Emergence of High-Avidity Melan-A-Specific Clonotypes as a Reflection of Anti–PD-1 Clinical Efficacy

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Abstract

Therapeutic strategies using anti–PD-1–blocking antibodies reported unparalleled effectiveness for melanoma immunotherapy, but deciphering immune responses modulated by anti–PD-1 treatment remains a crucial issue. Here, we analyzed the composition and functions of the large Melan-A–specific T-cell repertoire in the peripheral blood of 9 melanoma patients before and after 2 months of treatment with anti–PD-1. We observed amplification of Melan-A–specific Vß subfamilies undetectable before therapy (thereafter called emerging Vß subfamilies) in responding patients, with a predominant expansion in patients with a complete response. These emerging Vß subfamilies displayed a higher functional avidity for their cognate antigen than Vß subfamilies not amplified upon anti–PD-1 therapy and could be identified by a sustained coexpression of PD-1 and TIGIT receptors. Thus, in addition to the emergence of neoantigen-specific T cells previously documented upon anti–PD-1 therapy, our work describes the emergence of high-avidity Melan-A–specific clonotypes as a surrogate marker of treatment efficacy. Cancer Res; 77(24); 7083–93. ©2017 AACR.

Introduction

PD-1 (CD279) is an inhibitory receptor expressed on activated T and B cells, initially described for its role in the regulation of immune tolerance mechanisms. In addition to this physiologic context, PD-1 is also involved in immune tumor escape. The interaction between PD-1 and its ligands (PD-L1 and PD-L2) mainly occurs in peripheral tissues and tumor microenvironment and leads to the inhibition of effector functions of specific T cells, upon the dephosphorylation of T-cell receptor (TCR) proximal signals (1, 2). PD-1 expression by tumor-infiltrating lymphocytes (TIL) has been often associated with suboptimal immune responses and bad prognosis (3, 4). Nonetheless, in melanoma-infiltrating lymphocytes, PD-1 expression is increased on tumor-reactive CD8⁺ T cells (5). T-cell clonotypes specific for neoantigens have been identified exclusively in this PD-1–positive fraction (6). This highlights the dual role of PD-1, as a marker of reactive T-cell population and as an inhibitor of the immune response. Clinical trials targeting PD-1 in melanoma have shown impressive efficacy with about 30% of objective clinical responses (7–9). Combined therapies with anti–PD-1 and anti–CTLA-4 antibodies reported even better therapeutic efficiency (10, 11). Defining immune markers predicting clinical efficacy of anti–PD1/PD-L1 antibodies could further improve patient management and give keys for further combination strategies. In this respect, PD-L1 expression by melanoma cells has been proposed as a potential biomarker of treatment efficiency, although its predictive value might depend on the antibody used in IHC and the threshold of positivity defined (12). Furthermore, PD-L1 expression is known to be modulated by the tumor microenvironment, especially by IFNγ produced by activated T cells and is thus susceptible to vary during the course of the treatment, rendering the interpretation of a single-point-in-time analysis rather complex. In addition, it has been documented that pre-existing CD8⁺ PD-1⁺ T-cell infiltrate at the invasive tumor margin and inside tumors may predict the therapeutic response (13). In responding patients, these T cells also present a more clonal TCR repertoire, suggesting the accumulation of tumor-specific T cells at the tumor site reinvigorated upon PD-1 blockade. However, little is known about the tumor antigens recognized by T cells reinvigorated by checkpoint blockade. It has been suggested that tumor-specific mutant proteins are a major class of T-cell rejection antigens targeted by specific T cells following anti–PD-1 treatment in various cancers (14, 15). This is in agreement with the preferential efficiency of anti–PD-1 treatment in patients with mismatch repair deficiency (15, 16). Globally, blocking PD-1 signaling will induce the amplification of tumor reactive clonotypes, inhibited in the absence of treatment by PD-L1 expression on tumor cells, and among them clonotypes specific for neoepitopes. Nonetheless, at least in melanoma, the vast majority of neoantigens...
derived from patient-specific passenger mutations are considered as neutral in regard to cell functions and therefore are mainly patient specific (17, 18). Furthermore, the frequency of CD8+ T cells specific for these neoepitopes remain rather low in patient blood, although it appears technologically feasible to expand neoantigen-specific T cells from patient blood for some melanoma patients (19–21).

Thus, although technical issues to detect neoantigen-specific T cells will be overcome, to routinely follow-up such rare and patient-specific T-cell repertoires remains challenging.

