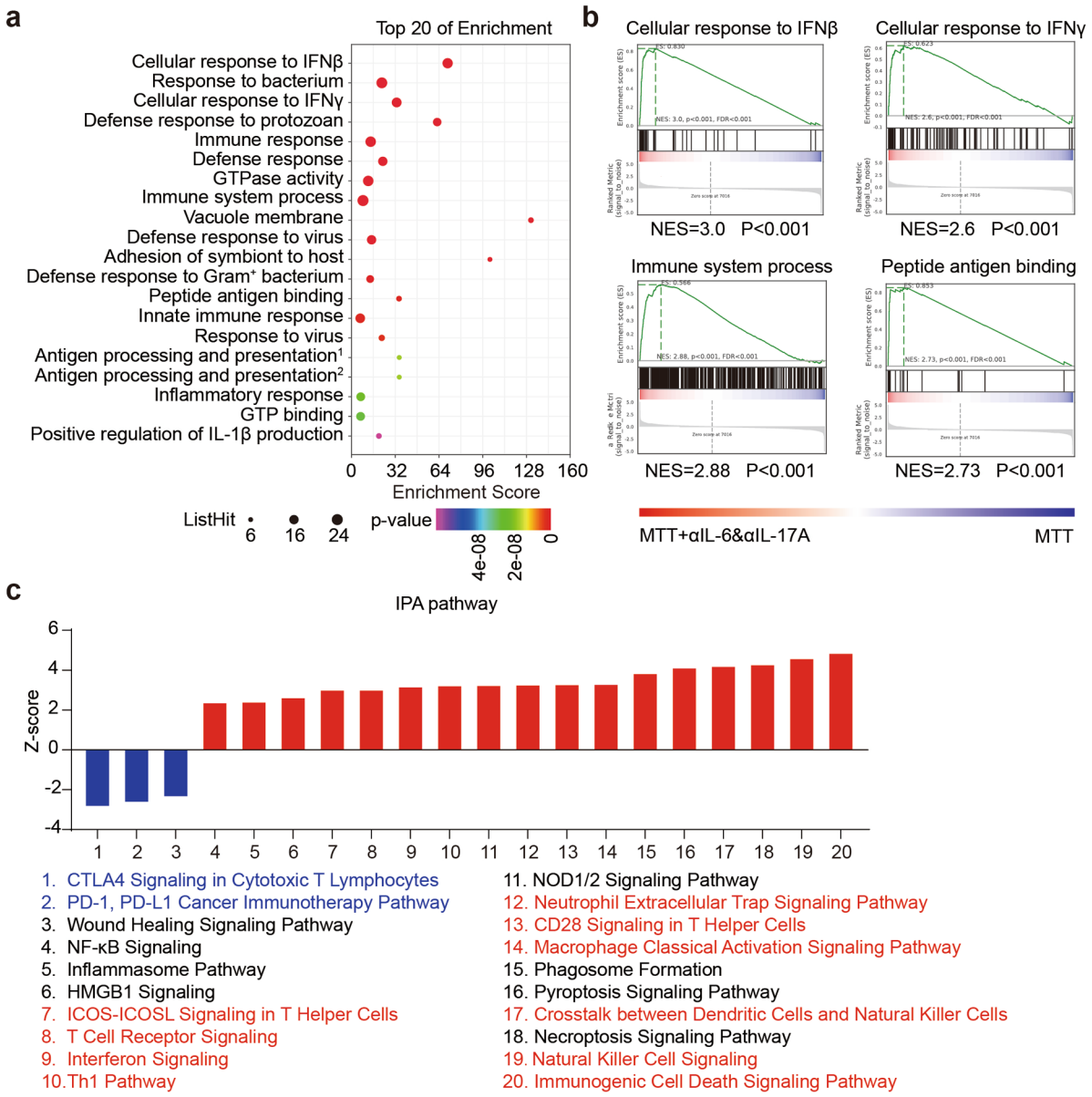


Fig. 6 Changes in lung cells in transcript levels after triple combination therapy (**a**) Bubble map for GO analysis presented top 20 enriched signalings in the lung cells from multi-mode thermal therapy and triple combination therapy. (**b**) Individual GSEA enrichment plots for cellular response to IFN-β and IFN-γ, immune system process and peptide antigen binding gene sets. (**c**) Top 20 enriched pathways predicted by ingenuity pathway analysis (IPA). NES, normalized enrichment score

