

# Tumor Antigen–Specific FOXP3<sup>+</sup> CD4 T Cells Identified in Human Metastatic Melanoma: Peptide Vaccination Results in Selective Expansion of Th1-like Counterparts

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## Abstract

We have previously shown that vaccination of HLA-A2 metastatic melanoma patients with the analogue Melan-A<sub>26-35</sub>(A27L) peptide emulsified in a mineral oil induces *ex vivo* detectable specific CD8 T cells. These are further enhanced when a TLR9 agonist is codelivered in the same vaccine formulation. Interestingly, the same peptide can be efficiently recognized by HLA-DQ6–restricted CD4 T cells. We used HLA-DQ6 multimers to assess the specific CD4 T-cell response in both healthy individuals and melanoma patients. We report that the majority of melanoma patients carry high frequencies of naturally circulating HLA-DQ6–restricted Melan-A–specific CD4 T cells, a high proportion of which express FOXP3 and proliferate poorly in response to the cognate peptide. Upon vaccination, the relative frequency of multimer+ CD4 T cells did not change significantly. In contrast, we found a marked shift to FOXP3-negative CD4 T cells, accompanied by robust CD4 T-cell proliferation upon *in vitro* stimulation with cognate peptide. A concomitant reduction in TCR diversity was also observed. This is the first report on direct *ex vivo* identification of antigen-specific FOXP3<sup>+</sup> T cells by multimer labeling in cancer patients and on the direct assessment of the impact of peptide vaccination on immunoregulatory T cells. [Cancer Res 2009;69(20):8085–93]

## Introduction

Specific immunotherapy is a promising approach (1–3) to the treatment of a wide range of medical conditions including chronic viral diseases, autoimmune inflammatory disorders, and various types of cancer. The discovery of tumor-associated antigens recognized by conventional  $\alpha\beta$  T cells provided the foundation for the design of defined antigen-based cancer vaccines (4). Important to the process of rational vaccine development,

monitoring of antigen-specific T-cell responses helps guiding vaccine optimization.

A large number of clinical trials using T-cell–defined antigens have been reported in practically all types of cancer (5). Metastatic melanoma is however the best studied tumor type in terms of immune reactivity and experimental vaccine testing (6). Among the numerous antigens expressed by cutaneous melanoma, Melan-A/Mart-1 (hereafter Melan-A) is one of the best characterized thus far. The *Melan-A* gene encodes a 118 amino acid long polypeptide expressed by normal melanocytes and the majority of primary and metastatic tumors (7). Interestingly, most of the Melan-A–specific T-cell responses identified in advanced tumor-bearing patients focus on the region spanning residues 20 to 40. In particular, the peptide 26 to 35 defines epitopes recognized by HLA-A2– and HLA-B35–restricted CD8 T cells (8–10). Slightly longer peptides containing the same core sequence are recognized by HLA-B45 (11) and -B44<sup>7</sup> restricted CD8 T cells. Moreover, the 25 to 36 dodecapeptide is recognized by HLA-DQ6–, HLA-DQ3–, and HLA-DR3–restricted CD4 T cells (12). This pronounced bias in processing and presentation of the Melan-A antigens is reminiscent of immunodominant protein regions and lends itself to detailed analysis of melanoma-specific CD8 and CD4 T-cell responses in defined clinical situations such as tumor progression, tumor cell death provoked by chemotherapy or radiotherapy, and in the course of immunotherapy. More importantly, such immunodominant region(s) are excellent candidates for peptide-based vaccines because of the promise to efficiently promote integrated protective T-cell responses (13–16).

We have shown that the natural Melan-A<sub>26-35</sub> decapeptide binds weakly to the HLA-A2 molecule because of the lack of a strong anchor residue at position P2. The replacement of Ala at position 27 for Leu results in enhanced binding to HLA-A2, in 2- to 3-log increase of its relative antigenicity and, more importantly, to an increased immunogenicity (17). Vaccination of metastatic melanoma patients with the peptide analogue Melan-A<sub>26-35</sub>(A27L) emulsified in mineral oil resulted in high frequencies of Melan-A–specific CD8 T cells that can be detected by direct *ex vivo* flow cytometry analysis using HLA-A2/Melan-A peptide multimers in ~60% of vaccinated patients (18, 19). Moreover, vaccination efficiency can be dramatically enhanced by inclusion of the TLR9 agonist ODN-CpG type B 7909/PF-3512676 in the immunizing emulsion. In this

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<sup>7</sup> P. Coulie, personal communication.

case, strong specific CD8 T-cell responses as measured by multimer labeling developed rapidly in all vaccinated patients (20).

A search for CD4 T-cell epitopes in the Melan-A polypeptide revealed that a dodecapeptide spanning the region 25 to 36 was optimally recognized by Melan-A-specific and tumor-reactive CD4 T cells isolated from melanoma patients (12). Moreover, HLA-DQ6/Melan-A<sub>25-36</sub> multimers directly identified specific CD4 T cells by flow cytometry. Interestingly, this optimal CD4 T-cell epitope fully encompasses the HLA-A2-restricted decapeptide Melan-A<sub>26-35</sub>. In this report, we used HLA-DQ6/Melan-A peptide multimers to analyze in detail Melan-A-specific CD4 T-cell responses in healthy individuals (HD) and a cohort of HLA-A\*0201 metastatic melanoma patients who shared expression of the HLA-DQB1\*0602 allele. We found relatively high frequencies of circulating HLA-DQ6/Melan-A multimer+ CD4 T cells in most melanoma patients studied. Fine specificity studies of a series of clones isolated from some of these patients showed efficient cross-recognition of the enhanced Melan-A<sub>26-35(A27L)</sub> decapeptide. The multimer+ CD4 T cells contained high proportions of FOXP3+ cells in the blood samples before peptide vaccination. In contrast, the proportions of FOXP3+ cells were markedly reduced among the multimer+ CD4 T cells, but not in the multimer- fractions, in blood samples obtained during the course of Melan-A peptide vaccination. Thus, although vaccination elicited the intended HLA-A2-restricted CD8 T-cell response, it also triggered an unexpected effect on specific CD4 T cells in the subset of HLA-DQ6-vaccinated patients.

## Materials and Methods

**Cells and tissues.** Peripheral blood and metastatic lymph nodes were obtained from melanoma patients upon informed consent. Clinical characteristics and treatments of the patients included in the study are described in Table 1. Treatments of the patients were approved by the institutional review board of the University Hospital of Lausanne, Swiss

Medic and the Ludwig Institute for Cancer Research Ltd. The healthy subjects were blood donors at the Blood transfusion Center in Bern, Switzerland. Mononuclear cells were purified by density gradient and immediately frozen, as previously described (21). Genomic DNA was extracted from frozen peripheral blood mononuclear cells (PBMC) and all individuals were molecularly typed for HLA-DQB1\*0602 by reverse PCR-sequence-specific oligonucleotides hybridization on microbeads arrays (LabType, OneLambda), using the luminex technology. Lymphocytes were maintained in culture as already described (12).

