IL-9 stimulates an anti-tumor immune response and facilitates immune checkpoint blockade in the CMT167 mouse model

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Abstract

There is mounting evidence that interleukin-9 (IL-9) is associated with cancer although its function in lung cancer remains elusive. This study aimed to elucidate the role of IL-9 in lung cancer and the mechanisms involved. We report the detection of interleukin-9 receptor (IL-9R) expression in CMT167 cells, but not in Lewis lung carcinoma (LLC) cells. LLC or CMT167 cells were inoculated in wildtype C57BL/6J immunocompetent mice. Tumor-bearing mice were randomized on day 3 post-inoculation (8 mice per group) to receive intraperitoneal treatment with murine recombinant IL-9 (50 ng/mouse on alternate days) ± anti-programmed cell death protein 1 (anti-PD-1, 10 mg/kg, every three days) or control until reaching humane endpoints whereby tumors were harvested. In LLC tumor-bearing mice, neither tumor growth nor intratumoral T cells were affected by IL-9 treatment. Nonetheless IL-9 decreased CMT167 tumor growth and enhanced anti-tumor T cell responses, both of which were absent in IL-9R knockdown CMT167 tumors. CD8+ T cells were identified as the key effector driving IL-9-induced tumor suppression in the CMT167 model. Increased dendritic cell population and MHC-I expression were observed in IL-9-treated CMT167 tumors. Simultaneously, PD-1 and programmed death ligand 1 (PD-L1) expression by CD8+ T cells and CMT167 cells was upregulated. The combination of IL-9 and anti-PD-1 antibody synergistically suppressed CMT167 tumor and enhanced tumor-infiltrating proportion of CD8+ T cells. Taken together, our study determined the role of IL-9 in anti-tumor immunity and proposes IL-9 as a promising adjuvant to immune checkpoint blockade in lung cancer.

Introduction

Lung cancer remains the leading cause of cancer mortality (1). Immune checkpoint blockade (ICB) has enabled great progress in cancer treatment over the past decades, of which programmed cell death protein 1 (PD-1)/programmed cell death protein ligand 1 (PD-L1) antibody is one of the most important (2). Although PD-1/PD-L1 inhibitors have been approved for clinical use, only a small percentage of patients may benefit (3), prompting the development of novel therapeutic targets and combined techniques to broaden the population who may benefit.

Interleukin-9 (IL-9) was initially described as a T cell growth factor and has pleiotropic functions in the immune system (4). Various immune cells such as mucosal mast cells, group 2 innate lymphoid cells (ILC2s), type 2 helper T (Th2) cells, regulatory T (Treg) cells, T follicular helper cells, and a part of memory B cells have been found to release IL-9 (5). IL-9-producing CD4+ T cells (Th9 cells), which are generated in vitro in the presence of TGF-β and interleukin-4 (IL-4), have attracted attention recently as another important cell source of IL-9 (6). Activities of IL-9 are mediated by a heterodimeric receptor that comprises a specific chain (IL-9Rα) and a common gamma chain (7). IL-9 is involved in inflammatory diseases by altering the functions of various interleukin-9 receptor (IL-9R)-expressing cells, such as promoting mast cell accumulation and activation (8), enhancing Treg cell survival (9), upregulating IL-5 and IL-13 in ILC2s (10), supporting B cell proliferation (11), facilitating airway epithelial cell mucus secretion (12), and boosting secretion of neutrophil-recruiting chemokines by airway smooth muscle cells (13).
In tumor biology, IL-9 plays two seemingly opposing roles. In hematologic tumors such as myeloid leukemia and lymphoma (14, 15), IL-9 plays a tumor promotion role by promoting the proliferation and survival of cancer cells. In models of breast and colon cancer, IL-9 inhibits adaptive immunity, preventing the establishment of immunologic memory in a developing tumor (16). Another study suggested that Th9 cells can boost hepatocellular carcinoma cell proliferation by promoting STAT3 activation and tumor cell synthesis of CCL20, which promotes tumor cell migration and tumor growth (17). Nonetheless IL-9 plays the opposite role in melanoma: it inhibits subcutaneous growth and metastasis in murine B16 melanoma (18, 19). IL-9 has been determined to act directly on the HTB-72 melanoma cell line, limiting cell growth and triggering apoptosis (20). IL-9 induced by DTA-1, which is an agonist of glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR), potentiates tumor-specific CD8+ T cell responses by enhancing dendritic cell (DCs) cross-presentation of tumor antigens (21). IL-9-secreting Th9 cells stimulate CCL20/CCR6-dependent DC recruitment, eliciting strong anticancer CD8+ T cell responses (18). Moreover, in another subcutaneous B16 model, IL-9 suppressed melanoma development through mast cell activation rather than through T or B cells (19).