Besides, the impact of PD-1 blockade on preexisting T cells specific for shared tumor antigens remains poorly documented. Starting from the premise that efficient PD-1 blockade will reinvigorate the whole antitumor immune response, we hypothesized that quantitative and qualitative variations within the large Melan-A-specific T-cell repertoire should be detected in anti-PD-1-treated patients. Indeed, in melanoma patients, Melan-A-specific T-cell frequency can reach up to 10–4 among CD8 T cells, with a large diversity of TCR B repertoire (22, 23), and the presence of Melan-A–specific T cells in peripheral blood has been associated with long-term survival of melanoma patient (24). Furthermore, changes in peripheral Melan-A–specific T-cell repertoire have been already observed upon adoptive cell transfer of Melan-A–specific T-cell clones, and associated with clinical outcome (25, 26). In addition, we already documented that blocking PD-1 signaling in vitro favored the amplification of Melan-A-specific T cells with a TCR repertoire biased toward a higher functional avidity (27). In this study, we sought to investigate the variations in peripheral Melan-A–specific T-cell repertoire in melanoma patients upon anti-PD-1 therapy, in terms of TCR diversity, functional avidity and phenotype, and correlation with therapeutic responses.

Materials and Methods

Peripheral blood mononuclear cells and cell lines

This study was performed in accordance with the Declaration of Helsinki and after approval by an Institutional Review Board (Nantes Ethic Committee). Peripheral blood mononuclear cells (PBMC) were isolated from metastatic melanoma patients under PD-1 therapy (Unit of Dermato-cancerology, Nantes Hospital, Nantes, France) after written informed consent (approval number: DC-2011-1399).

The human TAP deficient cell line T2 (174 x CEM.T2) used as a presenting cell was purchased from the ATCC (CRL-1992). All the cell cultures were weekly checked for mycoplasma contamination using HEK-Blue Detection Kit (hb-det3, Invivogen).

Amplification of Melan-A–specific T cells from patient blood

PBMCs were isolated from 40 mL of blood from HLA-A2 patients before and after 4 cures of anti-PD-1 therapy. PBMCs were seeded in 96-well/plates at 2 × 105 cells/well in RPMI1640 medium supplemented with 8% human serum, 50 IU/mL of IL2 (Proleukin, Novartis) and stimulated with 1 µmol/L of Melan-A27L peptide (ELAGIGILTV) purchased from Proteogenix. After 14 days, each microculture was evaluated for the percentage of specific CD8 T lymphocytes by double staining with the relevant HLA-peptide tetramer (from the SFR Sante recombinant protein facility) and anti-CD8 mAb (Clone RPA-T8, BioLegend) using a FACSCanto HTS. Microcultures that contained at least 1% of specific T cells were selected, pooled, and sorted with the relevant multimer-coated beads as described previously (28).

V8 repertoire of specific T cells

V8 diversity of sorted Melan-A–specific T-cell lines was analyzed by labeling with 24 anti-V8 mAbs included in the IOTest Beta Mark TCR V Kit (Beckman-Coulter, IM3497). These cytometric analyses were performed on a FACSCanto II (BD Biosciences).

Costimulatory receptor expression and functions of activated T cells

Phenotypic analyses were performed on activated T cells. Antigen-specific T cells were activated 6 hours in 96-well plates with coated anti-CD3 Ab (clone OTK3, CRL–8001, ATCC) at 1 µg/mL. PD-1, 4-1BB, and TIGIT expression was tested on specific T cells by quadruple labeling with Melan-A–specific tetramer (provided by the recombinant protein facility of the SFR Sante), anti-CD25 (clone M-A251, BD Biosciences) as activation marker, anti–PD-1 (Clone EH12, BD Biosciences) and anti-TIGIT (Clone MBSA-43, ebioscience) antibodies. All the antibodies were used at a concentration of 5 µg/mL. Analysis of receptor expression was performed on Tetramer™/CD25™ specific T cells.

CD107a mobilization and cytokine production were evaluated after coculture with TAP-deficient T2 cells loaded with 1 µmol/L of Melan-A27L peptide (ELAGIGILTV) at effector/target ratio 1/2. After a 6-hour stimulation period, in the presence of brefeldin A at 10 µg/mL (Sigma, B7651), T cells were labeled with PE-conjugated specific anti-V8 antibodies (Beckman Coulter) and fixed with PBS 4% paraformaldehyde (VWR, 100504-858). Lymphocytes were then stained for cytokine production using APC-conjugated anti-TNFα (clone cA2, Miltenyi Biotec), anti-IFNγ (clone 45-15, Miltenyi Biotec) and anti-IL2 (clone MQ1-17H12, BD Pharmingen). Concerning CD107a labeling, specific T cells were stimulated for 4 hours at 37°C in the presence of APC-conjugated mAb specific for CD107a (clone H4A3, BD Biosciences). T cells were then stained with selected anti-V8 antibodies (Beckman Coulter) and analyzed by flow cytometry.

