Dynamic Changes in the Immune Microenvironment in Tumor-Draining Lymph Nodes of a Lewis Lung Cancer Mouse Model After Microwave Ablation

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Purpose: Microwave ablation (MWA) is a minimally invasive technique for treating lung cancer. It can induce immune response; however, its effect on the immune microenvironment in tumor-draining lymph nodes (TdLN) is not well understood. This study aims to identify changes in the immune microenvironment in TdLN following MWA in a Lewis lung cancer (LLC) mouse model.

Methods: LLC mouse model was established and followed by MWA. TdLN were collected at various time points, including pre-MWA and days 1, 2, 4, and 8 post-MWA. Flow cytometry was used to determine the frequencies of CD4⁺ T cells, CD8⁺ T cells, regulatory T (Treg) cells, natural killer (NK) cells, dendritic cells (DCs) and other immune cells in the TdLN. Certain cytokines were also detected.

Results: Compared with pre-MWA, the frequency of CD4⁺ T cells significantly increased from day 1 to day 8 post-MWA. The frequency of CD8⁺ T cells decreased significantly on days 2 and 4, but no significant changes occurred on days 1 and 8. Significant decreases in the frequencies of Treg cells and KLRG1⁺ Treg cells were observed from day 1 to day 4. On days 4 and 8, there was a significant increase in the frequency of NK cells. The frequency of resident cDC2 significantly increased on day 4, whereas CD11b⁺ migratory cDCs increased on day 1. Additionally, on day 4, a notable rise was observed in the frequency of NK cells secreting IFN-γ, while on day 8, there was a significant increase in the frequency of CD8⁺ T cells secreting both IFN-γ and TNF-α.

Conclusion: MWA of lung cancer can alter the immune microenvironment in the TdLN, triggering immune responses. These changes are particularly evident and intricate within the initial 4 days post-MWA. Treatment combined with MWA within a certain period may significantly enhance anti-tumor immunity.

Keywords: microwave ablation, lung cancer, T cells, regulatory T cells, natural killer cells, dendritic cells

Introduction

Lung cancer persists as a major burden to society, accounting for a formidable portion of cancer-related morbidity and mortality worldwide. Recent global cancer data highlight the gravity of this malignancy as lung cancer accounts for 18% of total cancer-related deaths.¹ These dire statistics accentuate the need for more effective therapeutic strategies that transcend conventional treatment paradigms. Thermal ablation, such as microwave ablation (MWA), radiofrequency ablation (RFA), and cryoablation, has gained much attention as a super minimally invasive alternative therapy. Among these techniques, MWA is widely used for treating lung cancer, particularly in patients who are inoperable or cannot
tolerate conventional treatment because of comorbidities. It utilizes electromagnetic waves to induce localized hyperthermia and coagulative necrosis within the tumor. Compared with RFA, MWA has the advantages of faster ablation and a larger ablation volume because of its high convection and low heat deposition effects in the lung. Recent studies revealed promising outcomes with respect to safety, effectiveness, and localized tumor control in lung cancer patients treated with MWA. This minimally invasive approach offers an effective therapeutic option that enhances patient quality of life.

Recent evidence suggests that MWA not only provides localized tumor control, but also alters the tumor microenvironment, triggering a cascade of immunomodulatory events. Necrotic tumor debris induced by ablation has the potential to function as an in situ vaccine that induces an autologous antitumor immune response, including innate and adaptive immunity. The implications for such immunological effects are profound and may lead to synergistic treatment strategies that exploit the immune system.

Tumor-draining lymph nodes (TdLN) are the lymph nodes to which tumor cells travel through the lymphatic vessels. They are the first place that tumor-induced dendritic cells (DCs) migrate, serving as the initiation and maintenance site of the host antitumor immune response. Therefore, TdLN acts not only as a destination for tumor metastasis, but also as a hub for antitumor immune responses. However, as tumors progress, the persistent stimulation of tumor secretions results in the transformation of TdLN into an immunosuppressive state, which suppresses the antitumor immune response and results in immune tolerance. TdLN may be regarded as a dynamic compartment within the regional tumor microenvironment. Understanding its structure and function will provide important insights into tumor metastasis and immunity. Therefore, remodeling of the immune microenvironment in TdLN represents an opportunity to enhance tumor treatment.

