OPINION

A second chance for telomerase reverse transcriptase in anticancer immunotherapy

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Abstract | Telomerase reverse transcriptase (TERT) is a self-antigen that is expressed constitutively in many tumours, and is, therefore, an important target for anticancer immunotherapy. In the past 10 years, trials of immunotherapy with TERT-based vaccines have demonstrated only modest benefits. In this Perspectives, I discuss the possible immunological reasons for this limited antitumour efficacy, and propose that advances in our understanding of the genetics and biology of the involvement of TERT in cancer provides the basis for renewed interest in TERT-based immunotherapy. Telomerase and TERT are expressed in cancer cells at every stage of tumour evolution, from the cancer stem cell to circulating tumour cells and tumour metastases. Many cancer types also harbour cells with mutations in the *TERT* promoter region, which increase transcriptional activation of this gene. These new findings should spur new interest in the development of TERT-based immunotherapies that are redesigned in line with established immunological considerations and working principles, and are tailored to patients stratified on the basis of *TERT*-promoter mutations and other underlying tumour characteristics. Thus, despite the disappointment of previous clinical trials, TERT offers the potential for personalized immunotherapy, perhaps in combination with immune-checkpoint inhibition.

Telomerase reverse transcriptase (TERT) is a component of the ribonucleoprotein telomerase, a unique cellular enzyme: via reverse transcription of its own RNA template (TERC), telomerase synthesizes the tandem 5′-TTAGGG-3′ exonucleotide repeats of telomeric DNA, which prevents chromosome attrition resulting from incomplete semiconservative DNA replication at chromosomal ends[1]. Thus, the discovery of telomerase and telomerase-mediated extension of telomeric DNA solved the end-replication problem — that is, the mechanism by which telomeric DNA is maintained[2]. In addition, this discovery paved the way to elucidate the 'end-protection problem' and the mechanisms by which telomere elongation prevents the severe consequences of a cellular response to exposed DNA ends[3], such as end-to-end joining, DNA recombination, or DNA repair, which would lead to unstable chromosomes and, ultimately, aneuploidy[4]. As telomeres shorten progressively with successive cell divisions in the absence of TERT expression (which is restricted to certain cell types, predominantly stem or germ cells), telomere length is considered to mirror the replicative history of a cell lineage[5].

Telomerase imparts cells with the capacity to continuously replicate, which is often referred to as 'cell immortality' (REF. 6). When TERT is overexpressed together with simian virus 40 large T antigen and RAS oncoproteins in human cells, the cells become 'immortal', are malignantly transformed, and can form tumours *in vivo*[7]. Moreover, using the canonical telomeric repeat amplification protocol (TRAP) assay, telomerase activity is detected in >85% of human tumours of various histological types[8], but not in normal tissues. Modest levels of telomerase activity are detected in tissues with high self-renewal capacity, such as the bone marrow, testes, gastrointestinal crypt epithelium, and hair follicles[9–11]. In a minority of tumours of mesenchymal origin (soft-tissue sarcoma and osteosarcoma), as well as pancreatic neuroendrocrine neoplasms and gliomas[12], telomere length is maintained by one or more alternative, TERT-independent mechanisms. These mechanisms are referred to as 'alternative lengthening of telomeres' (ALT), and involve copying telomeric template DNA via homologous recombination[13]. Nevertheless, TERT is an important immunological target for anticancer therapy because of its widespread overexpression in human cancers, throughout the entire trajectory of the disease (see 'Recentring TERT in tumour evolution' section of this Perspectives).

TERT immunology in cancer

Human TERT is a self-antigen that consists of 1,132 amino acids[14]. Soon after its amino acid sequence was deduced more than 15 years ago, several laboratories probed the antigenicity and immunogenicity of TERT[15,16] — antigenicity refers to the property of being recognized by the adaptive immune system, whereas immunogenicity refers to the capacity to induce an adaptive immune response. As TERT is an intracellular protein that is not expressed on the external cell surface, it 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. The initial experiments into TERT immunology 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. The first two immunogenic TERT peptides discovered (p540 and p865) were identified based on a predicted high-affinity interaction with human histocompatibility antigen (HLA)-A*02 molecules, a MHC I serotype group. By probing the surface of cancer cells using $CD8^+$ T cells induced with these TERT peptides *in vitro*, investigators determined that the peptides are expressed on histologically distinct *HLA-A*02*$^{+/+}$, TERT-expressing cancer cell lines[15], and primary cancer cells[16]; antibodies targeting MHC I blocked T-cell killing of the cancer cells, implying that the reaction was mediated by the MHC I–peptide complexes. TCR-mimic antibodies directed at the same TERT peptides were also found to bind HLA-A*02$^+$/TERT$^+$ cell lines[17]. Other groups, however, were unable to detect the HLA-A*02-restricted, dominant TERT peptide (p540) on the surface of cancer cells[18–20]. This discrepancy could be attributed to a number of factors, including methodological variation between different laboratories (for instance, different groups have reported disparate results with the same anti-HLA-A*02–TERT-peptide complex antibody[17,19,21]); a lack of stringent specificity-control with regard to the $CD8^+$ T cells used to probe model cancer cells (cold target inhibition, a competitive assay that ensures the specificity of cell recognition, was used in only one study[15]); and the use of different cell lines, each with presumably different immunopeptidomes generated in the endoplasmic reticulum. In line with the latter interpretation, p540 was found to be preferentially destroyed during antigen processing, as it contains a proteasome cleavage site[18]. Thus, p540 might be expressed at only the low end of natural antigen-presentation levels (~10 copies per cell)[22,23]. Over the years, further evidence showed that TERT is processed endogenously, and that TERT peptides are presented by cancer cells that express HLA-A*03, HLA-A*24, and HLA-B*7 MHC I molecules[24–28].

Anticancer immunity can also be efficiently mediated by $CD4^+$ T cells that recognize antigens in the context of MHC class II (MHC II) molecules[29]. Predicting MHC II-restricted peptide antigens is more complex than predicting MHC I peptides, as 3,658 MHC II alleles are identified in the Immuno Polymorphism Database (IPD) and international Immunogenetics (IMGT)/HLA database: https://www.ebi.ac.uk/ipd/imgt/hla/). Indeed, few *bona fide* MHC II-binding TERT peptides have been identified and characterized; although some of those that have been identified are both promiscuous (bind to multiple MHC II alleles), and are produced endogenously in cancer cells[30–32]. Thus, cancer cells can present TERT peptides that can be recognized by either $CD4^+$ or $CD8^+$ T cells.

In vitro, TERT protein has been demonstrated to be readily immunogenic for peripheral blood T lymphocytes harvested from healthy individuals and patients with cancer, suggesting that TERT-reactive T-cell precursors exist in the blood and are not deleted in the thymus. This finding is important because immunization does not result in the *de novo* creation of antigen-specific T cells, but rather the selective expansion of reactive clones that pre-exist in the T-cell repertoire[15,16,24,26,28,33]. The *in vivo* immunogenicity of TERT has also been interrogated in preclinical models using a variety of approaches, including synthetic TERT peptides[15,24,28], dendritic cells (DCs) transfected with *TERT* mRNA[34–36], and DCs transduced with *TERT*-adenovirus[37], *TERT*-encoding lentivirus vectors[38], and plasmid DNA encoding *TERT*[39]. In selected instances, the induction of T-cell responses was associated with inhibition of tumour growth[34,38–41].

