

## Analysis of Spontaneous Tumor-Specific CD4 T-cell Immunity in Lung Cancer Using Promiscuous HLA-DR Telomerase-Derived Epitopes: Potential Synergistic Effect with Chemotherapy Response

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### Abstract

**Purpose:** To investigate the presence and impact of spontaneous telomerase-specific CD4 T-cell responses in cancer patients.

**Experimental Design:** A multistep approach was used to design novel pan-HLA-DR-restricted peptides from telomerase. T-cell clones isolated from cancer patients were used to characterize the polarization of telomerase-specific CD4 response. The presence of spontaneous CD4 T-cell response against telomerase was monitored in 84 metastatic non-small cell lung cancer (NSCLC) patients before first-line chemotherapy (CT) using IFN- $\gamma$  ELISPOT assay. Then we analyzed the impact of the pretherapeutic telomerase-specific CD4 T immunity on clinical outcome in patients according to their respective response to CT.

**Results:** We described four novel telomerase-derived CD4 epitopes referred as universal cancer peptides (UCP) that effectively bind to most commonly found human MHC class II alleles. UCP-specific CD4 T-cell repertoire is present in human and UCP-specific CD4 T-cell clones generated from cancer patients exhibited high avidity and are Th1 polarized. Significant frequency (38%) of naturally occurring UCP-specific T-cell responses were detected before CT in advanced NSCLC but not in healthy volunteers. This response was shown to significantly increase overall survival (OS) of patients responding to CT (Median OS: 53 vs. 40 weeks,  $P = 0.034$ ).

**Conclusions:** These results show for the first time a potential synergistic effect of telomerase-specific CD4 T-cell response with CT response in NSCLC and underline the potential role of tumor-specific CD4 T-cell response on the efficiency of conventional anticancer therapy. *Clin Cancer Res*; 18(10); 2943–53. ©2012 AACR.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Introduction

The recent introduction of immunotherapy in clinical practice (1, 2) emphasized the influence of immune responses on cancer prognosis and chemotherapy (CT) effectiveness. Among adaptive immune cells involved in antitumor responses, CD8 T cells (CTL) have been considered to be the main protagonists because they exhibit cytotoxic activity toward tumor cells expressing tumor-associated antigens (TAA). However, it is now clear that CD4 T helper 1 (Th1) lymphocytes also play a critical role in orchestrating the antitumor response. These cells mainly characterized by IFN- $\gamma$  production are critical for the induction and maintenance of CD8 T cells against tumors by providing help through multiple interactions (3, 4). CD4 Th1 cells can also exert antitumor activity that is independent of CD8 T cells by recruiting and activating innate immune cell such as natural killer and macrophages (5, 6). The IFN- $\gamma$  secreted by CD4 Th1 cells also mediates direct antitumor or antiangiogenic effect (7). A new dimension of CD4 helper T cells role during cancer is

### Translational Relevance

This article reports the role of naturally occurring telomerase-specific CD4 T-cell responses in lung cancer. We describe telomerase-derived CD4 epitopes referred as universal cancer peptides (UCP) that bind to most commonly found human MHC class II alleles. Spontaneous UCP-specific CD4 T-cell response was detected in 38% of patients' prior first-line chemotherapy (CT). The presence of this response significantly increases overall survival of patients responding to CT ( $P = 0.034$ ). These results provide a new tool for comprehensive monitoring of antitumor CD4 T-cell response and support the concept of immune modulation of CT efficacy. In addition, it could be used to provide compensatory measures to restore or improve anticancer immune responses.

also reported. It has been shown that CD4 T cells must pave the way for killer T-cell entry at tumor site (8) or infected mucosa (9). Furthermore CD4<sup>+</sup> T cells are required for the induction of cellular senescence and angiogenesis inhibition, resulting in sustained tumor regression upon inactivation of the MYC or BCR-ABL oncogene in a mouse tumor model (10). In human, high density of tumor-infiltrating Th1 cells has been shown as good prognostic marker in colorectal cancer (11). This study also showed that patients with high expression of the Th17 cluster gene had a poor prognosis. In non-small cell lung cancer (NSCLC) both immune cells infiltration and localization have been correlated with clinical outcome (12, 13).

On other hand, recent progress in the fields indicates that pretherapeutic immune parameters affect the efficacy of conventional chemotherapies (14, 15). Moreover, the efficacy of many of the currently used chemotherapeutic agents depends on the active contribution of both innate and adaptive immune effectors (16). For example, spontaneous and induced leukemia-specific CD4<sup>+</sup> Th1 cells in patients bearing chronic myelogenous leukemia and successfully treated with imatinib mesylate were associated with clinical benefit suggesting a synergy between the treatment tumor-specific CD4 T-cell response (17). However, little is known about the relationship between tumor-specific CD4 Th1 immunity and efficacy of CT. This could be related to technical reasons that limit the ability to easily monitor this response in patients. All above emphasize the critical function of CD4 Th1 responses in cancer immunosurveillance and strongly encourages to use/develop relevant tools to study tumor-specific CD4 helper T-cell responses and raise the interest to stimulate these cells during antitumor immunotherapy.

During the past 10 years, increasing attention has focused on identifying MHC class II epitopes from multiple TAAs (18). Nevertheless, only few CD4 helper epitopes derived from tumor antigens have been used in clinical settings.

Although HLA class II locus is very polymorphic, degenerate peptides capable of binding multiple allelic variants of HLA-DR have been described (19, 20). Identification of degenerate peptides of relevant TAAs may lead to improved cancer vaccine and for effective monitoring of CD4 T-cell immunity. On this view telomerase reverse transcriptase (TERT) has emerged as a promising universal target (21). TERT maintains telomere length in dividing cells and its overexpression is the predominant mechanism developed by malignant cells to escape telomere-dependent cell death (22). Therefore TERT activity has been observed in all studied cancer forms, including stem cell-like tumor cells (23) and is therefore a hallmark of cancer cells (24). Furthermore, recent clinical studies using a TERT-derived CD4 epitope vaccine called GV1001 was able to increase survival of cancer patients when combined with radio or CT (25, 26). These results suggested the interplay between anti-TERT-CD4 T-cell immunity and conventional anticancer therapy. However, the natural occurring TERT-specific CD4 helper T-cell responses in patients are unknown.