A correlation between MWA and the immune system has been observed in studies on various tumors, such as lung cancer, breast cancer, and hepatocellular carcinoma. However, current studies on the immune effects of MWA in lung cancer patients have primarily focused on peripheral blood immune cells, and have not considered changes in the immune microenvironment of the TdLN. Some studies only assessed the immune status at a specific time point after ablation, limiting the ability to track the dynamic effect of MWA on the immune response. Animal experiments involving lung cancer are scarce. The complexity of the in vivo immune network requires a more nuanced approach, for which animal models provide an indispensable tool. The Lewis lung cancer (LLC) mouse model serves as an archetype for this purpose. It has an important role in biological and translational studies of lung cancer with broad application in the field of immunology.

In this study, we examined the dynamic changes in the immune microenvironment in TdLN following MWA in the LLC mouse model. By elucidating the immunological changes post-MWA, we hope to provide insight into the development of combination therapies that enhance patient outcomes and survival rates.

**Materials and Methods**

**Animals and Cell Lines**

This study was reviewed and approved by the Animal Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (NSFC: No. 2020–497). All experimental procedures were complied with the national standard of Laboratory animal-Guideline for ethical review of animal welfare (GB/T 35892–2018). A schematic of the experimental design and timeline is shown in Figure 1A. C57BL/6 mice (6–8 weeks old) were purchased from Beijing Huafukang Biotechnology Co., Ltd. All mice were housed and maintained in specific pathogen-free conditions. The LLC cell line was purchased from Shanghai Fuheng Biotechnology Co., Ltd. and cultured in DMEM (C11995500BT, Gibco) supplemented with 10% fetal bovine serum (FBS, BS-1105, Inner Mongolia Opcel Biotechnology Co., Ltd) and 1% penicillin/streptomycin (P1400, Solarbio). The cells were incubated at 37°C in a 5% carbon dioxide (CO₂) incubator.
LLC Mouse Model

LLC cells in a logarithmic growth phase were collected, washed with PBS, and diluted to a concentration of $1 \times 10^7$ cells/mL. Subsequently, 0.1 mL of cell suspension was injected into the right axillary subcutaneous region of the mice. Tumor growth was carefully monitored and measured. After 8–10 days of inoculation, when the tumor had reached a maximum diameter of approximately 8–10 mm, treatment was initiated. The mice were randomly divided into five groups: pre-MWA group (day 0), post-MWA 1 day group (day 1), post-MWA 2 days group (day 2), post-MWA 4 days group (day 4), and post-MWA 8 days group (day 8), with 3–5 mice per group.

Ablation Procedures

MWA procedure was similar to that used clinically and aimed to completely ablate the tumor. Before surgery, the fur in the tumor area was removed using depilatory cream. The mice were anesthetized using a small animal anesthesia machine (R500, RWD). The mice were then placed in an induction chamber containing 3%–4% isoflurane (R510-22-10, RWD).
RWD) and an oxygen flow rate of 300–500 mL/min until fully anesthetized. After anesthesia, the mice were transferred to a nose cone with 1%–1.5% isoflurane and an oxygen flow rate of 100–200 mL/min for anesthesia maintenance. The tumor area was wiped with 75% alcohol, followed by treatment with an MWA device (KY-2000, Kangyou). The MWA needle was inserted along the longest axis of the tumor, with the needle tip positioned at the furthest end of the tumor. The ablation parameters were set at 3 W and 1 min. Preliminary experiments including pathological studies have confirmed that under these parameters, complete ablation of tumors within a maximum diameter of 1 cm can be achieved with minimal damage. The mice were monitored daily after ablation.

Sample Acquisition
At each time point, the mice were euthanized, and right axillary lymph node, namely TdLN, were collected for flow cytometry analysis.

