

Strong antigen-specific T-cell immunity induced by a recombinant human TERT measles virus vaccine and amplified by a DNA/viral vector prime boost in IFNAR/CD46 mice

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Abstract:	Cancer immunotherapy is seeing an increasing focus on vaccination with tumor-associated antigens (TAAs). Human telomerase (hTERT) is a TAA expressed by most tumors to overcome telomere shortening. Tolerance to hTERT can be easily broken both naturally and experimentally and hTERT DNA vaccine candidates have been introduced in clinical trials. DNA prime/boost strategies have been widely developed to immunize efficiently against infectious diseases. We explored the use of a recombinant measles virus (MV) hTERT vector to boost DNA priming as recombinant live attenuated measles virus has an impressive safety and efficacy record. Here, we show that a MV TERT vector can rapidly and strongly boost DNA hTERT priming in MV susceptible IFNAR/CD46 mouse models. The cellular immune responses were Th1 polarized. No humoral responses were elicited. The 4 kb hTERT transgene did not impact MV replication or induction of cell mediated responses. These findings validate the MV TERT vector to boost cell-mediated responses following DNA priming in humans.
Corresponding Author:	Elodie Pliquet, Ph.D INVECTYS/Institut Pasteur Paris, FRANCE
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	INVECTYS/Institut Pasteur
Corresponding Author's Secondary Institution:	
First Author:	Elodie PLIQUET, Ph.D
First Author Secondary Information:	
Order of Authors:	Elodie PLIQUET, Ph.D
	Claude RUFFIE
	Marie ESCANDE
	Jessie THALMENSI
	Valérie NAJBURG
	Chantal COMBREDET
	Thomas BESTETTI
	Marion JULITHE
	Christelle LIARD
	Thierry HUET
	Simon WAIN-HOBSON

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	Frédéric TANGY
	Pierre LANGLADE-DEMOYEN
Order of Authors Secondary Information:	
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Invectys
Pépinière Paris Santé Cochin
27, rue du Faubourg Saint Jacques
75014 Paris
France

FOR THE REVIEWERS

Reviewer #1: The authors have responded to comments in a satisfactory way.
Reviewer #2: The revised manuscript is substantially improved and therefore I am happy with it being accepted for publication without the need for any further revision.

Response: We are happy that our additional responses and modifications improved the manuscript and were satisfied for you.

FOR THE EDITORIAL OFFICE

4) "in IFNAR/CD46 mouse model" is not quite correct.
➔ Indeed, "IFNAR/CD46 mice" is more appropriate because there are two different mouse models in this manuscript with IFNAR/CD46 background.

7) Look at your "Author contributions" section.
➔ Done

9) please make sure that both the contents and the English of your entire paper (including all parts) are correct […] Maybe your co-author Simon Wain-Hobson is a native English speaker who could correct the entire final (!) version.
➔Done

Best regards,

Elodie Pliquet, PhD
Invectys
Pépinière Paris Santé Cochin
27**,** rue du Faubourg Saint Jacques
75014 Paris
France
Tel +33 181 800 524/520,
Fax +33 181 800 530
elodie.pliquet@invectys.com

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Strong antigen specific T-cell immunity induced by a recombinant human TERT measles virus vaccine and amplified by a DNA/viral vector prime-boost in IFNAR/CD46 mice

Elodie Pliquet[1,2], Claude Ruffie[3], Marie Escande[1], Jessie Thalmensi[1], Valérie Najburg[3], Chantal Combredet[3], Thomas Bestetti[1], Marion Julithe[1], Christelle Liard[1], Thierry Huet[1], Simon Wain-Hobson[1,2], Frédéric Tangy[3], Pierre Langlade-Demoyen[1,2]

[1] Invectys, Pépinière Paris Santé Cochin, Paris, France

[2] Molecular Retrovirology unit, Institut Pasteur, CNRS-URA 3015, Paris, France

[3] Viral Genomics and Vaccination Unit, Institut Pasteur, CNRS-UMR 3965, Paris, France

Corresponding author:

Elodie Pliquet
Invectys, Pépinière Paris Santé Cochin
27, rue du Faubourg Saint Jacques
75014 Paris
France
elodie.pliquet@invectys.com
+33 181 800 524

ORCID

Elodie PLIQUET: 0000-0003-4242-8569

Thierry HUET: 0000-0001-9842-591X

Simon WAIN-HOBSON: 0000-0002-6406-3858

Frederic TANGY: 0000-0002-5937-5654

Abstract

Cancer immunotherapy is seeing an increasing focus on vaccination with tumor-associated antigens (TAAs). Human telomerase (hTERT) is a TAA expressed by most tumors to overcome telomere shortening. Tolerance to hTERT can be easily broken both naturally and experimentally and hTERT DNA vaccine candidates have been introduced in clinical trials. DNA prime/boost strategies have been widely developed to immunize efficiently against infectious diseases. We explored the use of a recombinant measles virus (MV) hTERT vector to boost DNA priming as recombinant live attenuated measles virus has an impressive safety and efficacy record. Here, we show that a MV-TERT vector can rapidly and strongly boost DNA hTERT priming in MV susceptible IFNAR/CD46 mouse models. The cellular immune responses were Th1 polarized. No humoral responses were elicited. The 4 kb hTERT transgene did not impact MV replication or induction of cell mediated responses. These findings validate the MV-TERT vector to boost cell-mediated responses following DNA priming in humans.

Key words: Cancer, immunotherapy, hTERT, measles virus vaccine, T-cell responses

Précis

Heterologous prime/boost strategies provide exciting opportunities for use in tumor patients because higher immune response to the target antigen can be induced. MV vaccine has impressive safety record.

Abbreviations

AP	Alkaline phosphatase
ATU	Additional transcription unit
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CBA	Cytometric beads array
MOI	Multiplicity of infection
MV	Measle virus
NBT	Nitro blue tetrazolium chloride
PVDF	Polyvinylidene fluoride
ROI	Region of interest
Tg	Transgenic
TMB	3,3′,5,5′-Tetramethylbenzidine
Ubi	Ubiquitin
UCP	Universal cancer peptide

Introduction

Tumors manipulate their microenvironment and the adaptive immune response. With this understanding, immunotherapy has not surprisingly come to the fore as a means to treat cancer [1,2]. To stimulate anti-tumoral adaptive immunity, novel vaccine strategies are required. Attenuation and reverse genetics have allowed the development of viral vectors to deliver tumor–associated antigens (TAAs) [3]. Some live attenuated viral vectors can deliver transgenes directly to professional antigen-presenting cells (APCs) and dendritic cells (DCs) [4] allowing enhanced specific cytotoxic T-cells (CTLs) frequencies [5].

