*Cancer Therapy: Clinical*

# CTLA4 Blockade Broadens the Peripheral T-Cell Receptor Repertoire

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## Abstract

**Purpose:** To evaluate the immunomodulatory effects of cytotoxic T–lymphocyte-associated protein 4 (CTLA4) blockade with tremelimumab in peripheral blood mononuclear cells (PBMC).

**Experimental Design:** We used next-generation sequencing to study the complementarity-determining region 3 (CDR3) from the rearranged T-cell receptor (TCR) variable beta (V-beta) in PBMCs of 21 patients, at baseline and 30 to 60 days after receiving tremelimumab.

**Results:** After receiving tremelimumab, there was a median of 30% increase in unique productive sequences of TCR V-beta CDR3 in 19 out of 21 patients, and a median decrease of 30% in only 2 out of 21 patients. These changes were significant for richness ($P = 0.01$) and for Shannon index diversity ($P = 0.04$). In comparison, serially collected PBMCs from four healthy donors did not show a significant change in TCR V-beta CDR3 diversity over 1 year. There was a significant difference in the total unique productive TCR V-beta CDR3 sequences between patients experiencing toxicity with tremelimumab compared with patients without toxicity ($P = 0.05$). No relevant differences were noted between clinical responders and nonresponders.

**Conclusions:** CTLA4 blockade with tremelimumab diversifies the peripheral T-cell pool, representing a pharmacodynamic effect of how this class of antibodies modulates the human immune system. *Clin Cancer Res; 20(9); 2424–32. ©2014 AACR.*

## Introduction

Blockade of the negative immune regulatory checkpoint cytotoxic T–lymphocyte-associated protein 4 (CTLA4) results in long-lasting responses in a minority of patients with advanced melanoma. CTLA4 is a coinhibitory molecule in T cells and is thought to play a critical role in regulating natural immune responses by inhibiting the CD28-B7 costimulatory signaling. Two fully human CTLA4-blocking antibodies, the immunoglobulin G1 (IgG1) ipilimumab (Yervoy, formerly MDX010, Bristol-Myers Squibb) and the IgG2

tremelimumab (formerly CP-675,206, Pfizer Inc, now developed by MedImmune/AstraZeneca), have been tested in the clinic. Ipilimumab gained regulatory body approvals in many countries based on the demonstration of improved overall survival over a gp100 vaccine or dacarbazine in two randomized clinical trials (1, 2).

Phase I and II testing of the antitumor activity of tremelimumab demonstrated durable tumor regressions, most of them lasting beyond 5 years, in approximately 10% to 15% of patients with metastatic melanoma (3). The most common treatment-related serious toxicities with tremelimumab are skin rash and diarrhea/colitis, with a low percentage of patients experiencing endocrine abnormalities such as thyroiditis and hypophysitis (4–10). The objective response rate and the rate of grade 3–4 toxicities in patients treated with tremelimumab are very similar to the phase II clinical trial results with ipilimumab in a comparable population of patients (11, 12). However, tremelimumab did not lead to an improvement in overall survival in the reported phase III clinical trial (13). Median overall survival was 12.6 months in the tremelimumab arm compared with 10.7 months in the chemotherapy arm, with the differences being non-statistically significant. There was a high use of ipilimumab in patients randomized to the chemotherapy control arm, which is likely to be the major contributing factor resulting in this negative randomized trial (14, 15).

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

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## Translational Relevance

By applying next-generation sequencing of T-cell receptor (TCR) variable genes in blood of patients treated with an anti-CTLA4 antibody, this study demonstrated a remarkable effect of this therapy expanding the number of unique T-cell receptor (TCR) variable beta (V-beta) in blood. Therefore, cytotoxic T–lymphocyte-associated protein 4 (CTLA4) blockade impacts the human immune system expanding the pool of circulating T cells, which may be mechanistically related to the induction of autoimmune and inflammatory toxicities.

Studies in blood cells of patients treated with CTLA4-blocking antibodies have reported a mild increase in the absolute lymphocyte count (ALC), with a cutoff value of >1,000 lymphocytes correlating with patients with improved outcome after CTLA4 blockade (16, 17). The circulating lymphocytes have increased expression of activation markers mostly on $CD4^+$ T cells (5, 18–21), but most studies have failed to detect a consistent expansion of tumor or viral antigen-specific T cells (5, 19, 21, 22). Pathologic analysis of paired tumor biopsies demonstrated that tumor responses are mediated by the intratumoral infiltration of CTLs (18, 19). However, not all patients who had a significant increase in activated intratumoral T cells had a clinical tumor regression (19). Therefore, there remains a need to further understand the immunologic effects of CTLA4-blocking antibodies. In this context, we performed deep sequencing of the complementarity-determining region 3 (CDR3) of the T-cell receptor (TCR) variable beta (V-beta) as readout of the change in T-cell clonality and diversification in peripheral blood lymphocytes of patients with melanoma treated with tremelimumab.