Functional avidity of Melan-A sorted T cells

The relative avidity of sorted T cells was measured by CD107a membrane expression, in response to T2 cells loaded with a range of specific peptides (E/T ratio 1/2). Specific T cells were stimulated at an E/T ratio of 1/2 with peptide loaded T2 cells for 4 hours at 37°C in the presence of APC-conjugated mAb specific for CD107a (clone H4A3, BD Biosciences). T cells were then stained with selected anti-V8 antibodies (Beckman Coulter) and analyzed by flow cytometry.

Statistical analyses

We performed statistical analyses for the comparisons of EC50 (log EC50), PD-1, 4-1BB, and TIGIT expression (%) for the three groups of V8 T-cell subfamilies derived from patient blood before and after PD-1 treatment. Values for each group were compared using a nonparametric ANOVA Kruskal–Wallis analysis, followed by a Dunn posttest for multiple comparisons. We also compared the EC50 of two groups of V8 subtypes (highly or poorly coexpressing PD-1 and TIGIT), using Mann–Whitney test and
Results

Clinical outcome of PD-1–treated melanoma patients

All the patients included in this study were stage IIIc or IV unresectable metastatic melanoma patients receiving anti–PD-1 therapy as a first line of treatment (7/9), or after previous treatments (2/9) with anti-BRAF therapy, chemotherapy or ipilimumab (Table 1). Anti–PD-1 mAb (nivolumab, BMS) was administered intravenously every 2 weeks at 3 mg/kg, and the clinical evaluation was performed every 2 months after the start of anti–PD-1 therapy, by radiography according to RECIST criteria. We took into account the clinical evaluation performed at month 6 because it is now widely accepted that clinical responses to immunotherapy often occur later than those observed with chemotherapy or targeted therapies (29) (Fig. 1A). Melan-A–specific T-cell repertoire was analyzed before the first injection (T0) and after 4 injections of PD-1 mAb (M2). Fifteen patients were pre-selected for this study, but only 9 patients who could receive at least 4 doses of nivolumab were finally included in this study. After 6 months of anti–PD-1 therapy, 3 patients experienced a complete response according to RECIST criteria (P2, P8, and P10) and one patient experienced a partial response (67%; P7). Three patients remained stable at month 6 (P3, P6, and P9) and 2 patients progressed (P1 and P4). Age, BRAF mutation status, LDH, and ALC were not associated with clinical responses (Table 1).

Diversity of peripheral Melan-A–specific T cells in anti–PD-1–treated patients

We analyzed the diversity of Vß subfamilies among Melan-A–specific T-cell repertoire derived from the blood of these 9 HLA-A2 melanoma patients, before and after 2 months of anti–PD-1 (nivolumab) treatment, precisely 15 days after the fourth injection of this antibody at 3 mg/kg just before the 5th injection. We took into account the clinical evaluation performed at month 6 because it is now widely accepted that clinical responses to immunotherapy often occur later than those observed with chemotherapy or targeted therapies (29) (Fig. 1A). Melan-A–specific T-cell repertoire was analyzed before the first injection (T0) and after 4 injections of PD-1 mAb (M2). Fifteen patients were pre-selected for this study, but only 9 patients who could receive at least 4 doses of nivolumab were finally included in this study. After 6 months of anti–PD-1 therapy, 3 patients experienced a complete response according to RECIST criteria (P2, P8, and P10) and one patient experienced a partial response (67%; P7). Three patients remained stable at month 6 (P3, P6, and P9) and 2 patients progressed (P1 and P4). Age, BRAF mutation status, LDH, and ALC were not associated with clinical responses (Table 1).

two-tailed P value. All these analyses were performed using PRISM software.

Table 1. Patient’s characteristics and clinical outcome according to the RECIST criteria

<table>
<thead>
<tr>
<th>Age</th>
<th>Stage</th>
<th>Prior therapies</th>
<th>BRAF status</th>
<th>ALC Giga/L*</th>
<th>LDH µKat/L*</th>
<th>Clinical outcome (M6)</th>
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<tr>
<td>P1</td>
<td>58</td>
<td>IV Mbc</td>
<td>Iplimumab</td>
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<td>72</td>
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<td>Anti-BRAF chemotherapy</td>
<td>mut</td>
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<td>3.28</td>
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<td>III c</td>
<td>—</td>
<td>wt</td>
<td>2.14</td>
<td>2.80</td>
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<tr>
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<td>60</td>
<td>IV Mla</td>
<td>—</td>
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<td>3.24</td>
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<td>III c</td>
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<td>1.11</td>
<td>2.54</td>
</tr>
<tr>
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<td>54</td>
<td>IV Mla</td>
<td>—</td>
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</tr>
<tr>
<td>P8</td>
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<td>4.24</td>
</tr>
<tr>
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<td>—</td>
<td>wt</td>
<td>2.49</td>
<td>4.01</td>
</tr>
<tr>
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<td>58</td>
<td>III c</td>
<td>—</td>
<td>mut</td>
<td>2.32</td>
<td>3.65</td>
</tr>
</tbody>
</table>

