AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

72 (2024) 2, 109–115

DOI:
10.1556/004.2024.01037

RESEARCH ARTICLE

Antimetastatic effect of intratumoral Treg antagonists in mice with renal cancer

DOMINIK GULYÁS[1,2*], GÁBOR KOVÁCS[3], ISTVÁN JANKOVICS[2], MÁTÉ HIDVÉGI[4], BÉLA DÉNES[2], LILLA KÁLFALVY-MOLNÁR[1], REBEKA NAGYPÁL[1] and MÁRTA LŐRINCZ[1,2]

[1] Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, Budapest, Hungary

[2] National Laboratory of Infectious Animal Diseases, Antimicrobial Resistance, Veterinary Public Health and Food Chain Safety, University of Veterinary Medicine, Budapest, Hungary

[3] Central Hospital of Northern Pest – Military Hospital, Department of Urology and Andrology, Budapest, Hungary

[4] Jewish Theological Seminary – University of Jewish Studies (OR-ZSE), Budapest, Hungary

Received: 2 January 2024 • Revised manuscript received: 20 March 2024 • Accepted: 17 April 2024
Published online: 18 June 2024

ABSTRACT

The interplay of regulatory T cells (Tregs) within the tumour microenvironment presents a significant challenge in anticancer immunotherapy. This study investigates the potential of Treg blockade to enhance the efficiency of effector T cells. Two distinct treatment cocktails were examined: 3p-hpRNA (5′ triphosphate hairpin RNA) combined with unmethylated CpG oligonucleotide (CpG); CpG in combination with OX40 receptor-specific monoclonal antibody (anti-OX40). Treatment efficacy was assessed using a murine model of kidney adenocarcinoma.

Renca cells (renal cortical cells with adenocarcinoma) were subcutaneously engrafted in 30 BALB/c mice, then animals were allocated into three treatment groups: Group 1: CpG+anti-OX40, Group 2: CpG+3p-hpRNA, Group 3: untreated control. Treatment efficacy was evaluated based on tumour growth, the occurrence of metastases and overall survival.

On day 28 post-implantation, experiments had to be terminated due to tumour progression. Although comparisons of survival times and primary tumour sizes thus became inconsequential, histological examinations provided valuable insights. We observed distinct variations in primary tumour characteristics among the different groups: Groups 1 and 2 displayed demarcations, while Group 3 exhibited diffuse tumours with necrosis. Lung metastases were evident in 70% of untreated mice, whereas none were observed in either of the treated groups.

Our findings instil confidence in the potential efficacy of the treatments, thereby laying a solid foundation for future investigations.

KEYWORDS

immunotherapy, cancer therapy, companion animal, regulatory T cell, adjuvant, monoclonal antibody

INTRODUCTION

Maintaining peripheral tolerance, immune homeostasis, limiting autoimmune diseases and other excessively increased immune responses are essential roles that regulatory T cells (Tregs) play. They achieve this by suppressing multiple immune responses to self-antigens and restricting the immune response to foreign antigens, including oncoprotein-encoded ones (Grover et al., 2021). Myeloid-derived suppressor cells (MDSCs) are also present in tumour tissues. MDSCs inhibit the anticancer activity of effector T (Teff) cells and natural killer (NK) cells via impeding arginine metabolism (Rodriguez and Ochoa, 2008). Tregs and MDSCs within the tumour microenvironment thus block the effector function of immune

*Corresponding author. Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, Hungária krt. 23-25, 1143, Budapest, Hungary. Tel.: +36204579871.
E-mail: gulyas.dominik@univet.hu

cells and consequently may impair anticancer immunotherapy, including the action of checkpoint inhibitor drugs. Tregs and MDSCs may also dampen the tumour eradicating efficacy of cancer neoantigens containing therapeutic vaccines. Thus, by their tolerogenic immune function, Tregs and MDSCs may exacerbate the metastatic spread of tumour cells in the host. In fact, tissue-specific Tregs can also be held responsible for occasional therapeutic failures in eradicating metastatic tumours by immunotherapy (Huppert et al., 2022).