## Patients and Methods

### Clinical trial conduct and sample procurement

Peripheral blood samples were obtained by leukapheresis procedures from 21 patients with metastatic melanoma treated at the University of California at Los Angeles (UCLA) in an investigator-initiated phase II clinical trial of single agent tremelimumab (UCLA IRB# 06-06-093; IND# 100453; trial registration number NCT00471887) administered at 15 mg/kg every 3 months. Objective clinical responses were recorded following a modified Response Evaluation Criteria in Solid Tumors (RECIST). The modification in the RECIST criteria was to consider measurable disease lesions in the skin and subcutaneous lesions detectable by physical exam, but not by imaging exams, if they were adequately recorded at baseline using a camera with a measuring tape or ruler (23). Toxicities were graded according to the National Cancer Institute (NCI) common toxicity criteria version 2.0 during the first 3 months of therapy (one cycle of tremelimumab-based therapy), as the postdosing leukapheresis was performed only during the first cycle of therapy, most frequently between 30 and 60 days from the first dose of tremelimumab. Dose-limiting toxicities (DLT) were prospectively defined as any treatment-related toxicity equal or greater than grade 3, or the clinical evidence of grade 2 or higher autoimmune reaction in critical organs (heart, lung, kidney, bowel, bone marrow, musculoskeletal, central nervous system, and the eye). Peripheral blood mononuclear cells (PBMC) were collected from patients receiving tremelimumab by a leukapheresis procedure. Leukaphereses were planned as part of the predosing procedures. The postdosing leukapheresis were performed at a median of 41 days after the dose of tremelimumab (range, 28–81). In all cases, concentrations of tremelimumab in peripheral blood should have been above 10 µg/mL at the time of cell harvesting by leukapheresis, which is the minimum concentration of tremelimumab that stimulated a biologic effect consistent with CTLA4 blockade in preclinical studies (24). Processing of blood to obtain PBMCs has been previously described (25).

### Immunohistochemical and oncogenic analysis of tumor biopsies

Patients underwent baseline and postdosing biopsies as previously described (19). Biopsy samples were formalin fixed and paraffin embedded (FFPE) and stained by immunohistochemistry (IHC) for CD4 and CD8 T-cell markers as previously described (26), with the SimplePCI imaging system (version 5.2.1.1609; Compix Inc., Imaging Systems) to quantitatively evaluate T-cell infiltrations. The frequency of intratumoral and peritumoral lymphocytes was assessed by analyzing 10 tumor areas from each sample at ×200 magnification. The density was compared between pretreatment and posttreatment biopsies. All samples were analyzed without the knowledge of the patients' clinical outcomes (19). For 19 out of 21 patients, targeted oncogene mutational analysis was performed in baseline FFPE biopsies. After genomic-DNA (gDNA) extraction, they were sequenced with Oncomap v3, a mass spectrometry–based genotyping method, to detect 1,047 point mutations in 112 common oncogenes and tumor-suppressor genes (20)

### Analysis of human TCR V-beta CDR3 repertoire in PBMCs

gDNA extraction was performed following the DNeasy Blood & Tissue Kit (Qiagen). Proteinase K was used for digestion and DNA extraction was performed following animal blood or cell protocol with RNase treatment and using spin-column method. Samples were analyzed with high-throughput deep sequencing of the TCR V-beta CDR3 region with the Illumina Genome Analyzer from Adaptive Biotechnologies using the immunoSEQ immune-profiling system (27) at the deep level. This analysis results in a 5× sequence coverage for T cells from 3.6 µg of gDNA, which means an input of 200,000 T-cell genomes with an output of 1,000,000 sequences, depending on the proportion of T cells. The product was sequenced and organized providing in-frame and out-of-frame sequences. An algorithm was

applied to the in-frame sequences for collapsing reads and resulting in unique in-frame rearrangement of the CDR3 genes. In-frame unique sequences without stop codons are referred to as unique productive sequences and are object of this study. Results were compared with PBMCs from four healthy donors collected every 4 months from four different time points. The same amount of gDNA was loaded for deep sequencing of the TCR V-beta CDR3 region with subsequent immune profiling at the deep level. This was used as a comparison for patients with melanoma.

### Statistical analysis

Statistical measures such as richness (number of unique TCR V-beta CDR3 sequences), the Shannon diversity index (28), and the Pielou's evenness index (29) were calculated to evaluate the diversity of TCR V-beta CDR3 sequences for both patients and healthy donors. The Shannon diversity index was defined as $H = -\sum_{i=1}^{N} p_i \ln p_i$, where $p_i$ is the proportion of sequence $i$ relative to the total $N$ sequences. It accounted for both richness and relative abundance (evenness) of the TCR V-beta CDR3 sequences present in each sample. The larger the Shannon diversity index, the more diverse the distribution of the TCR V-beta CDR3 sequences. The Pielou's evenness index was defined as $J = H/\log(S)$, where $H$ is the Shannon index and $S$ is the number of unique TCR V-beta CDR3 sequences. The nonparametric Wilcoxon signed-rank test was used to compare the diversity measures before and after treatment among patients. Repeated measure analysis (30) was performed to assess if diversity changed over time for healthy donors. To test the hypothesis that patients with toxicity had higher diversity than those without toxicity, one-tailed Wilcoxon rank-sum test was used. Correlation between change in richness in PBMCs and density change observed in CD8 and CD4 in tumor biopsies was assessed by Spearman rank correlation coefficient (31), a robust, nonparametric correlation measure. Comparison of ALC pre- and posttreatment was done by Wilcoxon signed-rank test. For all statistical investigations, tests for significance were two-tailed unless otherwise specified. A $P$ value less than the 0.05 significance level was considered to be statistically significant. Data analyses were carried out using statistical software R version 3.0.0 (32). The reported $P$ values for toxicity are obtained from one-tailed Wilcoxon rank-sum test. Clonality values are calculated from entropy of the TCR V-beta CDR3 frequency distribution, and then normalized by log(# unique TCR V-beta CDR3). These values range from 0 (polyclonal distribution) to 1 (monoclonal distribution).