NOTE: Light gray lines illustrate patients experiencing a complete response after nivolumab therapy. Abbreviations: CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

*ALC, absolute lymphocyte count; normal value: 1.5–4 Giga/L.

**LDH, lactate dehydrogenase; normal value: 2.25–3.75 µKat/L.

Figure 2 illustrates the frequencies of each Vß subfamilies present in the Melan-A–specific T-cell repertoire, before and after four injections of anti–PD-1 therapeutic antibody. For each patient, we observed substantial changes in the Melan-A–specific TCRß repertoire upon PD-1 therapy, with either the predominant expansion of preexisting Vß subfamilies (gray bars) or the expansion of emerging Vß subtypes (red bars) that were not detectable before the treatment. This latter phenomenon was observed in the majority of treated patients (8/9). Interestingly, for the 3 patients exhibiting a complete response (P2, P8, and P10, Fig. 2), and for patient P9 with stable disease, a single emerging Vß subtype represented almost the entire Melan-A–specific repertoire after PD-1 therapy. For the other patient with partial response (P7), Melan-A–specific Vß subfamilies could only be partially identified (60%–70% of identified Vß subtypes before and after 4 cures of PD-1 therapy). Among identified Vß subtypes, two main TCR Vß chains were detected at month 2: an emerging one (Vß1, representing 20% of Melan-A–specific repertoire), and a preexisting one (Vß14, up to 40% of Melan-A–specific repertoire).

For P3 and P6 stable patients (Fig. 2), Melan-A–specific repertoire was much more diverse than that of responding patients after 2 months of treatment. The majority of Melan-A–specific repertoire was composed of various emerging Vß subfamilies, not detectable before the treatment, representing about 50% of Melan-A–specific T cells in each patient. The remaining repertoire is composed of preexisting amplified and nonamplified Vß subtypes.

In nonresponding patients P1 and P4 (Fig. 2, bottom), only minority or no emerging Vß subfamilies were detected after 4 cures of PD-1 treatment. In contrast, in these 2 patients, we observed the predominant expansion of a preexisting Vß subtype (respectively Vß13.1 and Vß7.1).

In conclusion, although quantitative and/or qualitative changes in Melan-A–specific T-cell repertoire were observed in each patient after PD-1 therapy, the predominant expansion of Melan-A–specific emerging clonotypes was only observed in patients exhibiting objective responses (3 CR and one PR).
PD-1 expression and functions of Melan-A–specific V8 subfamilies

We hypothesized that this biased TCR repertoire could reflect the preferential activity of anti–PD-1 antibody on specific T cells able to strongly induce PD-1 upon specific TCR activation. The proliferation of these T cells would then be restored upon PD-1 blockade, leading to their detection in patient blood. We thus measured PD-1 expression upon TCR activation on the main Vß subfamilies not amplified upon PD-1 therapy (n = 19, empty circles on Fig. 3A), on the preexisting Vß families that were amplified after PD-1 therapy (n = 5, gray circles) and on emerging ones amplified upon PD-1 treatment (n = 8, red circles). PD-1 expression was significantly higher on Vß subfamilies amplified upon PD-1 treatment (preexisting and emerging ones) than on Vß subfamilies that did not expand upon PD-1 therapy. This increased PD-1 expression is only detectable on peripheral antigen-specific T cells, and not at the level of whole peripheral CD8 T lymphocytes (Supplementary Fig. S2). Interestingly, PD-1 expression on emerging and preexisting amplified Vß subtypes is already significantly higher than that of nonamplified clonotypes in the absence of TCR stimulation (Supplementary Fig. S3). Thus, PD-1 blockade in vivo efficiently promotes the proliferation of PD-1hi antigen-specific T cells, presumably inhibited by PD-1 signaling before antibody treatment. Accordingly, anti–PD-1 therapy is less efficient at promoting expansion of PD-1hi–specific T cells.