Flow Cytometry Analysis
A cell dissociation solution, which was prepared by adding type IV collagenase (17,104–019, Gibco) to RPMI 1640 medium (C1187500BT, Gibco) containing 10% FBS, was added to homogenized lymph nodes and incubated at 37°C for 15 min to prepare a single-cell suspension. The cells were washed and resuspended in staining buffer. Staining was divided into transcription factor staining and intracellular cytokine staining. The staining process of transcription factors was roughly as follows: The cells were stained with Fixable Viability Stain 700 (FVS700, 564,997, BD, 1:1000) to exclude dead cells, followed by staining for cell surface markers, including anti-CD45 (564,225, BD, 1:400), anti-CD3e (612,771, BD, 1:400), anti-NK1.1 (564,144, BD, 1:400), anti-CD8a (563,234, BD, 1:400), anti-CD4 (47–0041-82, Invitrogen, 1:200), anti-CD44 (103,044, Biolegend, 1:400), anti-CD62L (104,448, Biolegend, 1:400), anti-Klrk1 (46–5893-82, Invitrogen, 1:400), anti-B220 (63–0452-82, Invitrogen, 1:400), anti-CD317 (127,039, Biolegend, 1:400), anti-CD11b (557,657, BD, 1:200), anti-Ly6C (48–5932-82, Invitrogen, 1:800), anti-CD11c (45–0114-82, Invitrogen, 1:200), anti-IA/IE (107,635, Biolegend, 1:400), and anti-CD326 (12–5791-82, Invitrogen, 1:1600). The cells were then fixed and permeabilized with a fixation/permeabilization solution (2,685,229, Invitrogen) for 45 min and stained with anti-Foxp3 (126,406, Biolegend). Finally, the cells were resuspended in PBS (P1020, Solarbio) and analyzed by flow cytometry (LSRFortessa™, BD). The process of intracellular cytokine staining was broadly similar to the aforementioned process. The difference lied in that before staining, single cells needed to be incubated at 37°C for 5 h after adding cell stimulation solution (00–4975-03, eBioscience). The fixation/permeabilization solution used was different (554,714, BD). Cytokines included IFN-γ (505,836, Biolegend, 1:200), IL-4 (554,389, BD, 1:200) and TNF-α (563,387, BD, 1:400). Cytometry data were analyzed using FlowJo software version 10.8.1 (TreeStar). Gating strategies for data analysis are shown in Figure 1B and C.

Statistical Analysis
Statistical analyses were performed using GraphPad Prism software (version 9.0, GraphPad). The measured data are expressed as the mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey’s multiple comparisons test or Kruskal–Wallis H-test. All statistical tests were two-sided, and P <0.05 was considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns indicates no significant difference.

Results
CD4+ T Cells and Memory CD4+ T Cells
Frequencies of total T cells, CD4+ T cells, CD8+ T cells, central memory T (TCM) cells, effector memory T (TEM) cells, and regulatory T (Treg) cells in the TdLN were determined by flow cytometry analysis. The results indicated that there were no significant changes in the frequency of total T cells at various time points post-MWA. However, compared with pre-MWA, the frequency of CD4+ T cells significantly increased from day 1 to day 8 and reached a peak on day 2 post-MWA. In contrast, from day 1 to day 8, the frequency of CD4+ TEM cells (marked CD4+Foxp3+CD44+CD62L-) significantly decreased; however, the frequency of CD4+ TCM cells (marked CD4+Foxp3-CD44+CD62L+) showed no
significant changes (Figure 2). The ability of CD4⁺ T cells to secrete cytokines IFN-γ and IL-4 was assessed, and it was found that the frequency of IFN-γ and IL-4 within CD4⁺ T cells exhibited an upward trend on day 8 post-MWA, although no significant difference was noted compared to pre-MWA levels (Figure 3).

**CD8⁺ T Cells and Memory CD8⁺ T Cells**

Compared with pre-MWA, the frequency of CD8⁺ T cells decreased significantly on days 2 and 4 post-MWA and showed no significant changes on days 1 and 8 (Figure 2). In addition, there were no significant changes in the frequencies of CD8⁺ TCM cells (marked CD8⁺CD44⁺CD62L⁺) and CD8⁺ TEM cells (marked CD8⁺CD44⁺CD62L⁻) from days 1 to 8 (Figure 2). Notably, on day 8 post-MWA, there was a significant increase in the proportion of CD8⁺ T cells secreting both IFN-γ and TNF-α(Figure 3).
The frequency of Treg cells (marked CD4$^+$ Foxp3$^+$) was significantly decreased from day 1 to day 4. The frequency of Klrg1$^+$ Treg cells (marked CD4$^+$ Foxp3$^+$ Klrg1$^+$), which exhibit stronger immunosuppressive effects, showed a similar trend to that of Treg cells, significantly decreasing from day 1 to day 4 and reaching their lowest level on day 4 (Figure 4).

