# A telomerase encoding DNA vaccine

The present invention relates to the field of anti-tumor vaccination. The invention more particularly provides a nucleic acid construct that encodes for an inactive enzymatic form of human telomerase reverse transcriptase protein.

## Background of the invention:

The stimulation of tumor-specific T-cell responses with active immunotherapy has several theoretical advantages over other forms of cancer treatment. In order to obtain clinical benefits T cell-based immunotherapy must stimulate both CD8 and CD4 tumor-reactive T cell responses which recognize tumor specific antigens. Consequently increasing attention has focused on identifying MHC class I and II epitopes from multiple tumor associated antigens (TAAs) (Cheever, et al, 2009). However, heterogeneous expression of most of the characterized tumor antigens among the different types of cancer limits the broad applicability of cancer vaccines that target such antigens. During the past few years, human telomerase reverse transcriptase (hTERT) has emerged as the first *bona fide* common tumor antigen and is actively investigated as a universal target for cancer immunotherapy. Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of the telomerase enzyme that synthesizes telomeric DNA at the chromosome ends. hTERT is overexpressed in most human tumors (>85%) and virtually all types of cancer. In addition, telomerase activation has become one of the most important tumor escape mechanisms to circumvent telomere-dependent pathways of cell death. It is well established that therapeutic strategies targeting antigens not involved in tumor growth can result in the selection of antigen-loss tumor mutants that are clinically progressive. Hence, down-regulation or loss of telomerase activity will severely impact the growth potential of the tumor cells. Moreover, telomerase is relatively specific of cancer cells as normal body cells express little or no telomerase for most of their lifespan and generally have longer telomeres than those in tumor cells. All these findings justify the clinical applications of hTERT for anticancer immunotherapy.

Broadly used in several anticancer vaccine trials, peptide vaccination is the most advanced strategy concerning hTERT antigen. However several factors could influence the optimal success of this peptide-based vaccine strategy, such as (1) the human leukocyte antigen (HLA) restriction, (2) the natural processing of peptides in tumor cells, (3) the loss of antigen

presentation on tumor cells, (4) the functionality of antigen-specific T cells, and (5) the long term persistence of the immune responses in the host after vaccination.

The memory response obtained with peptide vaccines and especially with short peptides is very low and not persistent. These suboptimal results can be explained in part by the absence of CD4 T-cell help. In addition, the half-life of MHC/peptide vaccine complex on presenting cells is only a few hours, the peptides then disappear. The dendritic cells then no longer present peptides to lymphocytes, and hence become tolerogenic. This defect in peptide presentation can be deleterious in some cases (Rosenberg et al., 2004).

**Summary of the invention:**

The inventors have now developed a DNA vaccine strategy which does not show the drawbacks of the peptide (even long peptide) vaccination, restricted to certain epitopes of hTERT. Particularly, DNA vaccination avoids expensive and complicated procedures for protein production and purification. Moreover a DNA vaccine encoding the hTERT protein makes it possible to induce both CTL and CD4 helper T-cells independently of the HLA-restriction of the patient, while being safe and inducing a better quantitative and qualitative immune response.

The invention provides a nucleic acid construct comprising a sequence that encodes a human telomerase reverse transcriptase (hTERT) protein which is devoid of telomerase catalytic activity and of a nucleolar localization signal.

In a preferred embodiment, the hTERT protein may be fused at N-terminus with a protein enhancing addressing of the hTERT protein to proteasome, such as ubiquitin.

The nucleic acid construct of the invention is useful in triggering an immune response in a subject, preferably a cellular immune response, against cells that overexpress telomerase, preferably dysplasia cells or tumor cells, as well as cells infected with an oncovirus.

It is herein described a method for preventing or treating a tumor in a patient, which method comprises administering said nucleic acid construct to a patient in need thereof.

Such treatment can be referred to as an active immunotherapy or a therapeutic vaccination, as it triggers an immune response against the tumor, especially a cytotoxic CD8 T-cell response, along with a specific CD4 T-cell response.

A broad cellular immune response is obtained because both CD4 and CD8 T-cell repertoires are stimulated by the epitopes available on hTERT. The number of CD4 and CD8 T-cells directed against many epitopes of hTERT is higher than in peptide vaccination. Production of interleukins is improved, further to the induction of CD4 T-cells, especially Th1 cytokines, allowing an optimal growth and differentiation of CD8 T-cells with the hallmark of anti-tumor cells.

In another aspect of the invention, it is provided nucleic acid constructs comprising sequences that derive from human telomerase reverse transcriptase (hTERT), wherein said sequences that derive from hTERT

i) encode all or substantially all epitopes of hTERT, in any order, and

ii) encode a protein that is devoid of telomerase catalytic activity and of a nucleolar localization signal.

Indeed the inventors evidenced that such nucleic acid constructs, herein also designated as "shuffled" telomerase constructs, also trigger a hTERT specific *in vivo* immune response, especially a cytotoxic CD8 T-cell response.

**Legends to the Figures**

**Figure 1A INVAC-1 plasmid map**

| Location (bases) | Sequence | Origin |
|---|---|---|
| 1-3478 | NTC8685-eRNA41H-HindIII-XbaI vector | NTC |
| 3479 - 3484 | HindIII cloning site: A.AGCTT | NTC/Invectys |
| 3485 - 6967 | Ubi-hTERT transgene | Invectys |
| 6968 - 6973 | XbaI cloning site: T.CTAGA | Invectys/NTC |
| 6974 - 7120 | NTC8685-eRNA41H-HindIII-XbaI vector | NTC |

**Vector Features**

eRNA11a RIG-I agonist: 7-532

trpA prokaryotic terminator: 535-564

Adenovirus serotype 5 VA RNAI (VA1): 568-761

Primosomal assembly site (PAS-BH) extended origin: 771-1055

pUC replication origin: 1056-2070

Sucrose selection marker (RNA-OUT): 2087-2231

SV40 enhancer: 2232-2451

CMV enhancer: 2452-2897

CMV promoter: 2898-3017

Untranslated leader (exon 1): 3018-3204

HTLV-1 R: 3089-3314

Synthetic Rabbit β-globin-based 3' intron: 3323-3429

Exon 2 (SR-protein binding sites-Kozak): 3430-3478

Ubi-hTERT transgene including HindIII-XbaI cloning sites– Invectys): 3479-6973

Eukaryotic terminator: 6980-7114

**Figure 1B Gel validation for INVAC-1**

INVAC-1 expression vector was verified by restriction mapping. The pattern corresponds to expected restriction map.

Lane 1: 1 kb Ladder

Lane 2: Undigested INVAC-1

Lane 3: INVAC-1 digested with BglII/NotI (3496, 3262, 220, 142 bp bands)

Lane 4: INVAC-1 digested with NcoI (4084, 3036 bp bands)

Lane 5: INVAC-1 digested with HindIII/XbaI (3631, 3489 bp bands)

**Figure 2A hTERT, INVAC-1 and INVAC-1 derivatives.**

Schematic alignment between wild-type hTERT and modified Ubi-hTERT proteins encoded by INVAC-1 and INVAC-1 derivatives: pUTD10Not (abbreviated as Δ10Not), pUTD10Cog (abbreviated as Δ10Cog) and pUTD23Tyn (abbreviated as Δ23).

**Sequence features:**

**VDD**: Deletion of amino acids 867-869 within the catalytic site

**DGLLLRL** (SEQ ID NO: 19): Additional deletion of amino acids 860-867; upstream VDD deletion

**FLLVTPH** (SEQ ID NO: 20): Additional deletion of amino acids 869-876; downstream VDD deletion

**IRR**: Additional deletion of amino acids 857-859; upstream DGLLLRL**VDD** (SEQ ID NO: 21): deletion

**LTH**: Additional deletion of amino acids 877-879; downstream **VDD**FLLVTPH (SEQ ID NO: 22): deletion

**Ubi**: human ubiquitin sequence (1-76 amino acids)

**V5**: C-terminal V5 tag for convenient protein detection

**Figure 2B Gel validation for INVAC-1 derivatives**

pUTD10Not, pUTD10Cog and pUTD23Tyn expression vectors (INVAC-1 derivatives) were verified by restriction mapping. The patterns correspond to expected restriction maps.

Lane M: 1 kb ladder

Lane 1: pUTD10Cog (5348, 3585 bp bands)

Lane 2: pUTD10Not (5348, 3585 bp bands)

Lane 3: pUTD23Tyn (5348, 3546 bp bands)

**Figure 3 Expression of wild-type hTERT, INVAC-1 and INVAC-1 derivatives *in vitro* into different cell lines assessed by western blotting**

Wild-type hTERT (pTRIP-CMV-hTERT), empty vector (pNTC8685-eRNA41H, INVAC-1 backbone with no foreign coding sequence), INVAC-1 and INVAC-1 derivative constructs (pUTD10Not/Δ10Not, pUTD10Cog/Δ10Cog and pUTD23Tyn/Δ23) were transfected into HEK293T cells (A, C). Wild type hTERT, pNTC8685-eRNA41H empty vector and INVAC-1 constructs were transfected into CrFK cells (B).

Protein expression was monitored for 18-96h post-transfection in HEK293T cells (A, C) and for 24-72h in CrFK cells (B).

The time of cell harvesting is indicated on the top of each lane. Fifteen µg of total protein from cell lysates were loaded per lane for membranes A, B, C (hTERT, INVAC-1) and 20 µg of total protein lysates were loaded per lane for membranes C (Δ10Not, Δ10Cog, Δ23). hTERT was detected with an anti-hTERT rabbit monoclonal antibody (hTERT, INVAC-1) or with an anti-tag V5 (Δ10Not, Δ10Cog, Δ23). β-actin protein detection was used as a loading control and detected with an anti-β-actin mouse monoclonal antibody. Detection of hTERT proteins from CrFK cells (B) and INVAC-1 derivative proteins from HEK293T cells (C) required a longer exposure time.

**Figure 4 Intracellular localization of hTERT and INVAC-1 constructs into different cell lines assessed by immunofluorescence**

Wild-type hTERT (pTRIP-CMV-hTERT), empty vector (pNTC8685-eRNA41H, INVAC-1 backbone with no foreign coding sequence) and INVAC-1 constructs were transfected into HEK293T (A) or CrFK cells (D) for 24h, and into HeLa (B) or QT6 (C) cells for 24h and 48h.

The cells were processed to immunofluorescence staining with an anti-hTERT rabbit monoclonal antibody and a goat Alexa Fluor 488® anti-rabbit secondary antibody (green). The nuclei were stained with DAPI (blue). Untreated cells were stained with DAPI only. The cells were analyzed upon fluorescence microscopy (x63).

**Figure 5 Telomerase activity of hTERT, INVAC-1 and INVAC-1 derivatives assessed by TRAP assay**

CrFK cells were transfected with wild-type hTERT (pTRIP-CMV-hTERT), INVAC-1 and INVAC-1 derivative constructs. Twenty-four hours later cells were collected, total cell proteins were extracted and telomerase (reverse transcriptase) activity was assessed by Telomeric Repeat Amplification Protocol (TRAP) assay. Absorbance measurements (OD450/690 nm) and Relative Telomerase Activity (RTA; sample/positive control ratio) of INVAC-1 (A, B) and INVAC-1 derivative constructs (C, D) compared to wild-type hTERT and untreated CrFK cells are displayed (n = 3 for 2.1 µg of total protein concentration samples), **: $p = 0.0016$, ***: $p < 0{,}0001$, unpaired t-test.

No telomerase activity was detected in CrFK cells transfected with INVAC-1 and INVAC-1 derivatives.

**Figure 6: Impact of electroporation to induce significant levels of hTERT specific CD8 T-cell secreting interferon-γ after ID administration of INVAC-1**

Seven week-old C57BL/6 female mice were immunized ID (2-8 mice per group) with 100 µg of INVAC-1 or 1X PBS. For half of the animals an electroporation was performed at each vaccination site directly after immunization. Fourteen days after vaccination, spleens of all mice were harvested. Splenocytes were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates with a pool of 2 hTERT peptides restricted to the $H2^b$ MHC (p429, p660) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of

hTERT specific CD8T cells secreting IFNγ/200,000 splenocytes. Kruskal-Wallis analysis with Dunn's multiple comparison test. *: p-value < 0.05. EP = electroporation.

**Figure 7: Evaluation of various administration routes for INVAC-1 vaccination followed by electroporation to induce hTERT specific CD8 T-cell secreting interferon-γ.**

Seven to ten week-old transgenic HLA-B7 mice were immunized *via* A) the ID or SC route (3-8 mice per group) and B) *via* the ID or IM route (4-5 mice per group) with 25µg of INVAC-1 or 1X PBS. All animals received an electroporation at each vaccination site directly after the immunization. Fourteen days after vaccination, spleens A) or peripheral blood B) of all mice were harvested. Splenocytes or PBMCs were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates with a pool of 3 hTERT specific peptides restricted to the HLA-B7 MHC (p351, p1123 and p277) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD8 T cells secreting IFNγ/200,000 splenocytes or PBMCs. Mann Whitney non parametric test, *: p-value < 0.05. A hatched line was voluntarily set at 10 hTERT specific CD8 T-cells secreting IFNγ/200,000 splenocytes as a cut-off threshold allowing the determination of responding animals.

**Figure 8: Impact of vaccine dose on hTERT specific CD8 T-cell response after a single ID immunization with INVAC-1 and electroporation**

Seven week-old C57BL/6 female mice were immunized ID A) with either 12.5, 25, 50 or 100 µg of INVAC-1 or 1X PBS (4-6 mice per group) and B) with either 100, 200, 400, 800 or 1200 µg of INVAC-1 or 1X PBS (3-5 mice per group). An electroporation was performed at each vaccination site directly after immunization. Fourteen days after vaccination, spleens of all mice were harvested. Splenocytes were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates with a pool of 2 hTERT peptides restricted to the $H2^b$ MHC (p429, p660) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD8 T-cells secreting IFNγ/200,000 splenocytes. Kruskal-Wallis analysis with Dunn's multiple comparison test. *: p-value < 0.05, **: p-value < 0.01. A hatched line was voluntarily set at 10 spots/200,000 splenocytes to allow determination of responding animals.

**Figure 9: Impact of a prime-boost vaccination regimen with INVAC-1 on hTERT specific CD8 T-cells secreting interferon-γ**

Seven to ten week-old transgenic HLA-B7 mice were immunized *via* the ID route (5 mice per group) with 25 µg of INVAC-1. All animals received an electroporation at each vaccine site directly after the immunization. Twenty one days later mice received a boost injection using the same procedure. Peripheral blood was collected before the first immunization, at day 7, 15 and 21 post-priming and at day 9, 16 and 22 post-boost.

PBMCs were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates a pool of 3 hTERT specific peptides restricted to the HLA-B7 MHC (p351, p1123 and p277) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD8 T-cells secreting IFNγ/200,000 splenocytes. Mann-Whitney non parametric test, *: p-value < 0.05. A hatched line was voluntarily set at 10 spots/200,000 splenocytes to allow determination of responding animals.

**Figure 10: Evaluation of ID vaccination (single immunization *vs.* prime-boost regimen) with INVAC-1, Δ10Not, Δ10Cog or Δ23 followed by electroporation to induce hTERT specific CD8 T-cell secreting interferon-γ.**

A) Seven week-old C57BL/6 female mice were immunized ID (4 mice per group) with 100 µg of INVAC-1, Δ10Not, Δ10Cog or Δ23 or 1X PBS. An electroporation was performed at each vaccination site directly after immunization. Half of the animals received a boost injection twenty one days after the first vaccination using the same procedure. Mouse spleens were harvested 14 days or 10 days after the last immunization respectively for the animals which received a single or a priming and boost injections. Splenocytes were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates with a pool of 2 hTERT peptides restricted to the $H2^b$ MHC (p429, p660) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD8 T-cells secreting IFNγ/200,000 splenocytes for animals which received a single injection (PRIME, black dots) or a prime and boost injections (PB, white dots). Mann Whitney non parametric test, *: p-value < 0.05. A cut-off was voluntarily set at 10 hTERT specific CD8 T cells secreting IFNγ/200,000 splenocytes (hatched line) to allow the determination of responding animals. PB= post-boost.

B) Seven to ten week-old transgenic HLA-B7 mice were immunized *via* the ID route (5 mice per group) with 100 µg of INVAC-1, Δ10Not, Δ10Cog or Δ23 or 1X PBS. All animals received an electroporation at each vaccination site directly after the immunization. Twenty one days after the first vaccination, mice received a boost injection using the same procedure. Splenocytes were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates with a pool of 3 hTERT specific peptides restricted to the HLA-B7 MHC (p351, p1123 and p277) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD8 T-cells secreting IFNγ/200,000 splenocytes or PBLs. Mann Whitney non parametric test, *: p-value < 0.05. A cut-off was voluntarily set at 10 spots/200,000 splenocytes in order to determine the frequency of responding animals (hatched line).

**Figure 11: Breadth of hTERT specific T-cell response after ID immunization(s) followed by electroporation: Comparison between INVAC-1, pNTC-hTERT and pNTC-hTERT-ΔVDD contructs**

Seven to 13 week-old transgenic HLA-B7 mice were immunized *via* the ID route (6 mice per group) with 25µg of INVAC-1, hTERTΔVDD (pNTC-hTERT-ΔVDD), hTERT (pNTC-hTERT) or empty vector NTC (pNTC8685-eRNA41H). Forty-eight animals received an electroporation at each vaccine site directly after the immunization. Half of the animals received a boost injection twenty one days after the first vaccination using the same procedure. Mice spleens were harvested 14 days or 10 days after the last immunization respectively for the animals which received a single or a priming and boost injections.

Splenocytes were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates, with a set of 269 purified peptides from hTERT (purity > 70%, GenScript) divided into 27 pool of 9-10 hTERT overlapping peptides (15mer peptides overlapping by 11 amino acids), during an over-night stimulation (19 hours). Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution.

For each mouse, the median number of spots was calculated per triplicates and per stimulation condition (medium or peptides pool). The frequency (F) of hTERT specific T-cells was then calculated after subtraction of the median number of spots in medium stimulated wells from the median number of spots in peptides pool stimulated wells. Negative values were set to 0 for subsequent analyses.

This analysis was performed for the animals which received a single (A) or a prime-boost (B) vaccination. (A and B) For each vaccination group (INVAC-1, hTERTΔVDD, hTERT, NTC), a median (n=6) of frequency (F) of telomerase specific T-cells secreting IFNγ/200,000 splenocytes was calculated per stimulation condition to obtained one value for each of 27 pools.

(C) Sum of total median of frequency (F) of telomerase specific T-cell detected for the 27 pools (269 purified peptides) after vaccination by INVAC-1, hTERTΔVDD, hTERT or NTC. Statistical analyses were performed with Prism 5 software using a non-parametric Kruskal-Wallis test with Dunn's correction. p-value < 0.05 was considered as statistically significant.

**Figure 12: Potency of INVAC-1 ID vaccination and electroporation to generate specific cytotoxic CD8 T-cells and Th1-CD4 T-cells**

A) Seven to 10 week-old transgenic HLA-B7 mice were immunized *via* the ID route (5 mice per group) with 25 µg of INVAC-1 or 1X PBS. All animals received an electroporation at each vaccine site directly after the immunization. At day 14 post injection, syngeneic splenocytes, pulsed with individual hTERT peptides restricted to the HLA-B7 MHC (either p351 or p1123) or left unpulsed were labeled with carboxyfuorescein-diacetate succinimidyl ester (CFSE) at three different concentrations: high = 1 µM (621), medium = 0.5 µM (987) and low = 0.1 µM (unpulsed). The same number of high, medium or low CFSE labeled cells was transferred IV to vaccinated mice. After 15-18 hours, the disappearance of peptide-pulsed cells was determined in the spleen by flow cytometry. The percentage of specific lysis was calculated by comparing the ratio of pulsed to unpulsed cells in vaccinated versus control mice. Data represent the percentage of specific lysis for each mouse against each individual peptide in the spleen after ID vaccination with INVAC-1. Horizontal bars show average percentage of lysis per peptide and per immunization route. Standard deviations are also plotted. (n=10 individual animals/group). Statistical analyses were performed with Prism 5 software using a non-parametric Kruskal-Wallis test with Dunn's correction. p-value < 0.05 was considered as statistically significant.