## Results

### Patient characteristics and outcomes

Among the 21 study patients (Table 1), 14 (66%) had M1c metastatic melanoma [visceral metastasis and/or high LDH (lactate dehydrogenase)], and the remaining had either in-transit (stage IIIc) or soft tissue and nodal metastasis (M1a). Toxicities were graded during the first 3 months of therapy, corresponding to the timing of leukapheresis to collect PBMCs for the current analyses. Among these patients, there were three with toxicities that met the definition of DLTs as defined in the clinical trial protocols. These included four cases of grade 3 diarrhea or colitis and 1 patient with symptomatic panhypopituitarism (grade 2 hypophysitis). None of these patients received corticosteroids before the collection of PBMCs for analysis from the postdosing leukapheresis. Four patients had an objective tumor response, resulting in sustained and durable complete tumor regressions in three of them (GA18, GA29, and GA33), while the other patient (GA5) had a partial response lasting 7 months followed by progression. All of them presented with normal levels of LDH and the 3 patients with a durable complete response (CR) had stage IIIc or M1a. There was no apparent association between the oncogenic driver mutation analysis for the tumor specimens and clinical response. Three of the patients with an objective response (GA5, GA29, and GA33) had $BRAF^{V600E}$ mutations, while 1 (GA18) had a $NRAS^{Q61K}$ mutation (Table 1).

### Changes in ALC

There was a median increase in ALC of 11.1% (ranging from decrease of 37.9% to increase of 80%) from baseline to days 30 to 60 without overall significant changes in this series of samples ($P = 0.18$). Three out of 21 patients started with a baseline count lower than 1,000 lymphocytes, and none of these patients had an objective response. The ALC after treatment with a cutoff value of >1,000 lymphocytes has been previously reported to be correlated with patients with improved outcome after CTLA4 blockade (17, 33). In our series, all four responders had baseline and postdosing counts above 1,000 lymphocytes. However, two of them (GA29 and GA33) experienced a decrease in ALC comparing baseline with the post-tremelimumab values.

### Increased TCR V-beta CDR3 richness and Shannon index diversity with CTLA4 blockade

To examine the dynamics of the CD8$^+$ T-cell repertoire upon anti-CTLA4 treatment, we first analyzed the general yields from the high-throughput TCR sequencing data (Supplementary Table S1). In this series of 21 patients, the average number of productive unique TCR V-beta CDR3 sequences was 87,867 per sample at baseline and 108,150 per sample 1 month after starting the treatment. The mean increase in the total number of productive sequences was 24%. This increase was consistent in 19 of 21 patients, experiencing increases between 11% and 73.6%. Only two patients treated with tremelimumab experienced a decrease (GA28 and GA23) in their total repertoire up to 30% (Fig. 1). Both these patients had rapid disease progression and died within 4 months of starting on study (Table 1). To visually understand the diversity and changes across patients, we generated rank–rank scatter plot figures for each patient (34). Rank–rank scatter plots show the degree of overlap and shift in the relative abundances from baseline to postdosing of each sequence. Sequences are ranked in descending order by frequency in the $x$-axis for baseline and $y$-axis