In this study, we described 4 novel TERT-derived CD4 epitopes referred as universal cancer peptide (UCP) that bind to most commonly found HLA-DR alleles. By prospectively studying a cohort of 84 advanced NSCLC patients, we found spontaneous UCP-specific CD4 immune response in 38% prior CT. Although CD4 T-cell response against TERT was not correlated with main clinical characteristics of the patient, the presence of this response was shown to be significantly associated with a better outcome in CT-responding patients. These results support the relationship between antitumor immune status and CT regimen in NSCLC.

## Materials and Methods

### Patients

Patients were enrolled at the university hospital Georges Pompidou (Paris, France) and university Hospital Jean Minjoz (Besançon, France) from January 2009 to September 2011. Tumor stage and grading were determined according to the International Union against Cancer (UICC) classification. After informed consent patients with histologically proven NSCLC were prospectively included in the clinical trial. This study was conducted in accordance with French laws and after approval by the local ethics committee. Blood cells were collected from anonymous healthy donors at the Etablissement Français du Sang (EFS, Besançon, France) as apheresis kit preparations after informed consent and following EFS guidelines. HLA-DR genotyping was done by using the Olerup SSP DRB1 typing kit (Olerup, Sweden).

### Telomerase-derived CD4 T epitopes selection and binding assay

The 4 peptides derived from TERT referred as UCP [UCP1 (PAAFRLVAQCLVCV), UCP2 (KSVWSKLQSIGIRQH), UCP3 (GTAQVQMPAHGLFPW), and UCP4

(SLCYSILKAKNAGMS)] were predicted to bind multiples HLA-DR molecules by using SYFPEITHI ([www.syfpeithi.de](http://www.syfpeithi.de)), NetMHCpan 2.1 (<http://www.cbs.dtu.dk/services/NetMHCpan/>) and NetMHCII 2.2 (<http://www.cbs.dtu.dk/services/NetMHCII/>) softwares (18). Synthetic peptides (>80% purity) were purchased from Activotec. The binding capacity to HLA-DR molecules was assessed by competitive ELISA as previously reported (27).

### Generation of UCP-specific T cell lines from healthy donors

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Sigma-Aldrich) and plated at  $4 \times 10^6$  cells per well in a 24-well plate in RPMI 5% human serum with 10  $\mu\text{mol/L}$  of pool of the 4 UCPs. Recombinants interleukin (IL) 7 (5 ng/mL; Peprotech) and IL-2 (20 UI/mL; Novartis) were added at days 1 and 3, respectively. At days 7 and 14, cells were stimulated with  $\gamma$ -irradiated autologous PBMCs pulsed with 10  $\mu\text{mol/L}$  of UCPs and 20 UI/mL IL-2 was added at days 8 and 15 as previously reported (27, 28). At day 21, CD4 T cells were purified (Miltenyi) and the specificity of T cell lines was investigated by IFN- $\gamma$  ELISPOT. Briefly, CD4 T cells ( $10^5$  per well) were cultured in anti-human IFN- $\gamma$  monoclonal antibody precoated ELISPOT plate with each UCP (5  $\mu\text{mol/L}$ ) in AIM V medium (Invitrogen) for 18 hours at 37°C. Cells cultured with medium alone or PMA (100 ng/mL; Sigma-Aldrich) and ionomycin (10  $\mu\text{mol/L}$ ; Sigma-Aldrich) were used as negative and positive controls, respectively. The IFN- $\gamma$  spots were revealed following the manufacturer's instructions (Gene Probe). The number of specific T cells expressed as spot-forming cells/ $10^5$  cells was calculated after subtracting negative control values (background). Spot-forming cells were counted using the C.T.L. Immunospot system (Cellular Technology Ltd.). For HLA-DR restriction, the following blocking antibodies anti-HLA class I (clone W6.32), HLA-DR (clone L243), and HLA-DP (clone B7/21; 10  $\mu\text{g/mL}$ ) were added in cell culture during the ELISPOT. All the experiments were conducted in triplicates.

### CD4 T-cell clones isolation and amplification

T-cells clones were isolated by limiting dilution and amplified after stimulation by PHA in presence of irradiated allogenic PBMCs, B-EBV cell line, and 150 UI of IL-2 according to previously described procedure (29). Functional analyses of UCP-specific CD4 T-cell clones were done by using intracytoplasmic TNF- $\alpha$  staining and Human Tenplex cytokines assay (Human Th1/Th2/Inflammation Diaplex; Diaclone).

### Assessment of spontaneous UCP-specific CD4 T-cell responses

Ficoll-isolated PBMCs from cancer patients or healthy volunteers were cultured with 10  $\mu\text{mol/L}$  of pool of UCPs in a 24-well plate ( $4 \times 10^6$  cells per well) in RPMI 5% human serum and IL-7 (5 ng/mL) and IL-2 (20 UI/mL) were added at days 1 and 3, respectively (27). For the recall response

against viruses, cells were similarly cultured with the mix of 32 peptides from cytomegalovirus, influenza virus, and Epstein-Barr virus (CTL). After 1 week of cell culture, the presence of UCP-specific T cells was measured by IFN- $\gamma$  ELISPOT as detailed above (30–32).

