Immunoprevalence and magnitude of HLA-DR4 versus HLA-DR-restricted spontaneous CD4+ Th1 responses against telomerase in cancer patients

Caroline Laheurte a,b,x, Jeanne Galaine a,c,x, Laurent Beziaud a,c, Magalie Dosset a,c, Jerome Kerzerho d, Claire Jacquemard b, Béatrice Gaugler a,b,c, Christophe Ferrando a,b, Anne Dormoy b, François Aubin b, Pascale Jacoulet b, Virginie Westeel b, Christophe Borg a,b,c,g, Eric Tartour b, Yann Godet a,c, Bernard Maillère e, and Olivier Adotévi a,c,x

aINSERM, UMR1098, LabEx LIPSTIC, Besançon, France; bEFS Bourgogne Franche-Comté, Plateforme de Biomonitoring, INSERM CIC1431, Besançon, France; cUniv. Bourgogne Franche-Comté, UMR1098, Besançon, France; dCEA, iBiTecS, Service d’Ingénierie Moléculaire des Protéines (SIMOPRO), Labex LERMIT, Labex VRI, Gil Sur Yvette, France; eDepartment of Dermatology, University Hospital of Besançon, EA3181, SFR4234, Besançon cedex, France; fDepartment of Pneumology, University Hospital of Besançon, Besançon cedex, France; gDepartment of Medical Oncology, University Hospital of Besançon, Besançon cedex, France; hINSERM, UMR970, Hôpital Européen Georges Pompidou, Paris, France, Department of Biological Immunology, Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France, University Paris Descartes, Sorbonne Paris Cité, Paris, France

ABSTRACT
Cumulative evidence supports that CD4+ Th1 cells play a key role in antitumor immunity. We previously reported the presence of spontaneous HLA-DR-restricted CD4+ Th1 responses against telomerase reverse transcriptase (TERT) in various cancers by using promiscuous HLA-DR epitopes. Here, we described novel highly immunogenic HLA-DR4-binding epitopes from TERT named TERT541–555, TERT575–587, TERT613–627, and TERT621–635 and addressed the question about the immunoprevalence and magnitude of the naturally occurring antitumor CD4+ T cell responses restricted by HLA-DR4 or HLA-DR, the two most common HLA class II. Direct comparative study of spontaneous anti-TERT CD4+ T cell responses in a cohort of 87 lung cancer patients showed that HLA DR4 and HLA DR-restricted specific Th1 responses in 10.1% and 25.2% of cancer patients respectively (p = 0.01). The magnitude of the HLA-DR-restricted responses was two to three times significantly higher than HLA-DR one (p = 0.005). Similar results were found in other cancers such as melanoma, breast cancer, renal cell carcinoma and colon cancer. Thus, our results describe for the first time in a large cohort of cancer patients a high immunoprevalence of HLA-DR-restricted spontaneous anti-TERT Th1 immunity compared to HLA-DR restriction. These results provide a new tool for comprehensive monitoring of antitumor CD4+ Th1 response in various cancers.

Introduction
Due to their central role in orchestrating the antitumor immunity, tumor-specific CD4+ T cells gained considerable interest for cancer immunotherapy during the past decade. Typically, CD4+ T cells recognize peptides of 12–15 amino acids in length presented by MHC class II molecules. Among the various subpopulations of CD4+ helper T cells regulating immune response, the CD4+ Th1 subset that produces IFNγ, TNF-α and IL-2, mainly controls cell-mediated immunity against tumors. Cumulative studies have shown that CD4+ Th1 cells mediate tumor protection by secreting IFNγ, which mediate cytotoxicity against tumor cells synergistically with TNF-α while also by inducing reactive oxygen species and nitric oxide inhibiting angiogenesis and by stimulating NK cells and cytotoxic type 1 macrophages. More importantly, only tumor-reactive CD4+ T cells have been found to ensure efficient effector cytotoxic T lymphocytes (CTL) recruitment at the tumor site and this effect was promoted by IFNγ-dependent production of chemokines such as CXCL9 and CXCL10. In cancer patients, CD4+ T cell responses against tumor antigens have been detected in periphery and at the tumor site. Accordingly, high density of tumor-infiltrating Th1 cells has been identified as a good prognostic marker in several human cancers. Studies in melanoma patients demonstrated the interest to use tumor reactive helper peptides rather than peptides derived from non-tumor antigen for vaccine efficacy. Furthermore, it has been shown that the presence of CD4+ T cells contributes to the efficacy of conventional chemotherapy suggesting a synergy between the treatment and antitumor CD4+ T cell immunity. More recently, it has been shown in a mouse model and melanoma patients that the majority of immunogenic neo-antigens or mutanome is frequently recognized by CD4+ T cells. In addition, the immunization with CD4+ T immunogenic neo-epitope confers strong antitumor response. This observation underlines the critical role of CD4+ T cells immunity in cancer immunosurveillance.
However, neo-epitopes targeting strategy meets several hurdles.\textsuperscript{22} Then, we believe that the identification of MHC class II-binding peptides from shared tumor antigens may contribute to a better analysis of CD4\textsuperscript{+} Th1 responses in cancer patients.

