Boosting Anti-Cancer Immunity with a Novel Chimeric Molecule

Damodar Gajurel, MD

Student ID: 5043056

School of Medical Science

Griffith University

Principal supervisor: Professor Ming Wei

School of Medical Science, Griffith University

Associate supervisor: Dr Alan Munn

School of Medical Science, Griffith University

A Report Submitted in Partial Requirement for the Award of the Master of Medical Research at Griffith University

July 2017

Abstract

High mortality, second only to cardiovascular causes, and high morbidity (physical as well as psychological) from cancer are unacceptable. Despite many years of multi-modality (conventional) treatment with surgery, radiotherapy, and chemotherapy, the status of cancer management, especially lung cancer, is still not satisfactory and alternate management strategies need to be developed.

Anti-tumour immunotherapy is being explored as a potential new form of cancer therapies. Cancer vaccines, as forms of immunotherapy, have been developed and tested in clinical trials. Unfortunately, almost all of them did not achieve expected clinical responses. One of the reasons for this failure has been attributed to the poor immunogenicity/antigenicity of those vaccines. It has been suggested that whole tumour cells, harbouring all known and unknown cancer antigens, would better serve as vaccine antigens by circumventing the probability of tumour antigen loss due to tumour immune editing than selected single antigens used in most of those failed trials. The fact that even histologically similar types of tumours can harbour divergent antigens in different patients also explains the failure of clinical trials using allogeneic cancer cell vaccines suggesting that personalized tumour vaccines using autologous whole tumour cells would most likely ensure clinical success of cancer vaccines. But, at the same time, studies have shown that, in contrast to isolated single antigens, whole cancer cells also contain self-antigens that could lead, if not to outright immune tolerance, then to poor immune response, necessitating the use of immune-potentiating adjuvants to garner sufficient anti-tumour immune stimulation, i.e. enhanced immunogenicity. Therefore, combining autologous whole cancer cells with the appropriate immune-potentiating adjuvant in various novel ways could ensure a highly immunogenic and clinically effective vaccine.

Considering the above facts, this study has been initiated to design a lung cancer vaccine with improved immunogenicity by conjugating a known strong and safe immune-potentiating adjuvant, i.e. unmethylated cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN) to whole A549 human lung cancer cell (as vaccine antigens) with the help of cross-linker bis-sulfosuccinimidyl suberate (BS^3) in a novel way. Studies have shown that bi-directionally active NHS-ester moiety of BS^3 covalently attach to the surface of the cancer cell membrane on one side and to one end of CpG ODN on the other side forming a novel chimeric molecule with 100-fold enhanced immune-potentiating capacity compared to their use in physical and temporal isolation. The formation of this covalently stable chimeric molecule using A549 whole lung cancer cells with CpG ODN, for the first time, was confirmed using fluorescein molecule tagged CpG ODNs and a scanning laser confocal microscope.

The immunogenicity of this novel chimeric molecule was tested *in vitro* by measuring the level of the cytokines interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) released after exposure to U937 differentiated macrophages.

The results of the level of the cytokines confirm that quantitatively more IL-6 and TNF-α, as surrogate markers for anti-cancer immune response, were released with the incorporation of the novel chimeric molecule than its control. The chimeric molecule induced the release of about 4500 pg/ml of TNF-α. This was very close to the amount of TNF-α (about 5000 pg/ml) released by 100 ng/ml of lipopolysaccharide (LPS) used as positive control. The negative control i.e. unconjugated CpG ODN and macrophages, released only around 500-600 pg/ml of TNF-α (i.e. our novel chimeric molecule induced nine times more cytokine release than its control. The difference in means of the cytokines released by them was also statistically significant ($p < 0.05$).

The results indicated that this novel chimeric molecule is highly immunogenic and could be further tested in animal models as the next step towards the development of a personalized anti-lung clinical cancer vaccine.

List of Abbreviations

A549	Human lung cancer cell line (adenocarcinoma)
Ab	Antibody
Ag	Antigen
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen presenting cells
ATCC	American Type Culture Collection
BCG	Bacillus Calmette–Guérin
BS^3	Bis-sulfosuccinimidyl suberate
CCM	Complete culture medium (+DMEM or +RPMI-1640)
CD	Clonal differentiation (+ Arabic numbering)
CpG-ODN	Cytosine-phosphate-guanine oligodeoxynucleotide
CTL	Cytotoxic T-lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
CV	Cancer vaccines
DAG	Diacyl glycerol
DAMP	Damage associated molecular patterns
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immuno-sorbent assay
GM-CSF	Granulocyte-monocyte colony stimulating factor

GVAX	Allogenic vaccine
IDO	Indoleamine 2,3-dioxygenase
IFN-α, γ	Interferon alpha, gamma
IgM, G, A	Immunoglobulin (M, G, A etc.)
ILs	Interleukins
IRAK	IL-1R-associated kinase
LSCM	Laser scanning confocal microscope
mAb	Monoclonal antibody
MAGE-3	Melanoma-associated antigen 3
MAGRIT	MAGE-A3 as Adjuvant Non-Small Cell Lung Cancer Immunotherapy trial
MAPK	Mitogen-activated kinases
MDSC	Myeloid-derived suppressor cells
MHC-I, II	Major histocompatibility complex I and II
MBV	Mixed bacterial vaccine
MUC1	Mucin 1 glycoprotein antigen
MyD88	Myeloid differentiation factor 88
NCI-USA	National Cancer Institute, United States of America
NF-kB	Nuclear factor kB
NK	Natural killer (cells)
NY-ESO-1	New York-oesophageal cancer-1 protein
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood monocytic cells
PD-1	Programmed death
pDC	Plasmocytic dendritic cells
PD-L	Programmed death ligand

PBS	Phosphate-buffered saline
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMT	Photo-multiplier tube
PRR	Pathogen recognition receptors
QoL	Quality of life
RCT	Randomised controlled trials
SCCC	Standard cell culture condition (95% humid air, 5% CO_2, 37^0 C)
SC-LFC	Sterile condition-laminar flow cabinet
START	The phase 3 Stimulating Targeted Antigenic Responses to NSCLC trial
STOP	Survival: tumour-free, overall and progression-free trial
TAM	Tumour-associated macrophages
TG4010	Recombinant modified poxvirus that codes for MUC1 antigen and IL-2
TGF-β	Transforming growth factor β
Th1& 2	T-helper cell response type 1&2
TLR-9	Toll-like receptors-9 (+ others in Arabic numerals)
TNF-α	Tumour necrosis factor alpha
TRAF6	Tumour necrosis factor receptor-associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	Regulatory T-cells
U937	Human monocytic tumour cell line
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WHO-IARC	WHO-International Agency for Research on Cancer

Contents

List of Tables, Figures, and Scans

Acknowledgement

I would like to acknowledge the important contributions made to completing this work by the following people.

First and foremost, my principal supervisor Professor Dr Ming Wei was the person to propose this research topic and suggest this design. He continuously supported and encouraged me during my literature search and experimentation. His positive, gentle, and approachable nature also helped in buffering the difficulties and frustrations associated with research for a novice like myself. He was always there to help and support me.

My associate supervisor Dr Alan Munn guided me with the revision of the thesis and helped me understand various concepts necessary for the successful completion of my thesis. He was always very supportive, helpful, and encouraging.

My experimental work would not have been successful without the support and help of Ph.D. candidate Tamim Mosaiab, who despite his own academic pressures, found time to technically guide me. I am very thankful to him. I would also like to thank Ph.D. candidates Kai Zhang, Guoying Ni, Mohsen Sohrabi, and Rushdi Fadhil for their help.

Though directly not related to this work, Associate Professor Dr Jing Sun helped me by giving me encouragement and guiding me on the proper use of statistical methods for this study.

I cannot forget the administrative support given by our Course Co-ordinator Associate Professor Jason Peart and one of the most supportive person I have ever met in my life, the School of Medical Sciences secretary Fran Humphries.

Finally, I would like to thank Griffith University for providing me the platform to hone my research skills in the pursuit of scientific knowledge.

Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, this dissertation contains no material that has previously been published or written by another person except where due reference is made in the dissertation itself.

Damodar Gajurel, MD

July 2017

Chapter 1: Introduction

1.1 Background

1.1.1 Cancer definition

Cancer is a generic term for a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs (WHO, 2017).

1.1.2 Cancer prevalence, incidence, and burden

Cancer is one of the most dreaded diseases. The basis for this fear relates to both genuine and exaggerated negative perception of its incidence, mortality, and morbidity (Vrinten, Wardle, & Marlow, 2016). There were 14.1 million new cancer cases, 8.2 million cancer deaths, and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide. Globally, nearly 1 in 6 deaths is due to cancer. It is expected that cancer cases will rise by about 70% from 14 million in 2012 to 22 million within the next 2 decades (*World Cancer Report 2014*, 2014). The estimated number of new cases and deaths from cancer in Australia in 2016 was 130,466 and 46,880 respectively (*Australia's health 2016*, 2016). Cancer mortality with 8.2 million deaths came second only to cardiovascular mortality with 17.5 million deaths in 2012 worldwide (WHO-IARC, 2014). It has been recognized that the diagnosis and treatment of cancer entails unusual physical and psychological morbidity (Stanton, Rowland, & Ganz, 2015) (Taqaddas, 2015) (Mehta & Roth, 2015).

1.1.3 Lung cancer prevalence, incidence, and burden

Among all cancers lung cancer is the second most common cancer in both men and women (not counting skin cancer). About 14% of all new cancers are lung cancers. The estimates for lung cancer in the United States for 2017 are: about 222,500 new cases of lung cancer (116,990 in men and 105,510 in women) and about 155,870 deaths from lung cancer (84,590 in men and 71,280 in women). Lung cancer is by far the leading cause of cancer death among

both men and women; about 1 out of 4 cancer deaths are from lung cancer. Each year, more people die of lung cancer than of colon, breast, and prostate cancers combined (Society, 2016).

1.1.4 Carcinogenesis: its causes and risk factors

1.1.4.1 Pathogenesis and risk factors for cancers

Cancer is a disease involving multiple time- and space-dependent changes in the health status of cells and tissues that ultimately lead to malignant tumours. Neoplasia (abnormal cell growth) is the biological endpoint of the disease. Tumour cell invasion into surrounding tissues and their spread (metastasis) to distant organs is the primary cause of morbidity and mortality of most cancer patients.

A major impediment in the effort to control cancer has been due in large part to the confusion surrounding the origin of the disease. Contradictions and paradoxes continue to plague the field. Much of the confusion surrounding cancer origin arises from the absence of a unifying theory that can integrate the many diverse observations on the nature of the disease. Without a clear understanding of how cancer arises, it becomes difficult to formulate a successful strategy for effective long-term management and prevention. The failure to clearly define the origin of cancer is responsible in large part for the failure to significantly reduce the death rate from the disease. Although cancer metabolism is receiving increased attention, cancer is generally considered a genetic disease. This general view is now under serious re-evaluation (Seyfried, Flores, Poff, & D'Agostino, 2014).

The prevailing paradigm in cancer research is the somatic mutation theory that posits that cancer begins with a single mutation in a somatic cell followed by successive mutations. Much cancer research involves refining the somatic mutation theory with an ever-increasing catalogue of genetic changes. But, various observations that are not accounted for by this paradigm need to be resolved. These are the unresolved paradoxes of the somatic mutation

theory of carcinogenesis: (1) the presence of large numbers of spatially distinct precancerous lesions at the onset of promotion, (2) the large number of genetic instabilities found in hyperplastic polyps not considered cancer, (3) spontaneous regression, (4) higher incidence of cancer in patients with xeroderma pigmentosa but not in patients with other comparable defects in deoxyribonucleic acid (DNA) repair, (5) lower incidence of many cancers except leukemia and testicular cancer in patients with Down's syndrome, (6) cancer developing after normal tissue is transplanted to other parts of the body or next to stroma previously exposed to carcinogens, (7) the lack of tumours when epithelial cells exposed to a carcinogen were transplanted next to normal stroma, (8) the development of cancers when Millipore filters of various pore sizes were inserted under the skin of rats, but only if the holes were sufficiently small (Baker & Kramer, 2007).

In an effort to explain these paradoxes and other issues in carcinogenesis, a metabolic cause for carcinogenesis has been proposed. Emerging evidence indicates that cancer is primarily a metabolic disease involving disturbances in energy production through respiration and fermentation. The genomic instability observed in tumour cells and all other recognized hallmarks of cancer are considered downstream epiphenomena of the initial disturbance of

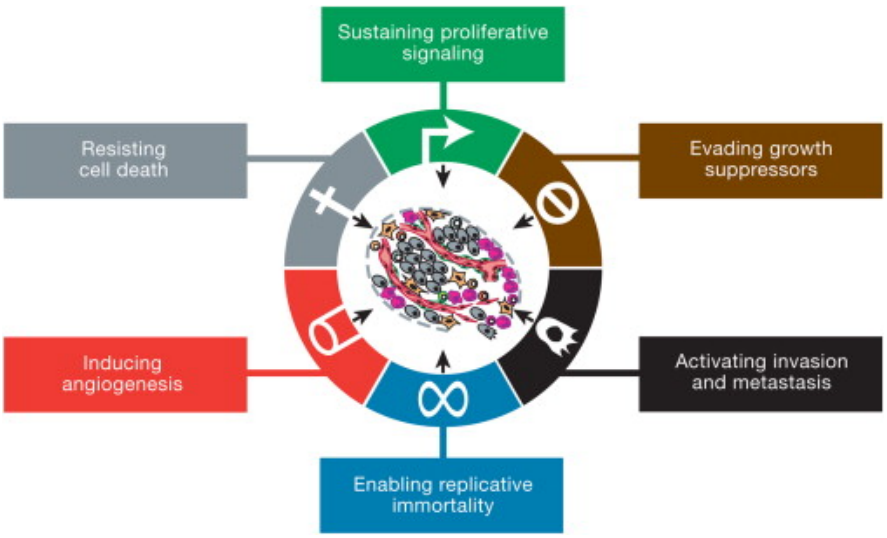


Figure 1: Hallmarks of cancer (Hanahan & Weinberg, 2011).

cellular energy metabolism. The disturbances in tumour cell energy metabolism can be linked to abnormalities in the structure and function of the mitochondria (Seyfried et al., 2014). Notwithstanding the existence of unresolved issues in carcinogenesis, for practical purposes, it is usually not possible to know exactly why one person develops cancer and another doesn't; but research has shown that certain risk factors may increase a person's chances of developing cancer. But, there are several established risk factors for cancer. These are: age, alcohol, cancer-causing substances, chronic inflammation, diet, hormones, immunosuppression, infectious agents, obesity, radiation, sunlight, tobacco, family history, and predisposition to hereditary cancer syndrome (National Cancer Institute, 2017).

1.1.4.2 Pathogenesis and the risk factors of lung cancers

Ever since a lung cancer epidemic emerged in the mid-1900s, the epidemiology of lung cancer has been intensively investigated to characterize its causes and patterns of occurrence. Many causes of lung cancer have been identified, including active cigarette smoking; exposure to second-hand cigarette smoke (passive smoking); pipe and cigar smoking; occupational exposure to agents such as asbestos, nickel, chromium, and arsenic; exposure to radiation, including radon gas in homes and mines; and exposure to indoor and outdoor air pollution. Despite the identification of this constellation of well-established causal risk factors, the global epidemic of lung cancer is primarily caused by a single factor: cigarette smoking.

The aetiology of lung cancer can be conceptualized as reflecting the joint consequences of the interrelationship between exposure to etiologic agents and individual susceptibility to these agents. Synergistic interactions among risk factors can have substantial consequences for lung cancer risk. Well-known examples include the synergistic effect of cigarette smoking on the lung cancer risk associated with exposure to asbestos and radon (Alberg, Brock, Ford, Samet, & Spivack, 2013).

The molecular basis of lung cancer is complex and heterogenous. Improvements in our understanding of molecular alterations at multiple levels (genetic, epigenetic, protein expression) and their functional significance have the potential to impact lung cancer diagnosis, prognosis, and treatment. Lung cancers develop through a multistep process involving development of multiple genetic and epigenetic alterations, particularly activation of growth promoting pathways and inhibition of tumour suppressor pathways. Greater understanding of the multiple biochemical pathways involved in the molecular pathogenesis of lung cancer is crucial to the development of treatment strategies that can target molecular aberrations and their downstream activated pathways. Specific molecular alterations that drive tumour growth and provide targets for therapy have been best defined in adenocarcinomas but there is increasing interest in the molecular landscape of squamous cell carcinoma highlighting new potential therapeutic targets. In lung cancer as in other malignancies, tumour-genesis relates to activation of growth promoting proteins (Cooper, Lam, O'Toole, & Minna, 2013).

There is great genetic diversity in lung cancer and lung cancer's harbour among the greatest numbers of genetic aberrations of all tumours. Understanding of the molecular biology of lung cancer has been revolutionised by next-generation sequencing technologies that provide a comprehensive means of identifying somatic alterations in entire cancer genomes or exomes (Cooper et al., 2013). This has to be taken into consideration while selecting cancer antigens for vaccines.

1.1.5 Diagnosis of lung cancer and other cancers

Adequate cancer management relies on both cancer diagnosis and staging. The suspicion of cancer (in our case lung cancer) is usually based on initial clinical and/or radiological findings in those with symptoms or risk factors for that cancer. This is usually followed by thorough clinical, biochemical, imaging, and histological and/or cytological examinations.

This multimodality diagnostic approach will ultimately lead to the correct diagnosis and staging and consequently to proper cancer management. Additionally, some biochemical tests are used to define disease burden and detect recurrences (Lawrence & Rosenberg, 2015; Silvestri et al., 2013).

1.1.6 **Cancer management (including lung cancer)**

Currently, the accepted cancer management strategy is a multi-disciplinary approach (Lawrence & Rosenberg, 2015) that entails the use of surgery, radiotherapy, chemotherapy, targeted therapy (if available), rehabilitation therapy, and immunotherapy in a few cases (e.g. prostate and bladder cancers) (Noguchi, Koga, Igawa, & Itoh, 2017; R.-F. Wang, 2017). The use of single modality of treatment is rarely successful for most cancers. Surgically removing cancer if limited to a defined anatomical location is theoretically plausible but there are as yet no accepted diagnostic methods to detect microscopic spread with certainty. Clinical experience has shown that the cancers thought to have been completely surgically removed have resurfaced in another location after many years. Thus, at present, cancer management strategies are generally based on the assumption that cancer is a systemic disease from the very outset (Heiss et al., 1995). Additionally, due to the lack of precise knowledge as to the origin and cause of cancer, targeted therapy for most cancers is lacking. Consequently, the main modalities of cancer management, surgery, radiotherapy, and chemotherapy being non-specific, it has been thought prudent to combine them to achieve maximum local and systemic anti-tumour effect and minimize tumour resistance and recurrence (Lawrence & Rosenberg, 2015). This approach, though more tumoricidal, also causes damage to normal tissues and leads to toxic morbidity. Despite decades of this multi-modality therapeutic approach, cancer management, by and large, remains unsatisfactory. The situation for lung cancer is even worse. It is mostly diagnosed when already advanced and too late for curative surgery either because it is unresectable or inoperable (Silvestri et al., 2013). The curative

potential of both radiotherapy and chemotherapy is negligible and their role in palliation is fraught with dangers of toxicities leading to decreased quality of life (QoL) (Pedersen, Koktved, & Nielsen, 2013).The above facts and the average 5-year survival rate of less than 15% , with almost no improvement in the last 40 years (UK, 2010-11), poignantly illustrates the failure of conventional therapy (surgery, radiation therapy, chemotherapy, and some targeted therapy) in the management of lung cancer and points to the need for novel therapies. This applies, to a greater or lesser extent, to most other common cancers as well (Guo et al., 2013).

1.1.7 Cancer immunity, cancer immunotherapy, and cancer vaccines

1.1.7.1 **Immunity definition**

It is the condition of being protected against an infectious disease, i.e. immunity can be caused by a vaccine, previous infection with the same agent, or by transfer of immune substances from another person or animal (National Cancer Institute, 2017).

1.1.7.2 Immunotherapy definition

It is a type of biological therapy that uses substances to stimulate or suppress the immune system to help the body fight cancer, infection, and other diseases, i.e. where some types of immunotherapy only target certain cells of the immune system while others affect the immune system in a general way (National Cancer Institute, 2017).

1.1.7.3 Vaccine definition

It is a biological preparation that improves immunity to a particular disease and typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe, its toxins or one of its surface proteins. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters (WHO, 2017).

Cancer vaccines are biological preparations directed against immunogenic cancer antigen(s) and stimulate the anti-tumour immune response. And there are two types of cancer vaccines: preventive and therapeutic. Preventive vaccines are applied to prevent tumour growth in healthy people from so-called “risk groups”. These vaccines induce specific immune response against oncogenic virus proteins. Curative (therapeutic) cancer vaccines are generally prepared from cancer cells, which serve as an antigen source. In some cases, these vaccines could be based on recombinant tumour-specific or tumour-associated proteins. The essential feature of curative vaccines is that they could not be used for cancer prevention because of wide diversity of tumour types and the impossibility to guess the individual-specific proteomics of tumour. But curative vaccines definitely could be used for treatment of patients with known cancer types and for prevention of recurrence and metastasis (Chekhun, 2008).

The role of immunity and immunotherapy in cancer, in contrast to infections, is still being defined (researched) and despite much research effort there have only been a few clinical applications (Noguchi et al., 2017; R.-F. Wang, 2017).

1.2 Statement of the problem

The foregoing section on cancer and lung cancer statistics clearly shows that mortality and morbidity is still too high to be satisfied with the preventive and management strategy in place today. Despite many decades of the application of surgery, radiotherapy, and chemotherapy in the management of cancers, mortality and morbidity has not changed much for many common cancers including lung cancer. Despite the potential for immunotherapy, recent clinical trials in lung cancer using single antigens have not produced clinical benefit. The analysis of these trials concludes that future trial ought to include whole cell antigens as vaccines (Mazza & Cappuzzo, 2016). This is what we propose to do in this project.

1.3 Study significance and the expected outcome

The immense suffering of people with cancer (including children) and minimum respite with serious toxicities from prevailing therapies mandates some serious work in the extremely potential and relatively safe field of cancer vaccines (immunotherapy). Among the various components of immunotherapy, cancer vaccines are supposedly the least toxic. The failure of individual antigens to garner clinically relevant clinical response as witnessed by the failure of several recent randomised controlled trials RCTs (Mazza & Cappuzzo, 2016), has brought whole cancer cells as antigenic material for use as vaccines to the forefront. We expect that this novel chimeric molecule with whole cancer cells as antigenic material will result in a strong anti-cancer immune response manifested by the adequate release of cytokines TNF-α and IL-6 as surrogate markers of such a response.

Chapter 2: Literature Review

2.1 Cancer immunotherapy

Immunotherapy could be the best novel option or addition to conventional therapy (J. Wang et al., 2012) for cancer because it relates to our inherent and natural immune capability with the additional benefit of it also being less toxic (Seledtsov, Goncharov, & Seledtsova, 2015) (Yu et al., 2017), cost-effective, easy to administer, relatively safe, and time-tested (there is a long history of the use of vaccines against infections) (Melief, van Hall, Arens, Ossendorp, & van der Burg, 2015).

The search for effective novel therapies, also as less toxic alternative or addition to conventional therapies for cancer, led researchers towards immunotherapy (Seledtsov et al., 2015). Historically, cancer occurrence and its outcome had been observed to be influenced by the immune status of the individual (Gatti & Good, 1971) as seen in patients with the acquired immunodeficiency syndrome (AIDS) and donor organ transplant (O. J. Finn, 2008).

Sufficient theoretical (biological plausibility) and empirical evidence to confirm the close, dynamic, and natural relationship between immunity and cancer had accumulated in the last 100 or so years (beginning with Paul Ehrlich's concept and prediction of "magic bullet" against cancer (Sathyanarayanan & Neelapu, 2015)) to scientifically back these observations. It was also noticed that vaccines against common infections, based on the principles of immunity, were tremendously successful in eradicating them (Banday, Jeelani, & Hruby, 2015). It was hoped that cancer also being harmful and arising *de novo* in the body could, like harmful microbes, be vigorously attacked if the immunity of the individual could be sufficiently mobilized to detect and destroy it (Zepp, 2016).