**Generation of pMHC I and II multimers; monoclonal antibodies and labeling.** The generation of HLA-DQB1\*0602 multimers was performed as already described using the Melan-A<sub>25-36</sub>, Melan-A<sub>26-35(A27L)</sub>, or the HA<sub>57-75</sub> peptides (12). Multimer labeling was performed for 1 h at 37°C before staining with fluorescent monoclonal antibodies (mAb) directed against surface molecules (20 min at 4°C). FOXP3 labeling was performed according to manufacturer's recommendations. Cells were analyzed by flow cytometry on a LSR II. For analysis, dead cells were excluded using either propidium iodide (Sigma-Aldrich) or ethidium monoazide (Molecular Probes), and all dot plots shown were gated on live CD3+ cells. All mAbs were purchased from BD Bioscience, with exception for anti-human FOXP3 and anti-human IL-17 mAbs (eBioscience). HLA-A\*0201/Melan-A<sub>26-35(A27L)</sub> multimers were produced as described (22). Multimer labeling was performed for 45 min at 4°C.

**Intracellular cytokine staining of Melan-A<sub>25-36</sub> CD4 T cells.** *In vitro* peptide stimulations were tested for the presence of cytokine-secreting CD4 T cells. Cells were stimulated for 6 h in the presence or absence of 2 µmol/L Melan-A<sub>25-36</sub>, Melan-A<sub>26-35(A27L)</sub>, or irrelevant peptide. Where indicated, purified anti-DR (L243), anti-DP (B7.21.7), or anti-DQ (SPVL3) were used to specifically block MHCII recognition (10 µg/mL). BrefeldinA was added after the first hour of incubation (10 µg/mL). After incubation and washing, cells were stained with anti-CD4 antibody, then fixed and stained intracellularly with anti-IFNγ, anti-IL-17 and anti-tumor necrosis factor (TNF)α or anti-interleukin (IL)-2 antibodies in the presence of Saponin 0.1%. Samples were analyzed by flow cytometry using an LSRII.

***In vitro* peptide stimulations with Melan-A<sub>25-36</sub> peptide.** Total PBMCs or enriched CD4 T cells in the presence of irradiated CD4 T cells were stimulated with 2 µmol/L Melan-A<sub>25-36</sub> peptide. Two days after addition of

**Table 1.** Clinical characteristics of melanoma patients

Patient	Sex	Age*	Stage <sup>†</sup>	Site of metastasis	Treatments	Clinical status <sup>‡</sup>
LAU 50	M	74	IV	LN <sup>§</sup>	Surgery, ILP, LUD 01-003 <sup>  </sup>	PD
LAU 203	F	62	III	LN	Surgery, ILP	CR
LAU 212	F	71	III	LN	Surgery, ILP, LUD 96-010 <sup>¶</sup>	SD
LAU 270	F	44	IV	LN, skin	Surgery, ILP, IFNα, LUD 96-010	†
LAU 353	F	49	III	LN	Surgery, LUD LAU 353	†
LAU 390	F	38	IV	LN	Surgery, IFNα, chemotherapy, IL-2, LUD 96-010	PD
LAU 470	F	35	III	LN, skin	Surgery, LUD 96-010	†
LAU 567	F	54	III	LN, skin	Surgery, LUD 96-010	†
LAU 618	F	73	III	LN, skin	Surgery, IFNα, ILP, LUD 96-010, LUD 00-018	PD
LAU 706	F	67	III	LN, skin	Surgery, ILP, p40, LUD 00-018 <sup>¶¶</sup>	PD
LAU 814	M	28	III/IV	LN	Surgery, LUD 98-009	SD

\*Age at diagnosis.

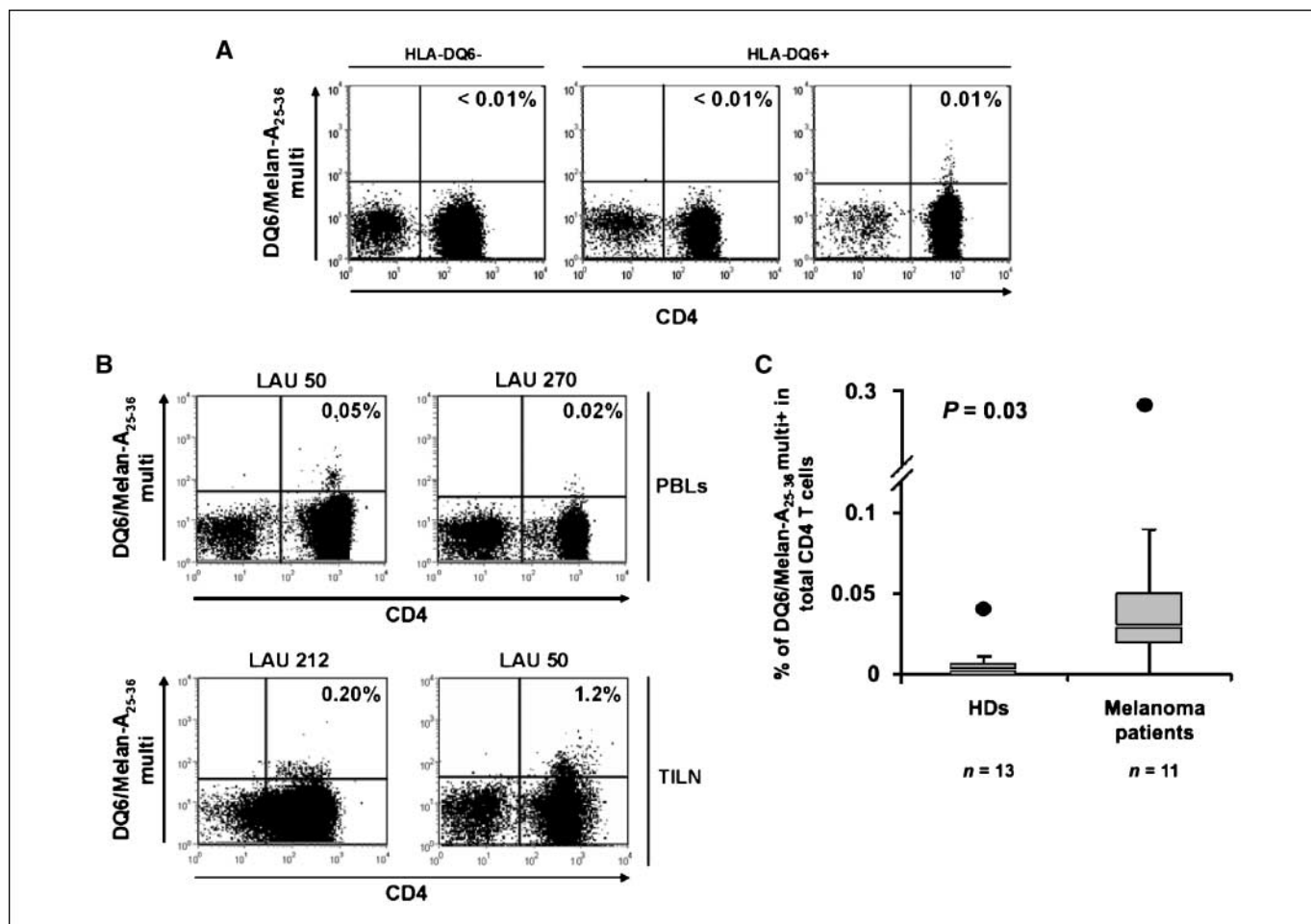
† Stage according to the American Joint Committee on Cancer staging system.

