Tumor formalin fixed vaccines and secondary immune response to elicit a therapeutic immune response in solid tumors

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To my mother and father

For their unlimited support.

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Abstract

In the fields of treatment of metastatic tumour disease and its recurrence there were not a lot of significant success. It seems that this was mainly due to complexity of tumour disease, effect of the tumour microenvironment and high adaptability of tumour cells as well as the most therapeutic approaches are targeting singular tumour protein or gene which can be easily self-modified that as result have therapy failure and progression of the tumour. In this study, by developing of the autologous tumour formalin fixed vaccines we tried to tackle these main issues in the treatment of solid tumours. We have used two murine models of the carcinogenesis in order to get better insight in the features of possible anti-tumour immune response. Tumour antigens were obtained from the animal's tumour tissue biopsies, processed and injected into the animals alone or along with the Rotarix. First immunisation was performed with the Rotarix or the AFFTV, while different combinations of the AFFTV and Rotarix were used for the second immunisation, so that the secondary immune response could be obtained. In the chemical carcinogenesis model, AFFTV made of tumour tissue biopsy in combination with a Rotarix induced a significant increase in the infiltration of lymphocytes in the primary tumour (liver), in vaccinated animals as compared to control. In the transplantable model of carcinogenesis, AFFTV made of the tumour cell line alone, when administered via subcutaneous route, have induced the highest score of lymphocyte infiltration in the subcutaneous tumour as well as in the lungs, as compared to other combinations. However, in the second experiment, the AFFTV made with the combination of tumour tissue biopsy and tumour cell line, injected with the Rotarix intraperitoneally, have induced highes lymphocyte infiltration in the subcutaneous tumour as compared to other combinations however the median survival of this group was the lowest as compared to other. The flow cytometry analysis was performed in order to quantify the $CD8^+$ T cells. It was observed that amount of these cells is highest in the control group as compared to vaccinated one, seven days after second immunisation with the combination of the AFFTV and the Rotarix, intraperitoneally. The small sample size and certain effect that AFFTV vaccine formulation induced provides the rationale for further study with larger sample size.

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4TI	Murine mammary tumour cell line
Ab	Antibody
AF	Alexa Fluor
AFFTV	Autologous formalin fixed tumour vaccine
ANOVA	Analysis of variance
APCs	Antigen presenting cells
b value	Point where the line of standard curve intercepts the y axis
B16	Murine melanoma cell line
BCG	Bacillus Calmette Guerine
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation
CD40	Cluster of differentation 40
CD40L	CD 40 ligand
CD80	Cluster of differentation 80
CD86	Cluster of differentation 86
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CO_2	Carbon dioxide
CPD	Citrate potassium dextrose

CpG oligo	Cytosine-phosphate-guanosine oligonucleotide
CT26	Cytosine-phosphate-guanosine oligonucleotide
CTLs	Murine colon tumour cell line
CTLs	Cytotoxic T lymphocytes
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DEN	Diethylnitrosamine
df	Degree of freedom
diH_2O	Deionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Dibuthylphtalate polystyrene xylene
dsRNA	Double-stranded ribonucleic acid
ELB	Erythrocyte lysis buffer
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme – linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot
etc.	And other similar things
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FC	Flow cytometry
FOXP3	Forkhead box P3 protein

FREC	Faculty research ethical committee
FSC	Forward scatter
GM – CSF	Granulocyte macrophage stimulating factor
H&E	Haematoxilin and eosin staining
H_0	Null hypothesis
H_1	Alternative hypothesis
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
ICS	Intracellular flow cytometry staining
IFN γ	Interferon γ
IL 10	Interleukin 10
IL-12	Interleukin 12
IL-2	Interleukin 2
k value	Kappa value
LAMP	Lysosomal associated membrane protein
m value	Slope that is multipled on the x axis
MDA 5	Melanosomal differentiation-associated protein 5
MDSCs	Myeloid derived suppressor cells
MHC	Major histocompatibility complex
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II

MT450	Murine mammary tumour cell line
n	Number of animals
n.d.	No date
n.s	Not significant
NK cells	Natural killer cells
NMOR	N – Nitrosomorpholine
NSAID	Non-steroidal anti – inflammatory drug
p	Probability value
p_0	Observed agreement
P60	Sixty days old
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
p_e	Expected agreement
PMT	Photomultiplier tube
poly I:C	Polyinosinic : polycytidylic acid
ppm	Parts per million
R^2 value	Coefficient of determination
RBCs	Red blood cells
RIG I	Retinoic acid inducible gene I-like receptor
rpm	Rounds per minute
RPMI	Rosswell Park Memorial Institute
s.c.	Subcutaneous
SD	Standard deviation

SSC	Side scatter
T reg	T regulatory cells
TAAs	Tumour associated antigens
TGF β	Tumour growth factor β
TILs	Tumour infiltrating lymphocytes
TLRs	Toll – like receptors
TNBC	Triple-negative breast cancer
TNF α	Tumour necrosis factor α
Trypsin/EDTA	Trypsin/ Ethylenediaminetetraacetic acid
TSAs	Tumour specific antigens
VEGF-C	Vascular endothelial growth factor C
VEGF-E	Vascular endothelial growth factor E
vs.	Versus
WHO	World Health Organisation
x value	Protein concentration
y value	Value on the y axis (absorbance)
Z score	Standard score

Chapter 1:

Introduction

1.1. Cancer

Cancer is the second leading cause of death in the world, with more than 9 million people estimated to die from this disease each year (World Health Organisation. 2018). Cancer cells can be defined as cells that are uncontrollably dividing and proliferating. These cells can invade normal surrounding tissue and organs and distant tissues and organs due to metastatic capability. All these features are a result of accumulated abnormalities in crucial regulatory mechanisms of those cells (Cooper *et al*., 2000; Hanahan and Weinberg. 2000). Along with abnormal proliferation, cancer cells are not differentiating properly and as such never become fully differentiated and don't undergo programmed cell death - apoptosis. They are blocked in the early stage of differentiation at which they retain the proliferative capacity and continue to reproduce (Cooper *et al*., 2000). These cells survive even in the absence of survival signals such are various growth factors that increase the survival of metastatic cells in distant tissue sites (Hannahan and Weinberg. 2000; Jiang *et al*., 2015). These cells can secrete growth factors that can induce the formation of new blood vessels, a process called angiogenesis, which can further support tumour growth and metastasis (Chaffer and Weinberg, 2011). As well, certain growth factors such as vascular endothelial growth factors C and E (VEGF C/E), can induce the formation of new lymph vessels, in the process called lymphangiogenesis (Quagliata *et al*., 2014). It is considered that metastatic disease is responsible for 90% of deaths in cancer patients (Seyfried. 2012; Chaffer and Weinberg, 2011). Cancer cells are in most cases successfully evade the immune response with the use of different strategies including the inhibition of the immune cells or the switching of their phenotype from effector to regulatory, etc. (Seyfried *et al*., 2014). It is known that a presence of regulatory T cells, increased presence of the immunosuppressive molecules such as interleukin 10 (IL - 10) and/or tumour growth factor β (TGF- β) can suppress anti-tumour immune response upon immunisation (Budhu *et al*., 2006; Li *et al.*, 2015). It is known that inflammation can be initiated due to invasion of the lung tissue by the tumour cells which further results in the disturbance of homeostasis (Chen *et al.*, 2018; Gomes *et al*., 2014; Chen *et al.*, 2018).

1.2. Overview of the immune system

Two main components of the immune system are innate and adaptive immunity (Marshall *et al*., 2018). Innate immunity presents the first line of defence and includes various types of cells that can recognise a broad specter of molecules present on pathogens or their products and act on them

(Chaplin. 2010). The innate immunity encompasses various levels of defence and includes various cells and molecules (Cruvinel *et al*., 2010). Cells include neutrophils, macrophages and natural killer cells (NK cells) (Chaplin. 2010). Specific pattern recognition receptors expressed both on the membrane surface and intracellularly, are part of innate immunity (Chaplin, 2010). These receptors recognise and interact with specific microbial patterns called pathogen-associated molecular patterns (PAMP) which further results in the initiation of the immune response (Medzhitov, 2007). These receptors can recognise the damage-associated molecular patterns (DAMPs) derived from the dying self- cells (Dear. 2016; Thompson *et al*., 2011). Some of the most important cell populations that form part of innate immunity are antigen presenting cells (APCs) that mainly consists of macrophages and dendritic cells (DCs). They are mediators between innate and adaptive immunity and as such are extremely important for the proper functioning of the immune system (Rogers *et al*., 2017). The main features of the innate component are a considerable immune response without the ability to form a memory and as such cannot be targeted with therapeutic approaches such as vaccination (Turvey and Broide, 2010). Opposite to the innate component, the adaptive component is characterised by having a memory function and high specificity and as such it is very adapted for vaccine strategies (Marshall *et al*., 2018). Cells of the adaptive component are present in a small number in normal conditions but have the ability to proliferate and increase number drastically upon adequate stimuli (Marshall *et al*., 2018). The adaptive component consists of both B and T cells (Akira *et al.*, 2009). T and B lymphocytes recognise specific antigens through receptors situated on their surface. Recognition of specific antigens by these receptors initiates a cascade that will result in the elicitation of a specific adaptive immune response (Medzhitov. 2007). Cells need to recognise both the pathogenic component and the self- structure on the cell surface in order to be activated. Therefore normal or antigen presenting cells express specific molecules on their surface which allow recognition, binding and presentation of foreign or aberrant self- antigens. These specific molecules belong to major histocompatibility complex (MHC) family that can bind peptide fragments that are a product of intracellular synthesis (MHC I) or peptides that bind to them extracellularly (MHC II) after which they are internalised, enzymatically processed and presented on the cell surface (Chaplin, 2010).

Presentation of endogenously synthesised peptides in cells can be up-regulated under the influence of interferon γ (IFN γ), which can be secreted in response to viral infection or as a consequence of the action of homeostatic mechanisms, in the case of aberrant or damaged proteins (Murphy *et al*., 2007). This is extremely important for the recognition of these cells by cytotoxic $CD8^+$ T (CTLs) lymphocytes that can eliminate these cells and prevent viral infection or tumour formation (Janeway

et al. 2001). Professional antigen presenting cells such as DCs bearing MHC II protein complex can ingest microbial protein or tumour - derived extracellular proteins and adequately process and present it to CTLs. This process is called cross-presentation (Rodriguez *et al*., 1999). Upon activation, the DCs will upregulate MHC II and secrete proinflamatory cytokines such are interleukin 2 (IL-2), IL-12 or interferon. As well, these cells will up-regulate the additional costimulatory molecules such as CD80 or CD86 and CD40 (Fuertes *et al*., 2011). These DCs will migrate and transport the antigen to the draining lymph nodes where they are able to prime the $CD8^+$ T cells. Upon priming, these $CD8^+$ T cells migrate to the tissue where the antigen was acquired, and upon recognition of antigen for the second time and stimulation from the infected cells or the APC through cytokines or cell surface contact T cells will become effector cells and try to eradicate infection (Banchereau and Steinman, 1998; Bennet *et al*., 1998; Curtsinger *et al*., 2005; Rescigno *et al*., 1999). The DCs can further represent these antigens to $CD4^+$ T cells which as a result can help elicitation of the immune response. (Bonilla and Oettgen *et al*., 2010; Turley *et al*., 2000). After exposure, $CD4^+$ T cells slowly transition from the naive to the effector phenotype (Abbas and Lichtman, 2003; Sallusto *et al*., 2009).

1.3. Conventional therapy

Although conventionally used treatments such as surgery, chemotherapy and radiotherapy are used with limited results, there is still a lot to be done in improving these statistics (Emens, 2008; Aldrich *et al*., 2010). Surgery as one of the most commonly used treatments, does not always eradicate tumour and due to incomplete tumour removal, it increases the chance of recurrence (Aldrich *et al*., 2010). Chemo- and radio - therapy although most commonly used treatment options for inoperable tumour, might increase immune tolerance and reduce or inhibit immune response (Aldrich *et al*. 2010).

1.4. Immunotherapy

The idea that the immune system plays a vital role in tumour destruction is very old and it is a foundation of immunotherapy (Couzin - Frankel. 2013). It is well known that the immune system is involved in the extremely complex process of interaction with tumour cells, in which it plays a crucial role in the detection, control and eradication of a tumour threat (Suckow. 2013; Galon *et al*. 2006). The presence of cancer suggests that the immune system failed in the eradication of neoplastic formation, yet there is still a possibility that the immune response could be activated and directed towards cancer eradication (Hanahan and Weinberg. 2011; Iwasaki and Medzhitov. 2004).

The immune response is primed with the presentation of antigens that are foreign, non-self or aberrant-self such as tumour associated antigens (TAAs) or tumour specific antigens (TSA) (Finn *et al*., 2017; Pan *et al*., 2018). TAAs and TSAs are essential for tumour propagation and survival (Schumacher and Schreiber. 2015). TAAs can be shared between tumours of similar pathology while TSAs are the ones that are unique for each tumour. TSAs are also called neo-antigens or mutated highly specific antigens (Budhu *et al*., 2014; Elliott. 2012; Rosenberg and Restifo, 2015). TAAs and TSAs can be used in cancer treatment by promoting and enhancing the interaction between the immune system and the tumour (Melief *et al*. 2015). In order for anticancer therapy to be effective in killing of cancer cells, various steps need to be initiated and accomplished (Chen and Mellman, 2013). According to Chen and Mellman (2013), the first step should be recognition and capturing of TAAs and TSAs by the APC. Processed antigens are then presented on MHC I or II to T cells. Along with this, in order to direct this process towards proinflamatory and antitumor immune response, specific proinflamatory signals should be released and recognized. These signals include DAMPs released by dying tumour cells or certain proinflamatory cytokines such as interleukin 1 (IL - 1), tumour necrosis factor α (TNF α), interleukin 6 (IL - 6) (Dear. 2016; Cho *et al*., 2013) .Combination of these two processes should overcome peripheral tolerance mechanisms and distinguish these antigens as aberrant or foreign ones so the proper antitumour response can be initiated. Antigens released as a consequence of cancer cell destruction tend to promote and enhance the antitumoural response (Chen and Mellman, 2013).

In most cancers, various obstacles in cancer biology and the biology of interaction between cancer and the immune system disrupt this process (Motz and Coukos, 2013). Immunotherapy tends to overcome obstacles led by low immunogenicity of aberrant antigens, immunotolerance, different evading systems that tumour use, and initiate an effective antitumour response. Different modalities of immunotherapy exist and include cancer vaccines, adoptive T cell therapies, targeted antibody therapy and immune - checkpoint blockade (Chen *et al*., 2012; Grupp *et al*., 2013; Predina *et al*., 2013; Wang *et al*., 2013).

1.5. Cancer vaccines

Cancer vaccination is one of the most promising immunotherapeutic modules. There are various approaches used in cancer vaccination (Schlom *et al*., 2014).

It was considered that regarding TAAs are increasingly expressed in tumours, and not significantly or not at all present in the normal adult tissue, they could be good targets to be used in the

construction of the cancer vaccines (Butterfield. 2015). However, most of these antigens have not provided results in actual trials. It is suggested that the main reason for this is the non - existence of the high affinity T cells that are highly specific for these antigens as they are probably eliminated or suppressed as a consequence of the immunetolerance mechanisms regarding that these antigens are present in normal tissue as well (Boon *et al*. 1994; Finn *et al*., 2017; Vollmer *et al*., 1999).

The TSAs are considered as only highly tumour specific antigens that are mostly products of crucial mutations in cancer cells and as that, perfect targets for the tumour specific therapy. It is thought that therefore these antigens can activate highly specific T cells characterized by high affinity (Vonderheide and Nathanson, 2013*).* The process of identification of these antigens is laborious and not promising in all settings, which makes isolation of these antigens difficult. However, there has been a lot of efforts to utilize the power of these highly specific tumour antigens without previously characterising them (Butterfield. 2015).

Many approaches are developed in order to utilise the power of TAAs and TSAs for the elicitation of the efficient anti - tumour response. These approaches include use of peptides derived from tumours that have high affinity to $CD8^+$ T cells, tumour tissue or cell lysates, antigen presenting cells, viral or oncolytic therapy (Butterfield. 2015; Ribas *et al*. 2003).

The approach using the tumour tissue or cell lysates is trying to use the immunogenicity of various antigens present in the tumour tissue or tumour cells (Butterfield. 2015). It is considered that the majority of these antigens are TAAs but as well that some of these antigens are TSA proteins (Hoover *et al*. 1993). This approach utilises the strategy of targeting mostly the TAAs and by doing that trying to induce a more generalised immune response that will eventually spread and direct the immune response towards the TSAs (Ribas *et al*. 2003). The use of these various TAAs or TSAs should increase the chance that the adequate anti-tumour response will be induced and will be able to eradicate the tumour (Ribas *et al*. 2003).

In the study by Kawahara and Takaku (2015), they have observed that the crucial mediators of the immune response in a syngeneic murine carcinoma model upon immunisation with the tumour lysate in combination with the baculovirus and the cytotoxic lymphocyte (CTL) peptide epitope, are $CD4^+$ T. It was observed that the tumour lysate itself might not be immunogenic enough to elicit a CTL response directly but it can stimulate the $CD4^+$ T cells that further contribute to the activation of the $CD8^+$ T cells. In their study, it was shown that this response is dependent on the tumour lysate and $CD4^+$ T cells. When the $CD4^+$ T cells were depleted, the immune response was impaired,

while when the $CD8^+$ T cells were depleted, the natural killer (NK) cells suppressed the tumour growth in combination with $CD4^+$ T cells for a certain time (Kawahara and Takaku, 2015).

A very important consideration is that tumour lysates are a source of the normal self- antigens as well, and these antigens can influence the immune response or even stimulate tolerance or auto-immune responses (Cohen *et al*., 2009). However, due to the mechanisms of central and peripheral tolerance, the chance for these antigens to induce autoimmune response should be minimal (Vigneron. 2015).

In this approach, autologous tumour cells and tissue or allogeneic tumour cell lines are used. Regarding the autologous cancer vaccines, it is considered that they are highly patient specific because they are made of the material isolated from a certain patient. Like that, it is thought that these vaccines should include patient specific TSAs that should be able to induce an effective immune response (Vonderheide and Nathanson. 2013). They have shown certain success in preclinical and clinical studies (Hanna. 2012; Overwijk *et al*., 2013; Kurtz *et al*., 2014). However, they have shown to be complicated to prepare and with limited feasibility regarding that they are specialised for the patient for who it is made (Ye *et al*., 2018). However, many studies have shown that the patient specific TSAs could be crucial factors determining the success of therapy in some types of cancer (Jiang *et al*., 2019).

In the study by Suckow *et al*. (2009), they have emphasised the fact that vaccines derived from tumour tissue biopsy, include the tumour specific antigens that are developed under the influence of the microenvironment. It further implicated that the tumour tissue biopsies contain a wider range of the tumour specific antigens as compared to cultured cancer cells. Besides the cancer cells, a vaccine made of the tumour tissue biopsy contains tumour tissue stroma antigens. The tumour stroma has been shown as a great source of the tumour specific antigens regarding that some features of the tumour stroma are specific to the tumour and as that might be recognised as suggested by Suckow. (2007). However, a study by Goldszmid *et al*. (2004), have suggested that syngeneic cell lines contain all the necessary TSA that can elicit an effective immune response. They have used a vaccine made of dying B16 melanoma cells exposed to DCs and have induced long-lasting cell immunity with both $CD4^+$ and $CD8^+$ T cells, in the mice (Goldszmid *et al*., 2004).

1.6. Improving the immunogenicity of tumour - derived antigens

As tumour antigens are aberrant self- antigens that mostly can be only mildly immunogenic, various approaches are used in order to improve their immunogenicity and immunogenicity of the cancer

vaccine itself. They include the use of various cytokines such as IL - 2 or granulocyte macrophage stimulating factor (GM - CSF), adjuvants and immunopotentiators (Le *et al*., 2012; Zerbini *et al*., 2006). They can also involve the use of DCs that are incubated with tumour cells, lysate, peptides and then once primed, reinjected in the patient from which they are derived. This approach is trying to overcome the problems related to the inefficient number of mature or dysfunctional DCs and problems of inadequate presentation of tumour antigens necessary for the activation of T cells (Bart *et al*., 2010; Geiger *et al*. 2000).

One of the emerging issues that should be addressed is the optimal clinical setting for the evaluation of the immunotherapeutics (Sondak *et al*., 2006). Vaccination in the metastatic setting has not yet proved satisfactory, most probably due to heavy metastatic burden that induces an immunosuppressive environment that impairs the anti-tumour response (Clifton *et al*., 2016). Some of the recent clinical trials have been conducted in patients with minimal residual disease and the vaccines have shown more efficient (Hale *et al*., 2014). It is known that the metastatic niche is a different environment compared to the primary tumour (Doglioni *et al*., 2019; Paolillo *et al*., 2019)

Some clinical trials have shown that in order to maintain immunity elicited with the cancer vaccines, booster vaccinations are necessary (Mittendorf *et al*., 2014). However, in other clinical trials, it was observed that the restimulation with the antigen can induce the exhaustion of CTLs (Xia *et al*., 2019). On the other side, in the advanced tumour setting, the general strong immune reaction can induce the narrowing and collapse of the lung airways which can further induce death of the patient (Lee *et al*., 2009; Nichols *et al*., 2012).

1.7. Viruses as immunopotentiators and non - specific immunotherapy

Introduction of a viral vector, whole attenuated virus or it's immunologically relevant parts in vaccine construction, together with TAAs, can markedly impact recognition of tumour antigens (Osman *et al*., 2011). Inducing the inflammation, specifically acute inflammation with microbial products that have high immunogenicity could provide a good environment for induction of an antitumor response (Thompson *et al*., 2011). Immunologically relevant parts of viruses can be used since these products can be recognised by Toll - like receptors (TLRs) positioned on the surface or inside immune cells (Wijewardana *et al*., 2013). These receptors are mostly expressed on APCs such are macrophages or dendritic cells. Furthermore, presentation of pathogen antigens on APCs to cells of the innate immune system activates the inflammation cascade (Nyschija *et al*., 2004).

A consequence of this cascade initiation is the activation of a specific adaptive immune response which further helps in eradication of infection (Nyschija *et al*., 2004; Osman *et al*., 2011). One of the most successfully used Toll-like receptors ligands is polyinosinic:polycytidylic acid (poly I:C), which mimics virus infection as it is a double - stranded RNA that binds to Toll - like receptor 3 (TLR3). This type of inflammatory stimulus can have a stimulatory effect on cells of innate immunity such as macrophages, neutrophils and NK cells (Salem *et al*., 2016). As well, triggering of the TLR signaling pathways, this can initiate a cascade that results in the secretion of proinflammatory cytokines, chemokines (Sivori *et al*., 2004). This forms the proper inflammatory environment which may result in non-specific anti-tumour responses (Osman *et al*., 2011). Pro-inflammatory mediators may induce maturation of APCs, and in combination with them can activate cytotoxic T lymphocytes and NK cells, which are key factors of the anti-tumour response (Sivori *et al*., 2004).

In a study by Cho *et al*. (2013), it was observed that the poly-IC stimulated a very robust T cell antigen specific immune response both when used as a prime and as a secondary boosting agent and when it was used as a secondary boosting agent alone regardless of which adjuvant was used for priming. They have suggested that this effect was probably due to the activation of the TLR3 receptor and as well as one of the retinoic acid inducible gene I-like receptors (RIGI). One of these RIGI receptors is the melanosomal differentiation - associated protein 5 (MDA5) which activation induces the production of the IFN type I cytokines which further stimulate the maturation of T cells (Cho *et al*., 2013). Cho *et al*. (2013) suggested that the ability of poly IC to stimulate the huge T cell response is more dependent on the MDA5 than TLR3. They have hypothesized that the antigen boosting event can activate the MDA5 receptors and induce the expression of the costimulatory molecules that will further activate the antigen specific T cells (McCartney *et al*., 2009). The TLR3, RIG1 and MDA5 can detect viral double-stranded RNA and activate the signal cascade which will result in the production of the IFN type I (Jiang *et al*., 2004). In the study by Kawahara and Takaku (2015), they have suggested that Baculovirus can be used as a good candidate as immunopotentiator for peptide - based cancer vaccines. In their study, they have observed that Baculovirus can provide a similar effect as the cytosine-phosphate - guanosine oligonucleotide (CpG oligonucleotide) which is used widely as immunopotentiator, where both were able to induce an effective cellular immune response when coinjected with the combination of peptide and tumour lysate (Kawahara and Takaku, 2015). On the other hand, it is interesting that in the study by Cho *et al*. (2013), poly I:C

have induced more effective cellular anti - tumour immune response when injected with the peptide cancer vaccine as compared to the CpG oligonucleotide. This further indicates that the immunopotentiators can differently interact with various cancer vaccine formulations which can have a significant impact on the capacity of the anti-tumour response.

During the construction of vaccines, it is very important to consider the effect of the adjuvant. The components of the adjuvant can direct the immune response towards the Th1 or Th2 immune response or the humoral or cellular response so it is very important to consider this factor (Hrouda *et al*., 1998). It is known that the metastatic niche is a different environment compared to the primary tumour (Doglioni *et al*., 2019; Paolillo *et al*., 2019)

There are also viruses that show a natural tendency towards infecting tumours, such as the measles virus, and as that they can be used in immunotherapy (Guillerme *et al*., 2013). Since cancer cells can express certain molecules or use certain mechanisms to evade the immune response, they present very suitable places for viral replication. Due to this feature, infection of cancer cells with live attenuated measles virus can induce cancer cell death and production of the DAMPs and PAMPs (Boisgerault *et al*., 2010; Guillerme *et al*., 2013). A similar effect was observed in the study by Ockert *et al*. (1996), where they have used the Newcastle Disease Virus (live attenuated).

1.8. Rotavirus

The Rotavirus is an double stranded ribonucleic acid (RNA) virus which comprises of several structural and non-structural proteins (Crawford *et al.*, 2017). It is causing a gastrointestinal infection which is followed with high level of diarrhoea, mostly in juvenile animals and infants, including rats and humans (Ciarlet *et al.*, 2002). Although it mainly infects the gastro-intestinal tract, the viral antigens can be spread and found in other organs since it can spread through the blood (Crawford *et al.*, 2017). It is observed that the virus is highly present in the gastro-intestinal tract in first three days after the infection (Istrate *et al*., 2014). One of the vaccine formulations constructed for the treatment of the Rotavirus infection, in humans, is the Rotarix (live Rotavirus attenuated vaccine) (Vetter *et al*., 2017). It is observed that the immune response on the vaccination is mainly humoural, with the cellular component present to a lesser extent (Desselberger and Huppertz, 2011). However, it is observed that in natural immune response, the cellular immune response, both $CD4^+$ and $CD8^+$ T cells, is important mediator of the viral infection eradication (Crawford *et al.*, 2017).

1.9. Importance of the anatomical site and the route of the vaccine administration

In several studies, it was observed that the anatomical site in which the vaccine will be injected matters in the priming of the immune response (Cho *et al*., 2009; Sultan *et al*., 2017). It is thought that this happens regarding that the administered antigens will interact with different APC subsets present in different amounts.

DCs are region-specific and their role is specified for the antigens that can naturally be presented there (Bode *et al*., 2008; Malik *et al*., 2014). For example, mesenteric lymph nodes have a different population of the DCs as compared to the axillary lymph nodes and this can affect the nature and extent of immune response even on the same pathogen (Bode *et al*., 2008; Tiede *et al*., 2008).

Studies have shown that different subpopulations of DCs are controlled by the microenvironment in which they are residing. The dendritic cells that are located in the axillary nodes are characterized by the presence of certain markers, suggesting that these are the same as the DCs present in the skin (Czeloth *et al*., 2005). The DCs present in the mesenteric lymph nodes are characterized with different markers which makes them the same as the subpopulation present in the *lamina propria* layer of the gut (Malik *et al*., 2010). It is important to consider the fact that the DCs from lymph nodes draining different tissues, can have influence on the expression of specific homing receptors on the T cells, and these receptors are crucial for delivery of T cells to the tumour site upon presentation by DC (von Adrian *et al*., 2000). It is suggested that the main factors that can influence on the level of the primary immune response are the delivery of the antigen or adjuvant to the DCs and the distribution that will target as many lymphoid organs as possible in order to prime high number of T cells (Sultan *et al*., 2017). However, the presence of different types of professional and unprofessional dendritic cells should be considered since they can influence the features of the immune response differently (Levin *et al*., 2015)

Cho *et al*. (2009), have observed that their cancer vaccine was highly dependent on the route of administration. The intravenous (i.v.) and intramuscular (i.m.) induced better and systemic response as compared to the subcutaneous (s.c.). They have observed that the i.v. administration of vaccine have delivered the antigen to various lymphoid structures and activated large numbers of antigen specific T cells. While s.c. had a local effect, and delivered antigen only to the draining lymph nodes (Cho *et al*., 2009).

Intraperitoneal (i.p.) administration of the cancer vaccine can be a good way of delivering the antigen of interest to the numerous lymph nodes present inside the peritoneal cavity. These include mediastinal, gastro-omental, hepatic, mesenteric and more (Malik *et al*., 2014). As well, the antigen can be delivered to the secondary lymphoid organs such as spleen and gut-associated ones such as gut-associated lymphoid tissue (Malik *et al*., 2010).

In the study by Head *et al*. (2017), whole cell vaccine containing irradiated 4TI mammary tumour cells with IL - 2 and GM - CSF induced significant prophylactic effect both when induced s.c. and i.p. They have suggested that the subcutaneous route of the cancer vaccine delivery can be effectively used for cancer treatment (Head *et al*., 2017).

Bonnotte *et al*. (2003), have observed that the tumorogenicity and immunogenicity of the syngeneic tumour cell line in mice depend on the route of the administration. They have observed that the s.c. route of inoculation allows the normal development of the tumour, without the influence of the immune cells. However, the i.d. route of inoculation stimulated the immune response of certain level that can suppress the formation of the tumour due to stimulation of the immune system. They have observed that the i.d. administration stimulated the DCs present in the epidermis and dermis which further stimulated the immune response (Head *et al*., 2017). It is known that in the epidermis and dermis there is a high amount of DCs, while in hypodermis and subcutaneous tissue there is a very low amount of these cells (Campton *et al*., 2000).

1.10. Cancer vaccine modifications - formalin fixation

Various modifications have been used in order to increase the immunogenicity of the cancer vaccines. One of the chemicals used in this purpose is formalin (Obata *et al*., 2004). Formalin is widely used for the fixation of tissue samples, usually biopsies taken during surgeries. The formalin fixation secures the preservation of the morphological and histological features of the sampled tissue. It induces the formation of various types of chemical bonds inside the deoxyribonucleic acid (DNA) as well as between the DNA and proteins or between protein polymers, which reduces the reactivity of the tissue and thus preserves it's features (Merck and Spelt. 1998, Obata *et al*., 2004). However, it is as well observed that the fixation of the tissue or cells in the formalin can have an impact on their antigenicity or immunogenicity (Obata *et al*., 2004).

It is suggested that formalin can provide increased immunogenicity of TAAs and TSAs as well as bacterial antigens (Obata *et al*., 2004). In the studies where the antigens were used for the treatment of bacterial infection, formalin has shown to be able to induce better immunogenicity of these

antigens (Åhrén *et al*., 1998; Raghavan *et al*., 2002). It is suggested that formalin can have an impact on the bonds made through interaction between protein polymers and as that can induce changes in their formation (Orlando and Paro, 1993). This can further have the crucial impact on the recognition of these antigens from the cells of the immune system, in the overcoming the immune tolerance mechanism. All this provides good conditions for the elicitation of the effective anti-tumour response (Obata *et al*., 2004; Orlando and Paro. 1993).

Studies by Peng *et al*. (2002) and Ohno (2005), have shown that the tissue sections derived from the hepatocellular tumour biopsy, can serve as a good platform for the activation of $CD8^+$ T lymphocytes derived from the same patient. In the study by Ohno (2005), it was observed that these $CD8^+$ T lymphocytes after reintroduction in the patient can initiate an effective immune response that can prevent recurrence of the tumour for a longer time period as compared to the control (surgical removal only). In the study by Ishikawa *et al*. (2007), it was observed that the vaccine made of the tumour tissue fragments derived from the tumour tissue biopsy that was paraffin-embedded and in combination with the formalin-fixed fragments can induce specific $CD8^+$ T cell immune response. They have observed that out of twelve patients that had a type of glioblastoma tumour, one patient has shown complete response.

In previously mentioned studies, authors have used immunopotentiators such as GM - CSF, IL - 2 and tuberculin, in order to enhance the immunogenicity of tumour-derived antigens. As well, it was observed that the level of fractioning of the tumour tissue can have a positive effect on the immunogenicity of antigens derived from this tissue (Ishikawa *et al*., 2007; Ohno. 2005; Peng *et al*., 2002). It is worth mentioning that in the study by Obata *et al*. (2004), it was observed that B16 melanoma cells processed in formalin could not elicit the significant immune response without the addition of the IL - 2.

1.11. Physico-chemical features of antigens that can have an impact on the distribution and immunogenicity of cancer vaccines

It is known that the electrostatic interactions of antigens or antigens and immunepotentiator or adjuvant can influence the type and strength of the immune response (Hamborg *et al*., 2014; Michelin *et al*., 2002).

The size of the vaccine antigens can have a significant impact on the biodistribution of these molecules upon injection (Bachmann *et al*., 2010). As well, it was observed that the large molecular

weight molecules tend to be delivered to the lymph nodes upon injection, while smaller molecules tend to be distributed through the systemic circulation due to the anatomy of lymph and blood vessels (Ali Khan *et al*., 2013; Bachmann *et al*., 2010). This further has a high impact on the nature of the possible immune response on these antigens (Bachmann *et al*., 2010).

When coinjecting two vaccines it is important to consider that the physico-chemical interactions or the interactions between the live attenuated vaccines can increase immunogenicity or cause loss of it (Gizurarson, 1998). Analysis of the structure, size and physico - chemical properties of the peptides constituting the vaccine can be achieved with use of techniques such as Western blot (Atascientific.com. 2019).

1.12. Murine cancer models

In order to thoroughly represent complicated histological, molecular and genetic changes underlying the process of carcinogenesis, experimental animal models were developed (Olson *et al*., 2018). The process of the tumour initiation and development is highly similar in murine and humans (Balmain and Harris. 2000). Biology and genetical background of cancer, mechanisms involved in its propagation and expansion as well as strategies used for the evasion of the immune response are highly similar to the ones observed in human (Balmain and Harris. 2000; Fearon and Vogelstein, 1990). All these features along with the fact that these animal models require easy maintenance, that they can be reproduced in large quantities and are suscceptible to genetical modifications make these models highly desirable in research (Heindryx *et al*., 2009).

1.13. Chemical carcinogenesis

It is suggested that the process of neoplastic transformation under the influence of a chemical carcinogen is a complex and multistep process. Steps included in this process are initiation, progression, promotion (Cohen. 1991; Hasegawa *et al*. 1998; Trosko. 2001).

The process of carcinogenesis is initiated when the chemical carcinogen induces DNA damage (Oliveira *et al*., 2007). Like that, these changes promote abnormalities in the functioning of normal cells which as the final result will have the formation of cancer cells. The DNA damage is a process that makes these cells susceptible to further mutations which will promote proliferation and consequently functional distortion of repair mechanism and as such, mutations will continue compiling and transferring to the future generation of cells (Farber. 1984; Klaunig *et al*. 2000,

Trosko. 2001).The second stage of this process is carcinogenesis promotion. Chemical agents used in this stage are further promoting the cell division, inducing more changes both on a genetic and epigenetic levels which further results in the establishment of mutations in crucial cell regulatory systems (Basu. 2018; Hanahan and Weinberg, 2000; Mehta. 1995). The process of progression is the one that is finally forwarding the cell to an irreversible state of malignancy. In this step various genetic and epigenetic changes occur and through them, cells are becoming malignant (Pitot *et al*., 1996; Shacter and Weitzman, 2002).

The main advantages of chemical carcinogenesis models is that animals after exposure to carcinogens develop tumors spontaneously (Santos *et al.,* 2017). Disadvantages of these models are that accuracy of tumour development is not high, long period for development, not easy to follow the development as compared to the palpation method that can be used in transplantable models (Oliveira *et al*., 2007). The imaging modalities for continuous live assessment such as optical imaging, ultrasonography, and magnetic resonance imaging could be used in order to improve this (Sandhu *et al*., 2010; Kumar and Pawaiya, 2010).

Diethylnitrosamine (DEN) can initiate and promote the carcinogenesis in mice or rats not older than 14 days, while in older mice or rats additional promoter such as N – Nitrosomorpholine (NMOR) is needed (Bakiri and Wagner, 2013; Espandiari *et al*., 2005). DEN is capable of inducing tumours in various organs especially in the liver (Santos *et al*., 2017). In order to be activated DEN needs to be metabolically processed in the liver (Heindryckx *et al*., 2009; Jin *et al*., 2007). Biological products of this process further interact and induce changes in cells on a genetic and epigenetic level, that initiate the carcinogenesis (Oliveira *et al*., 2007; Santos *et al*., 2017; Verna *et al*., 1996).

N - Nitrosomorpholine (NMOR) is considered as a complete carcinogen and similarly to DEN, it needs to be metabolically processed in order to be activated (Marra *et al*., 2011; Santos *et al*., 2016). However, it can be used as a promoter when the DEN is used as initiator which can further increase the potential of carcinogenesis (Santos *et al.,* 2017).

Both of these chemical carcinogens have an impact on liver and as a consequence on animal's metabolism, which can further possibly result in the animal weight loss (Oliveira *et al*., 2007, Patterson *et al*., 2012; Solaini *et al*., 2011; Tasaki *et al*., 2014).

1.13.1. DEN - NMOR chemical carcinogenesis model

The DEN - NMOR chemical carcinogenesis model was used in multiple studies where cancer biology as well as the effect of the various cancer therapeutic approaches were studied. Some of those studies include the study by Wang *et al*. (2009), Futakuchi *et al*. (1999) and Yoshino *et al*. (2005). In the study by Wang *et al*. (2009), they have used DEN in the concentration of 100 mg/kg of rats weight and NMOR as 100 ppm, in the Sprague-Dawley strain of rats. Futakuchi *et al*. (1999), have established the same model, however in the F344 lineage of rats and they have used DEN at the concentration of 100 mg/kg and NMOR as 120 ppm.

Studies by Futakuchi *et al*. (1999) and Wang *et al*. (2009), showed significant weight loss initially observed in the 16^{th} week and then continuously in between the 16^{th} and 22^{nd} weeks. In the study by Wang *et al*. (2009), they observed that specific morphological changes on the liver develop in different time frames. In the period between the 3^{rd} and 4^{th} month, they have observed the appearance of tumour nodules which looked like the start of cirrhosis. These smaller morphological changes were followed with the formation of unequal nodules and with the increase in the liver size in the 5^{th} and 6^{th} month (Wang *et al*., 2009). The cirrhosis was observed in some of the animals in a study by Yoschino *et al*. (2005) and Wang *et al*. (2009), and it was characterized as a feature that reflects the final stage of the liver cancer development. Authors of these studies have observed that in this stage the lung metastasis starts occurring. The Futakuchi *et al.* (1999) and Wang *et al*. (2009) have also observed that the weight loss was connected with the formation of lung metastasis.

As shown by Futakuchi *et al.* (1999), significant weight loss was connected with the increased liver tumour burden and lung metastasis. It was observed that this happens due to long term exposure to the NMOR. In their study, significant weight loss correlated with lung metastasis in 100% of animals. This kind of treatment has an effect on the increase in the number of metastatic lesions but not on their size (Futakuchi *et al.*, 1999). As they discuss, the high rate of metastasis (more than 80%) establishes this model as an adequate animal model of metastatic liver cancer. They have also observed that the multi-step process of metastasis has occurred in a specific time frame and that it encompassed prominent metastatic features (Futakuchi *et al.*, 1999; Wang *et al*., 2009).

Wang *et al*. (2009) have shown that histopathological features in the liver were resembling the actual changes in the development of inflammation and cancer. At the last stage, after 5-6 months, they have observed that the liver got enlarged, with rough surface and nodules appearance. As discussed by Yoshino *et al*. (2005), this model of hepatocellular carcinogenesis was easy to establish, in a relatively short period and with the high metastatic occurrence and potential. The

main processes included in tumour formation, dissemination were highly resembling the characteristics of the human hepatocellular cancer (Yoshino *et al*., 2005; Wang *et al*., 2009).

1.14. Transplantable tumour models

Different cancer cell lines that are suitable for growth in murine animals such as rat or mouse and can be used as transplantable tumor models (Olson *et al*., 2018). Syngeneic animals are a product of inbreeding, that results in the high genetic similarity. Furthermore, genetic similarity between these animals allows the inoculation or implementation of cells or tissue that was previously isolated from the animals of the same strain which allows the formation of syngeneic cancer models (Khanna and Hunter, 2005). The advantage of these models is that they allow easy and fast development of the tumour that adapts naturally to the tissue where it is inoculated (Olson *et al*., 2018). On the other hand, these models due to high genetic similarity can not fully reassemble the actual complexity of cancer disease that naturally occur (Khanna and Hunter, 2005).

The model in which cells are inoculated in a way that they can induce the spontaneous formation of tumours is called spontaneous transplantable model of cancer (Olson *et al*., 2018). These cells can be inoculated in the tissue that is different from the tissue from which these cells originated or in the tissue from which they originate (Erstad *et al*., 2018). Cells can be injected in the subcutaneous or intraperitoneal area, which makes these models very easy to apply. On the other hand, they can be injected or surgically inserted in the tissue of origin which can be complex (Kim *et al*., 2009; Tseng *et al*., 2010).

All transplantable models of cancer are dependent of the cell line used for the inoculation which further implicates that features of these cell lines can have an impact on the cancer formation (Fidler and Kripke, 1977). In the study by Hrouda *et al*. (1998), it was observed that it was hard to target the transplantable tumour with the immunotherapy regarding the extremely fast and high proliferation of these cells. The immune response can not act fast enough to eradicate the tumour (Hrouda *et al*., 1998).

Since the growth of these tumours can be fast and abnormal, it may result in the formation of an open wound (ulceration) in the skin layer covering the tumour (Arbiser, 2014; Panuncialman and Falamge, 2010). As well, the skin ulceration can be a result of a high pressure applied on that skin area or the inflammatory reaction (Bhattacharya and Mishra, 2015; Panuncialman and Falamge, 2010).

In the study by Cho *et al*. (2013) they have observed that the effect of the vaccine is highly dependent on the amount of the transplantable tumour cells used for the inoculation as well as the time of the first immunisation. When the immunisation was performed on the third day the percentage of mice that lived more than 100 days was 80 % as compared to when the immunization was performed on the 14^{th} day when this percentage was 60. This was dependent as well on the amount of B16 cells, 3×10^4 or 1×10^5. When the mice were inoculated with the $3x10^5$ B16 cells, they have all died by the 60^{th} day after the immunization (Cho *et al*., 2013).

In the study by Kawahara and Takaku (2013), it was observed that the combination of the vaccine made of the baculovirus and the tumour lysate, without the addition of peptide, was able to suppress carcinogenesis effectively in mice inoculated with the 5×10^4 CT26 colon carcinoma cells. Although, in their study from 2015, when the 4×10^5 CT26 cells were inoculated the vaccination with the baculovirus and the tumour lysate without the peptide was not able to suppress carcinogenesis effectively (Kawahara and Takaku, 2015).

1.14.1. MT450 syngeneic mammary gland tumour model

MT450 cell line is used for the development of the transplantable spontaneous tumour models in the Wistar-Furth strain of rats. Multiple authors, including Quagliata *et al*. (2014), Thiele *et al*. (2013) and Krishnan *et al*. (2003), have used this model their studies related to the tumour biology as well as in the assessment of the anti-tumour therapies.

Thiele *et al.* (2013) have used 5×10^5 MT450 cells for inoculation as well as Krishnan *et al.* (2003), while Quagliata *et al.* (2014) have used MT450 $1x10^6$ cells. In the study by Quagliata *et al*. (2014), it was determined that at the two weeks time period no cancer cells were observed in axillary lymph nodes or the lungs while the increase in pro - lymphangiogenic factors was observed. At the three weeks time period, MT450 started infiltrating the axillary lymph nodes and metastatic formation have appeared in the lungs. The amount of pro-lymphangiogenic factors increased (Quagliata *et al*., 2014). After the four weeks period, cancer cells have invaded and induced significant morphological changes in axillary lymph nodes. The blood and lymphoid vasculature in the tumour and its surrounding were highly expressed and infiltrated with the disseminated cancer cells. In the study by Quagliata *et al*. (2014), inhibition of certain vascular growth factor has significantly reduced the volume and the tumour burden of the axillary lymph node as compared to control while it did not have any effect on the lung metastasis (Quagliata *et al*., 2014).

Krishnan *et al*. (2003), observed that the size of the tumour correlates with the number and the expression of the lymphatic vessels which primarily correlates with the expression of growth factors such as VEGF-C/D. As well, Krishnan *et al*. (2003) discuss that due to morphological characteristics of lymphatic endothelium, its ability to pass large molecules and cells, increase of tumour induced angiogenesis can potentiate the metastasis of the MT450 cells. Therefore, increase in VEGF - C/D can increase metastasis to the draining lymph nodes and the lymphangiogenesis. As well they have observed, that the increased size and number of draining lymph node metastases were induced with the increase in the number of cancer cells in the lymphoid vessels. And thus, the number and size of these metastases directly correlate with the ability of a primary tumour to induce lymph node metastases (Krishnan *et al*., 2003). In the same study, they showed that the inhibition of the metastasis to the lymph nodes also suppressed the lung metastasis.

MT450 syngeneic tumour model has shown high efficiency as a metastatic model. As Thiele *et al.* (2013) showed, this model can be used in the assessment of different anti-tumour therapies. As demonstrated in their paper, important features of angiogenesis and lymphangiogenesis that induce metastatic formation and factor influencing on these features are successfully recapitulated in this model. Model provided metastasis to the draining lymph nodes as well as lungs (Thiele *et al.*, 2013).

1.15. H&E staining and prognostic significance of the immune infiltrate in solid tumours

Haematoxylin and eosin (H&E) staining method has been used widely and it is a very established approach in histopathology. It is relatively simple and easy to apply method that can provide very good insight inside the histopathological characteristics of the disease (Lahiani *et al*., 2018; Kumar and Pawaiya, 2010).

Regarding that there is no standardised methodology for the pathological assessment of the tumour infiltrating lymphocytes (TILs) in the solid tumour, most of the studies in breast cancer are following guidelines recommended by the International TILs Working group (Salgado *et al.*, 2015). In the recommendations from the International TILs working group (Salgado *et al*., 2015) it is stated that both the intratumoural and the stromal TILs should be evaluated. TILs that are in direct contact with tumour cells are considered as intratumoural and the ones in tumour stroma as stromal TILs. As more studies are showing, the stromal as well as intratumoural TILs are important as the prognostic factor in breast cancer (Vinayak. 2014).

Some of thee studies, including Pruneri *et al.* (2016), supported the evaluating capacity of the guidelines recommended by the International TILs Working group and indicated that each increament of 10% in TILs has strong positive implications on the survival of patients in triple-negative breast cancer (TNBCs). This was further confirmed in the study by Tian *et al*., (2016) where they suggested that both the intratumoral- and stromal- TILs were strong predictors of the survival in the breast cancer.

There are a lot of difficulties in subtyping the TILs and determining their spatial organisation. A lot of effort has been invested in immunophenotyping of the immune infiltrate by immunohistochemistry or immunofluorescence (Salgado *et al*., 2015). In colorectal cancer, in one of the studies examining the presence of the TILs in nine trials, it was observed that the presence of TILs is correlated with the positive clinical outcome regardless their specific location (Mei *et al*., 2014). Opposite, in a study by Jochems and Schlom (2011), there was a significant correlation observed depending on the type of infiltrate and position. The infiltration of the $CD8^+$ T cells have been strongly correlated with the positive outcome in breast cancer patients (Mahmoud *et al*., 2011), while the presence of the $CD4^+$ T regulatory cells have been correlated in some studies with good and in some studies with bad outcome (Bates *et al*., 2006; West *et al*., 2013).