In non-small cell lung carcinoma (NSCLC), the relevance of IL-9 is debatable. One clinical investigation found that patients with greater frequencies of IL-9-secreting Th9 cells had better recurrence-free survival (22), while another indicated that NSCLC patients with an increase in IL-9 levels after surgery had a shorter overall survival (23). In line with this observation, both pro- and anti-tumor effects of IL-9 have been reported in preclinical investigations. IL-9 facilitates the proliferation and migration of human lung cancer cell lines (24). Similarly, IL-9-secreting Th9 cells promote subcutaneous growth and metastasis of lung cancer by augmenting epithelial-mesenchymal-transition (25). Moreover, angiogenesis is promoted by IL-9 to facilitate tumor growth in a murine Lewis lung carcinoma (LLC) model (23). On the contrary, it also shows a tumor inhibitory function in an LLC model. IL-9-secreting Th9 cells increase the infiltration of tumor-specific IFN-γ-producing CD8+ T cells leading to suppression of LLC-OVA tumor growth (22). In another study, recombinant IL-9 suppressed LLC tumor growth through the activation of mast cells (19). To summarize, further research is needed to fully understand the role of IL-9 in lung cancer.

IL-9 is important in tumor biology and has pleiotropic effects, likely in a context- and tissue-specific manner, on multiple malignant diseases including NSCLC. In this study, we investigated the function of IL-9 utilizing two murine NSCLC models: LLC cells, which do not express IL-9R, and IL-9R-expressing CMT167 cells. In CMT167 cells, IL-9/IL-9R signaling increased MHC-I surface expression, enhanced CD8+ T cell responses, and ultimately led to tumor regression. LLC cells, on the contrary, showed a lack of direct IL-9 responses due to the absence of IL-9R. Furthermore, IL-9 rendered CMT167 tumors significantly more responsive to anti-PD-1 therapy. Our findings provide for the possibility that IL-9 can serve as an adjuvant immunotherapy in NSCLC.

Materials And Methods

Cell culture
LLC and CMT167 cells were purchased from the American Tissue Type Cell Collection (ATCC) and European Collection of Authenticated Cell Cultures (ECACC) respectively and maintained in DMEM supplemented with 10% FBS (Thermo Fisher Scientific) and GLUTAMAX I (Sigma-Aldrich). Cells were used within 15 passages after purchase without further cell line authentication. Cells were grown in a humidified incubator with 5% CO₂.

**The cell viability assay**

NSCLC cells were seeded in 96-well plates at a concentration of 2.5 x 10³/well. After treatment, 20 μl MTT solution (0.25 mg/mL) was added. DMSO was added to dissolve the purple crystals. The absorbance value at 570nm wavelength was quantified by a microplate reader (CLARIOstar®).

**Tumor-bearing mice with IL-9 and/or anti-PD1 treatment**

Female 6 to 8-week old (15-20 g) C57BL/6J mice were used for allograft experiments. All animal procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 5077-19). LLC or CMT167 cells (5 x 10⁵ per mouse) were subcutaneously inoculated into the right flank of mice. Tumor growth was monitored according to the formula: volume = (length x width x height)/2. Mice were randomized and intraperitoneally (i.p.) injected with IL-9 (50 ng per mouse, R&D) or PBS as control every other day when tumor size reached 50 mm³. For CD4⁺ or CD8⁺ T cell depletion study, mice were i.p. injected with anti-CD8b or anti-CD4 antibody, respectively. Equal amounts of isotype were given as a control. For immune checkpoint blockade experiments, anti-PD-1 antibody or isotype control was i.p. injected into the mice. *In vivo* antibodies used are listed in Supplementary Table 1. The endpoint was defined as the time when ulceration appeared, or tumor length reached 1.5 cm in its longest dimension.

**Flow cytometry**

For cell line analysis, collected cells were stained with appropriate antibodies for 30 minutes at 4°C in the dark before being analyzed. For *in vivo* studies, tumors were mechanically minced with scissors and digested in DMEM containing 2 mg/ml collagenase type IV (Gibco) and 10 unit/ml DNase I (Sigma-Aldrich) for 1 h at 37 °C. Tissue dissociates were passed through a 40 μm nylon mesh strainer (Corning) to collect a single cell suspension. LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) was used to discriminate between viable and dead cells. After incubation with appropriate antibodies (Supplementary Table 2) at 4 °C for 30 min in the dark, cells were analyzed on a Beckman Cytoflex. FlowJo software (BD Biosciences) was used to analyze flow cytometry data. Gating strategies are presented and described in Supplementary Fig. 1 and Supplementary Table 3.

