Clonotypic Diversification of Intratumoral T Cells Following Sipuleucel-T Treatment in Prostate Cancer Subjects

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Abstract

Sipuleucel-T is an autologous cellular therapy for asymptomatic or minimally symptomatic, metastatic castrate-resistant prostate cancer, designed to stimulate an immune response against prostate cancer. In a recent clinical trial (NCT00715104), we found that neoadjuvant sipuleucel-T increased the number of activated T cells within the tumor microenvironment. The current analysis examined whether sipuleucel-T altered adaptive T-cell responses by expanding pre-existing T cells or by recruiting new T cells to prostate tissue. Next-generation sequencing of the T-cell receptor (TCR) genes from blood or prostate tissue was used to quantitate and track T-cell clonotypes in these treated subjects with prostate cancer. At baseline, there was a significantly greater diversity of circulating TCR sequences in subjects with prostate cancer compared with healthy donors. Among healthy donors, circulating TCR sequence diversity remained unchanged over the same time interval. In contrast, sipuleucel-T treatment reduced circulating TCR sequence diversity versus baseline as measured by the Shannon index. Interestingly, sipuleucel-T treatment resulted in greater TCR sequence diversity in resected prostate tissue in sipuleucel-T–treated subjects versus tissue of nonsipuleucel-T–treated subjects with prostate cancer. Furthermore, sipuleucel-T increased TCR sequence commonality between blood and resected prostate tissue in treated versus untreated subjects with prostate cancer. The broadening of the TCR repertoire within the prostate tissue supports the hypothesis that sipuleucel-T treatment facilitates the recruitment of T cells into the prostate. Our results highlight the importance of assessing T-cell response to immunotherapy both in the periphery and in tumor tissue.

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Introduction

In recent years, immunotherapies have emerged as important and ground-breaking treatment approaches. They are designed to harness the immune system using a vaccine-like modality to attack chronic and preexisting conditions, such as cancer, infections, and other diseases (1). Like traditional vaccines, cell-based immunotherapy can activate the adaptive arm of the immune system, specifically T cells, and in cancer aim to produce or augment immune responses to tumor-associated antigens (2). However, unlike other forms of immunotherapies, immunotherapy both in the periphery and in tumor tissue that early clinical responses, such as prevention of disease progression, are not frequently observed soon after treatment (3). Similar to traditional vaccine approaches, cellular immunotherapy aims to engage the immune system by activating effector T cells and dampening immunosuppressive factors, ultimately facilitating the infiltration of lymphocytes into the tumor environment (4, 5). Sipuleucel-T is an autologous cellular immunotherapy, approved by the FDA for the treatment of asymptomatic or minimally symptomatic metastatic castrate-resistant (hormone-refractory) prostate cancer (mCRPC; ref. 6), that increases overall survival (7) and generates antigen-specific immune responses that correlate with increased overall survival (8). In addition, sipuleucel-T induced infiltration of T cells into prostate cancer tissue (9) in an open-label, phase II trial of neoadjuvant sipuleucel-T (NeoACT; ClinicalTrials.gov identifier: NCT00715104). The NeoACT trial was the first to demonstrate that sipuleucel-T induced a local immune effect (10). Neoadjuvant sipuleucel-T elicited a systemic antigen-specific T-cell response and recruited activated effector T cells into the prostate tumor microenvironment (10).

Although immunotherapy, such as sipuleucel-T, clearly alters the immune response to cancer, identifying the best method to measure and quantify such immune responses remains a challenge because of the difficulty in obtaining an adequate quantity of samples and the limitations of current functional assays. One method of analyzing the adaptive immune system response involves profiling the T-cell receptor (TCR) repertoire, thereby obtaining the relative abundance and diversity of T-cell clones (11). Novel emerging technologies, such as next-generation
sequencing (NGS), have recently begun to be used to assess TCR repertoires (12, 13) and may be useful in understanding immunotherapy-induced immune system changes (14, 15). The power of NGS is derived from its ability to examine unique sequences with inferred antigen specificity, or clonality, which mirrors the TCR repertoire, to enhance the speed of clonotyping analysis and to detect low-abundance clones (16).