**Figure 3** Dynamic changes of cytokines within CD4$^+$ T cells, CD8$^+$ T cells and NK cells in the TdLN post-MWA. (A) The percentage of defined cells in the TdLN, as illustrated in flow cytometry plots. (B) Statistical results for A (*P < 0.05, ns indicates no significant difference).

**Treg Cells**

The frequency of Treg cells (marked CD4$^+$ Foxp3$^+$) was significantly decreased from day 1 to day 4. The frequency of Klrg1$^+$ Treg cells (marked CD4$^+$ Foxp3$^+$ Klrg1$^+$), which exhibit stronger immunosuppressive effects, showed a similar trend to that of Treg cells, significantly decreasing from day 1 to day 4 and reaching their lowest level on day 4 (Figure 4).
From day 1 to day 4, the frequency of NK cells gradually increased from day 1 to day 4, followed by a decrease on day 8. Compared with pre-MWA, significant increases in the frequencies of NK cells on days 4 and 8 post-MWA were observed (Figure 4). Moreover, the frequency of NK cells secreting IFN-γ significantly increased on day 4 post-MWA (Figure 3).

Figure 4 Dynamic changes in Treg cells, Klrg1⁺ Treg cells and NK cells in the TdLN post-MWA. (A) The percentage of defined cells in the TdLN, as illustrated in flow cytometry plots. (B) Statistical results for A (*P < 0.05, **P < 0.01, ***P < 0.001, ns indicates no significant difference).
**DCs**

The frequencies of DCs as well as subsets of DCs were determined, such as plasmacytoid DCs (pDCs), classic DCs (cDCs), resident cDCs, and migratory cDCs. The results indicated that there were no significant changes in the frequencies of pDCs, cDCs, migratory cDCs, and resident cDC1 post-MWA (Figure 5); however, the frequency of resident cDC2 significantly increased on day 4. On day 1, the frequency of CD11b⁺ migratory cDCs increased, whereas that of CD11b⁻ migratory cDCs decreased (Figure 5).

**Other Immune Cells**

Other immune cells, including B cells, neutrophils and monocytes were also examined. There were no significant changes in the frequency of B cells and monocytes at different time points in the TdLN (Figure 5). Furthermore, compared to pre-MWA, the frequency of neutrophils in the TdLN significantly decreased from day 1 to day 8 post-MWA (Figure 5).

**Discussion**

To elucidate the dynamic changes in the immune microenvironment in TdLN after MWA in lung cancer, we established an LLC mouse model to examine the alterations in immune cells in TdLN at different time points after MWA. The immune response includes innate and adaptive immunity. Innate immunity is a primary defensive immunity induced within 96 h of exposure to antigens, whereas adaptive immunity is mediated by T cells and B cells, which are usually induced 4 to 7 days later. Therefore, we selected days 1, 2, 4, and 8 post-MWA as the time points. The immune cell types assessed included lymphoid immune cells (T cells, B cells and NK cells) and myeloid immune cells (DCs, neutrophils and monocytes).

