Summary of Ph.D. thesis

Modification of the Innate Immune Response using Low Molecular Weight Substances and its Effect on Different Tumor Types

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1. Introduction

In modern times, cancer-related mortality ranks as the second most common cause of death in developed countries, after cardiovascular diseases. Numerous currently employed methods are available for treating various types of tumors, including local procedures such as surgical removal or radiotherapy and systemic interventions like the use of cytotoxic agents. These so-called conventional therapeutic methods have many disadvantages in terms of both efficacy and cost-effectiveness. In cases of tumor cell proliferation, the host's innate immune system initially attempts to prevent the division of neoplastic cells. By evading the immune response, certain tumors can suppress T lymphocyte activity in the tumor microenvironment, thereby promoting the unchecked proliferation of cancer cells. Over the past two decades, immunotherapeutic approaches have become pivotal in the evolution of oncological interventions. For efficacy, these approaches are currently used in combination with traditional therapeutic methods (e.g., surgical intervention or radiotherapy) in cancer treatment. The long-term goal is to apply treatments achievable through immune system

manipulation as monotherapy in clinical practice. This paradigm shift is exemplified by awarding the 2018 Nobel Prize in Medicine to James P. Allison and Tasuku Honjo for their groundbreaking work in modern immunotherapy strategies. The 2011 Nobel Prize also recognized the role of the innate immune system, particularly dendritic cells, in the immune response against tumors. T lymphocytes capable of recognizing tumor-specific antigens are found in the neoplastic lesion microenvironment, but the established tumor can create an immunosuppressive environment, leading to effector T cell dysfunction. Immunotherapeutic interventions may have the ability to “strengthen” the immune system, enabling the destruction of tumors. This approach not only elicits a robust antitumor response at the primary tumor site but is also effective against metastases appearing elsewhere in the body. In recent years, various immunotherapeutic interventions have shown efficacy in treating numerous malignant tumors, including advanced melanoma, lung carcinoma, gastric adenocarcinoma, and renal cell carcinoma.

Several immunotherapeutic procedures are currently available, among which our research group investigated

the method and mechanism of *in situ* vaccination, where immune-stimulating substances were locally injected among tumor cells. *In situ* vaccination can induce a specific adaptive immune response in the tumor microenvironment by utilizing tumor-associated antigens.

2. Aims

In our inquiries, we investigated the impact of *in situ* vaccination as a therapeutic strategy on tumorigenesis, employing diverse murine models. Our study aimed to identify similarities and differences in treatments with different low molecular weight therapeutic agents for two distinct tumor types. The parameters examined encompassed survival rates, incidence of tumor demarcation, and metastatic progression. To accomplish this, we established the following objectives:

1) Based on the literature, we aimed to evaluate the efficacy of mouse-specific monoclonal anti-OX40 antibody and CpG (TLR-9 ligand) treatment in two previously untested cell lines using *in vivo* mouse models.

2) We aimed to test lipopolysaccharide (TLR-4 ligand) treatment in an aggressive tumor cell line to confirm or refute previous literature findings.

3) We aimed to conduct the first trial of harpin RNA (3p-hpRNA, RIG-I agonist) treatment as a TLR and RLR ligand.

4) We aimed to conduct the first application of various nucleic acids with hairpin termini (negative single-stranded RNA and single-stranded DNA) as treatments.

3. Materials and Methods

The fundamental framework of the investigations

At the onset of the study, tumor cells were cultured *in vitro*. Subsequently, based on prior experiments and literature, a predetermined number of cells were implanted subcutaneously into mice's left or right thighs. Following implantation, the subjects were randomly assigned to control and treatment groups. Treatment commenced with intratumoral injections once the tumor diameter reached 5-8 mm (or 8-12 mm). Treatments were repeated twice for each group, while the control group (except in the final experiment) received no treatment. Post-treatment, tumor size and physiological parameters were continuously monitored in both control and treated groups. The general condition and well-being of the mice were assessed daily. Necropsies and histological examinations were conducted on deceased animals. Treatment efficacy was evaluated by assessing survival, changes in primary tumor progression and morphology, and the presence of distant metastases.

Cancer Cell Lines

In our experiments, we used two different tumor cell lines. First, we worked with the MB49 murine bladder tumor cell line. Before the experiment, the MB49 carcinoma cell line was adapted to C57BL6/N mice. A total of $1x10^7$ cells in 0.2 ml suspension were injected subcutaneously into the left *musculus biceps femoris* region of the mice. The second cell line we used was the Renca CRL-2947 renal adenocarcinoma. In this case, a total of $3x10^5$ cells were implanted in the same manner described above.