### Flow cytometry

For intracytoplasmic cytokine staining, after a 5-hour stimulation period with or without 10  $\mu\text{mol/L}$  peptide, T cells were labeled with anti-CD4 (BD Bioscience) and anti-TNF- $\alpha$  (ebioscience) using Cytofix/Cytoperm KIT (BD Bioscience). For flow cytometry Treg analysis, PBMCs were first stained with surface antibodies (anti-CD4, anti-CD25), fixed, permeabilized, and then stained with anti-hFoxp3 (PCH101; eBioscience). Samples were acquired on a FACS Canto II (BD Biosciences) and analyzed with the DIVA software. Neutrophil-lymphocyte ratio (NLR) was defined as the absolute neutrophil count divided by the absolute lymphocyte count (13).

### Statistics

Statistical analyses were carried out with NCSS 2007 software (Number Cruncher Statistical Systems). The level of significance was set at  $P < 0.05$  for all tests. Variables were expressed as a mean  $\pm$  SD or median and tested with the Wilcoxon Rank-Sum test when suited. Survival curves were calculated with the Kaplan-Meier method and compared with the Log-rank test.

## Results

### Identification of universal HLA-DR-restricted CD4 T-cell epitopes from TERT

To predict the existence of CD4 epitopes within the amino acid sequence of TERT capable of binding to multiple HLA-DR molecules, we combined results from 3 algorithms Syfpeithi, NetMHCpan-2.1, and NetMHC2.2. We selected four 15-mers peptide sequences referred as UCP1 to UCP4 that scored high in the probability scale for their binding capacity to the commonly found human HLA-DR alleles (Supplementary Table S1). To confirm this result, we carried out an *in vitro* binding assay based on competitive ELISA as previously reported. The data were presented as relative affinities (RA) to easily compare their binding properties to high binder peptides that we used as references and the strong binders have a relative affinity less than 100. Results confirmed the ability of all the peptides to effectively bind to the most common alleles encoded by the HLA-DR (Table 1). The 4 peptides exhibited a strong capacity to bind 7 different HLA-DR molecules, including DR1, DR4, DR7, DR11, DR15, DRB3, and DRB5. Particularly, UCP1 and UCP2 were able to bind every HLA-DR molecules tested with RA from intermediate (100–500) to low RA ( $< 100$ ). Thus, according to phenotypic frequencies of the 10 prevalent HLA-DR antigens, these peptides could cover more than 90% of population (27). Furthermore, CD4 T-cell responses against UCPs were induced in humanized HLA-DR1\*0101/HLA-A2 transgenic mice following immunization with a DNA plasmid encoding the full-length TERT

**Table 1.** The binding capacity of predicted peptides was tested using competitive ELISA binding assays

Peptides	Sequences	Relative avidity									
		DR1	DR3	DR4	DR7	DR11	DR13	DR15	DRB3	DRB4	DRB5
<b>UCP1</b>	PAAFRALVAQCLVCV	<b>3</b>	<b>0.4</b>	<b>50</b>	<b>3</b>	<b>25</b>	<b>1</b>	<b>4</b>	<b>30</b>	<b>4</b>	<b>1</b>
<b>UCP2</b>	KSVWSKLQSIGIRQH	<b>0.2</b>	144	112	1	4	231	<b>8</b>	154	229	<b>1</b>
<b>UCP3</b>	GTAFVQMPAHGLFPW	<b>0.1</b>	173	<b>2</b>	<b>2</b>	<b>0.2</b>	134	<b>0.2</b>	<b>53</b>	>500	<b>0.2</b>
<b>UCP4</b>	SLCYSILKAKNAGMS	<b>0.3</b>	>500	<b>34</b>	<b>8</b>	<b>0.3</b>	>500	<b>3</b>	154	>500	<b>0.5</b>

NOTE: Data are expressed as relative activity RA (ratio of the IC<sub>50</sub> of UCPs to the IC<sub>50</sub> of the reference peptide) and are the means of 3 experiments. Good binders have a RA less than 100 and weak binders are RA more than 500.

protein (28, 33) and indicating that they are endogenously processed and presented to CD4 T-cell *in vivo* (Dosset and colleagues; manuscript in preparation).

Then the ability of UCP to stimulate human CD4 T cells was tested. For this purpose, lymphocytes isolated from peripheral blood of healthy volunteers were *in vitro* stimulated using a pool of UCP and the generation of UCP-specific CD4 T cell lines was screened using ELISPOT assay. As shown in Fig. 1A, CD4 T cells were able to recognize at least one UCP. The HLA-DR restriction of the UCPs specific CD4 T-cell response was confirmed with the inhibition of IFN- $\gamma$  secretion in presence of pan HLA-DR blocking antibody (Fig. 1B). The HLA-DR spectra typing revealed that the HLA-DR alleles of normal individual were not shared, supporting the promiscuous nature of the UCPs (Fig. 1C). Thus these results implied that precursor CD4 T cells against UCPs were present in human peripheral T repertoire and they recognized these peptides in multiple HLA-DR contexts. To further characterize these responses, we isolated CD4 T-cell clones specific for UCP2 and UCP4 from cancer patient. All the UCP4-specific CD4 T-cell clones were strongly reactive in the presence of cognate peptide and showed a half-maximal TNF secretion observed at very low peptide concentration (<0.1  $\mu$ mol/L; Fig. 2A and B). Similar results were obtained for UCP2-specific clones with a half-maximal TNF secretion observed at approximately 4  $\mu$ mol/L (Fig. 2C and D). In addition, we showed after peptide stimulation that the clones mainly produced IFN- $\gamma$  and TNF- $\alpha$ , but not IL-4 nor IL-17A, in agreement with a Th1 polarization (Fig. 2E). The reactivity of these CD4 T-cell clones were inhibited by HLA-DR blocking antibody indicating their HLA-DR restriction (data not shown). Thus, these results showed that high-avidity UCP-specific CD4 T-cell clones can be generated from cancer patients and were Th1 polarized. They also showed that these UCPs are naturally processed and presented to CD4 T-cell in the context of malignancies.