CD4⁺ T cells play an important role in the adaptive immune response. Recent data indicate that they can not only regulate and coordinate the function of other immune cells, but also directly kill tumor cells, thus making a significant contribution to tumor immunity.⁴⁰,⁴¹ We found that the frequency of CD4⁺ T cells significantly increased from day 1 to day 8 post-MWA, reaching its peak on day 2. During the “afferent phase” of the immune response, many naive T cells in the TdLN, which have highly specialized structures, recognize homologous antigens presented by antigen-presenting cells (APCs), and CD4⁺ T cells are primed.⁴² After activation, CD4⁺ T cells undergo clonal expansion and differentiate into CD4⁺ helper T (Th) cell subsets. Following MWA, the frequency of CD4⁺ T cells significantly increased, which occurred as early as the first day after treatment. This suggests the initiation of an adaptive immune response and the activation of CD4⁺ T cells occurs early after antigen recognition. IFN-γ and IL-4 are the primary cytokines secreted by CD4⁺ Th1 and Th2 cells, respectively.⁴³ In this study, despite an increase in the frequency of CD4⁺ T cells post-MWA, there was no significant change in the frequency of CD4⁺ T cells secreting cytokines, which may be related to the limited activation of the CD4⁺ T cells subpopulation.

Consistent with memory CD8⁺ T cells, memory CD4⁺ T cells were categorized as TCM and TEM cells. Upon reactivation, CD4⁺ TEM cells rapidly secrete effector cytokines and promote a secondary response to re-infection. CD4⁺ TCM cells exhibit higher proliferative activity and serve as a reservoir for CD4⁺ TEM cells, with less potential for rapid secretion of IFN-γ or IL-4.⁴⁴ In the present study, the frequency of CD4⁺ TEM cells was significantly decreased in the TdLN from day 1 to day 8 post-MWA; however, the frequency of CD4⁺ TCM cells showed no significant changes. These results may be attributed to the rapid response of CD4⁺ TEM cells in the TdLN to the release of tumor antigens. We observed the immune response within 8 days post-MWA and the CD4⁺ TCM response was slower; therefore, we failed to detect its changes.

Most notably, we found that the frequencies of Treg cells and Klrg1⁺ Treg cells decreased in the TdLN from day 1 to day 4 following MWA. Treg cells are a type of T cell subset with significant immunosuppressive functions. They are characterized by the expression of Foxp3, CD25, and CD4. Tregs accumulate in the TdLN and their number increases as tumor burden increases.⁴⁵ Klrg1⁺ Treg cells have stronger immunosuppressive functions compared with Klrg1⁻ Treg cells.⁴⁶ Therefore, reducing the number of Tregs or inhibiting their function can effectively reduce their immunosuppressive effects. The decrease in the frequency of Treg cells in the TdLN following MVA suggests that the adaptive
Figure 5 Dynamic changes in DCs, B cells, neutrophils and monocytes in the TdLN post-MWA. (A) The percentage of defined cells in the TdLN, as illustrated in flow cytometry plots. (B) Statistical results for A (*P < 0.05, **P < 0.01, ***P < 0.001, ns indicates no significant difference).
immune response is activated, and the immunosuppressive effect is weakened. This phenomenon has also been observed in clinical patients. Zhang et al. discovered that one month after MWA for lung cancer, the proportion of Treg cells in the peripheral blood of patients decreased.

DCs are professional APCs that can present antigens and subsequently activate an immune response. We found significant increases in the frequency of CD11b+ migratory cDCs on day 1 post-MWA. CD11b+ migratory cDCs are necessary for the induction of T follicular helper (Tfh) cells, a subset of CD4+ T cells. They can transport antigens to the T-B border of the lymph node where Tfh cells priming occurs. The spatial distribution of cDC subsets within the draining lymph nodes affects immune induction. Resident cDC2 cells are located in the peripheral area of the draining lymph nodes, whereas cDC1 cells are located in the center. This enables resident cDC2 to acquire antigens more efficiently compared with cDC1. In the present study, the increased frequency of resident cDC2, but not cDC1, on day 4 post-MWA in the TdLN is fully consistent with this theory.

NK cells are a unique group of innate immune lymphocytes with natural cytotoxicity and immunoregulatory abilities. NK cells not only serve as direct effector cells in the antitumor response, but also play an important role in assisting antitumor T cell responses and T cell-based checkpoint immunotherapy. We found that the frequency of NK cells in the TdLN gradually increased from day 1 to day 4 followed by a decrease on day 8. On day 4, the proportion of NK cells secreting IFN-γ has increased. This corresponds with the timing of the innate immune response. In a mouse model of breast cancer, Yu et al. found that the frequencies of splenic NK cells significantly increased 7 days post-MWA and were maintained up to 14 days post-MWA. Although our results were not entirely consistent, both studies confirmed that MWA activates NK cells. Additionally, we found that on day 1 post-MWA, the frequency of neutrophils in the in the TdLN decreased significantly. We hypothesize that the body promptly activates the innate immune response, recruiting neutrophils to the site of tumor ablation.

