Universal Cancer Peptide-Based Therapeutic Vaccine Breaks Tolerance against Telomerase and Eradicates Established Tumor

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

Results: The presence of a 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, UCP vaccinations induced high avidity CD4+ T11 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+ T cells.

Conclusions: Our results showed that UCP-based vaccinations strongly stimulate antitumor immune responses and could be used to design efficient immunotherapies in multiple types of cancers. Clin Cancer Res; 18(22): 6284–95. ©2012 AACR.

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 CTLs have been identified as the most powerful effector cells (2). As a consequence, most previous cancer vaccines target class I MHC-restricted peptides derived from tumor antigens 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 antitumor immunity and immunotherapy (5). The concept of CD4+ T-cell help initially emerged from studies showing 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, whereas 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 interleukin-2 (IL-2)] essential for the induction of cell-mediated immunity against tumors (10). One widely
Translational Relevance
The stimulation of CD4⁺ T-helper cell responses has gained considerable interest for cancer immunotherapy. This article reports a systematic analysis of CD4⁺ T-helper cell functions in response to universal cancer peptides (UCP), novel promiscuous HLA-DR–restricted, and 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-cell 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 the efficiency of cancer vaccines.

Accepted model shows the ability of CD4⁺ T cells to license dendritic cells (DC) for efficient CD8⁺ T-cell priming through the interaction of costimulatory receptors (11, 12). The cytokines secreted by CD4⁺ T_h1 cells also exert direct antitumor and antiangiogenic effects (13). More importantly, only tumor-reactive CD4⁺ T cells have been found to ensure efficient effector CTL recruitment at the tumor site (14). In human cancers, a high density of tumor-infiltrating CD4⁺ T_h1 cells has been shown as a good prognostic marker in patients with colorectal cancer emphasizing the role of these cells in cancer immunosurveillance (15). Altogether, these results underline the growing interest in stimulating tumor-specific CD4⁺ T_h1 cells for antitumor immunotherapy.

As a result, increasing attention has focused on identifying MHC class II epitopes from tumor antigens to actively stimulate 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 to prevent the induction of detrimental CD4⁺ T-cell responses.

Recently, we characterized potential immunogenic CD4⁺ epitopes referred as universal cancer peptides (UCP) derived from telomerase reverse transcriptase (TERT; ref. 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 (19). In the present study, we found naturally occurring CD4⁺ T-cell responses against UCPs in patients with various types of cancers. We then 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 UCP vaccinations stimulate CD4⁺ T_h1 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 4 peptides derived from TERT called UCPs: UCP1 (TERT44–58: PAAFRALVAQCLVCV), UCP2 (TERT578–592: KSVWSKLQSIGIRQH), UCP3 (TERT916–930: GTAFFQM-PAHGFLFW), and UCP4 (TERT1041–1055: SLCYSILKA-KNAGMS) have been described recently (19). The modified (first amino acid substitution with a tyrosine) HLA-A2–restricted pY988 (YLQVNSLQTV) and pY572 (YLFFYRKS) peptides derived from TERT have been described elsewhere as high-affinity forms of their cryptic counterparts (23, 24). The native forms of these 2 peptides are fully conserved in human and mouse TERT (23, 24). Synthetic peptides (>80% purity) were purchased from Activotec.

Detection of UCP-specific T-cell responses in cancer patients
Blood was collected from patients with cancer at the University Hospital of Besançon (Besançon, France) after informed consent. The study was conducted in accordance with the French laws and after approval by the local ethics committee. Ficoll-isolated lymphocytes were analyzed by ³H-thymidine incorporation as described previously (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 (GenIProbe). Concomitantly, cytokines production was measured after a 15-hour culture with or without UCPs using DIAplex Human Th1/Th2 kit (GenIProbe) 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 analysis 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 murine fibroblast were used as positive and negative controls, respectively. Telomerase activity was assessed by TRAP-ELISA assay using the TeloTAGG Telomerase PCR ELISA® kit (Roche Diagnostics) 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”. 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, 8- to 10-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 experiments, CD4 T cells were depleted with anti-CD4 monoclonal antibody treatment (clone GK1.5) before DNA immunization. For UCP immunization, mice were injected twice with 100 μg of each UCP emulsified in incomplete Freund adjuvant (IFA, Sigma-Aldrich). In some experiments, 50 μg of pY988 peptide was coinjected with 100 μg of each UCP in IFA. All peptide vaccinations were done subcutaneously in the right abdominal flank. All experiments were carried out according to the good laboratory practices defined by the animal experimentation rules in France.

