Universal Cancer Peptide-based therapeutic vaccine breaks tolerance against telomerase and eradicates established tumor

Magalie Dosset\textsuperscript{1,2,3}, Yann Godet\textsuperscript{1,2,3}, Charline Vauchy\textsuperscript{1,2,3}, Laurent Beziaud\textsuperscript{1,2,3}, Yu Chun Lone\textsuperscript{5}, Christine Sedlik\textsuperscript{6}, Christelle Liard\textsuperscript{7}, Emeline Levionnois\textsuperscript{8}, Bertrand Clerc\textsuperscript{1,2,3}, Federico Sandoval\textsuperscript{8}, Etienne Daguindau\textsuperscript{1,2,3}, Simon Wain-Hobson\textsuperscript{7}, Eric Tartour\textsuperscript{8}, Pierre Langlade-Demoyen\textsuperscript{7}, Christophe Borg\textsuperscript{1,2,3,4}, Olivier Adotévi\textsuperscript{1,2,3,4}

\textsuperscript{1}INSERM, UMR1098, F-25020 Besançon cedex, France. \textsuperscript{2}Université de Franche-Comté, UMR1098, SFR IBCT, F-25020 Dijon, France. \textsuperscript{3}EFS Bourgogne Franche-Comté, UMR1098, F-25020 Besançon cedex, France. \textsuperscript{4}CHU Besançon, Oncologie médicale F-25030 Besançon cedex, France. \textsuperscript{5}INSERM U1014, Hôpital Paul Brousse Bâtiment INSERM Lavoisier 14 Avenue Paul Vaillant Couturier F 94807 Villejuif Cedex. \textsuperscript{6}Institut Curie, Département de Transfert, INSERM U932; 26 rue d’Ulm, 75005 Paris. \textsuperscript{7}Invectys, Institut Pasteur Biotop, Paris, France. \textsuperscript{8}INSERM U970 PARCC, Université Paris Descartes, Hôpital Européen Georges Pompidou. Service d’Immunologie Biologique. (AP-HP). 75015 Paris France.

Corresponding authors: olivier.adotevi@univ-fcomte.fr

Running title: UCP-specific CD4 T cells help on antitumor CTL responses
**Statement of translational relevance:**

The stimulation of CD4+ T helper cell responses has gained considerable interest for cancer immunotherapy. The present manuscript reports a systematic analysis of CD4+ T helper cell functions in response to universal cancer peptides (UCP), novel promiscuous HLA-DR-restricted, TERT-derived peptides. Using a relevant preclinical HLA transgenic mouse model, we showed that UCP-specific CD4+ T cells induced after vaccination fulfilled helper features necessary to generate antitumor immune responses. UCP-based vaccinations break self tolerance against TERT and greatly increase primary and memory CTL responses. Furthermore the use of UCPs in therapeutic vaccination eradicates established mouse melanoma by promoting massive TERT-specific CD8+ T cells recruitment at the tumor site. Together with the presence of natural UCP-specific T cell responses in many human cancers, these results support that the stimulation of UCP-specific CD4+ helper T cells is a powerful method to improve cancer vaccines efficiency.
Abstract

**Purpose:** To evaluate CD4⁺ helper functions and antitumor effect of promiscuous universal cancer peptides (UCP) derived from telomerase reverse transcriptase (TERT).

**Experimental Design:** To evaluate the widespread immunogenicity of UCPs in human, spontaneous T cell responses against UCPs were measured in various types of cancer using T cell proliferation and ELISPOT assays. The humanized HLA-DRB1*0101/HLA-A*0201 transgenic mice was used to study CD4⁺ helper effects of UCPs on antitumor CTL responses. UCP-based antitumor therapeutic vaccine was evaluated using an HLA-A*0201positive B16 melanoma that express TERT.

**Results:** Presence of high number of UCP-specific CD4⁺ T cells was found in the blood of patients with various types of cancer. These UCP-specific T cells mainly produce IFN-γ and TNF-α. In HLA transgenic mice, UCPs vaccinations induce high avidity CD4⁺ Th1 cells and activated dendritic cells that produced interleukin 12. UCP-based vaccination breaks self tolerance against TERT and enhances primary and memory CTL responses. Furthermore, the use of UCP strongly improves the efficacy of therapeutic vaccination against established B16-HLA-A*0201 melanoma and promotes tumor infiltration by TERT-specific CD8⁺ TILs.

