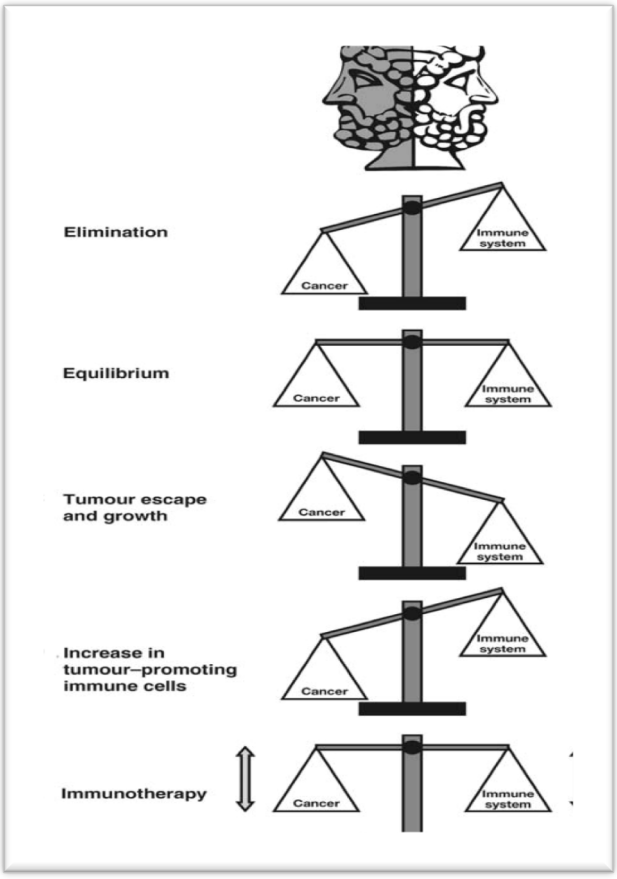
PhD Thesis of Dr Elisavet Papadimitraki

Establishment of the optimal administration schedule for cryptic telomerase peptides (hTERT) as cancer immunotherapy

Καθορισμός του βέλτιστου τρόπου χορήγησης τροποποιημένων κρυπτικών πεπτιδίων της Τελομεράσης (hTERT) σαν ανοσοθεραπεία σε ασθενείς με νεοπλασματική νόσο



Principal Supervisor Dimitrios Mavroudis, Professor of Medical Oncology

Co-supervisors Vasillios Georgoulias, Emeritus Professor of Medical Oncology
Joseph Papamatheakis, Emeritus Professor of Biology

Seven members committee
Dimitrios Mavroudis, Professor of Medical Oncology
Vasillios Georgoulias, Emeritus Professor of Medical Oncology
Joseph Papamatheakis, Emeritus Professor of Biology
Georgios Samonis, Professor of Internal Medicine
Sofia Agelaki, Associate Professor of Medical Oncology
Panayiotis Theodoropoulos, Associate Professor of Biochemistry
Athanasios Kotsakis, Associate Professor of Medical Oncology

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ABSTRACT

Establishment of the optimal administration schedule for cryptic telomerase peptides (hTERT) as cancer immunotherapy

Introduction

In most human cancers, activation of telomerase appears to be a hallmark, associated with unlimited cell proliferation of tumour cells (Blasco, 2005; Shay & Wright, 2000).

By ensuring maintenance of telomeres' length above a critically short point, telomerase prevents the induction of cellular senescence or apoptosis for the cancer cells, therefore allowing for tumour progression. Telomerase, and more specifically its catalytic subunit hTERT, is found to be overactive in 85–90% of cancers, marking it as a popular target for anticancer therapies.

$TERT_{572}$-based vaccine- Rationale

Nearly all human tumour-associated antigens, including telomerase, derive from non-altered self-proteins, thereby are subjects of the immune tolerance. The HLA-I molecules can bind both dominant and cryptic peptides. The dominant peptides have a strong affinity for HLA-I alleles, are abundant on the cell surface, and are strongly immunogenic, whereas cryptic peptides are not as abundant on the cell surface, have weak HLA-I affinity, demonstrating weak immunogenicity or complete lack of immunogenicity. In contrast to dominant peptides, cryptic peptides are poorly expressed, thereby do not induce immune tolerance escaping massive clonal deletion. These characteristics of the cryptic peptides make them a favourable target, candidate for the development of a specific, peptide antitumor vaccine therapy. Moreover, the use of tumour non-specific antigens may be a better choice for anticancer vaccines since they are not dependant on adjuvants or the efficacy of delivery (Mavroudis et al., 2006; Menez-Jamet & Kosmatopoulos, 2009; Ruden & Puri, 2013).

In our studies with the peptide-based vaccine (hTERT- based), we tried to overcome the tolerance-related blunting of T cell responses, by using cryptic (low affinity for HLA) peptides for the induction of an antitumor immune response. However, binding of wild type cryptic peptide antigens to HLA is usually unstable, with weak immunogenicity, and therefore

challenging in regard to immune response possibly hampering T cell priming and activation. More recent research has focused on the development of optimized cryptic peptides with higher affinity binding to HLA.

Based on this approach, our peptide-based anticancer vaccine, known as Vx-001 (Vaxon Biotech, Paris, France), consists of a low affinity cryptic peptide hTERT572 (RLFFYRKSV) and its optimized version, the hTERT572Y(1) (YLFFYRKSV), which has the first amino-acid residue replaced with a modified tyrosine (Y1) residue. This sequence aims to enhance the peptide's affinity for HLA-I molecules and potentially can circumvent the self-tolerance issue. The TERT572Y peptide has been found to induce tumour immunity in HLA-A∗0201 transgenic mice but luckily not autoimmunity (Gross et al., 2004). In addition, Vx-001 leads to enhanced immunogenicity of the cryptic peptide when presented by HLA-A∗0201 molecules (the most frequently expressed allele, present in 40–45% of population) without altering antigen's specificity (Mavroudis et al., 2006).

In the current study, our primary goal was to establish the optimal vaccination protocol, for administration of the two TERT peptides (the native $TERT_{572}$ and its optimized variant $TERT_{572Y}$) regarding its ability to elicit the best immunologic response in respect to ex vivo reactivity of peptide-induced CTLs. Following establishment of the best vaccination schedule, the study aims to 1) assess the safety profile of the TERT vaccine, 2) correlate the immunologic outcome with the clinical outcome of the patients who received the TERT vaccine.

Patients and Methods

Patients

In the first phase of the study for the establishment of the optimal vaccination schedule, 48 patients were enrolled, while overall 142 patients with various types of advanced solid tumours and previous exposure to standard treatment were enrolled in the telomerase peptide (hTERT) vaccination protocol. The inclusion criteria included HLA-A*0201 haplotype, histologically proven malignancy, advanced disease (Stage IV or locally advanced/unresectable), older than 18 years, performance status by WHO of 0-2, at least one chemotherapy regimen prior to vaccination, adequate bone marrow/liver/renal function.

Peptides

The 9-mer cryptic native $TERT_{572}$ (**R**LFFYRKSV) peptide and its optimized variant $TERT_{572Y}$ (**Y**LFFYRKSV), were synthesized initially by Epytop (Nimes, France) and later by Pepscan

(Lelystad, The Netherlands). Each peptide was prepared as a lyophilized powder (2 mg/vial) for reconstitution with 0.5 ml sterile water.

Blood samples for Immunomonitoring

Before each vaccination, 100ml peripheral blood in EDTA (ethylene diamine tetra acetic acid) was collected from each patient through a peripheral venous puncture. The time points of blood collection were set at baseline, prior to 3rd and 6th vaccination and before each boost administration of the peptide. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma, UK) density centrifugation and cryo-preserved in freezing medium at -80^{o}C until their future use the immune-assessment assays.

Vaccination protocol (Schemes A, B)

All HLA A*0201 patients (no= 48) received two subcutaneous (s.c) injections with 2mg of the optimized $TERT_{572Y}$ peptide followed by four s.c injections with 2mg of either the native $TERT_{572}$ peptide (scheme A) or the optimized $TERT_{572Y}$ (scheme B), depending on the randomization schedule, every three weeks until disease progression as indicated in each result section. 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.

Methods

The evaluation of interferon-γ enzyme linked-TERT-specific T cell immunologic response was performed mainly by the Enzyme-linked immunosorbent (ELIspot) assay. To ensure high accuracy, 3 independent experiments were performed for each test.

Results

Our results revealed that vaccination with the optimized $TERT_{572Y}$ followed by the native $TERT_{572}$ peptides can induce strong T cell responses, with higher avidity and frequencies of T cell responses, after the completion of 6-vaccinations. T cell responses after the sixth vaccination were detected more frequently (44% vs. 17%), and with higher number of peptide-specific reactive T cells (60 T cells/2×10^5 peripheral blood mononuclear cell vs. 10 T cells/ 2×10^5 peripheral blood mononuclear cell, $p = 0.04$), and higher avidity in the patients who received 4 more vaccinations with the $TERT_{572}$ peptide compared with patients who received only $TERT_{572Y}$ vaccinations. These results demonstrate that the best vaccination schedule involves

first the administration of the optimized $TERT_{572Y}$ followed by the native $TERT_{572}$ peptide in patients who are candidates for cancer immunotherapy.

The association between immunologic response and clinical outcome (PFS and OS) was evaluated. Overall, there was no significant difference in either PFS or OS for patients who developed an immunologic response at any time during vaccination between the 2 schemes. However, in the subgroup analysis of patients who enrolled in scheme A vaccination, those who developed an immune response had a significantly longer PFS compared with those without an immune response (13.5 vs. 3.5mo; log-rank test p=0.01).

In the next phase of the study, the best vaccination schedule was used in clinical trials with different tumour types and a cohort of NSCLC patients. Our studies confirmed a favourable toxicity profile of the TERT vaccine, without serious acute or late adverse events and without evidence of autoimmune reactions even after its administration for up to 2 years. Acute adverse events (AAE) were observed in 29 (52%) patients, and they were mild (grade 1). The most common AAE was grade 1 local skin reaction (n = 15; 27%).

In our study, those patients who developed an immunologic response at any time during vaccination had a significantly higher PFS (5.2 months; range, 0.9–51.8) compared with those who failed to develop any response following vaccination (2.2 months, range, 1.4–6.5; p = 0.0001. Multivariate analysis demonstrated that the development of immunological response was an independent factor associated with better PFS (HR = 3.35, 95% CI 1.7–6.7; p = 0.001), while there was a trend for worse OS in patients who did not develop immunologic response during the vaccination (HR = 2.0, 95% CI 1.0–4.0; P = 0.057).

ΠΕΡΙΛΗΨΗ

ΚΑΘΟΡΙΣΜΟΣ ΤΟΥ ΒΕΛΤΙΣΤΟΥ ΤΡΟΠΟΥ ΧΟΡΗΓΗΣΗΣ ΤΡΟΠΟΠΟΙΗΜΕΝΩΝ ΚΡΥΠΤΙΚΩΝ ΠΕΠΤΙΔΙΩΝ ΤΗΣ ΤΕΛΟΜΕΡΑΣΗΣ (hTERT) ΣΑΝ ΑΝΟΣΟΘΕΡΑΠΕΙΑ ΣΕ ΑΣΘΕΝΕΙΣ ΜΕ ΝΕΟΠΛΑΣΜΑΤΙΚΗ ΝΟΣΟ

Εισαγωγή

Η ενεργοποίηση της τελομεράσης συνδέεται με τον απεριόριστο κυτταρικό πολλαπλασιασμό των καρκινικών κυττάρων (Blasco, 2005; Shay & Wright, 2000).

Διασφαλίζοντας τη διατήρηση του μήκους των τελομερών πάνω από ένα κρίσιμο σημείο, η τελομεράση εμποδίζει την κυτταρική γήρανση ή απόπτωση των καρκινικών κύτταρων, επιτρέποντας έτσι την εξέλιξη του όγκου. Η τελομεράση, και ειδικότερα η καταλυτική υπομονάδα της, hTERT υπερεκφράζεται σε 85-90% των καρκίνων, γεγονός που την καθιστά δημοφιλή στόχο για αντικαρκινικές θεραπείες.

Σχεδόν όλα τα καρκινικά αντιγόνα συμπεριλαμβανομένης της τελομεράσης, προέρχονται από αυτό-αντιγόνα, και επομένως είναι υποκείμενα ανοσολογικής ανοχής. Τα μόρια HLA-I μπορούν να δεσμεύσουν τόσο κυρίαρχα όσο και κρυπτικά πεπτίδια. Τα κυρίαρχα πεπτίδια έχουν ισχυρή συγγένεια με τα μόρια HLA-I, βρίσκονται συχνά στην κυτταρική επιφάνεια και είναι ανοσογονικά, ενώ τα κρυπτικά πεπτίδια δεν είναι τόσο συχνά, έχουν ασθενή συγγένεια με τα μόρια HLA-I, επιδεικνύοντας ασθενή ανοσογονικότητα ή πλήρη έλλειψη ανοσογονικότητας. Σε αντίθεση με τα κυρίαρχα πεπτίδια, τα κρυπτικά πεπτίδια εκφράζονται ελάχιστα και δεν προκαλούν ανοσολογική ανοχή. Αυτά τα χαρακτηριστικά των κρυπτικών πεπτιδίων τα καθιστούν ιδανικό στόχο για την ανοσοθεραπεία με πεπτιδικά εμβόλια. Επιπλέον, η χρήση μη-ειδικών αντιγόνων όγκων αποτελεί καλύτερη επιλογή για αντικαρκινικά εμβόλια, αφού η αποτελεσματικότητα τους δεν εξαρτάται από την χρήση ανοσορυθμιστικών μορίων (adjuvants) (Mavroudis et al., 2006; Menez-Jamet & Kosmatopoulos, 2009; Ruden & Puri, 2013).

Στην παρούσα μελέτη προσπαθήσαμε να υπερνικήσουμε την ανοσολογική ανοχή χρησιμοποιώντας κρυπτικά πεπτίδια. Εντούτοις, η σύνδεση των κρυπτικών πεπτιδίων με τα μόρια HLA είναι συνήθως ασταθής, με ασθενή ανοσογονικότητα παρεμποδίζοντας την ανταπόκριση και ενεργοποίηση των Τ κυττάρων. Πιο πρόσφατες έρευνες επικεντρώνονται στην ανάπτυξη βελτιστοποιημένων κρυπτικών πεπτιδίων με υψηλότερη συγγένεια σύνδεσης με τα μόρια HLA.

Με βάση αυτή την προσέγγιση, το εμβόλιο μας, γνωστό ως Vx-001 (Vaxon Biotech,), αποτελείται από ένα κρυπτικό πεπτίδιο hTERT572 (RLFFYRKSV) χαμηλής χημικής συγγένειας και τη βελτιστοποιημένη του έκδοση, το hTERT572Y (YLFFYRKSV), στο οποίο έχει γίνει αντικατάσταση ενός αμινοξέος Αυτή η αλληλουχία στοχεύει στην ενίσχυση της συγγένειας του πεπτιδίου με τα μόρια HLA-I και μπορεί να παρακάμψει το ζήτημα της ανοχής. Έτσι αυξάνεται η ανοσογονικότητα του κρυπτικού πεπτιδίου όταν παρουσιάζεται από τα μόρια HLA-A∗ 0201 (το πιο συχνά εκφραζόμενο αλληλόμορφο, που υπάρχει στο 40-45% του πληθυσμού) χωρίς αλλοίωση της ειδικότητας του αντιγόνου (Mavroudis et al., 2006) .

Στόχος της μελέτης

Ο σκοπός της παρούσας μελέτης είναι ο προσδιορισμός του αποτελεσματικότερου τρόπου χορήγησης ενός βελτιστοποιημένου και ενός φυσικού πεπτιδίου που προέρχονται από ένα κρυπτικό επίτοπο της τελομεράσης με βάση την ανοσιακή απάντηση που αυτά επάγουν όταν χορηγηθούν σε ασθενείς με ανθεκτικά νεοπλάσματα.

Πρωταρχικός στόχος της μελέτης είναι να διευκρινισθεί ο βέλτιστος συνδυασμός ενεργητικής ανοσοποίησης με το τροποποιημένο πεπτίδιο $TERT_{572Y}$ και το φυσικό πεπτίδιο $TERT_{572}$ με βάση την ισχύ της χημικής συγγένειας (avidity) και την συχνότητα (frequency) της διέγερσης των ειδικών για το φυσικό πεπτίδιο TERT572 Τ λεμφοκυττάρων (CTL) που ενεργοποιούνται στους ασθενείς που έχουν εμβολιασθεί. Στη συνέχεια, ο βέλτιστος τρόπος χορήγησης χρησιμοποιήθηκε για τον εμβολιασμό ασθενών με διάφορα νεοπλάσματα. Οι δευτερογενείς στόχοι της παρούσας μελέτης είναι α) η μελέτη της τοξικότητας του εμβολίου της τελομεράσης, β) η συσχέτιση της in vivo ανοσολογικής ανταπόκρισης με την κλινική έκβαση των ασθενών.

Ασθενείς

Στην πρώτη φάση της μελέτης, για τον καθορισμό του πρωτοκόλλου εμβολιασμού, εντάχθηκαν 48 ασθενείς, ενώ συνολικά εντάχθηκαν 142 ασθενείς με διάφορα νεοπλάσματα. Όλοι πληρούσαν τα βασικά κριτήρια ένταξης, ήταν μεταξύ 18 και 80 ετών και σε γενική

κατάσταση που δηλωνόταν βάσει του ECOG performance status (PS) 0 – 2. Παρουσίαζαν όλοι έκφραση του HLA-A*0201. Είχαν επάρκεια αιμοποιητικού συστήματος ,φυσιολογική ηπατική και νεφρική λειτουργία και είχαν λάβει αποτελεσματικές θεραπείες εκλογής για το νεόπλασμά τους πριν την ένταξη στη μελέτη αυτή.

Κατά τη διάρκεια της μελέτης και τέσσερις εβδομάδες πριν και μετά από αυτή δεν έλαβαν καμία άλλη αντινεοπλασματική θεραπεία για τη νόσο τους είτε συστηματική (χημειοθεραπεία) είτε τοπική (ακτινοβολία). Επίσης κατά την ίδια περίοδο δεν ελάμβαναν κορτικοστεροειδή ή άλλη ανοσοκατασταλτική αγωγή.

Πεπτίδια

Και τα δύο πεπτίδια, το 9-μερές κρυπτικό φυσικό πεπτίδιο TERT572 (**R**LFFYRKSV) και το τροποποιημένο TERT572Y (**Y**LFFYRKSV), παράχθηκαν από την εταιρία Epytop (Nimes, France) και αργότερα από την Pepscan (Lelystad, The Netherlands). Η ποιότητά τους πιστοποιήθηκε με ειδικές μεθόδους και δεν αλλοιώθηκε ακόμα και μετά από δύο έτη βαθιάς κατάψυξης στους -80°C.

Δείγματα ασθενών

Η ανάλυση έγινε σε περιφερικό αίμα το οποίο ελήφθη από τους ασθενείς πριν την πρώτη χορήγηση, μετά την δεύτερη και την έκτη χορήγηση του πεπτιδίου, καθώς και πριν από κάθε αναμνηστική χορήγηση. Τα μονοπύρινα κύτταρα του περιφερικού αίματος (PBMCs) απομονώθηκαν με φικόλη (Ficoll-Hypaque ,Sigma, UK), φυγοκεντρήθηκαν και διατηρήθηκαν στην κατάψυξη (Gibco/Invitrogen, Paisley, Scotland, UK) στους -80^{o}C μέχρι τη μέτρηση της ανοσολογικής απάντησης.

Πρωτόκολλα εμβολιασμών (Σχήμα Α,Β)

Ολοι οι ασθενείς (no= 48) έλαβαν αρχικά δύο υποδόριες (s.c) ενέσεις του τροποποιημένου κρυπτικού πεπτιδίου $TERT_{572Y}$ στη δόση των 2mg κάθε τρεις εβδομάδες. Στη συνέχεια τυχαιοποιήθηκαν (1:1) σε δύο ομάδες. Η μία ομάδα έλαβε 2mg του φυσικού πεπτιδίου $TERT_{572}$, (σχήμα Α) ενώ η άλλη ομάδα συνέχισε τον εμβολιασμό με 2mg του τροποποιημένου πεπτιδίου $TERT_{572Y}$ (σχήμα Β). Ολοι οι ασθενείς έλαβαν έξι χορηγήσεις εκτός εάν παρουσίαζαν υποτροπή της νόσου, οπότε αποκλείονταν από τη μελέτη. Οι ασθενείς που δεν παρουσίασαν εξέλιξη της νόσου συνέχισαν με τις αναμνηστικές χορηγήσεις με το φυσικό πεπτίδιο $TERT_{572}$ και τις αιμοληψίες ανά 3 μήνες.

Μέθοδοι

Η μέτρηση της ανοσολογικής ανταπόκρισης στη χορήγηση του φυσικού πεπτιδίου $TERT_{572}$

έγινε με την μέτρηση της IFN-γ κυρίως με την μέθοδο Enzyme-Linked Immunosorbent Spot (ELISpot). Για την εξασφάλιση της μεγαλύτερης δυνατής αξιοπιστίας των αποτελεσμάτων, η μέθοδος επαναλήφθηκε τρεις φορές για κάθε ομάδα ασθενών σε ανεξάρτητες χρονικές στιγμές.

Αποτελέσματα

Ο βέλτιστος τρόπος εμβολιασμού αποδείχτηκε η διαδοχική χορήγηση του τροποποιημένου πεπτιδίου $TEPT_{572Y}$ ακολουθούμενου από το φυσικό πεπτίδιο $TEPT_{572}$. Συγκεκριμένα, μετά την έκτη χορήγηση στους ασθενείς που έλαβαν 4 χορηγήσεις με το φυσικό πεπτίδιο $TERT_{572}$, σε σχέση με τους ασθενείς που έλαβαν μόνο το τροποποιημένο πεπτίδιο $TERT_{572Y}$, παρατηρήθηκαν τα εξής:

Α) συχνότερα ανοσολογική ανταπόκριση (44% vs 17%) και με υψηλότερη χημική συγγένεια , και Β) υψηλότερη συγκέντρωση Τ κυττάρων ειδικών για το αντιγόνο (60 T cells/2x105 PBMC vs 10 T cells/2x105 PBMC, p=0.04).

Επιπροσθέτως αναλύθηκαν τα κλινικά δεδομένα των ασθενών που εντάχθηκαν στην πρώτη φάση της μελέτης, ώστε να συσχετισθεί η ανοσολογική ανταπόκριση με την κλινική πορεία των ασθενών. Δεν παρατηρήθηκαν σημαντικές διαφορές τόσο στην συνολική επιβίωση (overall survival) όσο και στο διάστημα ελεύθερο υποτροπής (progression free survival) ανάμεσα στους ασθενείς που ανέπτυξαν ανοσολογική απάντηση στις ομάδες Α και Β. Ωστόσο όσοι ασθενείς από την ομάδα Α εμφάνισαν ανοσολογική απάντηση είχαν σημαντικά μεγαλύτερο διάστημα ελεύθερο υποτροπής σε σχέση με όσους δεν ανέπτυξαν ανοσολογική απάντηση ((13.5 vs. 3.5 mo; log-rank test p=0.01).

Στην επόμενη φάση της μελέτης ο βέλτιστος τρόπος εμβολιασμού χρησιμοποιήθηκε σε ασθενείς με διάφορα νεοπλάσματα και σε πληθυσμό με Μη-μικροκυττταρικό καρκίνο του πνεύμονα (NSCLC). Το εμβόλιο αποδείχτηκε καλά ανεκτό με ήπιο προφίλ τοξικότητας, κυρίως δερματική αντίδραση στο σημείο της χορήγησης. Δεν παρατηρήθηκαν ανεπιθύμητες ενέργειες αυτοάνοσου τύπου κατά την διάρκεια της χορήγησης ή κατά την διάρκεια παρακολούθησης των ασθενών μετά το τέλος της μελέτης και για τουλάχιστον 2 έτη.

Οι ασθενείς που ανέπτυξαν ανοσιακή απάντηση είχαν καλύτερη κλινική έκβαση με μεγαλύτερο διάστημα μέχρι την υπότροπή (PFS 5.2 months; range, 0.9–51.8), σε σχέση με όσους δεν ανέπτυξαν (PFS 2.2 months, range, 1.4–6.5; P = 0.0001). Σε πολύπαραγοντική ανάλυση η ανάπτυξη ανοσιακής απάντησης στους ασθενείς που εμβολιάσθηκαν αποδείχτηκε ανεξάρτητος προγνωστικός παράγοντας για καλύτερο PFS (HR = 3.35, 95% CI 1.7–6.7; P = 0.001), ενώ όσοι απέτυχαν να αναπτύξουν ανοσιακή απάντηση στον εμβολιασμό παρουσίασαν τάση για χειρότερη επιβίωση (HR = 2.0, 95% CI 1.0–4.0; P = 0.057).

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1. CHAPTER 1: Introduction

1.1 The role of immune system

The immune system consists of various structures and bodies which work together to orchestrate and execute the body' s defense to external and internal attacks. It uses primarily specialized organs designed to filter out and respond to microbes entering the body's tissues and a mobile force of molecules and cells in the bloodstream to respond rapidly to such attacks and prevent disease. It is able to detect a wide variety of harmful agents, from parasitic worms to viruses, and distinguishes them from the organism's own healthy organisms and tissues. This detection and elimination is complicated due to pathogens' ability to evolve rapidly, adapting to avoid the immune system. At the same time, the immune system adapts accordingly its ability to recognize and distinguish between self and non -self's molecules (Sprent & Cho, 2008; Zou, Liu, & Chen, 2005). Despite the well-regulated and sophisticated function, the immune system occasionally fails giving rise to immunodeficiency, or over-reacts against foreign microbes, leading to tissue damage (Screiber, 2016).

1.2 Principles of Immunity

The study of the evolution of immunity provides a great insight of the natural selection pressure mechanisms driving the body's adaptation process in order to protect and maintain life as we know it (Litman & Cooper, 2007). Innate immunity is the defense mechanism chosen over many years of evolution, it is encoded in our germline, and passed down from generation to generation with only minor editing (C. A. Janeway, Jr. & Medzhitov, 2002).

The innate immunity components are able to detect and eliminate potentially harmful intruders but they also play substantial role in maintenance of homeostasis by reversing tissue damage and eliminating apoptotic or senescent cells. The caveat is that despite the immediate protection offered, this is incomplete and unfortunately demonstrates only short-term memory (C. A. Janeway, Jr. & Medzhitov, 2002; Netea, Quintin, & van der Meer, 2011). This lack of memory results in a sluggish process initiated all over again, with every encounter, even from a known thread. This is partly because the receptors utilized by the innate cells, such as the TLR are able to distinguish between self and non-self, but lack specificity, the skill to distinguish among non-selves between a friend and an enemy. With imprecise, impersonalized but powerful response, the innate immunity can impose significant harm to the entire organism itself. Therefore, in the

evolution of the immune system, new tools had to be created, the so- called adaptive immunity (Laird, De Tomaso, Cooper, & Weissman, 2000).

Table 1. 1 The types of the immune response (C. Janeway, 2005)

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

Any pathogen that breaches the host's anatomic and chemical barriers is able to encounter the cellular defenses conducted by the innate immunity components. This response is initiated whenever sensor cells detect inflammatory inducers. Sensor cells include different cell types that are able to detect inflammatory mediators through expression of many innate recognition receptors and which are encoded by a number of genes that remain constant over an individual's lifetime (C. A. Janeway, Jr. & Medzhitov, 2002). The intruders which play the role of inflammation inducers by triggering these receptors include molecular components unique to pathogens (bacteria or viruses), such as bacterial lipopolysaccharides, or molecules such as ATP. By triggering these receptors, the innate immune cells are activated to produce various mediators that either directly destroy invading pathogens, or induce the immune response by stimulating other cells (K. Murphy & Weaver, 2011).

While the innate immune response occurs rapidly on exposure to an intruder, response by the adaptive immune system take days rather than hours to develop. However, the adaptive immune system is capable of eliminating the threat more efficiently because of intense and delicate specificity of antigen recognition by its lymphocytes. More specifically, lymphocytes express highly specialized antigen surface receptors, Immunoglobulins (or antibodies) on B lymphocytes, and the T-cell receptors (TCRs) for antigen on T lymphocytes that, unlike receptors on innate immune cells, recognize non -self molecules with exquisite specificity. The

genes encoding these receptors are not embedded in the germline but are the product of gene recombination during lymphocyte development, an effective process that generates a very large number of unique antigen receptors by splicing, rearranging, and linking a set of adjacent genes. This enables the adaptive immune system to respond to virtually any pathogen and effectively focus resources to eliminate pathogens that have evaded or overwhelmed innate immunity. But the adaptive immune system interacts with, and relies on, cells of the innate immune system for many of its functions (Gearhart, 2004).

1.3 Innate immunity

Innate defense components include: a) mechanical barriers such as skin, mucosal surfaces, respiratory cilia, b) chemical barriers such as gastric acid, lacrimal lysozymes, c) cellular components such as Natural Killer Cells (NK cells), macrophages and Dendritic Cells (DCs) which all work together to prevent entry and proliferation of microorganisms (Fig 1.1). The cellular components such as the macrophages, granulocytes (the collective term for the white blood cells containing granules such as neutrophils, eosinophils, and basophils), mast cells, and dendritic cells of the innate immune system have a precursor cell, the common myeloid progenitor (CMP). Macrophages, granulocytes, and dendritic cells constitute the three types of phagocytes in the immune system.

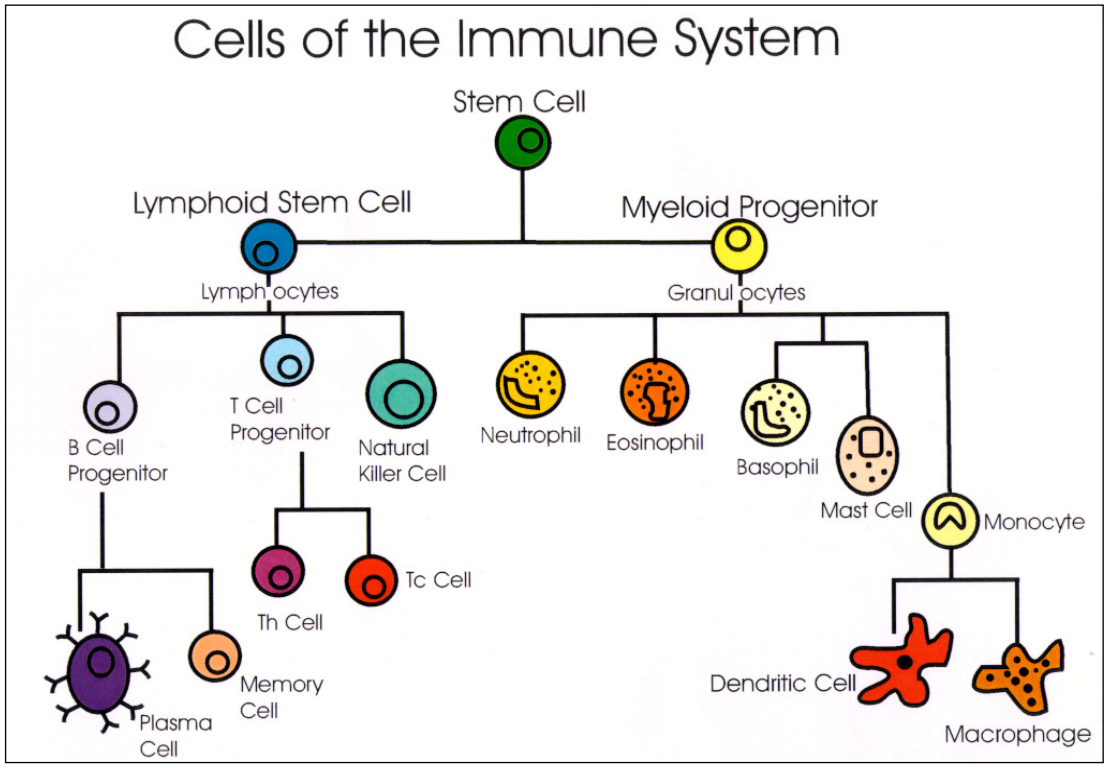


Figure 1.1 Origin and differentiation of cells of the immune system (Online Textbook of Bacteriology, Kenneth Todar)

Macrophages arising during embryonic development are resident in almost all tissues, while some macrophages arising from the bone marrow of the adult animal are the mature form of monocytes, which circulate in the blood and migrate continually into tissues, where they differentiate. Macrophages perform several different functions throughout the innate immune response and the subsequent adaptive immune response. The phagocytic function by engulfing and killing microorganisms is a first defense in innate immunity, while they are also engaged in the disposal process of pathogens and infected cells targeted by the adaptive immune response. Although both monocytes and macrophages are phagocytic, it is primarily macrophages that perform protective function, as most infections occur in the tissues. Moreover, macrophages orchestrate immune responses: they assist in inflammation induction, a crucial step for a successful immune response and they produce inflammatory mediators that stimulate/ activate other immune-system cells and engage them into an immune response. The phagocytosis is also triggered by the activation of complement system by bacterial surfaces, which in turn induces a cascade of proteolytic reactions that coat the microbes with fragments of specific proteins of the complement system. Subsequently, the coated microbes are recognized by specific complement receptors on macrophages and neutrophils, taken up by phagocytosis, and destroyed. (Alberts, Johnson, & Lewis, 2015; Gearhart, 2004; K. Murphy & Weaver, 2011).

Natural killer (NK) cells are cytotoxic lymphocytes playing major role in the rejection of tumours and cells infected by viruses. Their cytotoxic function is mediated through the release of small cytoplasmic granules of proteins called perforin and granzyme causing destruction of the target cells and 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, Lanier, Cwirla, & Phillips, 1989). Recent studies have demonstrated that NK cells may be able to cross over the traditional boundaries of innate and adaptive immunity with their capacity for memory-like responses. Although at present, the specificity of NK memory-like responses is not fully comprehended, their induction in response to cytokine stimulation suggests that they are nonspecific. NK cells can also specialize in the production of the T_H17 cytokine IL-22, and NK-22 cells seem to play role in maintenance of mucosal homeostasis during inflammation. NK cell development in the thymus and lymph nodes, source of traditionally adaptive immune cells, is suggestive of their role during the coordination of an adaptive immune response (Cooper, Colonna, & Yokoyama, 2009).

The granulocytes (also called polymorphonuclear leukocytes because of their oddly shaped nuclei) are named for the densely staining granules in their cytoplasm. Granulocytes are all relatively short-lived, surviving for only a few days. The three types of granulocytes— **neutrophils, eosinophils, and basophils**— are distinguished by the different staining properties of their granules, which serve distinct functions. They mature in the bone marrow, they are produced in higher numbers during immune responses, when they migrate to sites of infection or inflammation. The phagocytic neutrophils especially, play the most important role in innate immune response, by phagocytosing a variety of microorganisms and destroying them in intracellular vesicles by using degradative enzymes and other antimicrobial substances stored in their cytoplasmic granules. Eosinophils and basophils when activated, release enzymes and toxic proteins from their granules. Less abundant than neutrophils, they mostly play role in defense against parasites, which are too large to be ingested by macrophages or neutrophils. They are thought to contribute to allergic inflammatory reactions, producing a damaging rather than protective effect (C. Janeway, 2005; K. Murphy & Weaver, 2011).

Mast cells develop in the bone marrow, migrate as immature precursors and mature in peripheral tissues, especially skin, intestines, and airway mucosa. They also have granules containing inflammatory mediators, such as histamine and various proteases, which protect the internal surfaces from pathogens, including parasitic worms (K. Murphy & Weaver, 2011).

Dendritic cells (DCs) form the third class of phagocytic cells of the immune system and they are called after their membranous processes, which resemble the dendrites of nerve cells. They function as antigen presenting cells (APCs) and are present throughout the body. They develop in bone marrow, migrate in immature form through the bloodstream from the bone marrow to the peripheral tissues. Activation of immature DCs consists of MHC upregulation, increased expression of Lymph Nodes (LN)-homing chemokine receptors such as CCR7, T cell costimulatory molecules such as CD80 and CD86, and secretion of cytokines such as IL-12, IL-15, and type I IFNs (Sabado, Meseck, & Bhardwaj, 2016). 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. DCs may take up antigens from malignant cells through multiple mechanisms, namely phagocytosis, pinocytosis (a process by which they ingest large amounts of the extracellular fluid and its

contents leading to degradation of the pathogens), and receptor-mediated endocytosis, and migrate to the draining LNs, Fig 1.2 (Alvarez, Vollmann, & von Andrian, 2008).

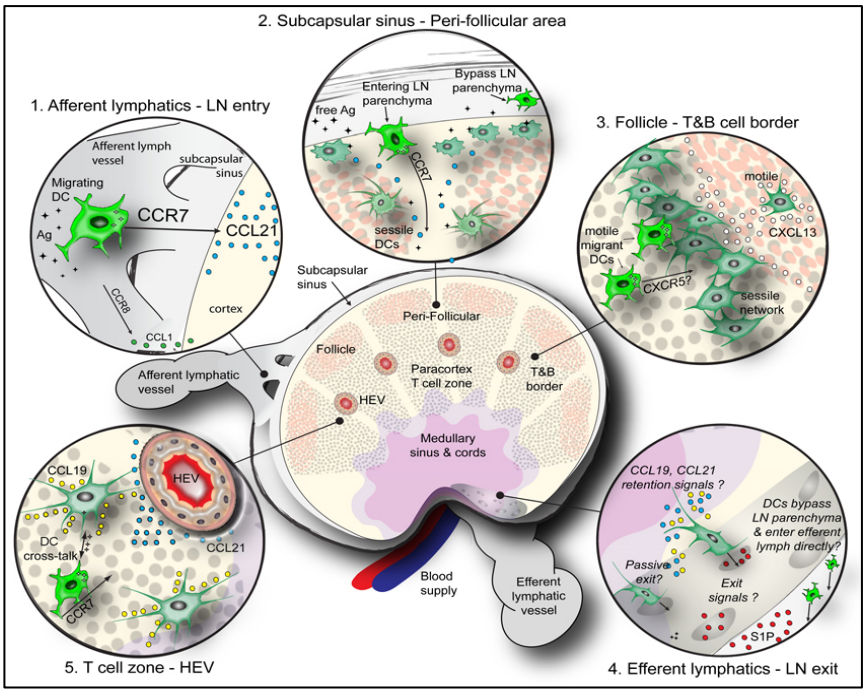


Figure 1.2 DC networks and migratory pathways of DCs within the LN. Picture of the DC network and anatomic features within the LN, including (clockwise from top left), the afferent lymphatics/LN entry point (inset 1), subcapsular sinus and the peri-follicular region (inset 2), the B cell follicle and T&B cell border (inset 3), the efferent lymphatics and LN exit point (inset 4), and the T cell zone/HEV (inset 5). Major structural features of the LN are depicted, in addition to T cells (grey circles), B cells (brown circles), and free flowing or processed Ag (black diamonds). The major trafficking pathways and chemotactic molecules that guide migratory and resident DC subsets within each zone are highlighted. Adopted from (Alvarez et al., 2008).

Within the LNs, DCs and naive T cells - with guidance from CCR7 and LN stroma- migrate to the paracortex region (Groom, 2015). Practically, they 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. This occurs while the DC is both maturing and migrating from the tissues toward the lymph nodes. More sophisticated imaging techniques have demonstrated that migratory DCs disperse in the peripheral paracortex, while the LN-resident DCs tend to accumulate in the central paracortex (Gerner, Kastenmuller, Ifrim, Kabat, & Germain, 2012). However, the main role of the DCs is

as sensor cells whose encounter with pathogens triggers the release of their mediators that in turn activate other immune cells. They were discovered by their role in activating T lymphocytes of the adaptive immunity and it was later found that dendritic cells and their products play a more critical role in controlling responses of cells of the innate immune system (Alberts et al., 2015; Banchereau & Steinman, 1998; K. Murphy & Weaver, 2011). More specifically, migratory DCs carrying viral antigens were found to travel to the LN, activate $CD4^+$ T cells in the paracortex, and recruit active $CD4^+$ T cells to promote cross-presentation by $XCR1^+$ DCs to $CD8^+$ T cells in the deep cortex DC-mediated T cell priming is a three-step process (Hor et al., 2015). In phase I, naive T cells sample DCs in short bursts. In phase II, T cells establish and maintain prolonged contact with DCs, initiating the activation and generation of memory $CD8^+$ T cells (Henrickson et al., 2013). In phase III, T cells resume transient DC contact and commence proliferation (Mempel, Henrickson, & Von Andrian, 2004). This process may take days to complete, which explains the delay in the appearance of an adaptive immune response. However, a dedicated subset of LN-DCs within the lymphatic sinus epithelium (LS-DCs) are speculated to capture LN draining antigens, vaccine components, or microbial factors, and activate T cells rapidly (Gerner, Torabi-Parizi, & Germain, 2015).

1.4 Adaptive Immunity

A unique feature of the adaptive immune system is its ability to generate immunological memory, enabling the individual, previously exposed once to an infectious agent, to mourn an immediate and stronger response against any subsequent exposure to it; that is, the individual will have protective immunity against it. One of the great challenges of immunotherapy today is to find ways to generate long-lasting immunity to pathogens/agents that do not naturally provoke immunity. The basic cellular components are the lymphocytes. There are two major types of lymphocytes in the vertebrate immune system, the B lymphocytes (B cells) and T lymphocytes (T cells). These express distinct types of antigen receptors and have quite different roles in the immune system. Lymphocytes remain relatively inactive until they encounter a specific antigen that interacts with an antigen receptor on their cell surface. Lymphocytes which have not yet been activated by an antigen are called naive lymphocytes, while those activated by the antigen, differentiate further into fully functional lymphocytes and are called effector lymphocytes. The lymphocytes are distinguished by the structure of the antigen receptor that they express on their surface. The B-cell antigen receptor, or B-cell receptor (BCR), is encoded by the same genes that encode antibodies, or immunoglobulins (Ig). The antigen receptor of B lymphocytes is also known as membrane immunoglobulin (mIg) or surface immunoglobulin (sIg). The T-cell antigen

receptor, or T-cell receptor (TCR), is related to the immunoglobulins but it differs in its structure and recognition properties (C. Janeway, 2005; K. Murphy & Weaver, 2011).

Lymphopoiesis (the production of new lymphocytes) takes place in specialized lymphoid tissues—the central or primary lymphoid tissues, the bone marrow for most B cells and the thymus for most T cells. Although their precursors originate in the bone marrow, the B cells complete most of their development there, while the precursors of most T cells migrate to the thymus, where they develop into mature T cells. Lymphopoiesis also generates a diverse repertoire of B-cell receptors and T-cell receptors on circulating B and T cells, respectively, thereby enabling an individual to make adaptive immune responses against the wide range of pathogens encountered during a lifetime. During early life development, the new lymphocytes originate from the central lymphoid tissues and migrate to the secondary lymphoid tissues or peripheral lymphoid tissues such as lymph nodes, spleen and mucosal lymphoid tissue. New B cells are constantly produced from the bone marrow, throughout life, while new T cells' development in the thymus slows down, and peripheral T-cell numbers are maintained by the division of mature T cells outside the central lymphoid organs (K. Murphy & Weaver, 2011).

Upon encountering antigens, lymphocytes proliferate and differentiate into specialized subsets. B lymphocytes into plasma cells, the source of antibodies, while T lymphocytes differentiate into helper and effector or cytotoxic subsets, each of which secreting distinct set of molecules, the cytokines. Helper T lymphocytes play a major role, orchestrating the mounting immune response for each intruder, whereas cytotoxic T lymphocytes directly attack and kill the cells harboring the intruder. Moreover, the immune responses are regulated by regulatory cells, specialized B and T lymphocytes (Josefowicz, Lu, & Rudensky, 2012; Mauri & Bosma, 2012). Upon response to the antigen, the majority of the antigen-specific lymphocytes involved will die, while those that survive become long-lived memory cells. These memory lymphocytes, unlike their naïve predecessors, ensure that any subsequent encounter with the same invader will effectively generate a more potent immune response (Mueller, Gebhardt, Carbone, & Heath, 2013). This response involves clonal expansion, differentiation, regulation, and memory, all features of the adaptive immunity generating higher number of lymphocytes -specific for each given antigen, with extended lifespan, faster response rate, superior proliferation capacity, and wider access to tissues (Yatim & Lakkis, 2015).

1.5 Linking Innate with Adaptive Immunity

The next step is the connection between the innate and the adaptive immunity. The phagocytic cells of the innate immune system capture the antigens, degrade them into small molecular blocks (peptides), and present them to the newly produced lymphocytes of the adaptive immune system which are able to recognize the antigens through their receptors. The subset of innate immune cells possessing the skill to process antigens are called antigen-presenting cells (APCs) and the most skilled among them are the dendritic cells (DCs) (Nussenzweig & Mellman, 2011). DCs process the antigenic peptides into major histocompatibility complex (MHC) proteins (human leukocyte antigens in humans), which ensures that all non -self peptides are presented to the T lymphocytes through their TCR which due to its high specificity and affinity is able to activate effectively the adaptive immune system. DCs are also capable of providing costimulatory signals, to ensure proliferation and differentiation of the T lymphocytes (Parham, 2005; Y. Zhu & Chen, 2009).

