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University of Veterinary Medicine Budapest

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Using low molecular weight substances to manipulate the innate immune response

Manipulation of innate immune response with viral RNA & DNA

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Abbreviations

(APCs)	Antigen-presenting cells
(DAMPs)	Damage-Associated Molecular Patterns
(DCs)	Dendritic Cell
(GM-CSF)	Granulocyte-macrophage colony-stimulating factor
(IFN)	Interferon
(IFN-γ)	Interferon-gamma
(IRAK)	Interleukin-1 receptor-associated kinases
(IL)	Interleukin
(MHC)	Major histocompatibility complex
(min)	Minutes
(MyD88)	Myeloid Differentiation Factor 88
(MDSCs)	Myeloid-derived suppressor cells
(NK)	Natural killer cells
(NRLS)	NOD-like receptors
(NF-KB)	Nuclear Factor Kappa-Light-Chain-Enhancer of activated B-cells
(PRRs)	Pattern Recognition Receptors
(PBS)	Phosphate-buffered saline solution
(Tregs)	Regulatory T-cells
(RCC)	Renal Cell Carcinoma
(RIG-1)	Retinoic acid-inducible gene-I
(RT)	Room temperature
(s.c)	Subcutaneously
(TIR)	Toll-interleukin-1 Receptor Domain
(TLRs)	Toll-Like Receptors
(TNFRS4)(OX40)	Tumour Necrosis Factor Receptor Superfamily Member 4
(TNF-a)	Tumour necrosis factor-alpha
(CpG)	Unmethylated cytosine–guanine dinucleotide

1. Literature Review

1.1 Cancer Immunotherapy

With cancer as the second leading cause of death in developed countries, the majority of patients receive treatment with a combination of surgery, radiotherapy and/or chemotherapy. The primary tumour, in most cases, can be treated effectively by a combination of these standard treatments. Prevention of metastatic spread through disseminated tumour cells proves more difficult to respond to the latter treatments. This area of unsolicited cancer development leads to the interest and further development of immunotherapy. The prime strategy of immunotherapy for cancer treatment is the exploitation of the therapeutic potential of tumour-specific antibodies and the cellular immune effector mechanisms.[1]. Exploiting the host's immune system in aid of cancer treatment dates back decades, relying on the ability of the immune system to eliminate malignant cells during transformation. [2] Spontaneous malignancy arises due to a combination of both genetic and epigenetic changes in a body. Malignant development of tissues results in foreign antigens being generated. These are known as neo-antigens. It is thanks to these proteins that render neoplastic cells detectable to the adaptive immune system, ultimately leading to their ruination and death. Nevertheless, in spite of the ability of the immune system to recognise of these antigens, certain cancer cells manage to escape immune patrol [3]. This is known as immune privilege, seen in particular in three regions of the body: the eye, the brain and the pregnant uterus. [4]

1.2 Neoantigen

Tumour neoantigens, a foreign protein, absent from physiologically normal human tissue, thus leading to a critical role in tumour-specific T cell-mediated response. Regarded as the ideal target for treatment due to its uncorrelated germline, the body can distinguish self from non-self [5]

It has been discovered that the T cell-based immune system is responding to these neoantigens as a consequence of DNA damage within the body. Recognition of these neoantigens is now known to be a significant driver of the clinical activity of both the T-cell checkpoint blockade and adoptive T-cell therapy used in onco-immunotherapy [6].

Numerous immunotherapies aim to approach these neoantigens, this may be seen to be applied uniquely to an individual's tumour. However, there are also advancements shown in a broader spectrum of neoantigen targeting, directed at recurrent mutations in

cancers. This latter approach is aimed at these target proteins that are necessary for malignant growth, therefore being applicable to treatment for many patients [7].

1.3 Damage-Associated Molecular Patterns (DAMPs)

In most tissues, when encountered with spontaneous malignancy followed by the replication of abnormal cells, the immune system relies on the release of damage-associated molecular patterns (DAMPs). DAMPs occur as a response to stress and cell death, both principal contributing factors in sterile inflammation. Encountering DAMPs aids in the proliferation of protective functions of our innate immune system [8]. In recognition of DAMPs, phagocytic cells, such as macrophages and neutrophils, bind to the latter, engulfing and digesting abnormal cells and destroying cancerous cells from the body. Macrophages are critical in the homeostasis of tissues, contorting the inclination of unwanted angiogenesis and metastasis [9].

Recognition of these DAMPs is crucial in the narration above. A major assembly aiding in this identification is termed pattern recognition receptors (PRRs), also known as toll-like receptors (TLRs) [10]. Initially discovered with regards to the innate immune reactions against microbial infection, followed later by the realisation that these TLRs also possessed an essential role in linking the innate and adaptive immune system, having numerous roles in inflammation, autoimmune diseases and cancer [11]. Acknowledgement of the stimulatory potential of TLRs and TLR-targeted schemes of treatment concerning cancer diseases proved effective in the regulation of the tumour microenvironment in connection with tumoricidal phenotypes. TLRs are type I transmembrane glycoproteins [12] with developmentally conserved structures varying throughout plants and vertebrates. [13] Following the discovery in 1966, 13 operative homologs of TLR have been identified in both humans and mice, with both TLR9 and TLR11 conserved in the two species [14–16].

1.4 Toll-Like Receptors

TLRs are vital to innate immunity, with their attention and ability to sense infection followed by the election of intracellular signalling cascades initiating an immune response, which ultimately results in the elimination of pathogens and infected cells. [15] Recognition of TLRs from almost all cells belonging to the immune system, including B-lymphocytes, macrophages, dendritic cells, neutrophils, and mast cells, as well as endothelial, epithelial cells, adipocytes and cardiomyocytes, recognition triggers a

downstream of signalling pathway [17]. This is followed by myeloid differentiation factor 88 (MyD88) and toll-interleukin-1 receptor domain (TIR) leading to the activation of nuclear factor kappa-light-chain-enhancer (NF-KB) of activated B cells, resulting ultimately in the transcription of pro-inflammatory cytokines, these include tumour necrosis factor-alpha (TNF-a), interleukin (IL)-1, and IL-6 [18]. This will be discussed in further detail below.

Recent studies have revealed that TLRs are expressed in tumour cell populations with the inclusion of tumour stem cells, alongside cancer-associated fibroblasts, tumour-associated macrophages, myeloid-derived suppressor cells (MDSCs), regulatory T-cells (Tregs) and adipocytes. These TLRs can be seen in the participation in both inhibition and promotion of tumour growth (Figure 1) [19–21].

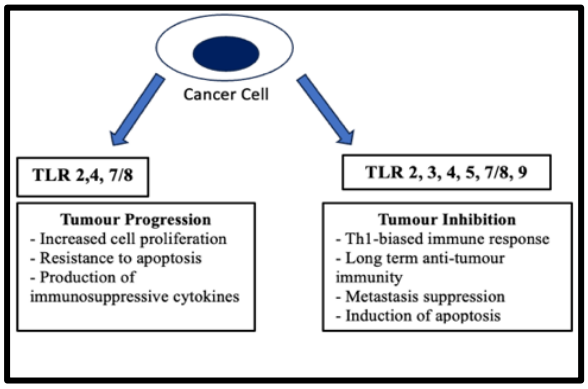

Figure 1. TLR stimulation in cancer, adapted from [22]

The exploitation of TLRs is one of the pillars in anti-tumour therapies currently, resulting in the enhancement of both the innate and adaptive immune system and, with succession, can result in the apoptosis in neoantigen-expressing tumour cells [23]. A challenge faced by TLRs is the characteristic of their inability to distinguish self from non-self, but instead responding to "danger signals"; these can be both exogenous or endogenous ligands [24]. TLR ligands fall into three categories these can be noted as, natural exogenous ligands, also regarded as PAMPs, natural endogenous ligands, read as DAMPs; and the third category being synthesised agents.

PAMPs originate from mostly elements seen in bacteria, fungi, parasites and viruses [20, 23, 25]. TLRs sense PAMPs as invading jeopardies, resulting in the direct activation of the innate immune system whilst indirectly stimulating the adaptive immune system to follow, thus providing invaluable protection against pathogens [26]. As

mentioned previously, DAMPs occur as a response to stress and cell death, both principal contributing factors in the sterile inflammation [8].