**Conclusions:** Ours results showed that UCP-based vaccinations strongly stimulate antitumor immune responses and could be used to design efficient immunotherapies in multiple types of cancers.
Introduction

The introduction of immunotherapy in the clinical cancer practice emphasizes the role of immune responses in cancer prognosis and has led to a growing interest to extend this approach to several human cancers (1). Considerable knowledge has been obtained on the elements that are relevant in antitumor immune responses, hence CD8 cytotoxic T lymphocytes (CTL) have been identified as the most powerful effector cells (2). As a consequence, most previous cancer vaccines target class I Major Histocompatibility Complex (MHC)-restricted peptides derived from tumor antigen in order to stimulate CTL responses. However, the clinical impact of CTL peptide-based cancer vaccines remains still modest, even if a recent gp100-derived peptide vaccination was shown to increase patient survival in melanoma (3, 4).

In the meanwhile, CD4 helper T cells have gained interest in anti-tumor immunity and immunotherapy (5). The concept of CD4 T cell help initially emerged from studies demonstrating that successful generation of antitumor CTL depends on the presence of CD4 T cells. Adoptive cell transfer with CD4 T cells induces tumor protection or regression while depletion of CD4 T cells inhibits vaccine-induced protective immunity (6-8). CD4 T cells have been thought to play a key role in “helping” antigen-specific CD8 T cells to undergo efficient activation and proliferation (9). In particular, tumor-reactive CD4 T helper 1 cells (Th1) produce several cytokines (such as IFN-γ, TNF-α and IL-2) essential for the induction of cell-mediated immunity against tumors (10). One widely accepted model demonstrates the ability of CD4 T cells to license dendritic cells (DCs) for efficient CD8 T cell priming through the interaction of costimulatory receptors (11, 12). The cytokines secreted by CD4 Th1 cells also exert direct antitumor and antiangiogenic effects (13). More importantly, only tumor-reactive CD4 T cells have been found to ensure efficient effector CTLs recruitment at the tumor site (14). In human cancers a high density of tumor-infiltrating CD4 Th1 cells has
been shown as a good prognostic marker in colorectal cancer patients emphasizing the role of these cells in cancer immunosurveillance (15). Altogether, these results underline the growing interest in stimulating tumor-specific CD4+ Th1 cells in antitumor immunotherapy.

As a result, increasing attention has focused on identifying MHC class II epitopes from tumor antigens in order to actively target antitumor CD4+ T cells in vivo (16). However, the CD4+ helper T cell subpopulation is known to be plastic (17, 18). Thus, the choice of tumor-reactive CD4 epitopes should require special caution in order to prevent the induction of detrimental CD4+ T cell responses.

Recently, we characterized potent immunogenic CD4 epitopes referred as universal cancer peptides (UCP) derived from telomerase reverse transcriptase (TERT) (19). TERT expression has been detected in all studied cancer forms, including stem cell-like tumor cells (20, 21). Thus TERT has emerged as a clinically relevant tumor antigen for cancer vaccines (22). These TERT-derived UCPs effectively bind to the most commonly found HLA-DR alleles and high frequency of spontaneous UCP-specific CD4+ T cell response was found in lung cancer patients. (19).

In the present study, we found naturally occurring CD4+ T cell responses against UCPs in patients with various types of cancers. Then we evaluated the potential of UCP for active immunotherapy in a preclinical tumor model. By using the humanized HLA-DRB1*0101/HLA-A*0201 transgenic mice, we found that UCPs vaccinations stimulate CD4+ Th1 cells that drastically improved antitumor CTL responses in vivo. Subsequently, UCP-based therapeutic vaccine was shown to inhibit tumor growth by mechanisms that involve CD8+ T cells.
Materials and Methods

**Synthetic peptides.** The four peptides derived from TERT called universal cancer peptides (UCPs): UCP1 (TERT\textsubscript{44-58}: PAAFRALVAQCLVCV), UCP2 (TERT\textsubscript{578-592}: KSVWSKLQSIGIRQH), UCP3 (TERT\textsubscript{916-930}: GTAFVQMPAHGLFPW) and UCP4 (TERT\textsubscript{1041-1055}: SLCYSILKAKNAGMS) have been described recently (19). The modified (first amino acid substitution with a tyrosine) HLA-A2-restricted pY988 (YLQVNSLQTV) and pY572 (YLFFYRKSV) peptides derived from TERT have been described elsewhere as high-affinity forms of their cryptic counterparts (23, 24). The native forms of these two peptides are fully conserved in human and mouse TERT (23, 24). Synthetic peptides (> 80% purity) were purchased from Activotec (UK).

**Detection of UCP-specific T cell responses in cancer patients.** Blood was collected from cancer patients at the university hospital of Besançon (France) after informed consent. The study was conducted in accordance with French laws and after approval by the local ethics committee. Ficoll-isolated lymphocytes were analyzed by \(^3\)H-thymidine incorporation as previously described (25). After a short *in vitro* stimulation of lymphocytes with UCPs as previously reported (19), UCP-specific immune responses were analyzed by human ELISPOT assay (GenProbe, France). Concomitantly, cytokines production was measured after a 15h-culture with or without UCPs, using DIAplex Human Th1/Th2 kit (GenProbe, France) according to the manufacturer’s instructions.