Antigenicity and immunogenicity are merely two aspects of a larger immunological equation. Any T-cell response *in vivo* also depends on the size of the available T-cell repertoire for a given protein. Indeed, the identification of TERT peptides with high-affinity for MHC molecules that are immunogenic for peripheral blood T cells *in vitro* provides no clue as to the actual size and breadth of the anti-TERT T-cell repertoire that exists *in vivo*. Using flow cytometry, investigators have detected $CD8^+$ T cells with specificity for TERT in the blood of variable proportions of patients with chronic myeloid leukaemia (~80%), breast cancer (~75%), lung cancer (~40%), colorectal cancer (~20%), and hepatocellular carcinoma (~10%)[42–46]. In one study in patients with solid tumours, limiting-dilution analyses resulted in estimated TERT-specific T-cell-precursor frequencies as high as 1:298–1:540 (REF. 47), suggesting the presence of spontaneous immune responses to TERT in some patients. Moreover, evidence indicates that spontaneous $CD4^+$-T-cell responses against promiscuous TERT peptides occur in 38% of patients with lung cancer[32]. The available T-cell repertoire for TERT in patients with cancer seems to be enriched over that of healthy individuals[32,43,47]; however, limited information — if any — exists on the functional status of these T cells, when in the course of cancer development their populations expand, and whether they could re-expand following TERT vaccination.

Immune tolerance is a major determinant of an individual's unique T-cell repertoire. During ontogeny, immune tolerance shapes the T-cell repertoire via elimination of T-cell precursors that express TCRs with high-affinity for MHC–peptide complexes (signal 1), while T-cell precursors with low–moderate-affinity TCRs are spared. Tumour growth can also promote peripheral immune tolerance if antigen-presenting cells activate T cells in the absence of co-stimulatory molecules (signal 2). In addition, certain T-cell specificities can be lost over time owing to T-cell senescence and exhaustion[48], or as a result of remodelling of cancer-cell immunogenicity by immune editing[49]. The roles that these factors have in determining the immunological responses to TERT in patients with cancer remain largely unknown.

Autoimmunity and TERT vaccines

At the outset of the decade-long quest for a successful therapeutic TERT-based vaccine against cancer, a recurrent concern raised was the potential for this approach to result in collateral damage to host tissues. For example, T lymphocytes and B lymphocytes are known to express telomerase during clonal expansion[50]; therefore, will these cells be attacked by TERT-specific T cells during such immunotherapy? The dynamics between clonal expansion of lymphocytes, telomerase activity, and TERT expression has been analysed, and telomerase expression in activated murine $CD4^+$ T cells was found to be induced in an antigen-specific and CD28–B7-mediated co-stimulation-dependent manner[51]. This finding highlights the possibility that CTLs specific for TERT could potentially engage in 'fratricidal' killing of T cells, with systemic consequences. In humans, however, T-cell activation through ligation of the CD3 subunit of the TCR results in TERT phosphorylation and relocation from the cytoplasm to the nucleus, without a net increase in TERT-protein levels[52]. Thus, following priming, the total amount of TERT protein in human T cells remains constant. On the other hand, human memory T cells have shorter telomeres compared with their naive counterparts, implying decreased telomerase activity[53]. Furthermore, terminally differentiated T cells, such as pre-senescent $CD27^-$/$CD28^-$ T cells, do not

express telomerase[48]. Taken together, these observations suggest an age-dependent decrease in telomerase, and hence TERT, expression in human T lymphocytes, with a diminished risk of autoimmunity.

Human B lymphocytes in germinal centres of lymph organs have markedly longer telomeres — presumably resulting from higher levels of telomerase and *TERT* expression — than naive and memory B cells[54,55]. Thus, one would predict that TERT-specific CTLs could eliminate B cells during the germinal-centre reaction; however, human CTLs with specificity for a low-affinity TERT peptide do not lyse autologous CD40-activated B lymphocytes *in vitro*[24]. Additionally, CTLs that target high-affinity TERT peptides do not kill bone-marrow-derived HLA-matched $CD34^+$ haematopoietic stem cells (HSCs)[15], despite evidence that most bone marrow HSCs express telomerase[56]. Nevertheless, caution is needed when using TERT-directed immunotherapy, as indicated by the results of a preclinical study in mice, in which three cycles of adoptive T-cell therapy with mouse TERT-specific $CD8^+$ T cells caused transient, self-resolving B-cell lymphopenia[57]. This effect might be attributable to either differences in the regulation of TERT expression between mouse and human tissues, or the aggressive therapeutic regimen used in the mouse model (transfer of 5×10^6 TERT-specific $CD8^+$ T cells, high-dose IL-2, and immunization of the mice with an adenovirus-vector encoding the TERT antigen recognized by the transferred T cells). In a prior report, the same group demonstrated that mice actively immunized with TERT had no B-cell abnormalities[39], suggesting that adoptive T-cell therapy could be associated with a greater risk of adverse events.

Collectively, the concerns that activated T cells, B lymphocytes, and HSCs would be targeted by TERT-specific T cells, albeit valid in principle, seem to be mitigated by the transient nature and low level of TERT expression in these cell types, and the fact that the induction of telomerase activity is not associated with a parallel increase in levels of TERT protein (at least in T cells). Congruently, preclinical studies revealed no abnormalities in the spleen and lymph nodes of *HLA-A*02*-transgenic mice vaccinated with a low-affinity TERT analogue peptide[40].

Therapeutic TERT-vaccine trials

Following the discoveries discussed in the previous sections, therapeutic TERT-based vaccination was rapidly pursued in patients with different types of cancer. Indeed, a total of 23 clinical studies with published data have investigated this anticancer strategy: 18 phase I/I–II and four phase II studies, and one phase III trial (TABLE 1). Various vaccination approaches were used in these studies.

Overall, synthetic peptides tailored to induce either $CD4^+$ or $CD8^+$ T-cell responses via their affinity for MHC II and MHC I molecules, respectively, have been the prevalent immunogen used: this approach was used in 13 of the phase I/I–II, three of the four phase II studies, and in the sole phase III trial. Many studies (13 out of 23) 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)[58]. Only five studies involved MHC II-restricted peptides. In six 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 only three phase I/I–II studies, but in three of the four phase II studies as well as in the sole phase III trial.

In the phase I/I–II studies, a TERT-specific T-cell response in ≥50% of the evaluable patients vaccinated was reported in 16 of the 18 studies (TABLE 1). Objective clinical responses were reported in four phase I/I–II studies with distinct designs, in a variable percentage (8–71%) of the evaluable vaccinees[59–63]. No objective responses were reported in the other phase I/I–II studies in which clinical outcomes were assessed ($n = 6$)[64–69]. Disease stabilization in >50% of the evaluable patients vaccinated was reported in two studies (rates of 67% and 83%)[59,69], whereas lower rates of disease stabilization (16–48%) have been observed in seven studies (TABLE 1). Cumulatively, the findings of the phase I/I–II studies showed that, although therapeutic TERT-based vaccination can induce specific T-cell responses in many of those vaccinated, the effect on tumour size was minimal: temporary disease stabilization was generally the best clinical result (TABLE 1).

Three of the four phase II clinical trials have been conducted in patients with a single type of cancer and vaccination was combined with chemotherapy (TABLE 1). Synthetic peptide immunogens were used in all but one of the phase II studies. Development of a TERT-specific T-cell response in >50% vaccinees (range 55–80%) was reported in three out the four studies[61,70,71]. Objective responses were reported in only one phase II study, comprising a complete response in a patient with breast cancer and liver metastases and a partial response in a patient with metastatic hepatocellular carcinoma[71]. Temporary disease stabilization was reported in three of the four studies, with rates that ranged from 33% to 57% in those vaccinated (TABLE 1)[70–72].

In the sole phase III trial[73], investigators evaluated the efficacy of TERT-peptide vaccination plus chemotherapy in patients with pancreatic cancer. In this three-arm randomized study that involved 1,062 patients, the median survival durations were not significantly different between the sequential or concurrent chemoimmunotherapy groups compared with the chemotherapy-only group, with an overall lack of benefit in terms of both median time to progression (4.5 months and 6.6 months, respectively, versus 6.4 months) and overall survival (6.9 months and 8.4 months, respectively, versus 7.9 months)[73]. Overall, objective responses were observed in 63 (18%) of 358 patients in the chemotherapy-alone group, 31 (9%) of 350 patients in the sequential chemoimmunotherapy group, and 55 (16%) of 354 patients in the concurrent chemoimmunotherapy group (TABLE 1).