1.5 MyD88 Pathway

TLR signalling can be separated into two pathways: the MyD88 dependent pathway, known as the MyD88 pathway and the MyD88 independent pathway, also recognised as the TIR pathway [27]. The toll-like receptors seen to have activated the two pathways are TLR4, TLR5, and TLR7-9, which can act independently with MyD88; contrasting to this, TLR-3 depends solely on TIR [27–29].

In the MyD88 pathway, a succession of activated molecules begins in a downstream series of events, which ultimately induces a wide range of pro-inflammatory cytokines (Figure 2) [30, 31].

On the basis of our research, we want to focus on the expression and manipulation of these TLRs using nucleic acid ligands in a vaccine model subsequent to renal carcinoma administration to murine models. Parallel to this research, there was also an experiment carried out in which the exploration of the synthetic unmethylated cytosine–guanine dinucleotide (CpG) and retinoic acid-inducible gene-I (RIG-I) were utilised to stimulate TLRs likewise. Almost half of TLRs recognise nucleic acid ligands. TLR3, TLR7/8, and TLR9 are intracellularly inhabitants and respond to double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and DNA, respectively [32], whereas, in comparison, TLR1, 2, 4, 5, 6 and TLR10 are expressed on the cell surface. With this information, TLR agonists are currently under investigation for anticancer therapy, being produced as vaccine adjuvants with the intention of creating an upregulation in desired immune cells. In humans, it has been discovered that TLRs are found on many cell types. However, it was noted that TLRs are primarily found on monocytes, mature macrophages and dendritic cells (DCs) [33].

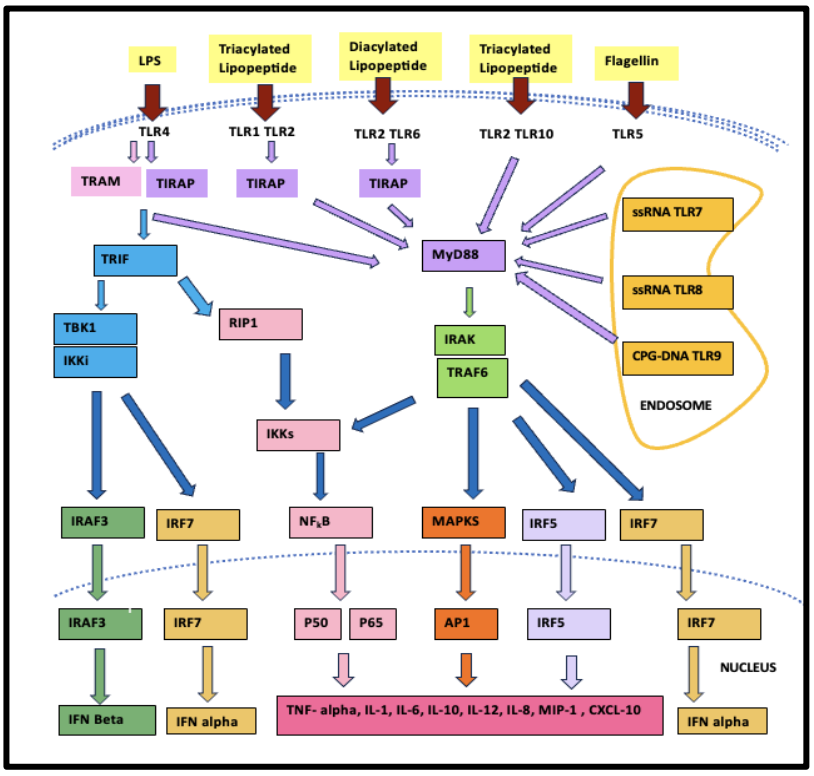

Figure 2: TLR ligand signalling pathway adapted from [34]

Figure 2: Comprehension of TLR ligands and signalling pathways. On the cell membrane, we see TLR expressed (TLR1,2,4,5,6,10). On the endosome, we can have TLR7,8,9. It is observed that TLRs can recognise a wide range of PAMPs. Upon ligation, dimerised TLRs activate either MyD88 or TIR pathways. TLR4 activates MyD88 with the addition of adaptor protein TRAM. The remaining TLRs indicated in Figure 1 can be seen to activate MyD88 alone, without the help of the coupling of the adaptor protein. As a result of this, IFNs are induced from the IRF3 and IRF7 activation alongside NF-KB, MAPK and IRF5. This initiates a large-ranging group of inflammatory and immune activities, resulting in cytokines, TNFs, and IL release, which leads to a concrete foundation for therapeutic exploration for tumour therapies. Adapted from [34]

1.6 T-cells, Regulatory T- cells

Stimulation of DC's results in the expression of many membrane-bound costimulatory molecules, including CD80, CD86, and CD40, alongside, also, cytokine IL-12, which is required for prime activation of T cells (CD4+ CD8+) [35, 36]. It has been proven that TLR agonists possess a fundamental role in the activation of innate and adaptive immune responses. In experimental mice, TLR agonist treatment was shown to reduce tumour growth size and, in some circumstances, alongside the additional treatment with combination therapeutic agents, such as chemotherapy drugs, monoclonal antibodies, and/or tumour antigen vaccination in the form of proteins, peptides or plasma DNA, showed further success, with results representing the destruction of established tumours. [37–39]. Theorised TLR agonists were selected based on their ability to activate antigen-presenting cells, namely, DCs. However, the relationship of TLRs on multiple T-cell

subsets has more recently been shown to elevate their responses and, with this, shows a promising strategy to enhance the effect of cancer immunotherapies [40].

$FOXP3^{+}CD25^{+}CD4^{+}$ regulatory T-cells (Tregs), play a crucial role in both our innate and adaptive immunity. Holding a significant role in the prevention of autoimmunity, this immunosuppressive activity can also aid in the promotion of tumour progressions [41].

This strong relationship can be seen as correlated between the quantity of '' Tregs and the progression of cancer. Tregs, found within tumours, ascites and the peripheral blood of patients with cancer are associated with poor prognosis. There has been evidence'' that is suggestive that the Tregs, protect tumours from effective immune responses, it is an objective in many recent oncotherapy trials, to interfere at this level [42].

1.7 Dendritic Cells

DCs were first identified in the epidermis, and these were named Langerhans cells. DCs are today acknowledged as a fundamental component of the lymphohematopoietic system, operating as protectors for the immune system. DCs are located in the interstitium of most organs, with the exclusion of the brain in low quantities. It is due to this absence that holds accountable for the infamous phenomenon known as immune privilege.

Setting in motion immune response, DCs capture a small amount of antigens, further then presenting it to antigen-specific T cells, beginning their maturation, fabricating typically the 3 phase process of the T cells whilst supporting the generation of not only lymphokine-secreting helper T cells but also effector cytotoxic T lymphocytes (CTLs) that subsequently migrate to tumour cells. No other APCs activate memory cells and naïve T cells, thus regarding DCs as professional APCs [43]. In regards to antitumour activity, dendritic cells can capture antigens from dying tumour cells and present them to adaptive immune cells, providing a more specific immune response [44].

For developmental vaccines against cancer, many researchers use dendritic cells, as these are seen to possess the most potent potential for antitumour activity [45]. As immature cells, DCs will be found residing in tissues and partly travelling through blood and lymph. DCs have a major histocompatibility complex (MHC) expression. DCs will only have a few costimulatory molecules, CD80 or CD86. On the journey throughout the body, DCs will come across antigens. When faced with this accoutrement, DCs will engulf said antigens through a variation of process. Mainly, they will show receptor-mediated endocytosis, phagocytosis or micropinocytosis. Following the ingestion of the antigens,

DCs will migrate towards the afferent lymphoid organs, and here they will metamorphize into mature cells. It is during this maturation process, and the undergo of various phenotypic and functional changes, that MHC expression is upregulated and with this, we see an increase of costimulatory molecules, which are CD80 and CD86 [46]. Along with this maturation of DCs, we also see an increase in of cytokines, including IL-6, IL-12, IL-13, and TNF-α. Additionally, we also have an increase in chemokines seen, such as CCL18. Acting together, we will see an attraction of naïve T cells to the location. Encountering takes the place of DCs and residing T cells with each other through their matching adhesion molecules. This forms immunological synapses. Resulting in the presentation of DCs antigens to T cells, which leads to the activation of T cells and the generation of antigen-specific responses [45]. This process is shown in Figure 3.