**Tumor cell lines and TERT expression analysis.** The HLA-A2.1-positive B16F10 murine tumor cell line (referred as B16-A2) was previously described (26). Telomerase detection in cell lines was achieved by western blot using anti-hTERT monoclonal antibody (clone 2C4) (Novus Biologicals) which cross reacts with mouse TERT. FaDu cell line (human head and neck squamous cell carcinoma) and murin fibroblast were used as positive and negative control respectively. Telomerase activity was assessed by TRAP-ELISA assay using the
TeloTAGGG Telomerase PCR ELISA**PLUS** kit (Roche Diagnostics, Germany) according to the manufacturer’s instructions.

**Mouse and vaccinations.** The HLA-DRB1*0101/HLA-A*0201-transgenic mice (A2/DR1 mice) have been previously described (25) and were purchased at the “Cryopréservation, Distribution, Typage et Archivage animal” (CDTA, CNRS France). These mice are H-2 class I and IA class II knockout, and their CD8 T and CD4 T cells are restricted by the sole HLA-A*0201 and HLA-DR1*0101 molecules respectively. To study the processing of UCP, eight to ten week-old A2/DR1 mice were immunized with a pTrip-TERT DNA (100 µg) at days 0 and 14 as previously reported (26). In some experiment CD4 T cells were depleted with anti-CD4 mAb treatment (clone GK1.5) prior DNA immunization. For UCP immunization, mice were injected twice with 100 µg of each UCP emulsified in incomplete Freund adjuvant (IFA, Sigma-Aldrich, France). In some experiments, 50 µg of pY988 peptide was co-injected with 100 µg of each UCP in IFA. All peptide vaccinations were done subcutaneously (s.c) at the right abdominal flank. All experiments were done according to the good laboratory practices defined by the animal experimentation rules in France.

**Pentamer staining and ELISPOT.** *Ex vivo* pentamer staining was performed as previously described (26, 27). Cells were stained with PE-conjugated pY988 and pY572 HLA-A2.1 pentamer (ProImmune, UK). After cell staining, samples were analyzed by flow cytometry on a FACS Canto II (BD Biosciences, France) and using Diva software. *Ex vivo* ELISPOT was performed as previously described (26, 27). Briefly, freshly ficoll-purified lymphocytes or spleen-isolated CD8+ or CD4+ T cells from immunized mice (T cell isolation kit, Miltenyi, Biotec, France) were incubated at 1 or 2.10^5/well (in triplicates) in Elispot IFN-γ or IL-2 plates in presence of the relevant or control peptides. Plates were incubated for 16 to 18 hours at 37°C, and spots were revealed following the manufacturer’s instructions (GenProbe,
France). Spot-forming cells were counted using the « C.T.L. Immunospot » system (Cellular Technology Ltd., USA).

**Cytotoxicity assays.** The in vivo CTL killing assays were performed using CFSE-labelled target cells (carboxyfluorescein-diacetate succinimidyl ester, Molecular Probes) as described previously (28). CFSE\textsuperscript{high} splenocytes from naive mice were pulsed with peptides at 10 μg/ml and non-pulsed CFSE\textsuperscript{low} splenocytes served as control. Equal numbers of each cell fraction (high or low) were injected intravenously into immunized and non-immunized mice. After 15h, cells were recovered from spleen or blood and analyzed by flow cytometry. The specific lysis was calculated as previously described (28). In vitro cytotoxicity assay was performed using a standard \(^{51}\)chromium-release assay as described previously (26). The cytolytic activity of CTL from immunized mice was tested against TERT-expressing tumor cells.

**Dendritic cells generation and activation.** Spleen or lymph nodes CD11c+ DCs from peptide-immunized mice were directly analyzed for co-stimulatory receptor expression. In some experiments, bone marrow cells from naive mice (8.106/mL) were cultured for 6-days in IMDM (Sigma-Aldrich, France) supplemented with 10% FCS, 2 mM L-glutamine (Sigma-Aldrich), 5 mM sodium pyruvate (Gibco), and 50 mM 2-mercaptoethanol (Gibco) with 30% conditioned medium from GM-CSF-producing NIH-3T3 (R1 medium). Isolated CD4 T cells from mice immunized with UCP or IFA alone were then culture 24-h in presence of UCP with immature bone marrow-derived DCs (iDC) from A2/DR1 mice. In some cases, blocking CD40L (MR1) or IFN-γ (XMG1.2) antibodies (20µg/mL) (Bio X Cell, USA) were added to the culture. Cells were then stained for cell surface expression of co-stimulatory receptors and cytokines production.