‡ PD, progressive disease; CR, chronic response; SD, stable disease; †, death.

§LN, lymph node; ILP, isolated lymph perfusion.

||Immunotherapy protocols (vaccination with): LUD 01-003 (Melan-A<sub>26-35(A27L)</sub>, NY-ESO-1<sub>157-165</sub>, Mage-A10<sub>254-262</sub>, Montanide, s.c.); LUD 96-010 (Melan-A<sub>26-35(A27L)</sub>, Influenza matrix<sub>58-66</sub>, PBS or AS02B or Montanide); LUD LAU 353 [Melan-A<sub>26-35(A27L)</sub>, Influenza matrix<sub>58-66</sub>, Montanide, s.c.]; LUD 00-018 [Melan-A<sub>26-35(A27L)</sub>, tyrosinase<sub>369-377</sub>, CpG 7909, Montanide, s.c.]; p40 [Melan-A<sub>26-35(A27L)</sub>, Klebsiella pneumoniae outer membrane protein p40, s.c.]; LUD 98-009 [Melan-A<sub>26-35(A27L)</sub>, Mage-A10<sub>254-262</sub>, Montanide, s.c.].

¶¶Melan-A<sub>26-35</sub> instead of Melan-A<sub>26-35(A27L)</sub>.



**Figure 1.** *Ex vivo* detection of Melan-A<sub>25-36</sub> CD4 T cells in PBLs and TILNs from healthy donors and melanoma patients, before immunotherapy. **A**, representative examples of direct *ex vivo* labeling of PBLs from HLA-DQ6- (left dot plot) and HLA-DQ6+ HDs (middle and right dot plots). **B**, representative examples of direct *ex vivo* labeling of PBLs (top dot plots) and TILN from HLA-DQ6+ melanoma patients (bottom dot plots). Percentages in the top right quadrant, frequency of multimer+ within total CD4 T cells. **C**, graph summarizing *ex vivo* frequencies of DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells in PBLs from HLA-DQ6+ HDs and melanoma patients, before immunotherapy.

peptide, 100 U/mL IL-2 was added to the cultures. After 2 wk, the presence of specific CD4 T cells was assessed by DQ6/Melan-A<sub>25-36</sub> multimers.

**cDNA preparation and TCR V $\beta$  analysis.** Five-cell aliquots of multimer+ cells were sorted with a FACS Vantage SE machine directly into wells of 96-V-bottomed plates. cDNA preparation, cDNA amplification, and PCR were performed as previously described (23). The quality of each sample was assessed as described before (24). Aliquots from each sample per patient and time point were pooled and subjected to individual PCR to assess the expression of the V $\beta$ s as previously published (25). Then, each five-cell sample was subjected to an individual PCR for each TCR BV chain positive in the pool.

**Statistical analysis.** The significance of the results was determined using Student's *t* test. *P* values of <0.05 were considered significant.

Results are represented graphically as box and whisker diagrams. Bottom and top of the boxes represent the 25th and 75th percentile, respectively; the line dividing the boxes indicate the median value, whereas the ends of the whiskers represent the maximal and minimum values, with exceptions of outliers that are shown as filled dots.

## Results

**Characterization of DQ6/Melan-A<sub>25-36</sub> CD4 T-cell clones.** We recently identified the Melan-A<sub>25-36</sub> peptide as a naturally processed epitope presented by the human HLA-DQB1\*0602 molecule

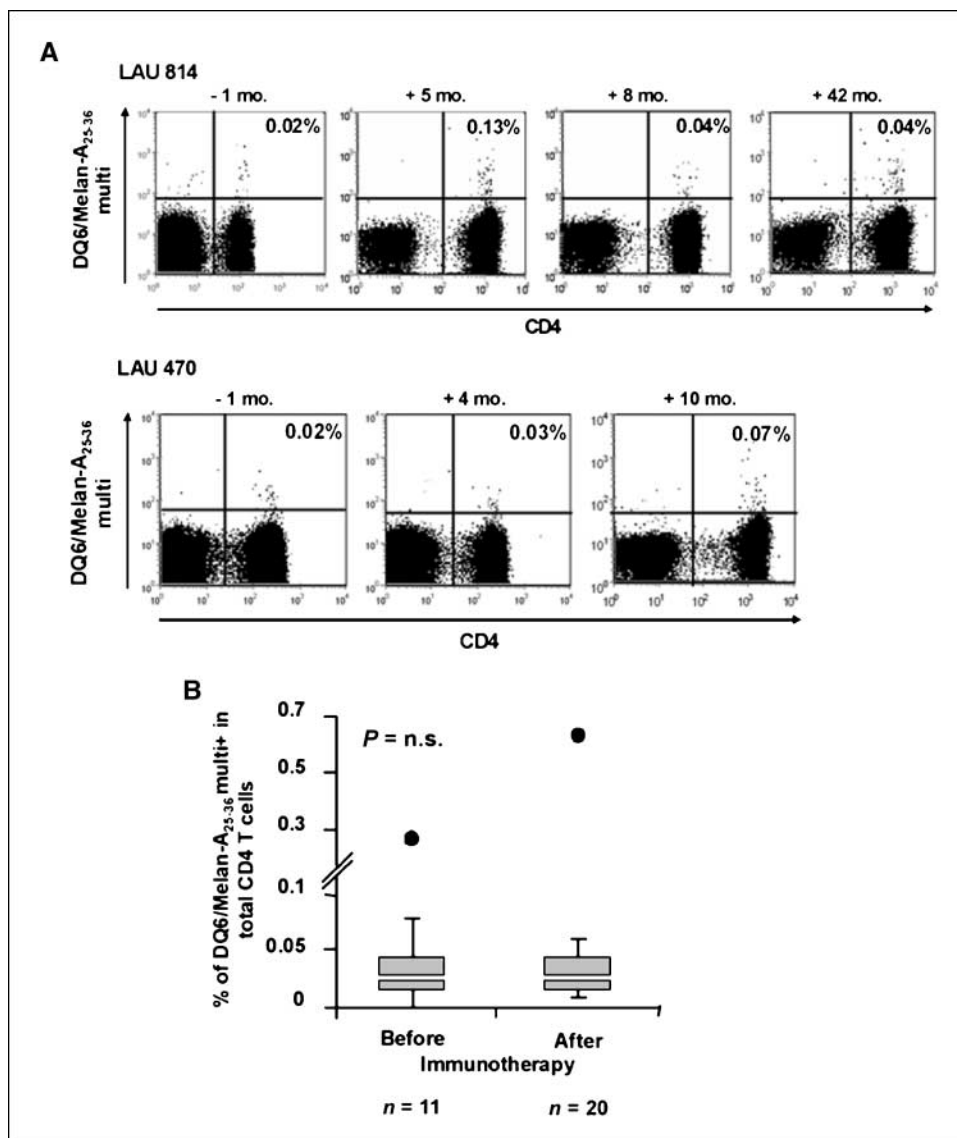
(12). We show here that CD4 T-cell clones generated from blood samples from different metastatic melanoma patients can be consistently labeled with DQ6/Melan-A<sub>25-36</sub> multimers (Supplementary Fig. S1A–C).