The utilisation of DCs in cancer vaccination plans requires a significant amount of cells to generate DCs. Protocols have been established to help in the generation of these large quantities in vitro. It has been proven that DCs can be generated from CD34-positive precursors from bone marrow or blood when these cells are incubated with cytokine cocktails that contain granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, or TNF-a [46–48]. An alternative approach used CD14-positive monocytes from blood and the cytokines GM-CSF and IL-4 or CD40 ligand to generate myeloid DCs [49–51]. These myeloid DCs are immature, with the expression of a few costimulatory molecules on their surface, but can be matured with different inflammatory stimuli like TLR ligands, TNF-a, or a combination of compounds that contain PGE2, TNF-a, IL-1beta and IL-6. [52]

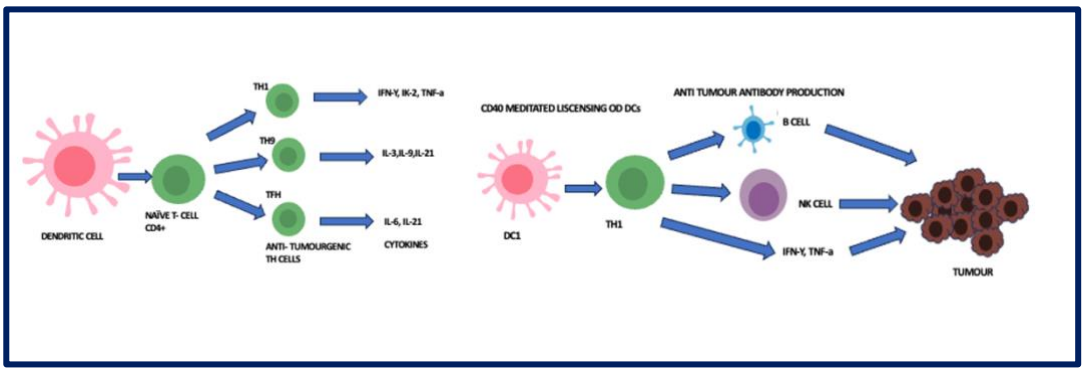

Figure 3; *Adaptation from [53]; Development of CD4+ T cells (induced by dendritic cells) and their function subsets in the immune system.*

Other lymphocytes with a profound role in the cessation of tumour growth are natural killer cells (NK). Upon activation, these cells possess both cytotoxicity and cytokine-producing effector functions, resulting in the release of cytotoxic molecules

inducing apoptosis of cancer cells. Exploration of the NK cells mechanism allowed for evidence showing NK cells to discriminate target cells from their own body healthy cells [34].

1.8 Nucleic Acid Vaccination in Cancer Immunotherapy

Using nucleic acids is a promising immunotherapeutic to manipulate the immune system, creating the desired high volume of DCs for cancer treatment. Over the past decade, there has been significant technological innovation, coupled with major research investment, that has led to the enablement of both DNA and RNA to become an auspicious tool in the field of vaccine development and protein replacement therapy [54–56]. The use of mRNA has various beneficial features over subunit, killed and live attenuated virus. The first and most desirable trait of mRNA vaccines is their safety. As mRNA is non-infectious along with a non-integrating platform, there is no probable risk of infection or no unwanted mutagenesis. In addition to this, mRNA will be degraded by the regular physiological process, and its in vivo half-life can be regulated by different modifications and delivery methods [54–57]. Favourably, the immunogenicity of the mRNA can be down-regulated, which will further enhance the safety of the vaccine [54, 57, 58]. The mRNA-based vaccines are becoming pillars in cancer treatments. Cancer vaccines can be designed to target tumour-associated antigens that are seen expressed in cancerous cells, also known as neo-antigens. Examples of these would be growth-associated factors or antigens that are unique to malignant cells [59]. These neoantigens, or it is the neuropeptides that are within them have been deployed as mRNA vaccine targets in humans [60]. Of course, these vaccines are utilised as therapeutic and not prophylactic in the case of tumour [61].

It is due to the central roles that DCs play in the initiation of antigen–specific immune responses that deem it logical to utilise them for cancer immunotherapy. This research was further successful with the inclusion of costimulatory molecules such as CD83 tumour necrosis factor receptor superfamily member 4 (TNFRS4- this is also known as OX40). The use of these molecules substantially leads to an increase in the immune stimulatory activity of DCs [62–65]. Proving advantages in multiple preclinical studies, increasing the DC activation and shifting CD4+ T cell phenotypes from Tregs to T helper cells [66–70]. It was notably apparent that the immunisation ‘’of patients with stage III or stage IV melanoma using DCs loaded with mRNA encoding melanoma-associated

antigens and mentioned adjuvants resulted in tumour regression in 27% of treated individuals'' [70].

In our research carried out, we utilised both influenza viral RNA and swine parvovirus 1 viral DNA individually in vaccine configuration to manipulate the immune system, leading to the upregulation of DCs. The influenza virus contains negative strand RNA that can activate RIG-1 signalling TLR7 and TLR8 pathways. Parvovirus is a single-strand DNA virus. An important similarity between the two viruses is that both contain a hairpin sequence that can also activate DCs. We think it is based on our immunological studies. The influenza we used was PR8, which was an H1N1 adapted to mice. The parvovirus we investigated was PPV1.

1.9 Vaccine Adjuvant; Tumour Necrosis Factor Receptor Superfamily Member 4 (TNFRS4) - also known as OX40

T cell manipulation for the enhancement of tumour immunotherapy is becoming more prevalent, as targeted Tregs therapy, either deliberately or inadvertently, can show that molecules specific to Tregs are promising candidates for Tregs depletion or moderating functionally. Such molecules can include CTLA-4, CCRA, and OX40 – which is of interest to this research carried out. An alternative approach is the utilisation of CD14-positive monocytes from blood and cytokines GM-CSF and IL-4 or CD40 ligand to generate myeloid.

Aiding in the reinforcement of successful treatment is the use of OX40. OX40 belongs to the tumour necrosis factor receptor family (TNFR). This was first said to be a receptor that was originally expressed on rat CD4 T cells isolated from the thymus and lymph nodes after being stimulated with concanavalin [71]. Further research showed us that both humans and mice expressed OX40 by CD8+ T-cells and CD4+ T-cells during antigen-specific priming [72–75]. Expression of OX40 is induced, following the TCR/DC3 cross-linking and with the presence of inflammatory cytokines, including ones such as IL-1, IL-2, and TNF-a [76]. Reinforcement of this proposition can be seen that with the absence of required activating signals, there will be a notably smaller volume of mature T-cell subsets expressing OX40 at relevant biological levels [77]. Preclinical research exhibited that the treatment of tumours in hosts with OX40 agonists, such as anti-OC4-mAB (mAB means monoclonal antibodies), resulted in tumour regression in several of these preclinical models [78–83]. The ability of OX40 agonists to regulate immune responses, alongside the regulation of the expression of CD4+ and CD8+ lymphocytes,

allowed for further examination and manipulation of OX40 as an immunological cancer treatment. With recent excursion, on Phase 1 trial in patients with solid tumours, with the results being promising [81, 83–85].

1.10 Renca cell line

Arising spontaneously as a renal cortical adenocarcinoma in BALB/cCr mice, the Renca cell line is an epithelial cell that was isolated. R. Wiltrout deposited the cell in 1969. The growth pattern of this tumour mimics that of adult human renal carcinoma, in particular with regard to lung and liver metastasis [86, 87]. This cancer line can be transplantable by various routes, including intramuscular, intraperitoneal, intravenous, subcutaneous, and intrarenal, which was proven to be the most effective [86]. Intra-renal, orthotopic tumours are implanted directly into the kidney through the translucent peritoneum. A minor incision in the flank grants visualisation into the spleen. This is used as a landmark for the left kidney, and using a small gauge needle, the Renca cells are injected directly. Compared to other administrations of this cancer, this allows for the throughput investigation of metastasis. It is also non–invasive, not requiring suture materials [88]. However, in most research, including our own, the Renca cell line is injected subcutaneously (s.c). This will result in an easy-to-measure local primary tumour and also as a focal treatment point [89–93]. The use of an s.c implantation of Renca cell tumour to model human Renal Cell Carcinoma (RCC) comes with a number of limitations, such as inaccurate angiogenesis of the tumour [94], variation in the microenvironment [95, 96]. Also, it can be quite commonly seen that many s.c tumours do not metastasise to distant organs. This impedes studies focusing on the clinically common characteristic of metastasis seen with RCC [97].