pathways after MTT + αIL-6&αIL-17A, including CTLA4 signaling in cytotoxic T lymphocytes, PD-1/PD-L1 cancer immunotherapy pathways, but increased activity related to the crosstalk between DCs and NK cells, the extracellular trap-mediated cell-killing capacity of neutrophils, the activation of macrophages and T cells (T cell receptor, CD28, ICOS and interferon signalings), and Th1 pathway response (Fig. 6c). These further revealed that double neutralization combining therapy systemically promoted immunostimulatory activity and downregulated tumor-suppressive activity in the lungs.

To further characterize the cytotoxicity of pulmonary immune cells, tumor-killing assay was performed. The killing capacity of T and NK cells in the lungs after MTT was slightly improved, whereas after MTT + αIL-6&αIL-17A, the killing capacity of whole lung cells, T and NK cells was significantly enhanced. In particular, T cells showed much stronger cytotoxicity than those after MTT (Fig. 7a-d). To further identify whether T cell-mediated cytotoxicity enhanced by MTT + αIL-6&αIL-17A inhibited tumor metastasis, pathological staining of the lungs from different groups of mice was performed on Day 14. Normal architectures of the lung from

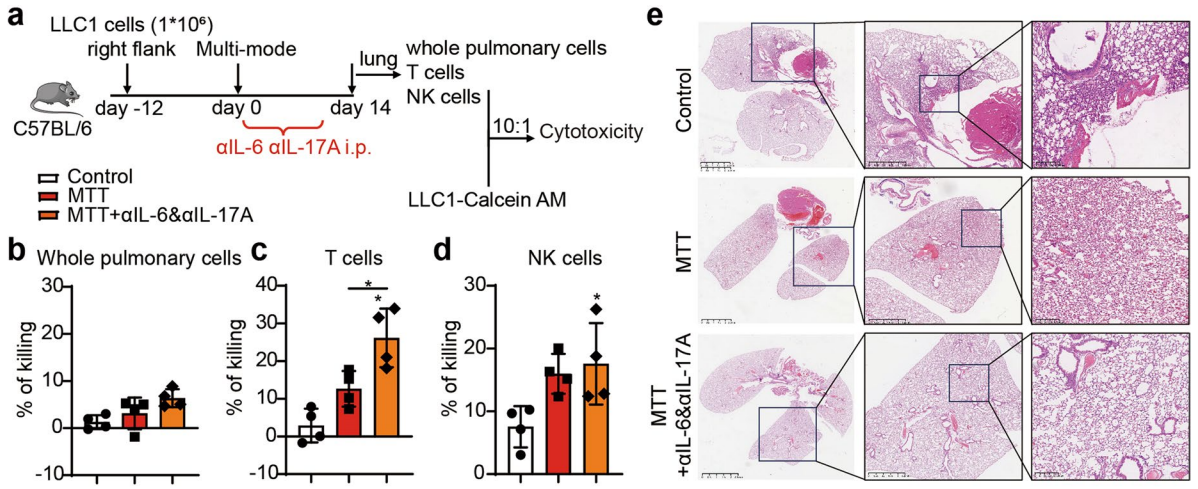


Fig. 7 Triple combination therapy enhanced the cytotoxicity of pulmonary immune cells (**a**) Study design scheme. Briefly, indicated pulmonary cells from different groups were isolated on day 14 after treatments by magnetic-bead sorting (MACS). The whole proportion of lung cells, and the isolated T cells and NK cells were then co-cultivated with calcein-stained LLC1 cells in a ratio of 10:1 for 4 h. The killing rate was detected by the leakage of calcein. (**b**-**d**) The killing rate of lung cells (**b**), T cells (**c**), and NK cells (**d**). (**e**) Pathological staining of the lungs from indicated treatment groups on Day 14. $n = 3$. One-way ANOVA was used for data analysis. $^*p < 0.05$ was regarded as significant

the MTT + αIL-6&αIL-17A treated mice were observed, while established micrometastases or the increased cell density could be observed in the lungs of the control or multi-mode treated mice, respectively (Fig. 7e). Collectively, these highlighted that triple combination therapy created an immunostimulatory environment in the lungs and significantly improved T cell-mediated cytotoxicity, contributing to the inhibition of tumor metastasis and achieving better survival in the LLC1 model.