**Lentivirus transduction**

Lentivirus carrying IL-9R shRNA plasmid was produced by co-transfecting HEK293T cells with the lentiviral vector and the packaging plasmid mix at a 1:1 ratio. Lentivirus supernatant collected at 48 h
was used directly to infect CMT167 cells, followed by selection with 5 μg/ml puromycin. Murine IL-9R shRNA vectors (PLKO.1) were obtained from Santa Cruz Biotechnology, and the other reagents used were purchased from Sigma-Aldrich.

**Real-time quantitative PCR (RT-qPCR)**

RT-qPCR was performed using TB green (TaKaRa, RR820A). Primer sequences are listed in Supplementary Table 4. PCR amplification and detection were performed on the Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Results were standardized to GAPDH and are shown as fold changes in gene expression in IL-9-treated CMT167 tumor compared with the control.

**Western blot analysis**

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (7.5-15%) and transferred onto a polyvinylidene difluoride membrane (GE Healthcare). The membranes were blocked for 1 hour at room temperature with 5% non-fat dry milk and incubated with specific primary antibodies (Supplementary Table 5) at 4°C overnight, followed by washing and further incubation with HRP conjugated secondary antibodies (Cell Signaling Technology). Bands were visualized using an enhanced chemiluminescence (ECL) kit (GE Healthcare). Images were captured by ChemiDocTM MP Imaging System (Bio-Rad).

**Statistical analyses**

Statistical analysis was performed using GraphPad Prism 8 software. Data are expressed as mean ± SEM and p values <0.05 were considered statistically significant. Comparisons between two groups were performed using an unpaired Student’s t-test. Multiple t-tests were utilized to analyze tumor growth delay at different time points.

**Results**

**IL-9 suppressed lung cancer growth in CMT167 but not in LLC tumor-bearing mice**

IL-9 acts on a variety of cells by interacting with IL-9R (4). To investigate the role of IL-9 in lung cancer, we first detected IL-9R expression in LLC cells, as well as another murine lung cancer cell line-CMT167 cells, a highly metastatic subline derived from CMT64 cells (26). Splenocytes from C57BL/6 mice were employed as a positive control. As shown in Fig. 1 A, IL-9R was expressed in CMT167 cells, but not in LLC cells. IL-9 has been shown to cause cytotoxicity in melanoma cells (20). Nonetheless neither LLC nor CMT167 cell viability was affected by IL-9 treatment in vitro (Fig. 1 B-C). To assess the effects of IL-9 on tumor growth in vivo, LLC or CMT167 cells were subcutaneously injected into the right flank of syngeneic C57BL/6 mice (Fig. 1 D). Smaller tumors were formed in IL-9-treated CMT167 tumor-bearing mice (Fig. 1 E-G), while IL-9 treatment did not alter the growth rate of LLC tumors (Fig. 1 H-J),

**IL-9 enhanced anti-tumor T cell response in CMT167 tumor**
To test the impact of IL-9 on anti-tumor immunity *in vivo*, we quantified T lymphocytes in IL-9-treated LLC and CMT167 tumors or control. Flow cytometry analyses confirmed significant increases in the proportion of intratumoral total CD3⁺ T cells (Fig. 2 A), CD4⁺ T helper (Th) cells (Fig. 2 B), and CD8⁺ cytotoxic T cells (Fig. 2 C) in IL-9-treated CMT167 tumors, while none was increased by IL-9 in the splenocytes of CMT167 tumor-bearing mice (Fig. 2 D-F). However, in LLC mouse model, no significant increase in the numbers of CD4⁺ or CD8⁺ T cells in the tumors or splenocytes was observed (Fig. 2 G-L). These findings indicate that IL-9 might suppress CMT167 tumor growth by promoting T cell responses, and the responses to IL-9 therapy existed locally inside the tumor microenvironment.

**IL-9/IL-9R interaction in CMT167 cells is critically involved in the anti-tumor immune responses triggered by IL-9 treatment**

IL-9 treatment attenuated the growth of IL-9R-expressing CMT167 tumor but had no effect on tumor growth in mice harboring LLC cells without IL-9R expression. To assess the involvement of IL-9/IL-9R interaction with CMT167 cells in the anti-cancer effect of IL-9 *in vivo*, subcutaneous mouse models were established with CMT167 cells carrying IL-9R shRNA (shIL-9R) and its scramble control (shCTL). Only around 5% of CMT167 cells expressed IL-9R following transduction (Fig. 3 A). IL-9R knockdown in CMT167 cells abolished tumor growth delay (Fig. 3 B-D) and reversed the antitumor T cell response triggered by IL-9 therapy (Fig. 3 E-G). These observations confirmed that tumor inhibition of IL-9 in CMT167 harboring mice is mediated via its effect on cancer cells.