2. Experiment Objective

The main objective of this experimental approach is to exploit the therapeutic potential of T-cell-mediated immunity against tumour antigens and tumour-specific antibodies. As part of anti-cancer immunity, neoantigens are expressed on the surface of cancer cells or cross-presented by dendritic cells and recognised by T-cell receptors on the surface of specific T-cells. The activation and clonal expansion of these cells can generate cytotoxic T-cells that will selectively kill the cancer cells. In order to achieve T-cell activation, the cancer or dendritic cells express co-stimulatory molecules, as mentioned, CD80 and CD86. Molecules that induce these co-stimulatory molecules are known as immune adjuvants in cancer immunotherapy. The single-stranded oligonucleotide CpG is a PAMP, which binds to the pattern recognition receptor TLR9, inducing CD80 and CD86. This cascade induces a highly effective anti-tumour response as part of a cancer vaccine/immunotherapy approach. In this project, we explored the ability of other PAMPs to show anti-cancer effects similar to CpG. In this approach, we also used an agonistic antibody against Ox40 to inhibit immunosuppressive pathways.

Two PAMPs were evaluated for anti-cancer adjuvancy. Firstly, negative-sense single-stranded RNA was obtained from the influenza virus. This acts as PAMP that can trigger TLR and RIG-I receptors. Secondly, DNA extracted from parvovirus was also assessed. This DNA has hairpin loops at its ends that stimulate TLRs and nucleotide-binding oligomerisation domain (NOD) receptors. In order to examine the anti-cancer vaccine effects of these molecules, a murine cancer model was used. Mice were then treated in the absence or presence of influenza-derived RNA or parvovirus-derived DNA and an anti-OX40 adjuvant antibody, with the objective that there should be a reduction in tumour growth, a decline or absence of metastases of cancer and also a longer survival time in those mice that received treatment.

3. Materials and Method

3.1 Manufacture of Vaccine

3.1.2 Viral Nucleic Acid Extraction

For the extraction of DNA and RNA, we used The Viral Nucleic Acid Extraction Kit II from Geneaid. This was designed uniquely for the systematic purification of viral RNA and viral DNA from cell-free samples, including serum, plasma, body fluids and supernatant of viral infected cell cultures. Utilising the efficient glass fibre spin column system, this is optimal for nucleic acid purification for a wide variety of both DNA and RNA viruses.

During this operation, a lab coat was worn, disposable gloves, protective goggles and an anti-fog facemask.

Before beginning this procedure, absolute ethanol was added to the AD buffer prior to the initial use. Also, the addition of absolute ethanol was added to the wash buffer prior to initial use.

Step one of the extraction was lysis. We used a PR8 influenza virus (mouse adopted H1N1 strain A/Puerto Rico/8/1934), which was propagated on Madin-Darby canine kidney cells (ATCC, USA), (I did not participate in this cellular work.). We transferred 200 µl of this sample to a 1.5 ml microcentrifuge tube. Following this, we added 400 µl of binding buffer (VB) lysis buffer to this sample. We mixed this by vortex, then incubated at room temperature (RT) for 10 minutes (min).

Step two was nucleic acid binding. This was initiated by adding 450 µl of AD buffer (this buffer was already mixed with ethanol) to the sample lysate. The tube was then shaken vigorously to mix. We placed the VB Column in a 2 ml collection tube, and we then centrifuged this at 15000 x g for 1 min. At the completion of this, the flow-through was discarded, and the VB column was placed back in the 2 ml collection tube. Transferring the remaining mixture to the VB Column. The 2 ml Collection tube containing the flow-through was discarded. Contents in the VB Column were then transferred to a new 2 ml collection tube.

Step three was washing the contents of the tube. This began with the addition of 400 µl of W1 Buffer to the VB Column, followed by centrifuging at 15000g x g for 30 seconds. The flow-through was discarded, and the VB Column was put back in the 2 ml collection tube. The wash buffer was then added (absolute ethanol was added to this buffer prior to the experiment). We then centrifuged it at 15000 x g for 30 seconds.

Step four consisted of the VB Column in a clean 1.5 ml microcentrifuge tube; the addition of 50 µl of RNase-free water was placed in the centre of the VB column. This was let stand for 3 minutes to ensure the RNase-free water was absorbed by the matrix. We centrifuged this then at 15000 x g for 1 min to elute the nucleic acid.

The same protocol was completed with regard the extraction of DNA from parvovirus. Parvovirus was isolated in the laboratory by Attila Csagola on swine testicular cells (ATCC, USA).

Because this method is expensive and the amount of nucleic acid is small we used another method. We mixed 200 µl cell supernatant (containing viruses) with 75 µl extraction buffer (50 mM Tris pH 7,5, 1 mM EDTA, 100 mM NaCl, 1% (w/v) SDS) and 1.5 µl (20 mg/ml) Proteinase K (Merck, USA). The sample is incubated for 120 min at 55 ºC (stirring occasionally). After the incubation, we add 75 µl phenol-clorophorm-isoamyl alcohol (ratio: 25:24:1, produced by Merck, USA) mix it and incubate 5 min in RT. After there is a step of centrifugation (5 minutes 7000 x g). The upper, aqueous phase is transferred to the new Eppendorf tube. Then 75 µl chloroform-isoamyl alcohol (24:1 ratio, Merck, USA) is added and the tube is mixed. There is another incubation period (5 min, RT) and centrifugation (3 min 7000 x g). The upper phase is placed in the new Eppendorf tube and is added 0.1 volume (7-8 µl) 3 M sodium-acetate (Merck, USA) and 2-2.5 volume (kb 150 µl) cold (-20 ºC) ethanol (96 %, Merck, USA) then incubate -20 ºC (overnight). After there is a centrifugation (5 min, 11000 x g). The supernatant is discarded and is added 150 µl 70% ethanol (Merck, USA). There is a centrifugation (5 min, 11000 x g). The supernatant is discarded and the nucleic acid is dissolved in 100 µl TE buffer (10 mM Tris pH 8, 1 mM EDTA pH 8; Merck, USA) [98].

3.1.2 Anti OX40

An OX40 receptor antibody was purchased from Cambridge, Abcam. We received this at a temperature of 4 ºC, and stored it at this temperature for short term until use. We mixed this with both our viral RNA/DNA extracts. This was mixed right before injecting, this was never stored in this mixture

Each mouse received the following;

- Group 1; 3,2 µg Influenza + 5 µg anti-CD134/ OX40L, in 200 µl pirogen-free water / mice / treatment
- Group 2 ; 1,2 µg PPV1 + 5 µg anti-CD134/ OX40L, in 200 µl pirogen-free water / mice / treatment
- Control Group ; 200 µl PBS

3.2 Cytotoxicity Effects

We did not know the cytotoxicity of the substances and the minimum effective dose. We performed an *in vitro* experiment on porcine white blood cells. Cell lysates are prepared according to the manual of Professor Tuboly. For cell culture 96-well plates (Greiner Bio-One, Austria) and 10000 cells/well were used. The cells were cultured in RPMI-1640 (Merck, USA) with 5 % foetal bovine serum (FBS; Merck, USA) and 10 ml/l antibiotic antimycotic Solution (Merck, USA). The plate was incubated for 3 days (conditions: 37 ^{0}C, 5% CO_2). After we measured the substances. The concentration was as follows; (seen in Figure 4, and the result seen Figure 5)