TERT, the catalytic subunit of the telomerase, represents the prototype of widely overexpressed and shared tumor antigens.\textsuperscript{23,24} The reactivation of telomerase during human tumorigenesis is the predominant mechanism developed by malignant cells to escape telomere-dependent cell death.\textsuperscript{25,26} Consequently, TERT is widely expressed in all types of studied cancers (>90% of all cancers).\textsuperscript{27} Besides maintaining telomere length, TERT also plays a crucial role in oncogenesis by providing proliferation, survival and anti-apoptotic signals.\textsuperscript{28} More recently, high frequency of TERT promoter mutations was found in several cancers in which they lead to an enhanced expression of TERT and an increase in telomere length.\textsuperscript{29-31} All these properties raise TERT to the rank of a hallmark of cancer.\textsuperscript{23,24} HLA-DR-restricted epitopes derived from TERT that stimulate specific CD4\textsuperscript{+} T cell responses have been previously described.\textsuperscript{32,33} More recently, we identified highly promiscuous HLA-DR-binding peptides derived from TERT called universal cancer peptides (UCP). These peptides have been designed to cover a large proportion of the population (around 80%) and to especially stimulate Th1-polarized CD4\textsuperscript{+} T cells.\textsuperscript{19,34} By using UCP, we detected naturally occurring HLA-DR-restricted CD4\textsuperscript{+} Th1 responses against TERT in several human cancers.\textsuperscript{19,34} Furthermore, in lung cancer patients, the presence of this spontaneous anti-TERT Th1 response was associated with a better outcome.\textsuperscript{19,35}

Previous studies suggested that HLA-DR molecules were more frequently involved in CD4\textsuperscript{+} T cell responses against tumor antigens than HLA-DP4 molecules.\textsuperscript{7,36-38} The HLA-DP4 allotype consists of two molecules, DP401 (DPA1 0103/DPB1 0401) and DP402 (DPA1 0103/DPB1 0402), which differ from each other by only three aa residues. One or both HLA-DP4 molecules are carried by around 75% of Caucasian individuals.\textsuperscript{39} However, the spontaneous HLA-DP4-restricted CD4\textsuperscript{+} T cell responses against TERT have not been extensively studied in cancer patients. In this study, we assessed if HLA-DP4 molecules, can sustain TERT-specific CD4\textsuperscript{+} Th1 responses and addressed the question about immunoprevalence and magnitude of HLA-DP4 versus HLA-DR-restricted anti-TERT CD4\textsuperscript{+} Th1 responses naturally occurring in cancer patients.

\textbf{Results}

\textit{Binding capacity and immunogenicity of TERT-derived HLA-DP4 peptides in healthy individuals}

A set of 22 peptides was first selected from the whole sequence of TERT according to their prediction score to bind to HLA-DP401 molecule.\textsuperscript{30} These predicted peptides were synthetized and then evaluated in binding assays specific to HLA-DP401 and HLA-DP402 molecules. We showed that 14 out of 22 predicted peptides (64%) bind with a good or moderate affinity to HLA-DP4001 (Table 1). Although two peptides only were expected to bind tightly to HLA-DP401 (IC50 inferior to 100 nM), the binding assays revealed seven good binders that we included in the same pool of peptides (pool A). Seven other peptides according to their prediction scores exhibited a moderate affinity and were retained to constitute a pool of moderate binders (pool B). Although other peptides were also predicted, they bound weakly to HLA-DP4001 (data not shown). A good correlation was observed between the binding values obtained with the two HLA-DP4 allotypes according to their minor sequence differences\textsuperscript{39} except for three out of the 14 peptides (TERT 456–470, TERT 559–673, and TERT 553–567).

To investigate whether HLA-DP4-restricted peptides could be recognized by human CD4\textsuperscript{+} T cells, we performed \textit{in vitro} peptide stimulation. Blood lymphocytes from healthy donors were cultured either with the pool of HLA-DP4 strong binder peptides (pool A) or with the pool of intermediate binders (pool B) for 2 weeks and specificity of T cells was assessed by IFN\textgamma ELISPOT assay. As shown in Fig. 1, the stimulation with pool A peptides induced specific T cells against two peptides referred as TERT 541–555 and TERT 573–587 in the four consecutive donors tested. From pool B peptides (intermediate

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{hTERT peptides} & \textbf{Sequences} & \textbf{Score peptide\textsuperscript{a}} & \textbf{Relative activity\textsuperscript{b}} \\
\hline
\textbf{Strong binders Pool A} & & & & & & & & \\
TERT 911–925 & D E A L G G T A F V Q M P A H & 30 & 11 & 22 \\
TERT 573–584 & L F K Y R K S V W S K L Q S I & 120 & 73 & 37 \\
TERT 541–555 & L A K F L H W L M S V Y V V V E & 160 & 32 & 17 \\
TERT 833–847 & Q G S I L S T L L C S L C Y G & 180 & 56 & 142 \\
TERT 357–371 & A R R V L V E T I F L G S R P W & 270 & 2 & 5 \\
TERT 386–400 & Y W Q M R P L F L E L L G N H & 360 & 77 & 17 \\
TERT 549–563 & M S V Y V V V E L L R S F F Y V & 360 & 65 & 43 \\
\textbf{Intermediate binders Pool B} & & & & & & & & \\
TERT 456–470 & S S P W Q V Y G F V R A C L R & 160 & 928 & 11 \\
TERT 659–673 & K A L F S V L N Y E R A R R P & 160 & 348 & 7 \\
TERT 93–107 & A K N V L A F G F A L L D G A & 220 & 308 & 371 \\
TERT 553–567 & V V E L L R S F F Y V T E T T & 240 & 374 & 21 \\
TERT 10–24 & V R S L L R S H Y R E V L P L & 240 & 3476 & 727 \\
TERT 613–627 & R P A L L T S R L R F I P K P & 360 & 106 & 311 \\
TERT 533–547 & E H R L R E E I L A K F L H W & 360 & 407 & 3583 \\
\hline
\end{tabular}
\caption{Prediction and binding capacities to HLA-DP4 molecules of TERT-derived peptides.}
\end{table}

\textsuperscript{a}Score peptide: binding prediction of TERT peptide to HLA-DP4 molecule was performed with algorithm prediction software.