Discussion

In our previous study, we provided strong evidence supporting that multi-mode thermal therapy induced robust antitumor immunity guaranteeing prolonged tumor-free survival of mice in various tumor models. However, here we only found slightly extended survival time after multi-mode thermal therapy but failed to improve the survival rate of mice carrying LLC1 tumor. Given that IL-17A plays a pivotal role in the advancement of NSCLC tumors, and IL-6 is essential for IL-17A inducing to enhance the invasion of NSCLC cells [20], we hypothesized that IL-6 and IL-17A modulate the therapeutic effect in mice with LLC1 after multi-mode thermal therapy. An in-depth study revealed that elevated serum IL-6 and IL-17A levels persisted following multi-mode treatment. To improve the therapeutic effect in LLC1-bearing mice, triple combination therapy was used to treat LLC1-bearing mice. Triple combination therapy displayed encouraging efficacy in improving the long-term survival rate in mice carrying LLC1, because it created an immunostimulatory environment, especially in the lungs, by preventing the accumulation of MDSCs, inducing the maturation of APCs, triggering the differentiation of Th1-dominant $CD4^+$ T cells and enhancing the cytotoxicity of pulmonary $CD8^+$ T cells and NK cells.

The novel multi-mode thermal therapy strategy developed previously achieved long-term survival of mice in various tumor types, such as the B16F10 melanoma [34], 4T1 triple-negative breast cancer [11], CT26 colorectal cancer [35], and KPC pancreatic cancer models (unpublished data). A reduced frequency of MDSCs and the accumulated $CD4^+$ T cell Th1 subset are proved to the key factors to determine multi-mode-induced long-term immune protection leading to long-term survival. In clinical application, multi-mode thermal therapy significantly improves the progression-free survival of colorectal cancer patients with liver metastasis [36]. However, this research found that while multi-mode thermal therapy extended survival, it failed to enhance the survival rate in mice carrying LLC1. Further researches indicated that that multi-mode thermal therapy inhibited MDSCs accumulation in the lungs, but had less impact on the induction of their maturation. At the same time, multi-mode thermal therapy failed to induce fully maturation of macrophages and DCs, $CD4^+$ Th1-dominant differentiation, or the modulating cytotoxicity of $CD8^+$ T cells and NK cells in the lungs. These results suggested that multi-mode thermal therapy alone could not systemically elicit sufficient anti-LLC1 immunity, though it could somewhat attenuate tumor immunosuppression by reducing the ratio of MDSCs.

LLC1-bearing mice are an NSCLC mouse model that develops metastasis extremely rapidly, developing pulmonary micrometastases within two weeks of subcutaneous inoculation, and have high levels of MDSC-mediated

immunosuppression [12, 37–39]. MDSCs stand out as the primary immunosuppressor in NSCLC and assist immune escape by protecting tumor cells against immune surveillance [40]. MDSCs inhibit the differentiation of Th1 cells and encourage the polarization of Tregs, thereby enhancing immune tolerance [41]. MDSC-derived tumor-associated macrophages (TAMs) induce the differentiation of fibroblasts into CAFs to further promote tumor growth [42, 43]. Patients with tumors exhibiting elevated MDSC levels face a markedly increased mortality risk [44]. Previous study have demonstrated that multi-mode thermal therapy contributed to the decreased proportion of MDSCs, but the residual MDSCs maintained a strong immunosuppressive function [45, 46]. Combining multi-mode with IL-6 signaling blockade significantly improved the functional remodeling of MDSCs and increased the level of $CD4^+$ Th1 cells, which ultimately led to better survival [45]. The findings indicate that combining with cytokine therapy could enhance the effectiveness of multi-mode thermal therapy in treating highly metastatic carcinoma.