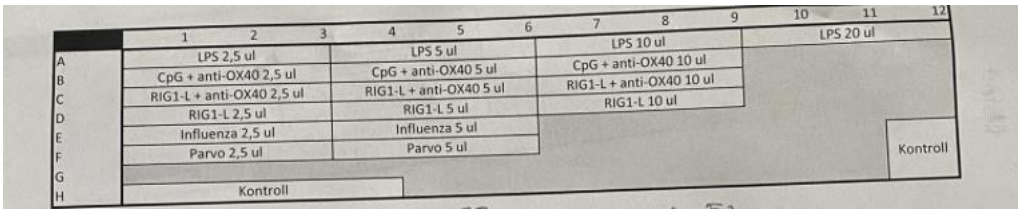

Figure 4 ; IV RNA + 2,5 ug AntiOX40, IV RNA + 5 ug AntiOX4040 and IV RNA + 10 ug AntiOX4040, ; PPV1 DNA + 2,5 ug AntiOX40, PPV1 DNA + 5 ug AntiOX4040 and PPV1 DNA + 10 ug AntiOX4040

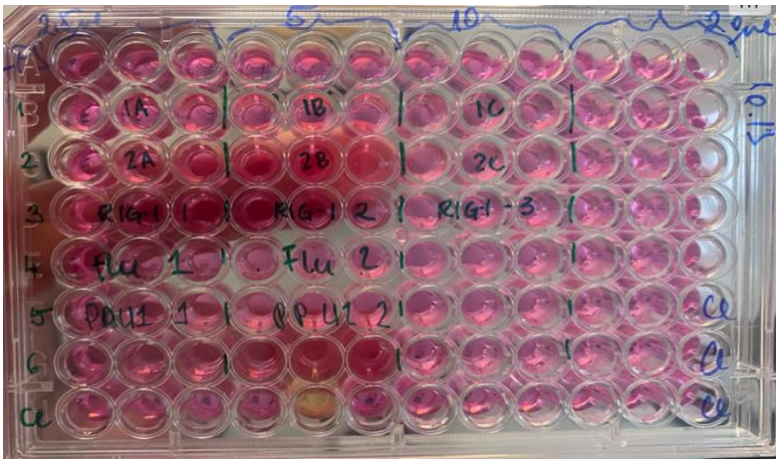

Figure 5; in vitro experiment on porcine white blood cells

3.3 Tumour Cell line used

Administration of Renca CRL-2947 mouse was used in this experiment. This is an epithelial cell isolated from a male mouse's kidney with renal adenocarcinoma. This was purchased from the American Type Culture Collection (ATCC), this is a private non-profit organisation dedicated to the authentication, acquisition, preservation and distribution of diverse biological materials for research.

The base medium for this cell is RPMI-1640 (Merck, USA). This modified medium contained 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate for use in incubators using 5% CO_2 in the air. When the cells overgrew the flask (Greiner Bio-One, Austria) these have to be passaged according to passage protocol for BIOE342 Laboratory.

3.4 Experimental Animals

In this research carried out, we had 86 murine models. These were adult, specific pathogen-free BALB/c female mice. They all weighed approximately 45-50 g. These mice were housed in our on-site in the animal house at 1143 Budapest Hungária krt. 23-25, the Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, Budapest. These mice were kept at a temperature of 20 ± 2 °C and a humidity of 50 ± 10%. The mice were housed in mouse boxes and separated into groups of 10. Ad libitum food and drinking water were provided throughout the entire experiment.

During the keeping of these animals, implementation of recommendations from the National Food Chain Safety Office, Directorate of Veterinary Diagnostics, with regards to the guidelines for the care and use of laboratory animals. These were strictly followed. The treatments that these animals received were in accordance to the experimental work plan. This was approved by the Workplace Animal Welfare Committee (MAB) (02.1/352-1/2018) and the decision of the Pest County Government Office (*PE/EA/922-7/2021)*

3.5 Inoculation of Renca Cell line - February 21, 2023 (Tuesday)

The Renca cell line was implanted at 3*10^5 cell/mice concentration. (in a 200 µl solution) s.c alongside 1 ml of the cell culture medium on the shaved and aseptic region of the right thigh muscle above the femoris muscle. 1 ml syringes with 25 gauge needles were used during administration. The mouse was appropriately fixated, and the skin was raised in a fold. Succeeding the needle penetration of the skin, an aspiration was performed with the syringe, ensuring the correct position (Figure 6). Following this confirmation, the cell

suspension was injected. Withdrawal of the needle was swiftly followed by the application of slight pressure to the injection site; this was a preventive measure aiming to cease the leakage of cells from under the skin. This was administered to 76 mice, allowing 10 to be kept for control, with phosphate-buffered saline solution (PBS) s.c administered. From this day, the mice underwent daily monitoring, which included taking note of tumour sizes, overall physical appearance, alertness, and any alteration in mannerisms or behaviour.

Figure 6; Injection of RENCA cell line adenocarcinoma

3.6 Course of treatment

After the administration of RENCA carcinoma, the mice received their first treatment 14 days later (March 3, 2023), at this stage of the cancer growth, we saw an increase in tumour size of 10 x 10 mm (± 1 mm). We had 2 treatment groups, alongside a control group, which received an equivalent dose of PBS as the treatment vaccines. Group one received influenza RNA (3 µg) alongside anti-OX40 adjuvant (5 µg), whilst Group Two received parvovirus DNA (3 µg) and anti-OX40 adjuvant (5 µg), and Group 3, being the control group, received PBS.

Treatment was administered intratumorally into the primary tumour. Following the initial treatment, the mice received an additional 2 treatments 3 days apart (March 7 and March 10, 2023). The composition of the treatments was the same as the first treatment. The control group received 3 treatments of PBS.

These groups received daily monitoring (twice a day, except weekends), comparison of median survival times alongside the average tumour growth was noted. Routine blood samples were taken, with cytokine levels being investigated. Following completion of all treatments, when there was a notable decline in animals' overall quality of life, euthanasia was performed intracardially.

3.7 Examination Process

3.7.1 Blood Sampling (March 3 and March 10, 2023)

With appropriate handling to avoid unnecessary stress, the mouse was anaesthetized with Isoflurane. Blood was retrieved from the heart. Disinfection of the skin was carried out with alcohol wipes. 27G needle was used, and a 1 ml syringe was used to obtain samples.

Blood taken was placed in a heparin tube, the plasma and the white blood cells were stored until used.

Cytokine levels, with a particular interest in IFN-γ, IL-6, and IL-10 were examined with an ELISA test (Thermo Fischer Scientic, USA). The ELISA tests were performed according to the manufacturer's instructions. The results were measured on a Multiskan Ex ELISA reader from Lab system (Thermo Fischer Scientic, USA).

3.7.2 Autopsy

With appropriate personal protective equipment worn. The dead mouse was removed from their group and brought to an aseptic room, here we had all the necessary sterile dissection tools, which included scissors and forceps.

The mouse was placed in a supine position on a clean dissection tray. External examination with interest in initial tumour size was noted. The dissection began with a midline cut on the ventral aspect of the mouse. This was continued up cranially. During this process, the skin was peeled off, which usually revealed the primary tumour at the femoris muscle. This is where our first sample was obtained – the tumour size, consistency, and surrounding demarcation were examined. The abdominal cavity was opened, inspection of organs was carried out, localisation of these organs where examined, and in case of metastatic spread of the tumour, visibly affected organs were studied also. The dissection was then continued, opening the chest cavity where the lungs and heart were examined. The primary objective of our post-mortem dissection was to find metastases and locate the cause of death. It is typical nature of renal adenocarcinoma to be an aggressive cancer, seen metastasising to the lungs and liver.

After the autopsy was performed, samples were taken from the tumour, spleen, liver, kidneys and lungs for histopathological examination. The organs of these mice were stored at 8 % formalin until the test was performed (as shown in Figure 7).

Tumour samples were obtained at the border of the healthy and the affected areas. This was to avoid necrotic central regions. The lungs were removed from the body and attached to the heart. These were separated *ex-situ*, and samples were placed into formalin.

3.7.3 Histopathological Examination

The sections made from the samples were processed in the laboratory of the Veterinary Diagnostic Directorate of the National Food Chain Safety Office. Hematoxylin – eosin was used for staining. Microscopic examination was performed by Márta Lőrincz and Béla Dénes, I also checked these.

