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MASTER THESIS

Assessment of the immune responses in HLA A*0201 patients with various types of chemo-resistant advanced solid tumours after vaccination with the therapeutic telomerase – specific vaccine Vx-001

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ABSTRACT

Background: The human telomerase reverse transcriptase is highly expressed in most human tumours and correlates with poor prognosis. The Vx-001 is an HLA-A*0201 restricted therapeutic telomerase-specific tumour vaccine which is composed of the 9-mer cryptic $hTERT_{572}$ peptide and its optimized variant $hTERT_{572Y}$. Based on previous studies supporting that Vx-001 is non-toxic, highly immunogenic and its specific immune response is correlated with prolonged survival in vaccinated NSCLC patients, its specific-T cell immune response in 77 HLA-A*0201 patients with various types of chemo-resistant advanced solid tumours, was further studied. The aim of the present study was to investigate the specific -T cell immune response against Vx-001.

Methods: All patients received 2 subcutaneous vaccinations with the optimized $TERT_{572Y}$ peptide followed by 4 vaccinations with the native $TERT_{572}$ peptide at 3 week intervals. Patients who completed the six-vaccination schedule and exhibited disease stabilization received booster vaccinations with the native peptide every 3 months until disease progression. Peripheral blood mononuclear cells (PBMCs), from patients were collected before vaccination, after the 2^{nd} and 6^{th} vaccination and before each boost vaccination and screened for reactivity to the $TERT_{572Y}$ and $TERT_{572}$ peptides by measuring IFN-γ production using ELISpot assay. Moreover, IFN-γ and IL-10-producing cells in response to the peptides were identified by intracellular staining (ICS) using flow cytometry.

Results: $TERT_{572Y}$-specific immune responses was induced in 21%, 27% and 32% of the vaccinated patients before the 1^{st} and after the 2^{nd} and 6^{th} vaccination, respectively. Similarly, $TERT_{572}$-specific immune responses was induced in 25%, 41% and 51% of the vaccinated patients before the 1^{st} and after the 2^{nd} and 6^{th} vaccinations respectively, as assessed by IFN-γ ELISpot. These responses were confirmed by Intracellular staining for IFN-γ. Moreover, it was observed that 40% of patients with a $TERT_{572Y}$-specific response after the 2^{nd} vaccination (early response) developed a $TERT_{572Y}^{+}/TERT_{572}^{+}$ immune response after the 6^{th} vaccination (late response). It was indeed a confirmation, of the initial hypothesis that the optimized $TERT_{572Y}$ peptide first generates peptide-specific T cells and then the stimulation with the native $TERT_{572}$ peptide selects among T cells those with the specificity for the native $TERT_{572}$ peptide which is presented by tumour cells.

Furthermore, boost vaccinations with $hTERT_{572}$ every three months in responding patients maintained the number of hTERT-specific cells in most cases. Surprisingly, patients without a $TERT_{572Y}$ and $TERT_{572}$ -specific pre-existing IFN-γ immune reactivity, responded more frequently after the 2nd vaccination than patients with a $TERT_{572Y}$ and $TERT_{572}$ -specific pre-existing IFN-γ immune reactivity. Furthermore, the presence of IL-10 either prior to vaccination or after the 2nd and 6th vaccination, was inversely correlated with the development of vaccine-induced $hTERT_{572}$-specific IFN-γ immune response.

Finally, in order to determine whether there was an association between the TERT-specific-IFN-γ immune reactivity and overall survival (OS), the outcome of the patients who enrolled the vaccination protocol with clinically documented stable (SD) or progressive disease (PD) was analyzed. It was observed that OS was significantly longer in late immune $TERT_{572}$-specific responding patients compared to non-late responding patients.

Conclusion: The results of the current study demonstrate that Vx-001 is able to induce a TERT-specific immune response in vaccinated patients with different types of solid tumours, as our vaccination strategy circumvented the immune tolerance of TERT. The mechanisms regulating the induction of Vx-001-specific immune response need to be further investigated as this vaccine seems to be promising anticancer vaccine and may pave the way for a new era in the field of cancer immunotherapy

ΠΕΡΙΛΗΨΗ

Θεωρητικό Υπόβαθρο: Η ανθρώπινη καταλυτική υπομονάδα της τελομεράσης (hTERT) έχει βρεθεί ότι είναι ένα αντιγόνο των όγκων που εκφράζεται σε πάνω από το 85% των καρκίνων του ανθρώπου. Το Vx-001, είναι ένα θεραπευτικό εμβόλιο της Τελομεράσης που αποτελείται από το 9μερές κρυπτικό-φυσικό πεπτίδιο $hTERT_{572}$ και την τροποποιημένη-βελτιστοποιημένη μορφή του, $hTERT_{572Y}$. Σύμφωνα με προηγούμενες μελέτες, το Vx-001 είναι μη τοξικό, ισχυρά αντιγονικό και η ανοσολογική απάντηση που επάγει συσχετίζεται με την παράταση επιβίωσης σε εμβολιασμένους ασθενείς με NSCLC. Έτσι, χρησιμοποιήσαμε για τον εμβολιασμό, δόσεις με το τροποποιημένο πεπτίδιο $hTERT_{572Y}$ και κατόπιν με το φυσικό πεπτίδιο $hTERT_{572}$ σε 77 ασθενείς με διάφορα προχωρημένα κακοήθη νεοπλάσματατα, οι οποίοι έχουν απλότυπο Αντιγόνων Ιστοσυμβατότητας (HLA)-A0201, για να εκτιμήσουμε την δυνατότητα πρόκλησης ειδικής ανοσολογικής αντίδρασης ενάντια στο «κρυπτικό» πεπτίδιο της τελομεράσης ($hTERT_{572}$) και δευτερευόντως την πιθανή αντινεοπλασματική δράση του θεραπευτικού εμβολίου. Η ανοσολογική ανταπόκριση αξιολογείται με την ανίχνευση της συχνότητας των $TERT_{572}$ –ειδικών $CD8^+$ κυτταροτοξικών Τ λεμφοκυττάρων (CTLs) στα μονοπύρηνα κύτταρα του αίματος (PBMC) των εμβολιασμένων ασθενών μετά από in vitro διέγερση των PBMC με το φυσικό πεπτίδιο $TERT_{572}$.

Μέθοδοι: Όλοι οι HLA-A*0201 ασθενείς εμβολιάσθηκαν ανά τρεις εβδομάδες με δύο υποδόριες χορηγήσεις των 2mg του $hTERT_{572Y}$ τροποποιημένου πεπτιδίου ακολουθούμενες από τέσσερις χορηγήσεις των 2mg του $hTERT_{572}$ φυσικού πεπτιδίου σε απουσία προόδου νόσου. Τα πεπτίδια αραιώνονται με Montanide (ISA51) ακριβώς πριν τον εμβολιασμό. Στους ασθενείς που δεν παρατηρήθηκε πρόοδος νόσου μετά τη συμπλήρωση των 6 εμβολιασμών, χορηγήθηκαν αναμνηστικές δόσεις με 2mg $hTERT_{572}$ ανά τρεις μήνες μέχρι εμφάνισης προόδου νόσου. Απομονώθηκαν μονοπύρηνα κύτταρα από το περιφερικό αίμα (PBMCs) των εμβολιασμένων ασθενών, το οποίο συλλέχθηκε πριν την πρώτη και μετά τη δεύτερη και έκτη χορήγηση του εμβολίου καθώς επίσης και πριν από κάθε αναμνηστική χορήγηση. Η απόκριση των PBMCs στα πεπτίδια, προσδιορίστηκε μετρώντας την παραγωγή της IFN-γ με τι μέθοδο ELISpot, πριν την πρώτη, μετά τη δεύτερη και μετά την έκτη χορήγηση του πεπτιδίου. Επίσης, προσδιορίστηκαν τα κύτταρα που παράγουν IFN-γ και IL-10 μέσω ενδοκυττάριας χρώσης, με τη χρήση του Κυτταρομετρητή Ροής.

Αποτελέσματα: Με τη χρήση της μεθόδου ELISpot υπολογίσθηκε ότι το 21% των ασθενών εμφάνισαν ειδική-$TERT_{572Y}$ ανοσολογική απόκριση πριν τον πρώτο εμβολιασμό, αντίστοιχα το 27% μετά τον δεύτερο εμβολιασμό και το 32% μετά τον έκτο εμβολιασμό. Όσον αφορά στο $TERT_{572}$ φυσικό πεπτίδιο, το 25% των ασθενών εμφάνισαν ειδική-$TERT_{572}$ ανοσολογική απόκριση πριν τον πρώτο εμβολιασμό, το 41% μετά τον δεύτερο εμβολιασμό και το 51% μετά τον έκτο εμβολιασμό. Τα αποτελέσματα αυτά επιβεβαιώθηκαν μέσω ενδοκυττάριας χρώσης για την IFN-γ. Επιπλέον παρατηρήθηκε ότι το 45% των ασθενών που εμφάνισαν ειδική -$TERT_{572Y}$ ανοσολογική απόκριση μετά τον δεύτερο εμβολιασμό, ανέπτυξαν ειδική - $TERT_{572}$ απάντηση μετά τον έκτο εμβολιασ μ ό. Το γεγονός αυτό, πιθανόν αποτελεί επιβεβαίωση της αρχικής υπόθεσης ότι το $TERT_{572Y}$ τροποποιημένο πεπτίδιο επάγει πρώτα τη δημιουργία ειδικών-Τ λεμφοκυττάρων και στη συνέχει το $TERT_{572}$ φυσικό πεπτίδιο επιλέγει ανάμεσα στα διεγερμένα Τ κύτταρα αυτά με την υψηλότερη ειδικότητα για το φυσικό πεπτίδιο, το οποίο είναι παρόν στα καρκινικά κύτταρα

Επιπλέον παρατηρήθηκε ότι η πλειοψηφία των ασθενών που έλαβαν αναμνηστικές χορηγήσεις, διατήρησε την ανοσολογική απάντηση. Μια σημαντική παρατήρηση ήταν ότι, οι ασθενείς οι οποίοι δεν εμφάνισαν $TERT_{572Y}$ ή $TERT_{572-}$ ειδική ανοσολογική απάντηση πριν τον πρώτο εμβολιασμό, παρουσίασαν πιο συχνή απόκριση μετά τον δεύτερο εμβολιασμό, σε σύγκριση με τους ασθενείς που δεν είχαν αναπτύξει πρόωρη $TERT_{572Y}$ ή $TERT_{572-}$ ειδική ανοσολογική απάντηση.

Επιπλέον, τα αποτελέσματα αυτά συσχετίστηκαν με τα αποτελέσματα που δόθηκαν από τη χρώση με IL-10 και παρατηρήθηκε ότι η ύπαρξη των ειδικών-CD8+ Τ-λεμφοκυττάρων που παράγουν IL-10 ήταν αντιστρόφως ανάλογη με τη ύπαρξη των ειδικών-CD8+ Τ-λεμφοκυττάρων που παράγουν IFN-γ, Τέλος, για να προσδιοριστεί εάν υπάρχει συσχέτιση μεταξύ της TERT-ειδικής-IFN-γ ανοσολογικής απόκρισης και της συνολικής επιβίωσης των ασθενών (OS), αναλύθηκε η κλινική έκβαση των ασθενών που εμφάνησαν πρόοδο νόσου (PD) ή σταθεροποίηση νόσου (SD) κατά την ένταξή τους στο πρωτόκολλο εμβολιασμού. Παρατηρήθηκε ότι οι ασθενείς με πρόοδου νόσου που ανέπτυξαν $TERT_{572-}$ ειδική ανοσολογική απάντηση μετά τον έκτο εμβολιασμό (late responders) είχαν μεγαλύτερη επιβίωση και το αποτελέσμα ήταν στατιστικά σημαντικό.

Συμπεράσματα: Η μελέτη αυτή απέδειξε την ικανότητα του Vx-001 να προκαλεί hTERT ειδική ανοσολογική απάντηση σε εμβολιασμένους HLA-A*0201 ασθενείς με διάφορα προχωρημένα κακοήθη νεοπλάσματατα. Ωστόσο, χρειάζονται επιπρόσθετες κλινικές μελέτες για να εξαχθούν ασφαλή συμπερασμάτα για το εάν βρισκόμαστε στο κατώφλι μιας νέας και πολλά υποσχόμενης εποχής στον τομέα της Ανοσοθεραπείας του καρκίνου.

ABBREVIATIONS

IFN-γ	Interferon- γ
IL-10	Interleukin-10
PBMCs	Peripheral Blood Mononuclear Cells
OS	Overall Survival
SD	Stable Disease
PD	Progressive Disease
NKs	Natural Killer Cells
DCs	Dendritic Cells
TCRs	T-Cell Receptors
AMI	Antibody-Mediated Immunity
CTLs	Cytotoxic T- cells
APCs	Antigen Presenting Cells
MHC	Major Histocompatibility Complex
GM-CSG	Granulocyte Macrophage-Colony Stimulating Factor
TGF	Tumour Growth Factor
NSCLC	Non Small Cell Lung Cancer
HCC	Hepatocellular Cancer
CRC	Colerectal Cancer
ADCC	Antibody-Dependent Cellular Cytotoxicity
HSPs	Heat Shock Proteins
TAAs	Tumour Associated Antigens
TNF	Tumour Necrosis Factor
T Regs	T Regulatory cells
TLRs	Toll-Like Receptors
BCG	Bacillus Calmette Guerin
DDR	DNA Damage Response
TERC	TElomerase RNA Component
TERT	TElomerase Reverse Transcriptase
DKC	Dyskerin

RNP	Ribonucleoprotein
LOH	Loss of Heterozygosity
ALT	Alternative Lengthening of Telomeres
EDTA	Ethylenediaminetetra acetic acid
RT	Room Temperature
HS	Human Serum
BSA	Bovine Serume Albumine
PBS	Phosphate Buffered Saline
FCS	Fetal Calf Serum
SEB	Staphylococcal Enterotoxin B
ConA	Concanavalin A
ER	Endoplasmic Reticulum
ICS	Intracellular Staining

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1. INTRODUCTION

1.1 PHYSIOLOGY OF THE IMMUNE SYSTEM

The immune system is a well organized system that is orchestrated at various levels (molecular, cellular, and systemic) in order to protect the organism. It consists of biological structures and processes within an organism that protects against disease by identifying and killing pathogens and tumour cells. It detects a wide variety of agents, from viruses to parasitic worms, and distinguishes them from the organism's own healthy cells and tissues in order to function properly. Detection is complicated as pathogens can evolve rapidly, producing adaptations that avoid the immune system and allow the pathogens to successfully infect their hosts. (Zou *et al.,* 2005; Sprent and Cho *et al.,* 2008).

The immune system adapts to recognize specific pathogens efficiently by distinguishing between self and non-self molecules of the organism's body (Mayer, 2006). Conversely, non-self molecules are those recognized as foreign molecules. One class of non-self molecules are called antigens (short for antibody generators) and are defined as substances that bind to specific immune receptors and elicit an immune response (immunogenicity) (Alberts *et al*., 2002). Immunogenicity causes the proliferation of specific T-cells or the production of particular antibodies by B-cells. There are three important features of an immunological response relevant to host defence and/or "immunity" to pathogenic microorganisms:

1) **Specificity**: An antibody will react specifically with the antigen which induces its formation and not with other antigens. Generally, this specificity is of the same order as that of enzyme-substrate specificity or a receptor-ligand interaction. However, cross-reactivity is possible. The specificity of the immunological response is explained on the basis of the clonal selection hypothesis: during the immune response, a specific antigen selects a pre-existing clone of specific lymphocytes and stimulates exclusively its activation, proliferation, and differentiation.

2) **Memory**: The immunological system has a "memory". During the first attack by a specific invading agent, only specific cells are able to recognize the antigen. This is called the "primary response". During the attack thousands of memory T-cells and memory B-cells are formed. When

the same antigen launches another attack weeks, months, or years later, memory cells, developed during the initial invasion, spring swiftly into action and therefore, a second rapid and vigorous response is prompted for the elimination of the antigen. This accelerated response is called a "secondary response". Antibodies produced in the secondary response have a higher affinity for the antigen than those produced in the primary response. Every time the same antigen is encountered there is a rapid proliferation of memory cells which may last for decades. The "memory" of the immune system is a key benefit of being exposed to pathogens as a child. In theory, exposure to antigens early on life will reduce the risk of the same antigen causing illness later in life (Janeway 2006).

3) **Tolerance:** It refers to the specific immunological non-reactivity to an antigen resulting from a previous exposure to the same antigen. While the most important form of tolerance is non-reactivity to self antigens, it is possible to induce tolerance to non-self antigens. When an antigen induces tolerance, it is called tolerogen. It can be either "self tolerance", or "induced tolerance". Self tolerance is a phenomenon in which the body does not mount an immune response to self antigens. When the immune system recognizes a self antigen and mounts a strong response against it, autoimmune disease develops. However, the immune system has to recognize self-MHC to mount a response against a foreign antigen.Therefore, the immune system is constantly challenged to discriminate self vers non-self and mediate the right response (Janeway 2006).

Tolerance can also be induced to non-self (foreign) antigens by modifying the antigen, by injecting the antigen through specific routes such as oral, as well as administering the antigen when the immune system is developing. Certain bacteria and viruses have devised some ways to induce tolerance so that the host does not kill these microbes. For instance, patients with lepromatous type of leprosy do not mount an immune response against *Mycobacterium leprae* (Janeway 2006).

Disorders in the immune system can result in disease. Immunodeficiency occurs when the immune system is less active than normal, resulting in recurring and life-threatening infections. Immunodeficiency can either be the result of a genetic disease, such as severe combined immunodeficiency, or be produced by pharmaceuticals or an infection, such as the acquired immune deficiency syndrome (AIDS) that is caused by the retrovirus HIV. In contrast, autoimmune diseases result from a hyperactive immune system attacking normal tissues as if they were foreign organisms.

Common autoimmune diseases include Hashimoto's thyroiditis, rheumatoid arthritis, diabetes mellitus type 1, and lupus erythematous (Janeway 2006).

1.1.1 PRINCIPLES OF INNATE AND ADAPTIVE SYSTEM

The immune response to pathogens and/or foreign molecules is divided to two types: innate and adaptive system (table 1.1) Innate defences include mechanical (skin, mucosal surfaces, respiratory cilia) and chemical (gastric acid, lacrimal lysozymes) barriers to entry and proliferation of micro-organisms as well as cellular components such as Natural Killer Cells (NK cells), macrophages and Dendritic Cells (DCs) (figure 1.1). NK cells are a type of cytotoxic lymphocytes that play a major role in the rejection of tumours and cells infected by viruses. They kill cells by releasing small cytoplasmic granules of proteins called perforin and granzyme that causes the target cell to go to apoptosis. They do not express T-cell antigen receptors (TCRs) but they usually express the surface markers CD16 (Fcγ RIII) and CD56 in humans (Nagler *et al.,* 1989**).**They were called "natural killers" because of the initial notion that they do not require activation in order to kill cells that are missing "self" markers of major histocompatibility (MHC) class I.

Table 1.1: **The types of the immune response** (Janeway 2006)

Components of the immune system	
Innate immune system	**Adaptive immune system**
Response is non-specific	Pathogen and antigen specific response
Exposure leads to immediate maximal response	Lag time between exposure and maximal response
Cell-mediated and humoral components	Cell-mediated and humoral components
No immunological memory	Exposure leads to immunological memory
Found in nearly all forms of life	Found only in jawed vertebrates

The macrophages and DCs are the first line of defence against many common pathogens in response to stimuli (e.g. bacterial lipopolysaccharide, carbohydrate and double stranded viral RNA), and are important for the control of common bacterial infections. Nevertheless, macrophages cannot always

eliminate infectious organisms as there are some pathogens that they cannot be recognized (Pashov *et al.,* 2007; Prestwich *et al.,* 2008). The cells of the innate immune system, however, play a crucial role in the initiation and subsequent direction of adaptive immune responses, as well as participating in the removal of pathogens that have been targeted by an adaptive immune response. Moreover, because there is a delay of 4 - 7 days before the initial adaptive immune response takes effect, the innate immune response has a crucial role in controlling infections during this period (Janeway 2006).

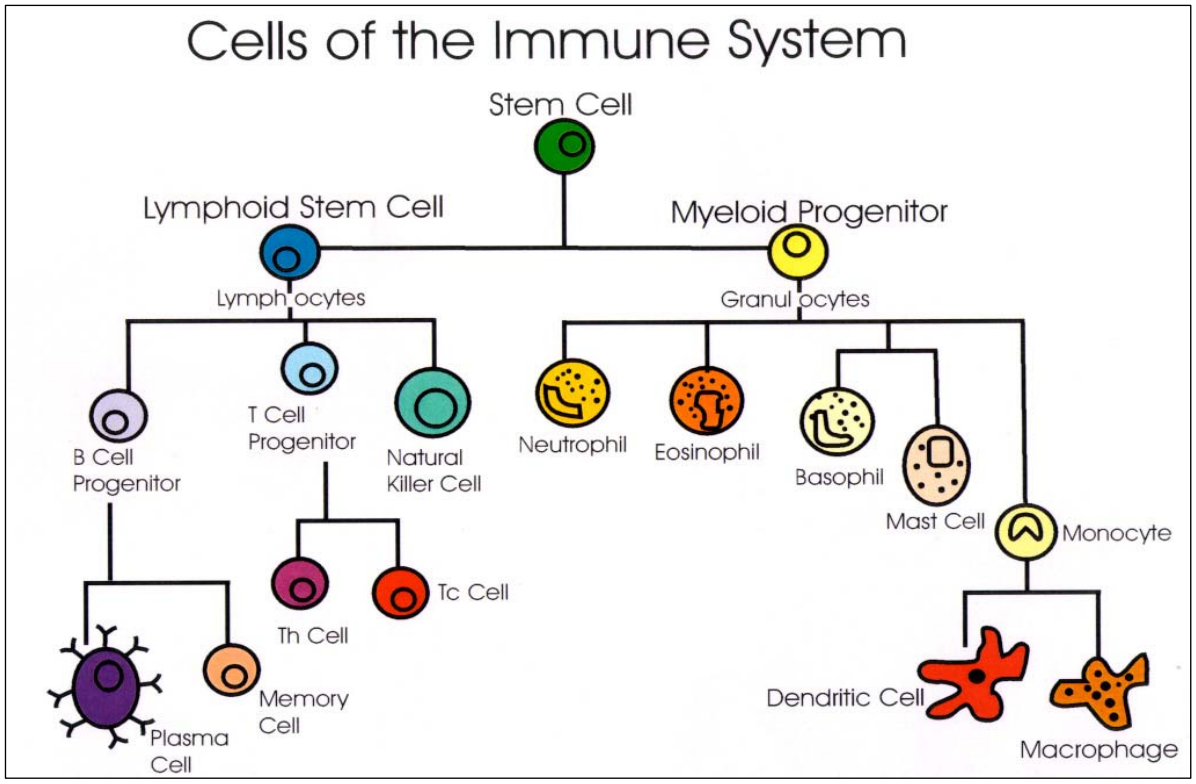


Figure 1.1: **Origin and differentiation of cells of the immune system** (Online Textbook of Bacteriology, Kenneth Todar)

Adaptive responses, by contrast, are slower to develop, are highly specific, and are activated when the host is exposed to an antigen. There are two types of adaptive (or acquired) immunity:

a) The antibody-mediated immunity (AMI) which includes B lymphocytes and is mediated by soluble host proteins called antibodies or immunoglobulins. Due to the presence of circulating antibody

molecules in the serum, it is also called circulating or humoral immunity. When a naïve (unstimulated) B cell encounters an antigen, become stimulated in order to be developed into a plasma cell which produces the antibodies that will react with the stimulating antigen. They also develop into clone of identical reactive B-cells called memory B-cells.

b) The cellular (cell-mediated) immunity which is mediated by specific subpopulations of T-lymphocytes called effector T-cells (Germenis and Karanikas *et al.,* 2007; Reiman *et al*., 2007). There are two T cell subsets: $CD8^+$ and $CD4^+$ T cells. CD8-expressing cytotoxic T cells (CTLs) recognize antigenic peptides which are presented to T cells via MHC class I molecules. CTLs mediate their effector functions by killing the antigen presenting cells (APCs) which present the specific antigens or secreting 'effector' cytokines such as interferon-γ (IFN-γ). CD4- expressing T cells, also referred to as T-helper cells (Th1-Th2), recognize antigenic peptides in association with MHC class II molecules and mediate their effector functions by enhancing the persistence of antigen-stimulated $CD8^+$ T cells, help for the production of the antibodies or through secretion of 'effector' cytokines, notably IFN-γ (Gilboa *et al.,* 2004). The lymphocytes of the adaptive immune system have evolved to provide a more versatile means of defence which, in addition, provides increased protection against subsequent re-infection with the same pathogen (immunological memory). This process of acquired immunity is the basis of vaccination.

1.1.2 DENDRITIC CELLS AS KEY ORCHESTRATORS OF THE IMMUNE RESPONSE

Dendritic cells (DCs) originate in the bone marrow, function as antigen presenting cells (APCs) and are present throughout the body. They are particularly prominent and active in skin and mucosal surfaces, sites of multiple and repeated exposure to antigens and microbial agents. The likelihood of an antigen being presented to its complementary lymphocyte is greatly increased by the presence of Antigen Presenting Cells (APCs) in the tissues (Banchereau and Steinman *et al.,* 1998). Dendritic cells reside within the tissues as immature cells, where they take up protein by micropinocytosis, process the protein within the intracellular organelles and present the constituent antigens with MHC molecules on the DC surface (figure 1.2). This occurs while the DC is both maturing and migrating from the tissues toward the lymph nodes.

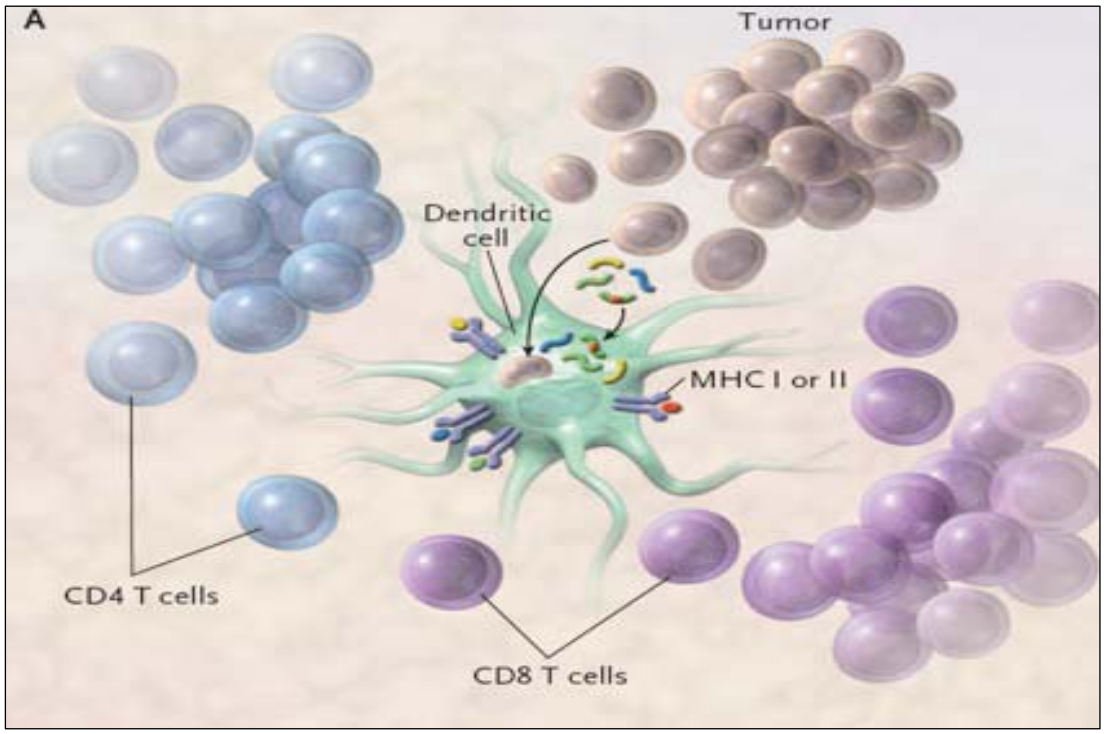


Figure 1.2: **Function of DCs**. Dendritic cells in the draining lymph node take up dying infected cells, proteins, and peptides and process and display them in their major-histocompatibility-complex (MHC) class I and class II molecules. If properly activated by immuno-stimulatory tumour products or other factors in the tumourinfected microenvironment, the dendritic cells induce effective -specific $CD4^+$ and $CD8^+$ T cells **(**Finn *et al*., 2008).

On arrival at the lymph node, the mature and activated DC has developed numerous cellular processes, an abundance of MHC class I and II molecules, as well as T cell binding and costimulatory molecules and actively secretes IL-12. In the lymph node, DCs present antigen to lymphocytes and stimulate clonal expansion of $CD4^+$ and $CD8^+$ T cells (figure 1.3). CTLs are then able to enter the circulation and are carried to the site of infection where they recognise and destroy infected cells.

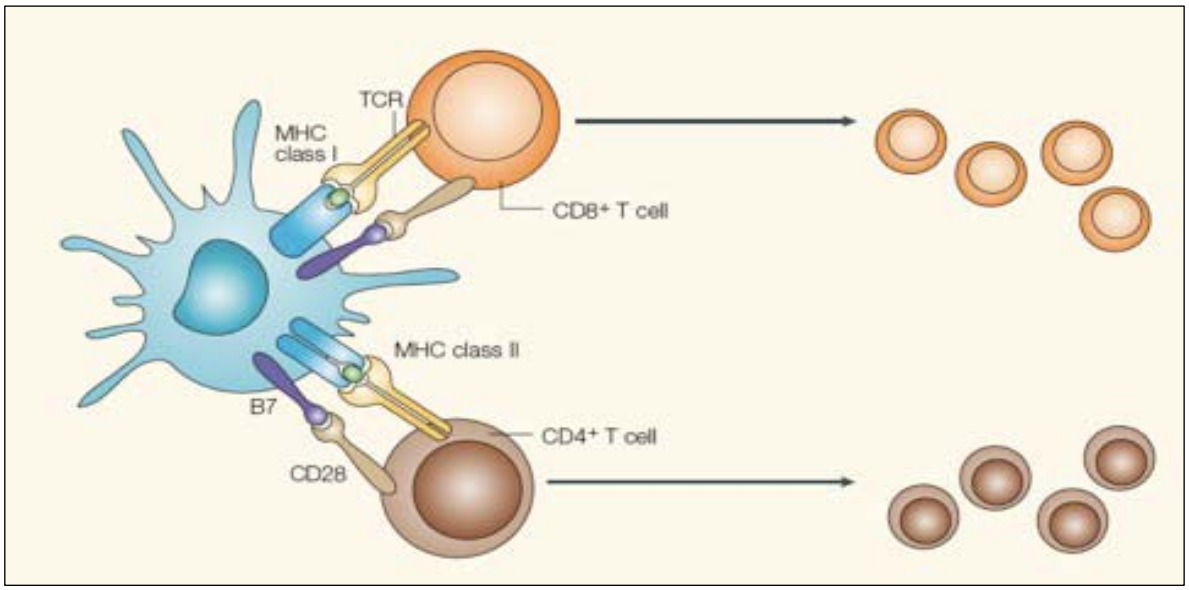


Figure 1.3: Activation of T cells by dendritic cells. Having acquired antigen in the periphery, the dendritic cell migrates to the draining lymph nodes, where it encounters a cognate $CD4^+$ or $CD8^+$ T cell, which is activated following presentation of antigen-derived peptides in association with major histocompatibility complex (MHC) class II or class I, respectively, to the T-cell receptor (TCR). Co stimulatory signals, mediated through B7–CD28 interactions, are needed in most instances to fully activate the T cell. Additional co-stimulatory signals, such as signals mediated through CD40L–CD40, or OX40L–OX40 interactions (not shown in the figure), are required to promote survival and proliferative capacity of the activated T cells and generation of memory T cells (Gilboa *et al.,* 2004).

There is considerable interest in the therapeutic potential of enhancing T cell migration into infected tissue. In general, DCs are considered to be key antigen presenting and immune-regulatory cells and represent a vital link between innate and adaptive immunity and the translation of innate activation into specific and biologically effective immune responses (Wilson *et al.,* 1970; Cronin and Penninger *et al.,* 2007; Gilboa *et al*., 2004).

1.2 THE IDEAL T-CELL MEDIATED IMMUNE RESPONSE TO CANCER

Immune responses in cancer patients are often far from ideal. Tumour cells are altered-self cells; therefore, it is expected that cancerous cells would elicit a cell-mediated response in order to target cancerous cells and destroy them. Three prerequisites are necessary for tumour elimination. Firstly, the immune system "see" the cancer, secondly the lymphocytes become activated and finally cancer cells are susceptible to killing.

In order for this to occur, the following procedure takes place. Lymphocytes infiltrate the tumour site, $CD4^+$ Th1 recognize tumour-specific antigens in association with MHC II molecules on the surface of APCs and receive signals from costimulatory molecules, such as B7 and GM-CSF (Granulocyte-macrophage colony stimulating factor). In turn, Th1 cells become activated and release appropriate cytokines such as, IL-2, IFN-γ and TNF-α. These cytokines, activate CTLs in order to lysis cancerous cells. Activation of B lymphocytes also occurs to secrete neutrilizing antibodies that aid in tumour cell phagocytosis by phagocytes, although their role in tumour immunity is less important (figure 1.4; Davis *et al*., 2003, Klein *et al*., 2005, Cronin *et al*., 2007).