**CD8⁺ T lymphocytes drive IL-9-induced tumor suppression in CMT167 tumor-bearing mice**

To determine the relative importance of different T cell subsets in regulating the growth of IL-9-treated CMT167 tumors, antibody-mediated CD4⁺ and CD8⁺ T cell depletion was performed as indicated in Fig. 4 A. Depletion efficacy was confirmed by analyzing T cells in peripheral blood using flow cytometry (Fig. 4 B). IL-9-mediated tumor suppression was completely abolished in the absence of CD8⁺ T cells (Fig. 4 F-H), while little or no effects were observed upon CD4⁺ T cell depletion (Fig. 4 C-E), confirming the role of CD8⁺ T cells as the main effector of IL-9-induced tumor suppression in the CMT167 model.

Mechanism(s) underlying the enhanced CD8⁺ T cell-mediated tumor suppression in IL-9-treated CMT167 tumors was then investigated. The expression of CD25 and CD69 was examined to measure the activation of CD8⁺ T cells. Neither revealed a significant change when IL-9 was given to CMT167 tumor-bearing mice (Supplementary Fig. 2). Moreover, T cell recruitment chemokines, CXCL9, CXCL10, and CXCL11 were not upregulated at the transcript level following IL-9 treatment (Supplementary Fig. 2). DCs, especially conventional type 1 dendritic cells (cDC1), were able to attract T cells, and promote the activation and proliferation of tumor-specific CD8⁺ T cells (27). In IL-9-treated CMT167 tumors, an increase in both total CD11c⁺ MHC-II⁺ DCs and CD103⁺ cDC1 was observed (Fig. 4 I-K), and these increased proportions could be reversed by knocking down IL-9R in CMT167 cells (Fig. 4 L-N). All these results indicate that the anti-tumor effects of IL-9 are mediated by CD8⁺ T cells, and DCs are involved in this process.
IL-9 promotes MHC-I expression in CMT167 cells *ex vivo* and *in vitro*

Defects in MHC-1 enable immune evasion and are commonly exhibited in solid tumors (28). To investigate the role of IL-9 in tumor immunogenicity, we examined MHC-I expression in CMT167 tumors treated with IL-9. CMT167 tumor harvested *ex vivo* displayed increased expression of MHC class I components H-2K, H-2D, and B2m at the transcription level, as well as an elevated protein level of H-2Kb upon IL-9 treatment (Fig. 5 A-B). Meanwhile, IL-9-mediated induction of H-2Kb was absent in IL-9R knockdown CMT167 tumors (Fig. 5 C). *In vitro*, H-2Kb surface expression was significantly enhanced on the IFN-γ-stimulated CMT167 cells in response to IL-9-treatment (Fig. 5 D). Next, we studied the downstream signaling proteins related to IL-9/IL-9R interaction in CMT167 cells, including STAT1, Akt, and ERK1/2. IL-9 induced the phosphorylation of ERK1/2 in CMT167 cells with or without stimulation by IFN-γ but did not activate the STAT1 or Akt pathways (Fig. 5 E-F). Overall, our findings suggest that IL-9 upregulates MHC-I surface expression on CMT167 cells through the activated ERK1/2 pathway.

**IL-9 treatment synergizes with PD-1 blockade**

Previously, others have reported increased expression of PD-1 on IL-9-secreting tumor-infiltrating lymphocytes (27), suggesting the potential synergism through combination of IL-9 and PD-1/PD-L1 blockade. We examined whether the expression of PD-1 could also be induced by IL-9 treatment in CMT167 xenografts. Indeed, the expression of PD-1 on tumor-infiltrating CD8+ T cells and PD-L1 expression on the intratumoral non-hematopoietic stromal cells was significantly increased by IL-9 (Fig. 6 A-B). We next investigated whether IL-9 could synergize with immune checkpoint blockade. Anti-PD-1 antibody and IL-9 were given to the established syngeneic tumors as indicated in Fig. 6 C. Consistent with previous studies (28), CMT167 tumors could respond to anti-PD-1 monotherapy. Importantly, the anti-PD1 antibody synergized with IL-9 in the CMT167 model in suppressing tumor growth (Fig. 6 D-G). Moreover, the combination of IL-9 and anti-PD-1 treatment further increased CD8+ T cell frequencies in CMT167 tumors compared with monotherapies (Fig. 6 H). These data indicate that IL-9 synergizes with anti-PD-1 in the CMT167 model.