As previously reported by us using a single Melan-A<sub>25-36</sub>-specific CD4 T-cell clone isolated from a melanoma patient, we further show here, using multiple independent DQ6/Melan-A<sub>25-36</sub> CD4 T-cell clones from additional patients, that they efficiently recognize the dodecapeptide Melan-A<sub>25-36</sub>, as well as the two amino acid shorter Melan-A<sub>26-35(A27L)</sub> analogue decapeptide, as determined in peptide titration assays (Supplementary Fig. S2A–C).

Because the cytokine responses to DQ6/Melan-A<sub>25-36</sub> and A2/Melan-A<sub>26-35(A27L)</sub> were comparable in all experiments, we then assessed whether DQ6/Melan-A<sub>25-36</sub> CD4 T-cell clones may also be labeled using DQ6 multimers loaded with the decapeptide analogue Melan-A<sub>26-35(A27L)</sub>. Interestingly, all the clones tested bound the DQ6/Melan-A<sub>26-35(A27L)</sub> multimers, whereas irrelevant clones did not (Supplementary Fig. S2D).

**Naturally acquired Melan-A<sub>25-36</sub> CD4 T-cell responses can be frequently detected in peripheral blood and tumor-infiltrated lymph nodes from HLA-DQ6 melanoma patients but only rarely in HDs.** To directly monitor HLA-DQ6-restricted CD4 T-cell





**Figure 2.** Ex vivo longitudinal monitoring of Melan-A<sub>25-36</sub> multimer+ CD4 T cells in PBLs from melanoma patients during CTL-based immunotherapy. **A**, representative examples of the longitudinal evolution of ex vivo Melan-A<sub>25-36</sub> CD4 T cells in PBLs from patients LAU 814 (top) and LAU 470 (bottom). Percentages in the top right quadrant, frequency of multimer+ within total CD4 T cells. **B**, graph summarizing ex vivo frequencies of DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells in PBLs from HLA-DQ6+ melanoma patients, before and after immunotherapy. n.s., not significant.

responses in humans, we first assessed the baseline frequencies of DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells in lymphocytes isolated from HDs. As expected, no detectable multimer+ CD4 T cells could be observed in blood samples from 13 HLA-DQB1\*0602- donors (Fig. 1A, left dot plots). In contrast, 3/13 HLA-DQB1\*0602+ HDs showed low but detectable frequencies of DQ6/Melan-A<sub>25-36</sub> CD4 T cells. The frequencies of positive cells were 0.01%, 0.01%, and 0.04% for the three donors, respectively (Fig. 1A, middle and right dot plots). Those cells could be expanded *in vitro* and secreted Th1 cytokines after stimulation with cognate peptide, suggesting that they represent circulating Melan-A-specific CD4 T cells in HDs (Supplementary Fig. S3A and B). Remarkably, 10/11 HLA-DQB1\*0602+ metastatic melanoma patients presented with variable, but detectable frequencies of DQ6/Melan-A<sub>25-36</sub> CD4 T cells in peripheral blood sampled before any specific immunotherapy (Fig. 1B, top). Overall, the mean frequency of DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells was 0.005% in DQ6+ HDs and 0.06% ( $P = 0.03$ ) in melanoma patients (range, 0–0.29%; Fig. 1C). In addition, we analyzed tumor-infiltrated lymph nodes (TILN) directly *ex vivo* from patients LAU 212 and LAU 50. This material was surgically removed in both patients before

any immunotherapy. We found an enrichment of multimer+ CD4 T cells compared with autologous peripheral blood lymphocytes (PBL). Indeed, the frequencies of multimer+ CD4 T cells in these TILNs were 0.2% and 1.2%, respectively (Fig. 1B, bottom).

**Frequencies of DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells *ex vivo* are not consistently modulated by CD8 T-cell-targeted immunotherapy.** As mentioned above, DQ6/Melan-A<sub>25-36</sub> CD4 T cells not only recognize the Melan-A<sub>25-36</sub>, but also the Melan-A<sub>26-35</sub>(A27L) peptide that is recognized by HLA-A2-restricted CD8 T cells. All except one (LAU 203) of the 11 DQ6+ melanoma patients studied were included in CD8 T-cell-targeted peptide immunotherapy trials (Table 1). We therefore determined whether vaccination with Melan-A<sub>26-35</sub>(A27L) had an effect on the frequencies and/or function of Melan-A-specific CD4 T cells. As exemplified for two patients in Fig. 2A and summarized for all the patients in Fig. 2B, higher levels of DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells could be measured during vaccination (range, 0.01–0.66). Moreover, patient LAU 618 with no detectable multimer+ CD4 T cells in prevaccine PBMCs had 0.03% and 0.02% of multimer+ CD4 T cells during immunotherapy. However, no significant overall increase in

DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells could be detected ( $P = 0.78$ ). Furthermore, the frequencies of multimer+ CD4 T cells did not correlate to the number of administered vaccines nor to the time elapsed after initiation of immunotherapy (data not shown).