In the few studies (six) with evaluable data, the immune response to TERT vaccination was generally found to correlate with clinical benefit, in terms of overall survival (TABLE 2). In fact, responders typically had overall survival durations that were significantly prolonged and approached or exceeded double that observed for nonresponders. Future studies will need to establish whether clinical benefit in immunological responders also correlates with the induction of T cells that infiltrate the tumour, as observed in a patient included in a phase I study[59].

Collectively, the data from these clinical trials performed to date indicate that therapeutic TERT-based vaccination has limited anticancer efficacy: the various immunogens reportedly induce T-cell responses to TERT in patients with cancer, but this effect is typically insufficient to control tumour growth or disease progression. In general, however, these clinical studies confirmed that the risk of adverse events following vaccination targeting TERT is minimal or nonexistent. For instance, no lymphopenia was observed in patients with prostate cancer who were vaccinated using a combination of high-affinity and low-affinity TERT peptides[74], nor in patients with myeloma

Table 1 | **Summary of reported therapeutic trials of TERT vaccines**

Study or trial	Setting	Vaccine approach	Combined with chemotherapy	IRR*	ORR	SD rate	Refs
Phase I/I–II							
Su *et al.* (2003)	Renal-cell carcinoma	DCs transfected with *TERT* mRNA (various HLA types)	No	6/7 (86%)	NR	NR	64
Parkhurst *et al.* (2004)	Multiple solid tumours	TERT p540 peptide (HLA-A*02)	No	7/13 (53%)	0%	NR	19
Vonderheide *et al.* (2004)	Multiple solid tumours	TERT p540 peptide (HLA-A*02)	No	4/7 (57%)	17% (1/6 evaluable patients)	67% (4/6 evaluable patients)	59
Su *et al.* (2005)	Prostate cancer	DCs transfected with *TERT* mRNA (various HLA types)	No	19/20 (95%)	0%	NR	184
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	74, 185
CTN-2000: Brunsvig *et al.* (2006/2011)	Non-small-cell lung cancer	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)	60, 61
Bernhardt *et al.* (2006)	Pancreatic cancer	TERT p611 (GV1001) peptide (MHC II) + GM-CSF	No	24/38 (63%)	NR	NR	65
Mavroudis *et al.* (2006)	Multiple solid tumours	TERT pY572 peptide (HLA-A*02)	No	13/14 (93%)	0%	21% (4/19 evaluable patients)	82
Bolonaki *et al.* (2007)	Non-small-cell lung cancer	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)	66
Berntsen *et al.* (2008)	Renal-cell carcinoma	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)	67
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	186
Schlapbach *et al.* (2011)	Cutaneous T-cell lymphoma	TERT p611 (GV1001) peptide (MHC II)	No	1/6 (17%)	0%	NR	68
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	187
Kyte *et al.* (2011)	Melanoma	TERT p611 (GV1001) peptide (MHC II)	Yes: temozolomide	18/23 (78%)	20% (5/25 evaluable patients)	24% (6/25 evaluable patients)	62
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 arm only)	NR	NR	75
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	63
Fenoglio *et al.* (2013)	Prostate or renal cancers	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)	83

Table 1 (cont.) | **Summary of reported therapeutic trials of TERT vaccines**

Study or trial	Setting	Vaccine approach	Combined with chemotherapy	IRR*	ORR	SD rate	Refs
Phase I/I–II (cont.)							
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)	69
Phase II							
Greten *et al.* (2010)	Hepatocellular carcinoma	TERT p611 (GV1001) peptide (MHC II) + GM-CSF	Yes: cyclophosphamide	0%	0%	46% (17/37 evaluable patients)	72
CTN-2006: Brunsvig *et al.* (2011)	Non-small-cell lung cancer	TERT p611 (GV1001) peptide (MHC II)	Yes: post-chemoradiotherapy with docetaxel	16/20 (80%)	NA	NA	61
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)	70
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)	71
Phase III							
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	73

Synthetic peptides used in the various trials were either restricted to the HLA-A*02 (MHC I) allele, MHC II alleles (not specified), or targeted both HLA-A*02 and MHC II alleles when used in combination. ASCT, autologous stem-cell transplantation; DC, dendritic cells; GM-CSF, granulocyte–macrophage colony-stimulating factor; IRR, immunological response rate; NA, not applicable; NR, not reported; ORR, objective response rate; pDNA, plasmid DNA; Refs, references; SD, stable disease; TERT, telomerase reverse transcriptase. *In evaluable patients, as assessed by T-cell cytoxicity assays, MHC–peptide tetramer staining, enzyme-Linked immunospot assay, depending on the study.

who received infusion of TERT-reactive $CD8^+$ T-cell expanded *ex vivo* and boosted by TERT-peptide vaccination[75].

Interrogation of the ClinicalTrial.gov (https://clinicaltrials.gov/) and WHO International Clinical Trial Registry Platform (http://www.who.int/ictrp/en/) databases reveals that an additional 16 new clinical trials of TERT vaccines are in progress, including phase I, phase II, and phase III trials. Most of these studies are, however, based on the same working principles as those used in the completed studies reviewed herein. Thus, a reasonable expectation is that similar trends will be found.

Lessons learned

Why isn't therapeutic vaccination against TERT more effective? Can lessons be learned from the results of the work conducted during the past decade? Is it possible to improve efficacy through better vaccine design, thus making therapeutic TERT vaccination a worthwhile option for patients with cancer? Multiple reasons might explain why the initial experience with therapeutic TERT vaccination has been largely disappointing; in the following paragraphs, I outline the immunological considerations that I believe received too little attention in previous approaches to TERT vaccination, but could potentially lead to marked improvements in future trials.

First, is TERT-peptide presentation altered in cancer cells? One might expect that patient-to-patient and cell-to-cell variability in the presentation of TERT peptides exists. The immunopeptidome, the multitude of peptides generated intracellularly and sorted in specialized organelles (for example, the endoplasmic reticulum), is not a mirror of the proteome or the transcriptome, and therefore, its content cannot be predicted. Indeed, the composition of the immunopeptidome is subject to, among other factors, plastic remodelling based on the cellular metabolic activity[76]. In addition, epigenetic changes might also affect the immunopeptidome. For instance, treatment of human tumour cells with the HDAC inhibitor trichostatin A has been shown to result in a threefold decrease in the levels of high-affinity TERT peptide–MHC I complexes displayed at the surface, and reduced cell killing by $CD8^+$ T cells[21].