Attenuated measles virus (MV), a member of *Morbillivirus* genus in the *Paramyxoviridae* family, is an enveloped virus with a non-segmented negative single-stranded RNA genome which replicates exclusively in the cytoplasm [6]. Today the attenuated Schwarz/Moraten vaccine strain has a longstanding efficacy and an excellent safety record [7,8]. This vaccine induces strong cellular and humoral immune responses after one or two low-dose injections. Indeed, MV specific CD8 T-cells and antibodies persist for life [9]. The cloned MV Schwarz strain has proved to be an excellent vector allowing the incorporation of up to an additional 6 kb, or ~40% of its genome [10,11]. Proof of concept in humans has recently been demonstrated for a measles vector-based Chikungunya vaccine (MV-CHIK). The phase I clinical trial of this candidate showed that the vaccine was well tolerated and induced robust and functional antibody responses in 100% of volunteers after 2 immunizations [12]. This trial also demonstrated that pre-existing measles antibodies (anti-vector immunity) did not impair the immunogenicity of the heterologous antigen, paving the way for using recombinant MV as a vaccine vector [13,12]. Some TAAs like carcinoembryonic antigen (CEA) have also been efficiently expressed in this vector [14,15].

Telomerase reverse transcriptase (TERT) has emerged as a near universal tumor antigen and is actively investigated as a target for cancer immunotherapy [16]. Human telomerase (hTERT) is the rate-limiting catalytic subunit of the telomerase enzyme that synthesizes telomere DNA at chromosome ends [17]. Telomerase transcriptional activation has become the most important tumor escape mechanism to circumvent telomere-dependent pathways of cell death [18]. Indeed, hTERT is overexpressed in >85% human tumors regardless of their origin [19] and is associated with poor prognosis [20,21]. Natural anti-hTERT immune responses in some cancer patients and a pre-existing anti-hTERT repertoire in healthy volunteers show that tolerance to telomerase may be readily overcome [22]. Towards this end, we have developed a hTERT

modified DNA plasmid (INVAC-1) vaccine candidate that elicited strong specific immune responses and reduced tumor growth in mice [23]. INVAC-1 is currently in an ongoing phase I clinical trial (NCT02301754).

DNA vaccines offer numerous advantages such as their capacity to readily incorporate multiple genes, their easy engineering and their stability [24]. Heterologous prime-boost strategies using a DNA prime followed by boosting with viral vectors have been shown to increase the magnitude of immune responses to HIV [25], Chikungunya virus [26], melanoma [27] or breast cancer [28]. Here, we have developed a recombinant MV vector expressing the insert of INVAC-1, which is a modified ubiquitin-hTERT fusion protein, as immunotherapeutic agent. In mouse models susceptible for MV infection, we demonstrate that immunization with a single low-dose of this construct referred to as MV-TERT, elicited strong hTERT specific cytotoxic cellular immune responses. The vector could expand primary hTERT memory responses induced by DNA priming.

Materials and Methods

Construction of measles virus telomerase construct

The pTM-MVSchw-ATU2 plasmid encodes the Ubi-hTERT insert of INVAC-1 [23] cloned into the additional transcription unit (ATU) inserted between the P and M genes of an infectious molecular clone of the Schwarz MV vaccine strain [11]. The INVAC-1 insert carries a 9 bp deletion that removes three amino acids (867VDD869) crucial to hTERT catalytic activity [29]. The first 47 residues encoding the nucleolar localization sequence (NoLS) [30] were replaced by human ubiquitin (76 residues) according to the ubiquitin-fusion approach [31] along with an HLA-A*0201 restricted influenza A virus epitope and V5 tag was added at the carboxy terminus to facilitate characterization. The entire transgene was codon optimized. The length of the insert (3576 bp) was such that the “rule of six” was respected - the number of nucleotides in a MV genome must be a multiple of 6 to allow efficient replication [32]. The transgene was synthetized by GeneCust (Luxembourg) and subcloned into the BsiWI/BssHII restriction sites of the pTM-MVSchw-ATU2 vector generating a plasmid designated pTM-MV-TERT.

Rescue of recombinant MV-TERT and MVSchw from cloned cDNAs

Recombinant MV-TERT and MVSchwarz (MVSchw) viruses were recovered using a helper cell-based rescue system [33,34]. HEK-293-T7-MV cells stably expressing both the T7-RNA polymerase and MVSchw N and P proteins, were co-transfected with 5 µg of pTM-MV-TERT

or pTM-MVSchw DNA and 20 ng of the pEMC-L plasmid that expresses the MV polymerase L gene. After incubation overnight at 37°C, the cells were heat shocked at 43°C for 3 hours and transferred onto Vero cell monlayers and incubated at 37°C for two days. Individual syncytia were isolated and transferred to 35 mm wells with a new monolayer of Vero cells and then expanded in 25 cm² and finally 150 cm² flasks. Viruses were harvested when syncytia reached 90% of the culture and titers were determined by an endpoint limit dilution assay on Vero cells. Viral titers were calculated using the Kärber method and were expressed as TCID50/mL.

Cell lines

HEK293-T7-MV helper cells were cultured in DMEM (Gibco) without sodium pyruvate supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (Life Technologies, Saint-Aubin, France). Vero cells were maintained in DMEM without sodium pyruvate supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (Life Technologies, Saint-Aubin, France). For co-culture, Vero cells were seeded at 5 x 10^6 cells and grown as monolayers in 10 cm dishes at 37°C and 5% CO_2 until single syncytia formation. For amplification, 3 x 10^5 Vero cells were seeded in 12 well plates and grown to 80-90% confluence, at which time each syncytium was then transferred.

Western blotting

Vero cells were seeded at 2 x 10^6 cells and grown as monolayers in T-25 flasks and infected at a multiplicity of infection (MOI) of 0.1. Forty-eight hours later, when syncytia reached 80-90% confluence, cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, USA) supplemented with a protease inhibitor cocktail (Roche Diagnostic, Indianapolis, USA). Proteins were separated on Nu-PAGE® Novex 4-12% Bis-Tris gels (Invitrogen, Carlsbad, USA) and electroblotted onto Polyvinylidene fluoride (PVDF) membranes (iBlot® device, Invitrogen, Carlsbad, USA). hTERT proteins were detected with primary mouse anti-V5 monoclonal antibody (R960-25; Invitrogen, Carlsbad, USA) and nucleoproteins with a primary mouse anti-MV N monoclonal antibody (Abcys, Courtaboeuf, France). A sheep anti-mouse IgG-HRP conjugate (NA931; GE Healthcare, Buckinghamshire, UK) was used as secondary antibody. Novex® Sharp Prestained Protein Ladder (Invitrogen, Carlsbad, USA) were used to determine molecular weight. β-Actin was used as loading control. Peroxidase activity was detected on films with chemiluminescence ECL HRP substrate reagent kit (GE Healthcare, Buckinghamshire, UK).