The maturation of DCs into potent APCs is induced by the same molecules that the innate immune system uses to sense non-self and trigger inflammation. Microbial proteins such as lipopolysaccharide (LPS), are recognised as nonself by innate immune components and by binding to its receptor TLR4 induces innate immune defence, while through the same receptor it primes DCs to present all the foreign antigens that the microbe carries, thus activating the appropriate T lymphocytes. The innate immune system is therefore linked in a sophisticated way to the adaptive immune system, which in turn orchestrates a maximal response to ensure the host is successfully protected (C. A. Janeway, Jr., 1989; Medzhitov, Preston-Hurlburt, & Janeway, 1997).

1.6 Lymphoid organs

The primary lymphoid organs are the sites of production and education of the immature immune cells. These are the bone marrow, where both innate and adaptive immune cells are born and where B lymphocytes are educated and the thymus, where the T lymphocytes are born and educated.

Education of the lymphocytes refers to the immune system's process of filtering and removing those lymphocytes that recognize self-antigens and could attack the organism by either killing them or putting them in a permanent state of unresponsiveness called anergy. This education

process, referred to as negative selection (Sprent, 2005) is crucial as the specificity of antigen receptors on B and T lymphocytes developed through random, somatic gene arrangement unlike the case with innate receptors and their development through a predetermined, germline processed route which was selected over years of evolution. This process ensures that the emerging B and T lymphocyte populations would not develop self-reactivity, unleashing the "horror autotoxicufs" described by Paul Ehrlich more than a century ago.

T lymphocytes in addition through a positive selection step taken place in thymus, are selected to survive based on their ability to recognize self-MHC molecules (Hedrick, 2012), to accommodate the predilection of TCR to engage only to peptides bound to MHC molecules. Therefore, in a sophisticated process, the T lymphocytes which are able to recognize self-MHC with a reasonable affinity are positively selected, whereas those that recognize self-MHC with too low affinity die by neglect and those with too high affinity die in the following negative selection step. The result is the emergence of mature lymphocyte repertoire capable of detecting an abundancy of nonself antigens but with a limited ability to develop immune response against self-antigens.

The secondary lymphoid organs or tissues have the ability to bring immune cells together at the right place and time by using synthesized molecules such as chemokines and adhesion molecules (Goodnow, 1997). While APCs, such as DCs, could live and move in either secondary lymphoid tissues or any non-lymphoid organ of the body, the secondary lymphoid organs such as lymph nodes, spleen, and Peyer's patches of the small intestine, are organized into T- and B-cell zones through which naïve T and B lymphocytes circulate constantly or reside for long time. Upon encounter with a nonself intruder' s antigens, the DCs migrate through lymphatic channels to the closest lymph node and, attracted by chemokine and adhesion molecules, move within the lymph node to activate antigen-specific T lymphocytes and subsequently, antigen-specific B lymphocytes. This process provides a continuous channel between innate and adaptive immune cells, generating ample effector and memory lymphocytes that then exit the lymph node and migrate through the bloodstream to the target tissues, the site of antigen entry. The movement of effector and memory cells to the target tissue is also guided by the action of the antigen-presenting DCs within the tissue, the chemokines and adhesion molecules. Some of the produced memory T lymphocytes remain in the non -lymphoid tissues as resident memory cells to prevent reinfection with the same pathogen.

1.7 Autoimmunity: an innate danger

Although in the majority of the cases, the immune system meets up our expectations, autoimmunity represents its major shortcoming. The innate cells detect pathogens such as bacteria, viruses, fungi, but they also respond to self-molecules such as proteins, nucleic acids, or chemicals which have the capacity to alarm the immune system about impeding or established tissue damage (Kono & Rock, 2008; Matzinger, 1998). Infected, ischemic, or injured tissues release damage-associated molecules in order to amplify the immune response to non-self (e.g., in infection), and engage in the tissue repair process. Components from both innate (e.g., macrophages) and adaptive immune system (e.g., regulatory T cells) actively participate in the tissue repair (Burzyn, Benoist, & Mathis, 2013).

Another important skill of the immune system is its ability to differentiate and stratify its response to the millions of non-harmful bacteria and other microbes which accompany us and support our well-being throughout life, by using promptly the regulatory mechanisms to ensure that DCs and lymphocytes at barrier surfaces (skin, gastrointestinal tract, vagina, lungs) are controlled to avoid attacks on helpful commensals (Hooper, Littman, & Macpherson, 2012). Interestingly, it also recognizes non-self which although, neither microbial nor pathogenic, it can still be harmful (e.g., a stem cell, a potentially transmissible tumour or ectopic fetus,) known as allogeneic non-self, against which it triggers powerful adaptive immune responses (Burnet, 1971; Oberbarnscheidt & Lakkis, 2014; Pearse & Swift, 2006).

Autoimmunity is the consequence of the activation of the very few self-reactive lymphocytes that the immune system failed to eliminate in the bone marrow or thymus during ontogeny, in the process of central tolerance taking place in central or primary lymphoid organs. Despite the additional regulatory mechanisms outside primary lymphoid organs, where peripheral tolerance is exercised in secondary lymphoid and non -lymphoid organs, as a safeguarding mechanism to edit the central tolerance, there are still a few events that could escape from time to time.

The key players of peripheral tolerance are the regulatory T lymphocytes, which are there to ensure that autoreactive lymphocytes are either prevented from reacting to self or are silenced early. However when some events occur, peripheral tolerance fails and the emergence of autoimmune disease occurs (Bluestone, 2011). These events include genetic mutations preventing regulatory T lymphocyte full development, maintenance, or function and inflammatory conditions such as infections by which cross-reactivity between self and non-self

antigens could occur, or during which interference with the function of regulatory T lymphocytes can happen.

In this context, different mechanisms have been recently described in which intracellular endogenous proteins are presented by MHC-II due to autophagy by antigen-presenting cells, in connection with infection with the herpes simplex virus and influenza viruses (Munz, 2012), or human proteins influence the activation of $CD4^+$ and $CD8^+$ T lymphocytes and on the formation of regulatory T cells (Arneth, 2018). Additionally, local tissue injuries may uncover hidden self-antigens previously ignored by the immune system but neither deleted in the process of central tolerance nor edited by the peripheral tolerance (Kurts, Panzer, Anders, & Rees, 2013; Yatim & Lakkis, 2015).

The evolution of the immune system is a dynamic process of trial and error, where the result comes from continuous adaptations and editing on chance and necessity, while maintaining the basic functional principles of the life as we know it (Ziauddin & Schneider, 2012). As such, continuous improvement of our knowledge regarding new paths or the discovery of new cells or new roles of them help us to understand the sophisticated interactions between the components of the immune system (Hwang & McKenzie, 2013; Min-Oo, Kamimura, Hendricks, Nabekura, & Lanier, 2013).

1.8 Immune system and cancer

The recognition that the immune system plays a crucial role in cancer has triggered exciting developments in cancer research in the last decades. Its ability to interact and influence the cancer development and progression has been the focus of research, looking on one hand, into the mechanisms leading to the development of cancer and the naturally occurring immune response to the developing tumours and on the other hand, investigating the immunotherapy-induced responses to the developed cancers (Grivennikov, Greten, & Karin, 2010; Mantovani, Allavena, Sica, & Balkwill, 2008; R. D. Schreiber, Old, & Smyth, 2011; Shankaran et al., 2001).

The unique properties of the immune system such as the specificity which could limit the off-target effects, and the immunologic memory which can control the cancer progression, both serve to optimise the immune therapies against cancer. It became apparent that the tumour-specific antigens play important role as targets of the immune response against cancer (Schumacher & Schreiber, 2015). This concept starts with the recognition of the neoantigens by

the immune system before they become clinically evident, proceeds with the modification of the cancer cells' immunogenicity in an immunocompetent environment, while orchestrating the immune elimination of the developing cancer with the appropriate immune-based therapy. This concept was dramatically accelerated in the recent years with the help of next generation sequencing and more sophisticated approaches able to predict cancer specific mutations, which play the role of neoantigens as targets of adaptive immunity (Gubin, Artyomov, Mardis, & Schreiber, 2015).

1.9 Cancer Immunoediting: Interaction between Immune system & Cancer

The term immunoediting is used to describe a dynamic process between host-protective actions (immunosurveillance) and tumour-promoting actions (tumour progression) which consists of three phases: elimination, equilibrium and escape, (Fig 1.3 from Screiber 2016), (Dunn, Old, & Schreiber, 2004b; Screiber, 2016; Shankaran et al., 2001)). The term was first introduced to stress out that an intact immune system not only protects against the development of cancer but it also edits the immunogenicity of the tumours rendering them unfit to grow in the immunocompetent host (R. D. Schreiber et al., 2011).

Cancer Immunoediting initiates after cellular transformation has occurred and the intrinsic tumour-suppressor mechanisms have been bypassed. It consists of three distinct phases: Elimination, Equilibrium, and Escape. In the Elimination phase, developing tumours are recognized by the innate and adaptive immunity which work together to eliminate emerging tumours before they become clinically apparent. This phase could be sufficient to complete tumour elimination, keeping the host cancer-free. If, however, the immune system fails to eliminate the tumour, the surviving cells may then enter the Equilibrium phase, in which although the tumour expansion is immunologically constrained, the tumour itself is not destroyed. Equilibrium may inhibit outgrowth of occult cancers for the lifetime of the host. However, in Equilibrium phase immunologic editing of the tumours leads to their alteration so they are no longer recognized as foreign by the immune system as a consequence of immune selection pressure. They become resistant to immune effector mechanisms and start growing progressively to induce an immunosuppressive tumour microenvironment favouring the cancer growth as we know it. As such, tumour cells enter the Escape phase, in which their outgrowth is no longer inhibited by the immune system, leading to a clinically apparent cancer. Prior to the introduction of the immunoediting concept, meta-analyses of clinical data showed that immunocompromised patients such as renal transplant patients had experienced higher

incidence of various types of cancers compared to non-transplanted immunocompetent individuals (Birkeland et al., 1995; Dunn, Bruce, Ikeda, Old, & Schreiber, 2002), while it was discovered that T cells and antibodies specific for tumours were expressed in cancer patients who harbour the tumours (Dunn, Old, & Schreiber, 2004a).

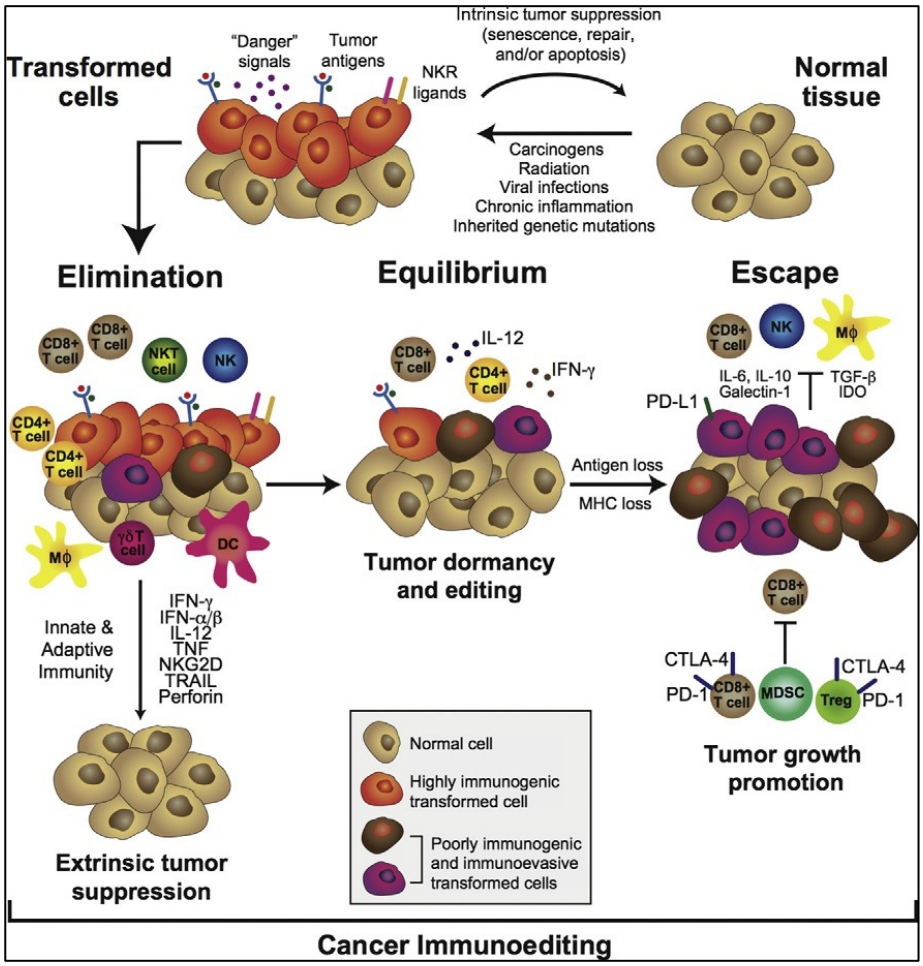


Figure 1. 3 Cancer Immunoediting. Cancer Immunoediting is an extrinsic tumour-suppressor mechanism that takes over after cellular transformation has occurred and the intrinsic tumour-suppressor mechanisms have failed. Cancer Immunoediting consists of three phases: Elimination, Equilibrium, and Escape. In the Elimination phase, innate and adaptive immunity work together to eliminate emerging tumours before they become clinically apparent. If, however, a cancer cell variant resists elimination, it may then enter the Equilibrium phase, in which editing of its immunogenicity occurs preventing its outgrowth. Equilibrium may inhibit outgrowth of occult cancers for the lifetime of the host. However, as a consequence of immune selection pressure, tumour cell variants may arise that are no longer recognized by adaptive immunity, become resistant to immune effector mechanisms, and may even induce an immunosuppressive tumour microenvironment. These tumour cells may then enter the Escape phase, in which their outgrowth is no longer inhibited by immunity, leading to a clinically apparent cancer. Figure adapted from Vesely, M. D., Kershaw, M. H., Schreiber, R. D., & Smyth, M. J. (2011). Natural innate and adaptive immunity to cancer. Annual Review of

Immunology, 29, 235–271. http://dx.doi.org/10.1146/annurev-immunol-031210-101324 and Schreiber, R. D., Old, L. J., & Smyth, M. J. (2011). Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion. Science, 331(6024), 1565–1570.

Moreover, it was found that cancer patients commonly have tumour-specific immune infiltrates in their tumours and the type, density and location of memory $CD8^+$ T cells in patients' cancer could have prognostic and predictive value for the clinical outcome (Galon et al., 2006).

1.9.1 Phases of Immunoediting: Elimination

Elimination is the first phase of Cancer Immunoediting, an expanded phase of Cancer Immunesurveillance. The key components here include cells of innate immunity such as macrophages, dendritic cells (DCs) and NK cells and cells of adaptive immunity such as $CD4^+$ cells, $CD8^+$ cells, NKT (Smyth, Godfrey, & Trapani, 2001; Teng, Galon, Fridman, & Smyth, 2015).

In this phase, host immune response is mediated through secretion and stimulation of other molecules such as tumour necrosis factor (TNF)-α, granzyme, perforin, Fas/FasL, TNF-related apoptosis-inducing ligand (TRAIL) and recognition molecules such as NKG2D (Diefenbach, Jensen, Jamieson, & Raulet, 2001; Mittal, Gubin, Schreiber, & Smyth, 2014; Smyth, Cretney, et al., 2001). The role of type I interferons IFN-α/β and IFN-γ in this phase was recently established. While the IFN-α/β has mainly action on the host cells enhancing cross-presentation activity of tumour antigens by $CD8^+$ /$CD103^+$ DCs, the IFN-γ targets both tumour and hematopoietic cells, promoting induction of $CD4^+$ T helper I (Th1) cells and $CD8^+$ cytotoxic lymphocytes (CTL) and plays a critical role in enhancing MHC I expression on tumour cells (Diamond et al., 2011; Fuertes et al., 2011). If the immune response in the Elimination phase achieves tumour elimination, the Immunoediting process is complete without the need to proceed to the next steps.

1.9.2 Phases of Immunoediting: Equilibrium

If some of the tumour cells develop resistance to host's immune response and survive, the second phase of Immunoediting process takes place. Equilibrium is the phase where although the tumour has not been destroyed, it is not clinically overt either – a state of balance between cancer and its host.

The first clinical hypothesis for the existence of this phase came from retrospective observation of clinical cases of cancer transfer following organ transplantation. MacKie, Reid and Junor (MacKie, Reid, & Junor, 2003) described the very interesting case of two kidney transplant recipients from the same cadaver donor, who both developed melanoma while their donor had been successfully treated for melanoma also 16 years before his death. It was later proposed that a possible explanation was that the melanoma cancer cells remained in equilibrium phase in the donor's body, but became clinically apparent when they were transferred to the recipients whose immune system was compromised to protect against graft-host disease.

This hypothesis was later tested in a preclinical model designed to reproduce the Equilibrium phase by Koebel et al (Koebel et al., 2007). In this study, they observed that mice previously treated with low doses of MCA (Monochroloacetic acid a hazardous chemical agent) remained cancer free for 200 days. After 200 days, the mice received a mixture of monoclonal antibodies against $CD4^+$ and $CD8^+$ cells which blocked the IFN-γ. Shortly after that, the mice developed sarcomas at the site of MCA injection. Consistently with these findings, it was later confirmed that the driver of the Equilibrium phase resides in the adaptive immunity components $CD4^+$ and $CD8^+$ cells, IFN-γ, the blockade of which allowed the dormant tumour cells to manifest clinically, producing cancers. On the other hand, monoclonal antibodies (mAb) against the innate immunity components [eg: (anti-NK1.1 inhibiting NK cells), (anti-NKG2D inhibiting NK cell recognition), (anti-TRAIL blocking NK cell effector function)], didn't seem to produce similar effect.

It was found that the dormant cancer cells have the ability to retain actively proliferating lymphocytes, suggesting that the cells in Equilibrium phase remain highly immunogenic and thus unedited, while the clinically apparent cancers have been immunologically edited thus displaying modified immunogenicity (Screiber, 2016). Further preclinical and clinical studies in other tumour types have shed more light in the role of other mechanisms involved in this phase such as the p53 depletion (Lai et al., 2012) and a IFN-γ and TNF - dependent mechanism promoting T cell- mediated cancer growth arrest (Braumuller et al., 2013).

1.9.3 Phases of Immunoediting: Escape

The third phase of cancer immunoediting is called Escape, marking the prevail of cancer escaping mechanism over the immunosurveillance of the host. The recent years more evidence came into light to explain the mechanisms which help cancer cells to survive and the underlying

pathways constituting one of the proposed hallmarks of cancer. Fig 1.4 outline of the six proposed hallmarks of cancer according to the work presented by Hanahan and Weinberg, (Hanahan & Weinberg, 2011).

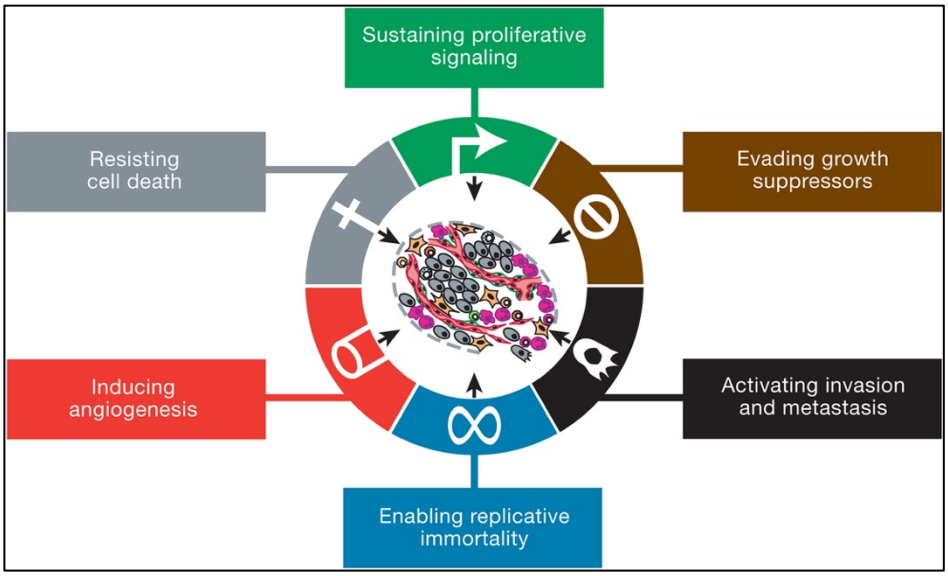


Figure 1. 4 The six hallmarks of cancer—distinctive and complementary capabilities that enable tumour growth and metastatic dissemination. The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumours. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. – Adapted from The Hallmarks of Cancer : The Next Generation (Hanahan & Weinberg, 2011).

Immune escape involves a wide range of mechanisms and pathways involving either the tumour cells or/and the microenvironment of the tumour. Firstly, the tumour cells can avoid the immune recognition by downregulating the MHC I, beta 2 microglobulin and calreticulin, through loss of NKG2D ligands, or through reduction of co-stimulatory molecules, or/and antigen loss (Dunn et al., 2004a; Vesely, Kershaw, Schreiber, & Smyth, 2011). Secondly, the tumours resist cell death (apoptosis) and promote survival through other pathways controlled by upregulation of proteins (eg: STAT-3, Bcl2 (an anti-apoptotic molecule) (Yu, Pardoll, & Jove, 2009).

Another important step for the survival of tumour cells is the development of an immunosuppressive microenvironment with the help of suppressive immune cells and cytokines

which are recruited in the tumour periphery. Such components include the myeloid-derived suppressor cells and regulatory T cells (T regs), the cytokines Il-10 and transforming growth factor beta (TGFβ) and the expression of immune checkpoints by the immune cells, or/and the tumour cells such as the cytotoxic T lymphocyte antigen -4 (CTLA-4), the programmed death ligand 1 (PD-L1)/PD-1, the lymphocyte activation gene 3 (LAG-3), the T cell immunoglobulin and mucin domain 3 (TIM-3) (Mellman, Coukos, & Dranoff, 2011).

Other molecules have been also identified to be implicated in the pathways of tumour-induced immune suppressing mechanisms such as B and T lymphocyte attenuator, CD73, V-domain Ig suppressor of T cell activation (VISTA), T cell Immunoglobulin and ITIM Domain (TIGIT), (Chauvin et al., 2015; Gavrieli, Watanabe, Loftin, Murphy, & Murphy, 2003; Jin et al., 2010; L. Wang et al., 2011).

The first among those molecules with negative regulatory effect identified and targeted in mice was the CTLA-4, a negative co-stimulatory receptor playing a critical role for the maintenance of immune homeostasis and the prevention of autoimmunity (Leach, Krummel, & Allison, 1996). Humans treated with high dose anti-CTLA-4 suffer from severe autoimmune conditions while the mice lacking CTLA-4 develop highly aggressive lymphoproliferative diseases (Gangadhar & Vonderheide, 2014). This was also the first molecule targeted in humans with a new category of drugs marking a new era for cancer immunotherapy as it was found that the attenuation of T cell activation by cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) further limits the potency of tumour immunity (Hodi et al., 2003). It was later found from preclinical and clinical studies that T cells' failure to respond to tumour antigens could be explained by the presence of CTLA-4 and that we can enhance the T cells' immune reactivity by treating the patients with antibodies against CTLA-4. It became evident that there is a potentially delicate balance between the anticancer activity of the immune system and the autoimmune consequences of this activity (Sharma & Allison, 2015a, 2015b; van Elsas, Hurwitz, & Allison, 1999; van Elsas et al., 2001).

Immune checkpoints regulate different components in the evolution of an immune response, Fig 1.5 (Pardoll, 2012). During T cell activation, CD28 interacts with CD80/86 (B7.1/B7.2) expressed on the surface of antigen presenting cells (APCs) and functions as a positive co-stimulatory molecule to the T cells responding to the presented antigen. The CTLA-4 expression on $CD4^+$ and $CD8^+$ T cells is delayed compared to the expression of CD28, its activating

counterpart, thus timing plays a critical role in the induction of T cell response. CTLA -4 displays higher affinity to CD80/86 than CD28 and therefore it engages preferentially with the CD80/86 on target cells (producing a negative co-stimulatory signal on T cell activation). More specifically, during the initial encounter of the T cell with the antigen, a CTLA-4 -mediated immune checkpoint is triggered in T cells. The magnitude of initial T-cell receptor (TCR) -mediated signalling affects the level of the CTLA-4 induction, in such way that high-affinity ligands are able to induce higher levels of CTLA-4, which in turn dampens the magnitude of the initial response. It is at this stage that the timing of surface expression regulates the T cell activation levels by the CD28–CTLA-4 system. Naive and memory T cells express high levels of cell surface CD28, while the CTLA-4 is located in intracellular vesicles and is transported to the cell surface, following TCR triggering by antigen encounter. The amount of CTLA-4 that is deposited on the T cell surface is directly related to the strength of the stimulation through the TCR and CD28. As a result, the CTLA-4's function is to regulate the signal transmission in order to maintain a consistent level of T cell activation despite the wide range of ligand's concentrations and affinities for the TCR.

The second inhibitory pathway revealed, was the programmed cell death protein 1 (PD-1)-mediated (H. Dong et al., 2002; H. Dong, Zhu, Tamada, & Chen, 1999; Freeman et al., 2000). In contrast to CTLA- 4 which blocks T cell priming at the initial stage of T cell activation, the PD-1 dampens T cell effector functions by regulating the antigen recognition by the effector T cells and the resulting inflammatory response in the peripheral tissues. PD -1- dependent T cell inhibition occurs upon engagement of its ligands PD-L1 (B7-H1) and PD-L2 (B7-H2) which are expressed on the surface of the tumour cells and the microenvironment of the host cells (Latchman et al., 2001). Following antigen stimulation, T cell receptor (TCR) signalling promotes PD -1 upregulation which then becomes highly expressed upon continuous TCR signalling (Barber et al., 2006). Thus, activated T cells upregulate PD1 and continue to express it in tissues.

In contrast, expression of PD-L1 by a wide variety of immune and non-immune cells (T cells, NK cells, monocytes, macrophages, DC, B cells, epithelial cells, vascular endothelial cells) induced by inflammatory signals in tissues (such as IFN-γ), downregulates the activity of T cells and thus limits collateral damage to the tissues due to potentially destructive T cell response to a microorganism infection (Loke & Allison, 2003). The most recognized signal for PD-L1 ligand 1 (B7-H1) induction is interferon- γ (IFN-γ), which is predominantly produced by T helper 1

(TH1) cells. Excessive induction of PD1 on T cells by chronic antigen exposure can induce an exhausted or anergic state in T cells. It seems that some tumours express high levels of PD-L1 which appears to be one of the escape mechanism during the Elimination phase of Immunoediting (Iwai et al., 2002).

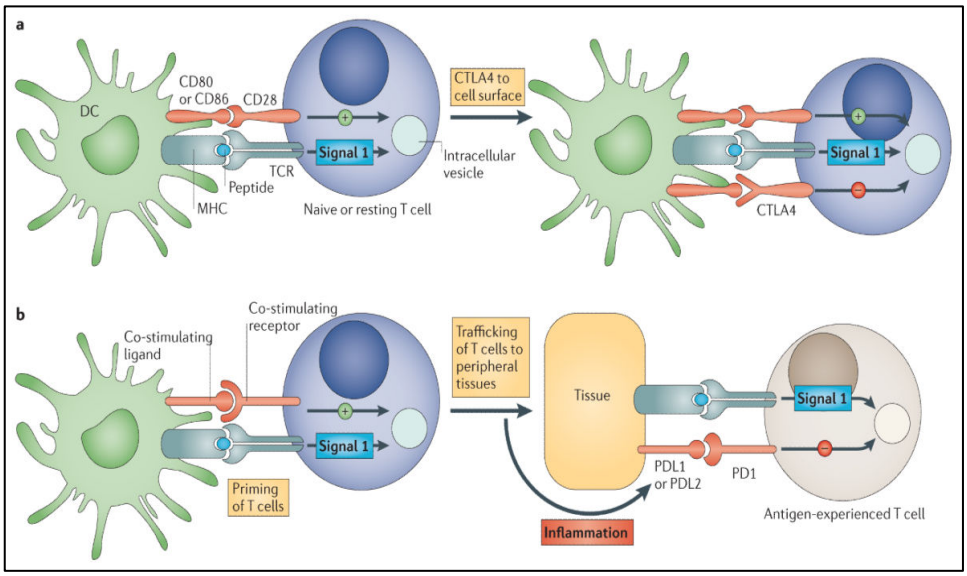


Figure 1. 5 Immune checkpoints regulate different components in the evolution of an immune response. (A) The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)-mediated immune checkpoint is induced in T cells at the time of their initial response to antigen. The level of CTLA-4 induction depends on the amplitude of the initial T cell receptor (TCR)-mediated signalling. High-affinity ligands induce higher levels of CTLA-4, which dampens the amplitude of the initial response. The key to the regulation of T cell activation levels by the CD28–CTLA4 system is the timing of surface expression. Naive and memory T cells express high levels of cell surface CD28 but do not express CTLA-4 on their surface. Instead, CTLA-4 is located in intracellular vesicles. After the TCR is triggered by antigen encounter, CTLA-4 is transported to the cell surface. The stronger the stimulation through the TCR (and CD28), the greater the amount of CTLA4 that is deposited on the T cell surface. Therefore, CTLA4 functions as a signal dampener to maintain a consistent level of T cell activation in the face of widely varying concentrations and affinities of ligand for the TCR. (B) By contrast, the major role of the programmed cell death protein 1 (PD1) pathway is not at the initial T cell activation stage but rather to regulate inflammatory responses in tissues by effector T cells recognizing antigen in peripheral tissues. Activated T cells upregulate PD1 and continue to express it in tissues. Inflammatory signals in the tissues induce the expression of PD1 ligands, which in turn downregulate the activity of T cells, limiting collateral tissue damage in response to a microorganism infection in that tissue. The best characterized signal for PD1 ligand 1 (PDL1; also known as B7-H1) induction is interferon- γ (IFNγ), which is predominantly produced by T helper 1 (TH1) cells, although many of the signals have not yet been defined completely. Excessive induction of PD1 on T cells in the setting of chronic antigen exposure can induce an

exhausted or anergic state in T cells. MHC, major histocompatibility complex. Adapted from Nat Rev Cancer (Pardoll, 2012).

This dynamic process of immune surveillance and immune editing is depicted in the of Janus, the Roman god of beginnings and transitions, adapted by (Finn, 2012) Fig 1.6. Janus' principle can be used to illustrate the two faces of scientific progress, the past accomplishments and the future opportunities. Equally, the two faces could be used to represent two sides of the same story, which in our immune system find the perfect paradigm, on one hand the immune function/ tumour rejection and on the other hand the immune dysfunction/tumour promotion. Through the process of immunosurveillance, the immune system can specifically identify and eliminate tumour cells on the basis of their expression of specific antigens.

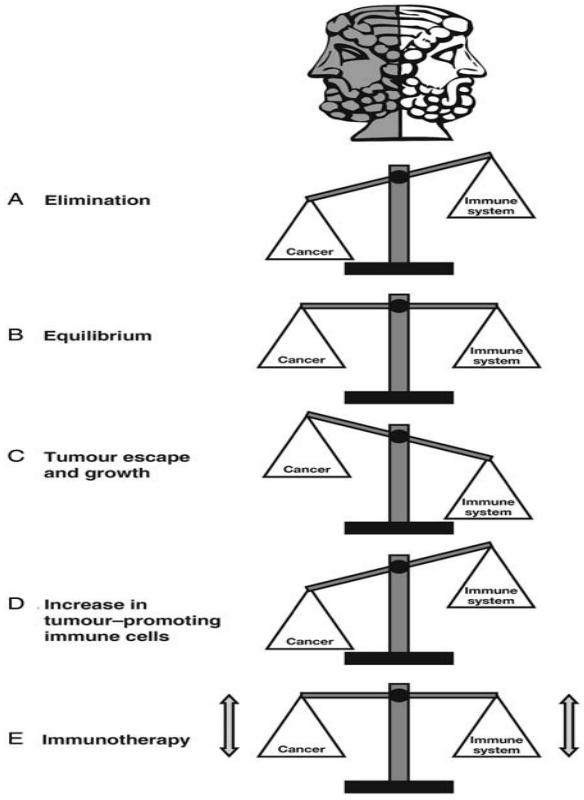


Figure 1. 6 Janus was the Roman god of beginnings and transitions, depicted as two-faced since he looks to the future and the past. A) When the immune system is not able to completely eliminate the cancer, but it can control it, a state of equilibrium develops whereby the tumour does not progress or further metastasize B) Eventually, if the immune response fails to completely eliminate the tumour, cancer cells that can resist, avoid, or suppress the anti-tumour immune response are selected, leading to the tumour escape and a progressively growing tumour C) Additionally, infiltration of tumours by inflammatory immune cells can result in a state of chronic inflammation maintaining and promoting cancer progression, while it suppresses

the innate anticancer immune response D) The aim of immunotherapy is to modulate tumour immunity to change the ongoing immune response from tumour-promoting to tumour-rejecting, thus providing durable and adaptable cancer control (E). Adapted from (Finn, 2012)

1.10 Immunotherapy: Active and Passive

Immunotherapy can be broadly divided into two major categories. Active immunotherapy which focuses on the stimulation of the host's immune system or a specific immune response to a disease or pathogen. This type of immunotherapy is widely used in the cancer treatment and in a variety of neurologic and neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, prion disease, and multiple sclerosis (Brody & Holtzman, 2008; Davis, 2000). Active immunotherapies induce an immune response through direct immune system stimulation and it is further categorized into specific and non-specific depending on the elicited response.

Non-specific active immunotherapy produces a general immune system response using cytokines and other stimulatory cell signalling molecules, whereas specific active immunotherapy focuses on the generation of cell-mediated and antibody immune responses targeting specific antigens expressed by the cancer cells, a concept which finds excellent application in the cancer vaccines (Monjazeb, Hsiao, Sckisel, & Murphy, 2012; Sheng & Huang, 2011).

Additionally, active immunotherapy has an immunological memory, in contrast to passive immunotherapy which produces a temporary anti-tumour effect and chronic administration is required to maintain the effect. In passive immunotherapy, the host immune's response is initiated by external antibodies or other immune components such as checkpoint inhibitors and cytokines that are generated in a laboratory.

One of the technologies developed to augment the immune response in passive immunotherapy is the adoptive transfer, which refers to transfer of immune components that can in turn induce a specific immune response readily available. This approach finds its best application in the monoclonal antibodies (MAbs) for cancer such as Trastuzumab (Herceptin) antibody targeting human epidermal growth factor receptor 2 (HER-2) proteins in breast and stomach cancer (Baxter, 2014).

1.11 Development of cancer vaccines

Theoretically, the ideal cancer vaccine has the ability to induce specific immunity against tumours, maintaining a sustainable and durable immune response leading to their death, while sparing the normal tissues. The generation of an effective immune response against tumours requires a cascade of events to take place: 1) presence of immunogenic tumour-antigens, 2) recruitment and maturation of antigen-presenting cells (APCs), mainly dendritic (DCs), 3) uptake of the antigens by mature APCs and their processing, 4) the presence of co-stimulatory signals and stimulatory cytokines in the absence of inhibitory molecules, 5) induction of T-helper-1 (Th1) cells, $CD8^+$ T cytotoxic lymphocytes (CTLs), antibodies of high specificity and high titre (H. Schreiber & Greenberg, 2015; Screiber, 2016).

In the early 80s researchers shed light in the existence and function of tumour-specific $CD8^+$ T cytotoxic lymphocytes (CTLs) in humans by showing that these T-lymphocytes, expanded in vivo, were capable of autologous tumour lysis in the presence of stimulation by IL-2 (Vose & Bonnard, 1982a, 1982b). In the late 80s more data supported the theory that tumour cells express antigens which are distinguishable from the normal cells, while adoptive transfer of tumour-infiltrating lymphocytes could induce an effective immune response leading to destruction of such tumours (Luescher, Romero, Cerottini, & Maryanski, 1991; Rosenberg, Schwarz, & Spiess, 1988; Rosenberg et al., 1998). The immunotherapy started focusing on the development of cancer vaccines that activate T cells to treat growing tumours (Rosenberg, Yang, & Restifo, 2004).

The immune response to cancer requires activation of specific T cells that recognize tumour antigen-derived peptides presented by MHC molecules. The cancer cell proteins are degraded to peptide fragments in the proteasome, transferred through the transporter associated with antigen processing (TAP) to the endoplasmic reticulum (ER), where they are complexed with the MHC class molecules to be transferred to the cancer cell surface with the help of the Golgi apparatus (Hansen & Bouvier, 2009; Leone et al., 2013).

1.11.1 Selection of tumour antigens

Tumour-associated antigens are expressed in tumour cells and can be recognized by T lymphocytes, resulting in activation of the immune system (Parmiani et al., 2002). Cancer antigens are broadly divided into four categories: 1) oncofetal antigens, fetal proteins which are not expressed normally in adult tissues but due to epigenetic alterations in tumours,

they are re-expressed, 2) viral antigens which typically occur in virus-triggered cancers, 3) neoantigens which are the results of somatic mutations, 4) differentiation antigens that are normal cellular proteins but overexpressed in cancers. The first three categories are ideal targets for cancer vaccines as they are almost exclusively expressed in cancer cells nut not in normal tissues. On the other hand, the differentiation antigens are self- antigens which are usually expressed in normal tissues albeit at low levels. The challenge here is that in order to develop effective immune response against them, we need to overcome the immune tolerance developed against self -antigens (Screiber, 2016).

The first human tumour antigens identified in nineties were in melanoma patients, using different approaches that not only lead in the discovery of the antigenic targets, but it also provided important information about the peptide-MHC interactions in tumour cells and the induction of immune responses with antigen -specific CTLs. These antigens were the MAGE-A1, a germline encoded gene of the family of cancer testis (C/T) antigens (Coulie et al., 1994), and a differentiation antigen MART-1 (van der Bruggen et al., 1991). They used an innovative approach of screening tumour-reactive autologous CTL clones against tumour-derived cDNA expression, which helped to identify those epitopes' sequences which are recognized by the CTL clones, an approach which later was confirmed to be at the core of immunotherapy (Coulie, Van den Eynde, van der Bruggen, & Boon, 2014). A biochemical approach, followed by Cox et al., used tandem mass spectrometry for screening of HLA-A2.1-bound -antigen peptides and discovered an antigenic peptide from the protein gp100 recognized by CTL clones from melanoma patients (A. L. Cox et al., 1994).

Subsequently, other researchers developed different strategies to identify tumour antigens eliciting antibody responses, such as the serological analysis of recombinant tumour-derived cDNA expression libraries (SEREX) by Sahin et al., (U. Sahin et al., 1995) and Chern et al., who discovered an antigen, highly expressed in different cancers but rarely- if at all- in normal tissues except the gonads ovary and testis, the germline-encoded antigen NY-ESO-1 (F. Chen, Liu, Zhang, Li, & Cai, 1997; H. Chen et al., 2000).

1.11.2 T cell Epitopes and Antigen Recognition by MHC

Prerequisite for an effective immune response and T cell activation is the successful processing of the antigen and a strong peptide-MHC interaction. Antigen recognition by T-cell receptors differs from recognition by B-cell receptors and antibodies. The immunoglobulin on B cells

binds directly to the intact antigen, and antibodies typically bind to the surface of protein antigens, coming in contact with the amino acids, whereas T cells respond to short, continuous amino acid sequences, which could be even buried within the native structure of the protein. Thus, antigens cannot be recognized directly by T-cell receptors unless the protein is unfolded and processed into peptide fragments to be presented by MHC molecule. Each allotype of the human MHC class I molecules binds a specific peptide motif, corresponding to a non-overlapping pattern of amino acids, (Carreno, Anderson, Coligan, & Biddison, 1990; Falk, Rotzschke, Stevanovic, Jung, & Rammensee, 1991). The T cells can recognize peptides and are stimulated by them, only when they are bound to an MHC molecule. The ligand recognized by the cell is a complex of peptide and MHC molecule. The T-cell receptor interacts with this ligand by making contacts with both the MHC molecule and the antigen peptide (Rivoltini et al., 1996). There are two classes of MHC molecules—MHC class I and MHC class II—and they differ in both their structure and their expression pattern in the tissues of the body. In regard to their structure, they mostly differ in their subunit composition. They both have two paired protein domains closest to the membrane, which resemble immunoglobulin domains, whereas the two domains furthest away from the membrane fold together to create a groove or cleft, which is the site of the peptide binding, Fig 1.7.

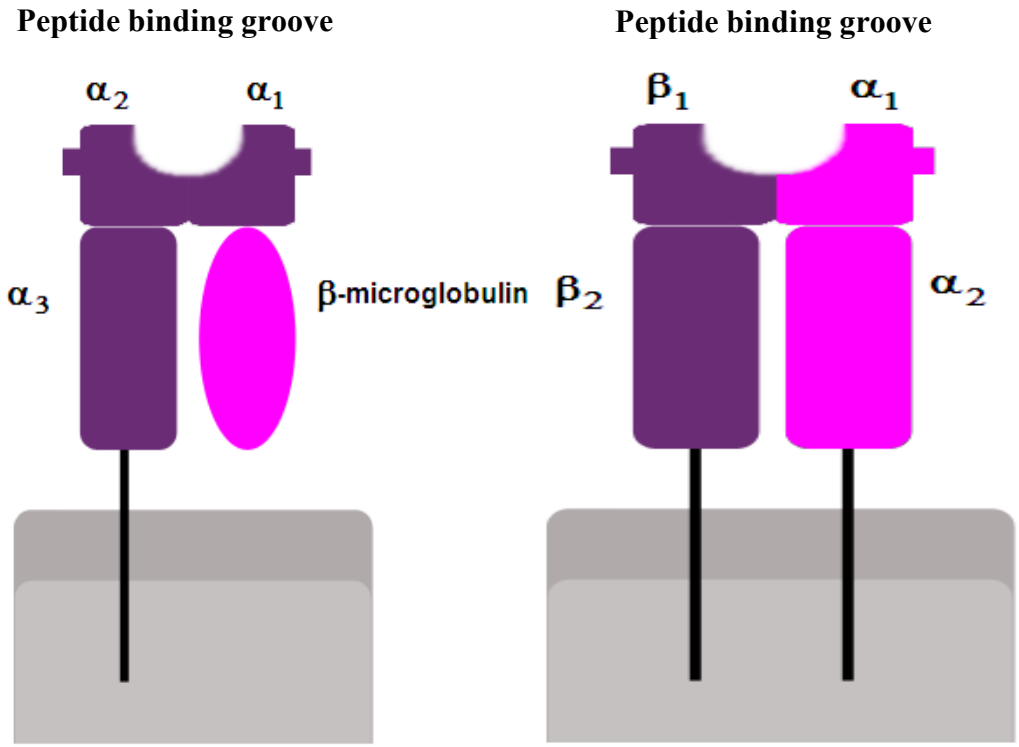


Figure 1. 7 The structure of MHC molecules. *Left*: The MHC class I molecule is a heterodimer of a membrane-spanning α chain bound non-covalently to β2-microglobulin, which does not

span the membrane. The α chain folds into three domains: α1, α2, and α3. The α3 domain and β2-microglobulin show similarities in amino acid sequence to immunoglobulin C domains and have similar folded structures, whereas the α1 and α2 domains are part of the same polypeptide and fold together into a single structure consisting of two separated α helices lying on a sheet of eight antiparallel β strands. The folding of the α1 and α2 domains creates a long cleft or groove, which is the site at which peptide antigens bind to the MHC molecules. For class I molecules, this groove is open at only one end. ***Right***: The MHC class II molecule is composed of two transmembrane glycoprotein chains, α and β. Each chain has two domains, and the two chains together form a compact four-domain structure similar to that of the MHC class I molecule on the left. The α2 and β2 domains, like the α3 and β2-microglobulin domains of the MHC class I molecule, have amino acid sequence and structural similarities to immunoglobulin C domains; in the MHC class II molecule the two domains forming the peptide-binding cleft are contributed by different chains and are therefore not joined by a covalent bond. Another important difference, not apparent in this diagram, is that the peptide-binding groove of the MHC class II molecule is open at both ends. Figure adapted from Murphy, Kenneth; Casey Weaver. Janeway's Immunobiology, 9th Edition (Pages 156- 157). Garland Science. Kindle Edition.

In vitro studies showed a direct association between MHC binding affinity and peptide immunogenicity (Salgaller, Marincola, Cormier, & Rosenberg, 1996). In the next years, research on cancer vaccines is more likely to focus on bioinformatics in order to identify potential target cancer antigens through algorithms and prediction -tool models comparing normal tissues with tumours for CTL epitopes (Kuksa, Min, Dugar, & Gerstein, 2015; Viatte, Alves, & Romero, 2006).