The goal of the current exploratory analysis of the NeoACT trial was to build upon the prior report showing antigen-specific T-cell response and T-cell infiltration into the prostate (10). We used NGS to characterize the complex changes in the TCR β-chain repertoire in paired sequential samples from peripheral blood mononuclear cells and posttreatment radical prostatectomy tissues from a small number of subjects with localized CRPC. These exploratory analyses sought to ascertain the extent to which sipuleucel-T modifies the diversity of the T-cell compartments both spatially and temporally. Herein, we report on the effects of sipuleucel-T therapy on the TCR repertoire of circulating T cells and on T cells within prostate tumor tissue.

**Patients and Methods**

**Sipuleucel-T treatment and trial design**

The manufacture of sipuleucel-T has been described previously (8). NeoACT (P07-1; NCT00715104) was a single-arm, multicenter phase II trial of open-label, neoadjuvant sipuleucel-T treatment in men with localized prostate cancer administered before planned radical prostatectomy (10, 17). NeoACT was conducted in accordance with applicable regulations of the FDA and the Good Clinical Practice guidelines of the International Conference on Harmonisation, and all subjects gave written informed consent to participate in the protocol approved by the Institutional Review Boards (IRB) of each participating institution. Subjects received sipuleucel-T [prepared by culturing freshly obtained leukapheresis peripheral blood mononuclear cells (PBMC) with a fusion protein of prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor (GM-CSF)] at the standard 2-week intervals for three planned doses (8). Radical prostatectomy was performed 2 to 3 weeks after the final sipuleucel-T infusion (Supplementary Fig. S1).

**DNA isolation**

Genomic DNA was extracted from PBMCs using the QIamp DNA Maxi Kit (Qiagen Inc.). Radical prostatectomy formalin-fixed, paraffin-embedded (FFPE) tissue was extracted using the QIamp DNA FFPE Tissue Kit (Qiagen Inc.) as per the manufacturer’s instructions (18). TCR sequence profiling was performed using DNA extracted from preculture PBMCs (as previously described in refs. 7, 8) derived from leukapheresis of sipuleucel-T–treated patients, from whole blood of healthy donors at weeks 0, 2, and 4, and from whole blood of nonsipuleucel-T–treated control subjects with prostate cancer at baseline. For tissue sequencing, the entire FFPE sections were used. Blood and/or tissue samples from sipuleucel-T–treated subjects, nontreated healthy donors, and nonsipuleucel-T–treated control subjects with prostate cancer were randomly selected from available samples.

**Healthy donors and nonsipuleucel-T–treated control subjects**

Whole blood was collected from healthy adult male donors who provided written informed consent in accordance with Western IRB approval. Non-sipuleucel-T–treated control subjects with prostate cancer were identified as described previously (10) and comprised patients who had undergone radical prostatectomy at the University of California San Francisco (UCSF; San Francisco, CA) during the same time period but had not received any preoperative therapy. Control subjects with prostate cancer were selected on the basis of a distribution of preoperative risk criteria that was analogous to the disease risk in the subjects participating in the NeoACT trial. The control subjects provided written consent for the use of their specimens for research (UCSF IRB-approved protocol for human subjects research #11-05226).

**High-throughput TCR sequencing**

The TCRβ CD3 (CDR3β) region was amplified and sequenced from a standardized 1,200 ng of DNA using the ImmunoSEQ assay (Adaptive Biotechnologies). In this assay, a multiplex PCR system was used to amplify the rearranged CDR3β sequences from sample DNA. The 87 base-pair fragment is sufficient to identify the VDJ region spanning each unique CDR3β (19, 20). Amplicons were sequenced using the Illumina platform, TCRβ V, D, and J gene definitions were provided by the IMGT database (www.imgt.org). The assay is quantitative, using a complete synthetic repertoire of TCRs to establish an amplification baseline and to adjust the assay chemistry to correct for primer bias. In addition, barcoded, spiked-in synthetic templates were used to measure the degree of sequencing coverage and residual PCR bias (21). This information was used for further PCR bias correction and to estimate the abundance of sequenceable templates in each sample. The resulting data are filtered and clustered using both the relative frequency ratio between similar sequences and a modified nearest-neighbor algorithm, to merge closely related sequences and remove both PCR and sequencing errors.