Immunization

MV susceptible IFNAR/CD46 and HHD/IFNAR/CD46 mice were obtained as described [34-36] and were housed under specific pathogen-free conditions at the Institut Pasteur animal facility. The IFNAR/CD46 strain has been accepted by the FDA as a model of measles toxicity prior to initiation of clinical trials of engineered MV strains. Transgenic mice between 6 and 16 weeks of age were inoculated intraperitoneally (i.p.) once or twice with 10^5 TCID50 of recombinant MV-TERT or MVSchw. To evaluate induction of humoral specific response, transgenic (Tg) mice were inoculated with 10^5 TCID50 (D0) and 8 x 10^4 TCID50 (D28) of MVSchw and MV-TERT at 1 month intervals. For the heterologous prime-boost, priming was performed via the intradermal (i.d.) route at the base of the tail (bilateral injections) using 25 µg of INVAC-1 plasmid coding for Ubi-ΔhTERT [23] or PBS, as control. Directly after DNA vaccination, electroporation was performed using CLINIPORATOR® 2 (IGEA, Carpi, Italy). The following train of pulses was applied using non-invasive plate electrodes (P-30-8G, IGEA, 0.5 cm apart); one high voltage pulse (100 µs duration; 1,000 V/cm) followed 1 s later by one low voltage pulse (400 ms duration; 140 V/cm). Twenty-one days later, mice received an i.p. boost injection with 10^5 TCID50 of MV-TERT or MVSchw.

ELISpot assays

HLA-A*0201 restricted hTERT peptides have been described [37,38]. H2-K/Db and H2-IAb restricted hTERT peptides were determined *in silico* using the following online epitope prediction algorithms: 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) and are described in Table 1. Peptides were dissolved in sterile water at 2 mg/mL and stored at -80°C or -20°C prior use.

Murine IFN-γ kits were purchased from Diaclone (Eurobio, Courtaboeuf, France) and used following the manufacturer's instructions. Ficoll-purified lymphocyte cell suspensions from peripheral blood or spleen were stimulated in triplicate at 2 x 10^5 cells/well with pools of HLA-A*0201, H2-K/Db or H2-IAb restricted hTERT peptides at 5 µg/mL, with serum free RPMI culture medium (as negative control), with MVSchw at MOI = 1 (as immunization control) or with PMA-Ionomycin as positive control (0.1 µM and 1 µM respectively). After 19 hours, spots were revealed with the biotin-conjugated detection antibody followed by streptavidin-alkaline phosphatase (AP) and 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium chloride

(BCIP/NBT) substrate solution. Spots were counted using the Immunospot ELISpot counter and software (Cellular Technology Limited, Bonn, Germany).

Bioluminescence imaging

Transgenic mice were immunized with 10^5 TCID50 of recombinant MV-Luc expressing the luciferase gene [39] or MVSchw as control. Immunized mice received i.p. 150 mg/kg body weight of a D-luciferin potassium salt solution at 30 mg/mL (Perkin Elmer Life Sciences, Villebon-sur-Yvette, France). Five minutes later, they were anesthetized with 2–3% isoflurane (Attane Isoflurane, JD Medical Dist. Co., Inc., Phoenix, AZ, USA) delivered in 100% oxygen at a flow rate of 0.8 liter/min and imaged using an IVIS Lumina while the results were analyzed using Living Image software (both Caliper Life Sciences, Hopkinton, MA, USA). Luciferin signals were followed during 8 days until the mice were euthanized using region of interest (ROI) drawing. Bioluminescence signal was expressed as average radiance (ph/sec/cm²/sr).

***In vivo* cytotoxicity assay**

The capacity of CD8 CTLs to kill peptide loaded target cells *in vivo* was assessed as described [40]. Briefly, splenocytes from naive IFNAR/CD46 mice were split in three and labeled with 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). Subsequently, $CFSE^{high}$-labeled cells were pulsed with the immunodominant hTERT p660 peptide and $CFSE^{medium}$-labeled cells were pulsed with the subdominant p1021 hTERT peptide for 1.5 hours whereas $CFSE^{low}$-labeled cells were left unpulsed. Cells were mixed in a 1:1:1 ratio and 6.8 x 10^6 cells in 50 µL of PBS were intravenously injected at day 7 into mice previously vaccinated with MV-TERT, MVSchw or PBS. Fifteen hours later, single-cell suspensions from immunized mice spleen were analyzed by MACSQuant® flow cytometer (Miltenyi, Germany). The percentage of specific killing was determined as follows:

$[1 - [\text{mean}(\%CFSE^{low}/CFSE^{high\ or\ medium})_{CONTROL}/(\%CFSE^{low}/CFSE^{high\ or\ medium})_{IMMUNIZED}]] \times 100$.

ELISA for humoral responses

Sera collected before immunization (D0) and 1 month after each vaccination (D28 and D49) were heat inactivated. MV-specific Ig antibodies were measured by using commercial ELISA kit (Trinity Biotech, USA). Briefly, plates were coated overnight with 50 ng of MV antigens and D28 and D49 sera were serially diluted to determine the end point positive limit dilution.

An anti-mouse antibody-HRP conjugate (Amersham) was used as secondary antibody with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate to obtain sample absorbance. Anti-MV titers were calculated as the highest serum dilution between maximum and minimum absorbance value of a 1/100 dilution of mixture control sera (mean OD+5SD).

The presence of hTERT-specific binding antibodies in sera was performed by Bertin Pharma (France) according to a validated qualitative ELISA immunoassay. Sera were incubated with a recombinant hTERT peptide (P165-S348, GenWay, San Diego, USA). A secondary goat anti-mouse IgG conjugated to AP was added. Antigen-Ab complexes were visualized by addition of a chromogenic substrate. Results are expressed as mean ratios (R) where R = OD values/cut off point; cut off point = normalized cut off x mean of eight determinations of the negative pool of matrices. Positive or negative results were obtained according the control (QC) ratio with an anti-hTERT monoclonal antibody (MAB6595, R&D Systems) diluted 1:4000 (Low QC) and 1:50 (High QC) in pool of negative serum samples from non-immunized mice.

T-cell cytokine secretion

For the heterologous prime-boost, at day 28, ficoll-purified splenocytes (6 x 10^5 cells) from vaccinated IFNAR/CD46 mice were cultured for 24 h at 37°C with H2-K/Db-restricted hTERT peptides (429, 660, 1021, 1034) at 5 µg/mL or with MVSchw at an MOI = 1. IL-2, IFN-γ, TNF-α, IL-4, IL-6, IL-17a and IL-10 were quantified simultaneously on supernatants using the Cytometric beads array mouse kit (CBA, BD biosciences) according to the manufacturer's instructions. Flow cytometry acquisition was performed using the FACScan LSR Fortessa flow cytometer (BD Biosciences); quantitative analyses were performed using the FCAP Array TM Software version 3.0 (BD Biosciences).