**Tumor challenge.** A2/DR1 mice were subcutaneously injected with 2.10^5 B16-A2 cells in 100µl of saline buffer on the abdominal flank. At day 5, groups of mice were immunized with either the mix of pY988 and pY572 peptides (100 µg) with or without UCP2 (100µg). A boost
injection was done at day 17. Control mice were treated with IFA in saline buffer. Tumor growth was monitored every 2-3 days using a calliper and mice were euthanized when the tumor mass reached a surface > 200 mm². The mice survival was assessed using the Kaplan-Meier model. For tumor infiltrative lymphocytes (TILs) analysis, tumor-bearing mice were treated as above and 7 days after the last immunization, tumors were recovered and treated with DNAse (Sigma-Aldrich) and collagenase (Roche) prior cell suspensions analysis by flow cytometry and antigen specificity of TILs was done ex vivo by ELISPOT assay.

**Statistics.** Data are presented as means ± SD. Statistical comparison between groups was based on Student t test using Prism 4 GraphPad Software. Mouse survival time was estimated using the Kaplan-Meier method, and the log-rank test was used. P values less than 0.05 (*) were considered significant.

**Results**

**Presence of naturally occurring UCP-specific CD4⁺ T cell responses in various human cancers**

Recently, we found frequent occurrence of spontaneous UCP-specific CD4⁺ T cell response in advanced lung cancer patients (19). Based on the broad expression of TERT in cancers, we sought to extend this study in patients with cancer of different histological origins. For this purpose, we measured ³H-thymidine incorporation of blood lymphocytes obtained from cancer patients or healthy donors directly stimulated with UCPs during 6 days. In contrast to healthy donors, blood lymphocytes from cancer patients specifically proliferate upon UCPs stimulation (Fig. 1A). Next, UCP-specific T cells were measured by IFN-γ ELISPOT after short-term in vitro stimulation. Accordingly, high number of IFN-γ-producing T cells directed against UCP was found in patients as compared to healthy donors (Fig. 1B). These responses included T cells specific of each UCP, supporting their immunogenicity (Fig. 1C).
Furthermore, the UCP-specific T cells mainly produce Th1 cytokines but no IL-4, IL-10 or IL-17 as previously reported in lung cancer (Fig. 1D). This result was also confirmed by the obvious Th1 polarization of UCP-specific CD4⁺ T cell clones isolated from a cancer patient (Supplementary Fig. S1). Thus, the UCP-specific T cell repertoire is spontaneously stimulated in various cancers such as colon, kidney, lung, stomach and leukaemia. This also underlined the universal nature of the promiscuous HLA-DR-restricted UCPs.

**UCPs are endogenously processed and induce high avidity Th1 polarized CD4⁺ T cell responses in vivo**

Based on the equivalent binding capacity of UCPs to HLA-DRB1*0101 molecules, we then used A2/DR1 mice to study the *in vivo* immunogenicity and natural processing of UCPs. To assess whether UCPs can be endogenously processed from the TERT protein, we performed immunizations with a plasmid DNA encoding the full length TERT sequence and the UCP-specific CD4 T cell proliferation was monitored by a five-day ³H-thymidine incorporation assay. As shown in Fig. 2A, all the UCPs stimulate proliferation of spleen lymphocytes from DNA-immunized mice. Especially, high T cell proliferation was measured in response to UCP2 and 3 as compared to UCP1 or UCP4. We confirmed these results by using *ex vivo* IFN-γ ELISPOT assay (Fig. 2B). These data clearly indicate that UCPs are differentially processed and presented to CD4⁺ T cells *in vivo* in the context of DRB1*0101 restriction.

Different populations of CD4⁺ T helper cells control the antitumor immune responses (9), thus we studied the polarization of the UCP-specific CD4⁺ T cell responses *in vivo*. To this end, freshly isolated CD4⁺ T cells from UCP-vaccinated mice were cultured in the presence of syngenic iDC pulsed or not with UCP and cytokines production was measured. In all cases, we showed that UCP-specific CD4⁺ T cells produce IFN-γ and IL-2 but not IL-4, IL-5, IL-10
nor IL-17 indicating that UCP immunization preferentially induces a Th1 polarized immune response in vivo (Fig. 2C).

Next, to assess the avidity of UCP-specific CD4$^+$ T cell, freshly purified CD4$^+$ T cells from UCP-immunized mice were cultured in the presence of increasing concentrations of peptide and the number of specific IFN-$\gamma$ producing CD4$^+$ T cells was measured. Results in Fig. 2D showed that mice immunized with UCP2 or UCP3 induced high avidity specific CD4 T cells ($< 10^{-7}$ $\mu$g/ml). By comparison CD4$^+$ T cells from mice vaccinated with UCP1 or UCP4 responded to $10^{-1}$ and $10^{-3}$ $\mu$g/ml of peptide concentration respectively. In addition, low doses of UCP2 or UCP3 peptides ($\sim 1\mu$g) stimulated potent IFN-$\gamma^+$ CD4$^+$ T cells in vivo (Fig. 2E). Collectively, these results show that UCPs are efficiently processed in vivo and stimulate high avidity Th1 polarized CD4$^+$ T cells in A2/DR1 mice.