Pentamer staining and ELISPOT

Ex vivo pentamer staining was conducted as previously described (26, 27). Cells were stained with phycoerythrin (PE)-conjugated pY988 and pY572 HLA-A2.1 pentamer (ProImmune). After cell staining, samples were analyzed by flow cytometry on a FACS Canto II (BD Biosciences) using Diva software. Ex vivo ELISPOT was conducted 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) were incubated at 1 or 2 × 10⁵ cells per well (in triplicates) in Elispot IFN-γ or interleukin (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 (Gen Probe). Spot-forming cells were counted using the « C.T.L. Immunospot » system ( Cellular Technology Ltd.).

Cytotoxicity assays

The in vivo CTL killing assays were conducted using CFSE-labeled target cells (carboxyfluorescein-diacetate succinimidyl ester, Molecular Probes) as described previously (28). CFSE<sup>high</sup> splenocytes from naïve mice were pulsed with peptides at 10 μg/mL and nonpulsed CFSE<sup>low</sup> splenocytes served as control. Equal numbers of each cell fraction (high or low) were injected intravenously into immunized and nonimmunized mice. After 15 hours, 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 conducted using a standard ⁵¹<sup></sup>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<sup>+</sup> DCs from peptide-immunized mice were directly analyzed for costimulatory receptor expression. In some experiments, bone marrow cells from naïve mice (8.10⁶/mL) were cultured for 6 days in Iscove’s modified Dulbecco’s medium (IMDM; Sigma-Aldrich) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine (Sigma-Aldrich), 5 mmol/L sodium pyruvate (Gibco), and 50 mmol/L 2-mercaptoethanol (Gibco) with 30% conditioned medium from granulocyte macrophage colony-stimulating factor (GM-CSF)-producing NIH-3T3 (R1 medium). Isolated CD4 T cells from mice immunized with UCP or IFA alone were then cultured for 24 hours in the 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) were added to the culture. Cells were then stained for cell surface expression of costimulatory receptors and cytokines production.

Tumor challenge

A2/DR1 mice were subcutaneously injected with 2.10⁵ B16-A2 cells in 100 μL of saline buffer in 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 (100 μg). A boost injection was done at day 17. Control mice were treated with IFA in saline buffer. Tumor growth was monitored every 2 to 3 days using a caliper and mice were euthanized when the tumor mass reached an area of more than 200 mm². The mice survival was assessed using the Kaplan–Meier model. For tumor infiltrative lymphocyte (TIL) 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) before cell suspension analysis by flow cytometry, and antigen specificity of TILs was done ex vivo by ELISPOT assay.

Statistics

Data are presented as mean ± 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. P values less than 0.05 (*) were considered significant.

Results

Presence of naturally occurring UCP-specific CD4<sup>+</sup> T-cell responses in various human cancers

Recently, we found frequent occurrence of spontaneous UCP-specific CD4<sup>+</sup> T-cell response in patients with advanced lung cancer (19). On the basis of the broad expression of TERT in cancers, we sought to extend this study in patients of different histologic origins. For this purpose, we measured ³H-thymidine incorporation of blood lymphocytes obtained from patients or healthy donors directly stimulated with UCPs during 6 days. In contrast with healthy donors, blood lymphocytes from patients with cancer specifically proliferate upon UCP 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 with 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 TGF-β cytokines but not IL-4, IL-10, or IL-17