Thermal ablation fragments cancer cells, triggering the release of antigens and prompting a remodeled tumor immune microenvironment that activates the antitumor immune response. Therefore, we inferred that dynamic changes in the immune microenvironment in the TdLN described above may be attributed to the release of tumor antigens induced by MWA. Theoretically, intratumoral migratory cDCs can recognize these released tumor antigens and migrate to the TdLN, where migratory cDCs activate CD4+ T cells to express CD40L, CD40L+CD4+ T cells license cDCs to fully activate CD8+ T cells. Our findings indicate a significant increase in the frequency of CD11b+ migratory cDCs, resident cDC2, and CD4+ T cells. The above theory effectively corroborates these observations. Additionally, the decrease in Treg cells proportion and the increase in NK cells proportion are plausible dynamics that arise from the interplay between these two cell types. Treg cells are known to exert inhibitory effects on NK cells, especially impairing the effector function of NK cells. A reduction in Treg cells diminishes their inhibitory effect on NK cells. Although we did not observe an increase in the proportion of CD8+ T cells, we found that the proportion of CD8+ T cells secreting IFN-γ and TNF-α increased, indicating that the function of CD8+ T cells may be enhanced. Furthermore, we found that the frequency of immune cells in the TdLN often changes within 4 days post-MWA. This alteration may be linked to the peak period of tumor antigen release.

Clinical research and practice have demonstrated that MWA combined with immunotherapy, such as anti-PD-1/PD-L1, anti-CTLA-4, has shown significant efficacy in the treatment of lung cancer. However, its mechanism is not yet clear. Our study confirms that MWA can induce immune response, alter the immune microenvironment in the TdLN. Therefore, the combination of MWA and immunotherapy may have a synergistic effect. It is worth noting that we detected more significant early immune changes post-MWA. Therefore, we speculate that selecting the appropriate timing for MWA combined with immunotherapy is crucial for enhancing immune efficacy.

Although we established some meaningful results, there are still some limitations to this study. First, only a fraction of immune cell types and immune indicators were examined, thus it is possible that other important immune cells and indicators are not involved. Second, we only focused on the short-term effects of MWA on the immune system, and future studies are needed to examine the long-term immune effects. Third, the method used for measuring immune cells was frequency, which may affect the accurate evaluation of immune cell changes. For future studies, a larger sample size, more optimal animal models, such as lung orthotopic implantation models and more reliable absolute counting methods should be used. We will also evaluate the impact of MWA on tumor-specific immune responses, including the number...
and function of tumor-specific T cells, and how MWA stimulates or inhibits tumor-specific immune reactions. In addition, the potential of MWA alone or combined with other therapies may be assessed by comparing it with surgery, radiation therapy, or chemotherapy.

**Conclusion**

MWA of lung cancer can alter the immune microenvironment in the TdLN, triggering an immune response, and the changes are particularly evident and intricate within the initial 4 days post-MWA. Treatment combined with MWA within a certain period of time may enhance anti-tumor immunity. These findings have implications for the optimization of treatment strategies based on MWA and the development of immunotherapies targeting the dynamic immune response post-MWA.

**Abbreviations**

MWA, microwave ablation; Treg cells, regulatory T cells; NK cells, natural killer cells; DCs, dendritic cells; TCM cells, central memory T cells; TEM, effector memory T cells; APCs, antigen-presenting cells; Tfh cells, T follicular helper cells; IFN-γ, interferon-gamma; IL-4, interleukin 4; TNF-α, tumor necrosis factor-α.

**Data Sharing Statement**

The authors confirm that the data supporting the findings of this study are available from the corresponding author on reasonable request.

**Ethics Statement**

This study was reviewed and approved by the Animal Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (NSFC: No. 2020-497).

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**Disclosure**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered a potential conflict of interest.

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