#### Presence of naturally occurring CD4 T cells against UCPs in NSCLC patients

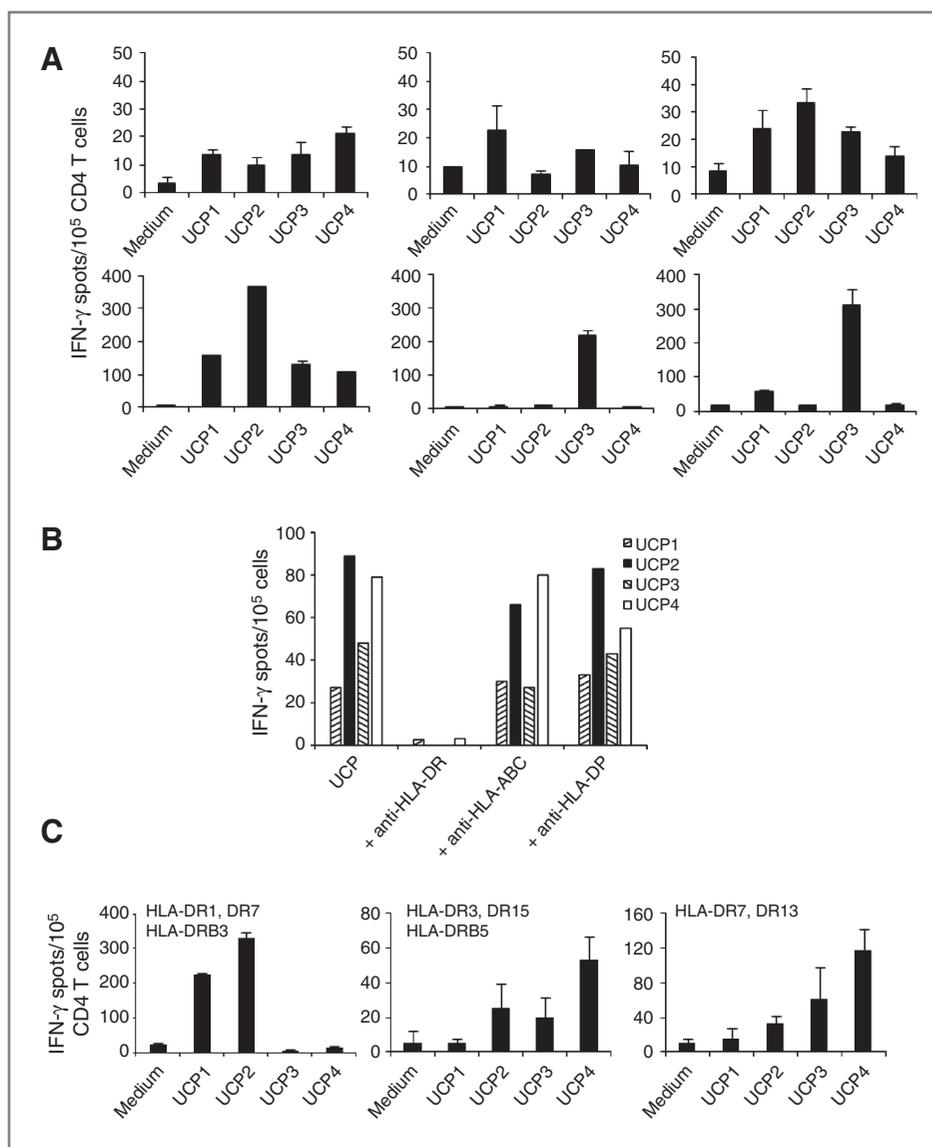
Telomerase gene polymorphisms have been associated with lung cancer susceptibility and TERT expression is found in all types of NSCLC (34, 35). Therefore, we carried out a comprehensive analysis of spontaneous UCP-specific

CD4 T-cell responses in NSCLC. Ficoll-isolated blood lymphocytes from 84 advanced NSCLC patients were collected before first-line CT and cultured shortly (one week) with the pool of UCPs, and the specific T cells were measured by IFN- $\gamma$  ELISPOT. Blood lymphocytes from 22 healthy volunteers were used as control. Responses were considered positive when the number of IFN- $\gamma$  secreting cells was at least 2-fold above the negative control. This experimental design enabled us to measure specific CD4 T-cell memory responses. As shown in Fig. 3A, UCP-specific memory immune responses were found in 32 of 84 patients (38%), whereas no specific IFN- $\gamma$  responses against UCPs were detected in 22 consecutive healthy donors (Fig. 3A). Analysis of the cytokine secretion profile of these responses revealed high production of TNF- $\alpha$  and IFN- $\gamma$  in the absence of IL-4, IL-17, and IL-10 indicating a Th1 polarization (data not shown). Analyzed individually, each of the 4 UCPs is able to generate a CD4 T-cell response in patients. However, the frequency of T-cell responses to UCP2 and UCP4 suggest that these peptides are more immunogenic (Fig. 3B). The absence of UCPs specific immune responses in patients could not be related to a global T-cell anergy, as illustrated by the presence of effective antiviral recall responses in patients without UCPs specific response (Fig. 3C). To exclude the influence of a number of immune parameters that have been reported to decrease antitumor response in NSCLC (13), we measured circulating CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells (Treg) and the plasmatic IL-10 in the patients with or without UCP-specific immune response. We showed similar level of circulating Tregs in the 2 groups (Fig. 3D) and absence of plasmatic IL-10 detection by ELISA was observed regardless of the UCP-immune status (data not shown). In addition, the total lymphocyte counts and NLR were quite similar in these 2 groups (Fig. 3D). Our results indicated that patients with NSCLC are able to spontaneously mount TAA-specific CD4 T-cell responses and that UCPs are prototypic peptides to monitor antitumor immune response in NSCLC.