Second, is the available T-cell repertoire skewed in response to TERT vaccination? T cells with the highest affinity for self-peptides are removed owing to the thymic immune-tolerance mechanisms. High-affinity anti-TERT T cells might preferentially interact with TERT peptides with high affinity for the MHC molecule (that is, as more-stable binders), resulting in their depletion from the T-cell repertoire, such that they can no longer

Table 2 | **Correlation between immune response to TERT vaccination and overall survival**

Study	Phase	Cancer type	Median OS		*P* value	Refs
			Responders	Nonresponders		
Bernhardt *et al.* (2006)	Phase I/II	Pancreatic cancer	216 days	88 days	$P=0.0001$	65
Bolonaki *et al.* (2007)	Phase I/II	Non-small-cell lung cancer	30 months	4.1 months	$P=0.012$	66
Kyte *et al.* (2011)	Phase I/II	Melanoma	396 days	250 days	NS ($P>0.05$)	62
CTN-2000: Brunsvig *et al.* (2006/2011)	Phase I/II	Non-small-cell lung cancer	19 months	3.5 months	$P<0.001$	60,61
Fenoglio *et al.* (2013)*	Phase I/II	Prostate or renal cancers	Not reached (>600 days)	~100 days	$P=0.0002$	83
Kotsakis *et al.* (2012)	Phase II	Multiple advanced-stage solid tumours	20 months	10.5 months	$P=0.041$	71

OS, overall survival; NS, not significant; Refs, references. *In this study, investigators compared 'full immunological responders' with 'weak immunological responders'; median OS durations are estimates based on the Kaplan–Meier curves.

contribute to the vaccine-induced T-cell responses. Evidence indicates that pruning of self-antigen-specific T-cell lineages, rather than outright deletion, occurs in humans[77], although no information exists on the size of the preimmune, high-affinity T-cell repertoire in cancer-free individuals. Nevertheless, a way around this obstacle would be to select low-affinity peptides, artificially increase their MHC-binding affinity, and then empirically identify the peptides with improved immunogenicity[24]. One such TERT peptide, pY572, is an analogue of a peptide with low affinity for HLA-A*02 (p572); pY572 induces TERT-specific CTL in humans, shares a crossreactive T-cell repertoire with the parental peptide, results in the lysis of tumour cells *in vitro*[24], and has been included in two vaccine formulations[66,74].

Third, were previous vaccination approaches optimal to maximize the induction of T-cell responses? In many published studies, TERT peptides were used straightforwardly, without consideration of $CD4^+$ T-cell help. The necessity to include peptides that activate both $CD4^+$ T-helper cells and $CD8^+$ CTLs in the same immunogen in order to achieve effective vaccination was first shown with a lipopeptide vaccine against the hepatitis B virus[78]. The induction of $CD8^+$ T cells in a 'helpless mode' has subsequently been found to yield $CD8^+$ T-cell responses that are poorly maintained, with low numbers of precursor T-cell that expand poorly after antigen restimulation[79,80]. Likewise, the induction of antitumour $CD4^+$ T-cell responses using a self-peptide alone, as used in many of the TERT-vaccine trials reported to date, would inevitably be ineffective. As recently reviewed elsewhere[29], cooperation between two $CD4^+$ T cells, which is based on associative recognition of antigen (the 'help for helpers' paradigm), is a basic immunological principle that should be an essential consideration in the design of therapeutic anticancer vaccines.

Fourth, was vaccination performed with nonpersisting vaccine in adjuvant formulations? Vaccination using peptides in adjuvants (incomplete Freund's adjuvant or Montanide® ISA adjuvants) that create antigen depots can result in sequestration of tumour-specific T cells at the injection site, thus diminishing tumour-infiltration and favouring apoptosis of the T cells, as shown in preclinical models[81]. Specifically Montanide® ISA adjuvants were used in six TERT-vaccine trials[19,66,71,75,82,83], with variable results with respect to the immune responses observed post-vaccination.

Fifth, is the class of responding T cells optimal for tumour-cell targeting? Both $CD8^+$ and $CD4^+$ T cells can induce antitumour responses. The effectiveness of the antitumour immune response might not only be dependent on the number of T cells activated; the quality of T cells induced by therapeutic vaccination is also relevant. Studies in mice have shown that while T cells at different stages of differentiation can mediate antitumour activity, central memory (T_{CM})[84] and memory T cells with stem-cell-like properties (T_{SCM})[85], confer superior protection, at least with respect to $CD8^+$ T cells. Few of the TERT-vaccine trials performed to date have used vaccines optimized to generate these classes of T cells, for example, through low-dose immunization in the context of associative recognition of antigen (that is, $CD4^+$ T-cell help to $CD8^+$ or $CD4^+$ T cells).

Sixth, are TERT-specific T cells induced by therapeutic vaccination 'anergized' systemically or locally in the tumour microenvironment? Even if all the prerequisites for optimal induction of protective antitumour T-cell responses are satisfied, a vaccine might not necessarily induce TERT-specific T cells if they are rendered anergic, or nonfunctional, *in vivo*. Early studies of anticancer vaccines revealed that circulating melanoma-antigen-specific T cells induced by peptide vaccination are quiescent cells, with low expression of genes associated with T-cell activation, proliferation, and effector function[86]. Whether this is also the case for the TERT-specific T cells generated in vaccinees in previous TERT vaccine trials is unknown, but this aspect of the immune response should be considered in future studies.

Finally, the timing of therapeutic vaccination needs to be gauged relative to the course of the disease. Vaccination of patients with very advanced-stage disease might limit both the immune response generated and antitumour effectiveness of any response. This consideration would favour the idea that immune intervention should take place early in the course of the disease, more as a 'pre-emptive' strike than as a curative approach in patients with advanced-stage disease. Preferably, patients should be vaccinated at the disease stage at which the tumour burden is much lower and more localized, and tumour immunosuppressive mechanisms might be less established. In the advanced-stage-disease setting, one could consider the adoptive transfer of TERT-specific T cells, followed by TERT vaccination[75], with the expectation that passively administered T cells would initiate a process of tumour destruction that would promote the development and improve the effectiveness of subsequently active anti-TERT immunity upon vaccination.

The tumour microenvironment has a major role in determining the success of therapeutic vaccination, although the

functional aspects of the tumour microenvironment in the anti-TERT immune response are, by and large, unexplored. In one report, investigators demonstrated prevalent tumour-specific regulatory T (T_{reg}) cells and T_{reg}-cell-dependent inhibition of memory responses to TERT antigens *ex vivo* in patients with colon carcinoma, but no information on tumour-infiltrating T cells was provided[87]. Theoretically, however, the tumour microenvironment would not be expected to have unique characteristics (for example, accumulation of cells able to inhibit T-cell function or upregulation of negative regulators on T cells) that would affect TERT vaccination. The tumour microenvironment is often enriched in T-cells that express 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[88–90]; regulatory/suppressor $CD4^+$ and $CD8^+$ T cells (T_{reg} cells)[91,92]; myeloid cells with both proinflammatory and immunosuppressive characteristics (such as tumour-associated macrophages and myeloid-derived suppressor cells)[93,94]; and B cells with tumour-promoting regulatory functions[95,96]. For example, we are now beginning to appreciate the negative effects of the endoplasmic-reticulum-stress response in the tumour microenvironment[97]. As a result of this response, bone-marrow-derived macrophages and DCs acquire proinflammatory/suppressive characteristics, secrete arginase 1, and have a reduced capacity for antigen presentation[98]. Together, these environmental changes negatively affect the activation and expansion of naive T cells[98]. Thus, controlling the tumour microenvironment, by either targeting these cell types directly or interfering with the pathways that result in their dysregulation, at the time of vaccination is likely to be not only important, but also necessary. The role of the tumour microenvironment in restraining antitumour immunity is exemplified by the results with single-agent[99–106] or dual-agent therapy[107,108] with immune-checkpoint inhibitors. The clinical responses observed with these treatments indicate that releasing the break on naturally acquired immune responses to tumour antigens is enough to result in deep and durable tumour responses in some patients, demonstrating the capacity for tumour antigens, which could theoretically include TERT, to evoke effective immunity.

Immunosuppressive cell types in the tumour microenvironment can be manipulated using various strategies. Immune-checkpoint inhibitors can restore the activity of exhausted T cells and regulate T_{reg}-cell activity — for example, via CTLA-4, glucocorticoid-induced TNF-receptor family related protein (GITR), or OX40 (REFS 109,110). Vaccination-induced T_{reg} cells might counter-suppress the anticancer T-cell response generated[111,112], but can potentially be opposed in a variety of ways: administration of a single low-dose of cyclophosphamide before anticancer vaccination with non-TERT vaccines; and treatment with thalidomide can reduce the frequency of T_{reg} cells[113]. Myeloid cells with proinflammatory immunosuppressive characteristics might be controlled with nitro-aspirin; doxorubicin; all-*trans* retinoic acid (ATRA); inhibitors of cyclooxygenase 2 (COX-2), arginase 1, or phosphodiesterase type 5 (PDE5); or selective inhibitors of the endoplasmic-reticulum-stress response[97,114,115]. Finally, tumour-promoting B cells within the tumour microenvironment could be targeted with anti-CD20 antibodies (such as rituximab) or B-cell-kinase inhibitors, such as the Bruton tyrosine kinase inhibitor ibrutinib. Combining such therapies with TERT-based vaccination might improve therapeutic responses and, thus, patient outcomes, and this approach will be interesting to explore in future studies (FIG. 1).