For T cells to be activated, two signals are required. The first one is antigen specific and is provided by the T cell receptor (TCR) through its interaction with peptide-MHC molecules on the membrane of the antigen presenting cells (APC). The second one is co-stimulatory, antigen nonspecific and it is provided by the interaction of the co-stimulatory molecules expressed on the membranes of the T cells and the APC. CD28 and ICOS (Inducible Costimulator) are examples of co-stimulatory molecules expressed by T cells. The CD28 interacts with CD80 (B7.1) and CD86 (B7.2) on the membrane of the APC, while the ICOS interacts with the ICOS-L. T cell co-stimulation is crucial for T cell proliferation, differentiation and survival, thus for an effective immune response. If this process fails, and T cells are activated without co-stimulation, this may ensue to T cell anergy, T cell deletion or the development of immune tolerance (C. Janeway, 2005; Seda & Mraz, 2015).

1.12 Cancer Vaccines

Recent advances in the development of cancer vaccines were enabled hugely by our improved knowledge of mechanisms underlying important steps in the process of tumour antigen presentation and the resulting activation of T cells. These include the role of DC and MHC molecules in the antigen presentation, the context of TCR recognizing specific antigenic epitopes and the ability of the cancer cells to produce cytokines which in turn have immunostimulatory effect in the host of the tumour, enhancing the immunogenicity of the cancer itself and the magnitude of the host's immune response (Colombo & Parmiani, 1991; Coulie et al., 1994; van der Bruggen et al., 1994; van der Bruggen et al., 1991).

Based on this knowledge, different cancer vaccine strategies have emerged. Thus, vaccine preparations can be based on: a) specific antigens in the form of peptides, full length proteins, or genetically encoded vectors, b) whole tumour cell and its associated stroma/vasculature, c) loading of the tumour cell or specific antigen onto autologous DCs ex vivo which in turn is administered to the patients.

1.12.1 Tumour cell vaccines

1.12.1.1 Autologous tumour cell vaccines

The concept of tumour cell vaccines was one of the first introduced in the field, in late seventies and it involved tumour cells, isolated from patients, irradiated and combined with immunomodulatory adjuvant such as BCG, before re-administered to the same individual (Berger, Kreutz, Horst, Baldi, & Koff, 2007; Hanna & Peters, 1978; Harris et al., 2000; Maver & McKneally, 1979; Schulof et al., 1988). One of the most important advantages of this approach is the potential to target a wide spectrum of TAA (Hanna, Hoover, Vermorken, Harris, & Pinedo, 2001) and hence its use in a variety of different neoplasms such as prostate cancer (Berger et al., 2007), renal cell cancer (S. J. Antonia et al., 2002; Fishman et al., 2008), colorectal cancer (de Weger et al., 2012; Hanna et al., 2001; Harris et al., 2000), lung cancer (Nemunaitis & Nemunaitis, 2003; Ruttinger et al., 2007; Schulof et al., 1988) and melanoma (Baars et al., 2002; Berd, Maguire, McCue, & Mastrangelo, 1990).

The immunogenicity of autologous tumour cells can be also modified to acquire higher immunostimulatory potential. Preclinical and clinical trials with Newcastle disease virus (NDV)-infected autologous tumour cells showed that they can induce tumour protective immunity in animal tumour models (Plaksin et al., 1994), but are also safe and effective inducing antitumor

immune memory in human cancer patients (Karcher et al., 2004; Schirrmacher, 2005; Steiner et al., 2004). Other modes of immunity engineering can also be effective, inducing tumour suppression in mice accompanied by high IFN-γ production and increased activation of cytotoxic T lymphocyte (CTL) and natural killer (NK) cells e.g., with the use of tumour cells engineered to express IL-12, a cytokine which is able to promote Th1 immunity (Asada et al., 2002) or with a co-stimulatory molecule B7-1 (Fishman et al., 2008).

An example of autologous tumour cell vaccines studied in multiple preclinical and clinical studies is the Granulocyte - Macrophage Colony Stimulating Factor (GM-CSF)-transduced autologous tumour cell vaccines (GVAX) which has been found to recruit DCs for antigen presentation and priming of $CD8^+$ T cells (Armstrong et al., 1996; Z. Dong, Yoneda, Kumar, & Fidler, 1998; Dranoff et al., 1997; Mach et al., 2000; Soiffer et al., 2003). Enhanced anti-tumour effect was observed when GVAX was combined with blockade of CTL-associated antigen 4 (CTLA-4), and the combination alters the intratumour balance of effector (Teff) and regulatory (T-reg) T cells, a strategy that could be proven promising for the future of immunotherapy (Leach et al., 1996; Peggs, Quezada, Korman, & Allison, 2006; Quezada, Peggs, Curran, & Allison, 2006).

Similar results were observed when a tumour cell vaccine designed to express Flt3 ligand (FVAX) was combined with blockade of CTLA-4 for the treatment of TRAMP prostate adenocarcinomas (Curran & Allison, 2009). In addition to CTLA-4, the programmed death-1 (PD-1) interaction with its ligand PD-L1/L2 or B7-1 also inhibits T cell activation and cytokine production (Butte, Keir, Phamduy, Sharpe, & Freeman, 2007). Apparently, the combination blockade of PD-1 and CTLA-4 have synergistic action with FVAX, but not GVAX, in maintaining control of the outgrowth of pre-established B16 tumours (Curran, Montalvo, Yagita, & Allison, 2010) suggesting that blockade of negative costimulatory pathways favours the expansion of tumour-specific T cells and maintenance of their effector functions, and results in shifting of the tumour microenvironment from an immunosuppressive state to an inflammatory/immunostimulatory state.

1.12.1.2 Allogeneic Tumour cell vaccines

Allogeneic whole tumour cell vaccines may be used to overcome certain limitations of autologous tumour cell vaccines and they usually contain two or three established human tumour cell lines. They may include multiple sources of tumour antigens and expression of

immunostimulatory molecules with standardized vaccine production, reliable analysis of clinical outcomes and cost-effectiveness. Canvaxin™ vaccine is an allogeneic whole-cell vaccine consisting of three melanoma lines combined with adjuvant BCG (Morton et al., 2002). In phase II trials for stage III and stage IV resected melanoma, patients who received Canvaxin™ as postoperative adjuvant therapy, had better outcome compared to standard groups (Hsueh et al., 2002; Morton et al., 2002).

Unfortunately these promising results were not confirmed in the phase III trials in patients with stage III and IV melanoma and the trials were discontinued (Sondak, Sabel, & Mule, 2006). Similarly, the good results from phase II trials using allogeneic GVAX vaccine for different tumours such as prostate (Simons et al., 2006) were not confirmed in the phase III (Antonarakis & Drake, 2010). In NSCLC ongoing research with an allogeneic tumour cell vaccine (belagenpumatucel-L) consisting of four NSCLC lines engineered to secret antisense oligonucleotide to immunosuppressive cytokine TGF-β2 provides a promising strategy for the treatment of NSCLC (Kelly & Giaccone, 2011; Nemunaitis et al., 2009).

1.12.2 Dendritic cell (DC) vaccines

The role of DCs as link between innate and adaptive immunity is well established and their biologic function as APCs has been extensively studied (Banchereau et al., 2000; Banchereau & Steinman, 1998). Many cancer immunotherapeutic strategies target DCs directly or indirectly and studies have showed that different DC subsets can direct in vivo development of distinct T cell populations and regulate different classes of immune responses (Pulendran et al., 1999). Interestingly, engagement of certain receptors on DCs may well induce immune suppression rather than stimulation (Li et al., 2012). Recent studies on the biology of DCs and their role in cancer immunotherapy demonstrate that in order to generate functional activation of DCs and subsequent innate and adaptive immunity against cancers, three interactive signals are required, focusing on adequate loading of MHC-peptide complexes to DCs for T cells priming, upregulation of co-stimulatory molecules such as CD40, CD80, and CD86, and production of cytokines capable of polarizing a Th1/Tc1 immune responses (Frankenberger & Schendel, 2012).

It was in the early nineties that researchers such as Steinman and Inaba provided the rationale for development of DC vaccines by culturing mouse DCs ex vivo from bone marrow precursors (Inaba et al., 1992), while human DCs were cultured from $CD34^+$ hematopoietic progenitors or

from peripheral blood -derived monocytes (Banchereau & Palucka, 2005). Tumour-associated antigens are loaded to patients' autologous DCs that are also stimulated by adjuvants and then administrated back into patients to induce anti-tumour immunity. These antigens include tumour-derived proteins or peptides, whole tumour cells, DNA/viruses and fusion of tumour cells and DCs and many of them have been tested in clinical trials for various neoplasms (Banchereau et al., 2001; G. Murphy, Tjoa, Ragde, Kenny, & Boynton, 1996; Palucka et al., 2006; Rosenblatt et al., 2011; Schuler-Thurner et al., 2002; Steele et al., 2011; Su et al., 2005).

The first of the cancer vaccines approved by the US FDA in 2010 was the Sipuleucel-T for the treatment of asymptomatic metastatic castrate-resistant prostate cancer based on the results of the clinical trial showing a survival benefit of 4.1 months (median OS 25.8 months for Sipuleucel-T treatment group vs 21.7 months for the placebo group) and a favourable toxicity profile. This autologous vaccine consists of APCs derived from PBMCs which have been intubated with PA2024 containing a prostate antigen, prostatic acid phosphatase (Longo, 2010). Despite its success and its approval by the health authorities marking a new era, the clinicians felt that the results were at best modest and research should focus on strategies to optimise the anti-tumour activity. In this context, modification of expression levels of activating or inhibitory molecules expressed on DCs which are involved in a pro-inflammatory or anti-tumour T cell response, could enhance the DC vaccine potency, while suppressive molecules can contribute to development of T tolerance or T cell suppression (Bonehill et al., 2008; Quezada, Jarvinen, Lind, & Noelle, 2004; Song et al., 2008). One strategy emerged recently is the one targeting SRA/CD204, an immune down-regulator of signalling pathways of DCs, attenuating the immunogenicity of DCs and CTL-mediated anticancer immunity (Yi et al., 2012). The absence or genetic silencing of SRA/CD204 enhances the immune-stimulating, antigen-presenting activity of DCs and the resulting immune response against cancer (Guo et al., 2012; Yi et al., 2011).

1.12.3 Peptide and Protein-based vaccines

1.12.3.1 The role of adjuvants in peptide-based cancer vaccines

The vast majority of the tumour antigens – except from the viral antigens and the neoantigens - are weakly immunogenic compared to foreign antigens. In such cases, adjuvants are important components of the vaccine preparations that have the ability to potentiate the immune response by helping in the activation, maturation and recruitment of the innate immunity cells, which will

subsequently be activated producing cytokines and other immunostimulatory molecules to elicit adaptive immune response.

Adjuvants are substances which are not antigenic themselves but help to induce strong and effective immune response against specific antigens (Wilson-Welder et al., 2009). Based on their mode of action, adjuvants can be classified into immune modulators, delivery vehicles or carriers with immunostimulatory effect (J. C. Cox & Coulter, 1997). They could enhance immune responses by the following mechanisms: 1) expedition of the immune response by activating innate immune responses or targeting antigens to antigen-presenting cells (APCs), 2) enhancement of the magnitude of the immune response, including the concentration and frequency of memory B and T cells, 3) maintenance of the immune response for a longer duration; 4) direction of the immune response toward the appropriate pathway (Th1, Th2, Th17, or balanced Th1/Th2), 5) modification of the specificity, affinity and isotype of the elicited antibodies. Different adjuvants have been used in the preparation of cancer vaccines with different effects in the immunologic outcomes (van Doorn, Liu, Huckriede, & Hak, 2016).

One of the most commonly used adjuvant is the pleomorphic cytokine Granulocyte-macrophage colony-stimulating factor (GM-CSF), an effective adjuvant for protein and peptide-based vaccine which affects the maturation and recruitment of DCs with impact in the stimulation of the CD^{+}T cells and the cross- priming of $CD8^{+}$T cells (Bowne et al., 1999; Disis et al., 1996). Moreover, recent studies showed that it could enhance tumour infiltration by vaccine-induced effector cells, e.g., virus-specific cytotoxic T lymphocytes can be efficiently expanded from granulocyte colony-stimulating factor-mobilized hemopoietic progenitor cell products ex vivo and transferred to stem cell transplantation recipients to enable immune reconstitution (Clancy et al., 2013).

Incomplete Freund's adjuvant (IFA) and similar oil-based adjuvants like Montanide combined with a triggering molecule of cytokine production can elicit $CD8^{+}$ T cell responses (Billiau & Matthys, 2001). Montanide ISA™ 51 (Seppic, France), a water-in-oil (W/O) emulsion composed of a mineral oil and a surfactant from the mannide monooleate family, is an adjuvant carrier with immune stimulatory effect (Aucouturier, Dupuis, Deville, Ascarateil, & Ganne, 2002; Wilson-Welder et al., 2009). When mixed with antigens in a ratio of 50/50 v/v (1:1), ISA 51 enhances antigen-specific antibody titers and cytotoxic T-lymphocyte (CTL) responses (Yamshchikov et al., 2001). The immune enhancing effect of ISA 51 is associated with its depot formation slowing

the release of antigens at the immunization site, the local inflammatory reaction stimulating the recruitment of APCs and the lymphocyte-trapping stimulating the accumulation of lymphocytes in draining lymph nodes (Aucouturier, Dupuis, & Ganne, 2001; Karbach et al., 2010). The combination of Incomplete Freund's adjuvant (IFA) and other oil- based adjuvants with an agonist for pattern recognition receptors (PRPs), which are expressed on the innate immune cells, could induce the production of proinflammatory cytokine to stimulate $CD8^+$ T cells (Billiau & Matthys, 2001).

More recent studies have combined the Montanide ISA-51, in peptide vaccines with synthetic agonists of different toll-like receptors (TLR) with variable results (Karbach et al., 2010). The choice of TLRs may have an impact on the type and the magnitude of the final immune response, as the expression of different TLRs varies in DC and myeloid cells (Dubensky & Reed, 2010; Sabado et al., 2015).

1.12.3.2 Tumour- associated antigens as targets of cancer vaccines

Recombinant vaccines are based on peptides from specific tumour-associated antigens (TAAs) and in most cases are combined with adjuvants or immune modulators. MAGE-1 was the first gene reported to encode a human tumour antigen recognized by T cells (van der Bruggen et al., 1991). The identification of TAAs has enabled researchers to design targeted therapeutic vaccines, using antigens that can be classified into several major categories.

As such, cancer-testis antigens (CT), such as MAGE, BAGE, NY-ESO-1 and SSX-2, are encoded by genes which are silenced in normal adult tissues but transcriptionally reactivated in tumour cells (De Smet et al., 1994; Gnjatic et al., 2010; Hofmann et al., 2008; Karbach et al., 2011). Tissue differentiation antigens on the other hand are of normal tissue origin but they are shared by both normal and tumour tissue, such as in melanoma (gp100, Melan-A/Mart-1 and tyrosinase) (Bakker et al., 1994; Kawakami et al., 1994; Parkhurst et al., 1998), prostate cancer (PSA, PAP) (Correale et al., 1997; Kantoff et al., 2010) and breast cancer (mammaglobin-A) (Jaramillo et al., 2002). Several tumour antigens that are highly expressed in tumours compared to normal tissues have been used in peptide-based vaccines such as MUC-1 (Finn et al., 2011), HER2/Neu (Disis et al., 2009), CEA (Tsang et al., 1995), tumor suppressor genes (p53) (Azuma et al., 2003) human Telomerase (hTERT) (Vonderheide, Hahn, Schultze, & Nadler, 1999) and anti-apoptotic proteins (i.e. livin/ survivin) (Schmidt et al., 2003).

A quite recent trend is targeting unique tumour-specific antigens, which are mutated oncogenes such as RAS and BRAF, an approach which seems very promising albeit very challenging, as these tumour-specific antigens are drivers of tumour proliferation, with higher chance to resist immunoselection, but equally are difficult to be identified (Brichard & Lejeune, 2008; Fox, Salk, & Loeb, 2009; Parmiani, De Filippo, Novellino, & Castelli, 2007).

Although the protein/peptide – based vaccines are considered cost effective, their main caveat is that in most cases they target only one or very few epitopes of the Tumour-associated antigens (TAA), restricting their capacity to induce both antigen-specific CTLs and antigen-specific helper T cells, a practice that seems to be more effective. In this context recent approaches use vaccines containing both CD4 and CD8 epitopes, or they alter the peptide sequence of TAAs in order to enhance the immunogenicity and increase potential for peptide binding to the MHC molecule or the T-cell receptor. This approach can theoretically induce higher levels of T-cell responses and or higher avidity of T cells (Dzutsev, Belyakov, Isakov, Margulies, & Berzofsky, 2007; Hodge, Chakraborty, Kudo-Saito, Garnett, & Schlom, 2005; Hou, Kavanagh, & Fong, 2008; Jordan, McMahan, Kemmler, Kappler, & Slansky, 2010; Rosenberg et al., 1998).

Despite the scientific rationale and the observed antigen-specific T cell responses, in clinical practice the results are far from satisfactory (Buonaguro, Petrizzo, Tornesello, & Buonaguro, 2011). That was reflected in the landmark phase III trial leading to the approval of ipilimumab (the first anti-CTLA-4 drug approved), where it was compared with the combination of ipilimumab and a gp100- based vaccine in advanced melanoma. In this trial there was no difference between the two arms in patients' outcome (overall survival) (Hodi et al., 2010). In contrast to these findings, more promising results were observed in another phase III clinical trial for locally advanced stage III and metastatic stage IV cutaneous melanoma, in which the patients were randomized to the combination of a melanoma vaccine [the gp100:209-217(210M) peptide vaccine] with high-dose interleukin-2 or to high-dose interleukin-2 (Schwartzentruber et al., 2011). The patients who received the combination had higher response rates, progression free survival and overall survival compared to the group of patients who received high-dose interleukin-2.

1.12.4 Genetic Vaccines

Another strategy for vaccines is to deliver the antigen or antigenic fragments in vivo by using viral or plasmid DNA vectors currying the expression cassettes. The major benefit of this

strategy is the easy delivery of multiple antigens thereby affecting multiple lines of immune response (Aurisicchio & Ciliberto, 2012). Although DNA vaccine platforms have shown promising results in preclinical studies, they haven't managed to translate this to humans' research landscape (M. A. Liu & Ulmer, 2005; Rice, Ottensmeier, & Stevenson, 2008).

1.13 The role of Telomerase in aging and cancer

Increasing evidence suggests that cancer is an aging-associated disease and that cancer and aging share many molecular pathways. One of the things aging and cancer seem to have in common, is the function of telomerase, a specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA and is thus responsible for *de novo* telomere extension in most adult tissues. Telomeres are repetitive DNA sequences at chromosome ends that are bound by shelterin, a protective protein complex, which prevents them from eliciting a DNA damage response (DDR). They appear to shorten with each cell division, eventually losing the ability to protect the ends of chromosomal DNAs from end-to-end fusions, mainly due to inability of the DNA replication machinery to fully replicate DNA ends, a process enabled due to silencing of telomerase. These fusions generate unstable dicentric chromosomes whose resolution results in a scrambling of karyotype threatening cell viability. Therefore, the length of telomeric DNA in a cell dictates how many successive cell generations its progeny can process through before telomeres are eroded and have consequently lost their protective functions, triggering entrance into apoptosis (Hanahan & Weinberg, 2011).

Telomerase activation could be used as a potential therapeutic target for age-related diseases, whereas abnormal telomerase expression and telomerase mutations have been associated with many different types of human tumours. Although some adult cell types, such as adult stem cells, have the ability to activate telomerase, this telomerase expression in stem cells is not sufficient to prevent progressive telomere shortening associated with increasing age (Bernardes de Jesus & Blasco, 2013; Blasco, 2007; Levy, Allsopp, Futcher, Greider, & Harley, 1992).

1.14 Telomerase and cancer

In most human cancers, activation of telomerase appears to be a hallmark, most likely associated with unlimited cell proliferation of tumour cells (Blasco, 2005; Shay & Wright, 2000). It is almost absent in non-immortalized cells but is expressed at significant levels in the vast majority of spontaneously immortalized cells, including cancer cells in humans. It appears to be an early

event in toumorigenesis, but not a prerequisite for generation of cancer (Hackett & Greider, 2002; Shay & Bacchetti, 1997). By ensuring maintenance of telomeres' length above a critically short point, telomerase prevents the induction of cellular senescence or apoptosis for the cancer cells, therefore allowing for tumour progression. In other words, the presence of telomerase activity is correlated with a resistance to induction of both senescence and apoptosis, whereas suppression of telomerase activity leads to telomere shortening and to activation of these two proliferation barriers.

Several activating mechanisms for telomerase have been proposed to work in cancer, such as different oncogenes including Myc and Wnt, which are believed to act as transcriptional regulators of telomerase, or alternative splicing and epigenetic alterations (Greider, 2012; Hoffmeyer et al., 2012; Kyo & Inoue, 2002; Wu et al., 1999). Recently in human melanomas, mutations have been described to increase transcriptional activity of the TERT promoter from generation of *de novo* consensus binding motifs for E-twenty-six (ETS) transcription factors (Horn et al., 2013; Huang et al., 2013). In addition to telomerase's role in maintaining telomeres above a critical length, other pathways (telomere-independent) may also be regulated by telomerase, and in particular its protein subunit TERT. As such, the ability of TERT to amplify signalling by the Wnt pathway, by serving as a cofactor of the β-catenin/LEF transcription factor complex, provides regulation of Wnt targets and metabolism, which in turn have an impact on cancer proliferation. Consistent with these broader roles suggesting cancer promotion and resistance to apoptotic mechanisms, TERT can be found associated with chromatin at multiple sites along the chromosomes, and not just at the telomeres (Masutomi et al., 2005; Park et al., 2009; E. Sahin et al., 2011).

On the other hand, the lack of telomerase is not a favourable event either, as it could also lead to increased chromosomal instability, driving cancer development when suppressor mechanisms fail, e.g. short telomeres due to lack of telomerase control mechanism could form the basis of genomic instability and cancer. A quite recent theory supports that the early event in the process of cancer could be the telomerase deficiency which causes genomic instability leading to cancer initiation, but it needs to be followed by activation of telomerase to allow for tumour cell survival and proliferation (Begus-Nahrmann et al., 2012; Ding et al., 2012; Feldser & Greider, 2007).

Our early knowledge regarding the role of telomerase in cancer has been extrapolated by studying the tumourigenesis in mice, a close model to human cancer process. In mice, the lack

of telomerase renders the mice resistant to spontaneous and induced tumourigenesis, except in the case of p53 deficiency cooperating with telomere dysfunction to promote carcinogenesis as observed when telomerase deficient mice are crossed with $p53^{+/-}$ or $p53^{-/-}$ (Artandi et al., 2000; Chang, Khoo, & DePinho, 2001; L. Chin et al., 1999; Gonzalez-Suarez, Samper, Flores, & Blasco, 2000). The short length of telomeres could induce their recognition as DNA double strand (dsDNA) breaks, which as a deleterious DNA aberration, results in a strong activation of DNA damage repair (DDR) pathways. If the DDR mechanism is intact and checkpoints cells are active, dsDNA breaks are able to activate a signalling cascade with p53 and tumour suppressor mechanisms.

However, if these cancer suppression mechanisms are bypassed or silenced, a commonly encountered tumour escape mechanism, chromosome fusions and genomic instability could lead to emergence of cancer. This could explain why telomerase regulation in adult tissues is crucial in the development of cancer and why in most adult cells telomerase expression is silenced. More support for the importance of transient telomerase deficiency in the process of cancer progression, comes from comparative analysis of premalignant and malignant breast lesions. Although the premalignant lesions did not express high levels of telomerase and were marked by telomere shortening and nonclonal chromosomal aberrations, the invasive carcinomas showed telomerase expression, concordant with reconstruction of longer telomeres and clonal outgrowth of the aberrant karyotypes. This net result was presumably acquired after telomere failure but before the acquisition of telomerase activity (K. Chin et al., 2004; Hanahan & Weinberg, 2011; Raynaud et al., 2010).

Not surprisingly telomerase has been considered a potential target for cancer immunotherapies. Interestingly, more recent evidence suggests that tumours which have lost telomerase function, may well adapt activating different pathways such as alternative telomere lengthening (Herrera, Martinez, & Blasco, 2000; Hu et al., 2012; Sachsinger et al., 2001).

1.15 Targeting the telomerase

Telomerase is a human ribonucleoprotein reverse transcriptase (hTERT) composed of two main subunits: the catalytic protein hTERT and the ribonucleoprotein template hTERT. Telomerase synthesizes telomeric DNA by adding continuously single stranded TTAGGG sequences onto the single stranded 3′ end of telomere in the 5′ to 3′ direction. Telomerase consists of 451 nucleotides but only the 11-base region, consisting of nucleotides 46 through

56 (5′ -CUAACCCUAAC-3′), serves as the template for telomere synthesis.
(Artandi & DePinho, 2010; Bisoffi, Heaphy, & Griffith, 2006; Blackburn, 2005; Dikmen, Wright, Shay, & Gryaznov, 2008; Tian, Chen, & Liu, 2010).

Telomerase, and more specifically its catalytic subunit hTERT, is found to be overactive in 85–90% of cancers, marking it as a popular target for anticancer therapies. In normal non-malignant cells telomerase is present in embryonic, male germline and some adult stem cells, in contrast to most somatic cells, where telomerase is present in nearly undetectable levels and is less active or inactive compared to cancer cells (Bisoffi et al., 2006).

There are two general strategies of telomerase targeting in cancer treatment. The one targets telomerase directly by inhibiting the activity of its catalytic subunit (hTERT) or its RNA template (hTER), leading to inhibition of telomerase activity (TA), telomere shortening and inhibition of cell proliferation. The second strategy targets the telomerase subunit indirectly thus blocking telomerase access to telomeres or inhibiting binding of telomerase-associated proteins leading to telomere uncapping and cell apoptosis (Harley, 2008; Ruden & Puri, 2013).

1.16 Telomerase-based immunotherapies

The immunotherapy approach, designed to induce $CD8^+$ cytotoxic T lymphocytes (CTL) response for hTERT antigens in different types of cancer, has shown more promising telomerase inhibition than other therapies (Shay & Keith, 2008). Due to telomerase's presence in most cancers, its peptides are universal telomerase-associated antigens (TAAs), capable of producing strong immune response. This response is manifested as induction of both $CD4^+$ and $CD8^+$ T-cell responses and stimulation of the hTERT peptide-specific CTL activity, a response which potentially lead to tumour cell lysis (Beatty & Vonderheide, 2008; Shay & Keith, 2008; Vonderheide, 2008). Although telomerase is normally considered a self-antigen against which development of tolerance is expected early in cell development, research suggests that immune tolerance to hTERT-specific antigens is not complete. Moreover, hTERT-specific CTLs are less detectable in the peripheral blood of healthy individuals compared to cancer patients (Vonderheide, 2008).

Additionally, in order to eliminate the self-tolerance and increase the immunogenicity of the hTERT, several strategies have been introduced in the development of vaccine, including the use of adjuvants like GM-CSF, and Montanide ISA^{TM}51 which potentiate the immunostimulatory

effect, and the use of cryptic peptide vaccines, in which one amino-acid residue of peptide has been replaced for another to enhance the affinity of that peptide to HLA molecules and stimulate the generation of peptide-specific CTLs.

Preclinical and clinical studies with hTERT peptides have shown promising results and many clinical trials are currently in phase III of development. The two most commonly used approaches in the hTERT-based immunotherapy appear to be the dendritic cell (DC) approach and the hTERT peptide vaccine approach, which we utilized in our present study (Ruden & Puri, 2013).

1.17 $TERT_{572}$-based vaccine- Rationale

Nearly all human tumour-associated antigens, including telomerase, derive from non-altered self-proteins, thereby are subjects of the immune tolerance. The HLA-I molecules can bind both dominant and cryptic peptides. The dominant peptides have a strong affinity for HLA-I alleles, are abundant on the cell surface, and are strongly immunogenic, whereas cryptic peptides are not as abundant on the cell surface, have weak HLA-I affinity, demonstrating weak immunogenicity or complete lack of immunogenicity. In contrast to dominant peptides, cryptic peptides are poorly expressed, thereby do not induce immune tolerance escaping massive clonal deletion. These characteristics of the cryptic peptides make them a favourable target, candidate for the development of a specific, peptide antitumor vaccine therapy. Moreover, the use of tumour non-specific antigens may be a better choice for anticancer vaccines since they are not dependant on adjuvants or the efficacy of delivery (Mavroudis et al., 2006; Menez-Jamet & Kosmatopoulos, 2009; Ruden & Puri, 2013).

In our studies with the peptide-based vaccine (hTERT- based), we tried to overcome the tolerance-related blunting of T cell responses, by using cryptic (low affinity for HLA) peptides for the induction of an antitumor immune response. However, as mentioned above, binding of wild type cryptic peptide antigens to HLA is usually unstable, with weak immunogenicity, and therefore challenging in regard to immune response possibly hampering T cell priming and activation. More recent research has focused on the development of optimized cryptic peptides with higher affinity binding to HLA.

Based on this approach, our peptide-based anticancer vaccine, known as Vx-001 (Vaxon Biotech, Paris, France), consists of a low affinity cryptic peptide $hTERT_{572}$ (RLFFYRKSV) and its optimized version, the $hTERT_{572Y(1)}$ (YLFFYRKSV), which has the first amino-acid residue

replaced with a modified tyrosine (Y1) residue. This sequence aims to enhance the peptide's affinity for HLA-I molecules and potentially can circumvent the self-tolerance issue. The $TERT_{572Y}$ peptide has been found to induce tumour immunity in HLA-A∗0201 transgenic mice but luckily not autoimmunity (Gross et al., 2004). In addition, Vx-001 leads to enhanced immunogenicity of the cryptic peptide when presented by HLA-A∗0201 molecules (the most frequently expressed allele, present in 40–45% of population) without altering antigen's specificity (Mavroudis et al., 2006).

Our group reported a dose-escalation phase I study, indicating that the administration of the optimized $TERT_{572Y}$ followed by vaccinations with the $TERT_{572}$ peptide was able to induce immune responses across all tested doses. Moreover, this vaccination protocol resulted in the induction of $TERT_{572Y}$-specific $CD8^+$ T cell immune response in the majority of the vaccinated patients and this peptide-specific immune response was correlated with prolonged survival (Hernandez et al., 2002; Mavroudis et al., 2006; Scardino et al., 2002).

Despite the fact that this immunization schedule was found to be effective in the induction of specific immune responses, it was not certain whether the administration of the 2 doses of the optimized TERT peptide ($TERT_{572Y}$) followed by 4 doses of the native peptide ($TERT_{572}$) was the best administration schedule for specific activation of the immune system or whether continued administrations of the optimized variant ($TERT_{572Y}$) would be preferable.

In the current study, our primary goal was to establish the best vaccination protocol, for administration of the two TERT peptides (the native $TERT_{572}$ and its optimized variant $TERT_{572Y}$) regarding its ability to elicit the best immunologic response in respect to ex vivo reactivity of peptide-induced CTLs. Following establishment of the best vaccination schedule, the study aims to 1) assess the safety profile of the TERT vaccine, 2) correlate the immunologic outcome with the clinical outcome of the patients who received the TERT vaccine.

2. CHAPTER 2: Patients & Methods

2.1 Patients

From 2007 to 2011, 142 patients with various types of advanced solid tumours and previous exposure to standard treatment including chemotherapy were enrolled in the telomerase peptide (hTERT) vaccination protocol.

The inclusion criteria were as follows: HLA-A*0201 haplotype, histologically proven malignancy, advanced disease (Stage IV or locally advanced/unresectable), older than 18 years, performance status by WHO of 0-2, at least one chemotherapy regimen prior to vaccination, adequate hematologic parameters (absolute neutrophil number ≥ 1,500/μl, absolute lymphocyte number ≥ 1,000/μl, platelets > 100,000/mm^3, hemoglobin > 10 g/dl, adequate renal function (creatinine < 2 mg/dl),adequate liver function (bilirubin < 1.5 times the upper normal value, transaminases <2.5 times the upper normal value). All patients were required to have failed prior standard treatment. The first line chemotherapy should have been finished at least 4 weeks before the enrollment in the study, any immunosuppressive or myelosuppressive treatment (chemotherapy, radiotherapy, systemic steroids) should have been discontinued at least 4 weeks prior to the study entry, capacity to understand and sign an informed consent for participation in the clinical trial, exclusion of pregnancy and commitment to use effective contraception method was ensured for women of childbearing age.

The exclusion criteria included: no prior standard treatment for their cancer, performance status >2, concurrent immunosuppressive or myelosuppressive therapy, significant co-morbidities including liver disease, renal disease or heart failure, co-morbidities requiring concurrent treatment with systemic immunosuppressive therapies.

The study complied with the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki, the updated ICH-GCP guidelines and was approved by the local ethics and scientific committees of the University Hospital of Heraklion (Greece), and the National Drug Administration (EOF) of Greece. The patients signed a written informed consent in order to participate in the study. The study was conducted in the Department of Medical Oncology of the University Hospital of Heraklion and the Laboratory of Translational Oncology of the Medical School of the University of Crete.

2.2 Patient Evaluation

Prior to the study entry, patients' medical history including information about their concurrent medications and previous therapies was taken by a member of the medical staff of the oncology department. Physical examination and requested FBC (full blood count) with differential count and serum biochemistry were also performed.

A baseline imaging assessment was performed prior to study entry and every twelve weeks (after the third and the sixth vaccinations) thereafter or when clinical signs of progression of disease indicated otherwise. FBC was repeated weekly, while clinical examination with serum chemistry every 3 weeks during the vaccination period and every month thereafter during the follow-up.

2.3 Response to treatment

The response to treatment was evaluated using the standard Response Evaluation Criteria in Solid Tumors Group (RESIST) (Therasse et al., 2000). Based on imaging studies, responses were categorised as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD) and were confirmed by an independent panel of radiologists. Time to progression (TTP) was determined by the time from the first treatment administration to the first date that disease progression was objectively documented. OS (overall survival) was calculated from the date of study entry to the date of death. Follow-up time was calculated from the day of first treatment administration to last contact or death. Immune responses were examined before the first injection, after the second and sixth injections, and after each boost vaccination for those patients continuing.

2.4 Peptides

The 9-mer cryptic native $TERT_{572}$ (RLFFYRKSV) peptide and its optimized variant $TERT_{572Y}$ (YLFFYRKSV), were synthesized initially by Epytop (Nimes, France) and later by Pepscan (Lelystad, The Netherlands). Quality assurance studies included confirmation of identity, sterility, and purity (95% for both peptides), as indicated by analytical high-performance liquid chromatography and were validated for identity by mass spectroscopy. No decrease in purity or concentration was observed after more than 2 years of storage at 80°C. Each peptide was prepared as a lyophilized powder (2 mg/vial) for reconstitution with 0.5 ml sterile water.

Two mg of each peptide in 0.5 mL of aqueous solution were emulsified with 0.5 mL of Montanide ISA51 immediately before being injected according to the manufacturer's instructions.

2.5 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 of either the native $TERT_{572}$ peptide or the optimized $TERT_{572Y}$, depending on the randomization schedule, every three weeks until disease progression as indicated in each result section. Both peptides were emulsified with Maintained 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.

2.6 Blood samples for immunomonitoring

Before each vaccination, 100ml peripheral blood in EDTA (ethylene diamine tetra acetic acid) we collected from each patient through a peripheral venous puncture. The time points of blood collection were set at baseline, prior to 3rd and 6th vaccination and before each boost administration of the peptide. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma, UK) density centrifugation and cryo-preserved in freezing medium (Section 2.9) at -80^{0}C until their future use the immune-assessment assays.

2.7 Isolation of peripheral blood mononuclear cells (PBMCs) from patients' blood

2.7.1 Principle of method

Isolation of PBMCs takes advantage of differences in cell density of the different blood components. Density gradient centrifugation of diluted whole blood layered over a density gradient medium yields PBMCs. Ficoll is a synthetic polymer, 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, which is the main advantage of its use. 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) Two subsequent washing steps remove remaining platelets. To store the cells for future assays, they

can be frozen and thawed when required. Dimethyl sulfoxide (DMSO) serves as a cryoprotectant for freezing PBMCs, but must be removed by washing after thawing, as it can become toxic to the cells on longer exposure. (Fig. 2.1) (Riedhammer, Halbritter, & Weissert, 2016)

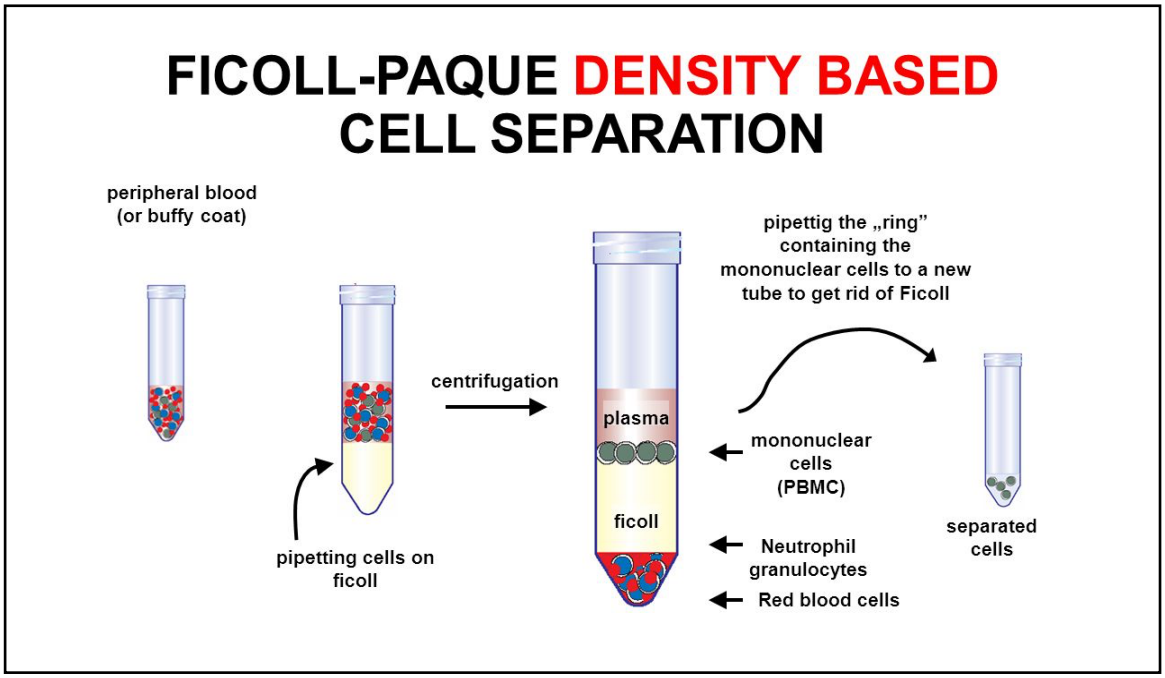


Figure 2. 1 Peripheral blood mononuclear cells can be isolated from whole blood by Ficoll-Hepaque centrifugation. Diluted anticoagulated blood (left panel) is layered over Ficoll-Hypaque and centrifuged. Red blood cells and polymorphonuclear leukocytes or granulocytes are more dense and centrifuge through Ficoll-Hypaque, while mononuclear cells consisting of lymphocytes together with some monocytes band over it can be recovered at the interface (right panel; adapted by Janeway, -Travers).

2.7.2 Experimental Procedure

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).

Firstly, blood was diluted with the washing solution in ratio1:1. 15ml of ficoll-hypaque were dispensed in sterile 50ml falcon tubes. 30ml diluted blood (2:1) was surfaced on the top gently, 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. Following centrifugation, the PBMCs-

containing interphase was collected carefully using a sterile pipette and it was transferred into a fresh 50ml falcon tube containing 15 ml washing solution. Subsequently, the cells were centrifuged at RT, at 2000 rpm for 15 min. The supernatant was discarded and the pellet was re-suspended in 15ml washing solution. The tubes were centrifuged at RT, at 1200 rpm for 10min. At the end, the pellet was re-suspended in 10ml complete medium and the cells were counted and frozen at -80ºC until future use.

2.8 Counting the PBMCs: Dye exclusion method.

2.8.1 Principle

Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. **The trypan blue dye exclusion test** is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue to penetrate, whereas dead cells do not. A viable cell will be clear in the centre whereas a nonviable cell will have a blue centre as the blue dye has penetrated through the membrane (Figure 2.2).

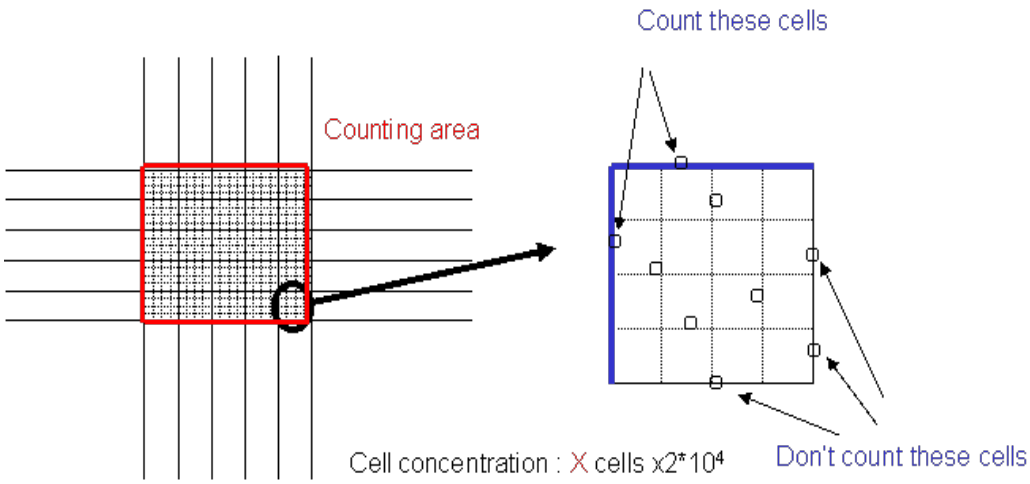


Figure 2. 2 PBMCs counting formula using dye exclusion method

2.8.2 Experimental Procedure

10µl PBMCs were mixed with 90µl Trypan Blue and were counted using the optical microscope (figure). The total number of PBMCs was estimated by using the following formula:

Total PBMCs number= Nº alive cells x 10^4 x dilution factor

2.9 Freezing and defrosting of PBMCs

2.9.1 Materials

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).

2.9.2 Freezing process

1ml of freezing medium was added in each cells-containing tube, so that the final concentration would be $10*10^6$ cells/ml. The tubes subsequently were placed into a Mr Frosty box, which was held at 4^0C and it is able to cause temperature reduction about 1^0C/min. The tubes-containing box was stored at -80^0C for future use.

2.9.3 Defrosting process

It took place each time prior to Elispot and Intracellular Staining (ICS) experiments. The frozen cell-containing tubes were removed from -80^0C and were placed into a water bath (at 37^0C) for a few minutes. In turn, the defrosted cells from each tube were dispensed in 20ml tubes containing 15ml washing solution and were centrifuged at 1200 rpm, at room temperature for 5 min. Following centrifugation, the supernatant was discarded, the pellet was resuspended in 1ml complete medium, and the cells were counted (Section 2.8).

2.10 Enzyme-linked immunosorbent spot (ELIspot) assay

2.10.1 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).

2.10.2 Principle

The enzyme-linked immunosorbent spot (ELISpot) assay is one of the most commonly used methods to measure antigen-specific T cells in both mice and humans. It is highly quantitative measuring a broad range of magnitudes of response from T and B cells and is capable of assessing critical cellular immune-related activities such as IFN-γ secretion and granzyme B release. Recent Phase I and II studies of cancer vaccines, tested in a variety of malignancies,

have suggested that ELISpot may be a useful biomarker assay to predict clinical benefit after therapeutic immune modulation. (Slota, Lim, Dang, & Disis, 2011).