\textsuperscript{b}Relative activity: peptides encompassing the whole sequence of TERT were submitted to binding assays specific for HLA-DP4 molecules by competitive ELISA (as described in the materials and methods section). Data are expressed as relative avidity RA (ratio of the IC\textsubscript{50} of TERT peptides to the IC\textsubscript{50} of the reference peptide) and are the means of three experiments. Good binders have an RA less than 100 and intermediate binders have a RA more than 100 and less than 500.
binders), only the TERT<sub>613–627</sub> peptide stimulated specific T cell responses but in three out of four healthy donors. We also derived T cell lines by 3 weekly rounds of in vitro stimulation of CD4<sup>+</sup> T cells collected from five HLA-DP4 healthy donors and co-cultured with autologous DC loaded with peptide pools. To confirm the HLA-DP4 restriction, IFN<sub>γ</sub> ELISPOT assay was performed with HLA-DP4 transfected L cells as antigen presenting cells (Fig. 2). Four T cell lines isolated from donors 214 and 215 (Fig. 2, left panel) reacted to TERT<sub>541–555</sub>. One T cell line retrieved from donor 247 (247.39) was specific to TERT<sub>911–925</sub> while two T cell lines generated from donor 298 were specific to peptide TERT<sub>613–627</sub> (Fig. 2, right panel). At least for these three peptides, we demonstrated their presentation to T cells on HLA-DP4. Together, these results imply that precursor CD4<sup>+</sup> T cells against these HLA-DP4-restricted peptides from TERT are present in the peripheral human T-cell repertoire.

The TERT-derived peptides are endogenously processed in HLA-DP4 transgenic mice

Then, we studied the processing of these peptides in HLA-A2/HLA-DP4 transgenic mice<sup>41</sup> (Fig. S1). Besides the lack of endogenous mouse MHC class I and II molecules, these mice
express human CD4+ co-receptor on CD4+ T cells that ensure a better interaction with the HLA-DP4 molecules and were successfully used to evaluate the immunogenicity of HLA-DP4-restricted epitopes. Mice were immunized with a plasmid DNA encoding the full-length human TERT and the specific CD4+ T cell responses were monitored by proliferation assay (as detailed in supplementary materials and methods). We showed that T cell proliferation was induced by in vitro stimulation with the HLA-DP4 restricted TERT peptides, TERT911–925, TERT541–555, TERT573–587 and TERT613–627 (Figs. S1A and B). The absence of T cell proliferation observed in presence of non-pulsed DC ruled out the recognition of a shared epitope derived from mouse TERT. Like in human, the T cells specific to HLA-DP4 strong binder peptides also produce a high amount of IFNγ suggesting their Th1 polarization (Fig. S1C). Altogether, these results indicated that all the four peptides are endogenously processed from TERT and presented to CD4+ T cells in an HLA-DP4 restricted manner.

**Naturally occurring HLA-DP4-restricted CD4+ Th1 responses against TERT are detected in several cancers**

We further evaluated spontaneous immune response against TERT-derived peptides in patients with various cancers before
any treatment or in healthy volunteers as controls. To this end, IFNγ ELISPOT assay was performed after a short term in vitro stimulation of blood lymphocytes with the mixture of the four peptides, named TERT541–555, TERT573–587, TERT911–925 and TERT613–627. In contrast to healthy donors, significant numbers of T cell responses against the HLA-DP4-binding peptides were detected in cancer patients such as melanoma, renal cell carcinoma, lung, breast, and colorectal cancers patients (Representative immune responders from various cancer cohorts are shown in Fig. 3A). In addition to IFNγ production, the analysis of a panel of cytokines in the supernatant of T cell cultures from responding patients showed high production of TNF-α but no production of IL-4, IL-10, and IL-17 supporting a Th1 polarization (Fig. 3B). No specific cytokines production was detected in the supernatants of T cell cultures from non-responding patients (data not shown). The HLA-DP restriction of the responses was assessed by using anti-HLA class II and class I blocking antibodies (Fig. 3C). We showed that the specific IFNγ production by T cells was abrogated in presence of a pan-HLA-DP blocking antibody but not in presence of an anti-HLA-DR blocking antibody or a pan anti-HLA class I antibody (Fig. 3C). To confirm that the spontaneous immune responses measured by IFNγ ELISPOT assay are mediated by CD4+ T cells, we also performed intracellular IFNγ staining assay (ICS) and showed that only CD4+ T cells are responsible of the immune responses (Fig. S2).

Immunogenicity of each peptide was further analyzed separately by stimulating T cells from responding cancer patients in IFNγ ELISPOT assay. As depicted in Fig. 4A, we found spontaneous CD4+ T cell responses against all the four HLA-DP4-restricted peptides. The frequency and magnitude of spontaneous T cell responses against TERT541–555 or TERT573–587 were significantly higher than those specific to TERT613–627 or TERT911–925 (Figs. 4A.
Like in healthy donors, TERT<sub>541–555</sub> and TERT<sub>573–587</sub> peptides appeared more immunogenic in cancer patients. We showed the ability of naturally occurring CD<sub>4</sub>T cells against these 4 peptides to produce both IFN-γ and TNF-α in several cancers revealing a polyfunctional capacity (Fig. 4C). The multi-cytokines analysis also confirmed the absence of Th2 and Th17-associated cytokines production by each peptide-specific CD<sub>4</sub>T cell supporting their Th1 polarization (data not shown). In addition, immune responses against multiple HLA-DP<sub>4</sub>-binding peptides were detected in responding patients and they were mostly directed against TERT<sub>541–555</sub> and TERT<sub>573–587</sub> peptides regardless the tumor type (Fig. 4C).