Chronic inflammatory cytokines are criminal in the progression of NSCLC [47]. IL-6 is recognized as the initiate cytokine during metastasis [48]. IL-6 induces the activation of signal transducer and activator of transcription 3 (STAT3) in tumor cells, which in turn drives the transcription of genes encoding cell cycle proteins and promotes tumor cell proliferation [49]. IL-6 enhance the level of TIM-4 on NSCLC cells, which acts synergistically with IL-6 to promote the migration, invasion and epithelial-mesenchymal transition (EMT) of NSCLC cells [50]. In addition, IL-6 is the major supervisor of MDSC-mediated suppression [51]. IL-6 promotes the accumulation of MDSCs through paracrine and autocrine mechanisms, induces reactive oxygen species (ROS) and NADPH oxidases 2 (NOX2) expression, enhances MDSC inhibitory function [52, 53], induces arginine (Arg)-1 expression in DCs, inhibits DC maturation [54], downregulates MHC-II expression on myeloid cells, as well as inhibits antigen presentation initiation and the T cell response [55]. This research we noticed that multi-mode thermal therapy combined with IL-6 neutralization significantly prolonged the survival time of LLC1-bearing mice. Furthermore, multi-mode thermal therapy combined with IL-6 neutralization supported the maturation of DCs, and subsequently promoted the proliferation and accumulation of $CD4^+$, $CD8^+$ T cells and NK cells. However, this strategy failed to further diminution the proportion of MDSCs and reprogram their function, which pointed that single IL-6 neutralization combination was still inadequate to remodel MDSC-mediated suppression. In addition, despite the increasing segment of T and NK cells, their cytotoxicity was unchanged after IL-6 neutralization combining, which ultimately led to tumor progression and the death of the mice.

IL-17A is another chronic inflammation-related cytokine which promotes NSCLC metastasis and is a poor prognostic marker in the clinic [20, 56]. Similar to IL-6, IL-17A participates in the progression of NSCLC cells, and induces the angiogenic chemokines secretion to ensure the self-seeding and colonization of tumors [57–60]. Moreover, IL-17A also participates in the accumulation of MDSCs in a granulocyte colony-stimulating factor (G-CSF)-dependent manner [61], and prevents the type I polarization of the $CD4^+$ T cell response [17]. Therefore, here we also observed that multi-mode thermal therapy combined with IL-17A neutralization presented immunostimulatory efficacy similar to that combining IL-6 neutralization by inducing the M1 polarization of macrophages, and the proliferation and accumulation of $CD4^+$, $CD8^+$ T cells and NK cells, but could not further decrease the proportion or suppressive phenotype of MDSCs or enhance the cytotoxicity of T cells or NK cells. These uncovered that single IL-17A neutralization combining could not successfully reshape MDSC-induced suppression or activate systemic antitumor immunity.

IL-6 activates the STAT3 pathway to induce the expression of IL-17A [48], which then promotes the expression of IL-6 [20] and thereby intensifies the suppression [62]. But there are reports that other multiple cytokines also participate in the induction of IL-6 and IL-17A production [63, 64], making single cytokine neutralization combination could not completely relieve tumor chronic inflammatory environment in NSCLC. Therefore, we hypothesized that multi-mode thermal therapy combined with double IL-6 and IL-17A neutralization would inhibit the IL-6/IL-17A axis, and further extended the survival rate of LLC1-bearing mice. This research proved that only triple combination therapy can exhibit significant improvement in long-term tumor-free survival. Consistently, compared with the tumor-bearing group, a notable decrease in the level of MDSCs but only an upward trend in the expression of the mature molecule (MHC-II) on MDSCs and IFN-γ in $CD4^+$T cells was exhibited in single neutralization combination therapy, while a significantly increased expression of the mature molecule on MDSCs and IFN-γ in $CD4^+$T cells was only observed in triple combination therapy. These suggest that single cytokine neutralization combination therapies could not trigger more robust antitumor immunity, which was insufficient to inhibit rapid tumor cell growth and progression of LLC1 leading to only prolong survival time, but not improve survival rate. However only triple combination therapy could establish strong antitumor immune protection characterized as significantly upregulated Th1-dominant immunity to achieve long-term survival rate. In our previous study, multi-mode thermal therapy combined with single IL-6 blockade was used to treat 4T1 triple-negative breast cancer. Our findings indicated that single anti-IL-6 combining markedly decreased the levels of MDSCs and promoted