Figure 7; Dissection of Mice, 8% Formalin Tubs

3.7.4 Recording Results

Cytokine result were submitted to Microsoft Excel, where bar charts demonstrating these results were generated.

We began comparing tumour sizes following the final treatment. This was day 17 after inoculation with the renca cell line. This was continued with observations and measurements every 7 days until day 38. At this point, there began to be no significant differences between the tumour growth.

We interpreted this information with the Mann-Whitney test. Simultaneously, whilst taking records of tumours, survival rates were also being noted and recorded. For this, we used the Log-rank test, also known as the Mantel-Cox test.

Results

4.1 Blood Test Results

Cytokine ELISA tests (preliminary results)

Table 1 shows the results of the cytokine measurements. The table processes the blood results taken on the 17th day after implantation (March 10, 2023).

Table 1*: Results of the cytokines*

	IFN-γ (pg/ml)	**IL-6 (pg/ml)**	**IL-10 (pg/ml)**
Group 1; RNA + anti-OX40			
Tumour -, IV-RNA + anti-OX40 I.	neg.	neg.	neg.
Tumour -, IV-RNA + anti-OX40 II.	20,5	neg.	neg.
Group 2; DNA + anti-OX40			
Tumour -, PPV1-DNA + anti-OX40 I.	42,5	56	1666
Tumour -, PPV1-DNA + anti-OX40 II.	neg.	12	neg.
Group 3; No Treatment – Control			
Tumour +, no treatment I.	neg.	neg.	350
Tumour +, no treatment II.	13,125	neg.	190

The IL-10 can be seen as negative in Group 1, the untreated group the cytokine production is higher than the normal range (4.8-9.8 pg/ml). In principle, the ELISA test measures an IL-10 level below 39 pg/ml as negative and gives a reliable result above 78 pg/ml. The result of the first sample of parvovirus DNA + anti-OX40 group turns out to be false positive due to the extremely high cytokine level. The second sample gave a negative result as expected. The investigation yielded the expected results.

IL-6 was seen to be most elevated in Group 2, treated with parvovirus RNA. This was an unexpected result, as in the control group, we had negative results for IL-6. In Group 2 it was also observed that the first of the two samples gave a consistently higher cytokine level, but the second was also higher than the others. This ELISA is much more sensitive than the previous one. In principle, it can detect 0.6 pg/ml (practically 1.25 pg/ml). The physiological value of the Il-6 level can vary within wide limits, and there is no uniform standard value in the literature. Regardless, we can suspect that inflammation develops after parvovirus treatment.

In the case of the IFN-γ level, one animal in each group gave a negative result, in the case of the other animal, group 2 gave the highest value, and the control had the lowest.

In a healthy animal, the physiological level of the cytokine remains below 10 pg/ml. In this case too, it can be said that the cytokine level should have been measured earlier.

4.2 Survival Times

Survival times fluctuated throughout the experiment. However, there was a pronounced difference in survival times for the RNA influenza treatment in comparison to the DNA parvovirus treatment and the control mice (Figure 8 present the survival times).

Using the Log-rank test, also known as the Mantel-Cox test, we fabricated this data. We calculated p values and significance, resulting in the below curve known as the Kaplan Meier curve. (Figure 8).

On day 38 post-implantation, the survival rates for the various groups were as follows;

Group 1; IV-RNA + anti-OX40 ; 60%
Group 2; IV-DNA + anti-OX40; 40%
Group 3; Untreated (PBS) 20%

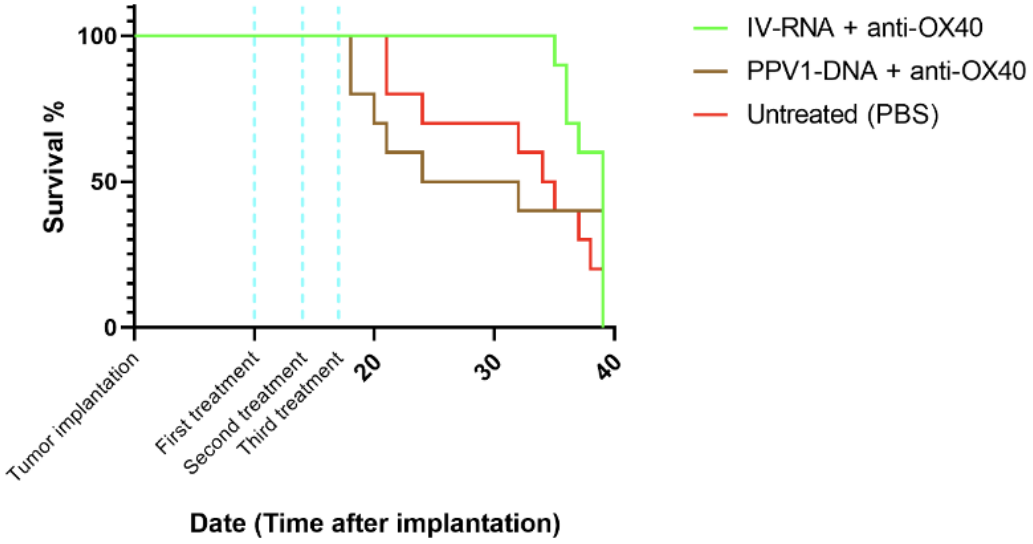

Figure 8; Survival times of the three groups

4.3 Tumour Size

Slight differentiation in tumour sizes was seen throughout the experiment, with the notable largest tumours seen in Group 3, the untreated (PBS) group, second largest seen in Group 2, PPV1 + DNA + anti-OX40. The most promising results are seen in Group 1, IV-RNA + anti-OX40.

To generate this data, we used the Mann-Whitney U statistical test, comparing the treated groups to those untreated.

Measurements of the primary tumour sizes were conducted following the final treatment on day 17 and then again every 7 days until day 38. On day 17, there were no significant disparities in primary tumour sizes within the treated groups, Group 1 and Group 2. However, a significant difference was noted in the untreated Group 3 to both Group 1 and Group 2. On day 24, there was a notable difference between Group 1, IV-RNA + anti-OX40, having a smaller tumour size in comparison to PPV1-DNA + antiOX40. At day 38, there began to be no significant differences in tumour sizes were evident among the groups. The primer tumour sizes are presented in Figure 9, the significance values are shown in Table 2.

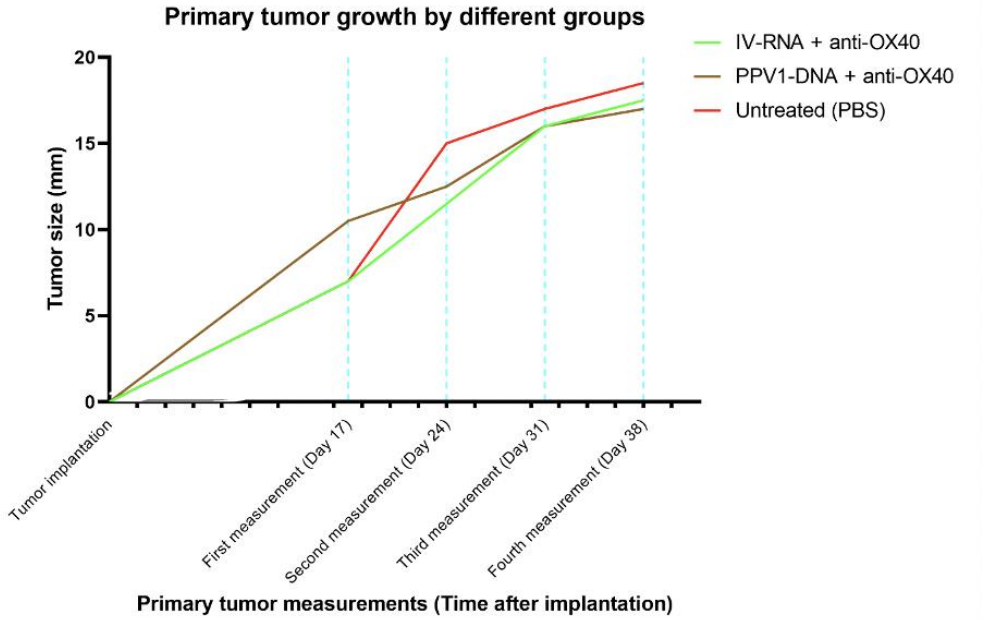

Figure 9 ; The average primary tumour size of the three groups

Table 2*: significant results of the groups compared to the control at the four measurement times*

	Day 17	Day 24	Day 31	Day 38
Group 1. IV-RNA + anti-OX40	0.9587	0.0002	0.1693	0.6000
Group 2 PPV1-DNA + anti-OX40	0.00014	0.0146*	0.3782	0.5238

*: Larger tumour size in the untreated group!
Red numbers show significant results

4.4 Metastases

Histological examination of our samples taken from the specimens were inspected for metastases and growth.