ELISpot assay is a very sensitive method and it is designed to enumerate cytokine producing cells in a single cell suspension. This method was chosen due to its 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)

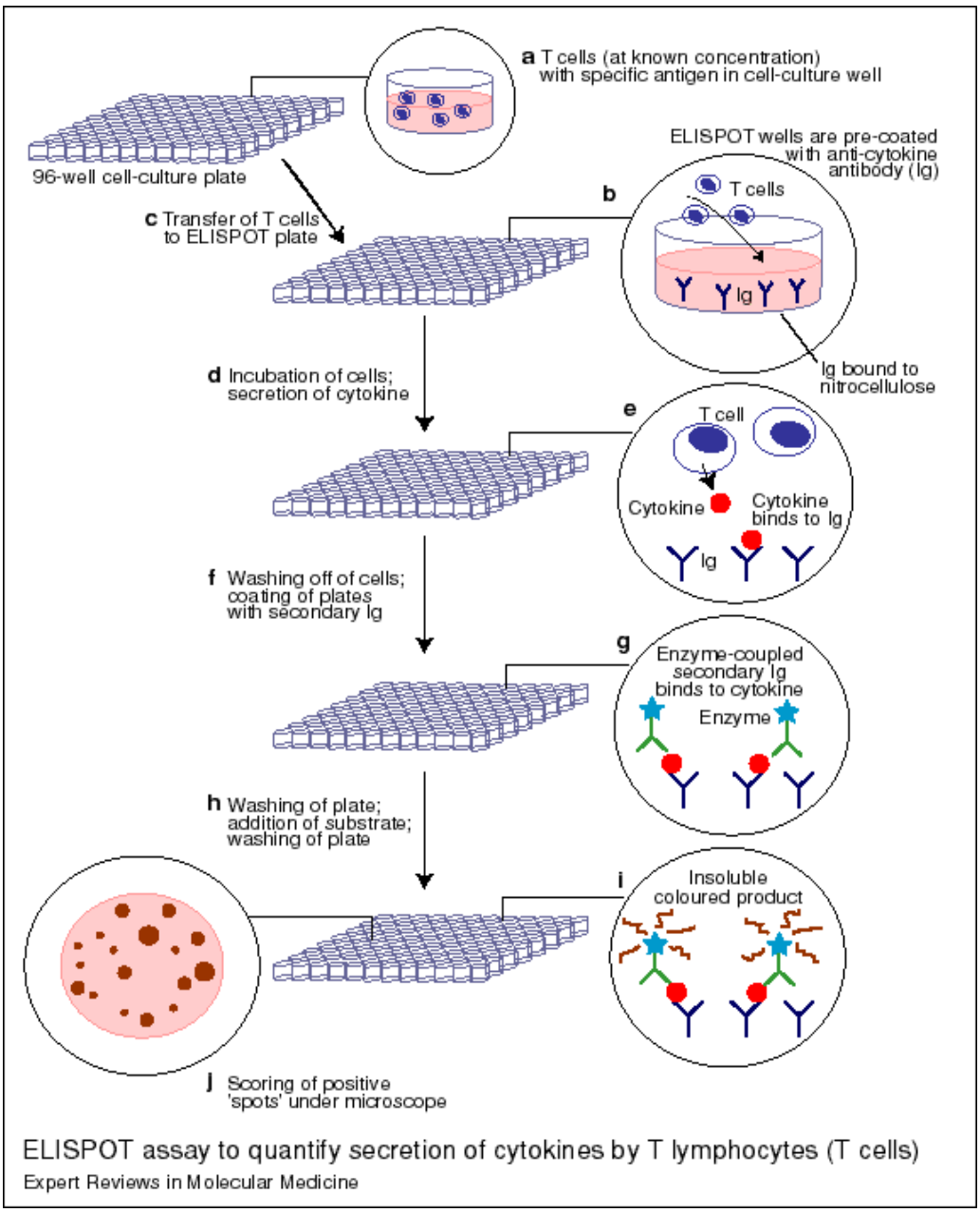


Figure 2. 3 ELISPOT assay to quantify the secretion of cytokines by T lymphocytes. (A) The T lymphocytes (T cells) are activated in vitro by being co-cultured with antigen. (B) The wells of the ELISPOT plate are coated with antibody (immunoglobulin; Ig) that is specific for the cytokine that is being assayed for. The Ig binds to the nitrocellulose base of the ELISPOT plate. (C) The activated T cells are transferred to the ELISPOT plate, and (D) cytokines are released during the incubation period. (E) Those cytokines that are released locally around each T cell bind to, and are therefore 'captured' by, the specific antibody. (F) The cells and any excess cytokines are washed off. (G) A second antibody that is also specific for the cytokine of interest is added; this antibody is coupled to an enzyme that is capable of converting a substrate into an insoluble coloured product. (H) The plates are washed once more, and the enzyme substrate is added. (I) The substrate is converted into the insoluble product, forming spots of colour that represent the areas of captured cytokines that were secreted by adjacent T cells. (J) The coloured spots are counted using a microscope or digital-imaging system.

The definition of a positive response at the IFN-γ ELISpot assay included 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. The number of the vaccine-reactive T cells above background was calculated as the difference between the numbers of the counted cells in peptide-stimulated and non-stimulated wells. Responses after the 2nd and 6th vaccination were normalized by subtracting the pre-vaccination responses. Results are presented as the number of peptide-reactive cells per 2×10^5 PBMCs. The definition of a positive response at the perforin ELISpot assay included a statistically significant difference ($P \leq 0.05$) between peptide-stimulated and negative control wells using the Student's *t* test. Results are presented as the number of peptide-reactive cells per 5×10^5 PBMCs.

2.10.3 Experimental Procedure

In this study, the IFN-γ and Perforin ELISpot assay was used to detect the production of specific-T cells, in response to $TERT_{572}$ and $TERT_{572Y}$ peptides. Both assays were performed according to the manufacturer's protocol (IFN-γ ELISPOT, Diaclone, Besancon, France; Perforin ELISPOT, Mabtech, Sweden). The kits provided all the reagents used, unless otherwise stated. In details, a nitrocellulose bottomed 96-well plate (MultiScreen MAIP N45; Millipore) was pre-wetted with 15µl / well of 35% EtOH for 3 min at RT. A pre-wetting protocol was used in order to increase spot number (better sensitivity) and more sharply defined spots (for more accurate quantification). 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) or 30 µg/ml coating anti-human perforin (Pf-80/164) in PBS overnight at 4ºC. The wells were washed once with 100µl of sterile PBS and blocked for 2 hours at room temperature with 100µl of 2% skimmed dry milk in PBS. This solution was used for the reduction of non-specific binding of antibodies. Following one wash with 100µl of sterile PBS, $2x10^5$ PBMCs in 100µl of complete medium were dispensed in each well in the absence or presence of different peptide ($TERT_{572Y}$ or $TERT_{572}$) concentration as stated in each result section. The plate was covered with a standard 96-well plate lid and the cells were incubated at 37ºC 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 the wells for 10 min at 4°C. Subsequently, the plates were washed with 100 µl PBS three times. After the final wash 100 µl of detection anti-human biotinylated IFN-γ 1% (v/v) BSA/PBS or 1 µg/ml in PBS/1% BSA anti-human biotinylated perforin (Pf-344-biotin) was added and the plate was incubated for 2 hours at room temperature. After 6 washes, 10µl of alkaline phosphatase-conjugated streptavidin diluted 1:1000 (v/v) in PBS, were added, and the plate was

incubated for 1 hour at room temperature. After washing, peroxidase substrate NBT/BCIP was added and the plate was incubated until the 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) and Bioreader 2000 (Bio-Sys, Karben, Germany).

In regard to ELISpot tests, six wells were tested for each group in three independent experiments. As negative controls were used the cells alone (spontaneous IFN-γ release and perforin release), whereas as positive controls were used the cells treated with 5 μg/ml Concanavalin A (ConA; Sigma,UK) or 5 μg/ml staphylococcus enterotoxin B (SEB; Sigma, UK) for IFN-γ and perforin ELISpot assays, respectively.. The experiment was considered complete and successful, as long as the spots in the positive control were at least twice as much as that in the negative control. The threshold for positive responses was set as the difference of more than 10 spot-forming cells and a statistically significant difference ($p \leq 0.05$) between peptide-stimulated wells and negative control wells using the Student's *t*-test.

The number of the vaccine-reactive T cells above background was calculated as the difference between the numbers of the counted cells in peptide-stimulated and non-stimulated wells. Responses after the 2nd and 6th vaccination were normalized by subtracting the pre-vaccination responses. Results are presented as the number of peptide-reactive cells per 2×10^5 PBMCs. The definition of a positive response at the perforin ELISpot assay included a statistically significant difference ($P \leq 0.05$) between peptide-stimulated and negative control wells using the Student's *t* test. Results are presented as the number of peptide-reactive cells per 5×10^5 PBMCs.

2.11 Flow cytometry & multi-parameter analysis for intracellular cytokine staining (ICS)

2.11.1 Materials

All the antibodies and reagents were purchased from BD Biosciences, 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

2.11.2 Principle

This method was chosen to identify peptide-specific $CD8^+$ T cells by measuring the cells-producing IFN-γ and IL-10 intracellular staining.

Flow cytometry is an optical, laser-based technology which analyses the physical and fluorescent properties of cells in suspension in real-time as they flow through the instrument. This approach has a number of advantages over other techniques that can be used for the characterization of cell populations in single-cell suspensions, acquiring data on the presence of different cell subpopulations and phenotypical changes within these populations in seconds. (Pockley, Foulds, Oughton, Kerkvliet, & Multhoff, 2015). This technique is used for counting and examining microscopic particles, such as cells and chromosomes suspended in a fluid stream which passes by an electronic detection apparatus.

The combination of scattered and fluorescent light is picked up by the detectors, and, by analyzing 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).

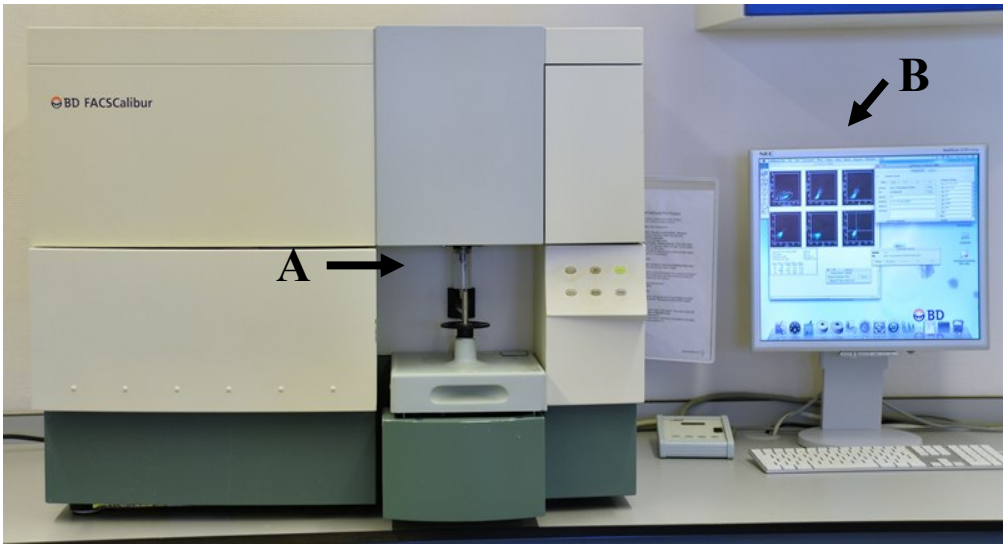


Figure 2. 4 Picture of the FACSCalibur used in our lab of cancer research at University of Crete. A: the compartment where the test tube is placed so the device can take up the fluid for analysis. B: the pictures of the blood. It shows how the blood can be branched into different cell types. The system is able to quantify cells and put them into groups.

2.11.3 Experimental Procedure

Peptide-specific $CD8^+$ T cells were identified by measuring the cells-producing IFN-γ and IL-10 intracellular staining using flow cytometry. At first, the cells were defrosted from -80 °C. Three tubes were prepared containing $1x10^6$/tube thawed PBMCs and were resuspended in 500 µl of complete medium. Then, in each a) 10µg/ml peptide ($TERT_{572Y}$ or $TERT_{572}$), b) no peptide or c) 5 µg/ml of Staphylococcal Enterotoxin B (SEB) was added, and the tubes were placed at 37°C in 5%CO_2 in air. 10µg/ml brefaldin A (BFA) was added 1h after the initial stimulation in order to inhibit the secretion of newly synthesized cytokines and the cells were incubated for further 18h at 37°C with 5% CO_2. The cells upon SEB activation were used as the positive control and cells with the medium only as the negative control. Brefeldin A is a lactoneantibiotic 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 the proteins to accumulating inside the ER.

The following day, 1ml FACs buffer was added in the cells and they were centrifuged for 5min. The cells were washed twice. After the centrifugation, the supernatant was discarded and the cells were vortexed gently. Subsequently, the cells were stained firstly for cell surface molecules (anti-CD3-APC and anti-CD8-PerCP) for 30min at 4 °C. Following 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, the cells were stained with conjugated anti–IL-10-PE and anti- IFN-γ-FITC. Following incubation for 1h at 4°C, 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 at 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 Cell Quest Pro software, Fig 2.4.

Results are expressed as the percentages of $CD8^+$ IFN-γ^+ or $CD8^+$ IL-10^+ T cells of the gated $CD3^+$ T cells in the dot plots. The number of T cells in the graphs was calculated as: $(2x10^5/100) \times$ [(experimental − spontaneous $CD8^+$ IFN-γ^+-releasing cells)].

A positive immune response was determined as:

1) Amount of $CD3^+CD8^+$ T or $CD3^+$ cells more than 10,000,

2) Response between positive control and stimulated cells of at least twice as much as that of the negative control, and

3) Percentage of $CD8^+$ IFN-γ^+ T or $CD3^+$cells greater than 0.02%.

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

2.12 $TERT_{572Y}$ Multimer Staining

2.12.1 Principle

This method was used to label TERT-specific T cells through formation of MHC:peptide complexes coupled to a fluorochrome, so that the binding to T cells can be monitored by flow cytometry.

Foreign antigen could not be used directly to identify T cells, since, unlike B cells, they do not recognize antigen alone but rather the complexes of peptide fragments of antigen bound to self MHC molecules. The affinity of interaction between the T-cell receptor and the MHC/peptide complex was in practice so low that attempts to label T cells with their specific MHC/peptide complexes routinely failed. The breakthrough in labelling antigen-specific T cells came with the idea of making multimers of the MHC/peptide complex, so as to increase the avidity of the interaction.

Peptides can be biotinylated using the bacterial enzyme BirA, which recognizes a specific amino acid sequence. Recombinant MHC molecules containing this target sequence are used to make MHC/peptide complexes which are then biotinylated. Avidin, or the bacterial counterpart streptavidin, contains four sites that bind biotin with extremely high affinity. Mixing the biotinylated MHC/peptide complex with avidin or streptavidin results in the formation of an MHC:peptide tetramer, four specific MHC/peptide complexes bound to a single molecule of streptavidin (Fig 2.5). Routinely, the streptavidin moiety is labeled with a fluorochrome to allow detection of those T cells capable of binding the MHC/peptide tetramer.MHC/peptide multimers have been used to identify populations of antigen-specific T cells (C. Janeway, 2005).

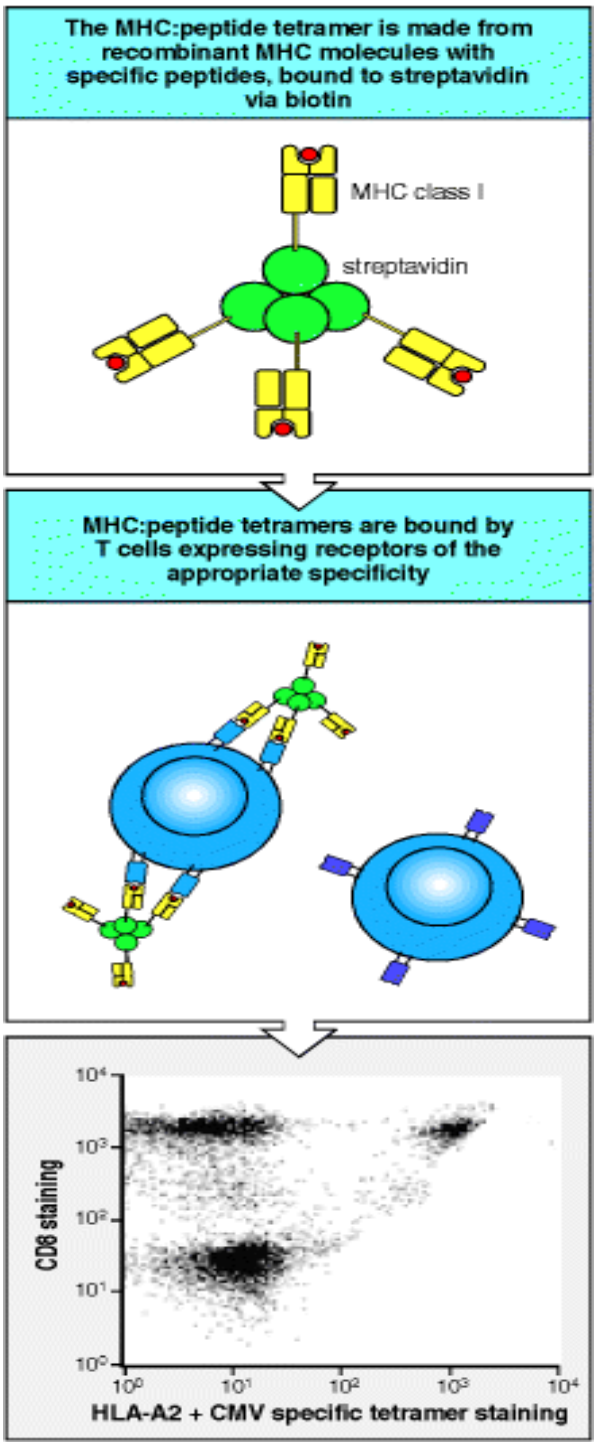


Figure 2. 5 MHC:peptide complexes coupled to streptavidin to form tetramers are able to stain antigen- specific T cells. MHC:peptide tetramers are formed from recombinant refolded MHC:peptide complexes containing a single defined peptide epitope. The MHC molecules can be chemically derivatized to contain biotin, but more usually, the recombinant MHC heavy chain is linked to a bacterial biotinylation sequence, a target for the *E. coli* enzyme BirA, which is used to add a single biotin group to the MHC molecule. Streptavidin is a tetramer, each subunit having a single binding site for biotin, hence the streptavidin/MHC:peptide complex creates a tetramer of MHC:peptide complexes (top panel). While the affinity between the T-cell receptor and its MHC:peptide ligand is too low for a single complex to bind stably to a T cell, the tetramer, by being able to make a more avid interaction with multiple MHC:peptide complexes binding simultaneously, is able to bind to T cells whose receptors are specific for the particular MHC:peptide complex (centre panel). Routinely, the streptavidin molecules are coupled to a fluorochrome, so that the binding to T cells can be monitored by flow cytometry. In the example shown in the bottom panel, T cells have been stained simultaneously with antibodies specific for CD3 and CD8, and with a tetramer of HLA-A2 molecules containing a cytomegalovirus peptide. Only the $CD3^+$ cells are shown, with the staining of CD8 displayed on the vertical axis and the tetramer staining displayed along the horizontal axis. The $CD8^-$cells (mostly $CD4^+$) on the bottom left of the figure show no specific tetramer staining, while the bulk of $CD8^+$ cells, on the top left, likewise show no tetramer staining. However, a discrete population of tetramer positive $CD8^+$ cells, at the top right of the panel, comprising some 5% of the total $CD8^+$ cells, can clearly be demonstrated. (Adapted from Janeway -Travers immunobiology).

2.12.2 Experimental method

10^6 thawed un-stimulated PBMCs were incubated with phycoerythrin- conjugated HLA-A*0201/$TERT_{572Y}$ or the control phycoerythrin (PE)-conjugated HLA-A*0201/human immunodeficiency virus p76 (HIVgag76) pentamer (Proimmune Ltd, Oxford, United Kingdom)

for 30 minutes at RT, and then with anti-CD8-APC and anti-CD3-FITC (BD Pharmingen, Mississauga, Canada) mAbs for 30 minutes at 4°C. Stained cells were analysed by flow cytometry (FACSCalibur; BD Biosciences, Mountain View, CA). The frequency of $TERT_{572Y}$ pentamer– positive cells was calculated according to the formula: (number of $TERT_{572Y}$-pentamer stained CD8 cells – number of HIVgag76-pentamer–stained CD8 cells)/10^5 CD8 cells.

In addition, $TERT_{572Y}$-specific $CD8^+$ T cells were obtained from the PBMCs of a responding vaccinated patient by sorting of $TERT_{572Y}$-$tetramer^+/CD8^+$ T cells using a cell sorter. 1×10^6 thawed unstimulated PBMCs were incubated with phycoerythrin-conjugated (PE)-HLA-A*0201/$TERT_{572Y}$ (Proimmune Ltd, UK) for 30 min at RT, and then anti-CD8-APC and anti-CD3-FITC (BD Biosciences, UK) were added and incubated for an additional 30 min at 4°C. Cells were washed once and sorted by a flow cytometry cell sorting. Sorted cells were used to set up limiting dilution cultures and in vitro expanded in the presence of 1 μg/ml PHA and 150 U/ml rIL-2 for 7 days and used for the chromium-release assay.

2.13 Chromium-release assay

2.13.1 Principle

Chromium-51 (51Cr) release assay was used due to its capacity for accurate quantification of T cell cytotoxicity. The assay is used to determine the number of lymphocytes produced in response to infection or after specific treatment. Target cells are labelled with 51Cr, the label is then released from the target cells by cytolysis. The label can be isolated by centrifuging the samples and collecting the supernatants. Supernatants from centrifugation can either be counted directly in a gamma counter or mixed with scintillation cocktail in a microplate (or dried on a LumaPlate™) and counted in a liquid scintillation counter. A summary of the assay principle is illustrated in Fig 2.6 below:

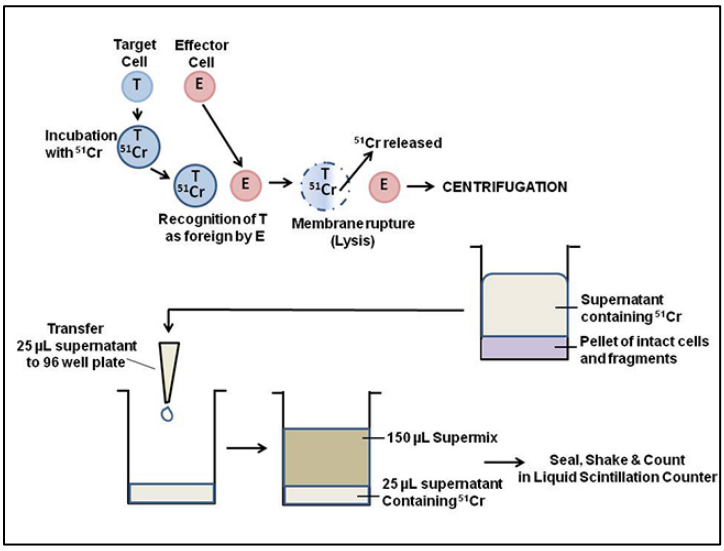


Figure 2. 6 Chromium-51 (51Cr) release assay principle.

2.13.2 Experimental procedure

Antigen recognition was assessed using target cell lines [$TERT^+$ (N18/TERT and NA8) and $TERT^-$(N418 and Me290) kindly provided by Prof. P. Romero, Ludwig Center for Cancer Research, Lausanne, Switzerland] labeled with ^{51}Cr for 1 h at 37°C and washed three times. Labeled target cells (1,000 cells in 50 µl) were then added to varying numbers of effector cells (100 µl) in V-bottom microwells. Chromium release was measured in the supernatant (100 µl) harvested after 4-h incubation at 37°C. The percentage- specific lysis was calculated as: 100 × [(experimental - spontaneous release)/ (total - spontaneous release)].

2.14 Statistical analysis

Owing to the exploratory nature of the study no formal sample size estimation was used. The primary objective of our study was to evaluate the optimal administration of the peptides in terms of eliciting the most effective immune response. Secondary objectives were: 1) the assessment of the safety profile of the telomerase hTERT vaccine, 2) the estimation of the overall survival of the patients participated in the study.

The overall survival was estimated from the date of study entry to the date of the last contact or death. Moreover, we analyzed the association between the development of TERT-specific immune response and the clinical outcome of the patients who were enrolled in the vaccination protocol with clinically documented stable (SD) or progressive disease (PD) prior to the study entry. The probability of survival was estimated using the Kaplan-Meier graph. The 95% confidence interval (95% CI) was calculated. The overall survival was compared using the

log-rank test, in each of the following 4 groups:

Group A) The patients who entered the study with SD and were "early responders" (who had developed a $TERT_{572Y}$- specific immune response after the 2nd vaccination) and "non-early responders",
Group B) The patients who entered the study with SD and were "late responders" (who had developed a $TERT_{572}$-specific immune response after the 6th vaccination) and "non-late responders".
Group C) The patients who entered the study with PD and were "early responders" and "non-early responders",
Group D) The patients who entered the study with PD and were "late responders" and "non - late responders".

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Institute Inc, USA). The frequencies of peptide-induced immune responses before and after vaccinations were compared using the Wilcoxon matched pairs test and Paired t-test. Statistical analysis of the frequencies of the peptide-responding patients after the completion of the 6-vaccinations in the 2 different vaccination groups was performed using Wilcoxon rank sum test. The total peptide response was obtained by subtracting the pre-vaccination response from the post-vaccination response for each group from 3 independent experiments. Finally, the frequencies at different time points after the completion of the 6-vaccinations were compared using the 2-way analysis of variance with Bonferroni post-test. All tests were considered significant when the resulting p value was <0.05.

3. CHAPTER 3: Randomized study to compare different schedules of vaccination

3.1 Introduction

Two vaccination schedules were compared in regard to induction of specific immune responses. The schedule of initial 2 doses of the optimized TERT peptide ($TERT_{572Y}$) followed by 4 doses of the native peptide ($TERT_{572}$) was compared to continuous administrations of the optimized variant ($TERT_{572Y}$) for 6 doses.

The aim of this study was to determine the best administration sequence of the optimized $TERT_{572Y}$ and native $TERT_{572}$ peptides for immunization in respect to ex vivo reactivity of peptide-induced CTLs.

3.2 Materials and Methods

3.2.1 Patients

Forty-eight HLA-A*0201 patients with various types of solid tumours were enrolled in this study. The inclusion criteria are described in the section 2.1.

3.2.2 Peptides

The 9-mer cryptic native $TERT_{572}$ peptide and its optimized variant $TERT_{572Y}$ were used in order to vaccinate the patients. More details are presented in the section 2.3.

3.2.3 Vaccination Protocols

All patients (N = 48) received initially 2 subcutaneous injections of 2 mg of the optimized $TERT_{572Y}$ peptide administered every 3 weeks. Then, the patients were randomized with 1:1 ratio into 2 groups and received 4 additional subcutaneous injections every 3 weeks. One group of patients received 2 mg of the native $TERT_{572}$ peptide (scheme A), whereas the other group continued the vaccinations with 2 mg of the optimized $TERT_{572Y}$ peptide (scheme B; Fig 4.1A). Both peptides were emulsified with Montanide ISA51 (Seppic Inc., Paris, France) immediately before injection. All patients completed the 6-vaccination protocol unless disease progression occurred, in which case treatment was discontinued.

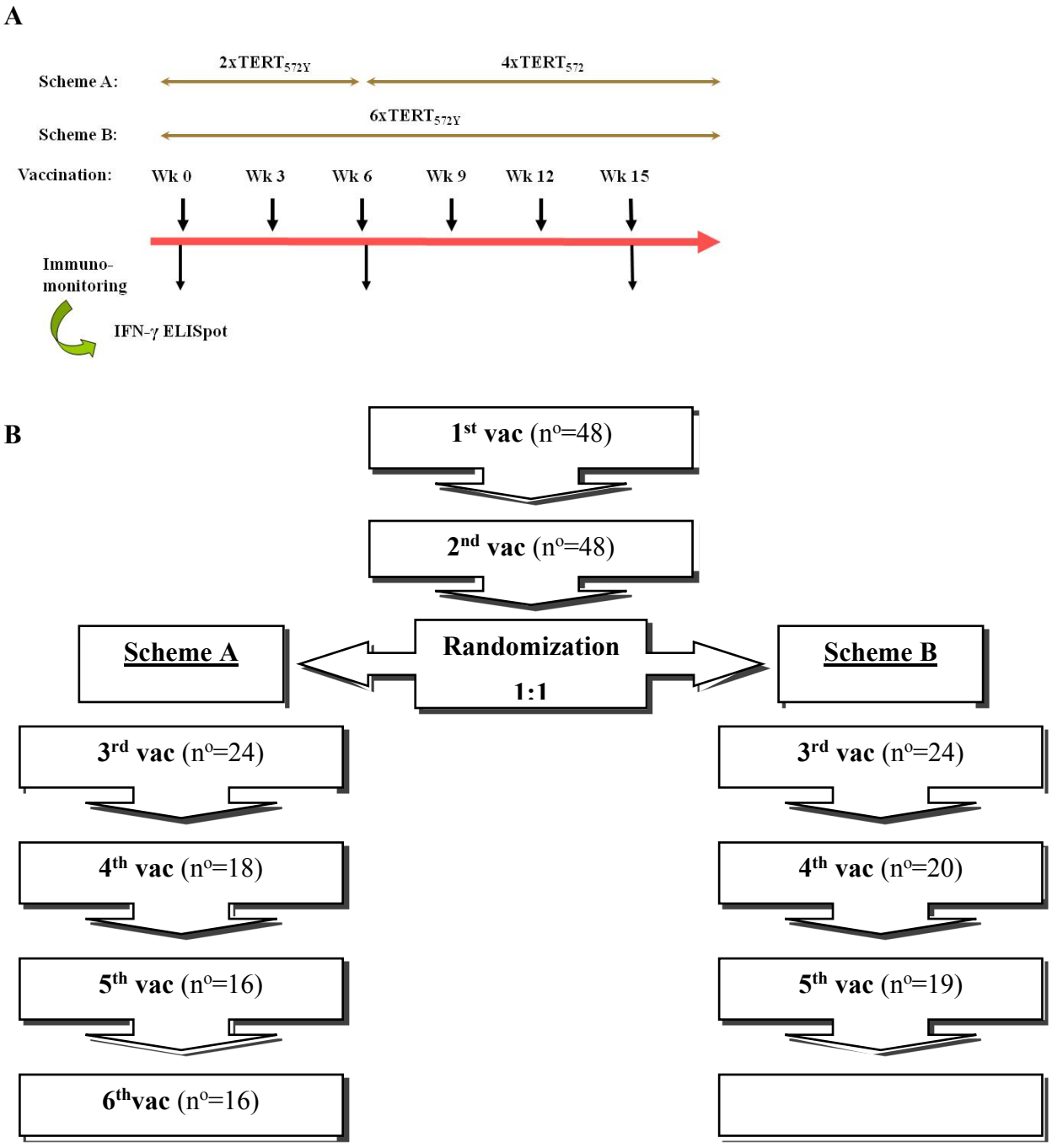


Figure 3.1 Schematic presentation of the vaccination schemes. A) All patients received 2 subcutaneous injections with 2 mg of the optimized $TERT_{572Y}$ peptide every 3 weeks. Then, the patients were randomised into 2 schemes and received 4 additional subcutaneous injections with different peptides, every 3 weeks. In scheme A, patients received 2 mg of the native $TERT_{572}$ peptide, whereas in scheme B, patients continued the vaccinations with 2 mg of the optimized $TERT_{572Y}$ peptide. Immuno-monitoring for reactive cells was performed in pre-scheduled time points as indicated at the figure B. Flowchart of the enrolled patients according to the vaccination scheme and the number of doses received. ELISpot, enzyme-linked immunosorbent spot; IFN, interferon; n^o, number of vaccinated patients; TERT, telomerase reverse transcriptase; vac, vaccination.

3.2.4 Patient Samples for Immunomonitoring

Patients' peripheral blood were (100 mL) collected before the first vaccination, after the second and sixth vaccination and peripheral blood mononuclear cells (PBMCs) were isolated as stated in material and methods section 2.5-2.7.

3.2.5 Enzyme-Linked Immunosorbent Spot (ELIspot)

The interferon (IFN)-γ ELISpot assay was performed as indicated under section 2.10.2. However, in this part of the study we used different doses of the peptide $TERT_{572}$ (10, 1, and 0.1 mg/mL $TERT_{572}$ or 10 mg/mL $TERT_{572Y}$) in order to measure the avidity of specific T cells. The T-cell avidity defined by the Ag dose-response of T cells is an indirect measurement of their effector function. The low avidity T cells are induced by the highest dose of the peptide while the high avidity T cells are induced by the low dose of the peptide. The clinical significance of this finding is discussed later in this chapter.

In all ELISpot tests, 6 wells were tested for each group in 3 independent experiments. Negative controls were the cells alone (spontaneous IFN-γ release), whereas positive controls were cells treated with 5 mg/mL Concanavalin A (Sigma, UK). Results are presented as the number of peptide reactive cells per 2×10^5 PBMCs.

3.2.6 Statistical Analysis

Wilcoxon matched pairs test was used to compare the frequencies of peptide- responding patients before and after the second vaccination. We used the Wilcoxon rank sum test for the statistical analysis of the frequencies of the peptide- responding patients after the completion of the 6-vaccinations in the 2 different vaccination groups. The total peptide response was obtained by subtracting the pre-vaccination response from the post-vaccination response for each group from 3 independent experiments. Finally, the frequencies at different time points after the completion of the 6 vaccinations were compared using the 2-way analysis of variance with Bonferroni post-test. All tests were considered significant when the resulting p value was <0.05. Progression-free survival (PFS) and overall survival (OS) was estimated as stated in section 2.12.

3.3 Results

3.3.1 Patients' Demographics and Vaccine Administration

Our aim was to evaluate the immune response in various cancer types and therefore forty-eight HLA-A*0201 patients with solid tumours were enrolled in this study. The baseline

characteristics of the patients are presented in Table 3.1. The enrolled patients had various tumours, including breast cancer (12.5%), Non-Small Cell Lung Cancer (31.3%), prostate cancer (8.3%) and ovarian cancer (8.3%) among others. At baseline, prior to the study entry, 64.6% of patients had distant metastases (stage IV) and 25% had presented with documented disease progression on the last chemotherapy regimen. Twenty-three (48%) patients had received at least 2 chemotherapy regimens before enrolment. All patients (100%) received at least the first 2 vaccinations and 28 (58.3%) completed the 6-vaccination scheme, while the remaining patients, due to disease progression were withdrawn from the study. The flow diagram of the study is shown in Fig 3.1B

Table 3. 1 Baseline characteristics of 48 patients enrolled

Characteristics	All Patients (n=48)		Scheme A (n=24)		Scheme B (n=24)	
	N⁰	%	N⁰	%	N⁰	%
Age (median, range)	63 (45-80)		63 (42-84)		63 (48-80)	
Sex						
Male	35	73	21	87.5	14	58.3
Female	13	27	3	12.5	10	41.6
Cancer type						
Breast	6	12.5	3	12.5	3	12.5
Colorectal	2	4.2	2	8.4	0	0
Ovarian	3	6.3	0	0	3	12.5
NSCLC	15	31.3	8	33.3	7	29.2
Thyroid	2	4.2	1	4.2	1	4.2
Melanoma	2	4.2	1	4.2	1	4.2
Hepatocellular	1	2.1	1	4.2	0	0
Renal	3	6.3	1	4.2	2	8.4
Prostate	4	8.3	3	12.5	1	4.2
Other	10	20.8	4	16.6	6	25
Disease Stage at study entry						
≤II	9	18.7	1	4.2	8	33.3
III	8	16.7	5	20.8	3	12.5
IV	31	64.6	18	75	13	54.2
Disease Status at study entry						
Progressive Disease	12	25	7	29.2	5	20.8
Stable Disease	36	75	17	70.8	19	79.2
Lines of Treatment prior to study entry						
1st line regimen	25	52.1	11	45.8	14	58.3
≥2nd line (range, 2-8) regimens	23	47.9	13	54.2	10	41.7

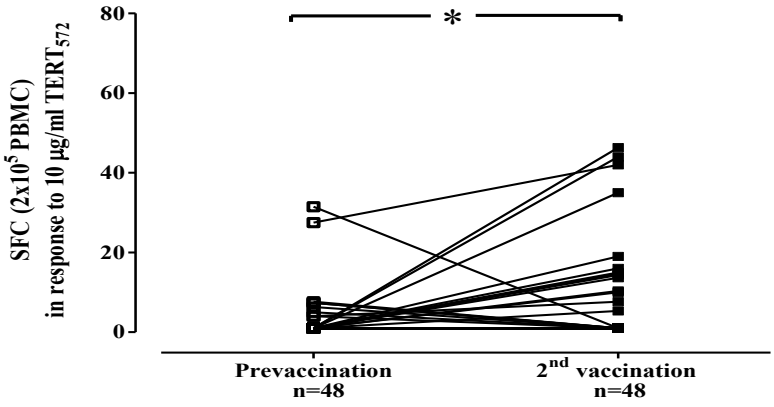
3.3.2 Assessment of induction of $TERT_{572}$-specific immune responses after vaccination with 2 doses of the optimized $TERT_{572Y}$peptide

For determination of the frequency of T cells capable of responding to a specific-stimulus by secretion of IFN-γ, ELIspot assay was used. The threshold for positive immune response was defined on the basis of the comparison of ELIspot results between the pre and post vaccination IFN-γ production as described in the section 2.10.2. The first assessment of the immune response occurred after the first 2 doses of the optimized peptide $TERT_{572Y}$.

After the second vaccination, 14/48 (29.2%) patients showed an increase in the frequencies of low-avidity (stimulated by the high dose 10mg/ml) $TERT_{572}$-specific T cells compared with the pre-vaccination frequencies, confirming that the T cells induced were able to recognize the native peptide. The mean (± standard deviation) frequency of low-avidity spot- forming cells was 2.9 ± 5.9 per 2×10^5 PBMCs before vaccination (background frequency), and 6.8 ± 11.9 per 2×10^5 PBMCs after the second vaccination (Fig 3.2A).

$TERT_{572}$-specific T cells with intermediate and high-avidity (as assessed by their reaction to 1 and 0.1 mg/mL of the peptide $TERT_{572}$ respectively), were detected in 12/48 (25%) and 16/48 (33.3%) patients, respectively, after the second vaccination. The mean (± standard deviation) frequency of intermediate and high- avidity reactive spot-forming cells was 3.7 ± 6.3 and 4.6 ± 7.5 per 2×10^5 PBMCs before vaccination (background frequency), and 6.9 ± 13.4 and 8.7 ± 15.7 per 2×10^5 PBMCs after the second vaccination, respectively (Fig 3.2B and 2C). In conclusion, the vaccination with the optimized $TERT_{572Y}$ peptide was able to induce the development of T cells of all avidities that were able to recognize the native peptide $TERT_{572}$.

A



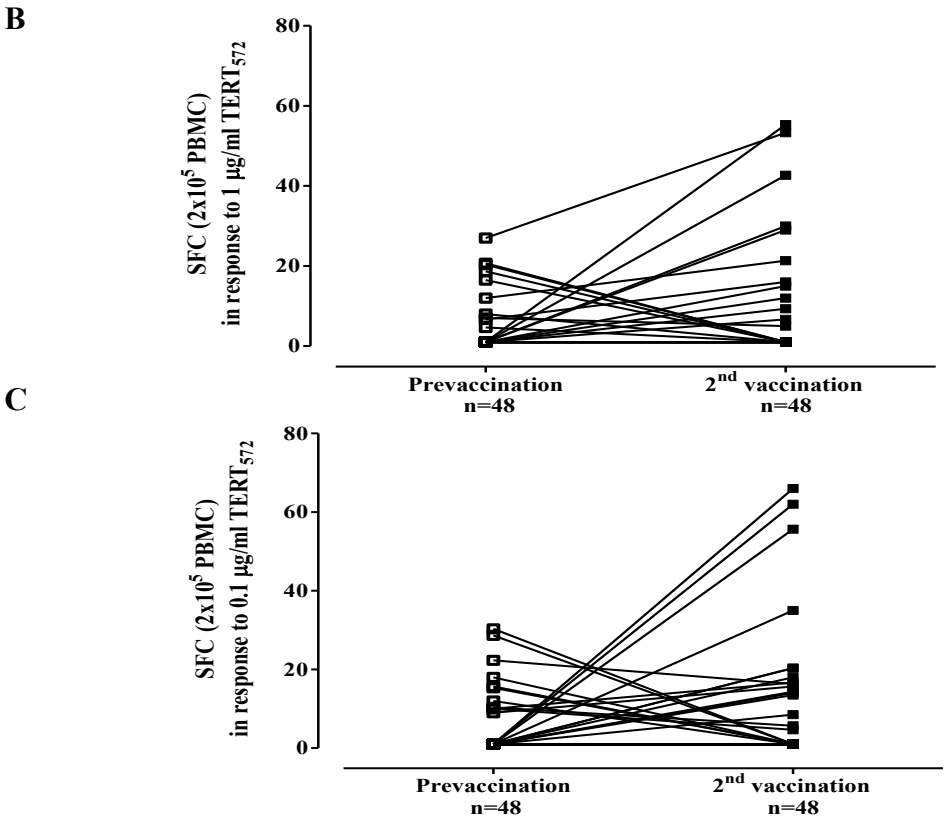


Figure 3.2 TERT-specific T cell responses in patients vaccinated with 2 doses of $TERT_{572Y}$ peptide. Frequencies of specific cells to $TERT_{572}$ peptides (A, B, and C), before vaccination and after the second vaccination with the optimized $TERT_{572Y}$ peptide, in vaccinated patients using interferon-γ enzyme-linked immunosorbent spot assay. Peripheral blood mononuclear cells from the vaccinated patients were stimulated with 10, 1, or 0.1 mg/mL $TERT_{572}$ peptide. The data in the graphs are presented as mean value of 3 independent experiments. Background frequencies have been subtracted. The frequencies of the specific cells were compared among vaccinated patients before and after the second vaccination (n = 48). *P < 0.05. SFC: spots-forming cells; TERT: telomerase reverse transcriptase.

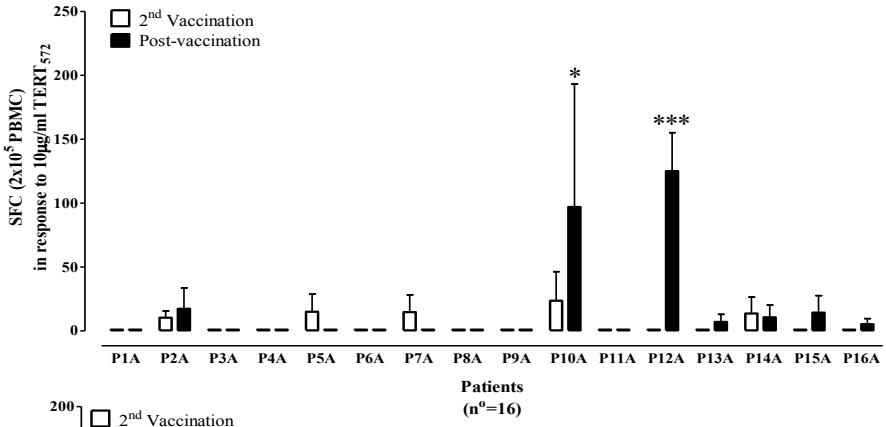
3.3.3 Assessment of the immune responses at the completion of the 6 vaccinations with 2×$TERT_{572Y}$ followed by 4×$TERT_{572}$ doses (Scheme A)

The second assessment of the immune response occurred in the end of the 6 vaccinations. The patients (n=24) who were randomised in the Scheme A received the first 2 doses with the optimized peptide followed by 4 doses of the native peptide.

$TERT_{572}$-specific immune responses were assessed in 16/24 (66.7%) patients after the completion of the 6-vaccination protocol (post-vaccination). Eight patients in this group (33, 3%) prematurely discontinued the vaccinations due to disease progression. Total immune responses of low-avidity $TERT_{572}$-specific IFN-γ -producing T cells were detected in 7/16 (43.8%) patients, post-vaccination. In two (12.5%) patients there was further increase of their immune response and in 2 (12.5%) a 2-fold decrease in peptide-specific frequencies was observed between the second and the sixth vaccination (Fig 3.3A). Furthermore, $TERT_{572}$-specific T cells of intermediate and high avidity were detected in 8/16 (50%) and 7/16 (43.8%) patients, respectively, after the completion of the vaccination scheme A (Fig 3.3B and C).

In conclusion, while the vaccination scheme A resulted in the induction of $TERT_{572}$-specific T cells of all avidities, the highest frequencies were observed in the induction of low–avidity-specific T cells (mean number = 47 cells/2 $\times 10^5$ PBMC) compared with the intermediate and high-avidity T cells (mean number = 20 and 25/2 $\times 10^5$ PBMC, respectively).