**Discussion**

IL-9 is shown by a growing body of data to be linked to lung cancer. Published findings are nonetheless incongruent (22, 23). In this study, LLC and CMT167 mouse models were utilized to explore the role of IL-9 in lung cancer. IL-9 inhibited tumor growth and enhanced anti-tumor CD8+ T cell immune responses in mice harboring IL-9R-expressing CMT167 cells, but no effect was evident in mice bearing LLC cells that lacked IL-9R expression. IL-9 therapy caused increased tumor-infiltrating DCs and elevated MHC-I surface expression in CMT167 tumors, which might explain the tumor suppression mediated by CD8+ T cells. Moreover, IL-9 could synergize with anti-PD-1 in CMT167 tumors.

This study is the first to discover IL-9R expression in CMT167 cells. LLC cells, on the contrary, did not express IL-9R, consistent with previous research (19). Despite the significance of IL-9R expression in
tumor suppression, results in this study indicated that IL-9 signaling is not directly linked to either apoptosis or proliferation of the two murine lung cancer cell lines. This observation differs to previous reports that showed IL-9 could promote the proliferation and migratory activity of human lung cancer cell A549 (24). Likely, such discrepancies are due to the different cell lines being tested in our study versus others. Indeed, in melanoma, variations have been reported in different cell lines. Growth suppression was observed only in HTB-72 and SK-Mel melanoma cells upon direct IL-9 exposure, while no responses were identified in HTB-65 and CRL-11147 cells (20). Nonetheless critical involvement of IL-9 through signaling upon IL-9R binding has been illustrated in in vivo models and knockdown experiments. In vivo, IL-9 inhibited the growth of IL-9R-expressing CMT167 tumors but not LLC tumors. Importantly, significant upregulation of CD4+ and CD8+ T lymphocyte frequencies were observed in IL-9-treated CMT167 tumors, indicating the ability of IL-9 to induce T cell immunity. In contrast, in the LLC model, neither tumors nor splenocytes showed significant T cell alteration upon IL-9 treatment. The unresponsiveness of LLC tumors to IL-9 in this study may be different to previous investigations although reports are inconsistent (19, 22, 23, 25). We believe such disparities could mainly arise from the experimental design. Some studies focused on the role of Th9 cells in LLC tumor-bearing mice instead of IL-9 (22, 25). Th9 cells not only secrete IL-9 but also other cytokines including IL-21 and IL-10 (29, 30), which could also directly contribute to inhibition of tumor growth and metastasis (29). As a result, compared with IL-9 injection, the adoptive transfer of Th9 cells into LLC-bearing animals may lead to more complicated outcomes. Furthermore, concomitant administration of IL-9 (19) or direct inoculation of IL-9 transfected LLC cells (23) would not be able to specifically delineate existing versus therapeutic function of IL-9 during LLC tumor development. In this study, IL-9 therapy was initiated only after the full establishment of tumor mass at a palpable size. We believe this study design is more clinically relevant and translatable, and the therapeutic potential of IL-9 could be specifically addressed.

Augmented sensitivity of CMT167 to IL-9-mediated immunotherapy could be due to the intrinsic characteristics of cancer cells (IL-9R positive versus IL-9R negative) as well as extrinsic contributions from the immune compartments in the tumor microenvironment (28). Again, results in this study highlighted the importance of IL-9R and thus IL-9 signaling in triggering anti-cancer immunity since blocking the IL-9/IL-9R axis by IL-9R knockdown abrogated tumor inhibition and anti-tumor T cell responses in CMT167 cells. Although both CD4+ and CD8+ T cells were elevated in the CMT167 tumor, CD8+ T cells were demonstrated to mediate growth control of the CMT167 tumor by IL-9 therapy. We further explored the molecular underpinnings of increased intratumoral CD8+ T cell responses. The T cell activation markers, CD69 and CD25, exhibited no change on the CD8+ T cells from IL-9-treated and control CMT167 tumors, indicating that a major effect of IL-9 is to enhance CD8+ T cell infiltration or proliferation rather than boosting its activation. CXCR3 ligand (CXCL9/10/11) are crucial chemokines that recruit T lymphocytes into tumors (31), and IL-9 increased CXCL9 expression in primary astrocytes during experimental autoimmune encephalomyelitis (32). Nonetheless neither CXCL9, CXCL10, nor CXCL11 exhibited any noticeable alteration in CMT167 tumors treated with IL-9. Instead, IL-9 increased the proportion of DCs, particularly cDC1, in CMT167 tumors, and played pivotal roles in CD8+ T cell priming (27) and recruitment into tumors (33). In line with our study, IL-9-secreting Th9 cells have been
shown to elicit strong anti-tumor cytotoxic T cell responses in a lung metastatic melanoma model by upregulating the recruitment of DCs in a Ccl20/Ccr6-dependent manner (18). Whether the increased intratumoral CD8⁺ T cell population is also a direct consequence of enhanced DC recruitment in an IL-9-treated CMT167 tumor warrants further investigation.