**Table 1.** Patient characteristics from patients included in the PBMC study for TCR V-beta CDR3 sequencing

| Patient study identification | Sex (M/F) | Ethnicity | Age | Active metastasis site | LDH | Stage | Mutational status | Toxicities | Response at EOS | PFS (mo) | OS (mo) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| GA5 | M | C | 65 | Skin, LN, adrenal | N | M1c | BRAF V600E | — | PR (but PD after EOS) | 7 | 20 |
| GA7 | M | C | 62 | Skin | N | IIIc | BRAF_V600E | G2 pruritus | PD | 2 | 67 |
| GA8 | F | C | 48 | SC | High | M1c | BRAF_V600E | G2 diarrhea | PD | 1 | 3 |
| GA9 | M | C | 52 | LN, bone | High | M1c | BRAF_V600E | — | PD | 3 | 14 |
| GA11 | M | C | 47 | LN | High | M1c | NRAS_Q61R | — | PD | 2 | 7 |
| GA12 | M | C | 76 | Skin | High | M1c | BRAF_V600E | G3 colitis | Off due to AE/PD | 2 | 20 |
| GA13 | M | C | 37 | LN | N | M1a | BRAF_V600E | G2 hypophysitis | PD | 3 | 13 |
| GA14 | M | C | 38 | SC, muscle | High | M1c | HRAS_G12D BRAF_V600E PTEN_N323fs | G1 diarrhea | PD | 3 | 15 |
| GA15 | M | C | 58 | Brain, bowel, liver | N | M1c | NRAS_Q61R | G3 rash | PD | 4 | 15 |
| GA18 | F | C | 49 | Skin | N | M1a | NRAS_Q61R | G1 diarrhea | CR | 62+ | 62+ |
| GA19 | M | C | 55 | LN, brain | N | M1c | NRAS_Q61R TP53_R175H CTNNB1_S37F | G3 diarrhea G2 rash | PD | 3 | 36 |
| GA21 | M | C | 71 | Skin, SC, LN, liver, spleen | High | M1c | KIT_L576P | — | PD | 3 | 8 |
| GA23 | M | C | 27 | Lung | N | M1b | | — | PD | 2 | 4 |
| GA24 | M | C | 81 | SC, lung | High | M1c | | G1 nausea | PD | 2 | 3 |
| GA25 | M | H | 71 | LN | High | M1c | KIT_N822K | — | PD | 3 | 8 |
| GA26 | M | C | 68 | LN, lung | N | M1b | BRAF_V600E | G3 diarrhea G1 Grover's | PD | 1 | 51 |
| GA27 | M | C | 52 | Axillary, clavicular | High | M1c | BRAF_V600E CDKN2A_E61[b] PIK3CA_3545K | G2 pruritus G1 diarrhea G1 hypophysitis | PD | 6 | 11 |
| GA28 | M | C | 48 | LN, Lung | High | M1c | BRAF_G466E EGFR_P753S ABL1_E255K | — | PD | 2 | 4 |
| GA29 | F | C | 79 | Skin, SC | N | IIIc | BRAF_V600E KIT_V559A | G3 diarrhea G1 pruritus | CR | 45 | 45 |
| GA32 | M | C | 36 | Muscle | N | M1c | | — | PD | 5 | 56 |
| GA33 | F | C | 49 | Skin | N | IIIc | BRAF_V600E | G2 adrenal insufficiency | CR | 55+ | 55+ |

Abbreviations: AE, adverse effects; C, Caucasian; EOS, end of study; H, Hispanic; LN, lymph nodes; N, normal; Neg, negative; OS, overall survival; PD, progressive disease; PFS, progression-free survival; SC, subcutaneous.

for postdosing, with the most highly abundant sequences proximal to the origin. Sequences not present in either the baseline or postdosing samples are assigned a frequency of 0 and are represented by the last rank on the axis. Increases in diversity after treatment are shown as an increase in the number of absent baseline sequences along the $x$-axis. Decreases in diversity after treatment are shown as an increase in the number of absent postdosing samples along the $y$-axis. Points are binned and colored on a log scale with red showing the greatest density of points. Fig. 2 shows three representative examples on how the TCR repertoire can

change after treatment toward an increase ($n = 14$ cases of 21; Fig. 2A), decrease ($n = 2$; Fig. 2B), or minimal change ($n = 5$; Fig. 2C) in the total repertoire (the complete scatter plots for all patients are shown in Supplementary Fig. S1). The sequencing method also displays the complete V and J segment usage for each TCR. However, there were no statistical differences in usage of V or J (data not shown).

We compared the results of TCR V-beta CDR3 sequencing in PBMCs from patients treated with CTLA4 blockade with the serial collection of PBMC from four healthy donors (four time points separated by 4 months each). For these
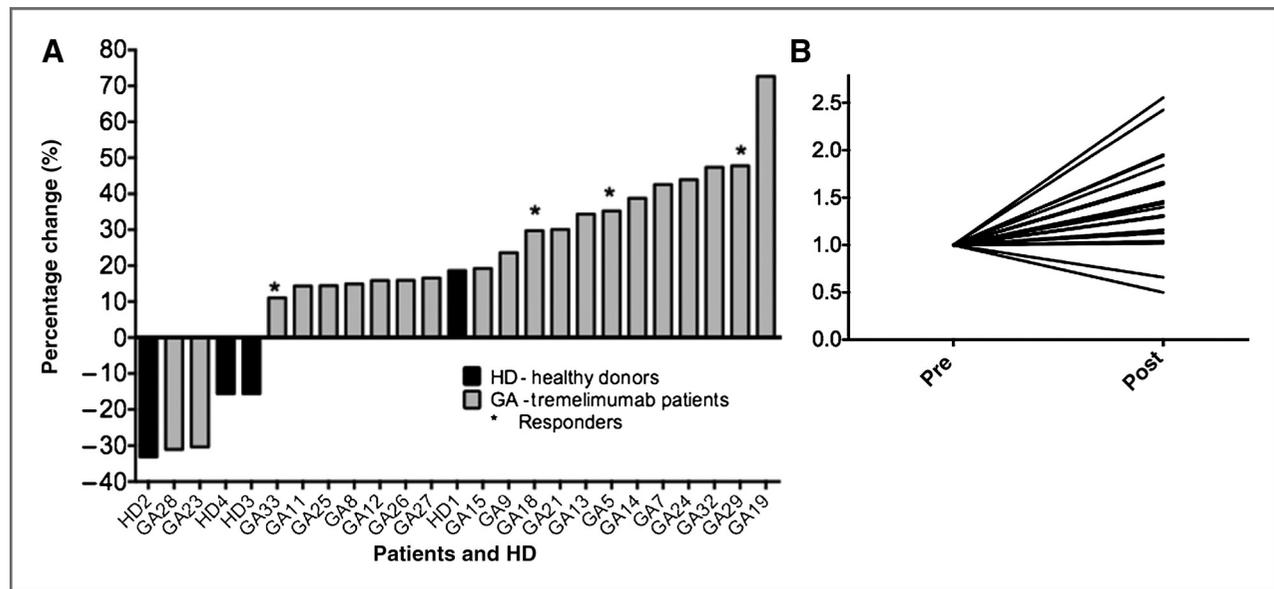
**Figure 1.** Change in absolute number of unique productive sequences. A, changes reported between baseline and post-tremelimumab samples from 21 patients with melanoma (GA, in black) and from four healthy donors (HD, in gray). B, normalized TCR V-beta CDR3 repertoire diversity. Analysis comparing baseline and post-tremelimumab PBMC samples. The values were normalized to 1, to show increase and decrease after treatment in a comparable manner.