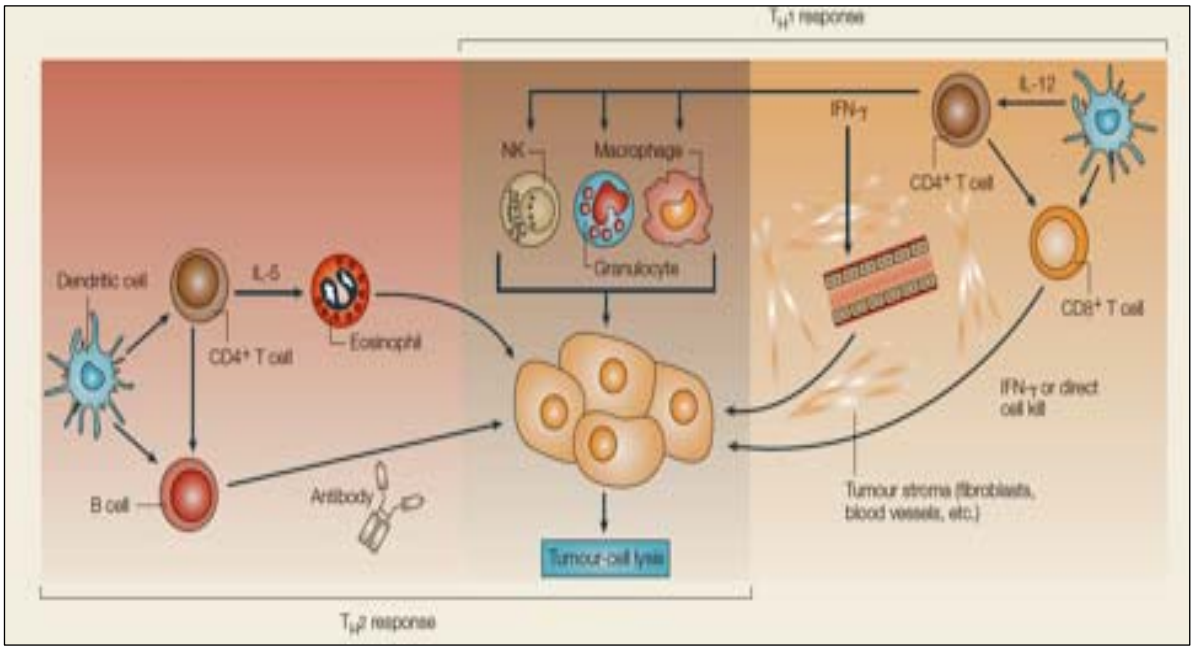


Figure 1.4: Antitumour effector arms of the immune response. The immune system can impact on tumour growth in several ways. It is thought that the most effective way of eradicating a tumour mass *in vivo* is through the combined action of $CD8^+$ and interferon-γ (IFN-γ)-secreting T-helper 1 (Th_1) $CD4^+$ T cells. Tumour specific $CD8^+$ T cells activated by dendritic cells (DCs) presenting tumour antigens can kill tumour cells directly. The survival and persistence of $CD8^+$ T cells as memory cells is regulated by tumour-specific $CD4^+$T cells. Both $CD8^+$ and, especially, $CD4^+$ T cells secrete IFN-γ, which can further sensitize tumour cells to $CD8^+$ T cells by upregulating major histocompatibility complex (MHC) class I and other components of the antigen-processing machinery, promoting the recruitment of natural killer (NK) cells, granulocytes or macrophages, and interfering with crucial functions of the tumour stroma — namely, angiogenesis. Tumours can also be controlled by Th $_2$-type immune responses, whereby DCs activate interleukin-5-secreting Th_2 $CD4^+$ T cells, which induce the accumulation of eosinophils in the tumour bed and/or provide 'T help' for the generation of a humoral, antibody-based antitumour response. Which pathway predominates depends on the biology of the tumour and/or the method of immunization (Gilboa *et al.,* 2004).

A main characteristic of activated CTLs is the existence of membrane-bound cytoplasmic granules which contain proteins such as perforin and granzymes that are used to kill other cells (figure 1.5). The CTL mediated lysis of cancerous cell is the ultimate action of an effective immune response against cancer. If any of the processes necessary for the induction of a cell-mediated response fail, tumour elimination may not be effective (Disis *et al*., 1996, Ye *et al.,* 2008).

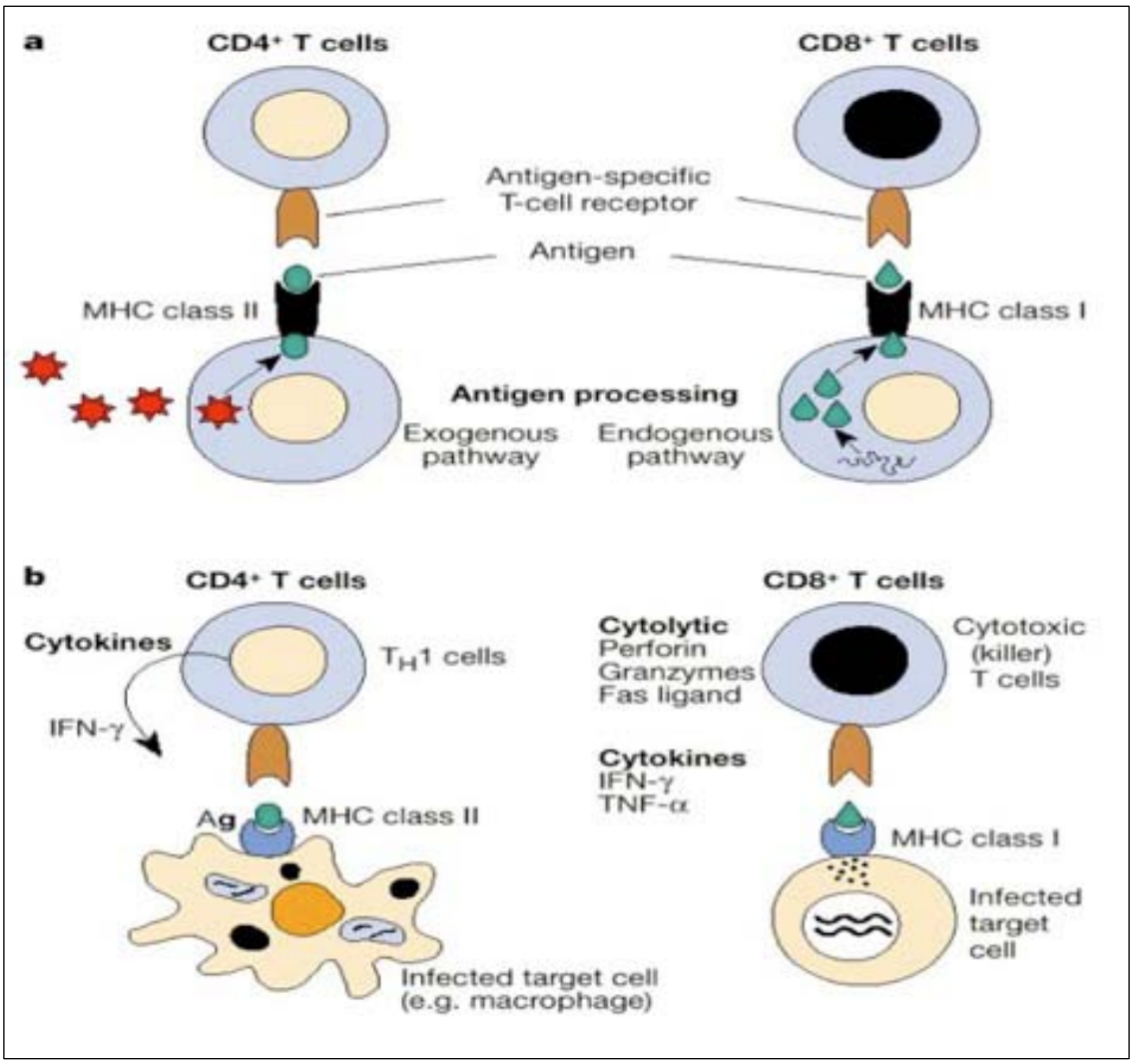


Figure 1.5: (a) T cells functional mechanism of antigen recognition by T cells (b) CD4+ (Th1) cells and CD8+ (cytotoxic) cells function

http://www.fruiting-bodies.co.uk/cancer_research/cr_appendix1.htm

1.2.1 CANCER IMMUNOSURVEILLANCE AND IMMUNOEDITING

In 1909, it was predicted by Paul Ehrlich that the immune system repressed the growth of carcinomas that would otherwise occur with great frequency. Therefore, it caused the initiation of a century of heated debate over immunologic control of neoplasia (Rescigno *et al.,* 2007; Gilboa *et al.,* 2004). The immune surveillance theory hypothesizes that cancerous cells arise regularly but the body eliminates them before they become harmful to surrounding tissue. Only those that escape surveillance develop into tumours. There is much evidence supporting the theory of immune surveillance. First of all, malignancy is most common in older people when immune system is poorest. Moreover, malignancy rates increase in patients with chronic immunosuppression (such as congenital immunodeficiency, AIDS). Last but not least, most cancer patients do not have substantive immune dysfunction; therefore, immune dysfunction in malignacy is tumour specific. If the immune surveillance theory is correct, there is a strong selective pressure favoring cancer cells that can avoid notice or somehow protect themselves from being killed by the immune system (Wilson *et al.,* 1970; Dunn *et al*., 2002; Rosenberg *et al.,* 2001).

However, there has been a growing recognition that immunosurveillance represents only one dimension of the complex relationship between the immune system and cancer (Dunn *et al.,* 2002; Dunn *et al.,* 2004**).** Recent work has shown that the immune system may also promote the emergence of primary tumours with reduced immunogenicity that is capable of escaping immune recognition and destruction (Armstrong and Dermime *et al.,* 2001; Klein and Klein *et al.,* 2005). These findings prompted the development of the cancer immunoediting hypothesis to broadly encompass the potential host-protective and tumour-sculpting functions of the immune system throughout tumour development (Dunn *et al.,* 2002; Dunn *et al*., 2004). Cancer immunoediting is a dynamic process composed of three phases as described in figure 1.6: 1) elimination, 2) equilibrium and 3)escape.

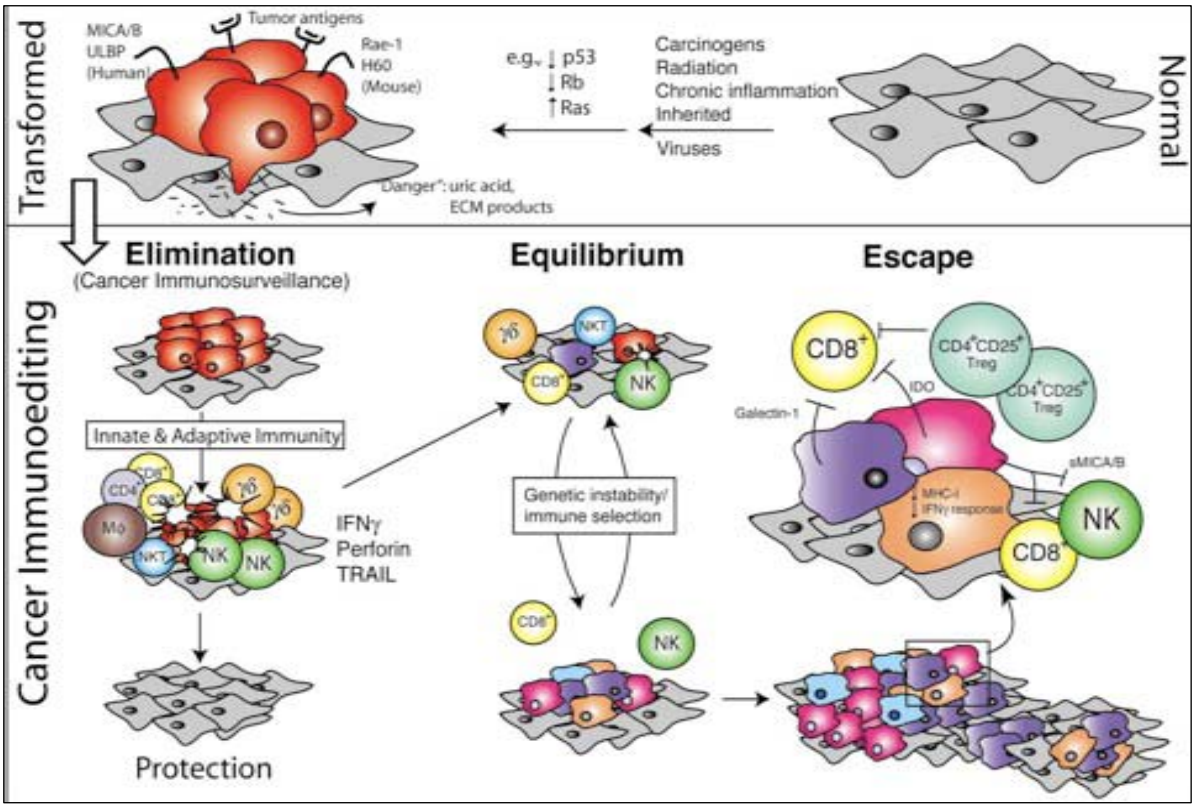


Figure 1. 6. The Three Phases of the Cancer Immunoediting Process. Normal cells (gray) subject to common oncogenic stimuli ultimately undergo transformation and become tumourtumour cells (red) (top). Even at early stages of tumourigenesis, these cells may express distinct tumourtumour-specific markers 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 immunosurveillance 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 maybe 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).

As far the "escape" phase is concerned, tumours escape immune surveillance in a variety of ways (low immunogenicity, antigenic modulation or tumour-induced immune suppression; figure 1.7). Depending upon the particular mutations accumulated and the location of the tumour, cancers can be quite diverse. The diversity of cancer applies to their immune evasion strategies as well (Murphy *et al.,* 2007)**.** The mechanisms that cancer uses to escape host immune responses may vary among

different types of cancers and even within a particular type. In general, tumours that are better at escaping host immunity may be more malignant than those that inefficiently evade immune responses.

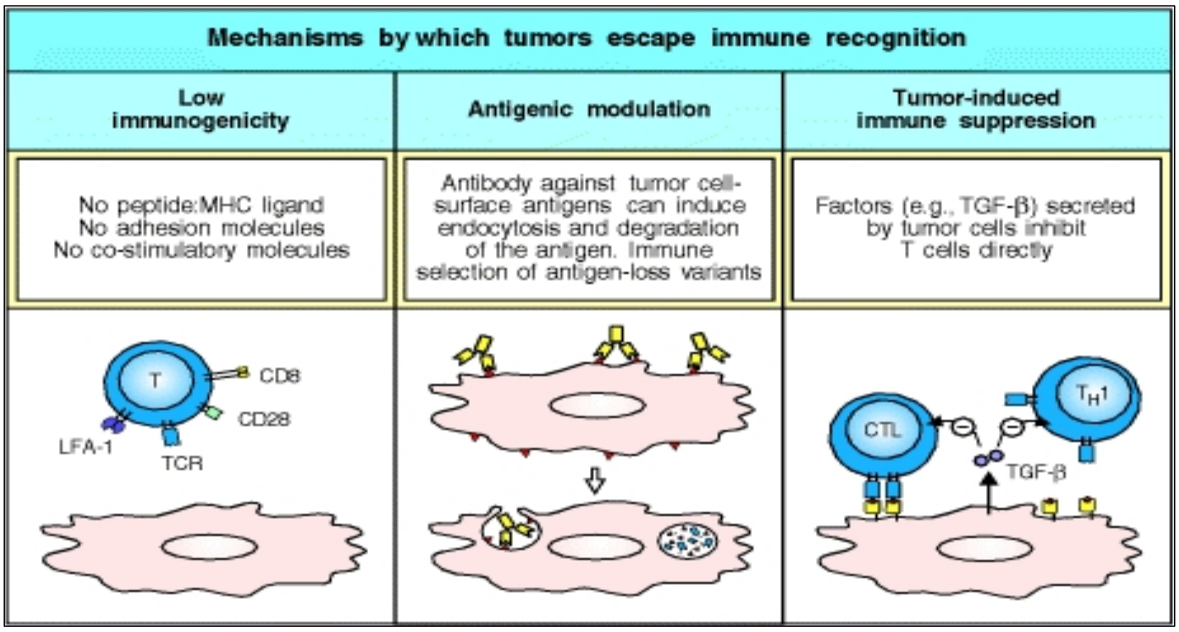


Figure 1.7: Tumours can escape immune surveillance in a variety of ways. First, tumours can have low immunogenicity (left panel). Some tumours do not have peptides of novel proteins that can be presented by MHC molecules, and therefore appear normal to the immune system. Others have lost one or more MHC molecules, and most do not express co-stimulatory proteins, which are required to activate naive T cells. Secondly, tumours can initially express antigens to which the immune system responds but lose them by antibody-induced internalization or antigenic variation. When tumours are attacked by cells responding to a particular antigen, any tumour that does not express that antigen will have a selective advantage (center panel). Thirdly, tumours often produce immunosuppressive substances, such as TGF-β, that suppress immune responses directly (right panel) (Janeway 2006).

1.3 CANCER IMMUNOTHERAPY

Cancer immunotherapy is mainly based on the activation of CTLs, which are considered chief mediators of tumour immuno-surveillance. Immunotherapy plays a pivotal role in the recognition and elimination of tumour cells, controls the tumour growth, and restricts cancer metastasis (Vonderheide *et al.,* 2004).

The potential of cancer immunotherapy was first documented by William Coley in 1890, when bacterial products (Coley's toxins) were administered for advanced inoperable cancers with dramatic response (figure 1.8). The idea of cancer immunotherapy was ignored until the latter years of the 20th century, when studies of chemically induced tumours of inbred mice showed transplantation resistance and spontaneous regression of melanoma, cast speculation that immunologic responses contributed to tumour regression (Quan *et al.,* 1997; Atanackovic *et al.,* 2008; Schmidt *et al.,* 2009)**.** While the non-specific immunotherapy was used in the past with representative examples the administration of IL- 2, IFN-α, και BCG for melanoma, kidney cancer and non-invasine bladder cancer respectively, in recent years scientists have started to use specific antigens that are expressed in tumour cells (tumour-associated antigens-TAAs). These new strategies have given satisfactory immune responses in patients with prostate cancer, melanoma (Zajac *et al.,* 2003; Quan *et al.,* 1997), and NSCLC (O'mahony *et al.,* 2005). Especially for NSCLC, many randomized studies with approved clinical efficiency have been conducted. For instance, the study of BLP-25 vaccine (MUC-1 lipopeptide; Biomira, Merck KGaA) offers a great approach in the MUC-1 target, which is overexpressed and glycozylated in NSCLC. This vaccine was designated so as to elicit an immune response, which it could lead to the immune rejection of cancer tissues that express the MUC-1 antigen (Butts *et al*., 2005). Other studies include the use of Lucanix vaccine (TGF- β antisense modified allogenic tumour cells; NovaRx) and melanoma antigen - 3 (MAGE - 3).

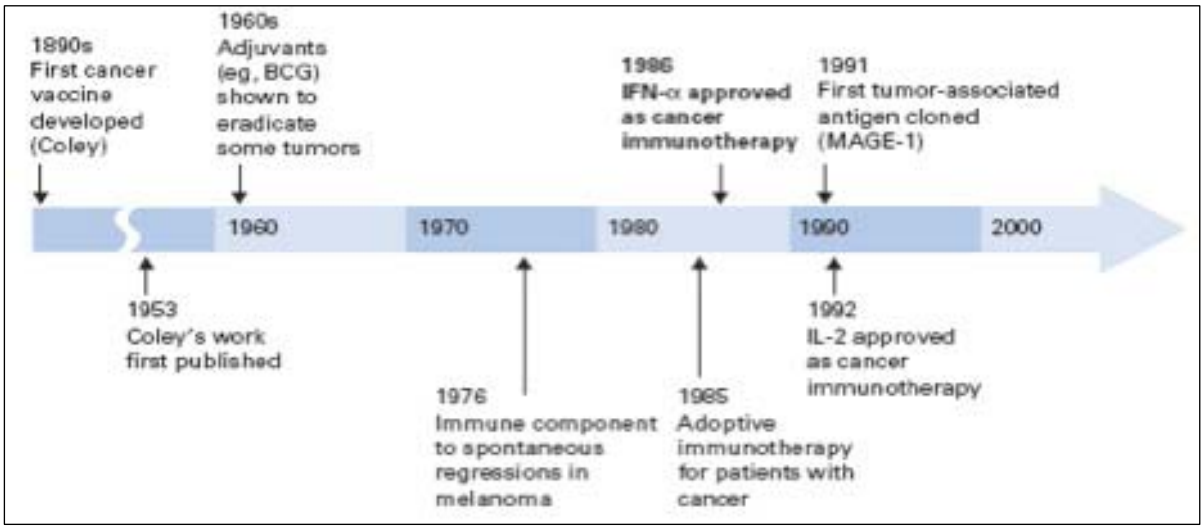


Figure 1.8: Key events in the history of cancer immunotherapy (Kirkwood *et al.,* 2008).

There are three types of cancer immunotherapy:

1) *Non-specific immunotherapy*: It includes the administration of recombinant cytokines such as IL-2, TNF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN or cytokine gene insertion and costimulatory molecules (Rescigno *et al.,* 2007).

2) *Passive immunotherapy*: It is based on the adoptive transfer of ex-vivo activated immune cells, immunomodulators (including cytokines) or tumour-specific antibodies. Up to now, antibody-based therapy has proven to be more beneficial than other immunotherapy approaches. Some antibodies can act either directly by blocking signal transduction pathways (i.e when targeted to growth factor receptors) or indirectly via the activation of NK-mediated killing (ADCC: antibody-dependent cellular cytotoxicity). Alternatively, antibodies can be conjugated to radioactive molecules, chemotherapy drugs or toxins and act as carriers to deliver in a highly specific manner the active compounds (Armstrong *et al.,* 2001).

Other passive immuno-therapies based on the selection of patient's specific anti-tumour T lymphocytes and the reinsertion in the same patient after *in vitro* expansion with cytokine cocktails and/or transduction with high affinity tumour-specific TCRs also hold promise (Greten *et al.,* 2001). However, only episodically beneficial effects on highly selected cohorts of patients have been

observed so far, suggesting that further improvements are still awaited. This strategy has the advantage that, if it is successful, can elicit long-lasting immunological memory that can protect the patient against minimal residual disease and tumour recurrence (Rescigno *et al.,* 2007).

3) *Active immunotherapy*: It is aimed at activating the patient's own immune system via the administration of cancer vaccines against tumour-specific antigens (therapeutic vaccination) or tumour viruses (prophylactic vaccination) (Sin *et al.,* 2009) (Table 1.2)

Table 1.2: **Types of cancer vaccines** (Janeway 2006)

PROPHYLACTIC		THERAPEUTIC
Viral Proteins	*Type of cancer*	Whole tumor cell-based
HPV	Cervical	Protein,peptide-based
Epstein-Barr virus	Burchitt's lymphoma nasopharyngeal cancer	Dendritic cell-based
		DNA vaccines

Tumour specific antigens cause immunological response and come from the modulation of tumour proteins in the cytoplasm of tumour cells. There are four categories of tumour antigens that are possible targets for the cancer patients' vaccination: 1) Specific antigens for melanoma, 2) Tumour-specific antigens, 3) Mutated antigens 4) overexpressed proteins (table 1.3)

Table 1.3. Class I antigens recognized by $CD8^+$ lymphocytes (Mayordomo *et al*., 1996; Quan *et al*., 1997; Kirkwood *et al.,* 2008)

(1) Specific antigens for melanoma	**(2) Tumour-specific antigens**
MART-1/Melan-A	MAGE-1,-2,-3
gp100	MAGE-12
Tyrosinase	BAGE
Tyrosinase related protein-1	GAGE-1,-2
Tyrosinase related protein-2	NY-ESO-1
Melanocyte-stimulating hormone receptor	
(3) Mutated antigens	**(4) Overexpressed proteins**
β- catenin	α- Fetoprotein
MUM-1	Telomerase catalytic protein
CDK-4	G-250
Caspase-8	MUC-1
KIA 0205	Carcinoembryonic antigen
	P53
HLA-A2-R1701	
	Her-2/neu

Therapeutic unlike prophylactic vaccines, must activate an immune response to an antigen that the organism has already been exposed and therefore, need to be more immunogenic than traditional immunizations.

1.3.1 TYPES OF THERAPEUTIC CANCER VACCINES

a) Antigen Vaccines

The antigen vaccines include the use of a protein (protein-based vaccines) or a peptide (peptide-based vaccines) (Disis *et al.,* 2009). Proteins are usually given in combination with adjuvant molecules, such as liposomes or GM CSF (Dranoff *et al.,* 1993). Moreover, a different strategy for cancer immunotherapy is the use of Heat Shock Proteins (HSPs) purified from tumour cells. HSPs are considered to be natural adjuvants that can bind antigenic peptides in the tumour cell and chaperone peptides to APCs in lymph nodes It has been shown that they induce tumour specific immunity when injected into animals. The immunogenicity of HSPs depends on tumour-derived peptides that are associated to HSPs along the purification process (Schmidt *et al*., 2009). The use of peptides as immunogens has many advantages: 1) it minimizes the potential for induction of autoimmunity when compared to the use of a whole protein, which might share fragments with normal cellular proteins, 2) the preparation of peptides is relatively easy and cost affordable, 3) peptides can be modified to increase their immunogenicity by generating peptide agonists and 4) the availability of *in vitro* or *ex vivo* assays that can assess patients' immune response to vaccine epitopes 5) the simplicity of peptide administration in a clinical setting (Belardelli *et al.,* 2004; Apostolopoulos *et al*., 2009).

b) Whole tumour cell vaccines

Whole tumour cell vaccines are divided into two categories: 1) Autologous vaccines in which cancer cells from a patient are used as a vaccine for the same patient. It provides patients with own antigenic repertoire for immunization. 2) Allogeneic vaccines in which tumour cells from other patients (usually from cancer cell lines), are used as a vaccine. Actually, it is an approach based on shared tumour antigens between individuals tumours. Allogeneic vaccines are relatively easy to prepare, comparing to autologous vaccines. Generally, a main advantage of whole tumour cell vaccines is that a range of tumour-associated antigens (TAAs) could be presented to the T cells, including antigens that are still unknown (Greten and Jaffee *et al.,* 2001)**.** Many TAAs can be delivered at the same time, eliminating the need to predetermine which antigens are the most immunogenic and mediate tumour rejection (Lehner and Cresswell *et al.,* 1996; Greten and Jaffee *et al.,* 2001). Extracellular proteins are presented on MHC class II molecules to $CD4^+$ T cells, while intracellular antigens are presented on MHC class I molecules to $CD8^+$ T cells. Nevertheless, it has been shown that exogenous antigens can

be taken up by DCs and presented by MHC class I molecules to $CD8^+$ T cells via so so-called *cross-presentation*, producing effector CTLs (Acherman and Cresswell *et al.,* 2004)**.** Therefore, whole tumour cell vaccines could activate $CD4^+$ and $CD8^+$. However, the preparation of this type of vaccines, requires high costs of production and quality control.

Tumour-cell vaccines can be genetically modified to enhance immunogenicity in many ways. These modifications have mainly focused on cytokines and costimulatory molecules. For instance, transduction of weakly immunogenic murine tumour cells to express IL-2 has shown to reduce tumourioenicity and to induce long lasting immunity against the parental tumour (Fearon *et al*., 1990). Moreover, in several mice tumour models, irradiated tumour cells expressing murine GM-CSF have shown to stimulate a long lasting antitumour immunity (Couch *et al*., 2003). Last but not least, modifications of tumour cells to express high levels of costimulatory molecules have also been used to enhance tumour immunogenicity. For example, adenocarcinoma cells in mice modified via infection with a recombinant vaccinia virus expressing B7-1 have shown to induce immunity against the parental tumour (Hodge *et al.,* 1994).

c) DC-based vaccines

DCs are considered as the most potent APCs and thus, one of the most powerful tools of immunization (Banchereau and Steinman *et al.,* 1998; Lapteva *et al.,* 2007; Gilboa *et al.,* 2007). The antigen loading techniques are numerous and include: 1) pulsing with MHC binding peptides, 2) pulsing with protein, 3) loading with idiotype Ab, 4) loading with tumour lysates or apoptotic tumour cells, 5) DNA and RNA transfection and 6) infection with a viral vector.

Melanoma is the most studied cancer type in DC immunotherapy, because a large variety of tumour-associated antigens have been characterized, consisting of tumour differentiation antigens such as gp100 and tyrosinase and tumour-specific antigens such as MAGE-3 (Atanackovic *et al.,* 2008). Among novel immunization strategies preclinical studies are being conducted on the use of DC tumour cell hybrid vaccines (Koido *et al.,* 2002). Finally, the disadvantage of DC-based vaccines is that clinical grade DCs are difficult to be produced. Large amounts of peripheral blood mononuclear cells (PBMCs) are required and should be cultured for several days in the presence of cytokines such as GM-CSF, IL-4 and/or TNF (tumour necrosis factor).

d) DNA Vaccines

The DNA vaccine is an example of a modern type of vaccine (also called a genetic vaccine). It is composed of a plasmid DNA that encodes the antigen of interest under the control of a mammalian promoter (i.e CMV intron A) and can be easily produced in the bacteria (Rice *et al*., 2008). With DNA vaccines, the subject is not injected with actual antigen but DNA encoding the antigen. This usually involves isolating one or more genes from a disease-causing agent with known antigenic properties and splicing those genes into plasmids (which are closed rings of self replicating DNA). The rings are then delivered into small groups of cells, often by injection, naked or formulated, into muscle cells. Once the plasmid DNA is administered *in vivo*, the encoded antigen is expressed in the host cells, processed, and finally presented by antigen presenting cells such as dendritic cells. This most likely occurs in the draining lymph nodes, whereby both humoral and cellular immune responses were elicited. The ability to introduce antigen to the host immune system, enabling it to elicit strong Th_1 type $CD4^+$ cells and $CD8^+$ cytotoxic T cells, is a unique feature of a DNA vaccine which is also free of the problems associated with producing recombinant protein vaccines as well as with the attenuated or inactivated vaccines which use infectious organisms. In addition, studies with DNA vaccines have shown that even after multiple immunizations, anti-DNA antibodies were not produced. Moreover, DNA vaccines can be easily manufactured and stored on a large scale (Coban *et al*., 2008, Rice *et al*., 2008). Some examples of cancer vaccines which are commercially available and are used in advanced clinical trials are shown in table 1.4.

Table 1.4: Commercial cancer vaccines in advanced stage clinical trials (Old *et al.,* 2008)

CANCER VACCINES IN ADVANCED STAGE CLINICAL TRIALS

Category	Defined Antigen	Tumour Type	Company	Trade Name
Whole Cell	No	Prostate	Cell Genesys	GVAX™
HSP-96	No	Melanoma,Renal	Antigenics	Oncophage
Dendritic cell	PAP	Prostate	Dendreon	Sipuleucel-T (Provenge)
Pox Vector(MVA)	5T4	Renal,Colon	Sanofi-Aventis	TroVax
Conjugate	MUC 1	NSCLC	Merck KGaA	Stimuvax
QS-21/CpG	MAGE-3	NSCLC	GlaxoSmithKine	MAGE-A3 ASCI

1.3.2 FACTORS FOR SUCCESSFUL CANCER IMMUNOTHERAPIES

Cancer immunotherapy rationale focuses on improving the tumour associated immune response either by boosting/enhancing immune elements (table 1.5a), inhibiting suppressors (table 1.5 b) or breaking the T cells tolerance.

The treatments indicated in the table 15a based on the hypothesis that immune elements are missing or are not sufficient in patients with tumours. The strategy for effective immunotherapy is to supplement whatever is missing or not sufficient, thereby boosting tumour-associated antigen (TAA)-specific immunity, and improving patient outcome (Zou *et al*., 2005)

Table 1.5a. Boosting/enhancing immune elements.

Immune elements	Enhancing 'the enhancers'
T cells	Injection of cytotoxic T lymphocytes
Dendritic cells (DCs)	DC vaccination
Natural killer cells	Injection of activated natural killer cells
TAA	Peptide, TAA-vector vaccination
Effector cytokines	Administration of interleukin-2, interferon-α and interleukin-12

In addition, another way to improve the immunotherapy is by blocking the suppressive mechanisms (table 15b), followed by immune boosting as detailed above with the aim of recovering and boosting TAA-specific immunity and improving patient outcome (Zou *et al*., 2005)

Table 1.5b: Inhibition of the immunosuppressive elements.

Suppressive elements	Inhibiting 'the inhibitors'
Regulatory T (T_{Reg}) cells	Blocking T_{Reg} cell function (for example, using denileukin diftitox to kills T_{Reg} cells)
Suppressive, dysfunctional DCs	Blocking suppressive pathways (for example, targeting cells that express B7-H1, B7-H4 or indoleamine-2,3-deoxygenase)
Self antigen or immodominant	Fostering antigen release by targeting the stroma antigen and/or improving antigen tumour priming
Suppressive cytokines	Blocking potential common cytokine signalling pathway (for example, targeting signal transducer and activator of transcription 3 and suppressor of cytokine signalling 1)

T regulatory Cells (Tregs) are the main modulators of the immunosuppression (Curiel *et al.,* 2007). These cells are usually $CD4^+$ and phenotypically they are distinguished by expression of CD25, high levels of cytotoxic T lymphocyte antigen-4 (CTLA-4), the glucocorticoid induced TNF-related receptor (GITR) and the transcription factor Foxp3 (Kiniwa *et al*., 2007, Mizukami *et al.,* 2008). Tregs arise in response to persistent antigen stimulation in the absence of inflammatory signals, in the presence of TGF-β and have been detected in high levels, in some cancer patients. Therefore, it is expected that the depletion of these regulatory T cells *in vivo* may facilitate the elaboration of effective antitumour T-cell responses. Studies in mice tumour models targeting all $CD25^+$ T cells for depletion have appeared promising. According to preclinical studies, reagents that can reduce the number or the function of Tregs are being investigated, which include CD25 antibodies (Kiniwa *et al.,* 2007), the fusion protein denileukin diftitox **(**a recombinant DNA-derived cytotoxic protein composed of the amino acid sequences for diphtheria toxin fragments A and B and the sequences for human interleukin-2; Ontak) (Mizukami *et al*., 2008) and cyclophosphamide (Germain, *et al*., 2008).

Targeting CD25 with monoclonal Aantibodies (mAb) has been shown to effectively deplete Tregs, but due to the long half-life of the antibody it can interfere also with T cell activation during the vaccination process. A way to get around this was the use of denileukin diftitox (Ontak), which has a very short half life (Foss *et al.,* 2000, Vieweg *et al.,* 2007).

Ontak was initially used to treat lymphomas expressing CD25 (LeMaistre *et al.,* 1998), but it has been recently demonstrated to be very efficient in eliminating Tregs in ovarian carcinoma patients (Barnett *et al.,* 2005), in renal cell carcinoma (Dannull *et al.,* 2005) and in melanoma patients (Mahnke *et al.,* 2007) and to favour the activation of tumour-specific immunity (Dannull *et al.,* 2005, Barnett *et al.,* 2005). A contrasting report was published by the group of Rosenberg showing no effective reduction of T regs after Ontak treatment of 12 melanoma patients (Attia *et al.,* 2005). This could be due to a different schedule of administration that seems to be more effective when limited overtime. This result poses another important issue in immunotherapy concerning the time and dose of administration. Indeed, it is extremely important that for each therapeutic approach,the best dose of drug and also the best timing of administration in order to achieve deletion of Tregs and the generation of a favourable environment in the tissue before the actual activation of the immune response has to be efficiently defined.

As far as cyclophosphamide is concerned, it is a DNA alkylating agent used to treat haematological and solid malignancies. The effect of cyclophosphamide is best appreciated at low dose acting as an immunostimulator via both the inactivation and elimination of T regs (Lutsiak *et al.,* 2005)**.** The combination of cyclophosphamide and immunotherapy has been tested in several tumour settings in humans (Nowak *et al*., 2006).