In the non-small lung, in the study by Geng *et al*. (2015), it was observed that the presence of $CD8^+$ T cells had been correlated with the better overall survival regardless the specific position. In the same study, the presence of forkhead box protein 3 positive T cells ($FOXP3^+$ T cells) had a negative influence on overall survival. In the study conducted by Huang *et al*. (2014) and Gabrielson *et al.* (2016), in the hepatocellular carcinoma, the same results were observed. Most of the data from the solid tumour studies suggests that the increased lymphocyte infiltration has positive implications on the survival (Hendry *et al*., 2017).

It is interesting that the relatively simple method of the assessment of lymphocyte infiltration, such as morphological evaluation of the H&E stained tissue slides can provide significant prognostic indications. Although it provides information regarding the general presence of lymphocytes in the tumour, and not about each subpopulation, it was proven valid in the prognostic and predictive evaluation of the patient's outcome (Denkert *et al*., 2010; Adams *et al*., 2014). In the paper by Salgado *et al*. (2015), it is discussed that this may further suggest that the histological evaluation of each lymphocyte subpopulation may be not so relevant. They suggest that the presence of different lymphocytes population can provide better insight into the interaction of the tumour and immune system in each patient and in different types of tumours. As example, the absence of the regulatory

T cells may indicate that the tumour is not at all recognized by the immune system, on the other side, presence of the regulatory T cells can indicate that the tumour is recognized and the immune response was elicitated, however, it is not powerful enough to completely eradicate the tumour (Salgado *et al*., 2015). However, as discussed by Harding *et al*. (2017), presence of the inflammation can suggest as well that the immunosuppressive environment has been established, so the more detailed insight into the subpopulation features can be beneficial.

1.16. Methods used for the quantification and assessment of the functionality of the cells of the immune system

A flow cytometry (FC) is widely used in preclinical and clinical studies regarding that it can provide researchers and clinicians with the information about multiple parameters of cells (Frelinger *et al*., 2010; Macchia *et al*., 2013). The flow cytometry can provide result about the cell morphology, including size, shape and granularity. As well, based on the markers (antibodies) used, it can provide very detailed information about the functionality and the cell phenotype (Frelinger *et al*., 2010).

Assays such as ELISpot or intracellular flow cytometry staining (ICS) can be used in quantification of cytokines or chemokines produced as a response on immune stimulation of $CD4^+$ or $CD8^+$ T cells with certain antigen (Keilholz *et al*., 2006). Intracellular flow cytometry (ICS) can be used to assess the production and presence of multiple cytokines or chemokines, such as IFN - γ, IL - 2 and TNF α, while Elispot can be used for the measurement of one cytokine or chemokine (Macchia *et al*., 2013). The complexity of disease should be considered when analysing the immune response through the presence and amount of these proteins. In some studies, although there was a significant production of the pro-inflammatory cytokines measured, the immune response did not actually eradicate the disease (McElrath *et al*., 2008). In order to get more reliable results, assay such as ICS and ELISA can be used in the measurement or identification of specific functions of cells of adaptive immunity that are crucial for their immune function (Chattopadhyay *et al*., 2009). The measurement of the lysosomal associated membrane protein (LAMP or CD107) can be used for the assessment of the degranulation of cytotoxic T cells. Its exposure indicate that cytotoxic agents such as perforin and granzyme that induce lysis of the aberrant or infected cell, are secreted (Betts *et al*., 2003). Quantification of the surface expressed CD107a can be used in quantification of antigen specific CTLs (Rubio *et al*., 2003). In order to assess the $CD4^+$ T helper cells with the FC, CD40L can be used regarding that it is one of the crucial ligands involved in the stimulation of the $CD8^+$ T cells and B cells (Chattopadhyay *et al*., 2005).

Proliferation of the effector T cells can be measured with the use of cell generation dyes that can provide the information about the number of cell division. One of these dyes is the carboxyfluorescein succinimidyl ester (CFSE) (Macchia *et al*., 2013). The isolated CTLs are labeled with the CFSE and cultured with a cancer cells or stimulant (antigen of interest), and the dye phosphorescence can be measured by flow cytometry (Keilholz *et al*., 2006).

When planning the flow cytometry analysis, one needs to be aware that the downregulation or upregulation of certain markers such as CD8 can be transient. In the study by Xiao *et al*. (2015), they have observed that the downregulation of the CD8 marker happens early after the immunisation with Listeria monocytogenes or Vaccinia virus, seven days after the immunisation. This down-regulation is transient regarding that the levels of CD8 are up-regulated after the formation of the memory pool of these cells. They have suggested that this mechanism is stimulated in order to protect the normal cells apart from target ones that are highly immunogenic (Xiao *et al*., 2015).

1.17. Aims and objectives

The aim of this study was the construction of the AFFTV and assessment of it's effectiveness in the murine tumour models in combination with the Rotarix or alone, upon second immunisation. Furthermore, main objectives were:

- Assessment of the potential anti-tumour immune response upon the immunisation with the AFFTV or/and Rotarix with use of the H&E staining and histological scoring system.
- Quantification of the $CD8^+$ T cells upon the immunisation of animals with the AFFTV and/or Rotarix.
- Assessment of potential differences in the features of the anti-tumour immune response upon the second immunisation of animals with the AFFTV made of the tumour tissue biopsy, AFFTV made of the MT450 cell line or with combination of both.
- Assessment of potential differences in the features of the anti-tumour immune response upon the second immunisation of animals with the AFFTV made of the MT450 cell line with standard or changed preparation protocol.

- Development of the AFFTV that is feasible and easy to manufacture and that can be used in the treatment of various solid tumours in murine models and eventually in humans.

Chapter 2: Materials

2.1. List of chemicals and small molecules used in this thesis:

Chemicals	Catalogue No.	Manufacturer
Ammonium Carbonate	11204	Sigma
Ammonium Chloride	AM02700500	Scharlau
Bovine Serum Albumine	A2153	Sigma-Aldrich
Calcium chloride	BDH9224	BDH Laboratories
Citric acid	C8532	Sigma
Dextrose (monohydrate)	IC90559405	BDH Laboratories
Dymethyl sulfoxide	276855	Sigma-Aldrich
Dibuthylphtalate polystyrene xylene	06522	Sigma-Aldrich
Ethylenediaminetetraacetic acid	28509-09	Aldrich Chemical
Ethanol	m/4065/17	Fischer Chemical
Foetal Bovine Serum	16000044	Sera Lab
Histopaque	10771	Sigma-Aldrich
Monobasic Sodium Phosphate	S00191000	BDH Laboratories
Paraffin Wax	10395900	Fischer Chemical
Potasium Chloride	20800-0	Aldrich Chemical
Phosphate Buffered Saline	P3813	Sigma-Aldrich
RPMI 1640 media	SLM-140	Sigma
Sodium azide	S-564	Sharlau
Sodium citrate (dihydrate)	S-464	Scharlau
Trypsin 10X	T4174	Sigma-Aldrich
Trypsin powder (porcine)	T4799	Sigma-Aldrich
Xylene	X5-1	Fischer Chemicals
Sodium chloride 0.9% solution	BAXTJB1323	Baxter
Paraformaldehide 37%	RSOF001010F	Ricca Chemical

2.2. List of chemicals used in animals:

Chemical/cream	Catalogue No.	Manufacturer
Eye drops	7610313304578	A.Vogel
Narketan 100 mg/ml	3605877535982	Vetoquinol
NSAID cream - Lidocaine	50488-6262	Alexso Inc.
Povidone iodine 10%	159257330	Mundipharma AG
Rotarix	146776	GlaxoSmithKline Biologicals
Surgical glue	084-1469SB	3M Vetbond
Xylapan 20 mg/ml	03605874090040	Vetoquinol

2.3. Commercially available kits and antibodies used in this thesis:

Kit/Antibody	Catalogue No.	Manufacturer
AF488 CD8-α (D-9) Alexa Fluor® 488	sc-7970	Santa Cruz Biotechnology
DC assay	5000112	BioRad
EasySep Rat $CD8^+$ T Cell Isolation Kit	19643	StemCell Technologies

2.4. List of potentially hazardous chemicals used in this study:

Chemical	**Catalogue No.**	**Manufacturer**
N-Nitroso diethylamine Isopac	258-1	Sigma
N-Nitrosomorpholine	466-25	TCI

2.5. Preparation of buffers and solutions used in this thesis:

10% Formalin:

1 liter of 10% Formalin was prepared by adding of the 100 ml the 37% formaldehyde to 900 ml of distilled water.

Phosphate buffered saline (PBS):

1 liter of PBS was prepared by dissolving the 9.6 g of the PBS powder in the 1 l of the distilled water. It was thoroughly mixed and autoclaved.

0.5% Trypsin stock solution:

100 ml of the 0.5% Trypsin stock solution was prepared by dissolving the 500 mg of the Trypsin powder in the 100 ml of distilled water.

0.5% $CaCl_2$ stock solution:

100 ml of the 0.5% $CaCl_2$ stock solution was prepared by dissolving of the 500 mg in the 100 ml of distilled water.

0.05% Trypsin/$CaCl_2$ working solution:

100 ml of the 0.05% Trypsin/$CaCl_2$ working solution was prepared by mixing the 10 ml of the Trypsin stock solution with the 10 ml of $CaCl_2$ stock solution and then mixing it with the 80 ml of distilled water.

0.5% Ethylenediaminetetraacetic acid (EDTA) stock solution:

100 ml of the 0.2% EDTA stock solution was prepared by dissolving the 500 mg of EDTA powder in the 100 ml of distilled water.

0.05 % Trypsin/EDTA working solution:

100 ml of the 0.05% Trypsin/EDTA solution was prepared by mixing the 10 ml of the 0.5% Tryspin stock solution with 10 ml of the 0.5% of the EDTA stock solution and then mixing it with 80 ml of distilled water.

Citrate Potassium Dextrose (CPD) buffer:

1 liter of CPD was prepared by dissolving of the 26.3 g of the sodium citrate (dehydrate), 25.5 g of dextrose (monohydrate), 3.27 g of citric acid and 2.22 g of monobasic sodium phosphate in 1 l of distilled water. It was autoclaved.

Erythrocyte - lysis buffer (EBL):

EBL was prepared by dissolving the 0.04 g of EDTA, 7.49 g of ammonium chloride, 0.745 g of potassium chloride and the 0.79 g of ammonium bicarbonate in 1 l of distilled water.

Blocking buffer:

50 ml of the blocking buffer was prepared by adding the 5 ml of the Fetal Bovine serum (FBS) and the 0.2 g of EDTA to 45 ml of PBS.

Surface staining buffer:

50 ml of the staining buffer was prepared by adding the 5 ml of FBS and the 0.05 ml of the 0.1% sodium azide to the 45 ml of PBS.

2X Trypsin EDTA solution:

2X Trypsin EDTA solution was prepared by diluting 20 ml of 10X Trypsin EDTA in 80 ml of PBS free of Ca^{-} and Mg^{+}.

Chapter 3: Methods

3.1. Animals

For this study, Wistar Furth and Sprague - Dawley strains of laboratory rat were used, both male and females with the average age of six to seven weeks. They were obtained from the internal University of Malta Animal Facility (Faculty of Medicine and Surgery). Conditions under which the rats were kept were in accordance with the Malta Animal Welfare Act (Chapter 439 Animal Welfare Act , 2001) guidelines. Two to three rats were kept per cage accordingly to their age, sex and size. Temperature and humidity in the Animal facility were set at 22°C with 40 - 60% humidity. A light/dark cycle of 12 hours was maintained and rats were provided with *ad libitum* food and water access.

3.2. Procedures on animals

All procedures on animals, including euthanasia and operational procedures, were performed in accordance to the Malta Animal Welfare Act and the Animal Experimental Regulations (Legal Notice 263, 2003), following approval of the procedures by the University FREC JFARSS committees (Faculty Research Ethics Comitee) (Ref No: FRECMDS_1819_088) (refer to Appendix A). Persons performing these procedures (myself included) were adequately trained and are in possession of appropriate certificates for laboratory animal handling (ScotPil certificate, Edinburgh 2018) (refer to Apendix B).

3.3. Animal restraint

Animals were restrained with a use of the three fingers grip technique (Manzoor *et al*., 2003). The rat was grasped with first two fingers below the front legs after which they were pushed towards the rat's head. In this way the front legs were extended so the rat's head and body movement were restricted (Manzoor *et al*., 2003). Additionaly, rat was supported with the other hand in order to avoid any spine injury. When the injections were inserted, two persons were included in restraint procedure. The Animal Facilty Officer and the student (myself). One person injected the animal while the other person kept the animal restrained by applying the previously mentioned restraining technnique. The procedure was performed as quickly and efficiently as possible in order to avoid animal distress and suffering.

3.4. Injections

Injections were used in multiple procedures including: anaesthetising of the animals, injection of the sterile sodium chloride solution 0.9% (Baxter, Malta), induction of the carcinogenesis agent (Diethylnitrosamine) or inoculation of the transplantable tumour cell line (MT450 mammary gland tumour cell line), immunisation with the Rotarix and the autologous formalin fixed tumour vaccine (AFFTV) alone or in combination. Prior to the injection, animals were restrained with a help of the Animal Facility Officer (refer to 3.3 section). Animals were injected intraperitoneally (i.p.) or subcutaneously (s.c) as explained in each procedure and each experimental design. For subcutaneous injection, the area between shoulder blades or the flank area was used as the skin is loose in those areas. In order to inject, the skin was lifted, and needle was inserted. The angle at which the injection was inserted was small in order to avoid damaging any underlying blood vessels or the tissue (Manzoor *et al*., 2003). In the case of the intraperitoneal injections, the insertion of the needle was performed in the posterior left quadrant of the abdomen (Waynforth *et al*., 1998). In both cases, subcutaneous or intraperitoneal, the needle was aspirated in order to check that the needle is positioned correctly (For example: if there was a blood it would suggested that the needle hit the blood vessel, and the substance was not injected) (Manzoor *et al*., 2003). For the injection purposes, sterile 25 gauge needles were used as well as 1 ml sterile syringes. Upon injection, the animals were followed up acutely for 2 - 3 hours, and over the following days, and any unusual behaviour was documented (bleeding, locomotion discrepancy, disorientation, stress features).

3.5. Surgical procedures

3.5.1. Preparation of the surgical area

Surgical procedures were performed in order to obtain tumour tissue biopsy *in vivo*. The required clean conditions were followed. The bench on which the surgical procedures were done was swabbed with 70% ethanol (Fischer Chemical, USA), after thorough cleaning as was the heating pad, which was positioned on the bench and covered with sterile paper sheets. The heating pad was set to the optimal temperature (around 27°C), in order to keep the animal at a comfortable temperature range, without the risk of falling into hypo- or hyper- thermic states. A new pack of gloves was prepared, sterile surgical swabs, solution of povidone iodine 10% (Mundipharma AG, Switzerland) and surgical instruments were sterilised on 300°C in a bead steriliser (Inotech, USA).

3.5.2. Preparation of animals for the surgical procedure

The weight of the animal was measured with use of digital scale (Salter, UK). The rats were first transferred to an open box, which weight was previously tarred and then measured. Based on their measured weight, the amount of the anaesthetic solution required was calculated. Prior to anaesthesia, the animals were observed, any unusual behaviour was documented and if signs of severe poor health were noticed, those animals were not followed up but euthanised.

3.5.3. Anaesthesia

A ketamine (Narketan 100 mg/ml, Vetoquinol, UK) xylazine (Xylapan 20 mg/ml, Vetoquinol, UK) mixture was used as an anaesthetic and was prepared as follows, 4 ml of narketan was added to 2 ml of xylapan and topped up until 8 ml with sterile sodium chloride solution 0.9%. This anaesthetic cocktail was applied intraperitoneally 100 mg/ml of narketan and 10 mg/ml of xylapane. Dosage that was used was 0.2 ml/100 g of rats bodyweight. Animals with signs of primary tumour which did not appear too sick were anaesthetized, kept in the dark for 10 minutes (depending on each animal's behaviour), after which general signs of anaesthesia or absence of reflexes were observed (tail pinch, pedal reflex, slower heart rhythm). Artificial eye drops (A. Vogel, Switzerland) were applied on eyes of treated animal multiple times during the operation, in order to avoid eyes drying and corneal damage (irritation and eye damage during time under anaesthesia may occur during the time under anaesthesia as the eye reflex of the animal is absent.

3.5.4. Operational procedure

Upon recognition of the general signs of anaesthesia or absence of reflexes, animals were transferred on the previously prepared sterile sheets on the heating pad. The surgical site was shaved, and cleaned with the 10% povidone iodine and an incision made using of a sterile scalpel. The animal's condition including breathing, heart rate, reflexes were observed and any change was noticed and acted upon (For example any signs of reaction would indicate insufficient anaesthesia and an additional dose of ketamine/xylazine given). Biopsy sampling was performed on different organs/structures (liver, subcutaneous tumor), depending on the experiment and carcinogenesis model. Following biopsy, the tissue was properly sutured or closed with surgical glue (3M Vetbond, USA) and the skin layers covering the spot were sutured (SMI sutures, Belgium) accordingly. A discontinuous suture technique was always applied in order to reduce the possibility of post-operational complications as a consequence of suture removal by animal.

3.5.5. Post-operational care

The surgical site was treated with NSAID (Non-steroidal anti - inflammatory drug) cream (Lidocaine, Alexso Inc.), at dose of 40 mg/ml and the animal was left to recover while still being kept in optimal temperature range (27 - 28 ^{0}C). Animal was injected subcutaneously with the sterile sodium chloride 0.9% solution while being under anaesthesia to maintain hydration, and during recovery from anaesthesia upon retrieval of the swallowing reflex, the animal was water nourished through a syringe. The sterile sodium chloride solution 0.9% and water were kept at the room temperature or warmed in order to aid prevention of hypothermia. The animals were constantly observed during the first couple of hours of the post-operational period until they appeared to have recovered from anaesthesia, and afterwards occasionally checked during the day and following days. If any unusual sign during animal recovery was noticed, it was followed by immediate action with regards to animal welfare. Very few complications were noted in the great majority of cases. The operated animals were treated with the Lidocaine cream, at dose of 40 mg/ml, two times daily in days following operation.

3.6. Animal euthanasia

Animal euthanasia was performed according to the Malta Animal Welfare (Act XXV, 2001) act and the Legal Notice 263 (Animal Experimental Regulations, 2003). It was performed according to the experimental design (refer to 3.14 section) or upon signs of critical health conditions being noticed. Features indicating deterioration in animal health upon which this procedure needs to be applied in order to relieve animal of pain and suffering include chronic pain, severe loss of function (locomotory, respiratory or other). In both cases, animals were euthanized in CO_2 chamber with slow increase of CO_2 concentration. After 15 minutes, animals were observed and death was confirmed if there were no pulse and animals were not breathing. This was followed with a neck dislocation as a certain death confirmation.

3.7. Organ collection

Soon after the death confirmation, relevant organs were isolated and documented (photography). Organs such as the liver and the lungs, were isolated by incising the abdominal peritoneal wall as well as the thorax area. Then the liver and the lungs were removed with the use of surgical scissors. The subcutaneous tumour was isolated with the separation of the skin from the right flank area

followed with the removal of the tissue around the tumour itself. Then it was incised with the use of surgical scissors. Organs were observed and any significant changes were documented and the photographs were taken. Following this, organs were transferred to a 50 ml falcon tubes containing 10% (v/v) formalin solution and kept for 24 hours after which formalin solution was discarded, organs washed with phosphate buffered saline solution (PBS) and transferred to the new 50 ml falcon tubes containing 70% ethanol, for long term preservation.

3.8. Histology

3.8.1. Tissue preparation

Isolated organs kept in 70% ethanol solution were processed further in the Pathology laboratory (Mortuary Facility, Mater Dei Hospital, Malta) where histological slides were made as follows. The 70% ethanol was discarded and the organs were transferred to sterile bench on the in the Pathology laboratory where they were sectioned into 3 - 5 mm slices with use of a scalpel, aiming to identify the relevant parts that can best represent tissue morphology and the morphological changes of importance. One or two slices were chosen, depending on the size of an organ, and transferred to a plastic embedding cassette.

3.8.2 Tissue dehydratation

Depending on the size of the sample and of the embedding cassette, sometimes samples of different organs were kept in the same cassette at an appropriate distance. Embedding cassettes containing samples were transferred into metal containers which were placed on the appropriate metal slide in the automatic tissue processing machine (Leica biosystems, Germany). Tissue samples underwent continued dehydratation through sub-sequent changes in ethanol (Fischer Chemical, USA) solution gradient, as follows: for 15 minutes in the 70% ethanol, 15 minutes in the 90% ethanol, in the absolute ethanol for 15 minutes, 30 and for 45 minutes.

3.8.3. Clearing

In order to remove the alcohol, rack with the embedding cassettes containing tissue samples was transferred automatically to the container filled with the clearing agent. The clearing agent used was with xylene (Fisher Chemical, USA). Tissue slides were incubated in xylene twice for 25 minutes and for 45 minutes.

3.8.4. Tissue paraffinisation

The rack containing the tissue slides was automatically transferred to the container containing the paraffin wax (Fisher Chemical, USA). Tissue slides were infiltrated with the paraffin as it was kept for 30 minutes and then twice for 45 minutes.

3.8.5. Tissue embedding

After this process, tissue samples were transferred to a paraffin bath where the previous wax was melted at 58 °C in order to get smooth blocks. Cassettes containing tissue samples were opened and tissue was transferred to appropriate sized metal moulds. Small amount of melted paraffin was added in order to cover the sample and the mould was transferred with warm forceps to an ice plate where tissue was gently pressed in order to reduce excess of covering wax as well as to position tissue as accurately as possible. Previously used labelled plastic cassette without a lid was positioned above and additional melted paraffin was added on top, covering the plastic completely. In this manner prepared samples were left on ice plate for 1 hour until wax was completely solidified and cooled.

3.5.6. Microtome

The tissue blocks were popped out and transferred on ice to the microtome (Leica Biosystems, Germany). The microtome was used for the sectioning of the waxed samples, the initial diameter (section thickness) was set to 30 µM for trimming purpose until well formed, smooth, tissue films were formed then the diameter was reset to 3 µM, and very thin tissue films were transferred to the water bath previously heated at 37°C and the tissue film was thus stretched out by surface tension and picked up on the glass slide. The tissue film was located centrally on the glass slide and these were left to dry after which they were translocated to the appropriate metal rack and left until staining.

3.8.7. Haematoxilin and eosin staining

Haematoxilin and eosin staining was performed automatically in the slide stainer (Leica Biosystems, Germany). First tissue slides were transferred to a ceramic rack inside the staining processor, then they were treated twice with xylene solution for 2 minutes. Then they were washed through different gradients of ethanol, first decreasing, from absolute ethanol, twice for two minutes, to 90% ethanol, once for 2 minutes. This was followed with water rinsing. This was performed in order to prepare tissue for haematoxylin staining since heamatoxilin is a water based

dye. Tissue samples were kept in haematoxylin for three minutes. After staining with haematoxylin, samples were rinsed with water shortly and treated with acetic acid in order to differentiate the colour of the haematoxylin. This step was repeated twice. Then tissue samples were passed through an increasing gradient of ethanol solution, 90 - 100%, each for one minute. This was performed in order to prepare tissue for eosin staining as eosin is an alcohol based dye. Slides containing tissue samples were immersed in eosin stain and then passed through an increasing gradient of ethanol again. This was followed with the incubation in xylene, twice for two minutes. In this way excess water was removed, and tissue slides were left to dry. Following this step, slides were transferred into a fume hood where the mounting process was performed.

3.8.8. Cover slip mounting

Cover slips were prepared as well as xylene solution and Dybuthylphtalate polystyrene xylene (DPX Mounting Medium, Sigma - Aldrich, USA). The cover slips were treated with DPX, with only a drop or two were placed on each cover slip. Tissue slides were immersed in xylene solution and transferred to a cover slip at a 45 degree angle cautiously, carefully avoiding the trapping of air bubbles. Slides were checked and any remaining air bubbles were extracted by applying gentle pressure with a rounded pencil top. Prepared tissue slides were left to dry and then transferred to the carton block where they were ordered by the labelling number and kept until the analysis.

3.9. Cell culture

MT450 mammary gland tumour cells were incubated in RPMI 1640 (Rosswell Park Memorial Institute) (Sigma - Aldrich, USA) medium, which was supplemented with the foetal bovine serum (FBS) (Sera Lab, USA) at 10% (v/v). Cells were incubated in a humidified incubator (Eppendorf, Germany) at 37°C and under 5% CO_2. Cell cultures were seeded in 25 ml tissue culture flasks at $1x10^6$ cells/ml in 5 ml, and depending on the cell number, sometimes subcultured to 75 ml flasks. Cultures were passaged two times per week in 75 ml culture flasks in sterile conditions and passaged to a maximal dilution of 1/10. In order to passage them into new culture flasks with fresh media, cells were counted on a haemocytometer, and 5 ml of medium with 5×10^5 cells/ml was subcultured into new flasks (25 ml). For long - time storage, cells were kept in liquid nitrogen in accordance with standard procedure. Cells were collected by centrifugation (1500 rpm, 5 minutes at a room temperature). The cell pellet was gently resuspended in 5 ml of freezing media (90% (v/v) foetal bovine serum, 10% (v/v) dimethyl sulfoxide (DMSO) (Sigma - Aldrich, USA) and transferred to cryovials at 1 ml per tube. Cells were frozen at concentration of 5×10^5 cells/ml.

When needed, cells were recovered by thawing the cryovials in a 37° C water bath upon which they were transferred to a 15 ml falcon tube containing the RPMI medium with the 10% of foetal bovine serum (FBS) and 1% of penicillin/streptomycin. The cells in falcon tubes were centrifuged, cell pellet was resuspended and cell count was determined. Following this step, cells were seeded in new 25 ml culture dishes containing a new RPMI media supplemented with foetal bovine serum (FBS) 10% (v/v) and penicillin/streptomycin 1% (v/v), at desired seeding density. After 24h they were ready for use in an experiment. Cells were inoculated into rats at various concentrations, 1 x 10^5 cells/500 μL, 5 x 10^5 cells/ 500 μL, 1 x 10^6 cells/ 500 μL and 5 x 10^6 cells/500 μL, depending on experimental design.

3.10. Chemical carcinogenesis

In order to construct the liver cancer model with metastasis to the lungs, two-step chemical carcinogenesis was applied. Two step carcinogenesis was comprised of the initiation step or the induction of the carcinogenesis, which was subsequently followed by the carcinogenesis promotion as a second step. As the initiator of the carcinogenesis, Diethylnitrosamine (DEN) (Sigma) was used and as the promoter N - Nitrosomorpholine (NMOR) (TCI Chemicals, Japan) was used. This model is called a DEN - NMOR model (Futakuchi *et al*., 1999; Yoshino *et al*., 2005). Prior to use, carcinogens were kept in the dark at 4° C in specifically designed areas. Any manipulation with carcinogens except for inoculation into animals was performed in the fume hoods and material that was used was carefully discarded in special waste containers specifically for this purpose that were sealed and transported to the appropriate waste disposal for hazardous material.

3.10.1. Preparation and application of the DEN

The DEN dose was prepared based on a rat's weight. Initial suggestion from published data indicated that 100 mg of DEN is used per 1 kg of rats weight. A ratio was set to 15 mg per 150 g of rats weight according to the size of the rats. A stock solution was prepared by dissolving 1 g of DEN in 50 ml of deionized water (diH20). The concentration used for injection was 10 mg per 0.5 ml. Prior to the injection, which was performed intraperitonealy, rats were measured and according to their weight, injection volumes were set and applied. Only one injection was given.

3.10.2. Preparation and application of the NMOR

The NMOR was first transferred to the water bath set on the 37° C and kept for 5 min due to its crystallization that occurs at a temperature below 37° C. This was done as quickly and efficiently as

possible as this compound is temperature sensitive and longer heat exposure can damage its properties. After it was noticed that it liquidized, 5 ml (1 g/ml) of NMOR were mixed with 41 L of the drinking water. This was done according to the number of rats per cage and to the average water intake of each rat (100 ml). In this way prepared NMOR was provided in drinking water as 120 parts per million (ppm) *ad libitum* to rats treated previously with DEN. Rats were given NMOR five days after they were injected with DEN, and for a period of one month.

3.11. Transplantable carcinogenesis model

The transplantable tumor model of carcinogenesis used with a rat mammary gland tumor cell line (MT450) derived from Wistar/Furth inbred rats (Thiele *et al*., 2013; Quagliata *et al*., 2014). Cells were cultivated like previously mentioned (refer to 3.9 section) and prior to injection, cell viability and number was determined using the standard protocol which included counting in the haemocytometer under the light microscope at a magnification of 400X. Cell count was set to a concentration of 1×10^5, 5×10^5, 1×10^6 and 5×10^6 cells per 0.5 ml of RPMI media supplemented with 10% of foetal bovine serum and 1% penicillin/streptomycin mixture. That volume was injected into each rat subcutaneously into the middle of the right flank. The injection site was observed and any leakage of injected material was documented and acted upon as well as formation of any sort of oedema or the tissue irritation.

3.12. Autologous formalin fixed tumour vaccine (AFFTV)

Depending on the experimental design and the carcinogenesis model, the AFFTV was prepared from the isolated tumour tissue or from the MT450 cells. Only initial steps in the preparation of the AFFTV differ among these two types.

3.12.1. Initial preparation of the AFFTV made from the isolated tumour tissue biopsy

The isolation of the tumor tissue was performed during surgical operation in which animal was anaesthetized. A small biopsy was sampled and transferred into a sterile tube previously filled with a 10% formalin solution (Ricca Chemical, USA) (refer to 2.5 section). The tumor tissue biopsy was kept in 10 % formalin for 24 to 48 hours. Following this period, the tumor tissue biopsy was transferred to a Petri dish previously placed in the sterile fume hood where it was mechanically processed. Prior to the step of mechanical processing, the tumor tissue was washed in the sterile phosphate buffered saline (PBS) (Sigma - Aldrich, USA). The mechanical processing included slicing of the tumor tissue with two surgical scalpels, one in each hand. Slicing was performed

thoroughly and the tumor tissue was sliced as much as possible, until it became a tissue suspension that can be collected with a 3 ml Pasteur pipette. When collecting, 2 to 3 ml of PBS were added to the tissue suspension and transferred into the 15 ml Falcon tube.

3.12.2. Initial preparation of the AFFTV made from the MT450 cells

In case of the MT450 cells, the cells were transferred from the 25 ml or 75 ml cell flasks into the 15 ml Falcon tube, centrifuged (Eppendorf 1580 centrifuge, Germany) at the 1500 rpm for 5 minutes. The supernatant was decanted and the cells were resuspended in 2 to 3 ml of PBS and left in the 15 ml Falcon tubes.

3.12.3. Further common steps in processing of the AFFTV, made of the tumour tissue biopsy or from the MT450 cells

The following step included heating of the samples in a microwave (DCG Eltronic MWG820, 800 W, Italy).The tubes containing samples mixed with PBS were transferred to a glass beaker (volume of 500 ml) that was previously filled with a bit of tap water (50 - 100 ml).The glass beaker with the tubes containing samples was transferred in to the microwave. Lids of the tubes were loose in order to prevent tubes bursting and loosing the sample. The microwave power level was set at the level five and the timing was set on two (changed protocol) or five minutes (standard protocol) and the microwave was turned on. During the heating cycle, PBS was added in order to prevent drying of a sample. Three heating cycles were performed both for changed and standard protocol. In between cycles, tubes containing samples were cooled under the tap water and the PBS was added if needed. After completion of the heating cycles, the tubes were transferred to the fume hood where 2 to 3 ml of the 2X Trypsin/EDTA solution (Sigma - Aldrich, USA) (refer to 2.5 section) was added to each tube. This step included enzymatic digestion of samples. Samples were left for 24h incubation in the cell incubator. After 24h incubation, tubes containing samples resuspended in trypsin/EDTA were transferred to the glass beaker and underwent the same heating cycle as previously but only once. Following this, tubes containing samples were centrifuged at 200 rpm for 5 minutes. They were transferred back to the fume hood where the supernatant was collected and filter sterilised with use of a 10 ml syringe and a syringe filter. Tubes containing sterilised supernatants were kept in the fridge at 4°C until immunisation.

3.13. Rotarix immunisation

Rotavirus oral vaccine (Rotarix, GlaxoSmithKline Biologicals, Belgium) was used for the first or/and the second immunization of animals depending of the experimental design. Before use, Rotarix was kept in the freezer at the 4°C and prior to the actual administration it was left for five to ten minutes at the room temperature in order to not be cold when administered. When used for the initial, first, immunisation, Rotarix was administered subcutaneously in between shoulder blades of the animal and the place was marked. The volume that was used for immunisation in general was around 0.2 ml. For the first and second immunisation, in initial experiments, chemical carcinogenesis model and the initial MT450 model, it was administered subcutaneously at the lower back side and in the other MT450 model experiments intraperitoneally. This was stated in each experimental design.

3.14. Assessing morphological changes in animals

3.14.1. Assessing morphological features of the tumour development in the liver and lungs

In both organs, greyish white nodules appearing on the organ surface were considered as tumours if their size was exceeding 1 mm in diameter (Fu *et al*., 2019; *Kuehl et al*., 2018). In the liver, this was usually followed with the increase of the connective tissue and loss of liver colour. In both organs, tissue texture started to become hard as the tumour were advancing (*Kuehl et al*., 2018).

3.14.2. Experimental design of the experiment in which the morphological features of the tumour metastasis in lungs of the rats inoculated with the 5 x 10^6 MT450 cells, after different time periods, were assessed

In order to determine the approximate time of metastatic formation in lungs after subcutaneous injection with the 5 x 10^6 MT450 cells, rats were divided into five groups. Under predetermined time frames, animals were sacrificed (refer to Table 3.1). Post mortem, animals were examined and any morphological change was documented. Additionally, the lungs were isolated and histologicaly analysed.

Groups	Time of sacrifice after inoculation
1	One and a half weeks
2	Two weeks
3	Two and a half weeks
4	Three weeks
5	Four weeks

Table 3. 1. Rats grouped based on the time of sacrifice after the inoculation with the 5 x 10^6 MT450 cells. Assesment of the morphological features of tumor metastasis in lungs of the rats inoculated with the 5 x 10^6 MT450 cells, after different time periods.

3.14.3. Experimental design of the experiment in which the morphological features of the tumour development in the rats injected with different amounts of the MT450 cells were assessed

In order to optimise the model of carcinogenesis and to provide better conditions for treatment with the AFFTV, an experiment was performed in which animals were grouped depending on the amount of the MT450 cells used for the inoculation. Both male and female animals were used in order to obtain data that will efficiently describe the tumour formation and it's progression in the animal model. Rats were injected with different amounts of the MT450 cells: 1 x 10^5, 5 x 10^5 and 1 x 10^6.

At certain time intervals after inoculation: seven, fourteen, twenty one, twenty eight and thirty five days, rats were sacrificed and organs including lungs, right axillary lymph node ,right brachial lymph node and the tumour tissue were isolated. Any morphological change, including the presence of the abnormal tissue on the organs, change in size and colour of the organs was documented.

3.15. Histological assessment

The tissue slides stained with haematoxilin and eosin were examined under the light microscope at different magnification including 40X, 100X and 400X. Total magnification used for the slide examination was calculated by multiplying the power of the objective (4X, 10X and 40X) by the power of the eyepiece (10X). In this study, magnification represented next to the photographs of the tissue slides is total magnification.

3.15.1. Histological assessment of the tumour infiltrating lymphocytes in liver, lung and subcutaneous tumour

The tumour area was selected and the assessment was performed on the lymphocytes present in the tumour stroma area or in the direct contact with the tumour cells. In this study, as opposed to the recommendation guidelines (Hendry *et al*., 2017, Salgado *et al*., 2015) for the assessment of the tumour infiltrating lymphocytes (TILs) in solid tumours, the lymphocytes present in the tumour were assessed as a whole and not separated as stromal and intratumoral. This was performed, as that it is an initial study and the emphasis was on the assessment of the general number of lymphocytes in the tumour after the immunisation.

3.15.2. Histological scoring of the lymphocyte infiltration

In order to derive data from the isolated tissue that can be used for analysis and comparison between different treatment groups of animals scoring was performed. As instructed in the literature related to the histopathological examination and scoring, any bias was restricted by 'blinding' of a researcher (myself) and my supervisor (prof. Pierre Schembri Wismayer) to a tissue origin (experimental groups or treatments). Strategy used for masking was so called 'comprehensive' masking strategy in which the tissue samples were labelled with no reference to the experimental group or the treatment (Gibson - Corley *et al*., 2013).Only what was provided was the source of the tissue, from which organ it was derived (lungs, liver, subcutaneous tumour etc.). In order to increase validity of the tissue examination and scoring, tissue samples were examined separately and scoring was provided as a mean in between researchers. In order to perform semi-quantative analysis, photomicrographs of the tissue slide sections were made under the magnification of 400X. Regarding that one block was prepared per sample, five micrographs of distinct tissue sections were captured and scored (Salgado *et al*., 2015). Scores were based on density/area covered and scored as a percentage of total cell number in one area (under the 400X magnification). Scores were as follows: score 0= absent (1% of TILs), score 1= minimal (from 1 - 10 % of TILs), score 2= mild (10 - 20 % of TILs), score 3= moderate (20 - 50 % of TILs) and score 4= strong (>50% TILs) (Grigoriadis *et al*., 2018; Salgado *et al*., 2015). The average score obtained from five non - overlapping images from one slide per block was used as the final score for that tissue sample. Based on this, the level of the lymphocyte infiltration in the isolated organs of each animal used in this study was assessed and it further provide indications of the therapy effect.

3.15.3. Morphological features of lymphocytes

Morphologic features of lymphocytes that were used in order to histologically distinguish them from the rest of the cells included size, shape, and colour of the nucleus and cytoplasm. So the lymphocytes were distinguished morphologically as cells of small to medium size that are round shaped with the round dark blue or purple nucleus and the cytoplasm that is pale coloured and barely existent (Histology Guide, n.d. ; Nguyen *et al*., 2017).

3.15.4. Morphological features of cancer cells

Morphological features of cancer that were used for their histological distinction were size, shape and colour of the nucleus and cytoplasm. Cancer cells were characterized morphologically as cells with irregular shape which have irregularly shaped and large nucleus that is usually not centrally positioned, with the cytoplasm that can be bright or colorless and which covers small surface (Kumar *et al*., 2015; Baba and Catoi, 2007). The cancer cells are usually different in size as compared to the normal cells in the same tissue (Kumar *et al*., 2015; Baba and Catoi, 2007).

3.16. Experimental designs of the experiments in which the lymphocyte infiltration was histologicaly assessed

3.16.1. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in the DEN - NMOR carcinogenesis model

In order to obtain initial information regarding the possible effect of the AFFTV and Rotarix on the initiation of the immune response we conducted the experiment in which we used the DEN - NMOR model of carcinogenesis. Initially, for the DEN - NMOR model of carcinogenesis Sprague Dawley rats, male and female, sixty days old (P60) were used. Animals were injected with the DEN i.p. and NMOR was provided them *ad libitum* for one month (refer to 3.10 section). After this, animals were occasionally measured (once per week) in order to determine any significant weight loss as a consequence of the possible tumour development. Animals in which signs of significant weight loss were observed, were weighed more frequently on every two to three days. This was done in order to determine the rate of weight loss. Animals with continuous weight loss were operated. During the operational procedure, in animals that were losing weight, examination was performed and any significant morphological change present on the liver tissue was documented. Based on those observations tumour tissue biopsies were obtained from animals in which changes were seen, processed and these animals were vaccinated with the AFFTV. Animals in which

significant morphological changes were not observed, were not vaccinated but were included as a control animals. During the operation, animals in which morphological changes were observed, were immunised with the Rotarix vaccine subcutaneously in between the shoulder blades and following three days they were immunised with the combination of the Rotarix and the AFFTV subcutaneously in the lower back. The animals that were not losing weight were operated as well eventually, regarding that a significant time period (8 - 9 months) since the initiation of the carcinogenesis passed. The same was applied as in the case of the animals that were losing weight.

Groups	First immunisation	Second Immunisation
Unvaccinated (n=5)	/	/
Vaccinated (control) (n=9)	Rotarix	Rotarix + AFFTV

Table 3. 2. Number of animals included in the control and the vaccinated group. Assessment of the features of immune response after the immunisation with the combination of the Rotarix and the AFFTV in the DEN - NMOR carcinogenesis model.

3.16.2 Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of the AFFTV and the Rotarix

In order to obtain initial information on the effect of the AFFTV and Rotarix on the immune response in the MT450 carcinogenesis mode when the Rotarix and AFFTV are applied separately or in combination. We conducted experiment in which we have used different combinations of the AFFTV and the Rotarix. As well, different timing for the second immunisation was applied, three and seven days after the first immunisation, in order to determine effect of timing on the immunisation. For this experiment, Wistar rats, male and female, sixty days old (p60) were used. Animals were separated in different groups, see Table. Animals were inoculated with the 5×10^6 MT450 cells in the right flank area subcutaneously. After the inoculation animals were observed any significant morphological change (palpable tumour, ulceration) were documented. After the four weeks period (when the tumours were obvious), all animals except the control group were immunised for the first time. All groups except the group that will be secondarily immunised with the AFFTV alone, were immunised with the Rotarix. In this experiment, AFFTV that was used, was the AFFTV made of the MT450 cells. Three or seven days after the first immunisation, animals were immunised for a second time. This time with the Rotarix or/and the AFFTV depending of the group, (refer to Table 3.3).

Groups	First immunisation	Second immunisation	Second vaccination timing (days after 1st immunisation)
Control (unvaccinated) (n=2)	/	/	/
Rotarix (n=2)	Rotarix	Rotarix	7
Rotarix (n=2)	Rotarix	Rotarix	3
Rotarix + AFFTV (n=3)	Rotarix	Rotarix + AFFTV (made of MT450 cell line)	3
Rotarix + AFFTV (n=3)	Rotarix	Rotarix + AFFTV (made of MT450 cell line)	7
AFFTV (n=3)	AFFTV	AFFTV (vaccine made of MT450 cell line)	3

Table 3. 3. Groups of animals separated based on the vaccine/Rotarix combination used for the first and second vaccination. Assessment of the features of immune response in the MT450 carcinogenesis model after the immunisation with various combinations of the AFFTV and the Rotarix.

For the first immunisation, animals were injected between the shoulder blades while for the second immunisation, animals were injected subcutaneously in the lower back area. After first and the second immunisation, animals were observed for any significant change, including ulcer formation (ulceration), general health decay, or any behavioural change.

3.16.3. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the Rotarix and AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cell line with the standard protocol

In order to determine is there a difference in the effect of the AFFTV made of the tumour tissue biopsy and/or the AFFTV made of the MT450 cell line with standard or changed protocol on the possible immune response in the MT450 carcinogenesis model, experiment was conducted. All Wistar rats were inoculated with 5×10^6 MT450 cells subcutaneously on the right abdominal side.

In this experiment, all animals except the ones from the control group were immunized with the Rotarix vaccine three weeks after the inoculation. The immunisation was performed earlier as compared to the previous experiment in order to target the primary subcutaneous tumour and lung metastasis at the earlier stage. The second immunisation was performed three days after the first immunisation with the Rotarix. For the second vaccination, different vaccination combination were used, refer to Table. 3.4)

Groups	**First immunisation**	**Second immunisation**	**Second vaccination timing (days after 1st immunisation)**
Control (unvaccinated) (n=3)	/	/	/
AFFTV made of the tumour tissue biopsy in the combination with the AFFTV made of MT450 cell line (n=3)	Rotarix	AFFTV made of the tumour tissue biopsy in the combination with the AFFTV made of MT450 cell line	3
AFFTV made of the tumour tissue biopsy (n=2)	Rotarix	AFFTV made of the tumour tissue biopsy	3
AFFTV made of the MT450 cell line) (n=5)	Rotarix	AFFTV (made of MT450 cell line)	3

Table 3. 4. Animals separated in groups based on the vaccine/Rotarix combination used for the first and second vaccination. Assesssment of the features of immune response in the MT450 carcinogenesis model after the immunisation with the Rotarix and AFFTV made from

the tumour tissue biopsy and/or the AFFTV made of the MT450 cell line with the standard protocol.

For the first immunisation, animals were injected between the shoulder blades while for the second immunisation, animals were injected intraperitoneally. After first and the second immunisation, animals were observed for any significant change, including ulcer formation, general health decay, or any behavioural change.

3.16. 4. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the Rotarix and AFFTV made of the MT450 cell line with the standard or changed protocol

In order to determine is there a difference in the effect of the AFFTV made of the MT450 cell line with standard or changed protocol on the possible immune response in the MT450 carcinogenesis model, experiment was conducted. All Wistar rats were inoculated with 5 x 10^6 MT450 cells subcutaneously on the right abdominal side. In this experiment, all animals except the ones from the control group were immunized with the Rotarix vaccine three weeks after the inoculation. The second immunisation was performed three days after the first immunisation with the Rotarix. For the second vaccination, different vaccination combination were used, refer to Table 3.5). A different protocol for the AFFTV made of MT450 cell line understood change in preparation of this vaccine, in heating retrieval timing (refer to the 3.12. section).

Groups	First immunisation	Second immunisation	Second vaccination timing (days after 1st immunisation)
Control (unvaccinated) (n=3)	/	/	/
AFFTV made of the MT450 cell line with changed protocol (n=2)	Rotarix	AFFTV made of the MT450 cell line with changed protocol	3
AFFTV made of the MT450	Rotarix	AFFTV (made of MT450 cell line)	3

cell line) (n=2)			

Table 3. 5. Animals separated in groups based on the vaccine/Rotarix combination used for the first and second vaccination. Assessmet of the features of the immune response in the MT450 carcinogenesis model after the immunisation with the Rotarix and AFFTV made of the MT450 cell line with the standard or changed protocol.

For the first immunisation, animals were injected between the shoulder blades while for the second immunisation, animals were injected intraperitoneally. After first and the second immunisation, animals were observed for any significant change, including ulcer formation, general health decay, or any behavioural change.

3.16.5. Survival analysis

Animals, both vaccinated and unvaccinated control, were followed and observed. For the experiment described in 3.16.1 section, for each animal the vaccination or surgery was used as a entry time point. For the experiments described in 3.16.2 and 3.16.3 sections, regarding that in all vaccinated groups vaccination was performed at the same time, as well as the operations, this was used as an entry time point for survival analysis. The same time was considered as the entry point for the control group.

In all experiments, the survival time was estimated as a time passed from the entry time point to the end point (euthanasia) and it was measured in days. Animals were euthanized when it was evident that they are suffering and that was considered as a humane end point of experiment. Post mortem, the organs (liver, lungs, subcutaneous tumour) were collected and further histologicaly processed and scored.

3.17. Isolation of blood

3.17.1. Experimental design

In order to get better insight in the amount $CD8^+$ T cells in the vaccinated and in the unvaccinated (control) animals, the peripheral blood of the animals was isolated. As well, in order to determine the effect of a different amount of the injected MT450 cells on the immune response after the AFFTV immunisation, we have injected subcutaneously animals with two different amount of the

MT450 cells. Rats were separated in two groups that will be vaccinated and two groups that will serve as a control. First group that will be vaccinated and first control group were inoculated with the 1 x 10^5 MT450 cells, while second group that will be vaccinated and the second control group were inoculated with the 5 x 10^5 MT450 cells. After a four weeks period, animals from two groups that were predetermined to be vaccinated were immunised with the Rotarix. Three days after the first immunisation, these animals were immunised with the AFFTV vaccine made of the MT450 cells. Seven days after the second immunisation, animals were euthanized and the blood was collected by cardiac puncture and further isolation of $CD8^+$ T cells was performed. The blood of one of the control animals was used for the isolation of the lymphocytes that will be unstained and used as an unstained control as well as for the isolation of $CD8^+$ T lymphocytes with the isolation kit that will be used as a positive control (refer to Table 3.6).