healthy donors, richness (number of unique productive sequences) and the Shannon diversity index (distribution of the sample) were calculated for each sample at each time point. There were no differences over time in either analysis ($P = 0.93$ and $P = 0.68$, respectively), suggesting that these four healthy donors had little change in their peripheral TCR V-beta CDR3 repertoire during the 1-year sampling time. The same analysis performed in samples of patients with melanoma treated with tremelimumab comparing baseline and posttreatment samples showed a significant increase in richness ($P = 0.001$ by Wilcoxon sign-rank test), which occurs with a corresponding increase in the total number of productive TCR V-beta CDR3 sequences. The Shannon diversity index was significantly larger posttreat-

ment ($P = 0.04$ by Wilcoxon sign-rank test; Fig. 3). The Shannon diversity index incorporates a combination of richness and evenness. Increases in the diversity index were not attributed to changes in the distribution itself because of a nonsignificant change in the Pielou's evenness index ($P = 0.5$), but instead an increase of the total TCR V-beta CDR3 repertoire itself.

Productive unique sequences were ranked according to their abundance and the top clones were selected up to the point that their sum was equal to 25%. These top 25% abundant clones have been reported to be the most frequent and feasible to track over time (35), avoiding the noise from the most infrequent clones present in blood. In our samples, this ranged from 4 to 5,185 sequences. In this subgroup of
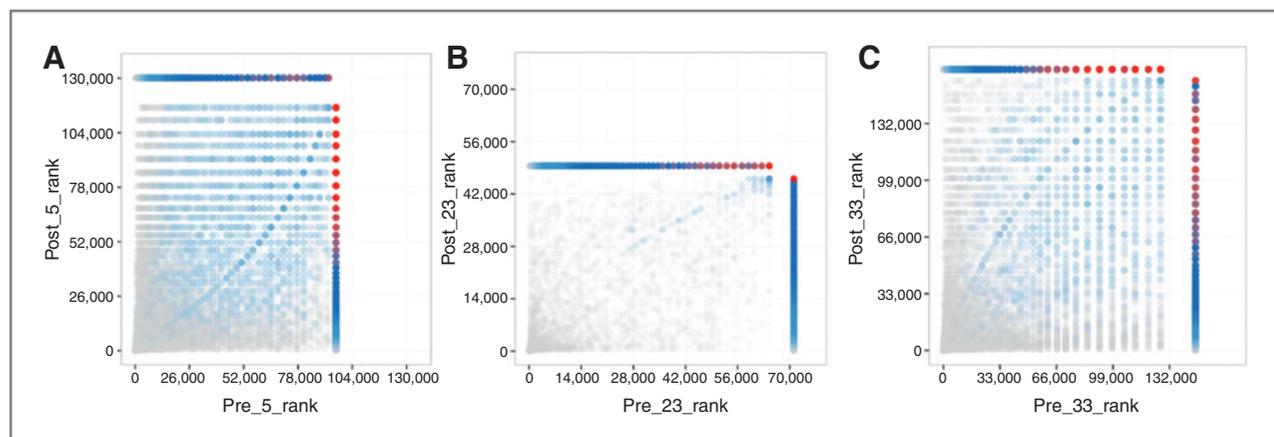


**Figure 2.** Rank–rank scatter plot analysis of all the TCR V-beta CDR3 sequences. Rank–rank scatter plot matching up baseline and post-tremelimumab samples from 3 representative patients. Each sequence was ranked according to clone abundance from highest frequency (rank 1 = 0) to lowest frequency. This provides a visual representation of the changes between baseline (x-axis) and posttreatment (y-axis) distributions. Example of an increase (GA5; A), a decrease (GA23; B), and of minimal change (GA33; C) in the TCR V-beta CDR3 repertoire. Scatter plots for all patients are shown in Supplementary Fig. S1.