A high PD-1 expression (often associated with that of other inhibitory molecules) has been associated with dysfunction and exhaustion of CD8+ specific T cells in chronic immune diseases and also in melanoma-infiltrating T lymphocytes (32, 33). As an exhausted phenotype is defined by failure to produce cytokines such as IFNγ, TNFα, and IL2, and ultimately to degranulate, we tested, for the three groups of clonotypes, their ability to produce these cytokines and to degranulate after activation with T2 cells loaded with 1 μmol/L of the Melan-A-A27L peptide. As shown on Fig. 3B, the emerging Vß subfamilies (indicated by red circles) and the preexisting ones (gray circles) displayed similar functional properties than nonamplified preexisting Vß subtypes (open circles), attesting that these PD-1–expressing specific T cells were not exhausted T cells. Interestingly, we even observed that emerging Vß subfamilies were in general among the most reactive ones, for the four tested function (red circles on Fig. 3B). Thus, we hypothesized that this PD-1 expression profile would reflect the high functional avidity of these T cells toward their cognate antigen, as it has been previously suggested for melanoma-infiltrating lymphocytes (5).

Functional avidity of Melan-A–specific V8 subfamilies

We thus tested the functional avidity of the main Melan-A–specific V8 subfamilies on T2 cells loaded with a range of Melan-A-A27L peptide, using double staining with a specific anti-Vß Ab together with CD107a labeling (Fig. 4A). Dotted lines represent the main Vß subpopulations present before PD-1 therapy and not amplified after treatment, solid black lines represent preexisting clonotypes amplified upon PD-1 blockade, and solid red lines illustrate emerging clonotypes. With the exception of patient P4, the EC50 could be estimated for each Vß subfamily and are reported in Table 2. As illustrated by Fig. 4A, for each individual patients, emerging Vß subfamilies are always in the high range of functional avidities compared with other Vß subfamilies from the same patients. In responding patients, emerging Vß subtypes are clearly of a higher avidity for P7 and P8 patients (EC50 of 5 and 6 nmol/L, respectively). For the other 2 responding patients, emerging Vß subfamilies exhibited a similar functional avidity than nonamplified ones (EC50 of 80 and 26 nmol/L). In patients

Figure 1.

Design and methodology of the study. A, Blood samples (60 mL) were collected from HLA-A2 melanoma patients, before the first injection (D0 = T0) with anti–PD-1 therapeutic antibody (nivolumab, 3 mg/kg), and 15 days after the fourth cure (D60 = M2). B, PBMCs were purified from blood and 2 × 10^5 PBMCs were stimulated in 96-well plates (2 × 10^5 cells/well) during 14 days with 1 μmol/L of Melan-A-A27L peptide. After 14 days, Melan-A–specific T cells were sorted with HLA-peptide multimer-coated beads and amplified 2 weeks on feeder cells. At the end of the amplification period (day 30), purity, Vß repertoire diversity, phenotype, and functional avidity of sorted Melan-A–specific T cells were documented.
with stable diseases (P3, P6, and P9), the emerging Vß subfamily was always one of the Vß subpopulation displaying the highest functional avidity, compared with preexisting amplified and nonamplified ones (Fig. 4A; Table 2). Of note, for patient P9 with stable disease, although the Vß16 emerging subfamily exhibited a rather high functional avidity (EC₅₀ = 67 nmol/L), CD107a mobilization reached a plateau of around 60%, suggesting suboptimal functional properties.

Finally, in nonresponding patients P1 and P4, predominant preexisting clonotypes amplified at month 2 (respectively Vß13.1 and Vß7.1) exhibited a poor functional avidity (EC₅₀ = 470 pmol/L for Vß13.1, and below calculation threshold for Vß7.1). The emerging subfamily detected in patient P1 (Vß2) exhibited a high functional avidity (EC₅₀ = 15 pmol/L), but represented only 8% of Melan-A-specific T-cell repertoire.

Overall, we clearly showed that the emerging Vß subfamilies (n = 8) displayed the highest functional avidities, with EC₅₀ ranging from 5 to 140 pmol/L (Table 2, and red circles on Fig. 4B). Furthermore, the range of functional avidities appeared relatively narrow for these emerging subpopulations. The