A



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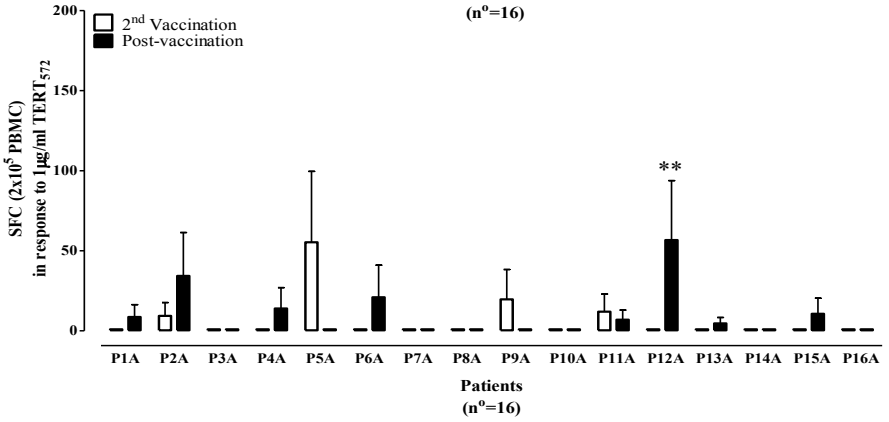

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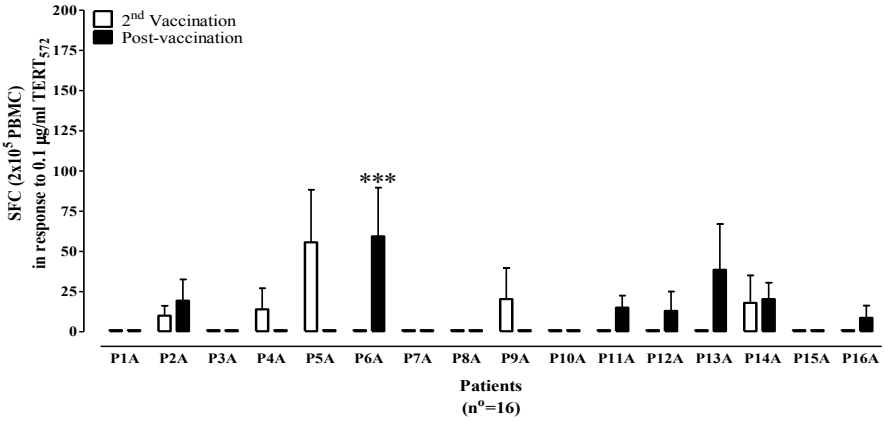


Figure 3. 3 TERT-specific T cell responses in patients vaccinated with 2 doses of $TERT_{572Y}$ peptide followed by 4 doses of $TERT_{572}$ peptide (Scheme A). TERT-specific immune response during the course of 6 cycles of vaccination in response $TERT_{572}$ peptide (A, B, and C), after the second and sixth vaccinations (post-vaccination), as assessed by interferon-γ enzyme-linked immunosorbent spot assay. Peripheral blood mononuclear cells from the vaccinated patients were stimulated with 10, 1, or 0.1 mg/mL TERT572 peptides. The data in the graphs are presented as the mean value of 3 independent experiments. Background and pre-vaccination frequencies have been subtracted (n = 16). *, **, ***P < 0.05, 0.01, 0.001 between second and sixth vaccination. SFC: spots-forming cells; TERT: telomerase reverse transcriptase.

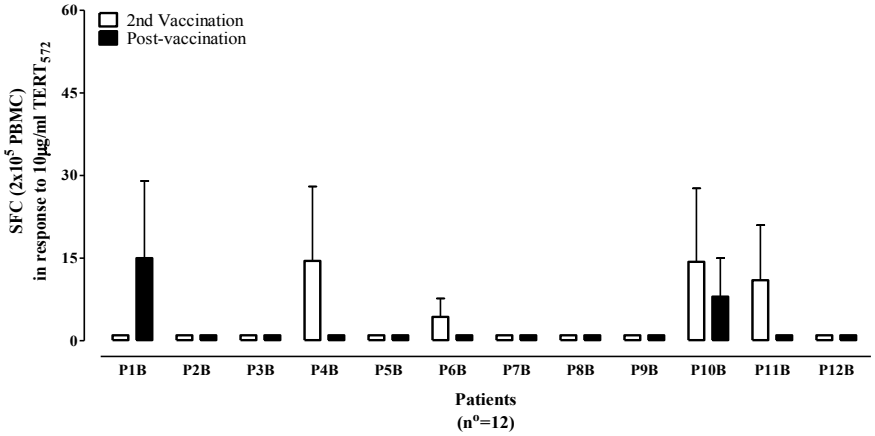
3.3.4 Assessment of the immune responses at the completion of the 6 vaccinations with $TERT_{572Y}$ ×6 (Scheme B)

Similarly, the patients (n=24) who were randomised in the Scheme B received the first 2 doses with the optimized peptide but they continued with 4 doses of the same, optimized peptide. $TERT_{572}$-specific immune responses were assessed in 12/24 (50%) patients after the completion of the 6-vaccination protocol (post-vaccination). Twelve patients (50%) discontinued early without completing the vaccination protocol due to disease progression.

Total immune responses of low-avidity $TERT_{572}$- specific IFN-γ-producing cells were detected in 2/12 (16.7%) patients at post-vaccination. Between the second and the sixth vaccination more than a 2-fold decrease in peptide-specific frequencies was observed in 4 (33.3%) patients (Fig 3.4A). Furthermore, $TERT_{572}$-specific T cells of intermediate and high avidity were detected in 4/12 (33.3%) and 2/12 (16.7%) patients, respectively, after the sixth vaccination (Fig 3.4B and C). Although numerically the frequency in the specific $TERT_{572}$ response at post-vaccination (between the second and the sixth vaccination) was higher in 4 patients, the 2-way analysis of variance test was unable to detect any significant difference between the second and the sixth

vaccination responses in any of these patients. What was even more interesting, was the observation in all patients that the frequencies of $TERT_{572}$-specific cells which developed after the second vaccination returned to the baseline or even decreased after the completion of the sixth vaccination with the modified $TERT_{572Y}$ peptide.

A



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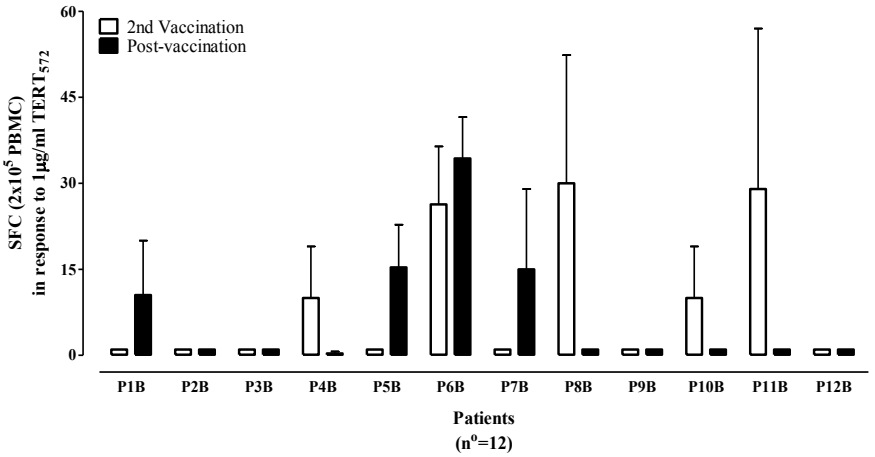

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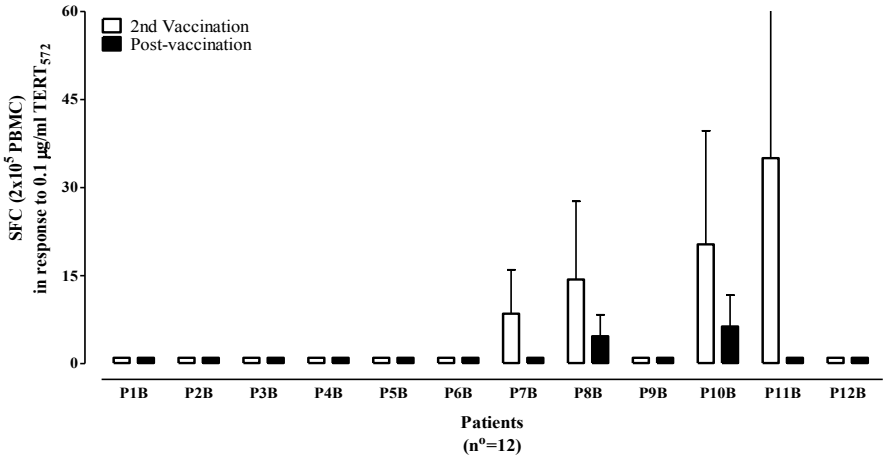


Figure 3. 4 TERT-specific T cell responses in patients vaccinated with 6 doses of $TERT_{572Y}$ peptide. TERT-specific immune response during the course of 6 cycles of vaccination in response to $TERT_{572}$ peptide (A, B, and C), after the second and sixth vaccinations (post-vaccination), as assessed by interferon-γ enzyme-linked immunosorbent spot assay. Peripheral blood mononuclear cells from the vaccinated patients were stimulated with 10, 1, or 0.1 mg/mL $TERT_{572}$ peptides. The data in the graphs are presented as the mean value of 3 independent experiments. Background and pre-vaccination frequencies have been subtracted (n = 12). SFC: spots-forming cells; TERT: telomerase reverse transcriptase.

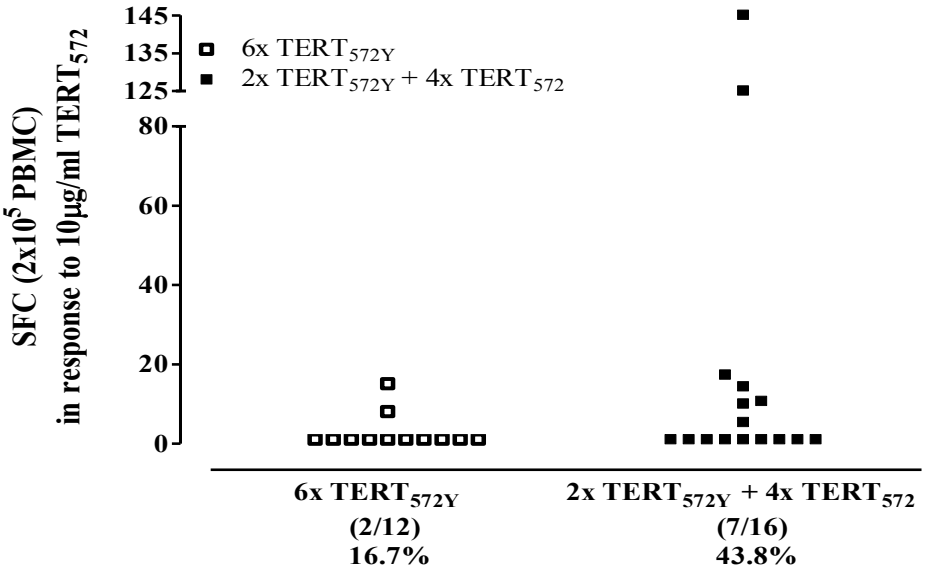
3.3.5 Comparison of the Immune Response to the native peptide ($TERT_{572}$) in patients vaccinated with the 2 different vaccination schemes

In order to establish the best vaccination schedule at the end of the 6 vaccinations, we compared the quantitative and qualitative characteristics of the immune response to the native peptide which was developed in the patients vaccinated with the two different vaccination schemes. More specifically, we compared the post-vaccination frequencies and the magnitude of the measured immune response of the peptide–induced-specific T cells between the 2 vaccination schemes.

The comparison revealed that the induction of low (Fig 3.5A), and intermediate avidity (Fig 3.5B) $TERT_{572}$-specific T cells was independent of the vaccination scheme at the completion of the 6-vaccinations. Immune responses of both low and intermediate-avidity $TERT_{572}$-specific T cells were detected more frequently in patients vaccinated with the native peptide (scheme A; 43.8% and 50%, respectively) compared with those vaccinated only with the modified peptide (scheme B; 16.7% and 33.3%). However, the magnitude of the immune response of the specific T cells was not significantly different between these two schedules (P = 0.1 and P = 0.5; Fig

3.5A and B). The main finding here was that at the end of the vaccination with the native peptide (scheme A) we observed the development of high-avidity $TERT_{572}$-specific T cells in 43.8% of the vaccinated patients compared with 16.7% of patients who received vaccinations with the optimized peptide only (scheme B). More importantly, the magnitudes of the immune responses of the high–avidity-specific T cells at post-vaccination in the patients enrolled in the scheme A were significantly higher compared with the patients in scheme B [11.5 T cells/2 $\times 10^5$ PBMC (scheme A) vs. 1.8 T cells/2 $\times 10^5$ PBMC (scheme B) cells, P = 0.03; Fig 3.5C].

A



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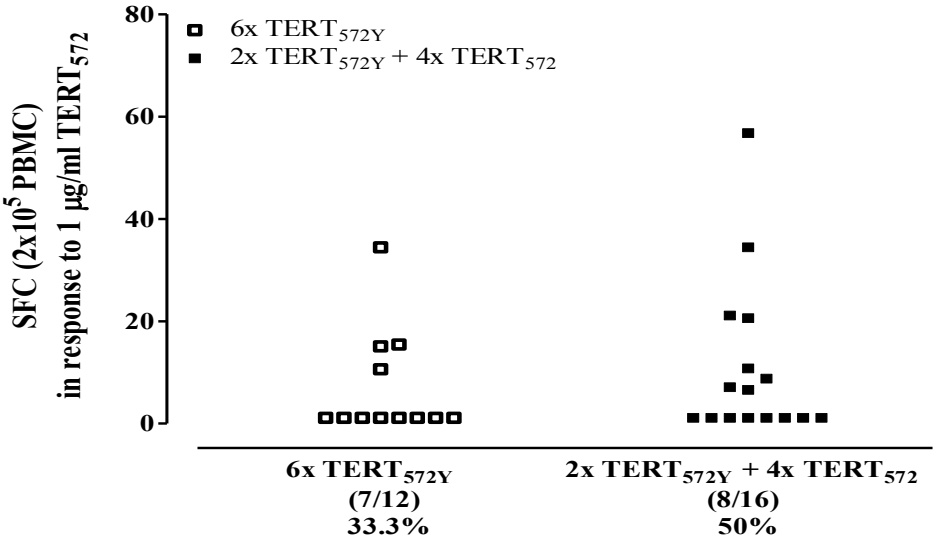

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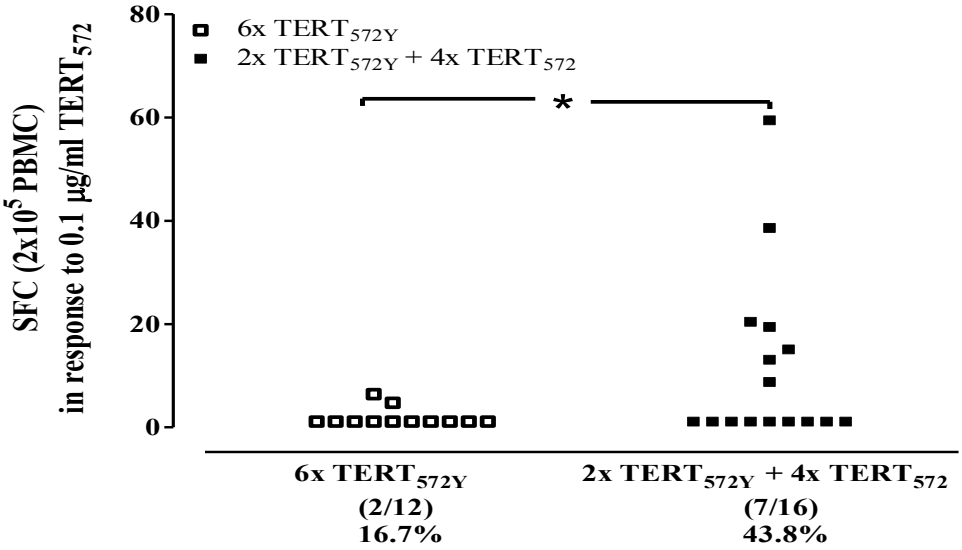


Figure 3. 5 Comparison of the frequencies of the $TERT_{572}$ responses in patients vaccinated with and without $TERT_{572}$ peptide. $TERT_{572}$-specific immune response in response to $TERT_{572}$ peptide (A, B, and C), at the completion of the sixth vaccinations, as assessed by interferon-γ enzyme-linked immunosorbent spot assay. Peripheral blood mononuclear cells from the vaccinated patients were stimulated with 10, 1, or 0.1mg/mL TERT572 peptides. The data in the graphs are presented as the mean value of 3 independent experiments. Background and pre-vaccination frequencies have been subtracted. The frequencies of the specific cells were compared among vaccinated patients who received 6 ×$TERT_{572Y}$ or 2 ×$TERT_{572Y}$+4 ×$TERT_{572}$. *P < 0.03; SFC: spots-forming cells.

3.3.6 Clinical outcome

In addition to the immunologic findings, we tried to establish whether there was any association between the development of $TERT_{572}$-specific IFN-γ immune reactivity and the patients' clinical outcome. For this purpose, we analyzed the Progression Free Survival (PFS) and the Overall Survival (OS) of the patients enrolled in the vaccination protocol. Overall, there was no significant difference in either PFS or OS for patients who developed an immunologic response at any time during vaccination between the 2 schemes (Tables 3.2 and 3.3). However, in the subgroup analysis of patients who enrolled in scheme A vaccination, those who developed an immune response had a significantly longer PFS compared with those without an immune response (13.5 vs. 3.5mo; log-rank test P=0.01; Table 3.2).

Table 3. 2 Progression-Free and Overall Survival of Responders vs Non-Responders in Schemes A and B

	Scheme A response	Scheme A Non-response	Scheme B response	Scheme B Non-response	p- value
PFS **Median** **Min-Max (months)**	N= 16, Events= 10 13.5 2 – 65	N=8, Events=8 3.5 2 – 19	N=11, Events=5 42 2 – 58	N=13, Events=10 4 1 – 51	**0.01** (Response vs non-response in Scheme A NS (between all other tested groups)
OS **Median** **Min-Max (months)** **1-year survival**	N=16, Events=4 Undefined 2 – 74 87.5 %	N=8, Events=4 9 2 – 38 62.5%	N=11, Events=3 Undefined 6 – 62 81.8%	N=13, Events=6 57 1 – 63 61.5%	NS (between all tested groups)

Table 3. 3 Progression-Free and Overall Survival of Responders vs Non-Responders at post-vaccination in Schemes A and B

	Scheme A Response	Scheme A Non-response	Scheme B Response	Scheme B Non-response	p- value
PFS **Median** **Min-Max**	N= 11, Events= 7 19 3 – 65	N=5, Events=3 8 3 – 42	N=6, Events=2 Undefined 7 – 49	N=6, Events=2 51 8 – 58	**NS** (between all tested groups)
OS **Median** **Min-Max** **1-year survival**	N=11, Events=3 Undefined 14 – 65 100 %	N=5, Events=1 51.5 28 – 42 100%	N=8, Events=1 Undefined 7 – 58 75%	N=4, Events=2 44.5 12 – 63 100%	**NS** (between all tested groups)

3.4 Conclusion

In this study, two vaccination schedules were compared using the native peptide $TERT_{572}$ and its optimized variant $TERT_{572Y}$. We tested the hypothesis that vaccinations of cancer patients with 2 doses of the optimized peptide ($TERT_{572Y}$) followed by 4 doses with the native peptide $TERT_{572}$ (scheme A) are able to induce CTLs with higher avidity and stronger antitumor efficacy than serial vaccinations with the optimized $TERT_{572Y}$ peptide alone (scheme B), as previously shown in *in vivo* preclinical studies using HLA-A*0201 transgenic HHD mice (Gross et al., 2004). In both schemes, we use an initial induction with the optimized peptide $TERT_{572Y}$ followed by a maintenance phase in which patients were randomized to receive either the native $TERT_{572}$ or the optimized peptide $TERT_{572Y}$. Our results revealed that vaccination with the optimized $TERT_{572Y}$ followed by the native $TERT_{572}$ peptides (scheme A) can induce strong T cell responses, with higher avidity and frequencies of T cell responses, after the completion of 6-vaccinations.

An interesting observation in this study was a shift in the avidity of the CTLs developed after the second and the sixth vaccination in regard to response to different peptide. More specifically, vaccinations of cancer patients with 2 doses of the optimized peptide ($TERT_{572Y}$) induced a significant number of CTLs of low avidity (Fig 3.2). Subsequently, those patients who received 4 more doses with the native peptide $TERT_{572}$ were able to induce CTLs with higher avidity than the ones who received serial vaccinations with the optimized $TERT5_{72Y}$ peptide alone who did not develop any response (Fig 3.5). This could be explained by our initial hypothesis that the optimized peptide ($TERT_{572Y}$) first may generate a pool of peptide-specific T cells of different avidities and then the stimulation with the native peptide $TERT_{572}$ selects among T cells those with highest avidities for the native peptide. These results were published in Journal of Immunotherapy in 2011 (Vetsika et al., 2011).

Therefore, our primary goal to establish the most efficient vaccination schedule using these two TERT peptides was successfully completed in this phase II randomized study. Subsequently, the best vaccination schedule was validated in patients with different tumour types in regards to its immunologic and clinical activity.

4. CHAPTER 4: Pilot NSCLC Study

4.1 Introduction

Following determination of the best vaccination schedule using the optimized and native cryptic peptide-based TERT vaccine we wanted to investigate whether this schedule could elicit sufficient immune responses in cancer patients. To this end a pilot study was designed in a homogenous population of NSCLC patients with advanced disease. In addition, we evaluated the safety profile and clinical efficacy of this particular vaccination strategy.

4.2 Materials and Methods

4.2.1 Patients

Twenty-two patients with locally advanced/unresectable stage III or metastatic stage IV NSCLC were enrolled in this phase. The eligibility criteria are summarized in the chapter 2, section 2.1.

4.2.2 Vaccination Protocol

Patients received six subcutaneous vaccinations administered every 3 weeks. The optimized $TERT_{572Y}$ peptide was used for the first and second vaccinations, and the native $TERT_{572}$ peptide for the remaining four vaccinations. Patients with no PD after six vaccinations received boost vaccinations with 2 mg of native TER_{T572} every 3 months until disease progression.

4.2.3 Peptides

The peptides used are described in section 2.4.

4.2.4 Enzyme-Linked Immunosorbent Spot Assay (ELIspot)

The IFN-γ ELIspot kit (Diaclone, Besancon, France) was used according to the manufacturer's recommendations. The principle and the experimental procedure are described in the section 2.10.2-2.10.3.

Six wells were tested for each group, and the standard deviation of replicates was 23% ± 14% of means in all groups for all tested samples. Statistical analysis for positivity was done using the *t* test. ELIspot assay was considered positive when there was (1) a difference of more than 10 spots between unstimulated and $TERT_{572}$- stimulated cultures and (2) a statistically significant difference between un-stimulated and $TERT_{572}$-stimulated cultures. $TERT_{572}$ reactive cells were calculated in ELIspot-positive assays according to the formula: number of spots in the $TERT_{572}$-

stimulated group – number of spots in the control group. Results are presented as the number of $TERT_{572}$ reactive cells per 10^5 CD8 cells calculated according to the formula: number of $TERT_{572}$ reactive cells ×percentage of $CD8^+$ cells (measured by double CD3/CD8 immunofluorescence staining).

4.2.5 $TERT_{572Y}$ Pentamer Staining

In this cohort of patients, $TERT_{572Y}$ Pentamer staining was performed as described in section 2.12.2

4.2.6 Statistical Analysis

The frequencies of $TERT_{572}$-reactive $CD8^+$cells detected by ELISPOT before and after vaccination were compared using the Student *t*- test. The probability of survival was estimated using the Kaplan-Meier method and tested for differences by the log-rank test. All tests were considered significant when the resulting $p =\leq 0.05$.

4.3 Results

4.3.1 Patients and vaccine administration

The patients' baseline characteristics at the time of the enrolment and the total number of vaccination administrations are summarized in the Table 4.1.

Table 4. 1 Patients' characteristics and vaccine administration (PS, performance status; F, female; LCC, large-cell carcinoma; M, male; CHT, chemotherapy; RT, radiotherapy; PR, partial response; SD, stable disease; AD, adenocarcinoma; SCC, squamous cell carcinoma; PD, progressive disease)

Table 3.1: Patients' characteristics and vaccine administration

Patient number	Age (years)	Sex	Histology	Previous treatment	Best Response to previous treatment	Time elapsed from previous treatment (months)	Stage	Status response before vaccination	PS	No. of vaccinations
1	55	F	LCC	1st line CHT/RT	PR	1	III	SD	1	6, 3*
2	48	M	LCC	1st line CHT/RT	PR	3	III	0	0	6,4*
3	56	M	AD	2nd line CHT	SD	3	IV	SD	1	6
4	61	M	SCC	1st line CHT	PR (6 months)	6	IV	PD	0	3
5	73	F	AD	1st line CHT	SD (5 months)	5	IV	PD	0	6, 3*
6	56	M	AD	1st line CHT	PD	1	IV	PD	2	3
7	61	M	AD	1st line CHT	SD	5	III	SD	0	6
8	65	M	SCC	2nd line CHT	PR (6 months)	7	IV	PD	0	3
9	55	M	LCC	3rd line CHT	PD	1	IV	PD	0	3
10	48	M	AD	2nd line CHT/RT	SD	7	IV	SD	0	6
11	60	M	AD	2nd line CHT	PD	1	IV	PD	1	6
12	60	M	SCC	6th line CHT	PD	2	IV	PD	1	4
13	46	M	AD	3rd line CHT	PD	1	IV	PD	0	6
14	73	M	Poorly differentiated	2nd line CHT/RT	PR (10 months)	10	IV	PD	0	6

15	47	M	SCC	1st line CHT	SD	1	IV	SD	0	6
16	60	M	SCC	1st line CHT/RT	PR (11 months)	11	III	PD	0	6, 4+*
17	58	M	Poorly differentiated	1st line CHT/RT	SD (4 months)	7	IV	PD	0	4
18	75	M	SCC	1st line CHT/RT	SD	2	III	SD	0	5
19	55	F	AD	1st line CHT/RT	PR	2	III	SD	0	6
20	55	F	AD	1st line CHT	PD	1	IV	PD	1	3
21	67	M	Poorly differentiated	1st line CHT	SD (5 months)	6	IV	PD	1	3
22	52	M	Poorly differentiated	1st line CHT	PD	3	IV	PD	2	2
*Four patients with stable disease lasting more than 3 months after the sixth vaccine administration received boost vaccinations with the native $TERT_{572}$ peptide every 3 months.										

All patients enrolled had received at least one chemotherapy regimen for the treatment of advanced/metastatic disease. Different numbers and types of chemotherapy regimens had been previously used according to the local policy and the physicians' choice. At the time of enrolment, 14 patients (63.6%) and eight patients (36.4%) had documented PD and SD, respectively, after the completion of the last chemotherapy regimen. Twelve patients (54.5%) had completed the vaccination protocol, and 10 patients (45.4%) were withdrawn after the second (patients 20 and 22), third (patients 4, 6, 8, 9, and 21), fourth (patients 12 and 17), and fifth (patient 18) vaccinations because of rapid disease progression (Tables 3.3.1 and 3.3.2). Four (patients 1, 2, 5, and 16) with SD lasting more than 3 months after the sixth vaccine administration received boost vaccinations with the native $TERT_{572}$ peptide every 3 months. The median follow-up period for the whole group of patients was 10.0 months (range, 2.4 to 40.0 months).

4.3.2 Toxicity

The toxicity was assessed every three weeks on the day of the vaccine administration, while the hematologic/biochemistry profile was additionally assessed every week with biochemistry and FBC. Sixteen patients (72.7%) developed grade 1 toxicity. The most common adverse events observed were local skin reaction (n 8; 36.4%), anaemia (n=3; 13.6%), thrombocytopenia (n = 3; 13.6%), and fever (n = 3; 13.6%). One patient developed grade 2 fatigue and nausea. All toxicities had recovered at the time of the vaccine administration and no additional delays needed for recovery.

4.3.3 Vaccine-induced immune response

The immune response elicited by the vaccination was evaluated with the detection of $TERT_{572}$-specific $CD8^+$ cells in the PBMCs using IFN-ELIspot assay and HLA-A*0201/$TERT_{572Y}$ pentamer staining. Eighty-eight samples were tested, and the background (unstimulated cultures) was 36 ± 23 spots/$2x10^5$ PBMCs. Assessment of the immune response by ELIspot or pentamer staining was performed in 21/22 patients after the second vaccination and in 11/22 patients after the sixth vaccination. 13/21 patients were monitored with both ELIspot assay and pentamer staining after the second vaccination and 7/11 patients were monitored with both ELIspot assay and pentamer staining after the sixth vaccination.

The immune response results of individual patients are presented in Table 4.2, and cumulative

results are presented in Figures 4.1A and 1B. Figure 4.1D shows also representative results of pentamer staining. $TERT_{572}$-specific IFN-γ- producing $CD8^+$ cells were not detected in any patient before vaccination, whereas they were detected in 12 (70.6%) of 17 patients after the second vaccination and 7 (87.5%) of 8 after the sixth vaccination (post-vaccination). The mean (± standard deviation) frequency of $TERT_{572}$-specific cells was less than $1/10^5$ $CD8^+$ cells pre-vaccination, 87 ± 112/105 $CD8^+$ cells after the second vaccination ($p < .005$), and 98 ± $81/10^5$ CD8 cells post-vaccination ($p < 0.00003$; (Fig 4.1A). HLA-A*0201/ $TERT_{572Y}$ pentamer–positive cells were detected in one (5.1%) of 17 patients before vaccination, in 14 (82.4%) of 17 patients after the second vaccination, and in 9 (90%) of 10 patients post-vaccination. The mean (± standard deviation) frequency of pentamer-positive cells was 11 ± $37/10^5$ $CD8^+$ cells before vaccination, 261 ± $212/10^5$ $CD8^+$ cells after the second vaccination ($p < .0002$), and 261 ± $229/10^5$ CD8 cells post- vaccination ($p < .01$; Fig 4.1B). There was a good correlation between the results of ELISpot and pentamer assays; the correlation coefficient R^2 was 0.5163 and 0.8013 after the second and sixth vaccinations, respectively. In all four patients who received boost vaccinations with the native $TERT_{572}$ peptide, the ELIspot assay confirmed that the immune response was maintained (Fig 4.1C). Six (37.5%) of the 16 immune responders versus two (40%) of the five immune non-responders had SD before entering the study (Table 4.2).

Table 4. 2 Peptide-induced Immune response and clinical outcome of vaccinated cancer patients (Abbreviations: $TERT_{572}$, ELIspot, enzyme-linked immunosorbent spot assay; SD, stable disease; PD, progressive disease; NA, not applicable (no blood sample because of PD; IS, inadequate sample).

Peptide-induced Immune response and clinical outcome of vaccinated cancer patients									
		$TERT_{572}$ –Specific cells /10^5 $CD8^+$ Cells							
Patient No.	Status before vaccination	Pre-vaccination		Second Vaccination		Post-vaccination		Clinical outcome (months)	Overall Survival (months)
		ELIspot	Pentamer	ELIspot	Pentamer	ELIspot	Pentamer		
1	SD	<1	<1	45	540	88	70	SD, 13.3*	19.7+
2	SD	<1	<1	58	560	80	190	SD, 17.7+*	17.7+
3	SD	<1	150	32	250	19	330	PD	19.9+
4	PD	<1	<1	40	120	NA	NA	PD	17.1
5	PD	<1	<1	200	170	140	250	SD, 20+*	20.0+
6	PD	<1	<1	85	340	NA	NA	PD	3.0
7	SD	<1	<1	357	130	237	260	SD,6.8+*	6.8+
8	PD	<1	<1	133	300	NA	NA	PD	4.3
9	PD	<1	<1	45	90	NA	NA	PD	5.5
10	SD	<1	<1	27	100	39	110	SD, 9.1+*	9.1+
11	PD	IS	<1	IS	350	IS	100	PD	30.0
12	PD	IS	<1	IS	450	NA	NA	PD	5.7
13	PD	IS	<1	IS	700	IS	700	SD, 9*	40.0+
14	PD	IS	<1	IS	350	IS	600	PD	21.4+
15	SD	<1	IS	313	IS	IS	IS	SD, 7.5*	15.0+
16	PD	<1	IS	220	IS	180	IS	SD, 18 +*	18.0+
17	PD	<1	<1	<1	<1	NA	NA	PD	8.0
18	SD	<1	<1	<1	<1	NA	NA	PD	3.5
19	SD	<1	<1	<1	<1	<1	<1	PD	10.9+
20	PD	<1	IS	<1	IS	NA	NA	PD	2.4
21	PD	<1	IS	<1	IS	NA	NA	PD	3.5
22	PD	IS	IS	NA	NA	NA	NA	PD	8.7+
*Eight of 22 vaccinated patients showed SD post-vaccination, with a median duration of 11.2 months (range, 6.8 to 20.0 months).									

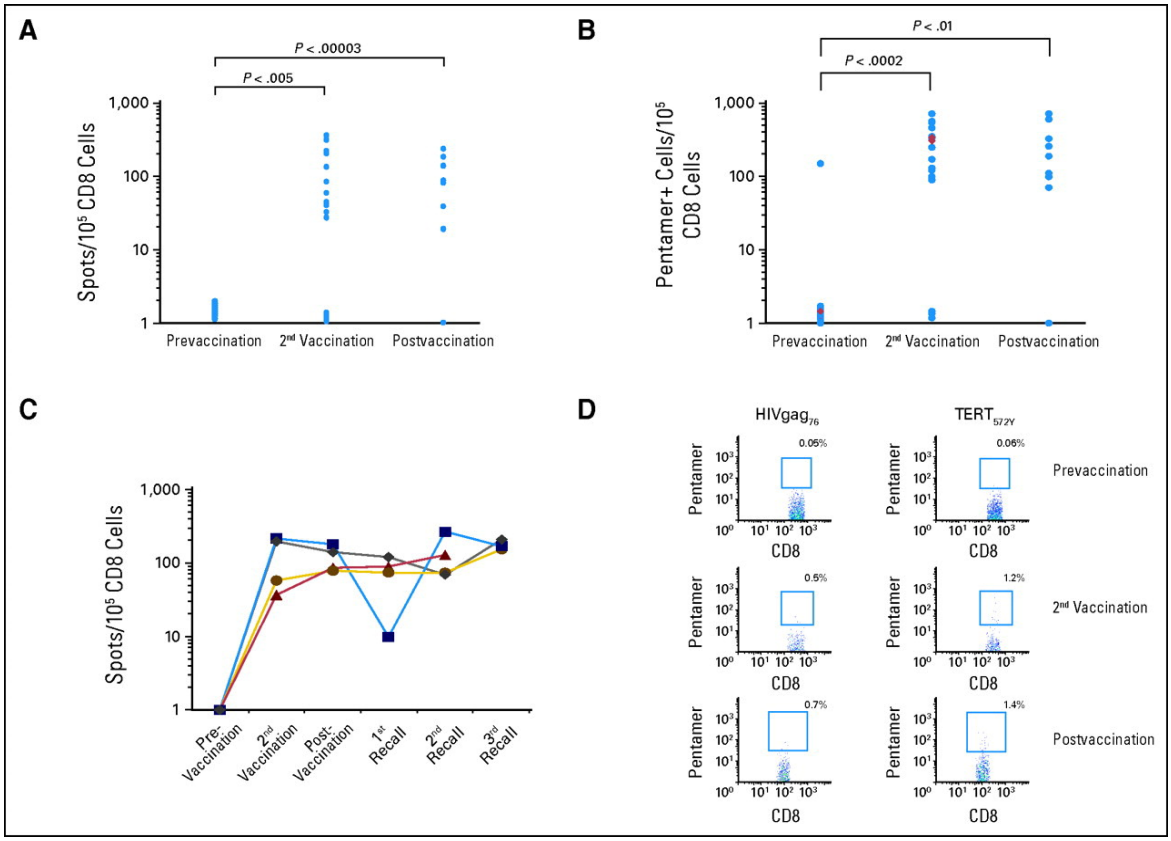


Figure 4. 1 Immune response developed in vaccinated patients (A, B) Immune response was evaluated by (A) enzyme-linked immunosorbent spot (ELIspot) assay (B) and pentamer staining (C) Kinetics of the immune response in patients 1 (♠), 2 (•), 5 (♦), and 16 (■) who received boost vaccinations as detected by ELIspot assay. (D) Pentamer staining of patient 13's peripheral blood mononuclear cells.

4.3.4 Clinical Outcome

Among the patients who participated in the study, 14 (63.6%) of 22 experienced PD and therefore discontinued the vaccine administration and were withdrawn from the study. From them, ten patients progressed during the vaccination (patients 4, 6, 8, 9, 12, 17, 18, 20, 21, and 22) and four following the completion of vaccination (patients 3, 11, 13, and 19). Seven of these patients with disease progression on the study, subsequently received other lines of treatment including chemotherapy and one patient received radiotherapy according to the local policy and physician's choice. Eight (36.4%; patients 1, 2, 5, 7, 10, 13, 15, and 16) of 22 vaccinated patients had SD post-vaccination, with a median duration of 11.2 months (range, 6.8 to 20.0 months; Table 2). Of the eight patients with SD post-vaccination, three (patients 5, 13, and 16) had PD and five (patients 1, 2, 7, 10, and 15) had SD before entering the study. Three (patients 1, 13, and 15, respectively) of them progressed with a TTP of 13.3, 9.0, and 7.5 months and received

chemotherapy, whereas five patients (patients 2, 5, 7, 10, and 16, respectively) were still in SD at the time of the report with a follow-up of 17.7, 20.0, 6.8, 9.1, and 18.0 months. The median TTP for the whole cohort of patients was 3.8 months (range, 1.4 to 20.0 months). Ten (45.4%) of 22 vaccinated patients had died at the time of the publication. Interestingly, 11 (91.7%) of 12 patients who managed to complete the vaccination protocol were alive at the time of analysis, with an estimated median OS of 18.0 months (range, 5.7 to 40.0 months; Table 4.2). The estimated median OS time for all 22 patients was 30.6 months (95% CI, 10.9 to 48.9 months), and the 1- and 2-year OS rates were 63.3% and 56.3%, respectively.

4.3.5 Immune Response and Clinical Outcome

The patients' characteristics in relation to the development of early immune response after the second vaccination are presented in Table 4.3. The correlation of clinical outcome and immune response developed after the second vaccination demonstrated that eight (50%) of 16 immune responders but none of the five non-responders experienced long-lasting (6 months) disease stabilization ($p<.04$). In addition, the overall strength of the immune response, as shown by ELIspot assay, was significantly higher in patients with SD (174 ± 134 $TERT_{572}$-specific cells/ 10^5 $CD8^+$ cells) than in patients with PD (34 ± 45 $TERT_{572}$-specific cells/10^5 $CD8^+$ cells; $p = .04$). Interestingly, the TTP and OS according to the immune response after the second vaccination demonstrated that early immune responders (n= 16) had a significantly longer TTP (4.2 versus 2.3 months; range, 1.6 to 20.0 vs 1.8 to 6.2 months; p:.046 and OS (30.0 versus 4.1 months (range, 2.8 to 40.0 versus 2.4 to 10.9 months; p: .012) than the immune non-responders.

Table 4. 3 Patients' characteristics for early immune Responders and Non-responders

Characteristics	**Early Responders (n=16)**		**Early Non-responders (n= 5)**	
	No	%	No	%
Age, years				
Median (Range)	58 (48-73)		58 (55-75)	
Sex				
Male	14	87.5	3	60
Female	2	12.5	2	40
Histology				
Squamous cell	5	31.2	1	20
Adenocarcinoma	7	43.7	2	40
Large cell	3	18.8	0	0
Poorly differentiated	1	6.3	2	40
Stage				
III	4	25	2	40
IV	12	75	3	60
Performance Status (WHO PS)				
0	11	68.7	3	60
1	4	25	2	40
2	1	6.3	0	0
Line of treatment				
Second	7	43.7	5	100
≥Third	9	56.3	0	0
Clinical status before vaccination				
Stable disease	6	37.5	2	40
Progressive disease	10	62.5	3	60

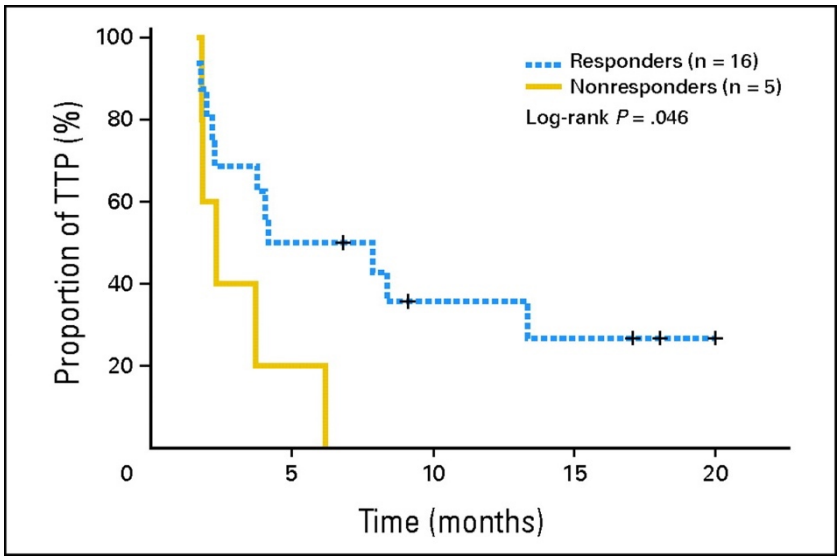


Figure 4. 2 Time to progression for patients with early immunological response (n = 16) and non-responders (n = 5)

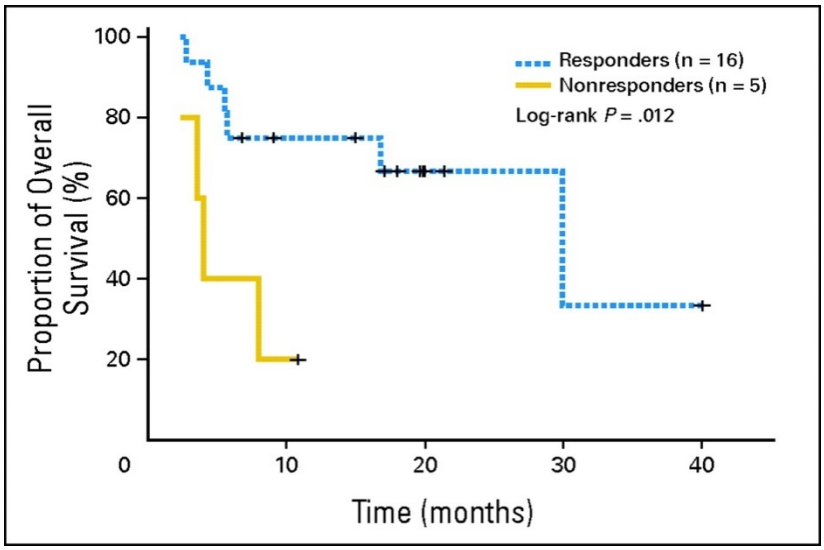


Figure 4. 3 Survival for patients with early immunological response (n = 16) and non-responders (n = 5)

4.4 Conclusion

The results of the current pilot study showed that the cryptic telomerase $TERT_{572}$ vaccine was safe and effective in terms of immunogenicity for almost all vaccinated patients. In 76% and 91% of evaluated patients, immune response was induced after the second and sixth vaccinations, respectively. $TERT_{572Y}$- generated CTLs recognized the native $TERT_{572}$ peptide (Bolonaki et al., 2007) and were maintained for at least 9 months in patients who received boost vaccinations with the native $TERT_{572}$ peptide. Although, objective response such as PR or CR was not observed based on RECIST criteria, eight patients had stable (SD) disease for 6.8-20 months. The patients who developed early immune response had a significantly better OS than patients who did not (30 vs 4.1 months; p= 0.012). The absence of immune response could be attributed to the rapid disease progression, which could explain the early death of non-responding patients. However, it is important to mention that among the five patients withdrawn from the study due to rapid disease progression who had developed an early immune response, three of them survived for 5.5, 5.7 and 17.1 months, respectively. The results of this study were published in Journal of Clinical Oncology (Bolonaki et al., 2007).

Following establishment of the best vaccination schedule, and the positive results of the pilot study, the next step was the investigation of the ex vivo reactivity and function of the CTLs induced by the vaccine.

5. CHAPTER 5: Immunologic and clinical outcome in patients vaccinated with the TERT vaccine. Expanded study of immunologic response in various tumour types

5.1 Introduction

Qualitative and quantitative characteristics of the CTLs induced by the vaccine. This was assessed in a cohort of patients with various tumour types, enrolled in this expanded study.

5.2 Patients and Methods

5.2.1 Patients

Fifty-five HLA-A*0201-expressing patients with various types of chemo-resistant advanced solid tumours (stages III and IV) were enrolled in this study. The eligibility criteria are summarised in the section 2.1.

5.2.2 Peptides

As stated in section 2.4

5.2.3 Vaccination protocol

All patients were vaccinated as previously described (Bolonaki et al., 2007; Vetsika et al., 2011) in section 2.5.