2.2 **A pioneering historical example of cancer immunotherapy**

Though also described by Busch, Fehleisen, and Dussosoy, the first ever well documented systematic use of immunotherapy to treat cancer (though anecdotal uses of cancer immunotherapy dates back to earlier periods in our Civilization) was that by William Coley, a New York surgeon in the 1890s. Based on his own observation of a number of his sarcoma patients improving spontaneously after contracting bacterial infections like erysipelas, he used MBV (mixed bacterial vaccine containing *Streptococcus pyogenes* and *Serratia marscecens*), popularly named "Coley`s Toxin" as a form of (vaccine) immunotherapy, as we understand it today, to induce fever to treat cancer (Kienle, 2012). This form of immunotherapy was partly successful with many of Coley's patients. It was also tried by a few others but with less success. The use of "Coley's Toxin" or MBV was largely abandoned after his death for the following reasons. Firstly, the effect of "Coley`s Toxin" or MBV was not reproducible by others largely because its production was not standardized. Secondly, it was a crude form of therapy with serious side-effects on top of its effect being unpredictable. Thirdly, the introduction of radiotherapy in cancer at around that period offered a more reliable and understandable alternative. Lastly, the mechanism of its workings was then

unclear (Ito & Chang, 2013). Despite its shortcomings, Coley's trials set the stage for the long and tumultuous journey in search of effective cancer immune-therapeutics.

The following chart (Fig. 2) demonstrates the milestones in the history of immunotherapy for cancer.

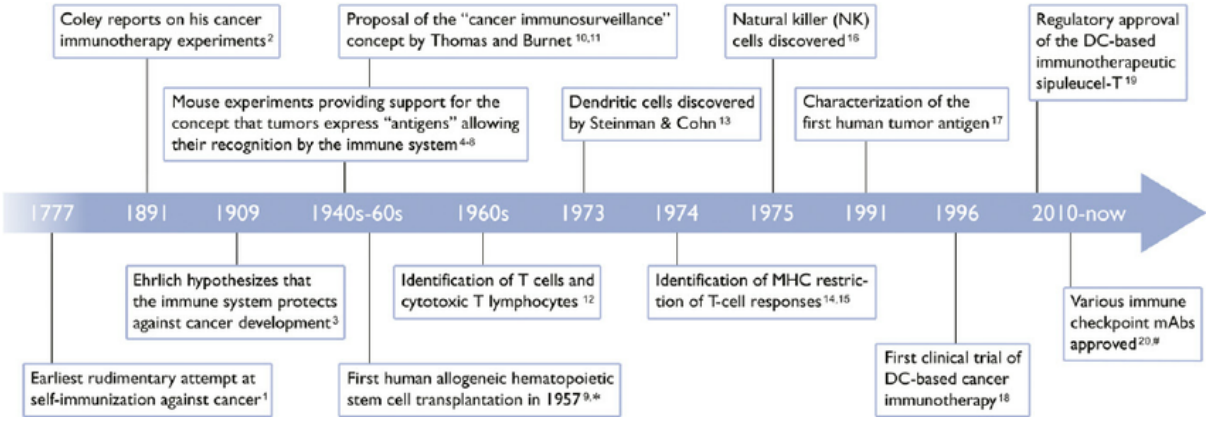


Figure 2. Milestones in the history of cancer immunotherapy (Lesterhuis, Haanen, & Punt, 2011).

The timeline depicts some of the pivotal milestones in the history of cancer immunotherapy, with a particular focus on dendritic cell (DC)-based cancer immunotherapy. References: 1 (Rosenberg, 1999); 2 (Coley, 1891); 3 (Ehrlich, 1909); 4 – 8 (Gross, 1943; Foley, 1953; Baldwin, 1955; Prehn and Main, 1957; Klein et al., 1960); 9 (Thomas et al., 1957); 10, 11 (Thomas, 1959; Burnet, 1967); 12 (Coulie et al., 2014); 13 (Steinman and Cohn, 1973); 14, 15 (Zinkernagel and Doherty, 1974a,b); 16 (Kiessling et al., 1975); 17 (van der Bruggen et al., 1991); 18 (Hsu et al., 1996); 19 (Kantoff et al., 2010); 20 (Pardoll, 2012).

2.3 **Establishment of the theoretical basis for cancer immunity**

The development of immunity to cancer occurs through certain specific steps that can be divided into seven steps. The first step starts with the release of cancer antigens following cancer cell death. These antigens are then captured and presented by antigen presenting cells (APCs) to T-lymphocytes in the lymph nodes. Following such priming and activation, the cytotoxic T-lymphocytes (CTLs) travel to the tumour tissues and infiltrate them. After recognizing the cancer cells, these CTLs kill them. Thus, more cancer antigens are produced to initiate another cycle of cancer-immunity (see Figure 3).

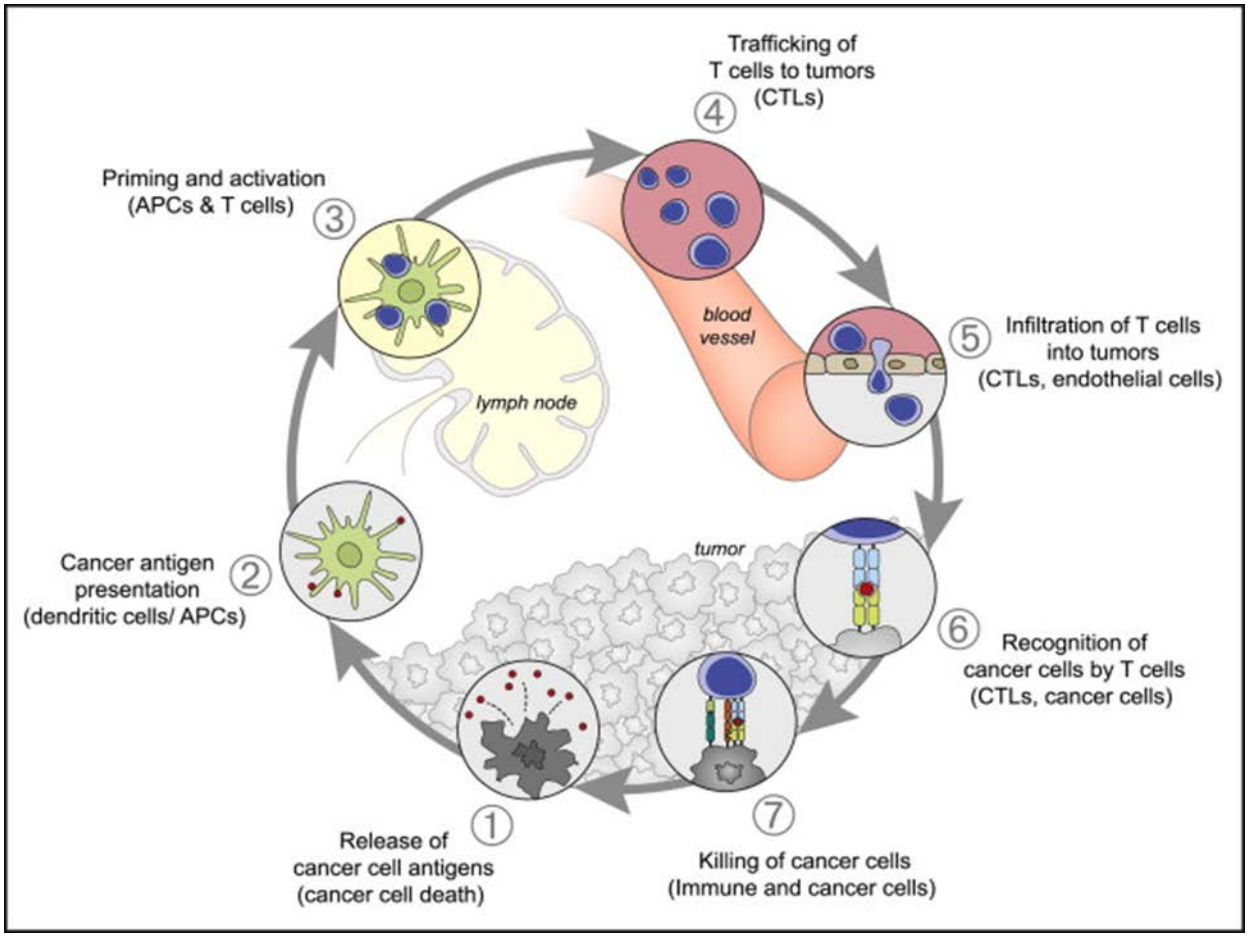


Figure 3. The cancer-immunity cycle (D. S. Chen & Mellman, 2013).

The generation of immunity to cancer is a cyclic process that can be self-propagating, leading to an accumulation of immune-stimulatory factors that in principle should amplify and broaden T cell responses. The cycle is also characterized by inhibitory factors that lead to immune regulatory feedback mechanisms, which can halt the development or limit the immunity. This cycle can be divided into seven major steps, starting with the release of antigens from the cancer cell and ending with the killing of cancer cells. Each step is described above, with the primary cell types involved and the anatomic location of the activity listed. Abbreviations are as follows: APCs, antigen presenting cells; CTLs, cytotoxic T lymphocytes (D. S. Chen & Mellman, 2013).

During these cancer-immunity cycles, both stimulatory and inhibitory factors are produced to either augment or weaken cancer immunity. Each step of the cycle is characterized by the presence of specific stimulatory and/or inhibitory factors (see Figure 4).

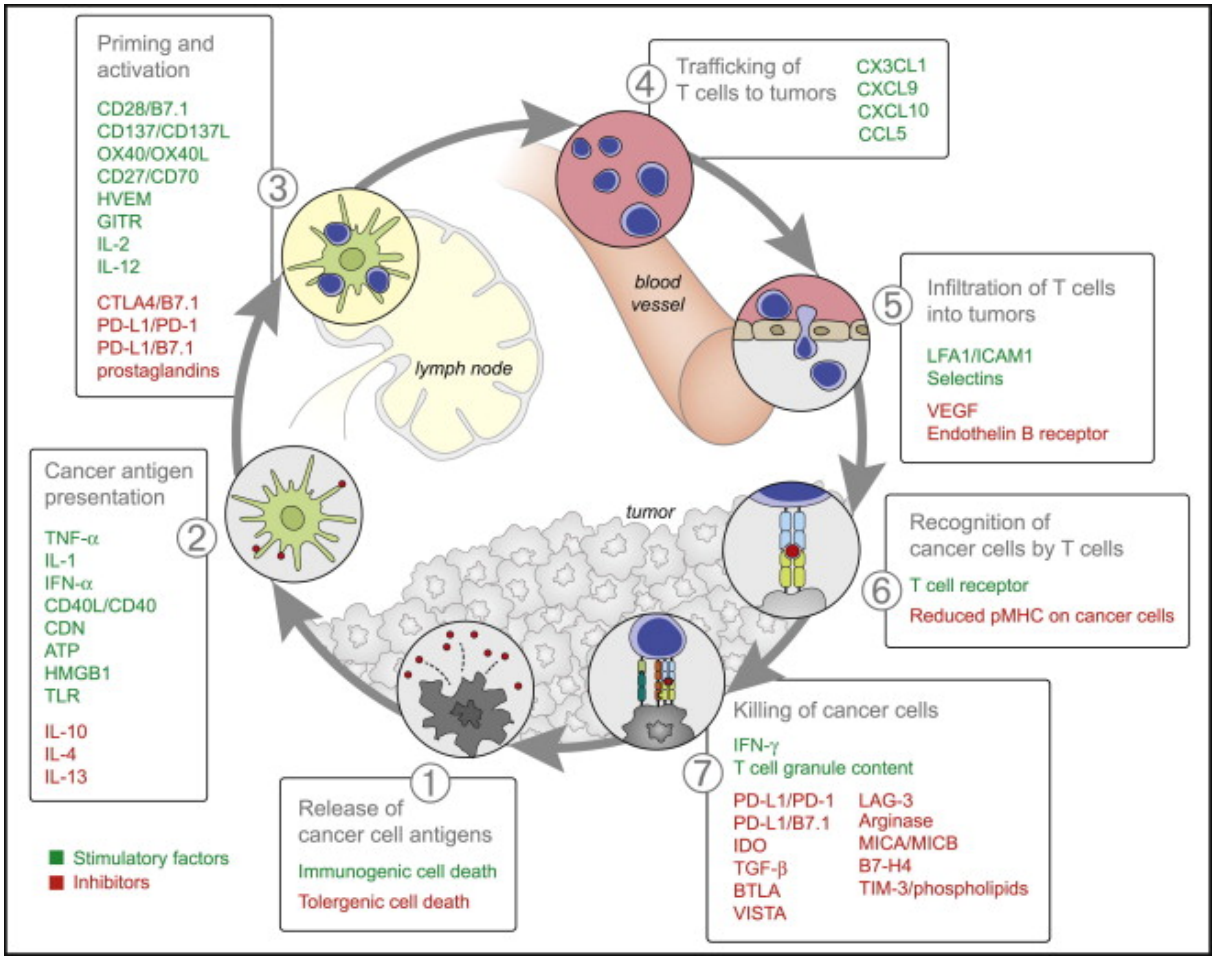


Figure 4. Stimulatory and inhibitory factors in the cancer-immunity cycle (D. S. Chen & Mellman, 2013).

Each step of the cancer-immunity cycle requires the coordination of numerous factors, both stimulatory and inhibitory in nature. Stimulatory factors shown in green promote immunity, whereas inhibitors shown in red help keep the process in check and reduce immune activity and/or prevent autoimmunity. Immune checkpoint proteins, such as CTLA4, can inhibit the development of an active immune response by acting primarily at the level of T cell development and proliferation (step 3). We distinguish these from immune rheostat factors, such as PD-L1, can have an inhibitory function that primarily acts to modulate active immune responses in the tumour bed (step 7). Examples of such factors and the primary steps at which they can act are shown. Abbreviations are as follows: IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; CDN, cyclic dinucleotide; ATP, adenosine triphosphate; HMGB1, high-mobility group protein B1; TLR, Toll-like receptor; HVEM, herpes virus entry mediator; GITR, glucocorticoid-induced TNFR family-related gene; CTLA4, cytotoxic T-lymphocyte antigen-4; PD-L1, programmed death-ligand 1; CXCL/CCL, chemokine motif ligands; LFA1, lymphocyte function-associated antigen-1; ICAM1, intracellular adhesion molecule 1; VEGF, vascular endothelial growth factor; IDO, indoleamine 2,3-dioxygenase; TGF, transforming growth factor; BTLA, B- and T-lymphocyte attenuator; VISTA, V-domain Ig suppressor of T cell activation; LAG-3, lymphocyte-activation gene 3 protein; MIC, MHC class I polypeptide-related sequence protein; TIM-3, T cell immunoglobulin domain and mucin domain-3. Although not illustrated, it is important to note that intra-tumoural T regulatory cells, macrophages, and myeloid-derived suppressor cells are key sources of many of these inhibitory factors (D. S. Chen & Mellman, 2013).

2.4 Potential targets in cancer-immunity cycle for immune-therapeutic intervention in cancer (cancer immunotherapy)

A range of factors that are characteristics of each step of the cancer-immunity cycle can become potential therapeutic targets that could either strengthen or weaken cancer immunity (see Figure 5).

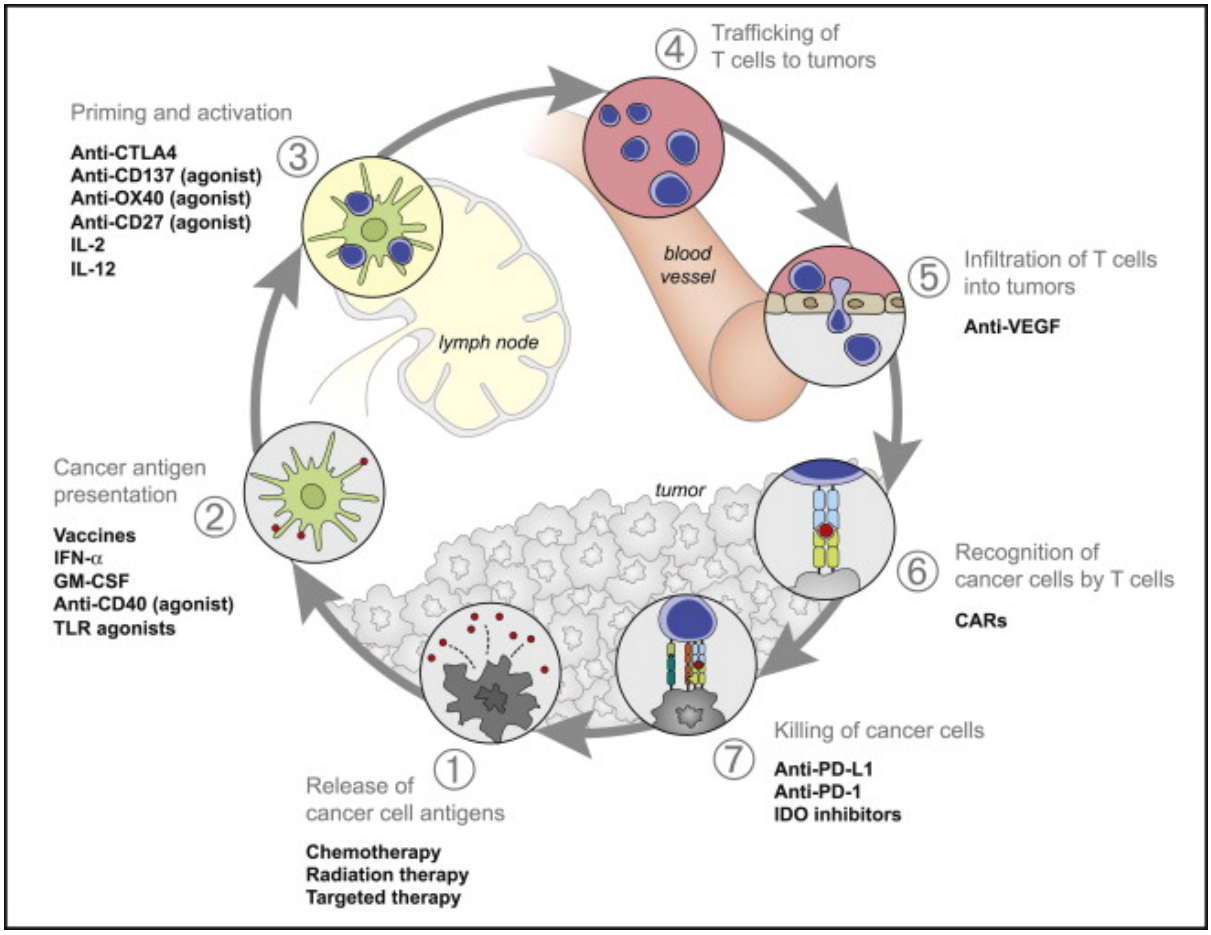


Figure 5. Therapies that might affect the cancer-immunity cycle (D. S. Chen & Mellman, 2013).

The numerous factors that come into play in the cancer-immunity cycle provide a wide range of potential therapeutic targets. This figure highlights examples of some of the therapies currently under preclinical or clinical evaluation. Key highlights include that vaccines can primarily promote cycle step 2, anti-CTLA4 can primarily promote cycle step 3, and anti-PD-L1 or anti-PD-1 antibodies can primarily promote cycle step 7. Although not developed as immunotherapies, chemotherapy, radiation therapy, and targeted therapies can primarily promote cycle step 1, and inhibitors of VEGF can potentially promote T cell infiltration into tumours—cycle step 5. Abbreviations are as follows: GM-CSF, granulocyte macrophage colony-stimulating factor; CARs, chimeric antigen receptors (D. S. Chen & Mellman, 2013).

2.5 Stages in the development of the scientific basis for cancer immunity and immunotherapy

2.5.1 Do cancers contain antigens to stimulate anti-cancer immunity?

The observation that, despite cancer arising from an aberrant cell and causing harm to the organism, the immune system seemed incapable of eliminating cancer and developing lasting immunity, made scientists curious. There were suspicions that, maybe, cancer did not possess appropriate antigens to stimulate the immune system. A series of experiments were then carried out to test the existence of appropriate antigens in cancer tissues. Although the existence of cancer antigens was prophesized by Paul Ehrlich as early as 1897 , a prerequisite for the development of a "magic bullet" (Sathyanarayanan & Neelapu, 2015), it was the animal experiment performed by Ludwig Gross in 1943 that produced the first experimental evidence of their existence in tumour tissues (Gross, 1943). However, it was the 1957 experiments by Richmond T. Prehn and colleagues and L.J. Old and colleagues in 1964 that convincingly confirmed the existence of cancer antigens (Prehn & Main, 1957) (Old & Boyse, 1964). Subsequently, many cancer antigens were discovered, classified, and compiled. Today, there is a special consortium whose responsibility it is to assemble, collect, and document all the available human tumour antigens (Cheever et al., 2009). Once the existence of cancer antigens was proved beyond doubt, it was necessary to find out how our immune system deals with cancers that arise in our body.

2.5.2 From cancer immuno-surveillance to cancer immunoediting

In 1909, Paul Ehrlich predicted that the immune system repressed the growth of carcinomas that he envisaged would otherwise occur with great frequency, thus initiating a century of contentious debate over immunologic control of neoplasia. Fifty years later, as immunologists gained an enhanced understanding of transplantation and tumour immunobiology and immuno-genetics, F. Macfarlane Burnet and Lewis Thomas revisited the

topic of natural immune protection against cancer. Burnet's thinking was shaped by a consideration of immune tolerance; he believed that tumour cell-specific neo-antigens could evoke an effective immunologic reaction that would eliminate developing cancers. In contrast, Thomas's early view was evolutionary in nature; he theorized that complex long-lived organisms must possess mechanisms to protect against neoplastic disease like those mediating homograft rejections. Despite subsequent challenges to this hypothesis over the next several decades, new studies in the 1990s—fuelled by technologic advances in mouse genetics and monoclonal antibody (mAb) production—reinvigorated and ultimately validated the cancer immune-surveillance concept and expanded it to incorporate the contributions of both innate and adaptive immunity (Dunn, Old, & Schreiber, 2004).

However, there has been a growing recognition that immune-surveillance represents only one dimension of the complex relationship between the immune system and cancer. Recent work has shown that the immune system may also promote the emergence of primary tumours with reduced immunogenicity that can escape immune recognition and destruction. These findings prompted the development of the cancer immune-editing hypothesis to more broadly encompass the potential host-protective and tumour-sculpting functions of the immune system throughout tumour development. Cancer immunoediting is a dynamic process composed of three phases: elimination, equilibrium, and escape. Elimination represents the classical concept of cancer immune-surveillance, equilibrium is the period of immune-mediated latency after incomplete tumour destruction in the elimination phase, and escape refers to the final outgrowth of tumours that have outstripped immunological restraints of the equilibrium phase (Dunn et al., 2004).

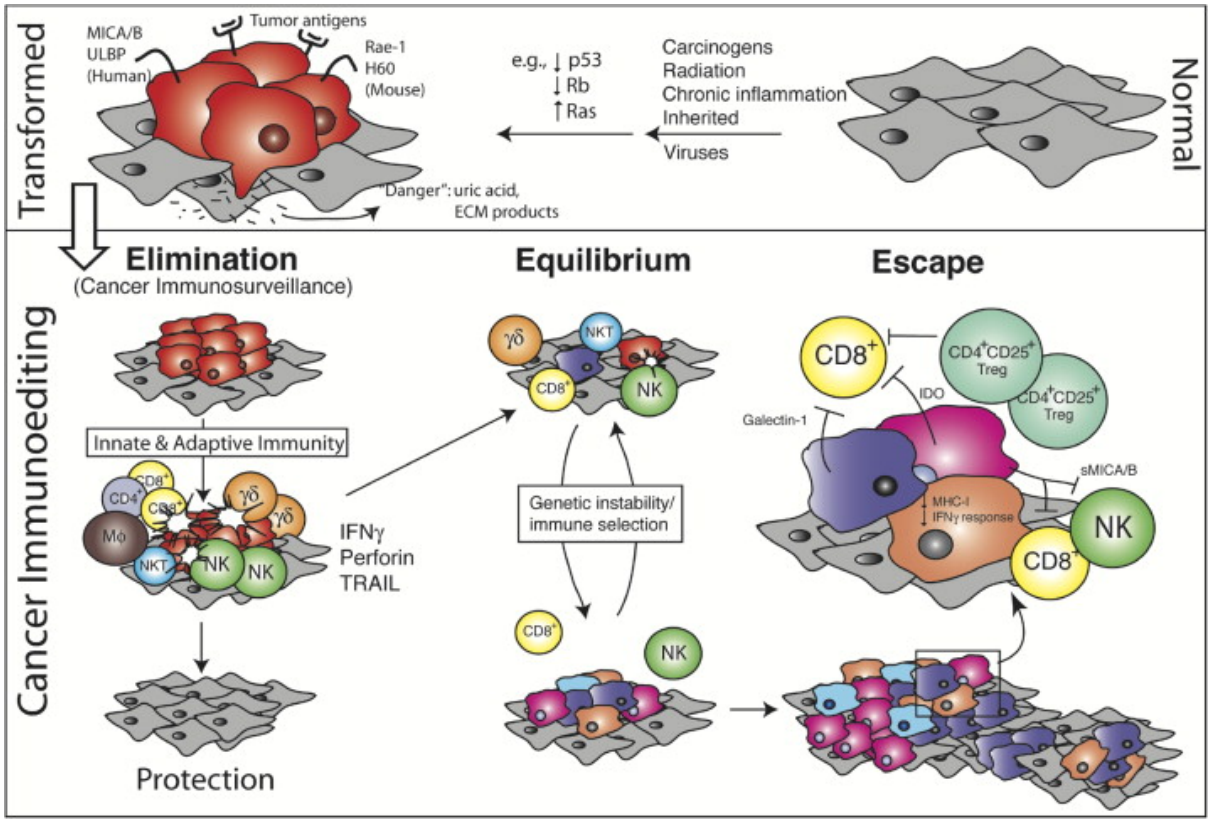


Figure 6. The three phases of the cancer immunoediting process (Dunn et al., 2004).