**UCP-specific CD4$^+$ Th1 cells provide help for optimal anti-self/TERT CD8$^+$ T cell responses in vivo**

CD4$^+$ T cell helper functions are thought to be important for the generation of potent and sustained CTL responses (29, 30). To address this question concerning UCP-specific CD4$^+$ T cells, we co-immunized mice with pY988 an HLA-A2$^+$ self/TERT peptide in the presence of UCP. The pY988-specific CTL response was measured ex vivo by pentamer staining and ELISPOT assays. As shown in Fig. 3A, a higher frequency of functional pY988-specific CD8$^+$ T cells was detected in mice immunized with pY988 plus UCPs compared to pY988/IFA group. Although UCP1 vaccination had little impact on the frequency of pY988/A2 pentamer$^+$ CD8 T cells-specific response, all UCPs were able to significantly increase the number of IFN-$\gamma$-secreting CD8$^+$ T cells against TERT (Fig. 3B). The magnitude of the pY988-specific CD8$^+$ T cells response was strongly correlated with the intensity of UCP-specific CD4 T cell responses concomitantly induced in mice (Fig. 3C, D). Furthermore,
these UCPs exerted similar helper effect on the self/TERT pY572-specific CTL responses in vivo (Supplementary Fig. S2). Thus, the addition of UCPs as helper peptides efficiently breaks immune tolerance against TERT in vivo.

We next sought out to study the impact of UCPs helper peptides on CTL avidity and memory, two critical functions for tumor eradication. To this end we focused on the UCP2 which induces potent Th1 immune responses in vivo. In addition, compared to HLA-DR1-restricted viral helper peptides such as Tax-derived peptide (16), UCP2 strongly enhanced CTL responses (supplementary Fig. S3). As shown in Fig. 4A, freshly isolated CD8+ T cells from mice immunized with pY988 + UCP2 were still reactive against very low concentrations of peptide (< 10⁻³ µg/ml). These cells also recognized the cryptic native counterpart p988 (data not shown), underlining their high avidity. Accordingly, mice vaccinated with pY988 + UCP2 displayed stronger in vivo cytotoxicity against CFSE-labelled target cells (Fig. 4B) than in pY988/IFA group. In addition, TERT-specific CTL from mice immunized in presence of UCP2 exhibit strong in vitro cytotoxicity against TERT-expressing B16-A2 cells (Fig. 4C, D). Furthermore, long-lasting TERT-specific CTLs were detected in mice co-injected with UCP2. This response was correlated to the sustained UCP2-specific CD4+ T cell response in vivo (Fig. 4E). Similar helper functions of UCP2 were obtained in other tumor antigen model such as E7 from HPV-16, (Supplementary Fig. S4). By using a second model of DNA immunization, we also showed in mice depleted or not of CD4+ cells that UCP-specific CD4 T cells are necessary for the induction of TERT-specific CD8 T cells (Fig. S4 C). Collectively, UCP2 helper immune responses enhance the magnitude and quality of antitumor CTL response.
**UCP-specific CD4^+ T cells promote dendritic cells activation in vivo**

The induction of dendritic cell activation represents one major helper mechanism used by CD4^+ Th1 cells to sustain antigen presentation and provide costimulatory signals to the CTLs. This is referred as the “ménage à trois” model (31). To test this mechanism, we analyzed the expression of co-stimulating receptors on DCs from mice immunized with the mix of pY988 +/- UCP2. As shown in Fig. 5A, lymph nodes CD11c^+ DCs from UCP2-immunized mice expressed higher level of HLA-DR molecules and slight increase of CD86 as compared to control mice. In a second set of experiments, CD4^+ T cells isolated from UCP2/IFA or IFA-injected mice were co-cultured with syngenic iDCs (Fig. 5B). Similar increase of DC activation was found in presence of UCP2-specific CD4 T cells (Fig. 5C, left). In addition, high rate of CD40L^+ CD4^+ T cells were detected in UCP2-immunized mice (Fig. 5C, middle) and significant amounts of Th1-associated cytokines such as IL-12, IFN-γ and GM-CSF were found in the supernatant of CD4_UCP2/DC co-culture (Fig. 5C, right). This DC’s activation could be partially inhibited by blocking CD40L and/or IFN-γ antibodies (Fig. 5D). Together, these results showed that the stimulation of UCP2-specific CD4^+ T cells shapes the phenotype and function of DC in vivo.