Conversely, in principle, it may be assumed that antagonists acting indirectly or directly on Tregs (and MDSCs) may have antimetastatic effects. One group of Treg antagonists consists of components of bacterial or viral origin, often nucleic acid (NA) derivatives (NA fragments) (Anz et al., 2010). Among other compounds, the latter also includes those specific natural or synthetic CpG motifs of microbial DNA which act indirectly or directly on Treg and MDSC functions. The unmethylated DNA moiety (the deoxycytidylate-phosphate-deoxy guanylate (CpG) motif) is present in viral and bacterial DNA (Thompson et al., 2011). CpG acts as a Toll-like receptor 9 (TLR9) agonist, which may increase the immune response against infectious microorganisms and cancer cells (Karapetyan et al., 2020). TLR9 activation by CpG can boost Th1 and proinflammatory cytokines, as well as antigen-specific B cells, and can also increase the production of T cells with long-lasting immune memory. By targeting TLR9, CpG can also reduce the frequency of Tregs and render immunosuppressive MDSCs in the tumour bed to differentiate into tumoricidal macrophages. In a clinical study with lymphoma patients, radiotherapy combined with intratumorally administered CpG resulted in a decrease of Tregs alongside tumour regression (Shirota et al., 2015).

Agonistic anti-OX40 compounds are immunostimulatory monoclonal antibodies that bind to and activate the surface OX40 receptor. OX40 is a member of the tumour necrosis factor receptor (TNFR) superfamily. It is upregulated on most recently activated T cells and constitutively expressed on Tregs (Croft, 2014). OX40-signaling by anti-OX40 agonist monoclonal antibodies may deplete Tregs and, concomitantly preventing Treg-mediated immune suppression, may promote an anticancer immune response in the host (Davis et al., 2022). It has been shown that by depleting tumour-infiltrating Tregs, an intratumorally administered cocktail of an anti-CTLA-4 (an immune checkpoint inhibitor) and anti-OX40, together with CpG, systemically eradicated CNS lymphoma with leptomeningeal and spinal cord metastases in mice (Marabelle et al., 2013).

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are cytosolic RNA sensors responsible for the induction of the production of type I interferons (IFN-Is) that generally play a pivotal role in the antiviral defence of the host (Rehwinkel and Gack, 2020). The three known members of RLRs are the RIG-I or DDX58, the melanoma differentiation-associated protein 5 (MDA5) and the laboratory of genetics and physiology 2 (LGP2). IFN-I controls the generation and functioning of Tregs in mice and humans. It has been shown that during immune activation, IFN-I delays the production of Tregs and promotes that of Teff cells (Golding et al., 2010). When RIG-I-signaling is activated in cancer cells, IFN-I-induced apoptosis of tumour cells and boosting of NK, dendritic and Teff cells can be observed. Well-known RIG-I agonists are the oncolytic viruses and the 5′ triphosphate terminal structure containing small hairpin dsRNA (5’ (3p)-hpRNA), which has been known to induce a robust innate anticancer and antiviral response in mammals (Goulet et al., 2013; Jiang et al., 2019). Host RNAs cannot typically contain the viral signature 5′(3p)-RNAs. Therefore, 5′(3p)-hpRNAs may mimic viral infection in hosts and thus, if exposed to RLRs, induce an antiviral immune cascade (Thompson et al., 2011).

Intratumoral delivery of immunomodulatory compounds, or the corresponding mRNA thereof, an innovative way of cancer drug administration, may result in systemic anticancer effects with complete tumour eradication from the host (Sagiv-Barfi et al., 2018; Haabeth et al., 2019). In experimental murine bladder cancer, we have been successful in inducing partial antimetastatic response when a CpG and an anti-OX40 agonist were simultaneously injected into the tumour microenvironment of cancer-bearing mice (Gulyás et al., 2022). Therefore, we decided to continue the search for injectable immunomodulatory molecular combinations with antimetastatic potential.