5.2.4 Patients' samples for immunomonitoring

Patients' peripheral blood in EDTA (100 ml) was collected before the first vaccination, after the 2nd and 6th vaccinations and before each boost dose for continuing patients. The process is described in detail in previous sections 2.5-2.8.

5.2.5 Enzyme-linked immunosorbent spot (ELISpot) assay

We used the IFN-γ and Perforin ELISpot assays to assess the reactivity and the cytotoxic activity respectively, of specific T cells produced in response in response to $TERT_{572}$ and $TERT_{572Y}$ peptides. Both assays were performed as described in previous section 2.10.

5.2.6 Intracellular cytokine staining and flow cytometry

Peptide-specific $CD8^+$T cells were identified by IFN-γ intracellular staining using flow cytometry. The principle and the method are described in the section 2.11.

5.2.7 $TERT_{572Y}$ tetramer staining and clones

$TERT_{572Y}$-specific $CD8^+$ T cells were sorted as described in section 2.12.

5.2.8 Chromium-release assay

The lytic activity of the sorted $TERT_{572Y}$-tetramer^{+}/$CD8^{+}$ T cells was assessed by a chromium-release assay against $TERT^{+}$ and $TERT^{-}$ cell lines as described in section 2.13.

5.2.9 Statistical analysis

As discussed in section 2.14.

5.3 Results

5.3.1 Patients' Demographics

Fifty-five patients were enrolled in this study. The patients' baseline characteristics are summarised in Table 5.1. The enrolled patients had various tumour types, including breast cancer (20%), melanoma (13%) and prostate cancer (20%) and pancreato-biliary (16%) among others. Prior to the study entry, the vast majority of patients (93%) had distant metastases (Stage IV) and 71% entered the study with documented disease progression (PD) on the last chemotherapy regimen. The majority of the patients (64%) had received one previous chemotherapy line but the rest 36% had received at least 2 chemotherapy regimens before enrolment. All patients (100%) received at least the first two vaccinations and 34 (62%) completed the 6-vaccination schedule. The vaccination protocol was prematurely terminated in the remaining patients, due to disease progression. Following completion of the 6 vaccinations, nine (16.4%) patients received at least one boost vaccination.

Table 5. 1 Patients' characteristics

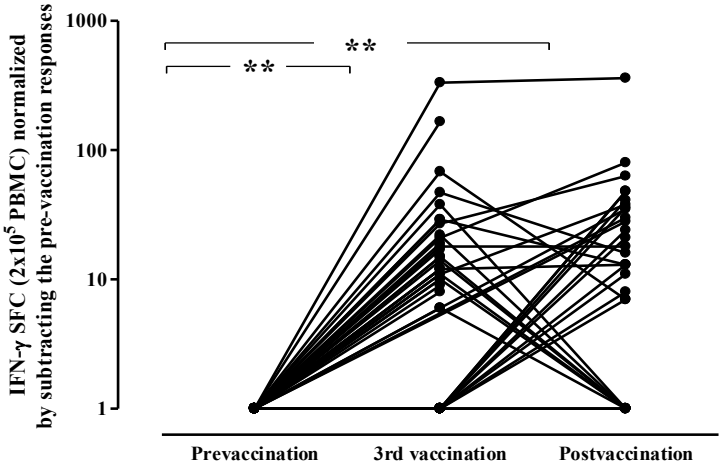
Characteristics	N^0	%
Age (median, range)	57 (31-84)	
Sex		
Male	37	67
Female	18	33
Cancer type		
Breast	11	20
Colorectal	3	5
Ovarian	1	2
Head & Neck	2	4
Pancreas/cholangio	9	16
Melanoma	7	13
Hepatocellular	2	4
Renal	7	13
Prostate	11	20
Other	2	4
Disease Stage at study entry		
III	4	7
IV	51	93
Disease Status at study entry		
Progressive Disease	39	71
Stable Disease	16	29
Lines of Treatment prior to study entry		
One chemotherapy regimen	35	64
≥2 (range, 2-9) chemotherapy regimens	20	36

5.3.2 TERT-specific T-cell responses

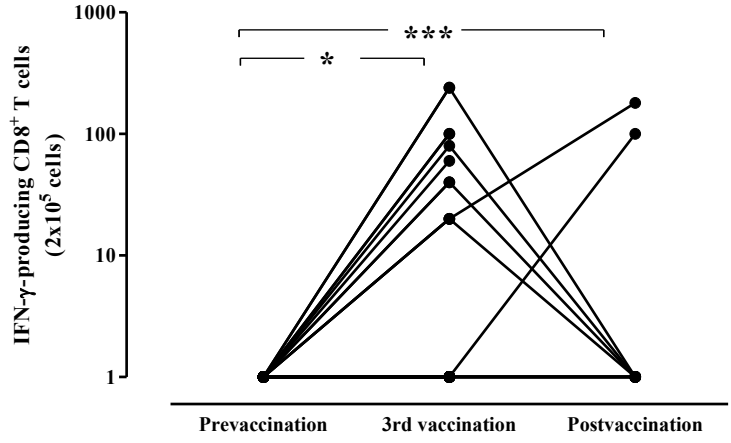
The baseline immune reactivity to $TERT_{572}$ peptide was assessed in the 55 patients and the vaccine-induced immune response after the 2nd vaccination in 53 out of the 55 patients. Lastly, the immune response was assessed in 32 (94%) out of 34 patients after the 6th vaccination (post-vaccination). For the evaluation of the immune response, the IFN-γ ELISpot assay was used to detect peripheral blood TERT-specific T cells. Moreover IFN-γ intracellular staining assay was performed in 35 out of 55 (63.6%) patients in whom sufficient material to detect PBMCs was available.

In Fig 5.2 there are ELIspot assay pictures of a "weak" and a "strong" responder. TERT-specific IFN-γ producing T cells were detected in 27 (51%) out of 53 patients after the 2nd vaccination and in 22 (69%) out of 32 after the completion of the 6 vaccinations (post-vaccination), using the IFN-γ ELISpot assay. The mean (± standard deviation) frequency of spot-forming cells was 6 ± 11 per $2x10^5$ PBMCs before vaccination (background frequency), 19.1 ± 50.7 per $2x10^5$ PBMCs after the 2nd vaccination, and 29 ± 62/ $2x10^5$ PBMCs post-vaccination. The TERT-specific frequencies after the 2nd and the 6th vaccinations were statistically different compared to the baseline (pre-vaccination; $p< 0.01$, paired *t*-test) (Fig 5.1A). It was found that while three (9.1%) did not change, nine (27.3%) had a >2 fold decrease in peptide-specific immunity observed between the 2nd and the 6th vaccination. Intracellular staining of PBMCs for TERT peptides-induced IFN-γ production confirmed these T-cell specific immune responses (Fig 5.1B and C).

A



B



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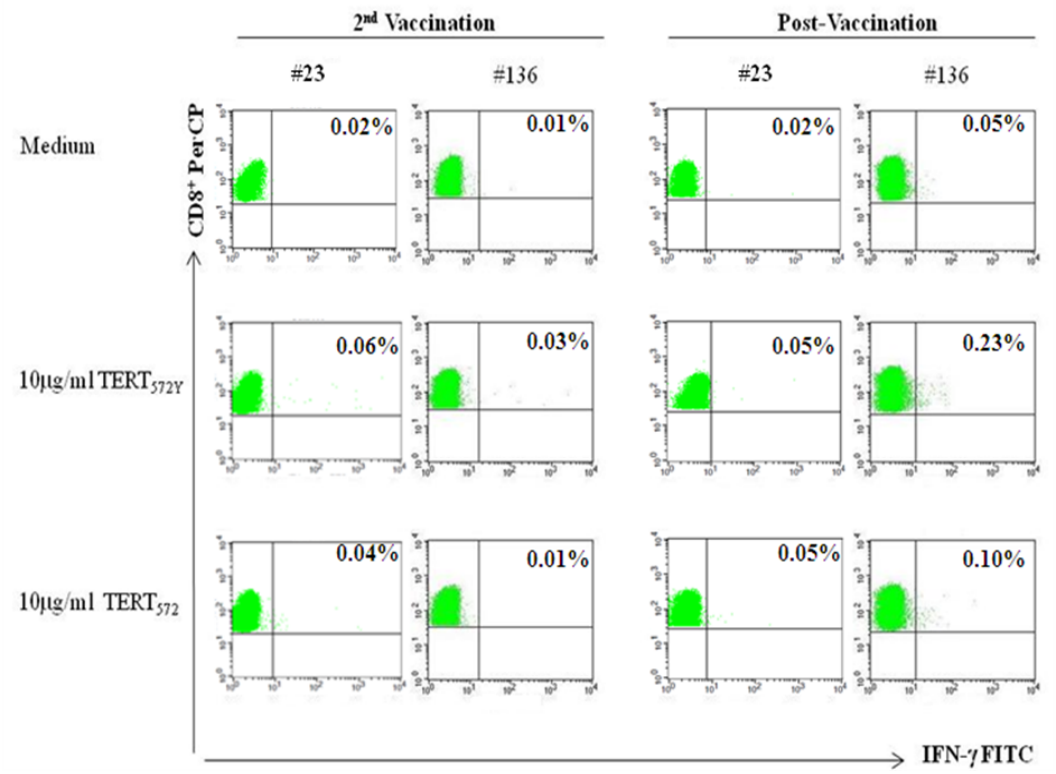


Figure 5. 1 $TERT_{572}$-specific T cell responses in patients vaccinated with the TERT vaccine. (A)Frequencies of specific-T cells to TERT-peptides in vaccinated patients prior to vaccination (background) and after the 2nd and 6th vaccination (post-vaccination) using IFN-γ ELISpot assay, and (B) IFN-γ intracellular staining, (C) $TERT_{572}$ and $TERT_{572Y}$ reactive $CD8^+$ T cells after the 2nd and 6th vaccination in two representative patients as assessed by IFN-γ ICS. Percentages in the dot plots are for $CD3^+CD8^+$ IFN-γ T cells [*** $p< 0.0001$; ** $p< 0.001$ and *$p< 0.05$; SFC= spots-forming cells].

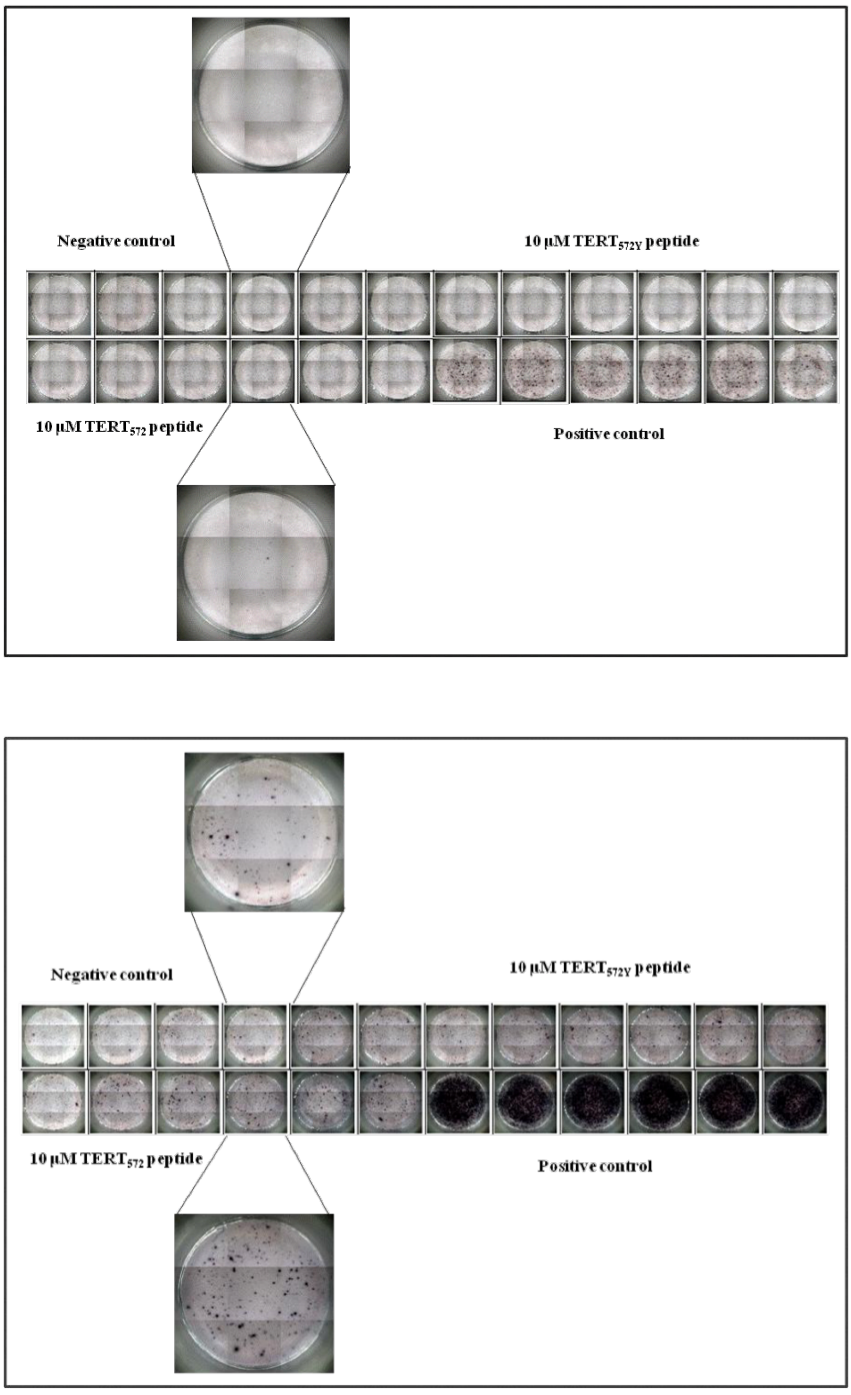


Figure 5. 2 ELISpot assay wells showing spots formed by secretion of IFN- γ, by 2x10^5 PBMC in response to ConA (positive control), TERT peptide and medium-only (negative control) of a (A) weak responder and (B) strong responder. The spots represent the IFN-forming cells. The stimulator peptides used are shown on the top and bottom of the wells. Two wells were enlarged so that the difference in the spot counts in the medium only and in the treated cells to be more distinct.

5.3.3 Functional analysis of TERT-specific T cells

It is clearly important to establish the ability of the vaccine to elicit immune response but it is also important to determine the cytotoxic activity of TERT- specific IFN-γ-producing T cells induced by the vaccine. As such, an ex vivo perforin ELISpot assay was used with PBMCs from 6 selected patients from whom biological material was available from post-vaccination time point.

Patients' demographics are presented in Table 5.2. For all those 6 patients, an immunological response had previously been demonstrated by the IFN-γ ELISpot assay. Results showed that five out of six (83%) patients showed CTL activity with the ability to specifically produce detectable levels of perforin ex vivo in the presence of $TERT_{572}$ peptide (Fig 5.2a). Three (50%) of these six patients were also able to produce perforin in response to the optimized $TERT_{572Y}$ peptide. In one patient however, T-cell-specific perforin release could not be detected in response to either peptide.

In addition, we assessed the functional specificity of sorted $hTERT_{572Y}$-$tetramer^{+}CD8^{+}$ T cells from one vaccinated patient in regard to recognizing and killing of TERT- expressing cells by a chromium-release assay. The $TERT^{+}$/ HLA-A*0201^{+}(N18/TERT and NA8) but not the $TERT^{-}$/ HLA-A*0201^{+}(N_{418} and Me_{290}) cells lines were lysed by $hTERT_{572Y}$-tetramer sorted $CD8^{+}$ T cells (Fig 5.3B).

Table 5. 2 Patients' characteristics in perforin assay

Characteristics	No	%
Age (median, range)	53 (45-57)	
Sex		
Male	5	83
Female	1	17
Cancer Type		
Breast	1	17
Head & Neck	1	17
Hepatocellular	1	17
Renal	2	32
Other	1	17
Disease Stage at study entry		
III	3	50
IV	3	50
Disease Status at study entry		
Progressive Disease	3	50
Stable Disease	3	50
Lines of Treatment prior to study entry		
One chemotherapy regimen	5	83
≥ 2 (range, 2-9) chemotherapy regimens	1	17

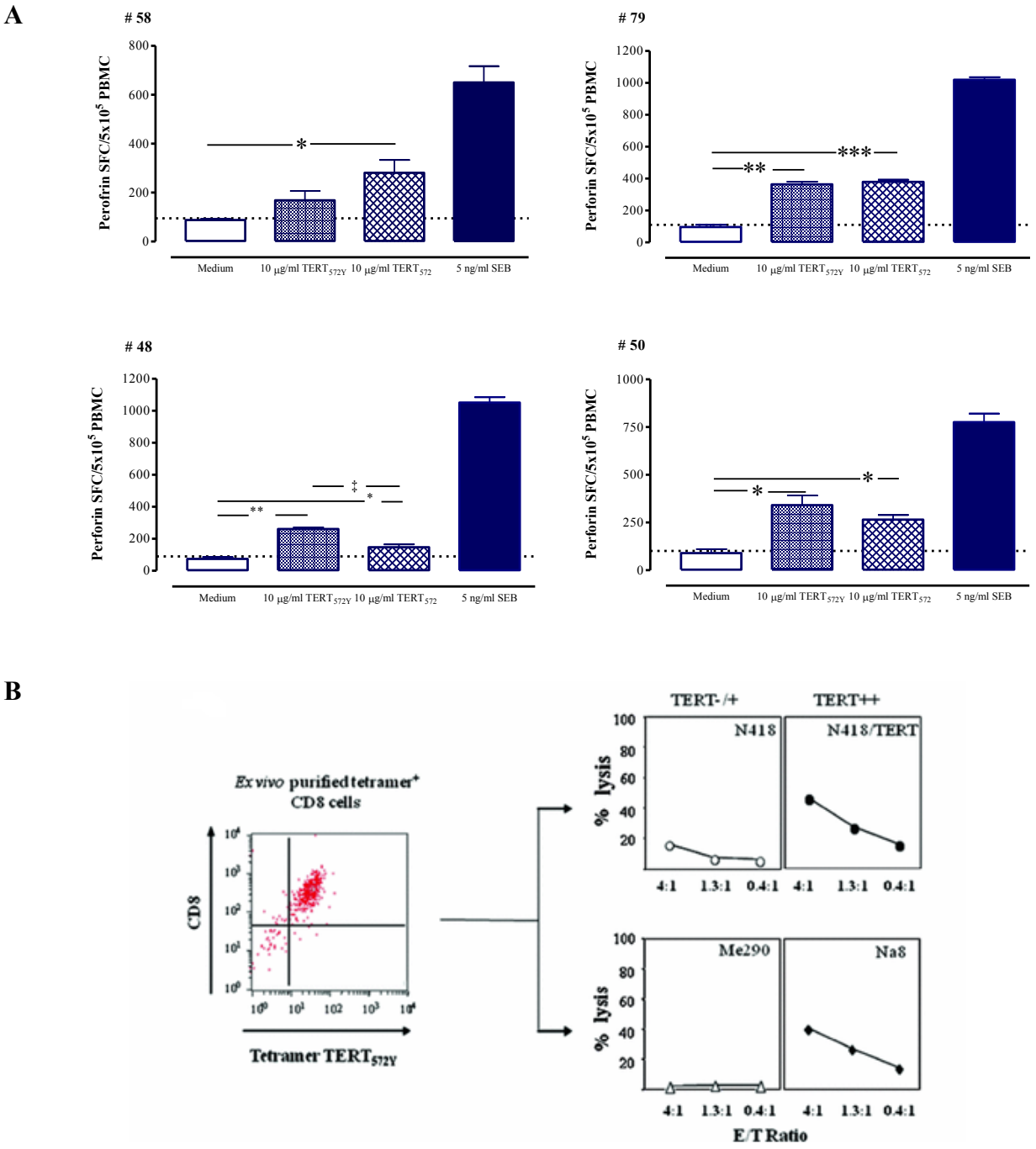


Figure 5. 3 Functionality of specific T cell to TERT$_{572}$ peptide. (A) Frequencies of cytotoxic specific T cell to TERT$_{572}$ peptide as assessed by perforin production in the post-vaccination samples of four representative patients using ELISpot. Dashed line represents the threshold for 'positive' response [*** p< 0.0001; ** p< 0.001 and *p< 0.05; SFC= spots-forming cells] (B) The lytic activity of the sorted TERT572Y-tetramer+/CD8+ T cells from one well-responding patient was assessed by a chromium-release assay against TERT+ and TERT- cell lines.

5.3.4 Immune responses according to disease stage and clinical status

The immune response was analysed in regard to the clinical stage that the patients had at the time of entry into the trial in an attempt to correlate the stage of the disease with the magnitude of the immune response. Among the patients who entered the trial with locally advanced disease (stage III, unresectable), TERT- specific IFN-γ-producing T cells were detected in 3 out of 4 (75%) patients after the 2nd vaccination and in 2 out of 3 (66%) patients after the completion of the 6-vaccination protocol. One patient significantly enhanced its immune response, whereas another one had a decrease in peptide-specific immunity after more than 3 vaccinations.

Among the patients who entered the trial with stage IV metastatic disease, TERT-specific IFN-γ-producing T cells were detected in 24 (49%) out of 49 patients after the 2nd vaccination and 20 (69%) out of 29 patients after the completion of the vaccination protocol (Fig 5.4A). Three (10%) patients increased further their immune response, three (10%) did not change, and seven (24%) had a twofold decrease in peptide-specific immunity observed between the 3rd and after the 6th vaccination. The TERT- specific frequencies after the 2nd and the 6th vaccination were statistically different compared to the baseline (pre- vaccination; $P < 0.001$, paired t test).

When the immune response was analysed based on the patients' response to the previous treatment (Stable Disease (SD) versus Progressive Disease (PD), we found that the TERT vaccine was similarly immunogenic in patients who entered the vaccination protocol with either stable (SD) or progressive disease (PD), as the majority almost 70% of the patients had developed TERT-specific IFN-γ-producing T cells after the completion of the 6-vaccination protocol (Fig 5.4B).

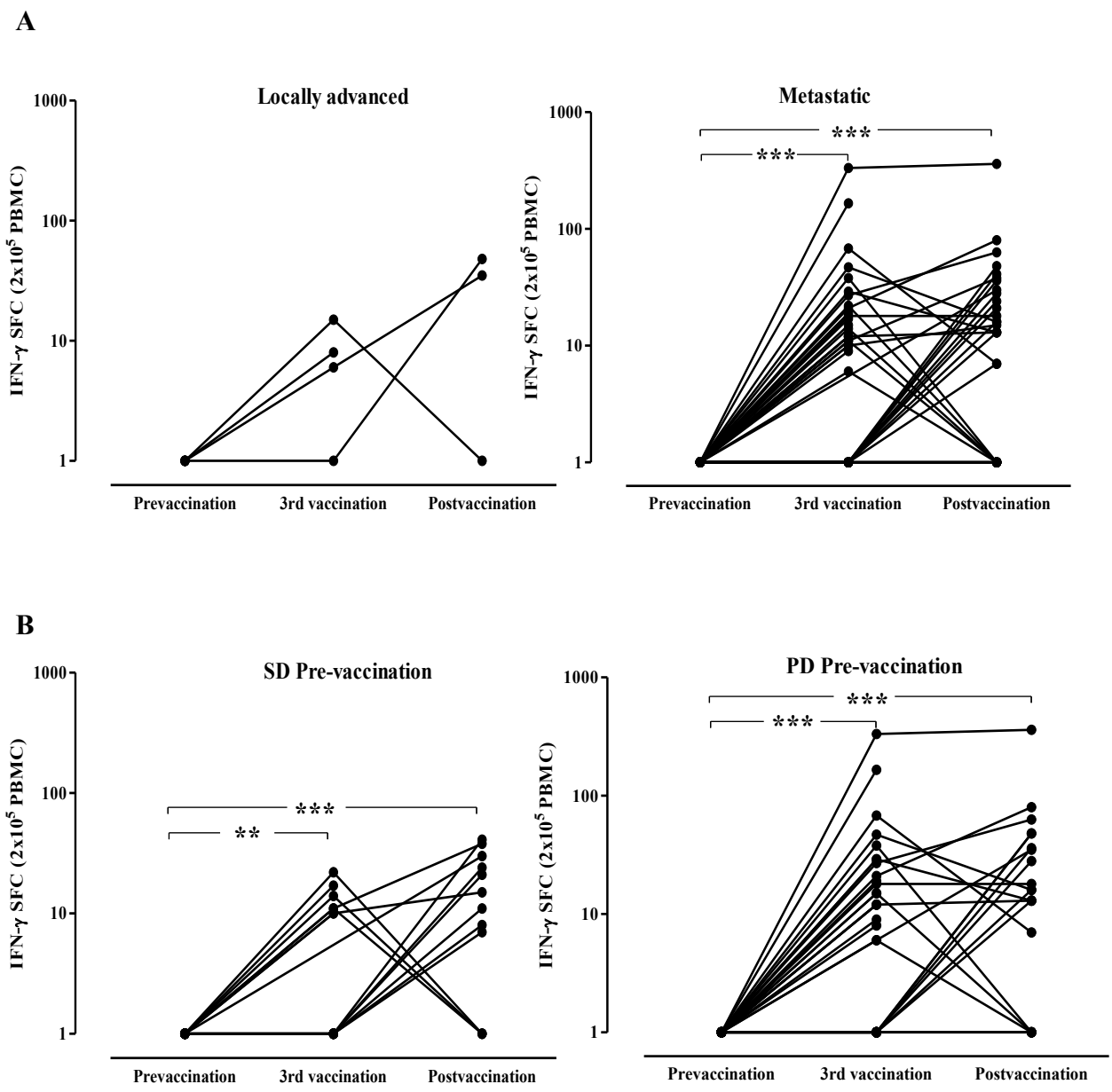


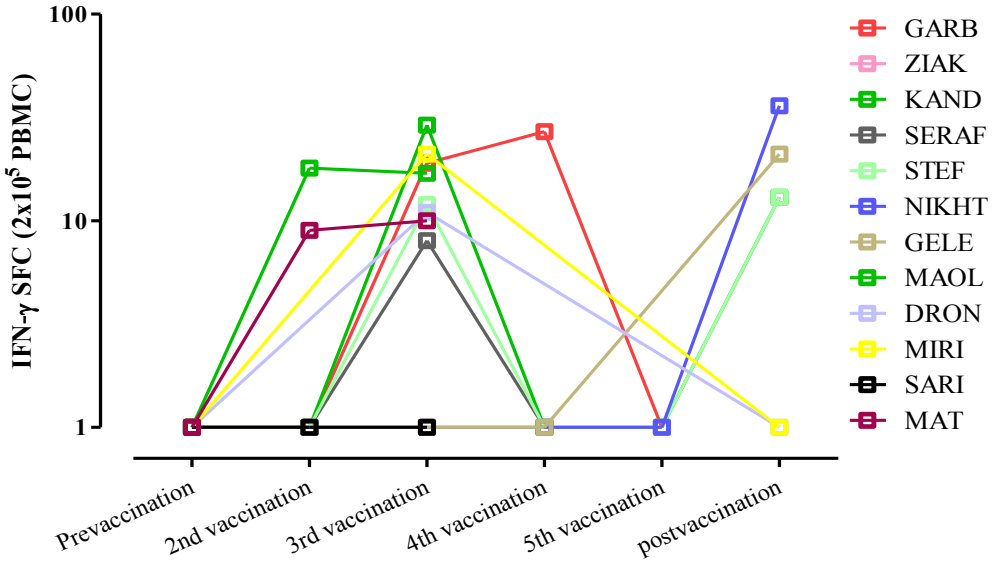
Figure 5. 4 $TERT_{572}$-specific T-cell responses according to disease stage and disease status before study entry and disease evolution in vaccinated patients. Magnitude of T-cell response to $TERT_{572}$ peptide in vaccinated patients prior to vaccination and after the 2nd and 6th vaccinations (post-vaccination) according to the: A) disease stage and B) disease status using ELISpot. [SFC = spot-forming cells; [***P <0.0001; **P <0.002, paired t test]

5.3.5 Kinetics of TERT-specific T-cell response

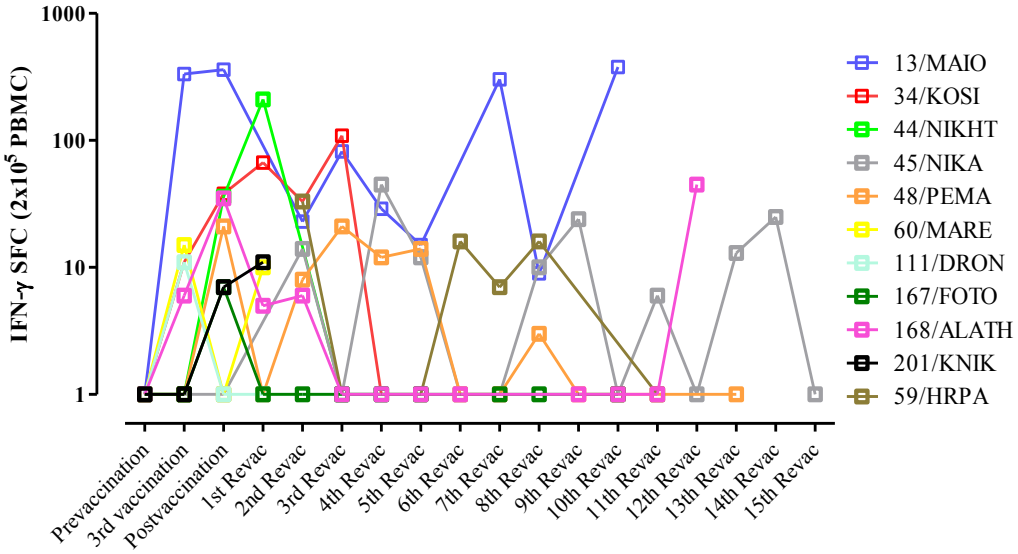
PBMCs were isolated from 12 random patients before each vaccination dose in order to assess the kinetics of induction of peptide-specific T-cell responses. As depicted in Fig 5.5A, there was variation in the induction of peptide-specific immune response from patient to patient; however,

the majority of patients developed an immune response after the 2nd administration of the $TERT_{572Y}$ peptide. Patients were also able to generate an immune response at different time points during the course of the 6-vaccination protocol, but not a single patient developed an immune response after the administration of only the first vaccination dose. What is also interesting is that the magnitude of vaccine-induced T-cell response after the completion of the 6 vaccinations was similar in all patients independently of the time of the induction of the TERT-specific immune response. Moreover, extended vaccination maintained the number of peptide-specific $CD8^+$ T cells in nine (82%) out of 11 patients who received boost vaccinations with the native $TERT_{572}$ peptide as assessed by IFN-γ ELISpot assay (Fig 5.5B) and IFN-γ intracellular staining (Fig 5.5C).

A



B



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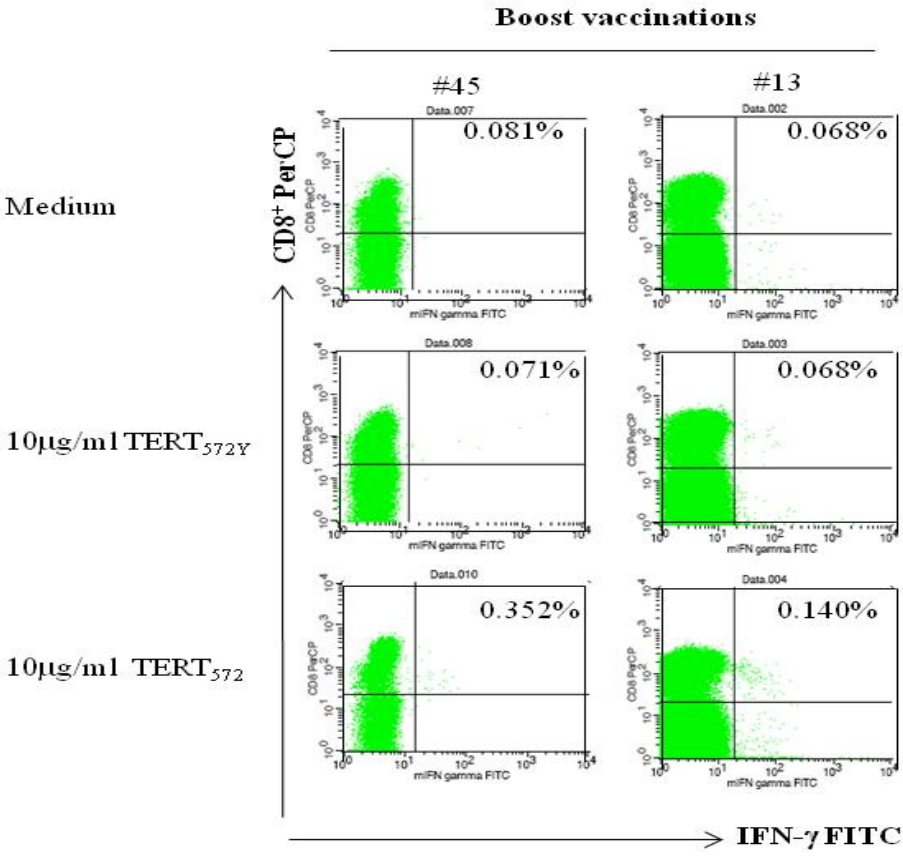


Figure 5. 5 Kinetics of TERT-specific T-cell response development after immunization. TERT-specific immune response A) during the course of six cycles of vaccination and B) in boosted patients as assessed by IFN-γ ELISpot assay, C) Immune response in two representative boosted patients after the 8th (#45) and 10th (#13) boosts as assessed by IFN-γ ICS. The data in the graphs are presented as the mean value of 3 independent experiments. Background frequencies have been subtracted. [SFC = spot-forming cells]

5.3.6 Immune response and clinical outcome

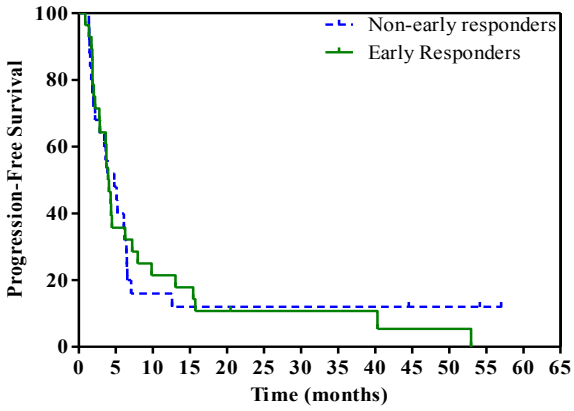
In addition to our primary goal, the assessment of the quantitative and qualitative characteristics of the immune response elicited by the vaccination, we tried to evaluate whether there was any correlation between the development of immune response and patients' outcome. More specifically, we analysed the association between the development of TERT-specific IFN-γ immune reactivity and PFS and OS.

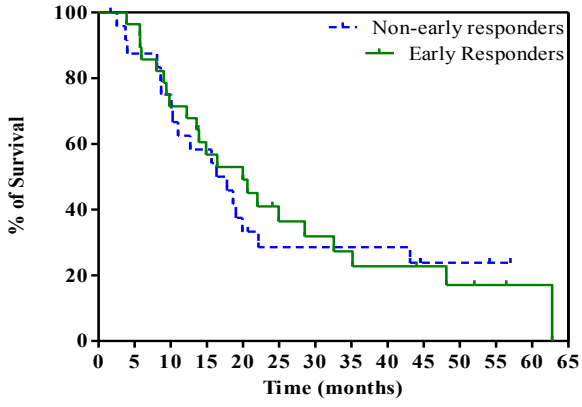
Overall, no significant difference observed in either PFS or OS between patients who developed an early (after the 2nd vaccination) or late (post-vaccination) immunological response during

vaccination versus the ones who did not (Fig 5.6A, B). An interesting observation however, was made when we analysed the subgroup of those patients who entered the study with progressive disease to the previous treatment, therefore had an overtly active disease. Among them, those who developed a late immune response had a significantly longer OS compared with that of those patients without a post-vaccination immune response (28.6 months vs. 13.1 months; log-rank test P = 0.01; Fig 5.6C). It is important to mention that this result was observed irrespectively of the cancer type which obviously could suggest different prognosis after progression to the previous lines of chemotherapy, but the estimated survival in most cases was 6 months or less. In addition, the induction of either early or late immune response versus no-response ($v^2 = 8.3$, P = 0.5 and $v^2 = 8.9$, P = 0.3, respectively) in the progressive disease patients was irrespective of their cancer type.

A Early Immune Response (all patients)

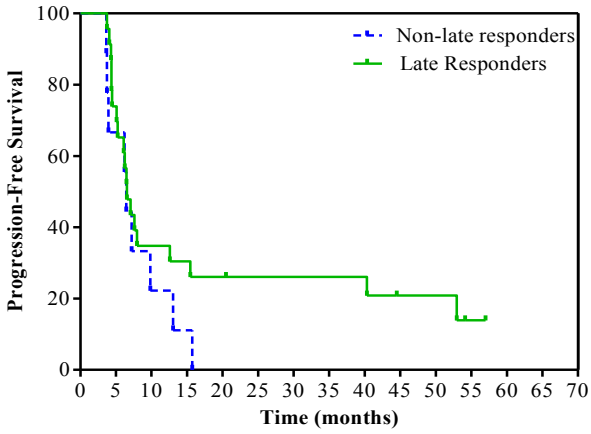
	Early Response	**Non- early Response**	**p- value**
PFS	N=28, Events=27	N=25, Events=22	
Median (mo)	4.1	4.8	0.9
Min-Max	1.4-53	1.4-57	
OS	N=28, Events=22	N=25, Events=22	
Median(mo)	20	17.1	0.8
Min-Max	3.9-62.8	1.7-57	
1-yr Survival	71.4%	60%	

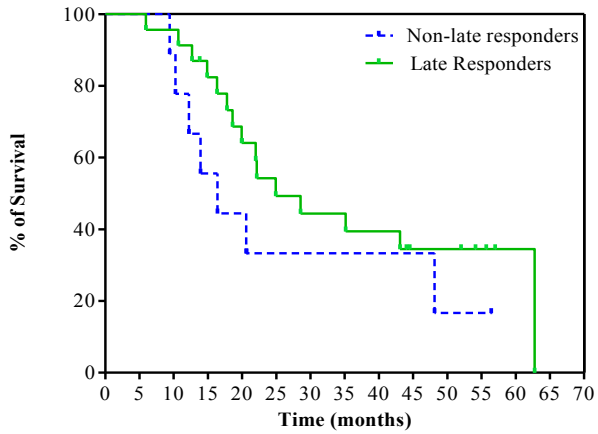




B Late Immune Response (all patients)

	Late response	Non-late response	p- value
PFS	N= 23, Events= 19	N=9, Events= 9	
Median	6.5	6.4	0.2
Min-Max	3.7 – 57	3.7 – 15.7	
OS	N=23, Events=15	N=9, Events=7	
Median	24.9	16.4	0.2
Min-Max	6 – 62.8	9.4– 56.4	
1-year survival	91.3 %	63 %	





C. Late Immune Response (patients with PD)

	Late response	**Non-late response**	**p- value**
PFS	N= 14, Events= 13	N=6, Events= 6	
Median	6.2	5.1	0.1
Min-Max	1.4 – 57	3.7 – 9.8	
OS	N=14, Events=10	N=6, Events=5	
Median	28.6	13.1	**0.01**
Min-Max	3.8 – 63	9.4– 20.7	
1-year survival	86.7 %	66.7 %	

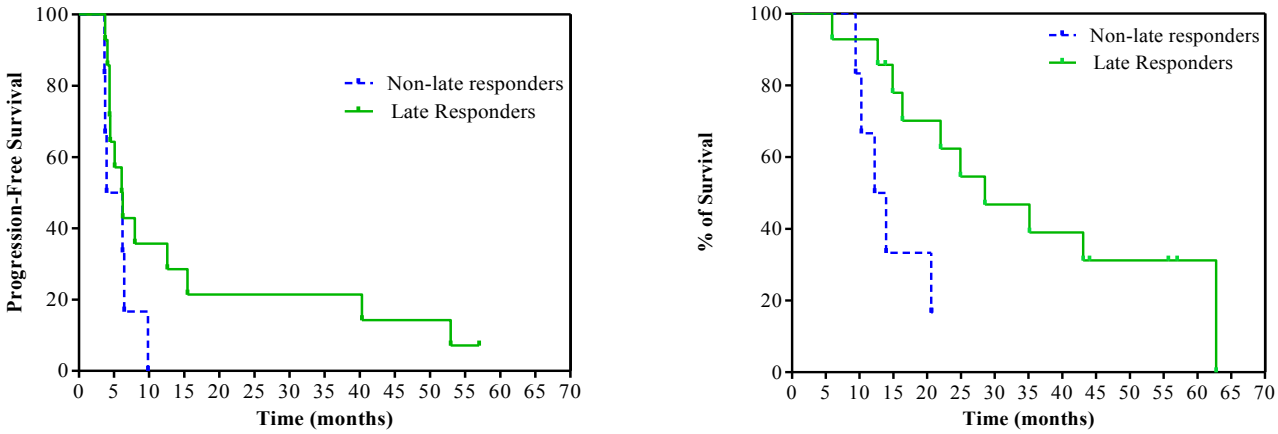


Figure 5. 6 Progression-free and overall survival of all vaccinated patients. The progression-free and overall survival of all patients was assessed according to the presence (green line) of absence (blue line) of TERT-specific immune response after the 2nd vaccination (A), and at the completion of the 6-vaccination protocol (B). Overall survival of vaccinated patients with PD at study entry according to post-vaccination immune response (C).

5.4 Conclusion

In a previous study, we showed that $CD8^+$ T-cell immune responses could be detected in 22 HLA-A*0201 patients with advanced NSCLC vaccinated with the TERT572-based vaccine Bolonaki et al. (2007). In the present study, we assessed a larger cohort of HLA-A*0201 patients with different (other than NSCLC) types of cancer, and we analyzed and further characterized the immune responses induced by the vaccine. The findings of the current study confirmed our previous observations regarding the ability of the vaccine to induce specific $CD8^+$ T cells against TERT peptides, which exhibited in vitro effector functions including IFN-γ and perforin production.

Furthermore, boost vaccinations with the native peptide resulted in the maintenance of specific immune response that had been induced by the 6-vaccination schedule (Fig 5.5B, C). These TERT-specific T cells appeared to be functional, releasing perforin following stimulation with TERT peptides (Fig 5.2A); it is well established that the perforin ELISpot correlates with cytotoxicity assays (Zuber et al., 2005). $hTERT_{572Y}$-$tetramer^+$ $CD8^+$T cells from one vaccinated patient were able to kill TERT-expressing tumour cells (Fig 5.2B).

In conclusion, the current study demonstrated that the TERT vaccine is able to induce a TERT-specific immune response in vaccinated patients with different types of solid tumours, irrespective of their disease stage and clinical status. The results of the current study were published in the Journal Cancer Immunology, Immunotherapy (Vetsika et al., 2012).

In addition to the ability of the vaccine to induce effective immune response, it is crucial to assess the clinical impact of the vaccination in the cancer outcome. Therefore, the current vaccination schedule was used subsequently in larger patients' groups and the clinical outcome was measured.

6. CHAPTER 6: Clinical outcome of patients with various advanced cancer types vaccinated with an optimized cryptic human telomerase reverse transcriptase (TERT) peptide: results of an expanded study

6.1 Introduction

In addition to the immunologic results presented in the chapter 5 we have also analysed and presented in more detail the safety data and the clinical outcome of the patients enrolled in this expanded study. In the era of personalized and targeted therapies, the immunotherapy is a popular approach especially for cancers that are refractory to conventional therapies. The aim of this study was to explore the clinical outcome of a larger cohort of patients with various malignant neoplasms vaccinated with the TERT vaccine.

6.2 Materials and methods

6.2.1 Patients and vaccine administration

The eligibility criteria are summarized in the section 2.1. All patients received six subcutaneous injections administered every 3 weeks. Two milligrams of each peptide in 0.5 ml of sterile water was emulsified with 0.5 ml of Montanide ISA51 (Seppic Inc., Paris, France) immediately before being injected. The optimized $TERT_{572Y}$ peptide was used for the first and second injections and the native $TERT_{572}$ peptide for the following four injections. Patients with no evidence of PD after the sixth vaccination were allowed to receive boost vaccinations with native $TERT_{572}$ peptide every 3 months until disease progression, consent withdrawal or unacceptable toxicity, whichever happens first.

The vaccine consisted of the HLA class I-restricted optimized $TERT_{572Y}$ (YLFFYRKSV) and the native $TERT_{572}$ (RLFFYRKSV) peptides. The vaccine's synthesis and preparation were previously presented in section 2.4.

6.2.2 Immunomonitoring

The immune response was evaluated using ELIspot assay as previously described in the section 2.10.

6.2.3 Patients' evaluation

Before study entry and at pre-specified time intervals after the enrolment, we performed clinical assessment of the patients and evaluation of their imaging and laboratory studies at the medical oncology Unit of the University Hospital of Heraklion. The monitoring protocol is presented in the section 2.2.