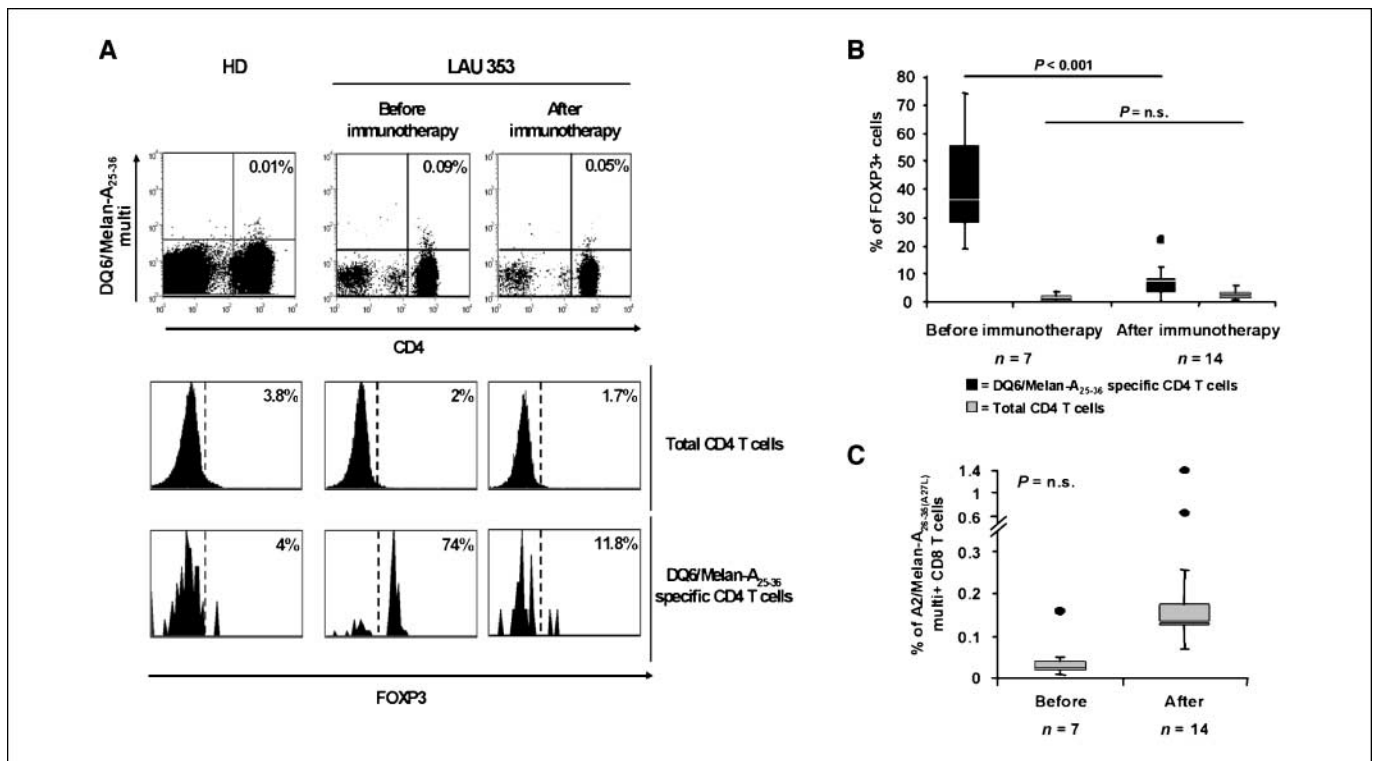
To confirm that the *ex vivo* detected DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells represent indeed specific cells, these cells were isolated by flow-cytometry guided sorting and cloned. Indeed, growing clones were specific for the peptide DQ6/Melan-A<sub>25-36</sub> [in both multimer labeling as well as peptide titration assays (data not shown)].

***Ex vivo* FOXP3+ DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells are consistently reduced by CD8 T-cell-targeted immunotherapy.** To assess if phenotype and function of Melan-A<sub>25-36</sub> CD4 T cells might be affected by CD8 T-cell-based vaccination, we combined *ex vivo* multimer labeling of CD4 T cells with additional phenotypic and activation markers. The majority of the Melan-A<sub>25-36</sub> CD4 T cells displayed a memory phenotype, already before vaccination, with different degrees of activation depending on the patients. We did not observe any significant change in the expression of differentiation markers (e.g., CCR7 and CD45RA) nor in the frequency of activated cells (i.e., HLA-DR and CD38) in the post-vaccination samples (data not shown).

However, remarkably, significant variations were observed in the frequency of multimer+ CD4 T cells positive for FOXP3, a marker that identifies regulatory T cells (26, 27), when comparing samples from a same patient, collected before and after vaccination. Before vaccination, large proportions (range, 19–74%) of Melan-A<sub>25-36</sub> CD4

T cells expressed FOXP3. In contrast, only 1% to 2% of the bulk CD4 T cells were FOXP3+ (Fig. 3A and B). Strikingly, the proportion of FOXP3+ among multimer+ CD4 T cells dramatically and significantly dropped over time during vaccination in all the patients tested (range, 0–21%;  $P < 0.001$ ; Fig. 3A and B), whereas the fraction of positive bulk CD4 T cells remained unchanged. In correlation with the significant decrease in FOXP3+ Melan-A<sub>25-36</sub> CD4 T cells overtime, we observed, in these same patients, an important expansion of Melan-A<sub>26-35</sub>(A27L) CD8 T cells during vaccination (Fig. 3C). When assessed in HDs' samples, FOXP3 expression in *ex vivo* detected Melan-A<sub>25-36</sub> CD4 T cells was low, being 7%, 4%, and 0% in the three PBL samples analyzed (Fig. 3A, representative example).

**The impaired *in vitro* proliferative CD4 T-cell response to Melan-A<sub>25-36</sub> is restored during the course of CD8 T-cell-based immunotherapy and correlates with the drop in FOXP3+ Melan-A<sub>25-36</sub> CD4 T cells.** In parallel to *ex vivo* detection of Melan-A<sub>25-36</sub> CD4 T cells, we assessed the functional capacities of these cells. We performed peptide *in vitro* stimulations (IVS) using the Melan-A<sub>25-36</sub> peptide of enriched CD4 T cells from prevaccination and postvaccination PBMC samples. As shown in Fig. 4A, in HDs, we expanded specific cells only in those samples where *ex vivo* detectable Melan-A<sub>25-36</sub> CD4 T cells were detected (HD2), whereas not in those with undetectable frequencies of Melan-A<sub>25-36</sub> CD4 T cells (HD1). In melanoma patients, significant expansions were observed only in the PBMC samples collected after vaccination (Fig. 4A and B), when the *ex vivo* frequency of FOXP3+ Melan-A<sub>25-36</sub> CD4 T cells was below 10% of multimer+ cells (Fig. 4A and B).