Next, we isolated three CD<sub>4</sub>T cell clones specific to TERT<sub>911–925</sub> from one responding HLA-DPB1<sup>0401</sup> lung cancer patient referred as CP001.08, CP001.16 and CP001.31 (Figs. 4D and E). The recognition of the cognate peptide by these clones was totally inhibited in presence of pan-HLA-DP
blocking antibody showing their HLA-DP4 restriction (Fig. 4D). The clonality of these three CD4+ T cell clones was assessed by spectratyping analysis and revealed that they share the same clonotype (Fig. S3). These clones also exhibited high TCR avidity as they were still reactive at very low peptide concentration (EC50 < 5 x 10^-2 µmol/L; Fig. 4E). Furthermore the CP001.31 clone recognized autologous DC loaded with recombinant hTERT protein in a HLA-DP-restriction manner supporting that the TERT911–925 epitope is endogenously processed (Fig. S4). Collectively, we showed that these novel HLA-DP4-binding peptides sustain spontaneous CD4+ Th1 responses against TERT in several cancers.

**Immunoprevalence and magnitude of spontaneous anti-TERT CD4+ Th1 responses restricted by HLA-DP4 vs. HLA-DR**

To address the question of the immunoprevalence (frequency of responders), we compared the frequency of anti-TERT CD4+ T cell responses restricted by HLA-DP4 or HLA-DR in a cohort of 87 advanced lung cancer patients. The monitoring of TERT-specific CD4+ T cell responses was performed before any treatment by IFNγ ELISPOT assay after short-term stimulation with the mixture of TERT-derived peptides restricted either by HLA-DP4 or HLA-DR molecules. The promiscuous HLA-DR-binding TERT-derived peptides (UCP) were previously used to monitor spontaneous anti-TERT CD4+ Th1 responses in several cancers.19,34 As shown in Fig. 5A, spontaneous anti-TERT CD4+ T cell responses were detected in 10% (8/79) of patients whose T cells were reactive against HLA-DP4-restricted peptides as compared to 25% (22/87) after HLA-DR/UCP stimulation (p = 0.01). Similar high prevalence of HLA-DR restricted anti-TERT CD4+ T cell responses was found in melanoma, breast cancer and renal cell carcinoma patients (data not shown). The HLA-DP4 genotyping in the lung cancer cohort (n = 29 patients) showed a frequency of 69% regardless the T cell reactivity against the HLA-DP4-restricted peptides. Fourteen out of these twenty-nine patients presented specific CD4+ T cell responses against the HLA-DP4-binding peptides. Among them, 10 patients are HLA-DP4 positive (71%) and 4 patients are HLA-DP4 negative (28%). Then, the HLA-DP4 genotyping of these patients revealed that they are HLA-DP*02 positive (Table S1) suggesting that other HLA-DP alleles are able to present these epitopes.

We also found that the magnitude of the HLA-DR-restricted immune response was significantly stronger than HLA-DP-restricted response (102+/−22 versus 37+/−7 mean+/− SEM number of specific IFNγ spots/10^5 cells respectively p = 0.005) (Fig. 5B). The frequency and intensity of recall antivirus T cell responses measured at the same time in patients by IFNγ ELISPOT assay was similar regardless of the HLA class II genotype (Fig. 5C). In patients without TERT-specific immune response, no other Th1, Th2 or Th17 cytokines production was detected in the supernatant of T cell cultured with HLA-DP4 or HLA-DR-binding peptides (data not shown). This ruled out the possible induction of TERT-specific immune response undetected by IFNγ ELISPOT assay. Furthermore, in lung cancer patients who presented both spontaneous HLA-DR and HLA-DP-restricted TERT-specific CD4+ Th1 cell responses, the magnitude of HLA-DR-restricted Th1 responses was also two to three times higher than HLA-DP4 ones in four out the six patients (Fig. 5D). This result was confirmed in other cancers (Fig. 5E). Collectively, these results showed that the HLA-DR locus sustained more prevalent and robust spontaneous CD4+ T cell responses against TERT than HLA-DP4 allotype in several human cancers.

**Discussion**

In this study, we characterized the HLA-DP4-restricted CD4+ T cell responses against TERT in various cancer patients. We identified four immunogenic HLA-DP4-binding peptides derived from TERT named TERT541–555, TERT573–587, TERT613–627 and TERT911–925. Unlike in healthy subjects, naturally occurring CD4+ T cell responses against these epitopes were detected in peripheral blood of patients from several cancer types. The spontaneous HLA-DP-restricted anti-TERT CD4+ T cells detected in cancer patients mainly produced IFNγ and TNF-α indicating their Th1 polarization. Nonetheless, differences in the peptide immunogenicity were observed at the individual setting. We found that spontaneous responses are more frequently specific to TERT541–555 and TERT573–587 peptides than TERT613–627 and TERT911–925 peptides. The TERT613–627 peptide extensively overlaps the HLA-DP4-binding peptide TERT611–625 known as GV1001 which induces specific CD4+ T cell responses after therapeutic vaccination.42,43 However, in absence of experiments with minimal peptide length from TERT613–627 peptide involved in HLA-DP-restricted responses, we could not conclude that this epitope differs from the GV1001 peptide.