Th9 cells were discovered to boost MHC-I expression on cervical cancer cells (34).

Impaired antigen presentation induced by mutations or loss of heterozygosity of MHC-I are common mechanisms of immune evasion by solid tumors (28, 35). Our study appears to be the first to demonstrate enhanced MHC-I expression on CMT167 cells in response to IL-9 therapy. Blocking the IL-9/IL-9R axis in CMT167 cells eliminated MHC-I upregulation by IL-9, demonstrating that MHC-I elevation was induced directly by IL-9. CMT167 cells had negligible basal MHC-I surface expression in vitro but were dramatically induced by IFN-γ and further augmented upon IL-9 stimulation, indicating enhanced tumor immunogenicity induced by IL-9. IL-9 has been reported to activate Jak-STAT, ERK/MAPK (36, 37), and PI3K/Akt pathways (38). In this study, phosphorylation of ERK1/2 was elevated by IL-9 in CMT167 cells, suggesting that IL-9 may upregulate MHC-I expression by activating the ERK1/2 pathway. This is in line with a recent study showing that upregulated ERK pathway activation increased MHC-I expression in NSCLC cells (36).

Previous studies have shown that preexisting CD8⁺ T cells correlate positively with the success of ICB (39). Besides, enhancing MHC-I levels in cancer cells is a promising strategy to improve ICB efficacy (40). In addition to the enhanced CD8⁺ T cell proportion and increased MHC-I surface expression, IL-9 has been observed to upregulate PD-1 expression on CD8⁺ T cells and PD-L1 expression on cancer cells in a CMT167 tumor. The interaction between PD-L1 and PD-1 leads to T cell dysfunction and exhaustion (41), and PD-L1 expression was shown to be positively correlated with ICB responses (42), leading us to hypothesize that IL-9 may synergize with anti-PD-1 in CMT167 tumor-bearing mice. We show here that the combination treatment indeed significantly delayed tumor growth and enhanced the proportion of CD8⁺ T cells in CMT167 tumors, indicating a synergistic effect of IL-9 and anti-PD-1 in mice bearing CMT167 tumors. Given that only a small population of NSCLC patients could potentially benefit from anti-PD-1 ICB, results from this study provide experimental evidence for the use of IL-9 as an adjuvant therapy to PD-1 blockade.

Our study has limitations that future studies should address. First, we based our findings on murine lung cancer cell lines and subcutaneous models that may not fully represent the situation in human lung cancer patients. Before proceeding to clinical trials with IL-9, more research is needed on human lung cancer cell lines and/or humanized in vivo models. Second, we focused on MHC-I due to its direct interaction and indispensable role in mounting cytotoxic T cell immune responses in tumors. It is worth noting that our findings may aid in the treatment of malignancies only in which MHC-I is repressed by gene regulatory mechanisms, not in those whose MHC-I components are genetically deleted or HLA genes are lost due to heterozygosity. Other mechanisms that may also contribute to the enhanced CD8⁺ T cell responses, such as DC presentation activity and clonal expansion, need to be further studied. Third,
increases in other immune cells such as CD4+ T cells and cDC1 were also observed upon IL-9 therapy. Future research should address the question of how these changes to other immune cell types are regulated and their potential contributions(s) to anti-cancer activities of IL-9 in CMT167 tumor. For instance, the possible involvement of cytokines and chemokines derived from both cancer cells and these immune cells in response to IL-9 therapy needs to be analysed. Finally, IL-9 is widely involved in immune diseases such as allergic asthma (43), thrombotic diseases (44, 45), and arthritis (38). Strategic and careful design of dose escalation and control of side effects are crucial if we intend to treat lung cancer patients with IL-9.