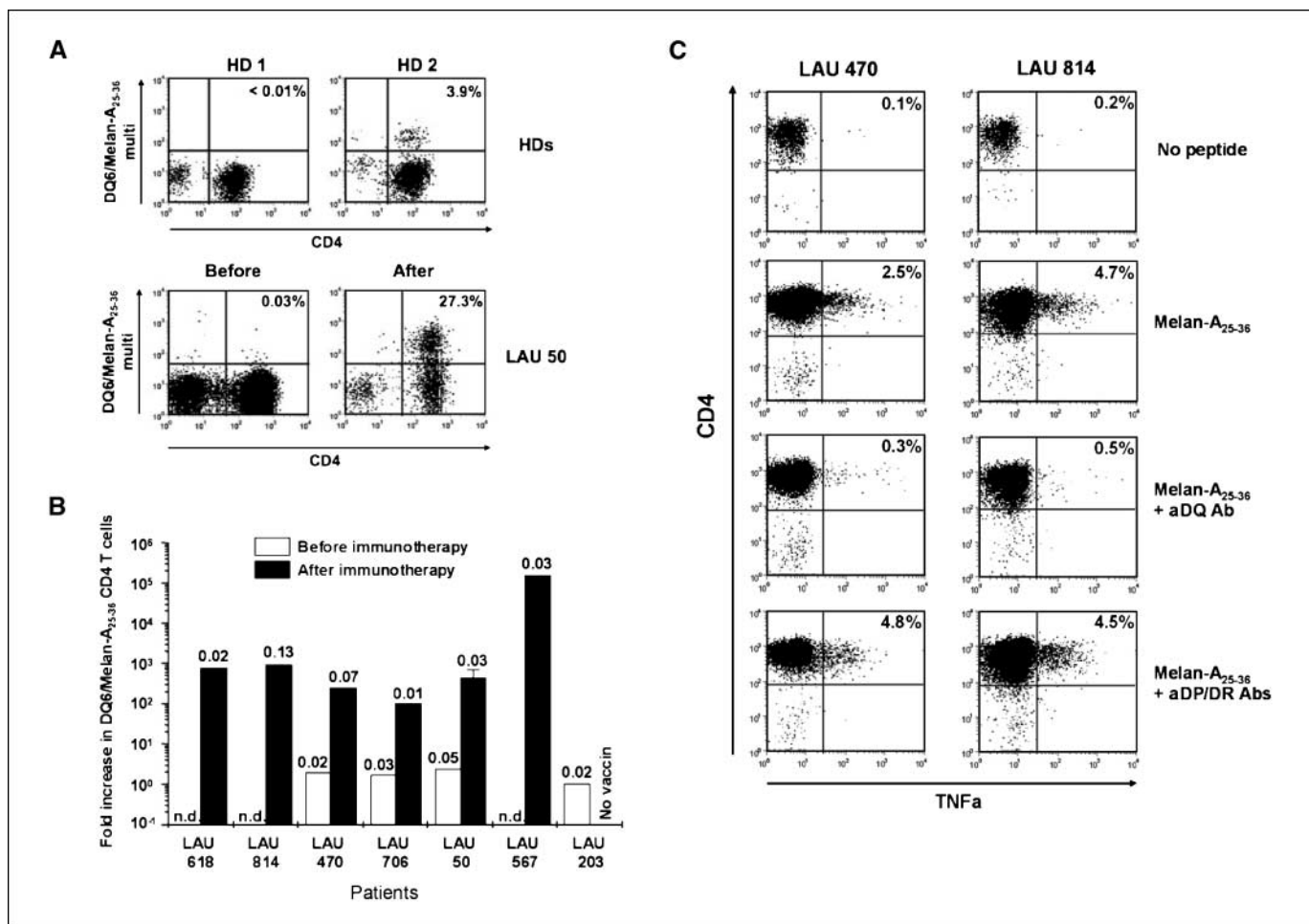
**Figure 3.** Significant decrease in *ex vivo* FOXP3+ Melan-A<sub>25-36</sub> CD4 T cells in PBLs from melanoma patients during CTL-based immunotherapy. **A**, representative example of DQ6/Melan-A<sub>25-36</sub> multimer labeling (top) on PBLs from a HD (left column) and patient LAU 353, before (middle column) and after immunotherapy (right column), combined with FOXP3 analysis in total (middle) or in Melan-A<sub>25-36</sub> CD4 T cells (bottom). Percentages in the top right quadrants of the dot plots, frequency of multimer+ cells within total CD4 T cells, before and after immunotherapy, in multimer+ or total CD4 T cells from PBLs from melanoma patients. **B**, graph summarizing frequencies of FOXP3+ cells, before and after immunotherapy, in multimer+ or total CD4 T cells from PBLs from melanoma patients. **C**, graph summarizing frequencies of A2/Melan-A<sub>26-35</sub>(A27L) multimer+ CD8 T cells in PBLs from melanoma patients, before and after immunotherapy.

Attempts to promote peptide-specific proliferation of Melan-A<sub>25-36</sub> CD4 T cells in prevaccination PBMCs by either depleting CD4+CD25+ T cells or by adding several stimulating or inhibitory molecules in the culture wells [e.g., CpG-ODNs, CD40L, high doses IL-2, IL-6, IL-15, IL-7, anti-transforming growth factor (TGF) $\beta$ , anti-IL-10 receptor] failed (data not shown).

Finally, the vigorous peptide-specific proliferative response of Melan-A<sub>25-36</sub> CD4 T cells from samples after vaccination was matched by their ability to secrete Th1 cytokines (e.g., TNF $\alpha$ ), when incubated with the specific peptide (Fig. 4C). Cytokine secretion is abolished selectively by anti-DQ antibody but remained unaffected in the presence of anti-DP and anti-DR antibodies.