#### Spontaneous UCP-specific T-cell immune response increase overall survival of patients responding to chemotherapy

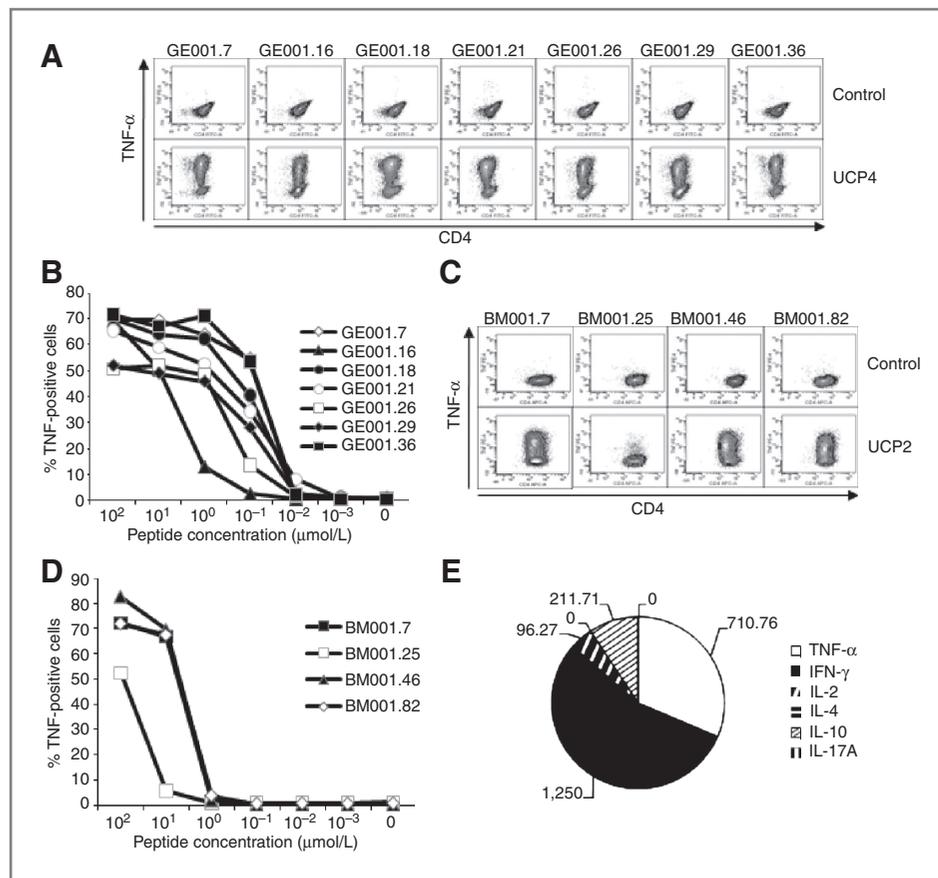
There is increasing knowledge that host immune status can modulate the efficiency of conventional CT. Then, the

**Figure 1.** UCP-specific T cell lines obtained from healthy donors. CD4 T cell lines were obtained from PBMCs of healthy donors after 3 round of stimulation with a mixture of the 4 UCP and IFN- $\gamma$ -producing CD4 T cells were assessed by ELISPOT. A, responses against individual UCPS are shown for 6 healthy donors. B, UCP-specific T cell lines were stimulated with the relevant peptide in presence of anti-HLA class I (W6.32), HLA-DR (L243), or HLA-DP (B7/21) blocking antibodies. C, responses against individual UCPS for 3 healthy donors with various HLA-DR genotype.



impact of naturally occurring UCP-specific CD4 immune response before CT on clinical outcome was analyzed in patients that responded or progressed after first-line CT. For this purpose, we focused on 55 stage IV NSCLC patients included between November 2009 and February 2011 with a median follow-up of 10 months. Patient's main clinical characteristics are shown in Table 2. T-cell responses against TERT were not correlated with clinical or prognostic variables such as age, tobacco, ECOG PS status, or histologic subtype (Table 2). Except 6 patients who received erlotinib therapy, all patients were treated with platinum doublet. Thirty of the 55 patients received a second-line CT mainly with erlotinib, pemetrexed, or taxanes. After first line, control disease (CD) based on RECIST criteria were achieved in 36 of 55 (65%), 25% of them achieved a partial response (PR; 14 of 55), and 40% a stable disease (SD; 22 of 55). Progressive disease (PD) was observed in 19 of 55

(35%). The frequency of spontaneous TERT-specific CD4 immune response was similar in patient with CD or PD after CT (Fig. 4A). In contrast, patients displaying a TERT-specific immunity before CT had an increased overall survival (OS) in the CD group compared with patients with no TERT-specific immunity [Median OS: 53 vs. 40 weeks,  $P = 0.034$ , HR = 0.54, 95% confidence interval (CI): 0.3–1]. The preexistence of UCP-specific immune response nonsignificantly increased the progression-free survival (PFS) of CD patients (Median PFS: 33 vs. 24 weeks,  $P = 0.391$ , HR = 0.76, 95% CI: 0.4–0.7; Fig. 4B). Similar results were observed when we focused on patients that received platinum-based CT, after excluding the erlotinib-treated patients (Median OS: 53 vs. 40 weeks,  $P = 0.049$ , HR = 0.52 95% CI: 0.3–0.9; Fig. 4C and D). No differences have been observed based on the second-line CT regimen received (data not shown). By contrast, in patients with



**Figure 2.** Functional characterization of UCP-specific CD4 T-cell clones. T-cell clones were obtained by limiting dilution of cancer patients T cell lines stimulated one time with the pool of UCPS. A and C, percentage of TNF-producing T cells and of T-cell clones isolated from patients GE001 in response to 10  $\mu\text{mol/L}$  of the relevant UCP;  $10^5$  T cells were incubated for 5 hours in the presence of Brefeldin A, stained with CD4 antibody, fixed, and stained with anti-TNF antibody in a permeabilization buffer;  $10^4$  T cells were then analyzed in flow cytometry. B and D, reactivity of the CD4 T-cell clones in response to relevant UCP. CD4 T-cell clones were culture with a range of the indicated peptide concentration. TNF secretion was assessed for 5 hours in the presence of Brefeldin A, by flow cytometry. E, detection of cytokines produced by GE001.36 T-cell clone in response to 10  $\mu\text{mol/L}$  of UCP4 using human 10-plex cytokines assay.