Mice

In the experiments forming the basis of this dissertation, we used 30 female C57BL6/N mice, aged 4-5 weeks and weighing 17 g, and 102 female BALB/c mice, also aged 4-5 weeks and weighing 17 g. The care and use of the animals complied with the guidelines outlined in the “Care and Use of Laboratory Animals” issued by the Animal Health Diagnostic Directorate of the National Food Chain Safety Office (NFCSO) in Hungary.

Therapeutic Agents

Once the diameter of the implanted tumors reached the size of 5-8 mm (or 8-12 mm), we initiated intratumoral treatments on the mice. The treatment cocktails contained various combinations and doses of commercially available unmethylated CpG oligodeoxynucleotide (TLR9 ligand), agonistic murine monoclonal anti-CD134/OX40L receptor antibody (anti-OX40), lipopolysaccharide (LPS – TLR4 ligand), and 5' triphosphate hairpin RNA (3p-hpRNA – RIG-I ligand). Additionally, we utilized nucleic acids that were purified in our laboratory, according to the "Molecular Genetic Analysis of Populations, a Practical Approach" protocol, including total RNA from influenza A virus (IV-RNA) derived from the PR8 mouse-adapted influenza strain and total DNA from parvovirus (PPV1-DNA) isolated from porcine parvovirus 1. All treatment combinations were dissolved in pyrogen-free water and injected into the central part of the tumors.

Humane endpoints

The animal experiments were conducted with the approval of the Ethics Committee of the Animal Health Diagnostic Directorate of the National Food Chain Safety

Office (NFCSO) in Hungary. The animal experimental protocols were approved by the Pest County Government Office, Food Chain Safety and Animal Health Department (permit number: PE/EA/00922-7/2021). Rapid euthanasia via CO_2 strictly followed the guidelines outlined in the "Guide for the Care and Use of Laboratory Animals" issued by the National Institute for Health, implemented when the tumor reached 20 mm in diameter or if the animal exhibited signs of chronic pain or distress, or any adverse changes in health or well-being.

Postmortem Data Collection

During the *in vivo* experiments, all deceased specimens were subjected to necropsy. The objectives were to determine the size of the primary tumor, assess any macroscopic changes in internal organs, and identify the presence of ascites. Metastases and other changes directly or indirectly related to the tumor were identified macroscopically and/or microscopically. Histological samples were taken from the primary tumor, spleen, liver, kidneys, brain, and lungs. Our investigations included data on the tumors' consistency, size, and relationships with their surroundings and the presence of necrotic or

inflammatory foci. Tissue sections from the organs were stained with hematoxylin and eosin for examination.

4. Results and Discussion

First experiment– MB49

In this study, we based our treatments on two pathogen-associated molecular patterns (PAMPs): unmethylated CpG oligonucleotide (CpG – TLR9 ligand) and lipopolysaccharide (LPS – TLR4 ligand). These were combined with an agonistic monoclonal antibody targeting the OX40 receptor (anti-OX40) expressed by activated T cells. Previous studies have reported favorable outcomes using the CpG + anti-OX40 combination and LPS treatment for various tumors. Based on these findings, we hypothesized that the combined use of anti-OX40 and CpG could induce an antitumor immune response capable of eradicating established bladder tumors and preventing metastasis. Additionally, building on promising results with LPS, we also combined it with anti-OX40 and tested this combination as a treatment.

Assessment of survival times

The experimental period lasted 120 days, corresponding to ten years in human comparison. By the sixtieth day, all animals in the control and LPS + anti-

OX40 groups had died, while five animals in the CpG + anti-OX40 group remained alive. Over the following 60 days, an additional two mice died (or were euthanized due to deteriorating health) without tumor-related findings. Notably, three members of the CpG + anti-OX40 group survived until the end of the experiment. The median survival in the CpG + anti-OX40 group (69.5 days) was significantly greater than that of the control group (28.5 days, $p<0.0042$) and the LPS + anti-OX40 group (32 days, $p=0.0018$). No statistically significant difference was observed between the LPS + anti-OX40 and control groups.

Primary tumor growth and appearance

Three days after the final treatment, we measured the size of the primary tumors in all experimental groups and compared these measurements to those taken before treatment (5-8 mm). The CpG + anti-OX40 treatment yielded promising results: in 4 out of 10 subjects, the tumor size decreased by 20-50%; in 1 case, the tumor completely disappeared, and in 2 cases, tissue proliferation ceased. In contrast, the LPS + anti-OX40 and control groups showed a notable increase in average

tumor diameter. In the control group, 5 subjects had to be euthanized following assessment as the tumor size exceeded the 20 mm threshold specified in the *“3. Materials and Methods, Humane Endpoints”* section.