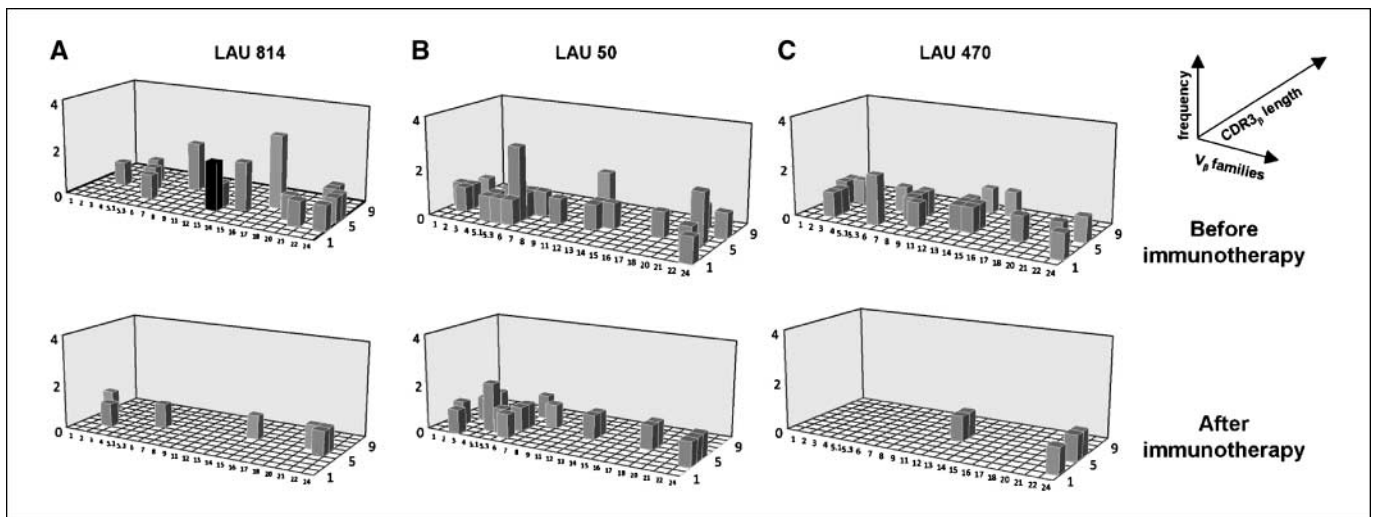
**Restriction of TCR V $\beta$  usage in Melan-A<sub>25-36</sub> CD4 T cells during CD8 T-cell-based immunotherapy.** The observation that the frequencies of FOXP3+ Melan-A<sub>25-36</sub> CD4 T cells decreases during CD8 T-cell-based immunotherapy prompted us to assess which mechanisms might underlie this phenomenon. To compare cells from the same antigen specificity at different time points, we analyzed their TCR V $\beta$  usage. We randomly selected three

patients, and isolated multiple five-cell samples of *ex vivo* DQ6/Melan-A<sub>25-36</sub> multimer+ CD4 T cells by flow cytometry-guided sorting. Next, we performed PCR for the known different human TCR V $\beta$ s on these samples and compared the results for the same patient, in samples before and after immunotherapy. As shown in Fig. 5, a large panel of TCR V $\beta$ s is used by Melan-A<sub>25-36</sub> CD4 T cells in all the patients, in samples before vaccination (13 by LAU 50, and 11 by both LAU 814 and LAU 470). In contrast, a smaller number of V $\beta$ s could be amplified in the sorted Melan-A<sub>25-36</sub> CD4 T cells from postvaccination PBMC samples (six for LAU 814, nine for LAU 50, and two for LAU 470), suggesting a vaccination-induced restriction of the repertoire. Additional analysis of the CDR3 sequence of the amplified PCR products revealed no sequence overlap between cells sharing the same V $\beta$ s, detected before and after vaccination, suggesting a *de novo* expansion of specific CD4 T cells with defined V $\beta$ s after immunotherapy (data not shown). Only in the case of patient LAU 814, the same clonotype (V $\beta$ 13, CDR3 sequence SPHYNQPQ) was identified in two independent five-cell samples from PBMCs collected before vaccination.



**Figure 4.** *In vitro* proliferative capacity and functional activity of Melan-A<sub>25-36</sub> CD4 T cells from HDs and melanoma patients. *A*, CD4 T cells from HDs (*top*) and melanoma patients, before and after immunotherapy (*bottom*) were stimulated for 14 d *in vitro* with Melan-A<sub>25-36</sub> peptide. Percentages in the top right quadrants, frequency of multimer+ cells within total CD4 T cells. *B*, summary of the expansion of Melan-A<sub>25-36</sub> CD4 T cells obtained by IVS of PBLs from different melanoma patients. The fold increase of specific cells has been calculated by dividing % of multimer+ cells after culture by % of multimer+ cells detected *ex vivo*. Values on the top of the bars, initial frequencies of *ex vivo* detected multimer+ CD4 T cells. *n.d.*, not determined. For LAU 50, multiple samples after vaccination have been analyzed. *C*, intracellular TNF $\alpha$  labeling on Melan-A<sub>25-36</sub> *in vitro* stimulated CD4 T cells from PBLs from patients LAU 470 (*left*) and LAU 814 (*right*). Percentages in the top right quadrants, frequencies of TNF $\alpha$ <sup>+</sup> within total CD4 T cells.





**Figure 5.** TCR V $\beta$  usage by *ex vivo* detected Melan-A<sub>25-36</sub> CD4 T cells in PBLs from melanoma patients, before and after CTL-based immunotherapy. Graphs indicate TCR V $\beta$  usage in *ex vivo* sorted five-cell samples from PBLs before (top) and after immunotherapy (bottom) for patient LAU 814 (A), LAU 50 (B), and LAU 470 (C). X axis, all the V $\beta$  tested; Y axis, CDR3 product length; Z axis, frequencies. Single filled bar, the only TCR clonotype (V $\beta$ 13, CDR3 sequence SPHYNQPQ) that was identified twice among the 5-multimer+ cell aliquots in patient LAU 814.

## Discussion

The main finding of our work is the direct *ex vivo* identification, using pMHCII multimers, of detectable frequencies of circulating Melan-A<sub>25-36</sub>-specific CD4 T cells in PBMCs of some DQ6+ HDs and the majority of DQ6+ metastatic melanoma patients. Remarkably, vaccination with an exact MHCII-restricted tumor-associated peptide modulates the phenotype, functionality, and TCR V $\beta$  usage of DQ6-Melan-A multimer+ CD4 T cells. Indeed, we describe for the first time major reductions in the levels of *ex vivo* detected FOXP3+ Melan-A<sub>25-36</sub>-specific CD4 T cells during the course of vaccination. The decline in antigen-specific FOXP3+ CD4 T cells is accompanied by a concomitant restoration of their peptide-specific proliferative potential and cytokine secretion. It is also associated with a significant restriction of their TCR V $\beta$  usage. Altogether our results suggest a previously unrecognized beneficial effect of therapeutic vaccination on a tumor antigen-specific CD4 T-cell population.

pMHCII multimers allow direct identification of antigen-specific CD4 T cells in various clinical situations in humans. However, due to the low frequencies of circulating antigen-specific CD4 T cells, this tool has not yet found the same wide usage as pMHCII multimers. In this regard, reports on the use of pMHCII multimers in the cancer field are still rare (28–30) and limited to the direct identification of specific cells after patients' immunization or after peptide IVS.