**Determination of TCR sequence diversity**

The Shannon diversity index reflects clonal or TCR repertoire diversity within and between populations (22) and was used to measure the diversity of the clonotype population at each time point. The Shannon diversity index was calculated using the formula \[ H = - \sum_{i=1}^{n} p_i \log_2(p_i) \], where \( p_i \) is the frequency of clonotype \( i \) for the sample with \( n \) unique clonotypes (22). The R package “Diversity Sampler” was used to calculate the Shannon diversity index (23, 24).

**Determination of TCR sequence commonality between two samples**

The Baroni–Urbaní and Buser (BUJB) index was applied as a measure of the commonality between TCR sequences in the blood and radical prostatectomy tissue across different time points for each subject. The BUJB index ranges from 0 to 1, with 0 denoting completely separate populations and 1 denoting two populations in complete concordance. The BUJB index was calculated in R using the formula \[ BUJB = \frac{a}{a + b + c + d} \], where \( a \) represents the number of unique sequences in one population, \( b \) represents the number of unique sequences in the second population, \( c \) represents the number of unique sequences that are found in both populations, and \( d \) represents the number of unique sequences that are not found in either population but are found in the rest of the populations (24, 25).
Determination of clonal frequency change
The fold change (FC) in the frequency count at week 2 or week 4 was computed to examine the change in TCR sequence frequency over time relative to week 0. FC was defined as the sequence frequency count at the later time point divided by the sequence frequency at week 0. For each time point comparison for each sequence, a sequence was categorized as increased if FC is $>2$, as decreased if FC is $<0.5$, and as unchanged if $0.5 < FC < 2$. All TCR sequences detectable at any time point at week 0, 2, or 4 were included in the analysis. For each subject and each time point comparison, the percentage of TCR sequences falling into each change category was computed.

Results
Sipuleucel-T treatment reduces TCR Shannon index in the blood of subjects with prostate cancer
We compared changes in the TCR sequence frequency between baseline (week 0) and week 2 in blood samples isolated from healthy donors and sipuleucel-T–treated subjects. The resultant sequence scatter matrices revealed a substantial increase in the number of productive sequences (each of them defining a unique T-cell clone) and greater differences in the sipuleucel-T–treated subject matrix compared with the healthy donor matrix (Fig. 1). In addition, a larger number of total productive sequences were evident in the blood of sipuleucel-T–treated subjects compared with healthy donors at all time-points examined. However, because this type of bulk visualization analysis fails to distinguish minute TCR repertoire alternations, we conducted additional analyses to address whether the complex observed changes in the TCR repertoire may reveal treatment-related effects.

We assessed the TCR repertoire in patient leukapheresis samples at each of the three sipuleucel-T treatment time points (weeks 0, 2, and 4). As a comparator for natural variance, or changes in TCR profiles, we also assessed the TCR repertoire from healthy subjects who provided blood samples over the same time points. Because each TCR sequence corresponds to a unique TCR transcript, we were able to determine the total number of unique TCR sequences in each group. Sipuleucel-T–treated subjects had a significantly higher number of unique TCR sequences at baseline compared with week 2 and week 4 ($P < 0.01$), but no such differences were observed in the healthy donors (Fig. 2A). These results demonstrate a significant decrease in the number of unique TCR sequences in the blood following sipuleucel-T treatment.

To evaluate the effect of the observed decrease in the number of unique TCR sequences on T-cell repertoire diversity over time, we next examined whether there was a difference in the TCR repertoire diversity over time using the Shannon diversity index (Fig. 2 and Supplementary Fig. S2). There was decreasing TCR diversity in the peripheral blood of sipuleucel-T–treated subjects between baseline (week 0) and weeks 2 and 4 ($P < 0.001$), but no such change in TCR diversity over time was evident in the peripheral blood of healthy donors (Fig. 2B). The Shannon diversity index was numerically greater at each time point in sipuleucel-T–treated subjects compared with healthy donors (Fig. 2B). Overall, these data suggest that the TCR repertoire is more diverse in subjects with prostate cancer compared with healthy donors and that sipuleucel-T treatment results in decreased TCR diversity.