1.3.3 BIOLOGICAL ADJUVANTS

The term biological adjuvant refers to a group of agents that improve the immunogenicity of an antigen. Each type has a different mechanism of action. For instance, classical adjuvants, such as incomplete Freud's adjuvant (IFA), allow the antigen to be maintained at the injection site so that infiltrating APCs and effector cells can initiate a stronger immune response (Atanackovic *et al.,* 2008, Coban *et al.,* 2008, Kirkwood *et al*., 2008). Another important adjuvant is the so-called Bacillus Calmette - Guerin (BCG). BCG therapy is thought to be the most successful immunotherapy against solid tumours in cases of human superficial bladder carcinoma particularly in preventing from its recurrence (Alexandroff *et al.,* 1999 and Higuchi *et al.,* 2009). Bacterial products, such as those present in IFA and BCG have strong immunostimulatory activity since they contain agonists of Toll like receptors (TLRs) and thus can activate the immune response of the host (Krieg *et al.,* 2002).

TLRs are a family of receptors that are expressed by DCs and other innate immunity cell types and bind to a number of different microbial components such as lipopolysaccharide (LPS), RNA species and CpG DNA motifs (Iwasaki and Medzhitov, 2004). CpG motifs are the most studied of the TLR agonists. In preclinical studies, when they are mixed with peptide immunogens they have shown to enhance immune responses (Cooke *et al.,* 2008) .Furthermore, according to clinical studies (Wooldridge and Weiner *et al.,* 2003)**,** CpG motifs have been used in tumour immunotherapy. Another biological adjuvant are some cytokines that are able to enhance immune responses by promoting the differentiation, activation or recruitment of APCs. In that way, they enhance the antigen presentation and activation of antigen specific T cells. For example, GM-CSF has been reported to enhance antigen specific T cell responses by modulating the activation of APCs populations such as macrophages and DCs (Disis *et al.,* 1996). Furthermore, it is known to stimulate growth and differentiation of myeloid progenitors and generate specific and long lasting tumour immunity in multiple murine studies (Dranoff *et al.,* 1993; Belardelli *et al.,* 2004 and O'mahony, 2005)**.** Moreover, there are some other cytokines that have also been shown to play a pivotal role in

enhancing T cell function. IL-2, a cytokine, with a wide variety of immunologic effects, is the most studied as a vaccine adjuvant (Rosenberg *et al.,* 2001)**.** Finally, the cytokines IL-7, IL-12 and IL-15, have also been shown to enhance T cell responses in experimental models and may also have clinical benefits (Dranoff *et al.,* 1993).

1.4 TELOMERES AND TELOMERASE

1.4.1 Telomere structure, function, and shortening

It was in the 1930s that telomeres (from the Greek telos: end and meros: part) were first recognized as essential structures at the ends of chromosomes and were shown to be important for chromosomal stability and integrity. However, it was only in 1978 that the first telomeric sequence was identified, in the protozoa Tetrahymena, which is a single cell organism that at a certain stage of development has many identical minichromosomes with twice as many telomeres. Telomeric DNA is characterized by being a G-rich double stranded DNA composed by short fragments tandemly repeated with different sequences depending on the species. Telomeres consist of many thousand repeats of the hexanucleotide TTAGGG giving rise to 4-15kb of repetitive non-coding DNA in humans and up to 50kb in mice, ending in a G-rich 3-single-strand overhang evolutionary conserved among eukaryotes that folds back to form a "T-loop" stabilized by several telomeric proteins (Wang *et al,*. 1997 and Callen *et al.,* 2004).

Telomeres have at least three major functions: a) protect natural chromosomal DNA ends from being inappropriately recognized as double-stranded breaks (DSBs) and therefore initiating an inappropriate DNA damage response (DDR), b) protect chromosomal ends from inappropriate enzymatic degradation and c) prevent chromosomal end-to-end fusions (Deng *et al.,* 2007). In general, they are required to maintain genome integrity, chromosome stability, nuclear architecture, and chromosome pairing during meiosis **(**Multani *et al*., 2006, Vallejo 2007). Telomeres have been reported to shorten as a function of age and *in vitro* and *in vivo* cell division (Verdun *et al.,* 2007). As somatic cells age, telomeres progressively shorten at each round of replication because of the "end replication problem" at the 5'-end of the DNA lagging strand. This shortening has been proposed to be the mitotic clock that sets the limit of a cell life span and the mechanism that regulates the number of times a cell can divide before entering senescence (Sedivy *et al.,* 1998). This is due to the fact that conventional DNA polymerase can only synthesize in 5'→3' direction and so the 5'-end of the lagging strand will be shortened when compared to its template strand once the most terminal Okazaki's fragment- RNA

primer is eliminated. The action of a 5'→3' exonuclease on the C-A rich strand also contributes to this phenomenon (Callen *et al.,* 2004).

1.4.2 Telomerase and its components

Unrestricted proliferation (characteristic of germ line cells, immortal and tumour cells), requires a mechanism that is able to counteract telomere shortening due to replication dependent telomere erosion. Telomere shortening is overcome by activating telomere maintenance mechanisms. In almost all eukaryotic cells, telomere repeats are generated by a cellular reverse transcriptase, known as telomerase (figure1.9) (Zimmermann *et al.,* 2007, Nugent *et al.,* 1998, Rivera *et al.,* 1998 and Tabori *et al.,* 2007). As far as telomerase is concerned it is a RNA-dependent DNA polymerase containing several components.

The core enzyme consists of:

1) Two molecules of **TE**lomerase **R**NA **C**omponent (TERC or hTR in humans) that is an essential RNA component that serves as the template for nucleotide repeat generation. It is expressed only in cancer, germ and regenerative cells (Wenz *et al*., 2001 and Carney *et al*. 2008).

2) Two molecules of **TE**lomerase **R**everse **T**ranscriptase (TERT), that encodes the catalytic component of telomerase for telomere repeat addition. Its expression in most cells is low, but it is upregulated in tumourogenesis **(**Wenz *et al.* 2001 and Carney *et al.,* 2008).

3) One molecule of dyskerin (DKC) that is responsible for the stabilization of telomerase complex (Greider *et al*., 1989, Boukamp *et al*., 2006 , Tsakiri et al., 2007 and Wenz *et al.,* 2001).

Moreover, many proteins are essential for a functional telomerase complex. First of all, many proteins have been implicated in the physiological function of telomerase, but the specific role of all but hTERT, telomerase-associated proteins remains unknown. According to studies (Witkin *et al*., 2004) both proteins, p65 and p45, have essential roles in the maintenance of telomeres length as part of a ciliate telomerase holoenzyme. The p65 subunit contains a La motif characteristic of a family of direct RNA binding proteins. It was found that p65 is associated specifically with telomerase RNA and that genetic depletion of p65 reduces telomerase RNA accumulation *in vivo*. These findings demonstrate that telomerase holoenzyme proteins play critical roles in ribonucleoprotein (RNP) biogenesis and function.

According to recent data (Venteicher *et al.,* 2008), the ATPases, pontin and reptin, have been identified as essential telomerase components, through biochemical purification of human TERT complexes. Pontin interacts with both known protein constituents of telomerase, dyskerin and TERT. The interaction of pontin and reptin with dyskerin and TERT occurs at the endogenous level in human cells. Moreover, the TERT-pontin-reptin complex is cell-cycle regulated and peaks during each S phase. Loss-of-function experiments have shown that pontin and reptin are critical for telomerase activity and for accumulation of TERC and dyskerin (Prathapam *et al.,* 2005).

In mammalian telomeres, two TTAGGG repeat binding factors, TRF1 and TRF2, have been discovered (Bachor *et al.,* 1999; Nikol *et al.,* 2001; Price *et al*., 2001**).***In vitro*, both TRF proteins bind to double-stranded telomeric DNA through a conserved myb domain located in their carboxy-terminal domains, but they differ in their N termini, which is rich in either acidic residues (TRF1) or basic residues (TRF2). TRF1 and TRF2 negatively regulate telomere length. Overexpression of TRF1 or TRF2 *in vivo* induces a gradual decline in telomere length (Chang *et al*., 1995 andVeziri *et al*.; 1994). The formation of the T- loop was proposed to involve both TRF1 and TRF2. TRF1 has the ability to induce binding and pairing of duplex telomeric DNA, whereas TRF2 induces the invasion of the 3-single-strand overhangs into duplex telomeric DNA. Thus, the T-loop based mechanism for telomere protection would predict that both TRF1 and TRF2 are crucial for length homoeostasis of human telomeres.

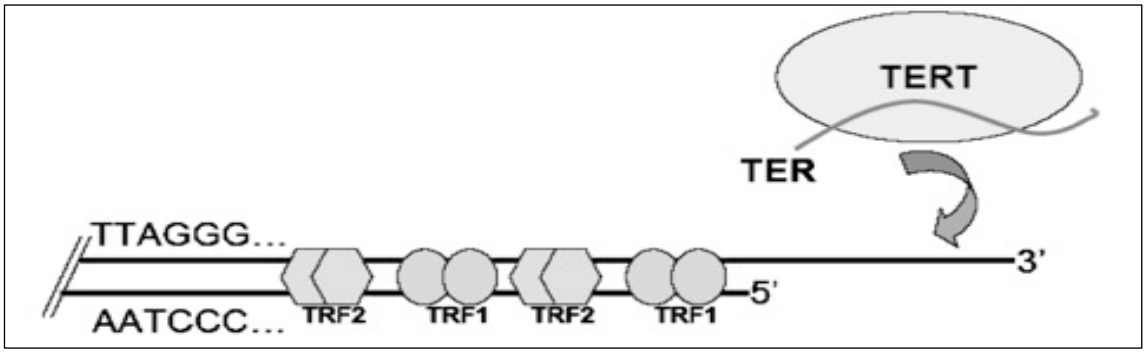


Figure 1.9: Schematic diagram of the human telomere and telomerase. Telomeres consist of TTAGGG sequences and associated binding proteins. The proteins TRF1 and TRF2 are depicted, but numerous other proteins bind directly or indirectly to telomeric DNA. The telomerase enzyme complex is minimally composed of two subunits: the telomerase reverse transcriptase protein (TERT) and telomerase RNA (TERC), which serves as the template according to which the enzyme adds TTAGGG repeats to telomeres. Other proteins that associate with telomerase are not depicted (Tabori *et al.,* 2007).

1.4.3 Role of telomerase

Telomerase elongates telomeres, allows cells to grow indefinitely and is expressed during embryonic development but is repressed in most adult tissues. Only regenerative tissues continue to show some level of activity. Its exact role is to add *de novo* telomeric sequences to the outermost ends of the telomeres and produce telomeric repeats via the catalytic subunit TERT. Therefore, it compensates for replication-or damage-dependent loss of telomere sequences or can even elongate the telomeres (Verdun *et al.,* 2007).

Telomerase activity in most human cells is down-regulated during embryogenesis leading to successive telomere shortening, which ultimately limits their proliferative capacity [mortality stage 1 (M1) or replicative senescence]. The growth arrest in M1 is mediated by DNA damage signalling of a few short telomeres and can be bypassed by inactivation of cell cycle checkpoint genes like p53, resulting in continued proliferation and eventually leading to critically short telomeres and massive cell death [mortality stage 2 (M2) or crisis **(**Tabori *et al*., 2007).However, sometimes single cells can escape M2 by maintenance of their telomeres and they turn into immortal cancer cells; this is observed in 90% of all human cancer cells by reactivation of telomerase. Even though mortality stages M1 and M2 are considered as tumour suppressor mechanisms, short telomeres may also promote genetic instability in a distinct genetic context (Greenwood *et al.,* 2003, Hackett *et al.,* 2001, Lores *et al.,* 2006).

1.4.4 Role of Telomeres and Telomerase in tumour initiation and progression

Telomerase was discovered in 1985 by Greider and Blackburn and first recognized as a unique and exciting anticancer target almost 5 years later (Harley 2008).Telomerase activity is present in 85-90% of human cancers, but is absent in most normal tissue. Therefore, telomerase expression is a key feature of tumour cell biology and according to translational studies telomerase was evaluated as an excellent tumour marker (Kim *et al.,* 2004, Collando *et al.,* 2007, Senior *et al.,* 2000, *Wu et al.,* 2003). Some data suggest, however, that telomere maintenance might be a stumbling block on the path to immortalization and that inhibition of telomere replication can attenuate human cancer cell growth (Zumstein *et al*., 1999**).** Telomere length operates as a mechanism to prevent uncontrolled cell growth and therefore defines the proliferation potential of a cell. *In vitro*, in somatic cells that have lost proliferation control, shortening of telomeres becomes the main source of genome instability leading

to genetic or epigenetic changes that may allow cells to become immortal and to acquire tumour phenotypes (Gandellini *et al.,* 2007, Ponder *et al*., 2001).

*M*ice models have indisputably shown both the protective and the promoting role of very short telomeres in cancer development (Raynaud *et al*., 2007). In humans, although telomere shortening and other types of telomere dysfunction probably contribute to the genome instability often detected in tumours, the specific contributions of such instability to the development of cancer remain still undetermined (Vallejo 2007, Stindl *et al*., 2008). The cells that lose p53 function could escape the senescence checkpoint and continue to shorten their telomeres and thus they enter into a phase of rampant chromosomal instability called crisis, characterized by chromosomal fusions and NRTs. Loss of heterozygosity (LOH) of tumour suppressors and/or amplification of oncogenes could lead to a pro-cancer genotype. Virally transformed human cells that eliminate p53 and/or pRB function escape crisis at extremely low frequencies, while those expressing hTERT are readily immortalized (Stewart *et al.,* 2000).

Cell-culture transformation assays showed that p53 null mouse cells with criticially shortened telomeres exhibit increased susceptibility to transformation by Myc and Ras oncogenes (Poynter *et al.*, 2006). Similar findings were observed *in vivo*, in which telomerase-null mice with dysfunctional telomeres and loss of p53 resulted in the selection of cells with a pro-oncogenic genome and early onset of cancer (Shawi *et al.,* 2007). Moreover, while 80-90% of human tumours possess telomerase activity, the remainder maintains telomeres via a recombination-mediated process termed ALT that is telomerase independent. Together, these observations reinforce the importance of an intact p53 pathway in tumour prevention, and support the view that crisis provides a potent barrier to tumour development and, that telomerere maintenance is an essential aspect of full malignant progression (Artandi *et al*., 2000, Deng *et al.,* 2007, Farazi *et al.,* 2003, Stewart *et al.,* 2000).

1.4.5 Telomerase and Vaccines

The fact that the majority of tumour cells exhibit telomerase activity in order to bypass the telomere checkpoint and to obtain unlimited growth potential makes telomerase an attractive target for selective cancer therapy. Expression of hTERT leads to telomere lengthening and cell immortalization (Shay *et al.,* 2006 ; Harley *et al*., 2008), whereas degradation of hTERT by proteasomes prompts telomere shortening, cancer cell senescence (ageing) and apoptosis (death) (Kim *et al.,* 2004; Pellicciotta *et al*., 2005). Degradation of hTERT occurs following ubiquitination of the protein mediated by E3 ubiquitin ligase (Kim *et al.,* 2004). The protein fragments or peptides of hTERT degradation are presented on the tumour cell surface as antigen by the major histocompatibility complex (MHC) class I pathway (Kim *et al.,* 2004; Vonderheide *et al.,* 1999; Minev et al., 2000; Lev et al., 2002). Targeting, by T cells, of these hTERT antigenic epitopes presented on the tumour cell surface represents a rapid and direct mechanism to destroy the tumour cells (Vonderheide *et al.,* 2002; Vonderheide *et al.,* 2008).

Several approaches have been described during the last decade on how to exploit this hallmark of cancer cells for therapeutic purposes. They can be divided into the following major categories: 1) Direct or indirect inhibition of the enzyme activity and consecutive telomeres decapping (Cerone *et al.,* 2006) 2) immunotherapy using hTERT as tumour-associated antigen (Bolonaki *et al.,* 2007) and 3) gene therapy with telomerase promoter driven suicide genes (introduction of proapoptotic genes whose expression is dependent upon the TERT promoter; Plumb *et al.,* 2001 and Zimmermann *et al.,* 2007).

As an ideal tumour-associated antigen (TAA), hTERT not only possesses tumour specificity with little or no expression in differentiated healthy somatic cells (Vonderheide *et al.,* 2002), but also produces epitopes for both MHC class I and II pathways to induce "help" for $CD8^+$ cytotoxic T lymphocyte (CTL) responses capable of attacking tumours. To enhance the anti-telomerase immune response, 26 hTERT peptides have been exploited as epitope-mimicking structures (mimotopes) for anti-tumour immunity (table 1.6) (Liu et al., 2009). Among these peptides, 23 are for MHC class I-mediated antigen presentation to induce $CD8^+$ T cell responses (Schroers *et al.,* 2002; Brunsvig *et al.,* 2006).This suggests that hTERT antigenic peptides represent potential molecular targets for the treatment of cancer.

Table 1.6 : **hTERT antigenic peptides tested for tumour immunity** (Liu *et al.,* 2009)

Epitope	Sequence	MHC	Avidity	CD8	CD4	Cell line	*In vivo*	Author	Year
I540	ILAKFLHWL	HLA-A2	High	+	−	+	+, human, mouse	Vonderheide et al.; Minev et al.	1999, 2000
R865	RLVDDFLLV	HLA-A2	High	+	−	+	+, human, mouse	Minev et al.	2000
R572	RLFFYRKSV	HLA-A2	Low	+	−	+	+, mouse	Hernandez et al.	2002
D988	DLQVNSLQTV	HLA-A2	Low	+	−	+	+, mouse	Scardino et al.	2001
R30	RLGPQGWR	HLA-A2	Low	+	−	+	+, mouse	Thorn et al.	2007
K973	KLFGVLRLK	HLA-A3	High	+	−	+	−	Vonderheide et al.	2001
Y325	YLEPACAKY	HLA-A1	Low	+	−	+	+, mouse	Schreurs et al.	2005
V324	VYAETKHFL	HLA-A24	High	+	−	+	−	Arai et al.	2001
V461	VYGFVRACL	HLA-A24	High	+	−	+	−	Arai et al.	2001
T1088	TYVPLLGSL	HLA-A24	High	+	−	+	+, human, mouse	Mizukoshi et al.	2006
C845	CYGDMENKL	HLA-A24	High	+	−	+	+, human, mouse	Mizukoshi et al.	2006
A167	AYQVCGPPL	HLA-A24	High	+	−	+	+, human, mouse	Mizukoshi et al.	2006
D637	DYVVGARTF	HLA-A24	High	+	−	+	+, human, mouse	Mizukoshi et al.	2006
M1	MPRAPRCRA	HLA-B7	High	+	−	+	+, mouse	Adotevi et al.	2006
A4	APRCRAVRSL	HLA-B7	High	+	−	+	+, mouse	Adotevi et al.	2006
A68	APSFRQVSCL	HLA-B7	High	+	−	+	+, mouse	Adotevi et al.	2006
R277	RPAEEATSL	HLA-B7	High	+	−	+	+, mouse	Adotevi et al.	2006
R342	RPSFLLSSL	HLA-B7	High	+	−	+	+, mouse	Adotevi et al.	2006
R351	RPSLTGARRL	HLA-B7	High	+	−	+	+, mouse	Adotevi et al.	2006
D444	DPRRLVQLL	HLA-B7	Low	+	−	+	+, mouse	Cortez-Gonzalez et al.	2006
F464	FVRACLRRL	HLA-B7	High	+	−	+	+, mouse	Cortez-Gonzalez et al.	2006
L1107	LPGTTLTAL	HLA-B7	High	+	−	+	+, mouse	Cortez-Gonzalez et al.	2006
L1123	LPSDFKTIL	HLA-B7	High	+	−	+	+, mouse	Cortez-Gonzalez et al.	2006
R672	RPGLLGASVLGLDDI	HLA-DR1, 7, 15	High	−	+	+	+, mouse	Schroers et al.	2002
L766	LTDLQPYMRQFVAHL	HLA-DR4, 11, 15	High	−	+	+	+, mouse	Schroers et al.	2003
E611	EARPALLTSRLRFIPK	HLA-DR, DQ, DP	High	−	+	−	+, human	Brunsvig et al.	2006

The hTERT 1540 (ILAKFLHWL) was the first telomerase immunogenic peptide identified by epitope prediction from melanoma patients (Vonderheide *et al.,* 1999). It binds to HLA-A*0201- the most frequently expressed MHC class I allele present in nearly 50% of Caucasians, Asians and Hispanics and 33% of African-Americans. According to experiments in human and HLA-A2 transgenic mice, hTERT 1540 fulfills the requirements of a TAA immunogenic epitope that is capable of triggering CTL responses (Vonderheide *et al.,* 2001; Amarnath *et al.,* 2004; Sievers *et al.*, 2004; Gannage *et al*., 2005). Recent clinical trials have demonstrated that induction of hTERT 1540-specific CTLs by peptide vaccination of patients suffering breast (Domchek *et al*., 2007), renal, prostate cancer or melanoma (Wenandy *et al.,* 2008) lead to increased immune responses against the tumour.

The peptide-targets of CTLs can be either dominant or cryptic. Dominant peptides have high HLA affinity and are often presented by tumour cells in contrast to cryptic peptides which have low HLA affinity and are rarely presented by tumour cells (Theobald *et al.,* 1997; Colella *et al*., 2000; Hernandez *et al.,* 2002; Gross *et al*., 2004; Finn *et al.,* 2008; Apostolopoulos *et al*., 2009; Schmidt *et al.,* 2009). All cancer vaccines that have been used until now and have targeted dominant peptides, with high HLA I affinity, have not given great results (Parkhurst *et al.,* 2004; Vonderheide *et al.,* 2004; Knutson *et al.,* 2002). According to studies conducted in mice, this was due to tolerance mechanisms (Cibotti *et al.,* 1992). Immune tolerance normally protects individuals from developing autoimmunity, but it is also represents a major obstacle in developing active T cell immunotherapy.

Expression of hTERT during fetal development may induce negative selection in the thymus to remove CTL precursors recognizing hTERT peptides

A way to overcome the tolerance mechanisms is to use "cryptic" peptides instead of "dominant". It has been shown that T lymphocytes are specific for cryptic peptides and avoid the tolerance mechanisms (Bolonaki *et al*., 2007). It means that cryptic peptides are appropriate to be used as vaccines against cancer. Cryptic peptides have low HLA affinity and are not immunogenic have to be optimized by altering their amino-acid sequence in order to increase their immunogenicity. In that way, HLA affinity is increased and specific CTLs are stimulated (Tourdot *et al*., 2000). According to clinical studies (Mavroudis *et al*., 2006) the vaccine Vx-001 (Vaxon Biotech,Evry,France) has caused immune response in NSCLC patients. This vaccine consists of two 9-mers peptides: the native $TERT_{572}$ (RLFFYRKSV) and the modified $TERT_{572Y}$ (YLFFYRKSV). The $TERT_{572Y}$ is an optimized cryptic human telomerase reverse transcriptase peptide presented by HLA-A* 0201 which differs from the native $TERT_{572}$ at position 1 where a tyrosine has been substituted for an arginine. This substitution enhances affinity for HLA-A*0201molecule (Tourdot *et al*., 2000). The $TERT_{572Y}$ peptide has been shown to induce tumour immunity but not autoimmunity in HLA- A*0201 transgenic mice (Gross *et al.,* 2004; Scardino *et al.,* 2002). In addition, *in vitro* studies in healthy donors and prostate cancer patients have shown that $TERT_{572Y}$ stimulated TERT-specific CTLs with anti-tumour activity (Hernandez *et al.,* 2002). Moreover, another study showed that, $TERT_{572Y}$ stimulated the specific CTLs *in vitro*, in both healthy donors and prostate cancer patients. Though the CTLs killed the tumour cells, they did not affect the normal ones (Hernandez *et al.,* 2002; Scardino *et al*., 2002). Furthermore, according to previous studies, $TERT_{572Y}$ is a non-toxic, highly antigenic vaccine and its use against patients with NSCLC, resulted in the induction of $TERT_{572Y}$ specific $CD8^+$ Tcell immune response in the majority of the vaccinated patients (Bolonaki *et al.,* 2007).

1.5 AIM OF THE STUDY

According to studies conducted in the laboratory of Tumour Biology, Vx-001 has recently been tested in a phase I/II clinical study in 116 patients with advanced cancer. Analysis of the first 19 patients who received increased doses of Vx-001 showed that the vaccine was non-toxic and highly immunogenic in all tested doses (Mavroudis *et al*., 2006). Moreover, subgroup analysis of 22 vaccinated patients with advanced Non-Small Cell Lung Cancer (NSCLC) showed that the vaccine-specific immune response correlated with prolonged survival (Bolonaki *et al*., 2007). The results of these studies revealed that the vaccination with Vx-001 stimulates $TERT_{572}$-specific reactive T cells in the peripheral blood in the majority of patients.

Based on the above results, the aim of the current study was to investigate the specific -T cell immune response against Vx-001 in a cohort of patients with various chemo-resistant types of solid tumours. Specifically, 77 HLA-A*0201 patients with various solid tumours were vaccinated with two doses of the -optimized peptide ($TERT_{572Y}$) followed by four doses with the native peptide ($TERT_{572}$). This vaccination schedule was based on *in vivo* preclinical studies which have shown that vaccination of HLA-A*0201 transgenic HHD mice with the optimized $TERT_{572Y}$ followed by the native $TERT_{572}$ peptide, induced CTLs with higher avidity and stronger anti-tumour efficacy than serial vaccinations with the optimized $TERT_{572Y}$ peptide alone (Gross *et al.,* 2004).

This could be explained by the hypothesis that the optimized $TERT_{572Y}$ peptide first generates peptide-specific T cells and then the stimulation with the native $TERT_{572}$ peptide selects among T cells those with the highest specificity for the native $TERT_{572}$ peptide which is presented by tumour cells.

As far as the methodology is concerned, the following approaches were employed:

a) MHC CLASS I 201 typing: A prerequisite for the patients' enrolment to the vaccination protocol was that the patient should have HLA-A*0201 haplotype. Patients' cells were stained with the HLA-A0201 antibody in order to test for the presence of the HLA-A201 molecules and analysed with the help of a flow cytometer. b) Isolation of Peripheral Blood Mononononuclear Cells (PBMCs) from vaccinated patients' blood c) Screening for reactivity to the $TERT_{572Y}$ and $TERT_{572}$ peptides using an IFN-γ ELISpot assay d) Identification of peptide-specific $CD8^+$ T cells by IFN- γ and IL-10 intracellular staining using flow cytometry.

Finally, in order to determine whether there was an association between the TERT-specific-IFN-γ immune reactivity and overall survival (OS), the outcome of the patients who enrolled the vaccination protocol with clinically documented stable (SD) or progressive disease (PD) was analyzed.

2. MATERIALS AND METHODS

2.1 PATIENTS

Seventy seven (77) patients with various types of chemo-resistant advanced solid tumours (stages III and IV) were enrolled in the Vx-001 vaccination protocol. Inclusion criteria were: 1) HLA-A*0201 haplotype, 2) at least one chemotherapy regimen prior to vaccination, , 3) age >18 years old, 4) performance status (WHO) of 0-2, 6) adequate hematologic parameters (absolute neutrophil number ≥ 1,500/μl, absolute lymphocyte number ≥ 1,000/μl, platelets > 100,000/mm^3, hemoglobin > 10 g/dl, and renal (creatinine < 2 mg/dl) and liver (bilirubin < 1.5 times the upper normal value, transaminases <2.5 times the upper normal value) and absence of heart deficiency, 6) the first line chemotherapy should have finished at least 4 weeks and no more than 8 weeks before the enrollment in the study.

Moreover, no other treatment with possible anti-tumour activity (i.e. chemotherapy, radiotherapy, hormonotherapy) or administration of immunosuppressive drugs was allowed four weeks before or during the course of vaccination. The protocol was approved by the Ethics and Scientific Committees of the University Hospital of Heraklion and the National Drug Administration (EOF) of Greece. All patients signed a written informed consent in order to take part to the study.

2.2 MHC CLASS I A201 TYPING

2.2.1 Experimental Procedure

100μl patient's venus blood were placed into a tube followed by addition of 5μl mouse anti-human HLA-A2 FITC antibody. In a second tube, 5μl mouse IgG2b Isotype FITC were added in 100μl blood which was used as a negative control. The tubes were incubated for 30 minutes (min). 2ml lysing buffer were added into tubes in order to lyse the red blood cells for 15 min at RT and then they were centrifuged. After that, the supernatant was discarded, the pellet was resuspended in 3ml PBS and the cells were centrifuged. Finally, 300μl cell fix solution (4% PFA) were added into the tubes and the tubes were placed at 4^0C until analysis. The tubes were analysed by flow cytometry using FACS Calibur (BD Bioscences, UK). The acquired graphic data were analysed using Cellquest Pro Software.

All incubations took place in dark at 4^0C, unless otherwise is stated.
All centrifuges performed at 1500 rpm at 4^0C.
All antibodies were purchased from BD Biosciences.

2.3 PEPTIDES

The Vx-001 vaccine consisted of the 9-mer native $TERT_{572}$ (RLFFYRKSV) peptide and its optimized variant $TERT_{572Y}$ (YLFFYRKSV). Both peptides were synthesized at the Faculty of Pharmacy, University of Patras (Greece) by means of solid-phase. Each peptide was prepared as a lyophilized powder and its reconstitution was in sterile water.

2.4 VACCINATION PROTOCOL

All HLA A*0201 patients received two subcutaneous (s.c) injections with 2mg of the optimized $TERT_{572Y}$ peptide followed by four s.c injections with 2mg native $TERT_{572}$ peptide, every three weeks until disease progression. Both peptides were emulsified with Montanide ISA51 (Seppic Inc, Paris, France) immediately prior to vaccination. Patients who completed the 6-vaccination schedule and experienced disease stabilization or objective clinical response, received boost vaccinations (re-vaccinations) with 2mg native $TERT_{572}$ peptide every three months until disease progression.

The 77 patients with various types of chemo-resistant advanced solid tumours are shown in the following table:

Table 2.1: The 77 patients various types of chemo-resistant advanced solid tumours, who were enrolled to the vaccination protocol

AGRI	**DILA**	**GERA**	**KNIK**	**MIRI**	**PATRA**	**STEDI**
AIMA	**DIMI**	**GIAN**	**KOSI**	**MUBA**	**PEMA**	**STEF**
ALAF	**DRON**	**GIEM**	**LAST**	**NAPO**	**PSIL**	**VAMP**
ALATH	**EKAF**	**GIBA**	**LUKI**	**NIKA**	**PYL**	**VAPOS**
ANTH	**FEM**	**GINI**	**MIOA**	**NIO**	**SAV**	**ZAHE**
ANTZ	**FIX**	**GONG**	**MARE**	**NITSA**	**SERAF**	**NIKHT**
BANI	**FOTO**	**HAI**	**MARI**	**PAKY**	**SFEP**	**VLAH**
DASK	**GARB**	**KAGI**	**MAT**	**PAM**	**SOFO**	**TYMA**
DEC	**GEDI**	**KANI**	**MAUR**	**PANAG**	**SPAG**	**TYR**
DEF	**GELE**	**KARGE**	**META**	**PAPAE**	**SPIR**	**RIST**
DELI	**GEOR**	**KAZI**	**MIMA**	**PAPI**	**STAN**	**EMMA**

2.5 PATIENT SAMPLES

100ml patients' peripheral blood in EDTA was collected before the first vaccination, after the 2nd and 6th vaccination and before each boost administration. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma, UK) density centrifugation and cryo-preserved at -80^{0}C until future use.

2.6 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) FROM VACCINATED PATIENTS' BLOOD

2.6.1 Principles of method

Ficoll is an uncharged, highly branched synthetic polymer made by co-polymerization of sucrose and epichlorohydrin. It is widely used for density gradient centrifugation. Due to its many hydroxyl groups, it is highly soluble in water. Densities of up to 1.2 g/ml can be attained. Its density is higher than that of monocytes and lymphocytes and lower than that of erythrocytes and granulocytes. This characteristic helps the isolation of red blood cells from PBMCs. Upon centrifugation, red blood cells and granulocytes come through ficoll-hypaque solution and they form a deposit at the bottom of the tube, while peripheral blood mononuclear cells remain above the ficoll layer forming the interphase and therefore, they can be collected (figure 2.1)

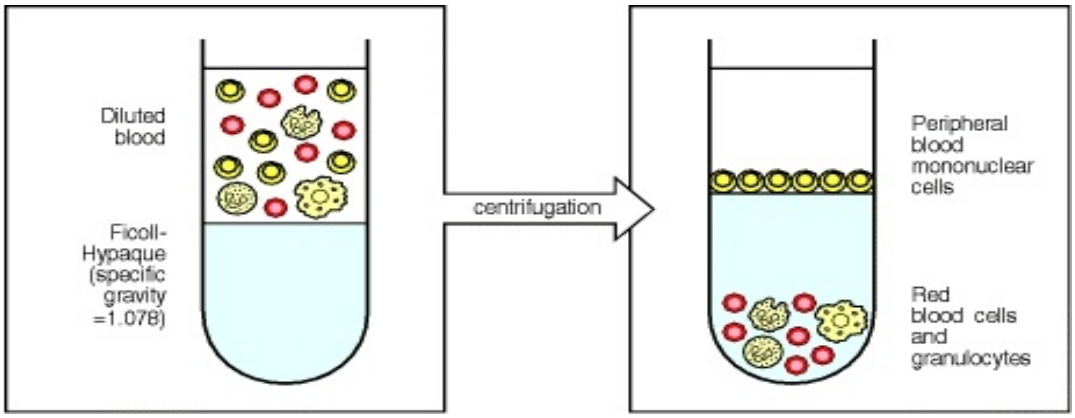


Figure 2.1: Peripheral blood mononuclear cells can be isolated from whole blood by Ficolle-Hepaque centrifugation. Diluted anticoagulated blood (left panel) is layered over Ficoll-Hypaque and centrifuged. Red blood cells and polymorphonuclear leukocytes or granulocytes are more dense and centrifuge

through Ficoll-Hypaque, while mononuclear cells consisting of lymphocytes together with some monocytes band over it can be recovered at the interface (right panel). (Janeway, 2006).

2.6.2 Experimental procedure

Firstly, blood was diluted with the washing solution in ratio1:1. Fifteen (15) ml of ficoll-hypaque were dispensed in sterile 50ml falcon tubes and overlayed with 30ml diluted blood (2:1) carefully, in order to avoid the mixture of the phases. In turn, the tubes were centrifuged at room temperature (RT) , at 1200 rpm for 30 min, without break. Upon centrifugation, the interphase (containing the PBMCs) was taken out carefully using a sterile pipette and it was transferred into a fresh 50ml falcon tube containing 15 ml washing solution and the cells were centrifuged at RT, at 2000 rpm for 15 min. The supernatant was discarded and the pellet was resuspended in 15ml washing solution. The tubes were centrifuged at RT, at 1200 rpm for 10min. At the end, the pellet was resuspended in 10ml complete medium and the cells were counted and frozen at -80^{o}C until future use.

Washing solution: RPMI-1640 (Gibco) supplemented with antibiotics P/G/S (40U/ml penicillin, 2Mm glutamine, 40U/ml streptomycin).

