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Immune Toxicities Elicted by CTLA-4 Blockade in Cancer Patients Are Associated with Early Diversification of the T-cell Repertoire

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Abstract

While immune checkpoint blockade elicits efficacious responses in many patients with cancer, it also produces a diverse and unpredictable number of immune-related adverse events (IRAE). Mechanisms driving IRAEs are generally unknown. Because CTLA-4 blockade leads to proliferation of circulating T cells, we examined in this study whether ipilimumab treatment leads to clonal expansion of tissue-reactive T cells. Rather than narrowing the T-cell repertoire to a limited number of clones, ipilimumab induced greater diversification in the T-cell repertoire in IRAE patients compared with patients without IRAEs. Specifically, ipilimumab triggered increases in the numbers of clonotypes, including newly detected clones and a decline in overall T-cell clonality. Initial broadening in the repertoire occurred within 2 weeks of treatment, preceding IRAE onset. IRAE patients exhibited greater diversity of $CD4^+$ and $CD8^+$ T cells, but showed no differences in regulatory T-cell numbers relative to patients without IRAEs. Prostate-specific antigen responses to ipilimumab were also associated with increased T-cell diversity. Our results show how rapid diversification in the immune repertoire immediately after checkpoint blockade can be both detrimental and beneficial for patients with cancer. *Cancer Res; 77(6); 1322–30. ©2016 AACR.*

Introduction

Among significant recent advances in cancer immunotherapy, antibodies blocking immunologic checkpoints such as CTLA-4 and PD-1 can potentiate immune responses to cancer leading to durable clinical responses. Ipilimumab (Bristol-Myers Squibb) is a monoclonal, fully human IgG1 antibody against the T-cell coinhibitory receptor CTLA-4 that is approved for the treatment of melanoma (1, 2), but may have activity in a subset of patients in other malignancies including metastatic castrate-resistant prostate cancer (mCRPC; ref. 3). While some patients respond to ipilimumab, some also experience organ-specific toxicities, which are the result of increased immune activation, termed immune-related adverse events (IRAE). These include diarrhea and colitis, transaminitis, rash and pruritus, panhypopituitarism, adrenal insufficiency, thyroiditis, and pneumonitis (4, 5), and are often serious, requiring cessation of therapy and initiation of immunosuppression. For instance, in the context of mCRPC, 13% of mCRPC patients treated with ipilimumab at the 10 mg/kg dose demonstrate >50% prostate-specific antigen (PSA) responses, while 26% of patients develop grade 3–4 IRAEs (3). Biomarkers associated with clinical response to ipilimumab have been described (6–12), but those predicting IRAEs have not.

We and others have previously shown that CTLA-4 blockade can remodel the pool of circulating T cells. T cells recognize antigens through their T-cell receptors (TCR), which are primarily comprised of a α and β chain. The antigenic diversity of T cells is the result of VDJ gene recombination, which can be used to identify individual T-cell clonotypes. In prior work, we used next-generation sequencing of TCRβ chains from peripheral blood mononuclear cells (PBMC) from patients with cancer to show that CTLA-4 blockade promotes active remodeling of the T-cell repertoire, with both gain and loss of clonotypes but net bias toward gains leading to increased repertoire diversity overall. However, overall survival was not correlated with the generation of new clones, but rather with the maintenance of preexisting, high frequency (greater than 1 in 1,000) clonotypes (13). Here we examined whether development of IRAEs is associated with changes in the T-cell repertoire. Because CTLA-4 blockade is associated with inflammation in specific tissues, we postulated that the treatment would expand tissue-reactive T-cell clones in patients that developed the corresponding toxicity, as has been observed in other tissue-specific autoimmune pathologies such as celiac sprue and gastrointestinal graft-versus-host-disease. Instead, we found that an early increase in diversity and the generation of new clones after administration of the combination of ipilimumab and granulocyte-monocyte colony-stimulating factor (GM-CSF) is specifically correlated with the development of IRAEs. These results indicate that increased T-cell diversity can be deleterious to patients via promotion of autoreactivity.