Metastatic events

We observed similarly favorable outcomes regarding pulmonary metastases following CpG + anti-OX40 treatment. In this group, metastases were detected in five cases (50%). In contrast, the LPS + anti-OX40 and control groups exhibited pulmonary metastases in all cases. Liver metastases were found in two cases (20%) of the CpG + anti-OX40 group, in six cases (60%) of the LPS + anti-OX40 group, and four cases (40%) of the control group. Renal tumors were recorded in a total of three cases: one in the CpG + anti-OX40 group and two in the LPS + anti-OX40 group. No such cases were observed in the control group. No signs of tumor cell proliferation were detected in the spleen or brain. Overall, these results support that the CpG + anti-OX40 treatment group had a reduced incidence of metastases compared to the LPS + anti-OX40 and control groups.

Second experiment – Renca I.

Based on the literature and our previous findings, we hypothesized that the simultaneous application of anti-OX40 and CpG could elicit an antitumor immune response, even though LPS did not yield positive results in the malignant tumors used in our study. Consequently, in addition to CpG, we aimed to evaluate the efficacy of a RIG-I ligand (3p-hpRNA). This ligand may activate the immune response suppressed by tumor cells through alternative pathways and potentially inhibit regulatory cells that obstruct the immune response.

Assessment of survival times

The experiment was concluded 28 days after tumor implantation, earlier than initially planned. This early termination was primarily due to the tumor's exceptionally aggressive growth. Additionally, in many cases, the tumors began to grow toward the abdominal cavity, complicating their detection. In most mice (28 out of 30), the primary tumor exceeded the 20 mm humane endpoint specified in the *“3. Materials and Methods, Humane Endpoints”* section by the 26th day, necessitating euthanasia. The final two mice (one from each treatment

group) were euthanized two days later. It is likely that the late initiation of treatment, starting 14 days after tumor implantation, contributed to the irreversible progression of the tumors.

Primary tumor growth and appearance

Primary tumor sizes were measured before the initiation of treatments and six days after the final treatment in all three experimental groups. None of the treatments demonstrated statistically significant effects on tumor growth. However, notable differences were observed between the treated and untreated groups concerning the morphology of the primary tumors and metastatic progression. In the CpG + anti-OX40 and CpG + 3p-hpRNA treated groups, primary tumors exhibited initial signs of demarcation, whereas, in the control group, primary tumors showed a diffuse appearance.

Metastatic events

According to the literature, Renca cell lines traditionally induce metastases only following intravenous or renal tissue injection. Our studies, however, demonstrated that metastases also occurred in tumors implanted

subcutaneously. Histological examinations were conducted in both treated and untreated groups to assess the presence of metastases in the lungs, kidneys, and liver. In the control group, metastases were detected in the lung tissue in 70% of the mice, whereas no metastases were observed in the treated groups. Metastases were found exclusively in the lungs.

Third experiment – Renca II.

In addition to the two treatments previously tested (CpG + anti-OX40 and CpG + 3p-hpRNA), we also evaluated three additional treatment combinations: 3p-hpRNA alone, IV-RNA + anti-OX40, and PPV1-DNA + anti-OX40. Given the aggressive nature of the Renca CRL-2947 cell line, the initiation of treatment was advanced compared to the previous experiment. Tumor sizes were measured not just once but four times following treatment, allowing for a temporal assessment of treatment efficacy. Results across all examined parameters were more favorable than those of the prior experiment.

Assessment of survival times

Thirty-eight days after tumor implantation, only 20% of the untreated mice were alive. The survival rates in the various treatment groups were as follows: CpG + anti-OX40: 70% (significant, p=0.0103), CpG + 3p-hpRNA: 50% (not significant), 3p-hpRNA: 50% (significant, p=0.0467), IV-RNA + anti-OX40: 60% (significant, p=0.0349), and PPV1-DNA + anti-OX40: 40% (not significant). Regarding median survival times, the CpG + anti-OX40, 3p-hpRNA, and IV-RNA + anti-OX40 groups demonstrated statistically significantly longer median survival times than the untreated group.

Primary tumor growth and appearance

In addition to median survival times, we also examined the changes in primary tumor sizes. Measurements were taken on days 17, 24, 31, and 38 post-implantation to assess differences between treated and control groups. On day 17, there were no significant differences in primary tumor sizes among the CpG + anti-OX40, CpG + 3p-hpRNA, 3p-hpRNA, and IV-RNA + anti-OX40 groups. In contrast, the PPV1-DNA + anti-OX40 group exhibited significantly larger tumors than the control group

(p=0.0014). On day 24, significant differences were observed: the CpG + anti-OX40 (p=0.0001), CpG + 3p-hpRNA (p<0.0001), IV-RNA + anti-OX40 (p=0.0002), and PPV1-DNA + anti-OX40 (p=0.0146) groups all demonstrated significantly smaller tumor sizes compared to the control group. The 3p-hpRNA group did not show significant differences. On day 31, a similar trend continued but with less pronounced results; only two treatment groups showed statistically significant differences compared to the control group: the CpG + anti-OX40 and CpG + 3p-hpRNA groups (p-values were 0.0007 and 0.0006, respectively). By day 38, the small sample size was insufficient to detect significant results. No significant differences were observed among the CpG + anti-OX40, CpG + 3p-hpRNA, 3p-hpRNA, IV-RNA + anti-OX40, and PPV1-DNA + anti-OX40 groups. In this context, it was noted that all treatments (except the 3p-hpRNA group) positively affected primary tumor size changes at least at one-time point. However, tumor growth resumed in treated mice after the cessation of treatment effects.