Normal cells (grey) subject to common oncogenic stimuli ultimately undergo transformation and become tumour cells (red) (top). Even at initial stages of tumorigenesis, these cells may express distinct tumour-specific markers and generate proinflammatory "danger" signals that initiate the cancer immunoediting process (bottom). In the first phase of elimination, cells and molecules of innate and adaptive immunity, which comprise the cancer immune-surveillance network, may eradicate the developing tumour and protect the host from tumour formation. However, if this process is not successful, the tumour cells may enter the equilibrium phase where they may be either maintained chronically or immunologically sculpted by immune "editors" to produce new populations of tumour variants. These variants may eventually evade the immune system by a variety of mechanisms and become clinically detectable in the escape phase (Dunn et al., 2004).

Thus, the theory of immune editing explained why despite early recognition of the *de novo* emergence of the cancer by the immune surveillance mechanism, the cancer still progressed to a clinical disease (and more so, in those with immunosuppression). It also explained the continued changes in antigen repertoire of cancers (consequent to mutations from selection pressure) resulting in escape from specific immune attack and the evolution of molecular mechanisms responsible for avoiding or actively suppressing host immunity. This, in practice, explains the difficulty in selecting the appropriate antigens in the design of anti-cancer vaccines and in circumventing the development of tumour immune evasion and

suppression, especially in advanced tumours, resulting in the failure of clinical response so often seen in most cancer vaccine trials (because only people with advanced cancers are included in trials as opposed to their testing in animals where tumours are of short duration and not so large or advanced). Thus, the existence and recognition of immune-editing should alert us to use as many antigens as is possible in our vaccine design with the inclusion of strong and effective adjuvants capable of mitigating tumour evasion and suppression. That is why we have chosen the whole cell, as opposed to single or a few specific antigens, as our source of antigens. Only in this way can we ensure quantitatively (number of antigens) and qualitatively (antigenicity) optimal antigenic material for the success of our cancer vaccine design.

2.5.3 "Danger" or "damage" signals: does their absence (quantitatively and qualitatively) really explain the poor anti-tumour immune response?

Given that cancer antigens exist, and our immune system recognizes them through ongoing immune surveillance mechanisms, it was appropriate to design cancer vaccines to augment cancer immunity for therapeutic purposes. To the surprise of many investigators, cancer vaccines prepared from cancer cells that were tested in animal models did not stimulate the type of immune response seen with microbial vaccines. In fact, cancer immune tolerance was observed. One aspect of the cancer immune-surveillance process that has been the subject of much controversy is whether the unmanipulated immune system can detect a developing tumour, even one that may express distinctive recognition molecules on its surface or contain tumour-specific antigens. In the past, it was argued that cellular transformation did not provide a sufficient proinflammatory or "danger" signal to alert the immune system to the presence of a developing tumour (Matzinger, 1994) (Pardoll, 2003). However, it was recently realized that (1) danger signals, such as uric acid, may arise from the inherent biology of the tumour itself and (2) induction of proinflammatory responses through the generation of

potential Toll-like receptor (TLR) ligands, such as heat shock proteins, or extracellular matrix derivatives, such as hyaluronic acid or heparan sulphates, may share similarities to the events that underlie activation of innate immune responses to microbial pathogens (Dunn et al., 2004).

The role of uric acid and other metabolic factors as “danger” signals has not been resolved conclusively (Pradeu & Cooper, 2012).Though Matzinger’s “danger” theory which hypothesized that the strength and direction of our immune response depended on the level of threat perceived by our body is a plausible explanation for the poor immune response elicited by cancer cells without adjuvants (immune-potentiators), this question has not yet been conclusively resolved (Pradeu & Cooper, 2012) (Matzinger, 1994).

2.5.4. The importance of the activation of the innate immune system for anti-tumour immunity

Therefore, cancer antigens exist, and the immune system recognizes them through immune-surveillance. Immune-editing is yet to occur and this takes place in the elimination or equilibrium phase. So, the next step in cancer immunity is the activation of the innate or primary immune system.

The activation of the innate immune system is a crucial step in cancer immunity as is the case with immunity in general (Coffman, Sher, & Seder, 2010). It is not clear whether this occurs because of “damage’ signals from the cancer cells themselves or from the adjuvants (immune-potentiators) administered with the cancer cells acting as “danger” or “damage” signal(s) (Pradeu & Cooper, 2012). Only by first activating the innate immune system, consisting mainly of DCs, macrophages, leukocytes, and certain cytokines, can the specific or adaptive immune system be mobilized. It was discovered that dendritic cells and macrophages were the vital link between the innate and adaptive immune responses (Steinman & Hemmi, 2006). Thus, adequately priming or stimulating these cells is the

necessary first step for an appropriate and desired anti-cancer immune response. In fact, the discovery of dendritic cells and their function in immunity by Ralph Steinman in 1973 was recognised by the Nobel Prize for Physiology in 2011 (Palucka & Banchereau, 2012).

After the discovery of dendritic cells, much research was devoted, and is still being devoted, to finding optimal ways of stimulating these cells and the innate immune system (Tacken & Figdor, 2011) (Steinman, 2011). In fact, our project is also testing whether our novel chimeric molecule will sufficiently stimulate the immune cells (macrophages and dendritic cells) responsible for the innate immune response to ultimately lead to specific anti-cancer immune response (Gardner, Alycia, & Brian, 2016).

So, how are these primary immune cells (dendritic cells and macrophages) of the innate immune system stimulated? Evolutionarily, humans evolved to recognize pathogens that were harmful to the body and destroy them. It was necessary, first, to urgently destroy and contain the pathogens until adaptive immunity for the pathogen developed later (Coffman et al., 2010). It was important to initially recognize broad categories of pathogens. Thus, pathogen recognition receptors (PRRs) evolved and these are found mainly in dendritic cells and macrophages (the first immune cells to encounter any invaders). These receptors recognize certain evolutionarily conserved molecular patterns common to most commonly invading organisms. These are collectively called pathogen-associated molecular patterns, PAMPs (Kaczanowska, Joseph, & Davila, 2013). One such PAMP is the specific unmethylated DNA found mostly in bacterial microbes. CpG ODNs that we are using in our experiment to create the novel chimeric molecule is a synthetic version of this PAMP (Scheiermann & Klinman, 2014). The binding of these PAMPS to PRRs (TLR 9 for CpG ODNs) then immediately triggers the primary immune attack, orchestrated by dendritic cells/macrophages (Palucka & Banchereau, 2012). It is thought that, unlike external pathogens harmful to us, cancers that arise internally do not possess PAMPs but rather

damage-associated molecular patterns, DAMPs. One example of a DAMP is uric acid, a product of cellular (including cancer cells) breakdown or necrosis. The workings of DAMP and their similarity in function to PAMP are still being investigated (Pradeu & Cooper, 2012). Since the function of PAMPs has been better elucidated and have been shown to work well in cancer vaccines, these are the types of molecules that are being tested as adjuvants in cancer vaccines. CpG ODNs are some of the most powerful PAMP-agonist in use today as adjuvant (H. Shirota & Klinman, 2014). Our novel vaccine design incorporates similar CpG ODNs. Intense research is ongoing to find an ideal DAMP for use as adjuvant for optimum anti-cancer immune stimulation (Temizoz, Kuroda, & Ishii, 2016).

After being primed by the interaction between PRRs and PAMPs/DAMPs/Adjuvants, dendritic cells and macrophages mature and then appropriately present cancer antigens in the context of the major histocompatibility complex (MHC) to T and B lymphocytes (mainly responsible for adaptive or specific immunity). The T- and B-lymphocytes the undergo activation to mount an effective and long-lasting adaptive cancer immune response (Gardner et al., 2016).

2.5.5 The specific anti-tumour immune system: the ultimate weapons against tumours represented by T-lymphocytes, B-lymphocytes, and their products

Ultimately, it is the adaptive immune system consisting mainly of T- and B-lymphocytes that can eradicate tumours and with the help of memory T-cells prevent their recurrence. Thus, the endgame of immunogenic cancer antigens, adjuvants, innate immune system and certain cytokines is to enhance and support the adequate functioning of this adaptive immune system (O. Finn, 2012; Palucka & Banchereau, 2012).

The central or bridging role of mainly dendritic cells between the innate and adaptive immune system has been established. The function of DCs in cancer immunotherapy has been extensively researched. In fact, active immunotherapy like cancer vaccines, works

mainly through DCs and/or macrophages. The optimization of vaccines, like our vaccine design aims to ensure adequate stimulation of DCs and macrophages (Palucka & Banchereau, 2012).

The adaptive immune system includes of two broad sets of antigen-responsive cells, the B and T lymphocytes. B lymphocytes are the precursors of antibody-producing cells (Spiering, 2015), and they use antibodies to target and destroy corresponding cancer antigens. Antibodies are capable of recognizing three-dimensional structures and thus can interact with and lead to the neutralization of pathogenic materials in extracellular fluid. B cell receptors recognize the same structures, and though it now appears that this recognition often occurs on cell surfaces, soluble molecules can certainly bind to B cell receptors and, when in a multivalent conformation, can elicit stimulatory signals in the B cell (W. E. Paul, 2011). By contrast, the T cell antigen recognition system is not adapted to the recognition of three-dimensional structures on the surface of pathogens. Rather, T cell receptors recognize a complex consisting of an antigen-derived peptide bound into a specialized groove in class I and class II major histocompatibility complex (MHC) molecules. As such, T cell recognition of antigen occurs on the surface of cells expressing these peptide/MHC complexes, often referred to as antigen-presenting cells (APCs) consisting mainly of DCs and macrophages (Madureira, de Mello, de Vasconcelos, & Zhang, 2015). These APCs or stimulated dendritic cells acquire enhanced capacity to process and present antigen, including the striking upregulation of class II MHC molecules; they increase their expression of potent costimulatory molecules such as CD80 and CD86 that allow them to efficiently activate those CD4 or CD8 T cells that have recognized antigen on their surface, and they secrete cytokines important for the differentiation of the activated T cells, such as IL-6, IL-12, and IL-23. Indeed, the pattern of cytokines that the dendritic cells produce and their efficiency in processing antigen, to a considerable extent, determine the phenotype that the differentiating

T cells will adopt. Among CD4 T cells, this would be whether they develop into Th1, Th2, Th17, or induced T regulatory cells and thus regulate immune responses designed to control distinct types of invasion. Cancer immunity relies mainly on a Th1-type response (William E. Paul).

2.5.6 Immune evasion and suppression: Counterattack by tumours

All is well and good for anti-cancer immunity with having immunogenic cancer tissue, enough intrinsic DAMP/PAMPs as “danger” signals/or extrinsic immune-stimulatory adjuvants capable of adequately priming DCs that could in turn lead to optimal activation of the specific or adaptive immunity as long as tumours have not entered the equilibrium or escape phase of immune-editing. Escape may result from the establishment of an immunosuppressive state within the tumour microenvironment or vice versa. Tumour cells can promote the development of such a state by producing immunosuppressive cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor–β (TGF-β), galectin, or an enzyme indoleamine 2,3-dioxygenase (IDO) and/or by recruiting regulatory immune cells that function as the effectors of immunosuppression. Regulatory T cells (Treg cells) and myeloid-derived suppressor cells (MDSCs) are two major types of immunosuppressive leukocyte populations that play key roles in inhibiting host-protective antitumor responses (Quezada, Peggs, Simpson, & Allison, 2011). Treg cells are CD4+ T cells that constitutively express CD25 and the transcription factor FoxP3. When stimulated, they inhibit the function of tumour-specific T lymphocytes by producing the immunosuppressive cytokines IL-10 and TGF-beta by expressing the co-inhibitory molecules CTLA-4, PD-1, and PD-L1; and by consuming IL-2, a cytokine that is critical for the maintenance of CTL function. MDSCs are a heterogeneous group of myeloid progenitor cells and immature myeloid cells that inhibit lymphocyte function by inducing Tregs cells, producing TGF-beta, depleting or sequestering the amino acids arginine, tryptophan, or

cysteine required for T cell function; or nitrating T cell receptors or chemokine receptors on tumour-specific T cells (Schreiber, Old, & Smyth, 2011).

Along with Tregs, MDSC, immunosuppressive cytokines, co-inhibitory molecules, tumour associated macrophages (TAM) also play a pivotal role in cancer immune suppression (Mantovani & Sica, 2010).

Maintaining the integrity and efficiency of cancer immunity requires the adequacy of all of the components listed above starting from cancer antigens to immune suppression. Thus, successfully designing cancer immune-therapeutics, as in this project, requires the consideration of all of the above elements.

2.6 Rationale for this particular cancer vaccine design

It has become quite clear that the future of cancer immunotherapy rests mainly on improving the three most vital and crucial components of cancer vaccines hopefully with clinical effect (Melief et al., 2015). These are:

i. cancer antigens
ii. cancer vaccine adjuvants
iii. preventing and reversing tumour immune evasion and suppression

2.6.1 Cancer antigens

The search for cancer antigens has led to the identification of hundreds of potential candidates (Cheever et al., 2009). It was also discovered that cancer antigens varied greatly among different cancers, even in the histologically same cancers in different people. Indeed cancer in the same person also varied depending on the site and there are suggestions that tumours at the same site may also contain different antigens. Immune editing is so powerful that it would not be surprising to have different antigens even at the same location or lesion (Ophir, Bobisse, Coukos, Harari, & Kandalaft, 2016). The ramification of such a finding is several fold, namely, the hope of finding a universal preventive or therapeutic cancer vaccine

became less probable and personalized therapeutic cancer vaccine seem now to be the most practical immunotherapeutic tool. Several recently completed randomised controlled trials with single antigens in lung cancer vaccines did not show any clinical benefit and an editorial published in response to this failure suggests the use of whole cell antigens in future trials (Mazza & Cappuzzo, 2016).

2.6.2 A549 lung cancer cell line as model antigen(s) for *in vitro* studies

One of the main challenges to cancer immunotherapy is finding the appropriate immune target (s) in the cancer cells/tissue itself. Most researchers have either focused on finding the ideal cancer antigen (s) and using them to design immune-therapeutics (Tagliamonte, Petrizzo, Tornesello, Buonaguro, & Buonaguro, 2014) or whole cancer cells/lysates with the aim of including all known and unknown cancer antigens (Ward et al., 2002). Based on the findings that not only do tumours (of the same type) differ in different people but also differ within a single lesion of the same person, it seemed to us that, at the present state of our knowledge on immunity, it was difficult to find universal cancer antigens for the cancer type (lung cancer) we are investigating (Ophir et al., 2016). Even if we found such a universal cancer antigen, one could not guarantee that the process of immune-editing would not result in its mutation and thus loss of antigenicity. Moreover, a meta-analysis of 173 published peer-reviewed immunotherapy trials in various solid tumor types revealed that patients immunized with whole tumour antigens had low but significantly higher rates of objective clinical response (8.1%) than patients immunized with molecularly defined tumour antigens (3.6%) (Mellman, Coukos, & Dranoff, 2011). Thus, our design is based on using whole cancer cells. The human lung cancer cell line A549 (Giard et al., 1973) is used in our design. It needs to be mentioned, that along with the advantage of including all possible antigens in the vaccine, there is also the disadvantage of including self-antigens that could cause tolerance (Ward et al., 2002) (Tagliamonte et al., 2014). It has been established that including

adjuvants (various kinds of molecules that enhance immunogenicity) with the whole cells or its components minimizes immune tolerance and enhances the quality of the immune response (Coffman et al., 2010).

The use of whole cancer cells as antigens was first pioneered by M.G. Hanna in 1978 (Hanna & Peters, 1978). Irradiated hepatocellular carcinoma cells were used as a vaccine in a guinea pig model and, admixed with *Bacillus* Calmette-guérin (BCG) as an immune adjuvant, they were found to generate protective immunity against subsequent challenge with syngeneic non-irradiated tumour cells. Based on this pre-clinical use of whole cell antigens, H.C. Hoover Jr and colleagues in 1993 carried out a phase III clinical trial. Patients with stage II/III colorectal cancer were vaccinated with irradiated autologous tumour cells and BCG and were randomised to be compared to surgery alone. Subgroup analysis revealed significant overall and disease-free survival for vaccinated patients. In addition, delayed type hypersensitivity (DTH) reactions to autologous tumour cells suggested the presence of tumour-specific immunity. Side-effects were minimal and consisted mostly of ulceration of the vaccination sites caused by the BCG adjuvant (Hoover Jr et al., 1993). Subsequent clinical trials of autologous cancer cells (including melanoma, renal cell carcinoma, and prostate cancer) with the use of additional adjuvants like GM-CSF gave mixed results. It was during one of these trials that the use of CpG ODN (type B) was used and there was no ulceration seen at the site of vaccination as was the case with use of BCG as adjuvant. Additionally, Jaffe and colleagues, also tested allogenic whole cell tumour (GVAX) vaccine from pancreatic cancer in patients with pancreatic cancer (Jaffee et al., 2001). GVAX vaccine from allogeneic prostate cells was also tested in a clinical trial. Generally, in all these trials using mainly whole cells as vaccine antigens, anti-tumour activity with a favourable median survival times and almost no side-effects were noted. The addition of check-point blockers, like the CTLA-4 blocker ipilimumab to the GVAX vaccine, was then tried to improve

response. Improved immune response was observed. Ultimately, since the cancer immune response depended on the appropriate activation of the innate immune system, targeting dendritic cells as the main link between cancer antigens and the adaptive immune system was crucial for any cancer vaccine design. Whole cell cancer vaccines with numerous ways of activating the dendritic cells have been tried and novel designs are still being experimented with as of today. Our project includes a novel design incorporating the adjuvant, CpG ODN, covalently attached to whole cancer cells for targeting dendritic cells/macrophages. Dendritic cells/macrophages are thought to be the main link to T-lymphocytes whose activation is required for the specific and long lasting anti-tumour immunity (De Gruijl, van den Eertwegh, Pinedo, & Scheper, 2008).

2.6.3 Cancer vaccine adjuvants

Vaccine adjuvants are immunological agents that function to enhance the magnitude, breadth, quality and/or longevity of specific immune responses generated against co-administered cancer antigens (Ag). Adjuvants are also used to reduce the dose and frequency of immunizations required to achieve protective immunity. Historically, vaccines were produced from live attenuated or heated-inactivated organisms. While not appreciated at the time, those original vaccines contained bacterial contaminants that served as adjuvants. There are several ways in which an adjuvant can promote immunity including:

(1) Stabilizing or entrapping the antigen (Ag) to extend release time and thus prolong immune stimulation; (2) promoting an inflammatory response at the site of Ag deposition thereby attracting activated macrophages and dendritic cells to improve Ag uptake and presentation;

(3) presenting co-stimulatory signals to T and B cells to enhance induction of Ag-specific immunity.

There is considerable interest in identifying safer and more effective adjuvants to enhance the utility of novel vaccines targeting infectious pathogens, allergy and cancer. In support of these goals, immunologists and microbiologists have sought to elucidate the mechanism(s) of action of adjuvants. Notable success was achieved in the discovery of Toll-like receptors (TLRs) and their role is promoting innate and adaptive immune responses, leading to a Nobel Prize for Physiology for Hoffmann and Beutler in 2011 (Hidekazu Shirota, Tross, & Klinman, 2015). Toll-like receptors (TLRs) are an important component of the host's pathogen sensing mechanism. The molecular structures recognized by TLRs have been evolutionarily conserved and are expressed by a wide variety of infectious microorganisms, and are termed pathogen-associated molecular patterns (PAMPs). The innate immune response elicited by TLR activation is characterized by the production of pro-inflammatory cytokines, chemokines, type I interferons and anti-microbial peptides. This innate response promotes and modulates the adaptive immune system.

Therefore, most vaccines contain adjuvants. They are mainly classified as either delivery systems (carriers) or immune-potentiators based on their dominant mechanism of action. CpG ODN adjuvant is classified as an immunopotentiator as it boosts the immune response of the innate immune system mostly by activating various classes of pattern recognition receptors (PRRs) specifically the toll-like receptors (TLRs). However, if the adjuvant promotes the uptake of antigen by antigen presenting cells (APCs), it is called a delivery system. Adjuvants that have been traditionally used for vaccines against common infections, like alum, poorly induce even a Th2-response, not to mention the Th1-response necessary for cancer eradication. Thus, they do not work for cancer immunity but for immunity to important chronic infections like malaria and AIDS. However, for our purpose, the most interesting class of adjuvants is that which includes compounds that serve as ligands for PRRs and induce innate immunity by targeting APCs leading ultimately to the indirect activation of the

adaptive immune system. Besides being effective, ideal adjuvants need to be safe. The difficulty of the immune system to target cancer cells lies primarily in the partial recognition of these cells and the production of signals by cancer cells which suppress the effect of B-cells and cytotoxic T-cells. Therefore adjuvants, the non-specific agents that enhance the immune response, are used in cancer vaccines to stimulate the immune system to fight against a specific target and break through the barriers that cancer cells build in order to protect themselves against B- and T-cells. Adjuvants used in cancer vaccines stimulate the immune system to produce a strong response against these cells (Banday et al., 2015).

On searching the literature, we found that CpG ODNs, among several potential adjuvants, are very promising as adjuvants due to their capacity, when included in vaccines, to induce a strong immune response with practically no toxicity (H. Shirota & Klinman, 2014).

2.6.4 CpG ODNs as cancer vaccine adjuvant

Among all the TLRs, only TLR 3, 7,8, and 9 recognize nucleic acids. Of these, TLR9 specifically recognizes CpG ODNs. TLR9 is activated by CpG motifs (consisting of a central unmethylated CG dinucleotide embedded within specific flanking regions) present at high frequency in bacterial DNA. In humans, TLR9 is expressed primarily by plasmocytoid DC (pDCs) and B cells. Reflecting the utility of TLR9 agonists as vaccine adjuvants, B lymphocytes exposed to TLR9 agonists become more susceptible to activation by antigens (Ag). Furthermore, TLR9 agonist-stimulated pDC produce type I interferons and more efficiently present Ag to T-lymphocytes. The signalling pathway activated when CpG interacts with TLR9 promotes the sequential recruitment of myeloid differentiation factor 88 (MyD88), IL-1R-associated kinase (IRAK), and tumour necrosis factor receptor-associated factor 6 (TRAF6). This signalling cascade subsequently leads to the activation of several mitogen-activated protein kinases (MAPK) and transcription factors (such as NF-kB and AP-1) and the transcription of pro-inflammatory chemokines and cytokines. Ultimately, these

molecular signalling pathways lead to the activation of both the primary and the adaptive immune systems (Hidekazu Shirota et al., 2015).