Published OnlineFirst October 2, 2012; DOI: 10.1158/1078-0432.CCR-12-0896

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Thus, the UCP-specific T-cell repertoire is spontaneously stimulated in various cancers such as colon, kidney, lung, stomach, and leukemia. 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**

On the basis of 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 conducted immunizations with a plasmid DNA encoding the full length TERT sequence, and the UCP-specific CD4 T-cell proliferation was monitored by a 5-day ^3^H-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 with 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^+^ Th1 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, or 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 cells, freshly purified CD4^+^ T cells from UCP-immunized mice were cultured in the presence of decreasing concentrations of peptide and the number of specific IFN-γ–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^ μg/mL). In comparison, CD4^+^ T cells from mice vaccinated with UCP1 or UCP4 responded to 10^-1^ and 10^-3^ μg/mL of peptide concentration, respectively. In addition, low doses of UCP2 or UCP3 peptides (~1 μg) stimulated potent IFN-γ–producing 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 coinmunized 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 with pY988/IFA group. Although UCP1 vaccination had little impact on the frequency of pY988/A2 pentamer\(^+\) CD8 T cell-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 and 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, 2 critical functions for tumor eradication. To this end we focused on the UCP2 that induces potent Th1 immune responses in vivo. In addition, compared with a HLA-DR1–restricted viral 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\(^{-3}\) \(\mu\)g/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-labeled target cells (Fig. 4B) than in pY988/IFA group. In addition, TERT-specific CTLs from mice immunized in the presence of UCP2 exhibit strong in vitro cytotoxicity against TERT-expressing B16-A2 cells (Fig. 4C and D).

Furthermore, long-lasting TERT-specific CTL response was detected in mice coinkjected 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 (Supplementary Fig. S4C). Collectively, UCP2 helper immune responses enhance the magnitude and quality of antitumor CTL response.

Figure 2. UCP vaccinations stimulate high avidity Th1-polarized CD4 T-cell responses. A and B, A2/DR1 mice (n = 8) were immunized twice with a DNA encoding TERT. A, proliferation of spleen lymphocytes in the presence of UCPs. B, CD8-depleted spleen lymphocytes from DNA-immunized mice were assayed in ex vivo IFN-\(\gamma\) ELISPOT. Columns, mean of triplicate from 4 mice; bars, SD. C and 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 cytokine 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-\(\gamma\) 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-\(\gamma\) ELISPOT.
UCP-specific CD4+ T cells promote DC activation in vivo

The induction of DC activation represents one major helper mechanism used by CD4+ T<sub>H1</sub> 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 costimulating 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 with control mice. In a second set of experiments, CD4+ T cells isolated from UCP2/IFA or IFA-injected mice were cocultured with syngenic idCs (Fig. 5B). Similar increase of DC activation was found in the 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 T<sub>H1</sub>-associated cytokines such as IL-12, IFN-γ, and GM-CSF were found in the supernatant of CD4<sub>UCP2/DC</sub>coculture (Fig. 5C, right). This DC 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; ref. 26). Mice were challenged with 2 × 10<sup>5</sup> B16-A2 cells and tumor bearing mice were then vaccinated twice either with the 2 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 of more than 200 mm<sup>2</sup> at day 25 in the control group injected with IFA alone. In this representative experiment, tumor regression was observed in 1 of 8 mice vaccinated with pY572 + pY988/IFA, whereas 2 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 of 8 mice. Accordingly, survival analysis out on day 50 after tumor cell injection showed that 63% of mice vaccinated in the presence of UCP2 were still alive as compared with 13% in the group of mice injected with pY988 + pY572/IFA (P < 0.05; Fig. 6B).