In summary, our findings indicate that IL-9 plays an important role in regulating cell-surface MHC-I expression and thereby promotes intratumoral CD8+ T cell infiltration or proliferation in CMT167 tumors. Our study is mechanistically new and has great translational potential. It suggests that IL-9 treatment can potentially be utilized in MHC-I-low lung cancer as an adjuvant to ICB therapy. In addition, IL-9R may be able to predict NSCLC patients' susceptibility to IL-9 therapy and ICB responses based on differences in IL-9R expression and responses in mice harboring LLC and CMT167 cells.

Declarations

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Data Availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval: All animal procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 5077-19).

References


**Figure 1**

**IL-9 suppresses lung cancer growth in CMT167 but not in LLC tumor-bearing mice.** (A) IL-9 receptor (IL-9R) was detected in CMT167 and Lewis lung carcinoma (LLC) cells by Western blot. Splenocytes (S) from C57BL/6 mice served as a positive control. (B-C) LLC cells and CMT167 cells were treated with different doses of IL-9 (ng/ml) and the optical density (OD) was measured. (D) CMT167 and LLC cells were inoculated intravenously into C57BL/6 mice. The tumor growth curve was monitored for 18 days. (E) Tumor volume was measured every 2 days in CMT167 cells treated with IL-9. (F) Control and IL-9-treated CMT167 cells showed similar tumor size. (G) Tumor weight was measured at 18 days post-inoculation. (H) LLC cells were treated with IL-9 and the tumor volume was monitored for 20 days. (I) Control and IL-9-treated LLC cells showed no difference in tumor size. (J) Tumor weight was measured at 20 days post-inoculation.

IL-9 receptor (IL-9R) was detected in CMT167 and Lewis lung carcinoma (LLC) cells by Western blot. Splenocytes (S) from C57BL/6 mice served as a positive control. (B-C) LLC cells and CMT167 cells were treated with different doses of IL-9 (ng/ml) and the optical density (OD) was measured. (D) CMT167 and LLC cells were inoculated intravenously into C57BL/6 mice. The tumor growth curve was monitored for 18 days. (E) Tumor volume was measured every 2 days in CMT167 cells treated with IL-9. (F) Control and IL-9-treated CMT167 cells showed similar tumor size. (G) Tumor weight was measured at 18 days post-inoculation. (H) LLC cells were treated with IL-9 and the tumor volume was monitored for 20 days. (I) Control and IL-9-treated LLC cells showed no difference in tumor size. (J) Tumor weight was measured at 20 days post-inoculation.
doses of IL-9 for 48 hours. Cell viability was measured using MTT assay and is presented as the absorbance value (OD value) at 570 nm. n = 6. (D) Schematic of experimental design. CMT167 or LLC cells (5 x 10^5 per mouse) were injected subcutaneously into C57BL6/J mice followed by IL-9 treatment as indicated. (E-G) Treatment of LLC tumor with IL-9 in mice. (E) Tumor growth curves from mice treated with IL-9 or PBS control for 2 weeks. Representative images (F), and weight (G) of LLC tumors retrieved from IL-9-treated and control mice sacrificed on day 18. n = 8 mice per group. (H-J) Mice bearing CMT167 tumor received IL-9 treatment. (H) Tumor growth curves from CMT167 tumor-bearing mice treated with IL-9 or PBS. Representative images (I), and weight (J) of CMT167 tumor harvested at the humane endpoint (day 15) by treatment group. n = 8. Data are shown as mean ± SEM. ns, not significant; *, p< 0.05. Two-tailed Student’s t-test, unpaired.
IL-9 enhances anti-tumor T cell response in CMT167 tumor. (A-C) Quantification of tumor-infiltrating CD3$^+$ total T cells, CD4$^+$ CD3$^+$ T cells, and CD8$^+$ CD3$^+$ T cells within CMT167 tumors by flow cytometry. (D-F) In the splenocytes of CMT167 tumor-bearing mice, CD3$^+$ total T cells, CD4$^+$ CD3$^+$ T cells, and CD8$^+$ CD3$^+$ T cell percentages were analyzed. (G-I) Tumor-infiltrating lymphocytes in LLC tumor, including CD3$^+$ total T
cells, CD4$^+$ CD3$^+$ T cells, and CD8$^+$ CD3$^+$ T cells, were analyzed by flow cytometry. (J-L) CD3$^+$ total T cells, CD4$^+$ CD3$^+$ T cells, and CD8$^+$ CD3$^+$ T cells in splenocytes of LLC tumor-bearing mice were analyzed by flow cytometry. Data are represented as mean ± SEM. n = 8. *, p < 0.05; **, p < 0.01; ns, not significant. Two-tailed Student’s t-test, unpaired.