In humans, four distinct classes of CpG ODN have been identified based on differences in structure and the nature of the immune response they induce. Although each class contains at least one motif composed of a central unmethylated CG dinucleotide plus flanking regions, they differ in structure and immunological activity. K-type ODNs (also referred to as B-Class ODN) contain from one to five CpG motifs typically on a phosphorothioate backbone. This backbone enhances resistance to nuclease digestion and substantially prolongs *in vivo* half-life (30–60 min compared to only 5–10 min for a phosphodiester backbone). K-type ODNs trigger pDC to differentiate and produce TNFα and stimulate B cells to proliferate and secrete IgM. D-type ODNs (also referred to as A-Class) have a phosphodiester core flanked by phosphorothioate terminal nucleotides. They carry a single CpG motif flanked by palindromic sequences that enables the formation of a stem-loop structure. D-type ODNs also have polyG motifs at the 3' and 5' ends that facilitate concatamer formation. D-type ODNs trigger pDC to mature and secrete IFNα but have no effect on B cells. C-type ODNs resemble K-type in being composed entirely of phosphorothioate nucleotides but resemble D-type in containing palindromic CpG motifs that can form stem loop structures or dimers. This class of ODN stimulates B cells to secrete IL-6 and pDC to produce IFNα. P-Class CpG ODN contains double palindromes that can form hairpins at their GC-rich 3' ends as well as concatamerize due to the presence of the 5' palindromes. These highly ordered structures are credited with inducing the strongest type I IFN production of any class of CpG ODN. CpG ODN should be particularly useful as adjuvants for vaccines targeting cancer that require the type of strong CTL response elicited by pDC activation.

TLR9 activation also induces human memory B cells to proliferate, undergo class switching to IgG2a and secrete antibodies in a T cell independent manner. By comparison, naive human

B cells express low levels of TLR9 and do not respond directly to CpG ODN. Ag stimulation via the B cell receptor induces naive B cells to up-regulate TLR9 expression and acquire responsiveness to CpG DNA. The requirement that naive B cells interact with cognate Ag before acquiring responsiveness to CpG prevents polyclonal B cell activation and reduces the risk of autoimmunity.

To optimize the efficiency of Ag presentation by DCs requires that they encounter CpG ODN in the presence of vaccine Ag. Co-delivery of ODN plus Ag to the same APC accelerates the induction, increases the maximal level and extends the duration of the induced immune response. It also supports modulation of Ab isotype and increases the immunogenicity of weak Ags. Murine studies show that conjugating CpG ODN directly to Ag can boost immunity by up to 100-fold over that induced by simply mixing CpG ODN with immunogen. The mechanisms by which CpG ODN-Ag conjugates enhance immunogenicity include insuring that both Ag and TLR agonist are taken up by the same APC and improving such uptake via DNA-binding receptors on the APCs (the latter effect is independent of the nature of the ODN but requires physical conjugation of DNA to target antigen).

Moreover, local i.e. intra-tumoural injection of CpG ODNs has been shown in many studies to also mitigate against tumour immune suppression (Hidekazu Shirota et al., 2015).

2.6.5 Addressing cancer immune evasion and suppression

Finally, though not directly related to our vaccine design, it is necessary to mention here that in human trials tumour evasion and immune suppression are a very pertinent and important issue that needs to be addressed for vaccines to be clinically effective. Most vaccines, though successful in animal models (where the tumour is small and of short duration) have subsequently failed in human clinical trials where participating patients have advanced and large tumours of long duration. The failures in clinical trials are due to both tumour evasion and suppression by the advanced cancer. There is evidence that CpG ODN, used as an

adjuvant in our vaccine design, also ameliorates cancer immune evasion and immune suppression (Hidekazu Shirota et al., 2015). Of course, in the future, when testing our vaccine design in advanced cancers, we will have to address the issue of cancer immune evasion and suppression more aggressively by incorporating the newly-discovered and clinically-proven immune check-point blockers as well.

To some extent, we are addressing tumour immune evasion and suppression by incorporating the vaccine adjuvant CpG ODN. This adjuvant, acting as an immunopotentiator, has shown in studies to also possess immune suppression mitigating effect (Y. Shirota, Shirota, & Klinman, 2012).

2.7 **Gaps, limitations, and conclusion of existing research**

During literature search, it was noted that despite the excellent safety profile of CpG ODN it was not commonly used as adjuvant. Moreover, research has shown that varying the sequences, motifs, and the lengths of the diverse types of CpG ODNs could produce adjuvants of varying degree of functional capacity. This could be further explored by computer modelling techniques (Aoshi, Haseda, Kobiyama, Narita, Sato, Nankai, Mochizuki, Sakurai, Katakai, & Yasutomi, 2015; Pohar, Krajnik, Jerala, & Benčina, 2015; Pohar, Lainšček, et al., 2015). Also, the discovery that physically combining CpG ODNs with antigens is almost hundred times more potent than when given apart, construction of such molecules has not been tried sufficiently (Tom, Mancini, & Esser-Kahn, 2013).

Apart from uric acid and a few other DAMP molecules as by-products of cancer metabolism, not enough is being done to search similar but potentially more potent DAMPs as adjuvants in cancer vaccines. Additionally, biological modification (using varying physical and chemical methods) of cancer cells to produce antigenically more potent cells has not been explored sufficiently (Herbáth et al., 2014).

The ever present immune-editing process, the ensuing resistant tumours, and thus antigenically distinct cancer cells even in the same person makes the use of single or even a few antigens as vaccines theoretically futile. So, there should be more experimentation using whole cells or various fragments of it in future designs (Mazza & Cappuzzo, 2016).

Chapter 3: Methodology

This chapter will elaborate on our research questions, the conceptual framework, hypotheses generated by them, the roadmap for action with the study aims and objectives, experimental design, and application.

3.1 Research questions

The following are our scientific queries in search for a vaccine design that could be used to cure lung cancer or improve its management:

1. Is it possible to covalently and stably attach immune-potentiating adjuvant, CpG-ODN (a potent and relatively safe adjuvant) onto A549 lung cancer cells to form a novel chimeric molecule?
2. And then, will this novel chimeric molecule induce a strong anti-tumour immune response?

3.2 Aims and objectives

This study intends to design and create a chimeric molecule possessing the capacity to induce strong anti-tumour immune response to human lung cancer cells ultimately leading to the creation of personalized therapeutic vaccine for lung cancer patients using their own (autologous) lung cancer cells/tissue.

The study foresees the following objectives:

1. To design, create, and test the formation of a chemically stable chimeric molecule using a synthetic analogue of a naturally occurring microbial adjuvant, CpG-ODN covalently bound to the membrane of the lung cancer cell line A549 with the help of a bi-directionally functional and covalently binding chemical linker BS^3.
2. To test the effectiveness of this chimeric molecule in inducing anti-cancer immune response by quantitatively measuring the levels of cytokines, IL-6 and TNF-α, as surrogate markers of anti-tumour immune response, released by macrophages resulting from their interaction with the chimeric molecule.

3.3 Hypotheses

*Alternate hypothesis 1***:** CpG-ODN will stably attach to A549 cancer cells with the help of linker BS^3 and form the envisaged chimeric molecule.

Null hypothesis 1: CpG ODN and A549 cells do not combine to form a stable chimeric molecule.

Alternate hypothesis 2: The interaction between this novel chimeric molecule and macrophage will produce strong anti-lung cancer immune response.

Null hypothesis 2: The interaction between the novel chimeric molecule and macrophage does not produce strong anti-tumour immune response.

3.4 Conceptual framework

Evolutionarily, in the struggle for survival, the human immune system has developed a mechanism to detect invading harmful microbes quite early in the course and diffuse them. The immune cells have receptors (e.g. PRRs like TLR-9) to detect microbial DNA in order to stimulate strong and prompt anti-microbial immune response while minimizing auto-immune sensitization (Venereau, Ceriotti, & Bianchi, 2015). These toll-like receptors (e.g.TLR-9) react specifically with microbial DNA containing unmethylated CpG motifs (human DNA is mostly methylated) to stimulate immune response against these invaders through the release

of cytokines, activation of primary immune cells (mainly DCs and macrophages), and consequently priming of the adaptive immune system (mainly T- and B-lymphocytes).

But, it has been suggested that cancer cells, arising in the body itself, in not seen as dangerous as foreign microbes. Thus, to boost anti-cancer immunity, it is useful to fool the body into believing (imitate microbial-like condition) that it is being invaded by microbes by synthetically designing specific oligodeoxynucleotides (ODN) to accompany cancer cells as antigens (O. J. Finn, 2008). It is thus hoped that our body will see cancer cells as dangerous antigens and mount a strong immune response to destroy them. Of course, the physical proximity of the adjuvant and the antigen is also important for such a response (Parish, 2003).

There are a few studies that have demonstrated that combining cancer cells or other antigens with such synthetic deoxyribonucleic acid (DNA) in varying ways strongly boosts anti-cancer immune response (Hidekazu Shirota & Klinman, 2011; Y. Shirota et al., 2012). It needs reconfirmation that this works equally well with other important cancers like lung cancer cells (A549 cell line).

Here the independent variables (categorical/nominal) are the chimeric molecules containing either live or apoptotic states of A549 cancer cells, type A or B CpG ODNs at different concentrations, and 1 mM or 2 mM concentrations of the chemical cross-linker bis[sulfosuccinimidyl] suberate (BS^3) in various combinations and the controls (positive and negative) and the dependent (outcome) variables (ratio/continuous)- the cytokines (Interleukin-6) IL-6 and (Tumour Necrosis Factor-alpha) TNF-α.

3.5 Study design

Our hypothesis testing is best done with an experimental design in a laboratory where we can culture almost identical cells, adequately characterize molecules, accurately measure variables, and adequately control experimental conditions using most precise and accurate instruments. Such a design (with good control of the experiment including the inclusion of

positive and negative controls) will likely be the most suitable in our case to prove the causal effect of our chimeric molecules (independent variables) on the release of anti-cancer immuno-stimulatory cytokines, IL-6 and TNF-α (dependent variables) from macrophages. Effects of other possible confounding variables apart from our independent and dependent variables will be controlled by tightly controlling the experimental conditions and by assuring similarity in other characteristics in the intervention and control groups except for the independent variables. This, along with the inclusion of negative and positive controls, will improve on the validity of the result of our study. And, by creating experimental conditions as similar to physiological conditions as possible (but practically very difficult to recreate exact physiological conditions), we will try to maximize generalizability of the experimental outcomes (David Machin, 2007; Geoffrey Marczyk, 2005; Montgomery, 2017).

The study sample and target population will comprise of macrophages (differentiated from U937 cells). These cells will be cultured from a single cell line under similar conditions to give almost identical cells. Apart from the intervention groups, there will be both positive (lipopolysaccharide from E. coli bacteria) and negative (A549 cancer cells, CpG ODN, and macrophages each alone or combined with each other) control groups. The data from the experiment will be collected using similar samples prepared at different time points and tested on separate occasions. Please see the experimental design in Tables 1,2, and 3 below (all abbreviations have been elaborated just below Table 3).

Table 1. Experimental design for the creation of the chimeric molecules in a 6-well plate

	1	2
A	Live A549 cells (LC)	LC+ODN2+BS3b (S2)
B	Apoptotic A549 cells (AC)	AC+ODN1+BS3a (S3)
C	LC+ODN1+BS3a (S1)	AC+ODN2+BS3b (S4)

Table 2. Experimental design for the interaction between chimeric molecules, controls, and macrophages in a 12-well plate

	1	2	3	4
A	LC+MP	S2+MP	ODN1+MP	LPS2+MP
B	AC+MP	S3+MP	ODN2+MP	MP only
C	S1+MP	S4+MP	LPS1+MP	

Table 3. ELISA (96-well plated) test for the quantitative measurement of TNF-α and IL-6 (column 1 and 12 are standards with corresponding blanks and column 3,4, and 5 are samples and controls in duplicate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	4000 pg/ml		LC+MP	LC+MP	LPS1+MP							4000 pg/ml
B	2000 pg/ml		AC+MP	AC+MP	LPS1+MP							2000 pg/ml
C	1000 pg/ml		S1+MP	S1+MP	LPS2+MP							1000 pg/ml
D	500 pg/ml		S2+MP	S2+MP	LPS2+MP							500 pg/ml
E	250 pg/ml		S3+MP	S3+MP	MP only							250 pg/ml
F	125 pg/ml		S4+MP	S4+MP	MP only							125 pg/ml
G	62.5 pg/ml		ODN1+MP	ODN1+MP								62.5 pg/ml
H	Blank diluent		ODN2+MP	ODN2+MP								Blank diluent

Note: LC= 1 million/mL live A549 cells; AC= 1 million/mL apoptotic A549 cells; ODN1=10 µg of CpG-ODN 7909/per $1x10^6$ cells; ODN2=40 µg of CpG-ODN D35/per 1 $x10^6$ cells; BS3a and BS3b=1mM and 2mM bis[sulfosuccinimidyl] suberate respectively; MP=1 million/mL macrophages differentiated from U937 cells; LPS1and LPS 2=lipopolysaccharide with 50 ng/ml and 100 ng/ml respectively.

There are eleven independent variables (samples and controls) and two dependent (outcome) variables (Il-6 and TNF-alpha). The independent variables are as designated in Table 1 and 2 and are used in this study as categorical variables of nominal/ordinal levels. The two dependent variables are of ratio/continuous level.

The data collection method is through laboratory tests. Concentrations of the chemicals and substances are measured in the metric scale using laboratory instruments. CpG ODNs is measured in microgram (µg)/per number of cancer cells and BS^3 is measured in millimoles (mM) and the desired concentration of the stock solutions of each is prepared in the

laboratory from the specified amount procured from the supplying company. The number of cancer cells and macrophages necessary for the experiment is cultured using respective specific culture medium, culture flasks, and placed in an incubator under standard conditions. The number of cells used for the experiment is counted using a Haemocytometer. Similarly, IL-6 and TNF-alpha is measured in picograms (pg)/ml based on the optical density (OD) readings of the tested samples or controls in wells on microplates with the help of the micro-plate reader in which absorbance is set at 450 nm. The mean absorbance for each standard, sample, and control is measured and the resulting concentration of samples and controls in pg/ml is derived from the plotted standard curve based on optical density and concentration readings of serially diluted standard solutions.

This being an (laboratory) experimental *in vitro* design, most errors and bias would result from the imprecision and inaccuracy of measuring instruments, improper maintenance of experimental conditions, and certain procedural errors. Thus, to minimize these errors, the calibration of measuring instruments was done on a regular basis and new instruments were validated before use. The maintenance of necessary experimental conditions was meticulously verified. Procedural errors were minimized by attentively and strictly following steps mentioned in the experiment protocols.

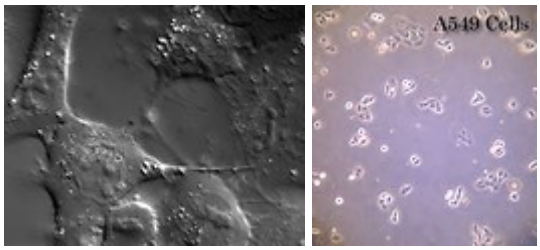
The limitations of the study stem mainly from the fact that this *in vitro* design will not be able to reflect the physiological conditions and responses as *in vivo* when this experiment is repeated in the next stage in animal models. For example, the physiological homeostasis is naturally maintained *in vivo* experiments but impossible to replicate fully in *in vitro* experiments. *In vitro* experiments cannot foresee or imitate *in vivo* stress-related influences (live organisms) on the outcome variables. The dose of the variables used for *in vivo* experiment in the future may not correlate with *in vitro* experimental doses. Thus, the

generalizability of the outcome of our experiment may be limited due to these limitations (David Machin, 2007; Geoffrey Marczyk, 2005; Montgomery, 2017).

3.6 Materials, reagents, and equipment

3.6.1 A-549 cancer cells

These cells were procured from American Type Culture Collection (ATCC), a not-for-profit, US based organisation. The A549 cell line (ATCC®: CCL-185™) was established in 1972.



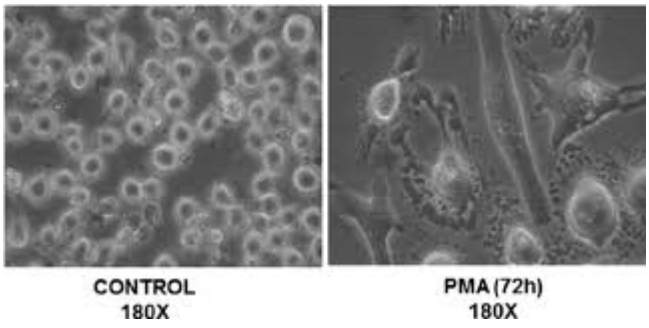
Scan 1. Microscopic images of A549 cells

The cells originate from an explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male (Giard et al., 1973). These cells grow as an adherent monolayer and can be used as a transfection host. The base medium for this cell line is Dulbecco's modified Eagle's medium (DMEM). The maintenance of cultures at a cell concentration between 6 x 10^3 and 6 x 10^4 cell/cm^2 and a sub-cultivation ratio of 1:3 to 1:8 with medium renewal of 2 to 3 times per week is recommended (ATCC, 2017). It is one of the major components (cancer antigens) constituting our novel chimeric molecule. The cells were cultured in CELLSTAR® T-25 and T-75 tissue culture flasks with 5-7 and 15-20 mL of complete culture medium (CCM) containing 90% DMEM, 10% foetal bovine serum (FBS), 2mM L-glutamine, and 1% Penicillin-Streptomycin respectively in an incubator at standard cell culture conditions (humidified air 95%, CO_2 5%, and at 37^0 C temperature), SCCC. These cells were harvested after the third passage and at about 70-80% confluency with no signs of contamination. Before starting any experiment A549 cells were passaged at least three times after thawing.

Furthermore, a maximum of 20 passages (after thawing) was not exceeded. It proved to be a robust cell line with an approximate population doubling time of 24 hours. This can be calculated from total cell counts during each sub-culturing procedure and monitored over time as a quality criterion. If this value changes or fluctuates over time cell viability after trypsinisation can be checked as an additional quality control parameter. A healthy culture should contain at least 80% viable cells and that was confirmed during the experiment. All procedures were carried out under sterile conditions in a laminar flow cabinet (SC-LFC) of the model TOPSAFE 1.8 ABC from Bio Air Company maintained on a regular interval. Only sterile equipment was used during cell handling and personal safety precautions were taken by the regular use of laboratory coat and gloves (Hirsch, 2014).

3.6.2 U937 cells

These cells were also procured from ATCC. U937 cell line (ATCC® CRL-1593.2™) was derived from the pleural effusion of a 37-year old male Caucasian patient with generalized histiocytic lymphoma (Sundström & Nilsson, 1976). They are suspension cells. This cell line is suitable as a transfection host. The base medium for this cell line to grow is Roswell Park Memorial Institute 1640 (RPMI-1640). Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at 1 to 2 x 10^5 viable cells/mL. Cell density is maintained between 1 x 10^5 and 2 x 10^6 viable cells/mL. Fresh medium is added every 3 to 4 days (depending on cell density) for cell renewal (ATCC, 2017).



Scan 2. Microscopic images of U937 cells before and after treatment with PMA

These cells were also cultured in CELLSTAR® T-25 and T-75 tissue culture flasks with 5-7 and 15-20 mL of complete culture medium (CCM) containing RPMI-1640 90%, foetal bovine serum (FBS)10%, 2mM L-Glutamine, and 1% Penicillin-Streptomycin (CCM-RPMI-1640) at SCCC.

The needed number of cells for the experiments were harvested after the third passage when the cell growth was optimum with no signs of contamination. These cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA) (Park et al., 2007).

3.6.3 Mitomycin C (MMC)

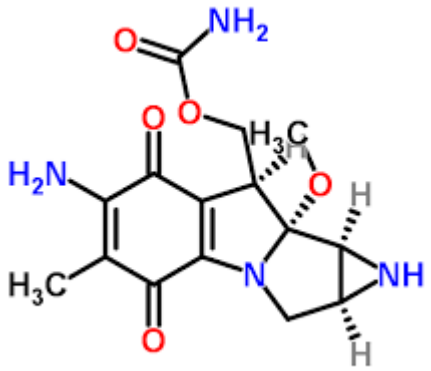


Figure 7. Chemical structure of mitomycin C

This molecule was purchased from MedChemExpress, a US based company. It belongs to a class of antibiotic with anti-cancer properties. MMC is also used for inducing cell apoptosis by enhancing TNF-related apoptosis-inducing ligand, TRAIL, through the downregulation of various cell survival proteins, upregulation of various apoptotic proteins, and upregulation of TRAIL receptors. The upregulation of death receptors by MMC is mediated by c-Jun N-terminal kinase (Cheng et al., 2012; Simamura, Hirai, Shimada, & Koyama, 2001). We used MMC to induce apoptosis of our A549 cancer cells (Simamura et al., 2001). Mitomycin C is dissolved in dimethyl sulfoxide, DMSO, and stored, and then diluted with appropriate

medium before use. Five milligrams (mg) of MMC was purchased and on receiving was diluted in one milliliter (mL) of DMSO making it into a concentration of 5 mg/ml as stock solution and stored at -80^{0}C. The concentration used for the apoptosis of our A549 cells was 10 µg/ml (Hidekazu Shirota & Klinman, 2011).

3.6.4 Phorbol 12-myristate 13-acetate (PMA)

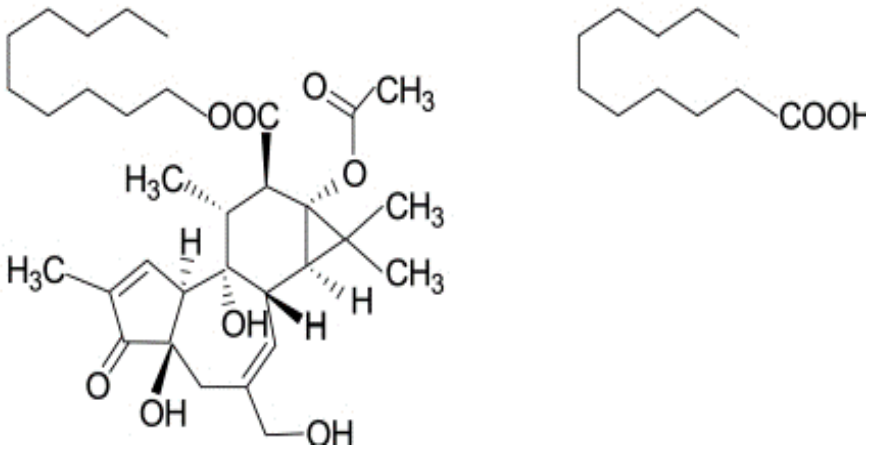


Figure 8. Chemical structure of PMA

PMA was also purchased from MedChemExpress. It is a well-studied differentiation-inducing chemical agent and an analogue of diacyl glycerol (DAG), which is a strong activator of protein kinase C (PKC). PMA treatment stimulates PKC signalling cascade in U937 to alter the expression of a wide range of genes via multiple transcription factors, including nuclear factor-κB (NFκB) and activator protein-1 (AP-1) (Song et al., 2015; Wasaporn & 2015). PMA is dissolved in DMSO and stored, and then diluted with appropriate medium before use. PMA should be stored in the freezer at – 20^{0}C and protected from light. Five milligram (mg) of PMA was dissolved in one milliliter of DMSO to make a stock solution of 5 mg/ml. We used 100 ng/ml concentration of PMA to induce differentiation of our U937 cells to macrophages. Since it is also a potent promoter of skin tumour in mice, precaution should be taken while handling it (Chang et al., 2005).

3.6.5 NH_2-modified and 6-FAM tagged cytosine-guanine triphosphate oligodeoxynucleotides (CpG ODNs)

NH_2-modified and 6-FAM tagged CpG ODNs 2006/7909 (Class B or type K) and D35 (Class A or type D) were ordered and purchased from GeneWorks Ltd, Australia.