B and C) Seven to ten week-old transgenic HLA-A2/DR1 mice were immunized *via* the ID route (7-10 mice per group) with 25 µg of INVAC-1 or 1X PBS. All animals received an electroporation at each vaccine site directly after the immunization. Fourteen days after vaccination, spleens of all mice were harvested. Splenocytes were Ficoll purified and B) half of them were stimulated in triplicates in an IFN-γ ELIspot assay with a pool of 3 hTERT

specific peptides restricted to the HLA-DR1 MHC (p1029, p578 and p904) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD4 T-cells secreting IFNγ/200,000 splenocytes. Mann Whitney non parametric test, ***: p-value < 0.001.

C) The second half of splenocytes was stimulated for 24 h with a pool of 3 hTERT specific peptides restricted to the HLA-DR1 MHC (p1029, p578 and p904). Supernatants from stimulated cells were recovered and tested in a CBA assay in order to evaluate the concentration of Th1/Th2 and Th17 cytokines secreted by hTERT specific CD4 T-cells. Results are the median cytokine concentrations in pg/mL. Kruskal-Wallis analysis with Dunn's multiple comparison test. *: p-value < 0.05.

**Figure 13: Impact of a therapeutic or preventive ID vaccination with INVAC-1 followed by electroporation in a syngeneic HLA-A2/DR1 transgenic mice tumor model.**

A) Five to ten week-old transgenic HLA-A2/DR1 mice were immunized *via* the ID route (5 mice per group) with 100 µg of INVAC-1 or 1X PBS. All animals received an electroporation at each vaccine site directly after the immunization. Twenty one days after priming, mice received a boost injection following the same procedure. One month after boosting, mice were inoculated *via* the SC route with 50,000 Sarc-2 tumor cells (mouse fibrosarcoma). Median tumor volume in each vaccinated group is shown at different days after tumor cell engraftment. A hatched line was drawn at 500 $mm^3$ to allow calculation of the tumor growth delay.

B) Twenty four week-old transgenic HLA-A2/DR1 mice (10 mice per group) were inoculated *via* the SC route with 20,000 Sarc-2 tumor cells (mouse fibrosarcoma). Four days after tumor cells engraftment, animals were immunized *via* the ID route with 25 µg of INVAC-1 or an empty plasmid (NTC, INVAC-1 backbone with no antigen sequence). All animals received an electroporation at each vaccine site directly after the immunization. Twenty one and 35 days after priming, mice received boost injections using the same procedure. Median tumor volume in each vaccinated group is shown at different days after challenge. A hatched line was drawn at 500 $mm^3$ to allow calculation of the tumor growth delay.

**Figure 14: Potentialization of INVAC-1-induced cellular immune responses by GM-CSF and *in vivo* efficacy in a syngeneic HLA-A2/DR1 transgenic mouse tumor model**

A) Seven week-old C57BL/6 female mice were immunized ID (5 mice per group) with 25 µg of INVAC-1, 25 µg of INVAC-1 and 0.5 µg mGM-CSF or 1X PBS. Electroporation was performed at each vaccination site directly after INVAC-1 immunization. Fourteen days after vaccination, spleens from all mice were harvested. Splenocytes were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates with a pool of 2 hTERT peptides restricted to the $H2^b$ MHC (p429, p660) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD8 T-cells secreting IFNγ/200,000 splenocytes. Kruskal-Wallis analysis with Dunn's multiple comparison test. **: p-value < 0.01.

B) Seven to ten week-old transgenic HLA-A2/DR1 mice were immunized *via* the ID route (5 mice per group) with 100 µg of INVAC-1, 100 µg of INVAC-1 and 5 µg mGM-CSF. All animals received an electroporation at each vaccine site directly after INVAC-1 immunization. Fourteen days after vaccination, spleens from all mice were harvested. Splenocytes were Ficoll purified and stimulated in triplicates with a pool of 3 hTERT specific peptides restricted to the HLA-DR1 MHC (p1029, p578 and p904) for 24 hours. Supernatants from stimulated cells were recovered and tested in a CBA assay in order to evaluate the concentration of Th1/Th2 and Th17 cytokines secreted by hTERT specific CD4 T-cells. Results are the median cytokine concentration in pg/mL. Kruskal-Wallis analysis with Dunn's multiple comparison test. *: p-value < 0.05. **: p-value < 0.01.

C) Seven to ten week-old transgenic HLA-A2/DR1 mice (10 mice per group) were inoculated *via* the SC route with 20,000 Sarc-2 tumor cells (mouse fibrosarcoma). Four days after tumor cells engraftment, animals were immunized *via* the ID route with 25 µg INVAC-1 and 0.5 µg mGM-CSF, an empty plasmid (NTC, INVAC-1 backbone with no antigen sequence) and 0.5 µg mGM-CSF or 1X PBS and 0.5 µg mGM-CSF. All animals received an electroporation at each vaccine site directly after INVAC-1 immunization. Twenty one and 35 days after priming, mice received boost injections with the same protocol. Median tumor volume in each vaccinated group is shown at different days after tumor cells engraftment.

A hatched line was drawn at 500 $mm^3$ to allow calculation of the tumor growth delay.

**Figure 15: Impact of IL-12 to potentiate INVAC-1 induced hTERT specific CD8 T-cell responses**

Seven to ten week-old transgenic HLA-A2/DR1 mice were immunized *via* the ID route (5 mice per group) with 100 µg of INVAC-1, 100 µg of INVAC-1 and 1 ng IL-12, 1X PBS or 1X PBS and 1 ng IL-12. All animals received an electroporation at each vaccine site directly after INVAC-1 immunization. Fourteen days after vaccination, spleens of all mice were harvested. Splenocytes were Ficoll purified and stimulated in triplicates in an IFN-γ ELIspot assay with a pool of 2 hTERT specific peptides restricted to the HLA-A2 (UCP4.1 and UCP2.1) for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD8 T-cells secreting IFNγ/200,000 splenocytes. A hatched line was set at 10 spots/200,000 splenocytes to allow the determination of responding animals.

**Figure 16** shows the complete nucleotide sequence of INVAC-1 plasmid expression vector (7120 bp). Vector features are detailed in Figure 1A legend. INVAC-1-encoded hTERT fusion protein (1158 AA) starts at position 3488 (ATG coding for M amino-acid) and ends at 6961 (GAC coding for D amino-acid). INVAC-1/hTERT protein was deleted of the 47 first amino-acids (1-47 AA) which were replaced by an ubiquitin polypeptide (76 AA). The catalytic site was inactivated by a 9 bp deletion (between nucleotides 6172-6173) coding for VDD (* in the Sequence) and corresponding to AA 867-869 of wild-type human telomerase (hTERT; Accession number NM_198253). First line is the nucleotide sequence; Second line is the corresponding amino-acid sequence. Annotations (see also Figure 1A) are given either above or below sequences."□": Stop codon.

**Figure 17** shows the insert sequence encoding the D10Not human ubiquitin-telomerase fusion protein (Ubi-hTERT). hTERT was deleted of the 23 first amino acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). An additional deletion was introduced between amino acids 912-913 (* see sequence), corresponding to AA 860-869 of wild-type human telomerase (hTERT; Accession number NM_198253). This 10 amino acids deletion includes the 3 AA deletion (ΔVDD) resulting in inactivation of human TERT enzymatic activity and the deletion of additional 7 AA upstream the VDD sequence. The 14 amino acids at the C-terminal sequence of the Ubi-hTERT code for the V5 epitope tag. First line is the nucleotide sequence; Second line is the corresponding amino acid sequence. Annotations are given either above or below sequences."□": Stop codon.

**Figure 18** shows the insert sequence encoding the D10Cog human ubiquitin-telomerase fusion protein (Ubi-hTERT). hTERT was deleted of the 23 first amino acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). An additional deletion was introduced between amino acids 919-920 (* see sequence), corresponding to AA 867-876 of wild-type human telomerase (hTERT; Accession number NM_198253). This 10 amino acids deletion includes the 3 AA deletion (ΔVDD) resulting in inactivation of human TERT enzymatic activity and the deletion of additional 7 AA downstream the VDD sequence. The 14 amino acids at the C-terminal sequence of the Ubi-hTERT code for the V5 epitope tag. First line is the nucleotide sequence; Second line is the corresponding amino acid sequence. Annotations are given either above or below sequences."□": Stop codon.

**Figure 19** shows the insert sequence encoding the D23Tyn human ubiquitin-telomerase fusion protein (Ubi-hTERT). hTERT was deleted of the 23 first amino acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). An additional deletion was introduced between amino acids 909-910 (* see sequence), corresponding to AA 857-879 of wild-type human telomerase (hTERT; Accession number NM_198253). This 23 amino acids deletion includes the 3 AA deletion (ΔVDD) resulting in inactivation of human TERT enzymatic activity and the deletion of additional 10 AA upstream and downstream the VDD sequence. The 14 amino acids at the C-terminal sequence of the Ubi-hTERT code for the V5 epitope tag. First line is the nucleotide sequence; Second line is the corresponding amino acid sequence. Annotations are given either above or below sequences."□": Stop codon.

**Figure 20 INVAC-1 shuffled derivatives plasmid maps**

| Location (bases) | Sequence | Origin |
|---|---|---|
| 1 - 882 | pcDNA™3.1 (+) vector | Invitrogen commercial vector backbone used by GeneCust |
| 883 - 922 | Multiple cloning site (MCS) containing HindIII cloning site: A.AGCTT | Invitrogen |
| 923 - 4474 | Ubi-hTERT shuffled transgenes | Invectys |
| 4475 - 4517 | Multiple cloning site (MCS) containing XbaI cloning site: T.CTAGA | Invitrogen |
| 4518 - 8918 | pcDNA™3.1 (+) vector | Invitrogen commercial vector backbone used by GeneCust |

**Figure 20A pUTScram: Vector features**

| Gene | Location (bases) |
|---|---|
| CMV promoter | 232-819 |
| T7 promoter | 863-882 |
| hUbi (human ubiquitin) | 923-1150 |
| 4xGly linker | 1151-1162 |
| Scrambled hTERT (scrambled human TERT) | 1163-4414 |
| hTERT fragment 7 | 1163-1372 |
| 6xGly linker | 1373-1390 |
| hTERT fragment 2 | 1391-1591 |
| 6xGly linker | 1592-1609 |
| hTERT fragment 6 | 1610-1921 |
| 6xGly linker | 1922-1939 |
| hTERT fragment 4 | 1940-2056 |
| 6xGly linker | 2057-2074 |
| hTERT fragment 9 | 2075-2650 |
| 6xGly linker | 2651-2668 |
| hTERT fragment 3 | 2669-2788 |
| 6xGly linker | 2789-2806 |
| hTERT fragment 1 | 2807-3064 |
| 6xGly linker | 3065-3082 |
| hTERT fragment 8 | 3083-3559 |
| 6xGly linker | 3560-3577 |
| hTERT fragment 10 | 3578-4093 |
| 6xGly linker | 4094-4111 |
| hTERT fragment 5 | 4112-4414 |
| 6xGly linker | 4415-4432 |
| tag V5 | 4433-4474 |
| BGH polyadenylation sequence | 4518-4742 |
| f1 ori (f1 origin) | 4788-5216 |
| SV40 early promoter and origin | 5221-5564 |
| Neomycin gene | 5626-6420 |
| SV40 pA (SV40 early polyadenylation signal) | 6594-6724 |
| pUC origin (complementary strand) | 7107-7777 |
| Ampicillin gene (complementary strand) | 7922-8782 |

**Figure 20B pUTInv: Vector features**

| Gene | Location (bases) |
|---|---|
| CMV promoter | 232-819 |
| T7 promoter | 863-882 |
| hUbi (human ubiquitin) | 923-1150 |
| 4xGly linker | 1151-1162 |
| Inverted hTERT (inverted human TERT) | 1163-4414 |
| hTERT fragment 10 | 1163-1678 |
| 6xGly linker | 1679-1696 |
| hTERT fragment 9 | 1697-2272 |
| 6xGly linker | 2273-2290 |
| hTERT fragment 8 | 2291-2767 |
| 6xGly linker | 2768-2785 |
| hTERT fragment 7 | 2786-2995 |
| 6xGly linker | 2996-3013 |
| hTERT fragment 6 | 3014-3325 |
| 6xGly linker | 3326-3343 |
| hTERT fragment 5 | 3344-3646 |
| 6xGly linker | 3647-3664 |
| hTERT fragment 4 | 3665-3781 |
| 6xGly linker | 3782-3799 |
| hTERT fragment 3 | 3800-3919 |
| 6xGly linker | 3920-3937 |
| hTERT fragment 2 | 3938-4138 |
| 6xGly linker | 4139-4156 |
| hTERT fragment 1 | 4157-4414 |
| 6xGly linker | 4415-4432 |
| tag V5 | 4433-4474 |
| BGH polyadenylation sequence | 4518-4742 |
| f1 ori (f1 origin) | 4788-5216 |
| SV40 early promoter and origin | 5221-5564 |
| Neomycin gene | 5626-6420 |
| SV40 pA (SV40 early polyadenylation signal) | 6594-6724 |
| pUC origin (complementary strand) | 7107-7777 |
| Ampicillin gene (complementary strand) | 7922-8782 |

**Figure 21A Gel validation for pUTScram**

pUTScram expression vector was verified by restriction mapping. The pattern corresponds to expected restriction map.

Lane M: 1 kb Ladder

Lane 1: pUTScram digested with HindIII/XbaI (3576, 5342 bp bands)

**Figure 21B Gel validation for pUTInv**

pUTInv expression vector was verified by restriction mapping. The pattern corresponds to expected restriction map.

Lane M: 1 kb Ladder

Lane 1: pUTInv digested with HindIII/XbaI (3576, 5342 bp bands)

**Figure 22 hTERT, INVAC-1, pUTScram and pUTInv constructs**

Schematic alignment between wild-type hTERT and modified Ubi-hTERT proteins encoded by INVAC-1 and INVAC-1 shuffled derivatives: pUTScram (Scrambled) and pUTInv (Inverted).

Modified hTERT sequence (ΔVDD) was divided into ten immunogenic fragments: **fragment 1** (258 bp; Leu24 – Gly109), **fragment 2** (201 bp; Phe115 – Ala181), **fragment 3** (120 bp; Trp203 – Ala242), **fragment 4** (117 bp; Ser255 – Arg293), **fragment 5** (303 bp; Pro320 – Thr420), **fragment 6** (312 bp; Ala423 – Val526), **fragment 7** (210 bp; Cys528 – Gln597), **fragment 8** (477 bp; Arg599 – Lys757), **fragment 9** (576 bp; Lys760 – Ile951), **fragment 10** (516 bp; Asn958 – Asp1129).

**Sequence features:**

**VDD:** Deletion of amino acids 867-869 within the catalytic site

**Ubi:** human ubiquitin sequence (1-76 amino acids)

**F (Phe):** Phenylalanine residue of hTERT (AA47)

**G (Gly):** C-terminal glycine residue of ubiquitin (AA76)

**R (Arg):** Arginine, first amino acid of INVAC-1 protein (AA 77)

**N (Asn):** Asparagine, first amino acid of artificial hTERT protein (Scrambled) encoded by pUTScram (AA 81)

**C (Cys):** Cysteine, first amino acid of artificial hTERT protein (Inverted) encoded by pUTInv (AA 81)

**V5:** C-terminal V5 tag for convenient protein detection

**Figure 23 *In vitro* expression of wild-type hTERT, INVAC-1 and INVAC-1 shuffled derivatives assessed by western blotting**

Wild type hTERT, INVAC-1, pUTScram and pUTInv were transfected into HEK293T cells. Protein expression was monitored for 18-96 h post-transfection. (A et C) Wild-type hTERT and INVAC-1 samples for 18 h and 72 h were loaded at 15 µg of total protein concentration. These samples were used as positive controls of protein expressions. (A) Scrambled and (C) Inverted proteins were loaded at 20 µg of total protein from the cell lysates per lane. hTERT was detected with an anti-hTERT rabbit monoclonal antibody (hTERT, INVAC-1) or with an anti-tag V5 mouse monoclonal antibody (Srambled, Inverted). Time of cells harvesting is indicated on the top of each lane. β-actin protein was used as a loading control and was detected with anti-β-actin mouse monoclonal antibody. Detection of INVAC-1 shuffled derivative products required a longer exposure time than wild-type hTERT and INVAC-1 proteins (10 sec to 30 min against less than 1 sec).

Shuffled protein signal intensities were normalized to β-actin signal on western blot (A and C) using ImageJ software. (B) Scrambled. (D) Inverted. Profile plots of loading control and protein bands were generated for each lane in order to obtain arbitrary numbers corresponding to the area under the curve profil. A ratio (relative density) is calculated by dividing the area value for each sample by the area value for the corresponding loading-control.

**Figure 24 Telomerase activities of hTERT, INVAC-1 and INVAC-1 shuffled derivatives assessed by TRAP assay**

CrFK cells were transfected with wild-type hTERT (pTRIP-CMV-hTERT), pUTScram and pUTInv constructs. Twenty-four hours later cells were collected, total cell proteins were extracted and telomerase (reverse transcriptase) activity was assessed by Telomeric Repeat Amplification Protocol (TRAP) assay. Absorbance measurements (OD450/690 nm) and Relative Telomerase Activity (RTA; sample/positive control ratio) of shuffled constructs (A and B respectively) compared to wild-type hTERT and untreated CrFK cells are displayed (n = 3 for 2.1 µg of total protein concentration samples), unpaired t-test was performed.

No telomerase activity was detected in CrFK cells transfected with pUTScram and pUTInv constructs.

**Figure 25: Evaluation of ID vaccination with INVAC-1, pUTScram and pUTInv followed by electroporation to induce hTERT specific CD8 T-cell secreting interferon-γ.**

Nine to fifteen week-old transgenic HLA-B7 mice were immunized *via* the ID route (3-5 mice per group) with 100 µg of INVAC-1, pUTScram, pUTInv or 1X PBS upon two immunization cycles (prime-boost regimen). An electroporation was performed at each vaccination site directly after each immunization. Mice spleens were harvested 10 days after the second immunization.

Splenocytes were Ficoll purified and stimulated in an IFN-γ ELIspot assay in triplicates with a pool of 3 specific hTERT peptides restricted to the HLA-B7 MHC (p277, p351 and p1123) or free medium for 19 hours. Spots were revealed with a biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Results are the median frequency of hTERT specific CD8 T-cells secreting IFNγ/200,000 splenocytes. Mann Whitney non parametric test was performed, *: p-value < 0.05. A cut-off was voluntarily set at 10 spots/200,000 splenocytes in order to determine the frequency of responding animals (hatched line).

**Figure 26: Potency of pUTScram and pUTInv to generate hTERT specific cytotoxic CD8 T-cells after ID vaccination and electroporation**

Fifteen week-old transgenic HLA-B7 mice were immunized *via* the ID route (4-6 mice per group) with 100 µg of INVAC-1, pUTScram, pUTInv or 1X PBS. All animals received an electroporation at each vaccine site directly after the immunization. At day 14 post injection, syngeneic splenocytes, pulsed with individual hTERT peptides restricted to the HLA-B7 MHC (either p351 or p1123) or left unpulsed were labeled with carboxyfuorescein-diacetate succinimidyl ester (CFSE) at three different concentrations: high = 5 µM (351), medium = 2 µM (1123) and low = 0.2 µM (unpulsed). A mix containing an equal number of CFSE labeled cells from each concentration was injected through the retro-orbital vein (IV) to vaccinated mice. After 15-18 hours, the disappearance of peptide-pulsed cells was determined in the spleen by flow cytometry. The percentage of specific lysis was calculated by comparing the ratio of pulsed to unpulsed cells in vaccinated versus control mice. Data represent the percentage of specific lysis for each mouse against each individual peptide in the spleen after ID vaccination. Horizontal bars show median percentage of lysis per peptide. Statistical analyses were performed with Prism 5 software using a non-parametric Kruskal-Wallis test with Dunn's correction. p-value < 0.05 was considered as statistically significant.