**Figure 3.** Richness and Shannon index for diversity. Differences in richness for total number of unique productive sequences ($P = 0.001$; A) and Shannon index for diversity of the repertoire ($P = 0.04$; B).
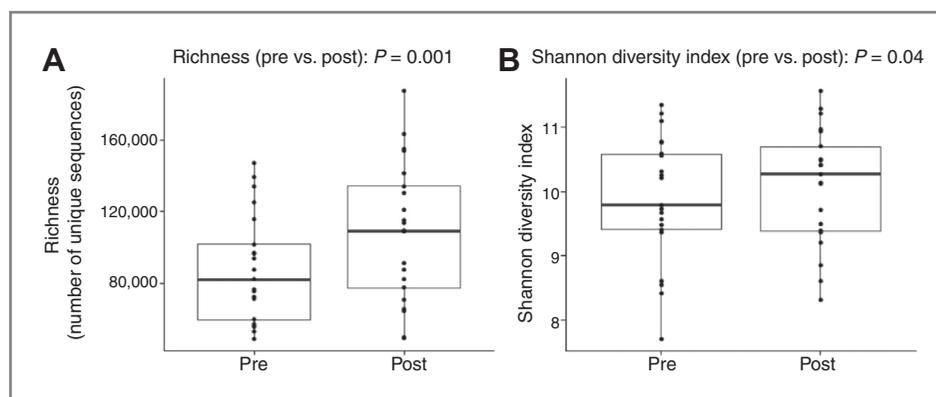
unique productive sequences, the increase was particularly impressive (Supplementary Table S3). Nine out of 21 (43%) of the paired samples had an increase of 2-fold or more, including three of the clinical responders (GA5, GA18, and GA29). The responding patient GA33 experienced a small decrease in TCR V-beta CDR3 diversity. Only 2 out of 21 (9%) had a decrease in the diversity of 2-fold or more. The changes in unique productive sequences reported in Table 2 correlate with a highly polyclonal population, with values of clonality ranging between 0.068 and 0.27. However, the increase in diversity (richness and Shannon diversity index) does not always correlate with a more polyclonal population. In fact, despite only 2 patients (GA23 and GA28) having a decrease in richness, 12 patients showed increase in clonality. In comparison, none of the samples from the four healthy donors had changes more than 2-fold when focusing on the top 25% abundant clones (except time point four in HD4, considered an outlier sample; Supplementary Table S2).

Considering that we observed an increase in ALC, we questioned whether the increase in richness was linked to the increase seen in peripheral lymphocytes counts. However, we identified 5 patients (GA8, GA12, GA24, GA29, and GA33) with decreases in the ALC who, nevertheless, demonstrated an increase in the number of unique productive sequences. Correlation analysis showed that there was no trend ($R = 0.001$) and no significant correlation ($P = 0.1$) between changes in ALC and changes in unique productive sequences (Supplementary Fig. S2). This analysis emphasizes the consistent trend of increased unique productive sequences upon treatment, and deemphasizes the more random and diverse changes in ALC.

### Weak correlation between TCR V-beta CDR3 diversity in blood and intratumoral CD8 T-cell infiltration

Figure 4 summarizes the changes in TCR V-beta CDR3 diversity for each patient and the fold-changes in density of T cells analyzed in paired baseline and post-tremelimumab biopsies from the same patients, as previously reported by our group (19). We had quantitated changes in density for CD4 and CD8 in the intratumoral and peritumoral sections of tumor biopsies stained by IHC. In that work, we noted between 1-fold and 100-fold increase in the intratumoral

infiltration by CD8^{+} T cells in 14 out of 18 evaluable cases regardless of clinical tumor response or progression. There was no difference between the absolute number, location, or cell density of infiltrating cells between clinical responding and nonresponding lesions. In the current context, our interest was to study whether the differences in TCR V-beta CDR3 diversity in peripheral blood would correlate with the differences in CD8^{+} T-cell infiltration in tumor biopsies. In patients with increased TCR V-beta CDR3 diversity in PBMCs, the Spearman correlation coefficient showed a positive trend in all cases comparing fold-changes in richness to increased T-cell infiltration. CD8 peritumoral infiltration was the strongest correlations and statistical significance (Spearman correlation coefficient; $\rho = 0.66$; $P = 0.01$; Fig. 4).