Table 4. Synthetic oligonucleotides used in this study

Oligo Name	Sequence	CpG motif	Backbone	Size
ODN2 (D35, Class A, D-type)	5'NH_2-GsGTGCAT**CpG**ATGCAGGGGsGsGs-6FAM 3'	1	Phosphodiester and phosphorothioate	20mer
ODN1 (7909, Class B, K-type)	5' NH_2-Ts**CpG**sTs**CpG**sTsTsTsTsGsTs**CpG**sTsTs TsTsGsTs**CpG**sTsTs-6FAM 3'	4	Phosphorothioate	24mer

Note: "s" indicates phosphorothiate backbone; "p" indicates phosphodiester backbone in the motif "CpG"; neither "s" nor "p" indicates natural phosphodiester bonds

The structural characteristic of CpG ODN class A (D-type) is comprised of phosphorothioate (synthetic) and phosphodiester (natural) bonds with single CpG motif and CpG flanking region forming a palindrome with poly G tail at 3' end. Consequently, ODN D-type has the propensity to aggregate, hindering its immune-stimulatory activity (Bode, Zhao, Steinhagen, Kinjo, & Klinman, 2011). CpG ODN D35 (A-class /D-type) mainly target plasmocytic dendritic cells (pDCs) to induce interferon alpha (IFN-α), thereby enhancing APCs maturation and natural killer (NK) cells activation (Klinman, Currie, & Shirota, 2006). CpG ODN D35 was modified with the addition of phosphorothioate at the 3' end to develop non-aggregating CpG ODN D35, but still maintaining its strong IFN-α inducing property (Aoshi, Haseda, Kobiyama, Narita, Sato, Nankai, Mochizuki, Sakurai, Katakai, Yasutomi, et al., 2015). Another study also demonstrated CpG ODN D35 to possess immune-stimulatory activity by stimulating peripheral blood monocytic cells (PBMC) with CpG ODN D35 *in*

vitro for 72h (Puig et al., 2006). This study showed that CpG ODN D35 enhances secretion of IFN-α, IL-6, and IFN-γ. According to this study, CpG ODN D35 also induced monocytes to mature to myeloid dendritic cells after 48h in culture by conferring expression of CD38, CD86, CD14 MHC II and CD40. However, the study of CpG ODN D35-conjugated whole tumour cell vaccines has not been studied before. Therefore, we utilised CpG ODN D35 to increase A549 immunogenicity aiming to maximise host's immunity against A549 lung cancer.

The immune-stimulatory effect of CpG ODN 7909/2006 (Class B/K type) as a cancer vaccine adjuvant was established from multiple clinical trials. In contrast to D-type ODN, K-type ODN have phosphorothioate backbone with multiple CpG motif, inducing the production of monocytes and B cells to promote IL-6, IL-12 and IgM (Verthelyi et al., 2002). Moreover, K-type ODN are rapidly transported to late endosomes, whereas D-type ODN are retained in the early endosomes interacting with MyD88/IRF-7 complexes, a signalling cascade that activate IFN-α production (Gan, Debra, & Dennis, 2011). These distinct activities of K-type ODN and D-type ODN have raised interests in our study to compare their immune-stimulatory activity as a cancer vaccine adjuvant. CpG ODN 7909 plus recombinant New York-oesophageal cancer (NY-ESO)-1 protein generated a stronger and more rapid CD8+ T-cell response against tumours expressing NY-ESO-1 protein. Therefore, the induction of tumour-reactive CD8+ T-cell is believed to play an essential role in eradicating tumours (Bode et al., 2011). Based on these publications, CpG ODN D-type and K-type are considered to be strong cancer vaccine adjuvant for our experiment.

CpG ODNs are synthetic oligodeoxynucleotides that mimic unmethylated bacterial DNA and act as PAMP to stimulate specific PRR, the TLR-9, on immune cells like macrophages and dendritic cells. Both classes of CpG ODNs will be used in our experiment to form our novel chimeric molecule together with live and apoptotic whole A-549 cancer cells. The

oligodeoxynucleotides on receipt were immediately kept at -20^{0}C and solutions for use made thereafter before the experiment. CpG ODNs 2006/7909 and D35 were named ODN-1 and ODN-2 respectively. Each vial of both types of ODN contained 474 µg. Thus, resuspending each ODN with 474 µl of water produced 1 mg/ ml concentrated solution (Technologies, 2017).

3.6.6 Bis[sulfosuccinimidyl] suberate, BS3

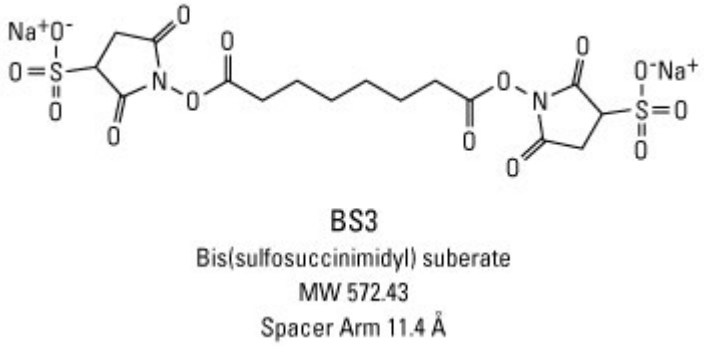


Figure 9. Chemical structure of BS3

It is the molecule that links CpG ODNs to A-549 cancer cells to form the chimeric molecule. It is supplied as a sodium salt and is water-soluble up to 10 mM. It was procured from CovaChem, a US-based company. The molecule being very moisture sensitive, it was immediately stored upon receipt in a dry condition at 4°C and opened for resuspension only after the vial had attained room temperature. This cross-linker is prepared immediately before use because the NHS-ester moiety readily hydrolyses and becomes non-reactive. The solution of BS3 was made immediately before the experiment by dissolving in water. To make a solution of 25 mM, 700 µL of water was added to 10 mg of BS3. The concentration of 1 and 2 mM that was needed for the conjugation reaction was prepared by further diluting it in ultra-pure distilled water.

3.6.7 Lipopolysaccharide (LPS)

It was purchased from Sigma-Aldrich, a US-based company. It is a major component of the outer membrane of gram-negative bacteria (Stromberg et al., 2015) and has long been

recognized as a key factor in septic shock in humans and, more generally, in inducing a strong immune response in normal mammalian cells (Rosenfeld & Shai, 2006).

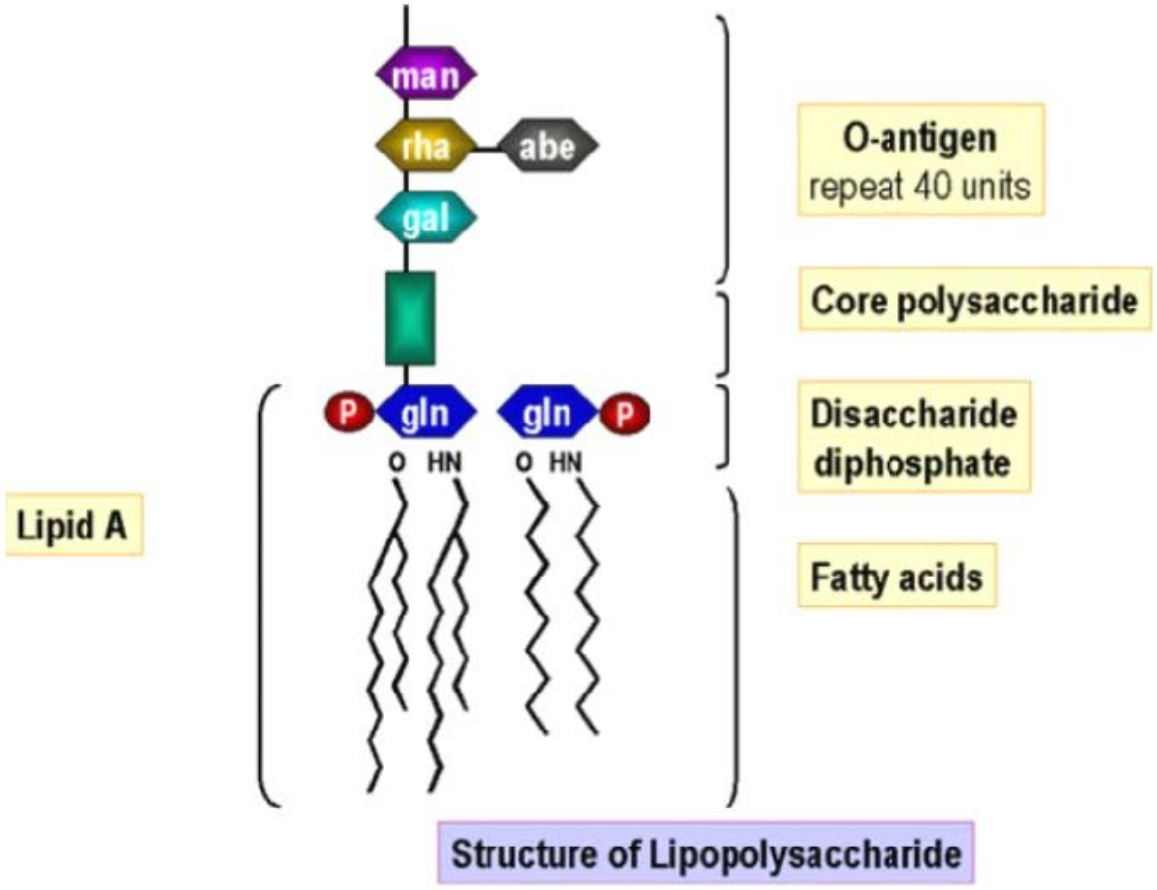


Figure 10. Chemical structure of LPS

(Source: South Carolina School of Medicine)

It is considered to be the most potent activator of the macrophage secretory response. Tumour necrosis factor alpha (TNF-α) is one of the earliest major proinflammatory mediators secreted by macrophages when stimulated with LPS in vivo and in vitro (Takashiba et al., 1999). PMA-differentiated macrophages from U937 cells were stimulated with 50 ng/mL and 100 ng/mL E. coli derived LPS (Park et al., 2007). This was used as a positive control in our experiment.

3.6.8 Laser Scanning Confocal Microscope, LSCM (Nikon)

Scan 3. Confocal microscope A1+ (Nikon)

Nikon confocal microscope system A1^{+} provides high-resolution imaging of up to 4096 x 4096 pixels with a galvano (non-resonant) scanner with diverse innovative optical and electronic technologies and superior image quality.
Source: https://www.microscopyu.com/tutorials/laser-scanning-confocal-microscopy

The above model confocal microscope from Nikon company, Japan was used for verifying the stable conjugation of CpG ODNs to A549 cancer cells.

Figure 11 shows a simplified diagram of the light path of an LSCM. This figure shows that laser light is directed to the sample through collimating and beam steering optics, scanning mirrors (which sweep the laser beam over the field of view) and an objective that focuses the light to a diffraction limited spot in the sample. Emission light from the sample is directed to light sensing detector(s) (typically photomultiplier tubes, also known as PMT's) through a pinhole that is in the conjugate image plane to the point of focus in the sample. After spatial filtering by the pinhole, the light is sensed by the detectors, and a proportionate voltage is produced and amplified and converted into digital levels for image display and storage.

At the heart of the confocal microscope is the pinhole. When placed in the conjugate image plane to the point of focus on the sample it enables optical sectioning. The pinhole optically sections by acting as a barrier to light originating from other focal planes in the sample. The

main advantage of LSCM is that one may optically section while still doing complex experiments. Another advantage is the versatility of imaging capabilities and types of experiments one can perform (Combs, 2010).

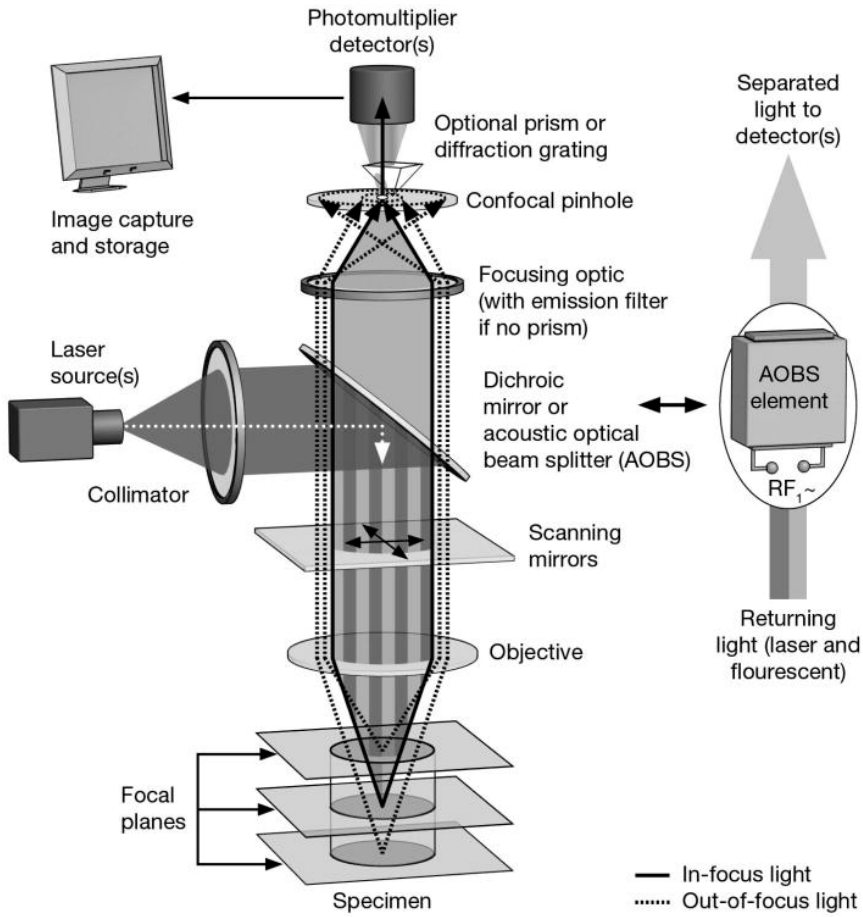


Figure 11. Basic architecture of a modern confocal microscope (Combs, 2010).

3.6.9 ELISA Kit for human IL-6 & TNF-α

The enzyme-linked immune-sorbent assay (ELISA) kits used for the testing of both the cytokines IL-6 and TNF-α was purchased from PeproTech company based in the US. The ELISA kit for IL-6 was: Human IL-6 Standard TMB ELISA Development Kit Catalog # 900-T16 Lot #0514T016 and for TNF-α was: Human TNF-α Standard TMB ELISA Development Kit Catalog # 900-T25 Lot #0414T025.

ELISAs are designed for detecting and quantitating substances such as peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface. The antigen is then complexed with an antibody that is linked to an enzyme. Detection is accomplished by incubating this enzyme-complex with a substrate that produces a detectable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. Most commonly, ELISAs are performed in 96-well polystyrene plates, which will passively bind antibodies and proteins.

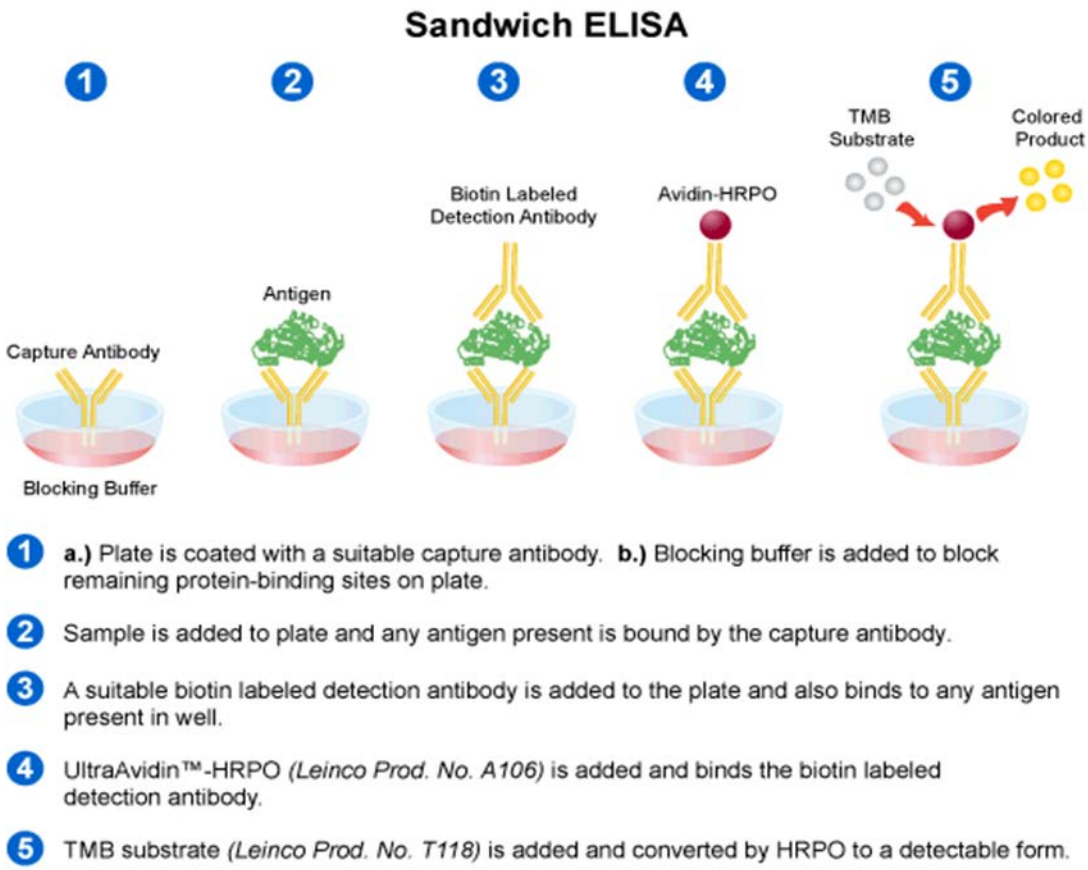


Diagram 1: *Illustration of Sandwich ELISA method.*

Source: https://www.leinco.com/sandwich_elisa

Figure 12. Sandwich ELISA method

It is this binding and immobilization of reagents that makes ELISAs so easy to design and perform, as first described by Eva Engvall, et al. Having the reactants of the ELISA

immobilized to the microplate surface makes it easy to separate bound from unbound material during the assay. This ability to wash away non-specifically bound materials makes the ELISA a powerful tool for measuring specific preparations (Engvall & Perlmann, 1972; Scientific, 2017). The most commonly used ELISA assay format is the sandwich assay (https://www.leinco.com/sandwich_elisa).

This type of assay is called a "sandwich" assay because the analyte to be measured is bound between two antibodies – the capture antibody and the detection antibody. The sandwich format is used because it is sensitive and robust (Palomäki, 1991).

3.6.10 Microplate Reader

Scan 4. Microplate reader POLARstar OMEGA

Microplate reader used for the optical density readings of the ELISA tests was POLARstar OMEGA model from BMG LABTECH company. The Omega Data Analysis software package installed therein was used to do derive the Standard Curve from the given concentrations of the Standard Solution and their corresponding optical density reading. The concentration of IL-6 and TNF-α in the samples and controls were derived from the Standard Curve using their optical density readings. The data of the duplicate concentration readings of cytokines in the samples and controls were then transferred to the GraphPad Prism version 7 software package to perform one-way ANOVA with Post-hoc tests and derive the

corresponding experiment results and their statistical significance. The graphical presentations of the results are depicted in Figure 13a and b in the Result section.

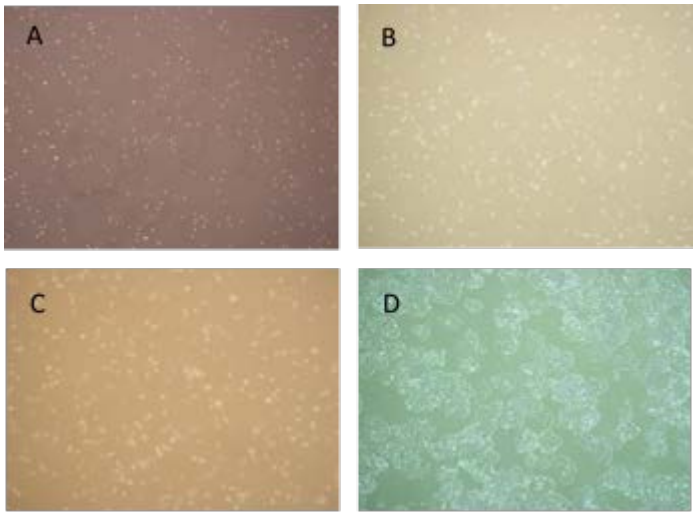
3.7 Methods (experimental procedures)

3.7.1 Hypothesis one: testing the formation of stable chimeric molecule

3.7.1.1 Creation of the chimeric molecule and slide preparation for examination under laser scanning confocal microscope (LSCM)

The following materials were collected: A-549 cells, CpG ODN-1 (2006/7909; class B)-tagged with 6-FAM fluorescein and NH2-modified; CpG ODN-2 (D35; class A)-tagged with 6-FAM fluorescein and NH2-modified, BS3, phosphate buffer saline (PBS), MMC, glass-slides, tweezer, 12-well plate with # 1.5 or 0.17 mm thick 3 mm coverslip.

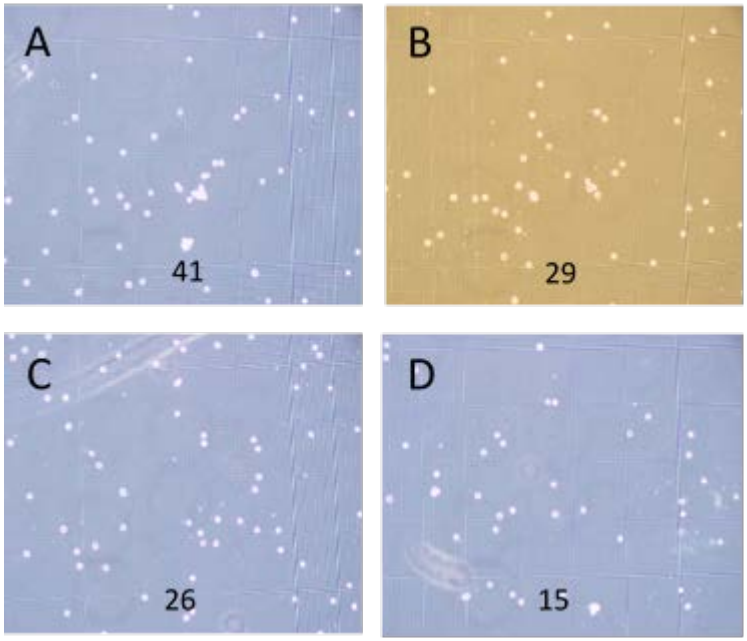
A549 cancer cells were retrieved from the nitrogen freezer (vapour phase) and thawed quickly over one and a half minute in a water bath at 37^0 C until tiny crystals of ice remained. The content of the vial was immediately transferred to a 25-cm^2 culture flask containing 5 ml CCM-DMEM and after checking the cells under the inverted microscope was incubated at SCCC. The next day, A549 cells were washed with 5 ml PBS and trypsinised with 2 ml of trypsin-0.5%-EDTA. After the dislodgement of A549 cells under the microscope CCM-DMEM was added to neutralize the trypsin and then centrifuged at 800 rpm over 5 minutes.



Scan 5. A549 cells culture on T75 flask

A.15min. after cells subculturing, cells at 10% confluency **B.** After 24h, cells confluence is 20%-30% **C.** After 48h, cells reached 40%-50% confluency **D.** After four days, cells confluence is 70%-80%**.**

After aspirating and discarding the supernatant, the cell-pellet was reconstituted in 10 ml CCM-DMEM and transferred to 75 cm^2 culture flasks and kept at SCCC. After around 70-80% confluent growth under the microscope, the cells were collected as described above and counted in a Haemocytometer to gather required number of A549 cells.



Scan 6. Cell count on a Haematocytometer

Around $1x10^6$ A549 per mL were then placed in 6 wells of 6-well microplate each containing 3 mm and # 1.5 or 0.17 mm thick coverslip for cell growth. The microplate with the coverslips was incubated for 24 hours at SCCC to ensure the attachment of the A549 cells on the coverslips. This was verified by observing under the inverted microscope. After confirming the attachment of the cells on the coverslips, 3 of the wells were then treated with 10 µg/ml of MMC for 12 hours to induce apoptosis and the remaining 3 of the wells were left intact with live A549 cells. After thoroughly washing the wells with ice-cold PBS, ODN 1 and ODN 2 at the concentration of 10 µg and 40 µg/$1x10^6$cells respectively were added to the respective wells with the coverslips and incubated at 4^0C for 1 hour. Then, according to the protocol, 1 and 2 mM and of freshly prepared BS^3 was added to the respective wells and

incubated at 4^0C for 30 minutes. The coverslips were then removed from the wells with tweezers and thoroughly washed with PBS to remove the unattached ODNs and BS^3.
The coverslips were placed in fresh wells on a 6-well microplate and treated with 100 % methanol for 30 minutes at -20^0 C for fixing the cells. The coverslips with fixed cells were then transferred to the prepared glass slides with the help of tweezers so that the side of the coverslips with the fixed cells faced down on the glass slide. These slides were then mounted and observed under confocal scanning laser microscopy with filters. The results of the confocal microscopy examination were scanned and photographed. The resulting photographs are depicted in Scan 7-10 of the Results section.