**Figure 27** shows the delineation of the immunogenic segments of Ubi-hTERT codon optimized sequence used for INVAC-1 shuffled derivative constructions. First line is the codon optimized nucleotide sequence of Ubi-hTERT (SEQ ID NO: 45) and second line is the corresponding amino acid sequence (SEQ ID NO: 46). Ubi-hTERT sequence was divided in ten fragments that include immunogenic sequences. These fragments are delineated by symbols (< ... >). Immunogenic sequences are highlighted in grey. Non-immunogenic inter-fragments hTERT sequences, which are not included in pUTScram and pUTInv constructs, are underlined. The 14 amino acids at the C-terminal sequence of the Ubi-hTERT code for the V5 epitope tag. Annotations are given either above or below sequences. (*) Indicates VDD sequence deletion. "□": Stop codon.

**Figure 28** shows the complete nucleotide sequence of pUTScram insert (3555 bp). Vector features are detailed in Figure 20 legend. Ubi-hTERT shuffled insert (Scrambled, 1184 AA) starts at position 923 (ATG coding for M amino acid) and ends at position 4474 (ACT coding for T amino acid) of pUTScram. hTERT protein was deleted of the 23 first amino acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). The catalytic site was inactivated by a 9 bp deletion coding for VDD (* in the sequence ) and corresponding to AA 867-869 of wild-type human telomerase (hTERT; patent WO 2007/014740 and hTERT isoform 1 Accession number NM_198253). hTERT sequence was divided into ten immunogenic fragments and reassembled in the following specific order: **fragment 7** (210 bp), **fragment 2** (201 bp), **fragment 6** (312 bp), **fragment 4** (117 bp), **fragment 9** (576 bp), **fragment 3** (120 bp), **fragment 1** (258 bp), **fragment 8** (477 bp), **fragment 10** (516 bp), **fragment 5** (303 bp). These 10 fragments are bridged with 6xGly linker (G linker; 18 bp). The 14 amino acids at the C-terminal sequence of the Ubi-hTERT shuffled insert code for the V5 epitope tag. First line is the nucleotide sequence (SEQ ID NO:47); second line is the corresponding amino acid sequence (SEQ ID NO:48). Annotations (see also Figure 20A) are given either above or below sequences."□": Stop codon.

**Figure 29** shows the complete nucleotide sequence of pUTInv insert (3555 bp). Vector features are detailed in Figure 20 legend. Ubi-hTERT shuffled insert (Inverted, 1184 AA) starts at position 923 (ATG coding for M amino acid) and ends at position 4474 (ACT coding for T amino-acid) of pUTInv. hTERT protein was deleted of the 23 first amino acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). The catalytic site was inactivated by a 9 bp deletion coding for **VDD** (* in the sequence) and corresponding to AA

867-869 of wild-type human telomerase (hTERT; patent WO 2007/014740; Accession number NM_198253). hTERT sequence was divided into ten immunogenic fragments and reassembled in the following specific order: **fragment 10** (516 bp), **fragment 9** (576 bp), **fragment 8** (477 bp), **fragment 7** (210 bp), **fragment 6** (312 bp), **fragment 5** (303 bp), **fragment 4** (117 bp), **fragment 3** (120 bp), **fragment 2** (201 bp), **fragment 1** (258 bp). These 10 fragments were bridged with 6xGly linker (G linker; 18 bp). The 14 amino acids at the C-terminal sequence of the Ubi-hTERT shuffled insert code for the V5 epitope tag. First line is the nucleotide sequence (SEQ ID NO:49); second line is the corresponding amino acid sequence (SEQ ID NO:50). Annotations (see also Figure 20B) are given either above or below sequences."□": Stop codon.

**Detailed description of the invention:**

Definitions

The telomerase complex consists of an RNA template and protein components including a reverse transcriptase, designated **"Telomerase Reverse Transcriptase" (TERT)**, which is the major determinant of telomerase activity. Unless otherwise specified, in the present specification, the term "telomerase" refers to TERT, including wild-type human telomerase, or variants thereof. Wild-type human telomerase (or hTERT) is known (GeneBank Accession number NM_198253), and has amino acid sequence SEQ ID NO: 2 (the cDNA is shown as SEQ ID NO: 1)

The **"telomerase catalytic activity"** refers to the activity of TERT as a telomerase reverse transcriptase. The term "devoid of telomerase catalytic activity" means that the nucleic acid sequence encodes a mutant TERT, which is inactive.

In the present invention, the term **"variant"** refers to allelic variants, splicing variants, natural or artificial mutants, which are homologous to the hTERT sequence of reference. Two amino acid sequences are "homologous", "substantially homologous" or "substantially similar" when one or more amino acid residue are replaced by a biologically similar residue or when greater than 80% of the amino acids are identical, or greater than about 90%, preferably greater than about 95%, are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin) pileup program, or any of the programs known in the art (BLAST, FASTA, etc.).

By "substituted" or "modified" the present invention includes those amino acids that have been altered or modified from naturally occurring amino acids.

Variants include proteins having a sequence that differs from wild-type hTERT protein by one or several mutations (i.e. substitutions, deletions, insertions), still preferably one or several single point substitutions. The variant may comprise conservative substitutions.

The term "conservative substitution" as used herein denotes the replacement of an amino acid residue by another, without altering the overall conformation and function of the peptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, shape, hydrophobic, aromatic, and the like). Amino acids with similar properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Neutral hydrophilic amino acids, which can be substituted for one another, include asparagine, glutamine, serine and threonine.

The term **"isolated polynucleotide"** is defined as a polynucleotide removed from the environment in which it naturally occurs. For example, a naturally-occurring DNA molecule present in the genome of a living bacteria or as part of a gene bank is not isolated, but the same molecule separated from the remaining part of the bacterial genome, as a result of, e.g., a cloning event (amplification), is isolated. Typically, an isolated DNA molecule is free from DNA regions (e. g., coding regions) with which it is immediately contiguous at the 5' or 3' end, in the naturally occurring genome. Such isolated polynucleotides may be part of a vector or a composition and still be defined as isolated in that such a vector or composition is not part of the natural environment of such polynucleotide.

The term **"immunogenic"** means that the composition or construct to which it refers is capable of inducing an immune response upon administration. "**Immune response**" in a subject refers to the development of an innate and adaptative immune response, including a humoral immune response, a cellular immune response, or a humoral and a cellular immune response to an antigen. A "humoral immune response" refers to one that is mediated by antibodies. A "cellular immune response" is one mediated by T-lymphocytes. It includes the production of cytokines, chemokines and similar molecules produced by activated T-cells, white blood cells, or both. Immune responses can be determined using standard immunoassays and neutralization assays for detection of the humoral immune response, which are known in the art.

In the context of the invention, the immune response preferably encompasses stimulation or proliferation of cytotoxic CD8 T-cells and/or CD4 T-cells and can be determined using

immunoassays such as the ELIspot assay, the *in vivo* cytotoxicity assay or the cytokine secretion binding assay.

As used herein, the term **"treatment"** or **"therapy" or "immunotherapy"** refers to any of the alleviation, amelioration and/or elimination, reduction and/or stabilization (e.g., failure to progress to more advanced stages) of a symptom, as well as delay in progression of the tumor or dysplasia, or of a symptom thereof. The term thus includes achievement of an efficient anti tumoral immune response observed in cancer patients.

As used herein, the term **"prevention"** or **"preventing"** refers to the alleviation, amelioration and/or elimination, reduction and/or stabilization (e.g., failure to progress to more advanced stages) of a prodrome, *i.e.* any alteration or early symptom (or set of symptoms) that might indicate the start of a disease before specific symptoms occur.

A cell that **"overexpresses telomerase"** refers to a cell in a subject, which either expresses telomerase, e.g. upon mutation or infection, especially infection by an oncovirus, whereas it does usually not, under normal conditions, or to a cell in a subject which expresses a higher level of telomerase (e.g. upon mutation or infection), when compared to normal conditions. Preferably the cell that overexpresses telomerase shows an increase of expression of at least 5%, at least 10%, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, or more.

The **"patient"** or **"subject"** is typically a mammal subject, preferably a human subject, of any age, sex, or severity of the condition.

Nucleic acid constructs

It is herein provided a nucleic acid construct that is designed to allow vaccination in patients. The nucleic acid construct encodes a telomerase that is devoid of telomerase catalytic activity (which abolishes its immortalizing activity) and devoid of a nucleolar localization signal (which prevents its transfer to the nucleolus).

The nucleic acid construct of the invention is in isolated form.

The nucleic acid may be DNA or RNA, but is preferably DNA, still preferably double stranded DNA.

The nucleic acid construct is not a naturally-occurring genomic nucleic acid, in particular it does not comprise introns.

As a first safety lock, the hTERT sequence is devoid of telomerase catalytic activity. In a preferred embodiment, the sequence that encodes hTERT contains mutations that provide inactivation of the catalytic activity of the hTERT protein. The term "mutation" includes a substitution of one or several amino acids, a deletion of one or several amino acids, and/or an

insertion of one or several amino acids. In a particular embodiment, the hTERT protein is devoid of telomerase catalytic activity by deletion of at least one amino acid.

Preferably the sequence shows a deletion, preferably a deletion of amino acids VDD, as shown in Figure 2A. Preferably the hTERT protein is devoid of telomerase catalytic activity by the sole deletion of amino acids 867-869 (VDD). In another particular embodiment, the hTERT protein is devoid of telomerase catalytic activity by a further deletion of 1 to 10, 11 or 12 amino acids upstream and/or downstream amino acids 867-869 (VDD).

As a second safety lock, the sequence encoding hTERT is further devoid of the nucleolar localization signal. This nucleolar localization signal is correlated with the subcellular localization of hTERT and thus its enzymatic activity. Preferably the hTERT protein is devoid of a nucleolar localization signal by deletion of at least amino acids 1-23, still preferably by deletion of amino acids 1-47.

In addition to the modifications that provide the first and second safety locks, the hTERT protein encoded by the nucleic acid construct of the invention may be a wild-type hTERT sequence, or a variant sequence.

In the sequence listing,

SEQ ID NO: 1 is the cDNA of the wild-type hTERT;

SEQ ID NO: 2 is the corresponding amino acid sequence;

SEQ ID NO: 3 is the cDNA of hTERT used in the INVAC-1 vector;

SEQ ID NO: 4 is the corresponding amino acid sequence;

SEQ ID NO: 5 is the cDNA of hTERT used in the pUTD10Not vector;

SEQ ID NO: 6 is the corresponding amino acid sequence;

SEQ ID NO: 7 is the cDNA of hTERT used in the pUTD10Cog vector;

SEQ ID NO: 8 is the corresponding amino acid sequence;

SEQ ID NO: 9 is the cDNA of hTERT used in the pUTD23Tyn vector;

SEQ ID NO: 10 is the corresponding amino acid sequence.

In a preferred embodiment, the invention employs a nucleic acid that encodes a protein of SEQ ID NO: 4.

Such nucleic acid may comprise sequence SEQ ID NO: 3.

In another embodiment, the nucleic acid construct encodes amino acid sequence SEQ ID NO: 6, 8 or 10, and preferably comprises SEQ ID NO: 5, 7 or 9.

In a preferred embodiment, the nucleic acid may further encode a protein which enhances the addressing of the hTERT protein to the proteasome (increasing class I presentation of the derived peptides). More particularly, the hTERT protein may be fused at the N-terminus with such protein enhancing addressing of the hTERT protein to the proteasome. Said protein may be preferably ubiquitin or it may be any chaperon protein, e.g. calreticulin.

In the sequence listing
SEQ ID NO: 11 is the full-length sequence of INVAC-1 plasmid including the cDNA of Ubi-hTERT encoded by INVAC-1;
SEQ ID NO: 12 is the corresponding amino acid sequence of Ubi-hTERT encoded by INVAC-1;

SEQ ID NO: 13 is the cDNA of the pUTD10Not insert;
SEQ ID NO: 14 is the corresponding amino acid sequence;

SEQ ID NO: 15 is the cDNA of the pUTD10Cog insert;
SEQ ID NO: 16 is the corresponding amino acid sequence;

SEQ ID NO: 17 is the cDNA of the pUTD23Tyn insert;
SEQ ID NO: 18 is the corresponding amino acid sequence.

In a particular embodiment, the nucleic acid construct encodes amino acid sequence SEQ ID NO: 12.

More particularly, the nucleic acid construct may comprise SEQ ID NO: 11, or nucleotides 3488 to 6961 of SEQ ID NO: 11.

In another embodiment, the nucleic acid construct encodes amino acid sequence SEQ ID NO: 14, 16, or 18, and preferably comprises SEQ ID NO: 13, 15, or 17.

In another embodiment, it is provided nucleic acid constructs comprising sequences that derive from human telomerase reverse transcriptase (hTERT), wherein said sequences that derive from hTERT

i) encode all or substantially all epitopes of hTERT, in any order, and

ii) encode a protein that is devoid of telomerase catalytic activity and of a nucleolar localization signal.

The nucleic acid construct of the invention is in isolated form.

The nucleic acid may be DNA or RNA, but is preferably DNA, still preferably double stranded DNA. The nucleic acid construct is not a naturally-occurring genomic nucleic acid, in particular it does not comprise introns.

These constructs are designated "shuffled constructs" or "polyepitope constructs" throughout the present description.

The term "epitope of hTERT" refers to any amino acid fragment of hTERT that is an antigenic determinant, i.e. it is recognized by cells of the immune system and is immunogenic, i.e. it can elicit an immune response. Preferably, it can be recognized, specifically by anti-hTERT T-cells. Several immunogenic epitope sequences of hTERT have been described. See e.g., international patent application WO07014740 for MHC class I restricted hTERT epitopes. Some others are described herein (see Figure 27, and Table below).

These "shuffled constructs" are capable of eliciting a specific immune response against hTERT, i.e. that cytotoxic T lymphocytes (CTLs) recognize the wild type epitopes.

None of these "shuffled constructs" coincides with the coding sequence of the full length hTERT.

The term "substantially all epitopes" means that the nucleic acid construct encodes a protein that comprises at least 80%, still preferably at least 85%, still preferably at least 90%, or at least 95% of the epitopes of wild-type hTERT.

The polynucleotide units encoding the multiple epitopes can be rearranged in any order, consecutively, i.e., the 3' end of the first polynucleotide unit is directly linked to the 5' end of the second polynucleotide unit (and so on), resulting in a polynucleotide encoding a peptidic sequence exclusively composed of consecutive epitopes. The multiple epitopes can alternatively be separated by a one-amino acid spacer or a peptide spacer, i.e., meaning that the different polynucleotide units are separated by one or several codon(s) encoding

respectively one or several amino acid(s). Typically, the immunogenic hTERT fragments can be separated by about four to six Gly amino acids.

The order in which the epitopes are rearranged can be determined by the man skilled in the art, according to the following criteria: some orders may facilitate either the transcription and/or the translation of the polynucleotide, may facilitate the transport of the resulting expressed polyepitope in the endoplasmic reticulum (ER), especially if the tridimensional conformation impacts the properties, and may facilitate the processing of the polyepitope in several epitopes or analogues and avoid the processing of overlapping epitopes.

In a preferred embodiment, all, or substantially all, immunogenic epitopes from amino acid 24 to amino acid 1132 of hTERT are encoded by the nucleic acid construct, although in any order.

The Table below shows immunogenic sequences that can be rearranged in a "Shuffle" construct:

| Immunogenic sequence | SEQ ID NO: |
|---|---|
| R R L G P Q G W R L V Q R G D P A A F R A L V A Q C L V C V P W D A R | 61 |
| V S C L K E L V A R V L Q R L | 62 |
| V L A F G F A L L | 63 |
| R S Y L P N T V T D A L R G S G A W G L L L R R V G D D V L V H L L A R C A L F V L V A P S C A Y Q V C G P P L Y | 64 |
| R E A G V P L G L | 65 |
| R R R G G S A S R S L P L P K R | 66 |
| G R T R G P S D R G F C V V S P A R P A E E A T S L E G A | 67 |
| Y A E T K H F L Y S S G D K E Q L R P S F L L S S L R P S L | 68 |
| A R R L V E T I F L G S R P | 69 |
| R R L P R L P Q R Y W Q M R P L F L E L L G N H A Q C P | 70 |
| V L L K T H C P L | 71 |
| R E K P Q G S V A | 72 |
| E E D T D P R R L V Q L L R | 73 |
| V Y G F V R A C L R R L V P P G L W G S | 74 |
| R R F L R N T K K | 75 |
| H A K L S L Q E L | 76 |
| S V R G C A W L R | 77 |

| | |
|---|---|
| EHRLREEILAKFLHWLMSVYVVELLRSF | 78 |
| ETTFQKNRL | 79 |
| KSVWSKLQSIGIRQH | 80 |
| AEVRQHREARPALLTSRLRFIPK | 81 |
| DYVVGARTFRREKRAERLTSRVKAL | 82 |
| YERARRPGLLGASVLGL | 83 |
| HRAWRTFVLRVRAQDPPPELYFVKVDVTGAYD TIPQDRLTEVIASIIKPQ | 84 |
| TYCVRRYAVVQKAAH | 85 |
| TLTDLQPYMRQFVAHL | 86 |
| SPLRDAVVIEQSSSLNEASSGLFDVFLR | 87 |
| AVRIRGKSY | 88 |
| ILSTLLCSLCYGDMENKL | 89 |
| IRRDGLLLRLFLLVTPHLTHAKTFLRTLVRGVP EYGCVVNLRKTVVNF | 90 |
| DEALGGTAFVQMPAHGLFPWCGLLLDTRTLEV QSDYSSY | 91 |
| AGRNMRRKLFGVLRLKCHSLFLDLQVNSLQT | 92 |
| IYKILLLQAYRFHACVLQLPFHQQV | 93 |
| NPTFFLRVISDTASLCYSILKAKNAGMS | 94 |
| GAKGAAGPL | 95 |
| WLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQL SRKLPGTTL | 96 |
| LEAAANPALPSDFKTIL | 97 |

Accordingly, the invention provides a polyepitopic nucleic acid construct, comprising all or substantially all of the immunogenic sequences shown as SEQ ID NO: 61 to 97, in any order.

The sequence is devoid of telomerase catalytic activity. In a preferred embodiment, the fragment that carries the hTERT catalytic activity contains mutations that provide inactivation of the catalytic activity. The term "mutation" includes a substitution of one or several amino acids, a deletion of one or several amino acids, and/or an insertion of one or several amino

acids. In a particular embodiment, the protein is devoid of telomerase catalytic activity by deletion of at least one amino acid.

Preferably the sequence shows a deletion, preferably a deletion of amino acids VDD, as shown in Figure 22. Preferably the hTERT protein is devoid of telomerase catalytic activity by the sole deletion of amino acids 867-869 (VDD). In another particular embodiment, the protein is devoid of telomerase catalytic activity by a further deletion of 1 to 10, 11 or 12 amino acids upstream and/or downstream amino acids 867-869 (VDD) of hTERT.

The sequence is further devoid of a nucleolar localization signal. This nucleolar localization signal is correlated with the subcellular localization of hTERT and thus its enzymatic activity. Preferably the protein is devoid of a nucleolar localization signal by deletion of at least amino acids 1-23, still preferably by deletion of amino acids 1-47 of hTERT.

In a preferred embodiment, the nucleic acid may further encode a protein which enhances the addressing of the protein to the proteasome (increasing class I presentation of the derived peptides). More particularly, the protein may be fused at the N-terminus with such protein enhancing addressing of the protein to the proteasome. Said protein may be preferably ubiquitin or it may be any chaperon protein, e.g. calreticulin.

ΔhTERT refers to hTERT deleted of VDD 867-869 amino acids.