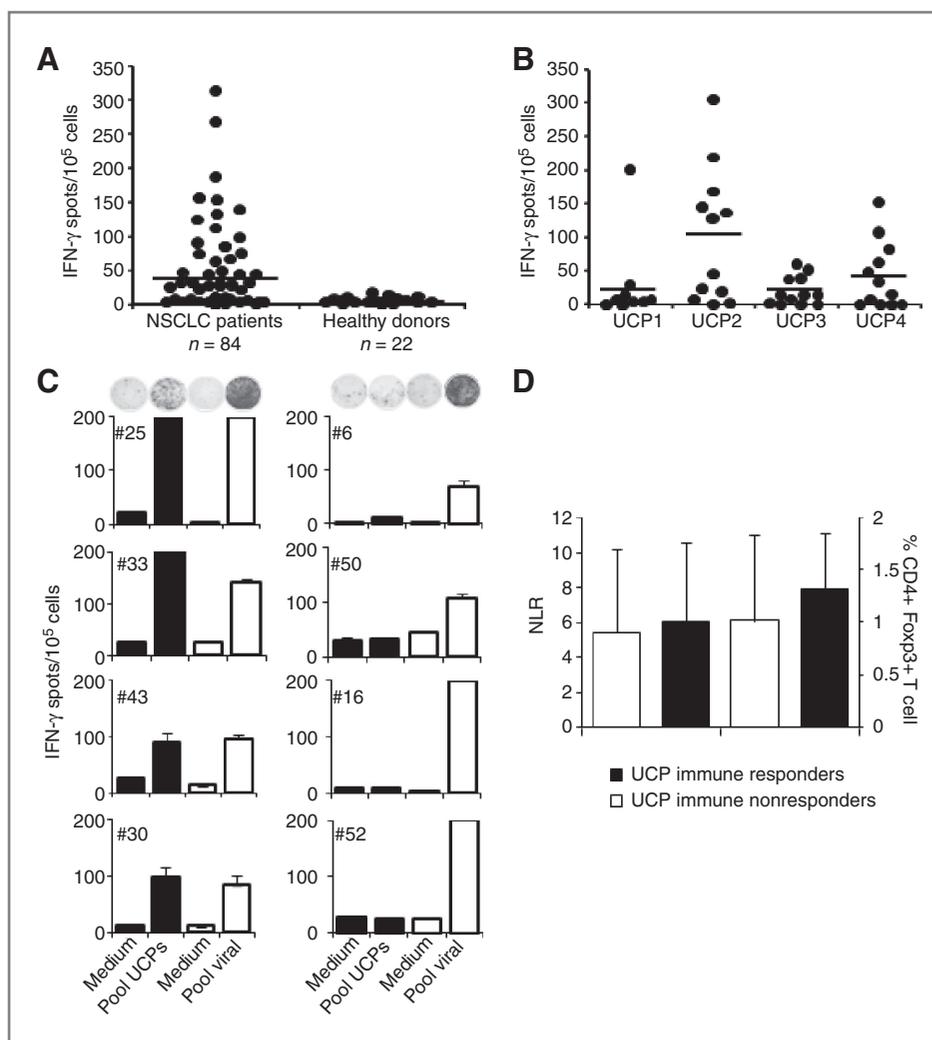
PD after first-line CT, we found no survival difference regardless of UCP-specific immune status (data not shown). Thus, the presence of natural TERT-specific CD4 Th1 responses in patients whose tumors are sensitive to CT is correlated to a higher OS. These results provided an innovative method to monitor antitumor CD4 immunity in cancer patients and emphasized the potential relationship between CT regimen and antitumor immune responses in NSCLC.

## Discussion

CD4 helper T cells play a major role in the generation and maintenance of effective antitumor immune responses (36). In this study, we characterized CD4 T-cell responses against novel pan HLA-DR-restricted epitopes derived from TERT and referred as UCP. The UCP-specific CD4 T-cell clones have generated a high avidity and are Th1 polarized. We found that IFN- $\gamma$  producing CD4 T-cell responses against UCPS are naturally present in advanced NSCLC patients. The presence of this anti-TERT Th1 immunity increased the survival in the group of patients that achieved disease control after CT. By contrast, the antiviral responses measured at the same time had no effect on survival (data not shown). Thus, our results showed for the first time to our knowledge an association between spontaneous TERT-specific CD4 T immunity and CT response in lung cancer.