Sipuleucel-T treatment drives change in the T-cell repertoire
To better understand the potential mechanism behind the observed changes in TCR sequence diversity, we examined how the circulating TCR repertoire was affected by sipuleucel-T treatment (i.e., loss and/or enrichment of TCR sequences). In each group, we examined the entire TCR repertoire that was present at any of the time points and found that a greater proportion of TCR sequences decreased at week 2 and week 4 in the sipuleucel-T–treated subjects (57.2% and 54.0%, respectively) compared with the healthy donors (32.1% and 31.9%, respectively; Fig. 3A), which further suggests narrowing of the TCR repertoire. We next examined only the TCR sequences that were common between all of the time points for each group and found that a greater proportion of TCR sequences remained unchanged at week 2 and week 4 in the healthy donors (52.2% and 52.4%, respectively) compared with sipuleucel-T–treated subjects (46.7% and 45.9%, respectively; Fig. 3B). In addition, there was a higher proportion of TCR sequences that increased at week 2 and week 4 in the sipuleucel-T–treated subjects (40.3% and 40.5%, respectively) compared with the healthy donors (27.1% and 23.8%, respectively; Fig. 3B). Taken together, these data demonstrate a greater degree of change over time in the TCR sequences isolated from sipuleucel-T–treated subjects.
Although changes were evident in the total TCR repertoire, there was no significantly increased TCR sequence diversity following sipuleucel-T treatment compared with healthy donors (Wilcoxon one-sided test, $P < 0.01$). As detected by the Shannon index, the TCR sequence diversity in the prostate tumor by calculating the Shannon index in resected prostate tissue from sipuleucel-T–treated and untreated subjects with prostate cancer. Because there was no difference in the BUB index between weeks 2 and 4, this suggests that commonality of TCR sequences between the blood and resected prostate tissue is maintained.

**Sipuleucel-T treatment is associated with increased TCR sequence diversity**

We next asked whether the observed changes in TCR sequence following sipuleucel-T treatment were compartment specific or were common to both T cells in the blood and those localized to the prostate tumor. Using the BUB index as a measure of commonality, we found that in subjects who received sipuleucel-T, the TCR sequence commonality between circulating T cells and resected prostate tissue significantly increased at week 2 compared with baseline (week 0; $P = 0.01$), and this increase was maintained at week 4 but was not significantly increased beyond the commonality observed at week 2 ($P = 0.65$, week 2 vs. week 4; Fig. 5A). Because there was no difference in the BUB index between weeks 2 and 4, this suggests that commonality of TCR sequences between the blood and resected prostate tissue is maintained.

**Discussion**

One of the hallmarks of cancer is the ability to evade or dampen immune recognition of tumors (26). As a result, several cancer vaccines and immunotherapies are being developed to activate the immune response against tumor cells using peptide-, tumor cell-, dendritic cell-, virus-, and immunoglobulin-based vaccines or to reverse negative immune regulatory mechanisms with anti-CTLA-4 and anti-PD-1 therapy (2). Although there has been extensive interest in developing cancer vaccines, there is still no clear guidance for how to best measure an effective immune response in the oncology setting (27, 28). However, NGS has recently been used in small groups of patients to examine how immune checkpoint inhibitors such as pembrolizumab and ipilimumab affect the peripheral T-cell repertoire (14, 15, 29). These preliminary investigations provide insights into how these drugs may work. Therefore, we have used a similar NGS technique to more deeply characterize both the peripheral and tissue-specific T-cell responses following neoadjuvant sipuleucel-T therapy, which has demonstrated a survival benefit in subjects with prostate cancer.

The proposed mechanism of action for sipuleucel-T involves the induction of T-cell–mediated immune responses that target prostate cancer cells (10, 30). We have previously shown that neoadjuvant sipuleucel-T elicited a systemic antigen-specific T-cell response and recruited activated effector T cells into the prostate tumor microenvironment (10). Here, we investigated the effects of sipuleucel-T therapy on both the circulating T-cell repertoire and the repertoire of prostate tumor-infiltrating T cells.
Figure 3. Alterations in TCR sequence frequency over time in healthy donors and sipuleucel-T–treated subjects. The FC in sequence frequency versus baseline was categorized as increased (FC ≥ 2), unchanged (0.5 < FC < 2), or decreased (FC ≤ 0.5). A, analysis of TCR sequences present at any time point (all clones). The percentage of sequences was calculated and categorized as increased, unchanged, or decreased, or based on the sequence FC for week 2 relative to week 0 (baseline) and week 4 relative to week 0. Data presented are the percentages in each category averaged over all subjects (healthy donors and sipuleucel-T–treated subjects) and included all sequences with a measurable frequency count at weeks 0, 2, or 4. B, analysis of TCR sequences present at all timepoints (common sequences). The percentage of sequences was calculated and categorized as increased, unchanged, or decreased based on the sequence FC for week 2 relative to week 0 (baseline) and week 4 relative to week 0. Data presented are the percentages in each change category averaged over all subjects and included only sequences with measurable frequency counts at both weeks 0 and week 2 (week 2 analysis) and both week 0 and week 4 (week 4 analysis).