**UCP2 helper peptide enhances the efficacy of self/TERT CD8 peptides vaccination against established HLA-A*0201^+ B16F10 melanoma**

To investigate the helper role of UCP2 in a therapeutic vaccination protocol, we used the aggressive and poor immunogenic B16F10-HLA-A*0201 melanoma (B16-A2) (26). Mice were challenged with 2.10^5 B16-A2 cells and tumor bearing mice were then vaccinated twice either with the two self/TERT CTL peptides (pY572 + pY988/IFA) alone or in presence of the UCP2. As shown in Fig 6A, the tumor growth reached an area > 200mm² at day 25 in the control group injected with IFA alone. In this representative experiment, tumor regression was
observed in 1/8 mice vaccinated with pY572 + pY988/IFA while two mice achieved a delay in tumor growth. In the group vaccinated with pY988 + pY572/IFA combined with UCP2, complete tumor regression was achieved in 5/8 mice. Accordingly, survival analysis out to day 50 after tumor cell injection showed that 63% of mice vaccinated in presence of UCP2 were still alive as compared to 13% in the group of mice injected with pY988 + pY572/IFA (p < 0.05) (Fig. 6B).

The density of tumor-infiltrating CD8 T cells was shown to be critical for tumor control (32). Therefore, we analyzed immune cell infiltration within tumor in mice treated with the same vaccination protocols. Higher total CD3⁺CD8⁺ T cells infiltration was observed in mice that received vaccine plus UCP2 helper peptide as compared to pY988 + pY572/IFA group (67% vs 40%, p < 0.05) (Fig. 6C). In contrast, UCP2 vaccination did not influence CD4⁺ TILs, NK or regulatory T cells, suggesting that UCP2-specific immunity mainly drive CTLs to the tumor microenvironment.

In line with this observation, we detected a large number of TERT-specific CD8⁺ TILs in mice that received UCP2-based vaccine (Fig. 6D lower panel). TERT-specific CTL response was also detected in spleen of mice which is correlated to UCP2-specific CD4 T cell response (Fig. 6D upper panel). However, UCP-specific CD4⁺ TILs were not detected at the tumor site. This could be due to the low level of CD4⁺ TILs or to the lack of HLA-DR expression on the B16/A2 model used. Together, our results clearly showed that UCP2 specific CD4⁺ T cells exert strong helper activity on tumor-specific CTL responses in vivo. Moreover the addition of UCP2 influences the homing of CD8⁺ T cells at the tumor site. All these data support the use of UCP for antitumor therapeutic vaccination.
Discussion

CD4 Th1 response against tumor is gaining considerable interest in cancer immunity. In this study we found spontaneous Th1 CD4⁺ T cell responses against recently described TERT-derived UCP in patients with different types of cancers. This observation underlines the great interest of these peptides for immunotherapy. To evaluate the potential applicability of UCP for cancer vaccine, we used the preclinical A2/DR1 mouse model. We have found that UCPs vaccination induces high avidity Th1 polarized CD4⁺ T cells that greatly increase CTL responses against self/TERT epitopes *in vivo* and promote potent antitumor immunity.

Different subpopulations of CD4⁺ T helper cells regulate host antitumor immune responses (10). Indeed, Th2 CD4⁺ T cells and Treg cells are often associated with an inhibitory environment within the tumor (10, 33). The role of Th17 cells in antitumor immune response is still controversial and seems to depend on the type of cancer (34). In contrast, Th1 immunity has a clear positive effect in cancer cell eradication. The CD4⁺ Th1 cells provide help for CTLs through multiple interactions during the induction and effector phases of antitumor immune responses (35, 36). Thus, there is a strong rational to develop cancer vaccines that stimulate antitumor Th1 immunity (5, 37). Nevertheless, in recent randomized trials, the use of melanoma-associated helper peptides paradoxically decreased CD8⁺ T cell responses to a melanoma vaccine (38). This could be related to the plasticity of CD4⁺ T helper cell responses (17). Consequently, the choice of tumor-reactive CD4 helper peptides for cancer vaccine needs to be done carefully.

Based on its expression profile and its role in multiple human tumors, TERT is an attractive target for cancer vaccination (22, 39). Schroers et al. have previously described TERT-derived promiscuous HLA-DR restricted peptides (40, 41). However their role on cell-mediated tumor immunity was not completely addressed neither in preclinical nor in clinical trials setting. Recently, a cancer vaccine using a TERT-derived CD4 helper peptide called
GV1001 was able to stimulate specific CD4 T cell immunity. Clinical trials using GV1001 suggest an increased survival in cancer patients when combined with cytotoxic agents (42, 43). Nevertheless, GV1001 vaccine also failed to induce specific immune responses and clinical benefit in other cancers (44). The impact of GV1001-specific CD4+ T cell help on antitumor CTL responses remains to be investigated.