Groups	Amount of MT450 cells used for the inoculation	First immunisation	Second immunisation	Time of sacrifice (in days)
Control 1 (n=4)	$1x10^5$	/	/	Seven days after the second immunisation
Control 2 (n=4)	$5x10^5$			
Unstained and positive control (n=1)	$1x10^5$			
Vaccinated 1(n=5)	$1x10^5$	Rotarix	AFFTV made of MT450 cells three days after the first immunisation	
Vaccinated 2(n=5)	$5x10^5$			

Table 3. 6. Animals separated in groups based on the amount of the MT450 cells used for the inoculation and whether they were immunized or not. Flow cytometry analysis. n - number of animals in each experimental group.

After isolation, cells were analysed on Flow cytometre on BD Cell Quest Pro Software after which data was further analysed on FlowJo software.

3.17.2. Blood letting

In order to get insight into the presence and distribution of lymphocytes, peripheral blood was extracted from the heart of the anaesthetized animals during the procedure that involved terminal cardiac puncture. Animals were anaesthetized with a ketamine/xylazine cocktail. After a certain time (around 10 minutes), reflexes and breathing rhythm were assessed. After determining that the animal was anaesthetized properly, the left thoracic side of the rat's body was punctured with a needle attached to a 10 ml syringe containing 1 ml of anticoagulant Citrate Phosphate dextrose (CPD) (refer to 2.5. section). This was followed by heart puncture and blood was very slowly collected because the heart can easily collapse during this procedure. After this, the blood was transferred into a 15 ml Falcon tube containing 2 ml of anticoagulant (CPD). Tube was properly closed and slowly shaken in order to enhance the anti - coagulative effect of the CPD buffer. At the end of the process, heart failure causes death of the animal. In order to ensure death, cervical dislocation was performed.

3.17.3. The lymphocyte isolation

Tubes containing blood samples were precisely labeled and then transferred to the centrifuge. Samples were centrifuged for 5 minutes at the 1500 rpm. After centrifugation, tubes containing samples were cautiously collected and transferred to the sterile laminar hood. The plasma layer from each tube was removed by pipeting and then the white turbid layer and the white layer that was directly on the top of the red blood cells (RBCs) were gently collected and transferred into another labeled 15 ml falcon tube. An equal amount of the PBS was added to what was collected. A new 15 ml Falcon tube was labeled and prepared with 3 ml of Histopaque (Sigma - Aldrich, Germany). Blood - PBS mixture was cautiously added dropwise, along the slantingly held tube, on the top of the Histopaque layer. Tubes were centrifuged at 2500 rpm for 25 minutes with brakes off/soft landing. Following the centrifugation, tubes were transferred to the sterile laminar hood where the upper clear layer (plasma/anticoagulant) was removed and discarded. The white layer above the Histopaque was collected cautiously and transferred into a new labeled tube. Special attention was dedicated to not disturbing the gradient layers and to not collect erythrocytes that were just below the white layer. Erythrocyte lysis buffer (ELB) : PBS solution was added to the sample in a ratio of 3:1, well mixed and incubated for 10 minutes under constant slow shaking. Following incubation, cells were centrifuged at 1500 rpm and for 5 minutes. This step was repeated until a clear white pellet was collected. The supernatant was then removed and 5 ml of media were added to the sample. This mononuclear cell sample was left for 2 hours in a tissue culture flask for

the monocytes to adhere in order to separate it from the lymphocytes. After 2 hours, supernatant containing lymphocytes was transferred into a new tube, cells were centrifuged at 1500 rpm for 5 minutes, supernatant was decanted and cells fixed with 500 µl of ice cold methanol, transferred to the polystyrene box previously filled with ice and left for 10 minutes. Following this step, cells were centrifuged at 1500 rpm for 5 minutes, the supernatant was decanted and the cells were left in 1 ml of the blocking buffer, transferred again into a polystyrene box containing ice and left in -20°C freezer until the surface staining. In case of the cells that were predetermined to be used as a positive control, prior to the surface staining, they have been further purified with the use of EasySep Rat $CD8^+$ T cell isolation kit (refer to 3.17.4 section).

3.17.4. Isolation of $CD8^+$T cells for positive control

A EasySep isolation kit (StemCell Technologies, USA) was used in order to obtain pure population of rat $CD8^+$ T cells from the cell suspension that was previously obtained from one rat's blood. The blood from this rat was isolated in the same way as from the other rats as previously explained in the section 3.17.3. A pure $CD8^+$ T cell population was used as a positive control for the flow cytometry analysis. This kit was used to target non - $CD8^+$ T cells for removal by using the antibodies that are specific for different cell surface markers in the process called "negative selection". Magnetic particles coated with antibodies were used to specifically recognize non - $CD8^+$ T cell and to separate them from the $CD8^+$ T cell population. In this way purified $CD8^+$ T cells were isolated for a positive control.

The assay was performed according to the manufacturer's guidelines. The recommended medium containing PBS with addition of 2% of foetal bovine serum (FBS) and 1 mM ethylenediaminetetraacetic acid (EDTA) (Aldrich Chemical, USA) was prepared and used for this assay. Tubes containing the fixed cells were transferred from the ice into the sterile laminar hood. Prior to that, the sample was passed through a sterile 70 µm mesh nylon cell strainer into 24 sterile well plate wells in order to prevent cell aggregation. Cells were collected and cell concentration was determined by haemocytometer counting. Since the cell concentration was much lower than 5×10^7 cells/ml in all samples, the total cell number was used. Cells were centrifuged at 1500 rpm for 5 min, the supernatant was decanted and cells were resuspended in 0.5 ml of medium. They were transferred into the 5 ml sterile polystyrene round bottom tubes and 25 µl (50 µl/ml of sample) of the isolation cocktail was added and mixed with cells. The isolation cocktail was previously vortexed shortly. The mixture was incubated at room temperature for 10 minutes. RapidSpheres (magnetic particles) were vortexed for 30 seconds and 12.5 µl (25 µl/ml of sample) was added to

the mixture, and mixed. The mixture was topped up to 2.5 ml with the medium and mixed gently by pippetting up and down. Cell separation was performed in two consequent steps which included use of three tubes. In each step negatively selected population was left in the tube while enriched targeted cell population was transferred into a new tube. The cell separation was performed as follows, the tube was placed without a lid into the EasySep magnet and incubated for three minutes at room temperature. The magnet was inverted along with the tube in one continuous move and the enriched negatively selected cell suspension was transferred into a new 5 ml tube, whilst the rest remained in the tube within the magnet. A new tube containing the enriched cell suspension was placed into the EasySep magnet instead of the one that was left within the magnet. The tube containing the enriched cell suspension was incubated at the room temperature for three minutes for a second separation. The magnet along with the tube was inverted in one continuous move and the enriched negatively selected cell suspension was poured into a new tube for further processing while the tube with the rest was discarded.

3.18. Surface staining for flow cytometry

3.18.1. Positive, negative and unstained control

For this experiment we had two biological (positive and negative) controls and one staining control (unstained cells). As a positive control, $CD8^+$ lymphocyte were used. They were obtained as previously explained in the 3.17.4 section.

As a negative control, the MT450 cells were used. Prior to staining, these cells were centrifuged at the 1500 rpm, for 5 minutes. The medium was decanted and the cells washed with the PBS.

As unstained control, cells isolated from the peripheral blood of one of the control animals were used. These cells were not stained as compared to the other cells (refer to 3.17 section).

3.18.2. Staining process

Cells isolated from the blood of vaccinated and unvaccinated animals, the cells that were additionally purified with EasySep isolation kit (positive control) and MT450 cells used as negative control were stained with an anti - CD8a antibody directly conjugated with the AlexaFlour 488 florophore (Santa Cruz Biotechnology, USA). Cells were transferred from ice and centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. Cells were additionally washed with PBS and centrifuged for at 1500 rpm for 5 minutes in order to remove any potential remains of

FBS. The supernatant was discarded and 500 µl of blocking buffer (refer to 2.5 section) was added and cells resuspended in it and incubated for 10 minutes at room temperature. Non - sterile BD FACS tubes were prepared and labeled for each sample as well as for positive and negative control. Additional tubes were labeled regarding that the samples were treated with different antibody dilution, 200 and 400 (refer to Table 3.7 and Table 3.8). Each sample, including positive and negative control was split by taking 250 µl and placing in the tube labeled with sample name and 200X and 250 µl to the tube labeled with sample name and 400X (400 times diluted antibody). In this way each sample was treated with 200X and 400X diluted antibody separately in order to include wider range of antibody concentration regarding that proper titration of antibody was not performed prior to the experiment. Addition of antibody and all steps following this one were done in the dark in order to avoid possible bleaching of the florophore conjugated to the antibody. The antibody mixture was prepared as stated below.

All tubes containing samples were centrifuged at 2000 rpm for 5 minutes, after which the supernatant was decanted and 50 µl of staining buffer - antibody mixture was added to each tube containing sample and mixed. Prior to this step, staining buffer - antibody mixture was vortexed and shaken. Tubes containing samples mixed with staining buffer - antibody mixture were placed in a polystyrene box previously filled with ice and kept in dark for 30 minutes on a shaking platform. Following incubation, 500 µl of washing buffer (PBS) was added to each tube and the tubes were vortexed shortly, placed again in polystyrene box containing ice and incubated for 10 minutes on a rotating platform. After incubation, tubes were centrifuged at 2000 rpm for 10 minutes, supernatant discarded and 500 µl of washing buffer (PBS) was added again. The washing step was repeated 2 times more, after which the final pellet was resuspended in the 250 µl of staining buffer and kept on ice, in dark, in -20 °C freezer until assessment by flow cytometry.

Antibody used	Dilution factor	Number of samples	Master mix depending of titration	Volume of the antibody and the staining buffer mixture added per tube
CD8a Alexa Fluor 488	200	12	3 µl of Ab 597µl of staining buffer	50 µl per tube
CD8a Alexa Fluor 488	400	12	3 µl of Ab 1200µl of staining buffer	100 µl per tube

Table 3. 7. Primary antibody directly conjugated to florophore and used in different dilution of 200 and 400 for staining of lymphocytes isolated from the blood of vaccinated animals.

Antibody used	Dilution factor	Number of samples	Master mix depending of titration	Volume of the antibody and the staining buffer mixture added per tube
CD8a Alexa Fluor 488	200	10	2.5 µl of Ab 497.5 µl of the staining buffer	50 µl per tube
CD8a Alexa Fluor 488	400	10	2.5 µl of Ab 997.5 µl of the staining buffer	100 µl per tube

Table 3. 8. Primary antibody directly conjugated to florophore and used at different dilution factor of 200 and 400 for staining of lymphocytes isolated from the blood of unvaccinated (control) animals.

3.19. Flow Cytometry

Flow cytometry was performed on FACS Canto machine (BD Science, USA). Prior to the assay initiation, levels of sheet fluid were checked and topped up, waste reservoir was checked and waste fluid was discarded. FACS machine was left for 15 minutes to warm up and computer was switched on.

Analysis was performed with the BD Cell Quest Pro software which is originally used by BD cytometers. The experiment document was set by construction of different dot plots and histograms that were required for analysis. Names of tubes containing samples were set, depending on the antibody dilution factor (200 and 400) and origin (vaccinated, control, positive and negative controls and unstained). In order to obtain accurate results and ensure that all expected cell populations are visible, individual forward scatter (FSC) and the side scatter (SSC) photomultiplier tube settings were adjusted (refer to Table 3.9). The FSC threshold was set in order to remove any debris, laser noise or air bubbles from analysis.

PMT voltages	Negative control (MT450)	FSC- E-1 4.26 SSC- 319 1.00
PMT voltages	Positive control (isolated $CD8^+$ T lymphocytes)	FSC- E 00 2.00 SSC- 427 1.00

Table 3. 9. PMT voltage values for negative control (MT450) and positive control (isolated $CD8^+$ T lymphocytes). Values include voltage and amplitude.

During the PMT settings, flow rate was set on LOW in order to provide enough time for proper setting and to avoid losing of cells. For the acquisition, flow rate was set on medium (MED) in order to get optimal resolution thus providing time for cells to settle. Required events were set on 10000 cells.

Parameters	
P1	Forward scatter (FSC)
P2	Side scatter (SSC)
P3	Alexa Fluor 488 (CD8a antibody)

Table 3. 10. Parameters used for the Cell Quest analysis.

Prior to the analysis, tubes containing samples were vortexed and then analyzed. After reaching 10000 events, tubes were replaced and analysis was continued. At the end, data was saved in the previously created folder, machine cleaning/rinsing process was performed and machine and computer were shut down. The complete saved data was transferred to a USB drive for backup and data was further analysed with the FlowJo software. The FlowJo software allows analysis of the data that was obtained through the BD Cell Quest Pro software, on the personal computer. The FlowJo software use the same same files (FCS 2.0) and is compatible to the BD Cell Quest Pro.

3.20. Determination of protein concentration in the AFFTV

In order to determine the protein (peptide) concentration in the standard AFFTV and in the one made with the changed protocol, the DC assay (BioRad, Germany) was used (Rossi *et al.*, 2015).That is a colorimetric assay based on the quantification of the product of the sample protein content solubilisation (BioRad, n.d.). The protein first reacts with the alkaline cooper tartrate solution and after with the Folin reagent which results in the production of reduced species (BioRad, n.d.). Their absorbance can be measured with the spectrophotometer at 750 nm.

3.20.1. Experimental layout

Additionally to the regular enzymatic and heat processing used for the tissue samples used for the AFFTV preparation, (please refer to the 3.12 section), we have used the modification of the heating time cycles and different enzyme combination. This was performed in order to determine the effect of the different conditions on the final protein concentration of the sample. We had applied four different combinations depending on the enzyme used, the duration of the enzyme digestion and the duration and number of heating cycles (refer to Table 3.11).

Enzyme	Duration of the enzymatic digestion	Number of heating cycles	Duration of heating cycles
0.05% Trypsin/EDTA	24h	3	2 minutes
0.05% Trypsin/$CaCl_2$	24h	5	5 minutes

Table 3. 11. Different conditions used for the tissue sample processing. Determination of the protein (peptide) concentration in the standard AFFTV and in the one made with the changed protocol.

Enzyme solutions were prepared as previously explained in the 2.5. section. The pH of working solution was set to 7.8 regarding that is optimal pH for trypsin activity (Cheison *et al*., 2011).

For each treatment combination we have used one tissue sample. These tissue samples were obtained from the liver tissue isolated from the rat that was not included in experiments. The tissue samples were sliced to be equal in weight. The weight of the samples was measured on the microbalance (Sartorius AG CPA26P, Germany) in the plastic dish that was previously weighed and its weight was tarred. Weight of the samples was approximately 120 mg. Samples were transferred to the tubes previously filled with a 10% formalin solution, and kept for 24h. After the formalin fixation, samples were mechanically processed (refer to 3.12 section). Following this step, each mechanically processed sample was washed with the PBS and transferred to the separate 12 ml Falcon tube. Tubes were filled to 3 ml with the PBS. Tubes containing samples were further processed under the different enzymatic and heating conditions, (refer to table. 3.11). For each tissue sample, each processing combination, the 0.05% trypsin (Sigma - Aldrich, USA) was processed in the same way. Trypsin was used as a technical control. The trypsin as a technical control was used in the same concentration as it is used in the combination with the EDTA and $CaCl_2$ (BDH Laboratories, Kuwait). This was performed in order to be able to deduct the concentration of trypsin from the final concentration of sample regarding that trypsin can be prone to auto-catalis and thus provide false result (Keil. 1971). The trypsin was prepared as explained in the 2.5 section. So for each treatment combination we had four tubes containing tissue samples and four tubes containing trypsin.

3.20.2. Preparation of the working reagent

A working reagent was prepared by mixing the 20 μL of the surfactant solution to the alkaline copper tartrate solution placed in the 12 ml Falcon tube. For each 1 ml of copper tartrate solution, 20 μL of surfactant solution was added. The amount of working agent was calculated based on the

number of samples. The mixture was subsequently vortexed for a short period of time and the tube containing the working agent mix was always wrapped in a foil due to a light sensitivity of these reagents.

3.20.3. Preparation of a protein standard (BSA) serial dilutions

Following this step, protein standard curve dilutions were prepared in a range from 0.2 mg/ml to 1.5 mg/ml. The bovine serum albumin (BSA) solution (BioRad, Germany) was used for the standard curve construction and was prepared as 1.5 mg of BSA per ml of PBS and serial dilutions were made from this working stock. The standard curve was prepared each time when the assay was performed and in the same buffer as the sample, which in this case was PBS. The PBS was used as a blank (negative control) as well.

3.20.4. DC assay and absorbance analysis

Into wells of a dry microtiter 96 well plate 5 μL of each standard dilution was pippeted and blank in triplicates. In order to determine the protein peptide concentration in the samples, final products of enzyme digestion and heating were used. From each sample 5 μL was transferred to the separate well of 96 microtiter well plate. Each sample was transferred in triplicate so for each sample three wells of 96 well plate were used. The same was applied for the trypsin samples. Subsequently, 25 μL of the working reagent was added to each well containing sample (tissue or trypsin) or blank. Following this, 200 μL of the Folin reagent was added in each well. The 96 well plate was transferred to a microplate absorbance reader (spectrophotometer). The spectrophotometer was adjust in a way that the 96 well plate was first shaken for a 5 seconds in order to mix the reagents and samples and then after 15 minutes absorbance was read at 750 nm. The same was applied on one more microtiter 96 well plate that contained trypsin samples along with the standards for each plate. Results were saved in an Excel file format and transferred to the USB drive.

3.20.5. Computational analysis of the DC assay results

Results were analyzed further in Excel. Since each sample and standard was done in triplicate, the mean absorbance for each standard and sample was obtained. Based on the mean absorbance of the standard and known concentration of standards, the standard curve was constructed. Furthermore, calculation of the concentration of samples and control (trypsin) was performed based on the two separate standard curves. Standard curves were prepared for each experiment (96 well plate) separately. The R^2 value of each standard curve had to be more than 0.95 in order to be valid. If not,

experiment was repeated. Equation obtained through the plotting of the standard curve was used for the assessment of the concentration of samples as well as control (trypsin). This equation was y= mx+b, where y represent the value on the y axis (absorbance), m represents the slope that is multiplied on the x axis, x represent a value on the x axis (protein concentration) and b represents a point where the line (of the standard curve) intercepts the y axis. From this equation in order to obtain the x value (protein concentration) of the sample, equation was transformed in the x=(y-b)/m. The x value was calculated for each sample, as well as for trypsin samples. Following this step, in order to calculate the final concentration of each sample, concentration of the trypsin control was deducted. It was performed for each treatment separately. In this way concentration of the sample was concentration of the pure sample without the addition of trypsin that was used for the sample digestion. The protein concentrations of samples processed under different conditions were compared with the concentrations of BSA (positive control) and represented in a graph.

3.21. Statistical analysis

The GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla California, USA) was used to perform statistical analysis.

Data from the experiments in which the scoring system was validated through the repeatability. The repeatability of the scoring system was evaluated through correlation between intra- and inter-observer. Cohen Kappa was used to check the agreement between two observers and their scoring values. The interpretation of Cohen Kappa that was used for analysis of results. It is mathematically represented with a formula: $k = (p_o-p_e)/(1-p_e)$, where p_o is observed agreement and p_e is an expected agreement. Obtained through the MedCalc Web site (MedCalc, n.d.). The inter-observer agreement was interpreted as exp;ained on the Figure 3.1.

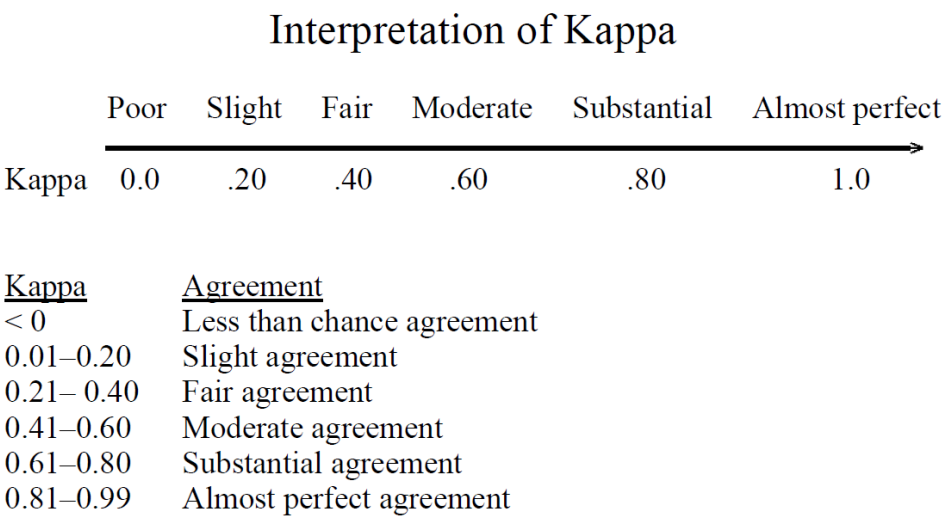


Figure 3. 1. The interpretation of the Cohen Kappa scale (Vierra and Garett. 2005).

The data obtained that was not ordinal, was statistically analyzed for normality using the Shapiro-Wilk's test. Ordinal data (histological score of the lymphocyte infiltration) was not analysed for normality since the ordinal data is not a normally distributed data. Where the data was not normally distributed or ordinal, non - parametric tests were used. The Mann Whitney test was used for the assessment of any mean differences in the histological score between two groups. In case of multiple groups, Kruskal Wallis test was applied. The Kruskal Wallis test was followed with the Dunn's post hoc test in order to compare each of the vaccinated groups with a control group. Where the data was normally distributed, the t-test was used for the assessment between means of two groups while for multiple groups One-way analysis of variance (ANOVA) was used. Survival analysis was applied in comparison of the median survival in each group and it was followed with the log rank test. A 95% confidence interval was applied and level of significance of 0.05. Hypotheses were postulated and if the probability value (p - value) was greater than 0.05, H_0 was accepted, if the p - value was less than or equal to 0.05 than the H_1 was accepted and the H_0 was rejected. The following are the study hypotheses divided by different experiments:

a) Histological assessment

Normality test in control and vaccinated groups:

Ho: The histological score for the lymphocyte infiltration in vaccinated and control groups follows the normal distribution.

H1: The histological score for the lymphocyte infiltration in vaccinated and control groups does not follow the normal distribution.

Mann Whitney U test in control and vaccinated groups:

Ho: The average difference in the histological score for the lymphocyte infiltration between control and any vaccinated group is not different then 0.

H_1: The average difference in the histological score for the lymphocyte infiltration between the control and any vaccinated groups is significantly different then 0.

b) Flow cytometry

Normality test in the control and vaccinated groups:

H_0: The number of $CD8^+$ T cells in the control and vaccinated groups follows the normal distribution.

H_1: The number of $CD8^+$ T cells in the control and vaccinated groups does not follow the normal distribution.

t-test:

H_0: The average difference in the number of $CD8^+$ T cells between the control and any of the vaccinated groups is not different than 0.

H_1: The average difference in the number of $CD8^+$ T cells between the control and any of the vaccinated groups is significantly different than 0.

c) Survival analysis

Log rank test:

H_0: The difference in median survival in between groups is not significantly different than 0.

H_1: The difference in median survival in between groups is significantly different than 0.

Chapter 4:

Results

4.1. Features of the immune response elicited by the combination of the Rotarix and the AFFTV vaccination in DEN - NMOR model of carcinogenesis

4.1.1. Pathology

After a period of seven to nine months after the DEN - NMOR carcinogenesis was initiated significant weight loss was observed in animals included in the study. Out of fourteen animals included in this study, eleven animals showed signs of significant weight loss, while three animals did not. Animals in which significant weight loss was observed were resected in order to determine presence of any abnormal morphological changes, tumour nodes on the liver. During the resection, in nine out of eleven animals that showed the significant weight loss, significant morphological changes on the liver (refer to Table 4.1) were observed while in two no significant change was observed. The animals in which significant morphological changes on the liver were observed were vaccinated while the animals in which there were no significant morphological changes were used as a control. The same was performed with the animals in which no significant weight loss was observed. Out of three animals that did not show signs of the significant weight loss, in all three animals small to significant changes were observed on the liver. These animals were included as a control (Refer to Table 4.1).

Number of animals in which significant weight loss was observed	Number of animals in which no significant weight loss was observed	Number of animals in which significant weight loss was observed along with the significant morphological change on the liver	Number of animals in which significant weight loss was observed but no significant morphological change on the liver was observed	Number of animals in which significant weight loss was not observed but significant morphological change on the liver was observed
11/14	3/14	9/11	2/11	3/3

Table 4. 1. Number of animals in which significant weight loss was observed along with or without significant morphological changes on the liver and opposite. Assessment of the features of immune response elicited by the combination of the Rotarix and the AFFTV vaccination in DEN - NMOR model of carcinogenesis.

Large abnormal change observed on the liver of one of the animals included in the study (refer to Figure 4.1).

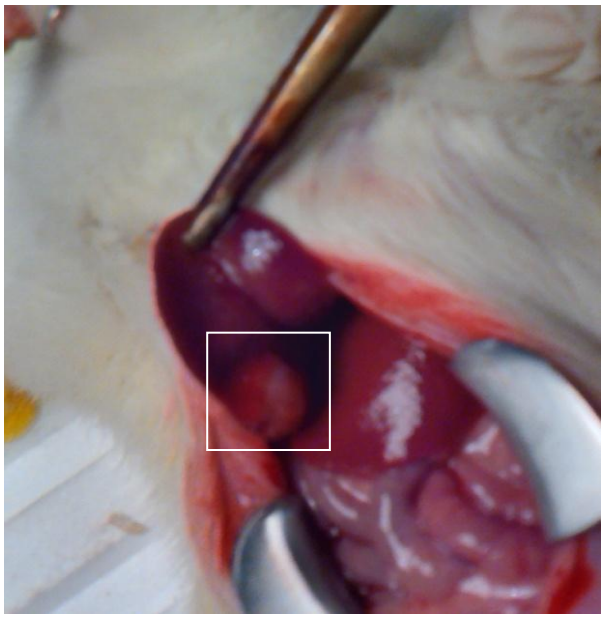


Figure 4. 1. Large abnormal change of the liver tissue, observed in one of the animals treated with the DEN - NMOR (inside the square). Only small part of the tissue was biopsied.

4.1.2. Survival analysis

Upon the death of the animals, survival curves were constructed and compared. It was observed that the median survival of the animals in the control group was 48 days, while the median survival of the animals in the vaccinated group was 34 days (refer to Figure 4.2).

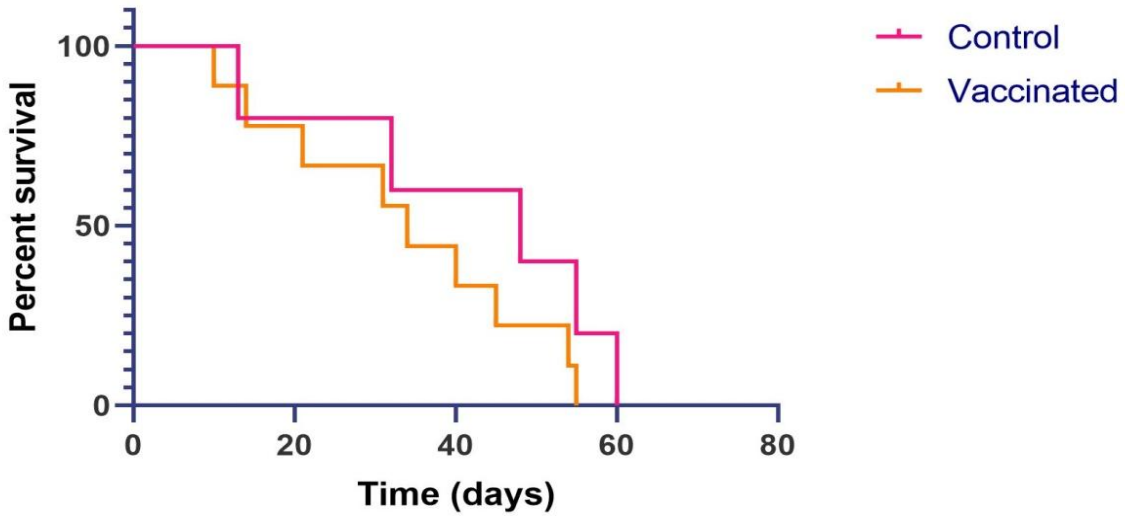


Figure 4. 2. Survival curves (days after vaccination) of animals that were vaccinated with the Rotarix and AFFTV and the animals that were not vaccinated (control) in DEN - NMOR carcinogenesis model.

With use of the log rank test it was determined that there was no significant difference between the survival curves (p= 0.23), thus the H_0 hypothesis was accepted.

After the death of the animals, organs (liver, lungs) and the tumour tissue were collected and histologically processed and analysed. As shown on the figure 4.3, significant morphological changes on the liver and the lungs were observed. The large tumour mass observed on the liver of one of the animals included in study and the multiple metastases visible on the lungs.

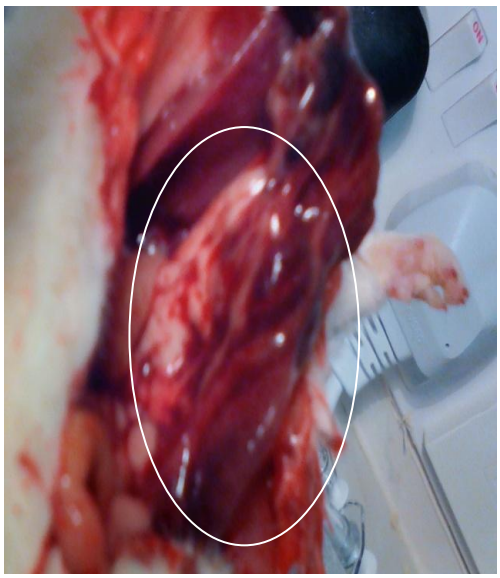
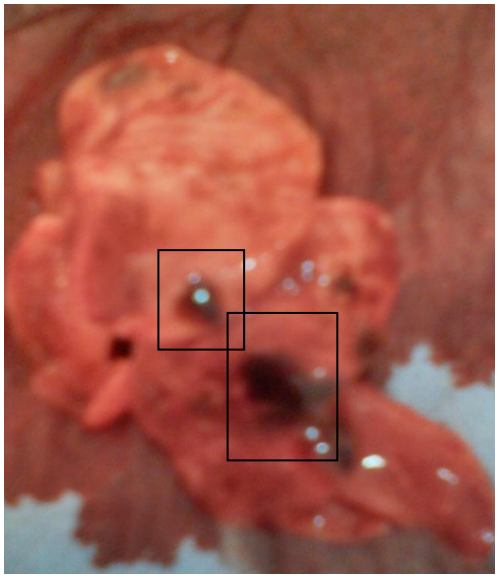
a)  b) 

Figure 4. 3. Organs isolated upon death of the animals that were vaccinated with the Rotarix and AFFTV or not in the DEN - NMOR carcinogenesis model. a) A liver tumour – white mass (area marked with a white circle); b) A lung metastasis – dark greyish masses (marked with rectangles).

Until the end of experiment, post mortem observations along with histological results have confirmed that all animals included in this experiment, vaccinated (100%) and control (100%) have developed liver tumours with lung metastases.

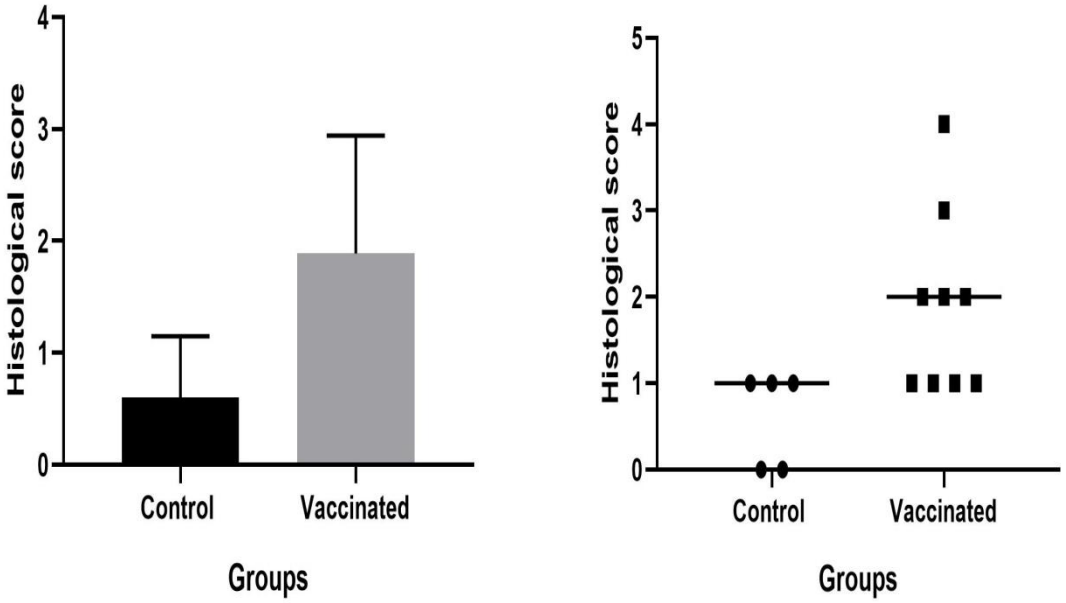
4.1.3. Histological assessment of the collected tissue samples

The collected histological samples of the liver and lung tissue were histologically assessed and the lymphocyte infiltration was scored (please refer to Table 4.4).

Groups		**Control group (n=5)**					**Vaccinated group (n=9)**					**p-value**
Histological score		**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	
Type of tissue specimen	Liver	2	3	0	0	0	0	4	3	1	1	0.028
	Lungs	0	3	2	0	0	0	5	1	2	1	0.65

Table 4. 2. Number of animals in each group with certain histological score of the lymphocyte infiltration in the lungs and in the liver. p - value as average difference and difference between medians obtained through Mann Whitney U test.

When comparing the histological score of the lymphocyte infiltration in the liver between the control and vaccinated group, it was observed that there is a difference between means and medians of two groups (mean control group: 0.6 and mean vaccinated group: 1.88; median control group: 1 and median vaccinated group: 2) (refer to Figure 4.4).

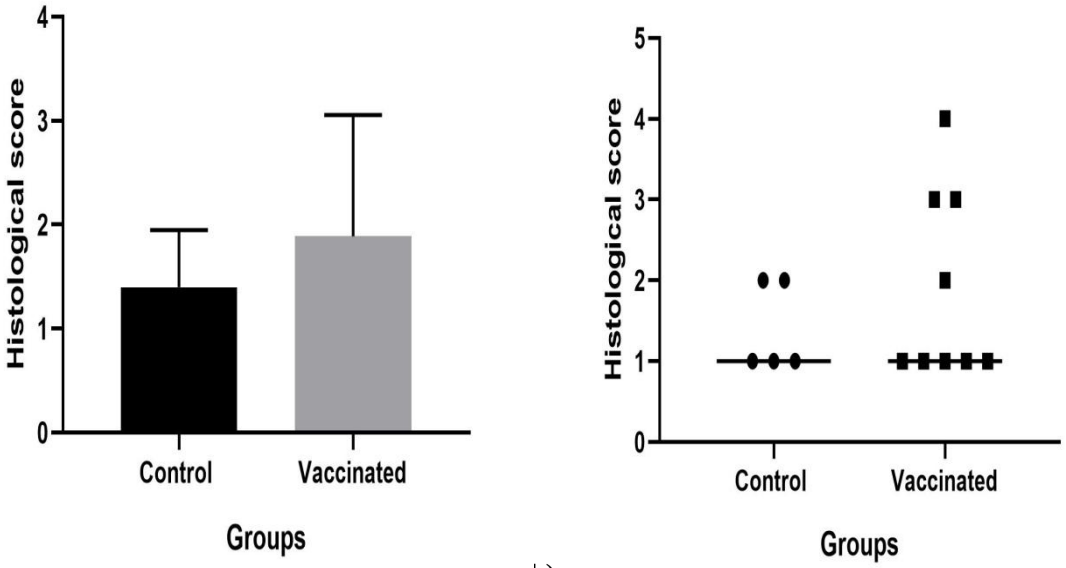


a) b)

Figure 4. 4. Histological score of the lymphocyte infiltration in the liver of control or vaccinated animals in the DEN - NMOR model of carcinogenesis. a) Medians of control and vaccinated group presented with straight lines; b) Means of control and vaccinated group.

When analysed with the Mann Whitney U test, it was observed that there was a significant difference in the histological score of the lymphocyte infiltration in the liver between control and the vaccinated group ($p = 0.028$). Regarding that there significant difference was observed the H_0 hypothesis was rejected and the H_1 hypothesis was accepted.

Means and medians of the histological score of the lymphocyte infiltration in the lungs were compared and there was a slight difference between the control and the vaccinated group (mean control: 1.4 and mean vaccinated group: 1.88; median control group: 1 and median vaccinated group 1) (refer to Figure 4.5).



a) b)

Figure 4. 5. Histological score of the lymphocyte infiltration in the lungs of control or vaccinated animals in the DEN - NMOR model of carcinogenesis. a) Medians of control and vaccinated group presented with straight lines; b) Means of control and vaccinated group.

When the Mann Whitney U test was applied, it was determined that there was no statistically significant difference in the histological score of the lymphocyte infiltration in the lungs between the control and vaccinated group ($p = 0.65$). Regarding that there was no statistically significant difference observed, the H_0 hypothesis was accepted

A small size of the sample and the statistical difference in means along with the difference in medians, in the histological score of the lymphocyte infiltration in the liver between these two groups provides an indication that this result can be considered promising and that the AFFTV derived from the tumour tissue biopsy in combination with the Rotarix should be tested in study with the larger sample size.

4.2. Features of the immune response elicited with different combinations of the Rotarix and the AFFTV in the transplantable rat mammary gland tumour model

4.2.1. Pathology

For the initial experiment all animals were inoculated with $5x10^6$ MT450 cells subcutaneously, based on data from previous experiments conducted by Pierre Schembri Wismayer *et al*. (2016) at the University of Malta (unpublished data). It was observed that under these conditions animals have developed palpable tumours on the injection site (right abdominal side), even after ten days (refer to Figure 4.6).

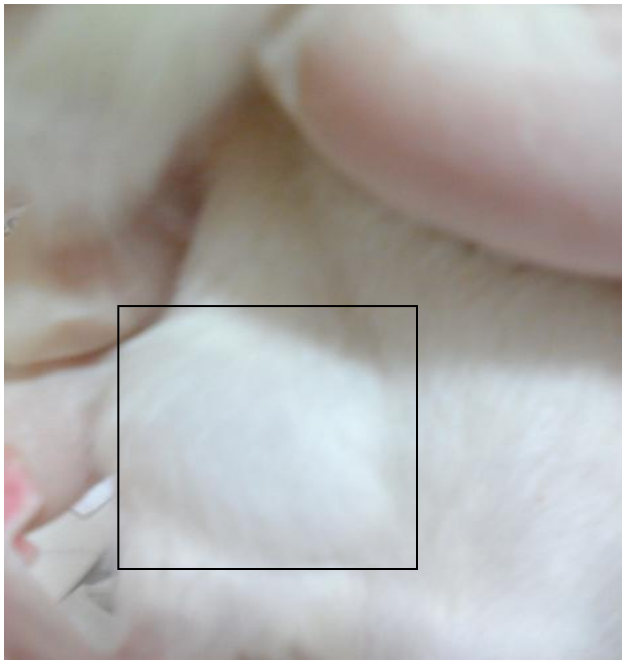


Figure 4. 6. Palpable tumour at the tumour inoculation site (inside the square) observed in the animal injected with the 5 x 10^6 MT450 cells.

In first three days after the first immunisation and before the second immunisation, half of the rats in every group have developed ulceration at the tumour inoculation site. After ten days, 100% of rats in all groups have developed ulceration at the tumour inoculation site (refer to Figure 4.7).

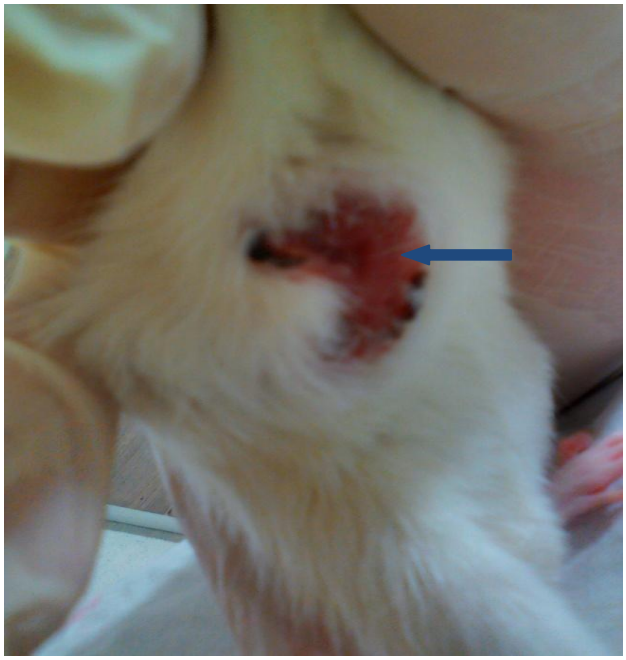


Figure 4. 7. Formation of large ulcerations (pointed out with blue arrows) at the tumour site of the animals injected with the 5 x 10^6 MT450 cells.

4.2.2. Survival analysis

Upon the death of the animals, survival curves were constructed and compared. It was observed that the median survival time in the control group was 34 days. In the group vaccinated with the Rotarix three days after the first immunisation, median survival time was 28 days. The median survival time in the group vaccinated with the Rotarix seven days after the first immunisation was 27 days. In the group vaccinated only with the AFFTV seven days after the first immunisation, median survival time was 30 days. The median survival time in the group vaccinated with the combination of the Rotarix and the AFFTV three days after the first immunisation was 28 days and the median survival time in the group treated with the combination of the Rotarix and the AFFTV seven days after the first immunisation was 24 days (refer to Figure 4.8).

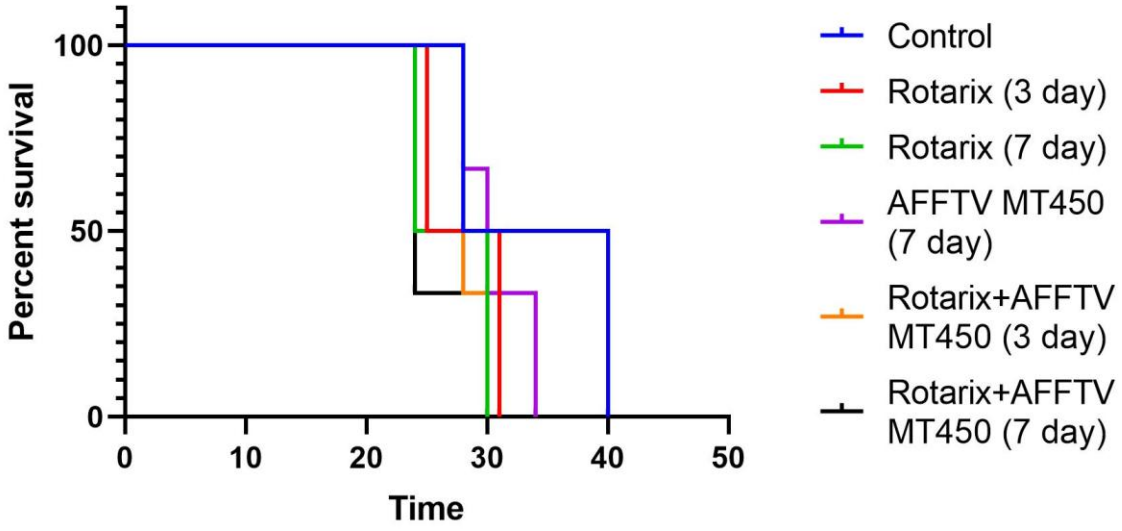


Figure 4. 8. Survival surves (days after vaccination) of animals that were vaccinated with different vaccine combinations and the ones that were not vaccinated (control). Analysis of the features of immune response elicited with different combinations of the Rotarix and the AFFTV in the transplantable rat mammary gland tumour model.

The log rank analysis showed that there was no significant difference in the survival curves between the groups ($p = 0.44$).

4.2.3. Histological assessment

Post mortem, it was observed that all animals, vaccinated and unvaccinated (control), have developed large tumours on the right abdominal side that were connected to the right axillary lymph node as well as lung metastases (refer to Figure 4.9).

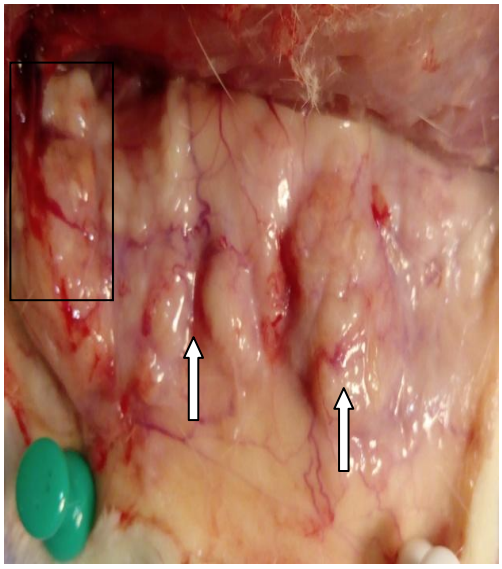
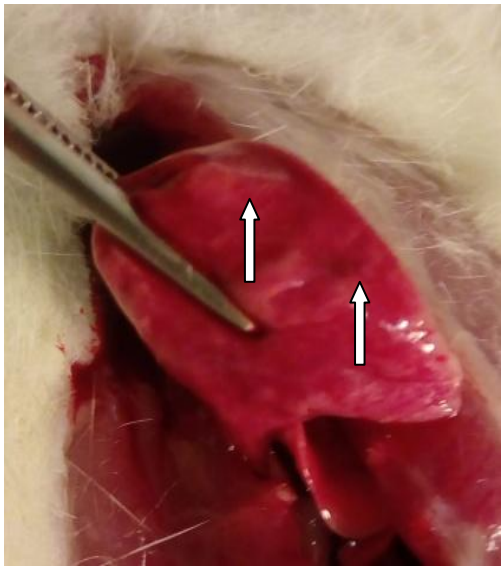
a)  b) 

Figure 4. 9. Photographs taken upon the death of the animals vaccinated with different vaccine combination or not (control). a) The subcutaneous tumour on the right side (pointed out with the white arrows) connected to the right axillary lymph node (marked with the black square); b) The lungs with several metastases (pointed out with the white arrows).

During the post mortem examination, it was observed that 100 % of animals included in this study have developed metastasis inside the lung tissue (refer to Figure 4.10).

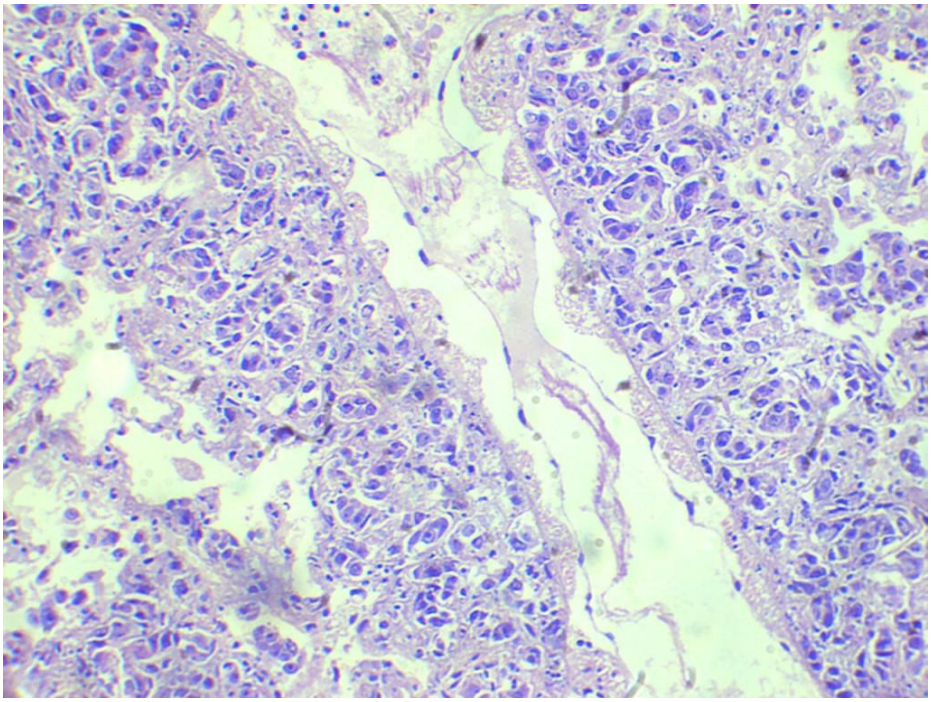


Figure 4. 10. Lungs intruded by tumor (400X magnification). Analysis of the features of immune response elicited with different vaccine combinations in the transplantable rat mammary gland tumour model.