Metastatic events

The incidence of metastases varied among the treatment groups: in the CpG + anti-OX40, CpG + 3p-hpRNA, IV-RNA + anti-OX40, and 3p-hpRNA groups, the rates of metastasis were 25.0%, 33.3%, 12.5%, and 37.5%, respectively. In the untreated (phosphate-buffered saline [PBS]-treated) group, metastases were observed in 77.7% of the mice. The PPV1-DNA + anti-OX40 group exhibited a lower incidence of metastases (62.5%) than the untreated group, although higher than other treated groups.

Discussion

Our studies have yielded promising results in treating mouse bladder carcinoma using CpG and the agonist anti-OX40, laying the groundwork for further research on TLR agonists and antagonists. Experiments with Renca cells also produced encouraging outcomes with various combinations of TLR and RLR ligands (CpG, 3p-hpRNA, IV-RNA, PPV1-DNA) and the agonist anti-OX40. These findings support the concept that such treatments, through targeted modulation of the immune response, may have potential roles in cancer therapy. The method

of *in situ* vaccination offers specificity and cost-effectiveness to cancer therapy despite our limited understanding of tumor antigens. It is applicable to both human and veterinary medicine. The results achieved — including increased survival, reduced primary tumor size, and decreased incidence of metastases — provide a solid foundation for further research and can be enhanced by refinement of treatment protocols.

5. Novel Scientific Findings

1. It was the first case to combine anti-OX40 and CpG (TLR-9 ligand) treatment in two *in vivo* murine models: 1) bladder carcinoma derived from MB49 cell line and 2) renal adenocarcinoma derived from the Renca CRL-2947 cell line. In both cases, significant improvements were observed: primary tumor sizes were significantly reduced, survival times were significantly extended, early signs of demarcation were observed, and the incidence of metastasis showed a decreasing trend.

2. Unlike other studies, we obtained negative results with the combination of anti-OX40 treatment and LPS (TLR-4 ligand). The treatments did not yield favorable outcomes in the mouse bladder cancer model, with no significant changes observed in tumor size, demarcation, metastasis incidence, or survival times.

3. This was the first case where 3p-hpRNA (RIG-I agonist) treatment alone and in combination with CpG was investigated in a mouse tumor tissue (renal adenocarcinoma model). Both treatments yielded positive results similar to those observed with the anti-

OX40 and CpG treatment, including reductions in primary tumor size and early signs of demarcation (tending), decreased incidence of metastasis (tending), and significantly extended survival times.

4. For the first time, we examined nucleic acids (RNA from PR8 mouse-adapted influenza virus strain and DNA from porcine parvovirus type 1 isolated by the Immunology Research Group) combined with anti-OX40 for treating a mouse renal adenocarcinoma model. Both treatments resulted in a significant reduction in primary tumor size, observable early demarcation, a trend towards decreased metastasis incidence, and extended survival times (significant for RNA, trending for DNA).

6. Scientific publications

1. Gulyás D., Kovács G., Jankovics I., Hidvégi M., Dénes B., Kálfalvy-Molnár L., Nagypál R., Lőrincz M.: **Antimetastatic effect of intratumoral Treg antagonists in mice with renal cancer.** Acta Vet Hung. 2024 Jun 18. *doi: 10.1556/004.2024.01037.*

2. Gulyás D., Földi D., Kovács G, Dénes B., Lőrincz M.: **A különböző immunológiai alapú terápiák alkalmazása a daganatok elleni küzdelemben (Utilization of various immunotherapies against cancer)**; Magyar Állatorvosok Lapja, 146 (3). pp. 167-180. ISSN 0025-004X. (2024.03). *doi: 10.56385/magyallorv.2024.03.167-180.*

3. Gulyás D., Kovács G., Jankovics I., Mészáros L., Lőrincz M., Dénes B.: **Effects of the combination of a monoclonal agonistic mouse anti-OX40 antibody and toll-like receptor agonists: Unmethylated CpG and LPS on an MB49 bladder cancer cell line in a mouse model.** PLoS One. 2022 Jul 8;17(7):e0270802. *doi: 10.1371/journal.pone.0270802.*