A particular nucleic acid construct comprises, in any order , fragment 1 encoding Leu24 to Gly109 of ΔhTERT (SEQ ID NO:51), fragment 2 encoding Phe115 to Ala181 of ΔhTERT (SEQ ID NO:52), fragment 3 encoding Trp203 to Ala242 of ΔhTERT (SEQ ID NO:53), fragment 4 encoding Ser255 to Arg293 of ΔhTERT (SEQ ID NO:54), fragment 5 encoding Pro320 to Thr420 of ΔhTERT (SEQ ID NO:55), fragment 6 encoding Ala423 to Val526 of ΔhTERT (SEQ ID NO:56), fragment 7 encoding Cys528 to Gln597 of ΔhTERT (SEQ ID NO:57), fragment 8 encoding Arg599 to Lys757 of ΔhTERT (SEQ ID NO:58), fragment 9 encoding Lys760 to Ile951 of ΔhTERT (SEQ ID NO:59), fragment 10 encoding Asn958 to Asp1129 of ΔhTERT (SEQ ID NO:60).

A preferred construct encodes SEQ ID NO:48 (also herein called "Scrambled"), also shown on Figure 28.

Another preferred construct encodes SEQ ID NO:50 (also herein called "Inverted"), also shown on Figure 29.

Genetic constructs, immunogenic compositions and administration

Preferably, the nucleic acid is a genetic construct comprising a polynucleotide sequence as defined herein, and regulatory sequences (such as a suitable promoter(s), enhancer(s), terminator(s), etc.) allowing the expression (e.g. transcription and translation) of the protein product in the host cell or host organism.

The genetic constructs of the invention may be DNA or RNA, and are preferably double-stranded DNA. The genetic constructs of the invention may also be in a form suitable for transformation of the intended host cell or host organism, in a form suitable for integration into the genomic DNA of the intended host cell or in a form suitable for independent replication, maintenance and/or inheritance in the intended host organism. For instance, the genetic constructs of the invention may be in the form of a vector, such as for example a plasmid, cosmid, YAC, a viral vector or transposon. In particular, the vector may be an expression vector, i.e. a vector that can provide for expression in *vitro* and/or in *vivo* (e.g. in a suitable host cell, host organism and/or expression system).

In a preferred but non-limiting aspect, a genetic construct of the invention comprises i) at least one nucleic acid of the invention; operably connected to ii) one or more regulatory elements, such as a promoter and optionally a suitable terminator; and optionally also iii) one or more further elements of genetic constructs such as 3'- or 5'-UTR sequences, leader sequences, selection markers, expression markers/reporter genes, and/or elements that may facilitate or increase (the efficiency of) transformation or integration.

In a particular embodiment, the genetic construct can be prepared by digesting the nucleic acid polymer with a restriction endonuclease and cloning into a plasmid containing a promoter such as the SV40 promoter, the cytomegalovirus (CMV) promoter or the Rous sarcoma virus (RSV) promoter. In a preferred embodiment, the TERT nucleic acid sequences are inserted into a NTC8685-eRNA41H expression plasmid (see Figure 1A).
Other vectors include retroviral vectors, lentivirus vectors, adenovirus vectors, vaccinia virus vectors, pox virus vectors, measles virus vectors and adenovirus-associated vectors.

Compositions can be prepared, comprising said nucleic acid or vector. The compositions are immunogenic. They can comprise a carrier or excipients that are suitable for administration in humans or mammals (i.e. non-toxic, and, if necessary, sterile). Such excipients include liquid,

semisolid, or solid diluents that serve as pharmaceutical vehicles, isotonic agents, stabilizers, or any adjuvant. Diluents can include water, saline, dextrose, ethanol, glycerol, and the like. Isotonic agents can include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others. Stabilizers include albumin, among others. Any adjuvant known in the art may be used in the vaccine composition, including oil-based adjuvants such as Freund's Complete Adjuvant and Freund's Incomplete Adjuvant, mycolate-based adjuvants, bacterial lipopolysaccharide (LPS), peptidoglycans, proteoglycans, aluminum hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), Pluronic® polyols.

The nucleic acid or composition can be administered directly or they can be packaged in liposomes or coated onto colloidal gold particles prior to administration. Techniques for packaging DNA vaccines into liposomes are known in the art, for example from Murray, 1991. Similarly, techniques for coating naked DNA onto gold particles are taught in Yang, 1992, and techniques for expression of proteins using viral vectors are found in Adolph, 1996.

For genetic immunization, the vaccine compositions are preferably administered intradermally, subcutaneously, intramuscularly, into the tumors or in any types of lymphoid organs by injection or by gas driven particle bombardment, and are delivered in an amount effective to stimulate an immune response in the host organism. In a preferred embodiment of the present invention, administration comprises an electroporation step, also designated herein by the term "electrotransfer", in addition to the injection step (as described in Mir 2008, Sardesai and Weiner 2011).

The compositions may also be administered *ex vivo* to lymphoid or myeloid cells using liposomal transfection, particle bombardment or viral transduction (including co-cultivation techniques). The treated cells are then reintroduced back into the subject to be immunized.

While it will be understood that the amount of material needed will depend on the immunogenicity of each individual construct and cannot be predicted *a priori*, the process of determining the appropriate dosage for any given construct is straightforward. Specifically, a series of dosages of increasing size, starting at about 5 to 30 µg, or preferably 20-25 µg, up to about 500µg to about 5mg, preferably up to 500-1500 µg, 500-1200 µg, or 500-1000 µg, for instance, is administered to the corresponding species and the resulting immune response is

observed, for example by detecting the cellular immune response by an IFNγ Elispot assay (as described in the experimental section), by detecting CTL responses using an *in vivo* lysis assay or a chromium release assay or detecting Th (helper T-cell) response using a cytokine release assay.

In a preferred embodiment, the vaccination regimen comprises one to three injections, preferably repeated three or four weeks later.

In a particular embodiment, the vaccination schedule can be composed of one or two injections followed three or four weeks later by at least one cycle of three to five injections.

In another embodiment, a primer dose consists of one to three injections, followed by at least a booster dose every year, or every two or years for instance.

These are examples only, and any other vaccination regimen is herein encompassed.

Prevention or treatment of tumors

The nucleic acid or immunogenic composition as described above is useful in a method for preventing or treating a tumor in a patient.

A method for preventing or treating a tumor in a patient is described, which method comprises administering an effective amount of said nucleic acid or immunogenic composition in a patient in need thereof. Said nucleic acid or immunogenic composition is administered in an amount sufficient to induce an immune response in the patient.

The tumor may be any undesired proliferation of cells, in particular a benign tumor or a malignant tumor, especially a cancer.

The cancer may be at any stage of development, including the metastatic stage. The cancer may be chronic or non-chronic (acute).

In a particular embodiment, tumor is a solid cancer or a carcinoma. Examples include melanoma, brain tumor such as glioblastoma, neuroblastoma and astrocytoma and carcinomas of the bladder, breast, cervix, colon, lung, especially non-small cell lung cancer (NSCLC), pancreas, prostate, head and neck cancer, or stomach cancer.

In another embodiment, the tumor may be a liquid tumor, e.g. a hematopoietic tumor or leukemia, such as a chronic or acute lymphocytic leukemia, chronic or acute myeloid leukemia, lymphoma including Hodgkin's disease, multiple myeloma, malignant myeloma.

In a particular embodiment, the treatment according to the invention may be combined with conventional therapy, including chemotherapy, radiotherapy or surgery. Combinations with

adjuvant immunomodulating molecules such as GM-CSF or a cytokine like IL-2 or IL-12, could also be useful.

The Figures and Examples illustrate the invention without limiting its scope.

## EXAMPLE I

### Abbreviations:

**AA**: Amino Acid, **APC**: Antigen Presenting Cell, **bp:** Base-pair, **CTL**: Cytotoxic T-Lymphocyte, **CMV**: Cytomegalovirus, **DNA**: Deoxyribonucleic Acid, **EP**: Electroporation, **HTLV-1**: Human T-lymphotropic virus Type I, **hTERT**: human Telomerase Reverse Transcriptase, **ID**: Intradermal, **IM**: Intramuscular, **IV**: Intravenous, **LTRs**: Long Terminal Repeats, **NoLS**: Nucleolar Localization Sequence, **PBMC**: Peripheral Blood Mononuclear Cells, **RIG-I**: Retinoic acid-Inducible Gene 1, **RNA**: Ribonucleic Acid, **RT**: Room Temperature, **RTA**: Relative Telomerase Activity, **SC**: Subcutanous, **TRAP**: Telomeric Repeat Amplification Protocol, **TERT**: Telomerase Reverse Transcriptase, **Ubi**: Ubiquitin, **VDD**: Valine-Aspartic Acid-Aspartic Acid

## MATERIALS AND METHODS

### Plasmid DNA vectors

### INVAC-1

INVAC-1 is a 7120 bp plasmid expression vector encoding a human ubiquitin-telomerase fusion construct of 1158 AA (Ubi-hTERT) corresponding to a protein of approximately 127.4 kDa (Figures 1A and 16). As INVAC-1 is intended to be used in human, the telomerase reverse transcriptase enzymatic activity has been inactivated for safety reasons. Indeed, the human TERT sequence encoded by INVAC-1 was modified in the catalytic site by a 9 bp deletion coding for three amino acid Valine-Aspartic Acid-Aspartic Acid (867-869 AA), abbreviated to VDD in the one letter code (Figure 2A). In addition, the 47 AA of the N-terminal part of the protein, which include the nucleolar localization sequence (NoLS) required for telomerase subcellular localization (Yang, 2002), was replaced by the ubiquitin (Ubi) coding sequence (1-76 AA).

The Ubi-hTERT transgene is inserted into a NTC validated vector backbone (Nature Technology Corporation, Lincoln, Nebraska) combining carefully designed synthetic genes for high yield bacterial production, increased expression in mammalian cells and consequently effective immune responses.

Target gene expression is driven from an optimized chimeric promoter-intron (SV40-CMV-HTLV-1 R synthetic intron) composed of a CMV promoter and start of exon 1, a HTLV-I R sequence which contains the 5' splice acceptor site, a synthetic 3' acceptor site based on the rabbit β-globin intron, an exon 2 splicing enhancer comprising a serine-arginine rich (SR) protein binding site to improve RNA export (Lavigueur et al., 1993) and an exon 2 Kozak sequence upstream of the start codon for the gene of interest. DNA between the stop codon and the terminator is limited to reduce the possibility of cryptic peptide expression or unintended microRNA-mediated expression alteration.

To improve cellular immune responses the vector encodes an RNA polymerase III transcribed double stranded RNA agonist of the retinoic acid inducible gene-1 (RIG-I) innate immune response activator.

There is no known virulence feature associated with this vector. The plasmid does not replicate in eukaryotic target cells. The vector backbone itself does not contain protein coding sequences and no alternative protein encoding open reading frames have been identified in the vector backbone, hence there is no antibiotic resistance gene. Plasmid selection is performed by the mean of an antibiotic-free sucrose selectable marker (RNA-OUT).

**Gene synthesis and cloning**

The Ubi-hTERT gene was *de novo* synthesized through an overlapping 40-mer oligonucleotides assembly process (GeneCust, Luxembourg). Several conservative base changes were made to eliminate restriction sites and attenuate GC rich sequences. The insert was cloned into the expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, USA) using HindIII-XbaI cloning sites and verified by sequencing.

**Subcloning of the Ubi-hTERT insert into the cloning vector NTC8685-eRNA41H-HindIII-XbaI**

The ubiquitin-telomerase insert was cloned into the NTC8685-eRNA41H-HindIII-XbaI expression vector designed by NTC. However, their best appropriate vector NTC8685-eRNA41H (ref. NTC-DV8685-41HLV) did not have restriction sites compatible with the Ubi-hTERT insert. Accordingly, this vector was digested with SalI and BglII and ligated to a synthetic double-stranded oligonucleotide which includes appropriate restriction sites for sub-cloning Ubi-hTERT, i.e., HindIII-XbaI:

SalI **HindIII** SmaI **XbaI** BglII
GTCGACAAGCTTCCCGGGTCTAGAAGATCT (SEQ ID NO: 23)

This new vector (NTC8685-eRNA41H-HindIII-XbaI) which now includes the above polylinker was verified by restriction digestion and sequencing using pVAC5' (GCTTTTCTGCCAGGTGCTGA SEQ ID NO: 24) and pVAC3' (GCCAGAAGTCAGATGCTCAA SEQ ID NO: 25) primers annealing to sequences upstream and downstream the polylinker site respectively.

The custom-made NTC8685-eRNA41H-HindIII-XbaI vector was digested with HindIII and XbaI and the 3631 bp vector was gel purified from the 12 bp linker. The pcDNA3.1-Ubi-hTERT construct was digested with HindIII and XbaI and the 3489 bp Ubi-hTERT insert transferred by ligating into NTC8685-eRNA41H-HindIII-XbaI acceptor to create NTC8685-eRNA41H-HindIII-XbaI-Ubi-hTERT (INVAC-1) (Figure 1A). The ligation product was transformed into antibiotic free selection host NTC4862 (DH5α $att_{\lambda}$::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR) (ref. NTC-DVU-CC1). The resultant vector was verified by restriction digestion (Figure 1B): BglII/NotI = 3496, 3262, 220, 142 bp bands; NcoI = 4084, 3036 bp bands; HindIII/XbaI = 3631, 3489 bp bands, and the termini of the Ubi-hTERT insert verified by DNA sequencing with pVAC5' and pVAC3' primers. No nucleotide alteration was identified.

**Plasmid production**

INVAC-1 was first produced by NTC under research grade quality conditions. Plasmid DNA was transformed into NTC4862 *E. coli* cells using electroporation. Cells were plated and propagated on 6% sucrose media as recommended by the manufacturer (NTC Instruction Manual, June 2011). After extraction, plasmid DNA was resuspended in endotoxin-free 1X PBS at a final concentration of 2 mg/ml.

INVAC-1 was subsequently manufactured by Eurogentec (Belgium) for GLP and GMP scale-up, and GMP production. Full-length sequencing of INVAC-1 plasmid was carried out at this point.

**INVAC-1 derivatives**

All INVAC-1 derivative constructs are double stranded DNA plasmids of approximately 8.9 kb encoding human ubiquitin-telomerase fusion proteins which are enzymatically inactive (Figure 2A). The Ubi-hTERT transgenes were inserted into Invitrogen pcDNA3.1(+) vector (5.4 kb) derived from pcDNA3.0 which was designed for high-level of stable and transient

expressions in mammalian cells. This vector contains the human cytomegalovirus immediate-early (CMV-IE) promoter and the bovine growth hormone polyadenylation (BHG-polyA) signal as termination sequence.

**pUTD10Not (abbreviated as Δ10Not)**

The hTERT coding sequence is located between the nucleotide 923 and 4492 bp of the pcDNA3.1 plasmid backbone. pUTD10Not encodes an 1189 AA human ubiquitin-telomerase fusion protein (Δ10Not) corresponding to approximately 130.8 kDa of molecular weight (Figure 2A). The hTERT was deleted of the 23 first amino acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). In the domain of the catalytic site, an additional deletion was introduced between amino acids 912-913 (* mark; Figure 17), corresponding to AA 860-869 (DGLLLRL**VDD**_ SEQ ID NO: 21) of wild-type hTERT (Accession number NM_198253). This 10 amino acids deletion includes the 3 AA deletion (ΔVDD) resulting in inactivation of hTERT enzymatic activity and the deletion of additional 7 AA upstream the VDD sequence. Fourteen amino acids at the C-terminal sequence of the Ubi-hTERT code for the V5 epitope tag (Figure 2A).

**pUTD10Cog (abbreviated as Δ10Cog)**

The hTERT coding sequence is located between the nucleotide 923 and 4492 bp of the pcDNA3.1 plasmid backbone. pUTD10Cog encodes an 1189 AA human ubiquitin-telomerase fusion protein (Δ10Cog) corresponding to approximately 130.8 kDa of molecular weight (Figure 2A). The hTERT was deleted of the 23 first amino acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). In the domain of the catalytic site, an additional deletion was introduced between amino acids 919-920 (* mark; Figure 18), corresponding to AA 867-876 (**VDD**FLLVTPH_ SEQ ID NO: 22) of wild-type hTERT (Accession number NM_198253). This 10 amino acids deletion includes the 3 AA deletion (ΔVDD) resulting in inactivation of hTERT enzymatic activity and the deletion of additional 7 AA downstream the VDD sequence. Fourteen amino acids at the C-terminal sequence of the Ubi-hTERT code for the V5 epitope tag (Figure 2A).

**pUTD23Tyn (abbreviated as Δ23)**

The hTERT coding sequence is located between the nucleotide 923 and 4453 bp of the pcDNA3.1 plasmid backbone. pUTD23Tyn encodes an 1176 AA human ubiquitin-telomerase

fusion protein (Δ23) corresponding to approximately 129.4 kDa of molecular weight (Figure 2A). The hTERT was deleted of the 23 first amino acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). In the domain of the catalytic site, an additional deletion was introduced between amino acids 909-910 (* mark; Figure 19), corresponding to AA 857-879 (IRRDGLLLRL**VDD**FLLVTPHLTH_ SEQ ID NO: 26) of wild-type hTERT (Accession number NM_198253). This 23 amino acids deletion includes the 3 AA deletion (ΔVDD) resulting in inactivation of hTERT enzymatic activity and the deletion of additional 10 AA upstream and 10 AA downstream the VDD sequence. Fourteen amino acids at the C-terminal sequence of the Ubi-hTERT code for the V5 epitope tag (Figure 2A).

**Genes synthesis and cloning**

The genes were *de novo* synthesized as ubiquitin-telomerase fusion constructs through an overlapping 40-mer oligonucleotides assembly process (GeneCust, Luxembourg). Gene synthesis included unique flanking restriction sites HindIII/XbaI to allow subcloning of the gene into desired expression system. The synthesized genes were cloned between HindIII and XbaI restriction sites of the pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, USA). The sequences of the plasmids were verified by sequencing using PEGFP-N5' CGGTGGGAGGTCTATATAAG (SEQ ID NO: 27) and BGH CAGGGTCAAGGAAGGCAC (SEQ ID NO: 28) primers.

**Plasmids production**

All INVAC-1 derivatives were transformed and produced in *E. coli* 5-alpha cells (fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) (Lucigen Corporation, Middleton, USA, ref. 60602-2) by RD Biotech (Besançon, France). Concentrated endotoxin-free gigaprep plasmid stocks (2 mg/mL) resuspended in 1X sterile PBS were prepared. The vectors were verified by restriction mapping (HindIII-XbaI; Figure 2B).

**pTRIP-CMV-hTERT**

pTRIP-CMV-hTERT encodes the 1132 AA (corresponding to approximately 124.5 kDa) wild-type human TERT (hTERT) protein with catalytic activity. This plasmid was used as a positive control for *in vitro* assays. The construct was first described in patent application WO 2007/014740. The pTRIP-CMV-hTERT was constructed by first subcloning an EcoRI-SalI hTERT insert derived from the pBABE-hygro-hTERT plasmid (kindly provided by Dr.

Robert Weinberg) into the pSP73 vector (Promega Life Science, Wisconsin, USA) to generate the pSPhTERT construct. A BglII-SalI fragment was then inserted into the pTRIP-CMV retroviral-derived vector cut with BamHI and XhoI to create pTRIP-CMV-hTERT. The hTERT expression is driven by the human cytomegalovirus (CMV) promoter.

The pTRIP-CMV-hTERT plasmid was transformed and produced in *E. coli* 5-alpha cells (fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) (Lucigen Corporation, Middleton, USA, ref. 60602-2) by RD Biotech (Besançon, France).

A 2 mg/ml concentrated endotoxin-free gigaprep plasmid stock resuspended in 1X sterile PBS was prepared. The produced vector was verified by restriction enzyme digestion (EcoRI+BamHI = 10286 + 2720 + 886 bp bands).

**pNTC-hTERT**

pNTC-hTERT encodes the 1132 AA wild-type human TERT (hTERT) protein with catalytic activity (SEQ. ID NO: 2). This plasmid was used to investigate the breadth of hTERT specific T-cell responses *in vivo* in comparison with INVAC-1 construct.