Here, we used a relevant mouse model to perform a systematic analysis of UCP-specific CD4+ T cell help on antitumor CTL responses in A2/DR1 mice. To this end, we selected two HLA-A2+ TERT peptides called pY572 and pY988 because they are self epitopes in mouse and also fully conserved in human TERT (23, 24). In addition, these peptides are already used for cancer vaccines in humans (45, 46). We found that the presence of UCP-specific Th1 cells drastically enhances self/TERT-specific CD8+ T cell responses as compared to mice immunized with CD8 peptides alone. The anti-self/TERT CTL induced in UCP-vaccinated mice displayed higher avidity and stronger cytotoxicity than the helper less counterpart. Furthermore, the addition of UCP2 to the CD8 TERT peptide vaccine led to B16-A2 tumor regression and improved the survival of mice.

Previous studies already showed the requirement of CD4 help for the generation of CTL against the self/TERT epitopes used in this study (23, 47). Gross et al. reported that vaccination of HHD mice with these peptides promote tumor protection only when they were coupled with a helper peptide derived from the hepatitis B virus. In this study, the vaccine was used prophylactically: ~25% of vaccinated mice were tumor free compared to 60% in our therapeutic vaccine study (47). This difference could be related to the nature of the help signal delivered by CD4 T cells. We used the tumor-reactive helper peptide UCP2 that mediates a better homing of CD8+ TILs than non tumor-antigen specific CD4 Th1 cells as previously reported (14, 48, 49). Indeed, we found that CD4 T cells specific for UCP2 cross-recognized its mTERT-derived counterpart peptide p568 (differing by one amino acid) (Fig.
Consequently, the contribution of xenogenic response in UCP2-mediated helper effect in mice studies seems to be weak. In agreement with previous studies, no sign of autoimmunity has been observed in all immunized mice suggesting the safety of TERT-based vaccination (26, 47).

Moreover, immunization with UCP2 stimulates specific CD4^+ T cells secreting high levels of IL-2 and GM-CSF which are known to be central components for the generation of CD8^+ T cell memory and DC licensing respectively (36, 50). Therefore, fully activated DCs and sustained self/TERT CTL responses were found in A2/DR1 mice co-immunized with UCP2.

Finally, we found that spontaneous UCP-specific Th1 responses are detected in patients with various cancers indicating the presence of a functional UCP-specific T cell repertoire. In our recent study, this pre-existing UCP-specific CD4^+ T cell immunity was shown to be associated with an increased overall survival of lung cancer patients responding to first line chemotherapy (19).

In conclusion, our study shows that the stimulation of UCP-specific CD4 T helper cells is a powerful method to improve cancer vaccine efficacy and also highlights the interest of TERT-derived UCPs for the monitoring of antitumor CD4^+ T cell responses.

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Disclosures:

P. Langlade-Demoyen is the Member of Advisory Board, INVECTYS Co. (current patent holder for UCPs).

Figures legends

Figure 1: Analysis of spontaneous UCP-specific T cell responses in human. A, Blood lymphocytes from cancer patients were directly cultured with pool of UCPs during five days and specific proliferation was measured by 3H-thymidine incorporation. Representative data from three healthy donors and nine responding patients are shown. Results are considered positive for a proliferation index > 2. B-D, Lymphocytes were cultured in vitro with pool of UCPs for one week. B, Detection of UCP-specific T cell by IFN-γ ELISPOT. Representative data from healthy donors and nine responding patients are shown. Columns, mean of triplicate; bars, SD. C, T cell responses against individual UCP for six responding patients. D, Detection of cytokine production by DIAplex assay in supernatant after 15h of culture in presence of UCPs. Columns, mean cytokine levels from three patients; bars, SD. (NSCLC: non small cell lung cancer; RCC: renal cell carcinoma; HNSCC: head and neck squamous cell carcinoma, AML: acute myeloid leukemia; CRC: colorectal carcinoma).

Figure 2: UCPs vaccinations stimulate high avidity Th1 polarized CD4 T cell responses. A-B, A2/DR1 mice (n = 8) were immunized twice with a DNA encoding TERT. A, Proliferation of spleen lymphocytes in presence of UCPs. B, CD8 depleted spleen lymphocytes from DNA-immunized mice were assayed in ex vivo IFN-γ ELISPOT. Columns mean of triplicate from 4 mice; bars, SD. C-D, Mice (3-4/group) were immunized once with each UCP in IFA. C, Ten days later, spleen-isolated CD4 T cells were cultured overnight in presence of DC loaded with UCP. The cytokines production was measured in the supernatant
by Luminex assay. Columns, mean of cytokine levels; bars, SD. D, Isolated CD4 T cells were cultured *ex vivo* with increasing concentrations of peptide as indicated. IFN-γ production was measured by ELISPOT. Curves, mean responses from 3 mice, bars, SD. E, Mice were vaccinated once with low dose of UCP as indicated. UCP-specific T cell responses were evaluated in spleen by *ex vivo* IFN-γ ELISPOT.