In HDs, both self- and tumor antigen-specific CD4 T cells have been identified by others. However, in contrast to the findings reported here, those specific CD4 T cells could not be directly identified *ex vivo* but only after multiple rounds of IVS of PBLs depleted of CD4+CD25+ T cells (31). The Melan-A<sub>26-35</sub> that is embedded in the HLA-DQ6-restricted epitope Melan-A<sub>25-36</sub> is recognized by HLA-A2-restricted CD8 T cells, and high frequencies of naïve precursors can be identified directly *ex vivo* with HLA-A2/Melan-A<sub>25-36</sub> multimers in a large proportion of HLA-A2+ HDs (21, 32). Such massive repertoire is generated by a high level of thymic output and limited turnover in the periphery. Similarly, it can be speculated that Melan-A<sub>25-36</sub> CD4 T cells might be able to escape

thymic-negative selection and accumulate at relative high frequencies in the circulation. In this regard, we failed to *ex vivo* detect CD4 T cells specific for other tumor (e.g., NY-ESO-1) or viral (e.g., HA) antigens when using pMHCII multimers (data not shown).

The finding that Melan-A<sub>25-36</sub> CD4 T cells are present at detectable levels in all but one of the patients' samples, before immunotherapy, and at significantly higher frequencies compared with HDs, argues for a tumor-induced expansion of these cells in the patients. The cells present a memory phenotype, and more interestingly, a large proportion expresses the transcription factor FOXP3. These features suggest that tumor progression selectively drives the amplification of memory tumor antigen-specific Tregs. Numerous reports have shown that Tregs are overrepresented in various cancer types (26, 33–35). Moreover, recent reports document on the existence of tumor-antigen specific Treg cells (36–40). However, no *ex vivo* data are currently available on the characterization of tumor antigen-specific Treg cells. We provide here evidence for the presence, in advanced stage melanoma patients, of relatively high numbers of circulating FOXP3+ Melan-A<sub>25-36</sub> CD4 T cells, with low *in vitro* proliferative potential. These cells may contribute to the progression of the disease and suppress an effective antitumor response.

Little is known on the effect of vaccination on antigen-specific CD4 T cells in cancer patients. Few clinical trials have been reported in which melanoma patients were immunized with MHCII- in conjunction with MHCII peptides. If one trial showed that addition of MHCII-restricted epitopes to the vaccine formulation did not seem to improve clinical responses (41), another study reported on Th1/Th2 responses induced in a small proportion of immunized patients (29). Furthermore, vaccination using full-length proteins of defined tumor antigens has resulted in the induction of integrated antibody, Th1, and CD8 T-cell responses (42). In a recent study on melanoma patients, it was reported that the use of *Salmonella typhimurium* engineered to deliver the antigen NY-ESO-1 elicits CD4 Th1 cells *in vitro*, which are able to recognize naturally processed antigen and are resistant to suppression by Tregs (43). Finally, in a clinical trial in breast cancer patients,

vaccination with a MHC-I-restricted HER2/neu peptide resulted in a significant decrease in circulating Tregs and TGF $\beta$  serum levels in the majority of patients (44). Here, we describe for the first time the effect of immunization using a MHC-I-restricted peptide on tumor antigen-specific CD4 T cells. We observe that the same Melan-A<sub>26-35(A27L)</sub> peptide that induces strong tumor-specific CD8 T-cell responses is able to influence circulating Melan-A<sub>25-36</sub> CD4 T cells in HLA-DQ6+ patients. Indeed, surprisingly, the Melan-A<sub>25-36</sub> FOXP3+ and helper CD4 T-cell ratio is reversed during the course of vaccination. This effect seems to be selective because the levels of FOXP3+ cells among the multimer- CD4 T cells remained stable during time. These observations are in contrast with results obtained in mouse tumor models, where immunization of tumor-bearing animals with tumor antigen recombinant vaccinia virus resulted in the expansion of tumor-induced Tregs (45, 46). These discrepancies might be explained by the fact that our vaccination protocol includes a peptide, Melan-A<sub>26-35(A27L)</sub>, which simultaneously targets CD4 and CD8 T cells. In this regard, it has been reported that immunization of animals bearing methylcholanthrene-induced sarcomas with the addition of a CTL epitope to the standard protocol consisting in plasmids encoding self-antigens resulted in a switch from tumor progression due to expansion of Tregs to an augmented CTL activity and T-cell help. (47).

In addition to the drop in FOXP3+ Melan-A<sub>25-36</sub> CD4 T cells, immunization also leads to a significant augmentation of the *in vitro* proliferative capacity of these cells, as well as to the ability to secrete Th1 cytokines (e.g., TNF $\alpha$ ). As suggested recently (27), the combined assessment of FOXP3 expression and cytokine profiles allows to distinguish regulatory from activated CD4+CD25+ T cells. The molecular and cellular mechanisms underlying this observation remain poorly understood. One possibility is that vaccine components, either acting directly on CD4 T cells, or primarily on CD8 T cells and indirectly on CD4 T cells, promote the generation/expansion of helper Melan-A<sub>25-36</sub> CD4 T cells. Thus far, our attempts to reproduce *in vitro* the *ex vivo* observations have failed, probably due to the complexity of the *in vivo* situation that cannot be efficiently mimicked in an artificial *in vitro* system. To gain further insights into the *in vivo* mechanism, we compared the TCR V $\beta$  usage of *ex vivo* detected Melan-A<sub>25-36</sub> CD4 T cells, in the same patient, before and after immunotherapy. The number of TCR V $\beta$  segments was significantly reduced in the

specific CD4 T cells from postvaccination samples compared with the prevaccination baseline values. Thus, it is possible that vaccination favored the selective expansion of FOXP3- specific CD4 T cells. Whether the FOXP3+ specific CD4 T cells underwent apoptosis (48) or simply became undetectable by the spectratyping technique remains to be addressed. In parallel, we have previously shown that specific CD8 T-cell responses expanded by vaccination are characterized by the persistence of only a small number of highly active and dominant clonotypes (49, 50).<sup>8</sup>

When feasible, additional *ex vivo* analyses directly on tumor-bearing tissues will allow to gain a more detailed picture on the redistribution of antigen-specific CD4 T cells upon immunization. Eventually, parameters to correlate immunologic responses with clinical benefit may be identified.

In conclusion, this study shows for the first time that vaccination with an exact MHC-I antigenic peptide leads not only to the efficient induction of MHC-I restricted CD8 T cells but may also drive the shift from a FOXP3+ MHC II-restricted peptide-specific CD4 T-cell population to a Th1-like specific response. Because the same peptide can be recognized by both CD8 and CD4 T cells, it is tempting to speculate that this class of peptides may be advantageous for vaccination. This hypothesis needs to be tested in appropriate animal models, in which the underlying mechanism(s) may be addressed.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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<sup>8</sup> Unpublished observations.

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## Tumor Antigen–Specific FOXP3+ CD4 T Cells Identified in Human Metastatic Melanoma: Peptide Vaccination Results in Selective Expansion of Th1-like Counterparts

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