The wild-type hTERT insert was synthesized *de novo* with HindIII-XbaI cloning sites through an overlapping oligonucleotides assembly process (GenScript, USA). The synthetic construct (3417 bp) was cloned in pUC57 (2710 bp) by HindIII and XbaI sites and then verified by sequencing using M13/pUC (-20) and M13/pUC (-26) primers and restriction mapping (HindIII/XbaI). Consequently, the hTERT insert was subcloned by NTC into the cloning vector NTC8685-eRNA41H-HindIII-XbaI as described above (see INVAC-1 construct). The resultant vector pNTC-hTERT was verified by restriction digestion (XmaI = 4375, 2041, 506, 120 bp bands; BamHI/XmnI = 6887, 155 bp bands; HindIII/XbaI = 3631, 3411 bp bands) and DNA sequencing using pVAC5', pVAC3' and hTERTseq (5' GGCAAGTCCTACGTCCAGTG 3', SEQ ID NO: 44) primers.

pNTC-hTERT plasmid was produced by NTC under research grade quality conditions as described before for INVAC-1 plasmid.

**pNTC-hTERT-ΔVDD**

pNTC-hTERT-ΔVDD encodes the 1129 AA human TERT (hTERT) sequence modified in the catalytic site by a 9 bp deletion coding for Valine - Aspartic Acid - Aspartic Acid (ΔVDD;

867-869 AA). This plasmid was used to investigate the breadth of hTERT specific T-cell responses *in vivo* in comparison with INVAC-1 construct.

hTERT-ΔVDD DNA sequence is identical to the wild-type hTERT except for a 3 amino acid deletion (ΔVDD). A 167 bp DNA insert including the 152 bp BamHI/XmnI fragment of hTERT, but with the ΔVDD deletion and additional EcoRV restriction sites was synthesized *de novo* by GenScript. This synthetic fragment was cloned in pUC57 vector (2710 bp) using EcoRV cloning sites. The synthesized gene was verified by sequencing using M13/pUC (-20) and M13/pUC (-26) primers and restriction digests (BamHI/NdeI). This vector was then digested using BamHI/XmnI sites and the ΔVDD-BamHI/XmnI fragment was cloned in the BamHI/XmnI predigested hTERT region of the pNTC-hTERT vector (6887, 155 bp bands). The resultant vector pNTC-hTERT-ΔVDD was verified by restriction digestion (XmaI = 4375, 2032, 506, 120 bp bands; BamHI/XmnI = 6887, 146 bp bands; HindIII/XbaI = 3631, 3402 bp bands) and DNA sequencing using pVAC5', pVAC3' and hTERTseq (5' GGCAAGTCCTACGTCCAGTG 3' SEQ ID NO: 44) primers.

pNTC-hTERT-ΔVDD was produced by NTC as described before for INVAC-1 and pNTC-hTERT constructs.

**Cell cultures and transient transfections for western blot and TRAP assays**

CrFK (Crandell Rees feline kidney), HEK293T (Human embryonic kidney) and HeLa (Henrietta Lacks' - Human cervical adenocarcinoma) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (PAA, Velizy-Villacoublay, France) and 1% penicillin/streptomycin (Life Technologies, Saint-Aubin, France).

QT6 (Quail Japanese fibrosarcoma) cell line was cultured in Ham's F10 (Eurobio, Courtaboeuf, France) supplemented with 10% heat-inactivated fetal calf serum (PAA), 1% penicillin/streptomycin (Life Technologies), 1% chicken serum (PAA), 10 mM L-glutamine (Sigma-Aldrich, St. Louis, USA), 0.5% tryptose broth (Sigma-Aldrich, St. Louis, USA).

Cells were grown as monolayers in 75 $cm^2$ flasks at 37°C in a humidified atmosphere containing 5% $CO_2$. The cells were grown until 70-80% confluence on the day of transfection. For western blot assays, $5x10^5$ cells were seeded in six-well tissue culture plates and incubated for 24 h. For TRAP assays, $7x10^5$ cells were seeded in six-well tissue culture plates and incubated for 24 h.

INVAC-1 and INVAC-1 derivative constructs were transfected into target cells using jetPrime cationic polymer transfection reagent according to manufacturer's instructions (Polyplus-transfection Inc., France). Cells transfected with pTRIP-CMV-hTERT plasmid was used as positive control and non-transfected cells or pNTC8685-eRNA41H empty plasmid transfected cells as negative control. Transfection mediums were removed 4 hours later and replaced by 2 mL of DMEM culture medium. After appropriate time of transfection – 18-96 hours for western blot assays and 24 hours for TRAP assays, cells were harvested and analyzed for telomerase expression and activity.

**Western blot**

For western blot analyses, transfected CrFK and HEK293T cells were lysed on ice for 10-20 minutes in RIPA buffer (Sigma-Aldrich, St. Louis, USA) supplemented with a protease inhibitor cocktail (Roche Diagnostic, Indianapolis, USA). Lysates were cleared by centrifugation at 14,000 rpm for 15 minutes at 4°C. The supernatants were harvested and the protein concentration was measured using the Bradford colorimetric assay. Protein samples were denatured 5 minutes at 95°C, separated on Nu-PAGE® Novex 4-12% Bis-Tris gels (Invitrogen, Carlsbad, USA) and electroblotted onto PVDF membranes (iBlot® transfer stack, Invitrogen, Carlsbad, USA) using the iBlot® device (Invitrogen, Carlsbad, USA). Novex® Sharp Prestained Protein Ladder (Invitrogen, Carlsbad, USA) were used to determine molecular weight. The membranes were cut approximately at 60 kDa and blocked with 1X PBS, 0.05% Tween®20, 3% milk. The upper part of the membrane was probed with an anti-hTERT rabbit monoclonal antibody (Abcam, Cambridge, UK) diluted at 1/2000 in blocking buffer or an anti-V5 mouse monoclonal antibody (Invitrogen, Carlsbad, USA) diluted at 1/5000. The lower part of the membrane was probed with an anti-β-actin mouse monoclonal antibody (Sigma Aldrich SARL, Saint-Quentin Fallavier, France) diluted at 1/5000. Finally, the relevant proteins were visualized by staining with the appropriate secondary horseradish peroxidase (HRP) conjugated antibody for 1 h at room temperature - anti-mouse HRP linked antibody (GE Healthcare, Vélizy, France) diluted at 1/5000 or anti-rabbit HRP linked antibody (Cell Signaling, Danvers, USA) diluted at 1/1000 in blocking buffer. The immunoblot signals were detected by enhanced chemiluminescence assay using ECL HRP chemiluminescent substrate Reagent Kit. The films and the corresponding cassette were purchased from GE Healthcare (Buckinghamshire, UK).

**TRAP assay**

Telomerase activity was assessed through Telomeric Repeat Amplification Protocol (TRAP) approach (Kim et al. 1994) using the TeloTAGGG Telomerase PCR ELISAPLUS kit (Roche Diagnostic GmbH Mannheim, Germany) according to the manufacturer's instructions. Twenty-four hours after transfection as described above, CrFK cells were harvested. Cells were washed with 1X PBS, followed by centrifugation at 1,600 rpm for 5 minutes at 4°C. Cells were resuspended in 0.2 ml of lysis buffer and incubated on ice for 30 minutes. Lysates were cleared by centrifugation at 14,000 rpm, 20 min at 4-8°C. The supernatants were harvested and the protein concentration was measured using the Bradford colorimetric assay. Supernatants were used for telomerase-mediated elongation of telomeric sequences and products were amplified by PCR using biotinylated primers. Each cellular supernatant was previously split into two aliquots before performing the assay: one was used to prepare a negative control by heat inactivation of telomerase for 10 min. at 85°C, the other one was used to evaluate the telomerase-mediated elongation of telomeric sequences. Furthermore, a 216 bp length internal standard, present in the reaction mixture, was simultaneously amplified to exclude false negative results due to Taq DNA-polymerase inhibitors that may be present in lysates. Lysis buffer was used as a negative control. All reaction mixtures were incubated 20 minutes at 25°C and then 5 minutes at 94°C. Telomerase products were amplified in 30 PCR cycles: 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 90 seconds ended by 1 cycle at 72°C for 10 minutes and held at 4°C.

2.5 µL of PCR amplification products were incubated for 10 minutes at RT with denaturation reagent provided in the kit. After incubation, 100 µL of hybridization buffer was added per well. Each solution was mixed and 100 µL was transferred to streptavidin pre-coated microplate and incubated for 2 hours at 37°C under gentle agitation (300 rpm). Then, the wells were washed with a washing buffer and incubated with an anti-digoxigenin horseradish peroxidase (HRP) linked antibody (1/50) for 30 minutes at RT. HRP substrate (TMB) was then added for 15 minutes at RT for colorimetric measurement. The reaction was stopped with ELISA stop reagent.

The level of telomerase activity in each sample was determined by comparing the signal from the sample to the signal obtained using a known amount of positive control template (template DNA with the same sequence as a telomerase product with eight telomeric repeats). Absorbance values were reported by $A_{450}$ reading against blank (reference wavelength $A_{690}$ nm). The relative telomerase activity (RTA) was obtained using the following formula:

$RTA = [(A_S - A_{S0})]/A_{S,IS}]/[(A_{TS8} - A_{TS8,0})/A_{TS8,IS}] \times 100$

where:

$A_S$ is the absorbance of sample,

$A_{S0}$, absorbance of heat-treated sample,

$A_{S,IS}$, absorbance of internal standard (IS) of the sample,

$A_{TS8}$, absorbance of control template (TS8),

$A_{TS8,0}$, absorbance of lysis buffer,

$A_{TS8,IS}$, the absorbance of internal standard (IS) of the control template (TS8).

**Immunofluorescence**

CrFK, HEK293T, HeLa and QT6 cells were seeded on 8-well Lab-Tek® chamber slides (Sigma-Aldrich, St. Louis, USA) at $2x10^4$ cells/well in 200 µL of culture medium and incubated overnight at 37°C, 5% $CO_2$. The next day, culture medium was discarded and 200 µL of fresh medium were added. Ten µL of a mix solution containing 0.2 µg of INVAC-1, pTRIP-CMV-hTERT or control empty plasmid pNTC8685-eRNA41H and 0.5 µL of Fugene HD (Promega France, Charbonnières-les-bains, France) in OptiMEM (Life Technologies, Saint-Aubin, France) were added to the corresponding chamber. $2x10^4$ untreated cells per chamber were used as a negative control. Chamber slides were incubated for 24 and 48 hours at 37°C, 5% $CO_2$. Transfected cells were carefully washed with 1X PBS and 200 µL 2% PFA were added to each well for 10 minutes at 4°C in order to fix and permeabilize the cells. Then, wells were washed twice with 1X PBS 0.05% Tween®20 and incubated 30 minutes at room temperature with 200 µL of blocking solution (0.5% Triton X100; Sigma-Aldrich, 3% BSA; Sigma-Aldrich, 10% Goat Serum; Invitrogen, in 1X PBS 0.05% Tween®20). Primary anti-hTERT rabbit monoclonal antibody (Abcam, Cambridge, UK) diluted at 1/100 in blocking buffer was applied on the cells for 1.5 hour at room temperature under agitation. After three washes in 1X PBS 0.05% Tween®20, a secondary goat anti-rabbit-Alexa Fluor 488® antibody (Life Technologies, Saint-Aubin, France) diluted in blocking solution (1/500) was applied for 45 minutes at room temperature under agitation. Wells were washed three times with 1X PBS 0.05% Tween®20 and mounted in VECTASHIELD® mounting medium containing DAPI (Vector laboratories, Cambridgeshire, UK). Cover slips were analyzed under fluorescence microscope (Axio observer Z1, Carl Zeiss MicroImaging GmbH, Jena, Germany) equipped with an image processing and analysis system (Axiovision, Carl Zeiss MicroImaging GmbH, Jena, Germany).

**Mice**

Female C57BL/6 mice (6-8 week old) were purchased from Janvier laboratories (Saint-Berthevin, France).

Two transgenic mouse strains were used: HLA-B*0702 and HLA-A2/DR1.

The HLA-B*0702 transgenic mice express the human HLA-B*0702 α1-α2 domains of the molecule and the murine α3 domain of the H2D molecule. These mice do not express the H2-$D^b$ and H2-$K^b$ molecules (Rohrlich et al., 2003).

The HLA-A2/DR1 transgenic mice express the human HLA-A*0201 α1-α2 domains, the murine α3 domain of the H2D molecule and the human β2-microglobulin. Moreover these transgenic mice express the human HLA-DRB1*0101 and HLA-DRA*0101 molecules. They are knock-out for murine H2-$D^b$, H2-$K^b$ and $IA^b$ genes (Pajot et al., 2004).

Both transgenic mouse strains were used between 6 and 10 weeks of age and were supplied by the Pasteur Institute of Paris. Animals were housed at the Specific Pathogen Free animal facility of the Pasteur Institute (Animal Facilities Lwoff n°22, agreement number B 75 15-07). Prior to intradermal (ID), intramuscular (IM) or subcutaneous (SC) immunizations or intravenous (IV) injection, mice were anesthetized with a mix solution of 2% xylazine (Rompun, Bayer Santé, Loos, France) and 8% Ketamine (Imalgen 1000, Merial, Lyon, France) in 1X Phosphate Buffer Saline (1X PBS, Life Technologies, Saint-Aubin, France) through the intraperitoneal route (IP) according to individual animal weight and duration of anesthesia. All animals were handled in strict accordance with good animal practice and complied with local animal experimentation (Directive 2010/63/UE).

**hTERT Peptides**

hTERT peptides restricted to HLA-B*0702, HLA-A*0201 or HLA-DR were previously described (see references in Table 1). hTERT peptides restricted to H2-$D^b$ and H2-$K^b$ were determined by *in-silico* epitope prediction in order to bind mouse MHC Class I molecules using four algorithms available online: Syfpeithi (http://www.syfpeithi.de/), Bimas (http://www-bimas.cit.nih.gov/), NetMHCpan and SMM (http://tools.immuneepitope.org/main/). All synthetic peptides were purchased lyophilized (>90% purity) from Proimmune (Oxford, United Kingdom). Lyophilized peptides were dissolved in sterile water at 2 mg/mL and stored at -20°C prior use. Details of peptide sequences and MHC restriction are shown in Table 1.

**Table 1: hTERT peptides and MHC restriction**

| Peptide Code (reference) | Sequence | MHC Restriction | Mouse Strain |
|---|---|---|---|
| 277 (Adotevi et al., 2006) | RPAEEATSL (SEQ ID NO : 30) | HLA-B*0702 | Transgenic HLA-B7 |
| 351 (Adotevi et al., 2006) | RPSLTGARRL (SEQ ID NO : 29) | | |
| 1123 (Cortez-Gonzalez et al., 2006) | LPSDFKTIL (SEQ ID NO : 31) | | |
| 540 (Firat et al., 2002) | ILAKFLHWL (SEQ ID NO : 32) | HLA-A*0201 | Transgenic HLA-A2/DR1 |
| Y572 (Firat et al., 2002) | YLFFYRKSV (SEQ ID NO : 33) | | |
| Y988 (Firat et al., 2002) | YLQVNSLQTV (SEQ ID NO : 34) | | |
| UCP2.1 (Dosset et al., 2012) | SVWSKLQSI (SEQ ID NO : 35) | HLA-A*0201 | |
| UCP4.1 (Dosset et al., 2012) | SLCYSILKA (SEQ ID NO : 36) | HLA-A*0201 | |
| UCP2 (Godet et al., 2012) | KSVWSKLQSIGIRQH (SEQ ID NO : 37) | HLA-DR | Transgenic HLA-A2/DR1 |
| UCP3 (Godet et al., 2012) | GTAFVQMPAHGLFPW (SEQ ID NO : 38) | | |
| UCP4 (Godet et al., 2012) | SLCYSILKAKNAGMS (SEQ ID NO : 39) | | |
| 429 | RPIVNMDYV (SEQ ID NO : 40) | $H2D^b$ | C57/BL6J |
| 660 | HAQCPYGVL (SEQ ID NO : 41) | $H2K^b$ | C57/Bl6J |
| 1034 | QAYRFHACVL (SEQ ID NO : 42) | $H2K^b$ | C57/Bl6J |
| 1021 | QTVCTINIYKI (SEQ ID NO : 43) | $H2D^b$ | C57/Bl6J |

**hTERT peptide library**

Lyophilized hTERT peptides (purity > 70%) were purchased from GenScript (USA). This set is composed of 269 peptides of 15 AA overlapping of 11 AA and recovering the whole protein sequence of INVAC-1 hTERT. Each peptide was resuspended in distilled water at 2 mg/mL prior use according to supplier recommendations and kept frozen at -20°C before use. Twenty-seven pools of 9-10 hTERT overlapping peptides (Table 2) were used to screen the breadth of hTERT specific T-cell response in an IFNγ ELISPOT assay.

**Table 2: Pools of hTERT overlapping peptides**

| P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 | P11 | P12 | P13 | P14 | P15 | P16 | P17 | P18 | P19 | P20 | P21 | P22 | P23 | P24 | P25 | P26 | P27 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 11 | 21 | 31 | 41 | 51 | 61 | 71 | 81 | 91 | 101 | 111 | 121 | 131 | 141 | 151 | 161 | 171 | 181 | 191 | 201 | 211 | 221 | 231 | 241 | 251 | 261 |
| 2 | 12 | 22 | 32 | 42 | 52 | 62 | 72 | 82 | 92 | 102 | 112 | 122 | 132 | 142 | 152 | 162 | 172 | 182 | 192 | 202 | 212 | 222 | 232 | 242 | 252 | 262 |
| 3 | 13 | 23 | 33 | 43 | 53 | 63 | 73 | 83 | 93 | 103 | 113 | 123 | 133 | 143 | 153 | 163 | 173 | 183 | 193 | 203 | 213 | 223 | 233 | 243 | 253 | 263 |
| 4 | 14 | 24 | 34 | 44 | 54 | 64 | 74 | 84 | 94 | 104 | 114 | 124 | 134 | 144 | 154 | 164 | 174 | 184 | 194 | 204 | 214 | 224 | 234 | 244 | 254 | 264 |
| 5 | 15 | 25 | 35 | 45 | 55 | 65 | 75 | 85 | 95 | 105 | 115 | 125 | 135 | 145 | 155 | 165 | 175 | 185 | 195 | 205 | 215 | 225 | 235 | 245 | 255 | 265 |
| 6 | 16 | 26 | 36 | 46 | 56 | 66 | 76 | 86 | 96 | 106 | 116 | 126 | 136 | 146 | 156 | 166 | 176 | 186 | 196 | 206 | 216 | 226 | 236 | 246 | 256 | 266 |
| 7 | 17 | 27 | 37 | 47 | 57 | 67 | 77 | 87 | 97 | 107 | 117 | 127 | 137 | 147 | 157 | 167 | 177 | 187 | 197 | 207 | 217 | 227 | 237 | 247 | 257 | 267 |
| 8 | 18 | 28 | 38 | 48 | 58 | 68 | 78 | 88 | 98 | 108 | 118 | 128 | 138 | 148 | 158 | 168 | 178 | 188 | 198 | 208 | 218 | 228 | 238 | 248 | 258 | 268 |
| 9 | 19 | 29 | 39 | 49 | 59 | 69 | 79 | 89 | 99 | 109 | 119 | 129 | 139 | 149 | 159 | 169 | 179 | 189 | 199 | 209 | 219 | 229 | 239 | 249 | 259 | 269 |
| 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 | 210 | 220 | 230 | 240 | 250 | 260 | |

**Tumor cell line**

The Sarc-2 tumor cell line used to assess the anti-tumor effect mediated by INVAC-1 was obtained from a spontaneous fibrosarcoma of a HLA-A2/DR3 mouse. The tumor mass was dissociated under sterile conditions and a primary cell suspension was generated. The cell line was shown to express the HLA-A*0201 molecule. Cells were cultured in RPMI glutamax medium (Life Technologies) supplemented with 10% FBS (Life Technologies) and 1% Penicillin/Streptomycin.