Figure 2.
Analysis of the Vß repertoire of Melan-A–specific T cells before and after anti-PD-1 therapy (n = 9). Melan-A repertoire diversity before PD-1 treatment (T0) and after 4 cures (M2) was assessed on sorted Melan-A–specific T cells using a panel of 24 anti-Vß antibodies. Empty stacks represent the fraction of Vß subfamilies not amplified upon PD-1 therapy, gray stacks illustrate preexisting Vß subfamilies amplified after therapy, and red stacks represent Vß subtypes only detected after treatment. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
preexisting clonotypes ($n = 4$, gray circles on Fig. 4B) amplified upon PD-1 therapy exhibited an EC$_{50}$ slightly higher than those of emerging ones, but not significantly different, ranging from 20 to 470 pmol/L (Table 2). Finally, V8 subfamilies that did not amplify upon treatment ($n = 18$) displayed lower and very diverse functional avidities, with EC$_{50}$ ranging from 12 to 6,620 pmol/L (Table 2, and empty circles on Fig. 4B), significantly different from those of emerging V8 subfamilies ($P < 0.01$). These results suggest that the preferential amplification of high avidity emerging V8 subfamilies could participate to the therapeutic response (as illustrated by patients P2, P8, and P10 exhibiting a complete response).

As we documented a similar PD-1 expression profile between amplified preexisting and emerging V8 subfamilies (Fig. 3A), we further investigated the expression of additional relevant markers that could be specifically associated with Melan-A–specific T-cell emerging subpopulations. We focused our study on two molecules that had been reported to be associated with PD-1 expression and antitumor reactivity, TIGIT and 4-1BB (5, 34, 35).

**TIGIT and 4-1BB expression on Melan-A–specific V8 subfamilies**

In addition to PD-1 expression on the different groups of T-cell subpopulations, we investigated the expression of TIGIT and 4-1BB upon TCR activation. Figure 5A is a representative example of the expression pattern of these molecules on a V8 subfamily only detected before treatment (left) and an emerging V8 subfamily (right) from patient P10.

Overall, 4-1BB expression was not differentially expressed between the three groups of Melan-A–specific subpopulations (Fig. 5B, top). Remarkably, emerging V8 subfamilies exhibited a high TIGIT expression ($n = 8$, 96% ± 4), significantly ($P < 0.001$) different from that of nonamplified subpopulations ($n = 19$, 30% ± 32). The mean percentage of TIGIT expression was also high for amplified preexisting clonotypes, although not significantly different from that of nonamplified clonotypes, probably due to a considerable dispersion and a restricted number of V8 subfamilies ($n = 5$, 65% ± 38; Fig. 5B, middle). Again, no difference could be observed concerning TIGIT expression on total CD8 T cells before and after treatment (Supplementary Fig. S2). Finally, the coexpression of TIGIT and PD-1 on Melan-A–specific T cells is significantly higher on emerging V8 subfamilies, compared with nonamplified ($P < 0.001$) and even preexisting amplified V8 subfamilies ($P < 0.05$; Fig. 5B, bottom). Thus, a sustained expression of these two molecules specifically identifies emerging V8 subfamilies among Melan-A–specific T-cell repertoire.

We further investigated whether the coexpression of PD-1 and TIGIT could be formally associated with functional avidity of specific T cells. On the basis of the mean fraction of all the V8 subtypes coexpressing these two molecules (Supplementary Fig. S4), we defined two groups of T-cell subpopulations highly ($>35$%) or poorly ($<35$%) able to express these molecules upon TCR activation. We further evaluated whether the functional avidities of these two groups of subpopulations could differ according to these expression profiles. As illustrated by Fig. 5C, PD-1$^{hi}$/TIGIT$^{hi}$ subpopulations exhibited a significantly better functional avidity than PD-1$^{lo}$/TIGIT$^{lo}$ subpopulations ($P < 0.05$). Thus, the coexpression of high levels of the inhibitory receptors PD-1 and TIGIT upon activation could be used to identify high-avidity emerging V8 families.

**Discussion**

Biomarker studies conducted as a part of anti–PD-L1/PD-1 clinical trials raise the hypothesis that these agents could be most effective in patients who have preexisting anticancer immunity (13). In line with this hypothesis, melanoma tumors are often enriched in melanoma-reactive TILs. Melan-A–specific T cells representing the most common specific T cells among melanoma-reactive TILs (36, 37). On the other hand, neoepitopes derived from mutated antigens have been described as a primary target of anti–PD-1 therapy in solid tumors, including melanomas (14, 38). These neoantigens have been postulated to be of particular relevance to tumor control, as the quality of
their specific T-cell repertoire would not be affected by central T-cell tolerance. Furthermore, it has been recently documented that T cells specific for neoepitopes could be detected in the periphery of melanoma patients, exclusively in the CD8⁺ PD-1⁺ compartment (6). Nonetheless, in melanoma, the majority of neoantigen-specific T-cell responses are directed against mutations unique to a single tumor, originating from patient-specific passenger DNA mutations (39). Consequently, the amplification of T-cell responses against neoepitopes, although fully relevant, could not be considered as a unique