### TCR V-beta CDR3 diversity analyzed in relation to clinical response and toxicity after CTLA4 blockade

There were no significant differences in the total unique productive TCR V-beta CDR3 sequences between the 4 patients with an objective response to tremelimumab compared with patients without a tumor response, either in baseline or in posttreatment samples ($P = 0.62$ and $0.39$ for baseline and posttreatment, respectively, two-tailed Wilcoxon rank-sum test). We thus reasoned that the changes in TCR V-beta CDR3 usage detected in peripheral blood may reflect a broader effect of CTLA4 blockade on the immune system, as opposed to the stimulation of melanoma-specific T cells. Therefore, the TCR V-beta CDR3 diversity changes may reflect expansion of autoreactive T cells leading to the clinically observed autoimmune toxicities of CTLA4-blocking monoclonal antibodies. This was studied by analyzing the potential correlation between TCR V-beta CDR3 diversity and the development of toxicities after administration of tremelimumab. Our results show that samples from patients with toxicity had richer and more diversely distributed TCR V-beta CDR3 sequences in the posttreatment samples compared with patients without toxicities ($P = 0.05$, one-tailed Wilcoxon rank-sum test; Fig. 5). Baseline samples had the same trend, but were not statistically significant as a predictive factor for toxicity (richness $P = 0.30$ and Shannon diversity index $P = 0.12$, by Wilcoxon rank-sum
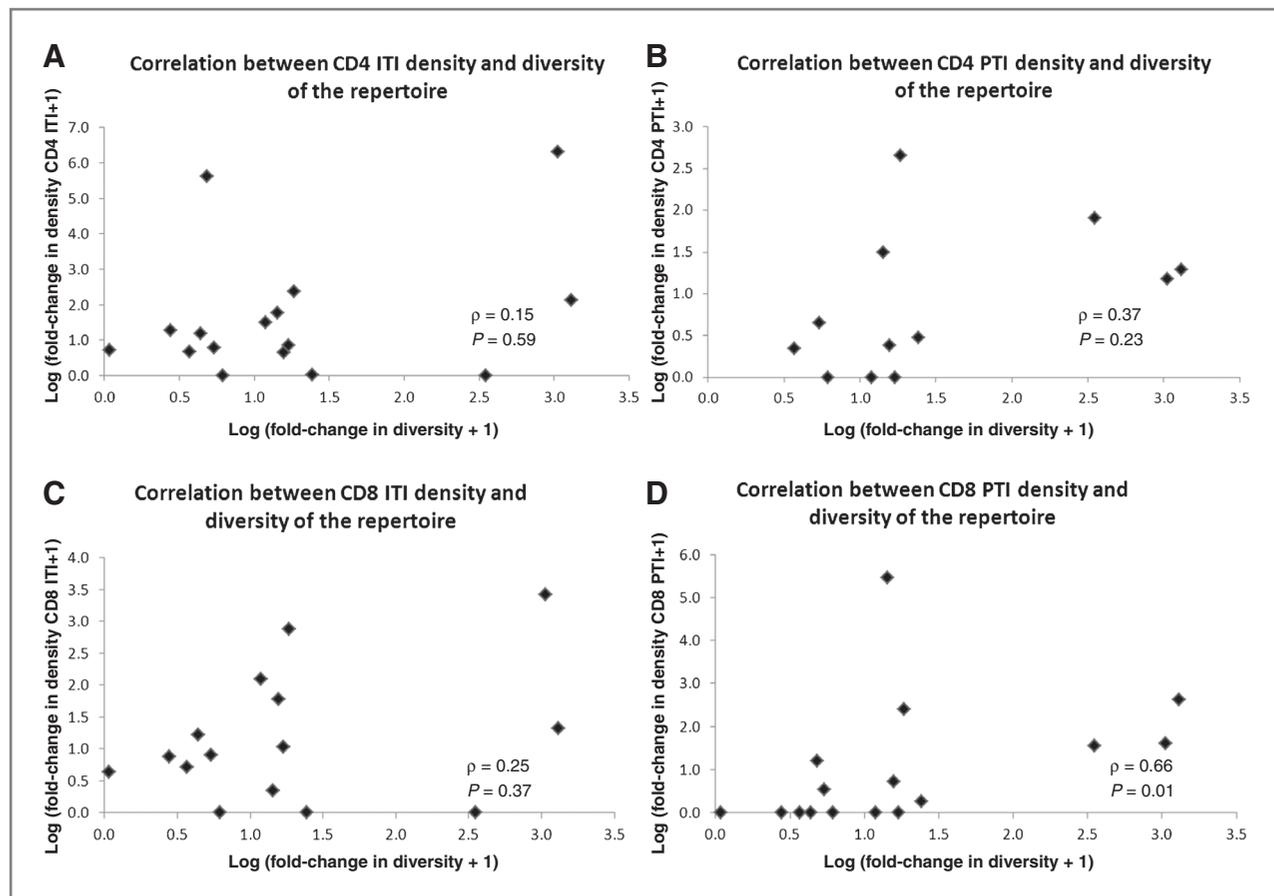
**Figure 4.** Correlation between fold-changes in top 25% abundant clones (x-axis) versus fold-changes in CD4/CD8 density (y-axis) at baseline and days 30 to 60, for CD4 intratumoral infiltration (ITI; A), CD4 peritumoral infiltration (PTI; B), CD8 ITI (C), and CD8 PTI (D).

test). Interestingly, patients with toxicity had higher increases in the total number of unique productive sequences after treatment ($P = 0.04$, one-tailed Wilcoxon rank-sum test).
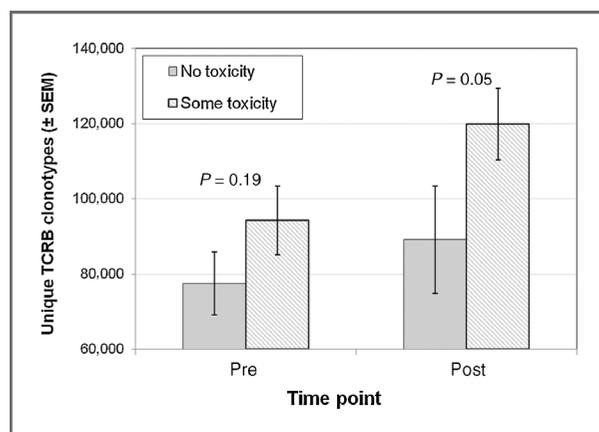


**Figure 5.** Changes in number of unique TCR V-beta CDR3 sequences related to toxicity during the first 3 months of therapy with tremelimumab.