Statistical analyses

GraphPad Prism 6.0 software was used for data handling, analysis and graphic representations. Data are represented as the mean ± standard deviation. Statistical analyses were performed using a two-tailed Mann Whitney test. Significance was set at p-value ≤ 0.05.

Results

Efficient expression of hTERT from recombinant MV vector

The Ubi-ΔhTERT-Flu-V5 transgene was cloned into pTM-MVSchw-ATU2 (Fig. 1a). Expression of hTERT fusion protein was assessed by Western blot using MV-TERT infected-

cell lysates with V5-specific antibodies (Fig. 1b). The full length Ubi-ΔhTERT-Flu-V5 fusion protein was identified at the predicted size of 130.9 kDa. The strongest band at 122.5 kDa corresponds to processing and removal of the ubiquitin moiety from the fusion protein as previously reported for INVAC-1 [23]. There is an additional, weaker N-terminal degradation product around 100 kDa, which is not surprising given that ubiquitin was added to facilitate degradation. Both MV-TERT and MVSchw constructs expressed the 57.7 kDa MV nucleoprotein to comparable levels (Fig. 1b) indicating the stability of MV-TERT construct.

Immunization elicited high frequencies of hTERT specific CD8 T-cells

The immunogenicity of MV-TERT recombinant vector was assessed using MV susceptible IFNAR/CD46 and HHD/IFNAR/CD46 mice. Mice were immunized i.p. with 10^5 TCID50 of each virus. Both hTERT and MV specific T-cell responses were monitored in splenocytes collected at 7 or 14 days post-immunization using an IFN-γ ELISpot assay with specific hTERT peptides for *ex vivo* re-stimulation and MVSchw virus for control stimulation (Fig. 2). To assess MV-TERT immunogenicity in HHD/IFNAR/CD46 mice, splenocytes were stimulated *in vitro* with a mix of HLA-A*0201 restricted hTERT peptides for a week. As expected, strong hTERT specific CD8 T-cell responses were observed in IFNAR/CD46 or HHD/IFNAR/CD46 MV-TERT immunized mice at 7 and 14 days after the last immunization compared to the MVSchw controls ($p<0.01$) (Figs. 2a and b). Similarly, hTERT specific CD4 T-cells were detected in MV-TERT immunized mice for all H2-restricted hTERT peptides tested, as compared to the MVSchw controls (Fig. 2c). Interestingly, the strength of MV specific T-cell responses was comparable for the two viruses indicating that the hTERT transgene did not impact the existing immunogenicity of MV (Figs. 2a and c).

Specific T-cells frequencies were highest on day 7 for control MVSchw and MV-TERT, while their frequencies decreased slightly by day 14 (i.e. Fig. 2a) indicating that both MVSchw and MV-TERT replicate efficiently and probably cleared after 6-7 days post-immunization due to the ramping up of immune responses. To corroborate this interpretation, vector replication and clearance were measured *in vivo* using a recombinant MV-luciferase vector (pTM2-MV/Luc [39]) and monitored by bioluminescence imaging (Fig. 2d). Luciferase activity was detected between 1-6 days post-infection (maximum average radiance at D3 = 2.01 x 10^5 p/s/cm^2/sr; Fig. 2d left) and had virtually disappeared by D8 when sacrificed. Even dissection of the animals failed to identify more luciferin activity at day 8 (D8) (Fig. 2d far right).

MV-TERT specific CD8 T-cells are cytolytic *in vivo*

To demonstrate that recombinant MV-TERT induces cytolytic hTERT specific CD8 T-cells, an *in vivo* killing assay was performed in IFNAR/CD46 mice using, CFSE–labeled and hTERT peptide pulsed (p660 and p1021) splenocytes or unpulsed splenocytes as target cells. Flow cytometry showed a strong decrease of p660 and p1021 pulsed CFSE cells in MV-TERT immunized mice compared to MVSchw controls illustrating that specific target cells were lysed when they were adoptively transferred into congenic recipient MV-TERT immunized mice (Fig. 3a). Human TERT specific CTLs from MV-TERT immunized mice killed ~50% of p660 pulsed cells and ~14% of p1021 pulsed cells (Fig. 3 b).

MV-TERT did not elicit hTERT antibodies

As MV infection is cytolytic, we wondered whether MV-TERT vaccination might induce hTERT antibodies. To explore this, IFNAR/CD46 and HHD/IFNAR/CD46 mice were immunized twice at a one month interval. MV and TERT specific ELISAs were performed using sera collected at D0, D28 and D49 (Fig. 4). The TERT ELISA plates were coated with a recombinant protein, residues 165-348, as target. As positive or negative results were determined with R = OD values/cut off point and according the control (QC) ratio (IFNAR/CD46: R QC high= 7.27-7.67; R QC low= 1.46-1.50; cut-off = 0.252. HHD/IFNAR/CD46: R QC high= 8.30-8.99; R QC low= 1.43-1.44; cut-off = 0.279), no anti-hTERT antibodies were detected in both mouse strains after one or two immunizations (Figs. 4a and b). By contrast, for both mouse strains, anti-MV antibodies were detected after one MV-TERT immunization (D28: IFNAR/CD46 = 81,000; HHD/IFNAR/CD46 = 6,800) followed by a 3-7-fold increase after the second immunization (D49: IFNAR/CD46 = 280,000; HHD/IFNAR/CD46 = 52,000) (Figs. 4c and d). These titers did not differ significantly to those generated by the control MVSchw virus (Figs. 4a and b). These findings confirm that the TERT transgene did not impact MV humoral immunogenicity.