Complete medium: RPMI-1640 (Gibco) supplemented with 10% Human Serum and antibiotics P/G/S (40U/ml penicillin, 2Mm glutamine, 40U/ml streptomycin).

2.7 COUNTING OF PBMCs

2.7.1 Experimental procedure

10μl PBMCs were mixed with 90μl Trypan Blue and were counted using the optical microscope (figure 2.2). **Trypan blue**,a diazo dye, is a vital stain used to selectively colour dead tissues or cells blue. Since cells are very selective in the compounds that pass through the membrane, in a viable cell, trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a **dye exclusion method**.

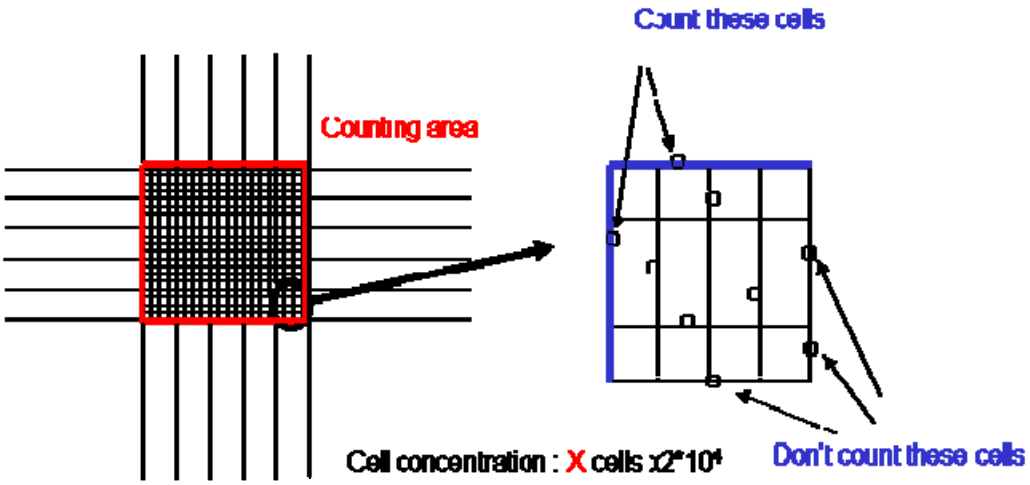


Figure 2.2. Way for counting the PBMCs

The total number of PBMCs is estimated by using the following equation:

Total PBMCs number= No live cells*10^4 * dilution factor.

2.8 FREEZING AND DEFROSTING OF PBMCs

2.8.1 Experimental procedure

Freezing medium was added into the cells so that the final concentration would be 10*10^6 cells/ml. 1ml of freezing medium was dispensed in each freezing tube. The tubes were placed into "Mr Frosty" box which is at 4^0C before its use and causes temperature reduction about 1^0C/min. The box containing the tubes was stored at -80^0C for future use.

The defrosting procedure performed when the cells were used in Elispot and Intracellular Staining (ICS). The freezing tubes were taken from -80^0C and were placed into a water bath (37^0C) for few minutes, in order to defrost them as quickly as possible. In turn, the defrost cells of each patient were dispensed in 20ml tubes containing 15ml washing solution and were centrifuged at 1200 rpm, at RT for 5 min. Upon centrifugation, the supernatant was discarded, the pellet was resuspended in 1ml complete medium, and the cells were counted (as described in section 2.7.1)

Freezing medium: RPMI 1640 (Gibco) + 50% FCS (Gibco) + 10% DMSO

Complete medium: RPMI-1640 (Gibco) supplemented with 10% Human Serum and antibiotics P/G/S (40U/ml penicillin, 2Mm glutamine, 40U/ml streptomycin).

Washing solution: RPMI-1640 (Gibco) supplemented with antibiotics P/G/S (40U/ml penicillin, 2Mm glutamine, 40U/ml streptomycin).

2.9 ENZYME-LINKED IMMUNOSORBENT SPOT (ELISPOT) ASSAY

2.9.1 Materials

All reagents were purchased from Diaclone, unless otherwise is stated. *Complete medium*: RPMI-1640 (Gibco) supplemented with 10% Human Serum and antibiotics P/G/S (40U/ml penicillin, 2Mm glutamine, 40U/ml streptomycin).

2.9.2 Principles of method

The ELISpot assay is a very sensitive method and it is designed to enumerate cytokine producing cells in a single cell suspension. This method has the advantage of requiring a minimum of in-vitro manipulations allowing cytokine production analysis as close as possible to in-vivo conditions in a highly specific way. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation, as well as the follow up of such frequency during a treatment and/or a pathological state.

Diaclone Elispot assay is based on sandwich immuno-enzyme technology. Cell secreted cytokines or soluble molecules are captured by coated antibodies avoiding diffusion in supernatant, protease degradation or binding on soluble membrane receptors. After cell removal, the captured cytokines are revealed by tracer antibodies and appropriate conjugates (figure 2.3)

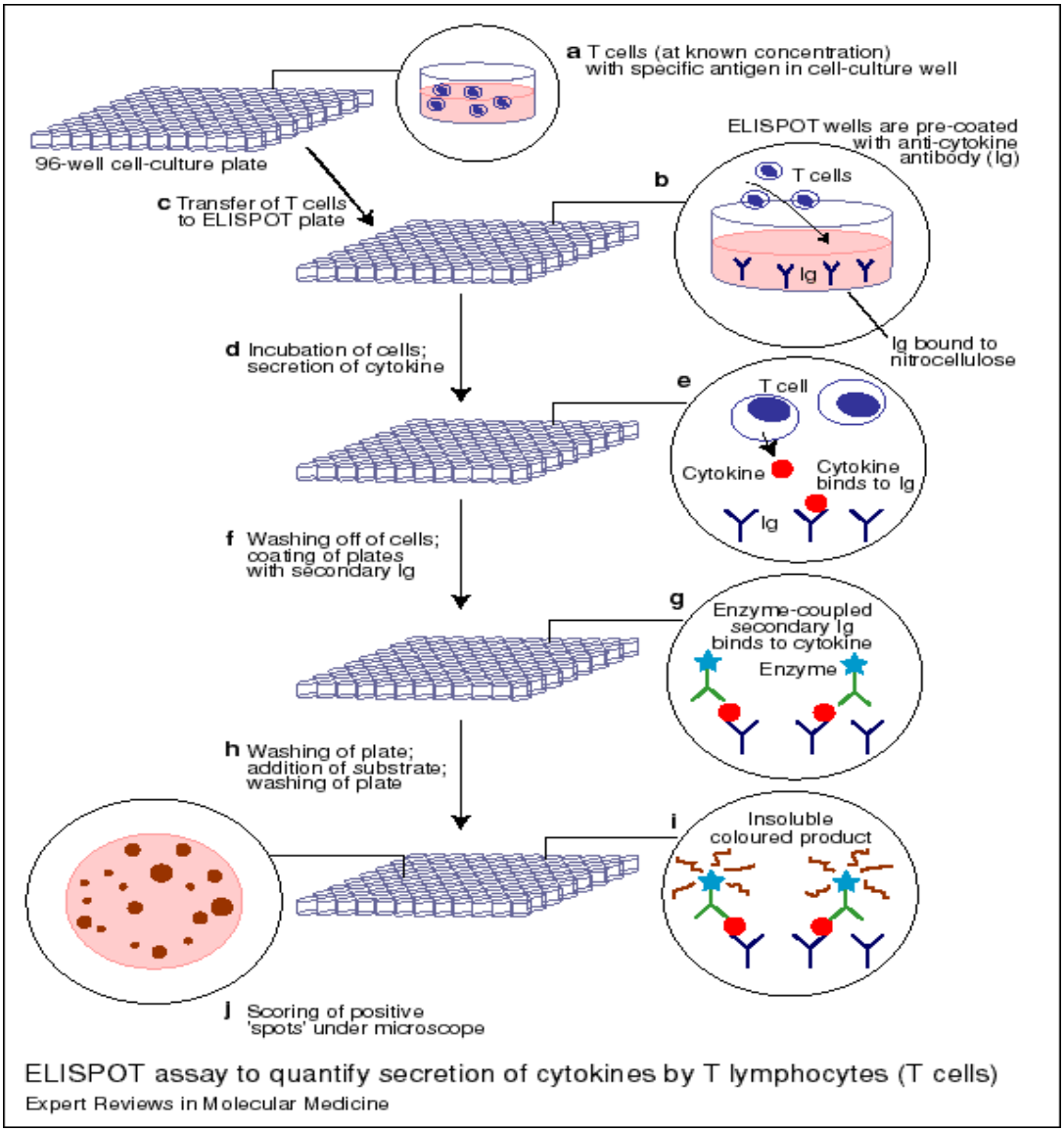


Figure 2.3: ELISPOT assay to quantify the secretion of cytokines by T lymphocytes

(Expert Reviews in Molecular Medicine, 2006)

2.9.3 Experimental procedure

The IFN-γ ELISpot assay was used to screen for specific-T cells, in response to $TERT_{572}$ and $TERT_{572Y}$ peptides. The assay was performed according to manufacturer's protocol (IFN-γ ELISPOT, Diaclone, Besancon, France). In brief, a PVDF- bottomed 96-well plate was pre-wetted with 15µl / well of 35% EtOH for 3 min at RT. The plate was washed three times with 100µl sterile PBS and coated with capture anti-human IFN-γ antibody (dilution in 1:100 in PBS) overnight at 4°C. The wells were washed once with 100µl of sterile PBS and blocked for 2 h at RT with 100µl of 2% skimmed dry milk in PBS. This solution is used for the reduction of non-specific binding of

antibodies. Upon one wash with 100μl of sterile PBS, $2*10^5$ PBMCs in 100μl of complete medium were dispensed in each well in the presence or absence of 10μM peptide ($TERT_{572Y}$ or $TERT_{572}$). The plate was covered with a standard 96-well plate lid and the cells were incubated at 37°C in a 5% CO_2 incubator ,overnight.

The following day, the cell suspension was discarded and 100 μl of PBS-0.1% Tween-20 (Sigma, UK) were added in wells for 10 min at +4°C. Afterwards, the plates were washed with 100 μl PBS, three times. After the final wash100 μl of detection anti-human biotinylated IFN-γ 1% (v/v) BSA/PBS was added and the plate was incubated for 2 h at RT. After 6 washes, 10μl of alkaline phosphatase-conjugated streptavidin diluted 1:1000 (v/v) in PBS, were added, and incubated for 1 h at RT. After washing, peroxidase substrate NBT/BCIP was added and incubated until appearance of dark spots in the negative control wells. The reaction was terminated by washing the plate with running tap water. The spots were counted using Axio Imager.M1 analyzer and KS Elispot software (Zeiss, Germany).

In all ELISpot tests, six wells were tested for each group in three independent experiments. Negative controls were the cells alone, whereas positive controls were cells treated with 5 μg/ml Concanavalin A (ConA) (Sigma,UK). The experiment was considered as successful, as long as the spots in the positive control was at least twice as much as that in the negative control. The threshold for positive responses was a difference of more than 10 spot-forming cells and a statistically significant difference ($p \leq 0.05$) between peptide stimulated and negative control wells using the Student's *t*-test. Moreover, pre-vaccination responses greater than observed after the 2^{nd} or 6^{th} vaccination were considered as no response.. The number of the vaccine - reactive PBMCs above background was calculated as the difference between the numbers of the counted in peptide-stimulated and the non-stimulated wells. The absence of the immune response was symbolised with the number 1 (1=no response). Results are presented as the number of vaccine reactive cells per $2*10^5$ PBMCs.

2.10 INTRACELLULAR CYTOKINE STAINING (ICS)

2.10.1 Materials

All the antibodies and reagents were purchased from BDBiosciences, UK, unless otherwise is stated. *Complete medium*: RPMI-1640 (Gibco) supplemented with 10% Human Serum and antibiotics P/G/S (40U/ml penicillin, 2Mm glutamine, 40U/ml streptomycin). *FACS buffer:* 0.05% azide and 1% FCS in PBS

2.10.2 Principle of method

Flow cytometry is a technique for counting and examining microscopic particles, such as cells and chromosomes by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multi-parametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Flow cytometry is routinely used in the diagnosis of health disorders, but has many other applications in both research and clinical practice. A common variation is to physically sort particles based on their properties, so as to purify populations of interest.

A beam of light (usually laser light) of a single length is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter SSC) and one or more fluorescent detectors. Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the light in some way, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source.

This combination of scattered and fluorescent light is picked up by the detectors, and, by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell size and SSC depends on the inner complexity of the particle

(i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Some flow cytometers on the market have eliminated the need for fluorescence and use only light scatter for measurement. Other flow cytometers form images of each cell's fluorescence, scattered light, and transmitted light.

2.10.3 Experimental procedure

Peptide-specific T cells were identified by measuring the cells-producing IFN-γ and IL-10 intracellular staining using flow cytometry. At first, the cells were defrozen from -80 °C. Thawed $1x10^6$/tube PBMCs were incubated in 500 μl of complete medium, in the presence or absence of 10μg/ml peptide (TERT $_{572Y}$ or TERT $_{572}$) or 5 μg/ml of Staphylococcal Enterotoxin B (SEB). The cells upon SEB activation, which is a super antigen, were used as a positive control and the cells with the medium only, as a negative one. , 10μg/ml brefaldin A (BFA) was added 1h after the initial stimulation and the cells were incubated for further 18h at 37°C with 5% CO_2. Brefeldin A is a lactone antibiotic produced by fungal organisms such as *Eupenicillium brefeldianum*. It inhibits transport of proteins from ER to Golgi and induces retrograde protein transport from the Golgi apparatus to the endoplasmic reticulum. This leads to proteins accumulating inside the ER.

On the following day, 1ml FACs buffer was added in the cells and they were centrifuged for 5min. All washes of the cells were performed twice. After the centrifugation, the supernatant was discarded and the cells were mixed gently. Then the cells were stained firstly for cell surface molecules (5μl anti-CD3-APC and 5μl anti-CD8-PerCP) for 30min at 4 °C. Upon the incubation, the cells were washed twice with 1ml FACS buffer. After the second centrifugation, the supernatant was discarded and the cells were mixed thoroughly in order to dissolve any cell aggregation prior to addition of cell fix/perm solution.

Then, 250μl Cytoperm/Cytofix was added to the cells for 20min at 4^0C. After the incubation, the cells were washed twice with 1ml Perm/wash buffer. After the centrifugation, the supernatant was discarded and the cells were mixed thoroughly for vortexing. Then, cells were stained with conjugated anti–IL-10-PE and anti- IFN-γ-FITC. Following incubation for 1h, 1ml Perm/wash solution was added in the cells and they were washed twice. Finally, the supernatant was discarded, and the cells were resuspended in cell fix solution and placed in 4^0C until analysis. The peptide-specific $CD8^+$ T cells were identified using BDFACS Calibur (FACs analysis) and the acquired

cytofluorographic data were analysed using Cellquest Pro software. Results are expressed as the percentages of $CD8^+$ IFN-γ^+ or $CD8^+$ IL-10^+ T cells of the gated $CD3^+$ $CD8^+$ T cells or $CD3^+$ IFN-γ^+ and $CD3^+$ IL-10^+ T cells. The positive immune response was determined as: 1) $CD3^+CD8^+$ T or $CD3^+$ cells should be more than 10.000, 2) The response between positive control and stimulated cells should be at least twice as great as that of the negative control and 3) the percentage of $CD8^+$ IFN-γ^+ T or $CD3^+$ cells should be greater than 0.02%.

All incubations took place in dark at 4^0C, unless otherwise is stated.
All centrifuges performed at 1500 rpm at 4^0C.

2.11 STATISTICAL ANALYSIS

Overall survival was estimated from the date of study entry to the date of last contact or death. The association between the development of TERT-specific immune response and the clinical outcome of the patients who enrolled the vaccination protocol with clinically documentated stable (SD) or progressive disease (PD) was analysed. In particular, overall survival was compared by the log-rank test, firstly between SD patients who were early responders (who had developed a $TERT_{572Y}$-specific immune response after the 2nd vaccination) and non early responders and secondly between SD patients who were late responders (who had developed a $TERT_{572}$-specific immune response after the 6th vaccination) and non-late responders. Respectively, overall survival was also compared in PD patients in the same way (early responders vs non-early responders, late responders vs non late responders). The probability of survival was estimated using the Kaplan-Meier graph. The 95% confidence interval (95% CI) was calculated. The frequencies of vaccine-induced immune responses before and after vaccinations were compared using the Paired t- test. All tests were considered significant when the resulting *p* value was <0.05.

3. RESULTS

3.1 MHC CLASS I A201 TYPING

A prerequisite for the patients' enrolment to the vaccination protocol was that the patient should have HLA-A*0201 haplotype. Patients' cells were stained with the HLA-A0201 antibody in order to test for the presence of the HLA-A201 molecules and analysed with the help of a flow cytometer as shown in figure 3.1 The positivity (HLA-$A2^{+}$) was determined by comparing the histogram of the cells labelled with the mouse anti-human HLA-2-FITC (red) with the histogram which indicates the isotype (green). According to the figure, the positivity is determined as a curve shift to the right compared to the isotype (c). The negative samples do not appear any curve shift (b).

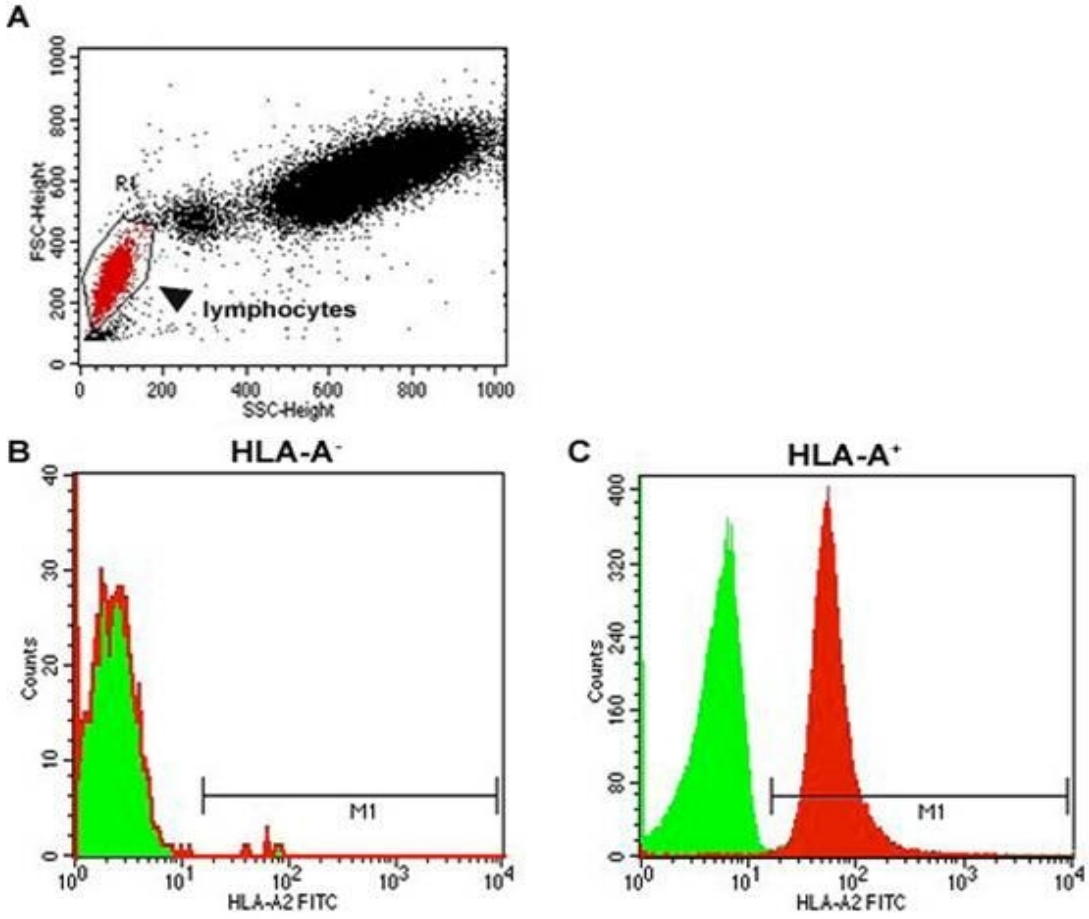


Figure 3.1: Detection of HLA-A2 haplotype. A) Dot plot indicates the populations of PBMCs in respect to their size (FSC) and granulation (SSC) **B)** histogram showing an HLA-$A2^{-}$ patient **C)** histogram showing an HLA-$A2^{+}$ patient

3.2 PATIENTS DEMOGRAPHICS

The patients' demographics are presented in table 3.1. The enrolled patients had various tumour types including breast cancer (9.6%), pancreato-billiar (2.7%), and NSCLC (38.4%) among others. At study entry 84.4 % of patients had distant metastasis (Stage IV) and 62.3% presented disease progression (PD) during the last chemotherapy. All patients had received at least the first two vaccinations with $TERT_{572Y}$ peptide and 49% completed the 6-vaccination protocol. The vaccination protocol was prematurely terminated in the remaining patients (51%) due to disease progression. Moreover 27.4% patients received at least one boost vaccination with $TERT_{572}$ peptide every 3 months until disease progression.

Table 3.1 Patients' demographics

Demographics	No Patients	%
Age (median)	61	
Sex		
Men	58	75.3
Women	19	24.7
Cancer Type		
NSCLC	28	38.4
Melanoma	7	9.6
Hepatocellular (HCC)	3	4.1
Prostate	9	12.3
Colerectal (CRC)	3	4.1
Renal	2	2.7
Thyroid	1	1.4
Pangreas	2	2.7
Cholangio	3	4.1
Breast	7	9.6
Ovarian	1	1.4
Other	11	14.3
Disease Stage at study entry		
III	12	15.6
IV	65	84.4
Disease Status at study entry		
Progressive Disease (PD)	48	62.3
Stable Disease (SD)	29	37.7
Lines of treatment prior to study entry		
≤1	24	31.2
≥ 2-3	53	68.8

3.3 ASSESMENT OF THE hTERT-SPECIFIC IMMUNE RESPONSE USING ELISPOT

The immune response was evaluated by detecting $TERT_{572}$ and $TERT_{572Y}$ -specific T -cells before the 1st and after the 2nd and 6th vaccination. The method used to screen the vaccine induced-specific CTLs was the IFNγ ELISpot. This method is designed to determine the IFNγ releasing cells in the form of spots (figure 3.3). Four patients were excluded from further analysis since no viable PBMCs could be recovered from the pre-vaccination samples after thowing. Therefore, baseline immune reactivity to $TERT_{572}$ peptide was assessed in 73 patients and the vaccine generated immune response after the 2nd vaccination was assessed in all of them. In addition the immune response was assessed in 37 patients (51%) after the 6th vaccination (post-vaccination)

A representative example of a patient who had an immune response against both peptides is presented in the figure 3.2. This is indicated by the presence of spots (specific cells) in the wells containing the cells with the peptides and the positive control (ConA) compared to the wells containing the cells without the peptides (negative control)

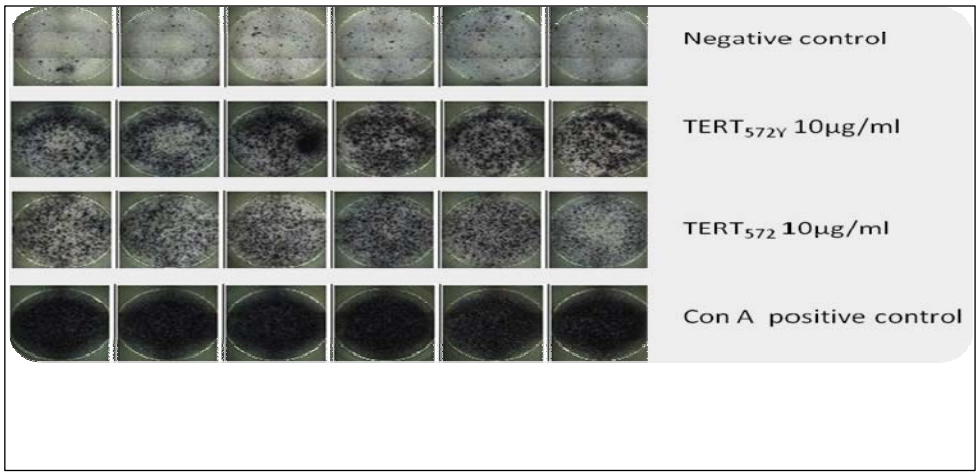


Figure 3.2: Picture taken from automatic microscope (Axio Imager M1-ZEISS) which indicates the spots upon activation of PBMCs by $TERT_{572Y}$, $TERT_{572}$ peptides and Concanavalin-A.

Moreover, graphs were generated using the measurements from the ELISpot program with the help of Graphpad (San Diego, California, USA). A representative example of these graphs either in the presence or in the absence of immune response against $TERT_{572Y}$ and $TERT_{572}$ peptides is shown in figure 3.3. It refers to patient's PSIL (NSCLC) immune response before the 1st (PSIL 1) after the 2nd (PSIL 2) and after the 6th (PSIL 3) vaccination.

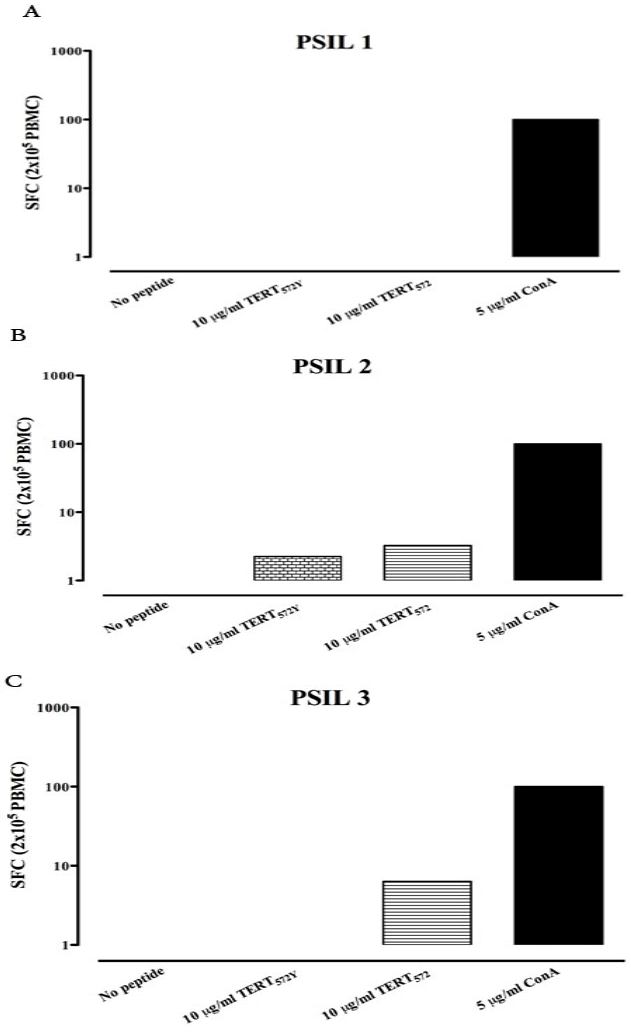


Figure 3.3: hTERT specific immune response (SFC-spot-forming cells)

3.3.1 Assessment of TERT- specific immune response in patients after the 2nd vaccination

3.3.1.1 Vaccine-induced $TERT_{572Y}$-specific immune response

Firstly, $TERT_{572Y}$ (modified) - specific immune response was assessed in patients who completed the 3rd vaccination (73/73, 100%) (Table 3.2)

Table 3.2. $TERT_{572Y}$ -specific immune response in patients who completed the 3rd vaccination, by using ELISpot assay (1=no response, 1> difference between pre and 2nd vaccination)

	Prevaccination	2nd vaccination
AGRI	1	1
AIMA	39	34
ALAF	17	1
ALATH	1	6
ANTH	1	1
ANTZ	1	26
BANI	1	15
DASK	1	24
DEC	1	1
DEF	1	11
DELI	1	15
DIMI	1	21
DILA	1	1
DRON	5	29
EKAF	1	1
EMMA	1	59
FEM	1	1
FIX	7	1
FOTO	1	1
GARB	1	10
GEDI	1	1
GELE	1	1
GEOR	1	1
GERA	1	1
GIAN	1	1
GIBA	1	1
GIEM	1	38
GINI	1	1
HAI	1	1
KAGI	35	23
KARGE	11	1
KANI	7	1

KAZI	1	1
KNIK	1	1
KOSI	1	13
LAST	1	1
LUKI	1	1
MARE	1	1
MAT	4	1
MAUR	42	32
MAX	1	1
META	1	1
MUBA	14	1
MIMA	1	13
MIRI	1	1
NAPO	1	1
NIKA	1	1
NIKHT	1	1
NIO	1	1
NITSA	1	1
PAKY	9	1
PAM	1	1
PANAG	1	1
PAPAE	46	1
PAPI	1	1
PATRA	12	1
PEMA	1	1
PSIL	1	1
PYL	1	1
RIST	1	1
SAV	11	1
SERAF	1	7
SOFO	1	1
SFEP	1	1
SPAG	1	1
SPIR	1	1
STAN	1	7
STEDI	1	29
STEF	1	1
VAMP	1	46
VAPOS	1	1
VLAH	1	1
ZAHE	1	1

According to the table above, 14 (19%) out of 73 patients had a $TERT_{572Y}$ –specific immune response prior to 1st vaccination and 20 (27%) out of 73 patients after the 2nd vaccination. The above data gave rise to the figure 3.4.

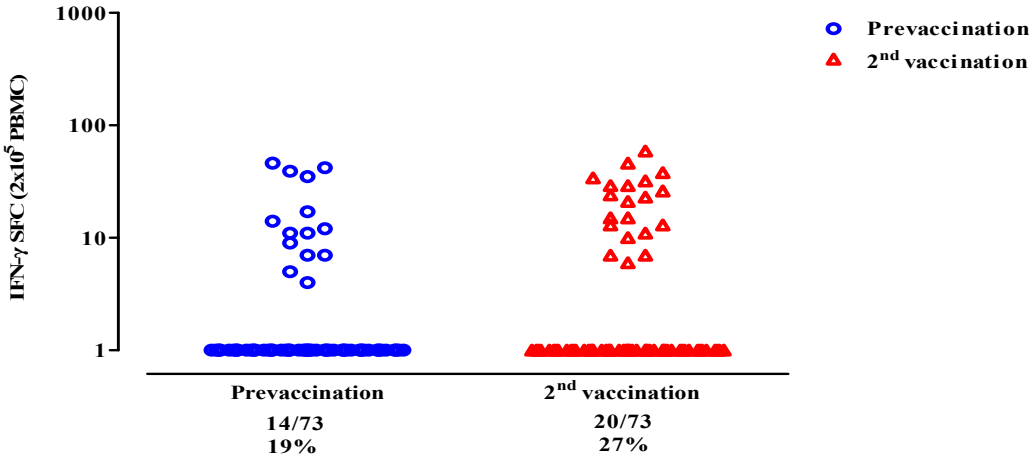


Figure 3.4. Frequencies of specific cells to $TERT_{572Y}$ peptide in vaccinated patients prior to vaccination and after the 2nd vaccination using IFN- γ ELISpot assay.

3.3.1.2 Vaccine-induced $TERT_{572}$-specific immune response

Furthermore, the $TERT_{572}$ (native) - specific immune response in patients who completed the 3rd vaccination was assessed (73/73,100%) (Table 3.3).

Table 3.3. $TERT_{572}$-specific immune response in patients who completed the 3rd vaccination by using ELISpot assay (1=no response, 1> difference between pre and 2nd vaccination)

	Prevaccination	2nd vaccination
ALAF	1	9
ANTH	1	1
BANI	1	1
DASK	1	46
DEC	21	8
DILA	1	1
DIMI	1	1
FIX	1	1
GARB	1	19
GEOR	1	1
GERA	1	60
GIAN	1	1
GIEM	1	1
GINI	1	1
KAGI	1	74
KANI	10	1
LUKI	1	5
MARE	1	5
MAT	6	16
MAUR	1	72
META	1	14
MIMA	1	1
MIRI	1	22
MUBA	31	1
NITSA	12	1
PANAG	1	1
PEMA	1	1
PYL	1	15
RIST	1	1
SAV	8	1
SERAF	1	8
SFEP	1	1
SOFO	1	1
SPIR	1	12
STAN	13	1
ZAHE	1	1
AGRI	1	1

AIMA	1	35
ALATH	1	1
ANTZ	1	1
DEF	1	1
DELI	1	1
DRON	1	11
EKAF	1	1
EMMA	1	1
FEM	9	10
FOTO	5	5
GEDI	1	6
GELE	11	10
GIBA	10	1
HAI	1	40
KARGE	9	1
KAZI	1	15
KNIK	9	1
KOSI	1	11
LAST	1	15
MAX	1	1
NAPO	47	28
NIKA	16	8
NIKHT	1	1
NIO	1	1
PAKY	4	1
PAM	1	22
PAPAE	1	1
PAPI	1	1
PATRA	1	1
PSIL	1	4
SPAG	6	1
STEDI	4	1
STEF	1	1
VAMP	1	1
VAPOS	1	14
VLAH	1	1

According to the table above, 18 (25%) out of 73 patients had a $TERT_{572}$ –specific immune response prior to 1st vaccination and almost doubled after the 2nd vaccination as 30 (41%) out of 73 patients developed $TERT_{572}$ –specific T cells. The vaccine-induced $TERT_{572}$ – specific immune response after the 2nd vaccination was statistically significant (p=0.01, *Student t-test*), compared to the baseline. The above data gave rise to the figure 3.5

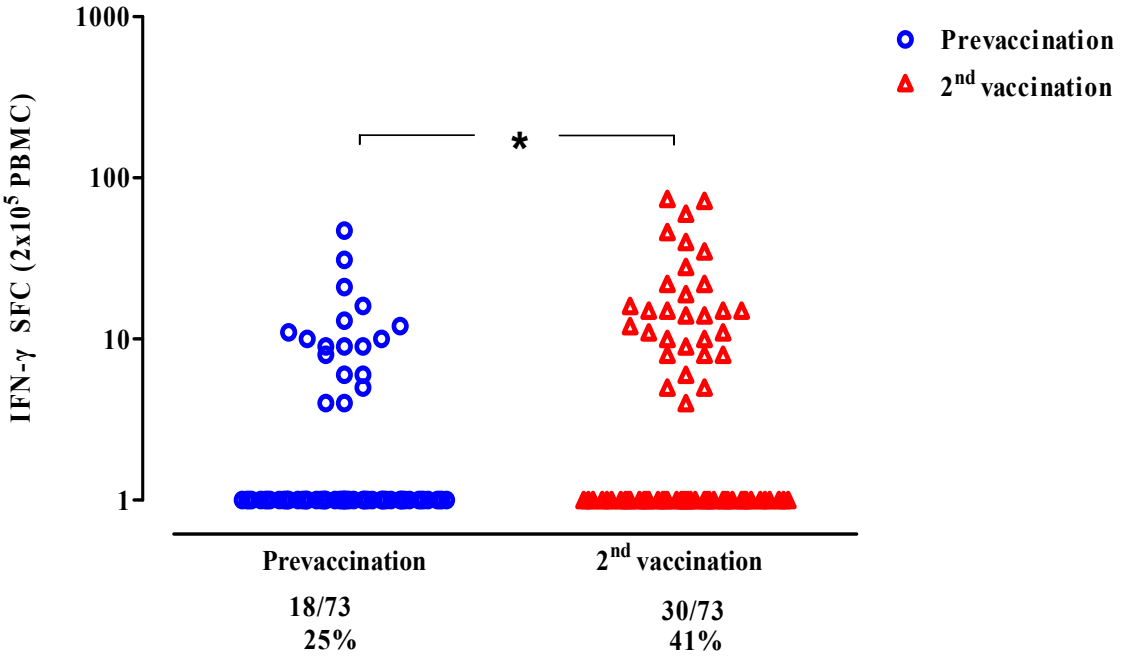


Figure 3.5. Frequencies of specific cells to $TERT_{572}$ peptide in vaccinated patients prior to vaccination and after the 2nd vaccination using IFN- γ ELISpot assay (p=0.01, *Student t-test*)

3.3.2 Assessment of TERT- specific immune response at the completion of the 6-vaccination protocol

3.3.2.1 Vaccine-induced $TERT_{572Y}$-specific immune response

In addition, $TERT_{572Y}$ – specific immune response was assessedin 37 out of 73 patients (51%) after the completion of the 6th vaccination (table 3.4)

Table 3.4 $TERT_{572Y}$ -specific immune response in patients who completed the 6th vaccination by using ELISpot assay (1=no response, 1> difference between pre,2nd and 6th vaccination)

	Prevaccination	2nd vaccination	postvaccination
AGRI	1	1	1
AIMA	39	34	33
ALATH	1	6	35
ANTZ	1	26	1
DEF	1	11	14
DELI	1	15	15
DRON	5	29	1
EKAF	1	1	126
EMMA	1	59	30
FEM	1	1	17
FOTO	1	1	15
GEDI	1	1	1
GELE	1	1	1
GIBA	1	1	1
HAI	1	1	1
KARGE	11	1	1
KAZI	1	1	1
KNIK	1	1	1
KOSI	1	13	1
LAST	1	1	13
MAX	1	1	1
NAPO	1	1	1
NIKA	1	1	1
NIKHT	1	1	1
NIO	1	1	1
PAKY	9	1	14
PAM	1	1	1
PAPAE	46	1	1
PAPI	1	1	63
PATRA	12	1	1
PSIL	1	1	1
SPAG	1	1	7
STEDI	1	29	1
STEF	1	1	1

VAMP	1	46	1
VAPOS	1	1	1
VLAH	1	1	1

6 (16.2%) out of 37 patients had pre-vaccination $TERT_{572Y}$ –specific reactivity. Following vaccination, 10(27%) out of 37 patients after the 2nd vaccination and 12 (32%) out of 37 patients developed peptide-specific cells after the 2nd and 6th vaccination, respectively. The frequencies of $TERT_{572Y}$ – specific immune response after the 6th vaccination were statistically significant (p=0.03, *Student t-test*) in only one patient.compared to the baseline.