A high immunoprevalence of MHC class II epitopes may result from their capacity to bind the prevalent MHC class II molecule HLA-DP4 or from their promiscuous binding affinity for HLA-DR molecules.39 Results from previous studies reported that HLA-DR molecules were more frequently involved in CD4+ T cell stimulation against tumor antigens such as survivin and NY-ESO-1 than HLA-DP4 molecules.7,36-38 However, this question was mainly addressed in healthy individuals and in a low number of patients. Here, we have extensively studied the immunogenicity of HLA-DP4 vs. HLA-DR-restricted tumor-specific CD4+ T cell responses by using TERT as relevant tumor antigen. As a correlation between the binding capacity and the immunogenicity of T cell epitopes has already been described,44 we used equal number of peptides (four peptides/allele) with equivalent binding affinity to their respective HLA class II molecules to monitor TERT-specific CD4+ Th1 immune responses. We have shown in a cohort of NSCLC patients that spontaneous anti-TERT CD4+ Th1 responses are two times more frequent in HLA-DR (25%) than in HLA-DP4 context (10%). Similar results were found in other cancers such as melanoma, breast cancer, renal cell carcinoma and colorectal cancer (data not shown). This supports the high immunoprevalence of HLA-DR-restricted CD4+ T cell response against telomerase. Although HLA-DP4 genotyping was not performed in all cancer patients cohort, the HLA-DP4 frequency observed in the lung cancer is 69%. Although this frequency is slightly lower than expected (75%) this could not explain the lower immunoprevalence of HLA-DP4-restricted responses. However, we found that around
28% of HLA-DP-restricted immune responders are HLA-DP4 negative suggesting that other HLA-DP molecules could present these peptides. Accordingly, binding peptide prediction showed that these HLA-DP4-binding peptides from TERT could be presented on several HLA-DP molecules (not shown).

Typically, it has been reported that tumor antigen-derived peptides which bind to HLA-DP4 are often promiscuous. For example, HLA-DP4-restricted peptides derived from NY-ESO-1 and MAGE-A3, have been shown to bind to multiple HLA-DR molecules. Epitope prediction revealed that these
novel TERT-derived HLA-DP4 peptides were low HLA-DR binders but efficiently bind to others HLA-DP alleles. In contrast, the TERT-derived HLA-DR-restricted peptides (UCP) were not predicted to bind HLA-DP4 molecules (data not shown). As a result, the low immunoprevalence of HLA-DP4 versus HLA-DR- restricted responses could not be related to a particular promiscuity of the UCP. We also found that the magnitude of the HLA-DR-restricted TERT-specific CD4\(^{+}\) T cell responses was higher than HLA-DP-restricted ones in cancer patients. Interestingly, in patients who had both MHC class II-restricted responses, the HLA-DR-restricted responses were commonly stronger than HLA-DP-restricted ones. The frequency and quality of antiviral T cell responses measured simultaneously in patients were similar in both HLA-DP and HLA-DR responding patients indicating that the difference observed could not be explained by a preferential T cell anergy of HLA-DP4 responding patients. As previously described, the HLA-DRB1 locus is called high expression locus because its product is abundant on the cell surface. In contrast HLA-DP locus, which product is sufficiently bind to others HLA-DP alleles. In contrast, HLA-DP allotypes are not considered. Thus, we speculated that the higher immunogenicity of HLA-DR-binding peptides could be explained by the higher expression of HLA-DR molecules on antigen presenting cells. We previously reported in NSCLC that patients with spontaneous HLA-DR-restricted anti-TERT Th1 response achieved a better survival when responded to chemotherapy. Hence, it would be interesting to evaluate the prognostic value of HLA-DP vs. HLA-DR-restricted TERT-specific CD4\(^{+}\) T cell responses in cancer patients.

In conclusion, we strictly demonstrated for the first time in a large cohort of cancer patients the immunoprevalence and immunodominance of anti-TERT CD4\(^{+}\) T cell responses against HLA-DR-binding peptides compared to HLA-DP4 ones. These results highlight the interest to consider the hierarchy of HLA class II-restriction for CD4\(^{+}\) T-cell-based antitumor immunotherapy and for the design of novel immunomonitoring tools.

### Materials and methods

#### Patients and healthy donors

Non-small cell lung cancer patients (NSCLC) \((n = 87)\) were involved at the University Hospital of Besançon (France) in the Telocap cohort, a prospective study on anti-telomerase immune responses in lung cancer. The other cancer patients were recruited from the department of oncology and dermatology of the University Hospital of Besançon. All patients were enrolled after the signature of informed consent, in accordance with the French regulation and after approval by the local and national ethics committee. Blood was collected before any cancer specific treatment. For healthy donors, blood cells were collected from anonymous donors at the Etablissement Français du Sang (EFS, Besançon, France) as apheresis kit preparation after the signature of the informed consent and following the EFS guidelines.

#### Synthetic peptides

Overlapping peptides and biotylated peptides were synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Advanced ChemTech Apex synthesizer (Advanced ChemTech Europe) and cleaved from the resin by 95% trifluoroacetic acid. If necessary, peptides were purified by reversed-phase-HPLC on a C18 Vydac column (Interchim). All synthetic peptides (>80 % purity) were purchased from ProImmune. The four promiscuous HLA-DR peptides derived from TERT called universal cancer peptides (UCP) UCP1 (PAARFLAVQCLVCV), UCP2 (KSYWSKLSQISIRQH), UCP3 (GTAFVQMPAHGLFPW) and UCP4 (SLCYSILKAKNAGMS) have been described previously and have been purchased from ProImmune (at > 80% purity).