their phenotypic maturation leading to Th1-dominated differentiation, which notably improved the survival rate [45]. The main reasons for the different therapeutic effects of the same administration strategy on LLC1 and 4T1 tumor models originate from the different characteristics of tumor cells. LLC1 presents more rapid tumor growth and progression compared to 4T1 triple-negative breast cancer model. Therefore, single IL-6 blockade combination therapy triggered anti-tumor immunity which was sufficient to inhibit tumor metastasis leading to the improved survival rate in 4T1 model. But in LLC1 model, only triple combination therapy could elicit a strong enough immune response to inhibit tumor metastasis resulting in promoting survival rate.

In this study, mice carrying LLC1 were experienced multi-mode ablation combined with cytokine therapy; however, single cytokine neutralization alone was not performed. Although clinical evidence supports that some patients benefit from IL-6 neutralization treatment alone, our previous study showed that IL-6 neutralization did not increase the survival rate while only extended the survival time in the 4T1 model, which also has an extremely high level of MDSCs. In addition, our studies showed that IL-6 neutralization had less of an impact on stimulating antitumor immunity at the late stage after treatment. Only by combining with IL-6 neutralization could entirely reprogram MDSC-induced suppression [45]. On the other hand, thermophysical therapy of tumors will promote the producing of IL-6, correlating with mortality [65]. These results suggest that single cytokine blockade would not be sufficient to induce strong and durable antitumor immunity, and multi-mode thermal therapy combined with cytokine blockade may maximize the immunostimulatory effect to promote long-term survival. In addition, the aim of this study is how to improve the therapeutic effect of multi-mode thermal therapy in LLC1 model, therefore, only multi-mode thermal therapy-based combination therapy was performed. This study was performed in LLC1 NSCLC model. Spontaneous NSCLC metastasis model has been considered in the future study to further estimate the efficacy of combination strategy in NSCLC.

In conclusion, our present studies demonstrated that multi-mode thermal therapy combined with IL-6 and IL-17A neutralization could efficiently prevent the accumulation of MDSCs, induce MDSC maturation and promote the maturation of DCs and M1-polarization of macrophages to remodel the immunosuppressive tumor environment in the lungs, thus increasing the proliferation of T cells and NK cells and facilitating a Th1-dominant $CD4^+$ T cell response; All of these effects ultimately led to a strong tumor-elimination power of T and NK cells thus improving the survival rate of LLC1 model mice. Our consequences emphasized the predominant role of MDSC-targeting therapy in LLC1 NSCLC treatment, and multi-mode thermal therapy combined with IL-6 and IL-17A neutralization could be an effective strategy for clinical NSCLC treatment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s44258-024-00016-4.

Acknowledgements The authors thank Jincheng Zou and Shiqing Zhao for providing and maintenance the multi-mode thermal therapy system, and thank prof. Weiliang Xia for providing the Lewis lung carcinoma cell line (LLC1).

Authors' contributions All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, J.Z., Y.L. and S.W.; Methodology, J.Z., Y.L. and S.W.; Machine maintaining, J.Z., A.Z.; Data curation, J.Z., S.W. and Z.Z.; Writing—original draft preparation, J.Z. and Y.L.; Writing—review and editing, J.Z., Y.L., S.W., Z.Z., J.W., P.D., Y.Z., J.Y., Y.Y., Y.H. and P.L.; Funding acquisition, P.L.

Funding This study was supported by the National Key Research and Development Program of China (Grant No. 2020YFA0909003), the National Natural Science Foundation of China (Grant No. 82072085), the Shanghai Science and Technology Commission of Shanghai Municipality (Grant No. 19DZ2280300 and No. ZJ2021-ZD-007).

Availability of data and materials Data are contained within the article or supplementary material or are available from the authors upon reasonable request.

Declarations

Ethics approval and consent to participate The animal study protocol was approved by the Ethics Committee of School of Biomedical Engineering and Med-X Research Institute, Shanghai Jiao Tong University (No.2020017).

Consent for publication Not applicable.

Competing interests The authors declare no conflict of interest.

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