There are several distinct mechanisms through which conventional CT modifies the interactions between tumor and immunologic environment (15). Through their action on cancer cells, chemotherapeutics can restore or enhance the expression of tumor antigens, making them more recognizable by the immune system (37, 38). The pioneering works from L. Zitvogel and G. Kroemer highlighted the capacity of some CT drug, such as anthracyclines and oxaliplatin, to induce immunogenic cell death resulting in activation of DC and priming of antitumor immune response (39). These drugs have the particular ability to facilitate the engulfment of the tumor cell by DCs through mechanism involving the chaperone calreticulin, HMGB1 or ATP pathway (40). However, little is known about the relationship between specific CD4 Th1 immunity and efficacy of CT in cancer patients. Nevertheless our results can be compared with those having shown the presence of leukemia-specific CD4<sup>+</sup> T cells in patients bearing chronic myelogenous leukemia and successfully treated with imatinib (17). Other reports also showed that antibody titers against antiphospholipids were associated with prolonged survival after retinoic acid treatment in promyelocytic leukemia (41). Although we have not studied the UCP-specific T-cell response after CT, we can speculate that, in responding NSCLC patients, the tumor lysis induced by CT releases TERT molecules that are available for antigen presenting cell

**Figure 3.** Naturally occurring UCPs specific response in metastatic NSCLC patients. **A**, spontaneous UCP-specific T-cell responses were assessed in 84 NSCLC patients and 22 healthy donors as control. After short time stimulation (one week) with a mixture of the 4 UCPs, the presence of specific T cells was detected using IFN- $\gamma$  ELISPOT assay. The results represented specific IFN- $\gamma$  spots after subtraction of background. Responses were positive when IFN- $\gamma$  spots were more than 10 and more than 2-fold the background. **B**, frequency of individual UCP-specific T-cell responses in 12 NSCLC patients was shown. **C**, illustration of UCPs versus viral specific immune responses in 8 NSCLC patients after one week *in vitro* stimulation. **D**, baseline neutrophils on lymphocytes ratio (NLR) and CD4<sup>+</sup> Foxp3<sup>+</sup> T-cell frequency in patients according to the UCP-specific immune status.



uptake and presentation of UCP to memory CD4 T cells. By contrast, when CT was ineffective, tumor lysis was low and consequently TERT antigen release was less available for the activation of the UCP-specific CD4 T naturally present *in vivo*. This would explain the lack of impact of UCP-specific immune response observed in patients with PD after CT. Ongoing monitoring of the UCP-specific immunity before and after CT would confirm the amplification of this immune response in patients that successfully respond to chemotherapeutic agents. Additional mechanisms exist through which chemotherapeutics increase the susceptibility of tumor cells to immune attack. DNA damaging agents may activate oncogene-based pathways that result in the upregulation of Fas or other death receptors such as the TNF-related apoptosis-inducing ligand (TRAIL) receptor (42), which can increase the susceptibility of tumor cells to lysis by immune effectors such as CD4 Th1 cells expressing CD95 ligand or TRAIL (5). The expression Fas/CD95 ligand and TRAIL on UCP-specific T cells was not evaluated in this study and need future investigations. Chemotherapeutic drugs have also direct effects on the immune system

that may contribute to an improved anticancer immunity. For example, low metronomic doses of cyclophosphamide or paclitaxel deplete and/or inhibit Tregs that have immunosuppressive properties (43). We reported recently a progressive reduction in Foxp3<sup>+</sup> Treg after sunitinib therapy (44). In this study, Tregs levels at baseline cannot explain the difference observed as both patients with control or PD have similar rate of circulating Treg cells. In addition, no difference was shown in the two groups with regard to the plasmatic level of IL 10, an immunosuppressive cytokine. According to the all aforementioned above, our results in advanced NSCLC patients suggest that patients responding to CT enter this positive circle and could benefit natural antitumor immune response targeting TERT. To address more precisely the cross-talk between UCP-specific CD4 T cells and CT, it would be interesting to evaluate the TERT-specific responses before and after CT. This purpose will be evaluated in future study.

They also support the synergy between CT and therapeutic vaccination targeting TERT that has been recently reported in lung cancer (25, 45).

**Table 2.** Baseline clinical characteristic of patient

Characteristics	Global	Spontaneous UCP-specific CD4 T-cell responses		p
		Positive	Negative	
All patients no.	55	22 (40)	33 (60)	n.s.
Age (y) median no. (%)	29	13 (45)	16 (55)	n.s.
<65	26	9 (35)	17 (65)	n.s.
≥65				
Sex no. (%)	37	15 (41)	22 (59)	n.s.
Male	18	7 (39)	11 (61)	n.s.
Female				
Smoking status no. (%)	45	17 (38)	28 (62)	n.s.
Current or former smoker	10	5 (50)	5 (50)	n.s.
Never smoker				
ECOG PS no. (%)	2	1 (50)	1 (50)	n.s.
0	25	10 (40)	15 (60)	n.s.
1	28	11 (39)	17 (61)	n.s.
2				
Histologic subtype no. (%)	38	19 (50)	19 (50)	n.s.
Adenocarcinoma (ADK)	13	1 (8)	12 (92)	n.s.
Squamous cell carcinoma	4	2 (50)	2 (50)	n.s.
Other				
Chemotherapy (%)	49	19 (39)	30 (61)	n.s.
Platinum doublet	6	3 (50)	3 (50)	n.s.
Erlotinib				