The results of the current analyses show that subjects with prostate cancer have greater circulating TCR sequence diversity in the peripheral blood at baseline compared with healthy donors, which suggests a preexisting immune recognition of prostate cancer. We demonstrate that neoadjuvant sipuleucel-T treatment narrows the diversity of circulating TCR sequences in the blood compared with baseline. Sipuleucel-T treatment also increases TCR sequence commonality between blood and resected prostate tissue and broadens the TCR repertoire within the prostate tissue. Interestingly, our findings are in contrast with the narrowing of the tumor-associated T-cell repertoire reported in patients with advanced melanoma who received pembrolizumab (anti-PD-1 mAb; ref. 14) and the broadening of the peripheral T-cell repertoire seen in patients who received ipilimumab (anti-CTLA-4 mAb) (15). This would be consistent with the different mechanisms at work in immune checkpoint inhibition as compared with vaccination.

In addition, we have shown that sipuleucel-T treatment induces substantial changes in the TCR repertoire. We observed a greater proportion of marked increases and decreases in the TCR repertoire among subjects who received sipuleucel-T treatment, whereas the TCR repertoire in healthy donors was relatively static over the same time points. Furthermore, we observed a considerable enrichment of certain sequences among the sipuleucel-T–treated subjects and very little change in the top 100 TCR sequences in the healthy donors. These data suggest that sipuleucel-T treatment induces a large degree of change in TCR sequences and is consistent with a narrowing of T-cell diversity over time.

Our results suggest that the narrowing of T-cell diversity in the blood following the first infusion of sipuleucel-T is a result of an expansion of T-cell clones specific for antigens associated with prostate cancer, as may be expected with a cancer vaccine. However, comparison of prostate cancer tissue of treated subjects to that of untreated subjects shows an unexpected increase in the T-cell diversity in the prostate cancer tissue of sipuleucel-T–treated subjects. The greater intratumoral T-cell diversity in the sipuleucel-T–treated subjects suggests a clear treatment effect. Taken together, the narrowing of circulating TCR sequences and the increase in TCR sequence commonality between blood and prostate tissue suggest that specific T-cell clones are recruited from the peripheral blood to the prostate tumor microenvironment. The current analyses support and extend our previous data showing that sipuleucel-T treatment facilitates the recruitment of T cells into the prostate tissue (10) and suggest that the broadening of the
immune response to prostate-specific antigens within the tissue is consistent with priming of new T cells.

We have previously shown that sipuleucel-T engages the immune system by activating antigen-presenting cells during the production process and induces long-lived immune responses after infusion (8). We have previously reported that after the first infusion of sipuleucel-T, cytokines known to facilitate T-cell expansion (IL2, TNFα, and IFNγ) were evident in abundant levels in the culture medium when making the second product (8). Available data indicate that sipuleucel-T prolongs patient survival through antigen-specific immune activation (8). Data from the metastatic prostate cancer setting suggest that sipuleucel-T induces humoral antigen spread (31). The current analysis takes this understanding one step further and demonstrates an inverse association between T-cell repertoire dynamics in the periphery and those within prostate cancer tissue. This inverse relationship, and the simultaneous enrichment of TCR clones common to the periphery and the prostate cancer tissue, suggests that prostate antigen-specific T cells from the peripheral blood may have been recruited to the prostate and may be recognizing other antigens.