These were stored in 8% formalin until they were stained with haematoxylin-eosin and investigated. In certain cases, we were unable to find any specific metastasises, an occurrence in the primary tumour disseminated within the confines of the abdominal cavity along with ascites was seen in certain cases. Although these cannot be categorised as traditional metastatic events, however, the untrammelled spread of the tumour occurred in these cases as well.

Ascites was seen in Group 2 and the control. In the Control Group, a tumour was found in the pancreas and in Group 2 cases a metastasis was found in an ovary (Figure 10). (The exception of these organs did not form a part of the normal protocol, they were included randomly.) Lung metastasis was found in 44% of the control mice, 38% of the mice treated with parvovirus DNA, and only 13% of influenza RNA treated mice. In the group 1 no tumour was visible in the abdominal cavity. Table 3 shows the results of metastasis.

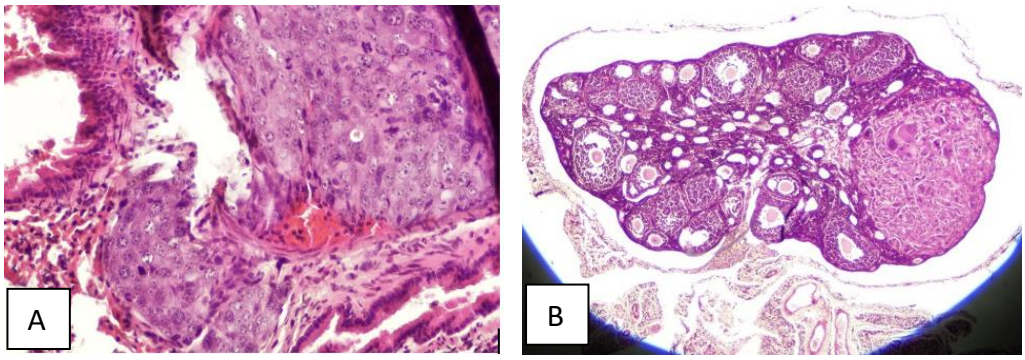

Figure 10; A: lung metastasis, B: tumour in an ovary

Table 3: *The results of the metastasis*

Treatments	Mice with metastases (%)			
	Lung	Kidney	*Abdominal cavity**	Total
IV-RNA + anti-OX40	13 (1/8)	0	*0*	**13 (1/8)**
PPV1-DNA + anti-OX40	38 (3/8)	0	*2***	**63 (5/8)**
Untreated (PBS)	44 (4/9)	0	*33 (3/9)*	**78 (7/9)**

Notably, discernible variations in primary tumour characteristics were observed: treated Group 1 IV-RNA + anti-OX40 demonstrated incipient demarcation, whereas the untreated (PBS) group exhibited diffuse primary tumours accompanied by necrosis. Figure 11 shows a piece of the primary tumour of an untreated animal. The lack of demarcation and blood vessels (1) penetrating the tumour can be observed, and the giant cells (2) characteristic of malignant tumours can also be observed.

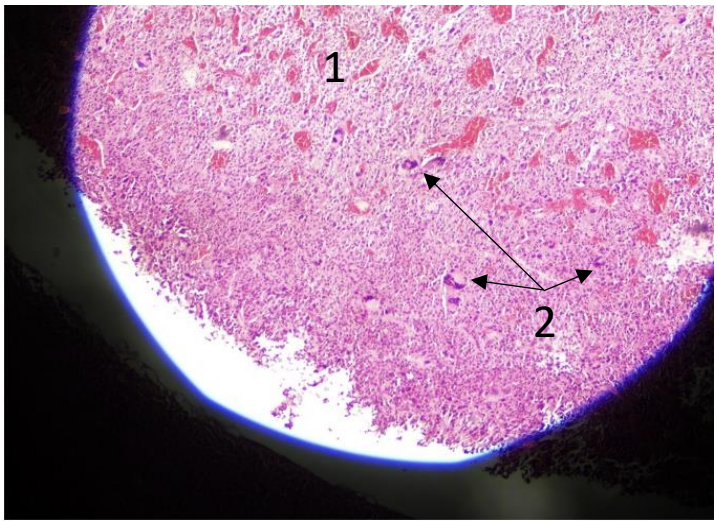

Figure 11 ; A piece of the primary tumour Number 1: blood vessels, number 2: giant cells

5. Discussion and Conclusion

Interleukin 10 (IL-10) belongs to the cytokine family, acting as a potent anti–inflammatory. This protein has a crucial role in limiting immune response. Therefore, it is an important molecule, preventing further damage to the host, and maintaining physiological tissue homeostasis. Upregulation of IL-10 is associated with enhanced immunopathology to infection or particular autoimmune diseases [99].

The results we used showed that the IL-10 level was much lower compared to the placebo (control group). According to our assumption, our treatment inhibited the function of the Tregs, and an immune response took place after the treatment. This result corresponds to what we expected.

Interleukin 6 (IL-6) is seen throughout the inflammatory process. Acute inflammation is mediated by IL-6. When this proinflammatory cytokine persists, we see a turn to chronic inflammation, thus leading to the elevation of circulating IL-6 seen in blood [100].

Based on the results, parvovirus DNA increased IL-6 levels. Since the study was performed after the last treatment of the animals, we do not know whether the treatment had an effect on the IL-6 level in the other two cases. Far-reaching conclusions cannot be drawn based on the small number of samples. It is conceivable that parvovirus treatment generates a longer-term inflammation, which can be an effective adjuvant.

Our last cytokine of interest was INF-γ, a pleiotropic cytokine with antiviral, antitumour and immunomodulatory characteristics, playing an important role in coordinating both innate and adaptive immune responses. INF- γ is seen to be an important adjuvant in oncotherapy, with the ability to inhibit angiogenesis in tumour tissue, induction T-cell apoptosis, stimulation of proinflammatory macrophages, overcoming tumour progression [101].

In the case of this study, it was clear that two animals per group is very few, and a clearly higher sample number is needed in order to draw any conclusions. Since one of the two animals was negative in all three groups, we would not even attempt to determine trends.

In the abdominal cavity, the tumours were lined up like a string of pearls. During the histological examination of the parvovirus-treated and control group, in several cases, the tumour did not invade the organs, but we found them in the case of the kidney and the liver. Presumably, if the animal survives a few more days, metastasis will occur here as well.

Pneumonia could also be seen in all three groups. We do not know the exact reason for this. Since it was also observed in untreated animals, it cannot be considered a side effect of the treatment. We did not observe any symptoms typical of other side effects.

The survival time and the size of the primer tumour make it clear that three treatments are not enough. More treatments or a prolonged effect are needed. The materials we use have both advantages and disadvantages. The long-term stability of natural nucleic acids is not guaranteed, so a solution must be found. Synthetic materials are stable, but because of this, they are also toxic.

With the conclusion of the experiment, it can be confidently assumed that the above treatment possesses a substantial promise for future treatments. Tangible outcomes were observed, with the most prominent being prolonged survival time of mice, reduction of primary tumour sizes, and mitigated metastatic formation. Following the final treatment,

there was a considerable decline in the animals' quality of life, with this, we decided to terminate the experiment.

It is also possible to combine treatments. Currently, influenza RNA testing offers promising opportunities. It may even be worth experimenting with combining other low molecular weight substances (natural CpG) and other types of alternative treatments (immunological cell death-inducing treatments, oncolytic viruses).