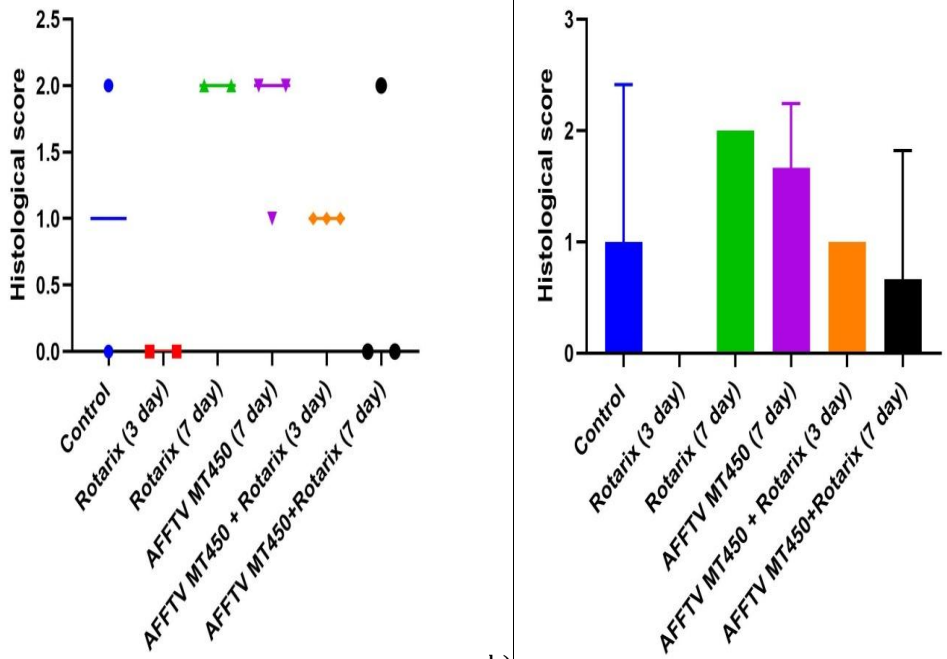
4.2.4. Histological scoring of the lymphocyte infiltration inside the subcutaneous tumour

The collected histological samples of the subcutaneous tumour tissue was histologically assessed and the lymphocyte infiltration was scored (refer to Table 4.3).

Groups	Histological specimen	Histological assessment of the lymphocyte infiltration					p value Dunn's test	p value Kruskal Wallis
		Score 0	Score 1	Score 2	Score 3	Score 4		
Control (n=2)	Subcutaneous tumour	1	0	1	0	0		
Rotarix (3 day) (n=2)	Subcutaneous tumour	2	0	0	0	0	>0.99	0.21
Rotarix (7 day) (n=2)	Subcutaneous tumour	0	0	2	0	0	>0.99	
AFFTV MT450 (7 day) (n=3)	Subcutaneous tumour	0	1	2	0	0	>0.99	
AFFTV MT450 + Rotarix (3 day) (n=3)	Subcutaneous tumour	3	0	0	0	0	>0.99	
AFFTV MT450 + Rotarix (7 day) (n=3)	Subcutaneous tumour	2	0	1	0	0	>0.99	

Table 4. 3. Number of animals in each group with certain histological score of the lymphocyte infiltration in the subcutaneous tumour of control or animals vaccinated with different vaccine combinations. p value after multiple comparison of means between control and each of the vaccinated groups (Dunn's test). p value after comparison of medians between all groups (Kruskal Wallis test).

When comparing the histological score of the lymphocyte infiltration in the subcutaneous tumour between the control groups and each of the vaccinated groups it was observed that there was a difference a slight difference between means (control group mean: 1, Rotarix 3rd day group mean: 0, Rotarix 7th day group mean: 2, AFFTV MT450 group 7th day mean: 1.6, AFFTV MT450 + Rotarix 3rd day group mean: 1, AFFTV MT450 + Rotarix 7th day group mean: 0.66). As well it was observed that medians of the control group and the vaccinated groups were different (control group median: 1, Rotarix 3rd day group median: 0, Rotarix 7th day group median: 2, AFFTV MT450 7th day group: median: 2 AFFTV MT450 + Rotarix 3rd day group median: 1, AFFTV MT450 + Rotarix 7th day group median: 0) (refer to Figure 4.11).



a) b)

Figure 4. 11. Histological score of the lymphocyte infiltration in the subcutaneous tumour of control or animals vaccinated with different vaccine combinations. a) Medians of every group presented with straight lines; b) Means of every group.

When the Kruskal Wallis analysis was performed and the groups were compared, it was determined that there was no any significant difference in the medians of the histological score of lymphocyte infiltration inside the subcutaneous tumour in between control and any of the vaccinated groups ($p = 0.21$). As a post hoc analysis, a Dunn's multiple comparison was performed and it was observed that average difference in the histological score in between the control and each of the vaccinated groups was $p > 0.99$. Regarding that there was no significant difference in the histological score of the lymphocyte infiltration in the subcutaneous tumour between the control and any of the vaccinated groups, H_0 hypothesis was accepted.

4.2.5. Histological scoring of the lymphocyte infiltration inside the lungs

The collected histological samples of lungs were histologically assessed and the lymphocyte infiltration was scored (refer to Table 4.4)

Groups	Histological specimen	Histological assessment of the lymphocyte infiltration					p value Dunn's test	p value Kruskal Wallis test
		Score 0	Score 1	Score 2	Score 3	Score 4		
Control (n=2)	Lungs	0	0	2	0	0		
Rotarix (3 day) (n=2)	Lungs	0	0	1	1	0	>0.99	0.16
Rotarix (7 day) (n=2)	Lungs	0	1	0	1	0	>0.99	
AFFTV MT450 (7 day) (n=3)	Lungs	0	0	0	2	1	=0.62	
AFFTV MT450 + Rotarix (3 day) (n=3)	Lungs	0	1	1	1	0	>0.99	
AFFTV MT450 + Rotarix (7 day) (n=3)	Lungs	1	1	1	0	0	>0.99	

Table 4. 4. Number of animals in each group with certain histological score of the lymphocyte infiltration in the lungs. p value after multiple comparison of means between control and each of the vaccinated groups (Dunn's test). p value after comparison of medians between all groups (Kruskal Wallis test).

When comparing the histological score of the lymphocyte infiltration inside the lungs, it was observed that there are slight differences in means and medians in between control and each of the vaccinated groups (Control group mean and median: 2, Rotarix 3rd day group mean and median: 2.5, Rotarix 7th day group mean and median: 2, AFFTV MT450 7th day group mean: 3.3 and median: 3, AFFTV MT450 + Rotarix 3rd day group mean and median: 2, AFFTV MT450 + Rotarix 7th day group mean and median: 1) (refer to Figure 4.12).

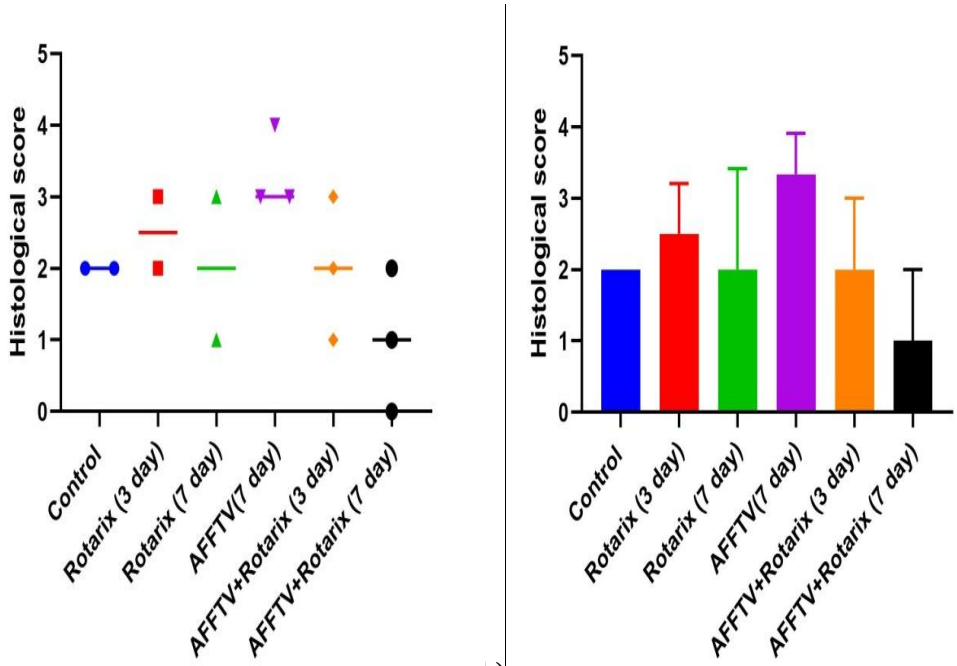


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Figure 4. 12. Histological score of the lymphocyte infiltration in the lungs of control or animals vaccinated with different vaccine combinations. a) Medians of every group presented with straight lines; b) Means of every group.

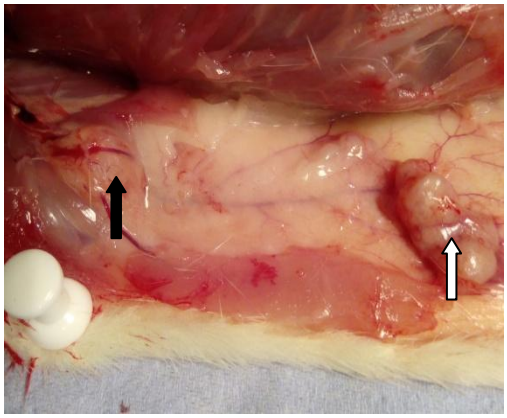
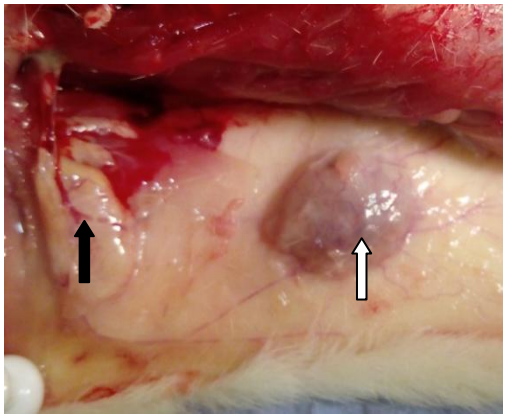
When the Kruskal Wallis analysis was performed and the medians of the groups were compared, it was determined that there was no any significant difference in the medians in between control and any of the vaccinated groups ($p = 0.16$). As a post hoc analysis, a Dunn's multiple comparison was performed and it was observed that average difference in the histological score of lymphocyte

infiltration between the control and the group vaccinated with the AFFTV made of MT450 cells seven days after the first immunisation with the AFFTV made of the MT450 cells was not significant ($p = 0.62$). When comparing the control with each of the rest vaccinated groups, there were no significant difference in the histological score of the lymphocyte infiltration ($p>0.99$). Regarding that there was no significant difference in the histological score of the lymphocyte infiltration in the lungs between the control and any of the vaccinated groups, H_0 hypothesis was accepted. However, regarding that the group vaccinated with the combination of the AFFTV made of the MT450 cells and Rotarix provided slight difference ($p = 0.75$; median = 3) in the histological score of the lymphocyte infiltration in lungs it should be considered for the future studies with larger sample size.

4.3. Determination of the lung metastases occurrence in the MT450 model under the conditions of $5x10^6$ cells inoculation

4.3.1. Observations

It was observed that already after one and a half weeks animals had enlarged axillary lymph node on the side of the tumour inoculation and that a palpable tumour was present at the inoculation site. The lungs of the animals sacrificed at one and a half weeks did not show any signs of metastatic occurrence. After a two weeks period, it was observed that tumours were expanding in size, the axillary lymph node was enlarged and tense. Lungs were still without any visible sign of metastasis. After a period of two and a half weeks, situation was similar, tumours were expanding in size, lymph nodes were similar size to previous ones and lungs were without any significant and visible changes. After a period of three weeks significant changes occurred. The axillary lymph nodes were getting twice enlarged in size and seemed connected to the brachial lymph node. The tumour was significantly enlarged and spreading to the axillary lymph nodes and invading the rib cage. Lungs started having small visible spots on the tissue surface (change in colour of the lung tissue). Following the four weeks period, tumour was almost connected to the enlarged axillary lymph node which was now compact in mutual formation with the brachial lymph node. The lymphatic and blood vasculature were visibly enlarged and certainly showing connection in between the axillary lymph node and the tumour itself (refer to Figure .4.13).

a)  b) 

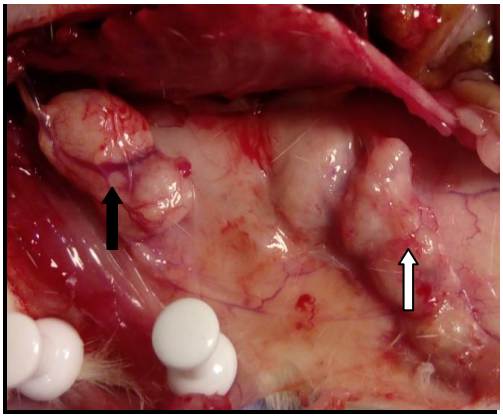
c) 

Figure 4. 13. Formed tumours (pointed out with white arrows) and enlarged axillary lymph node (pointed out with black arrows) in rats treated with 5 x 10^6 MT450 cells and sacrificed at different time periods: a) one and a half week; b) two and a half week; c) three weeks.

Multiple changes in the lung tissue colour were observed (refer to Figure 4.14)

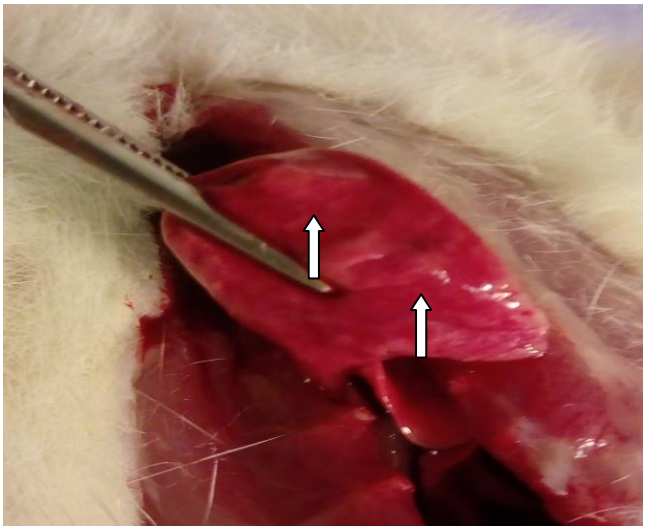


Figure 4. 14. Small changes on the lung tissue after three weeks period, shown as a white spots (pointed out with white arrows).

4.3.2. Histological assessment

Histological slides made of samples collected from these animals have shown that the occurrence of lung metastases is happening even before significant changes are visible on the lung tissue surface. As shown on the pictures below abnormal mass formations were present even after two weeks and getting increased in size and number in two and a half week period (refer to Figure 4.15 and Figure 4.16). Along with it, airspace were getting narrowed and collapsed (refer to Figure 4.15 and Figure 4.16)

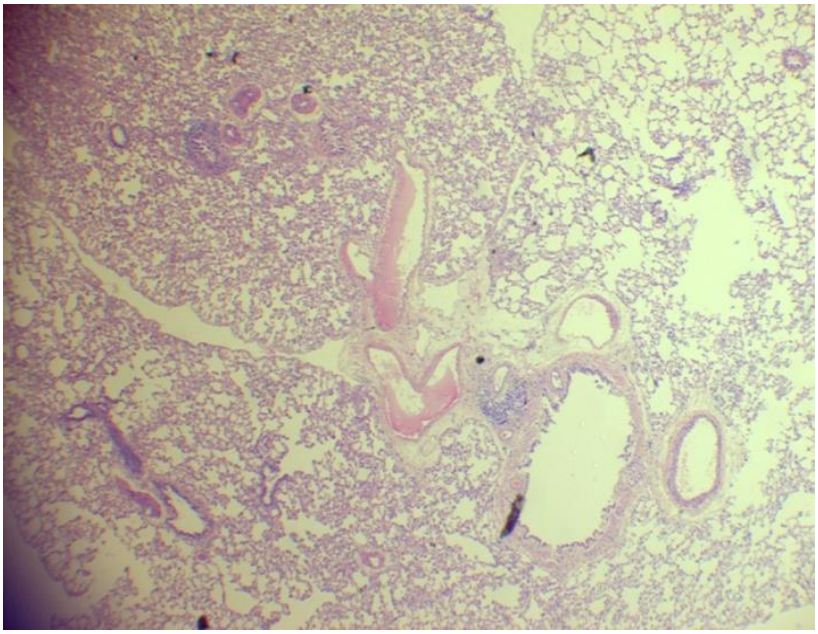


Figure 4. 15. Histology of the lungs of the rat inoculated with 5 x 10^6 MT450 cells and sacrificed after two weeks. Increased tissue density and reduced airspace in upper left part of the slide as opposed to more normal upper right part (40X magnification).

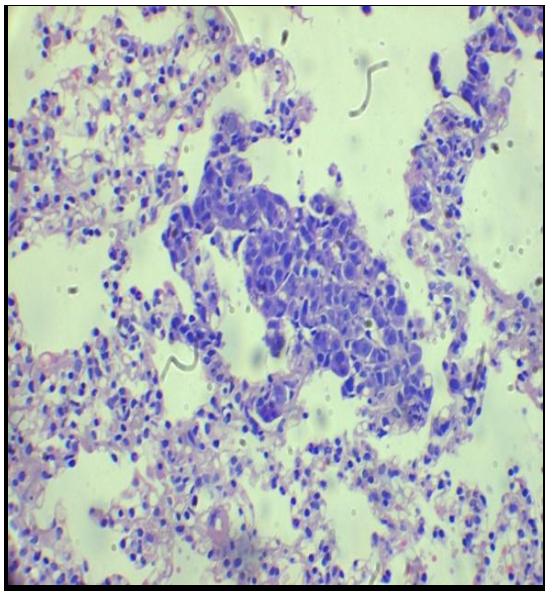
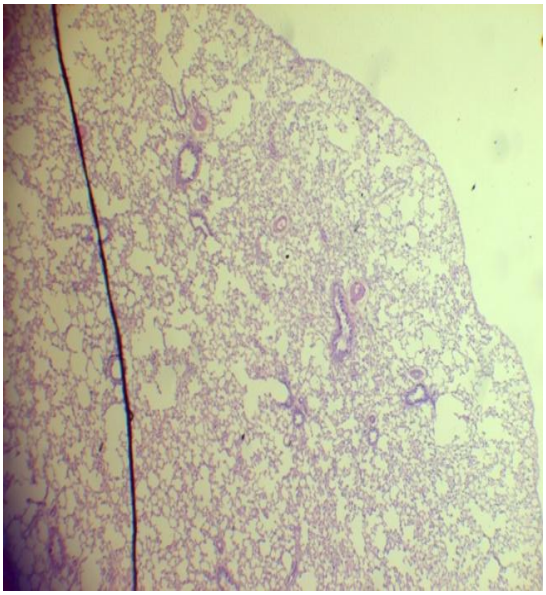
a)  b) 

Figure 4. 16. Histology of the lung tissue isolated from the rat inoculated with 5 x 10^6 MT450 cells and sacrificed after two and a half weeks. a) Abnormal mass formed in the centre of the tissue (100X magnification); b) Airspace getting narrowed and interstitial volume is increased (40X magnification).

As well it was observed that in the lungs of the animals sacrificed two or two and a half week after inoculation, there is a high interstitial volume and presence of lymphocytes in lung tissue and blood vessels (refer to Figure 4.17).

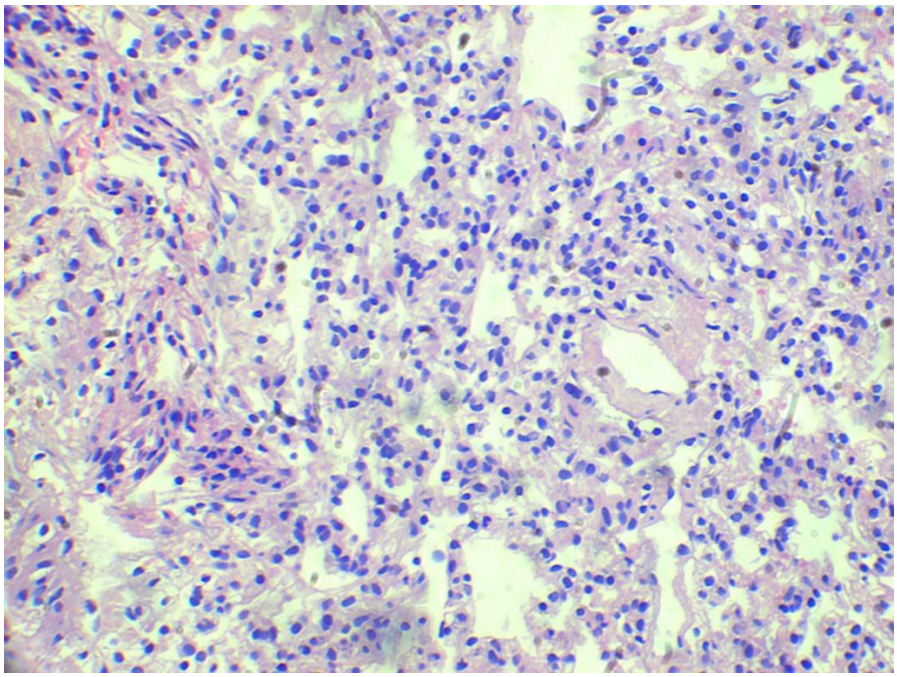


Figure 4. 17. Histology of the lung tissue isolated from the rats inoculated with 5 x 10^6 MT450. a) Presence of lymphocytes in lung tissue of the rat sacrificed after two weeks (400X magnification).

4.4. Features of the immune response elicited after the immunisation with the Rotarix and AFFTV made of the MT450 cell line or/and the AFFTV made of the tumour tissue biopsy

Following the pilot experiment in which we observed timing of the tumour tissue formation, its features and its metastatic ability towards the lung tissue we conducted experiment in which we used different treatment combinations.

4.4.1. Pathology

It was observed that all rats, vaccinated and unvaccinated (control), have developed subcutaneous tumours at the inoculation site three weeks after the inoculation. First immunisation was performed after three weeks and followed with the second immunisation after three days. Since rats that were treated with the AFFTV made of a tumour tissue biopsy required that those rats undergo operational procedure in which biopsy will be sampled, during the operation it was observed that tumours of these animals were liquid inside. It was hard to obtain any solid tumour tissue sample for further processing. This was performed prior to the first immunisation.

During the second immunisation with the Rotarix and different treatment combinations, ulceration formation at the tumour site were not observed. Five days following the second immunisation, rats were weighed and examined and it was noticed that 90% of animals that were vaccinated and 50% of control animals have developed smaller or larger ulcers at the site of tumour inoculation. Importantly, it was observed that in majority ulcers were formed in animals that were vaccinated with the Rotarix and AFFTV developed from tumour tissue biopsy alone or in combination with the AFFTV made of the MT450 cell line. Animals that were treated only with the Rotarix and AFFTV made of the MT450 cell line had not wounds observed (refer to Figure 4.18).

a)

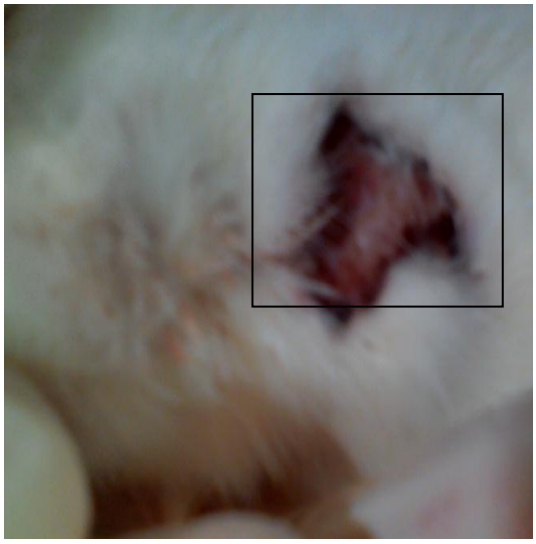


Figure 4. 18. Ulceration developed at the tumour site of the animal included in the assessment of the features of immune response elicited upon immunisation of the animals with the Rotarix and AFFTV made of the MT450 cell line or/and the AFFTV made of the tumour tissue biopsy (area marked with the rectangle).

4.4.2. Survival analysis

Rats were observed and upon their induced death, survival curves were constructed (refer to Figure 4.19) and organs including liver, lungs and subcutaneous tumour tissue were isolated and further histologicaly analyzed.

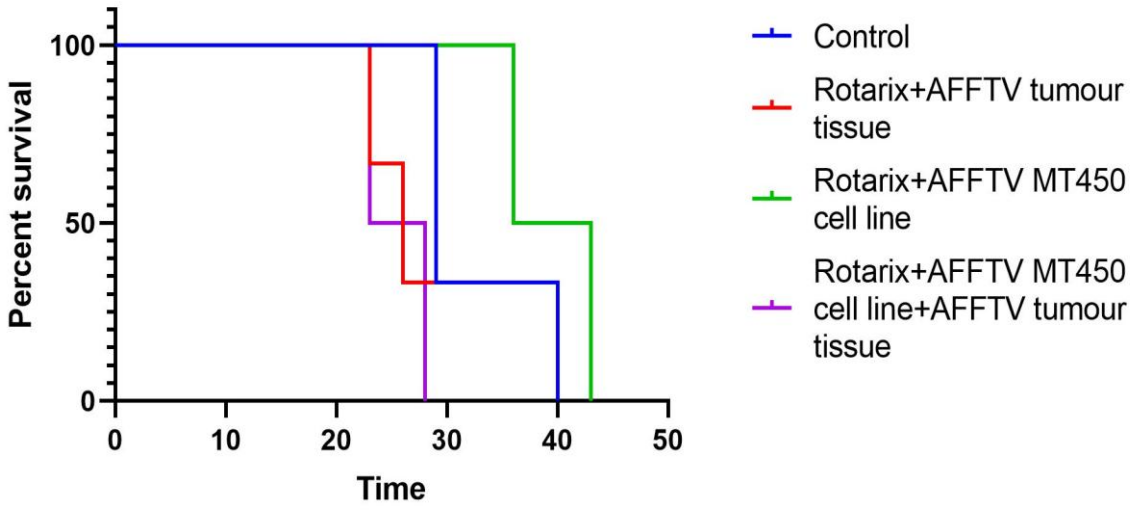


Figure 4. 19. Survival curves (days after vaccination) of animals that were vaccinated with different combination of the Rotarix and AFFTV made of the MT450 cell line and/or the AFFTV made of the tumour tissue biopsy and the animals that were not vaccinated (control).

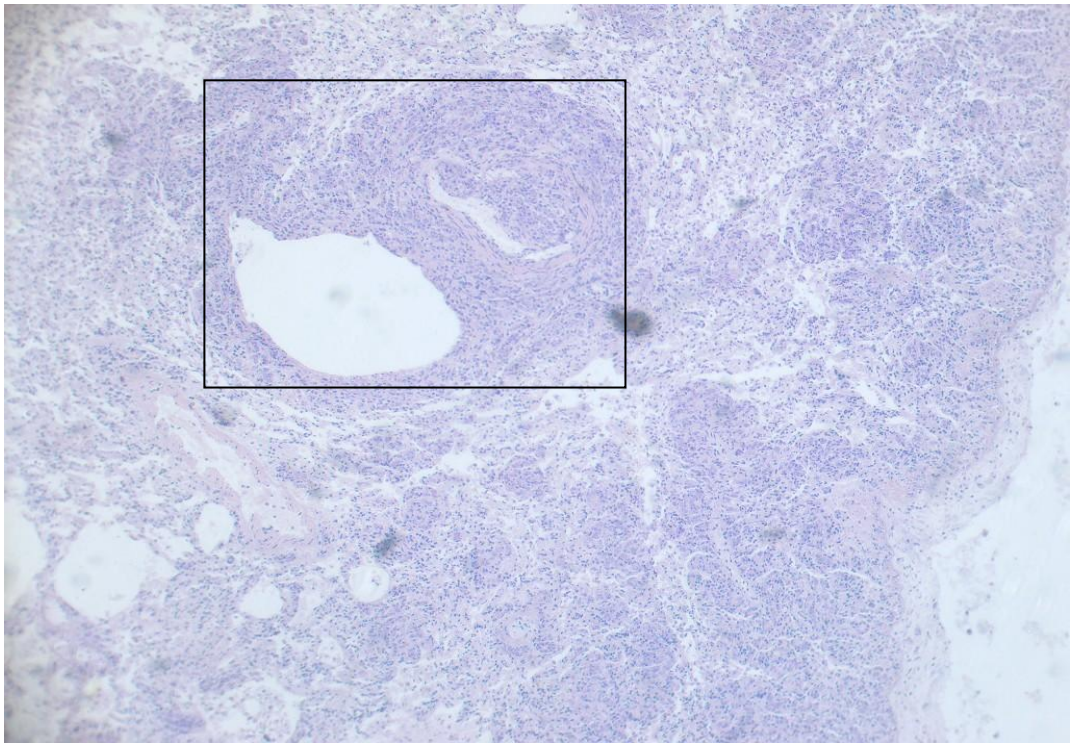
When the survival analysis was performed it was observed that there is difference in median survivals between groups (control group median: 29, Rotarix + AFFTV tumour tissue group median: 26, ARotarix + FFTV MT450 group median: 39.5, Rotarix +AFFTV MT450 +AFFTV tumour tissue group median: 25.5). The log rank test have shown that there was no significant difference in median survival between the survival curves ($p = 0.16$). Regarding that there was no significant difference in median survival between the vaccinated groups and the control group, H_0 hypothesis was accepted.

However, it should be noticed that the median survival of the group vaccinated with the Rotarix and AFFTV made of the MT450 cells was 39.5, and the control group 29. This further might suggest that there is an increase in the survival time in the group vaccinated with the Rotarix and AFFTV made of the MT450 cell as compared to the control group. As well, median survival in both the group vaccinated with the Rotarix and AFFTV made of the tumour tissue biopsy as well as the group vaccinated with the combination of the Rotarix, AFFTV made of the MT450 cell line and the AFFTV made of the tumour tissue biopsy was shorter as compared to control.

4.4.3. Histological assessment

During the post mortem examination, it was observed that 100 % of animals included in this study have developed metastasis inside the lung tissue (refer to Figure 4.20).

a)



b)

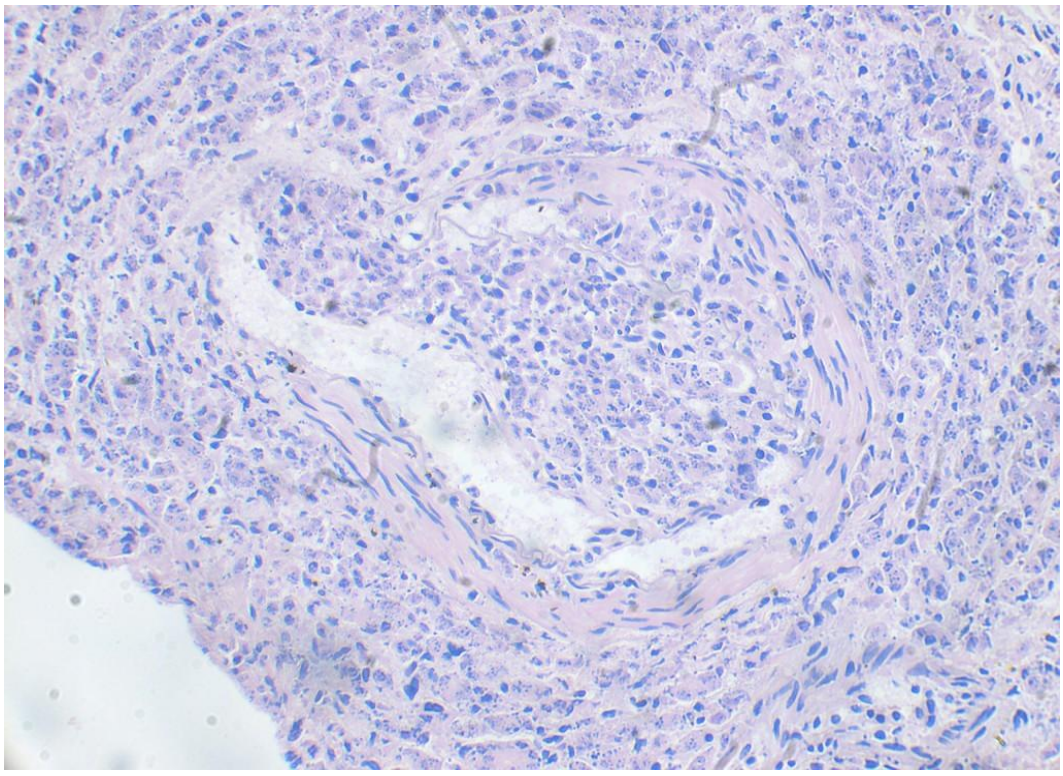


Figure 4. 20. Lung metastases of th animals includes in the assessment of the features of immune response elicited after the immunisation with the Rotarix and AFFTV made of the MT450 cell line or/and the AFFTV made of the tumour tissue biopsy. a) 100X (the area in squares); b) 400X (the same ame tumour formation).

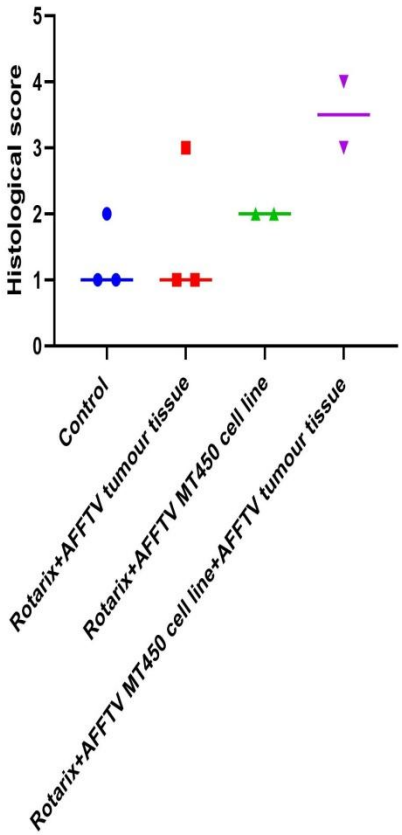
4.4.4. Histological scoring of the lymphocyte infiltration inside the subcutaneous tumour

The histological scoring of the lymphocyte infiltration inside the subcutaneous tumour was performed (refer to Table 4.5).

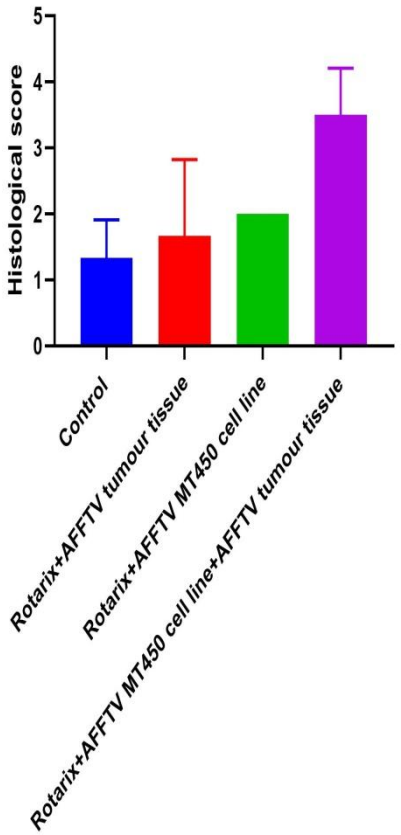
Groups	Histological specimen	Histological assessment of the lymphocyte infiltration					p value Dunn's test	p value Kruskal Wallis test
		Score 0	Score 1	Score 2	Score 3	Score 4		
Control (n=3)	Subcutaneous tumour	0	2	1	0	0		
Rotarix+ AFFTV MT450 cell line+ AFFTV tumour tissue biopsy (n=2)	Subcutaneous tumour	0	0	0	1	1	=0.10	0.15
Rotarix+ AFFTV made of tumour tissue (n=3)	Subcutaneous tumour	0	2	0	1	0	>0.99	
Rotarix + AFFTV made of the MT45(cell linen=2)	Subcutaneous tumour	0	0	2	0	0	>0.99	

Table 4. 5. Number of animals in each group with certain histological score of the lymphocyte infiltration in the subcutaneous tumour. p value after multiple comparison of means between control and each of the vaccinated groups (Dunn's test). p value after comparison of medians between all groups (Kruskal Wallis test).

When comparing the histological score of the lymphocyte infiltration inside the subcutaneous tumour, it was observed that there are slight differences in means and medians in between control and each of the vaccinated groups (Control group mean:1.33 and median: 1, Rotarix + AFFTV MT450 + AFFTV tumour tissue biopsy group mean and median: 3.5, Rotarix+AFFTV tumour tissue biopsy group mean: 1.66 and median: 1, Rotarix + AFFTV MT450 group mean and median: 2) (refer to Figure 4.21). When the Kruskal Wallis analysis was performed and the medians of the groups were compared, it was determined that there was no any significant difference in the medians in between control and any of the vaccinated groups ($p = 0.15$). As a post hoc analysis, a Dunn's multiple comparison was performed and it was observed that average difference in the histological score of lymphocyte infiltration between the control and the group vaccinated with the combination of the Rotarix, AFFTV made of the MT450 cell line and the AFFTV made out of tumour tissue biopsy was $p = 0.10$ and as that not significant. When comparing the control with each of other vaccinated groups, there were no significant difference in the histological score of the lymphocyte infiltration ($p>0.99$) .



a)



b)

Figure 4. 21. Histological score of the lymphocyte infiltration in the subcutaneous tumour: a) Medians of every group presented with straight lines; b) Means of every group.

Regarding that there was no significant difference in the histological score of the lymphocyte infiltration in the subcutaneous tumour tissue between the control and any of the vaccinated groups, H_0 hypothesis was accepted. However, regarding that the group vaccinated with the combination of the Rotarix, AFFTV made out of MT450 and the AFFTV made out of the tumour tissue biopsy, provided good indication ($p = 0.10$; median = 3.5) that it should be considered for the future studies with larger sample size.

4.4.5. Histological scoring of the infiltration inside the lungs

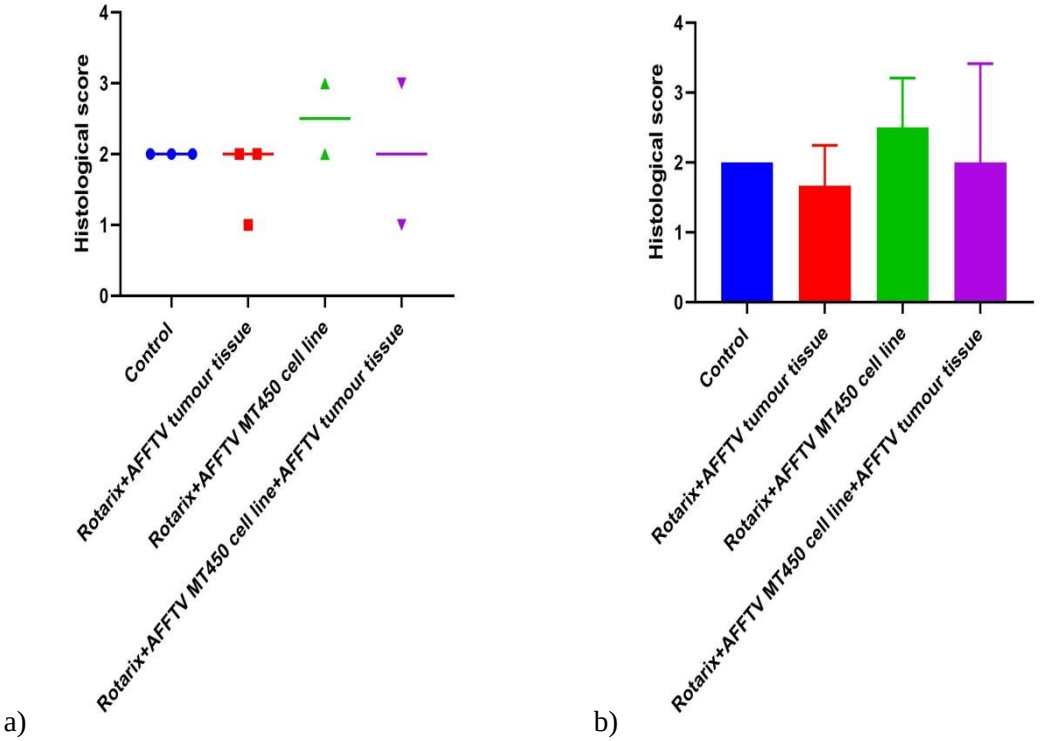
The lungs collected from the animals included in this study were histologicaly analysed and scored (refer to Table 4.5).

Groups	Histological specimen	Histological assessment of the lymphocyte infiltration					p value Dunn's test	p value Kruskal Wallis test
		Score 0	Score 1	Score 2	Score 3	Score 4		
Control (n=3)	Lungs	0	0	3	0	0		
Rotarix+AFFTV+AFFTV made of tumour tissue (n=2)	Lungs	0	1	0	1	0	>0.99	=0.84
Rotarix+AFFTV made of tumour tissue biopsy (n=3)	Lungs	0	1	2	0	0	>0.99	
Rotarix+AFFTV made of the MT450 cell line (n=2)	Lungs	0	0	1	1	0	>0.99	

Table 4. 6. Number of animals in each group with certain histological score of the lymphocyte infiltration in the lungs. p value after multiple comparison of means between control and each of the vaccinated groups (Dunn's test). p value after comparison of medians between all groups (Kruskal Wallis test).

When comparing the histological score of the lymphocyte infiltration inside the lungs, it was observed that there were slight differences in means and medians in between control and each of the

vaccinated groups (Control group mean and median: 2, Rotarix + AFFTV MT450 + AFFTV tumour tissue biopsy group mean and median: 2, Rotarix + AFFTV tumour tissue biopsy group mean: 1.66 and median: 2, Rotarix +A FFTV MT450 group mean and median: 2.5) (refer to Figure 4.22).



a) b)

Figure 4. 22. Histological score of the lymphocyte infiltration in the lungs of the animals that were vaccinated with different combinations of the Rotarix and the AFFTV made of the MT450 cell line and/ or the AFFTV made of the tumour tissue biopsy , and unvaccinated animals. a) Medians of every group presented with straight lines; b) Means of every group.

When the Kruskal Wallis analysis was performed and the medians of the groups were compared, it was determined that there was no any significant difference in the medians in between control and any of the vaccinated groups ($p = 0.84$). As a post hoc analysis, a Dunn's multiple comparison was performed and it was observed that there was no significant difference in the histological score of the lymphocyte infiltration in the lungs between the control and each of the other vaccinated groups ($p > 0.99$). Regarding that there was no significant difference in the histological score of the lymphocyte infiltration in the lungs between the control and any of the vaccinated groups, H_0 hypothesis was accepted.

4.5. Features of the immune response elicited by the AFFTV made with standard and changed protocol in combination with the Rotarix, in the mammary transplantable tumour model

4.5.1. Survival analysis

Rats were observed and upon their induced death, survival curves were constructed (refer to Figure 4.23) and organs including liver, lungs and subcutaneous tumour tissue were isolated and further histologicaly analysed.

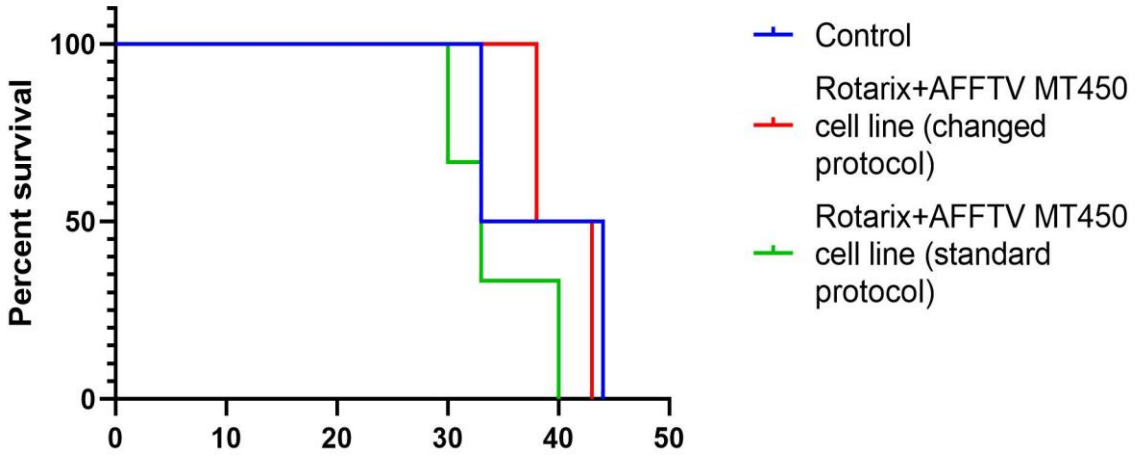


Figure 4. 23. Survival time (days after vaccination) of animals that were vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with standard or changed protocol, and the animals that were not vaccinated (control).

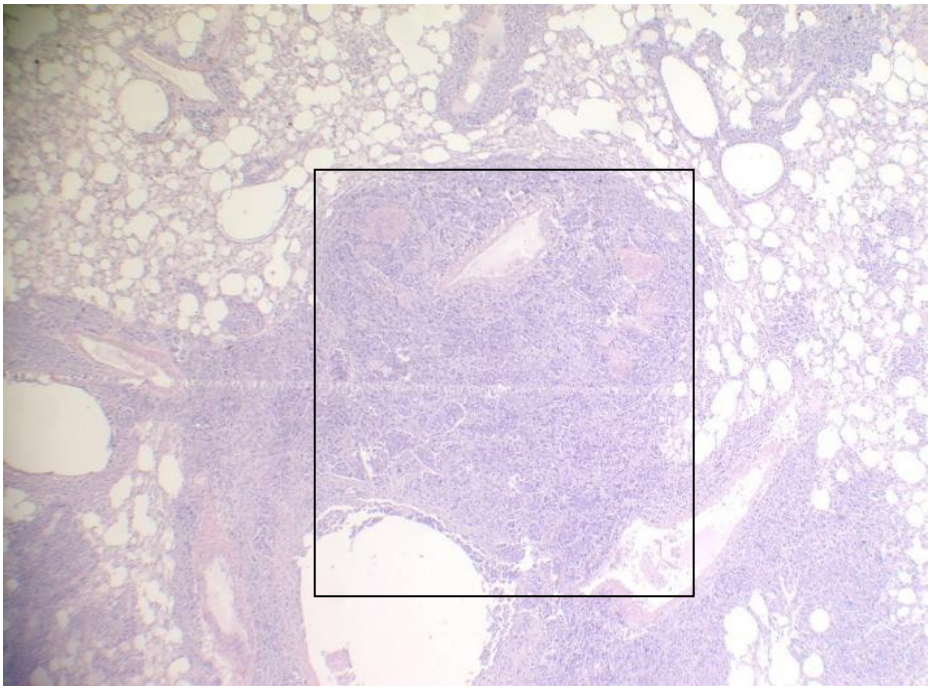
When the survival analysis was performed it was observed that there is difference in median survivals between groups (control group median: 33, Rotarix + AFFTV MT450 changed protocol group: 38.5, Rotarix + AFFTV MT450 standard protocol group: 40.5). The log rank test have shown that there was no significant difference in median survival between the survival curves ($p=0.38$). Regarding that there was no significant difference in median survival between the vaccinated groups and the control group, H_0 hypothesis was accepted.