Figure 4.
Change in abundance of TCR sequences during sipuleucel-T treatment. A, a ladder plot of the change in rank of the top 100 sequences at each time point during the course of treatment for representative examples of one healthy donor and one sipuleucel-T–treated subject. B, the SDs of the selected top 100 sequences were averaged for each sample across the three time points. For sequences with missing data points, the lower value observed in the same sample was imputed. The extent of rank shuffling of nucleotides is much greater in the sipuleucel-T–treated subjects compared with the healthy donors (P < 0.02, Wilcoxon one-sided test).

Figure 5.
Assessment of TCR sequences in blood and prostate tissue. A, BUB overlap index between blood and resected prostate tissue. Each point represents the BUB overlap index between the T-cell sequences in the blood samples at weeks 0, 2, and 4 with the radical prostatectomy tissue of the same subject. The horizontal lines represent the median value of the BUB index across the available samples at the corresponding time point. The BUB index is significantly greater at week 2 compared with week 0 (P = 0.01, Wilcoxon test). B, the Shannon diversity index of the TCR repertoire in resected prostate tissue samples from untreated and sipuleucel-T–treated subjects. TCR results were quantified globally across the biopsy/postsurgical tissue. The horizontal lines represent the median value of the Shannon diversity index across the available samples for each group of subjects. Resected prostate tissues from sipuleucel-T–treated subjects with prostate cancer had a significantly greater Shannon diversity index compared with resected prostate tissues from subjects with prostate cancer who did not receive sipuleucel-T (P = 0.01, Wilcoxon test). This represents an increase in T-cell sequence diversity in the prostate among sipuleucel-T–treated subjects.
released by a local cascading immune response (i.e., epitope spreading), thereby expanding the T-cell sequence diversity within the prostate. This broadening of T-cell responses may reflect processes similar to those seen in the immune response to infectious disease vaccinations (32–34). However, the specificity and type of T cells recruited to the prostate tissue remain unknown at this time. Taken together with our previous data (10), the current analyses suggest that sipuleucel-T increases the diversity of T cells within the prostate tumor, which could enhance immunologic containment of the tumor. This idea is consistent with observations in clinical trials of other prostate cancer vaccines and immunotherapies (35). Furthermore, our data demonstrate that sipuleucel-T therapy helps to establish a tumor-homing immune response. Regimens using sipuleucel-T in combination with other treatments that augment T-cell immunity, such as ipilimumab and PD-1/PD-L1-targeted therapies, could further increase clinical benefit (3).

The interpretation of these results is limited by the small sample number of sipuleucel-T–treated subjects with matched pre- and posttreatment peripheral blood samples and prostate cancer tissue on which extensive NGS analysis was performed. However, the optimal sample size has not yet been established for these types of analyses. Nevertheless, because of the sensitivity of the assays, these initial results have provided insight into the specific mechanism of action of neoadjuvant sipuleucel-T and are believed to be representative of the expected immune response in the larger population of subjects receiving neoadjuvant therapy for local prostate cancer in the NeoACT study. Other limitations, as stated previously (10), include the short time between sipuleucel-T therapy and radical prostatectomy (i.e., single tissue extraction), which does not allow for an adequate longitudinal evaluation of change in T-cell sequences within the prostate tissue, and the fact that the effects of neoadjuvant sipuleucel-T therapy on immune response may not be representative of what occurs during therapy for advanced metastatic disease.

The findings derived from these analyses are meant to generate hypotheses and should be expanded upon with additional studies. Our results highlight the importance of assessing immune responses in both blood and tissue and suggest that evaluating immunotherapies based on TCR sequence changes in the blood alone is likely not sufficient to understand the effects of immunotherapy on tumor tissue. Further studies are needed to identify the antigens associated with prostate cancer that are recognized through a sipuleucel-T–induced immune response and may help to identify biomarkers of clinical outcome for sipuleucel-T therapy.

Disclosure of Potential Conflicts of Interest

N.A. Sheikh has ownership interest (including patents) in Dendreon Pharmaceuticals. T. DeVries was the director of statistics at Dendreon Corporation. D. Hamm is a senior application scientist at Adaptive Biotechnologies. J.B. Trager is the vice president, research and has ownership interest (including patents) in Dendreon Pharmaceuticals Inc. L. Fong reports receiving commercial research support from Dendreon Pharmaceuticals Inc. No potential conflicts of interest were disclosed by the other authors.

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