3.7.2 Hypothesis two: testing the immune response of the macrophages treated with the above created chimeric molecule by measuring the IL-6 and TNF-α released with ELISA

U937 cancer cell was retrieved from Nitrogen Freezer (vapour phase) on the day of the initiation of the experiment. These cells were used to produce macrophages necessary for the experiment. They were immediately thawed in a water bath kept at 37^0C over one and half minutes till tiny ice crystals remained and the content was aseptically transferred in SC-LFC to a 25-cm^2 culture flask containing complete CCM-RPMI-1640. The culture flask was incubated over night at SCCC. The next day, U937 cells were aseptically transferred to 15-ml tube and centrifuged at 800 rpm for 5 minutes. The resulting cell pellet was reconstituted in CCM-RPMI 1640 and transferred to T-75 culture flask. Following a similar procedure, 12 million cells in 12 ml was collected after counting the cells in a Haemocytometer. PMA at a concentration of 50 ng/ml (600 ng) was added and after thorough mixing one ml each was transferred to designated 11 wells on a 12-well microplate and incubated for 48 hours. [PMA was dissolved in 100% DMSO to make a stock solution of 50 µg/ml (and stored in the dark at -20^oC) from which fresh 50 ng/ml solution was prepared and used to differentiate our U937

cells]. After 48 hours, the differentiation of U937 cells into macrophages was verified with the microscope. Thereafter, the medium with PMA was replaced with CCM-RPMI 1640 without PMA for a recovery period of 24 hours. These 11 wells containing around million macrophages per well (see Table 2) were then prepared for treatment with corresponding chimeric molecules and controls for 18 hours for the release of TNF-α and IL-6.

The chimeric molecules prepared above in 6-well plate (see Table 1) were scraped with a sterile scraper and transferred to the corresponding wells with the macrophages (see Table 2). Similarly, LPS1 and 2 with the concentration of 50 ng/ml and 100 ng/ml respectively were added to the corresponding wells with the macrophages as positive control. In the same manner ODN1 and ODN2 at a concentration of 10 and 40 µg per million macrophages respectively were added to the corresponding wells (see Table 2) as negative control.

The 12-well plate (as per Table 2) was then incubated at SCCC for 18 hours, after which the content of each well was aspirated into a designated microtube (Eppendorf tubes) and then centrifuged at 3000 rpm for 10 minutes. The supernatant was then used as samples for the measurement of cytokines, IL-6 and TNF-α by ELISA.

The stock solution preparation of all the reagents has been described in the Methodology section 3.6 and in Appendix A.

The sandwich ELISA kits used for both IL-6 and TNF-α was purchased from PeproTech® company, USA. The layout for standard, sample, and blank is as given below.

Table 5. Lay out for standards, blanks, and samples in duplicates on a 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	4000 pg/ml		LC+MP	LC+MP	LPS1+MP							4000 pg/ml
B	2000 pg/ml		AC+MP	AC+MP	LPS2+MP							2000 pg/ml

C	1000 pg/ml	S1+MP	S1+MP	MP	1000 pg/ml
D	500 pg/ml	S2+MP	S2+MP	LPS1+MP	500 pg/ml
E	250 pg/ml	S3+MP	S3+MP	LPS2+MP	250 pg/ml
F	125 pg/ml	S4+MP	S4+MP	MP	125 pg/ml
G	62.5 pg/ml	ODN1+MP	ODN1+MP		62.5 pg/ml
H	Blank diluent	ODN2+MP	ODN2+MP		Blank plain medium

Note*:* LC= one million live A549 cells; AC= one million apoptotic A549 cells; ODN1=10 µg of CpG-ODN 7909; ODN2=40 µg of CpG-ODN type A; BS3a and BS3b=1mM and 2mM bis[sulfosuccinimidyl] suberate respectively; MP=one million macrophages differentiated from U937 cells; LPS1and LPS 2=lipopolysaccharide of conc. 50 ng/ml and 100 ng/ml respectively.

The following procedures/steps were meticulously carried out for the sandwich ELISA of IL-6 and TNF-α:

1. Stock solutions of reagents were prepared as per PeproTech company delivered ELISA-kit- given protocol instructions and ten aliquots of each of them were stored at the temperature specified in the protocol.
2. On the day of the experiment, the designated 96-well microplates and aliquots of stock solution of reagents to be used were brought to room temperature from cold storage.
3. The total number of wells used for the whole experiment were 38 wells (see the table above). The total amount of reagents needed were calculated as for 50 wells (the company supplies reagents for 10 microplates assuming 100 wells in each

microplate) i.e. therefore half of the aliquoted stock of each of the reagent solution was prepared.

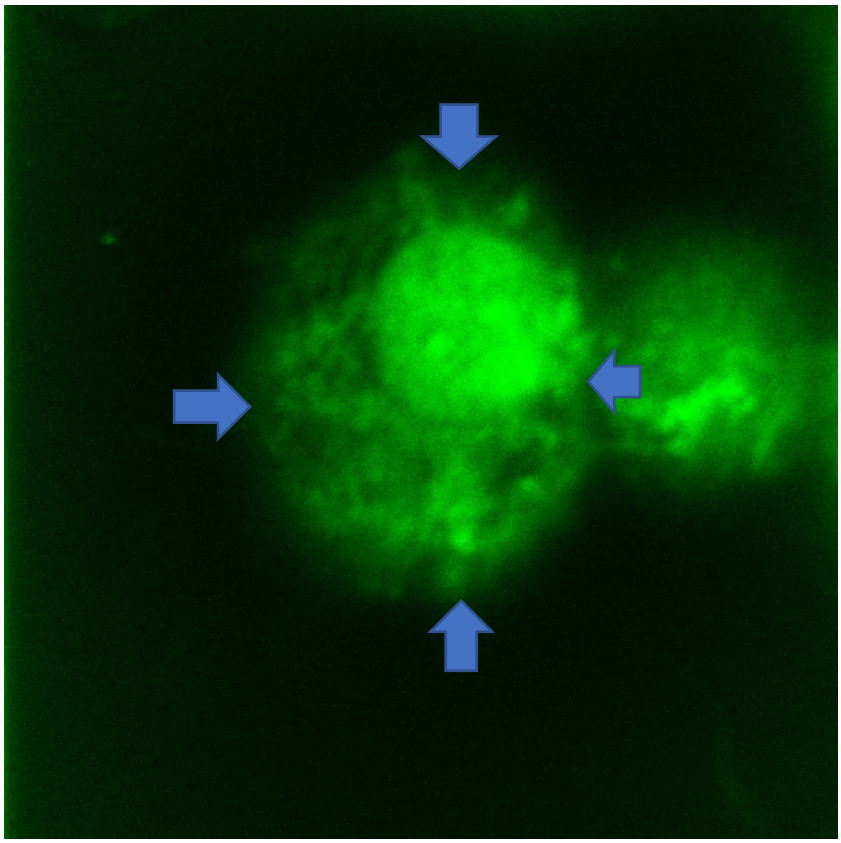
4. 100 µl of Capture Antibody solution of 0.1 µg/ml concentration was added to each designated well. After sealing the plate with the given plastic cover, it was kept at room temperature (RT) overnight.
5. Next day the contents of the wells were decanted and then each well washed four times with 300 µl per well of the given 1x wash buffer and dried on a paper towel.
6. Then 300 µl of the given Block buffer was pipetted into each well and incubated for at least one hour at RT.
7. After one hour, the wells were thoroughly washed as above with wash buffer and 100 µl of the Standard Antibody solution of a concentration from 4000 pg/ml to a final concentration of 62.5 pg/ml (serial dilution by factor two) was pipetted in duplicate into the respective wells. Following this 100 µl of each prepared experimental sample in duplicate was also pipetted into corresponding wells and after tightly sealing the plate was kept at RT for at least two hours.
8. After two hours, the wells were thoroughly washed and dried as above and 100 µl of Detection Antibody solution of 0.1 µg/ml concentration was pipetted into each well and incubated for two hours at RT.
9. After two hours, the wells were washed and dried as above and 100 µl of Streptavidin-HRP conjugate solution of 10 µg/ml concentration was added to each well and after sealing the plate kept for 30 minutes at RT.
10. After half-hour, the wells were thoroughly washed and dried as above and 100 µl of TMB solution added to each well and colour development was watched for 20 minutes after which 100 µl of Stop solution were added to each well.

11. The plate was then immediately taken to the plate reader and the optical density of the designated wells measured every five minutes and the readings saved for analysis.

Chapter 4: Results

This chapter presents the results of testing our hypotheses using the experimental designs and methodologies described in chapters 2 and 3.

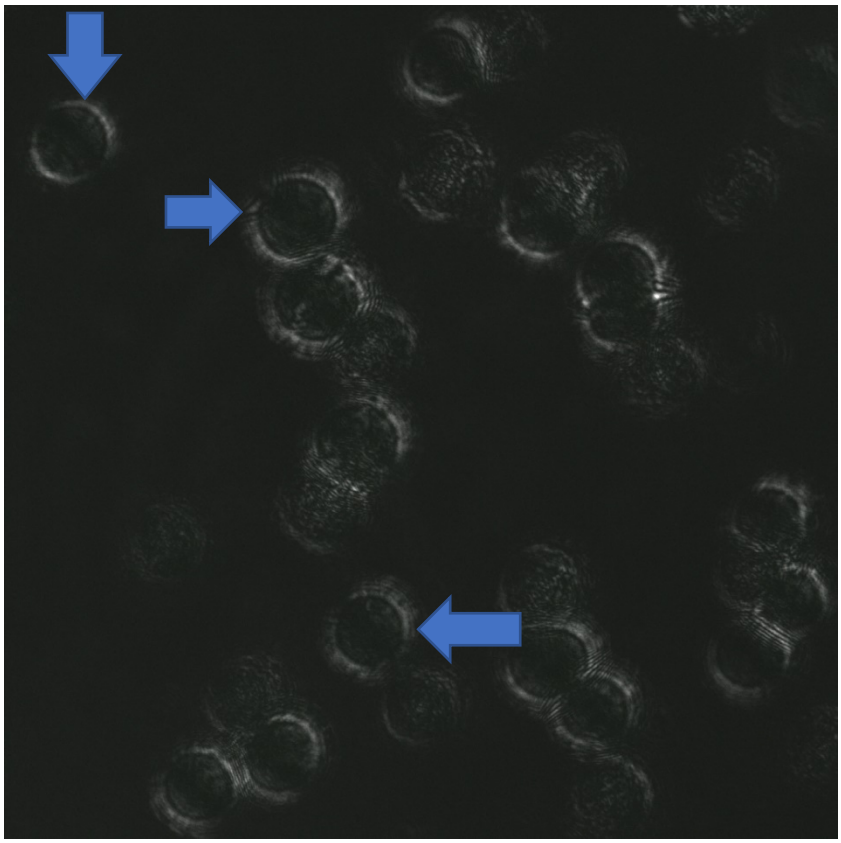
4.1 Formation of the stable chimeric molecule (hypothesis 1)



Scan 7. Fluorescence (green-yellow) of 6-FAM-tagged CpG-ODN1 (10 µg/10^6 cells) conjugated to a cell surface component of a A549 lung cancer cell (the thick blue arrows delineate the cell boundary of the A549 cell) with the help of cross-linker BS^3 (1 mM)

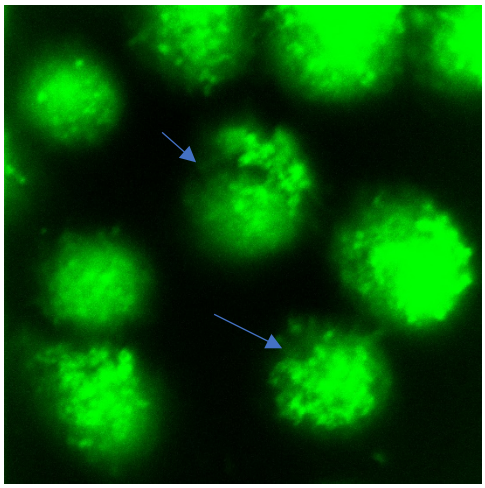
Research has shown that cancer cells alone do not adequately stimulate the immune system to produce robust anti-cancer immune response. Our experiment to conjugate CpG-ODN to cancer cells to create the chimeric molecule was designed to boost anti-cancer immune response. The novel chimeric molecule thus produced (as is shown in Scan 7) was stable and could be used to test our second hypothesis. Accordingly, stable conjugation of A549 lung

cancer cells and CpG-ODN with the aid of the bi-functionally active chemical cross-linker BS3 was verified with the help of laser scanning confocal microscope, LSCM (Nikon A1^{+} confocal microscope). See *Scans* 7,8, 9, &10. Controls using CpG-ODN with A549 cells, BS3 with A549 cells, and CpG-ODN with BS3 did not reveal any stable conjugation by the absence of fluorescence.

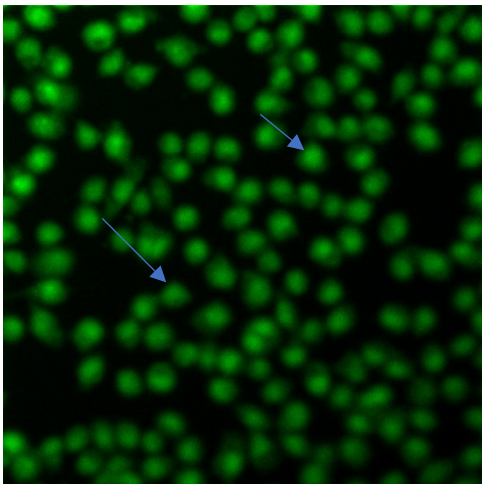


Scan 8. A549 lung cancer cells without the addition of any fluorescent conjugating reagent i.e. neither 6-FAM-tagged CpG-ODN nor BS3 (as negative control); the thick blue arrows point to individual A549 cells.

This image (Scan 7) clearly shows 6-FAM-tagged CpG-ODNs stably attached (conjugated) to surface components of the A549 cells. The absence of any green-yellow fluorescence signal in the spaces between A549 cells indicates that there is very low residual level of unbound 6-FAM-tagged CpG-ODN in the sample, i.e. only the stably conjugated CpG-ODN remains after washing the cells (Scan 9a and 9b).



Scan 9a



Scan 9b

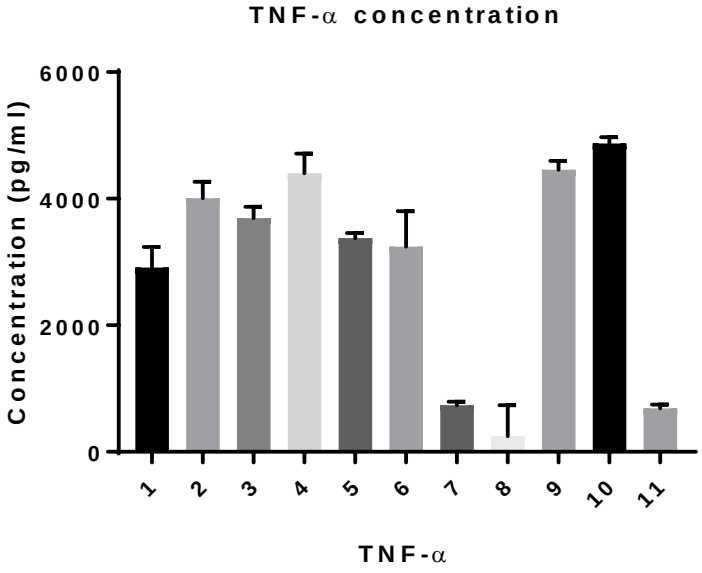
Scan 9a and 9b. The thin blue arrows indicate some of the A549 cells as chimeric molecules [stable conjugation of 6-FAM-tagged CpG-ODN1 (10 μg/10^6 cells) to A549 cells in the presence of 1 mM BS3] under lower magnification. The micrographs show distinct fluorescent cells. The scan also shows that there is no fluorescence in between the cells.

Thus, the null hypothesis that A549 cells and CpG-ODN would not stably conjugate and form a chimeric molecule in the presence of BS3 has been rejected. The alternative hypothesis that they will form such a chimeric molecule has been accepted. We can now use this chimeric molecule to test our second hypothesis.

4.2 Increased release of immune cytokines

Improved anti-cancer immune response could be anticipated if our chimeric molecule induced the immune system to produce more immune cytokines compared to controls using only cancer cells. The level of immune cytokines released in our experiment clearly show that the chimeric molecules induced more cytokine release than controls using only cancer cells. The details of the result of our experiment using the novel chimeric molecules and controls after exposure to the macrophages is described in detail below and show that the above so formed chimeric molecule did produce more immune cytokines (TNF-alpha and IL-6) compared to controls using only cancer cells (see also Figures 13a and 13b).

The statistical method used to analyse the ELISA data collected to test our second hypothesis was one-way ANOVA. ANOVA was performed using the software package GraphPad Prism 7. Differences observed between groups were considered statistical significant if $p < 0.05$. Post-hoc analysis (Tukey's) was also carried out.



Note: Samples and their contents denoted in Arabic numerals on the abscissa or x-axis **(**for both Fig.13a and 13b)

1: Live A549 cells + macrophages
2: Apoptotic A549 cells + macrophages
3: Chimeric molecule 1
4: Chimeric molecule 2
5: Chimeric molecule 3
6: Chimeric molecule 4
7: CpG-ODN 1 + macrophages
8: CpG-ODN 2 + macrophages
9: LPS 1 + macrophages
10: LPS 2 + macrophages
11: Macrophages only

Figure.13a. ELISA results of TNF-α levels in picogram per millilitre

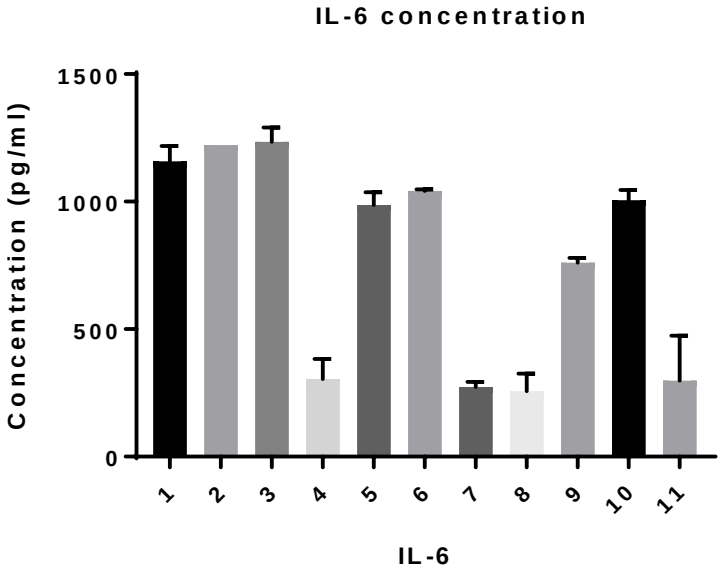


Figure 13b. ELISA results of IL-6 levels in picogram per millilitre

Briefly, (see details of the experiment in Chapters 2 and 3) the experiment entailed the formation of four types of chimeric molecules i.e. **Chimeric molecule 1** ***(sample 3***) contained $1x10^6$ live A549 cells conjugated to 10 µg per 10^6 A549 cells of CpG-ODN1 (7909/ type B) in the presence of 1 mM of BS^3 cross-linker and $1x10^6$ macrophages differentiated from U937 cells with PMA, **Chimeric molecule 2** ***(sample 4***) contained $1x10^6$ live A549 cells conjugated to 40 µg per 10^6 A549 cells of ODN2 (D_{35}/ type A) in the presence of 2 mM of BS^3 chemical linker and $1x10^6$ macrophages differentiated from U937 cells with PMA, **Chimeric molecule 3** ***(sample 5***) contained 10 µg/mL of mitomycin C treated $1x10^6$ apoptotic A549 cells conjugated to 10 µg/per 10^6 A549 cells of ODN1 (7909/ type B) in the presence of 1 mM of BS^3 chemical linker and $1x10^6$ macrophages, **Chimeric molecule 4** ***(sample 6***) contained 10 µg/mL of mitomycin C treated $1x10^6$ apoptotic A549 cells conjugated to 40 µg/per 10^6 A549 cells of ODN2 (D_{35}/ type A) in the presence of 2 mM of BS^3 chemical linker and $1x10^6$ macrophages differentiated. **LPS1** ***(sample 9***) and **LPS2** ***(sample 10***) of concentrations 50 ng/mL and 100 ng/mL respectively with $1x10^6$ macrophages were used as positive control in the experiment, and $1x10^6$ live and apoptotic A549 cells ***(sample 1*** & **2)** respectively with $1x10^6$ macrophages, 10 µg ODN1 & 40 µg ODN2 ***(sample 7*** & ***8***) respectively with $1x10^6$ macrophages, and $1x10^6$ macrophages alone ***(sample 11***) were used as negative controls (see Figure 13a and 13b).

4.3 Boosted anti-tumour immune response (hypothesis 2)

Analysis of the ELISA results for both TNF-α and IL-6 reveal the following (the analysis of the results of TNF-α and IL-6 is not done separately here because of their very similar trend):

1. Similar chimeric molecules were tested for the ability to induce quantitatively and qualitatively similar macrophage populations under similar experimental conditions in production of either TNF-α or IL-6 cytokines. It is clear from the results (Fig. 13a and 13b) that quantitatively, on average, almost four times more TNF-α released was

released compared to IL-6. This means that TNF-α is a more sensitive indicator of immune response than IL-6.

2. Macrophages alone (***sample 11***) and ODNs with macrophages (***sample 7 & 8***) i.e. the negative controls exhibited very low ability to induce cytokine TNF-α and IL-6 production compared to chimeric molecule 1 (***sample 3***) that was statistically significant ($p < 0.0001$). This means that whole cell antigens combined in appropriate ways with adjuvants is more potent immune stimulator.
3. The greatest level of TNF-α release (~5000 pg/mL) was observed for ***sample 10*** containing 100 ng/mL of LPS (LPS2) followed by ***sample 4*** (~4,500 pg/mL) containing chimeric molecule with 40 µg per 10^6 cells ODN2 (D35, type A) linked using 2 mM of BS^3. This indicates that both LPS2 (***sample 10)*** and the corresponding chimeric molecule ***(sample 4)*** were strong inducers of immune response ($p < 0.0001$). This means that this chimeric molecule has achieved the highest possible immune response at par with LPS considered to be a very potent immune stimulator. Since LPS is toxic to us, a safe adjuvant, CpG ODN, achieving that level of immune response is very important for cancer vaccine development.
4. When A549 cells alone were used, apoptotic A549 cells (***sample 2***) induced more TNF-α (~4,000 pg/mL) release than live A549 cells ***(sample 1***) (~3000 pg/mL) (statistically not significant, $p > 0.05$). However, the chimeric molecule with live A549 cells (***samples 3 & 4*** with 3,800 and 4,500 pg/mL respectively) induced more release of TNF-α than chimeric molecules with apoptotic cells (***samples 5 & 6*** with 3200 and 3,300 pg/mL respectively) (statistically not significant, $p > 0.05$). Although at a quantitatively lower level, IL-6 induction showed similar trends. This was a totally unexpected result because apoptotic cells have been considered to be more immunogenic than live cells (but we found no head to head comparative study to

confirm this). This means that may be attenuated (unable to multiply) cancer cells would be better antigens than apoptotic cancer cells for personalised cancer vaccines. One-way ANOVA statistical analysis was statistically significant ($F= 20.4$, $p <0.001$). And thus, post-hoc analysis (Tukey's) performed thereafter showed statistically significant difference in means between the following groups ($p <0.05$) [Table 6].

Table 6. List of groups whose means exhibited statistically significant differences in means (see complete details in Appendix D and E).