![Graph showing CD4 helper role of UCP vaccinations on the self/TERT-specific CTL responses.](https://example.com/figure3.png)

**Figure 3.** CD4 helper role of UCP 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 10 days later in the spleen. A, freshly isolated CD8 T cells were stained with TERT pY988/A2 pentamer. Representative flow cytometry dot plots (top) and mean percentages of pY988/A2+CD8 T cells (bottom) are shown. B, ex vivo detection of anti-pY988 CD8 T cells by IFN-γ ELISPOT. C and D, simultaneous UCP-specific CD4 T-cell responses were assessed in CD8-depleted fraction by IFN-γ (C) and IL-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 3 independent experiments.
Immunization in the 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 with 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 and /C0, untreated cells. D, cytotoxicity of T cells against TERT-positive B16 or B16-A2 tumor cells after 5 days of in vitro stimulation of splenocytes with pY988. Results represent the specificity (percentage) ± 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).
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-cell infiltration was observed in mice that received vaccine plus UCP2 helper peptide as compared with pY988 + pY572/IFA group (67% vs 40%, P < 0.05; Fig. 6C). In contrast, UCP2 vaccination did not influence CD4^+ TILs,
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 3 independent experiments.
natural killer, or regulatory T cells (Treg), 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, bottom). TERT-specific CTL response was also detected in spleen of mice, which is correlated to UCP2-specific CD4+ T-cell response (Fig. 6D, top). 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 T H1 response against tumor is gaining considerable interest in cancer immunity. In this study, we found spontaneous T H1 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 UCP vaccination induces high avidity T H1-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 H1 cells regulate host antitumor immune responses (10). Indeed, T H1,2 CD4+ T cells and Tregs are often associated with an inhibitory environment within the tumor (10, 33). The role of T H17 cells in antitumor immune response is still controversial and seems to depend on the type of cancer (34). In contrast, T H1 immunity has a clear positive effect in cancer cell eradication. The CD4+ T H1 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 T H1 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 H1 cell responses (17). Consequently, the choice of tumor-reactive CD4 helper peptides for cancer vaccine needs to be done carefully.

On the basis of its expression profile and its role in multiple human tumors, TERT is an attractive target for cancer vaccination (22, 39). Schroers and colleagues 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 patients with cancer 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 conduct a systematic analysis of UCP-specific CD4+ T-cell help on antitumor CTL responses in A2/DR1 mice. To this end, we selected 2 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 T H1 cells drastically enhances self/TERT-specific CD8+ T-cell responses as compared with 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 have already shown the requirement of CD4 help for the generation of CTL against the self/TERT epitopes used in this study (23, 47). Gross and colleagues 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: approximately 25% of vaccinated mice were tumor free compared with 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 nontumor antigen–specific CD4 T H1 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; Supplementary Fig. S5). 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 coimmunized with UCP2. Finally, we found that spontaneous UCP-specific T H1 responses are detected in patients with various cancers indicating the presence of a functional UCP-specific T-cell repertoire. In our recent study, this preexisting UCP-specific CD4+ T-cell immunity was shown to be associated with an increased overall survival of patients with lung cancer responding to the first-line chemotherapy (19).
In conclusion, our study shows that the stimulation of UCP-specific CD4 T1 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.

Disclosure of Potential Conflicts of Interest
P. Langlade-Demoyen is a member of advisory board, INVECTYS Co. (current patent holder for UCPS). No potential conflicts of interest were disclosed by other authors.

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References

Acknowledgments
The authors thank Dr. Nathalie Chapat for providing B16/HLA-A*0201 cell lines. The authors also thank Drs. E. Fabre, T. Nguyen, K. Stefano, and A. Thierry-Vuillemin for the recruitment of patients with cancer.

Grant Support
This work was supported by Ligue Contre le Cancer, Association pour la Recherche contre le Cancer (ARC), and BQR grant from university of Franche Comté.

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Received March 19, 2012; revised August 20, 2012; accepted September 16, 2012; published OnlineFirst October 2, 2012.
UCP-Specific CD4 T Cells Help with Antitumor CTL Responses

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doi:10.1158/1078-0432.CCR-12-0896

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