The above data gave rise to the figure 3.6

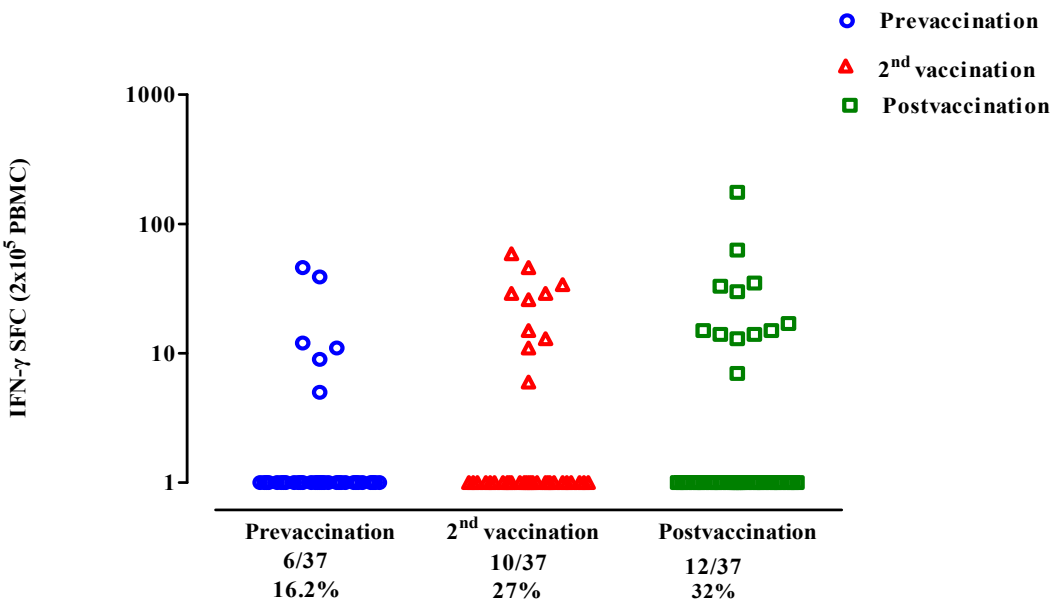


Figure 3.6. Frequencies of specific cells to $TERT_{572Y}$ peptide in vaccinated patients prior to vaccination and after the 2nd and 6th vaccination using IFN-γ ELISpot assay (p=0.03, *Student t-test*).

3.3.2.2 Vaccine-induced $TERT_{572}$-specific immune

Finally, $TERT_{572}$ (native) - specific immune response was assessed in 37 out of 73 patients (51%) after the completion of the 6th vaccination (table 3.5)

Table 3.5. $TERT_{572}$-specific immune response in patients who completed the 6th vaccination by using ELISpot assay (1=no response, 1> difference between pre, 2nd and 6th vaccination)

	Prevaccination	2nd vaccination	postvaccination
AGRI	1	1	1
AIMA	1	35	1
ALATH	1	1	9
ANTZ	1	1	38
DEF	1	1	16
DELI	1	1	1
DRON	1	11	1
EKAF	1	1	125
EMMA	1	1	1
FEM	9	10	17
FOTO	5	5	4
GEDI	1	6	1
GELE	11	10	31
GIBA	10	1	1
HAI	1	40	31
KARGE	9	1	1
KAZI	1	15	1
KNIK	9	1	11
KOSI	1	11	38
LAST	1	15	1
MAX	1	1	1
NAPO	47	28	35
NIKA	16	8	10
NIKHT	1	1	36
NIO	1	1	1
PAKY	4	1	12
PAM	1	22	9
PAPAE	1	1	28
PAPI	1	1	1
PATRA	1	1	1
PSIL	1	4	6
SPAG	6	1	7
STEDI	4	1	1
STEF	1	1	5
VAMP	1	1	1
VAPOS	1	14	1
VLAH	1	1	1

According to the table above, 11 out of 37 patients (30%) had a $TERT_{572}$ –specific immune response prior to 1st vaccination, 15 out of 37 patients (41%) after the 2nd vaccination and 19 out of 37 patients (51%) after the 6th vaccination. The frequency of patients who had a $TERT_{572}$ – specific immune response after the 6th vaccination was statistically significant (p=0.02, *Student t-test*) compared to the baseline

The above data gave rise to the figure 3.7

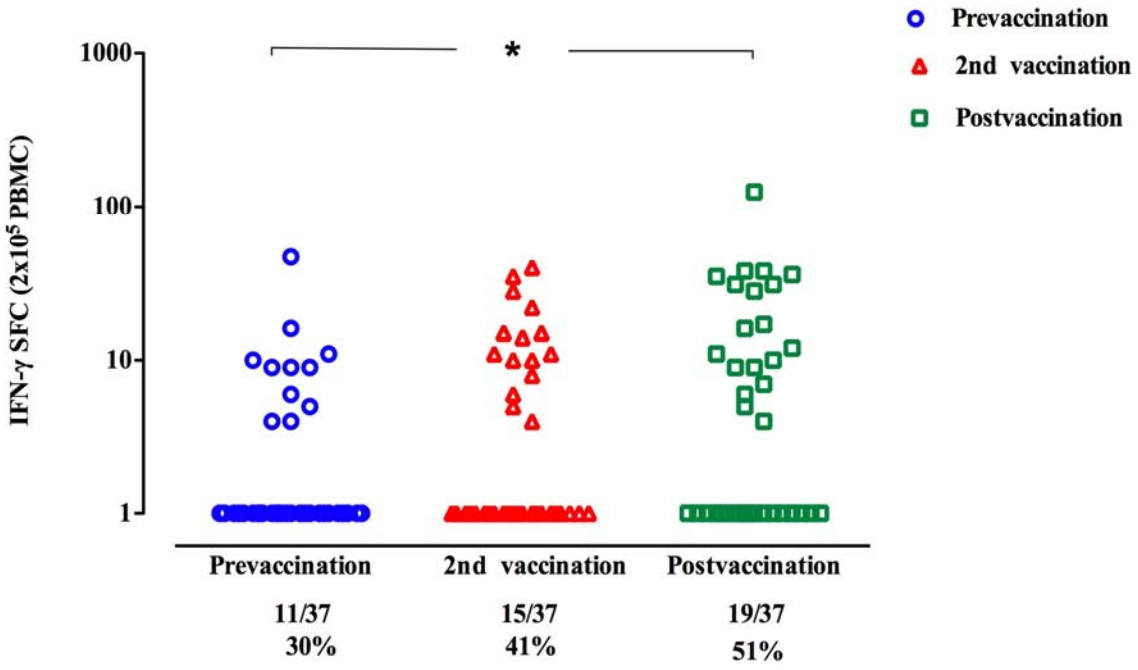


Figure 3.7. Frequencies of specific cells to $TERT_{572}$ peptide in vaccinated patients prior to vaccination and after the 2nd and 6th vaccination using IFN-γ ELISpot assay (p=0.02, *Student paired t-test*).

3.3.3 $TERT_{572Y}$ – specific versus $TERT_{572}$ –specific immune responses in early (after the 2nd vaccination) and late responders (after the 6th vaccination)

3.3.3.1 $TERT_{572}$-specific immune response after the 2nd vaccination in early $TERT_{572Y}$ responders.

Then, the next question that was needed to answer was the following. Do the T cells which recognise and become activated by the modified peptide are able to recognise the native peptide as well? As it was observed, in 5 out of 17 patients (29%) $TERT_{572Y}^{+}$/$TERT_{572}^{+}$ -specific T cells could be detected after the 2nd vaccination (DASK, GARB, SERAF, DRON, KOSI), whereas in 12 out of 17 patients (71%) $TERT_{572Y}^{+}$ -specific T cells were unable to recognize the native $TERT_{572}$ peptide, as well (ALATH, ANTZ, BANI, DIMI, GIEM, MIMA, DEF, DELI, EMMA, STEDI, VAMP, STAN).

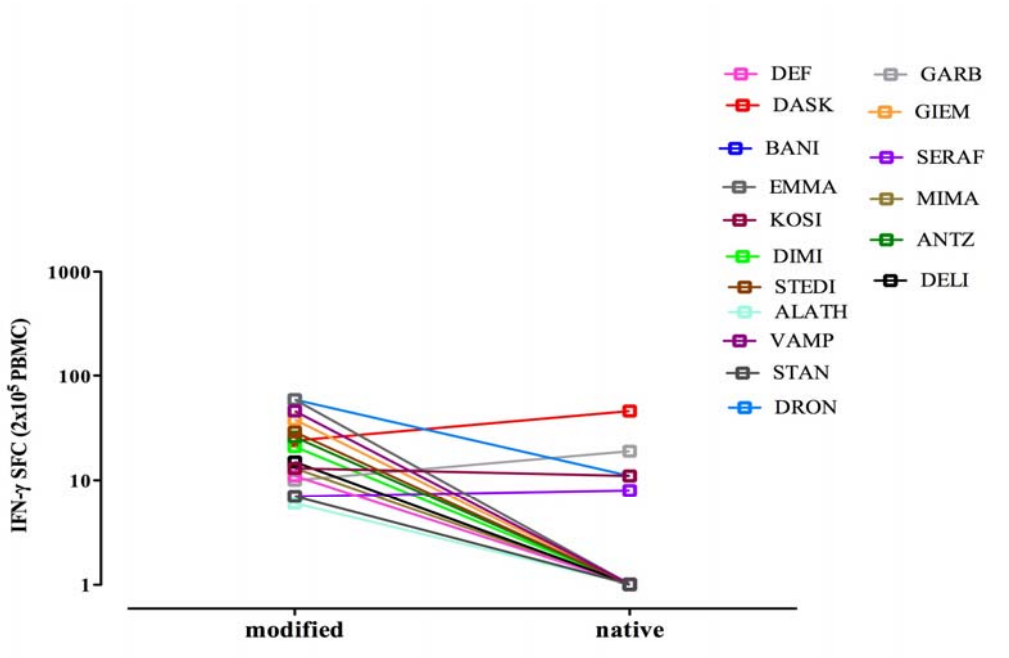


Figure 3.8: $TERT_{572/572Y}$ -specific immune response after the 2nd vaccination

3.3.3.2 $TERT_{572}$-specific immune response after the 6th vaccination in late $TERT_{572Y}$ responders

After the completion of the 6-vaccinations, $TERT_{572Y}^{+}/TERT_{572}^{+}$-specific cells were detected in 6 out of 10 patients (60%) (ALATH, EKAF, DEF, FEM, FOTO, SPAG), whereas, $TERT_{572Y}^{+}/TERT_{572}^{-}$-specific cells were found in 4 out of 10 patients (40%) (DELI, EMMA, LAST, PAPI).

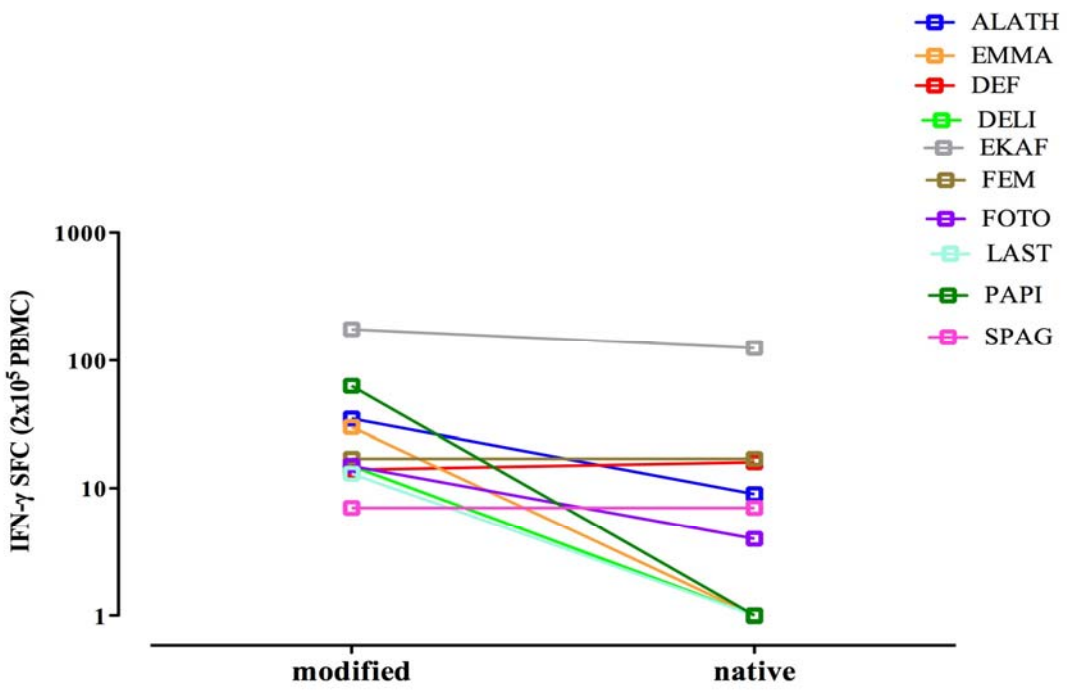


Figure 3.9: $TERT_{572572Y}$ /-specific immune response after the 6th vaccination.

3.3.3.3 $TERT_{572}$-specific immune response after the 6th vaccination in early $TERT_{572Y}$ responders

As it is observed, 4 out of 9 patients (45%) with a $TERT_{572Y}$-specific response after the 2nd vaccination developed a $TERT_{572Y}^{+}/TERT_{572}^{+}$ immune response after the 6th vaccination (ALATH, ANTZ, KOSI, DEF), whereas 5 out of 10 patients (55%) developed a $TERT_{572Y}^{+}/TERT_{572}^{-}$ immune response (DELI, DRON, EMMA, STEDI, VAMP).

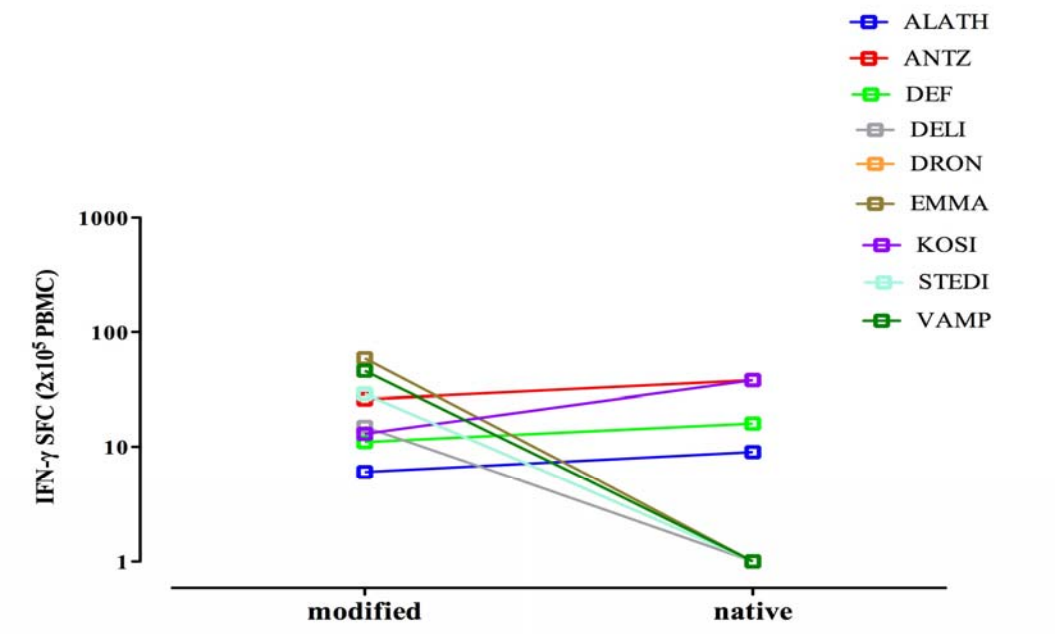


Figure 3.10. TERT572-specific immune response after the 6th vaccination in early responders with a TERT572Y- specific immune response

3.4 Patients with or without a pre-existing $TERT_{572Y}$ – specific immune response

3.4.1 Development of immune response in vaccinated patients with a $TERT_{572Y}$ - specific pre-existing immune reactivity

15/73 (21%) patients had a $TERT_{572Y}$ - specific pre-existing immune reactivity (table 3.6)

Table 3.6 $TERT_{572Y}$-specific immune responses after the 2nd and 6th vaccination in patients with a pre-vaccination immune response (- = no vaccination)

	Pre-vaccination	2nd Vaccination	Post-vaccination
AIMA	39	34	33
ALAF	17	1	-
DRON	5	29	1
FIX	7	1	-
KAGI	35	23	-
KANI	7	1	-
KARGE	11	1	1
MAT	4	1	-
MAUR	42	32	-
MUBA	14	1	-
PAKY	9	1	14
PAPAE	46	1	1
PATRA	12	1	-
SAV	6	1	-
SOFO	21	1	-

As it was observed, 4 out of 15 patients (27%) had a $TERT_{572Y}$-specific immune response after the 2nd vaccination and 2 out of 5 (40%) after the 6th vaccination. In particular, as far as AIMA is concerned, the $TERT_{572Y}$- specific immune response was not augmented further in response to vaccination between pre-vaccination and after the 2nd and 6th vaccination. Secondly, ALAF, FIX, KANI, KARGE, MAT, MUBA, PAPAE, PATRA, SAV and SOFO had a pre-existing $TERT_{572Y}$-specific immune response which was lost after the 2nd vaccination. Moreover, DRON showed an increase in $TERT_{572Y}$- specific immune response after the 2nd vaccination which was lost after the 6th vaccination. On the other hand, PAKY had a pre-existing $TERT_{572Y}$- specific immune response which

was lost after the 2nd vaccination and reappeared with a slight increase after the 6th vaccination. Finally, MAUR appeared a slight decrease in $TERT_{572Y}$- specific immune response after the 2nd vaccination. Generally, the majority of the patients (73%) with pre-existing $TERT_{572Y}$ immune reactivity a failed to mount an early immune response (Figure 3.11)

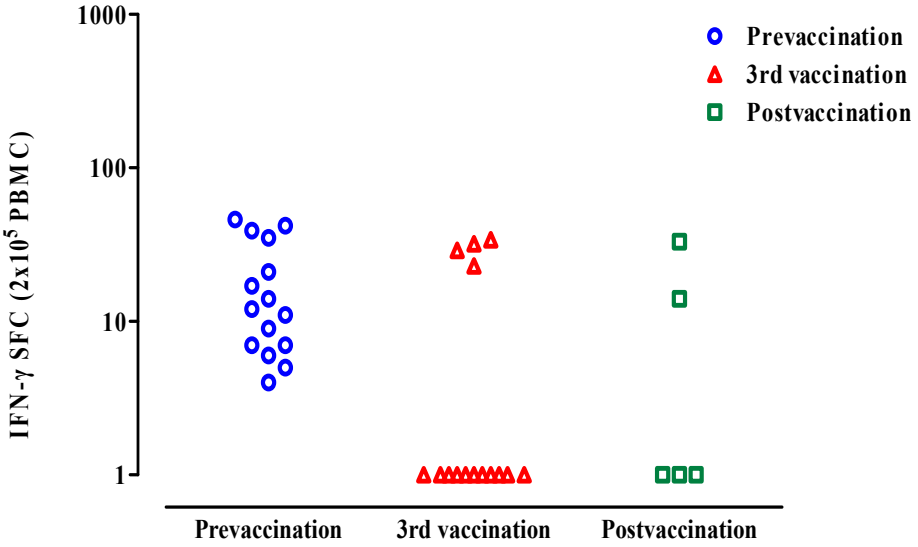


Figure 3.11. T cell responses to $TERT_{572Y}$ peptide after the 2nd and 6th vaccination in patients with $TERT_{572Y}$ – pre-existing reactivity.

3.4.2 Development of immune response following vaccination in patients without a pre-existing $TERT_{572Y}$ reactivity

58/73 (79%) of the vaccinated patients did not shown pre-existing $TERT_{572Y}$ - reactivity (table 3.7)

Table 3.7. $TERT_{572Y}$-specific immune responses in vaccinated patients without a prevaccination immune reactivity (- = no vaccination)

MIRI	1	1	-
NAPO	1	1	1
NIKA	1	1	1
NIKHT	1	1	1
NIO	1	1	1
NITSA	1	1	
PAM	**Pre-vaccination**	**2nd Vaccination**	**Post-vaccination**
AGRI	1	1	1
PANAG	1	1	18
ALATH	1	6	35
PAPL	1	1	63
ANTH	1	1	-
PEMA	1	1	1
ANTZ	1	26	1
PSIR	1	1	-
BANI	1	16	1
PYL	1	1	-
DASK	1	35	-
RIST	1	1	-
DEC	1	1	-
SERAF	1	7	
DET	1	11	14
SFEP	1	1	
DELI	1	10	15
SOFO	1	1	-
DILA	1	1	-
SPIR	1	1	-
DIMI	1	21	-
STAN	1	1	
EKAT	1	1	176
STEDI	1	39	1
EMMA	1	31	30
STEF	1	1	
FEM	1	1	19
VAMP	1	31	1
FOTO	1	1	15
VARDOS	1	1	1
GARB	1	10	-
VLAH	1	1	1
GEDI	1	1	1
ZAHE	1	1	1
GELE	1	1	
GEOR	1	1	-
GERA	1	1	-
GIAN	1	1	-
GIEM	1	38	-
GIBA	1	1	1
GINI	1	1	-
HAI	1	1	1
KAZI	1	1	1
KNIK	1	1	1
KOSI	1	13	1
LAST	1	1	14
LUKI	1	1	-
MARE	1	1	-
MAX	1	1	1
META	1	1	-
MIMA	1	13	-

$TERT_{572Y}$-specific immune response was mounted in 15 out

of 58 patients (26%) after the 2nd vaccination and 11 out of 32 patients (34%) after the 6th vaccination. In particular, ALATH, DEF and DELI, developed an immune response after the 2nd vaccination which was further increased after the 6th vaccination. EKAF, FEM, FOTO, LAST, PANAG and PAPI developed an immune response only after the 6th vaccination. Especially, EKAF showed the greatest increase in immune response after the 6th vaccination. Furthermore, ANTZ, KOSI, STEDI and VAMP developed an immune response after the 2nd vaccination, which could be detected after the 6th vaccination.

The frequency of patients without a $TERT_{572Y}$ pre-existing reactivity who developed an immune response after the 2nd and 6th vaccination was statistically significant (p= 0.0001, p=0.0006 respectively, *Student t-test*) compared to the baseline (figure 3.12).

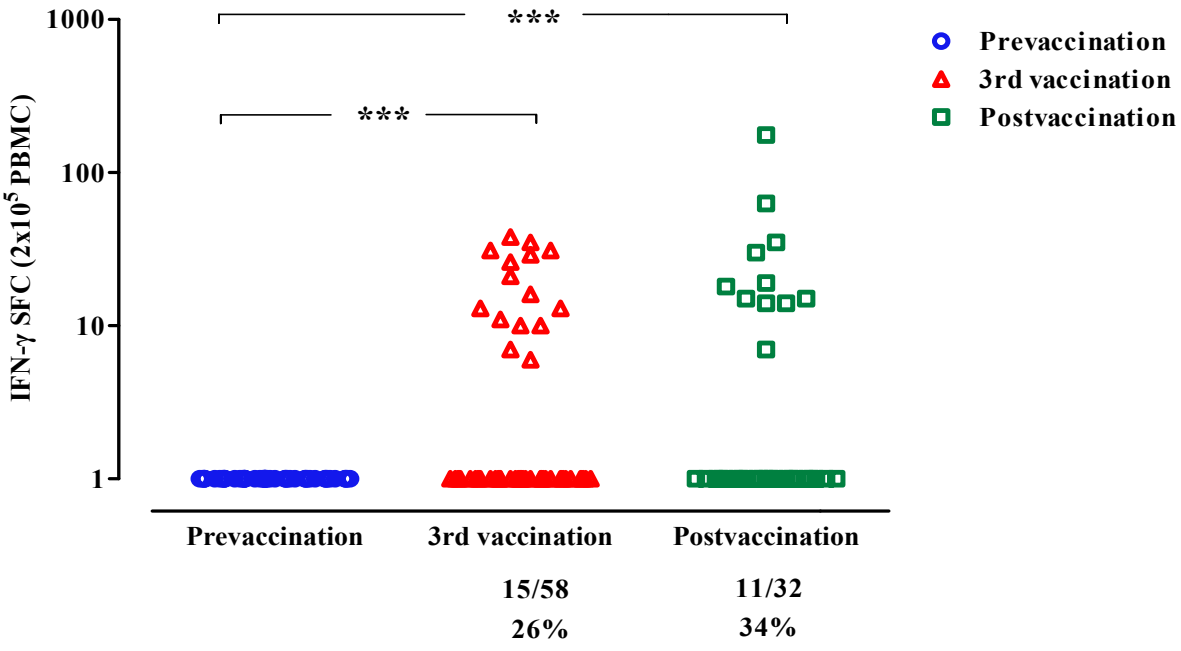


Figure 3.12: T cell responses to $TERT_{572Y}$ peptide after the 2nd and 6th vaccination in patients without $TERT_{572Y}$ pre-existing reactivity

3.5 Patients with or without a pre-existing $TERT_{572}$ – specific immune response

3.5.1 Development of immune response in patients with a $TERT_{572}$ -specific pre-existing immune reactivity

18/73 (25%) patients had a $TERT_{572}$ - specific pre-existing reactivity (table 3.8)

Table 3.8 $TERT_{572}$-specific immune responses prior to vaccination and after the 2nd and 6th vaccination in patients with a prevaccination immune response (- = no vaccination)

	Prevaccination	2nd Vaccination	Postvaccination
DEC	**21**	**8**	**-**
FEM	**9**	**22**	**34**
FOTO	**5**	**5**	**12**
GELE	**11**	**10**	**31**
GIBA	**10**	**1**	**1**
KANI	**10**	**1**	**-**
KARGE	**9**	**1**	**1**
KNIK	**9**	**1**	**11**

MAT	**6**	**16**	**-**
MUBA	**31**	**1**	**-**
NAPO	**47**	**28**	**35**
NIKA	**16**	**8**	**10**
NITSA	**12**	**1**	**-**
PAKY	**4**	**1**	**12**
SAV	**8**	**1**	**-**
SPAG	**6**	**1**	**7**
STAN	**13**	**1**	**-**
STEDI	**4**	**1**	**1**

$TERT_{572}$ –reactive cells were detected in 7/18 (39%) patients after the 2nd vaccination and in 8/11 (73%) after the 6th vaccination. In particular, an increase was observed after the 2nd vaccination in MAT and a gradual increase after the 2nd and 6th vaccination as far as FEM is concerned. FOTO and GELE, showed no increase in the immune response after the 2nd vaccination, but it was amplified after the 6th vaccination. Moreover, the immune response was decreased after the 2nd vaccination in DEC and NIKA and returned to the basal levels in GIBA, KANI, KARGE, MUBA, NITSA, SAV, STAN and STEDI. An important observation is that, in some patients there was a fluctuation in the immune response after the 2nd and 6th vaccination. For example, KNIK, PAKY and SPAG lost the immune response after the 2nd vaccination but it reappeared with a slight increase after the 6th vaccination. Moreover, as far as NAPO is concerned, there was a decrease after the 2nd vaccination which was followed by a slight increase after the 6th vaccination. Generally, 11 out of 18 patients (61%) lost their $TERT_{572}$ -specific pre-existing immune reactivity after the 2nd vaccination

The 60% of patients with pre-existing immune reactivity to $TERT_{572}$ peptide who failed to mount a $TERT_{572}$ –specific immune response after the 2nd vaccination and 30% of the patients after 6th vaccination. An interesting observation was that the larger decline induction of the immune response occurred after the 2nd vaccination compared to the post-vaccination numbers (Figure 3.13).

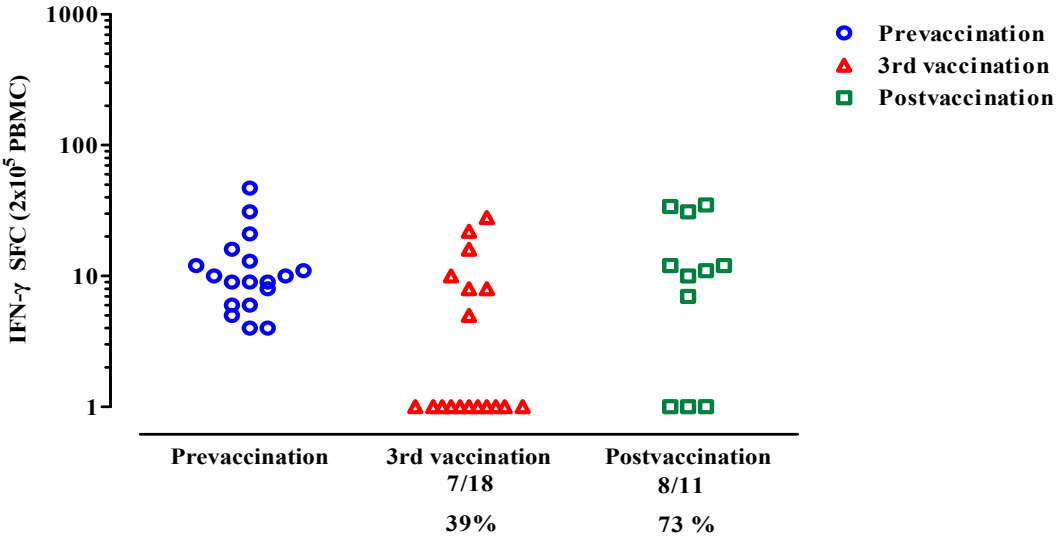


Figure 3.13: T cell responses to $TERT_{572}$ peptide after the 2nd and 6th vaccination in patients with $TERT_{572}$ – specific pre-existing immune reactivity

3.5.2 Development of immune response in patients without a $TERT_{572}$ -specific pre-existing immune reactivity

55/73 (75%) patients did not have a $TERT_{572}$ - specific immune reactivity prior to 1st vaccination (table 3.9)

Table 3.9 $TERT_{572}$-specific immune responses prior vaccination and after the 2nd and 6th vaccination in patients without a prevaccination immune response (- = no vaccination)

	Prevaccination	2nd Vaccination	Postvaccination
AGRI	1	1	1
AIMA	1	35	1
ALAF	1	9	-
ALATH	1	1	9

ANTH	1	1	-
ANTZ	1	1	38
BANI	1	1	-
DASK	1	55	-
DEF	1	1	16
DELI	1	1	1
DILA	1	1	-
DIMI	1	1	-
DRON	1	11	1
EKAF	1	1	125
EMMA	1	1	1
FIX	1	1	-
GARB	1	19	-
GEDI	1	6	1
GEOR	1	1	-
GERA	1	60	-
GIAN	1	1	-
GIEM	1	1	-
GINI	1	1	-
HAI	1	40	31
KAGI	1	74	-
KAZI	1	1	1
KOSI	1	11	38
LAST	1	15	1
LUKI	1	5	-
MARE	1	15	-
MAUR	1	72	-
MAX	1	1	1
META	1	1	-
MIMA	1	1	-
MIRI	1	22	-
NIKHT	1	1	36
NIO	1	1	1
PAM	1	22	9
PANAG	1	1	24
PAPAE	1	1	28
PAPI	1	1	1
PATRA	1	1	1
PEMA	1	1	41
PSIL	1	4	6
PYL	1	15	-
RIST	1	1	-
SERAF	1	8	-
SFEP	1	1	-
SOFO	1	1	-
SPIR	1	12	-
STEF	1	1	5

VAMP	1	1	1
VAPOS	1	14	1
VLAH	1	1	1
ZAHE	1	1	-

$TERT_{572}$-specific immune was mounted in 21 (38%) out of 55 patients after the 2nd vaccination and 13(46%) out of 28 patients after the 6th vaccination. More specifically, as far as DASK, GARB, GERA, KAGI, MARE, MAUR, MIRI, PYL are concerned, there was a significant increase in the immune response after the 2nd vaccination and a slighter increase in ALAF, LUKI, SERAF and SPIR. On the other hand, ALATH, ANTZ, DEF, EKAF, NIKHT, PANAG, PAPAE, PEMA and STEF developed an immune response only after the 6th vaccination. Moreover, AIMA, DRON, GEDI, LAST and VAPOS developed an immune response after the 2nd vaccination which was lost after the 6th vaccination. HAI developed an immune response after the 2nd vaccination, which was slightly decreased after the 6th vaccination. PSIL developed an immune response after the 2nd vaccination, which remained relatively constant after the 6th vaccination. Furthermore, there was a gradual increase in KOSI, after the 2nd and 6th vaccination. Last but to least, in PAM, there was a fluctuation in the immune response as it was increased after the 2nd vaccination and decreased after the 6th vaccination.