However, it should be noticed that the median survival of the group vaccinated with the Rotarix and AFFTV made of the MT450 cell line with standard protocol was 40.5, and the control group was 33. Which further might suggest that there is an increase in survival time in the group vaccinated with the Rotarix and AFFTV made of the MT450 cells made with the standard protocol

compared to the control group. As well, the group vaccinated with the AFFTV made of the MT450 cell line made with the changed protocol had higher median survival than the control group but less than the group vaccinated with the AFFTV made of the MT450 cell line but with the standard protocol.

4.5.2. Histological assessment

During the post mortem examination, it was observed that 100% of animals included in this study have developed metastasis inside the lung tissue (refer to Figure 4.24).

a) 

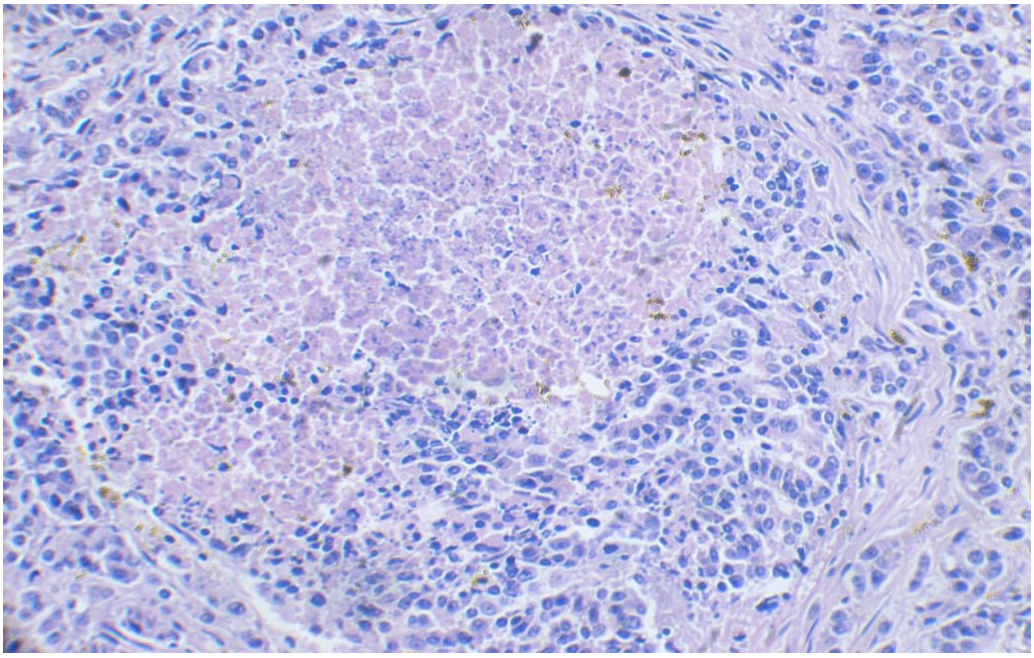
b) 

Figure 4. 24. Lung metastases of the animals included in the anssessment of the features of immune response elicited by the AFFTV made with standard and changed protocol in combination with the Rotarix, in the mammary transplantable tumour model. a) 100X (areas in squares); b) 400X (large tumour formation).

4.5.3. Histological scoring of the lymphocyte infiltration inside the subcutaneous tumour

The histological scoring of the lymphocyte infiltration inside the subcutaneous tumour was performed (refer to Table 4.7).

Groups	Histological specimen	Histological assessment of the lymphocyte infiltration					p value Dunn's test	p value Kruskal Wallis test
		Score 0	Score 1	Score 2	Score 3	Score 4		
Control (n=3)	Subcutaneous tumour	1	1	1	0	0		
AFFTV MT450 cell line (changed protocol (n=2)	Subcutaneous tumour	0	1	1	0	0	>0.99	0.54

Rotarix+AFFTV MT450 cell line (standard protocol) (n=2)	Subcutaneous tumour	0	0	2	0	0	=0.31	

Table 4. 7. Number of animals in each group with certain histological score of the lymphocyte infiltration in the subcutaneous tumour p value after multiple comparison of means between control and each of the treated groups (Dunn's test). p value after comparison of medians between all groups (Kruskal Wallis test).

When comparing the histological score of the lymphocyte infiltration inside the subcutaneous tumour, it was observed that there are slight differences in means and medians in between control and each of the vaccinated groups (Control group mean and median: 1, Rotarix + AFFTV MT450 cell line (changed protocol) group: 1.5, Rotarix + AFFTV MT450 cell line (standard protocol) group mean and median: 2) (refer to Figure 4.25). When the Kruskal Wallis analysis was performed and the medians of the groups were compared, it was determined that there was no any significant difference in the medians in between control and any of the vaccinated groups ($p=0.54$). As a post hoc analysis, a Dunn's multiple comparison was performed and it was observed that average difference in the histological score of lymphocyte infiltration between the control and the group vaccinated with the Rotarix and AFFTV made of the MT450 cell with standard protocol was $p = 0.31$ and as that not significant. When comparing the control with the group vaccinated with the Rotarix and AFFTV made of the MT450 cells with changed protocol, there was no significant difference in the histological score of the lymphocyte infiltration ($p > 0.99$). Regarding that there was no significant difference in the histological score of the lymphocyte infiltration in the subcutaneous tumour between the control and any of the vaccinated groups, H_0 hypothesis was accepted.

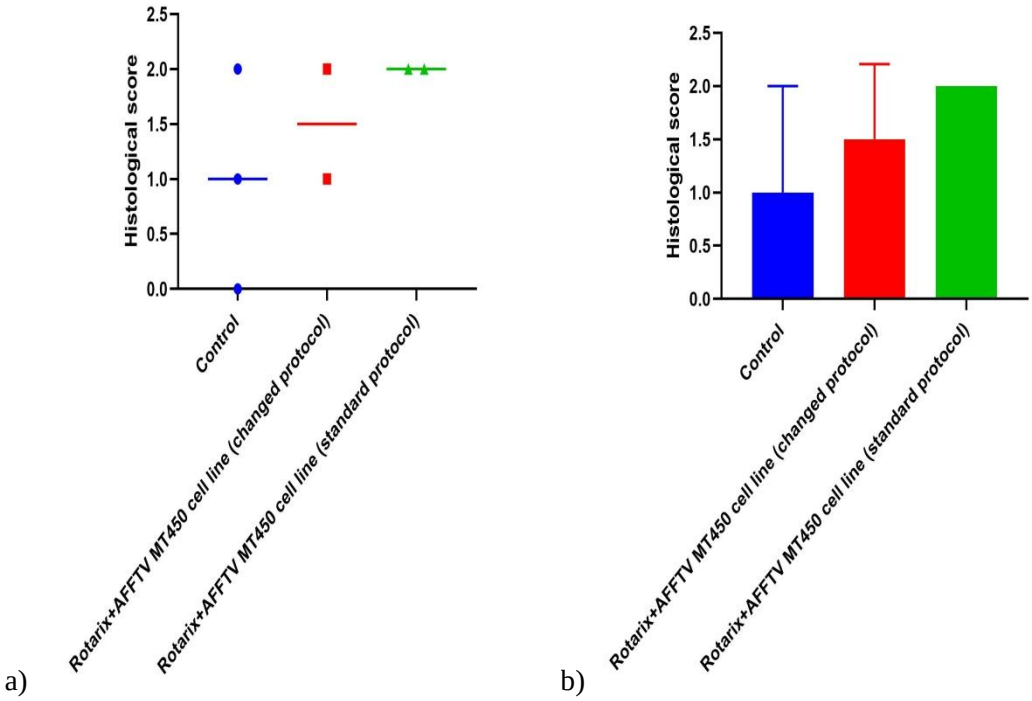


Figure 4. 25. Histological score of the lymphocyte infiltration in the subcutaneous tumour of animals that were vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with standard or changed protocol and control animals. a) Medians of every group presented with straight lines; b) Means of every group.

4.5.4. Histological scoring of the infiltration inside the lungs

The lungs collected from the animals included in this study were histologicaly analysed and scored (refer to Table 4.8).

Groups	Histological specimen	Histological assessment of the lymphocyte infiltration					p value Dunn's test	p value Kruskal Wallis test
		Score 0	Score 1	Score 2	Score 3	Score 4		
Control (n=3)	Lungs	0	0	3	0	0		
AFFTV MT450 3x2	Lungs	0	0	1	0	1	=0.41	0.42
AFFTV MT450 (n=2)	Lungs	0	0	1	1	0	=0.68	

Table 4. 8. Number of animals in each group with certain histological score of the lymphocyte infiltration in the lungs. p value after multiple comparison of means between control and each of the treated groups (Dunn's test). p value after comparison of medians between all groups (Kruskal Wallis test).

When comparing the histological score of the lymphocyte infiltration inside the subcutaneous tumour, it was observed that there are slight differences in means and medians in between control and each of the vaccinated groups (Control group mean and median: 2, Rotarix + AFFTV MT450 cell line (changed protocol) group: 2.5 ,Rotarix + AFFTV MT450 cell line (standard protocol) group mean and median: 2.5) (refer to Figure 4.26). When the Kruskal Wallis analysis was performed and the medians of the groups were compared, it was determined that there was no any significant difference in the medians in between control and any of the vaccinated groups ($p = 0.42$). As a post hoc analysis, a Dunn's multiple comparison was performed and it was observed that average difference in the histological score of lymphocyte infiltration between the control and the group vaccinated with the AFFTV made of the MT450 cell line with the standard protocol was $p = 0.68$ and as that not significant. The same was observed when the control and the group vaccinated with the AFFTV made of the MT450 cell line with the changed protocol were compared ($p = 0.41$). Regarding that there was no significant difference in the histological score of the lymphocyte infiltration in the lungs between the control and any of the vaccinated groups, H_0 hypothesis was accepted.

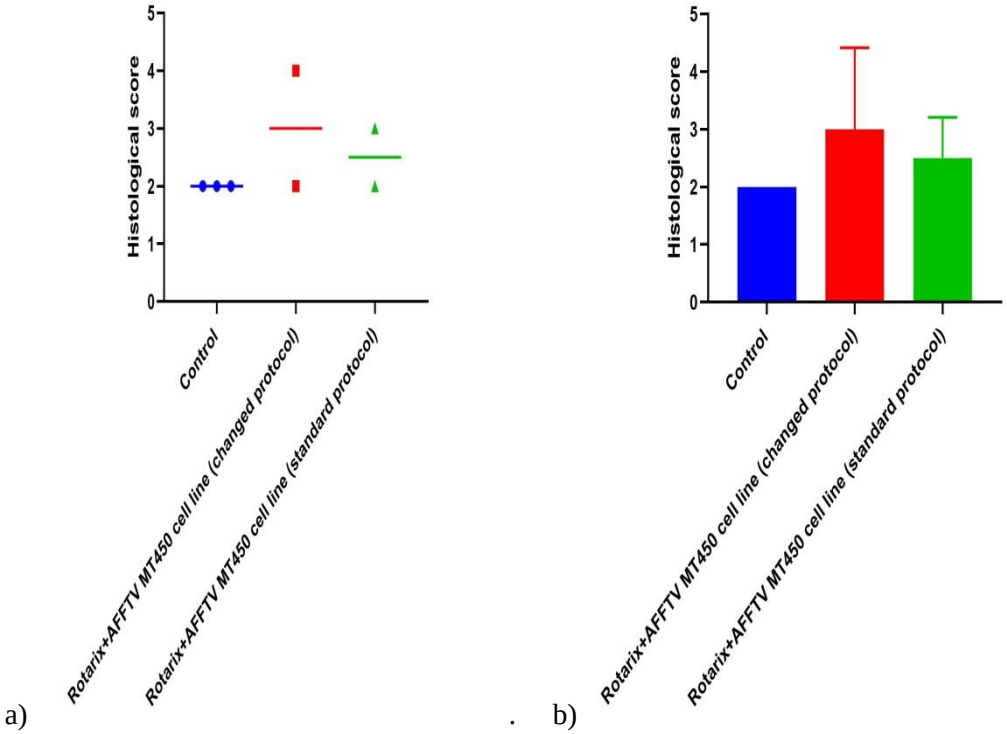
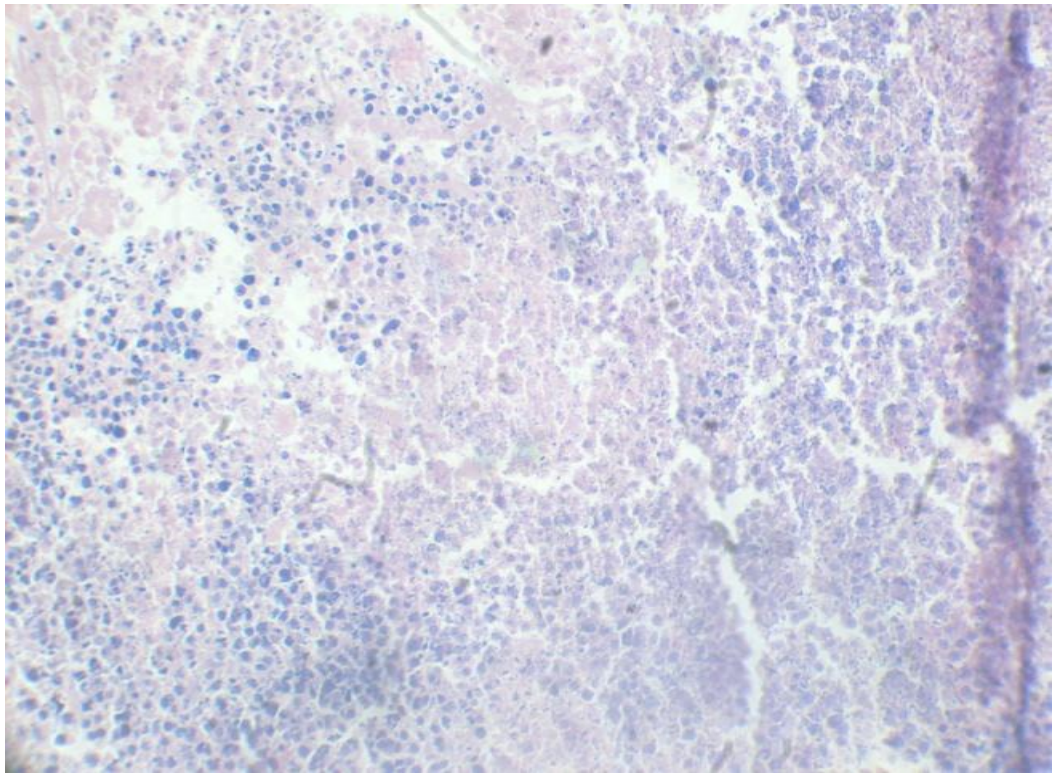


Figure 4. 26. **Histological score of the lymphocyte infiltration in the lungs of animals that were vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with standard or changed protocol and control animals. a) Medians of every group presented with straight lines; b) Means of every group.**

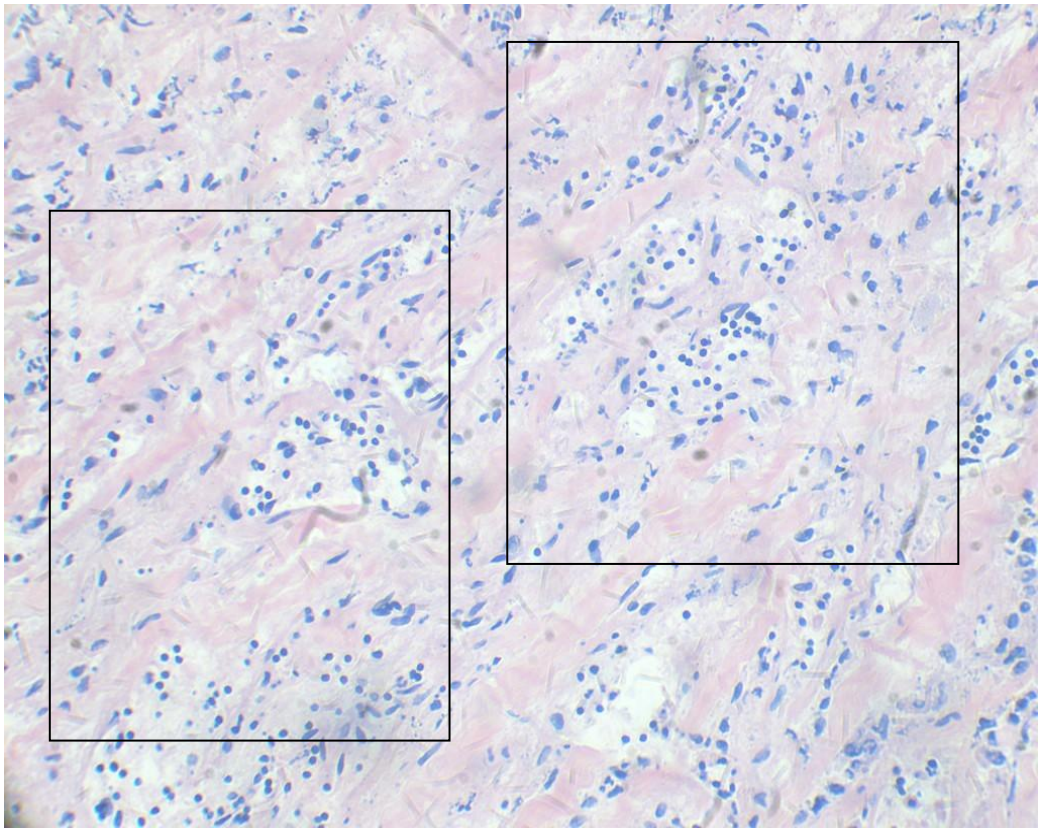
4.6. Photographs for each histological score of the lymphocyte infiltration

On the Figure 4.27 the histological scoring applied for the lymphocyte infiltration in the subcutaneous tumour is explained/ represented.

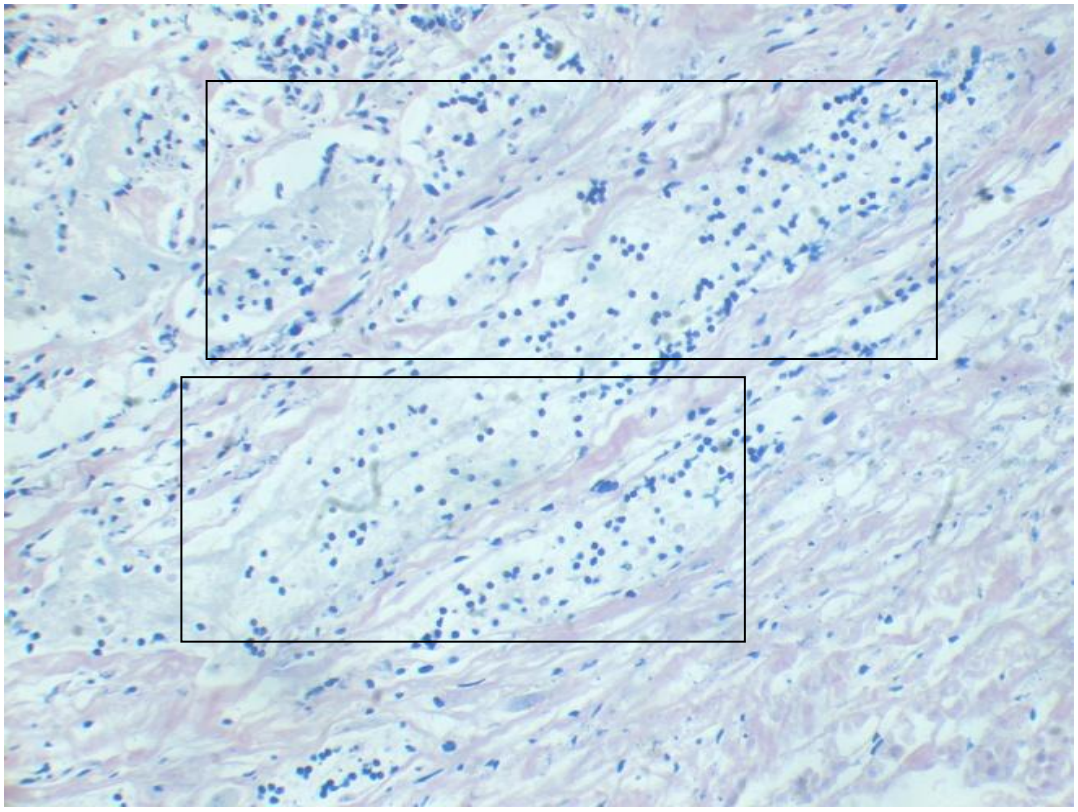
a)



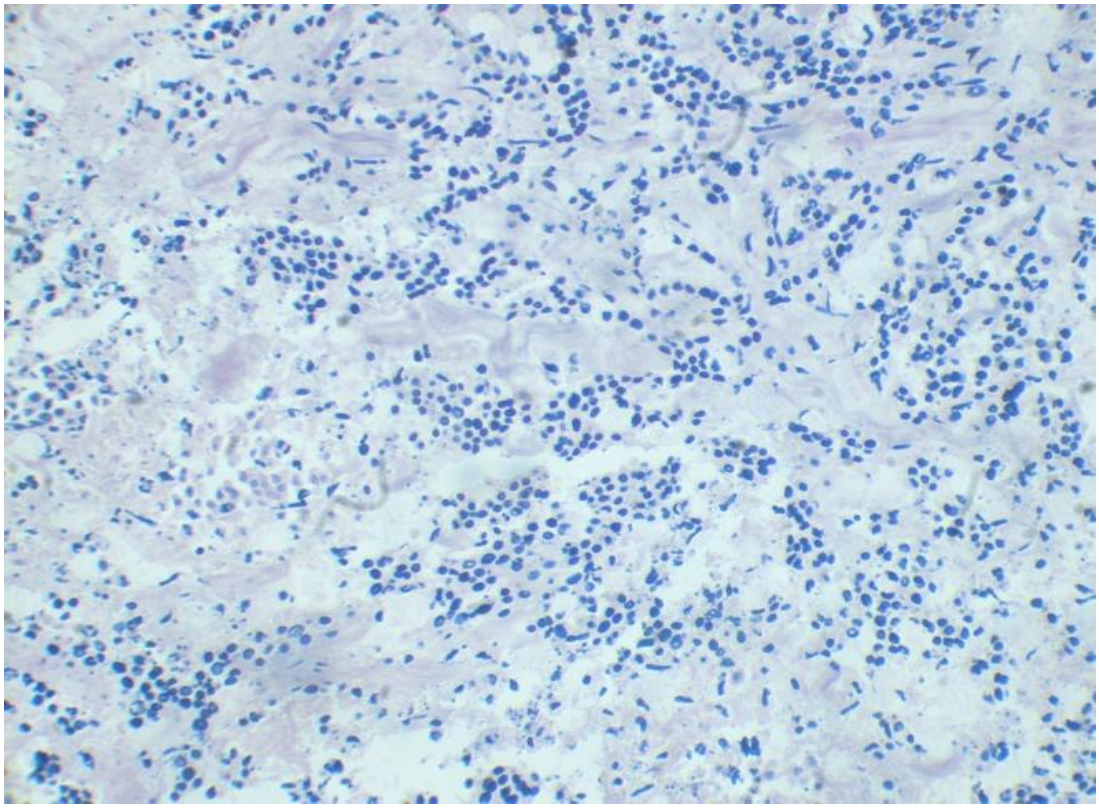
b)



c)



d)



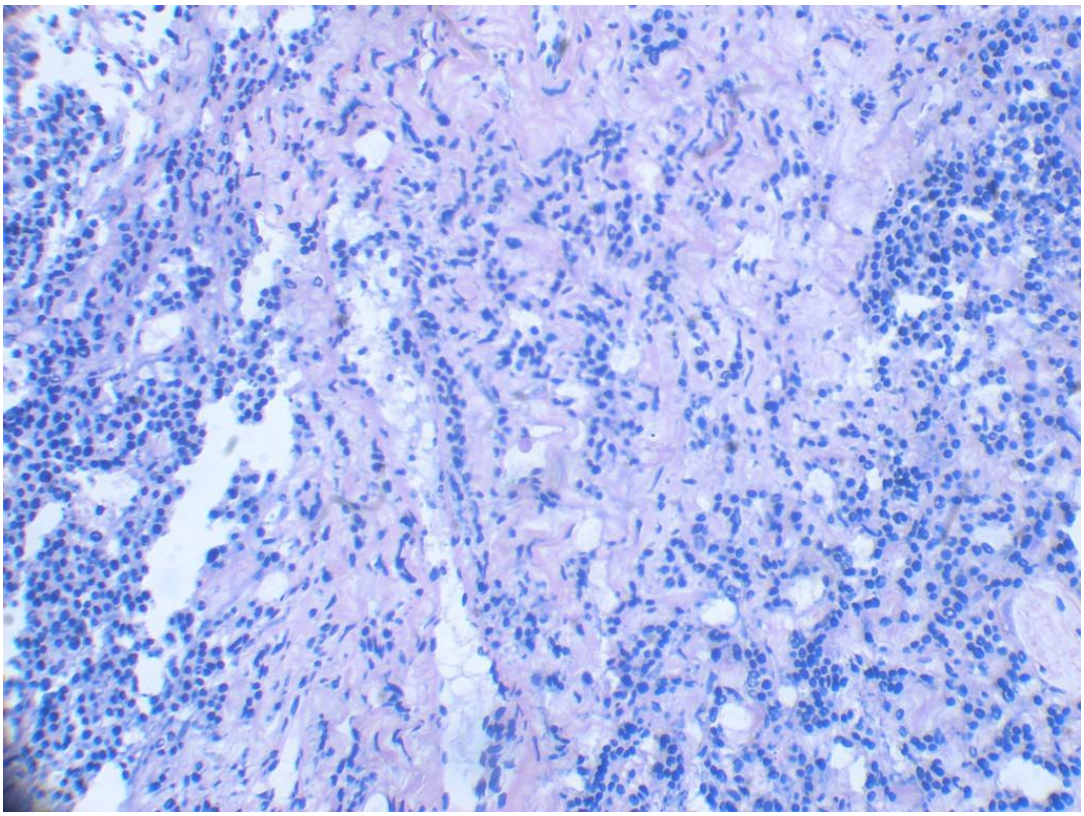
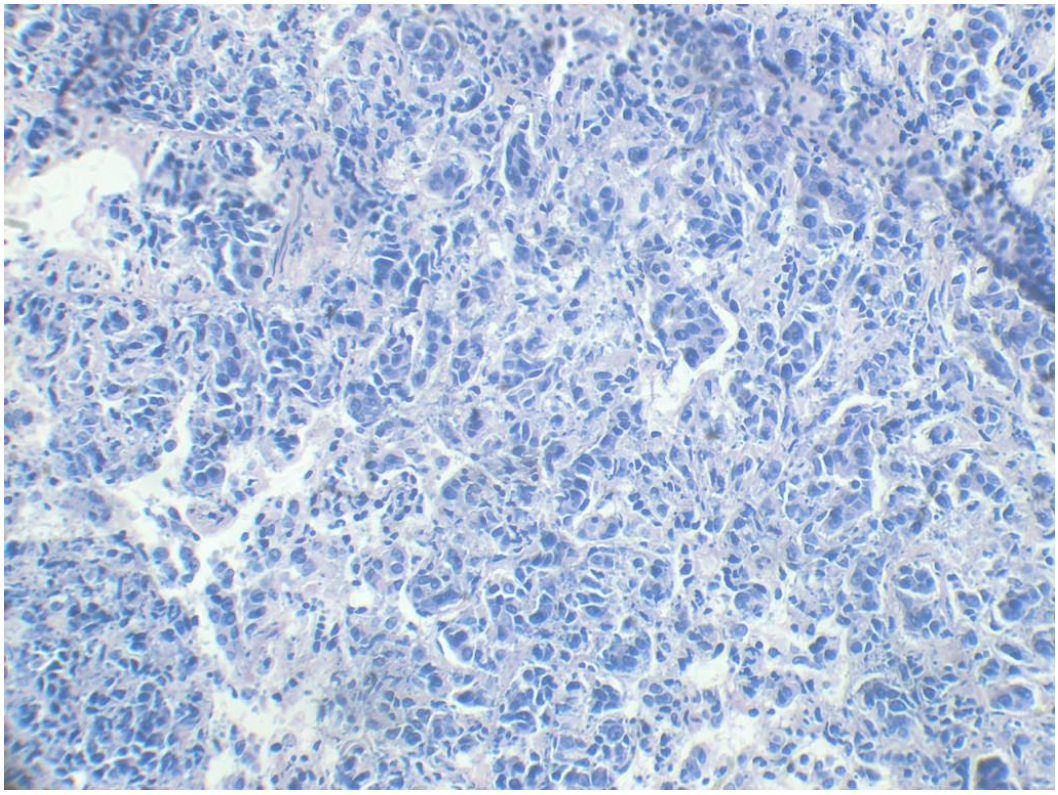
f) 

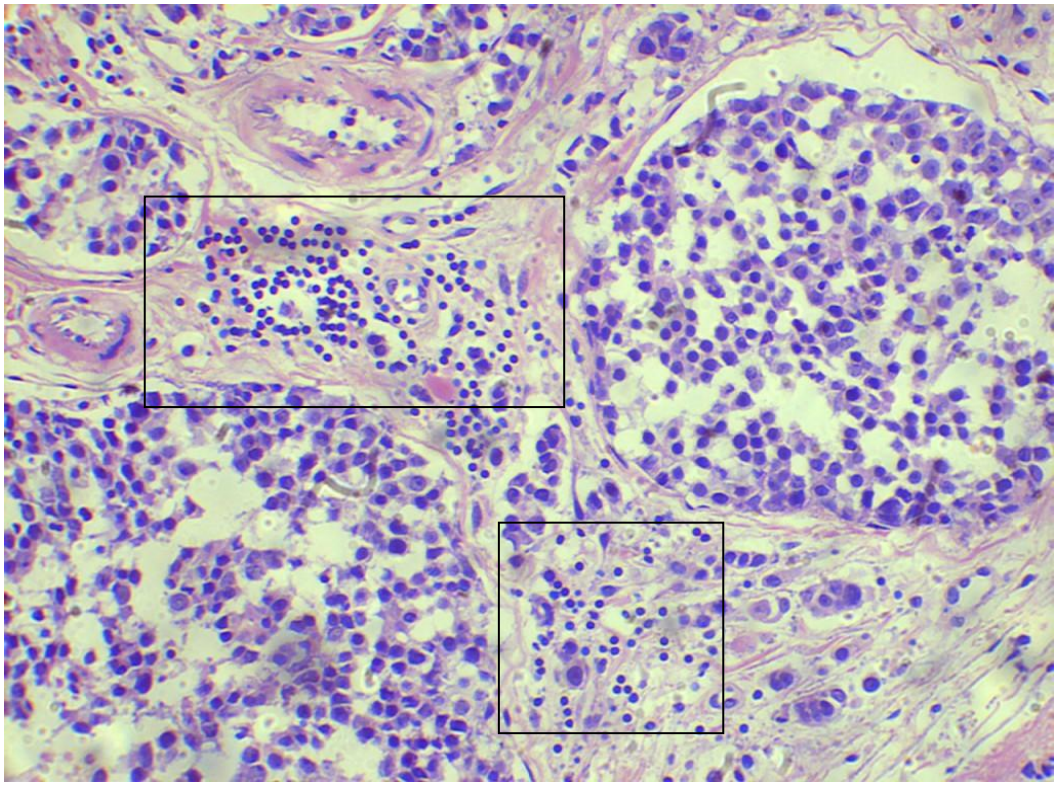
Figure 4. 27. The histological scoring of the lymphocyte infiltration in the subcutaneous tumour. a) Absent; b) Minimal infiltration (marked with rectangles); c) Mild infiltration (marked with rectangles); d) Moderate infiltration (throughout the tissue); e) Strong infiltration throughout the tissue).

On the Figure 4.28, the histological scoring applied for the lymphocyte infiltration in the subcutaneous tumour is represented/explained.

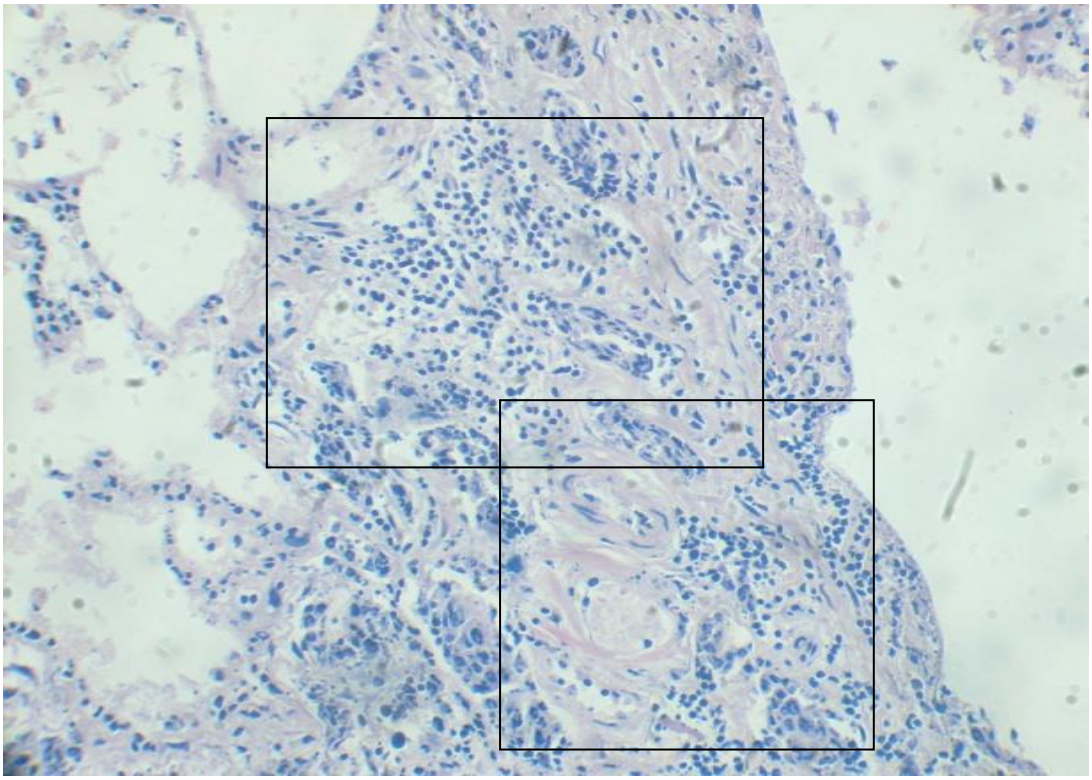
a)



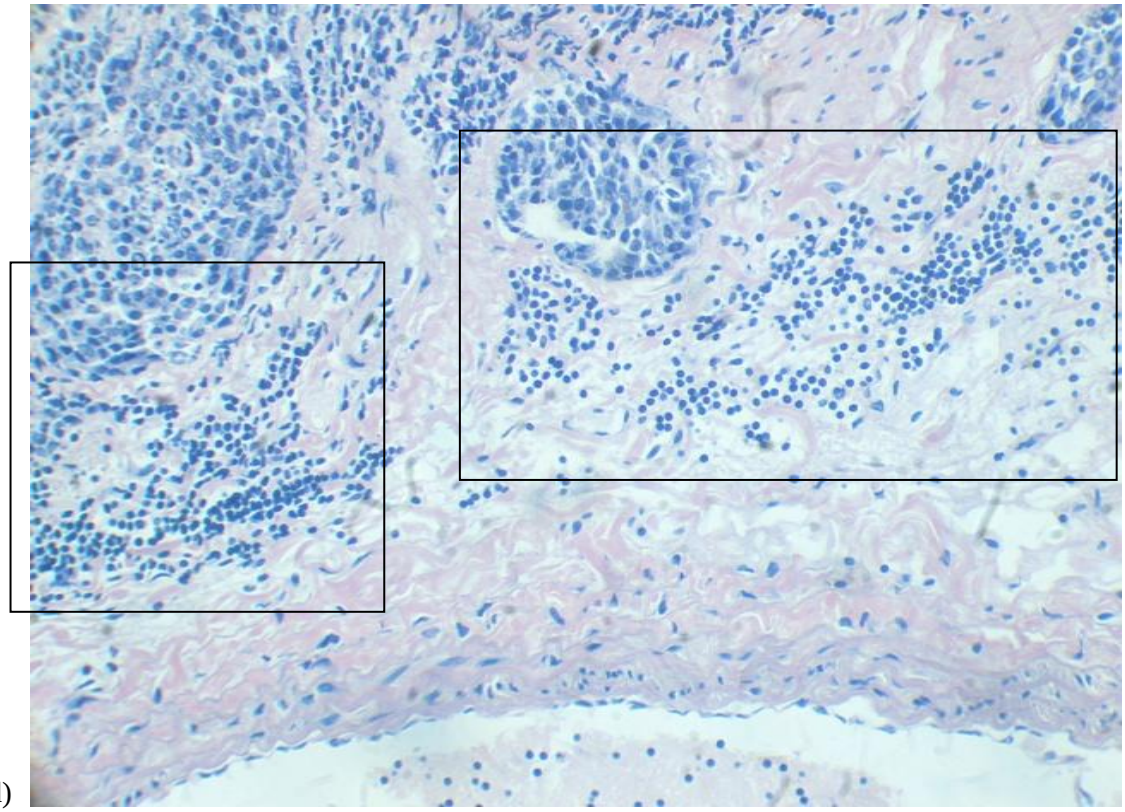
b)



c)



d)



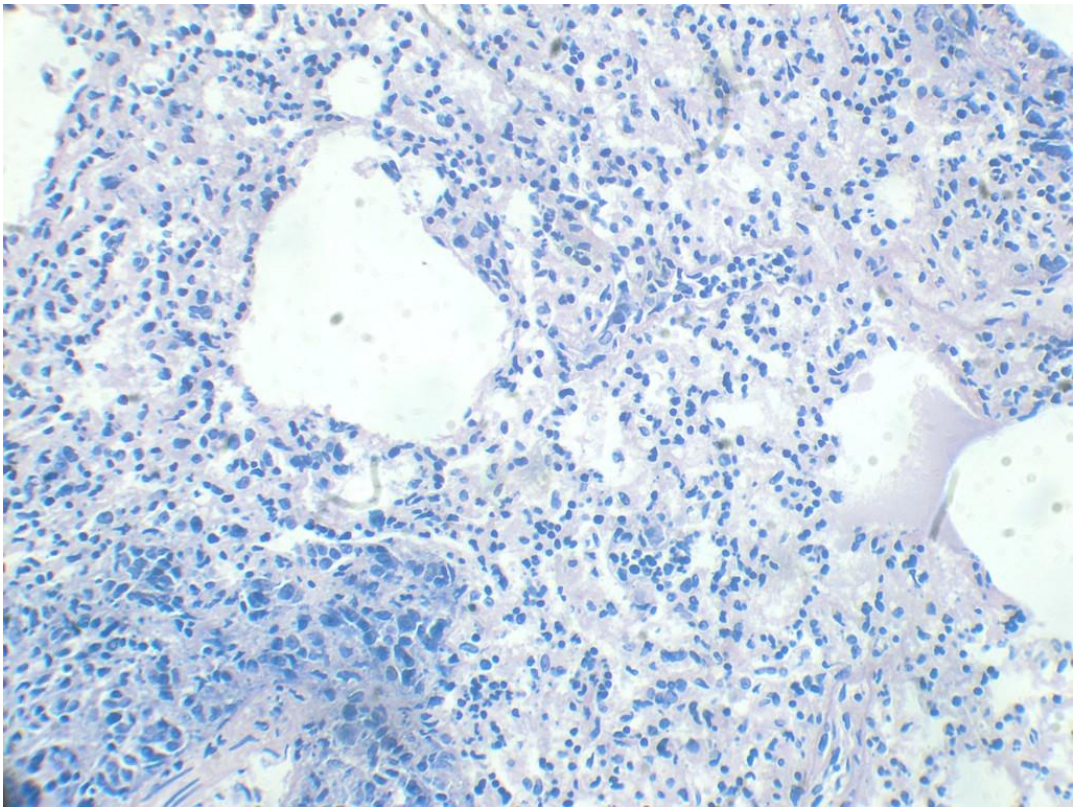
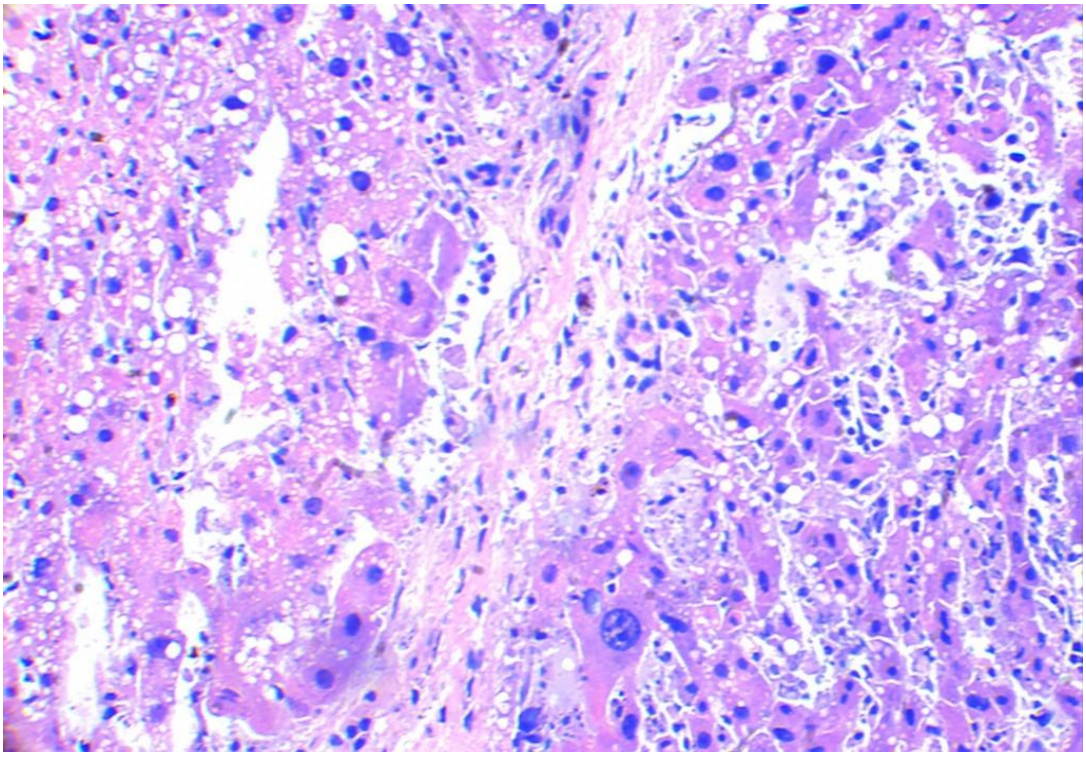
e) 

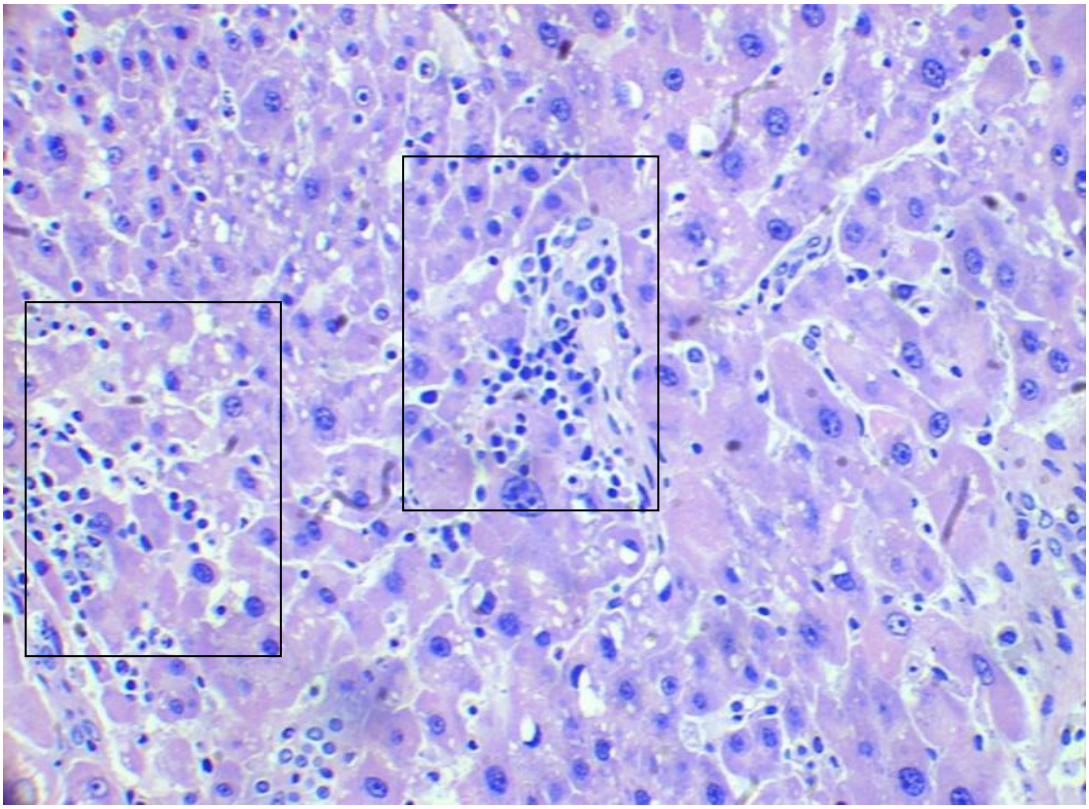
Figure 4. 28. The histological scoring of the lymphocyte infiltration in the lungs. a) Absent ; b) Minimal infiltration (marked with rectangles); c) Mild infiltration (marked with rectangles); d) Moderate infiltration (marked with rectangles); e) Strong infiltration (throughout the tissue).

On the Figure 4.29, the histological scoring applied for the lymphocyte infiltration in the subcutaneous tumour is represented/explained.