TNF-α	
Samples	1 vs. 4, 7-11
	2 vs. 7-8, 11
	3 vs. 7-8, 10-11
	4 vs. 6-8, 11
	5 vs. 7-8, 10-11
	6 vs. 7-11
	7 vs. 9-10
	8 vs. 9-10
	9 vs 11
	10 vs. 11

IL-6	
Samples	1 vs. 4, 7-9, 11
	2 vs. 4, 7-9, 11
	3 vs. 4,7-9, 11
	4 vs. 5-6, 9-10
	5 vs. 7-8,11
	6 vs. 7-9, 11
	7 vs. 9-10
	8 vs. 9-10
	9 vs. 11
	10 vs. 11

Chapter 5: Discussion

This is the first study that we know of, that has tested human lung cancer cell line A549 cells, both as live and chemically induced apoptotic whole cell forms, as antigens covalently linked to two types of CpG-ODNs (adjuvant) with the help of a chemical linker BS^3 as a potential antigenic material for a future clinical cancer vaccine. Justification and arguments for the need of such a vaccine for lung cancer and the methods used to create it has been described in detail in chapter one, two, and three of this thesis. In short, our research questions were related to whether the above combination would result in a stable chimeric molecule capable of inducing a strong anti-cancer immune response. We proposed two hypotheses to answer our research questions, namely, that a stable novel chimeric molecule would form (hypothesis 1) and that it would induce strong anti-cancer immune response (hypothesis 2). The results presented in chapter four, according to us, accepts both hypotheses. It needs to be mentioned that both the hypotheses are very closely inter-linked and their separation was for the ease of understanding the steps taken while carrying out the experiments. Thus, in this chapter, it would no longer be necessary to discuss them separately.

The successful creation of the immunogenic/antigenic chimeric molecule for personalised lung cancer vaccine (acceptance of hypothesis 1 and 2) using whole lung cancer cells in this project is a very important step for using whole cancer cells as vaccine antigens. Moreover, the failure of cancer vaccines incorporating selective cancer antigens to achieve meaningful clinical responses, especially in lung cancer, as witnessed by the failure of several recent well-powered and high profile clinical trials (MAGRIT, START, STOP, and TG 4010) has re-enforced the importance of this vaccine design using whole cancer cells (Mazza & Cappuzzo, 2016). Scientists who have analysed these failed trials have suggested that the use of selective (single) cancer antigens could be one of the important reasons for this failure. They have suggested that whole tumour cells, harbouring all known and unknown cancer

antigens, would better serve as vaccine antigens by circumventing the probability of tumour antigen loss due to tumour immune editing (Mazza & Cappuzzo, 2016). Thus, our chimeric molecule design coming at a time when cancer vaccines with selective (single) antigens have all failed could usher in a new era in the re-experimentation of whole (autologous) cancer cells as cancer vaccine antigens. But, past experimentation with whole cell cancer vaccines had been disappointing by the fact that they were not sufficiently immunogenic. This was attributed to immune-tolerance of whole cancer cells as antigens. Thus, mitigating immune-tolerance was equally important for the success of whole cell vaccines. The acceptance of both of our hypothesis, i.e. creation of the stable chimeric molecule capable of inducing strong anti-cancer immune response (prominent levels of cytokine release), points to the likelihood of our design achieving both enhanced antigenicity (multiple antigens in whole cells) and immune-tolerance mitigation (CpG ODN adjuvant induced immune stimulation). Past cancer vaccine designs with cancer cells, including allogeneic ones, and individual antigens with or without adjuvants have been explored and extensively tested both at the bench and bedside to no avail (Copier & Dalgleish, 2010; Makkouk & Weiner, 2015). Failed clinical trials of cancer vaccines using individual antigens suggests that we need to revisit whole cancer cell antigens using new adjuvants either as immune potentiators and/or vehicles (Mazza & Cappuzzo, 2016). The existence of immune-editing and the finding that a tumour at different sites (or even at the same site!) even in the same patient could harbour antigenically different cancer cells) has cast doubts on the feasibility of vaccines using single or few antigens (Chiang, Coukos, & Kandalaft, 2015; Schreiber et al., 2011). Consequently, using whole cell or whole cell derived antigens containing quantitatively more known and unknown antigens could be a better option to finding appropriate antigenic material for cancer vaccines (M. Chen et al., 2015). In this context, although allogeneic whole cancer cells (combining antigens from several cancer cell types) look theoretically attractive by

their, apart from quantitatively substantial number of antigens in them, qualitatively containing more immunogenic antigens derived from different cancer types, it also failed in practice, most likely, because the repertoire of cancer antigens in an individual cancer patient is unique. Therefore, vaccines made by using a person's own (autologous) cancer cells, would be the closest match to garner the required antigenicity to adequately stimulate anti-cancer immunity to that cancer (Ophir et al., 2016). But, the failure of autologous cancer cell vaccines in the past has been attributed to the tolerance of our immune system to these whole cells and , additionally, to the lack of the required immune stimulation (Keenan & Jaffee, 2012). Thus, methods to augment anti-tumour immunity to autologous cells needed to be extensively explored. One of the ways to do this was to find adjuvants and test varied designs to optimize their effectiveness (Tom et al., 2013). We have tested the adjuvant, CpG ODN, in a novel way to do just that and found it to be immuno-stimulatory. But, we need to continue to explore further adjuvants with novel designs and ways of combining them to ensure the success of personalized cancer vaccines.

And, also, another important lesson from the failure of one of the four trials using allogenic whole cell as cancer antigens (Belagenpumatucel-L1) was to use patient's own cancer cells in future vaccine designs/clinical trials. The fact that even histologically similar types of tumours can harbour divergent antigens in different patients explains the failure of allogenic vaccines suggesting that personalized tumour vaccines using autologous tumour cells would most likely ensure clinical success of cancer vaccines.

The results of our experiment also revealed that the cytokine TNF-α was quantitatively a better surrogate marker for immune response than IL-6. Future experiments like ours with other cancer cell lines should explore other cytokines instead of IL-6.

Similarly, the finding in our experiment that chimeric molecules with live A549 cells were more immunogenic than apoptotic A549 cells needs to be investigated further with other cancer cell lines.

Chapter 6: Limitations, implications, future directions, and recommendations

6.1 Limitations

There are several limitations to this study. Firstly, our results are based on artificially created *in vitro* conditions which are not reflective of the natural *in vivo* physiological and psychological conditions. Secondly, the concentrations of reagents used may or may not work in future animal and clinical trials. Thirdly, we are not sure how correlated the type and level of cytokine is to the actual anti-cancer immune response *in vivo*. Fourthly, we also do not know how the cancer cell line and its passage number we are using in our experiment will reflect the immunogenicity/antigenicity prevalent in the real cancer cells. And lastly, we do not know if the type of CpG ODN we used as adjuvant would also be suitable *in vivo* clinical trials, i.e. could CpG ODN D35 (Class A, D-type) we used in our design form aggregates *in vivo*?

6.2 Implications and future directions

At the outset, it needs to be made clear that our *in vitro* results need to be confirmed in animal models (like in murine models) and in humans (clinical trials) before it can be recommended as a therapeutic cancer vaccine.

We can conclude that we have come, albeit a little, closer to designing a safe and effective personalized cancer vaccine with the use of the patients own (autologous) cancer cells conjugated to a relatively safe and strong adjuvant CpG ODN. Though our design used lung cancer cell line as a model, options for experiments like our using other cell lines relating to

other common cancers could now be explored. If successful, design like ours will usher in an era of personalised cancer vaccines that could effectively prevent recurrences of cancers after their surgical removal. There is also a possibility that cancers which are inoperable or advanced could also be cured using such cancer vaccines.

It is necessary to mention here that, apart from poor immunogenicity/antigenicity, tumour immune-evasion and suppression are equally, if not more, important for the failure of cancer vaccines in clinical trials. Thus, further testing of our vaccine design in animal models and/or future clinical trials would need the addition of tumour immune-evasion and -suppression mitigating agents for clinical success. These could be various check-point blockers and other similar agents.

6.3 Recommendations

We recommend that researchers and cancer care-givers further explore such cancer vaccine designs in other cancers and, if successful, test them in animal models and clinical trials. Additionally, for the clinical success of such vaccines, it is equally important for researchers to also explore ways of mitigating tumour immune-suppression.

References

Alberg, A. J., Brock, M. V., Ford, J. G., Samet, J. M., & Spivack, S. D. (2013). Epidemiology of lung cancer: Diagnosis and management of lung cancer: American College of Chest Physicians evidence-based clinical practice guidelines. *CHEST Journal*, *143*(5_suppl), e1S-e29S.

Aoshi, T., Haseda, Y., Kobiyama, K., Narita, H., Sato, H., Nankai, H., . . . Yasutomi, Y. (2015). Development of nonaggregating poly-A tailed immunostimulatory A/D type CpG oligodeoxynucleotides applicable for clinical use. *Journal of Immunology Research, 2015*.

ATCC. (2017). A549 (ATCC® CCL-185™). Retrieved from https://www.atcc.org/Products/All/CCL-185.aspx#documentation

Australia's health (2016). Retrieved from http://www.aihw.gov.au/copyright/2016

Baker, S. G., & Kramer, B. S. (2007). Paradoxes in carcinogenesis: new opportunities for research directions. *BMC Cancer, 7*(1), 151.

Banday, A. H., Jeelani, S., & Hruby, V. J. (2015). Cancer vaccine adjuvants–recent clinical progress and future perspectives. *Immunopharmacology and Immunotoxicology, 37*(1), 1-11.

Bode, C., Zhao, G., Steinhagen, F., Kinjo, T., & Klinman, D. M. (2011). CpG DNA as a vaccine adjuvant. *Expert Review of Vaccines, 10*(4), 499-511. doi:10.1586/erv.10.174

Chang, M.-S., Chen, B.-C., Yu, M.-T., Sheu, J.-R., Chen, T.-F., & Lin, C.-H. (2005). Phorbol 12-myristate 13-acetate upregulates cyclooxygenase-2 expression in human pulmonary epithelial cells via Ras, Raf-1, ERK, and NF-κB, but not p38 MAPK, pathways. *Cellular signalling, 17*(3), 299-310.

Cheever, M. A., Allison, J. P., Ferris, A. S., Finn, O. J., Hastings, B. M., Hecht, T. T., . . . Weiner, L. M. (2009). The prioritization of cancer antigens: a National Cancer Institute pilot project for the acceleration of translational research. *Clinical Cancer Research, 15*(17), 5323-5337.

Chekhun, V. (2008). Cancer vaccines. *Exp Oncol, 30*(2), 90.

Chen, D. S., & Mellman, I. (2013). Oncology meets immunology: the cancer-immunity cycle. *Immunity, 39*(1), 1-10.

Chen, M., Xiang, R., Wen, Y., Xu, G., Wang, C., Luo, S., . . . Liu, N. (2015). A whole-cell tumor vaccine modified to express fibroblast activation protein induces antitumor immunity against both tumor cells and cancer-associated fibroblasts. *Sci Rep, 5*.

Cheng, H., Hong, B., Zhou, L., Allen, J. E., Tai, G., Humphreys, R., . . . El-Deiry, W. S. (2012). Mitomycin C potentiates TRAIL-induced apoptosis through p53-independent upregulation of death receptors: evidence for the role of c-Jun N-terminal kinase activation. *Cell Cycle, 11*(17), 3312-3323.

Chiang, C. L.-L., Coukos, G., & Kandalaft, L. E. (2015). Whole tumor antigen vaccines: where are we? *Vaccines, 3*(2), 344-372.

Coffman, R. L., Sher, A., & Seder, R. A. (2010). Vaccine adjuvants: Putting innate immunity to work. *Immunity, 33*(4), 492-503. doi:http://dx.doi.org/10.1016/j.immuni.2010.10.002

Combs, C. A. (2010). Fluorescence microscopy: a concise guide to current imaging methods. *Curr Protoc Neurosci, Chapter 2*, Unit 2.1. doi:10.1002/0471142301.ns0201s50

Cooper, W. A., Lam, D. C., O'Toole, S. A., & Minna, J. D. (2013). Molecular biology of lung cancer. *Journal of thoracic disease, 5*(5), S479-S490.

Copier, J., & Dalgleish, A. (2010). Whole-cell vaccines: A failure or a success waiting to happen? *Curr Opin Mol Ther, 12*(1), 14-20.

David Machin, M. J. C., and Stephen J Walters (2007). *Medical Statistics: A textbook for the Health Sciences* (Fourth ed.). Sussex, England: John Wiley & Sons Ltd.

De Gruijl, T. D., van den Eertwegh, A. J., Pinedo, H. M., & Scheper, R. J. (2008). Whole-cell cancer vaccination: from autologous to allogeneic tumor-and dendritic cell-based vaccines. *Cancer Immunology, Immunotherapy, 57*(10), 1569-1577.

Dunn, G. P., Old, L. J., & Schreiber, R. D. (2004). The immunobiology of cancer immunosurveillance and immunoediting. *Immunity, 21*(2), 137-148.

Engvall, E., & Perlmann, P. (1972). Enzyme-linked immunosorbent assay, ELISA. *The Journal of Immunology, 109*(1), 129-135.

Finn, O. (2012). Immuno-oncology: understanding the function and dysfunction of the immune system in cancer. *Annals of Oncology, 23*(suppl_8), viii6-viii9.

Finn, O. J. (2008). Cancer immunology. *New England Journal of Medicine, 358*(25), 2704-2715.

Gan, Z., Debra, T., & Dennis, M. K. (2011). TLR9 agonists *Novel Immunologic Adjuvants* (pp. 92-102): Future Medicine Ltd.

Gardner, A., Alycia, G., & Brian, R. (2016). Dendritic Cells and Cancer Immunity. *Trends in immunology*, *37*(12), 855-865.

Gatti, R. A., & Good, R. A. (1971). Occurrence of malignancy in immunodeficiency diseases: a literature review. *Cancer, 28*(1), 89-98.

Geoffrey Marczyk, D. D., and David Festinger. (2005). *Essentials of Research Design and Methodology*. Hoboken, New Jersey, USA: John Wiley & Sons, Inc.

Giard, D. J., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H., & Parks, W. P. (1973). In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *Journal of the National Cancer Institute, 51*(5), 1417-1423.

Gross, L. (1943). Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Research, 3*(5), 326-333.

Guo, C., Manjili, M. H., Subjeck, J. R., Sarkar, D., Fisher, P. B., & Wang, X.-Y. (2013). Therapeutic cancer vaccines: past, present and future. *Adv Cancer Res*, *119*, 421.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Hanna, M., & Peters, L. (1978). Immunotherapy of established micrometastases with Bacillus Calmette-Guérin tumor cell vaccine. *Cancer Research, 38*(1), 204-209.

Heiss, M. M., Allgayer, H., Gruetzner, K. U., Funke, I., Babic, R., Jauch, K.-W., & Schildberg, F. W. (1995). Individual development and uPA–receptor expression of disseminated tumour cells in bone marrow: a Reference to early systemic disease in solid cancer. *Nature medicine, 1*(10), 1035-1039.

Herbáth, M., Szekeres, Z., Kövesdi, D., Papp, K., Erdei, A., & Prechl, J. (2014). Coadministration of antigen-conjugated and free CpG: effects of in vitro and in vivo interactions in a murine model. *Immunology letters, 160*(2), 178-185.

Hirsch, C. (2014). Culturing A549 cells (protocol). from Project: VIGO of EMPA

Hoover Jr, H. C., Brandhorst, J. S., Peters, L. C., Surdyke, M. G., Takeshita, Y., Madariaga, J., . . . Hanna Jr, M. G. (1993). Adjuvant active specific immunotherapy for human colorectal cancer: 6.5-year median follow-up of a phase III prospectively randomized trial. *Journal of Clinical Oncology, 11*(3), 390-399.

Ito, F., & Chang, A. E. (2013). Cancer immunotherapy: current status and future directions. *Surgical oncology clinics of North America, 22*(4), 765-783.

Jaffee, E. M., Hruban, R. H., Biedrzycki, B., Laheru, D., Schepers, K., Sauter, P. R., . . . Donehower, R. C. (2001). Novel allogeneic granulocyte-macrophage colony-stimulating factor–secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation. *Journal of Clinical Oncology, 19*(1), 145-156.

Kaczanowska, S., Joseph, A. M., & Davila, E. (2013). TLR agonists: our best frenemy in cancer immunotherapy. *Journal of Leukocyte Biology, 93*(6), 847-863. doi:10.1189/jlb.1012501

Keenan, B. P., & Jaffee, E. M. (2012). Whole Cell Vaccines—Past Progress and Future Strategies. *Seminars in Oncology, 39*(3), 276-286. doi:http://dx.doi.org/10.1053/j.seminoncol.2012.02.007

Kienle, G. S. (2012). Fever in cancer treatment: Coley's therapy and epidemiologic observations. *Global advances in Health and Medicine, 1*(1), 92-100.

Klinman, D. M., Currie, D., & Shirota, H. (2006). Immunostimulatory CpG Oligodeoxynucleotides as Vaccine Adjuvants *Vaccine Adjuvants and Delivery Systems* (pp. 157-174): John Wiley & Sons, Inc.

Lawrence, T. S., & Rosenberg, S. A. (2015). *DeVita, Hellman, and Rosenberg's cancer: principles & practice of oncology*: Lippincott Williams & Wilkins.

Lesterhuis, W. J., Haanen, J. B. A. G., & Punt, C. J. A. (2011). Cancer immunotherapy--revisited. *Nature Reviews Drug Discovery, 10*, 591+.

Madureira, P., de Mello, R. A., de Vasconcelos, A., & Zhang, Y. (2015). Immunotherapy for lung cancer: for whom the bell tolls? *Tumor Biology, 36*(3), 1411.

Makkouk, A., & Weiner, G. J. (2015). Cancer immunotherapy and breaking immune tolerance: new approaches to an old challenge. *Cancer Research, 75*(1), 5-10.

Mantovani, A., & Sica, A. (2010). Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Current Opinion in Immunology, 22*(2), 231-237.

Matzinger, P. (1994). Tolerance, danger, and the extended family. *Annual review of immunology, 12*(1), 991-1045.

Mazza, V., & Cappuzzo, F. (2016). Immunotherapy and lung cancer: from therapeutic cancer vaccination to novel approaches. *Journal of thoracic disease, 8*(10), E1348.

Mehta, R. D., & Roth, A. J. (2015). Psychiatric considerations in the oncology setting. *CA: a cancer journal for clinicians, 65*(4), 299-314.

Melief, C. J., van Hall, T., Arens, R., Ossendorp, F., & van der Burg, S. H. (2015). Therapeutic cancer vaccines. *The Journal of Clinical Investigation, 125*(9), 3401-3412.

Mellman, I., Coukos, G., & Dranoff, G. (2011). Cancer immunotherapy comes of age. *Nature, 480*(7378), 480-489.

Montgomery, D. C. (2017). *Design and analysis of experiments*: John Wiley & Sons.

National Cancer Institute, U. (Ed.) (2017) NCI Dictionary of Cancer Terms USA: NCI, USA.

Noguchi, M., Koga, N., Igawa, T., & Itoh, K. (2017). Clinical development of immunotherapy for prostate cancer. *International Journal of Urology*.

Old, L. J., & Boyse, E. A. (1964). Immunology of experimental tumors. *Annual review of medicine, 15*(1), 167-186.

Ophir, E., Bobisse, S., Coukos, G., Harari, A., & Kandalaft, L. E. (2016). Personalized approaches to active immunotherapy in cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1865*(1), 72-82.

Palomäki, P. (1991). Simultaneous use of poly-and monoclonal antibodies as enzyme tracers in a one-step enzyme immunoassay for the detection of hepatitis B surface antigen. *Journal of immunological methods, 145*(1-2), 55-63.

Palucka, K., & Banchereau, J. (2012). Cancer immunotherapy via dendritic cells. *Nat Rev Cancer, 12*(4), 265-277.

Pardoll, D. (2003). Does the immune system see tumors as foreign or self? *Annual review of immunology, 21*, 807.

Parish, C. R. (2003). Cancer immunotherapy: the past, the present and the future. *Immunol Cell Biol, 81*(2), 106-113. doi:10.1046/j.0818-9641.2003.01151.x

Park, E. K., Jung, H. S., Yang, H. I., Yoo, M. C., Kim, C., & Kim, K. S. (2007). Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm Res, 56*(1), 45-50. doi:10.1007/s00011-007-6115-5

Paul, William E. Bridging Innate and Adaptive Immunity. *Cell, 147*(6), 1212-1215. doi:10.1016/j.cell.2011.11.036

Paul, W. E. (2011). Bridging innate and adaptive immunity. *Cell, 147*(6), 1212-1215. doi:10.1016/j.cell.2011.11.036

Pedersen, B., Koktved, D. P., & Nielsen, L. L. (2013). Living with side effects from cancer treatment–a challenge to target information. *Scandinavian journal of caring sciences, 27*(3), 715-723.

Pohar, J., Krajnik, A. K., Jerala, R., & Benčina, M. (2015). Minimal sequence requirements for oligodeoxyribonucleotides activating human TLR9. *The Journal of Immunology, 194*(8), 3901-3908.

Pohar, J., Lainšček, D., Fukui, R., Yamamoto, C., Miyake, K., Jerala, R., & Benčina, M. (2015). Species-specific minimal sequence motif for oligodeoxyribonucleotides activating mouse TLR9. *The Journal of Immunology, 195*(9), 4396-4405.

Pradeu, T., & Cooper, E. L. (2012). The danger theory: 20 years later. *Frontiers in Immunology, 3*, 287.

Prehn, R. T., & Main, J. M. (1957). Immunity to methylcholanthrene-induced sarcomas.

Puig, M., Grajkowski, A., Boczkowska, M., Ausín, C., Beaucage, S. L., & Verthelyi, D. (2006). Use of thermolytic protective groups to prevent G-tetrad formation in CpG ODN type D: structural studies and immunomodulatory activity in primates. *Nucleic Acids Research, 34*(22), 6488-6495. doi:10.1093/nar/gkl867

Quezada, S. A., Peggs, K. S., Simpson, T. R., & Allison, J. P. (2011). Shifting the equilibrium in cancer immunoediting: from tumor tolerance to eradication. *Immunological Reviews, 241*(1), 104-118.

Rosenfeld, Y., & Shai, Y. (2006). Lipopolysaccharide (Endotoxin)-host defense antibacterial peptides interactions: Role in bacterial resistance and prevention of sepsis. *Biochimica*

et Biophysica Acta (BBA) - Biomembranes, 1758(9), 1513-1522. doi:https://doi.org/10.1016/j.bbamem.2006.05.017

Sathyanarayanan, V., & Neelapu, S. S. (2015). Cancer immunotherapy: Strategies for personalization and combinatorial approaches. *Molecular oncology, 9*(10), 2043-2053.

Scheiermann, J., & Klinman, D. M. (2014). Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. *Vaccine, 32*(48), 6377-6389. doi:10.1016/j.vaccine.2014.06.065

Schreiber, R. D., Old, L. J., & Smyth, M. J. (2011). Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science, 331*(6024), 1565-1570.

Scientific, T.-F. (2017). *Thermo Scientific Pierce Assay Development Technical Handbook*

Seledtsov, V. I., Goncharov, A. G., & Seledtsova, G. V. (2015). Multiple-purpose immunotherapy for cancer. *Biomed Pharmacother, 76*, 24-29. doi:10.1016/j.biopha.2015.10.020

Seyfried, T. N., Flores, R. E., Poff, A. M., & D'Agostino, D. P. (2014). Cancer as a metabolic disease: implications for novel therapeutics. *Carcinogenesis, 35*(3), 515-527.

Shirota, H., & Klinman, D. M. (2011). CpG-conjugated apoptotic tumor cells elicit potent tumor-specific immunity. *Cancer Immunology, Immunotherapy, 60*(5), 659-669. doi:10.1007/s00262-011-0973-y

Shirota, H., & Klinman, D. M. (2014). Recent progress concerning CpG DNA and its use as a vaccine adjuvant. *Expert Rev Vaccines, 13*(2), 299-312. doi:10.1586/14760584.2014.863715

Shirota, H., Tross, D., & Klinman, D. M. (2015). CpG oligonucleotides as cancer vaccine adjuvants. *Vaccines, 3*(2), 390-407.

Shirota, Y., Shirota, H., & Klinman, D. M. (2012). Intratumoral injection of CpG oligonucleotides induces the differentiation and reduces the immunosuppressive activity of myeloid-derived suppressor cells. *The Journal of Immunology, 188*(4), 1592-1599.

Silvestri, G. A., Gonzalez, A. V., Jantz, M. A., Margolis, M. L., Gould, M. K., Tanoue, L. T., . . . Detterbeck, F. C. (2013). Methods for staging non-small cell lung cancer: diagnosis and management of lung cancer: American College of Chest Physicians evidence-based clinical practice guidelines. *CHEST Journal, 143*(5_suppl), e211S-e250S.

Simamura, E., Hirai, K.-I., Shimada, H., & Koyama, J. (2001). Apoptosis and epithelial phagocytosis in mitomycin C-treated human pulmonary adenocarcinoma A549 cells. *Tissue and Cell, 33*(2), 161-168.