6.2.4 Statistical analysis

As discussed in section 2.14. Disease control rate [DCR; complete response (CR) or partial response (PR) or SD] was analysed in addition to other clinical parameters.

In this phase we investigated the association of immune response with time-to-event end points using the log-rank test and we performed a uunivariate Cox regression analysis with hazard ratios (HRs) and 95% confidence intervals to explore the association between each potential prognostic factor with them using the SPPS Statistics 20 software (SPSS Inc, USA). Prognostic factors with significant univariate associations were then included in a multivariate Cox proportional hazards regression model with a stepwise procedure (unconditional backward procedure) evaluating their independent prognostic value on PFS and OS (Kirkwood & Sterne). All tests were considered significant when the resulting p value was ≤ 0.05.

6.3 Results

6.3.1 Patients' characteristics

Fifty-five patients were analyzed. The patients' baseline characteristics are listed in the Table 6.1. Thirty (55%) patients had previously received at least two chemotherapy regimens. Thirty-nine (71%) and 16 (29%) patients entered the study with documented PD or SD to previous treatment, respectively. All patients received the first two vaccinations and 34 (62%) completed the six- vaccination protocol. Twenty-one (38%) patients discontinued treatment before the sixth vaccination because of PD (14 patients after the third, 5 patients after the fourth and 2 patients after the fifth vaccination). Eight (15%) patients proceeded to receive boost vaccinations in the absence of clinical or imaging signs of progression and six of them continued the vaccination for more than 2 years. The clinical characteristics of the patients who received boost vaccinations are summarised in the Table 6.2.

Table 6. 1 Baseline patients' characteristics (N=55) (ECOG, Eastern Cooperative Group; PD, Progressive Disease; SD, Stable Disease)

Characteristics	N^0	%
Age (median, range)	57 (31-84)	
Sex		
Male	37	67
Female	18	33
ECOG, Performance Status		
0	34	62
1	19	34
2	2	4
Cancer type		
Breast	11	20
Colorectal	3	5
Ovarian	1	2
Head & Neck	2	4
Pancreas/cholangio	9	16
Melanoma	7	13
Hepatocellular	2	4
Renal	7	13
Prostate	11	20
Other	2	4
Disease Stage at study entry		
III	5	9
IV	50	91
Disease Status at study entry		
Progressive Disease	39	71
Stable Disease	16	29
Lines of Treatment prior to study entry		
One chemotherapy regimen	24	43.6
≥2 (range, 2-9) chemotherapy regimens	30	54.6
None	1	1.8

Table 6. 2 Clinical characteristics of patients who received boost vaccinations (RCC: renal cell carcinoma, SCCHN: squamous cell carcinoma of the head and neck, HCC: hepatocellular carcinoma, CRC: colorectal carcinoma, CT: chemotherapy, CRT: chemo-radiotherapy, M: male, F: female, PD: progressive disease, SD: stable disease, CR: complete response, PR: partial response)

ID	Type of neoplasm	Sex	PS	Stage	Prior therapy	Status Before vaccination	No of Boost Vaccinations	Status after vaccination	PFS (months)	OS (months)
1	RCC	M	0	IV	Surgery, 1st line CT, 2nd line CT	PD	11	SD	52	52
2	SCCHN	M	0	III	Surgery, CRT	SD	6	SD	37	37
3	Breast	F	0	IV	Surgery, adjuvant CT, hormone-therapy, 1st line CT	PD	2	SD	13	17
4	MEN2	M	0	IV	Surgery, 1st line CT	SD	12	SD	41	41
5	Breast	F	0	IV	Surgery, adjuvant CT, 1st line CT	SD	15	CR	29	29
6	HCC	M	0	III	None	PD	12	PR	28	28
7	Cholangio-carcinoma	M	0	IV	Surgery, 1st line CT, 2nd line CT	SD	1	SD	7	19
8	CRC	M	0	IV	Surgery, Adjuvant CRT,1st line CT, 2nd line CT	SD	4	SD	16	45

6.3.2 Toxicity

The toxicity was assessed in each clinical visit, prior to every vaccine administration while the FBC/biochemistry parameters were monitored weekly. The monitoring protocol is summarized in the section 2.2. The toxicity profile of the TERT vaccine was very favourable with minimal and reversible toxicity. The early adverse events (EAEs) were mild (grade 1) and occurred in 29 (52%) patients. The most common EAE was grade 1 local skin reaction (n = 15; 27%) at the site of the injection. Other reported side effects were fatigue grade 1(7%), anaemia (13%) and nausea (4%). One patient with extensive metastatic liver lesions experienced grade 3 transaminases elevation and discontinued vaccination. During the vaccinations period or throughout the follow up period until their disease progression or death, no symptoms or clinical and laboratory signs occurred to suggest late toxicity or an autoimmune reaction. Moreover, the booster vaccinations which in some patients were extended for up to 2 years were also proved safe without long term toxicity.

6.3.3 Response to treatment

The patients were assessed for response at pre-specified intervals (section 2.4) using the RECIST criteria. All objective responses and Stable Disease (SD) were confirmed by an external independent radiologist. Interestingly, in one (1.8%) patient a complete clinical response (CR) with disappearance of the disease was documented, in another one (1.8%) a partial response (PR) and in 18 (33%) patients, stable disease (SD) was observed (DCR = 36%; 95% CI 24% to 49%). The DCR was 56% for patients with SD at enrolment into the study (one CR and eight SDs) and 28% for those with PD at the same time point (one PR and 10 SDs) (p = 0.05; 95% CI 14% to 42%) (Table 6.3).

Some clinical cases merit further discussion due to the remarkable response and long clinical remission. A patient with ER positive metastatic breast cancer, previously treated with chemotherapy and hormonal therapy, entered the study with SD to the previous treatment but demonstrated CR of her hepatic disease after the sixth vaccination which was maintained after nine boost vaccinations. This patient remained in CR without radiological evidence of disease for 36 months (Fig 6.1).

Another patient with advanced hepatocellular carcinoma, unresectable predominantly due to extensive involvement of the locoregional lymph nodes presented with obstructive jaundice and early clinical signs of hepatic failure. Bearing in mind that the systemic chemotherapy treatment

at the time had offered marginal if any benefit, albeit with further hepatotoxicity, the patient denied chemotherapy, but he accepted best supportive care including biliary stenting followed by participation in the phase II study of the TERT vaccine. After the sixth vaccination, the size of the tumour decreased resulting in significant improvement of the signs of hepatic insufficiency. Moreover, he continued with 12 boost vaccinations, every 3 months, for 41 months before a clinical relapse was documented (Fig 6.2).

After a median follow-up period of 37 months (range, 2–52), the median PFS for the entire group of patients was 4 months (range, 0.9–51.8). The median PFS for the patients who entered the study with SD and PD was 7 months (range, 1.5–41.5) and 4 months (range, 0.9–51.8), respectively (P = 0.038). For the six out of the eight patients who received boost vaccinations, the PFS was >6 months from the time of the first booster vaccination and in three patients the PFS was >3 years. The median OS for all patients was 19 months (range, 2–52) and the 1-year survival was 66%. There was no difference in terms of median OS for patients enrolled with SD versus PD [20 months (range, 2.2–44.8) versus 15 months (range, 1.7–51.8), respectively; p = 0.116].

Table 6. 3 Clinical response of patients after the second and sixth vaccinations
(CR, complete response; NE, not evaluable; PD, disease progression; PR, partial response; SD, stable disease).

Pre-vaccination status	**Post -2nd vaccination status**	**Post -6th vaccination status**
SD (n=16)	PR=1	CR=1
	SD=9	SD=7, PD=2
	PD=3	Discontinued after PD
	NE=3	SD=1, PD=2
PD (n=39)	SD=20	PR=1, SD=10, PD=9
	PD=16	Discontinued after PD
	NE=3	PD=3

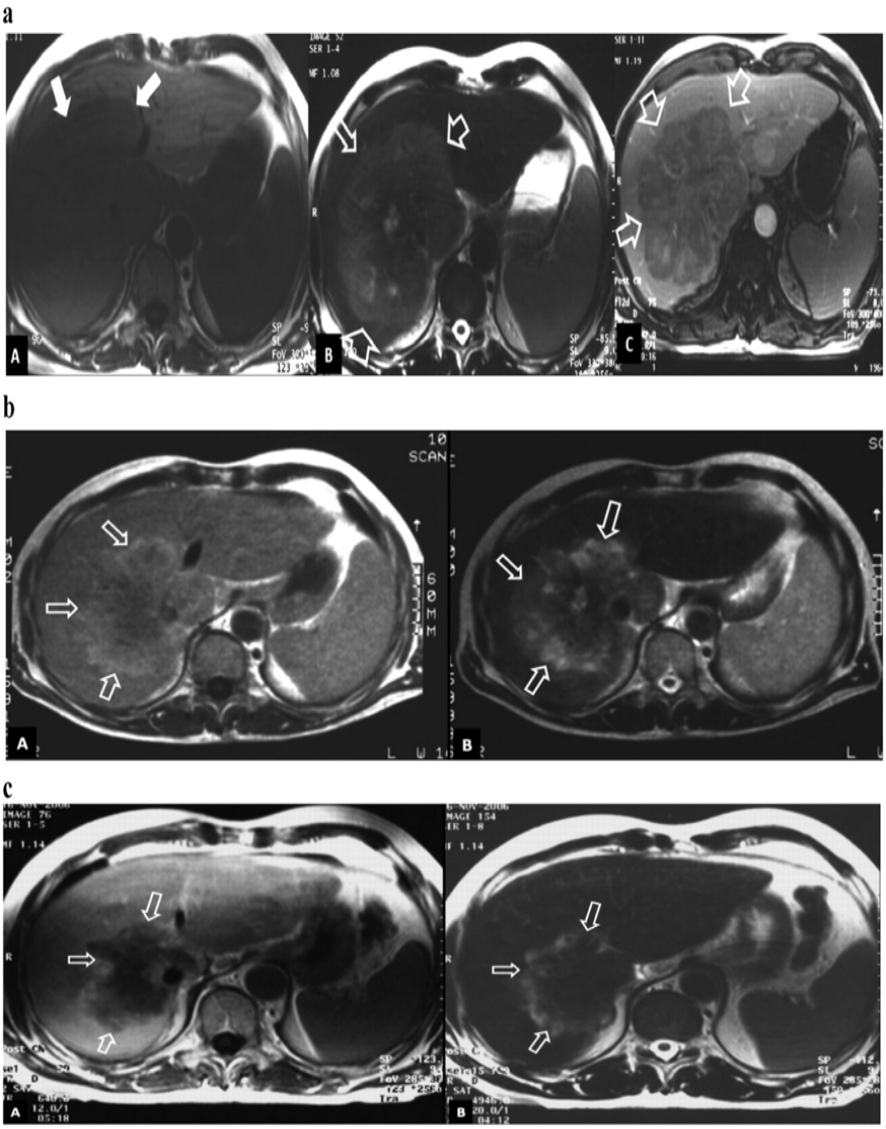


Figure 6. 1 Breast cancer patient with liver metastasis. The pre-vaccination plain (A) and contrast-enhanced (B, portal phase of I.V. contrast administration) axial computed tomography images show the hypodense lesion anterior to the portal vein (arrows). The post-treatment (1 year later) contrast enhanced T1-w magnetic resonance consecutive images at the same anatomical levels (C, D) show normal appearing hepatic parenchyma.

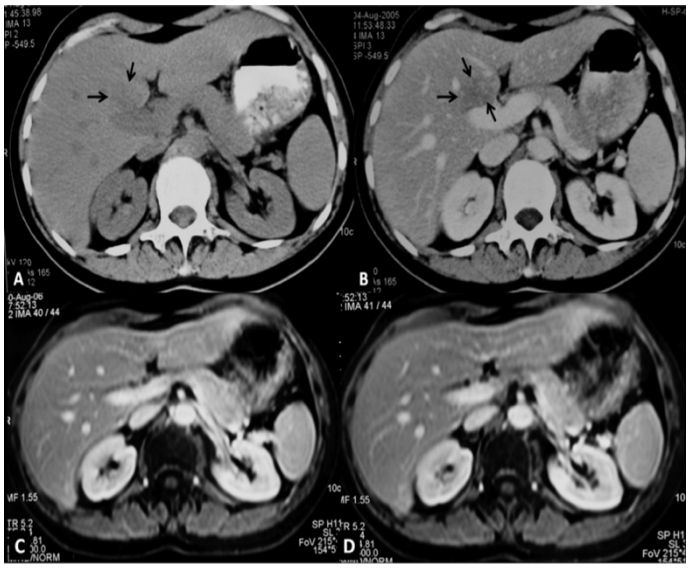


Figure 6. 2 Hepatocellular carcinoma patient. (a) Pre-vaccination imaging studies. The T1-w (A), T2-w (B) and contrast enhanced T1-w (C) axial magnetic resonance (MR) images show the initial size of the hepatic neoplasm (arrows). (b) The 6-month imaging follow-up study shows reduced size of the lesion on both the T1-w (A) and T2-w (B) axial MR images (arrows). (c) The 10-month imaging follow-up study shows a further reduction of the size of the lesion on both the T1-w (A) and T2-w (B) axial MR images (arrows).

6.3.4 Clinical outcome and immunological response

The primary diagnosis, the developed immune response after TERT vaccination, the pre-vaccination disease status and the clinical outcome are all summarized in the Table 6.4. Blood samples were available for immunomonitoring in 53 (96%) patients after the second vaccination and in all patients, who completed the six vaccinations (n = 34). Our results showed that the patients who developed an immunological response at any time during vaccination had a significantly higher PFS (5.2 months; range, 0.9–51.8) compared with those who failed to develop any response following vaccination (2.2 months, range, 1.4–6.5; p = 0.0001; (Table 6.4). This positive correlation of the development of immunological response with higher PFS was independent of disease status at study entry (SD: 7.2 versus 1.4 months; p = 0.008 and PD: 4.0 versus 2.0 months; p = 0.020) Table 6.4. Similarly, the development of immunological response was associated with a significantly higher OS (20 months; range, 3.8–51.8 versus 10.5 months; range, 1.7–30; p = 0.041) Table 6.4. The difference in the median OS of patients entering the

study with SD or PD, although was numerically higher for immune responders compared with non-responders, could not reach statistical significance (Table 6.4). Finally, the DCR was also higher in the immune responders (44.0% versus 14%; p = 0.047) compared to those who did not develop any response.

Quite interestingly, in fourteen patients (25%) immune reactivity against TERT was detected before the vaccination. This group was studied further in order to determine whether there was any correlation between the pre-existing TERT-specific immune reactivity and patients' clinical outcome (Table 6.5). Our results did not provide any evidence that such correlation exists for either PFS (P = 0.67) or OS (P = 0.26). However, we found that those patients who maintained immune reactivity after two and/or six vaccine administrations, had significantly higher OS compared to those who failed to maintain it after vaccination (P = 0.02). Overall, there was no significant difference in terms of PFS between these two groups (P = 0.09; (Table 6.6).

Table 6. 4 Immune monitoring and clinical outcome of patients vaccinated with TERT vaccine (NA= not applicable or inadequate specimen, SD= stable disease, PD= progressive disease, CR: complete response, PR: partial response, RCC: renal cell carcinoma, CRC: colorectal carcinoma, SCCHN: squamous cell carcinoma of the head and neck, HCC: hepatocellular carcinoma)

ID	Type of neoplasm	Stage	Status before vaccination	TERT-reactive cells/2x10^5 PBMC			Clinical outcome (months)	Overall survival (months)
				Pre-vaccination	2nd vaccination	Post-vaccination		
				Elispot	Elispot	Elispot		
1	RCC	IV	PD	1	333	360	SD (52)	52
2	CRC	IV	PD	1	136	NA	PD (3)	24.5
3	Biliary	IV	PD	1	362	1	PD (4)	9.5
4	Biliary	IV	PD	118	1	NA	PD (3.5)	4
5	Prostate	IV	PD	1	213	257	PD (4)	6
6	Breast	IV	PD	1	1	NA	PD (3)	9
7	Prostate	IV	PD	1	186	286	PD (4)	35.5
8	Biliary	IV	PD	1	1	104	PD (5)	13
9	Breast	IV	PD	1	145	316	PD (4)	22
10	SCCHN	IV	SD	1	120	127	SD (37)	37
11	Ovaries	IV	PD	1	1	NA	PD (2)	9
12	Pancreas	IV	SD	1	NA	92	SD (8)	11
13	SCCHN	III	PD	1	1	778	SD (6)	42
14	Breast	IV	SD	1	71	NA	PD (1.5)	9
15	Prostate	IV	PD	1	166	NA	PD (3)	6
16	Urothelial carcinoma	IV	PD	1	650	330	PD (4.5)	15
17	Breast	IV	PD	1	1	69	SD (13)	16.5
18	Melanoma	IV	PD	27	24	58	SD (44)	44
19	Prostate	IV	PD	1	1	1	SD (6.5)	21
20	Prostate	IV	PD	1	1	1	SD (6)	10.5
21	MEN2	IV	SD	1	1	240	SD (41)	41.5
22	RCC	IV	PD	165	NA	77	PD (4)	41
23	RCC	IV	PD	1	1	NA	PD (1.5)	16
24	Prostate	IV	PD	10	1	86	PD (1)	4
25	Breast	IIIB	PD	1	183	NA	PD (1)	14
26	Breast	IV	PD	1	292	120	SD (16)	29
27	Prostate	III	PD	1	242	1	SD (10)	21
28	RCC	IV	PD	1	680	333	SD (6)	14
29	Melanoma	IV	PD	1	1	NA	PD (4)	25.5
30	Melanoma	IV	SD	1	1	NA	PD (1.5)	8
31	RCC	IV	PD	1	1	NA	PD (3)	19
32	Breast	IV	SD	1	1	NA	PD (4)	30
33	Melanoma	IV	PD	53	42	NA	PD (2)	10
34	Melanoma	IV	PD	1	104	NA	PD (2)	8

35	RCC	IV	SD	1	61	1	SD (13)	16.5
36	RCC	IV	PD	1	460	100	SD (8)	25
37	Prostate	IV	PD	1	58	NA	PD (2)	29
38	Melanoma	IV	PD	221	1	NA	PD (2)	10
39	Cholangiocarcinoma	IV	PD	1	1	NA	PD (1.5)	2
40	CRC	IV	SD	1	1	300	SD (6.5)	22.5
41	Breast	IV	SD	26	27	50	CR (29)	29
42	HCC	III	PD	1	55	67	PR (28)	28.5
43	Cholangiocarcinoma	IV	PD	45	1	NA	PD (2)	11
44	Melanoma	IV	SD	56	1	68	PD (5)	18
45	Cholangiocarcinoma	IV	SD	96	1	172	SD (7)	19
46	Prostate	IV	PD	1	65	1	PD (4)	12.5
47	Pancreas	IV	PD	1	362	NA	PD (2)	4
48	Prostate	IV	PD	24	96	NA	PD (2)	6
49	Pancreas	IV	PD	50	1	NA	PD (2)	2.5
50	Breast	IV	SD	33	144	NA	PD (2)	20
51	CRC	IV	SD	1	60	1	SD (16)	45
52	Breast	IV	SD	95	135	281	SD (6.5)	20
53	Breast	IV	SD	1	133	1	PD (7)	39.5
54	Prostate	IV	SD	1	19	1	PD (4.5)	11
55	HCC	IV	PD	1	34	1	PD (4)	8

Table 6. 5 Survival data according to the pre-vaccination disease status and the development of immune response

A) SD at study entry

	Immune Responders	Immune No responders	P value
PFS	N= 14; (relapse n=11)	N=2; (relapse n=2)	
Median	7.2	1.4	**0.008**
Min – Max	1.4 – 41.4	1.4 – 3.9	
OS	N=14; (death n=8)	N=2; (death n=1)	
Median	20.2	8.2	0.926
Min – Max	9.2 – 44.8	8.2 – 29.9	
1-year survival	85.7%	50.0%	

B) PD at study entry

	Immune Responders	Immune Non responders	P value
PFS	N= 27; (relapse n=24)	N=12; (relapse n=12)	
Median	4.0	2.2	**0.020**
Min – Max	0.9 – 51.8	1.5 – 6.5	
OS	N=27; (death n=18)	N=12; (death n=10)	
Median	16.6	10.4	0.057
Min – Max	3.8 – 51.8	1.7 – 25.5	
1-year survival	69.8%	36.4%	

Table 6. 6 PFS and OS for the patients with pre-vaccination reactivity (IR: immune responder, INR: immune no-responders, PFS: progression-free survival, OS: overall survival)

	Post Vaccination IR	**Post Vaccination INR**	**p- value**
PFS	N=10; (relapse n=8)	N=4; (relapse n=4)	
Median	4.4	2.0	0.09
Min – Max	1.4 – 43.8	1.9 – 3.5	
OS	N=10; (death n=7)	N=4; (death n=4)	
Median	18.9	4.0	**0.02**
Min – Max	3.8 – 43.8	2.6 – 11.2	
1-year survival	70.0%	-	

6.3.5 Univariate and multivariate analysis

We conducted univariate and multivariate analysis in regard to patients' and disease parameters which could be potentially associated with the PFS and OS of the patients vaccinated. In the univariate analysis, the PS, the disease status after the prior treatment and the development of immunological response were significantly associated with better PFS and OS (Table 6.7). Multivariate analysis (Table 6.8) demonstrated that the development of immunological response was an independent factor associated with better PFS (HR = 3.35, 95% CI 1.7–6.7; p = 0.001), while the worse PS was associated with shorter OS (HR = 3.0, 95% CI 1.5–5.8; p = 0.001). There was a trend for worse OS in patients who did not develop immunologic response during the vaccination (HR = 2.0, 95% CI 1.0–4.0; p = 0.057).

Table 6. 7 Univariate analysis (CI, confidence interval; OS, overall survival; PFS, progression-free survival; indicate statistically significant correlations ($p \leq 0.05$)

	Log-rank test	P value	Hazard ratio	P value	95% CI
PFS (n=55)					
Sex (male vs female)	0.000	0.992	1.003	0.992	0.551–1.827
PS (1-2 vs 0)	5.178	**0.023**	1.955	**0.026**	1.084–3.526
Stage (IV vs IIB)	0.767	0.381	1.676	0.388	0.519–5.412
Pre-vaccination status (PD vs SD)	4.301	**0.0308**	1.949	**0.042**	1.024–3.709
Immune response at any time (no vs yes)	13.571	**0.0001**	3.346	**0.0001**	1.692–6.615
OS (n=55)					
Sex (male vs female)	0.010	0.919	1.037	0.919	0.520–2.066
PS (1-2 vs 0)	11.843	**0.001**	3.002	**0.001**	1.559–5.783
Stage (IV vs IIB)	0.997	0.318	2.037	0.328	0.489–8.482
Pre-vaccination status (PD vs SD)	2.470	0.116	1.813	0.121	0.854–3.851
Immune response at any time (no vs yes)	4.160	**0.041**	2.074	**0.046**	1.013–4.245

Table 6. 8 Multivariate analysis for PFS and OS (CI, confidence interval; OS, overall survival; PFS, progression-free survival; PD, disease progression; PS, performance status; SD: stable disease. The statistically significant correlations ($p \leq 0.05$) are shown in bold)

	Hazard ratio	P value	95% CI
PFS (n=55)			
PS (1-2 vs 0)	1.668	0.099	0.907–3.065
Pre-vaccination status (PD vs SD)	1.513	0.233	0.766-2.990
Immune response at any time (no vs yes)	3.346	**0.001**	1.692-6.615
OS (n=55)			
PS (1-2 vs 0)	2.970	**0.001**	1.536-5.743
Pre-vaccination status (PD vs SD)	1.283	0.538	0.580-2.837
Immune response at any time (no vs yes)	2.020	0.057	0.980-4.164

6.4 Conclusion

This study showed that the vaccine was effective in various cancers and that the development of immune response could be a surrogate for a better clinical outcome. Similar observations were made by other researchers using different vaccines (Domchek et al., 2007; Gonzalez et al., 2003; Lonchay et al., 2004; Nemunaitis et al., 2006), while it has been earlier demonstrated that the presence of specifically stimulated T cells in the tumour independently predicts better patients' clinical outcome (Clark et al., 1989; Galon et al., 2006; Marrogi et al., 1997; Zhang et al., 2003). Our study confirmed the importance of the immune response as a major predictor of the long-term outcome. It was also confirmed the favourable toxicity profile of this TERT vaccine, without serious acute or late adverse events and with no evidence of autoimmune reactions even after its administration for up to 2 years. These results were published in Annals of Oncology (Kotsakis et al., 2012).

It became evident that the group of NSCLC patients among the patients with other tumour types had experienced a remarkable clinical outcome, as it was shown in the pilot study results. Therefore, we decided to evaluate the immunologic and clinical outcome of the chosen vaccination schedule specifically in this cohort of patients with advanced NSCLC.

7. CHAPTER 7: A phase II trial evaluating the clinical and immunologic response of HLA-A2^{+} non-small cell lung cancer patients vaccinated with an hTERT cryptic peptide

7.1 Introduction

In the previous chapters we presented the data of patients with various tumour types who were enrolled in the study with the TERT vaccine. In addition, the data from the patients with NSCLC were analysed separately and are presented in this chapter. We focused on the long-term outcome of the vaccinated patients, comparing their survival with those of their counterparts according to the HLA-A2 haplotype but with similar age, PS and disease status.

7.2 Materials and methods

7.2.1 Patients and vaccine administration

The eligibility criteria are summarized in the section 2.1.

The vaccine consisted of the HLA class I-restricted optimized $TERT_{572Y}$ (YLFFYRKSV) and the native $TERT_{572}$ (RLFFYRKSV) peptides.

The vaccine's synthesis and preparation were previously presented in section 2.3.

7.2.2 Patients' evaluation

The monitoring protocol is presented in the section 2.2.

7.2.3 Statistical analysis

We used the same statistical plan as previously used for the other solid tumours and was described in chapter 5.

7.3 Results

Forty-six patients with histologically confirmed NSCLC were enrolled in the study. The eligibility criteria are summarized in previous section 2.1. The baseline patients' characteristics are summarised in Table 7.1

Table 7. 1 Baseline patients' clinicopathologic characteristics (SD: Stable Disease, PD: progressive Disease)

Characteristics	**Vaccinated patients, n (%)**	**Control patients HLA (-) n (%)**
No of patients	46	38
Age		
Median	59	61
Min-Max	38-80	42-82
Gender		
Men	40 (87)	32 (84.2)
Women	6 (13)	6 (15.8)
Performance Status (WHO)		
0	29 (63)	19 (50)
1	14 (30.4)	15 (39.5)
2	3 (6.5)	29 (76.3)
Stage		
IIIA	1 (2.2)	14 (36.8)
IIIB	9 (19.6)	24 (63.2)
IV	36 (78.3)	
Histologic type		
Squamous cell	15 (32.6)	14 (36.8)
Non- squamous cell (adenoca, large cell ca, NOS)	31 (67.4)	24 (63.2)
Prior Surgery	16 (34.8)	
Prior Chemotherapy		
Adjuvant	7 (15.2)	
1st line (Radical)	46 (100)	
Palliative	19 (41.3)	
Prior Radiotherapy		
Adjuvant	3 (6.5)	
1st line (Radical)	13 (28.2)	
Palliative	4 (8.7)	

Status before vaccination		
SD	27 (58.7)	
PD	19 (41.3)	

7.3.1 Toxicity

The toxicity profile of the vaccine was similar to the profile previously reported. In 25% of the patients vaccinated a local skin reaction was observed at the site of vaccination. Other low grade (grade ≤ 2) side effects observed were anaemia in 5 patients (11%), fatigue in 7 patients (15%), while 4 patients (8%) reported mild nausea. As previously reported, no late toxicity or autoimmune events were observed. Moreover, the extended vaccination for up to 2 years was equally safe with favourable and reversible toxicity profile.

7.3.2 Response to treatment

All tumour assessments were confirmed by an independent radiologist. In this cohort of the 46 vaccinated patients, 3 (7%) had PR, while 13 (28%) patients had SD as best response. After the completion of the 6- vaccination protocol, 14 (51.9%) out of 27 patients who entered the study with SD continued as having SD or presented with response, PR (n = 3; 12%).

However, among the 19 patients who entered the study with PD to the previous line of treatment, 2 patients (10.5%) experienced SD and 17 (89.5%) PD, during the 6-vaccination period (p = 0.004). Interestingly, among the vaccinated NSCLC patients the DCR for non-squamous histology was significantly higher compared to squamous-cell histology [n = 14 (45%) versus n = 2 (13%); p = 0.03]. The median PFS for the entire group of patients was 3.8 months (range, 0.7–99.4). The median PFS for the patients entering the study with SD was 5.7 months (range, 1.2–97.2), while for those with PD at study entry it was 2 months (range, 0.7–62.9; p = 0.0001). Similarly, the median OS for all vaccinated patients was 19.8 months (range, 0.7–99.4) and the 1-year survival rate 69%. A difference in OS according to the disease status at the time of enrolment was also observed. For those with SD, the OS was 31.2 months (range, 1.2–97.2) while for those with PD it was only 8 months (range, 0.7–99.4; p: 0.001).

The PFS for the 12 patients who received booster vaccination was 62.9 months (range, 6.7–97.2), while the OS was not estimated since most of the patients were still alive at the time of the assessment. However, among the patients who continued with booster vaccinations, 10 patients had long-standing disease control for over 20 months. The clinical characteristics of these

patients (n=10) were separately analysed (Table 7.2). Interestingly, all but one of these patients had a non-squamous histology. Among them, 5 patients had received prior radical locoregional treatment with SD and 9 of them entered the study with SD. These patients, who all received at least the 6 scheduled vaccinations, had significantly higher PFS and OS compared to the rest of the patients enrolled. Excluding those patients, the median OS for patients with SD remained significantly higher (19.8 months; range, 1.4–44.0) compared with that of patients with PD (5.8 months; range, 0.7–62.5) at the time of enrolment (p: 0.022). We compared the outcome of the vaccinated patients with random controls from our database matched in the baseline characteristics such as age, gender, PS, histologic type and previous treatment. The OS from the time of diagnosis was evaluated in a group of non-vaccinated patients with an HLA-A2-positive haplotype. Impressively, the OS for the non-vaccinated HLA-$A2^{+}$ patients was 9.2 months compared to 19.8 months observed in the vaccinated (Table 7.1; p: 0.0001).

Table 7. 2 Clinical characteristics of the patients received boost vaccinations and experienced long-term disease control (CHT: chemotherapy, CRT: chemo-radiotherapy, PR: partial response, SD: stable disease, PD: progressive disease, NED: non-evaluable disease)

Patient	Age	Sex	PS	Histology	Stage	Previous treatment	Pre-vac status	Post -vac status	PFS (months)	OS (months)
1	56	M	0	Non-squamous	IIIB	1st line CHT	PR	PR	13.7	67.1
2	49	M	0	Non-squamous	IIIA	CRT	NED	SD	96.6+	96.6+
3	59	M	0	Squamous	IV	Pneumonectomy Radical CRT Metastasectomy (Pt denied CT)	NED	SD	97.2+	97.2+
4	72	F	0	Non-squamous	IV	1st line CHT	PD	SD	62.9	99.4+
5	48	M	1	Non-squamous	IV	1st-2nd-3rd line CHT	SD	SD	43.8	53.1
6	60	M	0	Non-squamous	IIIB	Surgery Radical CRT Metastasectomy +CHT	SD	SD	46.1+	46.1+
7	74	M	0	Non-squamous	IV	1st line CHT	SD	PR	9.7	63.6
8	53	M	0	Non-squamous	IV	Surgery, Adj CHT, 1st-2nd-3rd line CHT	SD	SD	61.3+	61.3
9	60	M	0	Non-squamous	IV	Radical CRT for stIIIB Metastasectomy +CHT	SD	PR	52.1+	52.1+
10	61	M	0	Non-squamous	IIIB	Concomitant CRT	SD	SD	76.3+	76.3+

7.3.3 Immune response and clinical outcome

The immune response was evaluated in 35/46 patients, which was developed at any time during the vaccination course (Table 7.3). In total, 23 patients (66%) developed a specific immune response to the TERT vaccine. Among them, 8 patients maintained the disease control for long term. In the rest 11/46 patients, the immune response couldn't be assessed due to lack of blood samples at the pre-specified time points.

Six patients out of 46 responded to the first 2 administrations of the optimized peptide $TERT_{572Y}$ developing early immunologic response after 2 vaccine administrations but failed to develop late immunologic response, as they did not respond to the vaccination with the native peptide $TERT_{572}$ (Table 7.3). In contrary, 4 patients did not respond to the optimized but responded to the native peptide. A representative example of immune response is shown in Fig 7.1. It seems also that prolonged vaccination maintained the immune response against TERT-peptides in all, but one, patients as assessed by IFN-γ ELISpot assay (Table 7.4).

For those patients who developed any immune response at any time during the vaccination compared to those who did not, a difference in PFS was observed which was not statistically significant 6.7 months (range, 1.5–99.4) versus 2.7 months (range, 1.1–76.3; p = 0.090, Table 7.5). Similarly, among the patients who entered the study with SD (Table 7.6), those who developed immune response had a median PFS 13.4 months (range, 1.6–97.2) compared to 4.6 months (range, 1.7–76.3) for those who did not (p = 0.032). In contrast, among the patients who entered the study with PD, there was no difference in the PFS (2.1 versus 2.0 months; p = 0.2). The median OS was significantly prolonged in patients who developed immune response (40.0 months; range, 2.8–99.4 versus (9.2 months; range, 2.5–76.3; p = 0.02, Table 7.5) compared to those who did not. The median OS was also prolonged in patients responding immunologically compared to those who did not respond who entered the study with either SD (63.6 versus 19 months, respectively; p = 0.01, Fig 7.2A) or PD (16.5 versus 3.7 months, respectively; p = 0.03, Fig 7.2B).

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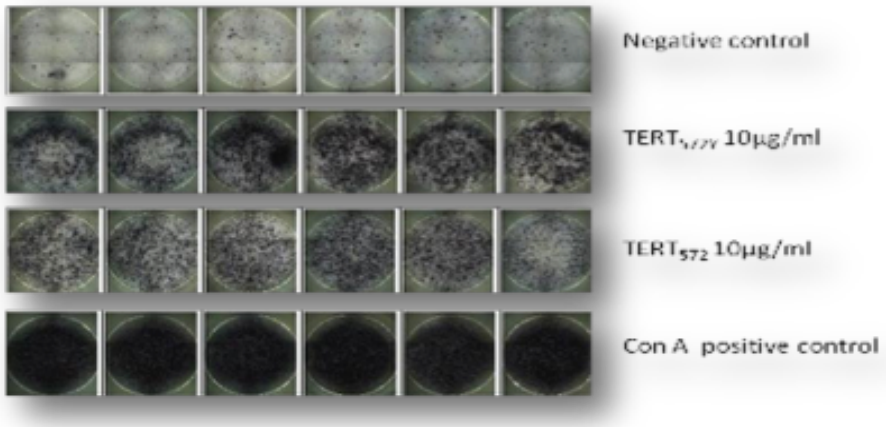

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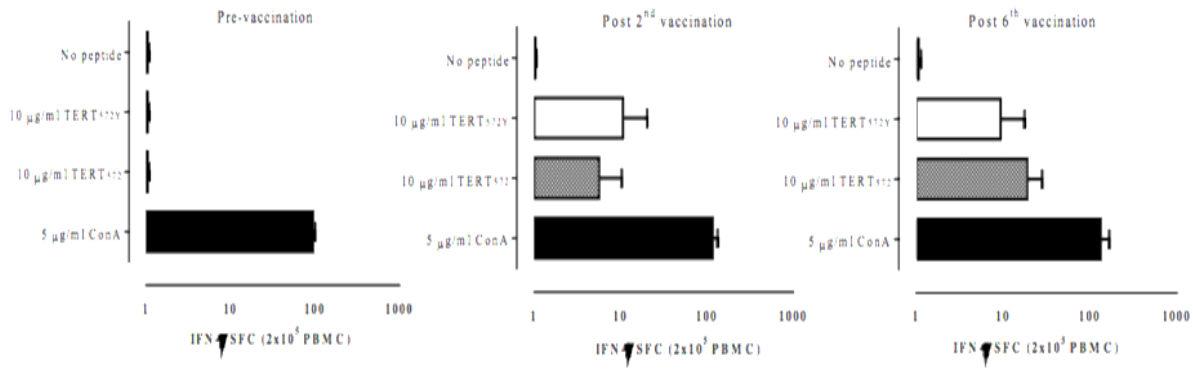

Figure 7. 1 $TERT_{572}$ and $TERT_{572Y}$ responders at the completion of 6-vaccinations protocol. (A) A representative ELISpot assay of a responder to $TERT_{572}$ and $TERT_{572Y}$ peptides. The spots represent the IFN- forming cells. The treatments of the cells are shown on the side of the wells. (B) $TERT_{572}$ and $TERT_{572Y}$ -reactive T cells after the 2nd and 6th vaccination in one patient as assessed by IFN- γ ELISpot. Peripheral blood mononuclear cells from the vaccinated patient were stimulated with 10 mg/ml TERT peptides. The data in the graphs are presented as the mean value of 3 independent experiments. SFC indicates spots-forming cells; TERT, telomerase reverse transcriptase.

Table 7. 3 Patients' demographics, clinical and immunological response (PS: Performance Status, M: male, F: female, PFS: Progression free survival, OS: Overall survival, PD: Progressive disease, NE: Non- evaluable, SD: Stable disease, PR: Partial response)

Age	Sex	PS	Histology	Stage	Early Immunological Response	Late Immunological Response	Immunological Response Any time	Pre-Vaccination Status	Post-Vaccination Status	PFS	OS
56	M	0	Non-Squamous	IIIB	NE	NE	NE	PR	PR	13.7	67.1
49	M	0	Non-Squamous	IIIA	Yes	Yes	Yes	NED	SD	96.6+	96.6+
59	M	0	Squamous	IV	Yes	No	Yes	NED	SD	97.2+	97.2+
72	F	0	Non-Squamous	IV	Yes	Yes	Yes	PD	SD	62.9	99.4+
48	M	1	Non-Squamous	IV	Yes	Yes	Yes	SD	SD	43.8	53.1
60	M	0	Non-Squamous	IIIB	Yes	No	Yes	SD	SD	46.1+	46.1+
74	M	0	Non-Squamous	IV	No	Yes	Yes	SD	PR	9.7	63.6
53	M	0	Non-Squamous	IV	Yes	No	Yes	SD	SD	61.3+	61.3+
60	M	0	Non-Squamous	IV	Yes	No	Yes	SD	PR	52.1+	52.1+
61	M	0	Non-Squamous	IIIB	No	No	No	SD	SD	76.3+	76.3+
49	M	0	Non-Squamous	IV	Yes	No	Yes	PD	SD	8.4	62.5
50	M	0	Non-Squamous	IV	No	Yes	Yes	SD	SD	30.5	44+
62	M	1	Squamous	IV	NE	NE	NE	PD	PD	2.3	5.8
75	M	0	Non-Squamous	IV	NE	NE	NE	PD	PD	3.8	24.0
51	M	0	Non-Squamous	IV	NE	NE	NE	PD	PD	1.9	14.1
61	M	0	Squamous	IIIB	NE	NE	NE	PD	PD	1.3	3.6
71	M	1	Squamous	IIB	NE	NE	NE	PR	PD	1.2	1.4 (L)

72	M	2	Squamous	IV	NE	NE	NE	PD	PD	0.7	0.7
38	M	0	Squamous	IV	NE	NE	NE	PD	PD	2.0	21.5
59	F	0	Squamous	IV	NE	NE	NE	PR	PD	1.3	17.7
51	F	0	Non-Squamous	IV	NE	NE	NE	SD	PD	3.1	4.2
61	M	1	Non-Squamous	IV	NE	NE	NE	PD	PD	2.5	4.5
60	M	1	Non-Squamous	IV	Yes	NE	Yes	PD	PD	4.0	30.0
54	F	0	Non-Squamous	IV	Yes	Yes	Yes	PR	SD	13.4	24.8
60	M	1	Non-Squamous	IV	Yes	No	Yes	SD	PD	4.2	30.6
50	M	0	Squamous	IV	Yes	No	Yes	SD	PD	5.2	18.4
61	M	0	Squamous	IV	Yes	NE	Yes	PD	PD	2.0	16.8
57	M	2	Non-Squamous	IV	Yes	NE	Yes	PD	PD	1.8	2.8
69	M	1	Squamous	IV	Yes	NE	Yes	PD	PD	1.6	4.3
54	M	1	Non-Squamous	IV	Yes	NE	Yes	PD	PD	1.5	5.6
59	M	0	Squamous	IIIB	Yes	Yes	Yes	PD	PD	4.2	16.5
55	M	1	Non-Squamous	IV	Yes	NE	Yes	PD	PD	2.1	13.4
58	M	0	Non-Squamous	IV	No	Yes	Yes	SD	PD	3.9	33.9+
54	M	1	Non-Squamous	IV	Yes	NE	Yes	SD	PD	1.6	40.0
58	M	0	Non-Squamous	IV	No	NE	No	PD	PD	2.3	8.0
74	M	1	Squamous	IV	No	NE	No	SD	PD	3.4	4.1
54	F	0	Non-Squamous	IIIB	No	No	No	PR	PD	5.7	18.2
55	F	1	Non-Squamous	IV	No	NE	No	PD	PD	1.1	3.7
66	M	2	Non-Squamous	IV	No	NE	No	PD	PD	2.0	2.5
80	M	1	Squamous	IIIB	No	NE	No	PR	PD	2.7	24.3
74	M	1	Non-Squamous	IIIB	No	No	No	SD	PD	4.7	38.9
69	M	0	Squamous	IV	No	NE	No	SD	SD	4.6	6.3
62	M	0	Squamous	IV	No	NE	No	SD	PD	2.1	31.2
51	M	0	Non-Squamous	IV	No	NE	No	SD	PD	1.7	9.2
53	M	0	Non-Squamous	IV	No	Yes	Yes	SD	SD	6.7	19.8

68	M	0	Non-Squamous	IV	No	NE	No	PR	SD	7.7	18.9

Table 7. 4 Kinetics of TERT-specific T-cell response development beyond the standard 6-cycles protocol of immunization. Immune responses in patients as assessed by IFN-γ ELISpot assay at each booster vaccination. (NE: Non-evaluate; -: no booster vaccinations were received)

Patient	Late Immunological Response	1st booster	2nd booster	3rd booster	4th booster	5th booster	6th booster	7th booster	8th booster	9th booster	10th booster	11th booster	12th booster	13th booster	14th booster	15th booster	16th booster	17th booster
1	NE	No	NE	Yes	NE	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Yes	No	Yes	Yes	No	NE	Yes	NE	NE	NE	NE	NE	No	No	No	Yes	Yes	-
3	No	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	No	Yes	No	No	-	-
4	Yes	NE	Yes	Yes	No	NE	NE	No	No	NE	No	No	No	-	-	-	-	-
5	Yes	No	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	No	Yes	NE	No	No	No	-	-	-	-	-	-	-	-	-	-	-	-
7	Yes	Yes	No	NE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	No	No	No	Yes	No	Yes	Yes	No	No	-	-	-	-	-	-	-	-	-
9	No	Yes	Yes	Yes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	No	No	No	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
12	Yes	Yes	Yes	Yes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	Yes	NE	Yes	Yes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	No	No	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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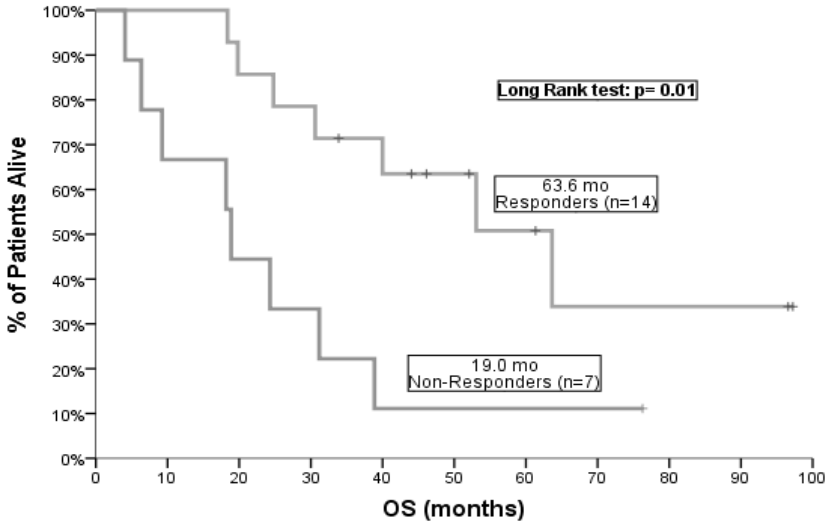

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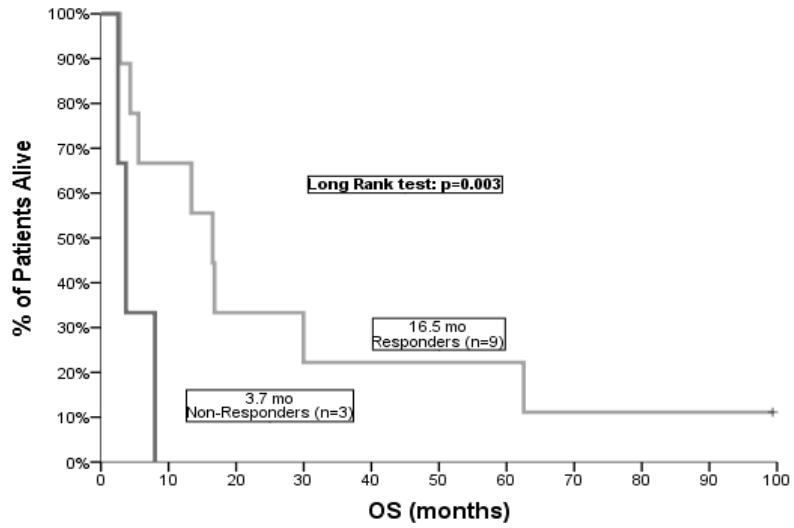

Figure 7.2 Overall survival according to disease status before vaccination. A) Pre - vaccination disease status: SD; B) pre-vaccination disease status: PD.