Figure 4.
Functional avidities of the main Vß subfamilies detected before and after therapy. A, T-cell functional avidities (dotted lines, Vß subtypes only detected before treatment; solid black lines, Vß subtypes present before and after treatment; and solid red lines, Vß subtypes only detected after treatment) were evaluated by measuring CD107a mobilization in response to T2 cells loaded with a range of Melan-A271 peptide, at an E:T ratio of 1:2. CD107a-positive fraction was evaluated by flow cytometry with a specific antibody, gated on the relevant Vß subtypes. B, EC₅₀ of the different Vß subfamilies was calculated using PRISM software for Vß subtypes not amplified upon PD-1 blockade (T0, empty circles, n = 19) and Vß subtypes amplified after anti–PD-1 treatment (M2, preexisting Vß subtypes, gray circles, n = 4 and emerging Vß subtypes, red circles, n = 8). Statistical analyses comparing log EC₅₀ of Vß subfamilies were performed with PRISM software, using a nonparametric Kruskal–Wallis test, followed by a Dunn multiple comparison posttest (**, P < 0.01, two-tailed P value).
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Table 2. Fraction and EC_{50} of dominant Vß subfamilies detected before and after PD-1 therapy

<table>
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<tr>
<th>Patient</th>
<th>T0</th>
<th>Vß subfamily</th>
<th>% of each Vß subfamilya</th>
<th>EC_{50} (pmol/L)b</th>
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<td>2 (CR)</td>
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NOTE: Light gray lines illustrate Vß subfamilies present before and after therapy, and dark gray lines represent Vß subtypes detected only after treatment (bold values).

aThe % indicates the proportion of each Vß subfamily among all Melan-A–specific T cells.
bEC_{50} were determined after activation of Melan-A–specific T-cell lines by T2 cells loaded with a range of Melan-A_{26-35} peptide.

marker of the clinical efficacy of PD-1 therapy. Moreover, it has been recently documented in NSLCC and melanoma patients that the immunologic effect of PD-1 blockade can be detected in patient blood through the identification of CD8^+/PD-1^+/K6^+^ T cells (40, 41). Reinvigorated CD8^+^ PD-1^+^ exhausted T cells contain T-cell clonotypes shared with autologous TILs, and the ratio T-cell invigoration/tumor burden appears associated with anti–PD-1 efficacy in melanoma patients (40). Nonetheless, the fine specificity of these reinvigorated shared clonotypes has not been addressed in this study.

Starting from the premise that PD-1 blockade could reinvigorate the whole antitumor T-cell response, we reckon that quantitative and qualitative variations among the vast and common Melan-A–specific T-cell repertoire could reflect the efficiency of anti–PD-1 treatments. Indeed, Melan-A–specific T-cell response has been neglected in these studies due to the fact that Melan-A was considered as a self-antigen, eliciting a suboptimal T-cell repertoire due to negative selection during thymic development (42). However, it has been recently reported that the immunodominant HLA-A2 Melan-A_{26-35} epitope was not presented by human medullary thymic epithelial cells, due to a misinitiation of gene transcription (43), thus leading to the evasion of central self-tolerance toward this epitope. This finding, associated with a specific combinatorial permissive pairing of TCRs and ß chains (44, 45), could explain the unusual abundance of Melan-A–specific T-cell repertoire in HLA-A2 individuals, and that makes this T-cell repertoire a relevant compartment to study in anti–PD-1 clinical trials. Another advantage of choosing Melan-A–specific T-cell response to monitor anti–PD-1 activity is that circulating Melan-A–specific T cells are relatively abundant in melanoma patient blood (between 1/10^3 and 1/10^4 among CD8^+^ T cells), and that Melan-A–specific T-cell repertoire is rather polyclonal (22, 45, 46).

We thus investigated the modifications of peripheral Melan-A–specific T-cell repertoire in terms of diversity, phenotype, and functions in melanoma patients before and after 4 cures of nivolumab. We clearly show that 4 cures of anti–PD-1 therapy induced substantial changes in Melan-A–specific T-cell repertoire, with the amplification of new Vß subfamilies undetectable before treatment in almost all the tested patients (Fig. 2), that become predominant in patients with objective response.

In accordance with the results obtained on neoepitope-specific T cells from patient blood (6), Melan-A–specific T cells amplified posttreatment (new or preexisting Vß subtypes) expressed significantly higher levels of PD-1 than specific Vß subtypes not amplified after therapy (Fig. 3A). This result, together with the fact that the polyfunctionality of these amplified T cells is well preserved (Fig. 3B), strongly suggested that PD-1 blockade reinvigorated and favored the expansion of highly reactive PD-1^{high} T cells within Melan-A–specific T-cell repertoire.