Among the relatively rare primary canine renal tumors, renal cell carcinoma (RCC) emerges as the most prevalent subtype (Kimata et al., 2022), while in humans, RCC represents around 3% of all malignancies (European Association of Urology Guidelines, 2023). Canine RCCs are malignant tumours with the potential to metastasize to the lungs, various abdominal organs and lymph nodes (Bryan et al., 2006). In humans, renal cell carcinoma (RCC) primarily spreads to the lungs (Cooley et al., 2021). In veterinary practice, the detection rate of pulmonary metastasis at presentation varies from 16% to 48%, underscoring the importance of prompt evaluation (Bryan et al., 2006; Klein et al., 1988). In recent years, thanks to advancements in comparative oncology and genome sequencing techniques, canine tumours have been discovered to share remarkable similarities with human neoplasms (Sobczuk et al., 2020). Given the notable prevalence of pulmonary involvement in renal cell carcinoma (RCC), there is an urgent need to explore adjuvant or alternative treatment strategies for metastatic RCC patients, regardless of whether they are human or canine.

Various Renca cell lines (among others, CRL-2947) are widely used kidney adenocarcinoma models adapted to mice. Based on data published so far, the metastasizing ability of Renca cells varies according to the application procedures. In the case of subcapsular kidney injection and intravenous administration, metastases with a course similar to human cases have been described (mainly in the surrounding lymph nodes, lungs, and liver) (Hrushesky et al., 1973; Salup et al., 1986). However, in the case of subcutaneous administration, localized cancerous lesions unsuitable for metastases have been described (Chakrabarty et al., 1994; Sobczuk et al., 2020).

In the present study, we chose an experimental murine kidney adenocarcinoma (Renca), an established analogue of the human metastatic renal cell carcinoma (RCC), to test the antimetastatic effect of two immunomodulating combinations: CpG plus either an anti-OX40 or a RIG-I agonist.

MATERIALS & METHODS

Cell line

We used the Renca Renal Adenocarcinoma (CRL-2947) cell line (obtained from ATCC, Manassas, Virginia, USA) in the experiment. Tumour cells were cultured at 37°C, 95% humidity and 5% CO_2 concentration in RPMI-1640 medium (Sigma-Aldrich, St Louis, Missouri, USA), containing 10% inactivated foetal bovine serum (Sigma-Aldrich, St Louis, Missouri, USA), 2 mM glutamic acid, penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}). Confluent cell cultures were treated with trypsin-EDTA, suspended and washed twice with serum- and antibiotics-free RPMI-1640. We have instilled 3×10^5 CRL-2947 cells subcutaneously, in a 200 µL suspension, to the region of each mouse's right musculus biceps femoris. Only 90% viable cell suspension was used for implantation. Cell viability was determined by trypan blue (TB) staining.

Mice

In this study, we used 30, 4–5 weeks old, BALB/c female mice (Charles River, Germany), weighing 17 g. We formed three groups: two treated groups and a control group. Animals of the control group received no treatment at all. Animals were housed in mouse boxes (10 per box) at 20 ± 2°C, 50 ± 10% humidity and exposed to a 12-hours light/dark cycle, with ad libitum food and drinking water. All animals were kept strictly under the guidelines for the Care and Use of Laboratory Animals, issued by the Veterinary Diagnostic Directorate, National Food Chain Safety Office, Hungary (VDD, NFCSO).

Reagents

In the two experimental groups, mice were treated intratumorally with the following cocktails:

Group 1: 50 µg of unmethylated CpG oligodeoxynucleotide (TLR9 ligand – InvivoGen, San Diego, California, USA) and 5 µg agonistic rat monoclonal anti-CD134/OX40L receptor antibody (anti-OX40 – Abcam, Cambridge, UK) dissolved in 100 µL of pyrogen-free water.
Group 2: 50 µg of unmethylated CpG oligodeoxynucleotide (TLR9 ligand – InvivoGen, San Diego, California, USA) and 3 µg 3p-hpRNA (5′ triphosphate hairpin RNA) – a specific agonist of RIG-I (InvivoGen, San Diego, California, USA) dissolved in 100 µL of pyrogen-free water.