Heterologous prime-boost elicited enhanced and multifunctional hTERT responses

Heterologous prime-boost vaccination can enhance vaccine specific immune responses in infectious diseases and cancer [41]. To test this strategy, we vaccinated a group of ten IFNAR/CD46 mice with INVAC-1 DNA and boosted six with MV-TERT and four with MVSchw 21 days later (Fig. 5). TERT specific CD8 T-cells were monitored by an IFN-γ ELISpot assay in PBMCs over time (Fig. 5a) and in splenocytes at each end-point (Fig. 5b). Priming with a single shot of INVAC-1 resulted in weak to no hTERT specific CD8 T-cell

responses at 14 days post-immunization; only 2/10 mice developed weak responses in blood (Fig. 5a). This is not surprising given that DNA priming is known to be poor in IFNAR KO mice [42,43]. Animals were boosted with MV constructs 21 days post DNA prime immunization. One week later strong hTERT specific CD8 T-cell responses were detected in the PBMCs of MV-TERT boosted group in comparison of MVSchw boosted mice (Fig. 5a, mean #spots: 118.5; n=6). In the spleen, significant numbers of hTERT specific CD8 T-cells were detected after boosting with MV-TERT compared to controls (Fig. 5b, mean #spots: 453.7 (DNA+MV-TERT; n=6) versus 0.7 (DNA; n=6) or 22.5 (DNA+MVSchw; n=4); p=0.0022 and 0.0095) and compared to only one MV-TERT injection (Fig. 5b, mean #spots: 453.7 (DNA+MV-TERT; n=6) versus 160.4 (MV-TERT; n=13); p=0.0047). A significant difference in MV specific T-cell responses was observed between a MV-TERT prime and DNA+MV-TERT boost as expected from the immunization strategy. In keeping with this, no difference in MV specific T-cell responses between DNA+MVSchw or DNA+MV-TERT boost were found (Fig. 5b).

In order to demonstrate the functionality of the hTERT and MV specific T-cells after heterologous prime-boost vaccination, secreted cytokines were assessed using CBA with overnight stimulation supernatant. Significant concentrations of IL-6, IFN-γ and TNF were secreted by hTERT specific CD8 T-cells induced by MV-TERT compared to MVSchw, (Fig. 5c). MV specific T-cells secreted the same cytokine, IL-2, IL-6, IL-10, IFN-γ and TNF, whether boosted by MV-TERT or MVSchw (Fig. 5d). Taken together, these results demonstrated that priming with INVAC-1 DNA and boosting by MV-TERT elicit a powerful TERT specific Th1 response while a more mixed polarized response was induced to MV.

Discussion

With a heterologous prime-boost strategy in mind, we developed a recombinant MV vaccine vector expressing the modified ubiquitin-hTERT fusion protein, to increase the magnitude of hTERT specific immune responses. This choice reflects promising vector properties of the MV vaccine that is characterized by long standing safety and efficacy with immunity persisting for up to 25-30 years [8,14]. As MV targets the human CD46 receptor, it allows hTERT antigen production directly within macrophages, DCs and lymphocytes [8]. The recombinant MV-vectored Ubi-ΔhTERT-Flu-V5 fusion protein was efficiently expressed and presented a similar degradation profile compared to native INVAC-1 reflecting ubiquitin-fusion [44]. As the construct carried a deletion in the catalytic site resulting in complete activation, there was no

risk of cellular transformation. This is further compounded by the fact that MV infection is lytic while infected cells are marked out for destruction by viral antigen presentation. Bioluminescence imaging data demonstrated efficient vector replication two days after immunization and its partial clearance six days later by the ramping up of MV specific immune response in Tg IFNAR/CD46 mice, confirming the transitory nature of MV transgene delivery *in vivo*.

The MV-TERT construct induced high levels of MV and hTERT specific CD8 and CD4 T-cells seven and fourteen days after a low dose (10^4-10^5 TCID50) immunization. The hTERT specific CD8 T-cells were able to recognize and lyse specific target cells *in vivo,* which is crucial to the induction of antitumor immunity. An important consideration in this study for the recombinant MV kinetics is the choice of IFNAR KO mice model that allows powerful and rapid viral replication in the absence of IFN type I context and subsequently the induction of specific immune response within seven days [45]. Similar experiments using a recombinant wild-type MV-EGFP in macaques have demonstrated that the levels of MV-infected lymphocytes increase exponentially during the first 7–9 days due to viral replication but decrease rapidly during the subsequent week with the appearance of MV specific T-cells [46].

The primary goal of the present study was to test a heterologous INVAC-1 DNA prime – MV-TERT boost strategy to expand hTERT specific immune responses. As expected for IFNAR KO mice, the present findings reiterate the observation that DNA vaccination is weak in this background [42,43] compared to our previous work with INVAC-1 DNA on syngeneic or HLA Tg mice only [23]. It also should be noted that hTERT specific CD8 T-cell response induced by heterologous prime-boost was 2-3 fold higher than the response after one MV-TERT immunization (Fig. 5b vs. Fig. 2a). This suggests that INVAC-1 DNA vaccination in IFNAR/CD46 mice induced hTERT specific CD8 T-cell repertoires but did not enable their expansion, in contrast to MV-TERT immunization. Indeed, low dose of DNA and its adjuvant properties are generally efficient to initiate and expand primary immune responses but they are less relevant to raise the level of secondary memory responses in contrast of live attenuated viral vectors [47].

This, along with our results, shows that INVAC-1 DNA prime and MV-TERT boost elicit hTERT specific and effective CD8 T-cells with Th1 polarization in a mixed response to MV [48]. In addition, differences in MV specific T-cell responses observed between a MV-TERT

prime and DNA+MV-TERT prime-boost but not observed between MVSchw or MV-TERT boost confirm a DNA-activated-immune context. These results open the way to undertaking a phase I combination DNA prime, MV boost clinical trial.

Author contributions

Simon Wain-Hobson, Frédéric Tangy and Pierre Langlade-Demoyen conceived the project. They also shared their knowledge and experience to help Elodie Pliquet with the experimental program design.

Elodie Pliquet performed and analyzed the experiments under the supervision of Christelle Liard and Thierry Huet. Elodie Pliquet and Claude Ruffie carried out mice experiments. Valérie Najburg and Chantal Combredet constructed the recombinant measles-telomerase virus. Marie Escande, Jessie Thalmensi, Thomas Bestetti and Marion Julithe provided technical help.

Elodie Pliquet analysed all the data and wrote the manuscript together with Simon Wain-Hobson and Frédéric Tangy. All authors viewed and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval and ethical standards

All protocols have been submitted and approved by the Pasteur Institute office of laboratory animal care CETEA N°89 under the references 2013-0026 on February 01st, 2013 and 2014-0061 on October 07th, 2015 and by French Committee for Hygiene, Safety and Working

Conditions (CHSCT) under the reference 07.157 on April 10th, 2007 and 16.0013 on January 8th, 2016.

All *in vivo* experiments were conducted in strict accordance with good animal practice and complied with local animal experimentation and ethics committee guidelines of the Pasteur Institute (agreement N°75-15-01 delivered on the 06th September 2013 and N°A75-15-01-1 on 18th August 2014) and Directive 2010/63/EU on the harmonization of laws, regulations and administrative provisions relating to the protection of animals.