**Mouse immunization and *in vivo* electroporation procedure**

Intradermal (ID) immunization was performed on the lower part of the mouse flank with insulin syringes and specific needles (U-100, 29GX1/2''-0.33x12 mm, Terumo, Belgium) after shaving. No erythema was observed after shaving, during and after the immunization procedures. Intramuscular immunization (IM) was performed in the anterior *tibialis cranialis* muscle, also using insulin syringes and specific needles U-100. Subcutaneous immunization (SC) was performed at the base of the tail, also using insulin syringes and specific needles U-100. Each animal received a priming IM, ID or SC injection of plasmid (INVAC-1, NTC, pUTD10Not, pUTD10Cog or pUTD23Tyn) corresponding to either 12.5, 25, 50, 100, 200, 400, 800 or 1200 µg of DNA or 1X PBS, depending on the experiment. According to the vaccine regimen, mice could receive a similar second or third injection of DNA or 1X PBS.

*In vivo* DNA electroporation was performed using the CLINIPORATOR® 2 electroporation system and software (IGEA, Italy) equipped with plate electrodes (P-30-8G, IGEA). Directly after ID or SC vaccination, a skin fold was made at the injection site, entirely covered with

conductive gel (Labo FH, blue contact gel, NM Médical, France) and placed between the plate electrodes. Two pulses of different voltages were applied (HV-LV): HV: 1250 V/cm, 1 Hz, 100 µs; 1 pulse, 1000 ms break; LV: 180 V/cm, 1 Hz, 400 ms, 1 pulse. Directly after IM injection, each muscle was entirely covered with conductive gel and placed between the plate electrodes. Two pulses of different voltages were applied (HV-LV): HV: 750 V/cm, 1 Hz, 100 µs; 1 pulse, 1000 ms break; LV: 100 V/cm, 1 Hz, 400 ms, 1 pulse.

In certain experiments, 18 hours before DNA vaccination or concomitantly to the administration of INVAC-1, mice were injected ID with 0.5 µg of murine GM-CSF or 1 ng of murine IL-12 in a final volume of 25 µl/flank. Both cytokines were purchased from Miltenyi (Germany).

**ELispot assay**

Spleens from immunized mice were taken and mashed, and cell suspensions were filtered through a 70 mm nylon mesh (Cell Strainer, BD Biosciences, France) to isolate splenocytes. Blood from immunized mice was collected through retro-orbital puncture under anaesthesia in order to isolate peripheral mononuclear blood cells (PBMC). Splenocytes or PBMC were purified using Ficoll (Lymphocyte Separation Medium, Eurobio, France). Ficoll-purified lymphocytes from blood or spleen were numerated using the Cellometer® Auto T4 Plus counter (Ozyme, France).

ELIspot PVDF microplates (IFNγ Elispot kit, Diaclone, Abcyss, France, ref. 862.031.010P) were coated overnight with capture antibody (anti-mouse IFN-γ) and blocked with 1X PBS-2% milk. Cell suspensions were added to the plates in triplicates at $2\times10^5$ cells/well and stimulated with 5 µg/ml of HLA or H2 restricted hTERT derived peptides with serum free culture medium or with PMA-ionomycin (respectively 0.1 µM and 1 µM). After 19 hours, spots were revealed with the biotin-conjugated detection antibody followed by streptavidin-AP and BCIP/NBT substrate solution. Spots were counted using the Immunospot ELIspot counter and software (CTL, Germany). When analyzing ELIspot data, a vaccinated animal is considered as a responder if the frequency of spots, corresponding to hTERT specific CD8 or CD4 T-cells, is superior to the cut-off value of 10 spots.

***In vivo* cytotoxicity assay**

For target cell preparation, splenocytes from naive HLA-B7 mice were labeled by a 1X PBS solution containing high (5 µM), medium (1 µM) or low (0.2 µM) concentrations of CFSE (Vybrant CFDA-SE cell-tracer kit; Life Technologies, Saint-Aubin, France). Naive

splenocytes labeled with 5 and 1 μM CFSE were pulsed respectively with 2 different HLA-B7 peptides, 1123 and 351 at 5 μg/mL for 1.5 hour at room temperature. CFSE low labeled splenocytes were left unpulsed. Each mouse previously vaccinated with INVAC-1 or 1X PBS received, at day 14 post-prime or at day 10 post-boost injection, $10^7$ CFSE-labeled cells of a mix containing an equal number of cells from each fraction through the retro-orbital vein. After 15–18 hours, single-cell suspensions from spleens were analyzed by flow cytometry using MACSQUANT® flow cytometer (Miltenyi, Germany).

The disappearance of peptide-pulsed cells was determined by comparing the ratio of pulsed (high/medium CFSE fluorescence intensity) to unpulsed (low CFSE fluorescence intensity) populations in INVAC-1 immunized mice versus control (1X PBS) mice. The percentage of specific killing per test animal was established according to the following formula:

$[1 - [\text{mean } (CFSE^{low}PBS/CFSE^{high/medium}PBS)/(CFSE^{low}pDNA/CFSE^{high/medium}pDNA)]] \times 100.$

**Cytokine Binding Assay (CBA)**

Splenocytes ($6x10^5$ cells) from vaccinated HLA-A2/DR1 mice were cultured 24 h at 37°C with HLA-DR-restricted hTERT derived peptides (578, 904, and 1029) at 5 μg/mL. Cytokine culture supernatants were recovered and kept frozen at -20°C until testing. A commercially available kit, the mouse Th1/Th2/Th17 Cytometric Beads Array (CBA, BD biosciences) kit was used to quantify respectively the concentration of IL-2, IFNγ, TNFα, IL-4, IL-6, IL-17a and IL-10. The CBA immunoassay was carried out according to the manufacturer's instructions. Flow cytometry acquisition was done using the FACScan LSRII flow cytometer (BD Biosciences); analyses were performed using the FCAP Array TM Software version 3.0 (BD Biosciences).

***In vivo* anti-tumor effect**

For therapeutic vaccination experiments, 24 week-old HLA-A2/DR1 mice were subcutaneously engrafted with $2.10^4$ Sarc-2 cells on the right abdominal flank. Then, animals were immunized with DNA vaccines *via* the ID route followed by electroporation as described above at day 4, 21 and 35 post-engraftment. Every 2 to 3 days, tumor growth was monitored using a caliper. Mouse weight was also monitored every 2 to 3 days. Mice were euthanized when tumors reached 2000 $mm^3$. The guidelines for the welfare and use of animals in cancer research were followed, especially for monitoring of clinical signs necessitating immediate intervention (Workman *et al.* 2010, BJC). Tumor volume was

calculated using the following formula: $(L*l^2)/2$. Results are expressed in $mm^3$ (L= length; l= width).

For prophylactic vaccination, 5-10 week-old HLA-A2/DR1 mice were vaccinated twice (days 0 and 21) as described above. Thirty two days after the last immunization, animals were subcutaneously engrafted with $5.10^4$ Sarc-2 cells. Mice weight and tumor growth were monitored every 2 to 3 days as described before. Mice were euthanized when tumors reached 2000 $mm^3$.

The tumor growth delay (TGD) criterion was used to assess vaccine efficacy. It compares the time to reach a defined tumor volume (500 $mm^3$) in control and treated groups.

**Statistical analysis and data handling**

Prism-5 software was used for data handling, analysis and graphic representations. Data are represented as the mean ± standard deviation or as median. Statistical analyses of ELISpot assays were performed using a Mann Whitney non parametric and/or a Kruskal-Wallis analysis with Dunn's multiple comparison test. Significance was set at p-value < 0.05.

## RESULTS

**Characterization and sequence analysis of INVAC-1 plasmid DNA**

Ubi-hTERT transgene was successfully inserted into pNTC8685-eRNA41H-HindIII-XbaI as shown by restriction mapping using various restriction endonucleases (Figure 1A & 1B). The resulting pNTC8685-eRNA41H-HindIII-XbaI-Ubi-hTERT (INVAC-1) vector was also partially sequenced at junctions using pVAC5' and pVAC3' primers. Sequences confirmed that the cloning process was successfully achieved.

Full-lenght sequencing of INVAC-1 plasmid has been performed on the Master Cell Bank plasmid material (SEQ ID NO: 11 & Figure 16). The result matched the expected sequence except for one base. Indeed, this full sequencing identified a silent mutation (G6064C; GGG glycine to GGC glycine) when compared to the human telomerase gene filed in databases (Accession number NM_198253). This silent mutation could be considered as an additional signature of INVAC-1 as this base change destroys a unique BamHI site (GGATCC to GCATCC) present in the wild-type telomerase gene.

**Characterization and sequence analysis of INVAC-1 derivative constructs**

Three INVAC-1 derivative DNA plasmids expressing different Ubi-hTERT fusion proteins were synthesized and cloned (Figure 2A). All Ubi-hTERT transgenes were successfully

ligated into pcDNA3.1(+) Invitrogen expression vector as shown by HindIII and XbaI digestion and electrophoresis (Figure 2B). Inserts and junctions were sequenced using PEGFP-N5'and BGH primers matching the vector sequence flanking the DNA insert. Sequencing results confirmed that transgenes have been correctly cloned (SEQ ID NO: 13, 15, 17 & Figures 17 to 19).

**INVAC-1 and INVAC-1 derivative proteins are correctly expressed *in vitro* and degraded by the proteasome pathway**

Western blot assay was performed to provide information about the global expression of wild-type hTERT, INVAC-1 and INVAC-1 derivative proteins after 18 h to 96 h of *in vitro* transient transfection into HEK293T and CrFK cell lines. The bands of wild-type hTERT protein corresponded to the size of unmodified hTERT at 124.5 kDa (Figures 3A and 3B, left part of the Figure). Wild-type hTERT protein expression appeared to be stable over the time, especially in HEK293T cells. By contrast, INVAC-1 (Figures 3A and 3B, right part of the Figure and Figure 3C, upper part of the Figure) and INVAC-1 derivative proteins (Figure 3C, lower part of the Figure) were rapidly degraded over the time.

In contrast to wild-type hTERT (pTRIP-CMV-hTERT), INVAC-1 construct produced two distinct bands: A weak upper band corresponding to the Ubi-hTERT fusion protein at the predicted size of 127.4 kDa and a lower band corresponding to INVAC-1-encoded hTERT protein lacking the ubiquitin polypeptide (119 kDa). These two forms of INVAC-1-encoded hTERT protein were detected in both cell lines, HEK293T and CrFK (Figures 3A and 3B). The same pattern was observed for INVAC-1 derivative constructs, Δ10Not, Δ10Cog and Δ23 (Figure 3C). Taken together, the weaker expression of INVAC-1 and INVAC-1 derivatives proteins as compared to wild-type hTERT, their expression patterns and their kinetics of disappearance over time suggest that these proteins were rapidly degraded by the ubiquitin-dependent proteasome pathway in accordance with the proposed model for the degradation of ubiquitin fusion proteins (Bachmair, 1986). The rapid appearance of INVAC-1 band of 119 kDa indicates that the majority of the protein was cotranslationally cleaved or nearly so by ubiquitin-specific processing proteases at the Ubi-hTERT junction. Consequently, the protein entered into a rapid proteasome-dependent degradation pathway according to the so-called N-end rule for protein degradation (Tasaki, 2012; Varshavsky, 1996).

These results validate the *in vitro* expression pattern and identity of Ubi-hTERT fusion proteins encoded by INVAC-1 and INVAC-1 derivatives. The ubiquitin polypeptide fused to hTERT-derived proteins played its role by enhancing the degradation of the proteins in

accordance with the N-end rule. According to this N-end rule, hTERT became an unstable protein rapidly degraded by the proteasome system involved in the production of peptides for antigen presentation by major histocompatibility complex (MHC) class I molecules (Cadima-Couto, 2009; Michalek et al, 1993). Thus, these data indicate that the Ubi-hTERT fusion constructs which undergo enhanced degradation in mammalian tissue culture cells, could also be rapidly degraded *in vivo* and can effectively induce higher CD8+ T-cell responses than wild-type hTERT.

**INVAC-1 protein has a predominant cytoplasmic distribution and a nucleolar exclusion pattern**

With the idea to delocalize INVAC-1-derived hTERT protein to enhance its degradation, the nucleolar localization signal (N-terminal part of hTERT) was removed. Therefore, the cellular localization of hTERT encoded by INVAC-1 was assessed by immunofluorescence analysis after transfection into CrFK, HEK293, HeLa, QT6 cell lines (Figure 4).

Wild-type hTERT (pTRIP-CMV-hTERT) was shown to predominantly localize into the nucleus and nucleolus in transfected HEK293T cells at 24h (Figure 4A). In contrast, INVAC-1 protein was distributed between nucleus and cytoplasm with, first and foremost, a clear nucleolar exclusion pattern (Figure 4A). Transient transfection of wild-type hTERT (pTRIP-CMV-hTERT) and INVAC-1 plasmids into HeLa cells showed similar localization patterns at 24 and 48 hours post-transfection for both proteins (Figure 4B).

The weak anti-hTERT fluorescence signal which could be observed in HEK293T and HeLa cells after transfection of pNTC8685-eRNA41H empty backbone vector was probably due to the cross reactivity with endogenous hTERT.

To overcome the non-specific fluorescence background due to endogenous hTERT protein expression, non-human cell lines, QT6 quail fibrosarcoma and CrFK feline kidney cells, were used for immunostaining. No background signal was observed in both cell lines after transient transfection with pNTC8685-eRNA41H empty backbone vector (Figures 4C & D). As expected, exogenous wild-type hTERT protein (pTRIP-CMV-hTERT) was mainly detected in nucleus and nucleolus of both cell lines (Figures 4C & D). INVAC-1 protein, as already observed in HEK293T and HeLa cells, had a nuclear and cytoplasmic distribution into CrFK cells at 24h (Figure 4D). Interestingly, expression of INVAC-1 into QT6 cells at 24 and 48h was only cytoplasmic suggesting that the deletion of the nucleolar localization signal drastically altered the distribution of the protein in this cell line.

Taken together, these results showed that INVAC-1-derived hTERT protein has a modified subcellular distribution as compared to wild-type hTERT in different cell lines. This alteration may be an advantage for enhancing proteasomal degradation of the protein into peptides for MHC class I presentation to generate specific cellular immune responses (Andersson and Barry, 2004).

Transfection of QT6 and CrFK cells (without non specific hTERT background) with INVAC-1 derivatives (pUTD10Not, pUTD10Cog and pUTD23Tyn) confirmed a nucleolar exclusion pattern of these hTERT-derived proteins (data not shown). Their subcellular distribution was mostly cytoplasmic as compared to wild-type hTERT.

**INVAC-1 and INVAC-1 derivatives have no enzymatic activity**

Human telomerase plays a critical role in tumor growth by participating to immortalization and preventing senescence of tumor cells. Therefore, the use of wild-type telomerase as a vaccine product may lead to safety concerns.

A TRAP assay was performed to evaluate the telomerase activity of Ubi-hTERT constructs in telomerase negative CrFK cell line. Telomerase activity was only detected in CrFK cells transfected with wild-type hTERT using pTRIP-CMV-hTERT plasmid. No telomerase activity was detected in CrFK cells transfected with INVAC-1 or INVAC-1 derivatives (Figure 5).

As shown in Figures 5A and 5C, raw absorbance data demonstrated that the level of telomerase activity of INVAC-1 and INVAC-1 derivatives is comparable to the level of untreated cells.

Relative Telomerase Activity (RTA) data (Figures 5B and 5D) which represent fully analyzed results taking into account the specificity of the assay by using various negative controls including heat-inactivated samples, shown that INVAC-1 and INVAC-1 derivatives are completely devoid of any telomerase activity.

All samples treated with the internal amplification standard (IS) control were highly positive confirming the absence of Taq DNA polymerase inhibitors in CrFK lysate samples and thus reemphasizing the specificity of the assay.

In conclusion, these results confirmed that INVAC-1 and INVAC-1 derivatives do not have any enzymatic activity. Therefore, with regards to the telomerase activity, there is no safety concern for using INVAC-1 in human.

**Electroporation is advantageous to induce significant levels of hTERT specific CD8 T-cells secreting interferon-γ after ID administration of INVAC-1**

The intensity of hTERT specific CD8 T-cell responses was assessed in C57BL/6 mice previously immunized with INVAC-1 *via* the intradermal route followed or not by skin electroporation (Figure 6). Fourteen days post-injection, mouse spleens were harvested and the induced immune responses were monitored *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to H2. A significant difference in the frequency of $IFN\gamma^+$ CD8 T-cells was observed between the group of mice which received an electroporation after the ID injection of INVAC-1 and the group which did not ($p< 0.05$). Thus, these results suggest that electroporation is advantageous to induce significant levels of hTERT specific CD8 T-cell responses after ID vaccination with INVAC-1.

**INVAC-1 vaccination *via* different administration routes followed by electroporation induces hTERT specific CD8 T-cell secreting interferon-γ. The ID vaccination route appears to be the best route**

Conventional vaccines are commonly administered via the SC or IM route. However, the intradermal route of immunization is now regaining interest in the field of vaccination (Combadiere and Liard, 2011). Consequently, the ID route was tested for the administration of INVAC-1 and compared to the conventional SC and IM routes.

In a first set of experiments different groups of transgenic HLA-B7 mice were immunized with INVAC-1 *via* the ID or SC route immediately followed by electroporation (Figure 7A). Fourteen days after vaccination/electroporation, mouse spleens were harvested and the induced immune response was monitored in the spleen *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to HLA-B7. In a second set of experiments one group of transgenic HLA-B7 mice was immunized with INVAC-1 *via* the ID route and the other one via the IM route immediately followed by electroporation (Figure 7B). The frequency of hTERT specific CD8 T-cells was monitored in PBMCs *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to HLA-B7. It was established that the vaccination with INVAC-1 followed by electroporation was able to induce hTERT specific CD8 T-cell responses in HLA-B7 mice whatever the route of vaccination used (Figure 7A and 7B).

Moreover, as shown in Figure 7A, the number of responding animals was higher in the group of mice vaccinated *via* the ID route as compared to group vaccinated *via* the SC route, with 6 out of 8 *vs.* 3 out of 8 responders respectively. A significant difference was also observed in

the frequency of hTERT specific CD8 T-cells between the group of mice vaccinated ID in comparison with the animals vaccinated IM ($p<0.05$) (Figure 7B).

Both experiments demonstrated that the ID route of vaccination was more efficient than the IM and SC routes for the INVAC-1-mediated induction of hTERT specific CD8 T-cells. Similar data were obtained using other mouse models *i.e* HLA-A2-DR1 mice (data not shown). Consequently, all subsequent immunogenicity studies performed with INVAC-1 were then designed with an ID administration of the vaccine followed by electroporation.

**Impact of the vaccine dose on hTERT specific CD8 T-cell response after a single ID immunization with INVAC-1 and electroporation**

Another important parameter to be tested was the impact of the vaccine dose on hTERT specific CD8 T-cell responses. C57BL/6 mice were immunized *via* the ID route followed by electroporation in both lower flanks with increasing doses of INVAC-1. The vaccine volume remained constant at 50 µL/site. Animals were vaccinated in 2 or 4 sites depending on the final vaccine dose received. Fourteen days after vaccination/electroporation, mouse spleens were harvested and the specific cellular immune responses were monitored *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to H2.

In a first set of experiments, C57BL/6 mice received a single ID injection of INVAC-1/electroporation with doses ranging from 12.5 µg to 100 µg (Figure 8A). A significant difference in the frequency of hTERT specific CD8 T-cells was observed in the group of animals vaccinated with 100 µg of INVAC-1 in comparison with control animals vaccinated with PBS ($p<0.01$) (Figure 8A). It was also observed that the median number of hTERT specific CD8 T-cells increased in proportion to the dose of vaccine received (from 12.5 µg to 100 µg). The number of responding animals also increased along with the vaccine dose with respectively 4 out of 6 responders for the 12.5 µg dose, 4 out of 5 for the 25 µg dose and 6 out of 6 responders for the 50 and 100 µg doses.