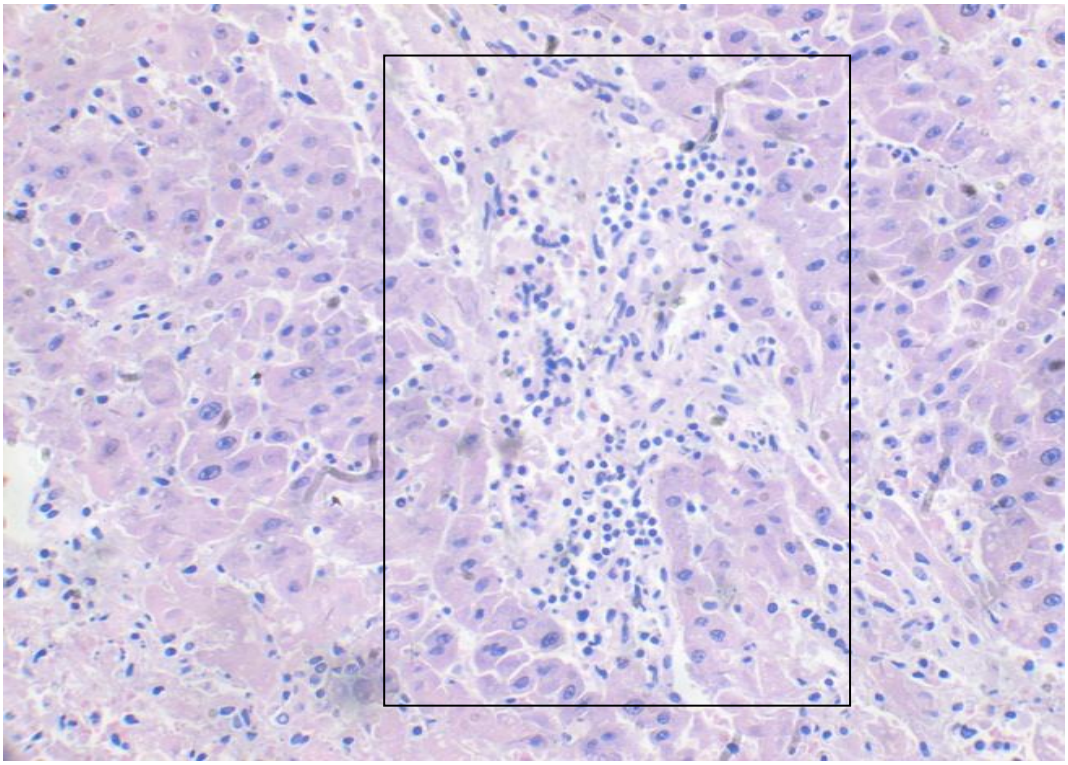
a)



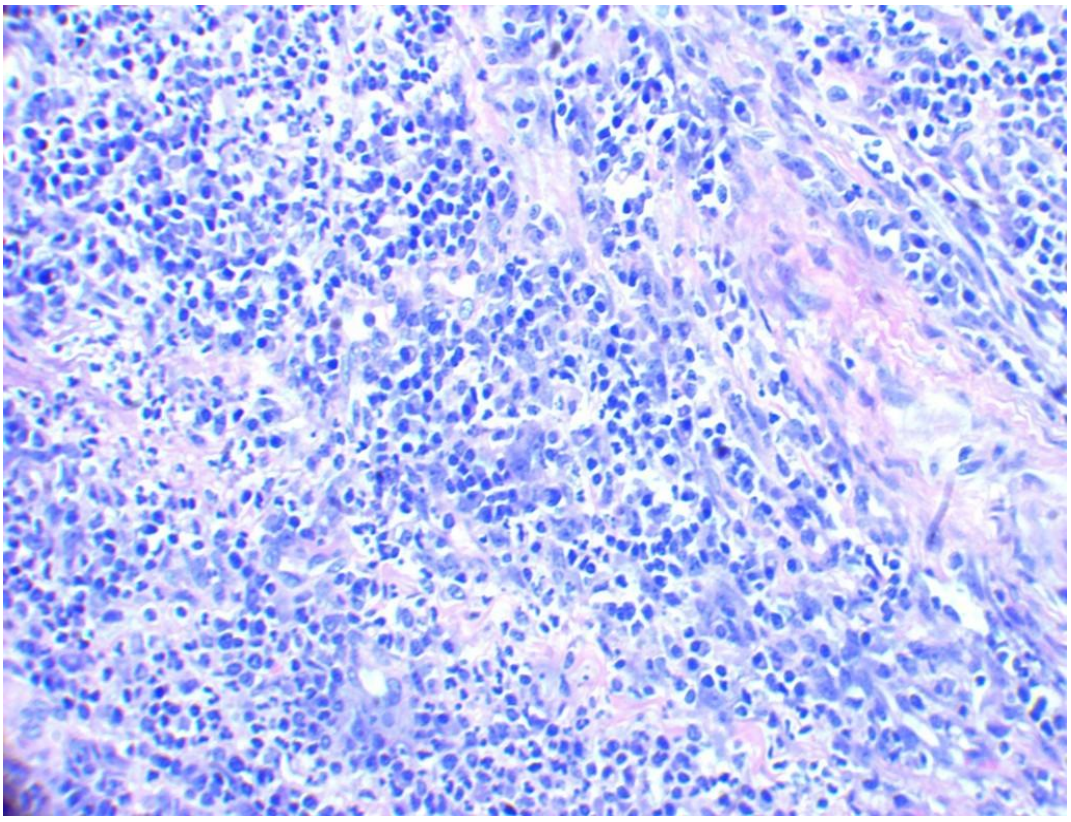
b)



c)



d)



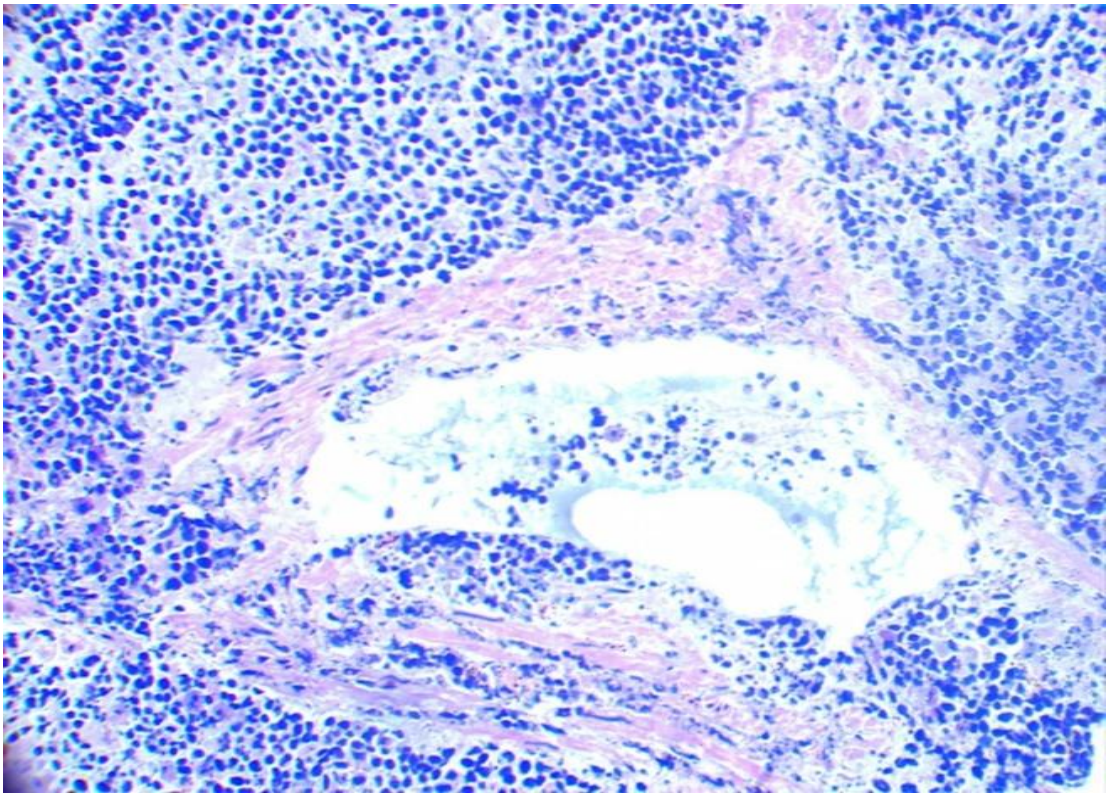
e) 

Figure 4. 29. The histological scoring of the lymphocyte infiltration in the liver. a) Absent ; b) Minimal infiltration (marked with rectangles); c) Mild infiltration (marked with rectangles); d) Moderate infiltration (throughout the tissue); e) Strong infiltration (throughout the tissue).

Cohen Kappa (k) was applied to assess the overal interobserver agreement of lymphocyte infiltration. First observer (myselsf) and a second observer prof. Pierre Schembri Wismayer. The Cohen Kappa resulted in substantial agreement (k = 0.66) (refer to Table 4.9).

	Observer A vs Observer B
kappa	0.66
Standard error	0.66
95% CI	0.531to 0.790

Table 4. 9. Interobserver agreement (kappa). Observer A (myself); Observer B (prof. Pierre Schembri –Wismayer); CI - Confidence interval.

4.7. Morphological features of a tumour development in the MT450 carcinogenesis model

After seven days it was observed that rats inoculated with the different MT450 cell amounts all have developed tumour formations which size correlated to the amount of cells used. It was observed that animals inoculated with the 1 x 10^6 MT450 cells had proportionally larger tumour and more expressed/visible blood vessels around the tumour tissue as compared to the other animals inoculated with a smaller amount of the MT450 cells. As well it was observed that in all animals after seven days axillary/brachial lymph nodes were not enlarged. A tumour was encapsulated and liquid inside. Lungs were without any significant change (refer to Figure 4.30).

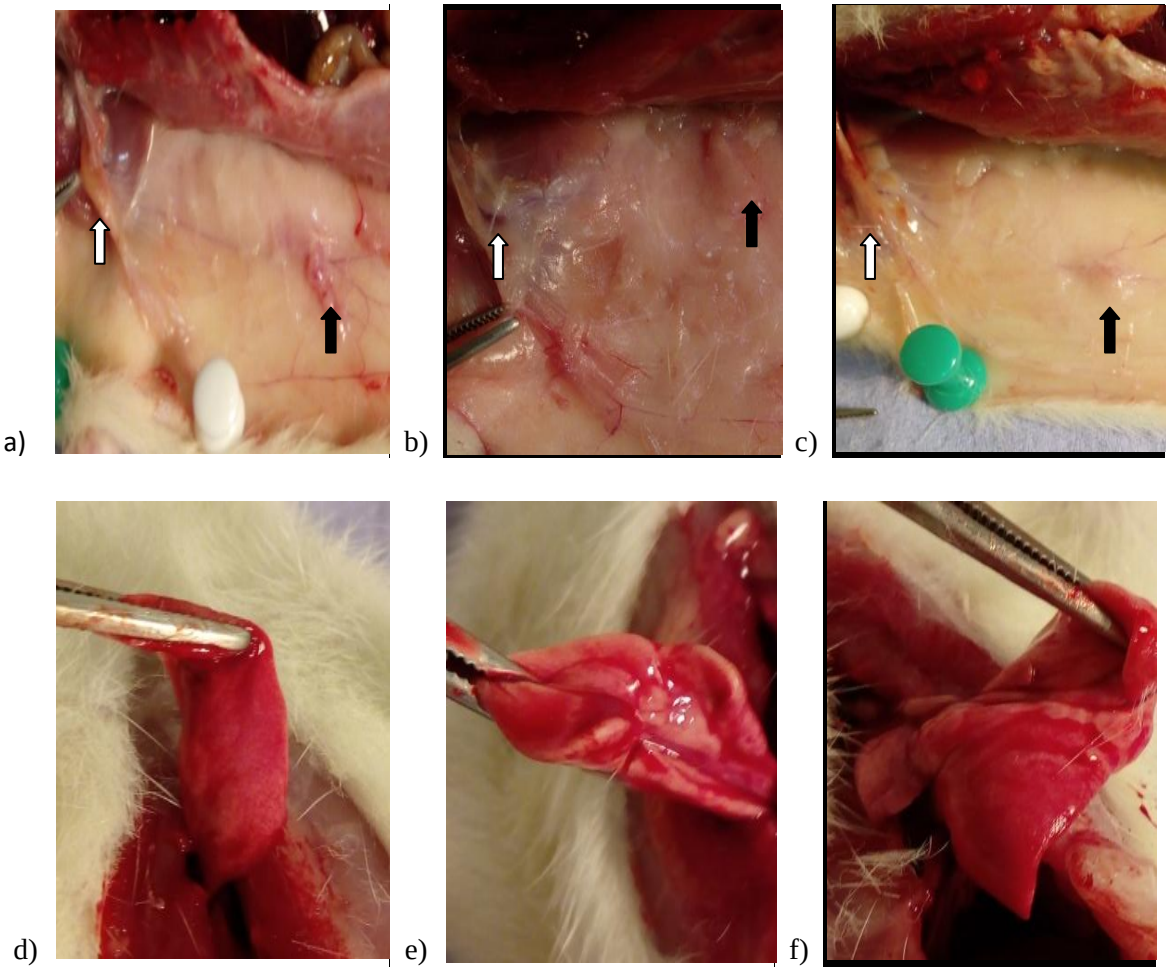
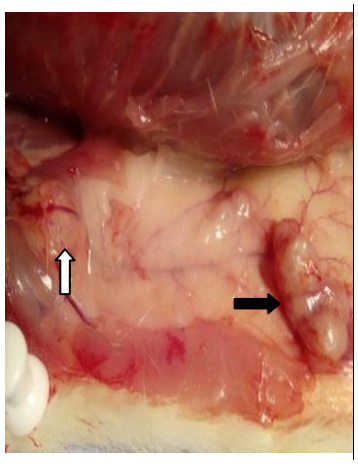
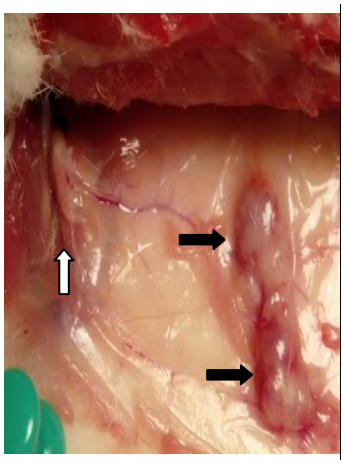
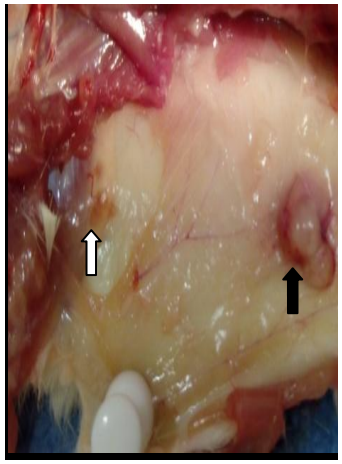


Figure 4. 30. Observations made after post mortem organ isolation of animals injected with different amounts of the MT450 cells, sacrificed after seven days: a) Subcutaneous tumour and the axillary lymph node in animals injected with the 1 x 10^6 MT450 cells, b))

Subcutaneous tumour and the axillary lymph node in animals injected with the 5 x 10^5 MT450 cells, c) Subcutaneous tumour and the axillary lymph node in animals injected with the 1 x 10^5 MT450 cells; d) Lungs of the animals injected with the 1 x 10^6 MT450 cells; e) Lungs of the animals injected with the 5 x 10^5 MT450 cells; f) Lungs of the animals injected with the 1 x 10^5 MT450 cells. The axillary lymph node pointed out with a white arrow. The subcutaneous tumour pointed out with a black arrow.

After fourteen days, in animals injected with the 1 x 10^6 MT450 cells tumour was expanding towards the mammary glands, blood vessels were more expressed. Animals injected with the 5 x 10^5 MT450 cells had tumours larger in size that were still circular, not significantly spread through the tissue. Blood vessels were visibly expressed around the tumour. Animals injected with the 1 x 10^5 MT450 cells had tumours of smaller size, round shaped, not spread,with surrounding blood vessels less expressed. Fourteen days following the MT450 cell inoculation any enlargement of the axillary/brachial lymph nodes on the side of inoculation or on the opposite side was not noticed. The lungs seemed normal and without any significant change in size/colour/texture (refer to Figure 4.31).

a)  b)  c) 

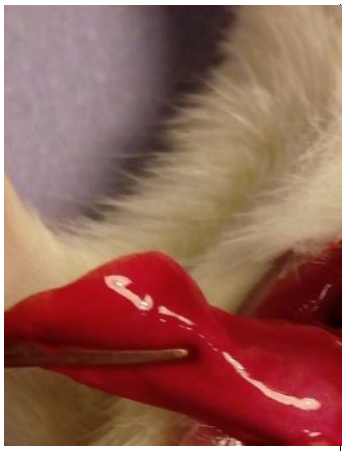
d)  e)  c) 

Figure 4. 31. Observations made after post mortem organ isolation of animals injected with different amounts of the MT450 cells, sacrificed after fourteen days. a) Subcutaneous tumour and the axillary lymph node in animals injected with the 1 x 10^6 MT450 cells; b)) Subcutaneous tumour and the axillary lymph node in animals injected with the 5 x 10^5 MT450 cells; c) Subcutaneous tumour and the axillary lymph node in animals injected with the 1x10^5 MT450 cells; d) Lungs of the animals injected with the 1 x 10^6 MT450 cells, e) Lungs of the animals injected with the 5 x 10^5 MT450 cells; f) Lungs of the animals injected with the 1 x 10^5 MT450 cells. The axillary lymph nodes pointed out with white arrows. The subcutaneous tumour pointed out with black arrows.

Following twenty one day period, significant changes in all groups were observed. Tumours were double in size and the axillary lymph node on the same side was enlarged while the brachial lymph node was still normal sized. The subcutaneous tumour was getting ticker and spread towards the inner tissue layers and the rib cage as well as towards the right axillary and brachial lymph node. The blood vasculature in between the tumour and the axillary lymph node was still very expressed (refer to Figure 4.32).

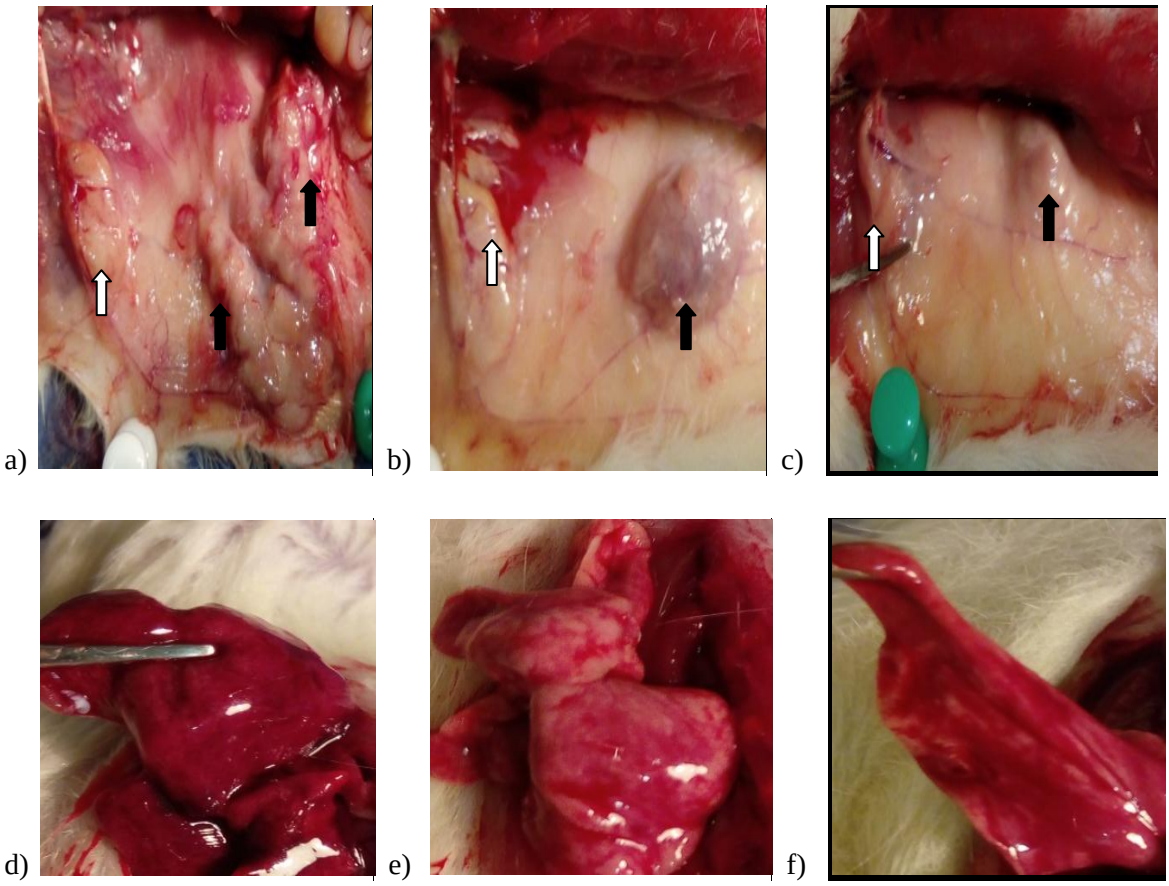


Figure 4. 32. Observations made after post mortem organ isolation of animals injected with different amounts of the MT450 cells, sacrificed after twenty one days. a) Subcutaneous tumour and the axillary lymph node in animals injected with 1 x 10^6 MT450 cells; b)) Subcutaneous tumour and the axillary lymph node in animals injected with 5 x 10^5 MT450 cells; c) Subcutaneous tumour and the axillary lymph node in animals injected with 1x10^5 MT450 cells; d) Lungs of the animals injected with 1 x 10^6 MT450 cells; e) Lungs of the animals injected with 5 x 10^5 MT450 cells; f) Lungs of the animals injected with 1 x 10^5 MT450 cells. The axillary lymph nodes pointed out with white arrows. The subcutaneous tumour pointed out with black arrows.

At the twenty eight days, in animals treated with 1 x 10^6 MT450 cells, tumours were quite massive and almost connected to the right axillary lymph node which was much larger in size as compared to the one isolated at the 21st day. The brachial lymph node on the inoculation side was getting larger and almost forming a compact structure with the axillary lymph node on the same side. Axillary and brachial lymph node on the opposite side were enlarged but on a small scale as compared to the right side ones. No significant change was seen on the lung tissue. Same situation

was observed in animals injected with the $5x10^5$ MT450 cells, where only change as compared to animals treated with 1×10^6 MT450 cells was smaller tumour size. In animals injected with 1×10^5 MT450 cells, tumours were larger in size as compared to the one isolated on the 21st day and a blood vasculature was significantly expressed around the tumour. A tumour tendency of spreading towards the right axillary lymph node was observed in this model and the right lymph node was enlarged but no significant change in size of the brachial lymph node on the same side or the axillary/brachial lymph node on the opposite side was observed. The lungs were without any significant morphological change (refer to Figure 4.33).

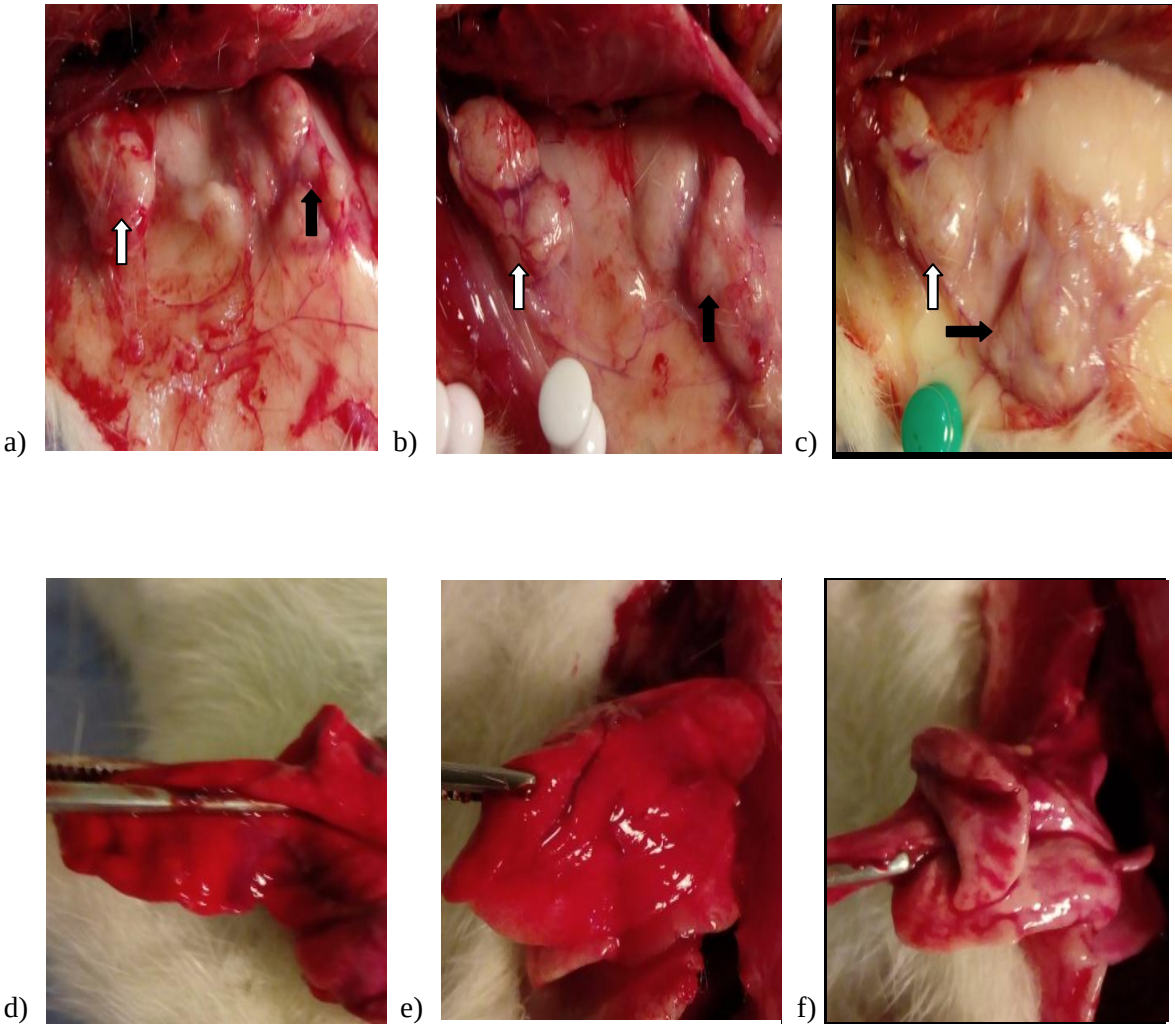


Figure 4. 33. Observations made after post mortem organ isolation of animals injected with different amounts of the MT450 cells, sacrificed aftr twenty eight days: a) Subcutaneous tumour and the axillary lymph node in animals injected with 1×10^6 MT450 cells; b)

Subcutaneous tumour and the axillary lymph node in animals injected with 5 x 10^5 MT450 cells; c) Subcutaneous tumour and the axillary lymph node in animals injected with 1 x 10^5 MT450 cells; d) Lungs of the animals injected with 1x10^6 MT450 cells; e) Lungs of the animals injected with 5 x 10^5 MT450 cells; f) Lungs of the animals injected with 1 x 10^5 MT450 cells. The axillary lymph nodes pointed out with white arrows. The subcutaneous tumor pointed out with black arrows.

Significant change in the tumour development in between different sexes, male and female, was observed at this time period and in the animals treated with 1 x 10^6 and 5 x 10^5 MT450 cells. It was shown that tumours formed in male rats are losing continuity in a tumour development rate in terms of size and tumour appearance as compared to female rats from the same group. Some of the male rats injected with the same amount of cells and which were sacrificed later were seen having smaller and less developed tumours as compared to the ones sacrificed earlier. As well it was observed that features related to tumour formation such as the enlargement of lymph nodes were less pronounced in some of them. This tendency was not observed in female rats from the same groups after the 21 days time frame. In female rats, size and rate of the tumour development along with features related to it seemed continuous and in accordance with the time frame.

Following thirty five days, rats inoculated with 1 x 10^6 MT450 cells had extremely large tumours,that were spread and connected physically with the axillary and brachial lymph node structure. It was observed that these tumours had as well invaded the tissue in between ribs. Both the axillary and brachial lymph node on the opposite side were enlarged and larger in size comparing to the ones isolated on the 28^{th} day. Lungs had a few visible changes in the colour of the tissue. Animals inoculated with the 5 x 10^5 MT450 cells had large tumours spread towards the rib cage and the axillary lymph node on the same side but the tumour was not physically connected to the axillary – brachial lymph node structure. The opposite lymph nodes were enlarged but less as compared to the ones isolated from the rats injected with the 1 x 10^6 MT450 cells. Lungs had small changes in the colour of the tissue. Animals inoculated with the 1 x 10^5 MT450 cells had large tumours that showed tendency of spreading towards the axillary and brachial lymph nodes on the same side as well as towards the rib cage. The tumour was not physically connected to the lymph nodes and the axillary and brachial lymph nodes on the opposite side were enlarged but significantly, less as compared to the ones isolated from animals inoculated with 1 x1 0^6 and 5 x 10^5 MT450 cells. For lungs it was hard to determine did they have any significant change (refer to Figure 4.34).

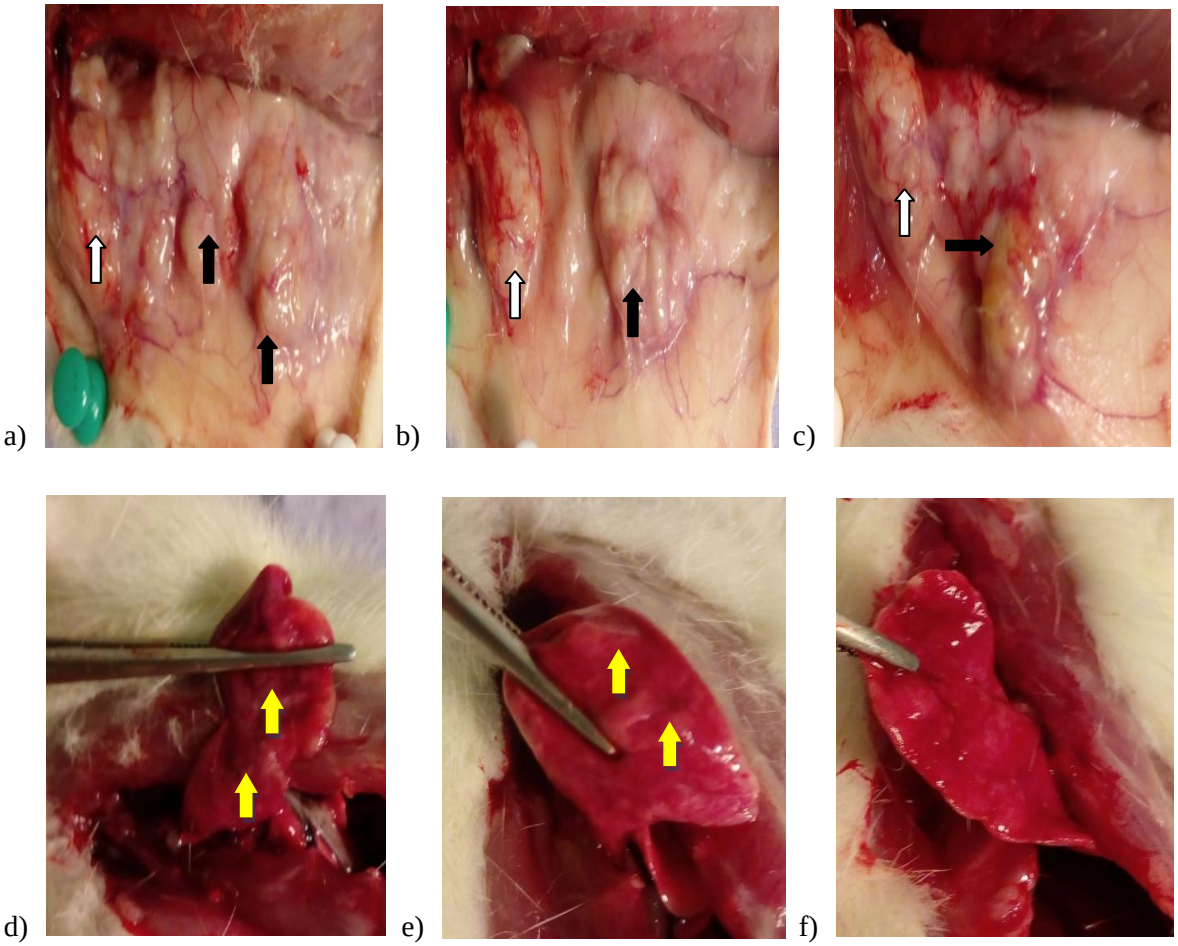
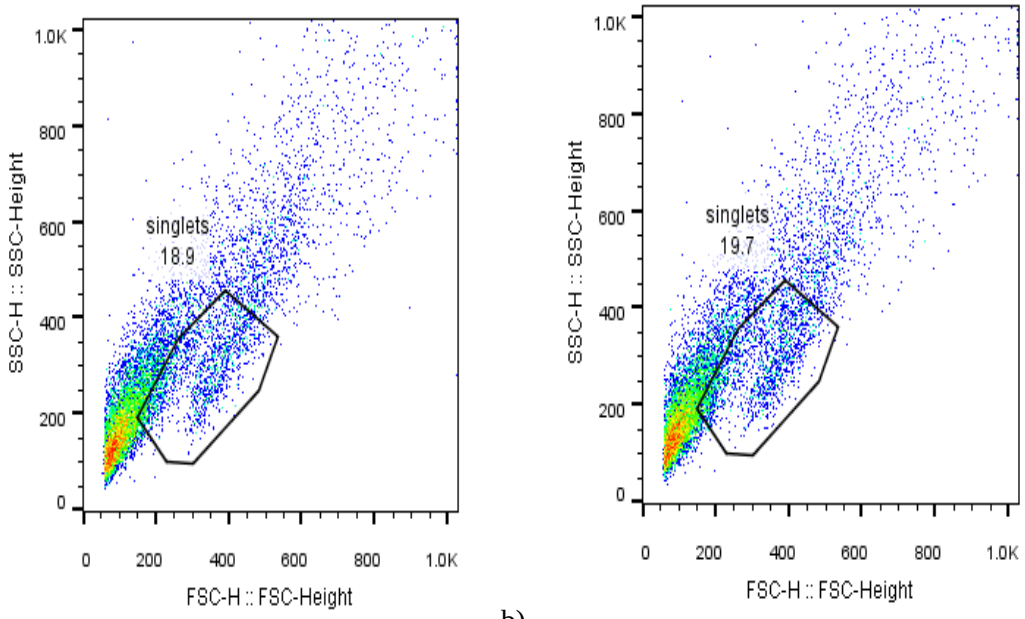


Figure 4. 34. Observations made after post mortem organ isolation of animals injected with different amounts of the MT450 cells, sacrificed after thirty five days. a) Subcutaneous tumour and the axillary lymph node in animals injected with 1 x 10^6 MT450 cells; b)) Subcutaneous tumour and the axillary lymph node in animals injected with 5 x 10^5 MT450 cells; c) Subcutaneous tumour and the axillary lymph node in animals injected with 1 x 10^5 MT450 cells; d) Lungs of the animals injected with 1 x 10^6 MT450 cells; e) Lungs of the animals injected with 5 x 10^5 MT450 cells; f) Lungs of the animals injected with 1 x 10^5 MT450 cells. The axillary lymph nodes pointed out with white arrows. The subcutaneous tumour pointed out with black arrows. Lung tumour pointed out with yellow arrows.

4.8. Flow cytometry analysis

The difference in the fluorescence of the cells stained with the 200x and 400x dilution factor of the CD8a primary conjugated antibody with fluorescent florophore (CD8a AlexaFluor 488) was not

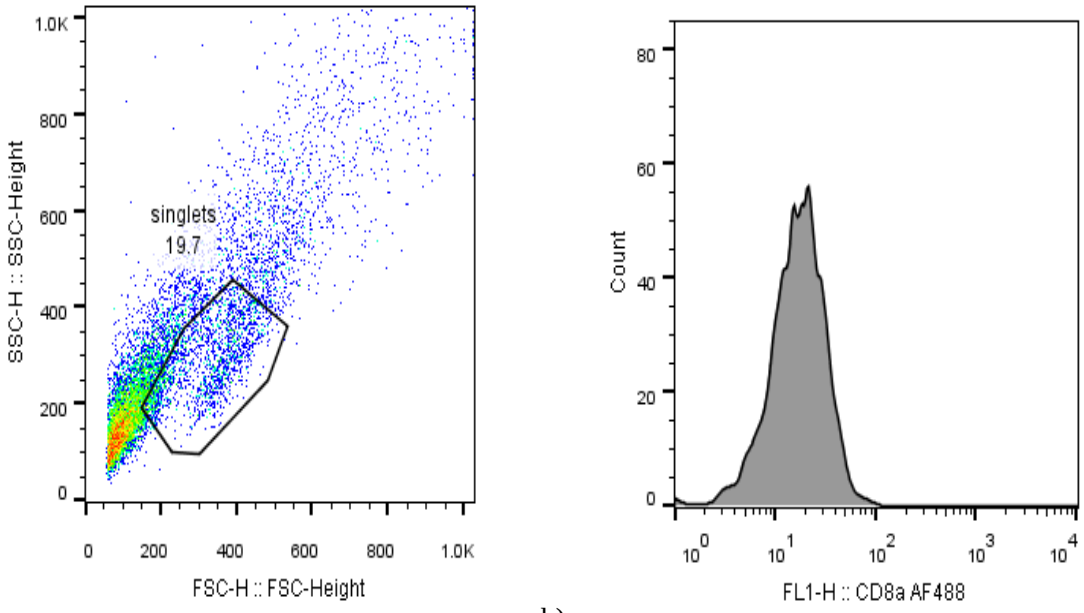
observed during analysis (refer to Figure 4.35). Therefore, the samples stained with the 400x diluted CD8a antibody were further analysed.



a) b)

Figure 4. 35. The Difference in staing of a sample stained with the 200x and 400x Ab dillutions. a) 200x Ab dillution; b) 400x Ab dillution.

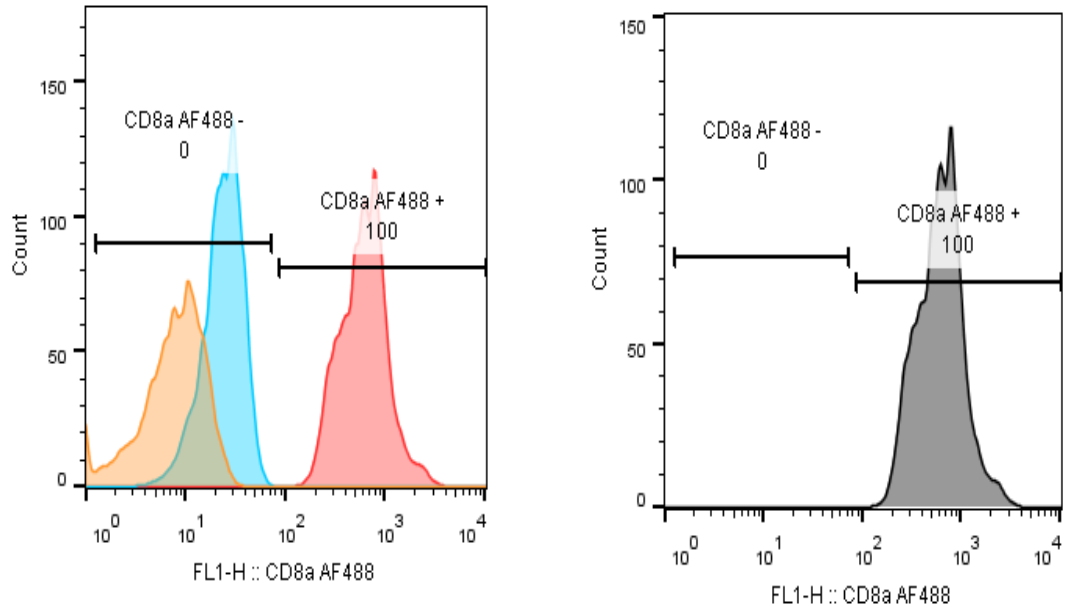
Gating strategy that was used included first gating SSC - Height versus FSC-Height in order to separate the lymphocyte population. The very low FSC events were excluded regarding that they are usually representing the erythrocytes, cell debri or dead cells. Similar was performed with the SSC were the very low and high events were excluded. Lower to intermediate FSC and SSC areas were used for gating of lymphocytes due to their small size and lower granularity. This was followed with plotting of the CD8a AF488 histogram gated on the lymphocyte population (refer to Figure 4.36).



a) b)

Figure 4. 36. Gating strategy. A) FSC vs SSC; b) Histogram of the CD8a AF488 on the lymphocyte population.

In order to determine which cells should be considered as a positive CD8a AF488 and what as a negative CD8a AF488, histograms of the unstained control, negative and positive were visualised and based on the observation made the gates were set (refer to Figure 4.37).



a) b)

Figure 4. 37. Gating strategy applied. A) Visualisation of the histograms of positive (pink colour), negative (blue colour) and unstained (orange colour) control; b) CD8a AF488 positive and negative population cell positive and negative cell population (orange colour- unstained cells, blue colour – negative control, pink colour – positive control).

Cell concentration was counted by dividing number of events with volume of blood that was analysed. Prior to that volume of blood that was analysed was counted as a product of multiplication of blood sample flow rate with the time passed during sample analysis. Then, based on total cell number and the percentage of $CD8^+$ T positive cells previously obtained through FlowJo analysis, number of $CD8^+$ T positive cells in each blood sample was determined. Based on these calculations, statistic analysis was performed in a GraphPad Software and appropriate graphs were constructed. In the control group, blood was not collected from two animals (one from the group injected with the 1×10^6 and one from the group injected with the 5×10^6). This happened because of the problems with the blood extraction during the cardiac puncture (most probably because of a heart failure).

In order to determine if the data, in the group previously inoculated with the 1×10^5, was normally distributed, the Shapiro-Wilk's test was applied. It was determined that data was normally distributed ($p = 0.74$; $p = 0.55$), so the H_0 hypothesis was accepted. Since the data was normally distributed it is further analysed with the t-test. Since the sample size was small, t-test with Welch's

correction was applied. Significant difference in number of $CD8^+$ T positive cells in between animals of the group that was previously inoculated with 1 x 10^5 MT450 cells, that were not vaccinated (control) and animals that were vaccinated was not observed (p = 0.08) (refer to Figure 4.38).

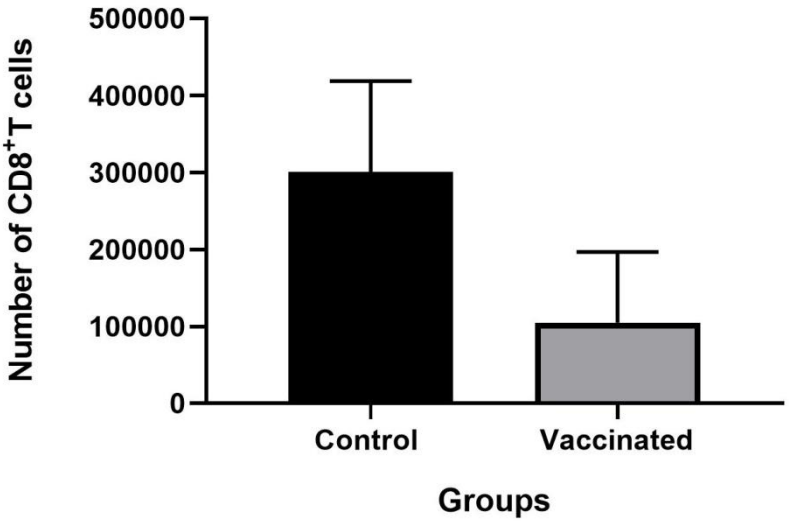


Figure 4. 38. Difference in number of $CD8^+$ T cells between unvaccinated (control) group and vaccinated group that were previously inoculated with 1 x 10^5 MT450 cells.

In order to determine if the data, in the group previously inoculated with the $1x10^5$, was normally distributed, the Shapiro-Wilk's test was applied. It was determined that data was normally distributed (p = 0.33; p = 0.33), so the H_0 hypothesis was accepted.

In order to compare amount of the $CD8^+$ T cells in between animals that were not vaccinated (control) and animals that were vaccinated, in the group previously inoculated with the 5 x 10^5 MT450 cells, t test with the Welch's correction was performed. Results of the t test indicated that there was no significant difference in the amount of the $CD8^+$ T cells in between vaccinated and control group (p = 0.35) (refer to Figure 4.39).

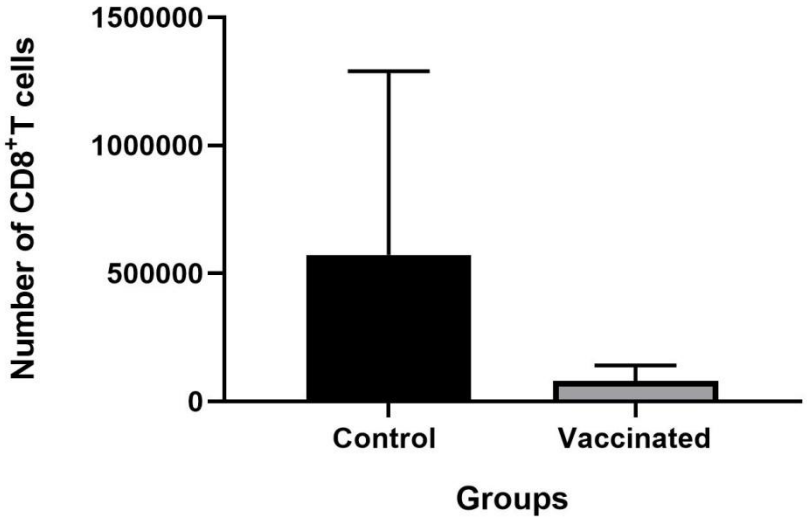


Figure 4. 39. Difference in number of $CD8^+$ T cells between unvaccinated (control) and vaccinated group that were previously inoculated with 5 x 10^5 MT450 cells.

4.9. Determination of protein concentration in the AFFTV and comparison in between different processing

In order to determine the protein (peptide) concentration in the AFFTV prepared in standard way DC assay was performed. As well, the protein concentration of the AFFTV prepared in the standard way was compared to the protein concentration of the AFFTV (tissue samples) prepared in different ways as described in the 3.12 section.The protein concentration of samples was determined based on the measure of the absorbance and the standard curve constructed based on the known protein concentrations as explained previously.

Regarding that each sample was tested in technical triplicates, based on the absorbance of each of the replicates we have counted the mean concentration of each sample (refer to Figure 4.40 and Table 4.10). In order to determine is there a significant difference in between the samples differently processed, we have compare them with the one - way ANOVA test. It was observed that there is no significant difference in the protein concentration between differently processed tissue samples (p=0.77).

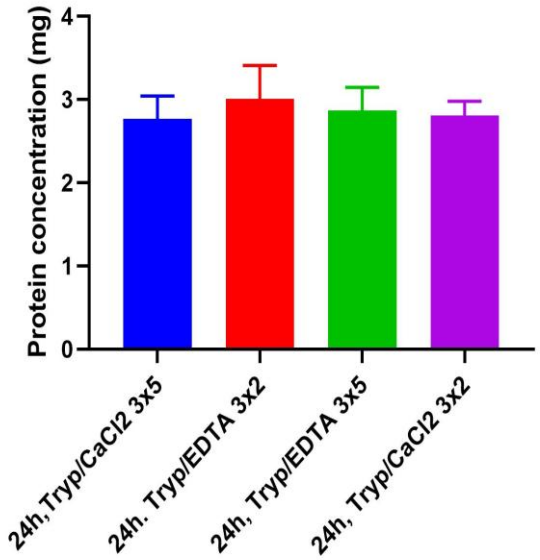


Figure 4. 40. Comparison of average protein concentration in between AFFTV differently processed.

Different treatments			Average protein concentration in sample (mg)
Enzyme	**Incubation time**	**Heating cycles**	
Trypsin/$CaCl_2$	24h	3x5	2.76
Trypsin/EDTA	24h	3x5	2.86
Trypsin/$CaCl_2$	24h	3x2	2.80
Trypsin/EDTA	24h	3x2	3.00

Table 4. 10. **Average protein concentrations in each AFFTV sample**.

Chapter 5: Discussion

5. Discussion

In our study we have developed and tested the cancer vaccine which is derived from the solubilised peptides of the tumour tissue and tumour cell line. This construction aimed to encompass the TAA and TSAs and increase their immunogenicity by incorporating the virus particles. In order to test the effect of this vaccine we have used two model of carcinogenesis.

The Sprague Dawley rats used in the DEN - NMOR model of carcinogenesis (p60 at exposure) developed signs of tumour presence after a period of seven to nine months. As an indicator of tumour formation, significant weight loss over a short period was used. At the time when the experiment was performed, the imaging modalities for continuous live assessment such as optical imaging, ultrasonography and magnetic resonance imaging were not available. The weight follow up method was backed up by the fact that both of these chemical carcinogens have an impact on liver and as a consequence on animal's metabolism (Oliveira *et al*., 2007, Patterson *et al*., 2012; Solaini *et al*., 2011; Tasaki *et al*., 2014). This method has proven to be partially successful. In order to determine whether the weight loss is continuous, the animals were weighed every two to three days after the first significant weight loss was noticed. As described previously, some of the animals showed signs of significant weight loss, but did not actually form tumours. As described by Futakuchi *et al*. (1999) significant weight loss was associated with increased liver tumour burden and lung metastasis. It was observed that this happens on long term exposure to the NMOR (Futakuchi *et al*., 1999). In their study, significant weight loss correlated with the lung metastasis in 100% of the cases while in our study this was observed in 65% of the cases. Fifteen percent of the animals in our study exhibited signs of significant weight loss but no lung metastasis. Twenty percent of the animals have not exhibited any signs of significant weight loss, but post-mortem lung metastasis were observed in these animals. Use of more reliable diagnostic tests, such as the ultrasonography or magnetic resonance imaging as well as the optical imaging techniques (Kumar and Pawaiya. 2010; Sandhu *et al*., 2010) would be preferred in order to better track the tumour formation and dissemination. Studies by Futakuchi *et al*. (1999) and Wang *et al*. (2009), showed significant weight loss initially observed in the 16^{th} week and then continuously in between the 16^{th} and 22^{nd} weeks. In our study, on the other hand, significant weight loss was observed in between the 28^{th} and 36^{th} week. We hypothesised that this may be partially explained by the individual differences in the particular rat colonies. Out of fourteen animals included in study, in twelve significant morphological changes on the liver were observed. Some of the animals have developed smaller changes and some larger changes or both. This was similar to the study of Wang *et al*.