Animal source

IFNAR/CD46 (IFN-α/βR$^{-/-}$ CD46$^{+/-}$) mouse strain used for immunization experiments was generated by Frédéric Tangy's team by cross-breeding and backcross between FVB/CD46$^{+/-}$ mice (gift from F. Grosveld, Erasmus University, Rotterdam, The Netherlands) and 129sv IFN-α/βR$^{-/-}$ mice which lack the type I IFN receptor (gift from M. Aguet, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland).

HHD/IFNAR/CD46 (HHD$^{+/+}$ CD46$^{+/-}$ IFN-α/βR$^{-/-}$) mouse strain was generated by Frédéric Tangy's team by cross-breeding and backcross between HHD mice (human β2m–HLA-A2.1 α1α2-H-2D^{b} α3-transmembrane and H-2Db$^{-/-}$ β2mb$^{-/-}$ double knockout; mice generated at Pasteur Institute by François Lemonnier) and IFNAR/CD46 mice.

Cell line authentication

HEK-293-T7-MV cell line was produced, provided and patented by Frédéric Tangy and the Institut Pasteur. Vero cells were provided by the American Type Culture Collection (ATCC®) under reference number CCL-81™.

Figure legends

Fig. 1 Recombinant MV-TERT construction and expression

(a) Schematic maps of the pTM-MV-TERT plasmid. The MV orfs are: N, nucleoprotein; P, phosphoprotein including C and V proteins; M, matrix; F, fusion; H, hemagglutinin; L, polymerase. T7, T7 RNA polymerase promoter; hh, hammerhead ribozyme; h∂v, hepatitis delta virus ribozyme; T7t, T7 RNA polymerase terminator. **(b)** Western blot of hTERT transgene and MV nucleoproteins. β-Actin served as loading control.

Fig. 2 Induction of hTERT specific CD8 and CD4 T-cells

(a) MV and hTERT CD8 specific T-cell responses were evaluated seven or fourteen days post-immunization (MVSchw n=4/day; MV-TERT n=6/day) by IFN-γ ELISpot assay with MV or a pool of H2-K/Db restricted hTERT specific peptides. **(b)** IFN-γ ELISpot assay was performed using a mix of hTERT specific peptides restricted to HLA-A*0201 on HHD/IFNAR/CD46 mice seven days post-immunization (MVSchw n=4; MV-TERT n=6) after one week of *in vitro* stimulation with the same peptides. **(c)** Seven days post-immunization (n=3 mice/group), MV, hTERT CD8 and CD4 specific T-cell responses were evaluated using MV, pool of H2-K/Db restricted hTERT peptides or individual H2-IAb restricted hTERT peptides. **(a, b, c)** MV stimulation was used as immunization control. Data are represented as mean±SD. Mann Whitney non-parametric test against mice control (MVSchw), **p<0.01. **(d)** Kinetics of recombinant MV-Luc replication cycle on IFNAR/CD46 mice (MVSchw n=1; MV-TERT n=5, data shown 2/5 mice).

Fig. 3 MV-TERT induces hTERT specific cytotoxic T-cells *in vivo*

(a) The disappearance of naive peptide-pulsed-splenocyte in spleens of IFNAR/CD46 mice immunized once with PBS, MVSchw or MV-TERT was analyzed by flow cytometry (mean data shown per group). Gating was based on FSC/SSC parameters with doublet discrimination. Viable single stained cells were analyzed with FITC channel corresponding to CFSE fluorescence. **(b)** Percent killing for p660 (black bars) and p1021 (hatched bars) was calculated using cytometry data and was presented as mean±SD (MVSchw n=3; MV—TERT n=5). Mann Whitney non-parametric test against mice control (MVSchw), *p<0.05.

Fig. 4 Antibody responses following MV-TERT immunization

(a, b) Detection of anti-hTERT antibody after MV-TERT prime-boost immunization (before immunization (D0) and one month after last immunization (D28 and D49)). Results are expressed as mean ratio (R)±SD. Mann Whitney non-parametric test against MVSchw control was performed for each day. **(c, d)** Anti-MV antibody titers detected according the same schedule. Results are expressed as mean values±SD determined in serial dilutions of sera. Mann Whitney non-parametric test against MVSchw control was performed. **(a, c)** Data on IFNAR/CD46 mice (MVSchw n=3; MV-TERT n=5) and **(b, d)** on HHD/IFNAR/CD46 (MVSchw n=4; MV-TERT n=6).

Fig. 5 Heterologous DNA prime / MV-TERT boost induced multiple patterns of T-cell responses

(a) Individual hTERT CD8 specific T-cell response at days 0, 7, 14 and 21 post-priming and at day 7 post-boost (D28) on PBMCs stimulated with pool of hTERT specific peptides restricted to H2-K/Db. Black arrows indicate vaccination. **(b)** At day 28, MV specific T-cells and hTERT specific CD8 T-cells were detected on ficoll-purified splenocytes with MV or pool of hTERT specific peptides restricted to H2-K/Db. Data for prime with PBS (n=4), INVAC-1 (n=6) or MV-TERT (3 experiments, n=13) (hatched bars) and prime-boost with INVAC-1 + MVSchw (n=4) or MV-TERT (n=6) (grey bars) are represented as mean±SD. MV specific T-cell stimulation was used as immunization control. Mann Whitney non-parametric test between INVAC-1+MV-TERT and INVAC-1 DNA or MV-TERT alone, $^{**}p<0.01$. **(c)** At day 28, concentration of different cytokines secreted by hTERT specific CD8 T-cells and **(d)** by MV specific T-cells was evaluated using CBA assay. Cytokine concentrations in pg/mL are represented as mean±SD. Mann Whitney non-parametric test against mice control (MVSchw), $^{*}p<0.05$; $^{**}p<0.01$.

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Tables

Table 1. hTERT peptides

Peptide	Numbered to	Sequence	MHC	Mouse Strain
429	Ubi-ΔhTERT	HAQCPYGVL	H2-Kb	IFNAR/CD46
1034		QAYRFHACVL		
660		RPIVNMDYV	H2-Db	
1021		QTVCTNIYKI		
85		VCVPWDARPPPAAPS	H2-IAb	IFNAR/CD46
86		CVPWDARPPPAAPSF		
87		VPWDARPPPAAPSFR		
329		GRQHHAGPPSTSRPP		
1080		MSLGAKGAAGPLPSE		
1082		LGAKGAAGPLPSEAV		
1137		TLTALEAAANPALPS		
1138		LTALEAAANPALPSD		
540 [37]	hTERT	ILAKFLHWL	HLA-A*0201	HHD/IFNAR/CD46
Y572 [37]		YLFFYRKSV		
Y988		YLQVNSLQTV		
UCP2.1 [38]		SVWSKLQSI		
UCP4.1 [38]		SLCYSILKA		