**Figure 3: CD4 helper role of UCPs vaccinations on the self/TERT-specific CTL responses.** Mice (3/group) were immunized either with pY988 plus each UCP in IFA or with pY988/IFA alone and the immune responses were monitored ten days later in the spleen. A, freshly isolated CD8 T cells were stained with TERT pY988/A2⁺ pentamer. Representative flow cytometry dot plots (upper panel) and mean percentages of pY988/A2⁺ CD8 T cells (lower panel) are shown. B, *Ex vivo* detection of anti-pY988 CD8 T cells by IFN-γ ELISPOT. C-D, simultaneous UCP-specific CD4 T cell responses were assessed in CD8-depleted fraction by IFN-γ (C) and interleukine-2 (D) ELISPOT assays. DR1-restricted Tax191-250 was used as irrelevant peptide. Columns, mean of spots from 3 mice; bars, SD. Data are representative of three independent experiments.

**Figure 4: Immunization in presence of UCP2 enhances the quality of self pY988-specific CTL responses.** Mice (3-4/group) were immunized once either with pY988 plus UCP2 (UCP2 + pY988/IFA) or with pY988/IFA alone. A, Ten days later, freshly isolated spleen CD8 T cells were cultured with increasing pY988 peptide concentration and IFN-γ-secreting CD8 T cells were detected by *ex vivo* ELISPOT. B, *In vivo* cytotoxic assay. Representative flow cytometry histograms showing lysis of CFSE-labeled pY988-loaded target cells compared to unpulsed (UP) and the mean of in vivo percentage lysis are shown. C, TERT expression by western-blot (left) and activity by TRAP-ELISA assay (right) in B16-A2
melanoma cells. (HT): Heat-Treated cells, (-): untreated cells.** D,** Cytotoxicity of T cells against TERT-positive B16 or B16-A2 tumor cells after 5 day of in vitro stimulation of splenocytes with pY988. Results represent the specific lysis (percentage) plus or minus SD in each immunized group of mice. **E,** Long-term T cell responses were evaluated 30 days after immunization. Frequencies of pY988/A2 pentamer+ CD8 T cells gated on CD44hiCD62lo cells (left) and by IFN-γ secretion assay (middle). UCP2-specific CD4 T cell response measured in CD8-depleted fraction by ex vivo IFN-γ ELISPOT (right).

**Figure 5: UCP2-specific CD4 Th1 cells active dendritic cells.** A,** Mice (3/group) were immunized once either with UCP2 + pY988/IFA or pY988/IFA alone. Ten days later, the expression of activation markers CD80, CD86 and HLA-DR were analyzed on lymph nodes CD11c+ DC by flow cytometry. Representative flow cytometry histograms (upper panels) and the mean of MFI (lower panels) are shown. Columns, mean of MFI; bars, SD. B-E: Analysis of DC and CD4 T cells cross talk. **B,** Schema of the in vitro DC-CD4 T cell co-culture. **C,** Expression of CD86 and HLA-DR on CD11c+ DC (left). CD40L expression on CD4 T cells (middle). IFN-γ, GM-CSF and IL-12p70 production measured by ELISA in the supernatant (right). **D,** Expression of CD86 on CD11c+ DC co-cultured with CD4 T cells from UCP2-immunized mice in presence or not of blocking CD40L and/or IFN-γ antibodies. Representative flow cytometry histograms (left) and mean of percentage from 3 mice (right) are shown. Data are representative of two independent experiments.

**Figure 6: Therapeutic antitumor effect of UCP-based vaccination.** Tumor-bearing mice (6-8 mice/group) were therapeutically vaccinated with peptides as described (materials and methods). **A,** Follow-up of tumor size. The numbers in parentheses indicate mice with tumor regression per group. **B,** Survival curves recorded until 50 days. **C,** Tumor-bearing mice were
vaccinated as above and tumor-infiltrating immune cells were analyzed by flow cytometry. Columns, mean of percentages of cells from 4 mice; bars, SD. D, TERT-specific T cells in spleen and in tumor were analyzed by ex vivo IFN-γ ELISPOT. Columns, mean of spots from 5 mice; bars, SD. All data are representative of three independent experiments.
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Figure 3

A

B

C

D

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Figure 4

A

B

C

D

E

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Universal cancer peptide-based therapeutic vaccine breaks tolerance against telomerase and eradicates established tumor

Magalie Dosset, Yann Godet, Charline Vauchy, et al.

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