The cocktails were injected into the middle of each tumor.

Humane endpoints

The animal experiments were approved by the ethics committee of VDD, NFCSO. Approvals of animal experiments were issued by the Government Office of Pest County Division of Food Chain Safety, Animal Health, Plant Protection Department (license number: PE/EA/00922-7/2021). Mice were euthanized by CO_2 anoxia when the mean tumour diameters (MTD) reached 20 mm, or the animals were in chronic pain or distress, or there were adverse changes in the animals' health and/or well-being (e.g., impaired mobility, inability to remain upright and interference with a vital physiological function). After tumour implantation, mice did not receive any anaesthetic and/or analgesic treatment, as this would have likely caused more stress (handling animals and needle puncture) than the pain itself. The rapid CO_2 sacrifice was humanely performed in compliance with the Guide for the Care and Use of Laboratory Animals, issued by the National Institute for Health (National Research Council, 2011). The health conditions of the animals were monitored daily.

Histopathological assessment

Following postmortem procedures, specimens were collected for histological analysis. Three vital organs – specifically, the liver, lung, and kidney – were selected from each subject for comprehensive evaluation. Additionally, samples were obtained directly from the primary tumour itself. These organs were preserved in an 8% formalin solution until the conclusion of the experimental period, at which point the sections underwent uniform processing. Tissue from the primary tumour was meticulously acquired from the periphery of the neoplastic region, excluding areas displaying necrotic changes. Following staining with hematoxylin-eosin, the tissues underwent microscopic scrutiny.

RESULTS

Based on our previous results (Gulyás et al., 2022), we supposed that treatment with the agonistic anti-OX40 antibody alongside the TLR9 ligand, unmethylated CpG, may induce an antitumour immune response. Furthermore, together with CpG, we also tested a RIG-I ligand (3p-hpRNA), which may also enable the initiation of the immune response suppressed by tumour cells via another pathway. Our goal was to test the inhibition of tumour dissemination and the reduction of tumour size (CRL-2947). We implanted Renca CRL-2947 cells subcutaneously in the right thigh of mice, allowing the tumours to grow, reaching an average of 8–12 mm in mean tumour diameter (MTD). Mice were then inoculated with the combination of CpG and anti-OX40 or 3p-hpRNA. After treatments, survival times, tumour dissemination, tumour growth and regression were monitored. After sacrifice, an autopsy was performed on all animals. The appearance of the primary tumour and the

histology of the primary tumour plus relevant organs, i.e. lungs, liver and kidneys, were examined.

Survival times

Due to tumour progression, experiments had to be terminated 28 days after tumour implantation. The reason was that on day 26 after implantation, in most mice (28/30), the size of the developed (and then treated) primary tumours exceeded the previously defined humane endpoint of 20 mm MTD. The remaining two mice (1-1 from the treated groups, resp.) were euthanized two days later. We think the reason for the irreversible tumour progression was that the treatments were started late (on the 14th day after tumour implantation). Therefore, the comparison of survival times had no relevance.

Primary tumours

Primary tumour sizes were measured at baseline and six days after the last treatment in all three groups. As mentioned, none of the treatments had any statistically significant effect on the growth of the primary tumour. Interestingly, however, differences between the treated and untreated groups in terms of the appearance of the primary tumour and the development of metastases were found. The primary tumours showed incipient demarcation in the CpG + anti-OX40 and CpG + 3p-hpRNA treated groups. In contrast, the primary tumours showed a diffuse appearance with necrosis in most of the control group mice (Fig. 1).

Metastases

According to the literature, Renca cell lines only form metastases after intravenous (i.v.) or subcapsular kidney injection. We, however, found that they also form metastases when injected subcutaneously (s.c.). Histological examinations were performed in both treated and in the non-treated (control) group, to analyse the presence of metastases in the lungs, kidneys and liver. Metastases were only found in the lungs (Fig. 2). In the control group, lung metastases were found in 7 mice out of 10 (70% incidence), while none were found in either of the treated groups (Table 1).