## Discussion

CTLA4 blockade provides long-lasting tumor remissions resultant from a T-cell response to cancer, but it is not fully understood how these antibodies impact on the human immune system to induce durable tumor responses or autoimmune toxicities. Our studies show an expansion of the total number of unique productive TCR V-beta CDR3 sequences in blood after CTLA4 blockade, reflecting a larger T-cell diversity in blood after treatment. There was an increase in the total amount of unique productive sequences, but no expansion of specific clones toward predominance on top of the others, suggesting that this mode of therapy does not result in the specific clonal expansion of T cells to cancer detectable in blood.

Our data are consistent with an immunologic effect of CTLA4 blockade mediated by a nonspecific T-cell proliferation and increased release to circulation, which is congruent with prior data from the literature. Evidence from many groups shows that the anti-CTLA4 antibodies tremelimumab and ipilimumab do not generally expand the number or function of tumor antigen–specific CD8 or CD4 T cells in peripheral blood (4, 5, 25, 36), even though this has been

reported anecdotally in occasional patients (22, 37–39). The release of the CTLA4 checkpoint, which limits T-cell replication, with CTLA4-blocking antibodies was demonstrated in 10 of the patients included in this same series who underwent positron emission tomography (PET) imaging using the PET tracer [$^{18}$F] fluoro-L-thymidine ([$^{18}$F]FLT; ref. 21). [$^{18}$F]FLT PET provides a noninvasive imaging for cell replication at the whole-body level. Postdosing [$^{18}$F]FLT scans demonstrated increased uptake in the spleen, which is the largest lymphoid organ, consistent with the release of the CTLA4 cell-cycle checkpoint in patients treated with tremelimumab. This prior finding suggests the induction of proliferation of T lymphocytes in lymphoid organs, which may contribute to the observation of increased ALC in blood after treatment with CTLA4-blocking antibodies. CTLA4-mediated checkpoint release may also account for the longstanding detection of increase in T-cell activation markers on T cells after the administration of these antibodies, including HLA-DR and inducible costimulator (ICOS; refs. 4, 5, 36, 40–42), and our current finding of increased diversity of the TCR repertoire after CTLA4 blockade.

The increase in diversity of TCR V-beta CDR3 in peripheral blood may reflect a pharmacodynamic effect of the anti-CTLA4 antibody therapy that is more related to a general ability of that patient's immune system to be activated, rather than a correlation with tumor responses. As such, we report that the diversity change has an association with the development of toxicities after receiving tremelimumab. Prior studies to understand the mechanism of the autoimmune and inflammatory toxicities resultant from CTLA4 blockade have focused on the evidence that in some patients there is an increase in IL-17–producing T cells (Th17 cells), as interleukin (IL)-17 is a known mediator of colitis (43). Our findings suggest that the patients more likely to develop toxicity expanded the number of unique productive sequences, which may in part reflect mobilization of auto-reactive T cells.

In conclusion, we report an increased diversity of T cells (richness and Shannon diversity index) in blood after CTLA4 blockade, with no specific expansion of clonal populations. This increased T-cell diversity, together with their increased T-cell activation state, may be mechanistically linked to the development of tumor responses and toxicities in patients treated with CTLA4-blocking antibodies.

## Disclosure of Potential Conflicts of Interest

R. Emerson is an employee of and has ownership interest (including patents) in Adaptive Biotechnologies. H. Robins is employed as a consultant for, is an advisory board member for, and has ownership interest (including patents) in Adaptive Biotechnologies. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** L. Robert, J. Tsoi, X. Wang, R. Emerson, A.J. Cochran, B. Comin-Anduix, R.C. Koya, T.G. Graeber, H. Robins, A. Ribas
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# Correction: CTLA4 Blockade Broadens the Peripheral T-cell Receptor Repertoire

In this article (Clin Cancer Res 2014;20:2424–32), which was published in the May 1, 2014, issue of *Clinical Cancer Research* (1), an author's affiliation was incorrectly printed. The full, corrected author and affiliation list should read as follows:

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The authors regret this error.

## Reference

1. Robert L, Tsoi J, Wang X, Emerson R, Homet B, Chodon T, et al. CTLA4 blockade broadens the peripheral T-cell receptor repertoire. Clin Cancer Res 2014;20:2424–32.

# Clinical Cancer Research

# CTLA4 Blockade Broadens the Peripheral T-Cell Receptor Repertoire

Lidia Robert, Jennifer Tsoi, Xiaoyan Wang, et al.

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