Figure 3
CMT167 cells are involved in the anti-tumor immune responses triggered by IL-9 treatment. (A) CMT167 cells were transduced with lentivirus containing IL-9R shRNA to knock down IL-9R expression. Flow cytometry was used to measure IL-9R expression level in parental, scramble control (shCTL), and IL-9R knockdown (shIL-9R) CMT167 cells. Light grey, isotype staining control. (B-D) shCTL and shIL-9R CMT167 cells were inoculated into immunocompetent C57BL/6J mice at the right flank and treated with IL-9 or PBS. Tumor growth curves (B), representative image (C) and weight (D) of tumors harvested on day 13. (E-G) Tumor-infiltrating lymphocytes, including CD3+ total T cells, CD4+ CD3+ T cells, and CD8+ CD3+ T cells, were quantified by flow cytometry. Data are represented as mean ± SEM. n=8 mice in each group. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant. Two-tailed Student’s t-test, unpaired.
Figure 4

CD8+ T lymphocytes drive IL-9-induced tumor suppression in CMT167 tumor-bearing mice. (A) Schematic of experimental design. CMT167 cells were injected subcutaneously into C57BL6/J mice followed by anti-CD4/CD8 antibodies and IL-9 treatment as indicated. (B) Representative FACS images of CD4+CD3+ and CD8+CD3+ T lymphocytes in the peripheral blood from anti-CD4/CD8 antibody-treated mice. (C-E) CMT167 tumor-bearing mice were treated with anti-CD4 antibody and IL-9. Tumor growth curves (C),
representative image (D), and weight (E) of tumors harvested from mice at the humane endpoint. (F-H) CD8+ T cell depletion in CMT167 tumor-bearing mice treated with IL-9. Tumor growth curves (F), representative image (G), and weight (H) of the tumor. (I-K) Total dendritic cells (DCs) and conventional dendritic cell 1 (cDC1) in IL-9 or PBS control treated CMT167 tumors were analyzed by flow cytometry. Representative FACS image of DCs (I) and quantitative statistics of DCs (J) and cDC1 (K). (L-N) Total dendritic cells (DCs) and conventional dendritic cell 1 (cDC1) in IL-9R knockdown CMT167 tumors were analyzed by flow cytometry. Representative FACS image and quantitative statistics are shown. Data are represented as mean ± SEM. n = 8. *, p< 0.05; **, p< 0.01; ***, p < 0.001; ns, not significant. Two-tailed Student’s t-test, unpaired.
Figure 5

IL-9 promotes MHC-I expression in CMT167 cells \textit{ex vivo} and \textit{in vitro}. (A) Relative mRNA expression of H-2K, H-2D and B2m in control and IL-9-treated CMT167 tumor. n=8 tumors per group. (B) Expression of H-2Kb on the surface of subcutaneously grown CMT167 cells in control and IL-9-treated tumors by flow cytometry. n=8 tumors per group. (C) Representative FACS image and pooled analysis of H-2Kb expression on tumor cells harvested from shCTL and shIL-9R CMT167 bearing mice. n=8 tumors per
group. (D) Flow-cytometry and pooled analysis of H-2Kb on the surface of CMT167 cells \textit{in vitro}. Cells were treated with IL-9 for 2 days, followed by 10 h treatment with IFN-γ. $n=4$. (E-F) Signaling proteins in CMT167 cells treated as in (D) were evaluated by Western blot analysis. $n=4$. Data were represented as mean ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant. Two-tailed Student’s t-test, unpaired. MFI: mean fluorescence intensity.

\begin{figure}[h]
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\end{figure}
**IL-9 synergizes with anti-PD-1 antibody.** (A) The expression of PD-1 by CD8$^+$ T cells in control and IL-9 treated CMT167 tumors was evaluated by flow cytometry. (B) Representative FACS image and quantitative estimates of PD-L1 expression on the subcutaneously grown CMT167 cells in control and IL-9-treated tumors. (C) Injection schematic of anti-PD-1 antibody and IL-9 in subcutaneously inoculated CMT167 tumor-bearing mice. (D) Tumor growth curves in IL-9 and/or anti-PD-1 antibody administered mice. Representative images (E), final volume (F), and weight (G) of tumors retrieved from IL-9-treated and control mice. (H) Quantitative measurement of tumor-infiltrating CD8$^+$ CD3$^+$ T cells by flow cytometry. Data are represented as Mean ± SEM. n=8 mice. *, p< 0.05; **, p< 0.01; ***, p < 0.001; ns, not significant. Two-tailed Student’s t-test, unpaired.

**Supplementary Files**

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