The frequency of patients without a $TERT_{572}$ pre-existing reactivity who developed an immune response after the 2nd and 6th vaccination was statistically significant ($p= 0.0001$, $p=0.0005$ respectively, *Student t-test*) compared to the baseline (figure 3.14).

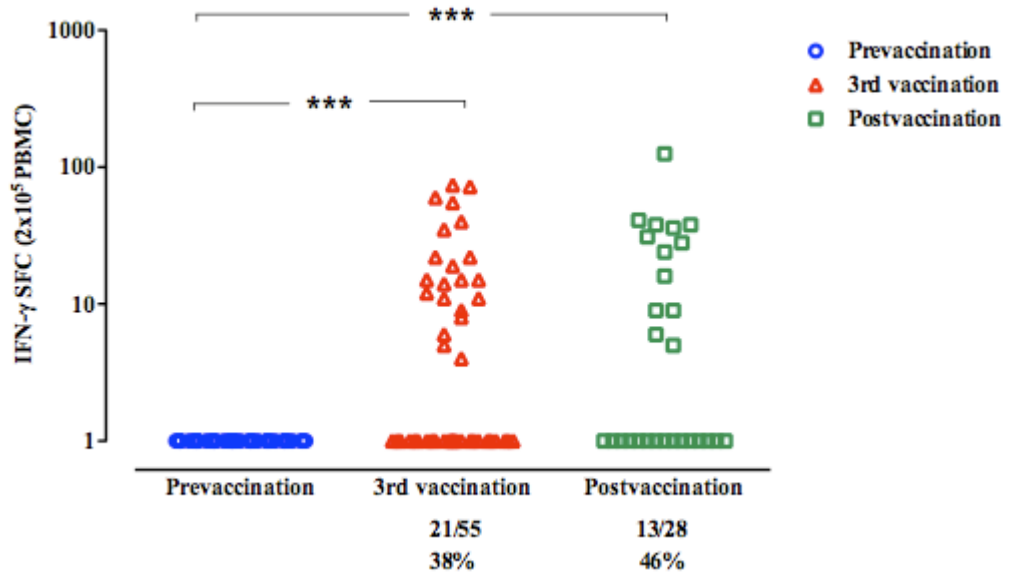


Figure 3.14. T cell responses to $TERT_{572}$ peptide after the 2nd and 6th vaccination in patients without $TERT_{572}$ - specific pre-existing immune reactivity

In summary, 21% patients had a $TERT_{572Y}$-specific and 25% a $TERT_{572}$-specific pre-existing immune reactivity. Moreover, 27% patients developed a $TERT_{572Y}$-specific immune response after the 2nd vaccination (early responders) and 32% patients after the 6th vaccination (late responders). Furthermore, 41% patients developed a $TERT_{572}$-specific immune response after the 2nd vaccination and 51% after the 6th vaccination.

Table 3.10 Overall percentages of patients who developed a $TERT_{572Y}$ specific and $TERT_{572}$-specific immune response prior to 1st vaccination and after the 2nd and the 6th vaccination

	$TERT_{572Y}$-specific immune response	
	% Responders	% Non-responders
Prevaccination	21	79
After the 2nd vaccination	27	73
After the 6th vaccination	32	68

	$TERT_{572}$-specific immune response	
	% Responders	% Non-responders
Prevaccination	25	75
After the 2nd vaccination	41	59
After the 6th vaccination	51	49

A significant observation is the difference between the percentages of early responders with a $TERT_{572Y}$-specific immune response and late responders with a $TERT_{572}$-specific immune response. In particular, the percentage of patients who developed a $TERT_{572}$-specific immune response after the 6th vaccination (51%) is much greater than the percentage of patients who developed a $TERT_{572Y}$-specific immune response after the 2nd vaccination (27%). This could be explained by the hypothesis that the optimized $TERT_{572Y}$ peptide first generates peptide-specific T cells and then the stimulation with the native $TERT_{572}$ peptide selects among T cells those with the highest specificity for the native $TERT_{572}$ peptide which is presented by tumour cells. Therefore, it is normally expected that an increase in $TERT_{572}$ –specific immune response would occur after the 6th vaccination

3.6 ASSESMENT OF THE hTERT-SPECIFIC IMMUNE RESPONSE USING INTRACELLULAR STAINING (ICS)

3.6.1. Phenotypic Characterization

The phenotypic characterization of stimulated IFN-γ –PBMCs was determined by using the surface molecules $CD3^+$ and $CD8^+$ along with the intracellular cytokine IFN-γ. The intracellular staining was used in order as to determine the phenotype of the activated cells in response to the peptides, as was shown by using ELISpot method.

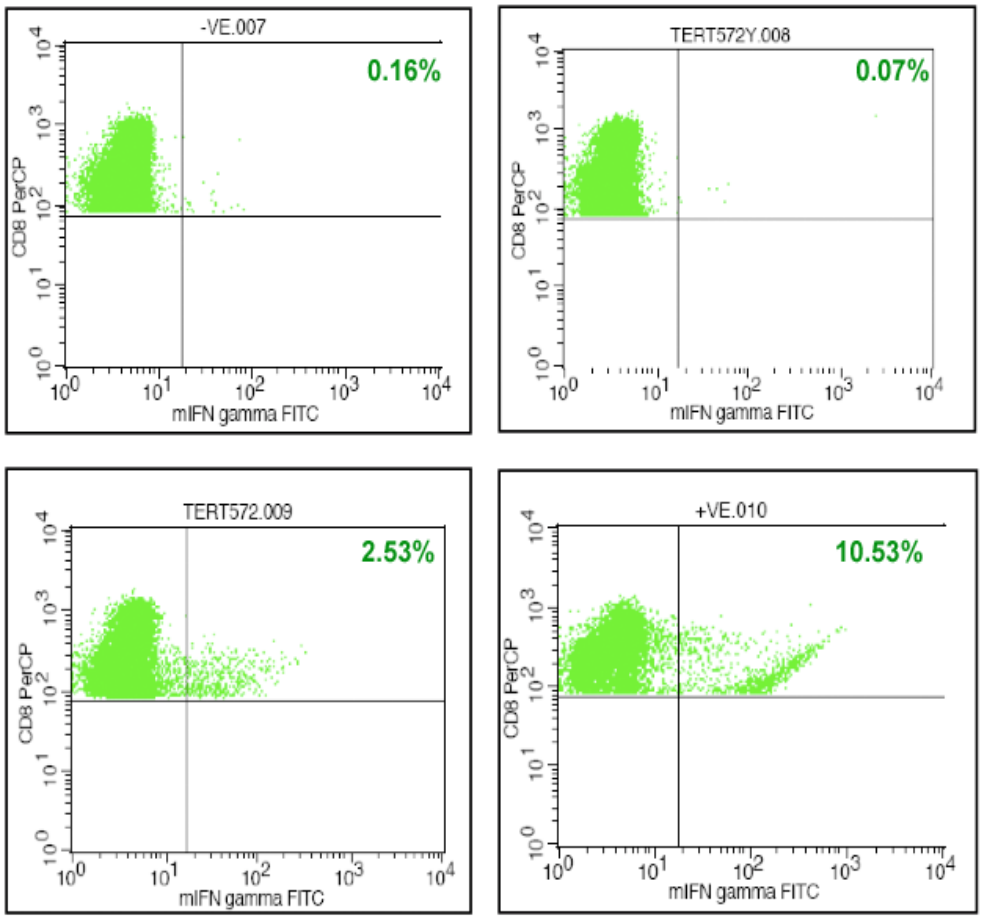


Figure 3.15. **Analysis of IFN-γ-specific-$CD3^+$ $CD8^+$ T-lymphocytes (CTLs) stimulated by the peptides**. The percentage of $CD3^+CD8^+$ IFN-γ+ cells is shown on the right corner of the boxes.

Thus, firstly it was examined if the activated cells were T cells**,** by using the surface molecule $CD3^+$ and then whether they were cytotoxic T cells (CTLs), by using the surface molecule $CD8^+$**.**

Table 3.11. Number of $TERT_{572Y}$-specific $CD3^+$ T cells of patients before the 1st and after the 2nd and 6th vaccination, by using ICS (1=absence of immune response, n>1=number of stimulated $TERT_{572Y}$-specific $CD3^+$ cells, **-**= no vaccination)

	Pre-vaccination	3rd Vaccination	Post-vaccination
AGRI	80	1	20
ALAF	1	1	-
ALATH	1	250	-
ANTH	1	1	-
DASK	1	1	-
DIMI	1	170	1
EMMA	1000	260	1
FEM	1	1	15
FOTO	1	150	-
GEDI	1	1	1
GEOR	60	1	-
GIEM	1	100	40
KARGE	15	1	1
KAZI	1	1	20
KNIK	1	40	1
LAST	1	1	1
LUKI	400	1	-
MAT	1	1	-
MIRI	1	50	80
NAPO	1	40	-
NIKA	70	20	-
PAPI	1	1	1
PEMA	60	1	1
PSIL	75	1	1
PANAG	20	1	1
SPAG	1	1	10
SPIR	1	1	-
STEDI	150	10	-
STEF	1	1	-
ZAHE	1	1	-

As it can be observed, 10 out of 30 patients (33.3%) had a $TERT_{572Y}$ - specific pre-existing immune reactivity, 10 out of 30 patients (33.3%) developed a $TERT_{572Y}$ - specific immune response after the 2nd vaccination and 6 out of 12 patients (50%) after the 6th vaccination. In particular, AGRI, GEOR, KARGE, LUKI, PEMA, PSIL and PANAG had a $TERT_{572Y}$ - specific pre-existing immune reactivity, which was totally lost after the 2nd vaccination, with the exception of AGRI, in whom it reappeared after the 6th vaccination. Moreover, EMMA, NIKA and STEDI, had a $TERT_{572Y}$ - specific pre-existing immune reactivity, which was decreased after the 2nd vaccination. Furthermore, ALATH, DIMI, FOTO, GIEM, KNIK, MIRI and

NAPO developed a $TERT_{572Y}$ - specific pre-existing immune response only after the 2nd vaccination; In particular, as far as GIEM is concerned, it was maintained after the 6th vaccination but in lower levels while in MIRI, it was further amplified after the 6th vaccination. Finally, FEM, KAZI and SPAG developed a $TERT_{572Y}$ - specific pre-existing immune response only after the 6th vaccination.

The above data gave rise to the figure 3.16

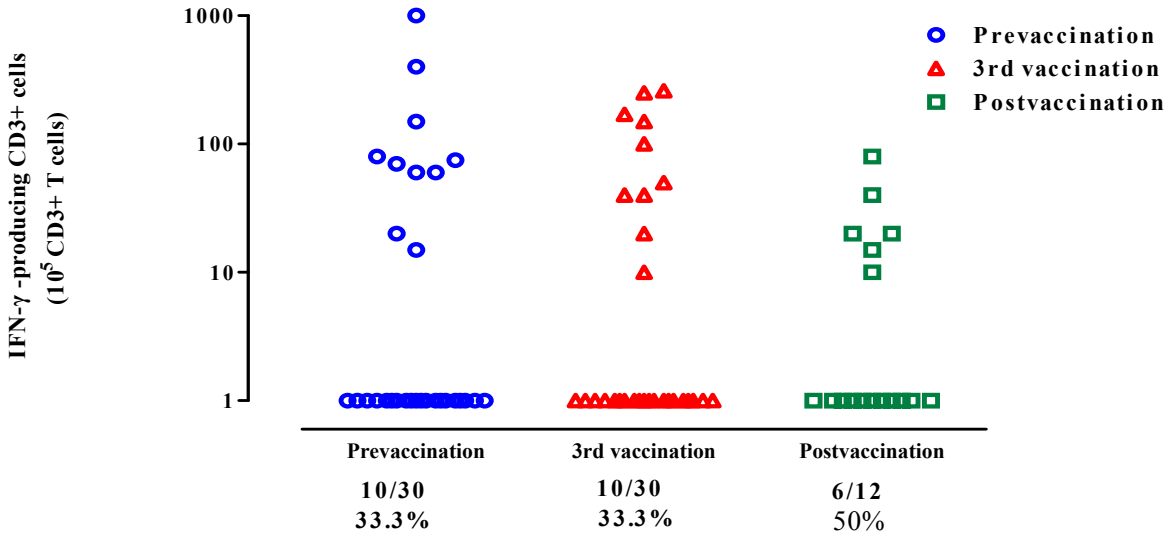


Figure 3.16. Frequencies of IFN-γ-producing CD3+ cells specific to $TERT_{572Y}$ peptide in vaccinated patients prior to vaccination and after the 2nd and 6th vaccination using ICS

Table 3.12 Number of $TERT_{572}$-specific $CD3^+$ cells of patients before the 1st and after the 2nd and 6th vaccination, by using ICS (1=absence of immune response, n>1=number of stimulated $TERT_{572}$-specific $CD3^+$ cells, **-** = no vaccination)

	Prevaccination	3rd Vaccination	Postvaccination
AGRI	**250**	**200**	**1**
ALAF	**1**	**18**	**-**
ALATH	**1**	**240**	**-**
ANTH	**1**	**1**	**-**
DASK	**1**	**1**	**-**
DIMI	**1**	**1**	**1**
EMMA	**430**	**178**	**1**
FEM	**1**	**1**	**16**
FOTO	**1**	**100**	**-**
GEDI	**1**	**1**	**1**
GEOR	**1**	**100**	**-**
GIEM	**1**	**100**	**1**
KARGE	**1**	**1**	**1**
KAZI	**1**	**18**	**16**
KNIK	**10**	**45**	**1**
LAST	**1**	**12**	**1**
LUKI	**1**	**1**	**-**
MAT	**1**	**1**	**1**
MIRI	**1**	**1**	**1**
NAPO	**1**	**1**	**-**
NIKA	**54**	**5**	**-**
PAPI	**1**	**20**	**1**
PEMA	**1**	**1**	**1**
PSIL	**1**	**5**	**1**
PANAG	**1**	**1**	**1**
SPAG	**1**	**1**	**1**
SPIR	**1**	**1**	**-**
STEDI	**215**	**1**	**-**
STEF	**1**	**1**	**-**
ZAHE	**1**	**1**	**-**

As it can be observed, 5 out of 30 patients (13%) had a $TERT_{572}$ - specific pre-existing immune reactivity, 13 out of 30 patients (33.3%) developed a $TERT_{572}$ - specific immune response after the 2nd vaccination and 2 out of 9 patients (11%) after the 6th vaccination. In particular, AGRI, EMMA and NIKA had a $TERT_{572}$ - specific pre-existing immune reactivity, which was decreased after the 2nd vaccination and lost after the 6th vaccination. KNIK had a $TERT_{572}$ - specific pre-existing immune reactivity which was further amplified after the 2nd vaccination while STEDI had a $TERT_{572}$ - specific pre-existing immune reactivity which was lost after the 2nd vaccination. Some of the patients

developed a $TERT_{572}$ – specific immune response after the 2nd vaccination which was either lost after the 6th vaccination (ALAF, ALATH, GIEM, LAST, PAPI, PSIL) or remained relatively constant (KAZI). Finally, FEM was the only patient who developed a $TERT_{572}$ – specific immune response after the 6th vaccination.

The above data gave rise to the figure 3.17

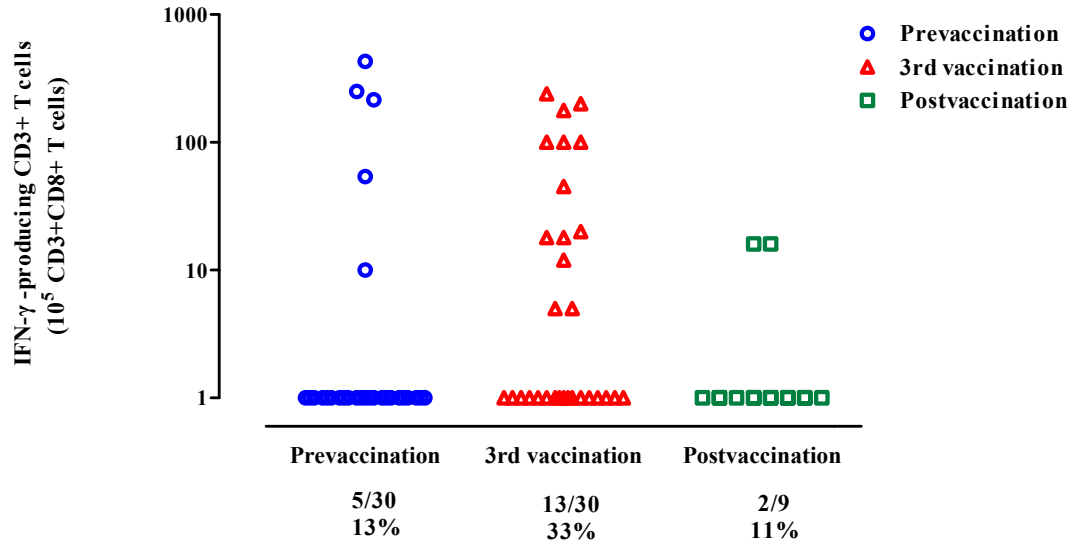


Figure 3.17. Frequencies of IFN-γ-producing CD3+ cells specific to $TERT_{572}$ peptide in vaccinated patients prior to vaccination and after the 2nd and 6th vaccination using ICS

3.6.2 Correlation between ELISpot and ICS results

In order to determine if there was a correlation between ELISpot and ICS results, there was a comparison between the presence of IFN- γ - SFC cells identified by using ELISpot assay and IFN- γ - releasing CD3+ T cells identified by using ICS, in patients to whom the number of CD3+ T-cells was measured in response to peptides before the 1st and after the 2nd and 6th vaccination

3.6.2.1 $TERT_{572Y}$- specific CD3+ cells after the 2nd vaccination

Table 3.13. Number of patients who developed a $TERT_{572Y}$-specific immune response after the 2nd vaccination, either by using ELISpot assay or ICS.

	ELISPOT	
	+	-
+	**5**	**3**
ICS		
_	**4**	**18**

As it is observed, in 5 out of 30 patients (17%) a $TERT_{572Y}$-specific immune response after the 2nd vaccination was observed by using both ELISpot assay and ICS. Moreover, in 4 out of 30 patients (13%), a $TERT_{572Y}$-specific immune response after the 2nd vaccination was identified only by using ELISpot assay, in 3 out of 30 patients only by using ICS (10%) and in 18 out of 30 patients (60%) a $TERT_{572Y}$-specific immune response was not observed neither by using ELISpot assay nor ICS (correlation coefficient r= 0.03 *Spearman test,* no significant correlation).

3.6.2.2. $TERT_{572Y}$- specific CD3+ cells after the 6th vaccination

Table 3.14. Number of patients who developed a $TERT_{572Y}$-specific immune response after the 6th vaccination, either by using ELISpot assay or ICS.

	ELISPOT	
	+	-
+	**2**	**2**
FACS		
_	**3**	**4**

As it is observed, in 2 out of 11 (18%) patients a $TERT_{572Y}$-specific immune response after the 2nd vaccination was observed by using both ELISpot assay and ICS. Moreover, in 3 out of 11 (27%) patients, a $TERT_{572Y}$-specific immune response after the 2nd vaccination was identified only by using ELISpot assay, in 2 out of 11 patients (18%) only by using ICS and in 4 out of 11 patients (37%) a $TERT_{572Y}$-specific immune response was not observed neither by using ELISpot assay nor ICS (correlation coefficient r= 0.02, *Spearman test*, no significant correlation)

3.6.2.3 $TERT_{572}$ specific CD3+ cells after the 2nd vaccination

Table 3.15. Number of patients who developed a $TERT_{572}$-specific immune response after the 6th vaccination, either by using ELISpot assay or ICS.

	ELISPOT	
	+	-
+	**6**	**7**
FACS		
–	**8**	**9**

As it is observed, in 6 out of 30 (20%) patients a $TERT_{572}$-specific immune response after the 2nd vaccination was observed by using both ELISpot assay and ICS. Moreover, in 8 out of 30 (27%) patients, a $TERT_{572}$-specific immune response after the 2nd vaccination was identified only by using ELISpot assay, in 7 out of 30 patients (23%) only by using ICS and in 9 out of 30 patients (30%) a $TERT_{572}$-specific immune response was not observed neither by using ELISpot assay nor ICS (correlation coefficient r= 0.02, *Spearman test,* no significant correlation).

3.6.2.4 $TERT_{572}$ specific CD3+ cells after the 6th vaccination

Table 3.16. Number of patients who developed a $TERT_{572}$-specific immune response after the 6th vaccination, either by using ELISpot assay or ICS.

	ELISPOT	
	+	-
+	**1**	**1**
FACS		
_	**3**	**6**

As it is observed, in 1 out of 11 (9%) patients a $TERT_{572}$-specific immune response after the 2nd vaccination was observed by using both ELISpot assay and ICS. Moreover, in 3 out of 11 (27%) patients, a $TERT_{572}$-specific immune response after the 2nd vaccination was identified only by using ELISpot assay, in 1 out of 11 patients (9%) only by using ICS and in 6 out of 11 patients (55%) a $TERT_{572}$-specific immune response was not observed neither by using ELISpot assay nor ICS (correlation coefficient r= 0.01, *Spearman test,* no significant correlation*)*.

3.6.3 Characterization of vaccine-induced $CD3^+CD8^+$ T cells

3.6.3.1 IFN-γ

The IFN-γ intracellular staining was used to further characterize the IFN-γ-releasing $CD3^+$ T cells ofden einai 30 - 16 patients before the 1st vaccination and after the 2nd and 6th vaccination. As it was observed, $CD3^+CD8^+$ cytotoxic T cells were developed in response to vaccination. The data are shown in tables 3.17 and 3.18

Table 3.17. Number of $TERT_{572Y}$-specific IFN-γ –producing CTLs of patients before the 1st and after the 2nd and 6th vaccination, by using ICS (1=absence of immune response, n>1=number of stimulated $TERT_{572Y}$-specific CTLs , -= no vaccination)

	Prevaccination	3rd Vaccination	Postvaccination
AGRI	**50**	**1**	**20**
ALATH	**1**	**241**	**-**
DIMI	**1**	**100**	**1**
EMMA	**1000**	**260**	**1**
FOTO	**1**	**100**	**-**
GEOR	**40**	**1**	
GIEM	**1**	**100**	**40**
KAZI	**1**	**1**	**20**
KNIK	**1**	**40**	**1**
MIRI	**1**	**20**	**80**
NAPO	**1**	**40**	**-**
NIKA	**60**	**20**	**-**
PEMA	**40**	**1**	**1**
PSIL	**40**	**1**	**1**
PANAG	**20**	**1**	**1**
STEDI	**133**	**1**	**-**

As it is observed, 8 out of 16 patients (50%) had a $TERT_{572Y}$ - specific pre-existing immune reactivity (AGRI, EMMA, GEOR, NIKA, PEMA, PSIL, PANAG, STEDI). In addition, IFN-γ- producing CTLs were detected in 9 out of 16 patients (56%) in response to $TERT_{572Y}$ peptide after the 2nd vaccination (ALATH, DIMI, EMMA, FOTO, GIEM, KNIK, MIRI, NAPO, NIKA) and in 4 out of 10 patients (40%) after the 6th vaccination (AGRI, GIEM, KAZI, MIRI)

The above data gave rise to the figure 3.18

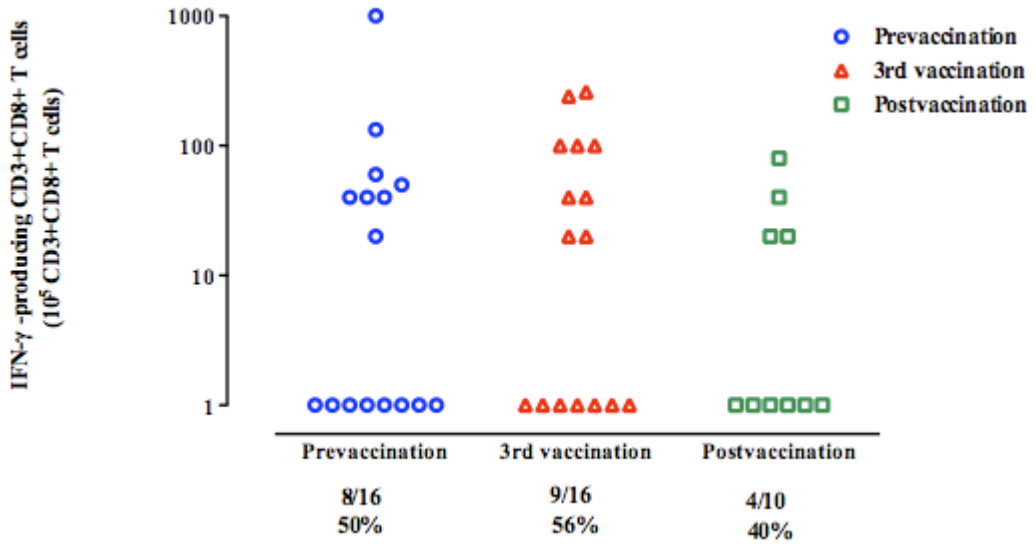


Figure 3.18. Frequencies of IFN-γ-specific CTLs to $TERT_{572Y}$ peptide in vaccinated patients prior vaccination and after the 2nd and 6th vaccination by using ICS

Table 3.18. Number of $TERT_{572}$-specific IFN-γ –producing CTLs before the 1st and after the 2nd and 6th vaccination, by using ICS (1=absence of immune response, n>1=number of stimulated $TERT_{572}$-specific CTLs, -= no vaccination)

	Prevaccination	3rd Vaccination	Postvaccination
AGRI	**230**	**100**	**1**
ALAF	**1**	**10**	**-**
ALATH	**1**	**240**	**-**
EMMA	**427**	**160**	**1**
FEM	**1**	**1**	**11**
FOTO	**1**	**100**	**-**
GEOR	**1**	**80**	**-**
GIEM	**1**	**80**	**1**
KAZI	**1**	**13**	**15**
KNIK	**1**	**40**	**1**
NIKA	**50**	**1**	**-**
PAPI	**1**	**20**	**1**
STEDI	**207**	**1**	**-**

As it is observed, 4 out of 13 patients (31%) had a $TERT_{572}$ - specific pre-existing immune reactivity (AGRI, EMMA, NIKA, STEDI). Furthermore, IFN-γ- producing CTLs were detected in 10 out of 13 patients (77%) in response to $TERT_{572}$ peptide after the 2nd vaccination (AGRI, ALAF,ALATH, EMMA, FOTO,GEOR, GIEM,KAZI, KNIK, PAPI) and in 2 out of 7 patients (29%) after the 6th vaccination (FEM, KAZI)

The frequency of patients who had a $TERT_{572}$ -specific immune response after the 6th vaccination was statistically significant (p= 0.04 *Student t-test*).

The above data gave rise to the figure 3.19.

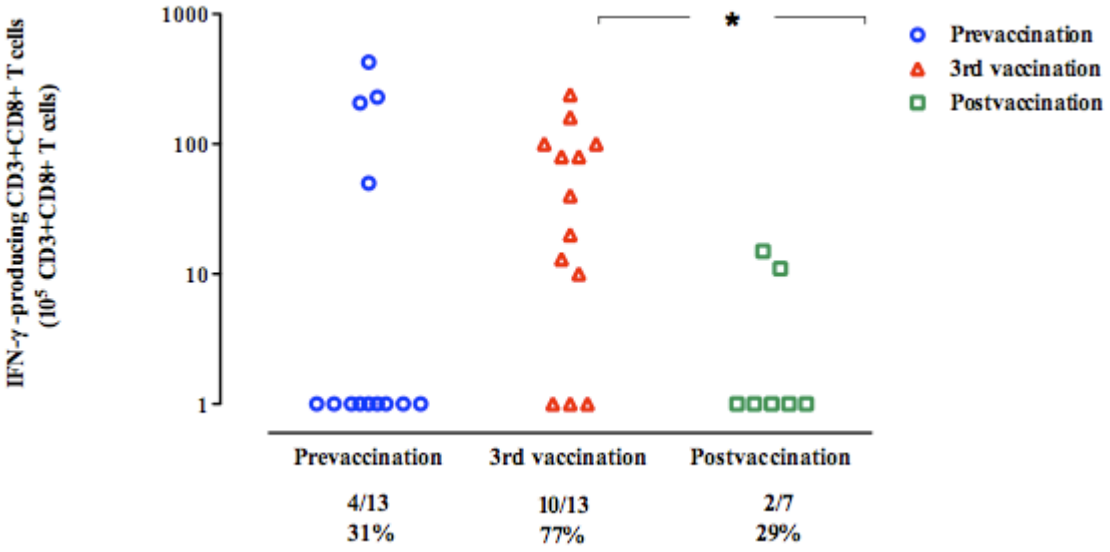


Figure 3.19. Frequencies of IFN-γ-specific CTLs to $TERT_{572}$ peptide in vaccinated patients prior to vaccination and after the 2nd and 6th vaccination using ICS (p=0.04, *Student's t-test*)

3.6.3.2 Interleukin – 10

The presence of IL-10-producing $CD3^+CD8^+$ T cells was also assessed in response to the peptides. IL-10 acts inversely to IFN-γ; it inhibits IFN- γ production and suppresses the antigen presentation.

Table 3.19 Number of $TERT_{572Y}$-specific IL-10-producing CTLs of patients before the 1st and after the 2nd and 6th vaccination, by using ICS (1=absence of immune response, n>1=number of stimulated $TERT_{572Y}$-specific CTLs, -= no vaccination)

	Pre-vaccination	2nd Vaccination	Post-vaccination
AGRI	1	1	1
ALAF	1	1	-
ALATH	-	1	1
ANTH	67	60	-
DASK	1	20	-
DIMI	-	1	-
EMMA	1	1	1
FEM	1	1	1
FOTO	-	1	-
GEDI	1	1	-
GEOR	1	20	-
GIEM	1	60	1
KARGE	1	1	1
KAZI	1	1	1
KNIK	1	40	1
LAST	1	1	1
LUKI	1	13	-
MAT	1	1	1
MIRI	1	1	1
NAPO	1	1	-
NIKA	1	40	-
PAPI	20	1	1
PEMA	40	1	1
PSIL	1	1	1
PANAG	50	40	1
SPAG	1	1	1
SPIR	-	1	-
STEDI	1	1	-
STEF	1	1	-
ZAHE	1	1	-

As it is observed, 4 out of 30 patients (13%) had a $TERT_{572Y}$ - specific pre-existing immune reactivity (ANTH, PAPI, PEPA and PANAG). Moreover IL-10-producing CTLs were detected in 8 out of 30 patients (27%) in response to $TERT_{572Y}$ peptide after the 2nd vaccination (ANTH, DASK, GEOR, GIEM, KNIK, LUKI, NIKA, PANAG) and in 0 out of 8 patients (0%) after the 6th vaccination.

The above data gave rise to the figure 3.20

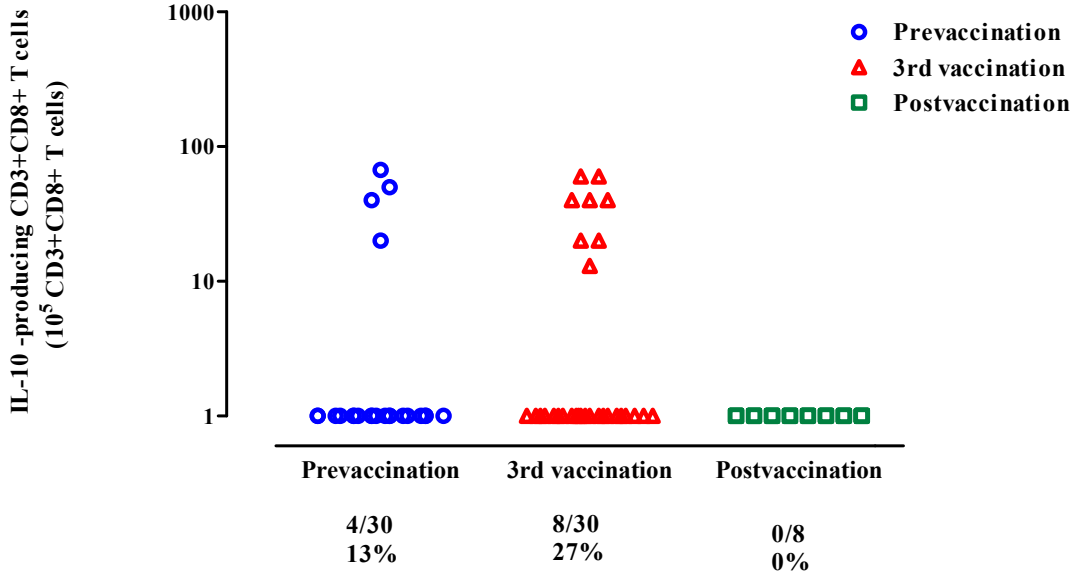


Figure 3.20: Frequencies of IL-10-specific CTLs to $TERT_{572Y}$ peptide in vaccinated patients prior to vaccination and after the 2nd and 6th vaccination using ICS

Table 3.20: Number of $TERT_{572}$-specific IL-10-producing CTLs of patients before the 1st and after the 2nd and 6th vaccination, by using ICS (1=absence of immune response, n>1=number of stimulated $TERT_{572}$ specific CTLs

	Prevaccination	2nd vaccination	Postvaccination
AGRI	1	1	1
ALAF	1	120	-
ALATH	-	1	40
ANTH	33	1	-
DASK	1	1	-
DIMI	-	1	-
EMMA	1	1	1
FEM	1	1	1
FOTO	-	1	-
GEDI	1	1	1
GEOR	1	1	-
GIEM	1	40	1
KARGE	1	1	1
KAZI	33	20	1
KNIK	60	1	1
LAST	20	20	1
LUKI	1	13	-
MAT	1	1	1
MIRI	1	1	1
NAPO	1	1	-
NIKA	1	40	-
PAPI	1	1	1
PEMA	1	1	1
PSIL	1	1	1
PANAG	1	1	1
SPAG	1	1	1
SPIR	-	30	-
STEDI	1	1	-
STEF	1	1	-
ZAHE	1	1	-

As it is observed, 4 out of 30 patients (13%) had a $TERT_{572}$ - specific pre-existing immune reactivity (ANTH, KAZI, KNIK, LAST). Moreover IL-10-producing CTLs were detected in 7 out of 30 patients (23%) in response to $TERT_{572}$ peptide after the 2nd vaccination (ALAF, GIEM, KAZI, LAST, LUKI, NIKA, SPIR) and in 1 out of 9 patients (12.5 %) after the 6th vaccination (ALATH).