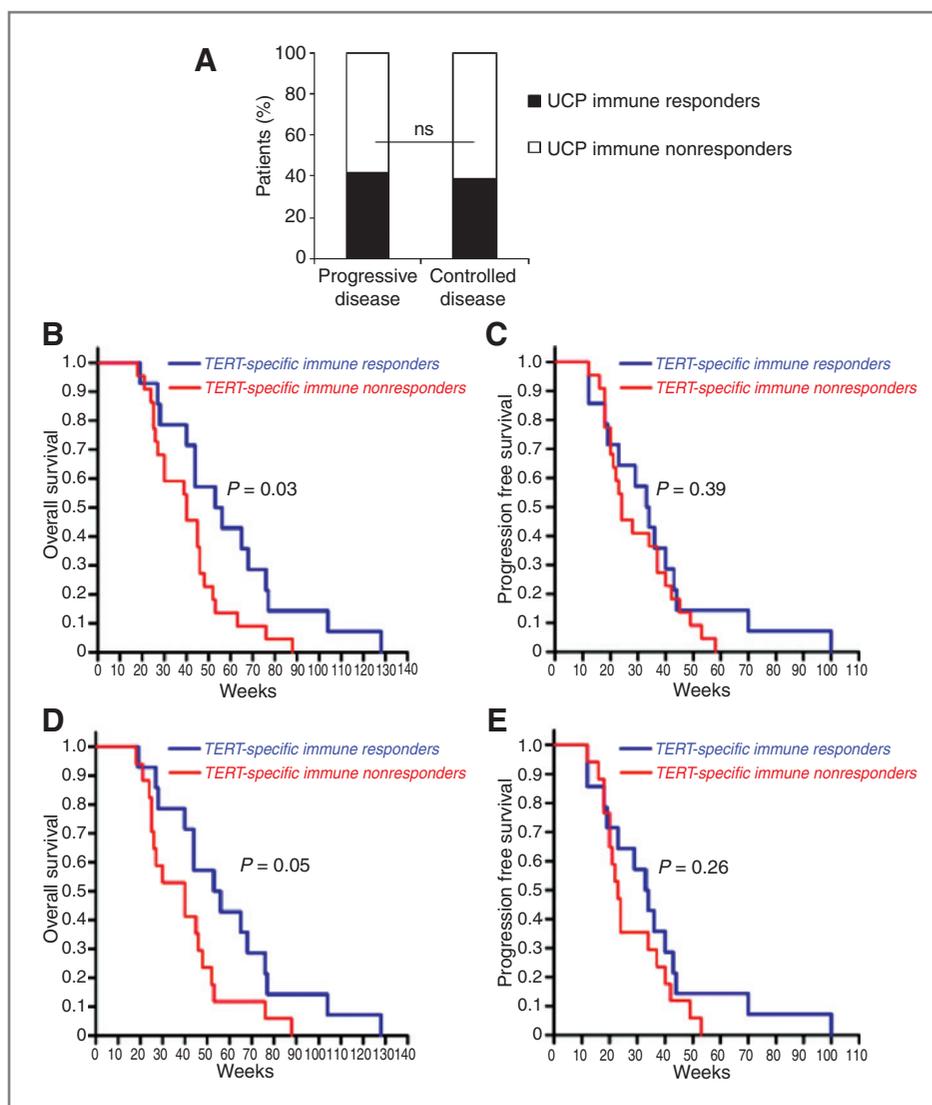
NOTE: Spontaneous UCP-specific T-cell responses measured by standardized IFN- $\gamma$  ELISPOT assay as detailed in Material and Method section. ECOG indicate Eastern Cooperative Oncology Group.

For technical reasons, there are very few studies that address the frequency of tumor-specific effector or memory T cells or antibody titers before and after CT. During the past years, different groups have focused on the identification of CD4 T-cell epitopes from TAA that could be used to improve anticancer immunotherapy and for the monitoring of anti-tumor CD4 T-cell responses (18). This study illustrates the potential of TERT-derived UCPs for a dynamic monitoring of tumor-specific CD4 T-cell responses in cancer patients. It also underlines the importance of tumor antigen selected to monitor the tumor-specific immune responses in cancer patients. It also underlines the importance of tumor antigen selected to monitor the tumor-specific immune responses in cancer patients. On this view TERT present several advantages: (i) it is expressed in most human cancers, (ii) its oncogenic role is essential for cell immortality and tumor growth and this prevents the antigenic loss tumor escape mechanism, (iii) its constitutively high expression in cancer cells and cancer stem cells, and (iv) its immunogenicity (22, 24). Schroers and colleagues have first reported 2 TERT-derived promiscuous HLA-DR-restricted peptides hTERT672 and hTERT766 (46, 47). However, the capacity of these 2 peptides to bind several HLA-DR alleles was not evaluated using binding assay and the presence in cancer patients of spontaneous CD4 T-cell

response against these peptides was not clearly studied. Another promiscuous TERT-derived CD4 epitope called GV1001 was reported by Gaudernack and colleagues (48). This peptide has been used in clinical trials with encouraging results when combining to CT in melanoma and NSCLC (25, 26). Nevertheless, the impact of spontaneous GV1001-specific immune response on vaccination efficiency and clinical outcome has not been investigated yet. Note that in our cohort the present GV1001-specific immune response was less frequent than UCP's (data not shown).

In this article, we report widely promiscuous HLA-DR TERT-derived peptides recognized by CD4 T cells, and naturally occurring CD4 T-cell responses against these peptides were found in advanced NSCLC patients before treatment. Interestingly, this TERT-specific, DR-restricted T-cell response positively impact OS in CT responding patients. In a complementary study, the spontaneous T-cell response against UCPs was confirmed in other malignancies such as colon cancer, leukemia, and renal carcinoma, and the helper role of UCP-based antitumor vaccine was also established in a HLA-DR1\*0101/HLA-A2 transgenic mice (Dosset and colleagues; manuscript in preparation). Taken together, this study identified UCP that should be used as a powerful tool to evaluate the

**Figure 4.** Impact of spontaneous UCPs CD4 T-cell response in metastatic NSCLC patients. A, UCPs responder and nonresponder frequencies in patients with PD or CD. B, Kaplan–Meier estimates of OS and (C) PFS of CD patients. D, OS and (E) PFS of CD patients treated with platinum-based first-line CT.



interplay between conventional anticancer therapy and tumor-specific CD4 T-cell responses. These findings also indicate that targeting TERT with promiscuous immunogenic CD4 T epitopes is of particular interest for cancer vaccine.

#### Disclosure of Potential Conflicts of Interest

P. Langlade-Demoyen is a Member of Advisory Board, INVECIYS Co. (current patent holder for UCPs). The other authors disclosed no potential conflicts of interest.

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# Clinical Cancer Research

## Analysis of Spontaneous Tumor-Specific CD4 T-cell Immunity in Lung Cancer Using Promiscuous HLA-DR Telomerase-Derived Epitopes: Potential Synergistic Effect with Chemotherapy Response

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