#### HLA-DP4-specific binding assay

The binding to DPB1*0401 and DPB1*0402 molecules was assessed by competitive ELISA as previously reported. Briefly, experiments were performed in 10 mM phosphate, 150 mM NaCl, 1 mM n-dodecyl b-D-maltoside, 10 mM citrate, and 0.003% thimerosal (pH 5) buffer with 10 mM of Oxy271–287 peptide, an appropriate dilution of HLA-DP4 molecules, and serial mid-dilutions of competitor peptides. After 24 h incubation at 37°C, samples were neutralized and applied to B7/21-coated plates for 2 h. Bound biotylated peptide was detected by means of streptavidin–alkaline phosphatase conjugate (GE Healthcare) and 4-methylumbelliferyl phosphate substrate (Sigma-Aldrich). Emitted fluorescence was measured at 450 nm upon excitation at 365 nm in a victor II spectrofluorometer (PerkinElmer Instruments). Data were expressed as the peptide concentration that prevented binding of 50% of the labeled peptide (IC50). IC50 values of the Oxy271–287 peptide for HLA-DP401 and HLA-DP402 were 6 nM and 4 nM, respectively.

#### In vitro immunogenicity of peptides in healthy donors

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Sigma-Aldrich) and plated at 4 × 10^6 cells per well in a 24-wells plate in RPMI 5% human serum with 10 mmol/L of pool of TERT peptides from pool A and B. Recombinant interleukins, IL-7 (5 ng/mL; Peprotech, 200–07), and IL-2 (20 UI/mL; Novartis) were added at days 1 and 3, respectively. At days 7 and 14, cells were stimulated with γ-irradiated autologous PBMCs pulsed with of UCPs (10 mmol/L) and IL-2 (20 UI/mL; Novartis) were added at days 8 and 15 as previously reported. At day 21, the T cell specificity was investigated by IFN\(\gamma\) ELISPOT (Diaclone, 856 051 020P).

#### Generation of peptide-specific CD4\(^{+}\) T cell lines from healthy donors

Monocyte-derived dendritic cells (moDC) were generated from plastic-adherent cells of PBMC as described previously. CD4\(^{+}\) T lymphocytes were isolated by positive selection using magnetic microbeads (human CD4\(^{+}\) isolation Kit Miltenyi Biotec,
130-045-101), as recommended by the manufacturer. Mature DC (5 \( \times 10^5 \)) were incubated for 4 h in 1 mL complete IMDM medium containing a mixture of TERT peptides, with each peptide being at a concentration of 10 \( \mu \text{g/mL} \). The CD4\(^+\) T lymphocytes were restimulated on days 7, 14, and 21 with autologous DC freshly loaded with the TERT peptides in complete IMDM medium supplemented with 10 UI/mL IL-2 (Novartis) and 5 ng/mL IL-7 (Peprotech). Specificity of the T cell lines was tested by IFN\(\gamma\) ELISPOT assays at days 28 using HLA-DP4 transected L cells as described previously.\(^{19,34}\)

**Assessment of spontaneous antigen-specific T-cell responses in cancer patients**

Spontaneous responses were assessed by IFN\(\gamma\) ELISPOT after a short-term in vitro stimulation of PBMC with the mixture of the four selected peptides (5 \( \mu \text{g/mL} \)) during 7 days as previously described. A peptide mixture derived from virus influenza (Flu), Epstein barr virus (EBV), cytomegalovirus (CMV) was used to evaluate antiviral recall response (CTL, PA-CEF-001). The presence of peptide-specific T cells was measured by IFN\(\gamma\) ELISPOT assay as detailed above.\(^{19,34}\)

**Isolation and functional analysis of CD4\(^+\) T cell clones**

HLA-DP4 restricted TERT peptide reactive T cells were isolated by IFN\(\gamma\) T cell sorting according to manufacturer’s instruction (Miltenyi Biotec, 130-054-201). Specific CD4\(^+\) T cell clones were generated by IFN\(\gamma\) limiting dilution and amplified after stimulation with PHA in presence of irradiated autologous PBMCs, EBV-transformed cell-lines according to the previously described procedure.\(^{19}\) Functional analysis of CD4\(^+\) T cell clones was performed by using intracytoplasmic IFN\(\gamma\) staining (ICS). Briefly, after a 12-h stimulation period with or without 10 \( \mu \text{g/mL} \) peptide, T cells were labeled with anti-CD4\(^+\) (Diaclone, 954.031.010), anti-IFN\(\gamma\) (BD Biosciences, 554702) using Cytofix/CytoPerm KIT (BD Biosciences, 555809) for 1 h before the addition of peptide.