DISCUSSION

While primary renal tumours are infrequent in dogs, renal cell carcinoma (RCC) stands out as the most prevalent

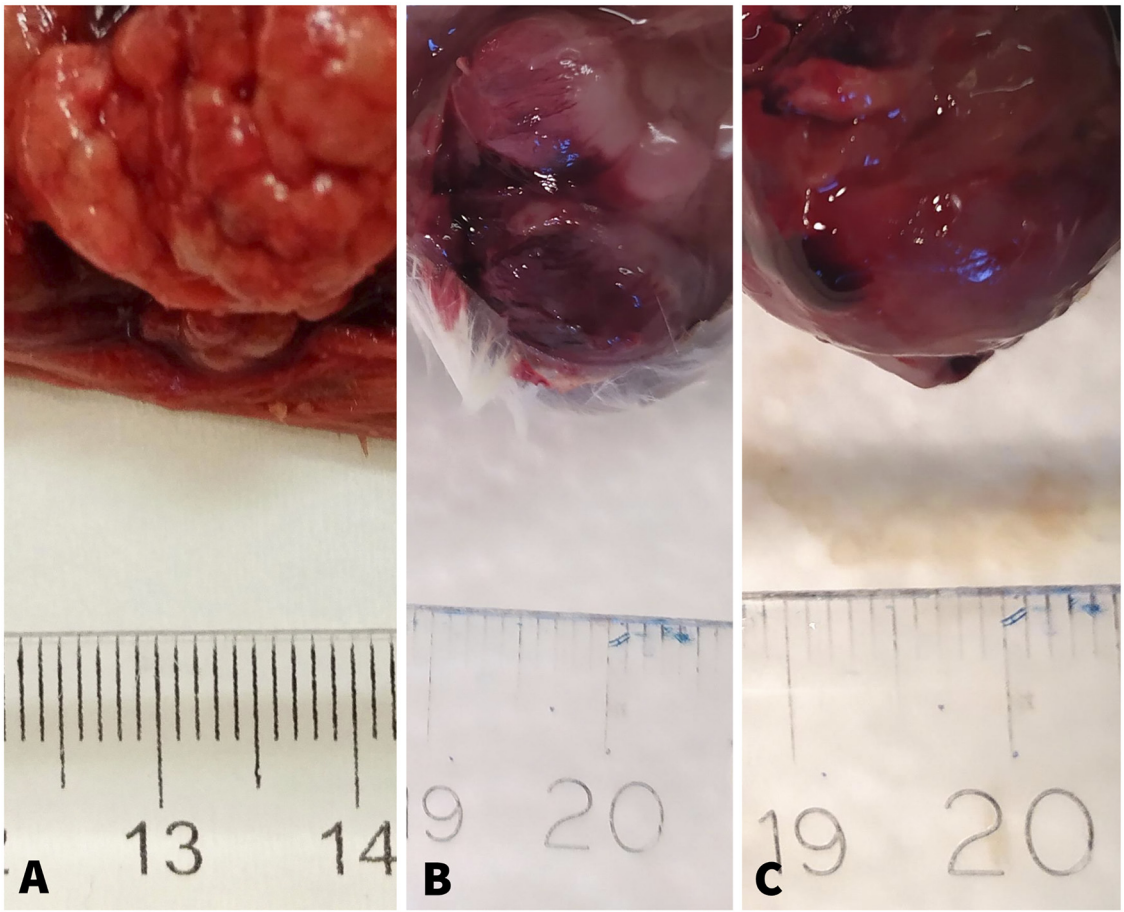

Fig. 1. **Primary tumour morphology.** Notable differences in primary tumour morphology were observed across various experimental groups: A) untreated group, B) CpG+anti-OX40, and C) CpG+3p-hpRNA. In groups B) and C), the primary tumours exhibited early demarcation, whereas in group A), primary tumours displayed a diffuse appearance with necrosis in most cases