In a second series of experiments, C57BL/6 mice received a single ID injection of INVAC-1/electroporation with doses ranging from 100 µg to 1200 µg (Figure 8B). A significant difference in the frequency of hTERT specific CD8 T-cells was observed in the group of animals vaccinated with 800 µg of INVAC-1 administered at 4 mg/mL in comparison with control animals vaccinated with PBS ($p<0.05$) (Figure 8B). It was noticed that the median number of hTERT specific CD8 T-cells increased proportionally to the dose of vaccine received from 100 µg to 800 µg and that this median number decreased when injecting 1200 µg. The number of responding animals increased with the vaccine dose with respectively 4

out of 5 responders for the 100 µg dose, 5 out of 5 or 4 out of 4 responders for the doses superior to 200 µg. For the 1200 µg dose, even if all animals were responders, there were still 2 out of 5 animals with a level of specific responses close to the cut-off value.

In conclusion, for the vaccine specific CD8 T-cell criterion in C57BL/6 mice, a dose response was observed as a consequence of the administration of different quantities of INVAC-1. Interestingly, no sign of vaccine toxicity was observed in animals injected with the highest doses of vaccine (800 and 1200 µg) in comparison with control mice (monitoring of body weight and macroscopic autopsy at sacrifice). Similar data were obtained in transgenic HLA-B7 mice (data not shown).

**A prime-boost regimen is recommended for INVAC-1 vaccination in order to increase the level of hTERT specific CD8 T-cell response**

Most of vaccination protocols recommended for conventional vaccines (BCG, measles, influenza...) include a prime-boost regimen in order to improve the frequency of vaccine specific immune responses. Consequently, the impact of a prime-boost regimen on the generation of hTERT specific CD8 T-cell responses was tested for INVAC-1 ID vaccination and electroporation. Towards this aim, transgenic HLA-B7 mice were immunized ID with INVAC-1 and skin vaccination sites were electroporated directly after vaccine administration. Twenty one days after the first immunization, mice received a second injection of INVAC-1 using the same vaccination procedure. At different time points after prime and boost immunizations, peripheral blood was collected in order to monitor hTERT specific CD8 T-cell responses *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to HLA-B7 (Figure 9). A peak of hTERT specific CD8 T-cell response was observed at 14 days post-priming. However, the median frequency of hTERT specific CD8 T-cells in the group of vaccinated animals was relatively low (11.3 spots/ 200,000 PBMCs) and there were 2 out of 5 animals which did not respond to the vaccine. After boosting, a peak of hTERT specific CD8 T-cells was observed at day 10 post-injection. The median frequency of hTERT specific CD8 T-cells in the group of vaccinated animals at this time point (D31 post-prime, D10 post-boost) was significantly different from the median frequency of hTERT specific CD8 T-cells in pre-immune samples ($p<0.05$). There were 4 out of 5 responders after boosting.

In conclusion, a prime-boost vaccination regimen is recommended for INVAC-1 ID vaccination/electroporation because first it allows increasing the frequency of hTERT specific CD8 T-cells circulating in the blood (effector T-cells) and secondly it shortens the time

necessary to reach the peak of the specific cellular immune response, which is an important parameter in the context of an anti-cancer vaccination.

**ID vaccination with Δ10Not, Δ10Cog or Δ23 constructs followed by electroporation also induces hTERT specific CD8 T-cell response. A prime-boost vaccination regimen is recommended to increase the frequency of vaccine specific CD8 T-cells.**

Together with the development of INVAC-1, 3 other DNA plasmid constructs (INVAC-1 derivatives) were designed: Δ10Not (pUTD10Not), Δ10Cog (pUTD10Cog) or Δ23 (pUTD23Tyn). Three deletions were engineered into the catalytic site of the hTERT enzyme. They ranged from 10-23 amino acid residues and spanned the crucial trio of Valine-Aspartic acid-Aspartic acid residues (Val-Asp-Asp, or VDD in the one letter code) (Figure 2A). These constructs were designed to show that any deletion eliminating the activity of the enzyme could retain immunogenicity.

In order to confirm this hypothesis, C57BL/6 mice were immunized *via* the ID route followed by electroporation with INVAC-1, Δ10Not, Δ10Cog, Δ23 or PBS (Figure 10A). Half of the animals received a second injection of DNA or PBS 21 days after the first immunization using the same procedure. Animals were sacrificed fourteen days (group of mice which received a single injection) or ten days (group of mice which received 2 injections) after the last vaccination/electroporation. Mouse spleens were harvested and the induced CD8 T-cell response was monitored *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to H2 (pool of 4 peptides).

For the animals which received a single DNA injection, a significant difference in the frequency of hTERT specific CD8 T-cells was observed only in the group of mice vaccinated with 100 μg of INVAC-1 in comparison with control animals vaccinated with PBS ($p<0.05$) (Figure 10 A, dark dots). When analysing the frequency of responders, there were 3 out of 4 responders in the group of mice vaccinated with INVAC-1 and Δ10Cog. However, for Δ10Cog, animals were low responders with hTERT specific CD8 T-cell responses inferior to 50/200,000 splenocytes. There was only 1 out of 4 responders in the group of animals vaccinated with Δ23 and no responder with animals treated by Δ10Not. For the animals which received two vaccinations (Figure 10A, white dots), a significant median frequency of hTERT specific IFN-γ secreting CD8 T-cells was observed in the spleen of mice immunized with INVAC-1, Δ10Not, and Δ10Cog in comparison with control mice injected with PBS ($p<0.001$). There were only 2 out of 4 responding animals in the group of mice vaccinated

with Δ23 which was not statistically significant. In conclusion, after one or two rounds of vaccination, INVAC-1 and INVAC-1 derivative constructs allowed the induction of hTERT specific CD8 T-cells, INVAC-1 being the more immunogenic one in C57BL/6 mice.

In a second set of experiments, transgenic HLA-B7 mice were vaccinated ID with INVAC-1, Δ10Not, Δ10Cog, Δ23 or PBS (Figure 10B) followed by electroporation and received a second injection 21 days after the first one using the same procedure. Spleens were collected 10 days after the last injection and the induced CD8 T-cell response was monitored *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to B7. As shown in Figure 10B a significant median frequency of hTERT specific IFN-γ secreting CD8 T-cells was observed in the spleen of mice immunized with INVAC-1, Δ10Not, Δ10Cog and Δ23 in comparison with control mice injected with PBS ($p<0.001$).

As shown for INVAC-1, the 3 INVAC-1 derivatives Δ10Not, Δ10Cog and Δ23 were also capable of inducing hTERT specific CD8 T-cells *in vivo* after ID vaccination and electroporation in two different mouse strains. A prime-boost vaccination regimen was also recommended for INVAC-1 derivatives to reach significant levels of hTERT specific CD8 T-cell responses. Taken together, these results demonstrate that INVAC-1 is the construct which allows the induction of the best hTERT specific CD8 T-cell response. This is probably due to the difference observed in ΔhTERT protein expression levels after plasmid transfection as shown by western blotting (Figure 3).

**The breadth of hTERT specific T-cell response after ID vaccination(s) followed by electroporation is different according to the hTERT plasmid construct used for vaccination (INVAC-1, pNTC-hTERT or pNTC-hTERT-ΔVDD)**

The impact of hTERT sequence modifications engineered within the INVAC-1 construct, i.e, (1) the deletion of the nucleolar localization signal, (2) the addition of the ubiquitin sequence and (3) the deletion within the catalytic site, on the repertoire of the T-cell immune response against hTERT has been assessed. INVAC-1 hTERT specific cellular immune responses were screened after ID immunization(s)/electroporation(s) with INVAC-1 and compared to responses induced by a DNA encoding the native/wild-type sequence of the human TERT (pNTC-hTERT) and a DNA encoding the hTERT sequence only deleted in the VDD region (pNTC-hTERT-ΔVDD). Control animals received ID injection(s) of 25 μg of pNTC empty vector followed by electroporation.

A first series of HLA-B7 transgenic mice received a single injection of either one of the 4 constructs using the vaccination protocol described before (25 µg/mouse). A second series of animals received a prime injection and a boost 21 days after the first vaccination with either one of the 4 constructs using the vaccination protocol described before (25 µg/mouse).

Fourteen days after a single injection or 10 days post-boost, splenocytes from vaccinated and control mice were tested in an IFNγ ELIspot assay using 269 peptides of 15 AA overlapping of 11 AA and recovering the whole protein sequence of hTERT (27 pools composed of 10 peptides each).

Immunization with INVAC-1 induced a large repertoire of T-cells against numerous hTERT epitopes since after the priming, about 12 pools of peptides were recognized (Figure 11A). These data suggest that a minimum of 12 epitopes restricted to HLA-B7 were expressed after processing on the surface of dendritic cells with a density of MHC peptide complexes allowing the induction of a strong T-cell response. These important results show the capacity of INVAC-1 for processing and expression of numerous hTERT peptides on the surface of APC. The difference obtained with the other constructs (hTERT and hTERTΔVDD) validates the optimization features made in INVAC-1 leading to increase the breadth of the T-cell repertoire against hTERT. In addition, these results stress out the advantage of DNA vaccination versus peptide immunization.

The advantage for a second cycle of immunization (prime-boost) with INVAC-1 in transgenic mice was confirmed in this study. *In vivo* T-cell repertoire was improved as at least 5 new epitopes were revealed (Figure 11B). A total of at least 17 epitopes were recognized after the boost. These data confirm that several injections in the patient will be beneficial to obtain a better anti-tumor response.

Analyzing these data globally by doing the sum of total median of frequency of specific T-cell detected for the 27 pools of peptides, no major differences were observed after one (prime) or two (prime-boost) cycles of immunizations between the three hTERT constructs (Figure 11C). This suggests that the modifications made in INVAC-1 hTERT had no impact on the breadth of the immune response, even though a significant higher T-cell mediated immune response was observed after the boost with INVAC-1.

In conclusion, INVAC-1 vaccination mediated a large repertoire of T-cell immune response against numerous hTERT epitopes different from wild-type hTERT and hTERTΔVDD constructs in terms of peptides/epitopes recognized by T-cells.

**ID vaccination with INVAC-1 followed by electroporation induces hTERT specific T-cell responses with the hallmark of an anti-cancer immune response: cytotoxic CD8 T-cells and Th1 CD4 T-cells**

Among immune cells that are relevant in antitumor immune responses, cytotoxic CD8 T lymphocytes (CTL) and Th1 CD4 T-cells have been identified as the most powerful effector cells (Vesely et al., 2011) (Braumuller et al., 2013).

In a first step, the cytotoxic activity of hTERT specific CD8 T-cells was investigated *in vivo* after ID vaccination/electroporation with INVAC-1. Indeed, this activity is necessary to kill tumor cells. In order to measure the *in vivo* cytolytic strength of the hTERT specific $CD8^+$ T-cell response elicited by INVAC-1 immunization, an *in vivo* cytotoxicity assay was performed using carboxyfuorescein-diacetate succinimidyl ester (CFSE)–labelled, peptide-pulsed splenocytes as target cells. HLA-B7 transgenic mice which received a prime or a prime-boost vaccination with INVAC-1 (or PBS as control) *via* the ID route as described before were intravenously injected with $7.10^6$ target cells. Target cells were splenocytes from naive congenic mice independently labelled with 3 different concentrations of CFSE and pulsed with either a hTERT peptide restricted to HLA-B7 (p351, immuno-dominant peptide or p1123, sub-dominant peptide) or left unpulsed as an internal control. After 15-18 hours, spleen cells were harvested and the disappearance of peptide-pulsed cells in immunized *vs.* control mice was quantified by flow cytometry.

Results show that all mice developped specific CTLs against the immuno-dominant peptide p351 after a single injection (Figure 12A, white dots) with a median specific lysis of 35%. One third of the animals developed specific CTLs against the sub-dominant peptide p1123 (Figure 12A, black dots). It can be expected that multiple injection cycles would allow increasing the number of animals which develop a specific CTL lysis against the sub-dominant peptide 1123.

It has been recently described that a hTERT specific CD4 T-cell response may be associated with a better chemotherapy response in NSCLC patients (Godet et al., 2012). Therefore, the presence of a hTERT specific CD4 T-cell response after INVAC-1 ID injection was investigated. To this aim HLA-A2/DR1 transgenic mice were ID immunized with INVAC-1 followed by electroporation and the hTERT specific CD4 T-cell response was monitored in the spleen 14 days after vaccination *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to DR1. As shown in Figure 12B, a significant median frequency of hTERT specific IFN-γ secreting CD4 T-cells was observed in the spleen of ID vaccinated mice in comparison with control mice injected with PBS ($p<0.001$).

It has been emphasized that Th1 immunity had a clear positive effect on cancer cell elimination *in vivo* (Braumuller et al., 2013). As a matter of fact, $CD4^+$Th1 cells produce several cytokines (such as IFN-γ, TNF-α and IL-2) essential for the induction of cell-mediated immunity against tumors. Consequently, after INVAC-1 ID vaccination, the different cytokines secreted by hTERT specific CD4 T-cells were investigated. To this aim, splenocytes from INVAC-1-vaccinated HLA-A2/DR1 transgenic mice were stimulated *in vitro* for 24 hours with a pool of hTERT peptides or left unstimulated. Supernatants were recovered and assayed in a Cytokine Binding Assay (CBA) in order to evaluate the concentration of Th1, Th2 and Th17 cytokines secreted by hTERT specific CD4 T-cells.

As shown in Figure 12C, significant concentrations of Th1 cytokines IL-2, TNFα and IFNγ were detected in supernatants from splenocytes recovered from mice vaccinated with INVAC-1 in comparison with supernatants from control mice ($p<0.05$).

Thus, ID vaccination/electroporation with INVAC-1 is able to promote the expansion of hTERT specific CD8 T-cells which exhibit a cytotoxic activity *in vivo* along with specific CD4 T-cells with a Th1 profile. Both types of response are the hallmark of a favourable anti-cancer immune response.

**Therapeutic and preventive ID vaccination with INVAC-1 followed by electroporation delay tumor growth after syngeneic tumor inoculation in HLA-A2/DR1 transgenic mice**

Up to this point, results have shown that an ID injection of INVAC-1 followed by electroporation was able to induce cytotoxic CD8 T-cells and Th1 CD4 T-cells in mice. The next step was then to evaluate the protection of transgenic HLA-A2/DR1 mice conferred by INVAC-1 ID vaccination and electroporation after Sarc-2 (fibrosarcoma) tumor cells inoculation. In a first attempt, transgenic HLA-A2/DR1 mice were vaccinated ID with INVAC-1 followed by electroporation in a prime-boost strategy or mock vaccinated with PBS. One month after preventive vaccination, mice were challenged *via* the SC route with 50,000 Sarc-2 cells. Tumor volume was measured every 2-3 days. Figure 13A shows the kinetics of the median tumor volume after challenge according to mice treatment. Tumor growth delay (TGD) at 500 $mm^3$ was then calculated. This criterion allows measuring a vaccine treatment effect on tumor growth by comparing the time to reach a defined tumor volume in control and treated groups. An eleven-day tumor growth delay was observed between the group of mice vaccinated with INVAC-1 and the group of animals which received PBS. Thus, preventive vaccination with INVAC-1 was responsible for a slow-down in tumor growth. Because tumor inoculation was performed one month after the last

vaccination, anti-tumor effects could be to some extent attributed to the presence of hTERT specific memory T-cells.

In a second series of experiments, transgenic mice were engrafted with 20,000 Sarc-2 cells and were ID vaccinated with INVAC-1 followed by electroporation 4 days after cell inoculation (Figure 13B). Control animals received an ID injection of an "empty" plasmid (NTC) which has the same backbone as INVAC-1 but which does not encode any tumor antigen. Two boost vaccinations were performed with the same procedure 21 and 35 days after tumor engraftment. Tumor growth delay at 500 $mm^3$ was calculated. A 4-day tumor growth delay was observed between the group of mice vaccinated with INVAC-1 and the group of animals which received the NTC empty plasmid. In conclusion, therapeutic vaccination with INVAC-1 allowed a relatively weak, nevertheless repeatedly observed, slow-down in tumor growth.

**Administration of murine GM-CSF along with INVAC-1 ID vaccination/electroporation improves the intensity and quality of hTERT specific cellular immune response and delays tumor growth after a syngeneic tumor challenge in HLA-A2/DR1 transgenic mice.**

Different cytokines have been used so far as immunomodulators to facilitate antigen recognition and T-cell expansion in anti-cancer vaccination studies both in animal models and in humans. One of the most frequently used cytokine is the GM-CSF (Granulocyte macrophage Colony Stimulating Factor). This cytokine is known to help the maturation of Antigen Presenting Cells and to favor the Th1 cellular immune responses (Parmiani et al., 2007). Regarding the major role played by GM-CSF in the context of anti-tumor vaccines, the impact of the addition of murine GM-CSF (mGM-CSF) on hTERT specific T-cell responses after INVAC-1 ID vaccination and electroporation was tested. To this aim, C57BL/6 mice received an ID injection of mGM-CSF 18 hours prior being vaccinated with INVAC-1 *via* the ID route followed by electroporation (Figure 14A). Another group of mice was vaccinated ID with INVAC-1/electroporation without addition of mGM-CSF. Control animals were mock vaccinated with PBS and electroporation. Fourteen days post-injection, mouse spleens were harvested and the induced immune responses were monitored *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to H2. A significant difference in the frequency of $IFN\gamma^+$ CD8 T-cells was observed between the group of mice which received mGM-CSF before the ID injection of INVAC-1 and the group which did not ($p< 0.001$). Thus, addition of mGM-

CSF allowed a major increase in the frequency of hTERT specific CD8 T-cells. A second step consisted in investigating the impact of this immunodulator on the quality of hTERT specific CD4 T-cells, and especially on the generation of Th1 specific T-cells. To this aim, splenocytes from INVAC-1 or INVAC-1/mGM-CSF vaccinated HLA-A2/DR1 transgenic mice were stimulated *in vitro* for 24 hours with a pool of hTERT peptides restricted to DR1 or left unstimulated. Supernatants were recovered and assayed in a Cytokine Binding Assay (CBA) in order to evaluate the concentration of Th1, Th2 and Th17 cytokines secreted by hTERT specific CD4 T-cells. As shown in Figure 14B, significant concentrations of Th1 cytokines IL-2, TNFα and IFNγ were detected in supernatants from splenocytes recovered from mice vaccinated with INVAC-1/mGM-CSF in comparison with supernatants from mice vaccinated with INVAC-1 only. When adding mGM-CSF, there was a major increase in the concentration of TNFα ($p< 0.01$), IFNγ ($p< 0.05$) and IL-2 ($p< 0.05$) which are Th1 anti-tumoral cytokines.

Thereafter, the combination mGM-CSF/INVAC-1 was studied in the Sarc-2 animal tumor model in order to evaluate if mGM-CSF could potentiate anti-tumor effects.

To this aim, HLA-A2/DR1 transgenic mice were engrafted with 20,000 Sarc-2 cells and were vaccinated ID with INVAC-1 and mGM-CSF followed by electroporation 4 days after cell engraftment (Figure 14C). Control animals received an ID injection of an empty plasmid (NTC) and mGM-CSF or PBS and mGM-CSF. Two boost vaccinations were performed with the same procedure 21 and 35 days after tumor engraftment. Tumor growth delay (TGD) at 500 $mm^3$ was calculated. A 14-day TGD was observed between the group of mice vaccinated with INVAC-1/mGM-CSF and the group of animals which received NTC/mGM-CSF; 10-day TGD was observed between INVAC-1/mGM-CSF and PBS/mGM-CSF group. These results demonstrate that a therapeutic vaccination with INVAC-1 combined with mGM-CSF allowed a slow-down in tumor growth.

**Administration of murine IL-12 along with INVAC-1 ID vaccination/electroporation improves the intensity of hTERT specific CD8 T-cell response**

The impact of the IL-12 cytokine on hTERT specific CD8 T-cell response after INVAC-1 ID vaccination and electroporation was also investigated. To this aim, HLA-A2/DR1 mice received an ID injection of IL-12 along with the ID administration of INVAC-1 followed by electroporation (Figure 15). Another group of mice was vaccinated ID with INVAC-1/electroporation without addition of IL-12. Control animals were mock vaccinated with PBS and IL-12 or PBS alone followed by electroporation. Fourteen days post-injection, mouse

spleens were harvested and the induced immune responses were monitored *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to A2. The frequency of responding mice was increased when adding IL-12. Indeed, there were 2 out of 5 and 4 out of 5 responding animals for the INVAC-1 vaccinated group and the INVAC-1/IL-12 vaccinated group respectively.