**ELISPOT and DIAplex assays**

IFN\(\gamma\) ELISPOT was conducted as previously described.\(^{19}\) Briefly, cells (10\(^5\) per well) were cultured in triplicates in ELISPOT plate with HLA-DP4 restricted peptides (mixture or individual at 5 \( \mu \text{g/mL} \) in X-vivo 15 medium, Ozyme, BE04–418) for 15 h. The IFN\(\gamma\) spots were revealed following the manufacturer’s instructions (Diaclone, 856 051 020P). Spot-forming cells were counted using the C.T.L. Immunospot system (Cellular Technology Ltd). The number of specific T cells expressed as spot-forming cells per 10\(^5\) cells was calculated after subtracting negative control values (background). Responses were positive when IFN\(\gamma\) spots were more than 10 and more than two times the background. In some experiments, a panel of cytokines production analysis was measured using DIAplex Human Th1/Th2/Th17 kit (Diaclone, 880.100.010) in the supernatant of lymphocyte culture according to the manufacturer’s instructions. To determine HLA restriction, PBMC were treated with 10 \( \mu \text{g/mL} \) of anti-HLA-DP antibody (B7/21) (Leinco, H260) or anti-HLA-class I antibody (hybromida supernatant from clone W6/32) for 1 h before the addition of peptide.

**Statistics**

Statistical differences between CD4\(^+\) T cell responses were calculated using Mann–Whitney or Student t test using Prism 4 GraphPad Software. \( p \) values less than 0.05 were considered significant (\( ^{\circ}<0.05, ^{\#}<0.01 \)).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

The authors also thank all medical doctors for the recruitment of patients at medical Oncology department, chest disease and thoracic oncology, and dermatology department of the University Hospital of Besançon. The authors thank Dr. Lone Yu Chun for providing the HLA-A2.1\(^{+}\)/HLA-DP4\(^{+}\) hCD4\(^{+}\) transgenic mice. The authors thank the Biomonitoring platform of CIC-1431 for their technical support.

**Funding**

This work was supported by grants from the French National Institute of Cancer (INCA), la Ligue contre le cancer, the Conseil Régional de Franche-Comté.

**References**

1. Kim H-J, Cantor H. CD4\(^+\) T-cell subsets and tumor immunity: the helpful and the not-so-helpful. Cancer Immunol Res 2014; 2:491–8; PMID:24778273; http://dx.doi.org/10.1158/2326-6066.CIR-13-0216
2. Disis ML, Watt WC, Cecil DL. Th1 epitope selection for clinically effective cancer vaccines. Oncoimmunology 2014; 3:e954971; PMID:25941610; http://dx.doi.org/10.4161/21624011.2014.954971
3. Kennedy R, Celis E. Multiple roles for CD4\(^+\) T cells in anti-tumor immune responses. Immuno Rev 2008; 222:129–44; PMID:18363998; http://dx.doi.org/10.1111/j.1600-606X.2008.00616.x
4. Marzo AL, Kinnear BF, Lake RA, Frelinger JJ, Collins EJ, Robinson BW, Scott B. Tumor-specific CD4\(^+\) T cells have a major “post-licensing” role in CTL mediated anti-tumor immunity. J Immunol 2000; 165:6047–55; PMID:11086036; http://dx.doi.org/10.4049/jimmunol.165.11.6047
5. Bos R, Sherman LA. CD4\(^+\) T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8\(^+\) T lymphocytes. Cancer Res 2010; 70:8368–77; PMID:20940398; http://dx.doi.org/10.1158/0008-5472.CAN-10-1322
6. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Toselini M, Camus M, Berger A, Wind P et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006; 313:1960–4; PMID:17008531; http://dx.doi.org/10.1126/science.1129139
7. Wang X-F, Kerzerho J, Adotevi O, Nuyttens H, Badoual C, Munier G, Oudard S, Tu S, Tartour E, Maillere B. Comprehensive analysis of HLA-DR- and HLA-DP4-restricted CD4\(^+\) T cell response specific for the tumor-shared antigen survivin in healthy donors and cancer patients. J Immunol 2008; 181:431–9; PMID:18566409; http://dx.doi.org/10.4049/jimmunol.181.1.431
8. Tsuju T, Altorki NK, Ritter G, Old LJ, Gnajtic S. Characterization of preexisting MAGE-A3-specific CD4\(^+\) T cells in cancer patients and
healthy individuals and their activation with protein vaccination. J Immunol 2009; 183:4800-8; PMID:19734225; http://dx.doi.org/10.4049/jimmunol.0900903

9. Kudela P, Sun Z, Fourcade J, Janjic B, Kirkwood JM, Maillere B, Zarour HM. Epitope hierarchy of spontaneous CD4+ T cell responses to LAGE-1. J Immunol Baltim MD 1950 2011; 186:312-22; PMID:21133412; http://dx.doi.org/10.4049/jimmunol.0901988

10. Ayyoub M, Pignon P, Classe J-M, Odunsi K, Valmori D. CD4+ T effectors specific for the tumor antigen NY-ESO-1 are highly enriched at ovarian cancer sites and coexist with, but are distinct from, tumor-associated Treg. Cancer Immunol Res 2013; 1:303-8; PMID:24777968; http://dx.doi.org/10.1158/2326-6066.CIR-13-0062-T

11. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumors: impact on clinical outcome. Nat Rev Cancer 2012; 12:298-306; PMID:22419253; http://dx.doi.org/10.1038/nrc3245

12. Widmeyer M, Griesemann H, Stevanovic S, Feyerabend S, Klein R, Attig S, Hennenlotter J, Wernet D, Kuprash DV, Saizykin AY et al. Promiscuous survivin peptide induces robust CD4+ T-cell responses in the majority of vaccinated cancer patients. Int J Cancer J Int Cancer 2012; 131:140-9; PMID:21858810; http://dx.doi.org/10.1002/ijc.26365