The above data gave rise to the figure 3.21.

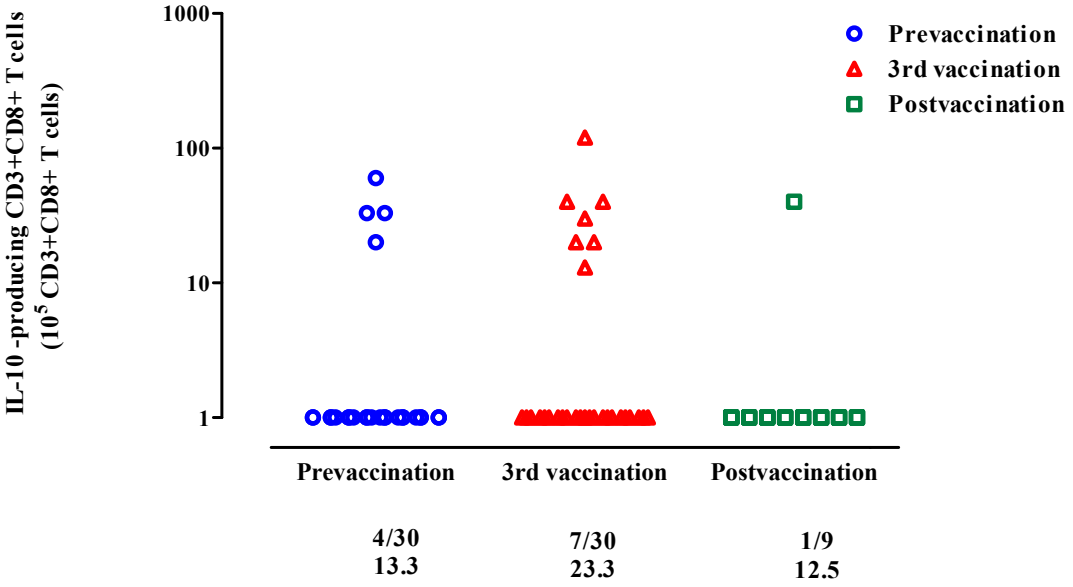


Figure 3.21: Frequencies of IL-10-specific CTLs to $TERT_{572}$ peptide in vaccinated patients prior to vaccination and after the 2nd and 6th vaccination using ICS

Furthermore, we compared the presence of the IFN-γ-releasing or IL-10-releasing $CD3^+CD8^+$ T cells in response to the peptides before the 1st and after the 2nd and 6th vaccination.

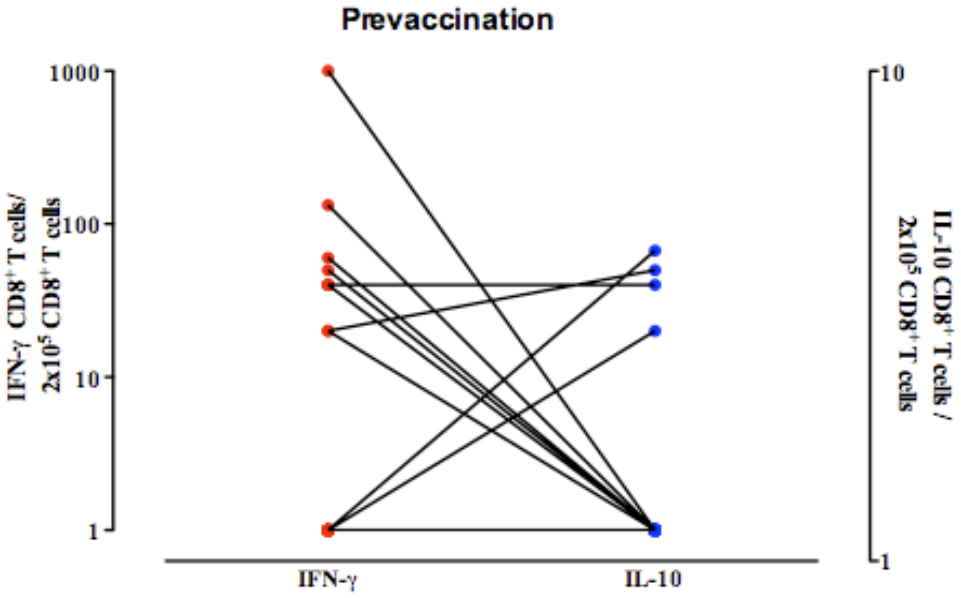


Figure 3.22. Comparison between IFN-γ and IL-10 produced $TERT_{572Y}$-specific $CD3^+CD8^+$ T cells before 1st vaccination (correlation coefficient r= -0.06, *Spearman test,* no significant correlation).

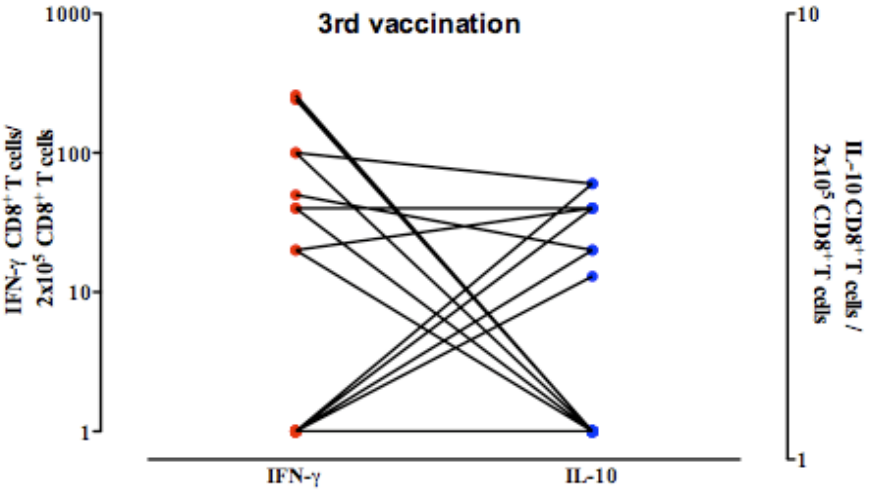


Figure 3.23. Comparison between IFN-γ and IL-10 produced $TERT_{572Y}$-specific $CD3^+CD8^+$ T cells after the 2nd vaccination (correlation coefficient r= 0.02, *Spearman test,* no significant correlation).

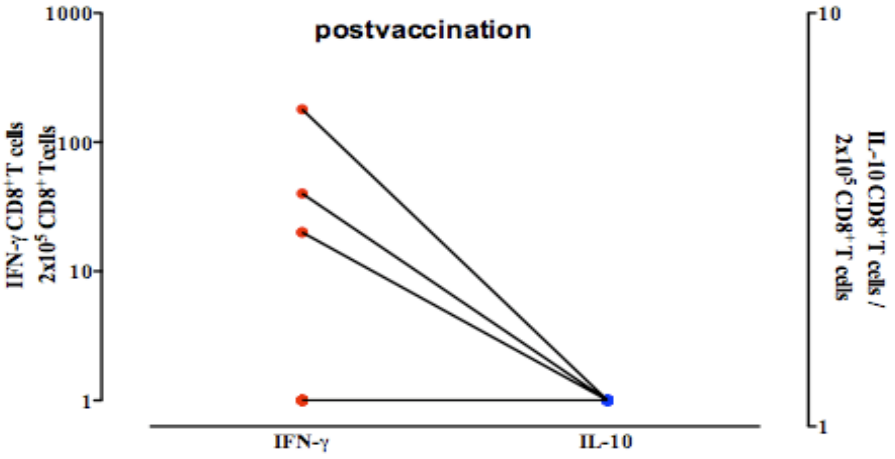


Figure 3.24. Comparison between IFN-γ and IL-10 produced $TERT_{572Y}$-specific $CD3^+CD8^+$ T cells after the 6th vaccination (correlation coefficient r = -0.06, *Spearman test,* no significant correlation).

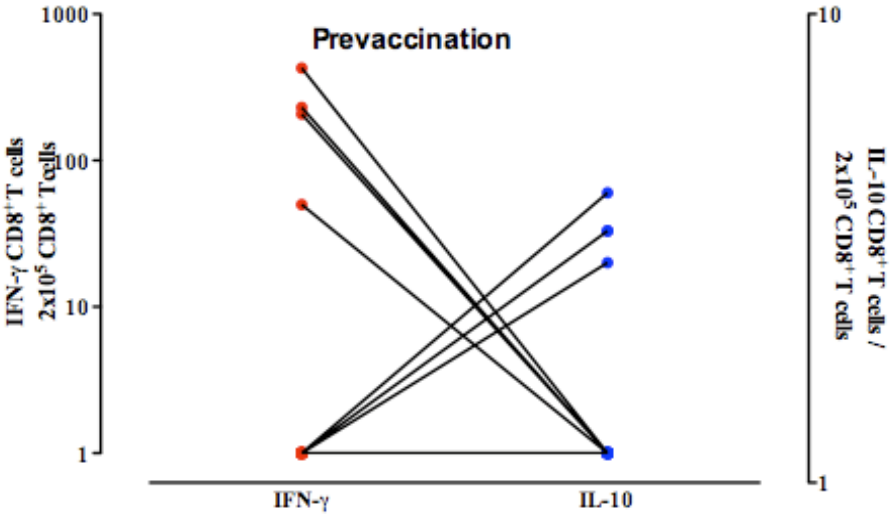


Figure 3.25: Comparison between IFN-γ and IL-10 produced $TERT_{572}$-specific $CD3^+CD8^+$ T cells before 1st vaccination (correlation coefficient r = -0.1, *Spearman test,* no significant correlation)

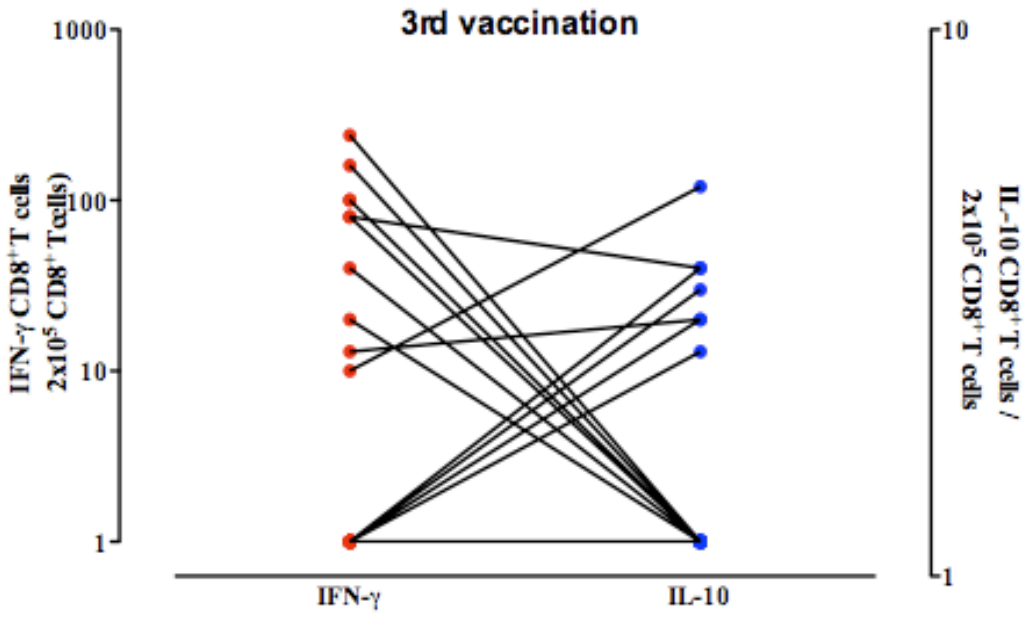


Figure 3.26. Comparison between IFN-γ and IL-10 produced $TERT_{572}$-specific $CD3^+CD8^+$ T cells after the 2nd vaccination (correlation coefficient r = -0.08, *Spearman test,* no significant correlation).

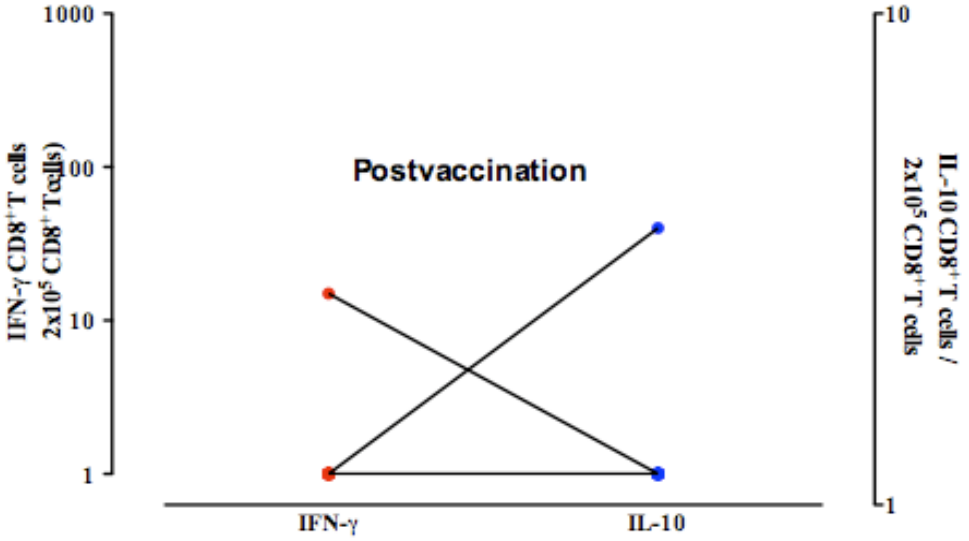


Figure 3.27. Comparison between IFN-γ and IL-10 produced $TERT_{572}$-specific $CD3^+CD8^+$ T cells after the 6th vaccination (correlation coefficient r = -0.06, *Spearman test,* no significant correlation).

Via the above graphs, it is observed that the existence of $TERT_{572Y}$-specific and $TERT_{572}$-specific $CD3^+CD8^+$ T cells which produce IL-10 was inversely proportional to the existence of $TERT_{572Y}$-specific and $TERT_{572}$-specific $CD3^+CD8^+$ T cells which produce IFN-γ. This observation was expected; as IL-10 is a cytokine which acts inversely to IFN-γ (it suppresses the immune response)

3.7 h-TERT –specific immune response beyond the completion of the sixth vaccination (Boost vaccinations)

After the completion of the 6th vaccination, 19 patients without progression disease (PD) received boost vaccinations (revaccinations) with 2mg of $TERT_{572}$ peptide every three months until progression disease. Their immune response was assessed by the production of IFN-γ $TERT_{572Y}$ and $TERT_{572}$ − specific cells by using ELISpot method.

Table 3.21. Number of $TERT_{572Y}$- specific cells in patients who received boost vaccinations, by using ELISpot method (- = no vaccination)

.

	Post vaccination	**1st revac**	**2nd revac**	**3rd revac**	**4th revac**	**5th revac**	**6th revac**	**7th revac**	**8th revac**
AIMA	33	40	26	-	-	-	-	-	-
ALATH	35	5	1	1	1	1	1	-	-
ANTZ	1	-	6	12	-	-	-	-	-
DEF	14	1	8	1	1	-	9	-	-
EKAF	176	92	-	-	-	-	-	-	-
EMMA	30	1	7	1	4	-	-	-	-
FEM	17	-	-	1	1	1	1	1	-
HAI	1	-	33	21	1	-	-	1	1
KAGI	1	1	1	-	-	-	-	-	-
KARGE	1	1	1	1	1	31	1	1	1
KAZI	1	1	1	1	1	1	18	-	-
KOSI	1	67	33	68	1	1	-	-	-
NIKA	1	-	14	1	1	12	1	1	10
NIKHT	1	-	-	-	-	-	-	1	-
PAKY	14	1	1	-	-	-	-	-	-

PAM	1	12	50	1	1	1	1	14	1
PEMA	1	1	8	4	12	14	1	1	1
STEDI	1	26	16	1	-	-	-	-	-
VAMP	1	10	1	1	-	-	-	-	-

	9th revac	**10th revac**	**11th revac**	**12th revac**	**13th revac**	**14th revac**	**15th revac**	**16th revac**
AIMA	-	-	-	-	-	-	-	-
ALATH	1	1	1	45	1	1	1	1
ANTZ	-	-	-	--	-	-	-	-
DEF	-	-	-	1	1	1	1	9
EKAF	-	-	-	-	-	-	-	-
EMMA	-	-	-	-	-	-	-	-
FEM	-	-	-	-	-	-	-	-
HAI	-	1	1	1	1	1	-	-
KAGI	-	-	-	-	-	-	-	-
KARGE	1	1	1	1	11	19	22	-
KAZI	-	-	-	-	-	-	-	-
KOSI	-	-	-	-	-	-	-	-
NIKA	24	1	6	1	13	25	1	-
NIKHT	1	-	-	-	-	-	-	-
PAKY	-	-	-	-	-	-	-	-
PAM	1	1	1	1	5	1	1	-
PEMA	1	1	1	-	28	-	-	-
STEDI	-	-	-	-	-	-	-	-
VAMP	-	-	-	-	-	-	-	-

It is observed that KAGI did not develop a $TERT_{572Y-}$ specific immune response despite having received boost vaccinations and FEM AND PAKY lost their $TERT_{572Y-}$ specific immune response beyond the completion of the 6th vaccination. On the other hand, KARGE did not develop a $TERT_{572Y-}$ specific immune response during first four revaccinations but only during the fifth boost vaccination. This immune response disappeared after the following revaccinations, reappeared in the13th, and remained until the 15th revaccination. KAZI developed a $TERT_{572Y-}$ specific immune response only during 6th vaccination. In the majority of the rest patients, a $TERT_{572Y-}$ specific immune response was induced during the 1st or 2nd revaccination, which was maintained, decreased, or lost upon the further boost vaccinations. Generally, it was observed that each patient who received boost

vaccination developed $TERT_{572Y}$- specific immune response at different time points. In particular, in about 13 out of 19 patients (68%) the $TERT_{572Y}$- specific immune response was maintained.

The above data gave rise to the figure 3.28:

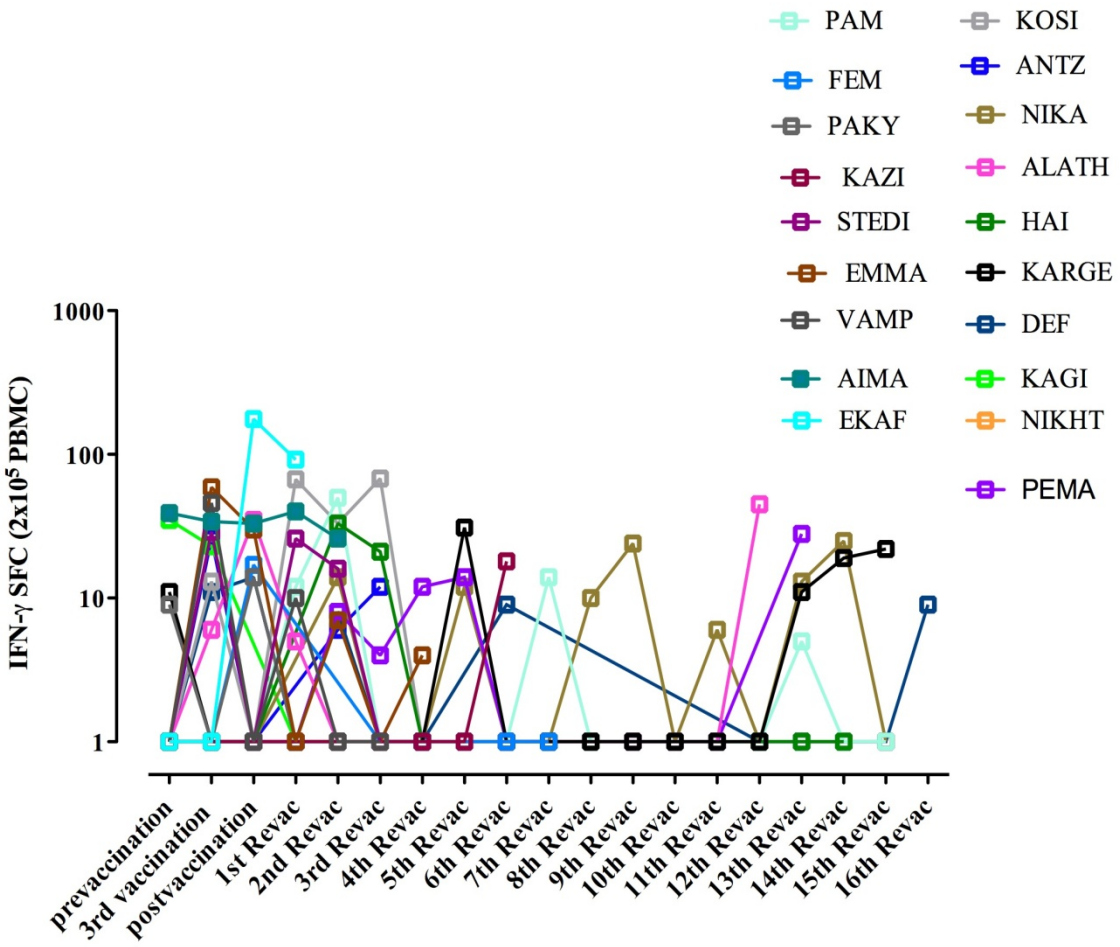


Figure 3.28. Assessment of the $TERT_{572Y}$ specific immune response in patients who received boost vaccinations,by using ELISpot method.

Furthemore, the number of IFN- γ - CTLs cells in response to $TERT_{572}$ peptide ,in patients who received boost vaccinations, was assessed by ELISpot method.

Table 3.22. Number of $TERT_{572}$- specific cells in patients who received boost vaccinations, by using ELISpot method (- = no vaccination)

	Post vaccination	**1st revac**	**2nd revac**	**3rd revac**	**4th revac**	**5th revac**	**6th revac**	**7th revac**	**8th revac**
AIMA	1	1	1	-	-	-	-	-	-
ALATH	9	1	6	1	1	1	1	-	-
ANTZ	38	-	12	1	-	-	-	-	-
DEF	1	1	9	12	1	-	1	-	-
EKAF	125	61	-	-	-	-	-	-	-
EMMA	1	1	1	1	8	-	-	-	-
FEM	17	-	-	1	1	1	1	1	-
HAI	31	-	1	26	1	-	-	1	1
KAGI	1	1	1	-	-	-	-	--	
KARGE	1	1	1	23	23	1	1	19	10
KAZI	1	1	1	5	1	8	1	-	-
KOSI	38	14	1	109	1	1	-	-	-
NIKA	10	-	9	1	45	1	1	1	1
NIKHT	36	210	-	1	-	210	-	1	-
PAKY	12	16	19	-	-	-	-	-	-
PAM	9	1	63	9	1	1	23	1	1
PEMA	1	1	1	21	9	1	1	1	1
STEDI	1	1	1	1	-	-	-	-	-
VAMP	1	1	1	1	-	-	-	-	-

	9th revac	10th revac	11th revac	12th revac	13th revac	14th revac	15th revac	16th revac
AIMA	-	-	-	-	-	-	-	-
ALATH	1	1	1	1	-	-	-	-
ANTZ	-	-	-	-	-	-	-	-
DEF	-	-	-	-	-	-	-	-
EKAF	-	-	-	-	-	-	-	-
EMMA	-	-	-	-	-	-	-	-
FEM	-	-	-	-	-	-	-	-
HAI	-	1	1	1	1	1	-	-
KAGI	-	-	-	-	-	-	-	-
KARGE	1	1	10	8	1	47	39	-
KAZI	-	-	-	-	-	-	-	-
KOSI	-	-	-	-	-	-	-	-
NIKA	10	1	1	1	1	14	1	-
NIKHT	-	-	-	-	-	-	-	-
PAKY	-	-	-	-	-	-	-	-
PAM	1	1	1	1	1	1	1	-
PEMA	1	1	1	-	22	-	-	-
STEDI	-	-	-	-	-	-	-	-
VAMP	-	-	-	-	-	-	-	-

It is observed that some of patients, maintained their $TERT_{572}$-specific immune response during 1st vaccination, 2nd or 3rd vaccination (ANTZ, DEF, EKAF, KARGE, KOSI, NIKHT, PAKY, PAM, PEMA) whereas others lost it (AIMA, FEM, KAGI, STEDI, VAMP. In general, 12 out of 19 patients (63%) maintained their $TERT_{572}$-specific immune response.

The above data gave rise to the figure 3.29:

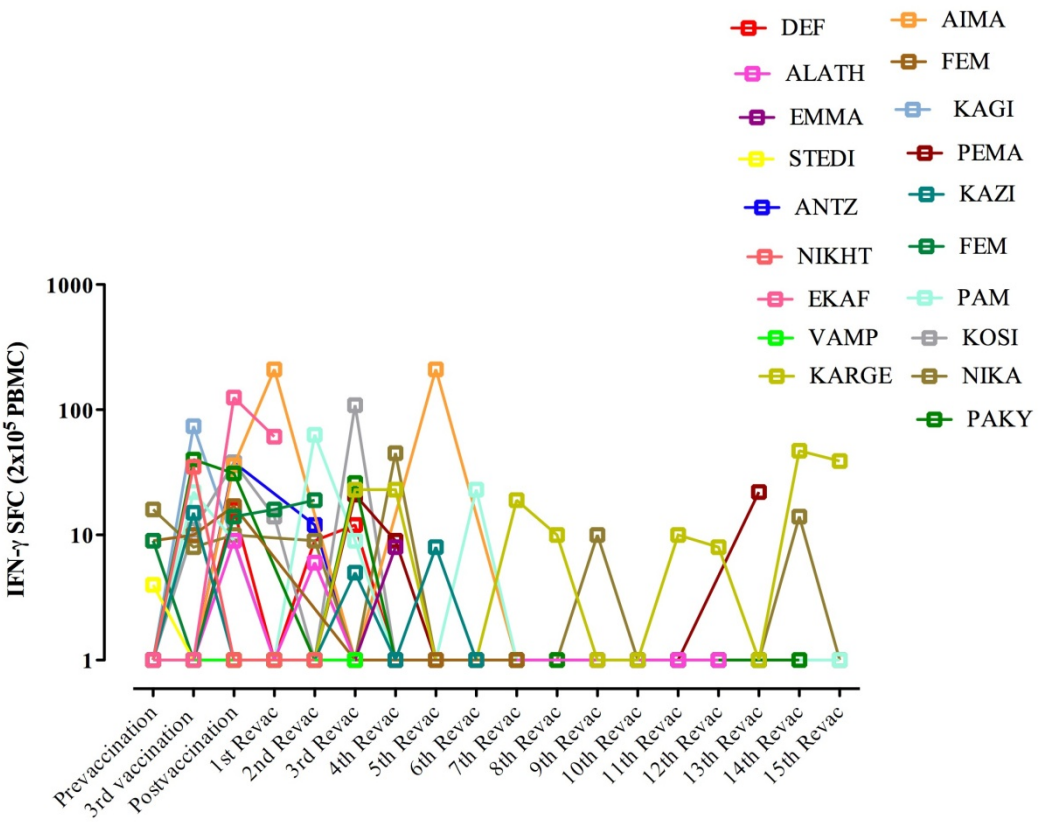


Figure 3.29. Assessment of the $TERT_{572}$ - specific immune response in patients who received boost vaccinations,by using ELISpot method.

Furthermore, ICS was used in patients who received boost vaccinations, in order to confirm the data coming from ELISpot assay.

Table 3.23. Number of $TERT_{572Y}$- specific $CD3^+CD8^+$ T cells in patients who received boost vaccinations, as assessed by ICS

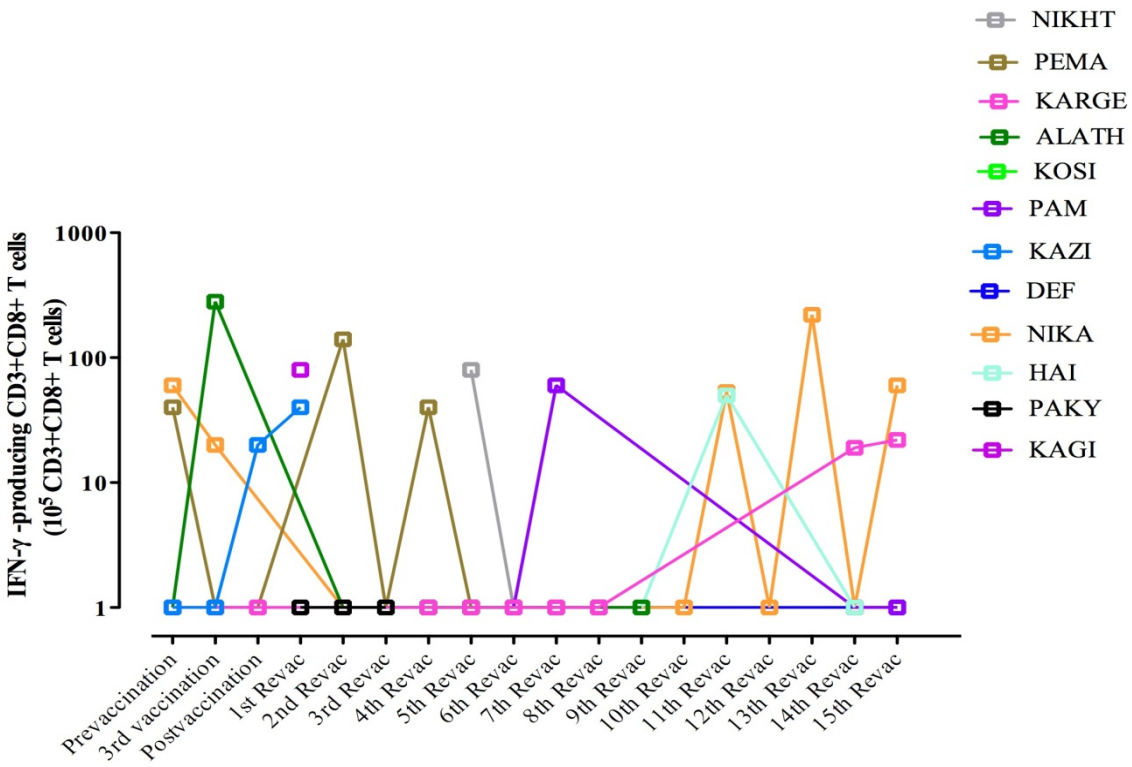
	Post vaccination	1st revac	2nd revac	3rd revac	4th revac	5th revac	6th revac	7th revac	8th revac
DEF	-	-	-	-	1	-	-	-	1
KAGI	-	80	-	-	-	-	-	-	-
KOSI	-	-	-	-	1	1	1	-	-
NIKHT	-	-	-	-	-	80	1	-	-
NIKA	-	-	1	-	-	-	-	1	1
PEMA	1	-	140	1	40	1	1	1	-
PAM	-	-	-	-	-	-	1	60	
HAI	-	-	-	-	-	-	-		
ALATH	-	-	2	-	1	1	-	-	1
KARGE	1	1	1	1	1	1	1	1	1
PAKY	-	1	1	1	-	-	-	-	-
KAZI	20	40	-	-	-	-	-	-	-

	9th revac	10th revac	11th revac	12th revac	13th revac	14th revac	15th revac
DEF	-	-	-	-	-	1	1
KAGI	-	-	-	-	-	-	-
KOSI	-	-	-	-	-	-	-
NIKHT	-	-	-	-	-	-	-
NIKA	-	1	53	1	220	1	60
PEMA	-	-	-	-	-	-	-
PAM	-	-	-	-	-	1	1
HAI	1	-	50	-	-	1	-
ALATH	1	-	-	-	-	-	-
KARGE	-	-	-	-	-	19	22
PAKY	-	-	-	-	-	-	-
KAZI	-	-	-	-	-	-	-

Moreover, the ICS revealed that KAZI maintained the $TERT_{572Y}$-specific immune response during the 1st revaccination. KAGI developed a $TERT_{572Y}$-specific immune response during the 1st revaccination whereas NIKHT during the 5th revaccination. There was a fluctuation concerning PEMA and NIKA. Furthermore, KARGE developed a $TERT_{572Y}$-specific immune response during 14th and 15th boost

vaccinations. In general terms, 6 out of 12 patients (50%) who received boost vaccination, maintained their $TERT_{572Y}$-specific immune response.

The above data gave rise to the figure 3.30



.Figure 3.30. Assessment of the $TERT_{572Y}$ - specific immune response in patients who received boost vaccinations, by using ICS.

Moreover, the number of $CD3^{+}CD8^{+}$ T cells, in patients who received boost vaccinations, in response to $TERT_{572}$ peptide was assessed by ICS

Table 3.24: Number of $TERT_{572}$- specific **$CD3^+CD8^+$ T** cells in patients who received boost vaccinations, **as assessed by** ICS

	Post vaccination	**1st revac**	**2nd revac**	**3rd revac**	**4th revac**	**5th revac**	**6th revac**	**7th revac**	**8th revac**
DEF	-	-	-	-	1	-	-	-	1
KAGI	-	1	-	-	-	-	-	-	-
KOSI	-	-	-	-	1	1	160	-	-
NIKHT	-	-	-	-	-	1	20	-	-
NIKA	-	-	1	-	-	-	-	1	1
PEMA	1	-	1	160	60	1	280	1	-
PAM	-	-	-	-	-	-	1	1	
HAI	-	-	-	-	-	-	-		
ALATH	-	-	60	-	80	1	-	-	1
KARGE	1	20	1	1	1	20	1	1	1
PAKY	-	1	1	1	-	-	-	-	-
KAZI	15	1	-	-	-	-	-	-	-

	9th revac	**10th revac**	**11th revac**	**12th revac**	**13th revac**	**14th revac**	**15th revac**
DEF	-	-	-	-	-	-	-
KAGI	-	-	-	-	-	-	-
KOSI	-	-	-	-	-	-	-
NIKHT	-	-	-	-	-	-	-
NIKA	-	1	67	1	1	30	160
PEMA	-	-	-	-	22	-	-
PAM	-	-	-	-	1	1	1
HAI	1	-	1	-	1	1	-
ALATH	1	-	-	-	-	-	-
KARGE	-	-	-	-	1	47	39
PAKY	-	-	-	-	-	-	
KAZI	-	-	-	-	-	-	-

As is shown in the table KAZI developed an immune response after the 6th vaccination which was not further detected. Moreover, KOSI and NIKHT developed a $TERT_{572}$-specific immune response after the 6th vaccination which was then lost. ALATH developed an immune response after the 2nd re-vaccinaton and maintained it until the 4th vaccination. NIKA developed an immune response after the 11th revaccination, which disappeared and reappeared during 15th and 16th vaccination. Finally, there was a fluctuation as far as KARGE is concerned; the immune response was developed after the 1st revaccination, then it was lost and reappeared during the 5th vaccination , then it disappeared again and finally it reappeared in increased levels during the 14th and 15th vaccination. In general terms, 6 out of 12 patients (50%) who received boost vaccination, maintained their $TERT_{572}$-specific immune response.