These considerations are not unique to TERT vaccination, and apply to vaccines against other tumour antigens expressed in a high proportion of human cancers, such as MUC1, mutated p53, mutated KRAS, and NY-ESO1 (also known as cancer/testis antigen 1), which are observed in >60%, 5–48%, 9–30%, and 20–40% of all tumours, respectively[116–119]. Some of these antigens have undergone intense clinical experimentation with regard to vaccine development in the past 10–15 years. For these antigens, a reassessment of vaccine and trial design along the lines discussed in this Perspectives would be timely, in order to capitalize on knowledge and expertise accrued to date.

Taken together, these considerations indicate that, if cancer immunotherapy targeting TERT is justified, optimization of immunogenicity and the quality of T-cell responses (BOX 1) should be the focus of new research efforts. In addition, the importance of assessing and quantifying the existence of T-cells precursors specific for TERT before immunotherapy should be a key consideration. A new generation of *TERT*-DNA vaccines delivered via electroporation has already been tested successfully in nonhuman primates, and induced a broad spectrum of anti-TERT responses[120]. Nevertheless, renewed interest in other forms of TERT immunotherapy is now warranted on the basis of two important developments: firstly, TERT upregulation has been associated with every stage of tumour evolution; secondly, whole-genome sequencing (WGS) of cancers has revealed mutations and rearrangements that affect *TERT* transcription. The former finding makes TERT an ideal target antigen on tumour cells based not only on expression level, percentage of positive cells, and number of patients with antigen-positive cancers, but also owing to antigen expression on cancer stem cells (CSCs)[121]. The latter observations might permit selection of patients for TERT-based therapy on the basis of genomic abnormalities that increase TERT expression. In addition, the developments in our understanding and therapeutic targeting of tumour immunosuppressive mechanisms made over the past 5 years could potentially be leveraged to exploit the associations of TERT with cancer and thus advance TERT-based immunotherapy.

Recentring TERT in tumour evolution

To date, TERT immunotherapy has been based on the early discovery that bulk tumours are generally telomerase positive. Importantly, more-recent findings indicate that TERT is expressed at every stage of the cancer process, from the incipient CSCs and/or tumour-initiating cells to advanced metastatic cancer cells, and has essential roles at each stage of tumorigenesis.

TERT is expressed in CSCs. Studies in mice and humans point to the fact that telomerase and TERT are important in enabling the self-renewal of stem and progenitor cells. Mice deficient in the telomerase RNA component (TERC) demonstrate defective stem-cell function, which can be rescued by reintroducing functional telomerase and by abrogating p53 activity[122]. Furthermore, in a subset of patients with dyskeratosis congenita, mutations in telomerase (TERT or TERC) that inactivate its enzymatic activity lead to bone-marrow failure[123]. These observations suggest that telomerase expression underlies the self-renewal capacity of stem cells.

Abundant evidence indicates that telomerase is associated with cancer, but which cells account for telomerase

positivity in a bulk tumour mass is not entirely clear (a tumour with a volume of 1 cm^3 might comprise a mixture of 10^9 heterogeneous cells), and whether these TERT-positive cells derive from a population of cells with tumour-initiating properties is unknown. CSCs are hypothesized to possess the exclusive ability to self-renew and propagate the tumour[124]. Paradoxically, CSCs have been shown to have short telomeres, compared with those in bulk tumour cells[125]. This finding suggests that altered telomerase levels in CSCs that formed early in tumour evolution maintain telomeres at low levels sufficient to oppose quiescence and/or senescence, or the DNA-damage response that can result in apoptosis. Of note, *TERT* overexpression has been demonstrated in $CD133^+$ CSCs derived directly from $CD133^+$ primary astrocytic glioblastoma[126]; however, these cells were found to give rise to $CD133^-$ progenitor cells with low telomerase levels, which were nontumorigenic and had progressive telomere shortening during repeated rounds of cell division. TERT overexpression has also been identified in androgen-receptor-negative and androgen-insensitive $CD44^+$ prostate cancer cells derived from surgical specimens (early localized tumours)[127,128]; the authors hypothesized that these cells represent tumour-initiating cells that underlie tumour growth and resistance