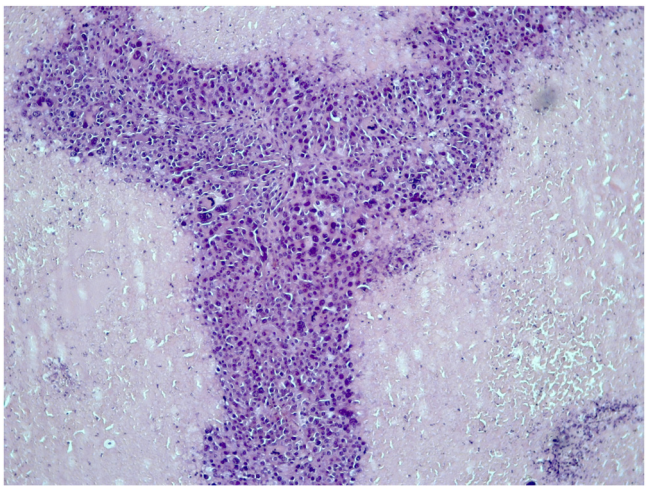

Fig. 2. **Histological examinations.** Histological examinations were conducted in both groups, assessing the presence of metastases in the lungs, kidneys, and liver. Metastases were exclusively detected in the lungs, particularly notable in the untreated group where Renca cells exhibited a pronounced tendency for lung metastasis. Sections were stained using hematoxylin-eosin

Table 1. Histopathological results of intratumoral treatments

Treatment	Mice with metastases (%)		
	Lung	Liver	Kidney
CpG+anti-OX40	0	0	0
CpG+3p-hpRNA	0	0	0
Control	70	0	0

subtype. In men and women, kidney cancer constitutes approximately 5% and 3% of all malignancies, respectively. Remarkably, kidney cancer ranks as the sixth most common cancer type in men and the tenth most common in women (Miller et al., 2018). RCC accounts for over 90% of human kidney cancers (Hsieh et al., 2017). This juxtaposition highlights the significance of RCC in both human and veterinary oncology, despite its relatively rare occurrence in dogs. In patients with renal cell carcinoma (RCC), metastases can develop in the early stages of the disease. In humans, approximately 30–50% of localized tumours progress to metastatic disease, and even after surgery, nearly 40% of patients with localized RCC experience distant metastases (Pavlakis et al., 2004). Similarly, in canine patients, metastases are observed in 69% of cases with renal carcinomas at the time of death (Kimata et al., 2022). This highlights the aggressive nature of RCC and emphasizes the critical need for discovering novel treatment interventions in both human and veterinary medicine.

In this regard, leveraging the anticancer potential of the immune system emerges as a promising avenue for exploration. However, recent concerns have been raised regarding the inhibition of immune checkpoints in advanced human RCC (Pal et al., 2023). A combined approach involving a low-mass tyrosine kinase inhibitor and a monoclonal antibody-targeting programmed cell death ligand was implemented. Notably, both the tyrosine kinase inhibitor and the combination therapy elicited adverse effects, with survival outcomes showing no encouraging trends.

However, our approach to Treg inhibition was based on a distinct methodology. Instead of systemic inhibition, we opted for intratumoral injections containing Treg antagonists, specifically, CpG + anti-OX40 and CpG + 3p-hpRNA. Although these treatments did not lead to a cure or a reduction in primary tumour size, we observed promising results in two aspects of anticancer efficacy.

Firstly, the experimental immunomodulatory cocktails yielded favourable changes in the appearance and structure of the primary tumours, with evident signs of demarcation. Conversely, primary tumours in the control group of mice exhibited a diffuse appearance accompanied by necrosis.

Secondly, both experimental treatments demonstrated a significant antimetastatic effect. In the control group, 70% of mice developed lung metastases. However, mice treated with the experimental intratumoral cocktails remained metastases-free, which is promising.

Based on these preliminary results, the intratumorally administered Treg antagonist cocktails we utilized may possess antimetastatic potential, laying the groundwork for further experiments to refine the concept of transforming incurable cancers into treatable ones.