The above data gave rise to the figure 3.31

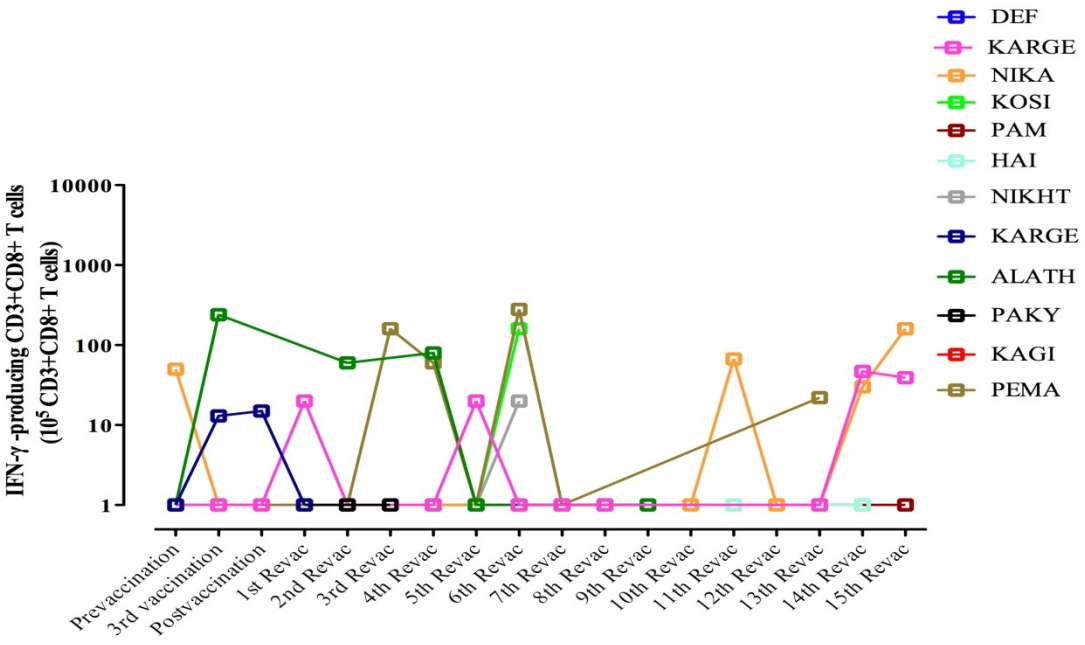


Figure 3.31. Assessment of the $TERT_{572}$ - specific immune response in patients who received boost vaccinations, by using ICS.

In general, boost vaccinations with the native peptide resulted in the maintenance of the specific immune response which had been induced by the optimized peptide. In particular, 68% patients maintained their $TERT_{572Y}$-specific immune response as assessed by ELISpot assay and 50% as assessed by ICS. Similarly, 63% patients maintained their $TERT_{572}$-specific immune response as assessed by ELISpot assay and 50 % as assessed by ICS. Thus, it was observed, that the percentage of patients who maintained their $TERT_{572Y}$ or $TERT_{572}$-specific immune response upon the completion of the 6th vaccination, as assessed by ELISpot assay was greater than the respective result assessed by ICS. This was not expected due to the fact that ICS was used in order to confirm the data coming from ELISpot assay.

3.8 Overall Survival of vaccinated patients

Finally, the association between the development of TERT-specific immune response and the clinical outcome of the patients who enrolled in the vaccination protocol with clinically documentated stable (SD) or progressive disease (PD) was analysed. In particular, overall survival was compared by the log-rank test, firstly between SD patients who were early responders (who had developed a $TERT_{572}$-specific immune response after the 2nd vaccination) and non early responders and secondly between SD patients who were late responders (who had developed a $TERT_{572}$-specific immune response after the 6th vaccination) and non-late responders. Respectively, overall survival was also compared in PD patients in the same way (early responders vs non-early responders, late responders vs non late responders). The probability of survival was estimated using the Kaplan-Meier method.

Firstly, there was a comparison between the overall survival of SD patients who were early responders (who developed $TERT_{572}$- specific immune response after the 2nd vaccination) and non early responders (figure 3.32)

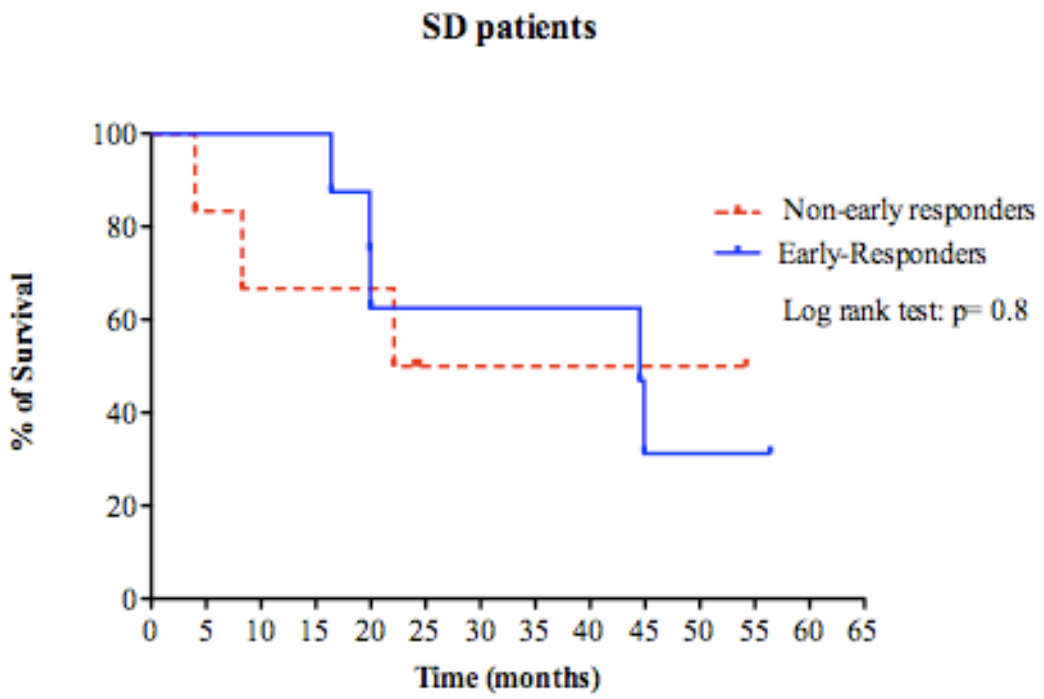


Figure 3.32. Overall survival of early responders vs non-early responders in SD patients.

The overall survival was greater in SD patients who had developed a $TERT_{572}$ – specific immune response after the 2nd vaccination (early responders) comparing to non-early responders but the result was not statistical significant (p=0.8) according to *Student's t-test*

Secondly, there was a comparison between the overall survival of SD patients who were late responders (who developed $TERT_{572}$ –specific immune response after the 6th vaccination) and non late responders (figure 3.33)

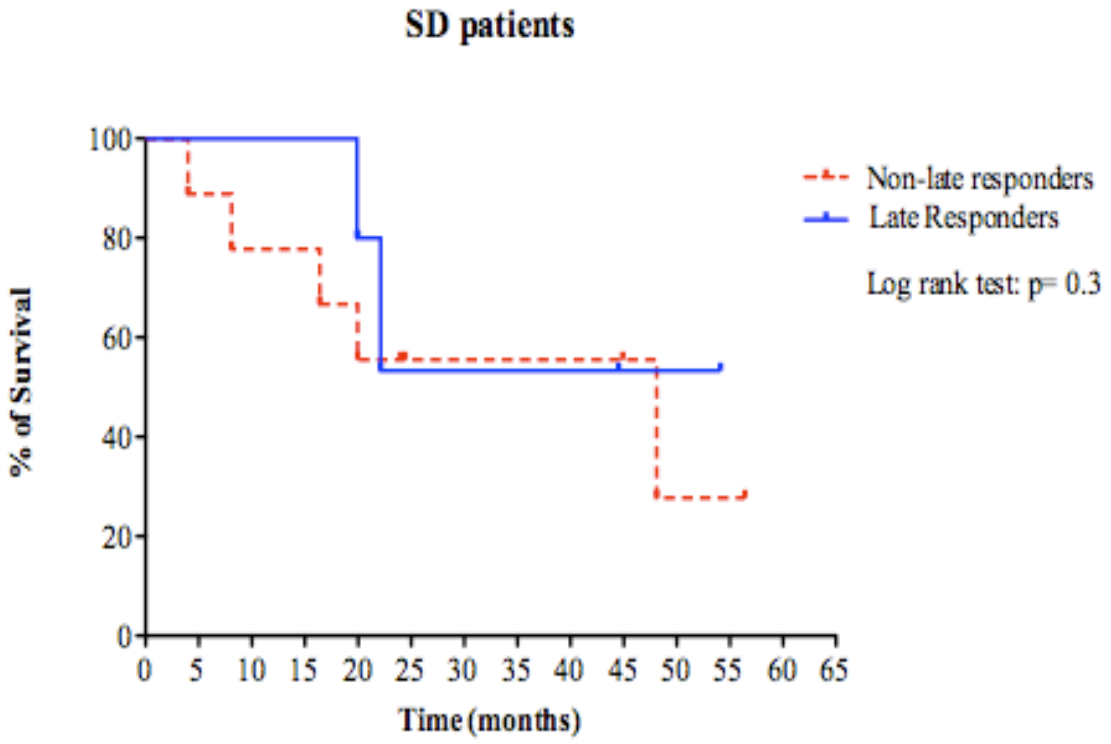


Figure 3.33: Overall survival of late responders vs non-late responders in SD patients.

The overall survival was greater in SD patients who had developed a $TERT_{572}$ – specific immune response after the 6th vaccination (late responders) comparing to non-late responders but the result was not statistical significant (p=0.3) according to *Student's t-test*

Furthermore, there was a comparison between the overall survival of PD patients who were early responders (who developed $TERT_{572}$- specific immune response after the 2nd vaccination) and non early responders (figure 3.34)

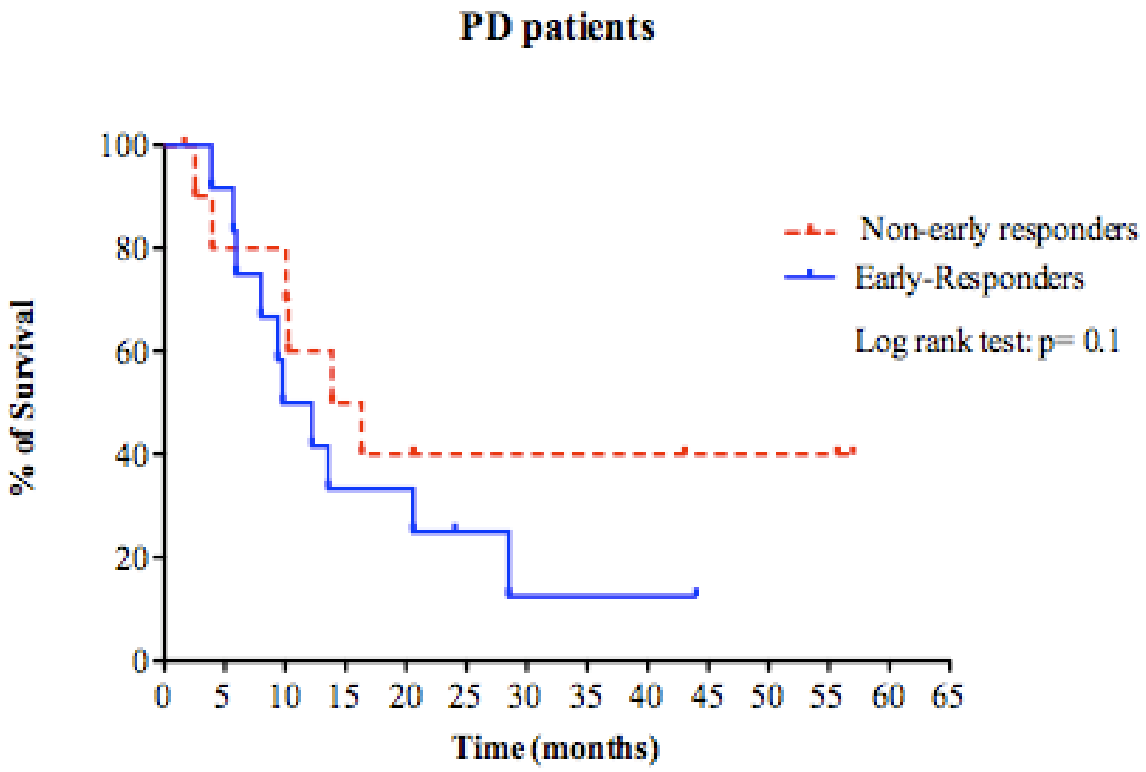


Figure 3.34. Overall survival of early responders vs non-early responders in PD patients.

The overall survival was greater in PD patients who had not developed a $TERT_{572}$ – specific immune response after the 2nd vaccination (non- early responders) comparing to early responders but the result was not statistical significant (p=0.1) according to *Student's t-test*

Finally, there was a comparison between the overall survival of PD patients who were late responders (who developed $TERT_{572}$- specific immune response after the 2nd vaccination) and non late responders (figure 3.35)

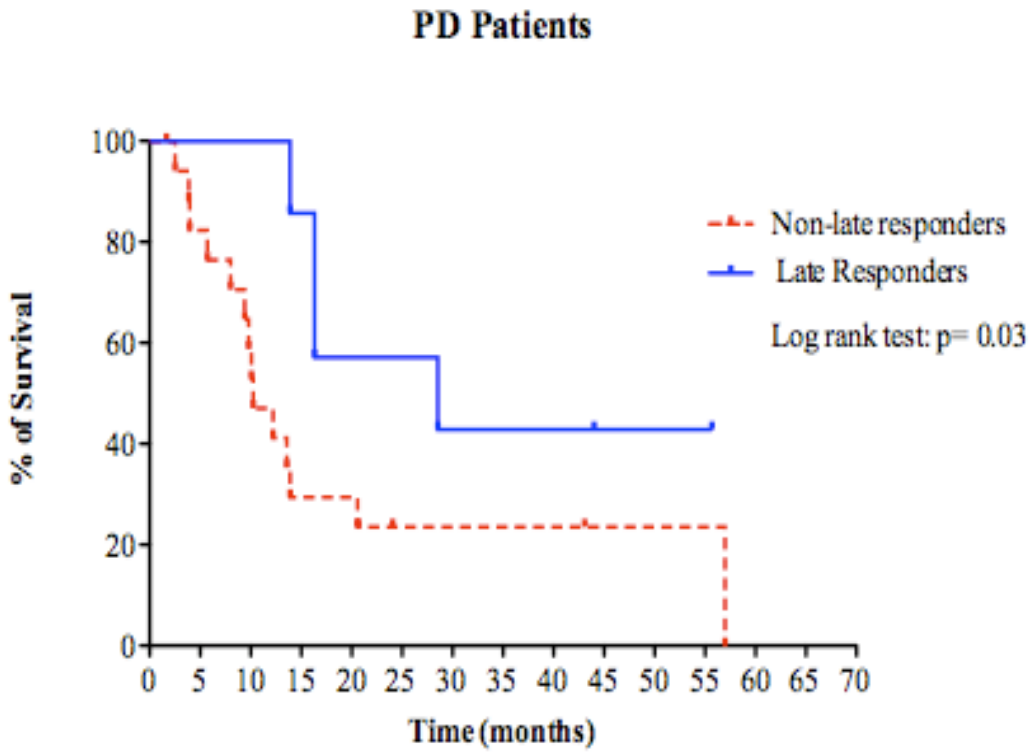


Figure 3.35: Overall survival of late responders vs non-late responders in PD patients.

As it was observed, the overall survival was greater in PD patients who had developed a $TERT_{572}$ – specific immune response after the 6th vaccination (late responders) comparing to non-late responders and the result was statistical significant (p=0.03) according to *Student's t-test*

4. DISCUSSION

In a previous study, it has been shown that $CD8^+$ T cell immune responses could be detected in 22 HLA-A*0201 patients with advanced NSCLC vaccinated with the Vx-001 vaccine (Bolonaki *et al*., 2007**).** In the current study a larger cohort of HLA A*0201 patients with various solid tumour types was assessed and the immune responses induced by the Vx-001 vaccine were analyzed and further characterized. The findings of the present study, confirm previous observations since the administration of Vx-001 vaccine induced specific $CD8^+$ T cells against TERT peptides which exhibited effector functions *in vitro*.

Researchers' perspectives as far as cancer vaccines are concerned, are to enhance the existed interaction between the host and the tumour and restrict or even suppress tumour growth. In general, a successful vaccination protocol includes two major aspects: a) an antigen and b) an adjuvant. The antigen represents the target which should be eliminated and the adjuvant is an enhancer of the immune response caused by the antigen itself **(**Mads Hald Andersen *et al.,* 2008).

Before referring to the results of the current study, it is essential to mention some major things about interferons and interleukins. Interferons (IFNs) are proteins made and released by lymphocytes in response to the presence of pathogens –such as viruses, bacteria, parasites, or tumour cells. They allow the communication between cells to trigger the protective defences of the immune system that eradicate pathogens or tumours. IFNs belong to the large class of glycoproteins known as cytokines and they are named after their ability to "interfere" with viral replication within host cell (Varesio *et al.,* 1984). Their functions are: a) to activate immune cells, such as NKs and macrophages, b) to increase recognition of infection or tumour cells by up-regulating antigen presentation to T lymphocytes and c) they increase the ability of uninfected host cells to resist new infection by virus.

About ten distinct IFNs have been identified in mammals and seven of these have been described for humans. There are two types of IFNs: 1) type I (IFN-α, IFN-β), 2) type II (IFN-γ). IFN-α is expressed by white blood cells that have been activated by viruses or microbes. IFN-β is a glycoprotein which is derived from fibroblasts that are cultivated upon exposure to microorganisms. IFN-γ is expressed by activated T-lymphocytes and stimulates macrophages. It used for the treatment of various immunodeficiencies and infections (Varesio *et al*., 1984).

Interleukins are a group of cytokines that were first seen to be expressed by white blood cells (leukocytes). The term interleukin derives from (inter-) as a means of communication and (-leukin)

"deriving from the fact that many of these proteins are produced by leukocytes and act on leukocytes". It has since been found that interleukins are produced by a wide variety of body cells. The function of the immune system depends on a large part of interleukins. The majority of them are synthesized by helper $CD4^+$ T lymphocytes, as well as through monocytes, macrophages, and endothelial cells.

The first interleukins which were identified were IL-1 (macrophages, B cells, monocytes, dendritic cells) and IL-2 (Th1 cells). Totally about thirty five interleukins have been identified (Mizel *et al.,* 1989).The table 4.1, shows the cytokines used in the current study, their source and their function.

Table 4.1: Cytokines (Parkin and Cohen, 2001**)**

Cytokine	Source	Function
Interleukin 10	$CD4^+$ cells and activated monocytes	Inhibition of IFN- γ and IL-1 production and suppression of the antigen presentation
Interferon- γ	T and NK cells	Regulation and activation of the immune system

Since many tumour antigens are normal non-mutated self proteins, dominant peptides derived from these proteins are often affected by T-cell tolerance; thus, cryptic peptides derived from these proteins could be better candidates for including an anti-tumour immune response since, due to their low avidity and recognition efficiency, are not affected by tolerance and/or ignorance. In previous studies, it was shown that the substitution of an arginine by a tyrosine at the position 1 of the native $TERT_{572}$ peptide enhances the immunogenicity of the cryptic peptide (Tourdot *et al*., 2000). Moreover, such modified peptides have also been used by other groups in order to induce a better T cell activation than that induced by the native peptides (Meijer *et al*., 2007; Rosenberg *et al.,* 1998; Parkurst *et al.,* 1996; Valmori *et al.,* 1998).

In a study conducted in murine models, it was observed that the vaccination with two modified cryptic peptides derived from murine TERT peptide ($TERT_{572Y}$ and $TERT_{988Y}$) stimulated specific CTLs which induced a successful anti-cancer immunity (Gross *et al.,* 2004). On the contrary, the vaccination with the dominant peptides did not induce any immune response (Scardino *et al.,* 2002). In the current study, patients with various solid tumours were vaccinated with two doses of the modified peptide ($TERT_{572Y}$) followed by four doses with the native peptide ($TERT_{572}$).

This vaccination schedule was based on *in vivo* preclinical studies which have shown that vaccination of HLA-A*0201 transgenic HHD mice with the optimized $TERT_{572Y}$ followed by the native $TERT_{572}$ peptide induced CTLs with higher avidity and stronger antitumour efficacy than serial vaccinations with the optimized $TERT_{572Y}$ peptide alone (Gross *et al.,* 2004). This could be explained by the hypothesis that the optimized $TERT_{572Y}$ peptide first generates peptide-specific T cells and then the stimulation with the native $TERT_{572}$ peptide selects among T cells those with the highest specificity for the native $TERT_{572}$ peptide which is presented by tumour cells.

Indeed, the present data demonstrate that $TERT_{572Y}$-specific immune responses could be induced in 27% and 32% of the vaccinated patients after the 2^{nd} and 6^{th} vaccination, respectively. Furthermore, $TERT_{572}$-specific immune responses could be induced in 41% and 51% of the vaccinated patients after the 2^{nd} and 6^{th} vaccinations respectively, as assessed by IFN-γ ELISpot. Intracellular staining was used in order to confirm the above data. Moreover, it was observed that 45% of patients with a $TERT_{572Y}$-specific response after the 2^{nd} vaccination developed a $TERT_{572}^{+}$ immune response after the 6^{th} vaccination (fig 3.10). Furthermore, the existence of $TERT_{572Y}$-specific and $TERT_{572}$-specific $CD3^{+}CD8^{+}$ T cells which produce IL-10, was inversely proportional to the existence of $TERT_{572Y}$-specific and $TERT_{572}$-specific $CD3^{+}CD8^{+}$ T cells which produce IFN-γ. This observation was expected, as IL-10 is a cytokine which acts inversely to IFN-γ. These findings clearly indicate that our vaccination strategy circumvented the immune tolerance of TERT (Robbind *et al.,* 1996; Van *et al.,* 1995; Tourdot *et al.,* 2000).

Moreover, boost vaccinations with the native peptide resulted in the maintenance of the specific immune response which had been induced by the optimized peptide. In particular, 68% patients maintained their $TERT_{572Y}$-specific immune response as assessed by ELISpot assay and 50% as assessed by ICS. Similarly, 63% patients maintained their $TERT_{572}$-specific immune response as assessed by ELISpot assay and 50 % as assessed by ICS. Thus, it was observed, that the percentage of patients who maintained their $TERT_{572Y}$ or $TERT_{572}$-specific immune response upon the completion of the 6^{th} vaccination, as assessed by ELISpot assay was greater than the respective result assessed by ICS. This was not expected due to the fact that ICS was used in order to confirm the data coming from ELISpot assay.

Several possible explanations could be given, among others: 1) By using ELISpot assay, immune response is assessed according to the number of stimulated IFN- γ-PBMCs without any further characterization of these cells. For instance, these cells could be NK cells, DC cells or monocytes. On the other hand, by using ICS, the estimation of the number of specific-reactive cells is restricted only to $CD3^{+}CD8^{+}$ T-lymphocytes (CTLs) 2) the patients' samples which were used for ICS were less than the samples used for ELISpot assay, due to the lack of biological material 3) the two methods have different sensitivity. In particular, ELISpot assay is considered to be a much more sensitive method than ICS. Due to the above reasons, there was not a perfect correlation between ELISpot and ICS results, as it was initially expected.

An interesting observation in the present study was that 19% and 25% of the patients had a $TERT_{572Y}$ and $TERT_{572}$-specific pre-vaccination immune reactivity, respectively. Moreover, the $TERT_{572Y}$ or $TERT_{572}$-specific pre-existing immune reactivity could not be further amplified after the 2nd vaccination in most of these patients; on the contrary, patients who had no $TERT_{572Y}$ or $TERT_{572}$ - specific pre-existing immune reactivity at the baseline mounted more efficiently an early immune response (after the 2^{nd} vaccination) to the vaccination. T his observation seems to indicate that patients with pre-existing T cell reactivity against $TERT_{572}$ and $TERT_{572Y}$ peptides are less likely to develop an early vaccine specific immune response as has already been shown (Bercovici *et al.,* 2008).

In the current study, it was observed that pre-existing $TERT_{572}$-reactive T cells in the blood of vaccinated patients disappeared after the 2^{nd} administration of Vx-001 which suggests that either cytotoxic cells migrated to the tumour sites or were subjected to apoptosis. Moreover, a significant immune response could be observed after the 2nd vaccination in patients who did not have a

$TERT_{572Y}$ or $TERT_{572}$ –specific pre-existing immune reactivity. It has been previously reported that tumour-reactive T cells could be easily detected in the skin and lymph node biopsies but not in the blood of patients after vaccination (Slingluff *et al*., 2004).

Recent studies have proposed that one mechanism of immune escape used by the tumours is the production of immunosuppressive type II cytokines at the tumour sites (Yang *et al.,* 2003). IL-10, has been shown to be present in a variety of human cancers and its ability to suppress an effective type I response is well documented (Kruger-Krasagakes *et al.,* 1994, Huang *et al.,* 1995**).** A $TERT_{572Y}$ – specific immunity (27%) and $TERT_{572}$ – specific immunity (23%) were mounted after the 2nd vaccination in patients who did not have IL-10-producing CD8+ T cells, prior to vaccination.

Therefore, it could be supposed that the presence of IL-10 might be involved in the regulation of the development of the $TERT_{572}$-specific IFN-γ producing T cells by downregulating the development of the IFN-γ- producing T cells. However, other mechanisms may also be involved, such as enhanced expression of surface CTLA-4 (cytolytic T lymphocyte-associated antigen 4) molecule which has higher affinity and effectively competes with CD28 for B7.1 and B7.2 binding, inducing inhibitory signals in effector T cells (Gabriel *et al*., 2007; Hodi *et al*., 2003; Korman *et al.,* 2005; Phan *et al.,* 2003; Sun *et al.,* 2008) or an increased expansion of T regulatory cells (Tregs) which can suppress effector T cells (Ling *et al.,*2007; O' Mahony *et al.,* 2007; Sakaguchi *et al.,* 2005; Yamaguchi *et al.,* 2006). Therefore, considering all the above possible mechanisms, those responsible for our findings need to be further investigated. This could not be achieved in the current study, due to the complete utilization of the available biological material.

In the field of cancer vaccination, scientists have tried to estimate the tolerance and immunogenicity of each vaccine, as well as determine the correlation between immune response and patients' clinical outcome. Phase II clinical studies have been conducted, using the anti-cancer vaccine G-VAX (granulocyte-macrophage colony-stimulating factor gene modified), the MVA-MUC1-IL2 (Modified Vaccinia Ankara), a recombinant virus which is associated with bovine disease and the vaccine MAGE-3 **(**Nemunaitis *et al.,* 2004; Nemunaitis *et al.,* 2006; Liu *et al.,* 2004; Atackanovic *et al.,* 2004). Moreover, specific antigens such as MART-1/Melan-A and gp100 have been used in order to stimulate specific CLTs (Scheibenbogen *et al*., 1997).

As far as NSCLC is concerned, the use of the vaccine BLP-25 has been proved to be very effective. According to a study conducted in patients who were vaccinated with BLP-25, the overall survival was increased (13 months rather than 5 months as was expected), while only 16 out of 78 (21%) patients developed an immune response (Butts *et al*., 2005).Furthermore, it has been reported that the use of the vaccine BEC2, which is expressed in the membrane of the cells in the majority of NSCLC patients, failed to improve the overall survival of the patients in phase III of the clinical study (Giaccone *et al.,* 2004). According to previous studies (Mavroudis *et al*., 2006)**,** the immune response was induced in 76% and 91% of NSCLC patients after the 2^{nd} and after the 6^{th} vaccination, respectively. Comparing to other vaccines used against NSCLC, Vx-001 has induced a higher immune response in vaccinated patients. Indeed, in vaccinated patients with BLP-25 and GV1001, an immune response was observed only in 20.5% and 54.2% of them, respectively (Butts *et al.*, 2005; Brunsvig *et al.,* 2006).

Due to ethical reasons, the patients enrolled to these studies, were in the last stage of disease and they were not the best candidates for this type of immunotherapy (Mavroudis *et al.,* 2006; Bolonaki *et al.,* 2007). It is generally accepted, that immunotherapy would be more effective in patients who are not in an advanced stage of disease and the primary aim should have been the prevention of the clinical relapse of the disease and not the cure of the advanced tumours. The inability of the vaccines to eliminate the advanced tumours drastically, has been shown with the use of animal models (Cheever *et al*., 1997).

A significant observation is that, immune response was not induced in all patients. Similar results of other studies, suggest that immune response was not induced in all of vaccinated patients with BLP-25 but however, their overall survival was numerically and statistically higher comparing to the overall survival of non-vaccinated patients (Palmer *et al*., 2001). Similarly, immune response was not induced in all vaccinated patients with Vx-001 but however they showed a statistically significant prolonged overall survival (Mavroudis *et al.,* 2006).

According to the current study, a significant correlation between late $TERT_{572}$-specific IFN-γ immune response and overall survival of vaccinated patients who enrolled the study with clinically documented progressive disease (PD) was observed. Indeed, late immune $TERT_{572}$-specific responding patients had a better overall survival compared to non-late responding patients, which was also statistically significant ($p=0.03$). This observation seems to indicate that the patients who did not

have a $TERT_{572}$-specific immune response upon the completion of the 6th vaccination may not have had a clinical benefit from the vaccination. However, conclusions should be drawn with caution, since the patients had different types of heterogeneous solid tumours and have received several different chemotherapy regimens. However, a significant correlation between immune response and patients' clinical outcome is rarely observed (Nemunaitis *et al.,* 2008; Gonzalez *et al.,* 2003; Lonchay *et al.*, 2004**).** This may be due to the low development of the produced CLTs, the existence of the tumour and the criteria used for the assessment of the immune response.

Finally, after many years of effort, a 'proof of principle' for boosting antitumour immunity in humans has been established. However, it might not be sufficient to passively supplement the essential immune elements, including adoptively transferring tumour antigens, T cells, cytokines, and DCs. According to Zou *et al*., a '3S' therapeutic strategy is suggested: subversion of tolerizing conditions (S1), supplementation of immune elements (S2), and suppression of tumour angiogenesis and growth (S3

Clinical studies are needed to investigate further the many hTERT antigenic epitopes, as well as to characterize hTERT-specific cytotoxic and helper T cell clones, and the ability of these cells to effect tumour regression. Moreover, further studies are required to investigate the intracellular trafficking, processing and presentation of hTERT antigen in different types of tumours. A better understanding of hTERT peptide processing along MHC class I and class II- pathways in tumours may lead to novel strategies for a better approach to immunological treatments of cancer. Studies of cytokine controls of telomere remodelling (Cassar *et al*., 2008) in activated lymphocytes may promote longer and more persistent anti-telomerase immunity.

Several approaches have been proposed for the development of successful and efficient immunotherapy protocols, including vaccination coupled to inhibitory molecules, mAbs, bacteria or combined with radiotherapy or chemotherapy **(**Rescigno *et al*.,2007**).** Some crucial factors should be taken into consideration when designing a cancer immunotherapy clinical trial in order to achieve better clinical outcome. Firstly, the timing and dose of the administration of combined protocols are of crucial importance for the success of the therapy. Furthermore, correlation studies of immunological/clinical response should also be implemented particularly to understand failure and successes of tested therapies. In this regard, the analysis of the tumour microenvironment before and

after the treatment is essential in order to evaluate protocol efficacy and the immunological profile of the clinical responders.

In summary, the results of the current study demonstrate that Vx-001 is able to induce a TERT-specific immune response in vaccinated patients with different types of solid tumours. The mechanisms regulating the induction of Vx-001-specific immune response need to be further investigated as this vaccine seems to be a promising anticancer vaccine and may pave the way for a new era in the field of cancer immunotherapy. We should bear in mind that lab findings are often simplistic and that it is a long way to bring efficient therapies from bench to bedside. Especially cancer is such a complex disease that demystifying it is like travelling over a new and more realistic landscape.

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Ευχαριστίες..

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Το μεγαλύτερο ευχαριστώ το οφείλω στα άτομα της ομάδας μου: Δρ. Κέλλυ Βέτσικα, Δέσποινα Αγγουράκη και Γιώργο Κονσολάκη με τα οποία είχαμε μια άριστη συνεργασία , η οποία δεν περιορίστηκε στις τυπικές σχέσεις, αλλά επεκτάθηκε σε φιλικό επίπεδο. Ήταν πάντα ευγενικοί και πρόθυμοι να με εκπαιδεύσουν και χωρίς της παρουσία τους, αυτή η εργασία δε θα είχε ολοκληρωθεί. Ευχαριστώ ιδιαιτέρως την Κέλλυ για τις συμβουλές και τις γνώσεις που μου μεταβίβασε καθ'ολη τη διάρκεια της παραμονής μου στο εργαστήριο, την υπομονή της, την προθυμία της και την πολύτιμη συμβολή της στη συγγραφή της παρούσας εργασίας. Τη Δέσποινα επίσης για την καλή της διάθεση να μου λύσει οποιαδήποτε απορία μου και την προθυμία της να με εκπαιδεύσει όσον αφόρα στην ανάλυση των αποτελεσμάτων. Το Γιώργο, τόσο για την βοήθεια του σε τεχνικό επίπεδο και στην ανάλυση των αποτελεσμάτων , όσο και για την καλή του διάθεση και πολύτιμη συμβολή του στην επίλυση οποιουδήποτε προβήματος προέκυπτε. Τέλος, τη Σταυρούλα Μαλανδράκη, η πτυχιακή

εργασία της οποίας αποτέλεσε σημαντικό βοήθημα για τη συγγραφή της παρούσας εργασίας.

Επιπλέον, ευχαριστώ ειλικρινά, όλα τα άτομα του εργαστηρίου για το πολύ ευχάριστο και φιλικό κλίμα που επικρατούσε και όλους τους φίλους που έκανα στην Κρήτη, εντός και εκτός εργαστηρίου, με τους οποίους έχουμε μοιραστεί πολύ όμορφες στιγμές και θα κουβαλάω πάντα τις πιο ευχάριστες αναμνήσεις.....I will miss you…

Τέλος ευχαριστώ ολόψυχα τους γονείς μου, για τη συμπαράστασή τους και την ψυχολογική τους στήριξη σε κάθε μου βήμα. και όλους τους δικούς μου ανθρώπους που είναι πάντα δίπλα μου και στα εύκολα και στα δύσκολα, και μου δίνουν δύναμη να συνεχίσω...