Society, A. C. (2016). *Key Statistics for Lung Cancer*. Retrieved from http://www.cancer.org/cancer/non-small-cell-lung-cancer/about/key-statistics.html#references

Song, M. G., Ryoo, I. G., Choi, H. Y., Choi, B. H., Kim, S. T., Heo, T. H., . . . Kwak, M. K. (2015). NRF2 Signaling Negatively Regulates Phorbol-12-Myristate-13-Acetate (PMA)-Induced Differentiation of Human Monocytic U937 Cells into Pro-Inflammatory Macrophages. *PLoS One, 10*(7), e0134235. doi:10.1371/journal.pone.0134235

Spiering, M. J. (2015, 2015 Summer). Primer on the immune system. *Alcohol Research: Current Reviews, 37*, 171+.

Stanton, A. L., Rowland, J. H., & Ganz, P. A. (2015). Life after diagnosis and treatment of cancer in adulthood: contributions from psychosocial oncology research. *Am Psychol, 70*(2), 159-174. doi:10.1037/a0037875

Steinman, R. M. (2011). Decisions about dendritic cells: past, present, and future. *Annu. Rev. Immunol.*

Steinman, R. M., & Hemmi, H. (2006). Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol, 311*, 17-58.

Stromberg, L. R., Stromberg, Z. R., Banisadr, A., Graves, S. W., Moxley, R. A., & Mukundan, H. (2015). Purification and characterization of lipopolysaccharides from six strains of non-O157 Shiga toxin-producing Escherichia coli. *Journal of microbiological methods, 116*, 1-7.

Sundström, C., & Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *International Journal of Cancer, 17*(5), 565-577.

Tacken, P. J., & Figdor, C. G. (2011). Targeted antigen delivery and activation of dendritic cells in vivo: steps towards cost effective vaccines. *Semin Immunol, 23*(1), 12-20. doi:10.1016/j.smim.2011.01.001

Tagliamonte, M., Petrizzo, A., Tornesello, M. L., Buonaguro, F. M., & Buonaguro, L. (2014). Antigen-specific vaccines for cancer treatment. *Human vaccines & immunotherapeutics, 10*(11), 3332-3346.

Takashiba, S., Van Dyke, T. E., Amar, S., Murayama, Y., Soskolne, A. W., & Shapira, L. (1999). Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor κB. *Infection and immunity, 67*(11), 5573-5578.

Taqaddas, A. (2015). Assessing Chemo-Radiotherapy Induced Toxicity and Quality of Life of Cancer Patients.

Technologies, I. D. (2017). *Oligonucleotide Yield, Resuspension, and Storage* Retrieved from

Temizoz, B., Kuroda, E., & Ishii, K. J. (2016). Vaccine adjuvants as potential cancer immunotherapeutics. *Int Immunol, 28*(7), 329-338. doi:10.1093/intimm/dxw015

Tom, J. K., Mancini, R. J., & Esser-Kahn, A. P. (2013). Covalent modification of cell surfaces with TLR agonists improves & directs immune stimulation. *Chemical Communications, 49*(83), 9618-9620. doi:10.1039/C3CC45468A

UK, C. R. (2010-11). lung cancer survival statistics. Available from Cancer Research UK lung cancer survival statistics http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/lung-cancer/survival#heading-Zero

Venereau, E., Ceriotti, C., & Bianchi, M. E. (2015). DAMPs from Cell Death to New Life. *Front Immunol, 6*, 422. doi:10.3389/fimmu.2015.00422

Verthelyi, D., Kenney, R. T., Seder, R. A., Gam, A. A., Friedag, B., & Klinman, D. M. (2002). CpG Oligodeoxynucleotides as Vaccine Adjuvants in Primates. *The Journal of Immunology, 168*(4), 1659-1663. doi:10.4049/jimmunol.168.4.1659

Vrinten, C., Wardle, J., & Marlow, L. A. (2016). Cancer fear and fatalism among ethnic minority women in the United Kingdom. *Br J Cancer, 114*(5), 597-604. doi:10.1038/bjc.2016.15

Wang, J., Zou, Z.-H., Xia, H.-L., He, J.-X., Zhong, N.-S., & Tao, A.-L. (2012). Strengths and Weaknesses of Immunotherapy for Advanced Non-Small-Cell Lung Cancer: A Meta-Analysis of 12 Randomized Controlled Trials. *PLoS One, 7*(3), e32695. doi:10.1371/journal.pone.0032695

Wang, R.-F. (2017). A special issue on cancer immunotherapy: Nature Publishing Group.

Ward, S., Casey, D., Labarthe, M. C., Whelan, M., Dalgleish, A., Pandha, H., & Todryk, S. (2002). Immunotherapeutic potential of whole tumour cells. *Cancer Immunol Immunother, 51*(7), 351-357. doi:10.1007/s00262-002-0286-2

Wasaporn, C. V., P. ; & Harry, W. , & (2015). THP-1 and U937 Cells In K. V. e. al. (Ed.), *The Impact of Food Bio-Actives on Gut Health*.

WHO-IARC. (2014). *World Cancer Report 2014*.

WHO. (2017). Vaccines. Retrieved from http://www.who.int/topics/vaccines/en/

World Cancer Report 2014. (2014). Retrieved from http://publications.iarc.fr/Non-Series-Publications/World-Cancer-Reports/World-Cancer-Report-2014

Yu, L. Y., Tang, J., Zhang, C. M., Zeng, W. J., Yan, H., Li, M. P., & Chen, X. P. (2017). New Immunotherapy Strategies in Breast Cancer. *Int J Environ Res Public Health, 14*(1). doi:10.3390/ijerph14010068

Zepp, F. (2016). Principles of vaccination *Vaccine Design: Methods and Protocols: Volume 1: Vaccines for Human Diseases* (pp. 57-84).

Appendix A: Calculations of doses of reagents used in this study

1. **U937 Cells**
   $1x10^6$ cells per ml
2. **A549 Cells**
   $1x10^6$ cells per ml
3. **PMA**

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration **(C1)**
1 mg	20 ml DMSO		50 ug/ml

V1	C1	V2	C2
12 ul	50 ug or 50,000 ng/ml	1 ml per well 12 X 1 ml = 12 ml	50 ng/ml

V1 = V2*C2/C1=12 ml *50 ng.ml/ 50, 000 ng/ml = 0.01 ml = 12 ul
Add 12 ul of stock PMA solution to 12 ml RPMI-CCM (containing $1x10^6$ MP cells/ml)

4. Mitomycin C

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration (C1)
5 mg	1 ml DMSO	1000 ul	5 mg/ml

V1	C1	V2	C2
20 ul	5000 ug/ml	1 ml per well 2 well x 1 ml = 2 ml	10 ug/ml

V1 = V2*C2/C1=2000 ul*10 ug.ml/5000 ug.ml= 4 ul
Add 2 ul of stock solution of MMC in each designated well (containing 1 ml of DMEM-CM with $1x10^6$ A549 cells)

5. (CpG) ODN 1 & 2

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration (C1)
474 ug	474 ul sterile H2O	474 ul	1 mg/ml

10 ug/$1x10^6$ A549 cells as ODN1 i.e. 10 ul (conc. 1 ug/ul) in each of 2 wells (CL & CA B1 and B2)
40 ug/ $x10^6$ A549 cells as ODN2 i.e. 40 ul (conc. 1 ug/ul) in each of 2 wells (CL&CA i.e.C1 and C2)

6. BS3

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration (C1)
10 mg	700 ul sterile (H2O) (70 ul/ per 1 mg to make a conc. of 25 mM)	700 ul	25 mM (MW of BS3 = 572.43)

V1	C1	V2	C2
700 ul	25 mM	1 ml per well BS3a = 2 ml	1mM
		1 ml per well BS3b = 2 ml	2 mM

BS3a: V1 = V2*C2/C1=1000 ul*1 mM/25 mM = 40 ul in each well
Add 40 ul of stock BS3 into each designated well i.e B1 and B2
BS3b: V1 = V2*C2/C1=1000 ul*2 mM/25 mM = 80 ul in each well
Add 80 ul of stock BS3 into each designated well i.e. C1 and C2

7. LPS

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration **(C1)**
2 mg	20 ml	1 ml	100 ug/ml

V1	C1	V2	**C2**
0.5 ul	100 ug/ml	1 ml	50 ng/ml
1 ul	100 ug/ml	1 ml	100 ng/ml

V1= V2*C2/C1= 1000 ul*50 ng.ml/100,000 ng/ml = 0.5 ul

Reagents used in ELISA:

8. Wash buffer (total for experiment)

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration (C1)
--	--	500 ml	20x

V1	C1	V2	C2
15 ml	20x	300 ul per well 50 x 300 (x 4 times x 5 steps) = 300, 000 ul = 300 ml	1x

V1 = V2*C2/C1=300 ml*1x/20x = 15 ml stock of wash buffer solution

Diluent (sterile water) = V2-V1= 300 ml – 15 ml = 285 ml

9. Capture Antibody

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration (C1)
50 ug	Sterile water 500 ul = 0.5 ml	500 ul (total) 50 ul (aliquot)	100 ug/ml

V1 = V2*C2/C1=5000 ul*0.5 ug.ml/100 ug.ml= 25 ul

V1	C1	V2	C2
	100 ug/ml	100 ul per well 50 x 100 ul = 5000 ul = 5 ml	0.5 ug/ml

Vol. of 20xPBS needed= 5000 ul/20 = 250 ul

Vol. of distilled water needed = 5000-250= 4,750

10. Block Buffer

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration (C1)
--	--	400 ml	1x

V1	C1	V2	C2
400 ml	1x	300 ul per well 50 well x 300 ul = 15,000 ul = 15 ml	1x

11. **Diluent** (total for experiment)

Total 1xDiluent needed: 1000 ul (Standard Ab) + 5000 ul (Detection Ab) + 5000 ul (S-HRP) = 11,000 ul.

Thus, 11,000 /20 = 550 ul of 20xDiluent that needs to be added to 10,450 ul of water.

12. Standard Antibody

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration (C1)
1 ug	1 ml sterile H2O	1000 ul (total) 100 ul (aliquot)	1 ug/ml

V1	C1	V2	C2
2 ul	1 ug/ml	100 ul per well 5 wells x 200 ul = 1000 ul = 1 ml But only 2 wells	2000 pg/ml

V1 = V2*C2/C1=1 ml*2000 pg.ml/1000,000 pg/ml = 0.002= 2 ul
Add 2 ul of stock Standard Ab to 1000 ul or 1 ml of diluent

13. **Sample**

There are 22 wells (11 samples in duplicates) with 100 ul per well without dilution.

14. **Detection Antibody**

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration **(C1)**
10 ug	0.1ml sterile PBS	100 ul (total) 10 ul (aliquot)	100 ug/ml

V1	C1	V2	C2
5 ul	100 ug/ml	100 ul per well 50 well x 100 ul = 5000 ul = 5 ml	0.1 ug/ml

V1 = V2*C2/C1=5000 ul*0.1 ug.ml/100 ug.ml= 5 ul
Add 5 ul of stock Detection Ab to 5000 ul or 5 ml of Diluent

15. Streptavidin-HRP Conjugate

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration **(C1)**
17 ul vial	153 ul of sterile 1xPBS	170 ul (total) 16 ul (aliquot)	100 ug/ml

V1	C1	V2	C2
2.5 ul	100 ug/ml	100 ul per well 50 wells x 100 ul = 5000 ul = 5 ml	0.05 ug/ml

V1 = V2*C2/C1=5000 ul*0.05 ug.ml/100 ug.ml= 2.5 ul
Add 2.5 ul of stock Streptavidin-HRP to 5000 ul or 5 ml of Diluent

16. TMB Liquid Substrate

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration **(C1)**
--	--	110 ml	1x

V1	C1	V2	C2
5 ml --	1x --	100 ul per well 50 wells x 100 ul = 5000 ul = 5 ml	1x --

V1 = V2*C2/C1=5000 ul*1x/1x= 5000 ul = 5 ml
V1 (no dilution needed) = 5 ml

17. Stop Solution (1 M HCL)

Stock amount	Diluent amount	Stock volume (V1)	Stock concentration (C1)
--	--	110 m**l**	1M (g/L)

V1	C1	V2	C2
5 ml	1M	100 ul per well	1M
--	--	50 wells x 100 ul = 5 ml	--

V1 = V2*C2/C1=5000 ul*1M/1M= 5000 ul = 5 ml

Appendix B: Results of TNF-α ELISA in Excel

User: USER			Path: C:\Program Files\BMG\Omega\User\Data\							Test ID: 6737		
Test Name: DAMO ELISA-30-06-17			TNF-alpha					Date: 6/30/2017		Time: 7:28:11 PM		
Absorbance			Absorbance values are displayed as OD									
	Linear regression fit based on Blank corrected in pg/ml (450)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	4830.285		3141.234	2687.482	4358.322							2540.278
B	3481.169		4189.872	3824.139	4557.123							1569.036
C	1795.154		3818.068	3564.635	4944.102							755.621
D	763.209		4622.378	4183.802	4801.451							239.649
E	294.281		3434.124	3324.86	2265.599							-48.689
F	59.059		2846.826	3637.478	2012.165							-229.279
G	8.979		775.35	694.919								-183.752
H			596.277	-97.251								

Appendix C: Results of IL-6 ELISA in Excel

User: USER			Path: C:\Program Files\BMG\Omega\User\Data\							Test ID: 6742		
Test Name: DAMO ELISA-30-06-IL6								Date: 6/30/2017		Time: 9:49:40 PM		
Absorbance			Absorbance values are displayed as OD									
	Linear regression fit based on Blank corrected in pg/ml (450)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	3891.44		1476.365	1395.791	1016.249							3121.754
B	2449.604		1321.579	1224.043	850.862							3452.528
C	1669.316		1268.571	1260.089	1050.175							787.252
D	643.068		-283.523	140.546	1126.507							32.408
E	144.787		1041.693	1028.971	-306.847							-279.283
F	1058.656		975.963	990.805	0.603							-279.283
G	-232.635		-251.718	-253.838								-584.613
H			-308.967	-39.683								

Appendix D: One-way ANOVA with Tukey's post-hoc analysis of TNF-α ELISA results in Notepad

Number of families 1

Number of comparisons per family 55

Alpha 0.05

Tukey's multiple comparisons test	Mean Diff.	95 % CI of diff.	Significance	Summary	Adjusted P Value	
1 vs. 2	-1093	-2222 to 37.11	No	ns	0.0608	A-B
1 vs. 3	-777	-1907 to 352.8	No	ns	0.2962	A-C
1 vs. 4	-1489	-2618 to -359	Yes	**	0.0077	A-D
1 vs. 5	-465.1	-1595 to 664.6	No	ns	0.8405	A-E
1 vs. 6	-327.8	-1458 to 802	No	ns	0.9766	A-F
1 vs. 7	2179	1049 to 3309	Yes	***	0.0003	A-G
1 vs. 8	2665	1535 to 3795	Yes	****	<0.0001	A-H
1 vs. 9	-1543	-2673 to -413.6	Yes	**	0.0058	A-I
1 vs. 10	-1958	-3088 to -828.7	Yes	***	0.0008	A-J
1 vs. 11	2227	1097 to 3357	Yes	***	0.0003	A-K
2 vs. 3	315.7	-814.1 to 1445	No	ns	0.9817	B-C
2 vs. 4	-396.1	-1526 to 733.7	No	ns	0.9279	B-D
2 vs. 5	627.5	-502.2 to 1757	No	ns	0.5428	B-E
2 vs. 6	764.9	-364.9 to 1895	No	ns	0.3127	B-F
2 vs. 7	3272	2142 to 4402	Yes	****	<0.0001	B-G
2 vs. 8	3757	2628 to 4887	Yes	****	<0.0001	B-H
2 vs. 9	-450.7	-1580 to 679	No	ns	0.8618	B-I
2 vs. 10	-865.8	-1996 to 264	No	ns	0.1949	B-J
2 vs. 11	3320	2190 to 4449	Yes	****	<0.0001	B-K
3 vs. 4	-711.7	-1841 to 418	No	ns	0.3930	C-D

3 vs. 5	311.9	-817.9 to 1442	No	ns	0.9831	C-E
3 vs. 6	449.2	-680.6 to 1579	No	ns	0.8639	C-F
3 vs. 7	2956	1826 to 4086	Yes	****	<0.0001	C-G
3 vs. 8	3442	2312 to 4572	Yes	****	<0.0001	C-H
3 vs. 9	-766.4	-1896 to 363.4	No	ns	0.3106	C-I
3 vs. 10	-1181	-2311 to -51.67	Yes	*	0.0380	C-J
3 vs. 11	3004	1874 to 4134	Yes	****	<0.0001	C-K
4 vs. 5	1024	-106.2 to 2153	No	ns	0.0874	D-E
4 vs. 6	1161	31.18 to 2291	Yes	*	0.0424	D-F
4 vs. 7	3668	2538 to 4798	Yes	****	<0.0001	D-G
4 vs. 8	4154	3024 to 5283	Yes	****	<0.0001	D-H
4 vs. 9	-54.63	-1184 to 1075	No	ns	>0.9999	D-I
4 vs. 10	-469.7	-1599 to 660.1	No	ns	0.8335	D-J
4 vs. 11	3716	2586 to 4846	Yes	****	<0.0001	D-K
5 vs. 6	137.3	-992.4 to 1267	No	ns	>0.9999	E-F
5 vs. 7	2644	1515 to 3774	Yes	****	<0.0001	E-G
5 vs. 8	3130	2000 to 4260	Yes	****	<0.0001	E-H
5 vs. 9	-1078	-2208 to 51.52	No	ns	0.0656	E-I
5 vs. 10	-1493	-2623 to -363.5	Yes	**	0.0075	E-J
5 vs. 11	2692	1562 to 3822	Yes	****	<0.0001	E-K
6 vs. 7	2507	1377 to 3637	Yes	****	<0.0001	F-G
6 vs. 8	2993	1863 to 4122	Yes	****	<0.0001	F-H
6 vs. 9	-1216	-2345 to -85.82	Yes	*	0.0317	F-I
6 vs. 10	-1631	-2760 to -500.9	Yes	**	0.0038	F-J
6 vs. 11	2555	1425 to 3685	Yes	****	<0.0001	F-K

7 vs. 8	485.6	-644.1 to 1615	No	ns	0.8079	G-H
7 vs. 9	-3723	-4852 to -2593	Yes	****	<0.0001	G-I
7 vs. 10	-4138	-5267 to -3008	Yes	****	<0.0001	G-J
7 vs. 11	47.8	-1082 to 1178	No	ns	>0.9999	G-K
8 vs. 9	-4208	-5338 to -3078	Yes	****	<0.0001	H-I
8 vs. 10	-4623	-5753 to -3494	Yes	****	<0.0001	H-J
8 vs. 11	-437.8	-1568 to 691.9	No	ns	0.8795	H-K
9 vs. 10	-415.1	-1545 to 714.7	No	ns	0.9076	I-J
9 vs. 11	3770	2641 to 4900	Yes	****	<0.0001	I-K
10 vs. 11	4185	3056 to 5315	Yes	****	<0.0001	J-K

Appendix E: One-way ANOVA with Tukey's post-hoc analysis of IL-6 ELISA results in Notepad

Number of families 1

Number of comparisons per family 55

Alpha 0.05

Tukey's multiple comparisons test	Mean Diff.	95 % CI of diff.	Significant	Summary	Adjusted P Value	
1 vs. 2	-64.05	-340.7 to 212.6	No	ns	0.9952	A-B
1 vs. 3	-77.1	-353.8 to 199.6	No	ns	0.9820	A-C
1 vs. 4	852.8	576.1 to 1130	Yes	****	<0.0001	A-D
1 vs. 5	170.8	-105.9 to 447.5	No	ns	0.4168	A-E
1 vs. 6	116.2	-160.5 to 392.9	No	ns	0.8258	A-F

1 vs. 7	884.9	608.2 to 1162	Yes	****	<0.0001	A-G
1 vs. 8	900.3	623.6 to 1177	Yes	****	<0.0001	A-H
1 vs. 9	397.3	120.6 to 674	Yes	**	0.0039	A-I
1 vs. 10	151.8	-124.9 to 428.5	No	ns	0.5573	A-J
1 vs. 11	859.9	583.3 to 1137	Yes	****	<0.0001	A-K
2 vs. 3	-13.05	-289.7 to 263.6	No	ns	>0.9999	B-C
2 vs. 4	916.9	640.2 to 1194	Yes	****	<0.0001	B-D
2 vs. 5	234.8	-41.85 to 511.5	No	ns	0.1222	B-E
2 vs. 6	180.3	-96.41 to 457	No	ns	0.3546	B-F
2 vs. 7	949	672.3 to 1226	Yes	****	<0.0001	B-G
2 vs. 8	964.4	687.7 to 1241	Yes	****	<0.0001	B-H
2 vs. 9	461.4	184.7 to 738.1	Yes	**	0.0011	B-I
2 vs. 10	215.9	-60.83 to 492.6	No	ns	0.1805	B-J
2 vs. 11	924	647.3 to 1201	Yes	****	<0.0001	B-K
3 vs. 4	929.9	653.2 to 1207	Yes	****	<0.0001	C-D
3 vs. 5	247.9	-28.8 to 524.6	No	ns	0.0928	C-E
3 vs. 6	193.3	-83.36 to 470	No	ns	0.2800	C-F
3 vs. 7	962	685.3 to 1239	Yes	****	<0.0001	C-G
3 vs. 8	977.4	700.7 to 1254	Yes	****	<0.0001	C-H
3 vs. 9	474.4	197.7 to 751.1	Yes	***	0.0009	C-I
3 vs. 10	228.9	-47.78 to 505.6	No	ns	0.1383	C-J
3 vs. 11	937	660.4 to 1214	Yes	****	<0.0001	C-K
4 vs. 5	-682	-958.7 to -405.3	Yes	****	<0.0001	D-E
4 vs. 6	-736.6	-1013 to -459.9	Yes	****	<0.0001	D-F
4 vs. 7	32.07	-244.6 to 308.8	No	ns	>0.9999	D-G

4 vs. 8	47.49	-229.2 to 324.2	No	ns	0.9996	D-H
4 vs. 9	-455.5	-732.2 to -178.8	Yes	**	0.0013	D-I
4 vs. 10	-701	-977.7 to -424.3	Yes	****	<0.0001	D-J
4 vs. 11	7.117	-269.6 to 283.8	No	ns	>0.9999	D-K
5 vs. 6	-54.56	-331.2 to 222.1	No	ns	0.9986	E-F
5 vs. 7	714.1	437.4 to 990.8	Yes	****	<0.0001	E-G
5 vs. 8	729.5	452.8 to 1006	Yes	****	<0.0001	E-H
5 vs. 9	226.5	-50.15 to 503.2	No	ns	0.1452	E-I
5 vs. 10	-18.98	-295.7 to 257.7	No	ns	>0.9999	E-J
5 vs. 11	689.2	412.5 to 965.8	Yes	****	<0.0001	E-K
6 vs. 7	768.7	492 to 1045	Yes	****	<0.0001	F-G
6 vs. 8	784.1	507.4 to 1061	Yes	****	<0.0001	F-H
6 vs. 9	281.1	4.408 to 557.8	Yes	*	0.0455	F-I
6 vs. 10	35.58	-241.1 to 312.3	No	ns	>0.9999	F-J
6 vs. 11	743.7	467 to 1020	Yes	****	<0.0001	F-K
7 vs. 8	15.42	-261.3 to 292.1	No	ns	>0.9999	G-H
7 vs. 9	-487.6	-764.3 to -210.9	Yes	***	0.0007	G-I
7 vs. 10	-733.1	-1010 to -456.4	Yes	****	<0.0001	G-J
7 vs. 11	-24.96	-301.6 to 251.7	No	ns	>0.9999	G-K
8 vs. 9	-503	-779.7 to -226.3	Yes	***	0.0005	H-I
8 vs. 10	-748.5	-1025 to -471.8	Yes	****	<0.0001	H-J
8 vs. 11	-40.37	-317.1 to 236.3	No	ns	0.9999	H-K
9 vs. 10	-245.5	-522.2 to 31.17	No	ns	0.0976	I-J
9 vs. 11	462.6	185.9 to 739.3	Yes	**	0.0011	I-K
10 vs. 11	708.1	431.4 to 984.8	Yes	****	<0.0001	J-K