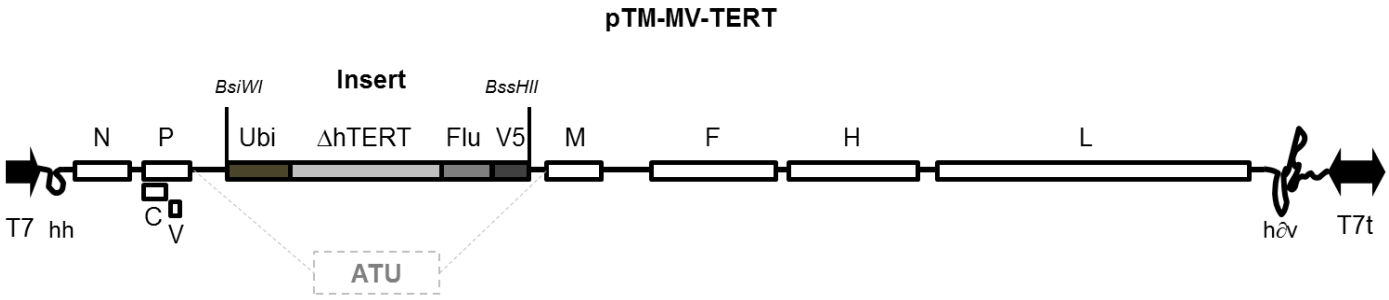
Following H2 restricted Ubi-ΔhTERT peptides have been predicted *in silico*; HLA-A*0201 restricted hTERT peptides were previously described [37,38]. Peptides were used either in ELISpot assay, CBA or *in vivo* cytotoxicity assay according to the strains of mouse.