## EXAMPLE II

### Abbreviations:

**AA**: Amino Acid, **bp:** Base-pair, **CTL**: Cytotoxic T-Lymphocyte, **CMV**: Cytomegalovirus, **DNA**: Deoxyribonucleic Acid, **EP**: Electroporation, **ID**: Intradermal, **NoLS**: Nucleolar Localization Sequence, **RNA**: Ribonucleic Acid, **RTA**: Relative Telomerase Activity, **TRAP**: Telomeric Repeat Amplification Protocol, **TERT**: Telomerase Reverse Transcriptase, **Ubi**: Ubiquitin, **VDD**: Valine-Aspartic Acid-Aspartic Acid

### MATERIALS AND METHODS

### Plasmid DNA vectors

### INVAC-1

The INVAC-1 construct was already described in EXAMPLE I.

### INVAC-1 shuffled derivatives

pUTScram and pUTInv constructs are double stranded DNA plasmids of approximately 8.9 kb encoding human ubiquitin-telomerase-based fusion proteins which are enzymatically inactive. The Scrambled and Inverted transgenes were inserted into Invitrogen pcDNA3.1(+) vector (5.4 kb) derived from pcDNA3.0 which was designed for high-level of stable and transient expression in mammalian cells. Transgene expression is driven from human cytomegalovirus immediate-early (CMV) promoter to allow efficient high-level expression in a wide range of mammalian cells. The vector contains multiple cloning sites (MCS) to facilitate cloning. Efficient transcription termination is driven by the Bovine Growth Hormone (BGH) polyadenylation signal.

**pUTScram (named Scrambled)**

The Ubi-Scrambled hTERT insert (Scrambled, 1184 AA) starts at position 923 and ends at position 4474 of the pUTScram plasmid (Figure 20A). pUTScram encodes a human ubiquitin-telomerase-based fusion construct (Scrambled) of 1184 AA corresponding to a protein of approximately 130.2 kDa. hTERT protein was deleted of the 23 first amino-acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). The catalytic site was inactivated by a 9 bp deletion coding for **VDD** (* mark; Figure 28) and corresponding to AA 867-869 of wild-type human telomerase (hTERT; patent WO 2007/014740 and hTERT isoform 1 Accession number NM_198253). hTERT sequence was divided into ten immunogenic fragments and reassembled in the following specific order: **fragment 7** (210 bp), **fragment 2** (201 bp), **fragment 6** (312 bp), **fragment 4** (117 bp), **fragment 9** (576 bp), **fragment 3** (120 bp), **fragment 1** (258 bp), **fragment 8** (477 bp), **fragment 10** (516 bp), **fragment 5** (303 bp). These 10 fragments are bridged with 6xGly linker (G linker; 18 bp). Consequently, 76 non-immunogenic AA (228 bp) were deleted from hTERT sequence. The 14 amino acids at the C-terminal sequence of the Ubi-hTERT shuffled insert code for the V5 epitope tag (Figure 22).

**pUTInv (named Inverted)**

The Ubi-inverted hTERT insert (Inverted, 1184 AA) starts at position 923 and ends at position 4474 of the pUTInv plasmid (Figure 20B). pUTInv encodes a human ubiquitin-telomerase-based fusion construct (Inverted) of 1184 AA corresponding to a protein of approximately 130.2 kDa. hTERT protein was deleted of the 23 first amino-acids (1-23 AA) which were replaced by an ubiquitin polypeptide (76 AA). The catalytic site was inactivated by a 9 bp deletion coding for **VDD** (* mark; Figure 29) and corresponding to AA 867-869 of wild-type human telomerase (hTERT; patent WO 2007/014740; Accession number NM_198253). hTERT sequence was divided into ten immunogenic fragments and reassembled in the following specific order: **fragment 10** (516 bp), **fragment 9** (576 bp), **fragment 8** (477 bp), **fragment 7** (210 bp), **fragment 6** (312 bp), **fragment 5** (303 bp), **fragment 4** (117 bp), **fragment 3** (120 bp), **fragment 2** (201 bp), **fragment 1** (258 bp). These 10 fragments were bridged with 6xGly linker (G linker; 18 bp). Consequently, 76 non-immunogenic AA (228 bp) were deleted from hTERT sequence. The 14 amino acids at the C-terminal sequence of the Ubi-hTERT shuffled insert code for the V5 epitope tag (Figure 22).

**Genes synthesis and cloning**

The genes were *de novo* synthesized as ubiquitin-telomerase-based fusion constructs through an overlapping 40-mer oligonucleotides assembly process (GeneCust, Luxembourg). Several conservative base changes were made to eliminate restriction sites and attenuate GC rich sequences. Gene synthesis included unique flanking restriction sites HindIII/XbaI to allow subcloning of the gene into desired expression system. The synthesized genes were cloned between HindIII and XbaI restriction sites of the pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, USA). The sequences of the plasmids were verified by sequencing using PEGFP-N5' CGGTGGGAGGTCTATATAAG (SEQ ID NO: 27) and BGH CAGGGTCAAGGAAGGCAC (SEQ ID NO: 28) primers.

**Plasmids production**

These INVAC-1 shuffled derivatives synthetized by GeneCust were transformed and produced in *E. coli* 5-alpha cells (fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) (Lucigen Corporation, Middleton, USA, ref. 60602-2) by RD Biotech (Besançon, France). Cells were plated and propagated on Lenox Broth media containing ampicillin (#EU04000D, Euromedex). After extraction and purification, concentrated endotoxin-free gigaprep plasmid stocks (2 mg/mL) resuspended in 1X sterile PBS were prepared. The vectors were verified by restriction mapping (HindIII-XbaI; Figure 21).

**pTRIP-CMV-hTERT**

This DNA plasmid was already described in EXAMPLE I.

**Cell cultures and transient transfections for western blot and TRAP assays**

CrFK (Crandell Rees feline kidney) and HEK293T (Human embryonic kidney) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (PAA, Velizy-Villacoublay, France) and 1% penicillin/streptomycin (Life Technologies, Saint-Aubin, France).

Cells were grown as monolayers in 75 $cm^2$ flasks at 37°C in a humidified atmosphere containing 5% $CO_2$. The cells were grown until 70-80% confluence on the day of transfection. For western blot assays, $5x10^5$ cells were seeded in six-well tissue culture plates and

incubated for 24 h. For TRAP assays, $7x10^5$ cells were seeded in six-well tissue culture plates and incubated for 24 h.

INVAC-1, pUTScram and pUTInv constructs were transfected into target cells using jetPrime cationic polymer transfection reagent according to manufacturer's instructions (Polyplus-transfection Inc., France). Cells transfected with pTRIP-CMV-hTERT plasmid was used as positive control and non-transfected cells as negative control. Transfection mediums were removed 4 hours later and replaced by 2 mL of DMEM culture medium. After appropriate time of transfection – 18-96 hours for western blot assays and 24 hours for TRAP assays, cells were harvested and analyzed for telomerase expression and activity.

**Western blot**

Western blot analyses were performed using transfected HEK293T cells. The western blot procedure is as described in EXAMPLE I.

**TRAP assay**

This procedure is as described in EXAMPLE I.

**Mice**

HLA-B*0702 transgenic mouse strain was used in these experiments.

The HLA-B*0702 transgenic mice express the human HLA-B*0702 α1-α2 domains of the molecule and the murine α3 domain of the H2D molecule. These mice do not express the H2-$D^b$ and H2-$K^b$ molecules (Rohrlich, Cardinaud et al. 2003).

Mice were used between 9 and 15 weeks of age and were supplied by the Pasteur Institute of Paris. Animals were housed at the Specific Pathogen Free animal facility of the Pasteur Institute (Animal Facilities Lwoff n°22, agreement number B 75 15-07). Prior to intradermal (ID) or intravenous (IV) injection, mice were anesthetized with a mix solution of 2% xylazine (Rompun, Bayer Santé, Loos, France) and 8% Ketamine (Imalgen 1000, Merial, Lyon, France) in 1X Phosphate Buffer Saline (1X PBS, Life Technologies, Saint-Aubin, France) through the intraperitoneal route (IP) according to individual animal weight and duration of anesthesia. All animals were handled in strict accordance with good animal practice and complied with local animal experimentation (Directive 2010/63/UE).

**hTERT Peptides**

hTERT peptides restricted to HLA-B*0702, were previously described in EXAMPLE I. Lyophilized peptides were dissolved in sterile water at 2 mg/mL and stored at -20°C prior use.

**Mouse immunization and *in vivo* electroporation procedure**

Intradermal (ID) immunization was performed on the lower part of the mouse flank with insulin syringes and specific needles (U-100, 29GX1/2''-0.33x12 mm, Terumo, Belgium) after shaving. No erythema was observed after shaving, during and after the immunization procedures. Each animal received a priming ID injection of plasmid (INVAC-1, pUTScram or pUTInv) with 100 µg of DNA or 1X PBS. According to the vaccine regimen, mice could receive a similar second injection of DNA or 1X PBS.

*In vivo* DNA electroporation was performed using the CLINIPORATOR® 2 electroporation system and software (IGEA, Italy) equipped with plate electrodes (P-30-8G, IGEA). Directly after ID vaccination, a skin fold was made at the injection site, entirely covered with conductive gel (Labo FH, blue contact gel, NM Médical, France) and placed between the plate electrodes. Two pulses of different voltages were applied (HV-LV): HV: 1250 V/cm, 1 Hz, 100 µs; 1 pulse, 1000 ms break; LV: 180 V/cm, 1 Hz, 400 ms, 1 pulse.

**ELISpot assay**

ELISpot Assay was performed according to the method described in EXAMPLE I. Only a pool of three specific hTERT peptides restricted to HLA-B*0702 (p277, p351 and p1123) was used in Example II.

***In vivo* cytotoxicity assay**

*In vivo* lysis assay was performed according to the procedure described in EXAMPLE I. Only two specific hTERT peptides restricted to HLA-B*0702 (p351 and p1123) were used respectively as immunodominant and subdominant peptides in Example II.

**Statistical analysis and data handling**

GraphPad Prism 5 software was used for data handling, analysis and graphic representations. Data are represented as the mean ± standard deviation or as median. Statistical analyses of

ELISpot assays were performed using a Mann Whitney non parametric and/or a Kruskal-Wallis analysis with Dunn's multiple comparison test. Significance was set at p-value < 0.05.

## RESULTS

### Characterization and sequence analysis of INVAC-1 plasmid DNA

The characterization and sequence analysis of INVAC-1 plasmid DNA were already described in EXAMPLE I.

### Characterization and sequence analysis of INVAC-1 shuffled derivative constructs (pUTScram and pUTInv)

Two INVAC-1 shuffled derivative genes were synthesized and cloned (Figure 20). These constructs were based on INVAC-1 nucleotide sequence described in EXAMPLE I and wild-type hTERT amino acid sequence described in international patent application WO 2007/014740.

Codon optimization was carried out for high level expression in mammalian cells (Figure 27). Scrambled and Inverted Ubi-hTERT shuffled transgenes were successfully ligated into pcDNA3.1(+) Invitrogen expression vector as shown by HindIII and XbaI digestion and electrophoresis (Figure 21). Inserts and junctions were sequenced using PEGFP-N5'and BGH primers matching the vector sequence flanking the DNA insert. Sequencing results confirmed that transgenes have been correctly cloned (Figures 28 and 29).

### INVAC-1 shuffled derivative proteins are correctly expressed *in vitro* and degraded by the proteasome pathway

Western blot assay was performed to provide information about the global expression of wild-type hTERT, INVAC-1, pUTScram and pUTInv proteins after 18 h to 96 h of *in vitro* transient transfection into HEK293T cell lines. The bands of wild-type hTERT protein corresponded to the size of unmodified hTERT at 124.5 kDa (Figures 23A and 23C, left part of the Figures). In EXAMPLE I, INVAC-1 proteins have been shown to be rapidly degraded over time contrariwise to the wild type hTERT proteins expressed at a stable level. Specific bands for Scrambled and Inverted shuffled proteins were detected over time (Figures 23A and 23C, right part of the Figures). For both, these bands were observed at a smaller size (<110 kDa) than predicted size for entire proteins (130.2 kDa). These forms of Scrambled and Inverted proteins correspond to degraded products. Indeed, Scrambled and Inverted

expression non-degraded products were not detectable on western blot analysis. These constructs gave respectively from 1 to 3 specific bands suggesting a fast degradation of these proteins just after production. As INVAC-1, the same pattern of degradation over time for Scrambled degraded products was demonstrated after normalization to β-actin loading control (ImageJ analysis; Figure 23B). Inverted degraded products have a pattern more similar to the others INVAC-1 derivatives proteins (Figures 23C, 23D and Figure 3C: pUTD10Not, pUTD10Cog and pUTD23Tyn, see EXAMPLE I).

**INVAC-1 shuffled derivatives have a predominant cytoplasmic distribution and a nucleolar exclusion pattern**

As demonstrated for INVAC-1 and INVAC-1 derivatives (pUTD10Not, pUTD10Cog and pUTD23Tyn, see EXAMPLE I), Scrambled and Inverted shuffled proteins encoded by pUTScram and pUTInv were distributed between nucleus and cytoplasm with a nucleolar exclusion pattern (data not shown).

**INVAC-1 shuffled derivatives have no enzymatic activity**

A TRAP assay was performed to evaluate the telomerase activity of Ubi-hTERT shuffled constructs in telomerase negative CrFK cell line. Telomerase activity was only detected in CrFK cells transfected with wild-type hTERT using pTRIP-CMV-hTERT plasmid.

As shown in Figures 24A, raw absorbance data demonstrated that the level of telomerase activity of Scrambled and Inverted proteins is comparable to the level of untreated cells. Relative Telomerase Activity (RTA) data (Figure 24B) which represent fully analyzed results taking into account the specificity of the assay by using various negative controls including heat-inactivated samples, confirmed that these shuffled proteins are completely devoid of any telomerase activity.

**Shuffled hTERT constructs induce hTERT specific CD8 T-cell response**

pUTScram and pUTInv constructs were designed to induce antigen presentation of multiple hTERT epitopes increasing the scope of INVAC-1 features. Immunogenicity comparison of pUTScram, pUTInv and INVAC-1 was assessed in HLA-B7 mice ID immunized with the different constructs followed by skin electroporation after two cycles of immunization (prime-boost regimen). Animals were sacrificed ten days after the second vaccination/electroporation. Mouse spleens were harvested and the induced CD8 T-cell response was monitored *via* an IFN-γ ELISPOT assay using hTERT peptides restricted to

HLA-B7 MHC class I (pool of 3 peptides p277, p351 and p1123). A significant difference in the frequency of hTERT specific CD8 T-cells was observed in mice vaccinated with INVAC-1, pUTScram (Scrambled) and pUTInv (Inverted) in comparison with control animals (Figure 25).

These results demonstrate that artificial hTERT shuffled constructs, pUTScram (Scrambled) and pUTInv (Inverted), were capable to induce significant high levels of hTERT specific CD8 T-cell responses after two immunization cycles as INVAC-1 did. Indeed, as previously demonstrated for INVAC-1, the advantage of a prime-boost vaccination regimen is to selectively boost the previous activated specific T-cells and broaden epitope presentation in order to generate secondary hTERT specific T-cell involving new specific TCRs.

**Vaccination with artificial shuffled hTERT constructs pUTScram and pUTInv induce *in vivo* cytotoxic hTERT specific CD8 T-cells**

Among immune cells that are relevant in antitumor immune responses, cytotoxic CD8 T lymphocytes (CTL) and Th1 CD4 T-cells have been identified as the most powerful effector cells (Vesely, Kershaw et al. 2011) (Braumuller, Wieder et al. 2013).

The cytotoxic activity of hTERT specific CD8 T-cells was investigated *in vivo* after ID vaccination/electroporation with INVAC-1, pUTScram and pUTInv. In order to measure the *in vivo* cytolytic strength of the hTERT specific $CD8^+$ T-cell response elicited by DNA immunization, an *in vivo* cytotoxicity assay was performed using carboxyfluorescein-diacetate succinimidyl ester (CFSE)–labelled and peptide-pulsed splenocytes as target cells. HLA-B7 transgenic mice which received one vaccination with DNA constructs (or PBS as control) *via* the ID route, as described before, were intravenously injected with $10^7$ target cells. Target cells were splenocytes from naive congenic mice independently labelled with 3 different concentrations of CFSE and pulsed with either a hTERT peptide restricted to HLA-B7 (p351, immunodominant peptide or p1123, subdominant peptide) or left unpulsed as an internal control. After 15-18 hours, spleens of immunized mice were harvested and splenocyte suspensions were analysed by flow cytometry. The percentage of specific lysis was evaluated by comparing the ratio of pulsed to unpulsed CFSE labeled cells in vaccinated mice versus control mice.

Results show that all mice immunized with the different constructs developed hTERT specific cytotoxic T lymphocytes (CTLs) after one immunization.

As expected, the cytotoxicity against the immunodominant peptide p351 was higher than against the subdominant peptide p1123 for the three groups (Figure 26).

Immunization with INVAC-1 and pUTInv led to a specific lysis of telomerase immune-dominant (p351) epitopes-bearing target cells of 37% and 35%, respectively (Figure 26, black dots). In comparison, immunization with pUTScram led to a specific lysis of 20%. Two INVAC-1 immunized mice out of five and one pUTScram out of six developed specific CTLs against the subdominant peptide p1123 (Figure 26, grey dots).

As stated previously, it can be expected that multiple injection cycles would allow increasing the number of animals that develop a specific CTL lysis against both immunodominant and subdominant peptides. Indeed, previous results (see EXAMPLE I) demonstrated that a second immunization widens the breadth of the immune response against subdominant epitopes.

In conclusion, as INVAC-1, artificial shuffled hTERT Scrambled or Inverted-mediated immunization can generate hTERT specific CD8 T-cells that exhibit *in vivo* cytolytic activity.

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## CLAIMS

1. A nucleic acid construct comprising a sequence that encodes a human telomerase reverse transcriptase (hTERT) protein which is devoid of telomerase catalytic activity by deletion of amino acids 867-869 (VDD), and of a nucleolar localization signal by deletion of at least amino acids 1-23, wherein the hTERT protein is fused at N-terminus with a protein enhancing addressing of the hTERT protein to proteasome.

2. The nucleic acid construct of claim 1, wherein the hTERT protein is devoid of telomerase catalytic activity by a further deletion of 1 to 12 amino acids upstream and/or downstream amino acids 867-869 (VDD).

3. The nucleic acid construct of claim 1 or 2, wherein the hTERT protein is devoid of a nucleolar localization signal by deletion of amino acids 1-47.

4. The nucleic acid construct of any of claims 1 to 3, wherein the protein enhancing addressing of the hTERT protein to proteasome is ubiquitin.

5. The nucleic acid construct of any of claims 1 to 3, wherein the protein enhancing addressing of the hTERT protein to proteasome is a chaperon protein, such as calreticulin.

6. The nucleic acid construct of any of claims 1 to 5, which is a DNA, preferably a DNA plasmid.

7. The nucleic acid construct of claim 6, which encodes amino acid sequence SEQ ID NO: 12.

8. The nucleic acid construct of claim 7, which comprises SEQ ID NO: 11 or nucleotides 3488 to 6961 of SEQ ID NO: 11.

9. The nucleic acid construct of claim 6, which encodes amino acid sequence SEQ ID NO: 14, 16 or 18, and preferably comprises nucleotide sequence SEQ ID NO: 13, 15, or 17.

10. The nucleic acid construct of any of claims 1 to 9, for use in triggering an immune response in a subject, against cells that overexpress telomerase, preferably dysplasia cells, tumor cells, or cells infected by an oncovirus.

11. The nucleic acid construct of any of claims 1 to 9, for use in preventing or treating a tumor in a patient.

12. The use of the acid nucleic construct of any of claims 1 to 9, in the manufacture of a medicament for the prevention or treatment of a tumor in a patient.