In a previous study, we demonstrated that in vitro PD-1 blockade during the expansion of Melan-A–specific T cells favored the amplification of high-avidity specific T–cell clonotypes (27). We thus evaluated the functional avidities of the main T-cell Vß...
subfamilies present in patient blood before and after therapy. Our results showed that emerging Vß subfamilies exhibited a higher functional avidity than Vß subtypes not amplified after therapy (Fig. 4B). Clinically, we observed that the predominant expansion of a single high-avidity Vß subfamily occurred in the 3 patients undergoing a complete response at month 6 (P2, P8, and P10) and in the patient P9 experiencing a stable disease. For this latter patient, although Vß16 emerging subfamily exhibited a high and in the patient P9 experiencing a stable disease. For this latter patient, although Vß16 emerging subfamily exhibited a high functional avidity than Vß subtypes not expanded after PD-1 therapy (left) and an amplified emerging Vß subfamily (right). The expression of these molecules was evaluated by multiple labeling on the fraction Vßpos/CD25pos after anti-CD3 activation. B, 4-1BB (top), TIGIT (middle) expression, and PD-1/TIGIT coexpression (bottom) were assessed on the main Vß subtypes from all Melan-A–specific T-cell populations, upon TCR activation, through multiple labeling with anti-Vß, anti-CD25, and specific antibodies. Empty circles represent preexisting nonamplified Vß subfamilies (T0, n = 19). Gray and red circles illustrate respectively amplified preexisting (n = 5) and emerging Vß subfamilies (n = 8). Statistical analyses were performed using a nonparametric Kruskal–Wallis test, followed by a t test (***, P < 0.001; two-tailed P value).

C, Calculated EC50 of Melan-A–specific T-cell subpopulations were reported according to the two profiles of PD-1/TIGIT coexpression based on the mean value measured on all subpopulations (Supplementary Fig. S4). Statistical analyses were performed using a nonparametric Mann–Whitney t test (*, P < 0.05; two-tailed P value).

Thus, we analyzed additional markers (LAG-3, Tim-3, 4-1BB, and TIGIT) that could identify specifically the subgroup of high avidity clonotypes. Indeed, each of these markers has been associated either with PD-1 expression and T-cell antitumor reactivity (5, 34, 35). Among these potential markers, LAG-3, Tim-3, and 4-1BB expression profiles did not allow to discriminate between the
three groups of clonotypes (Fig. 5B, top and data not shown). The similar expression pattern of 4-1BB molecule by the different Vß subfamilies resonates with previous results showing that 4-1BB was poorly able to define tumor-reactive repertoire in tumors (52). TIGIT expression was previously shown to be upregulated on circulating melanoma antigen-specific CD8+ T cells and TILs, and associated with PD-1 expression (34). This immunoreceptor controls T-cell specific responses through its interaction with CD155 constitutively expressed on melanoma cells, during the effector phase (47). Interestingly, TIGIT expression was significantly enhanced on emerging Vß subfamilies, compared with nonamplified ones, but did not allow to specifically differentiate emerging Vß subfamilies from preexisting amplified ones (Fig. 5B, middle).

Finally, PD-1hi-TIGIThi coexpression was significantly higher on emerging Vß subtypes compared with Vß subtypes not amplified upon PD-1 blockade, and this coexpression also enables emerging T-cell populations to be differentiated from preexisting amplified ones. Furthermore, a sustained PD-1/TIGIT coexpression was significantly associated with a better functional avidity (Fig. 5C). Altogether, we demonstrated that a high PD-1/TIGIT coexpression on melanoma antigen-specific T cells could be a valuable marker of high-avidity T-cell clonotypes (including a high majority of emerging clonotypes), and potentially of PD-1 blockade efficiency.

In conclusion, and even if it requires to be confirmed on a larger patient cohort, within the peripheral Melan-A-specific T-cell compartment, the predominant expansion of high-avidity emerging T-cell populations, identified by a sustained coexpression of TIGIT and PD-1 molecules could be a surrogate marker of the clinical efficiency of PD-1 therapy for melanoma patients.

Disclosures of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Simon, N. Labarriere
Development of methodology: S. Simon, N. Gervois
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Simon, V. Vignard, A. Khammari, B. Dreno
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Simon, V. Vignard, F. Lang, B. Dreno, N. Labarriere
Writing, review, and/or revision of the manuscript: S. Simon, A. Khammari, F. Lang, B. Dreno, N. Labarriere
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Varey, T. Parrot, A.-C. Knol, A. Khammari
Study supervision: A. Khammari, N. Labarriere

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