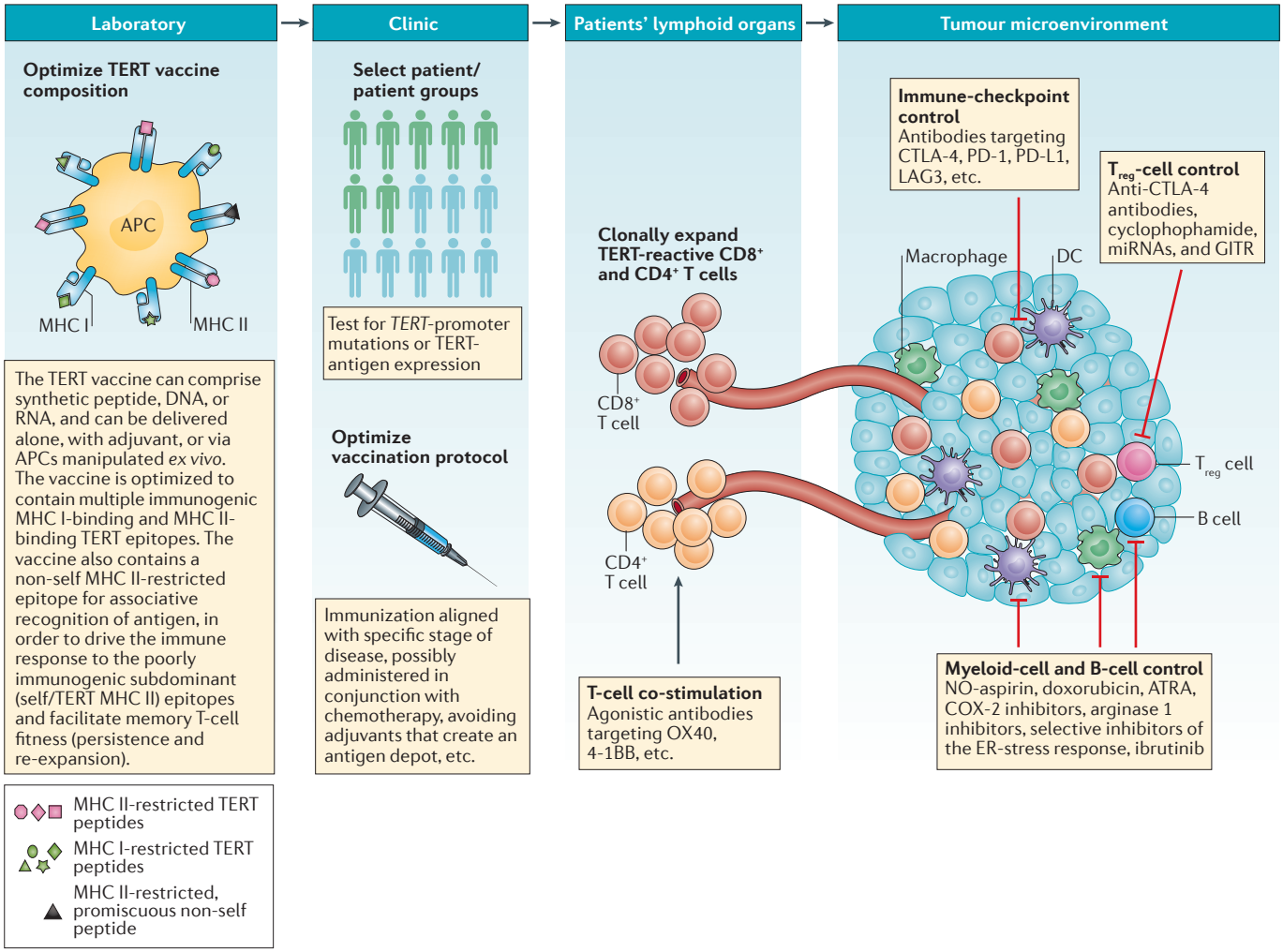

Figure 1 | **Four steps in TERT-based therapeutic vaccination.** In the laboratory: vaccine formulation and optimization based on established immunological principles to maximize the expansion of $CD8^+$ and $CD4^+$ T cells from the pool of *TERT*-specific precursors in the available T-cell repertoire, leveraging vaccine design and delivery features — collectively, the vaccine platform. In the clinic: patient selection for vaccination on the basis of genetic alterations in the *TERT*-promoter region, and further prioritized based on tumour tissue of origin and the related rate of stem-cell division, telomerase-expressing circulating tumour cells, and the stage of disease. Ideally, TERT vaccination should be used as a 'pre-emptive strike', rather than a curative approach. In the patients: the aim of immunization is to induce a wide spectrum anti-TERT T-cell response, involving both $CD8^+$ and $CD4^+$ T cells, in secondary lymphoid organs. Consideration should be given to the generation of long-term memory responses, preferentially of the central memory or stem-like memory type, by modulating antigen dose or administering an agonistic antibody to OX40 or 4-1BB, co-stimulatory molecules expressed by T cells. In the tumour microenvironment: the best objective clinical responses are expected when TERT immunization is associated with control of the tumour microenvironment. Thus, efforts should be made to modify the immunosuppressive tumour microenvironment and its pleiotropic effects, which are known to derail autochthonous and vaccine-induced antitumour T-cell responses. Several approaches can be considered, used concomitantly or sequentially with the vaccine. APC, antigen-presenting cell; ATRA, all-*trans* retinoic acid; COX-2, cyclooxygenase 2; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; ER, endoplasmic reticulum; GITR, glucocorticoid-induced TNFR-family-related gene; LAG3, lymphocyte-activation gene 3; MHC, major histocompatibility complex; miRNA, microRNA; NO-aspirin, nitro-aspirin; PD-1, programmed cell-death protein 1; PD-L1, programmed cell death 1 ligand 1; TERT, telomerase reverse transcriptase; T_{reg}, regulatory T.

to androgen-deprivation therapy[127,128]. Interestingly, probing of induced pluripotent stem cells (iPS) generated from human prostate cancer cells with TCR-mimic antibodies demonstrates that these cells process and present the dominant p540 or p865 TERT peptides (M. Zanetti, unpublished data). The implications are profound. The demonstration that prostate-cancer-derived iPS cells process and present TERT peptides implies that CSCs, similarly to differentiated cancer cells, are susceptible to immune attack. This finding is important, as CSCs and progenitor cells are widely believed to be resistant to conventional therapies. Thus, the development of immunotherapies that effectively eradicate these cell types, through targeting of TERT, would represent a major step towards preventing cancer relapse.

TERT in CTCs. Circulating tumour cells (CTCs) can be detected in the blood of patients with various cancers, and are thought to be shed from the primary tumour into the general circulation via the lymphatics, or through ruptures in the walls of capillaries and small blood vessels. Increasing attention is being placed on the use of CTCs for diagnostic and prognostic purposes, and these cells harbour information for genomic interrogation. Telomerase expression has been demonstrated in CTCs in the blood of patients with prostate, ovarian, breast, and metastatic bladder cancer, or non-small-cell lung cancer[129–132]. No report exists that demonstrates TERT-antigen presentation by CTCs, although one could reasonably assume that CTCs process and present TERT peptides, and would, consequently, be recognized by $CD8^+$ T cells once they seed a distal tissue. If this assumption proves to be correct, TERT vaccination might promote removal of CTCs, thus preventing colonization of other tissues — in turn, reducing the likelihood of relapse and improving survival. Moreover, discriminating CTCs on the basis of high and low *TERT* expression or TERT-protein levels might be important in efforts to stratify patients with cancer for immunotherapy (FIG. 1). Hence, whether TERT is expressed in CTCs should be a key question for further studies.

TERT is required for epithelial-to-mesenchymal transition. Epithelial-to-mesenchymal transition (EMT), a process whereby differentiated neoplastic cells undergo transcriptional phenotypic inversion, occurs in premetastatic cancer cells, and is also associated with the acquisition of stem-cell-like characteristics[133]. Cells that have undergone EMT display not only enhanced motility, but also a CSC phenotype (self-renewal) that manifests through a process of cell dedifferentiation[134]. Thus, a plausible prediction is that factors that promote EMT also promote the transcriptional activation of TERT, and vice versa. Indeed, findings indicate that TERT overexpression promotes, and its inhibition suppresses, EMT[135]; EMT mediated by transforming growth factor-β1 and β-catenin is abolished by small interfering RNA silencing of *TERT* expression and, therefore, EMT seems to require TERT activation. Clearly, this requirement renders TERT an important target in efforts to control EMT immunologically.

Box 1 | **Issues relating to the basic and applied science of TERT vaccination**

- Several hurdles must be overcome to successfully induce TERT-specific $CD8^+$ and $CD4^+$ T cells through vaccination, including self-tolerance, the limited size of the precursor-T-cell repertoire, and the immunosuppressive tumour microenvironment (involving, for example, regulatory T cells and myeloid-derived suppressor cells)
- Induction of $CD8^+$ T cell using MHC class I (MHC I) TERT peptides might not yield immunogenicity and immune protection in the absence of T-cell help via linked recognition of MHC I-binding and MHC II-binding peptides on the antigen-presenting cell at the time of T-cell priming: cooperation between $CD8^+$ and $CD4^+$ T cells enables the expansion of memory $CD8^+$ T cells that persist and have maximal capacity to undergo clonal recall expansion upon antigen re-encounter
- The efficacy of TERT vaccination could potentially be enhanced by using analogue ('mutant') peptides. For example, wild-type peptides with low affinity for MHC class I molecules could be modified to introduce amino acid substitutions that increase the MHC I-binding affinity. Theoretically, the repertoire of T-cell precursors that can recognize these modified 'low-affinity' MHC I-binding peptides is greater than that comprising precursors that recognize high-affinity peptides because the latter cells are generally removed during ontogeny, owing to immune-tolerance mechanisms in the thymus
- Cooperation between two $CD4^+$ T cells is essential to generate a $CD4^+$ T-cell response to a self-antigen. Thus, MHC II-binding TERT peptides should be used in association with another MHC II-binding peptide derived from a nonself-antigen. The immune response to the latter would not be subject to self-tolerance mechanisms, and would readily activate a TERT-independent $CD4^+$ T-cell response that could drive the $CD4^+$ T-cell response to the TERT self-antigen
- Both the quantitative and qualitative aspects of the T-cell response should be considered in the approach to TERT vaccination. For instance, protective antitumour T-cell responses are now appreciated to be mediated preferentially by memory T cells with stem-cell characteristics (central memory and stem-like memory T cells)
- TERT vaccination should be focused at patients with early stage disease, wherein the immune system might remain relatively intact and able to respond to immunization, thus avoiding the negative effects of immunosuppressive tumour microenvironments on T cells

Genetic aberration of *TERT*

The transcriptional regulation of *TERT* expression is complex, involving multiple factors and a broad range of mechanisms, and has been extensively reviewed elsewhere[136]. The human *TERT* promoter lacks both TATA and CAAT boxes that are involved in the regulation of many genes, but is highly GC-rich. The promoter is inactive in normal and 'pre-immortal' cells, but is activated by derepression in 'immortal' cells, such as stem and progenitor cells, and a subset of tumour cells. The *TERT* promoter contains binding sites for transcription factors putatively involved in its regulation, including the oncoproteins MYC, Sp1, the human papillomavirus 16E6 protein, and steroid-hormone receptors (oestrogen and androgen receptors). On the other hand, numerous negative regulators of *TERT* transcription have been identified, including p53, E2F1/4/5, hypomethylated retinoblastoma-associated protein, and Wilms tumour protein[136].