(2009), where they observed that specific morphological changes on the liver develop in different time frames. In the period between the 3rd and 4th month, they have observed appearance of tumour nodules which looked like start of cirrhosis (Wang *et al*. (2009). These smaller morphological changes were followed with the formation of unequal nodules, small and large changes, and with the increase in the liver size in the 5th and 6th month (Wang *et al*. (2009). In our study, in one rat, the liver appeared cirrhotic which might be a consequence of the carcinogen uptake through drinking water. The cirrhosis was observed in some of the animals in the study by Yoschino *et al*. (2005) and Wang *et al* (2009), and it was characterised as a feature that reflects the final stage of the liver cancer development. Authors of these studies have observed that in this stage the lung metastasis start occurring. However, in our study the lung metastasis occurred in animals regardless the liver cirrhosis, which might suggest that the lung metastasis can be correlated with the earlier stages of the liver cancer development. As compared to our study, the previous studies observed the weight loss in animals quite early on (Futakuchi *et al.*, 1999; Wang *et al*., 2009). The studies of Futakuchi *et al.* (1999) and the Wang *et al*. (2009) have also observed that the weight loss was connected with the formation of lung metastasis this may indicate that in our study metastasis occurred late.

As shown by Wang *et al.* (2009) and Santos (2017) the histopathological features in the liver resembled the actual changes in the development of liver cancer and inflammation along with the formation of significant morphological changes. In our tissue slides of the liver and the lung tissue, we have observed signs of the inflammation resembling high interstitial activity with increased presence of inflammatory cells along with aberrant ones.

This was observed both in unvaccinated (control) animals and vaccinated ones. Therefore it may partially explain the fact that in our study, in three out of five animals in the control group, minimal lymphocyte infiltration has been observed in the liver and in the lungs. Furthermore, this may have an impact on results obtained from the tissue slides of the vaccinated animals since in four out of nine animals, the lymphocyte infiltration in the liver was scored as minimal. The same was observed in the lungs in five out of nine animals. The lymphocyte infiltration was scored as mild in two out of five animals in lungs of the control animals, while in the liver in none of the control animals it was scored as mild. In the vaccinated animals, lymphocyte infiltration was scored as mild in the lungs of three out of nine animals and in the livers of one out of nine animals. This might indicate that the minimal and weak infiltration is present both in control and vaccinated animals probably as a consequence of the tumour development itself and the processes following it.

In the control group, the lymphocyte infiltration was scored as moderate or strong in none of the animals. On the other hand, in the vaccinated group, the lymphocyte infiltration was scored as moderate in the liver of one out of nine animals and in the lungs of two out of nine animals. As strong infiltration was noted in the liver of one out of nine animals and in the lungs in one out of nine vaccinated animals. When the average scores of the lymphocyte infiltration were compared, there was a significant difference between the control and vaccinated group in liver ($p = 0.028$) while in the in the lungs there was no statistically significant difference observed ($p = 0.65$). This result might suggest that the immunisation have been more efficient in the elicitation of the immune response in the primary tumour than in lung metastases. It may also suggest that the metastatic cancer cells have certain suppressive effect on the lymphocytes (Budhu *et al*., 2006; Li *et al* 2015). However, existence of the statistical difference may suggest that it is worth studying effect of the AFFTV derived from tumour tissue biopsy and Rotarix with a larger sample.

Although a significant difference in the histological score of the lymphocyte infiltration was observed in between the control and vaccinated group, there were no significant difference observed in the median survival time ($p = 0.23$). Although most of the data suggests that the increased lymphocyte infiltration has positive implications on the survival (Hendry *et al*., 2017), in our study that was not observed. As discussed by Harding *et al*. (2016), it is also possible that due to the present inflammation in these organs, the immunosuppressive environment has been established. That would implicate that the presence of regulatory T cells, MDSCs, increased production of IL - 10 or TGF - β can suppress the potential anti-tumour immune response from the lymphocytes activated by the immunisation (Budhu *et al*., 2006; Li *et al* 2015). As mentioned by Seyfried *et al*. (2014) and Li *et al*. (2015), it is well known that the cancer cells can utilize different strategies that can induce the inhibition of the effector immune cells or changing their phenotype from effector to regulatory.

It is known that the presence of the $CD8^+$ T cells is correlated with the positive prognosis in cancer, while the presence of Treg cells is correlated with the negative prognosis in most solid cancers. However, as H&E staining by itself can not distinguish between the functional subpopulations of lymphocytes, therefore more advanced techniques such as immunohistochemistry are necessary to investigate the actual presence of these different lymphocyte subpopulations. There are a lot of difficulties in subtyping the TILs and determining their spatial organisation. Lots of effort has been invested in immunophenotyping of the immune infiltrate by immunohistochemistry or immunofluorescence (Salgado *et al*., 2015). Although H&E staining provides information regarding

the general presence of lymphocytes in the tumour, and not about each subpopulation, it was proven valid in the prognostic and predictive evaluation of patient outcome (Adams *et al*., 2014; Denkert *et al*., 2010). In the paper by Salgado *et al*. (2015), it is discussed that this may further suggest that the histological evaluation of each lymphocyte subpopulation may be not so relevant. They suggest that the presence of different lymphocytes population can provide better insight in the interaction of the tumour and immune system in each patient and in different types of tumours (Salgado *et al*., 2015). As example, the absence of the regulatory T cells may indicate that the tumour is not at all recognised by the immune system, on the other side, presence of the regulatory T cells can indicate that the tumour is recognized and the immune response was elicitated, however it is being suppressed and thus not powerful enough to completely eradicate the tumour (Salgado *et al*., 2015). This can further help in deciding which therapeutic approach should be applied in order to provide better results.

It is worth mentioning that although no statistically significant difference was observed in the median survival between the control and vaccinated group, the median survival of the control group was 48 days while the median survival of the vaccinated group was 34 days. This may suggest that the stronger immune response that was observed in the liver and the lungs of the vaccinated group as compared to the control group might have been responsible for shorter median survival of this group.

The other model of carcinogenesis used in our study was the syngeneic MT450 model. This model has been previously used in cancer research as demonstrated by Thiele *et al*. (2013) and Quagliata *et al*. (2014). In our study we have initially inoculated the Wistar rats with the 5×10^6 MT450 cells. This amount of MT450 cells has proved very efficient in forming the subcutaneous tumour in a short time in Wistar rats in a previous study conducted by Pierre Schembri Wismayer *et al.* (2016; unpublished data). After the inoculation with 5×10^6 MT450 cells, visually it was observed that already after one and a half weeks, animals had enlarged axillary lymph node on the side of the tumour cell inoculation and a palpable tumour was present at the inoculation site.

It was observed that in the lungs of the animals sacrificed two or two and a half weeks after inoculation, there is a high interstitial volume in the lung tissue and the presence of lymphocytes in lung tissue. It is known that inflammation can be initiated due to invasion of the lung tissue and the disturbance of homeostasis (Chen *et al.*, 2018; Gomes *et al*., 2014; Zhang *et al*., 2018). The invasion of the lymph node or the lung tissue by the MT450 cells could have induced inflammation

of a certain level. However, the use of methods such as IHC, in future studies should provide more detailed information regarding this.

The presence of inflammation, might explain the fact that in the experiment where the features of the immune response elicited with different combinations of the Rotarix and the AFFTV made of the MT450 cell line, in control group both in the subcutaneous tumour and in the lungs, lymphocytes were present. The lymphocyte infiltration in the subcutaneous tumour was scored as mild in one out of two animals, and it was absent in one out of two animals, , while in the lungs it was scored as moderate in both animals.

It is interesting that the infiltration in the subcutaneous tumour and the lungs had the highest average and median values in the group vaccinated with the AFFTV made of the MT450 cell line only, both in the first and the second immunisation after seven days. In this group, in the subcutaneous tumour, the lymphocyte infiltration was averagely scored as moderate while in the lungs it was scored as strong. It seems that the AFFTV made of the MT450 cell line alone induced an increase in the number of lymphocytes. That may suggest that formalin fixation increased the immunogenicity of peptides obtained from the MT450 lysis. In other studies, where a formalin fixed vaccine against pathogenic bacteria was tested, it was observed that this formalin fixation can increase the immunogenicity of antigens (Åhrén *et al*., 1998; Raghavan *et al*., 2002). However, in a study by Obata *et al*. (2004), it was observed that B16 melanoma cells processed in formalin could not elicit significant immune response without addition of the IL-2 (Obata *et al*., 2004). With the addition of the IL-2 they induced a good cellular immune response. Studies by Ohno *et al*. (2005) and Peng *et al*. (2002), have shown that cytotoxic lymphocytes can be expanded *in vitro* with the use of the autologous formalin fixed tumour tissue. In fact when these CTLs are injected *in vivo*, they induce successful cellular responses in patients (Ohno *et al*., 2005; Peng *et al*., 2002). In the study by Ishikawa *et al*. (2007), it was observed that the vaccine made of the tumour tissue fragments derived from the tumour tissue biopsy that was paraffin-embedded and in combination with the formalin-fixed fragments can induce specific $CD8^+$ T cell immune response in glioblastoma cancer patients. Although no statistically significant difference was observed, it is important to emphasise the fact that in our study, the solubilised peptides derived from the MT450 cell line, that were treated with formalin, have induced the increase in the amount of lymphocytes without addition of an adjuvant or immunopotentiator. This is important as a lot of pre-clinical and clinical trials have shown that the immunogenicity of the antigens derived from the whole tumour

cell or tissue lysates is low and due to that, they can not elicit the immune response against the tumour (Kurtz *et al*., 2014; Obata *et al*., 2004; Zerbini *et al*., 2006).

In the same experiment, the groups vaccinated with the combination of the Rotarix and the AFFTV made of the MT450 cell line had a lower average histological score of lymphocyte infiltration in the lungs, as compared to the group vaccinated with the AFFTV alone or the Rotarix alone, at both the three or seven days period. While in the subcutaneous tumour the group vaccinated with the Rotarix alone, in a period of three days, had lower histological score of lymphocyte infiltration as compared to the group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line both in a period of three and seven days. However, the group vaccinated with the Rotarix alone, after seven days, had a higher histological score of lymphocyte infiltration as compared to both of these groups. This result might also suggest that the addition of Rotarix in the model of the AFFTV where the MT40 cells were used did not improve the effect of AFFTV vaccine as compared to the AFFTV made of the MT450 cell line given alone.

It was interesting that the Rotarix viral vaccine alone also induced the increase in the amount of the lymphocytes in the subcutaneous tumour and in the lungs. This could partially be explained by the fact that Rotarix contains live attenuated Rotavirus, and it is known that certain live attenuated viruses can exhibit tropism towards the cancer cells (Boisgerault *et al*., 2010). One of the live attenuated viruses that have natural preference towards cancer cells is attenuated measles virus (MVA) (Guillerme *et al*., 2013). Regarding that cancer cells can express certain molecules or use certain mechanisms to evade the immune response, they present very suitable places for the viral replication (Boisgerault *et al*., 2010; Guillerme *et al*., 2013). Due to this feature, infection of cancer cells with MVA can induce cancer cell death and production of the DAMPs and PAMPs (Boisgerault *et al*., 2010). These signals can stimulate TLRs and RLRs on and in DCs that can further activate innate and adaptive immune response through cross-presentation, which can further induce the eradication of the viral infection as well as the cancer (Guillerme *et al*., 2013). There is also a possibility that the Rotavirus can induce a non - specific immune response, thus enhancing the amount of lymphocytes. The ability of viruses or the bacteria to cause a non-specific inflammation is known (Wijewardana *et al*., 2013). The virus or it's relevant part can be recognised by the TLRs on the cells of the innate immune system like macrophages, dendritic cells, neutrophils and NK cells (Salem *et al*., 2016; Dear. 2016). Activation of these receptors can induce the production of various pro - inflamatory cytokines and chemokines that can induce the pro-inflamatory environment (Osman *et al*., 2011). This can then further induce the maturation of the

APCs and activation of the $CD8^+$ T cells or NK cells (Sivori *et al*., 2004). This was observed in the study by the Ockert *et al*. (1996), where they have used the Newcastle Disease Virus (live attenuated).

On the other hand, it seems that the Rotavirus vaccine does not interact positively with the antigens derived from the MT450 cells, while on the other hand it seems that interact better with the antigens derived from the tumour biopsy. This can be observed through the histological score of infiltration which was higher in both experiments where the AFFTV made of the tumour tissue biopsy was injected along with the Rotarix, as compared to the ones where the AFFTV made of the MT450 cell line was injected along with the Rotarix.

It is possible that there is a negative interaction between vaccines, this is known phenomenon that can occur when the vaccines are injected simultaneously or in combination. Physico-chemical interactions or the interactions between the live attenuated vaccines can increase immunogenicity or cause loss of it, as well as induce the adverse effects (Gizurarson, 1998). However, it is more likely that the specific APCs or other types of the immune cells that are activated with the Rotarix do not interact/respond well to the antigens of MT450 cells, or impair their presentation, when these antigens are coinjected subcutaneously.

The group that was vaccinated only with the Rotarix three days after the first immunisation had lower mean and median score values of lymphocyte infiltration in the subcutaneous tumour as compared to the group vaccinated only with Rotarix seven days after the first immunisation. However the group vaccinated with the Rotarix three days after the first immunisation had a higher median and mean score of the lymphocyte infiltration in the lungs as compared to the group vaccinated with the Rotarix seven days after. It is possible that this can be a result of the the Rotavirus antigens spreading through the blood to different organs with different speed and susceptibility (Crawford *et al.*, 2017).

It is interesting that the highest mean and median histological scores of infiltration were observed in groups of animals vaccinated only with the Rotarix in the first and second immunisation or only with the AFFTV made of the MT450 cell line in a first and second immunisation. Regarding that the live attenuated viruses can infect the cancer cells, there is a possibility that the the Rotavirus itself infected the tumour tissue faster as compared to the health tissue. It would be interesting to apply the immunostaining assay and examine the tumour tissue and see if there are Rotavirus

proteins present. As well, it would be interesting to compare the effect of the Rotavirus with the conventionally used viral immunopotentiators such as poly I:C, when coinjected with the AFFTV made of the tumour tissue biopsy or with the one made of the MT450 cell line. Poly I:C is a potent stimulator of immune response regarding that it can stimulate TLR3 and MDA5 (RLR). When both of these receptors are activated, the cells are stimulated to secrete large amounts of IFN type I and proinflammatory cytokines (IL - 1, TNF α, IL - 6) (Cho *et al.*, 2013). In the study by Cho *et al*. (2013), it was observed that poly I:C stimulates a very robust T cell antigen specific immune response both when used as a prime and as a secondary boosting agent and when it was used as a secondary boosting agent regardless of which adjuvant was used for priming. It is interesting that the poly I:C used in their study as immunopotentiator along with the peptide cancer vaccine induced more effective immune response as compared to the CpG oligonucleotide, which is also one of the used immunopotentiators (Cho *et al*., 2013). On the other hand, in the study by Kawahara and Takaku (2015), they have suggested that the CpG oligo is a potent immunopotentiator able to induce effective cellular immune response when co-injected with a combination of peptides and tumour lysate. This further indicates that the immunopotentiators can differently interact with various cancer vaccine formulations which can have the significant impact on the capacity of the anti-tumour response.

In the experiment where the features of the immune response elicited with different combinations of the AFFTV made of the MT450 cell line and the Rotarix, no significant difference was observed between the mean or median scores of lymphocyte infiltration in the subcutaneous tumour ($p = 0.21$), or the lungs ($p = 0.16$), in the vaccinated and the control group. However it is worth considering that the group vaccinated only with the AFFTV made of the MT450 cell line, had a high trend of scores of the infiltration both in the subcutaneous tumour and the lungs and thus provides rationale for the further investigation with larger animal group size.

Some of the studies, including Pruneri *et al*. (2016), supported the evaluating capacity of the guidelines proposed by Salgado *et al*. (2014), and indicated that each increase of 10% in TILs has strong positive implications on the survival of patients in triple negative breast cancer (TNBCs) . In the non-small lung, in the study by Geng *et al*. (2015), it was observed that the presence of $CD8^+$ T cells had been correlated with the better overal survival regardles the specific position. In the same study, the presence of $FOXP3^+$ T reg cells had negative influence on overal survival (Geng *et al*., 2015). However, in our study no significant difference was observed in the median survival between vaccinated and control groups. This may suggest that the anti-tumour immune response on

the AFFTV made of the MT450 cells, Rotarix or the combination of two, was not effective enough to suppress the tumour development or to eradicate it. One of the reasons might be that that the tumour microenvironment was well established and thus the immune response was suppressed as discussed previously.

When the median survival time was compared between the groups, no statistically significant difference was observed ($p = 0.44$). However, it is worth mentioning that the median survival time in any of the vaccinated groups was lower as compared to the control group. This may further suggest that the immune response elicited with different vaccination combination may influence on the survival of animals.

In the second experiment with the MT450 carcinogenesis model, we have inoculated animals with the $5x10^6$ MT450 cells the same as in previous one. However, in this experiment as compared to the previous one, animals were first vaccinated with Rotarix subcutaneously three weeks after the inoculation, and after three days vaccinated intraperitoneally with the combination of Rotarix and the AFFTV made of the tumour tissue biopsy or/and the one made of the MT450 cell line. In this experiment the features of the anti-tumour immune response upon the immunisation with the AFFTV of different origin (from tumour tissue biopsy or/and from MT450 cell line) were observed.

Similarly to the previous experiment, in the control group, both in the subcutaneous tumour and in the lungs lymphocytes were present. The lymphocyte infiltration in the subcutaneous tumour was averagely scored as minimal to mild, while in the lungs, it was scored as mild.

The lymphocyte infiltration was averagely scored as moderate to strong in the subcutaneous tumour in the group vaccinated with Rotarix and after three days with the combination of the AFFTV made of the MT450 and the AFFTV made of the tumour tissue biopsy. While in the lungs, in the same group, it was scored as mild. This may suggest that although these tumours are derived from the syngeneic tumour cell line, there are specific antigens or the modifications of antigens happening during development of the tumour in the animal. This was suggested in the study by Suckow *et al*. (2009), where they emphasised the fact that the vaccines derived from the tumour tissue biopsy, include the tumour specific antigens that are developed during the *in vivo* growth of the tumour under the influence of the microenvironment. It further implicates that the tumour tissue biopsies contain wider range of the tumour specific antigens as compared to the cultured cancer cells. Beside the cancer cell vaccines made of the tumour tissue biopsy contains tumour tissue stroma

antigens (Suckow *et al*., 2009). The tumour stroma has been shown as a great source of the tumour specific antigens regarding that some features of the tumour stroma are specific to the tumour and as that might be recognised as suggested by Suckow. (2007). However, a study by Goldszmid *et al*. (2014), suggested that the syngeneic cell lines contain all the necessary TSAs that can elicit an effective anti-tumour immune response. They have used the vaccine made of dying B16 melanoma cells exposed to DCs and have induced long lasting cellular immunity, involving both $CD4^+$ and $CD8^+$ T cells, in the mice (Goldszmid *et al*., 2014). This may partly explain the results of the previous experiment in which the AFFTV made of the MT450 cells only, have also increased number of lymphocytes both in the subcutaneous tumour and in the lungs. On the other hand, in this experiment the AFFTV made of the tumour tissue biopsy and the MT450 cells have caused the averagely moderate to strong lymphocyte infiltration in the subcutaneous tumour while in the lungs the infiltration was scored as mild, possibly due to changes in stromal antigens.

Although the score of the lymphocyte infiltration was not significantly different from the control group, when the median survival times were compared, the median survival time of the group vaccinated with the AFFTV made of the tumour tissue biopsy and the MT450 cells was 25.5 days. This was the shortest median survival time as compared to other vaccinated groups and control group. This may suggest that the strong infiltration in the primary tumour tissue and minimal to mild infiltration in the secondary tumour can impair the survival of these animals. This may be partially explained by the fact that in the advanced tumour setting, the general strong immune reaction can induce the narrowing and collapse of the lung airways which can further induce death of the animal (Nichols *et al*., 2012; Lee *et al*., 2009). However, narrowed and collapsed airways have been observed in the control animals as well which may suggest that the infiltration of the tumour cells itself is causing this. It might also suggest that the immune response in the primary tumour can somehow impact the immune response on the metastatic tumour. It is known that the primary tumour cells are orchestrating the formation of the secondary tumour and that they are responsible for it's main features and that these two formations are closely connected (Doglioni *et al*., 2019; Paolillo *et al*., 2019). This may further have influence on the possible anti tumour response. However, it is known that the metastatic niche is a different environment compared to the primary tumour (Doglioni *et al*., 2019; Paolillo *et al*., 2019).

This might also suggest that the combination of the AFFTV made from the MT450 cells and the AFFTV made from the tumour tissue biopsy in combination with the Rotarix increase the immunogenicity of antigens. It is known that the electrostatical interactions of antigens or antigens

and immunopotentiator can influence the type and strength of the immune response (Hamborg *et al*., 2014; Michelin *et al*., 2012).

The size of the vaccine antigens can have a significant impact on the biodistribution of these molecules upon injection (Bachman *et al*., 2010). As well, it was observed that the large molecular weight molecules tend to be delivered to the lymph nodes upon injection, while smaller molecules tend to be distributed through the systemic circulation due to the anatomy of lymph and blood vessels. This may further have a high impact on the nature of the possible immune response on these antigens (Bachmann *et al*., 2010). Therefore, it would be interesting to get better insight in the structure, size and physico-chemical properties of the peptides constituting the AFFTV vaccine made of the MT450 cells and the tumour tissue biopsy. This could be achieved with use of techniques such as Western blot (Atascientific.com. 2019).

It would be interesting to compare the presence of the cells at the injection site, regarding that the presence of different types of professional and unprofessional dendritic cells can influence the features of the immune response (Levin *et al*., 2015). Thus, the interactions of these vaccines and the treatment/digestion of the peptides might be influencing the physico - chemical properties of the antigens and therefore influencing on their presentation and processing.

It is interesting that ulcerations at the tumour site were mainly developed in the groups secondary immunised with the AFFTV made of the tumour tissue and Rotarix or in combination with the AFFTV made of the MT450 cell line and Rotarix. This may suggest that these ulcerations were formed as a result of moderate to strong immune reaction in the subcutaneous tumour. It is known that ulcerations can be formed as a consequence of the inflammation in the certain area (Panuncialman and Falamge, 2010). The ulcerations can be formed as a result of the prolonged pressure on the certain skin area or as a result of the abnormal tumour growth which leads to the impaired blood supply in the area (Arbiser, 2014; Bhattacharya and Mishra, 2015). This could explain formation of ulceration both in vaccinated and control animals throughout the studies with the MT450 transplantable tumour model. However, since the tumour tissue biopsy was obtained from these animals, it is possible that they were scratching or biting the wound to remove the suture.

When the median survival times were compared, no statistically significant difference was observed ($p=0.16$). However, it is worth mentioning that only the group vaccinated with the combination of

the AFFTV made of the MT450 cell line and the Rotarix had median survival time of 39.5 days, while other vaccinated groups had shorter survival time as compared to control group, 29 days. This can be partially explained with the fact that as in previous experiment, the animals that were vaccinated with the combination of the Rotarix and the AFFTV made of the MT450 cell line did not have high scores of the lymphocyte infiltration in the subcutaneous tumour, which was averagely scored as mild. However in this experiment, where the features of the anti-tumour immune response elicted upon immunisation with the AFFTV of different origin in combination with the Rotarix or alone, were examined, in the group vaccinated with the combination of the AFFTV made of the MT450 cells and Rotarix, lymphocyte infiltration was averagely scored as mild to moderate in the lungs. This may suggest that the immunisation via the intraperitoneal route as compared to the previously used subcutaneous route improved the effectiveness of the AFFTV vaccine made from the MT450 cell line. This might be explained by the fact that the antigens administered in different anatomical sites, will interact with different APCs subsets present in different amounts (Bode *et al*., 2008; Malik *et al*., 2014; Tiede *et al*., 2008). Intraperitoneal administration of the cancer vaccine can be a good way of delivering the antigen of interest to the numerous lymph nodes present inside the peritoneal cavity. These include the mediastinal, gastro-omental, hepatic, mesenteric and more (Malik *et al*., 2014; Tiede *et al*., 2008). As well, the antigen can be delivered to the secondary lymphoid organs such as spleen and gut associated ones such as gut-associated lymphoid tissue (Malik *et al*., 2010). This further suggests that the antigens delivered through this route can have higher chance of eliciting the immune response.

However, in the study by Head *et al*. (2017), whole cell vaccine containing irradiated 4TI mammary tumour cells with IL-2 and GM-CSF induced significant prophylactic effect both when injected subcutaneously and intraperitonealy. They have suggested that the subcutaneous route of the cancer vaccine delivery can be effectively used for the breast cancer treatment. As well, they have suggested that the intraperitoneal route should be considered for the treatment of the various solid tumours including the breast cancer (Head *et al*., 2017).

On the other hand, the study by Bonnotte *et al*. (2003), observed that the tumorogenicity and immunogenicity of the syngeneic tumour cell line in mice depended on the route of the administration. They have observed that the s.c. route of inoculation allows the normal development of the tumour, without the influence of the immune cells. However, the i.d. route of inoculation stimulates the immune response to a certain level that can suppress the formation of the tumour due to stimulation of the immune system. They have observed that the i.d. administration

stimulates the DCs present in the epidermis and dermis, which further stimulates the immune response (Bonnotte *et al*., 2003). It is known that in the epidermis and dermis there is a high amount of DCs, while in hypodermis and subcutaneous tissue there is a very low amount of these cells (Campton *et al*., 2000; Grabe *et al*., 1991). This further suggests that the route of the vaccine administration can have a decisive role in the stimulation and direction of anti-tumour response in murine models. Although the i.d. route of delivery is very difficult to apply in the murine models especially if the amount of injected material is larger due to the thin layer of skin, it would be a very applicable route in human treatment. This further suggests that the i.d. route should be investigated as one of the delivery routes of the AFFTV and Rotarix if the volume of injection can be decreased.

The important factor that should be considered when choosing the right administration route for delivery of the vaccine is that dendritic cells are region-specific and their role is specified for the antigens that can naturally be presented there (Bode *et al*., 2008; Malik *et al*., 2014). For example, as previously mentioned, mesenteric lymph nodes have a different population of the dendritic cells as compared to the axillary lymph nodes and this can affect the nature and extent of immune response even on the same pathogen (Bode *et al*., 2008; Tiede *et al*., 2008). This can influence the action of the Rotarix vaccine considering that Rotavirus is naturally invading the gastro/intestinal tract and as such the i.p. route of administration can be more appropriate than other routes. However, as the dendritic cells from lymph nodes draining different tissues can have influence on the expression of specific homing receptors on the T cells, and these receptors are crucial for trafficking of T cells to the tumour site upon presentation by DC (von Adrian *et al*., 2000), the delivery of the AFFTV alone and especially together with the Rotarix to the subcutaneous tumour for example, could be impaired after i.p. injection. For example, the T cells that are primed in the mediastinal lymph nodes express certain receptors that are directing them to the lung tissue while the ones primed in the skin are expressing different receptors that are priming them to the skin (Bachmann *et al*., 2010; Bode *et al*., 2008). However, it is suggested that the main factors that can influence the level of the primary immune response are the delivery of the antigen or adjuvant to the DCs but equally importantly distribution that will target as many lymphoid organs as possible in order to prime high number of T cells (Sultan *et al*., 2017). This is the additional reason to further analyse the effect of different administration routes on the AFFTV and Rotarix effectiveness.

It is interesting that in both the group vaccinated with the combination of the Rotarix and AFFTV made of the tumour tissue biopsy and the Rotarix and AFFTV made of the MT450 cell line, as well as in the group vaccinated only with the Rotarix and AFFTV made of the tumour tissue biopsy in

second immunisation, median survival time was shorter as compared to control. Along with it, the average histological score of the lymphocyte infiltration both in the subcutaneous tumour and in the lungs, was different in these two groups. While the group vaccinated with the combination of the AFFTV made of the tumour tissue biopsy and AFFTV made of the MT450 cell line, had higher histological scores of the lymphocyte infiltration, the group vaccinated with the AFFTV made of the tumour tissue biopsy alone had lower scores. This further might suggest that the antigens derived from the tumour tissue biopsy possibly have specific influence on the anti-tumour immune response and that they might activate other immune cell types beside the CTLs. Therefore, it would be interesting to further analyse the presence of different immune cell types in the tumour, lungs as well as in different immunologically relevant organs such as spleen, axillary lymph nodes. Techniques such as IHC and Flow cytometry could be used. In the third experiment with the MT450 carcinogenesis model, we have inoculated animals with the $5x10^6$ MT450 cells the same as in previous two. In this experiment the same as in the previous one, animals were first vaccinated with Rotarix subcutaneously three weeks after the inoculation, and after three days vaccinated intraperitoneally with the Rotarix and the AFFTV made of the MT450 cell line with standard protocol or with the Rotarix and the AFFTV made of the MT450 cell line with changed protocol (3x2).

In the group vaccinated with the Rotarix and AFFTV made with the MT450 cell line with the standard protocol, the lymphocyte infiltration in the lungs was scored as mild to moderate while in the subcutaneous tumour it was scored as mild. In the group vaccinated with the Rotarix and AFFTV made with shorter (changed) protocol, the lymphocyte infiltration in the subcutaneous tumour was scored as minimal to mild , while in the lungs it was scored as moderate. There was no statistically significant difference observed in the score of the lymphocyte infiltration between the group vaccinated with the Rotarix and AFFTV made of the standard protocol and the one made with the shorter protocol as compared to control.

When the median survival times were compared, no statistically significant difference was observed between the groups ($p = 0.38$). However, it is worth mentioning that the median survival of the group vaccinated with the Raotarix and the AFFTV made of the MT450 cell line with the standard protocol was 40.5 days and the one vaccinated with the Rotarix and the AFFTV made of the MT450 cell line but with shorter protocol was 38.5 days.While the median survival of the control group was 33 days.

This might suggest that there is no significant influence if the AFFTV is prepared through microwave heating cycles of two minutes as compared to the standard five minute cycles. However, regarding that the shorter heating intervals can make the preparation of this vaccine less time consuming and might save some of the peptides from the heat denaturation it might be worth while studying it further. As well, it would be interesting examining the effect of the AFFTV made of the tumour tissue biopsy, injected with the Rotarix or injected alone, prepared with shorter heating intervals, both in first and second immunisation and compare the effect with the AFFTV made with standard protocol.

It is important to mention that although two observers have participated in the analysis of the histological slides and the substantial agreement was observed (k=0.66), the participation of the professional pathologist would be highly desirable in the future study.

In order to get insight into the presence of the $CD8^+$ T cells in the blood upon the immunisation of the animals with the AFFTV made of the MT450 cell line in combination with Rotarix, flow cytometry analysis was performed. Animals were first immunised with the Rotarix subcutaneously and after three days with AFFTV made of the MT450 cell line and the Rotarix intaperitoneally. Flow cytometry analysis has shown that there is no statistically significant difference between the amount of $CD8^+$ T cells in the peripheral blood of the control and vaccinated animals. However, it was observed that there are a lot more $CD8^+$ T cells present in the control group as compared to the vaccinated group regardless of the amount of MT450 cells used for the inoculation. This may suggest that seven days after the second immunisation, some sort of the depletion of $CD8^+$ T cells happened.

This may suggest that other types of immune cells such as $CD4^+$ T cells, NK cells or B cells, are mediators of this immune response. In the study by Kawahara and Takaku (2015), they have observed that the crucial mediators of the immune response in syngeneic murine carcinoma model upon immunisation with the tumour lysate in combination with the Baculovirus and the CTL peptide epitope are $CD4^+$ T cells. In their study it was shown that this response is dependent of the tumour lysate and $CD4^+$ T cells. When the $CD4^+$ T cells were depleted, immune response was impaired, while when the $CD8^+$ T cells were depleted, the NK cells suppressed the tumour growth in combination with $CD4^+$ T cells for a certain time (Kawahara and Takaku, 2015). As well, since the Rotarix is a vaccine made of the live attenuated Rotavirus, it is possible that the possible infection of cancer cells with the live attenuated virus can induce cancer cell death and production

of the DAMPs and PAMPs (Boisgerault *et al*., 2010; Guillerme *et al*., 2013). This effect is observed with the MVA (Guillerme *et al*., 2013) and with the live attenuated Newcastle Disease Virus (Ockert *et al*., 1996). The TLRs can DAMPs and PAMPs and activate the signal cascade which can result in the secretion of proinflammatory cytokines, chemokines, that can further have a stimulatory effect on cells of the innate immunity such as macrophages, neutrophils and NK cells (Sivori *et al*., 2004; Jiang *et al*., 2004; Salem *et al*., 2016). So these cells can be mediators of the anti-tumour immune response. It is observed that the immune response elicitated through the vaccination of animals with the Rotarix is mainly humoural with cellular component present in lesser degree, so the specific antibodies could be more prevalent. In future studies, the presence of different immune cells in should be analysed.

However, other factors, such as transient down-regulation of the antigen sensitivity during the differentiation of effector $CD8^+$ T cells, could have induced the decrease presence of $CD8^+$ T cells after the immunisation. In the study by Xiao *et al*. (2015), they have observed that the down - regulation of the CD8 marker happens early after the immunisation with *Listeria monocytogenes* or *Vaccinia virus*, seven days after the immunisation. This down-regulation was transient since the levels of CD8 were up-regulated after the formation of the memory pool of these cells. They have suggested that this mechanism is stimulated in order to protect the normal cells apart from target ones that are highly immunogenic (Xiao *et al*., 2015). One of the factors that should be considered and that can have an effect on the functionality of these cells is the restimulation caused by the booster injection or in our case second injection of Rotarix. Some clinical trials have shown that in order to maintain immunity elicited with the cancer vaccines, booster vaccinations are neccessary (Mittendorf *et al*., 2014). However, in some clinical trials it was observed that the restimulation with the antigen can induce the exhaustion of CTLs (Salusto *et al*., 2010).
Thus, beside the amount, multifunctionality of T cells, especially $CD8^+$ T cells, was shown as crucial in the immune response (Macchia *et al*., 2013). In the future study, multiparametric flow cytometry including surface and intracellular staining should be performed in order to determine the functionality of T cells. Assays such as ELISpot or intracellular flow cytometry staining can be used in quantification of cytokines or chemokines produced as a response on immune stimulation of $CD4^+$ or $CD8^+$ T cells with certain antigen (Keilholz *et al*., 2006, Macchia *et al*., 2013). The measurement of the CD107a can be used for the assessment of the degranulation of cytotoxic T cells which results in secretion of cytotoxic molecules such as perforin and granzyme that can be used as a measure of cytotoxic capacity of these cells. Quantification of the surface expressed CD107a can be used in quantification of antigen specific CTLs (Rubio *et al*., 2003). The

quantification of the enzymes such as perforin and granzymes can be performed with use of the ICS and ELISA (Chattopadhyay *et al*., 2009). In order to assess the $CD4^+$ T helper cells, CD40L can be used regarding that it is one of the crucial ligands involved in the stimulation of the $CD8^+$ T cells and B cells (Chattopadhyay *et al*., 2005). Proliferation of the effector T cells can be measured with the use of carboxyfluorescein succinimidyl ester (CFSE) (Keilholz *et al*., 2006).

In the other studies we have used smaller amounts of MT450 cells ,from 1×10^5 to 1×10^6 , in order to optimize our model and get insight in the progress of the tumour and its features in this model. Smaller amounts of MT450 cells were used as well in the studies by Thiele *et al*. (2013) and Quagliata *et al*. (2014). In our study, after seven days it was observed that the animals iinjected with different MT450 cell amounts all have developed tumour formations which size correlated to the amount of cells used. This correlates with the data from the previously published work, where the size of the tumour correlated to the amount of cells used (Thiele *et al*., 2013; Quagliate *et al*., 2014). Although tumours were formed, there were no enlargement of axillary lymph nodes on the side of the tumour injection, as well lungs were without any visible change. After two weeks, we have observed the enlargement of the tumour, while the size of the axillary lymph nodes remained the same, regardless the different MT450 cell amounts used. No significant change on lungs were observed. These results correlate with the observations made in the study by Quagliata *et al*. (2014), where they have inoculated animals with $1x10^6$ MT450 cells, and after two weeks no significant enlargement of the lymph nodes was observed. They have shown that in this period, MT450 cells were not observed in the lymph nodes or the lungs (Quagliata *et al*., 2013). In our study, after three weeks period, significant change in size of the tumour as well as the axillary lymph node were observed in the group of animals inoculated with the $1x10^6$ MT450 cells, however no obvious change was observed in the lung tissue. Similarly, in the study by Quagliata *et al*. (2013), they have observed the enlargement of the axillary lymph nodes. Histologically they have observed that in this period, MT450 cells started infiltrating the axillary lymph nodes and forming metastases in the lungs (Quagliata *et al*., 2013). This further suggests that the isolated organs and tissues in our study should be analysed histologically in future work. As well, it might suggest that the less than three weeks after the inoculation could be optimal timing for immunisation, considering that the metastasis will have started forming but are not overly developed. At the four weeks period after the injection of these cells, the axillary lymph nodes were enlarged in all animals regardless the amount of MT450 cells with which they were inoculated. Although more detailed histological evaluation is needed in order to determine the possible differences between the presence of metastasis in the axillary lymph nodes and the lungs in animals inoculated with different MT450 amounts, data from

previous studies suggest that the obvious change in the size of the axillary lymph nodes indicates that the cancer cells have metastasised to it and high possibility that they are starting to invade the lungs as well. However, the amount of cells used for the inoculation can be a very important factor to consider regarding the vaccination effect. In the study by Kawahara and Takaku (2013), they have observed that the combination of the vaccine made of the baculovirus and the tumour lysate was able to suppress carcinogenesis effectively in mice injected with the 5×10^4 CT26 colon carcinoma cells. However, in their study from 2015, when the animals were inoculated with the 4×10^5 CT26 cells , the vaccination with the baculovirus and the tumour lysate was not able to suppress carcinogenesis effectively (Kawahara and Takaku, 2015). This was as well suggested in the study by Cho *et al*. (2013), where they have observed that the effect of the vaccine is highly dependent on the amount of the tumour cells used for the inoculation but as well as the time of the first immunisation. When the immunisation was performed on the third day, the percentage of mice that lived more than 100 days was 80% as compared to when the immunisation was performed on the 14^{th} day when this percentage was 60. This was dependent as well on the amount of B16 melanoma cells, $3x10^4$ or $1x10^5$. When the mice were inoculated with the 3×10^5 B16 cells, they have all died by the 60^{th} day after the immunization (Cho *et al*., 2013). This further suggests that the syngeneic transplantable tumour models are highly dependable of these factors and as such these models should be carefully optimised.

This can further imply that in future work, lower amounts of the MT450 cells, such as 1×10^5 cells should be used for the inoculation of animals. Changes observed in our study in after four and five weeks suggest that the tumour is drastically advancing and spreading. It was observed that after five weeks period in all groups, beside the axillary lymph nodes on the side of the tumour inoculation, the axillary lymph nodes on the opposite side are as well enlarged. This might suggest that at the five weeks period the tumour is in highly advanced phase and as that highly untreatable. This is one of the issues emerging and that should be addressed ,the issue of optimal clinical setting for the evaluation of the immunotherapeutics (Sondak *et al*., 2006). The vaccination in metastatic settings has not yet proved satisfactory, most probably due to the heavy metastatic burden that induces an immunosuppressive enviornment that impairs the anti-tumour response (Clifton *et al*., 2016). Some of the recent clinical trials have been conducted in the patients with minimal residual disease and the vaccines has been shown to be more efficient (Hale *et al*., 2014). Thus, this might suggest that in the future study, we could vaccinate the animals at the earlier stages of the tumour development. As well, it should be noticed that the syngeneic tumour models are hard to treat regarding the fast pace of development. In the study by Hrouda *et al*. (1998), it was observed that the it was hard to

target the syngeneic transplantable tumour with the immunotherapy regarding the extremely fast and high proliferation of these cells. The immune response can not act fast enough to eradicate the tumour. Due to this fact, these tumours are not fully compaerable with the actual human replicates (Hrouda *et al*., 1998). Hrouda *et al*. (1998), suggested that the spontantaneous tumour models induced with chemical carcinogenes or the ones occuring in the animal models prone to certain tumours are more comperable to human ones and better models for the immunotherapy. However, our study as much as the other studies in the field suggest that in the moment there is no perfect model that could be used for immunotherapy. As such we suggest use of different models in the study, regarding that each model has its advantages and disadvantages. However, these features should be considered when planing a study as well as when interpreting the results and the models should be carrefully optimised.

However, it would be interesting to observe the influence of the AFFTV in different stages of tumour development in the syngeneic tumour models, and observe the correlation between the size and stage of tumour development and the effect of the AFFTV. The effect of the AFFTV as a prophilactic vaccine should be observed as well, regarding that in many syngeneic models cancer vaccines were more effective as prophylactic agents as compared to therapeutic. This could provide further insight into the potential mechanisms that this vaccine uses and hence provide solutions for the improvement of its effect.

As shown in the study by Quagliata *et al.* (2014), inhibition of certain vascular growth factors significantly reduced the volume and the tumour burden of the axillary lymph node as compared to control while it did not have any effect on the lung metastasis. However, in the study by Krishnan *et al*. (2003), they showed that the inhibition of the metastasis to the lymph nodes also suppressed the lung metastasis. Krishnan *et al*. (2003), observed that the size of the tumour correlates with the number and the expression of the lymphatic vessels which primarily correlates with the expression of growth factors such as VEGF-C/D. As well, Krishnan *et al.* (2003) discuss that due to morphological characteristics of lymphatic endothelium, including its ability to pass large molecules and cells, increase of tumour induced angiogenesis can potentiate the metastasis of the MT450 cells. Therefore, increase in VEGF-C/D can increase metastasis to the draining lymph nodes and the lymphangiogenesis (Krishnan *et al.*, 2003). As well they have concluded, that the increased size and number of draining lymph node metastases were induced with the increase in the number of cancer cells in the lymphoid vessels (Krishnan *et al.*, 2003). And thus, number and size of these metastasis directly correlates with the ability of a primary tumour to induce lymph node

metastases (Krishnan *et al.*, 2003). Due to this fact, it would be interesting to study the effect of the AFFTV on these pro-lymphangiogenic factors as well as to study the effect of the AFFTV on the number and size of the lymph node and lung metastasis.

As Thiele *et al.* (2013) showed, the MT450 syngeneic transplantable tumour model, can be used in assessment of different anti-tumour therapies. As demonstrated in their paper, important features of angiogenesis and lymphoangiogenesis that induce metastatic formation and factors influencing on these features can are successfully recapitulated in this model (Thiele *et al.*, 2013).

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Appendix A: FREC Approval

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Monday 16th September 2019

Mr Srdjan Tadic
Country View Flats, Flat 2,
Triq Il Qbajjar,
Marsalforn. Gozo.

Dear Mr Srdjan Tadic,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Tumour formalin fixed vaccines and secondary immune responses to elicit therapeutic immune responses in solid tumours

The Faculty Research Ethics Committee granted ethical approval for the above mentioned protocol after JFARSS Animal Research Committee has reviewed such an application and recommended for approval.

Yours sincerely,

Professor Pierre Mallia
Chairman
Research Ethics Committee

Appendix B: ScotPIL Certificate

S A B

SCOTTISH ACCREDITATION BOARD

This is to certify that

Srdjan Tadic

has successfully achieved the learning outcomes of the following modules as required under UK and EU training frameworks.

E1/L

National Legislation	(EU 1)
Ethics, Animal Welfare, and the 3Rs – level 1	(EU 2)

PIL A

Basic and Appropriate Biology Theory/Skills	(EU 3.1/3.2)*
Animal Care, Health and Management	(EU 4)*
Recognition of Pain, Suffering and Distress	(EU 5)*
Humane Methods of Killing Theory/Skills	(EU 6.1/6.2)
Minimally Invasive Procedures Without Anaesthesia Theory	(EU 7)*
Minimally Invasive Procedures Without Anaesthesia Skills	(EU 8)*

* Species specific

PIL B

Anaesthesia for Minor Procedures	(EU 20)

PIL C

Advanced Anaesthesia	(EU 21)
Principles of Surgery	(EU 22)

Species covered: Mouse; Rat***

Training organised by: SCOTPIL TRAINING COMMITTEE
Number: SAB/SCT-E/185/2018 DATE: 25/09/2018

Signature 1
For Course Organiser

Signature 2
For Scottish Accreditation Board

Please note, this Certificate allows you to apply for a Licence, but it is not a licence to perform procedures under the Animals (Scientific Procedures) Act 1986

References to EU Modules relates to requirements arising from Directive 2010/63/EU – *http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Endorsed_E-T.pdf*

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Appendix C: Equipment

- •Cover slips (Leica, Germany)
- •Fume cupboard (Arredi technici villa, Italy)
- •Incubator (Windsor, Raymond A. Lamb Ltd, England)
- •Measuring cylinders
- •Micropipette tips
- •Microscope (Olympus, Japan)
- •Oven
- •Pasteur pipette
- Analytical microbalance (Sartorius AG CPA26P, Germany)
- Automatic tissue processing machine (Leica biosystems, Germany
- bead steriliser (Inotech, USA)
- Centrifuge (Eppendorf, Germany)
- CO_2
- digital scale (Salter, UK
- Embedding cassettes
- FACS Canto (BD Science, USA)
- Forceps
- Fume hood
- Glass volume beaker (500 ml)
- Haemocytometer
- Heating pads
- Humidifiedincubator (Eppendorf, Germany)
- Metal rack holders
- Micropipettes (Socorex, Swizerland)
- Microtome (Leica Biosystems,Germany)
- Microwave (DCG Eltronic MWG820, 800 W, Italy).
- Needle Clamp Holder

- Needles 25 G
- Refrigerator (Angelantoni life science, Italy)
- Scalpel
- Serological pippetes
- SMI sutures, Belgium
- Surgical scissors
- Surgical sutures
- Syringe filter
- Syringes 1 ml, 10 ml and 20 ml
- Tips for serological pipett
- Tissue culture flasks 25 ml, 75 ml (ThermoFischer, USA)

Appendix D: Raw data - Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Raw data: histological score of the lymphocyte infiltration.