13. Aarntzen EJHG, De Vries IJM, Lesterhuis WJ, Schuurhuis D, Jacobs JFM, Boel K, Schreibelt G, Mus R, De Wilt JHW, Haanen JBAG et al. Targeting CD4+ T-helper cells improves the induction of antitumor responses in dendritic cell-based vaccination. Cancer Res 2013; 73:19-29; PMID:23087058; http://dx.doi.org/10.1158/0008-5472.CAN-12-1127

14. Adotévi O, Dosset M, Galaline J, Beziaud L, Godet Y, Borg C. Targeting antitumor CD4 helper T cells with universal tumor-reactive helper peptides derived from telomerase for cancer vaccine. Hum Vaccines Immunother 2013; 9:1073-7; PMID:23537860; http://dx.doi.org/10.4161/hv.23587

15. Woods K, Cebon J. Tumor-specific T-cell help is associated with improved survival in melanoma. Clin Cancer Res Off J Am Assoc Cancer Res 2013; 19:4021-3; PMID:23656225; http://dx.doi.org/10.1158/1078-0432.CCR-13-0349

16. Sluggfl CL, Lee S, Zhao F, Chianese-Fridman C, Galon J. The immune contexture in human tumors: impact on clinical outcome. Nat Rev Cancer 2012; 12:298-306; PMID:22419253; http://dx.doi.org/10.1038/nrc3245

17. Chen C, Maecker HT, Lee PP. Development and dynamics of CD8 T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. Proc Natl Acad Sci U S A 2003; 100:8862-7; PMID:12853579; http://dx.doi.org/10.1073/pnas.1133324100
39. Castelli FA, Buhot C, Sanson A, Zarour H, Pouveller-Moratille S, Nonn C, Gahery-Ségard H, Guillet J-G, Ménez A, Georges B et al. HLA-DP4, the most frequent HLA II molecule, defines a new supertype of peptide-binding specificity. J Immunol Baltim Md 1950 2002; 169:6928-34; PMID:12471126; http://dx.doi.org/10.4049/jimmunol.169.12.6928

40. Busson M, Castelli FA, Wang X-F, Cohen WM, Charron D, Ménez A, Maille B. Prediction of CD4(+) T cell epitopes restricted to HLA-DP4 molecules. J Immunol Methods 2006; 317:144-51; PMID:17107686; http://dx.doi.org/10.1016/j.jim.2006.10.002

41. Ru Z, Xiao W, Pajot A, Kou Z, Sun S, Maille B, Zhao G, Ojcius DM, Lone Y-C, Zhou Y. Development of a humanized HLA-A2.1/DP4 transgenic mouse model and the use of this model to map HLA-DP4-restricted epitopes of HBV envelope protein. PloS One 2012; 7:e32247; PMID:22403638; http://dx.doi.org/10.1371/journal.pone.0032247

42. Brunsvig PF, Kyte JA, Kersten C, Sundström S, Møller M, Nyakas M, Hansen GL, Gaudernack G, Aamdal S. Telomerase peptide vaccination in NSCLC: a phase II trial in stage III patients vaccinated after chemoradiotherapy and an 8-year update on a phase I/II trial. Clin Cancer Res Off J Am Assoc Cancer Res 2011; 17:6847-57; PMID:21918169; http://dx.doi.org/10.1158/1078-0432.CCR-11-1385

43. Inderberg-Suso E-M, Trachsel S, Lislerud K, Rasmussen A-M, Hansen GL, Gaudernack G, Aamdal S. Telomerase peptide vaccination in NSCLC: a phase II trial in stage III patients vaccinated after chemoradiotherapy and an 8-year update on a phase I/II trial. Clin Cancer Res Off J Am Assoc Cancer Res 2011; 17:6847-57; PMID:21918169; http://dx.doi.org/10.1158/1078-0432.CCR-11-1385

44. Sette A, Vitiello A, Reherman B, Fowler P, Nayersina R, Kast WM, Melief C, Oseroff C, Yuan L, Ruppert J et al. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J Immunol 1994; 153:5866-91; PMID:7527444

45. Sidney J, Steen A, Moore C, Ngo S, Chung J, Peters B, Sette A. Five HLA-DP molecules frequently expressed in the worldwide human population share a common HLA supertypic binding specificity. J Immunol 2010; 184:2492-503; PMID:20139279; http://dx.doi.org/10.4049/jimmunol.0903655

46. Consogno G, Manici S, Facchinetti V, Bachi A, Hammer J, Conti-Fine BM, Rugarli C, Traversari C, Protef MP. Identification of immunodominant regions among promiscuous HLA-DR-restricted CD4+ T-cell epitopes on the tumor antigen MAGE-3. Blood 2003; 101:1038-44; PMID:12393675; http://dx.doi.org/10.1182/blood-2002-03-0933

47. Nuñez G, Ball EJ, Myers LK, Stastny P. Allostimulating cells in man. Quantitative variation in the expression of HLA-DR and HLA-DQ molecules influences T-cell activation. Immunogenetics 1985; 22:85-91; PMID:3160658; http://dx.doi.org/10.1007/BF00430597

48. van Vreeswijk H, Ruiter DJ, Bröcker EB, Welvaart K, Ferrone S. Differential expression of HLA-DR, DQ, and DP antigens in primary and metastatic melanoma. J Invest Dermatol 1988; 90:755-60; PMID:3283252; http://dx.doi.org/10.1111/1523-1747.ep12560951

49. Nagler A. HLA-DP1 matching: are we there yet? Blood 2014; 124:2476-7; PMID:25323686; http://dx.doi.org/10.1182/blood-2014-09-599225