In the past 3 years, WGS studies have revealed a disease-segregating, highly-penetrant, causal germ-line mutation in the noncoding promoter region of *TERT* in a family with hereditary melanoma, as well as somatic *TERT*-promoter mutations in >70% of melanoma cell lines and tissues from patients with metastatic melanoma[137–139]. These unexpected findings led to the speculation that *TERT*-promoter mutations might be responsible for increased *TERT* expression and telomerase activity in many cancers. Indeed, the *TERT*-promoter mutations generate a sequence with affinity for ETS/TCF family transcription factors[140], with data indicating that the mutated regions are

bound predominantly by the transcription factor GA-binding protein (GABP)[141]. Prevalent *TERT*-promoter mutations have been reported in other cancer types, including glioblastoma (~80%), bladder cancer (>60%), hepatocellular carcinoma (~50%), grade II–III gliomas (10–44%), and thyroid carcinoma (11–21%)[142–147]. In fact, the results of WGS analyses have established that *TERT*-promoter mutations are the most-prevalent mutations in noncoding regions of cancer genomes[148,149]. The effects of promoter mutations on *TERT* transcription have been investigated using *TERT*–luciferase-gene reporter constructs, revealing that the mutations result in a twofold-to-fivefold increase in *TERT* transcriptional activity[138,145]. Similarly, *TERT* transcription is increased in hepatocellular carcinoma cells harbouring promoter mutations at this locus, compared with those from the normal liver, or cirrhotic lesions[144]. Notably, this study also revealed that *TERT*-promoter mutations are among the earliest genetic alterations associated with neoplastic transformation[144], highlighting the potential importance of such mutations in tumorigenesis. This finding also indicates that *TERT*-promoter aberrations might be clonal mutations — that is, mutations that are common to almost all cancer cells within a tumour. Of note, earlier in 2016, neoantigens arising from clonal mutations were shown to be the key targets of immunotherapy[150]; thus, whether clonal expression of TERT antigens can be exploited for immunotherapy should be established in future studies.

In patients with cancer, the most-frequent, mutually exclusive *TERT*-promoter mutations are −124C>T and −146C>T, but other promoter sites can harbour somatic mutations (CC>TT tandem mutations at −124/−125 and −135/−139 positions). The exact mechanism(s) by which *TERT*-promoter mutations are generated is not known; however, these novel findings underscore the possible role of these mutations in adaptive mechanisms that drive tumorigenesis. In fact, in the setting of telomerase-deficiency and telomere dysfunction, conditional re-expression of the *Tert* gene exacerbates tumour growth and metastasis in a mouse model of *Tp53*$^{-/-}$/*Pten*$^{-/-}$ prostate cancer[151], and facilitates the progression of T-cell lymphoma that arises spontaneously in *Atm*$^{-/-}$ mice[152]. Interestingly, ALT was noted in the context of telomerase suppression in the *Atm*$^{-/-}$ mouse model[152], and this finding might highlight a potential resistance mechanism to TERT-based immunotherapy. Of relevance, in humans the *TERT* promoter is a common integration site for several viruses, including hepatitis B virus[153,154], hepatitis C virus[155], and human papillomavirus[156] — which can lead to activation of the *TERT* gene in *cis*.

A causal nexus between *TERT*-promoter mutations and tumorigenesis has been investigated. In human bladder cancer cell lines the presence of promoter mutations was found to correlate with higher levels of *TERT* mRNA[157]; in turn, patients with *TERT*-promoter mutations had worse disease-specific survival than those without such mutations in two independent cohorts of patients with bladder cancer[157]. The results of additional studies showed that patients with *TERT*-promoter mutations have a more-aggressive disease course and shorter survival, compared with those without these mutations[142,146,158]. The mechanism by which *TERT*-promoter mutations promote tumorigenesis has been further clarified by demonstrating that they prevent silencing of the *TERT* gene, thus increasing its transcriptional levels and suppressing telomere shortening *in vivo*[159].

In addition to *TERT*-promoter mutations, recurrent genomic rearrangements in a chromosomal region proximal to *TERT* have been reported[160]. These rearrangements are present in some patients with high-risk neuroblastoma, induce strong transcriptional upregulation of TERT, and correlate with a poor clinical outcome[160]. Moreover, associations between *TERT*-promoter mutations and genetic polymorphisms at the *TERT* promoter have also been reported. For example, some patients with glioblastoma who are homozygous for the rs2853669 C-allele, which is situated in a putative ETS2 binding site in the *TERT* promoter close to the C228T and C250T mutation hotspots, also have tumours with *TERT*-promoter mutations[161]. These patients had markedly shorter overall survival durations than those with the wild-type allele (11 months versus 20 months, $P = 0.002$, and 12 months versus 20 months, $P = 0.04$, for the C228T and C250T mutations, respectively)[161]. This relationship, therefore, warrants further investigation.

Collectively, genomic investigations indicate that in some patients with cancer, *TERT*-promoter mutations yield greater *TERT* expression and possibly higher levels of TERT protein. From an immunological viewpoint, these mutations are relevant to the composition of the 'HLA ligandome' — that is, the number and range of MHC–peptide complexes presented at the cell surface. Any correlation that exists between mRNA transcription and the HLA ligandome in cancer tissues might only be weak[162,163], and a single human cell can display ~120,000 MHC I molecules on its surface[22]; nevertheless, a target cell need only express 1–3 specific MHC–peptide complexes to be functionally recognized by the corresponding CD8$^+$ T cells[164]. High-resolution mass-spectroscopy-based approaches will be needed to address this issue, in the context of cells with *TERT*-promoter mutations.

TERT immunotherapy in rebound

Upregulation of TERT in cancer cells at all stages of differentiation is well established. Cells that accumulate initial cancer-promoting mutations are 'immortalized' by the activation of telomerase, and tumour-initiating cells and progenitor cells require telomerase for self-renewal[126–128]. Telomerase reactivation is not only necessary for tumour-cell resistance to apoptosis, but also for the initiation of local invasion and cancer progression. Mobilization and extravasation require TERT, and its overexpression promotes EMT[134,165]. Thus, TERT is a potential immunological target throughout the evolution and progression of cancer. TERT overexpression promotes the canonical functions of telomerase — telomere elongation and prevention of the DNA-damage response — that are critical for the survival and proliferation of cancer cells. Via its noncanonical functions, for example, the activation of β-catenin, TERT also confers resistance to antigrowth signals, and can affect all nine recognized hallmarks of cancer[165]. Thus, I feel that we should continue in our efforts to pursue TERT as a target for anticancer therapy, exploiting our improved understanding of both TERT and cancer immunology.

The discovery that mutations in the *TERT* promoter are frequently associated with certain types of cancer, and are, overall, the most-prevalent mutations in noncoding regions of cancer genomes, only strengthens the idea that TERT is a critically important therapeutic target for anticancer therapy. *TERT*-promoter mutations prevent silencing of the *TERT* gene, increasing its transcription; although TERT-protein expression might not be directly proportional to the level of heightened transcription[157], even a twofold increase in TERT levels would generate more peptides, potentially making tumour

cells more susceptible to T-cell killing. Experiments will need to interrogate, on a quantitative basis, TERT-peptide processing and presentation in the context of *TERT*-promoter mutations.