Further investigations are warranted to confirm the treatment's efficacy in eradicating the primary tumour. Successful validation could enable surgical excision post-treatment to prevent recurrence without supplementary therapeutic interventions, such as chemotherapy. This query holds paramount significance for malignancies, given their propensity for early metastasis, thus highlighting the critical importance of early detection.

Additionally, an important aspect to consider is the treatment's impact on metastatic lesions. It is presumed that no metastatic growths had occurred before administering interventions (as per our findings, only histologically discernible tumours were detected in the lungs of untreated subjects). An intriguing avenue for exploration lies in investigating whether treating the primary tumour, even in cases where tumours have already metastasized to other organs, can localize these growths and impede the development of new tumours.

This objective can only be achieved by significantly reducing the cell count of the primary tumour in Renca cells. However, animal welfare imperatives must be considered, as the excessive proliferation of primary tumours severely compromises mice's quality of life. Therefore, future research efforts should strike a balance between therapeutic efficacy and the well-being of experimental subjects.

We have attained promising outcomes in our investigations utilizing Renca cells, employing various combinations of CpG, 3p-hpRNA and agonistic anti-OX40, as therapeutic mixtures. These results serve as a robust foundation for further explorations involving Toll-like and Rig-like receptor agonists and antagonists, which can modulate the immune response along the cellular pathway. The

primary objective of this research endeavour is to harness its potential application within the realm of cancer therapies for both human and companion animal subjects. By advancing our understanding of immune modulation and its influence on tumour progression, our goal is to contribute to the development of more effective and targeted treatment strategies that benefit both human and veterinary patients.

Ethics approval: Mice were housed and maintained at the animal house of the Department of Microbiology and Infectious Diseases, University of Veterinary Medicine Budapest, Budapest, Hungary. All animal experiments were carried out by the Experimental Work Plan, approved by the Animal Welfare Committee, and with the decree of the Government Office for Pest.

Competing interests: The authors declare that they have no conflict of interest regarding the publication of this manuscript.

Availability of data and materials: The datasets used and/or analysed during the current study are included in the manuscript. All data generated or analysed are available from the corresponding author on reasonable request.

Funding: Project nr. 2019-1.2.1-Egyetemi-Öko-2019-00010 has been implemented with the support provided by the National Research, Development and Innovation Fund of Hungary, financed under the 2019-1.2.1-Egyetemi-Öko funding scheme.

Project no. RRF-2.3.1-21-2022-00001 has been implemented with the support provided by the Recovery and Resilience Facility (RRF), financed under the National Recovery Fund budget estimate, RRF-2.3.1-21 funding scheme.

Authors' contributions: BD, ML, DG and IJ designed the experiments. BD, DG, GK, LKM, RN and LM carried out the experiments. ML, BD, IJ and DG contributed to the interpretation of the results. MH, DG, BD and ML wrote the manuscript. All authors have read and approved the final manuscript.

Conceptualization, Dominik Gulyás, István Jankovics, Béla Dénes and Márta Lőrincz; Data curation, Máté Hidvégi and Béla Dénes; Funding acquisition, Márta Lőrincz; Investigation, Dominik Gulyás, Gábor Kovács, István Jankovics, Béla Dénes, Lilla Kálfalvy-Molnár, Rebeka Nagypál and Márta Lőrincz; Methodology, Dominik Gulyás, István Jankovics, Béla Dénes and Márta Lőrincz; Project administration, Gábor Kovács; Resources, Gábor Kovács and István Jankovics; Software, Dominik Gulyás; Supervision, István Jankovics and Márta Lőrincz; Visualization, Dominik Gulyás, István Jankovics, Béla Dénes and Márta Lőrincz; Writing – original draft, Dominik Gulyás, Máté Hidvégi, Béla Dénes and Márta Lőrincz; Writing – review & editing, Dominik Gulyás, István Jankovics, Máté Hidvégi, Béla Dénes and Márta Lőrincz.

ACKNOWLEDGMENTS

The authors greatly appreciated the assistance provided by Józsefné Herbák, Andrea Katona and Annamária Gásparné Stoll.

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