Table 7. 5 Clinical outcome according to the immune response to hTERT at any time (PFS: Progression free survival, OS: Overall survival)

	Responders (n= 23)	**Non-responders** (n= 12)	***p*- value**
PFS			
Median (min– max)	6.7 (1.5 – 97.2)	2.7 (1.1 – 76.3)	0.090
OS			
Median (min– max)	40.0 (2.8 – 99.4)	9.2 (2.5 – 76.3)	0.021
1-year survival	87.0%	50.0%	

Table 7. 6 Clinical outcome according to immunological response at any time among patients with stable disease at the time entered into the study (PFS: Progression free survival, OS: Overall survival)

	Response	**No response**	***p*- value**
PFS	N= 14, Events=9	N=9, Events=8	
Median	13.4	4.6	0.032
Min – Max	1.6 – 97.2	1.7 – 76.3	
OS	N=14, Events=7	N=9, Events=8	
Median	63.6	18.9	0.010
Min – Max	18.4 – 97.2	4.1 – 76.3	
1-year survival	100 %	66.7%	

7.4 Conclusion

The results from this study were concordant with the results from the pilot (Bolonaki et al., 2007) and the expanded studies (Kotsakis et al., 2012; Vetsika et al., 2012), which investigated the same vaccine protocol in patients with NSCLC and other solid tumours. A randomised phase IIb trial (Georgoulias et al., 2013) was later conducted to further investigate the role of the TERT vaccine in the NSCLC population.

A favourable toxicity profile of the TERT vaccine was confirmed in all the above studies. In terms of clinical response, three patients had PR to the TERT vaccine, while in most cases stabilisation of the disease was the best response achieved. Interestingly, among the immune-responders a DCR of 52% was observed. The role of immune response as a good surrogate for improved clinical outcome was also confirmed in this study. The results of this study were published in Lung cancer (Kotsakis et al., 2014).

8. CHAPTER 8: Discussion

Over the last two decades our increasing understanding of the role of immune system in cancer and the need for a personalized and targeted therapeutic approach has brought immunotherapy - a hot albeit challenging therapy- in the spotlight of the clinical research. In the past ten years, different trials of various immunotherapeutic approaches have been conducted across the world. Among them, active immunotherapy with peptide-based vaccines using different tumour-associated antigens gained a lot of attention but has not yet become part of standard clinical practice.

The discovery of telomerase (TERT) and its role in the DNA replication revealed a unique cellular enzyme and an ideal target for immunotherapy. Human TERT is a self-antigen and several laboratories probed its natural properties of being recognized by the adaptive immune system (antigenicity) and its ability to induce an adaptive immune response (immunogenicity) (Minev et al., 2000; Vonderheide et al., 1999).

In our study, two TERT-based peptides ($TERT_{572}$ and its optimized variant $TERT_{572Y}$) were used and combined with the immunoadjuvant Montanide ISA-51 to formulate a peptide-based vaccine given subcutaneously every three weeks to patients with various, advanced solid tumours.

TERT is not expressed on the external cell surface, but as an intracellular protein can only be recognized by T cells as short peptides comprising 8–16 amino acids, which are processed inside the cell before being exported to, and presented at, the cell surface in the context of major histocompatibility complex (MHC) molecules. Our work regarding TERT immunotherapy, like most of the researchers', focused on TERT-peptide binding to MHC class I (MHC I) molecules, which are expressed by almost all cell types and, when bound to a target antigen, can induce the activity of $CD8^+$ cytotoxic T lymphocytes (CTLs) expressing a complementary T-cell receptor (TCR). Thus, the initial questions were essentially whether endogenous TERT could be processed and presented in the context of MHC I to become the target of $CD8^+$ T lymphocytes, and thereby activate cytotoxic T-cell responses.

In the first part of our research, two different schedules of vaccination were compared in order to identify the optimal sequence of peptide administration in terms of eliciting the best immune response. Subsequently, the most effective vaccination schedule was tested in patients with advanced various tumour types in pilot and expanded studies, and their immunologic data were correlated with their clinical outcome.

8.1 Sequential peptide administration is the optimal vaccination schedule

We tested the hypothesis that vaccinations of cancer patients with 2 doses of the optimized peptide ($TERT_{572Y}$) followed by 4 doses with the native peptide $TERT_{572}$ (scheme A) are able to induce CTLs with higher avidity and stronger antitumor efficacy than serial vaccinations with the optimized $TERT_{572Y}$ peptide alone (scheme B), as previously shown in *in vivo* preclinical studies using HLA-A*0201 transgenic HHD mice (Gross et al., 2004). In both schemes, we used an initial induction with the optimized peptide $TERT_{572Y}$ followed by a maintenance phase in which patients were randomized to receive either the native $TERT_{572}$ or the optimized peptide $TERT_{572Y}$.

Our results revealed that vaccination with the optimized $TERT_{572Y}$ followed by the native $TERT_{572}$ peptides (scheme A) can induce strong T cell responses, with higher avidity and frequencies of T cell responses. The maintenance vaccinations with the native $TERT_{572}$ peptide may favour the selection and expansion of T cells with the highest avidity for the native $TERT_{572}$ peptide from the peptide-specific T cell pool primed by the induction of vaccinations with the optimized $TERT_{572Y}$ peptide. Our focus on the generation of high-avidity $TERT_{572}$-specific T cells is based on the fact that only the native peptide is presented by tumour cells and therefore these cells could be effective in tumour recognition and elimination. In addition to previous studies (Rosenberg et al., 1998) focused on the induction of responding cells to modified peptides, in this study immune monitoring assessed by IFN-γ ELISpot (Fig 3.3 and 3.4), was performed in response to the native $TERT_{572}$ peptide. Indeed, the present data demonstrate that $TERT_{572}$-specific immune responses were stronger in patients enrolled in scheme A compared with the responses observed in patients of scheme B, after the completion of 6-vaccinations. These findings clearly indicate that the scheme A is more efficient vaccination strategy than scheme B and could potentially overcome the immune tolerance against TERT (Robbins & Kawakami, 1996), offering a more comprehensive immune response.

In addition to the development of specific T cells, the immune response is considered successful when it manages to induce high antigen-specific T cell avidity. The avidity of T cells is defined as the measurement of the sensitivity of responding T cells to a peptide antigen. T cells of high functional avidity are able to respond to very low levels of cognate Ag and high functional avidity has been linked with enhanced clearance of viral infections and tumours (Alexander-Miller, 2005; Alexander-Miller, Leggatt, & Berzofsky, 1996; Yee, Savage, Lee, Davis, & Greenberg, 1999). Both pre-clinical and clinical trials have shown that high-avidity CTLs are more effective

at eliminating cancer cells (Gattinoni, Powell, Rosenberg, & Restifo, 2006; Nugent et al., 2000; Yee et al., 1999).

This is an important finding of the present work since the vaccination with scheme A was able, not only, to induce specific T cells, but moreover to increase the number of high- avidity T cells in contrast to scheme B (Fig 3.5). Interestingly, the vaccination with the native peptide selectively expanded T cells displaying higher avidity for the native peptide, as revealed by testing them against low peptide concentrations. This increased number of responding cells was significantly superior compared with the responses obtained in patients vaccinated only with the optimized peptide. Recent studies have shown higher expansion of central and memory CTLs after antigen exposure both in vivo and in vitro, with higher sensitivity to low concentration of antigen stimulation (Alexander-Miller, 2005; Gattinoni et al., 2006; Nugent et al., 2000; Yee et al., 1999; Zeh, Perry-Lalley, Dudley, Rosenberg, & Yang, 1999). In clinical practice, this strategy could be used for the design of anticancer vaccines that would be able to induce a potent immune response following stimulation by low antigenic concentrations and thus being more effective.

An interesting observation in this study was a shift in the avidity of the CTLs developed after the second and the sixth vaccination in regard to response to different peptide. More specifically, vaccinations of cancer patients with 2 doses of the optimized peptide ($TERT_{572Y}$) induced a significant number of CTLs of low avidity (Fig 3.2) and subsequently, those patients who received 4 more doses with the native peptide TERT572 were able to induce CTLs with higher avidity than the ones who received serial vaccinations with the optimized ($TERT_{572Y}$) peptide alone who did not develop such response (Fig 3.5). This could be explained by our initial hypothesis that the optimized peptide ($TERT_{572Y}$) first may generate a pool of peptide-specific T cells of different avidities and then the stimulation with the native peptide $TERT_{572}$ selects among T cells those with highest avidities for the native antigen.

Interestingly 19% of the patients had a pre-vaccination immune reactivity to $TERT_{572}$ peptide. Moreover, the pre-existing $TERT_{572}$-specific immune reactivity could not be further amplified after the second vaccination in most of these patients; on the contrary, patients who had no pre-existing $TERT_{572}$ immune reactivity at the baseline sample, mounted more efficiently an early immune response to the vaccination (Fig 3.2). This observation indicates that patients with pre-existing T cell reactivity against $TERT_{572}$ are in fact less likely to develop a detectable early

vaccine-specific immune response as already shown by Bercovici et al. (Bercovici et al., 2008) and that this effect may perhaps be due to CTLs migrating to the tumour sites or subjected to cell death. Indeed, it has been previously reported by 2 different groups 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 (de Vries et al., 2005; Slingluff et al., 2004).

Alternatively, Zaks et al. (Zaks, Chappell, Rosenberg, & Restifo, 1999) reported that the re-stimulation of T cells at the peak of their expansion or activation may cause activation-induced cell death. In contrast to our results, two previous studies have indicated that patients responding to either a Human Epidermal Growth Factor Receptor 2 dominant peptide vaccine or to a melanoma dominant peptide vaccine (Melan-A/MART- 126-35 peptide and influenza matrix protein58-66 peptide) were primarily those who had a pre-existing antigen-specific immune response (Salazar et al., 2007; Speiser et al., 2003).

More studies have suggested that one possible mechanism of immune escape used by the tumour cells, is the production of immunosuppressive type II cytokines at the tumour sites (Aruga et al., 1997; Lattime, Mastrangelo, Bagasra, Li, & Berd, 1995; Yang & Lattime, 2003). Recent research has shed more light on immune escape mechanisms 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, thus inducing inhibitory signals to effector T cells (Chauvin et al., 2015; Gabriel & Lattime, 2007; Hodi et al., 2003; Korman, Yellin, & Keler, 2005; Krummel & Allison, 1995; Phan et al., 2003; Sun et al., 2008) or an increased expansion of T regulatory cells (Tregs) that can suppress effector T cells (Bercovici et al., 2008; Gattinoni et al., 2006; Nugent et al., 2000; Zeh et al., 1999).

In addition, the decreased immune response observed in a proportion of patients who completed the six vaccinations could be due to initial stimulation of the immune response by the modified peptide that was subsequently faded off due to inadequate further stimulation by the native peptide. Future studies could investigate in depth these inhibitory mechanisms of immune response.

8.2 Use of optimal vaccination schedule in the pilot NSCLC study

Following establishment of the vaccination strategy, we tested the vaccine in different tumour types. The first results reported was from the pilot study with 22 patients with NSCLC and a follow up period of nearly 2 years.

The results of the pilot study showed that the cryptic telomerase $TERT_{572}$ vaccine was effective, eliciting immune response after the second and sixth vaccinations, in 76% and 91% of evaluated patients, respectively. These results in addition to previously published data by our group (Mavroudis, Bolonakis et al. 2006), confirmed that $TERT_{572Y}$- generated CTLs recognized the native $TERT_{572}$ peptide and were maintained for at least 9 months in patients who received boost vaccinations with the native $TERT_{572}$ peptide.

In the pilot study, no objective clinical response (PR or CR) was observed based on RECIST criteria. However, for eight patients their disease remained stable (SD) for 6.8-20 months. An interesting observation came from this early phase that there was a correlation between the development of an early immune response and a favourable clinical outcome. The patients who developed early immune response had a significantly better OS than those patients who did not (30 vs 4.1 months; p= 0.012). Even among the five patients withdrawn from the study due to rapid disease progression who had developed an early immune response, three of them survived for 5.5, 5.7 and 17.1 months, respectively.

Following the promising results of the pilot study, the next step was to assess the toxicity, the immunologic and clinical efficacy of the TERT vaccine in a bigger cohort of patients with various tumour types. An expanded study was designed using the previously established vaccination schedule with 2 doses of modified $TERT_{572Y}$ peptide followed by 4 doses of native $TERT_{572}$ peptide. The boost vaccinations were with the native peptide.

8.3 Immune response and clinical outcome of vaccinated patients in the expanded studies

Fifty-five HLA-A*0201-expressing patients with various types of chemo-resistant advanced solid tumours (stages III and IV) were enrolled in these studies. The expanded study consolidated the results of the pilot study, confirming that the vaccination strategy was effective. TERT-

specific immune responses were induced in 51% and 70% of the vaccinated patients after the 2nd and 6th vaccinations, respectively, as assessed by both IFN-γ ELISpot and intracellular cytokine staining assays (Fig 5.1). The induction of $TERT_{572}$-specific immune response was independent of the stage of disease or the disease clinical status at enrolment. The kinetics of immune response varied from patient to patient, and in some patients more than two doses were required to induce a detectable immune response (Fig 5.5A), an observation made also by other research groups (Bercovici et al., 2008).

Furthermore, boost vaccinations with the native peptide resulted in the maintenance of specific immune response that had been induced by the 6-vaccination schedule (Fig 5.5B, C). These TERT-specific T cells appeared to be functional, releasing perforin following stimulation with TERT peptides (Fig 5.3A); it is well established that the perforin ELISpot correlates with cytotoxicity assays (Zuber et al., 2005). $hTERT_{572Y}$-tetramer$^+$ $CD8^+$T cells from one vaccinated patient were able to kill TERT-expressing tumour cells (Fig 5.3B).

A significant correlation was observed between late (after the 6th vaccination) TERT-specific IFN-γ immune response and overall survival of vaccinated patients who entered the study with progressive disease. Indeed, late immune responders had a significantly better overall survival compared to that of non-responding patients as depicted in (Fig 5.6C). This observation suggests that the failure of induction of immune response at the end of vaccination protocol may define a subgroup of patients who are less likely to derive a clinical benefit from the vaccination. However, this observation should be interpreted with caution taking into consideration that this study was not designed to investigate this question and that our patient population was widely heterogeneous in terms of cancer type which is obviously associated with different estimated survival, but also with different numbers of previous treatments which may have also played a role in the development of immune response. The patients' ability to mount an immune response could equally reflect their better clinical status which could be correlated with better outcome.

In this population we also observed that in some patients the vaccine-induced TERT-reactive T cells detected in the blood after the 2nd vaccination disappeared after the completion of the 6-vaccination protocol (Fig 5.1A). This could be explained by the presence of other pathways that exert immunosuppressive effect, as discussed before.

The main concern of the immunotherapeutic strategies is the risk of autoimmunity. In case of TERT-based vaccines, the risk of T and B lymphocytes being the target of TERT-specific T cells was raised due to the fact that they both express telomerase during clonal expansion (Hodes, Hathcock, & Weng, 2002). However, in humans T-cell activation is through ligation of the CD3 subunit of the TCR, leads to TERT phosphorylation and movement from the cytoplasm to the nucleus, without increase in TERT-protein levels (Liu, Hodes, & Weng, 2001). Therefore, the total amount of TERT protein in human T cells remains constant following priming. On the other hand, human memory T cells have shorter telomeres compared with their naive counterparts, implying decreased telomerase activity (Weng, Levine, June, & Hodes, 1995). Moreover, terminally differentiated T cells, (e.g. pre-senescent $CD27^{-}/CD28^{-}$ T cells) do not express telomerase (Akbar & Henson, 2011). These findings are suggestive of an age-dependent decrease in telomerase, and TERT expression in human T lymphocytes, with a decreased risk of autoimmunity as collateral damage.

Despite the theoretical risk that the TERT-specific CTLs could attack B cells during the germinal-center reaction (where B lymphocytes seem to have longer telomeres and higher levels of telomerase and TERT, compared to naïve and memory B cells) (K. Liu et al., 1999; Weng, Granger, & Hodes, 1997), it appears that human CTLs with specificity for a low-affinity TERT peptide do not lyse autologous CD40-activated B lymphocytes in vitro, while CTLs for high affinity TERT peptides do not attack bone-marrow-derived HLA-matched $CD34^{+}$ haematopoietic stem cells (HSCs), although they do express telomerase (Hernandez et al., 2002; Minev et al., 2000; Morrison, Prowse, Ho, & Weissman, 1996).

In accordance with the above observations, the expanded study confirmed the favourable toxicity profile of this TERT vaccine, without serious acute or late adverse events and with no evidence of autoimmune reactions even after its administration for up to 2 years. Acute adverse events (AAE) were observed in 29 (52%) patients, and they were mild (grade 1). The most common AAE was grade 1 local skin reaction (n = 15; 27%). Other grade 1 AAEs possibly related to vaccination included asthenia (7%), anaemia (13%) and nausea (4%). One patient with extensive metastatic liver lesions experienced grade 3 transaminases elevation. No symptoms or laboratory findings suggesting late toxicity or an autoimmune syndrome were observed. Similarly, the booster vaccinations for up to 2 years were also proved safe with minimal toxicity.

The expanded study showed that the vaccine was effective in various cancers, in addition to NSCLC (Bolonaki et al., 2007), and that the development of immune response could be a surrogate for a better clinical outcome. Similar observations were made by other researchers using different vaccines (Domchek et al., 2007; Gonzalez et al., 2003; Lonchay et al., 2004; Nemunaitis et al., 2006), while it has been earlier demonstrated that the presence of specifically stimulated T cells in the tumour independently predicts better patients' clinical outcome (Clark et al., 1989; Galon et al., 2006; Marrogi et al., 1997; Zhang et al., 2003). Our study confirmed the importance of the immune response as a major predictor of the long-term outcome.

In our study, those patients who developed an immunologic response at any time during vaccination had a significantly higher PFS (5.2 months; range, 0.9–51.8) compared with those who failed to develop any response following vaccination (2.2 months, range, 1.4–6.5; P = 0.0001; (Table 6.4). This positive correlation of the development of immunological response with higher PFS was independent of disease status at study entry (SD: 7.2 versus 1.4 months; P = 0.008 and PD: 4.0 versus 2.0 months; P = 0.020) (Table 6.4). Similarly, the development of immunological response was associated with a significantly higher OS (20 months; range, 3.8–51.8 versus 10.5 months; range, 1.7–30; P = 0.041) (Table 6.4).

The difference in the median OS of patients entering the study with SD or PD, although was numerically higher for immune responders compared with non-responders, could not reach statistical significance (Table 6.4). Finally, the disease control rate (DCR) was also higher in the immune responders (44.0% versus 14%; P = 0.047) compared to those who didn't develop any response.

In the univariate analysis, the PS, the disease status after the prior treatment and the development of immunological response were significantly associated with better PFS and OS (Table 6.7). Multivariate analysis (Table 6.8) demonstrated that the development of immunological response was an independent factor associated with better PFS (HR = 3.35, 95% CI 1.7–6.7; P = 0.001), while the worse PS was associated with shorter OS (HR = 3.0, 95% CI 1.5–5.8; P = 0.001). There was a trend for worse OS in patients who did not develop immunologic response during the vaccination (HR = 2.0, 95% CI 1.0–4.0; P = 0.057).

It is clear that only a subset of patients responds immunologically, but it remains unclear whether we can predict with clinical or other criteria this group of patients who is more likely to derive

benefit from this approach. In the last decades, research has also focused on the mechanisms contributing to immune tolerance, induced by immune-suppressive pathways and cells which appear active in cancer patients (Almand et al., 2001; Bronte, Serafini, Apolloni, & Zanovello, 2001; Gabrilovich, 2004; S. Kusmartsev, Nagaraj, & Gabrilovich, 2005; S. A. Kusmartsev, Li, & Chen, 2000; Melani, Chiodoni, Forni, & Colombo, 2003; Pandit, Lathers, Beal, Garrity, & Young, 2000). Regulatory T cells (Tregs), myeloid-derived suppressor cells and others have been proposed to play important role in the immune system's failure to mount an efficient response against cancer cells (Hoechst et al., 2008; Mandruzzato et al., 2009; Rodriguez et al., 2009; Srivastava et al., 2008; Vuk-Pavlovic et al., 2010). Futures studies should prospectively investigate the role of various suppressive cells in the development of immune responses after vaccination with the TERT vaccine.

In this patients' cohort immune reactivity against TERT was detected before the vaccination in fourteen patients (25%). This group was studied further in order to determine whether there was any correlation between the pre-existing TERT-specific immune reactivity and patients' clinical outcome (Table 6.6). Our results did not provide any evidence that such correlation exists for either PFS ($P = 0.67$) or OS ($p = 0.26$). However, we found that those patients who maintained immune reactivity after two and/or six vaccine administrations, had significantly higher OS compared to those who failed to maintain it after vaccination ($p = 0.02$). Overall, there was no significant difference in terms of PFS between these two groups ($p = 0.09$; (Table 6.6).

Objective clinical responses are not frequently observed with vaccine-based immunotherapy (Rosenberg et al., 2004; Rosenberg et al., 1998; Simon et al., 2001). There is ongoing discussion regarding the optimal end points in the evaluation of immunotherapy strategies and some patients may also benefit from delay in tumour progression even in the absence of an objective response based on the currently used RECIST criteria for solid tumours (Nishino et al., 2013). More recently, endpoints such as durable response rate [DRR; a continuous response (complete or partial objective response) beginning within 12 weeks of treatment and lasting ≥6 months] has been validated in clinical practice to be associated with clinical benefits such as improved OS and QoL and prolonged TFI, thus supporting the usefulness of DR as a meaningful immunotherapy clinical trial endpoint (Kaufman et al., 2017). The Cancer Vaccine Trial Working Group concludes that tumour shrinkage may be a less relevant measure of vaccine efficacy in the treatment of solid tumours and recommends the duration of SD as an indicator of antitumor activity (Hoos et al., 2007; Wolchok et al., 2009). Interestingly, in the present study,

two objective clinical responses were documented in addition to a significant rate of SD (overall DCR = 36%).

In this study we observed among eight patients who received boost vaccinations, six experienced SD for over than 6 months since the first booster vaccination and three of them continued to receive boost vaccinations for more than 2 years, with no evidence of disease progression. Allowing for the limitation of the small number of patients, this long-term stabilization of disease could be explained also by the small tumour burden, or their excellent performance status (Colombo & Piconese, 2007; Lechner, Liebertz, & Epstein, 2010). Moreover, the disease status (SD versus PD) at the time of study enrolment was associated with the DCR ($p = 0.050$). Both finding suggest that patients without progressive disease, or with limited burden of disease may be better candidates for vaccine-based immunotherapy and that the clinical setting of maintenance treatment could be more appropriate for the vaccine-based strategy.

With increasing evidence suggesting the immune-mediated effects of cancer and the emerging role of immunotherapy in NSCLC, we studied separately the results of NSCLC patients enrolled in the expanded study. Three patients had partial response to the TERT vaccine, while in most cases stabilization of the disease was the best response achieved. Interestingly, among the immune-responders a disease control rate of 52% was observed. Our results showed that despite the development of immune response in the majority of the patients, progression of disease by RECIST criteria was documented.

In accordance with the findings in other tumour types, among NSCC patients the immune-responders had a significantly prolonged survival of 40 months compared to 9 months for non-responders ($p = 0.02$). Moreover, all objective clinical responses, the long- lasting disease control and the long-term survival were observed in those patients who were able to develop immunological response to the TERT vaccine. In twenty-two patients (47.8%) the vaccination was discontinued due to early progression of disease, within 2 months after their enrolment. It is unlikely that such a short interval of vaccination could be sufficient to elicit durable immunologic response, hence we believe that this may have some impact in the final survival results reported. Based on similar observations from other groups, many investigators consider as appropriate candidates for vaccine trials, patients with SD and low tumour burden rather than rapidly progressive, bulky disease (Hoos et al., 2007).

Although the PFS difference was not statistically significant between the responders and non-responders, the OS was significantly prolonged for the immune responders. In most cases these patients were treated with subsequent lines of therapy (chemotherapy) following disease progression to the TERT vaccine, therefore the prolonged survival could be explained on the basis of synergistic effect of cellular immunity and chemotherapy for the immune responders. This hypothesis has been proposed by other groups, as well (Ardizzoni et al., 2003; Gribben et al., 2005; Wheeler, Das, Liu, Yu, & Black, 2004). In one study with extensive small cell lung cancer (SCLC), the clinical response observed to subsequent chemotherapy was closely associated with the induction of immunologic response to the prior vaccination (S. J. Antonia et al., 2006). Newer approach in this field proposes combination of various antineoplastic treatments including chemotherapy with immunotherapy (Baxevanis, Perez, & Papamichail, 2009).

The role of the HLA-A2 status has been previously linked with the long- term prognosis of patients with various malignancies. HLA-$A2^+$ overrepresentation has been associated with worse prognosis in tumours such as breast cancer (Biswal, Kumar, Julka, Sharma, & Vaidya, 1998), squamous- cell carcinoma (SCC) of the cervix (Montoya, Saiz, Rey, Vela, & Clerici-Larradet, 1998) and chronic myelogenous leukaemia (Cortes et al., 1998). More specifically for NSCLC diagnosis, patients with HLA-$A2^+$ haplotype have worse prognosis compared to those with HLA-$A2^-$ haplotype (So et al., 2001). In the current study we compared the survival of the historical control HLA-$A2^+$ patients from our database matched with the patients enrolled in the study in regard to the histology, sex, stage, treatment status. Interestingly, the overall survival of the control patients was significantly lower compared to that of the vaccinated patients. This comparison should be however interpreted with caution as it lacks the unbiased selection of a prospective randomised clinical trial.

In clinical practice it is crucial to identify the ideal candidates for immunotherapy, who are more likely to achieve a long term durable disease control. Specifically, for patients with NSCLC, recent investigation of the genomic landscape showed that inactivating somatic mutations in squamous cell lung cancer (SCC) are likely to lead to impaired immune response to antigens produced by cancer cells. These somatic loss-of-function alterations of HLA-A gene were reported in patients with SCC (Cancer Genome Atlas Research, 2012). Consistently with these findings, our study results showed that patients with NSCLC and non-squamous histology were more likely to developed immune response and benefit from better response rates compared to

those with squamous histology. Failure of T-cells to recognize the administered $TERT_{572}$ peptide could be attributed to the presence of HLA-A gene alterations explaining the poor response of our SCC patients to the TERT vaccine. Future immunotherapy trials could use a genotype-based selection to identify the ideal patient-candidates.

Various synthetic TERT peptides tailored to induce either $CD8^+$ or $CD4^+$ T-cell responses via their affinity for MHC I and MHC II molecules, respectively, have been used as prevalent immunogens in many clinical trials, most of them phase I/I–II and II, and only phase III (Table 8.6.1). Many studies have been conducted in HLA-A*02^+ patients, as this is the most-frequent MHC I allele in white individuals (~45% of whom express this HLA serotype) (Sette & Sidney, 1999) and only a few used MHC II-restricted peptides. In some studies, cells (dendritic cells or B lymphocytes) transfected with RNA or DNA, or cultured with apoptotic tumour cells, were used to vaccinate patients. Concomitant chemotherapy was used in very few early studies (phase I/I–II), but in three of the four phase II studies as well as in the sole phase III trial presented in the Table 8.1.

Table 8. 1 Summary of clinical trials with TERT vaccines

Study	Cancer type	Vaccine	Chemo	IRR	ORR	SD rate
Su et al. (2003)	Renal-cell	DCs transfected with TERT mRNA (various HLA types)	No	6/7 (86%)	NR	NR
Parkhurst et al. (2004)	Multiple solid tumours	TERT p540 peptide (HLA-A*02)	No	7/13 (53%)	0%	NR
Vonderheide et al. (2004)	Multiple solid tumours	TERT p540 peptide (HLA-A*02)	No	4/7 (53%)	17% (1/6)	67% (4/6)
Su et al. (2005)	Prostate cancer	DCs transfected with TERT mRNA (various HLA types)	No	19/20 (95%)	0%	NR
Millard et al. (2005)	Prostate cancer	B lymphocytes transfected with pDNA encoding two TERT peptides: p540 and pY572 (HLA-A*02)	No	12/15 (80%)	NR	NR
CTN-2000: Brunsvig et al. (2006/2011)	NSCLC	Two TERT peptides: p611 (GV1001) and p540 (MHC II and HLA-A*02 mixture) + GM-CSF	No	13/24 (54%)	8% (2/24 evaluable patients)	16% (4/24 evaluable patients)
Bernhardt et al. (2006)	Pancreas	TERT p611 (GV1001) peptide (MHC II) + GM-CSF	No	24/38 (63%)	NR	NR
Mavroudis et al. (2006)	Multiple solid tumours	TERT pY572 peptide (HLA-A*02)	No	13/14 (93%)	0%	21%(4/19 evaluable patients)

Bolonaki et al. (2007)	NSCLC	TERT pY572 peptide (HLA-A*02)	No	16/21 (76%) after 2nd vaccination; 10/11 (91%) after 6th vaccination	0%	36% (8/22 evaluable patients)
Berntsen et al. (2008)	Renal cell ca	DCs loaded with multiple TERT and survivin peptides, or tumour lysate (HLA-A*02, or MHC II mixture) + low-dose IL-2	No	6/6 (100%)	0%	48% (13/27 evaluable patients)
Kitawaki et al. (2011)	Acute Myeloid leukaemia	DCs pulsed with apoptotic cells and injected with killed Streptococcus pyogenes OK-432 to induce maturation	No	2/4 (50%)	0%	NR
Schlapbach et al. (2011)	Cutaneous T-cell lymphoma	TERT p611 (GV1001) peptide (MHC II)	No	1/6 (17%)	0%	NR
Hunger et al. (2011)	Cutaneous melanoma	TERT p611 (GV1001) and p540 peptides (MHC II and HLA-A*02 mixture) + GM-CSF	No	7/10 (70%)	NR	NR
Kyte et al. (2011)	Melanoma	TERT p611 (GV1001) peptide (MHC II)	Yes Temozolamide	18/23 (78%)	20% (5/25 evaluable patients)	24% (6/25 evaluable patients)
Rapoport et al. (2011)	Multiple myeloma	TERT p540, pY572 and pY988 mixed with survivin peptides (HLA-A*02) in only HLA-A*02-positive patients (n = 28); all patients (n = 54) received Pneumococcal-conjugate vaccine immunizations, ASCT, and adoptive transfer of post-vaccination autologous T cells activated and expanded ex vivo	No	10/28 (36%; TERT vaccine only	NR	NR

Vik-Mo et al. (2013)	Glioblastoma	DCs transfected with mRNAs from tumour-cell lysates, and TERT and survivin mRNA (various HLA types)	Yes: standard postoperative chemoradiotherapy	7/7 (100%)	71% (5/7 evaluable patients)	NA
Fenoglio et al. (2013)	Prostate or Renal cell	Four TERT peptides p540, p672, p766 and p611 (HLA-A*02 and MHC II mixture)	No	9/14 (64%)	0%	40% (4/10 evaluable patients)
Staff et al. (2014)	Pancreatic cancer	TERT p611 (GV1001) peptide (MHC II) + GM-CSF	Yes: gemcitabine concurrently (groups A/B), or added at disease progression (group C)	Group A/B: 8/12 (67%) Group C: 2/5 (40%)	0%	Group A/B: 83% (10/12) Group C: 20% (1/5)
Greten et al. (2010)	HCC	TERT p611 (GV1001) peptide (MHC II) + GM-CSF	Yes: cyclophosphamide	0%	0%	46% (17/37 evaluable patients)
CTN-2006: Brunsvig et al. (2011)	NSCLC	TERT p611 (GV1001) peptide (MHC II)	Yes: post-chemoradiotherapy with docetaxel	16/20 (80%)	NA	NA
Ellebeck et al. (2012)	Melanoma	DCs loaded with TERT, survivin and p53 peptides in HLA-A*02-positive patients, or DCs pulsed with tumour lysates in HLA-A*02-negative patients, plus IL-2	Yes: metronomic cyclophosphamide	9/15 HLA-A*02-postive patients (60%)	0%	57% (16/28 evaluable patients)
Kotsakis et al. (2012)	Multiple advanced-stage solid tumours	TERT pY572 peptide (HLA-A*02)	No	30/55 (55%) after 2nd vaccination; 24/36 (70%) after 6th vaccination	3.6% (2/55 evaluable patients)	33% (18/55 evaluable patients)

Middleton et al. (2014)	Pancreatic cancer	TERT p611 (GV1001) peptide (MHC II) + GM-CSF	Yes: gemcitabine and capecitabine, sequentially or concurrently with vaccination	NR	Sequential: 9% (31/350) Concurrent:16 % (55/354) Chemotherapy: 18% (63/358)	NR

There is no doubt that in the last two decades, great progress has been made in cancer immunotherapy. In case of the TERT-based vaccines, despite the good scientific rationale and their success achieving the target of immunologic response, their overall clinical effect remained modest. There is ongoing discussion addressing the possible caveats of the design and strategy followed so far and research on how to improve the clinical efficacy of the vaccines (Zanetti, 2017). The following aspects should be considered in the design of clinical trials with TERT-based vaccines:

A. Choice of peptide

One of the important steps is the choice of the peptide in order to bypass the tolerance developing when peptides with high affinity for MHC molecules are used in the vaccines. High-affinity anti-TERT T cells preferentially interact with TERT peptides with high affinity for the MHC molecules, resulting in their depletion from the T-cell repertoire, flattening the induced immune response. A suggested approach to overcome this obstacle and increase their MHC-binding affinity, is the selection of low-affinity peptides, and then empirically identify the peptides with improved immunogenicity (Hernandez et al., 2002). Our study, along with the work from another group (Cortez-Gonzalez & Zanetti, 2007) used this specific approach by selecting a TERT peptide, p572Y, which is an analogue of a peptide with low affinity for HLA-A*02 (p572). The pY572 induces TERT-specific CTL in humans, shares a cross-reactive T-cell repertoire with the parental peptide and thus resulting in the lysis of tumour cells in vitro (Hernandez et al., 2002).

B. The use of adjuvants

Another consideration is that the vaccines using adjuvants such as incomplete Freund's adjuvant or Montanide ISA adjuvants may create antigen depots leading to sequestration of tumour-specific T cells at the injection site, potentially hampering tumour infiltration and promoting apoptosis of the T cells (Hailemichael et al., 2013). In many trials with TERT-vaccine (including our study) Montanide® ISA adjuvants were used (Bolonaki et al., 2007; Fenoglio et al., 2013; Kotsakis et al., 2012; Mavroudis et al., 2006; Parkhurst et al., 2004; Rapoport et al., 2011), with variable immune responses observed post-vaccination.

C. Targeting T cells

To date, most of the clinical trials using TERT– based vaccines target the $CD8^+$ T cells without cooperation with $CD4^+$ T cells. The need to include peptides that are capable of activating both

$CD4^+$ T-helper cells and $CD8^+$ CTLs in the same immunogen for more effective vaccination was introduced with a lipopeptide vaccine against the hepatitis B virus. Subsequently, it was shown that when $CD8^+$ T cells are solely stimulated without the help of T helper cells, the $CD8^+$ T-cell response is poorly maintained, with low numbers of precursor T-cell and poor expansion after antigen re-stimulation. Similar results should be expected with vaccines targeting only $CD4^+$ T-cell using a self-peptide alone (Langlade-Demoyen et al., 2003; Shedlock & Shen, 2003; Vitiello et al., 1995). This could explain the suboptimal response following re-stimulation for those patients who had already detectable immunity to TERT-peptides, as depicted in our study.

Recently, it was proposed that cooperation between two $CD4^+$ T cells enables the activation and expansion of $CD4^+$ T cells specific for poorly immunogenic determinants and /or tolerized $CD4^+$ T cells, which otherwise would be unable to expand or may expand to a limited extent. This process was named Th–Th cooperation or "help for helpers" (Gerloni & Zanetti, 2005). According to the researchers, Th–Th cooperation drives the activation of $CD4^+$ T cells specific for a self-tumour Ag, providing more comprehensive, durable, and specific immune response against tumour re-challenge. This is based on associative recognition of Ag, where self and non-self Th cell determinants are presented by the same APC. For future trials, this essential immunologic paradigm merits consideration for design of the vaccines (Cohn, 2005; Zanetti, 2015).

It is undisputable that the class of responding T cells is important for optimization of tumour response. Both $CD8^+$ and $CD4^+$ T cells can induce anti-tumour responses and the quality of T cells, in addition to their quantity, matters. Especially regarding $CD8^+$ T cells, preclinical studies have shown that although T cells at different stages of differentiation can induce anti-tumour activity, central memory (TCM) and memory T cells with stem-cell-like properties (TSCM) provide superior protection against cancer (Gattinoni et al., 2011; Klebanoff et al., 2005). As such, an approach using low-dose immunization of antigen for induction of $CD4^+$ T-cell help to $CD8^+$ or $CD4^+$ T cells could be considered. It seems that very few of the TERT-vaccine trials to date have used similar approach to generate these classes of T cells.

D. Timing of vaccination

The timing of vaccination in the course of the disease may affect the immune response induced. In our study, those patients who entered the program with PD, thus very advanced-stage disease,

developed limited immune response and progressed clinically shortly after the onset of vaccination, before they even had sufficient time to develop adequate immune response. A possible explanation is that the advanced cancer status might limit both the immune response generated and anti-tumour effectiveness of any response. In other words, although initially cancer possesses immunogenic properties capable of stimulating the immune response, its further growth and progression result in immunosuppression due to escape mechanisms discussed in the chapter 1.

It has been suggested by other researchers that vaccination should be considered at the disease stage when the tumour burden is low and the tumour is localized, and immunosuppressive mechanisms might be less established. This approach is explored in our phase IIb clinical trial, where patients with NSCLC are vaccinated with the TERT-vaccine in the maintenance setting after completion of their 1st line treatment and before progression of their disease occurs (Georgoulias et al., 2013).

For the advanced-stage disease setting, a possible approach could consider the adoptive transfer of TERT-specific T cells, followed by TERT vaccination (Rapoport et al., 2011). The rationale behind this approach is that passively administered T cells could initiate a process of tumour destruction which in turn can promote the development and potentiate the effectiveness of subsequently active anti-TERT immunity given with the vaccination.

E. Targeting the tumour microenvironment

The tumour microenvironment may also play a critical role in the success of therapeutic vaccination, although its interaction with the TERT vaccines has been largely unexplored in clinical trials to date. This consideration is based on the fact that the tumour microenvironment may be enriched with T-cells expressing the immune-checkpoint proteins cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell-death protein 1 (PD-1) or its ligand programmed cell death 1 ligand 1 (PD-L1), and/or other inhibitory molecules (Chambers, Kuhns, Egen, & Allison, 2001; Keir, Butte, Freeman, & Sharpe, 2008; Sharma & Allison, 2015a). Among them special consideration should be given to the regulatory/suppressor $CD4^+$ and $CD8^+$ T cells (Treg cells) (Mougiakakos, Choudhury, Lladser, Kiessling, & Johansson, 2010; R. F. Wang, 2008), the myeloid cells with both immunosuppressive and pro-inflammatory characteristics (such as tumour-associated macrophages and myeloid-derived suppressor cells)

(Gajewski, Schreiber, & Fu, 2013; Mahadevan & Zanetti, 2011), and B cells with tumour-promoting regulatory functions (Affara et al., 2014; Shalapour et al., 2015).

The effect of stress due to cancer and/or inflammation may alter the capacity of the bone-marrow-derived macrophages and DCs to present the antigen, which can have further impact in the activation and expansion of naïve T cells. Therefore, the control of tumour microenvironment, either by targeting these cells directly or by interfering with the pathways resulting in their dysregulation, is likely to be necessary at the time of vaccination (Mahadevan et al., 2012; Rodvold, Mahadevan, & Zanetti, 2016). An example of this important interaction between the immune system and the immunosuppressive microenvironment is provided by the results with single-agent or dual-agent therapy with immune-checkpoint inhibitors (S. Antonia et al., 2016; Gettinger et al., 2015; Hamid et al., 2013; Hodi et al., 2010; Postow et al., 2015; Powles et al., 2014; Robert et al., 2015; Schadendorf et al., 2015; Topalian et al., 2012; Topalian et al., 2014). The significant clinical response observed when we aim to release the break on naturally acquired immune responses to tumour antigens (such as TERT), can be used in the design of the future vaccines.

Immunosuppressive cell types in the tumour microenvironment can be targeted with different strategies. For example, immune-checkpoint inhibitors (such as anti-CTLA-4) can restore the exhausted activity of T cells and regulate Treg -cell activity (Nishikawa & Sakaguchi, 2014; Wing et al., 2008). Treg cells induced by vaccination may counter-suppress the T-cell response developed against cancer, but their action can be opposed with different approaches including a single low-dose of cyclophosphamide before anticancer vaccination or thalidomide (Filaci et al., 2007; Giannopoulos et al., 2008; Klebanoff, Gattinoni, & Restifo, 2006), while other agents have been used to control the action of myeloid cells with proinflammatory immunosuppressive properties (Marvel & Gabrilovich, 2015; Wesolowski, Markowitz, & Carson, 2013). Agents such as anti-CD20 or B-cell-kinase inhibitors targeting tumour -promoting antibodies within the tumour microenvironment can be combined with TERT-based vaccines to improve immunologic and clinical responses in future studies.

F. Selection of cancer types

We know that telomerase complex and telomerase reverse transcriptase (TERT) are expressed at every stage of tumour evolution (Beier et al., 2011; Finones et al., 2013). Recent whole-

genome sequencing studies have identified mutations in the TERT promoter which are associated with certain types of cancer, and are the most prevalent mutations in noncoding regions in human cancers (X. Liu et al., 2013; Melton, Reuter, Spacek, & Snyder, 2015; Weinhold, Jacobsen, Schultz, Sander, & Lee, 2014), leading to the transcriptional upregulation of TERT and high TERT-protein expression (Nault et al., 2013). This could increase TERT-antigen presentation by cancer cells, making them more susceptible to T-cell recognition and attack. Therefore, patients could be assessed for TERT-promoter alterations through quantitative real-time PCR analysis of DNA from tumour specimens, while future TERT-based immunotherapy can be focused on patients with presence of these alterations.

Moreover, candidate patients for TERT-based immunotherapy could be further selected based on other clinicopathologic criteria including tissue origin of cancer and potential virus-induced carcinogenesis (some viruses such as hepatitis B virus, hepatitis C virus, and human papillomavirus are known to integrate into the TERT promoter) (Ferber et al., 2003; Horikawa & Barrett, 2001; Paterlini-Brechot et al., 2003; Z. Zhu et al., 2010).

Taking into account parameters such as the frequency of TERT-promoter mutations in different types of cancers, and associated mechanisms increasing the expression of TERT, we could potentially identify cancer types in which TERT immunotherapy would have the highest likelihood of clinical success.

In conclusion, the effectiveness of TERT vaccination could be augmented by concomitant immune-checkpoint inhibition, particularly in patients who have detectable TERT-specific T cells before immune intervention. TERT vaccination, vice versa, could also help potentiating the activity of immune-checkpoint inhibitors in patients with responsive cancer types (such as melanoma and lung cancer), and provide a treatment option to cancers that, to date, have proven refractory to immunotherapy agents such as the immune- checkpoint inhibitors.

Our study answered the scientific and clinical question for which it was originally designed and developed a strategy which was proven effective. Our results in accordance with similar studies shed light, addressing the major caveats in the design and strategies of active immunotherapy. This study contributes to the better understanding of TERT-based vaccination and shares important lessons for the design of immunotherapy in the future.

9. References

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