In light of our findings, it can be ascertained that these treatments hold the potential for a solid foundation in onco-immunotherapy. With further exploration, modification of composition, and adjustments in treatment frequency, there exists a high possibility for enhancement of these achieved results.

6. Abstract

Nowadays, immuno-oncotherapy is an innovative treatment for tumours. The exploitation of the therapeutic potential of tumour-specific antibodies, along with the manipulation of cellular mechanisms of the immune system, prove to be the most important direction. The application of nucleic acid vaccines is intended to intervene in the latter form.

Negative, single-stranded RNA of the influenza virus and the single-stranded, hairpin DNA of the parvovirus act as pathogen-associated molecular patterns (PAMPs), which are connected to the body's toll-like receptors (TLRs). Innate immunity is activated, while the treatment also indirectly stimulates the adaptive immune system, thus providing invaluable anti-tumour protection. Influenza virus RNA also results in the activation of retinoic acid-inducible gene 1 (RIG-1), while parvovirus hairpin activates nucleotide-pair oligomerisation action domain receptors, the NOD-like receptors (NRLS). The activation of RIG1 and NRLS - in any of its different forms - inhibits the formation of tumours.

In our experiment, we examined the effect of the nucleic acid of these viruses on tumours. Renal adenocarcinoma was implanted subcutaneously in BALB/C mice, and then an intratumoral vaccine treatment was applied to the animals. Group 1 received purified influenza RNA and an anti-OX40 adjuvant. Group 2 individuals received purified parvovirus DNA and an anti-OX40 adjuvant as treatment. Mice were monitored daily, taking note of the median survival time and the change in the mean primary tumour over time. We also took blood for the examination of cytokine levels. The metastases of the primary tumour were followed by histological examinations. We compared our results with a group of untreated tumour (control) mice (Group 3).

Our results showed that Group 1 was significantly more promising than Group 2. The group treated with influenza virus RNA achieved a significantly higher median survival time than the other two groups, and the tumour sizes were significantly smaller. Although lung metastases could be detected in all three groups, the lowest occurrence rate was observed in Group 1. No significant changes in cytokine levels were observed comparing the three groups.

Based on the generated data, we can establish that our treatments provide an effective basis for future research. Through modification of composition and adjustments in treatment frequency, there exists the potential for further enhancement of efficacy.

6.1 Összefoglaló

Az immun-onkoterápia napjainkban egy újszerű daganatkezelési módszer lehetőségével kecsegtet. A daganatspecifikus ellenanyagok terápiás potenciáljának kiaknázása mellett az immunrendszer celluláris effektor mechanizmusai bizonyulnak a legfontosabb irányvonalaknak. A nukleinsav-oltások alkalmazása az utóbbi formában kíván beavatkozni.

Az influenzavírus negatív, egyszálú RNS-e és az egyszálú, hajtűvégű parvovírus DNS a kórokozóval összefüggő molekuláris mintázatként (PAMP-ként) működnek, melyek a szervezet toll-like receptoraihoz (TLR-ek) is kapcsolódnak. A veleszületett immunválaszt beindítja a kezelés, miközben közvetetten az adaptív immunrendszert is stimulálja. Így felbecsülhetetlen daganatellenes védelmet nyújt. Az influenzavírus RNS a retinsavval indukálható gén I (RIG-I) aktiválását is eredményezi, míg a parvovírus hajtűje aktiválja a nukleotidkötő oligomerizációs domén receptorokat (NRLS). A RIG-I és az NRLS aktivációja – bár különböző formákban – gátolja a daganatok kialakulását.

Kísérletünkben ezen két vírus nukleinsavának a daganatokra kifejtett hatását vizsgáltuk. Veseadenokarcinómát BALB/C egerek bőre alá implantáltunk, majd intratumorális vakcinakezelést alkalmaztunk az állatokon. Az 1. csoport tisztított influenza RNS-t és anti-OX40 adjuvánst, a 2 csoport egyedei tisztított parvovírus DNS-t és anti-OX40 adjuvánst kaptak kezelésként. Az egereket naponta ellenőriztük a túlélési idő mediánjának és az átlagos primer tumor időbeni változásának követése céljából. Vért is vettünk citokinszintek meghatározása miatt. A daganat metasztázisait szövettani vizsgálatokkal követtük. Az eredményeinket kezeletlen daganatos (kontroll) egerek csoportjával (3. csoport) hasonlítottuk össze.

Eredményeink szerint az 1. csoport lényegesen ígéretesebb volt, mint a 2. csoport. Az influenzavírus RNS-sel kezelt állatok szignifikánsan magasabb medián túlélési időt éltek meg, mint a másik két csoport, és a tumoraméreteik is lényegesen kisebbek voltak. Ugyan mindhárom csoporban ki lehetett mutatni tüdőmetasztázisokat, de a legalacsonyabb előfordulási arányt az 1. csoportban észleltük. A citokinszintek jelentős változását nem tapasztaltuk a három csoportot összehasonlítva.

A keletkezett adatok alapján megállapíthatjuk, hogy kezeléseink a jövőbeli kutatásokhoz hatékony alapot adnak. Az összetétel- és a kezelési gyakoriság módosításával lehetséges az eddig elért eredmények javítása.

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Köszönöm Szépen

9. Supporting Documents

9.1. Supervisor Approval

Témavezetői nyilatkozat TDK dolgozathoz

Alulírott, **Lőrincz Márta**, mint témavezető nyilatkozom, hogy **Katie Commins**, 6. évfolyamos hallgató „**Using low molecular weight substances to manipulate the innate immune response**" című dolgozatát átolvastam és jóváhagytam, részvételét támogatom az Állatorvostudományi Egyetem 2023. évi Tudományos Diákköri Konferenciáján. Továbbá nyilatkozom, hogy a feltöltött TDK dolgozat plágiumellenőrzésen sikeresen átesett és az esetlegesen feltárt egyezőség az Egyetemi iránymutatásoknak/szabályoknak megfelel.

Budapest, 2023. október 20.

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témavezető

Témavezetői nyilatkozat TDK dolgozathoz

Alulírott, **Gulyás Dominik Ádám**, mint témavezető nyilatkozom, hogy **Katie Commins**, 6. évfolyamos hallgató „**Using low molecular weight substances to manipulate the innate immune response**" című dolgozatát átolvastam és jóváhagytam, részvételét támogatom az Állatorvostudományi Egyetem 2023. évi Tudományos Diákköri Konferenciáján. Továbbá nyilatkozom, hogy a feltöltött TDK dolgozat plágiumellenőrzésen sikeresen átesett és az esetlegesen feltárt egyezőség az Egyetemi iránymutatásoknak/szabályoknak megfelel.

Budapest, 2023. október 20.

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témavezető

DECLARATION

I hereby declare that the thesis entitled ‘’ Using low molecular weight substances to manipulate the innate immune response. Manipulation of innate immune response with viral RNA & DNA ‘’ is identical in terms of content and formal requirements to the TDK research paper submitted in 2023.
Date: 5th November 2023

Katie Teresa Commins

Thesis progress report for veterinary students

Name of student: Cummins Katie Teresa

Neptun code of the student: LK1P09

Name and title of the supervisor: Mária Lőrinczi, Senior Lecturer / Dominika Gulyás, Department Engineer

Department: Department of Microbiology and Infectious Diseases

Thesis title: Using low molecular weight substances to manipulate the innate immune response

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	02	22	Designing experimental plan	
2.	2023	03	02	Designing experimental plan	
3.	2023	04	25	Execution of toxicity pre-experiment	
4.	2023	05	05	Evaluation of the results of toxicity pre-experiment	
5.	2023	06	01	Execution of animal experiment	

Grade achieved at the end of the first semester: excellent (5)

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	06	05	Execution of animal experiment	
2.	2023	07	15	Evaluation of the results of animal experiments	
3.	2023	09	25	Discussion of thesis content	
4.	2023	10	01	Discussion of thesis content	

5.	2023	1a	10	Discussion of thesis contents	[illegible]

Grade achieved at the end of the second semester: excellent (5)

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

[illegible]
signature of the supervisor

Signature of the student: Katie A. Lonning

Signature of the secretary of the department: [illegible]

Date of handing the thesis in……