Figure 1

Click here to access/download;Figure;MVTERT-submitted version Figure 1-CII.ppt

FIGURE 1

a



b

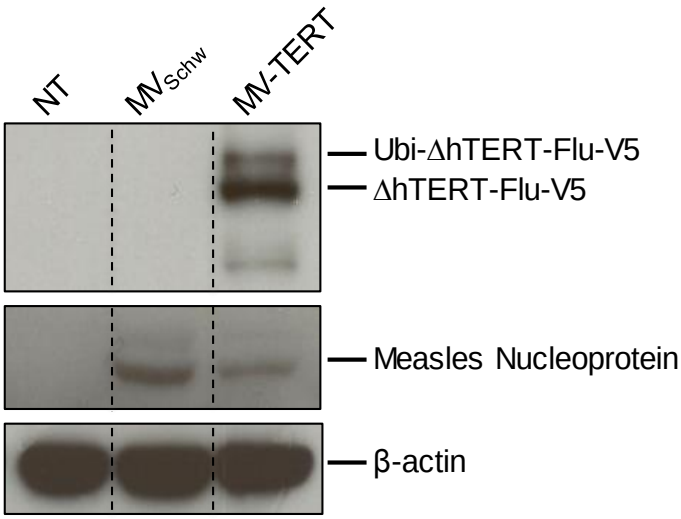


Figure 2

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FIGURE 2

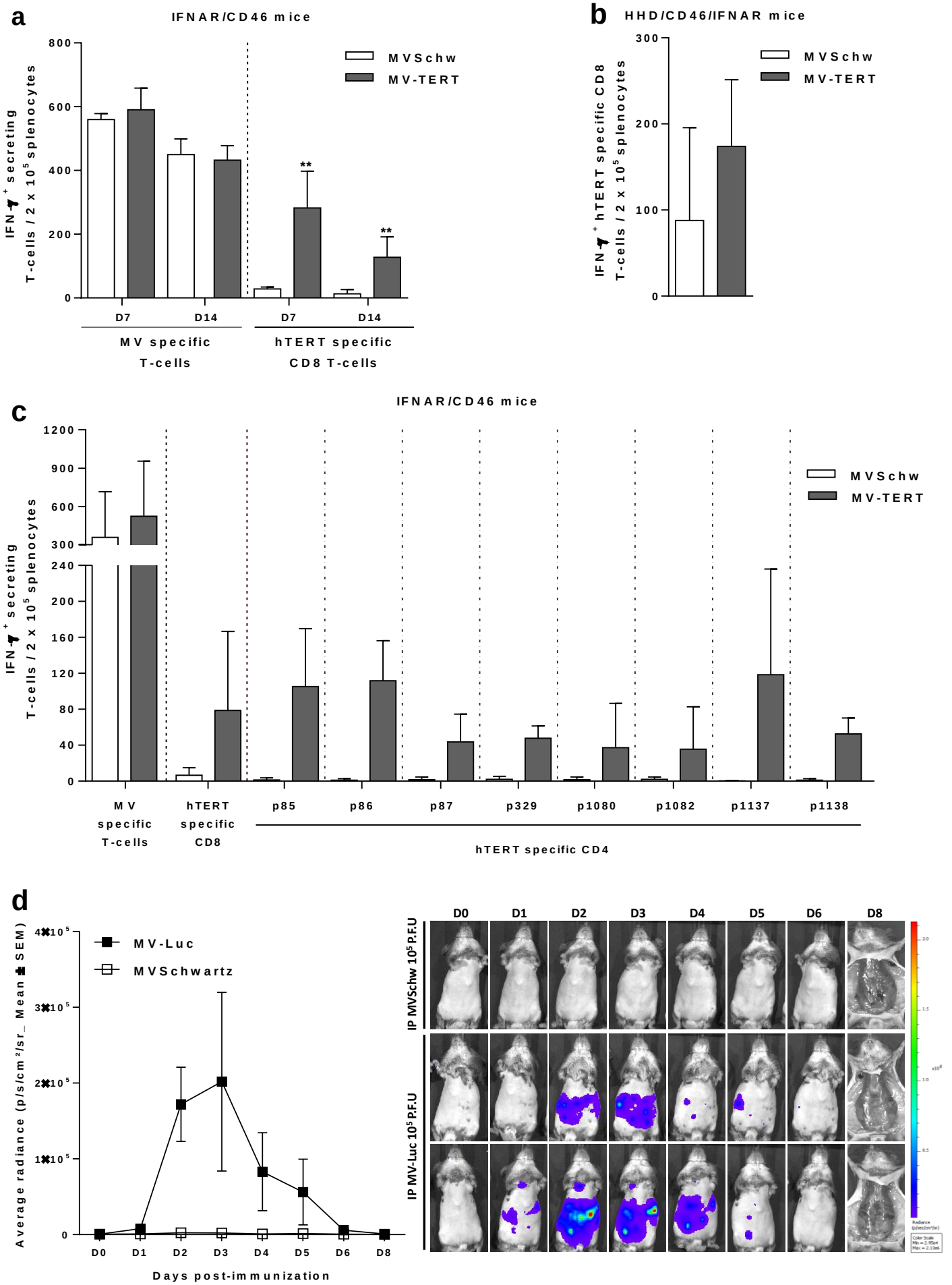


Figure 3

Click here to access/download;Figure;MVTERT-submitted version Figure 3-CII.ppt

FIGURE 3

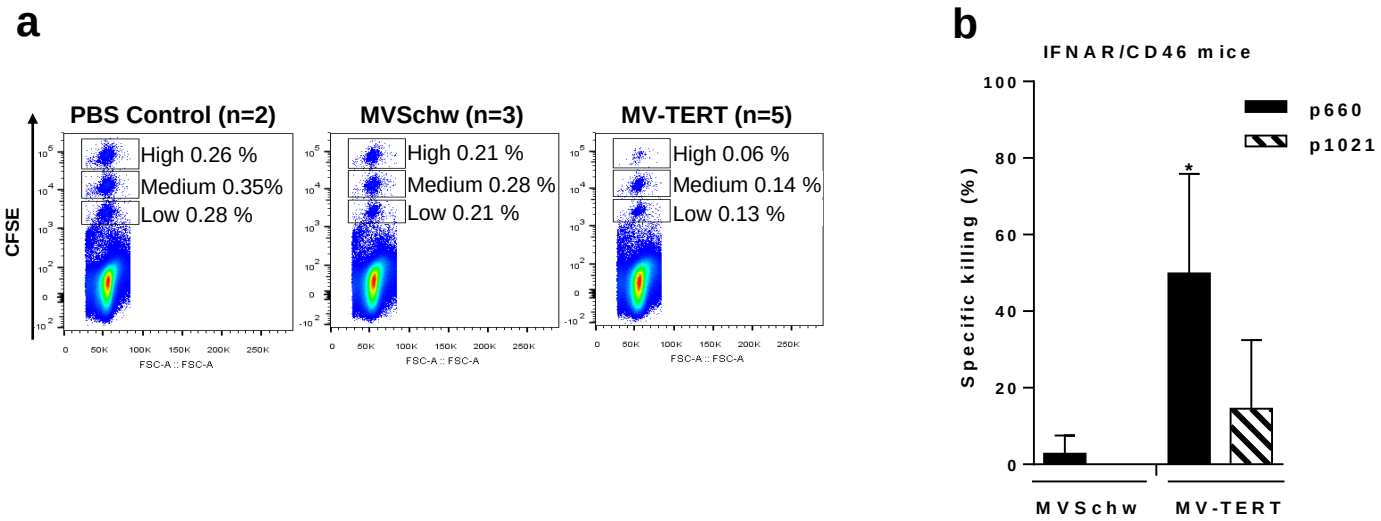


Figure 4

Click here to access/download;Figure;MVTERT-submitted version Figure 4-CII.ppt

FIGURE 4

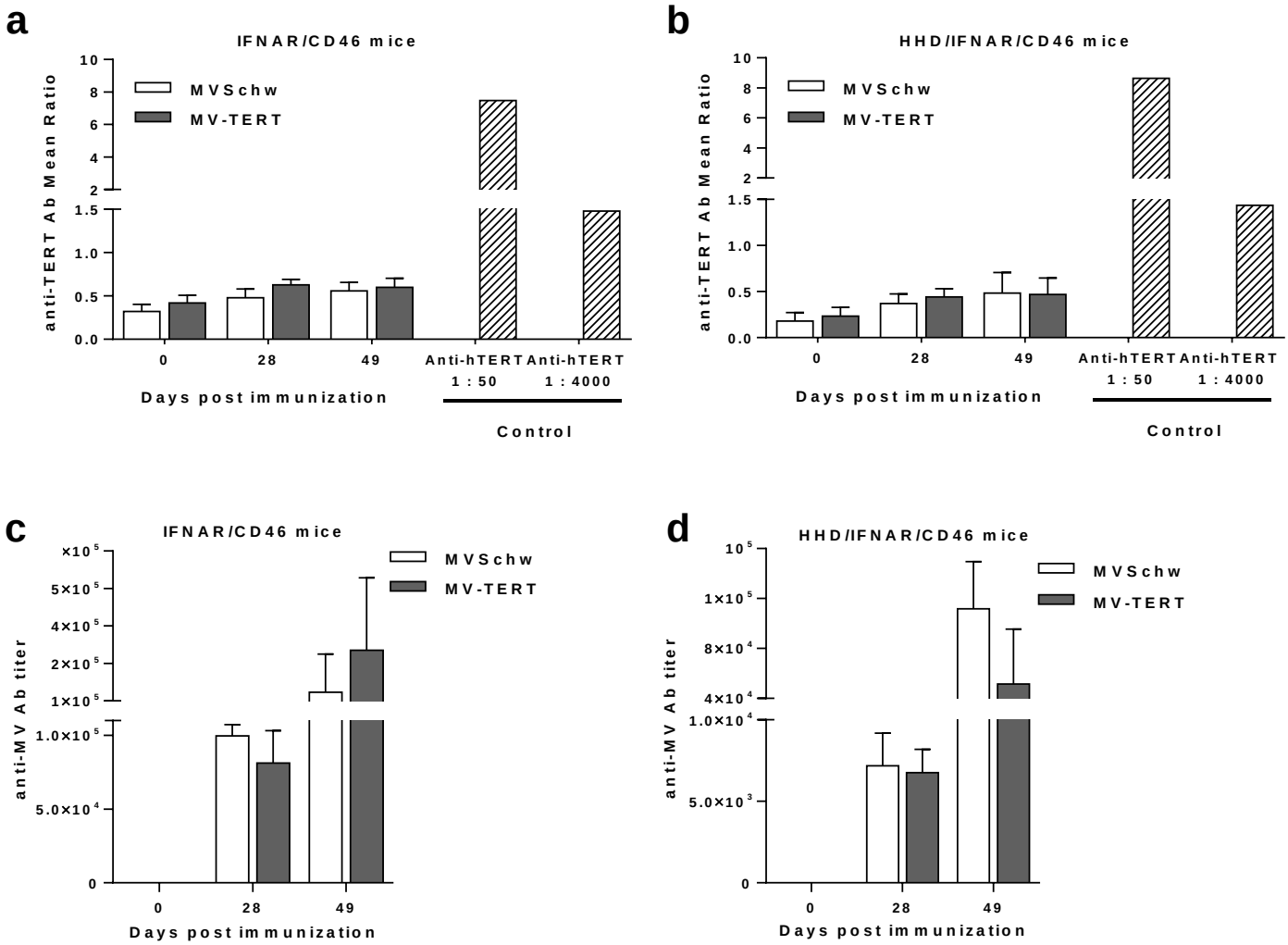


Figure 5

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FIGURE 5