Animal	Group	Type of tissue specimen	1st observer liver scores (for each photomicrograph) (%)	1st observer lungs scores (for each photomicrograph) (%)	2nd observer liver scores (for each photomicrograph) (%)	2nd observer lungs scores(for each photomicrograph) (%)
1	1	1	5,5,10, 5,5		5,10,10,10,10	
2	1	1,2	15,10,10,10,15	35,30,35,30,25	15,15,20,10,10	40,35,40,45,45
3	1	1	5,10,5,5,10		5,10,10,10,10	
4	1	2		0,0,5,5,10		0,1,1,5,10
5	1	1,2	15,20,5,5,15	15,15,10,5,5	5,5,20,15,15	5,5,0,1,15
6	1	2		10,5,0,15,5		1,10,15,10,20
7	1	1	50,60,60,55,60		25,60,65,55,60	
8	1	1,2	5,1,0,1,10	40,50,65,50,60	1,0,10,10,10	50,55,55,45,60
9	1	2		15,5,10,15,20		20,30,10,15,30
10	1	1	1,0,10,10,5		1,10,10,10,5	
11	1	1,2	20,10,30,20,25	25,15,35,35,20	25,15,35,40,25	10,15,30,30,35
12	1	2		5,10,10,15,1		10,5,15,5,1
13	1	1	5,1,1,10,15		10,10,20,5,15	
14	0	1,2	0,0,1,1,1	1,0,1,0,1	0,1,0,1,1	0,1,1,1,1
15	0	1,2	0,1,0,1,1	0,1,1,1,1	0,0,1,1,1	0,1,1,1,1
16	0	1,2	5,10,10,5,1	10,5,5,15,10	5,10,5,5,15	10,10,15,5,15
17	0	1,2	1,5,15,10,10	1,5,15,5,5	10,15,5,5,20	5,1,1,5,5,15

18	0	1,2	10,15,15,5,10	10,5,15,5,10	10,15,15,1,1	1,5,10,5,5
19	1	1	10,20,5,15,5		10,20,5,20,5	

Table D. 1. Histological score per each tissue slide photomicrograph, for each observer. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Animal	Treatment group	Type of tissue specimen	Average score for liver infiltration 1st observer	Average score for lungs infiltration 1st observer	Average score for liver infiltration 2nd observer	Average score for lungs infiltration 2nd observer	Average score between observers (liver) (%)	Average score between observers (lungs) (%)	Average score between observers (liver)	Average score between observers (lungs)
1	1	2	6			9	7.5			1
2	1	1,2	13	31	14	41	13	36	2	3
3	1	1	7		9		5.2		1	
4	1	2		4		3.4		3.7		1
5	1	1,2	12	10	12	5.2	12	8	2	1
6	1	2		7		11.2		9.1		1
7	1	1	57		53		55		4	
8	1	1,2	3.4	52	6.2	53	4.8	53	1	4
9	1	2		13		21		17		2

10	1	1	5.2		7		6.2		1	
11	1	1,2	21	26	28	24	25	25	3	3
12	1	2		8.2		7.2		7.7		1
13	1	1	6.4		12		9.2		1	
14	0	1,2	0.6	0.6	0.6	0.8	0.6	0.7	0	0
15	0	1,2	0.6	0.8	0.6	1	0.6	0.8	0	0
16	0	1,2	6.2	9	5	11	7.1	10	1	1
17	0	1,2	8.2	6.2	11	5.3	9.6	5.7	1	1
18	0	1,2	10	5	8.4	5.2	9.7	7.1	1	1
19	1	1	11		12		11.5		2	

Table D. 2. Average histological score per each tissue slide, for each observer and between observers. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Interpretation of the numbers used for type of tissue specimen and group to which certain animal belong:

-Treatment group:

0- Control group
1- Vaccinated group

-Type of tissue specimen:

0 -Liver
1 -Lungs

Raw data: AFFTV in DEN-NMOR model of carcinogenesis, survival data

	Animal from group	Survival (days from entry to end point)
1	1	40
2	1	55
3	1	45
4	1	31
5	1	54
6	1	14
7	1	21
8	1	10
9	1	34
10	0	13
11	0	48
12	0	55
13	0	32
14	0	60

Table D. 3. Survival time (days from entry to end point), for each animal. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Appendix E: Raw data - Assessing the features of immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix

Raw data: histological score of the lymphocyte infiltration in the MT450 model of carcinogenesis. Analysis of the features of AFFTV made of the MT450 cell line and/or Rotarix.

Animal	Treatment group	Type of tissue specimen	1st observer liver scores (for each photomicrograph) (%)	1st observer lungs scores (for each photomicrograph) (%)	2nd observer liver scores (for each photomicrograph) (%)	2nd observer lungs scores (for each photomicrograph) (%)
24	1	0	5,5,10,10,1		10,15,20,1,1	
25	4	0	0,1,1,1,0		1,1,1,1,0	
26	2	0	10,5,20,20,5		10,5,10,20,5	
27	4	1		5,5,15,15,10		5,5,15,10,5
28	3	1		1,5,1,10,10		10,15,10,1,5
29	0	0	1,1,1,0,0		5,1,1,0,0	
30	5	0	20,20,15,10,5		20,10,15,10,1	
31	2	0		25,25,25,15,15		20,20,15,15,10
32	4	0	0,1,1,1,1		0,0,0,1,5	
33	1	0	0,5,0,0,1		1,1,0,1,1	
34	5	0	20,25,25,10,15			20,25,25,20,15
35	5	1		30,25,25,10,30		25,30,15,15,15
36	5	0	1,10,15,5,10			1,5,5,15,20
37	0	1		15,5,10,20,20		15,20,20,5,25
38	5	1		30,30,35,25,15		30,10,35,25,15
39	4	1		15,10,15,20,5		20,25,25,10,15

40	2	1		35,30,35,15,25		35,35,10,15,35
41	4	0	15,10,20,5,10		10,5,5,15,15	
42	1	1	35,40,15,30,30		20,15,15,40,30	
43	5	1		60,25,60,60,55		60,30,50,60,55
44	0	1		5,25,20,20,15		10,10,5,20,10
45	2	1		5,5,10,15,1		10,15,15,10,10
46	0	0		1,5,15,20,15		20,1,10,5,20
47	3	1		35,40,20,15,35		40,35,35,15,35
48	3	1		1,5,15,20,20		5,5,15,10,15
49	4	1		0,1,1,1,1		1,5,0,0,0
50	3	0	10,15,5,5,5		10,15,15,10,5	
51	3	0	5,15,20,5,1		10,10,15,1,15	
52	1	0	1,1,0,1,1		1,1,1,1,0	
53	1	1		25,15,10,30,30		20,15,30,10,15

Table E. 1. Histological score per each tissue slide photomicrograph, for each observer. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and the Rotarix.

Animal	Treatment group	Type of tissue specimen	Average score for liver infiltration 1st observer	Average score for lungs infiltration 1st observer	Average score for liver infiltration 2nd observer	Average score for lungs infiltration 2nd observer	Average score between observers (liver) (%)	Average score between obsevers (lungs) (%)	Average score between observers (liver)	Average score between obsevers (lungs)
24	1	0	6.2		9.4		7.8	1	1	
25	4	0	0.6		0.8		0.7	0	0	
26	2	0	12		10		11	2	2	

27	4	1		10		8		9		1
28	3	1		5..4		8.2	6.8	6.8		1
29	0	0	0.6		1.4		1		0	
30	5	0	14		11.2		12.6		2	
31	2	0	21		16		18.5		2	
32	4	0	0.8		1.2		1		0	
33	1	0	1.2		0.8		1		0	
34	5	0	19		21		20		2	
35	5	1		24		20		22		3
36	5	0	8.2		9.2			8.7	1	
37	0	1		14		17		15.5		2
38	5	1		27		23		25		3
39	4	1		13		19		16		2
40	2	1		28		26		27		3
41	4	0	12		10		11		2	
42	1	1		30		24	27			3
43	5	1		52		51		51.5		4
44	0	1		17		11		14		2
45	2	1		7.2		12		9.6		1

46	0	0	11.2		11.2			11.2	2	
47	3	1		29		32		30.5		3
48	3	1		12.2		10		11.1		2
49	4	1		0..8		1.2		1		0
50	3	0	8		11		9.5		1	
51	3	0	9.2		10.2		9.7		1	
52	1	0	0.8		0.8		0.8		0	
53	1	1		22		18		20		2

Table E. 2. **Average hustological score per each tissue slide, for each observer and between observers. Assessiement of the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix.**

Interpretation of the numbers used for type of tissue specimen and group to which certain animal belong:

- Group:

0- Control
1- Group vaccinated with the Rotarix 3rd day
2- Group vaccinated with the Rotarix 7th day
3- Group vaccinated with the combination of Rotarix and AFFTV made of the MT450 cell line (3rd day)
4- Group vaccinated with the combination of Rotarix and AFFTV made of the MT450 cell line (7^{th} day)
5- Group vaccinated with the AFFTV made of the MT450 cell line (7th day)

Type of tissue specimen:

0- Subcutaneous tumour
1- Lungs

Raw data: Survival data

	Animal from group	Survival (days from entry to end point)
1	3	34
2	4	24
3	2	24
4	4	30
5	3	28
6	0	40
7	2	30
8	4	24
9	1	31
10	5	28
11	5	34
12	5	30
13	0	28
14	3	28
15	1	25

Table E. 3. Survival time(days from entry to end point), for each animal.

Appendix F: Raw data - Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol

Raw data: histological score of the lymphocyte infiltration

Animal	Treatment group	Type of tissue specimen	1st observer liver scores (for each photomicrograph) (%)	1st observer lungs scores (for each photomicrograph) (%)	2nd observer liver scores (for each photomicrograph) (%)	2nd observer lungs scores (for each photomicrograph) (%)
54	3	1		10,10,15,5,5		10,15,20,1,5
55	0	1		10,20,20,15,10		10,10,5,15,5
56	3	0	60,60,40,65,55		65,65,65,40,40	
57	2	0	10,5,5,1,15		10,15,15,5,10	
58	0	1		15,20,10,5,20		5,10,15,10,5
59	0	0	20,15,20,10,20		5,15,20,15,10	
60	1	1		35,10,5,35,30		5,20,30,35,35
61	0	1		15,15,20,5,5		10,20,5,5,10
62	2	1	5,5,1,10,15		10,10,15,15,5	
63	3	0	30,20,5,30,25		20,15,5,25,30	
64	1	0	15,15,10,20,5		5,5,15,20,15	
65	3	1		30,25,20,5,25		20,20,30,15,25
66	1	0	25,5,10,5,15		15,5,20,25,15	
67	2	0	30,35,30,10,15		30,30,25,20,10	
68	0	0	0,5,5,10 ,0		0,5,0,1,1	
69	1	1		20,5,20,25,15		10,15,15,1,5
70	2	1		15,20,20,10,5		15,15,10,5,5

71	2	1		5,10,10,25,25		5,5,15,15,10
72	2	0	5,10,1,10,10		5,5,15,15,10	
73	0	0	1,1,5,10,10		5,10,5,15,5	
74	2	0		5,15,20,5,5		5,5,20,10,10

Table F. 1. Histological score per each tissue slide photomicrograph, for each observer. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol.

Animal	Treatment group	Type of tissue specimen	Average score for liver infiltration 1st observer (%)	Average score for lungs infiltration 1st observer (%)	Average score for liver infiltration 2nd observer (%)	Average score for lungs infiltration 2nd observer (%)	Average score between observers (liver) (%)	Average score between obsevers (lungs) (%)	Average score between observers (liver)	Average score between obsevers (lungs)
54	3	1		9		10.2		9.6		1
55	0	1		15		9		12		2
56	3	0	56		55		55.5		4	
57	2	0	7.2		11		9.1		1	
58	0	1	17	14		9		11.5		2
59	0	0			13		15		2	
60	1	1		23		25		24		3
61	0	0	7.2	12		10		11		2
62	2	1				11		9.1		1
63	3	0	22		19		20.5		3	

64	1	0	13		12		12.5		2	
65	3	1		21		22		21.5		3
66	1	0	12		15		13.5		2	
67	2	0	24		23		23.5		3	
68	0	0	4		1..4		2.7		1	
69	1	1		17		9.2		13.1		2
70	2	1		14		10		12		2
71	2	1		15		10		12.5		2
72	2	0	7.2		10		8.6		1	
73	0	0	5.4		8		6.7		1	
74	2	0	10		10		10		1	

Table F. 2. Average hustological score per each tissue slide, for each observer and between observers. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol

Interpretation of the numbers used for type of tissue specimen and group to which certain animal belong:

-Group:

0- Control
1- Group vaccinated with the Rotarix and the AFFTV made of MT450 cell line
2- Group vaccinated with the Rotarix and the AFFTV made of the tumour tissue biopsy
3- Group vaccinated with the combination of the Rotarix, AFFTV made of the MT450 cell line and the AFFTV made of the tumour tissue biopsy

-Type of tissue specimen:

0- Subcutaneous tumour

1- 1- Lungs

Raw data: Survival data

	Animal from group	Survival (days from entry to end point)
1	3	23
2	0	29
3	2	23
4	0	29
5	0	40
6	1	36
7	3	28
8	1	43
9	2	26
10	2	40

Table F. 3. Survival time (days from entry to end point), for each animal. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol.

Appendix G: Raw data - Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol

Raw data: Histological score of the lymphocyte infiltration

Animal	Treatment group	Type of tissue specimen	1st observer liver scores (for each photomicrograph) (%)	1st observer lungs scores (for each photomicrograph) (%)	2nd observer liver scores (for each photomicrograph) (%)	2nd observer lungs scores (for each photomicrograph) (%)
75	0	1		10,15,20,20,15		10,10,20,20,5
76	2	0	0,20,15,5,5		15,15,10,10,5	
77	0	0	15,20,15,20,5		15,10,15,5,5	
78	0	0	1,5,10,15,10		15,5,10,15,10	
79	0	1		20,20,10,10,5		30,25,25,20,5
80	0	0	1,1,0,0,0		1,0,1,0,1	
81	2	0	5,10,15,5,5		5,15,15,10,10	
82	0	1		20,20,15,15,5		25,25,25,20,10
83	2	1		15,20,25,20,10		10,20,25,20,5
84	2	1		50,55,60,60,45		50,40,60,50,45
85	1	0	10,5,5,20,20		15,5,15,20,20	
86	1	1		35,20,25,20,20		30,15,20,15,10
87	1	1		30,25,20,15,10		10,20,15,5,5
88	1	0	25,25,10,15,10		20,10,10,5,15	

Table G. 1. Histological score per each tissue slide photomicrograph, for each observer. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.

Animal	Treatment group	Type of tissue specimen	Average score for liver infiltration 1st observer	Average score for lungs infiltration 1st observer	Average score for liver infiltration 2nd observer	Average score for lungs infiltration 2nd observer	Average score between observers (liver) (%)	Average score between observers (lungs)	Average score between observers (liver)	Average score between observers (lungs)
75	0	1		16		13		14.5		2
76	2	0	13		11		12		2	
77	0	0	15		10		12.5		2	
78	0	0	8.2		11		9.6		1	
79	0	1		13		25		20		2
80	0	0	0.4		0.6		0.5		0	
81	2	0	8		11		9.5		1	
82	0	1		15		21		18		2
83	2	1		18		16		17		2
84	2	1		54		49		51.5		4
85	1	0	12		15		13.5		2	

86	1	1		24		18		21		3
87	1	1		20		15		17.5		2
88	1	0	17		12		14.5		2	

Table G. 2. **Average histological score per each tissue slide, for each observer and between observers. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.**

Interpretation of the numbers used for type of tissue specimen and group to which certain animal belong:

-Group:

0- Control
1- Group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with standard protocol
2- Group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with changed protocol (3x2)

-Type of tissue specimen:

0- Subcutaneous tumour
1- 1- Lungs

Raw data: Survival data.

	Animal from group	Survival (days from entry to end point)
1	0	30
2	2	44
3	0	33
4	0	40
5	2	33
6	1	43
7	1	38

Table G. 3. Survival time (days from entry to an end point), for each animal. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol

Appendix H: Raw data - Flow cytometry analysis

Animals	Time (in min)	Flow rate (ml/min)	Time x flow rate (in ml)	Events	Initial volume of blood (in ml)	(Time x flow rate) x initial volume	Number of total cells	$CD8^+$ T cells as a freq. of total cell number	Number of $CD8^+$ T cells
1	0.5	0.035	0.0175	10000	2	20000	1142857.143	0.89 %	10171.4286
2	0.016	0.035	0.00056	10000	4	40000	71428571.43	0.080 %	57142.8571
3	0.016	0.035	0.00056	10000	5	50000	89285714.29	0.17 %	151785.714
4	0.016	0.035	0.00056	10000	6	60000	107142857.1	0.060 %	64285.7143
5	0.016	0.035	0.00056	10000	5	50000	89285714.29	0.27 %	241071.429

Table H. 1. Vaccinated animals, group injected with 1 x 10^5 MT450 cells. Flow cytometry analysis.

Animals	Time (min)	Flow rate (ml/min)	Time x flow rate (ml)	Events	Initial volume of blood (ml)	(Time x flow rate) x initial volume	Number of total cells	$CD8^+$ T cells as a freq. of total cell number (%)	Number of $CD8^+$ T cells
1	0.016	0.035	0.00056	10000	2	20000	35714285.7	0.48	171428.5
2	0.03	0.035	0.00115	10000	2	20000	17316017.	0.35	60606.0

	3		5				3		
3	0.033	0.035	0.001155	10000	2	20000	17316017.3	0.63	109090.9
4	0.016	0.035	0.00056	10000	2	20000	35714285.7	0.08	28571.4
5	0.033	0.035	0.001155	10000	2	20000	17316017.3	0.18	31168.8

Table H. 2. Vaccinated animals, group injected with 5 x 10^5 MT450 cells. Flow cytometry analysis.

Animals	Time (min)	Flow rate (ml/min)	Time x flow rate (ml)	Events	Initial volume of blood (ml)	(Time x flow rate) x initial volume	Number of total cells	$CD8^+$ T cells as a freq. of total cell number (%)	Number of $CD8^+$ T cells
1	0.16	0.035	0.0056	10000	2	20000	3571428.571	4.86	173571.429
2	0.15	0.035	0.00525	10000	4	40000	7619057.619	4.18	318476.191
3	0.15	0.035	0.00525	10000	4	40000	7619047.619	5.36	408380.952

Table H. 3. Control animals, group injected with 1 x 10^5 MT450 cells. Flow cytometry analysis

Animals	Time (min	Flow rate (ml/mi	Time x flow rate	Events	Initial volume of	(Time x flow rate)	Number of total cells	$CD8^+$ T cells as a	Number of $CD8^+$ T cells

	)	n)	(ml)		blood (in ml)	x initial volume		freq. of total cell number (%)	
1	0.116	0.035	0.00406	10000	6	60000	14778325.12	9.39	1387684.73
2	0.1	0.035	0.0035	10000	6	60000	17142857.14	1.69	289714.286
3	0.15	0.035	0.00525	10000	3	30000	5714285.714	0.67	38285.7143

Table H. 4. Control animals, group injected with 5 x 10^5 MT450 cells. Flow cytometry analysis

Interpretation:

Number of CD8+ T cells= (time x flow rate/events x initial volume)/freq of total

Total cell amount

Number of total cells= time x flow rate/events x initial volume

Appendix I: Raw data - Protein concentration in the AFFTV

Absorbance per replicate	Absorbance per replicate divided with m	(Absorbance per replicate/m) minus b	Protein concentration of replicate minus average protein concentration of trypsin sample for the same treatment (mg/ml)
0.415	3.042	2.981	2.540
0.472	3.460	3.399	2.998
0.425	3.115	3.054	3.054

Table I. 1. Tissue samples treated with the Tryp/EDTA 3 x 5, 24h.

Absorbance per replicate	Absorbance per replicate divided with m	(Absorbance per replicate/m) minus b	Protein concentration of replicate minus average protein concentration of trypsin sample for the same treatment (mg/ml)
0.544	3.988	3.926	3.464
0.440	3.225	3.164	2.702
0.460	3.372	3.311	2.848

Table I. 2. Tissue samples treated with the Tryp/EDTA 3 x 2, 24h.

Absorbance per replicate	Absorbance per replicate divided with m	(Absorbance per replicate/m) minus b	Protein concentration of replicate minus average protein of trypsin sample for the same treatment (mg/ml)
0.467	3.423	3.362	3.012
0.441	3.233	3.171	2.821
0.393	2.881	2.819	2.469

Table I. 3. Tissue samples treated with the Tryp/CaCl2 3 x 5, 24h.

Absorbance per replicate	Absorbance per replicate divided with m	(Absorbance per replicate/m) minus b	Protein concentration of replicate minus average protein concentration of trypsin sample for the same treatment (mg/ml)
0.425	3.115	3.054	2.653
0.388	2.844	2.783	2.783
0.416	3.049	2.988	2.988

Table I. 4. Tissue samples treated with the Tryp/CaCl2 3 x 2, 24h.

Absorbance per replicate	Average absorbance per treatment	Average absorbance per treatment divide with m	Average protein concentration of trypsin per treatment minus b (mg/ml)
0.079	0.076	0.501	0.440
0.075			
0.074			

Table I. 5. Trypsin samples treated with Tryp/EDTA 3 x 5, 24h.

Absorbance per replicate	Average absorbance per treatment	Average absorbance per treatment divide with m	Average protein concentration of trypsin per treatment minus b (mg/ml)
0.063	0.062	0.411	0.35
0.061			
0.063			

Table I. 6. Trypsin samples treated with Tryp/CaCl2 3 x 5, 24h.

Absorbance per replicate	Average absorbance per treatment	Average absorbance per treatment divide with m	Average protein concentration of trypsin per treatment minus b (mg/ml)
0.08	0.079	0.523	0.462
0.079			
0.079			

Table I. 7. Trypsin samples treated with the Tryp/EDTA 3 x 2, 24h.

Absorbance per replicate	Average absorbance per treatment	Average absorbance per treatment divide with m	Average protein concentration of trypsin per treatment minus b (mg/ml)
0.066	0.07	0.462	0.400
0.072			
0.072			

Table I. 8. Trypsin samples treated with the Tryp/CaCl2 3 x 2, 24h.

Absorbance per replicate	Average absorbance per treatment
0.001	0.001
0.003	
0.001	

Table I. 9. Blank control samples.

Interpretation:

b= Point where the line of standard curve intercepts the y axis

m= Slope that is multipled on the x axis

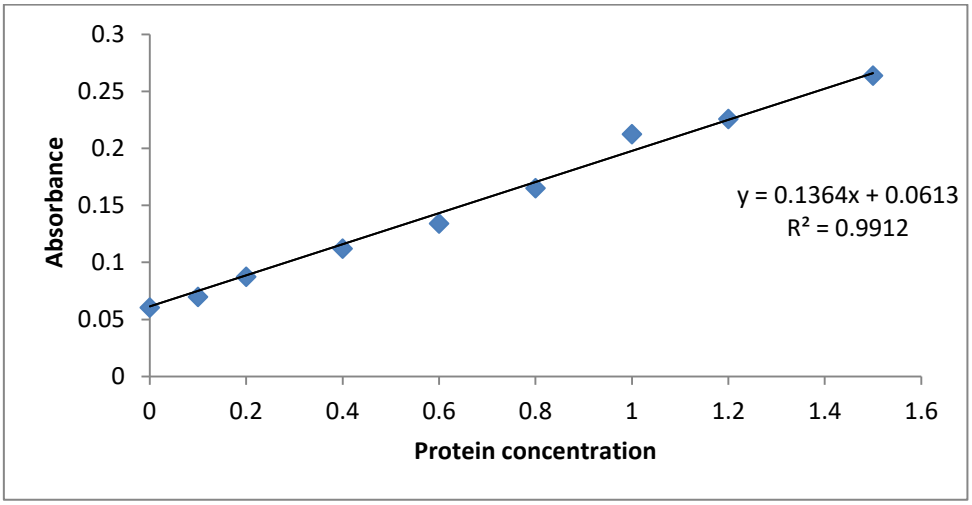
Concentration of BSA standard (mg/ml)	Absorbance 1st replicate	Apsorbance 2nd replicate	Absorbance 3rd replicate	Mean absorbance
0	0.062	0.055	0.064	0.060
0.1	0.07	0.071	0.068	0.069
0.2	0.084	0.093	0.085	0.087
0.4	0.115	0.108	0.113	0.112
0.6	0.136	0.133	0.133	0.134
0.8	0.159	0.163	0.173	0.165
1	0.181	0.223	0.233	0.212
1.2	0.225	0.227	0.225	0.225
1.5	0.277	0.261	0.253	0.263

Table I. 10. Values used for the construction of the standard curve for tissue samples based on the BSA standard concentrations.

Concentration of BSA standard (mg/ml)	Absorbance 1st replicate	Apsorbance 2nd replicate	Absorbance 3rd replicate	Mean
0	0.067	0.066	0.069	0.067
0.1	0.079	0.076	0.078	0.077
0.2	0.101	0.102	0.113	0.105
0.4	0.135	0.145	0.116	0.132
0.6	0.18	0.144	0.156	0.160
0.8	0.184	0.164	0.191	0.179
1	0.204	0.209	0.209	0.204
1.2	0.253	0.248	0.233	0.244
1.5	0.291	0.28	0.29	0.287

Table I. 11. Values used for the construction of the standard curve for trypsin samples based on the BSA standard concentrations.

a)



b)

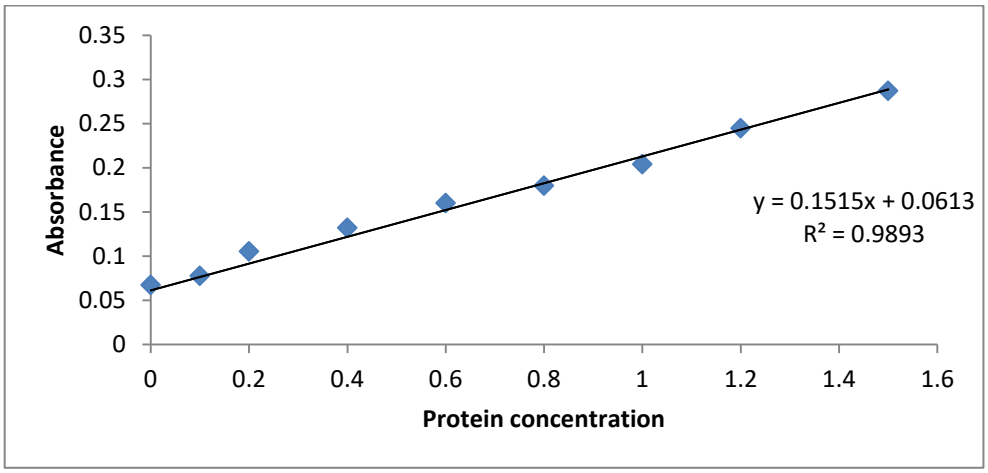


Figure I. 1. Standard curves constructed for: a) Tissue samples; b) Trypsin samples.

Appendix J: Statistics – Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Descriptive statistics:

Group	0	1
Number of values	5	9
Minimum	0	1
25% Percentile	0	1
Median	1	2
75% Percentile	1	2.5
Maximum	1	4
Mean	0.6	1.889
Std. Deviation	0.547	1.054
Std. Error of Mean	0.244	0.351
Lower 95% CI	-0.080	1.079
Upper 95% CI	1.28	2.699
Mean ranks	4.2	9.333

Table J. 1. Descriptive statistics for the chemical carcinogenesis experiment, the average histological score of the lymphocyte infiltration in liver per group. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Group	0	1
Number of values	5	9
Minimum	1	1
25% Percentile	1	1
Median	1	1
75% Percentile	2	3
Maximum	2	4
Mean	1.4	1.889
Std. Deviation	0.54	1.167
Std. Error of Mean	0.244	0.388
Lower 95% CI	-0.080	0.992
Upper 95% CI	0.719	2.786
Mean ranks	2	9.167

Table J. 2. Descriptive statistics for the chemical carcinogenesis experiment, the average histological score of the lymphocyte infiltration in the lungs, per group. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model.

Interpretation

Treatment group:

0- Control group

1- Vaccinated group

Mann-Whitney U test:

	Group 1 vs Group 0
P value	0.028
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in group 0	21
Sum of ranks in group 1	84
Mann-Whitney U	6
Median of group 0	1.000, n=5
Median of group 1	2.000, n=9
Difference: Actual	1
Difference: Hodges-Lehmann	1
95.80% CI of difference	0.000 to 2.000
Exact or approximate CI?	Exact

Table J. 3. Mann Whitney U test, comparison of the average histological score of the infiltration in the liver between groups. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Interpretation:

Group 0 – Control

Group 1- Vaccinated group

n- Number of animals

	Group 1 vs Group 0
P value	0.65
Exact or approximate P value?	Exact
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in group 0	33.5
Sum of ranks in group 1	71.5
Mann-Whitney U	18.5
Median of group 0	1.000, n=5
Median of group 1	1.000, n=9
Difference: Actual	0
Difference: Hodges-Lehmann	0
95.80% CI of difference	-1.000 to 2.000
Exact or approximate CI?	Exact

Table J. 4. Mann Whitney U test, comparison of the average histological score of the infiltration in the lungs between groups. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Interpretation

Treatment group:

0- Control group

1- Vaccinated group

n - Number of animals

Comparison of the survival curves – log rank test:

	Comparison of Survival Curves
Chi square	1.433
df	1
P value	0.231
P value summary	ns
Are the survival curves sig different?	No
Median survival Group 0	48
Median survival Group 1	34
Median survival Ratio (and its reciprocal) Group 0	1.412
Median survival Ratio (and its reciprocal) Group 1	0.708
Median survival 95% CI of ratio Group 0	0.473 to 4.213
Median survival 95% CI of ratio Group	0.237 to 2.114
Hazard Ratio (and its reciprocal) (logrank) 0/1	0.552
Hazard Ratio (and its reciprocal) (logrank) 1/0	1.81
95% CI of hazard ratio 0/1	0.193 to 1.575
95% CI of hazard ratio 1/0	0.635 to 5.161

Table J. 5. Comparison of survival curves. Assessing the features of the immune response after the immunisation with the combination of the Rotarix and the AFFTV in DEN - NMOR carcinogenesis model

Group 0- Control group;

Group 1- Vaccinated group; 0/1 – Group 0/Group 1;

1/0 – Group 1/Group 0.

Appendix K: Statistics - Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix

Descriptive statistics:

	Group					
	0	1	2	3	4	5
Number of values	2	2	2	3	3	3
Minimum	0	0	2	1	0	1
25% Percentile	0	0	2	1	0	1
Median	1	0	2	1	0	2
75% Percentile	2	0	2	1	2	2
Maximum	2	0	2	1	2	2
Mean	1	0	2	1	0.6667	1.667
Std. Deviation	1.414	0	0	0	1.155	0.5774
Std. Error of Mean	1	0	0	0	0.6667	0.3333
Lower 95% CI	-11.71	0	2	1	-2.202	0.2324
Upper 95% CI	13.71	0	2	1	3.535	3.101
Mean ranks	7.75	3	12.5	7.5	6.167	10.83

Table K. 1. Descriptive statistics for the the average histological score of the lymphocyte infiltration in the subcutaneous tumour, per group. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix.

	Group					
	0	1	2	3	4	5
Number of values	2	2	2	3	3	3
Minimum	2	2	1	1	0	3
25% Percentile	2	2	1	1	0	3
Median	2	2.5	2	2	1	3
75% Percentile	2	3	3	3	2	4
Maximum	2	3	3	3	2	4
Mean	2	2.5	2	2	1	3.333
Std. Deviation	0	0.707	1.414	1	1	0.577
Std. Error of Mean	0	0.5	1	0.577	0.577	0.333
Lower 95% CI	2	-3.853	-10.71	-0.484	-1.484	1.899
Upper 95% CI	2	8.853	14.71	4.484	3.484	4.768
Mean ranks	7	9.5	7.5	7.333	3.667	13

Table K. 2. **Descriptive statistics for the the average histological score of the lymphocyte infiltration in the lungs, per group. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix.**

Interpretation:

0- Control
1- Group vaccinated with the Rotarix 3rd day
2- Group vaccinated with the Rotarix 7th day
3- Group vaccinated with the combination of Rotarix and AFFTV made of the MT450 cell line (3rd day)
4- Group vaccinated with the combination of Rotarix and AFFTV made of the MT450 cell line (7^{th} day)
5- Group vaccinated with the AFFTV made of the MT450 cell line (7th day)

Kruskal-Wallis test:

Kruskal-Wallis test	
Number of treatments (columns)	6
Number of values (total)	15
P value	0.2132
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)?	No
Number of groups	6
Kruskal-Wallis statistic	7.101

Table K. 3. Kruskal - Wallis test for the comparison of the average histological score of the lymphocyte infiltration in the subcutaneous tumour. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix

Kruskal-Wallis test	
Number of treatments (columns)	6
Number of values (total)	15
P value	0.181
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)?	No
Number of groups	6
Kruskal-Wallis statistic	7.579

Table K. 4. Kruskal - Wallis test for the comparison of the average histological score of the lymphocyte infiltration in the lungs. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix

Interpretation:

0- Control
1- Group vaccinated with the Rotarix 3rd day
2- Group vaccinated with the Rotarix 7th day
3- Group vaccinated with the combination of Rotarix and AFFTV made of the MT450 cell line (3rd day)
4- Group vaccinated with the combination of Rotarix and AFFTV made of the MT450 cell line (7^{th} day)
5- Group vaccinated with the AFFTV made of the MT450 cell line (7th day)

Dunn's multiple comparisons test:

Test between groups	Dunn's multiple comparisons test						
	Mean rank diff.	Significant?	Summary	Adjusted P Value	n1	n2	Z
0 vs.1	4.75	No	ns	>0.9999	2	2	1.13
0 vs 2	-4.75	No	ns	>0.9999	2	2	1.13
0 vs 3	0.25	No	ns	>0.9999	2	3	0.065
0 vs 4	1.583	No	ns	>0.9999	2	3	0.412
0 vs 5	-3.083	No	ns	>0.9999	2	3	0.803

Table K. 5. Dunn's multiple comparison test for the comparison of the average histological score of the lymphocyte infiltration in the subcutaneous tumour. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix

Test between groups	Dunn's multiple comparisons test						
	Mean rank diff.	Significant?	Summary	Adjusted P Value	n1	n2	Z
0 vs.1	-2.5	No	ns	>0.9999	2	2	0.5824
0 vs 2	-0.5	No	ns	>0.9999	2	2	0.1165
0 vs 3	-0.3333	No	ns	>0.9999	2	3	0.08506
0 vs 4	3.333	No	ns	>0.9999	2	3	0.8506
0 vs 5	-6	No	ns	0.6288	2	3	1.531

Table K. 6. Dunn's multiple comparison test for the comparison of the average histological score of the lymphocyte infiltration in the lungs. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix

Interpretation:

0- Control
1- Group vaccinated with the Rotarix 3rd day
2- Group vaccinated with the Rotarix 7th day
3- Group vaccinated with the combination of Rotarix and AFFTV made of the MT450 cell line (3rd day)
4- Group vaccinated with the combination of Rotarix and AFFTV made of the MT450 cell line (7^{th} day)
5- Group vaccinated with the AFFTV made of the MT450 cell line (7th day)

Comparison of survival curves-log rank test:

	Comparison of Survival Curves
Chi square	4.781
df	5
P value	0.4432
P value summary	ns
Are the survival curves sig different?	No
Median survival group 0	34
Median survival group 1	28
Median survival group 2	27
Median survival group 3	28
Median survival group 4	30
Median survival group 5	24

Table K. 7. Comparison of survival curves. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with various combinations of AFFTV and/or the Rotarix

Appendix L: Statistics - Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol

Descriptive statistics:

	Group			
	0	**1**	**2**	**3**
Number of values	3	2	3	2
Minimum	1	2	1	3
25% Percentile	1	2	1	3
Median	1	2	1	3.5
75% Percentile	2	2	3	4
Maximum	2	2	3	4
Mean	1.333	2	1.667	3.5
Std. Deviation	0.577	0	1.155	0.707
Std. Error of Mean	0.333	0	0.666	0.5
Lower 95% CI	-0.100	2	-1.202	-2.853
Upper 95% CI	2.768	2	4.535	9.853
Mean ranks	3.667	6	4.5	9.25

Table L. 1. Descriptive statistics for the the average histological score of the lymphocyte infiltration in the subcutaneous tumour, per group. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol.

Group:

4- Control
5- Group vaccinated with the Rotarix and the AFFTV made of MT450 cell line
6- Group vaccinated with the Rotarix and the AFFTV made of the tumour tissue biopsy
7- Group vaccinated with the combination of the Rotarix, AFFTV made of the MT450 cell line and the AFFTV made of the tumour tissue biopsy

	Group			
	0	1	2	3
Number of values	3	2	3	2
Minimum	2	2	1	1
25% Percentile	2	2	1	1
Median	2	2.5	2	2
75% Percentile	2	3	2	3
Maximum	2	3	2	3
Mean	2	2.5	1.667	2
Std. Deviation	0	0.707	0.577	1.414
Std. Error of Mean	0	0.5	0.333	1
Lower 95% CI	2	-3.853	0.232	-10.71
Upper 95% CI	2	8.853	3.101	14.71
Mean ranks	5.5	7.5	4.167	5.5

Table L. 2. Descriptive statistics for the the average histological score of the lymphocyte infiltration in the lungs, per group. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol.

Kruskal-Wallis test:

Kruskal-Wallis test	
Number of treatments (columns)	4
Number of values (total)	10
P value	0.156
Exact or approximate P value?	Exact
P value summary	ns
Do the medians vary signif. (P < 0.05)?	No
Number of groups	4
Kruskal-Wallis statistic	5.005

Table L. 3. Kruskal - Wallis test for the comparison of the average histological score of the lymphocyte infiltration in the subcutaneous tumour.

Kruskal-Wallis test	
Number of treatments (columns)	4
Number of values (total)	10
P value	0.844
Exact or approximate P value?	Exact
P value summary	ns
Do the medians vary signif. (P < 0.05)?	No
Number of groups	4
Kruskal-Wallis statistic	1.875

Table L. 4. Kruskal - Wallis test for the comparison of the average histological score of the lymphocyte infiltration in the lungs. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol.

Interpretation:

Group:

0- Control
1- Group vaccinated with the Rotarix and the AFFTV made of MT450 cell line
2- Group vaccinated with the Rotarix and the AFFTV made of the tumour tissue biopsy
3- Group vaccinated with the combination of the Rotarix, AFFTV made of the MT450 cell line and the AFFTV made of the tumour tissue biopsy

Dunn's multiple comparisons test:

Test between groups	Dunn's multiple comparisons test						
	Mean rank diff.	Significant?	Summary	Adjusted P Value	n1	n2	Z
0 vs 1	-2.333	No	ns	>0.9999	3	2	0.885
0 vs 2	-0.833	No	ns	>0.9999	3	3	0.353
0 vs 3	-5.583	No	ns	0.102	3	2	2.119

Table L. 5. Dunn's multiple comparison test for the comparison of the average histological score of the lymphocyte infiltration in the subcutaneous tumour.

Test between groups	Dunn's multiple comparisons test						
	Mean rank diff.	Significant?	Summary	Adjusted P Value	n1	n2	Z
0 vs 1	-2	No	ns	>0.9999	3	2	0.821
0 vs 2	1.333	No	ns	>0.9999	3	3	0.612
0 vs 3	0	No	ns	>0.9999	3	2	0

Table L. 6. Dunn's multiple comparison test for the comparison of the average histological score of the lymphocyte infiltration in the lungs. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol.

Interpreatation:

Group:

0- Control
1- Group vaccinated with the Rotarix and the AFFTV made of MT450 cell line
2- Group vaccinated with the Rotarix and the AFFTV made of the tumour tissue biopsy
3- Group vaccinated with the combination of the Rotarix, AFFTV made of the MT450 cell line and the AFFTV made of the tumour tissue biopsy

Comparison of survival curves – log Rank test:

	Comparison of Survival Curves
Chi square	5.137
df	3
P value	0.162
P value summary	ns
Are the survival curves sig different?	No
Median survival group 0	29
Median survival group 1	39.5
Median survival group 2	26
Median survival group 3	25.5

Table L. 7. Comparison of urvival curves in the MT450 transplantable tumour model. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made from the tumour tissue biopsy and/or the AFFTV made of the MT450 cells with the standard protocol.

Appendix M: Statistics - Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol

Descriptive statistics:

	Group		
	0	**1**	**2**
Number of values	3	2	2
Minimum	0	2	1
25% Percentile	0	2	1
Median	1	2	1.5
75% Percentile	2	2	2
Maximum	2	2	2
Mean	1	2	1.5
Std. Deviation	1	0	0.707
Standard Error of Mean	0.577	0	0.5
Lower 95% confidence interval	-1.484	2	-4.853
Upper 95% confidence interval	3.484	2	7.853
Mean ranks	3	5.5	4

Table M. 1. Descriptive statistics for the the average histological score of the lymphocyte infiltration in the subcutaneous tumour, per group. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.

	Group		
	0	2	1
Number of values	3	2	2
Minimum	2	2	2
25% Percentile	2	2	2
Median	2	2.5	3
75% Percentile	2	3	4
Maximum	2	3	4
Mean	2	2.5	3
Std. Deviation	0	0.707	1.414
Std. Error of Mean	0	0.5	1
Lower 95% confidence interval	2	-3.853	-9.706
Upper 95%	2	8.853	15.71
Mean ranks	3	4.5	5

Table M. 2. Descriptive statistics for the the average histological score of the lymphocyte infiltration in the lungs, per group. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.

-Group:

0- Control
1- Group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with standard protocol
2- Group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with changed protocol (3x2)

Kruskal - Wallis test:

Kruskal-Wallis test	
Number of treatments (columns)	3
Number of values (total)	7
P value	0.542
Exact or approximate P value?	Exact
P value summary	ns
Do the medians vary signif. (P < 0.05)?	No
Number of groups	3
Kruskal-Wallis statistic	2

Table M. 3. Kruskal - Wallis test for the comparison of the average histological score of the lymphocyte infiltration in the subcutaneous tumour. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.

Kruskal-Wallis test	
Number of treatments (columns)	3
Number of values (total)	7
P value	0.428
Exact or approximate P value?	Exact
P value summary	ns
Do the medians vary signif. (P < 0.05)?	No
Number of groups	3
Kruskal-Wallis statistic	1.833
Number of treatments (columns)	3
Number of values (total)	7

Table M. 4. Kruskal-Wallis test for the comparison of the average histological score of the lymphocyte infiltration in the lungs. Assessing the features of the immune response in the

MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.

Interpretation:

-Group:

0- Control
1- Group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with standard protocol
2- Group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with changed protocol (3x2)

Dunn's multiple comparisons test:

Test between groups	Dunn's multiple comparisons test						
	Mean rank diff.	Significant?	Summary	Adjusted P Value	n1	n2	Z
0 vs. 1	-2.5	No	ns	0.314	3	2	1.414
0 vs. 2	-1	No	ns	>0.9999	3	2	0.565

Table M. 5. Dunn's multiple comparison test for the comparison of the average histological score of the lymphocyte infiltration in the subcutaneous tumour. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.

Test between groups	Dunn's multiple comparisons test						
	Mean rank diff.	Significant?	Summary	Adjusted P Value	n1	n2	Z
0 vs. 1	-1.5	No	ns	0.685	3	2	0.948
0 vs. 2	-2	No	ns	0.411	3	2	1.265

Table M. 6. Dunn's multiple comparison test for the comparison of the average histological score of the lymphocyte infiltration in the lungs. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.

Interpretation:

-Group:

0- Control
1- Group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with standard protocol
2- Group vaccinated with the Rotarix and the AFFTV made of the MT450 cell line with changed protocol (3x2)
3-

Comparison of survival curves:

	Comparison of Survival Curves
Chi square	1.902
df	2
P value	0.386
P value summary	ns
Are the survival curves sig different?	No
Median survival group 0	33
Median survival group 1	40.5
Median survival group 2	38.5

Table M. 7. Comparison of survival curves. Assessing the features of the immune response in the MT450 carcinogenesis model after the immunisation with the AFFTV made of the MT450 cell line with the standard or changed protocol.

Appendix N: Statistics – Flow cytometry analysis

Descriptive statistics:

	Control	Vaccinated
Number of values	3	5
Minimum	173571	10171
25% Percentile	173571	33657
Median	318476	64286
75% Percentile	408381	196429
Maximum	408381	241071
Mean	300143	104891
Stdandard Deviation	118474	91712
Standard Error of Mean	68401	41015
Lower 95% confidence interval	5838	-8985
Upper 95% confidence interval	594447	218767

Table N. 1. Descriptive statistics flow cytometry data. Control and vaccinated groups, injected with 1x 10^5 MT450 cells.

	Control	Vaccinated
Number of values	3	5
Minimum	38286	28571
25% Percentile	38286	29870
Median	289714	60606
75% Percentile	1387685	140259
Maximum	1387685	171429
Mean	571895	80173
Standard Deviation	717593	60438
Standard Error of Mean	414302	27028
Lower 95% confidence interval	-1210704	5130
Upper 95% confidence interval	2354494	155216

Table N. 2. Descriptive statistics flow cytometry data. Control and vaccinated groups, injected with 5 x 10^5 MT450 cells.

Shapiro-Wilks test:

Shapiro-Wilk test	Control	Vaccinated
Statistic	0.982	0.923
P value	0.743	0.555
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns

Table N. 3. Shapiro - Wilk's test for normality, flow cytometry data. Data obtained from animals injected with the 1x10^5 MT450 cells, control and vaccinated. W

Shapiro-Wilk test	Control	Vaccinated
Statistic	0.884	0.885
P value	0.336	0.335
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns

Table N. 4. Shapiro - Wilk's test for normality, flow cytometry data .Data obtained from animals injected with the 5 x 10^5 MT450 cells, control and vaccinated.

Unpaired t test with Welch's correction:

Unpaired t test with Welch's correction	
P value	0.080
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=2.448, df=3.472
Difference between means (Vaccinated - Control) ± standard error of mean	-195251 ± 79755
95% confidence interval	-430611 to 40108
R squared (eta squared)	0.633

Table N. 5. Unpaired t test with Welch's correction, flow cytometry data. Control and vaccinated groups, injected with 1 x 10^5 MT450 cells.

Unpaired t test with Welch's correction	
P value	0.357
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=1.184, df=2.017
Difference between means (B - A) ± SEM	-491722 ± 415183
95% confidence interval	-2263724 to 1280280
R squared (eta squared)	0.410

Table N. 6. Unpaired t test with Welch's correction, flow cytometry data Control and vaccinated groups, injected with 5 x 10^5 MT450 cells.

Appendix 0: Statistics – Protein concentration in the AFFTV

Descriptive statistics:

	Treatment group			
	0	1	2	3
Median	2.822	2.849	2.998	2.783
75% Percentile	3.012	3.465	3.055	2.989
Maximum	3.012	3.465	3.055	2.989
Mean	2.768	3.005	2.865	2.809
Std. Deviation	0.275	0.404	0.281	0.1688
Std. Error of Mean	0.158	0.233	0.162	0.097
Lower 95% CI	2.084	2	2.165	2.389
Upper 95% CI	3.452	4.01	3.564	3.228

Table O. 1. **Descriptive statistics, protein concentration data.**

Interpretation:

Treatment group (samples treated with):

0- Tryp/CaCl2, 3x5 protocol,24h
1- Tryp/EDTA 3x2 protocol, 24h
2- Tryp/EDTA, 3x5 protocol, 24h
3- Tryp/CaCl2 3x2 protocol, 24h

Shapiro - Wilk's test:

	Group			
	0	1	2	3
W	0.971	0.887	0.830	0.983
P value	0.675	0.348	0.190	0.751
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns

Table O. 2. Shapiro - Wilk's test for normality, protein concentration data.

Interpretation:

Treatment group (samples treated with):

0- Tryp/CaCl2, 24h standard protocol
1- Tryp/EDTA changed protocol, 24h
2- Tryp/EDTA, standard protocol, 24h
3- Tryp/CaCl2 changed protocol, 24h

ANOVA test:

ANOVA test	
F	0.371
P value	0.776
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R square	0.122

Table O. 3. **One - way ANOVA analysis. Protein concentration determination (DC assay).**

	Test between groups					
	Group 0 vs.Group 1 24h. Tryp/EDTA 3x2	Group 0 vs. Group 2	Group 0, vs. Group 3	Group 1 vs. Group 2	Group 1 vs. Group 3	Group 2 vs. Group 3
Mean Diff.	-0.237	-0.096	-0.04	0.14	0.1966	0.056
95.00% CI of diff.	-1.008 to 0.533	-0.867	-0.811	-0.629	-0.573	-0.714
Significant?	No	No	No	No	No	No
Summary	ns	ns	ns	ns	ns	ns
Adjusted P Value	0.761	0.976	0.998	0.934	0.844	0.995
Mean Diff.	-0.237	-0.096	-0.04	0.14	0.196	0.056
SE of diff.	0.24	0.24	0.24	0.24	0.24	0.24
q	1.395	0.568	0.238	0.826	1.156	0.329
DF	8	8	8	8	8	8

Table O. 4. Multiple comparisons. Protein concentration determination (DC assay).

Interpretation:

Treatment group (samples treated with):

0- Tryp/CaCl2, 24h standard protocol
1- Tryp/EDTA changed protocol, 24h
2- Tryp/EDTA, standard protocol, 24h
3- Tryp/CaCl2 changed protocol, 24h