Subsequently, a reasonable proposal would be to use WGS or promoter-targeted sequencing to guide the selection of patients on the basis of *TERT*-promoter mutations, on the assumption that the tumour cells present in these patients have a constitutively higher content of the target antigen and more-aggressive growth characteristics. A way to test this hypothesis would be to vaccinate patients with bladder cancer or hepatocellular carcinoma (where the frequency of *TERT*-promoter mutations is on average one in two), and see if, at comparable levels of induction of anti-TERT T-cell responses, patients with *TERT*-promoter mutations have a more-favourable clinical outcome, or at least a greater degree of improvement (as such patients might already have a poorer prognosis), than vaccinees without such mutations.

At a time of burgeoning interest in personalized immunotherapy[166,167], a practical approach based on the systematic identification of *TERT*-promoter mutations, on a cancer-type and individual-patient basis, followed by therapeutic TERT vaccination, could represent a convenient and effective alternative option (BOX 2). By taking into account the frequency of *TERT*-promoter mutations in different cancer types[142–144,149], variations in the propensity of cancer formation in different tissues (which are related to the rates of stem-cell division in the particular tissues)[168], genomic rearrangements proximal to *TERT*[160], and high numbers of telomerase-expressing cancer progenitor cells[127], one could potentially identify cancer types in which TERT immunotherapy would have the highest likelihood of clinical success. On the basis of these criteria, TERT-based immunotherapies should be tested for efficacy, on a prioritized basis, in sporadic melanoma, hepatocellular carcinoma, bladder carcinoma, glioblastoma, neuroblastoma, and prostate adenocarcinoma. Ideally, one would want to probe for TERT expression and presentation on an individual-patient basis. TERT-protein abundance in tumour samples can be assessed using commercially available antibodies, although the performance of these antibodies in biomarker assays should be validated by reference laboratories. TERT presentation by MHC molecules could be assessed with anti-MHC–TERT-peptide-complex antibodies, but this would require generating antibodies for TERT peptides bound to each main HLA serotype[169]. CTCs should be systematically probed for telomerase expression — an approach that will require the development of new technology. Furthermore, prospective studies should use massively parallel sequencing of T-cell receptor CDR3 Vβ amplicons to analyse spectratypes in vaccinees, on an individual basis[170].

The efficacy of therapeutic vaccination will also depend on controlling negative immune regulation at the time of vaccination. A combined approach with immune-checkpoint inhibitors, such as antibodies targeting CTLA-4, PD-1, or PD-L1[171–173], could result in expansion of TERT-specific T-cell precursor populations, and/or reactivation of anergic or exhausted autochthonous T-cells in the tumour microenvironment. By 'releasing the brakes and simultaneously stepping on the accelerator', this combination-therapy approach could unleash the full potential of TERT vaccination, particularly in view of the fact that chemotherapy-induced tumour regression is synergized by a pre-existing TERT-reactive T cells[174], and that tumour regression after therapeutic PD-1 blockade requires pre-existing $CD8^+$ T-cells that are negatively regulated by PD-1–PD-L1 interactions[175]. Checkpoint-inhibitor monotherapy is effective in selected cancer types and only a fraction (17–40%) of patients have objective responses[100,101,105,176], and response has been shown to depend on the tumour mutational burden (>100 mutations per tumour)[176–178]. A higher objective response rate (~60%) has been observed in patients with melanoma treated with two checkpoint inhibitors — the anti-PD-1 antibody nivolumab and the anti-CTLA-4 antibody ipilimumab — in combination, but drug-related adverse events of grade 3 or 4 also occurred at a high frequency (in ~55% of patients)[107]. In this context, determining if immune-checkpoint inhibition used in combination with TERT vaccination generates similar or better results would be of interest, not only in cancers in which non-synonymous mutations are prevalent[179], but also in cancers with low mutation rates. In patients with high levels of TERT and TERT-antigen presentation, an alternative approach might be to use adoptive T-cell approaches, such as chimeric antigen receptor (CAR) T-cell therapy that enables effective and selective targeting of antigens expressed or presented prominently on the surface of tumour cells — with or without immune-checkpoint inhibition[180,181]. Limited data is available, however, on this approach in patients with solid tumours,

Box 2 | The basis for renewed interest in TERT-based immunotherapy for cancer

- In the past decade, studies have revealed that telomerase complex and telomerase reverse transcriptase (TERT) are expressed at every stage of tumour evolution, including in tumour-initiating cells[126–128]; thus, TERT is a 'first-in-class' near-ubiquitous tumour antigen
- Whole-genome sequencing studies have resulted in the identification of mutations in the *TERT* promoter. Such mutations are associated with certain types of cancer, and are the most-prevalent mutations in noncoding regions in human cancers[148,149]. Mutations in the *TERT* promoter lead to the transcriptional upregulation of *TERT* and, consequently, high TERT-protein expression[144]; in principle, this could increase TERT-antigen presentation by cancer cells, making them more susceptible to T-cell recognition and attack. Of note, abundant evidence indicates that the antigen-processing machinery is functional in tumour cells at various stages of tumour evolution
- Consequently, future TERT-based immunotherapy can be focused on patients with *TERT*-promoter alterations, the presence of which can be assessed through quantitative real-time PCR analysis of DNA from tumour specimens. Genetic mutational analysis should be complemented with proteomic analysis of the levels and distribution of TERT protein in cancer cells
- In addition to genetic profiling, candidate patients for TERT-based immunotherapy could potentially be further selected based on criteria including a viral cancer pathogenesis (hepatitis B virus, hepatitis C virus, and human papillomavirus are known to integrate into the *TERT* promoter)[153–156], tissue of origin and the penetrance of cancer in different tissues (which relate to the number of stem-cell divisions)[168], and the detection of circulating tumour cells expressing high levels of TERT
- The effectiveness of TERT vaccination might be augmented by concomitant immune-checkpoint inhibition, which can enhance T-cell responses in patients with cancer, particularly in patients who have detectable TERT-specific T cells before immune intervention. Vice versa, TERT vaccination might increase the objective response rates to immune-checkpoint inhibitors in patients with responsive cancer types (such as melanoma and lung cancer), and expand the indications for immune-checkpoint inhibitors to cancers that, to date, have proven refractory to such agents

suitable T-cell therapies will need to be developed, and such a strategy is costly and logistically challenging.

Conclusions

Despite the limited success of past clinical trials, TERT-based immunotherapy might offer opportunities for personalized intervention if vaccine design and immunization modalities are optimized based on the immunological and more general considerations discussed herein; patients selection is prioritized on the basis of *TERT*-promoter mutations and genomic rearrangements proximal to *TERT* (molecular profiling); and perhaps in combination with immune-checkpoint inhibitors. I believe that this approach could elevate cancer immunotherapy to new standards of success, possibly beyond the limits of immune-checkpoint inhibition alone. The type of precision/personalized intervention proposed herein should be pursued as an alternative to contemporary approaches to personalized immunotherapy that are based on neoantigen peptides identified by whole-exome sequencing; although initial proof of concept in humans has already been provided for the latter approach[182,183], one can predict that it will have more limited large-scale clinical applicability, with higher costs and considerable organizational challenges.

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Competing interests statement
M.Z. is a named inventor for the Issued US patent #7388071 (Telomerase as cancer antigen), and serves as Medical Advisor to Invectys, a Paris-based biotechnology company that is developing a TERT vaccine.

FURTHER INFORMATION
The Immuno Polymorphism Database (IPD) and international Immunogenetics (IMGT)/HLA database:
https://www.ebi.ac.uk/ipd/imgt/hla/
The US NIH ClinicalTrials.gov database:
https://clinicaltrials.gov/
The WHO International Clinical Trials Registry Platform:
http://www.who.int/ictrp/en/

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