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Materials and Methods

Study design

Cryopreserved PBMCs were sequenced from mCRPC patients treated with anti–CTLA-4 (ipilimumab; Bristol-Myers Squibb) and GM-CSF (sargramostim; Sanofi) in a phase I/II clinical trial (ClinicalTrials.gov identifier: NCT00064129) as described previously (14, 15). Up to 4 doses of ipilimumab were given ranging from 1.5 to 10 mg/kg every 4 weeks, and GM-CSF was given at 250 $\mu g/m^2$ per day on the first 14 days of every cycle. Patient characteristics for the entire 42-patient cohort were previously reported (15). Ipilimumab was administered every 4 weeks for 4 planned doses. Serial PBMCs obtained at baseline and six posttreatment timepoints from 35 of these patients were sequenced; of these, 21 patients had available sequence at both pretreatment (week 0) and immediate posttreatment (week 2) timepoints and were utilized for the analyses at these early timepoints unless otherwise stated. Patients were not restricted by HLA alleles. Informed consent was obtained for all investigations. We restricted our initial analysis to consensus IRAEs listed in the manufacturer's prescribing information for ipilimumab (http://packageinserts.bms.com/pi/pi_yervoy.pdf).

Flow cytometry

PBMCs were thawed into FACS wash (PBS 2% BSA) and washed twice with FACS wash. Samples were stained with designated panels for 20 minutes at 4°C and washed twice with FACS wash. Cells requiring intracellular staining were fixed and permeabilized with BD Cytofix/Cytoperm buffer (catalog no. 554722) according to the manufacturer's protocol. Intracellular staining with antibodies was carried out for 30 minutes at 4°C and washed twice with FACS wash. Cells were acquired on a LSRII flow cytometer (BD Biosciences) and data were analyzed with FlowJo analysis software version 9.7.5 (FlowJo, LLC). Absolute counts (per microliter of blood) for each immune subset is calculated by multiplying the percentage of each subset with the preceding parent subset and with the absolute lymphocyte count quantitated on the day of blood drawn.

TCRβ amplification and sequencing, clonotype identification, and counting

The amplification and sequencing of TCRβ repertoire from RNA, read mapping to clonotypes via identification of V and J segments, and counting of the number of unique clonotypes have been previously described in detail (13). Of note, after filtering for read quality, reads were mapped to a clonotype if at least 2 identical reads were found in a given sample. Clonotype frequencies were calculated as the number of sequencing reads for each clonotype divided by the total number of passed reads in each sample.

Statistical analysis

Demographic and clinical characteristics were summarized by descriptive statistics. In general, frequency distribution and percentages were used to summarize categorical variables, and median with interquartile range was used to describe continuous variables. Comparison of continuous variables between two groups was performed using the Wilcoxon t test. χ^2 test was applied to determine statistical association between two categorical variables. Statistical significance was declared at $\alpha < 0.05$ and no multiple testing adjustment was done. All statistical analysis was done with the statistical computing software R (https://www.r-project.org/).

Clonality was used to measure the diversity of the clonotype population for each patient at each time point. The clonality was calculated using the formula $1 - \sum_{i=1}^{n} p_i \log_e(p_i)/\log_e(n)$, where p_i is the frequency of clonotype i for a sample with n unique clonotypes. Of note, this metric is normalized to the number of unique clonotypes, and in our dataset, clonality was found to be a robust metric and was not significantly correlated with the number of unique clonotypes found in each sample (P values for correlation with clonality at week 0 = 0.263 for molecules, 0.852 for counts; P values for correlation with clonality at week 2 = 0.048 for molecules but this is a positive correlation, 0.309 for counts). Clonality was compared between week 0 and 2 by paired Wilcoxon test, and clonality comparisons between patients with IRAEs versus patients without IRAEs, or between responders versus nonresponders at each timepoint were performed using two-sample Wilcoxon test. To determine the relative change in diversity over time, relative clonality was calculated as the ratio of the clonality at two consecutive timepoints; comparisons of this metric between patients with versus without IRAEs were done by two-sample Wilcoxon test. To explore the effect of other adverse events (AE) on the change of clonality from week 0 to 2 for each type of AE, the relative clonality (week 2/week 0) between patients with that AE versus those without that AE was compared by two-sample Wilcoxon test.

To measure the commonality between TCR sequences in week 0 (pretreatment) and week 2 (posttreatment) for each subject, the proportions of clones only present at week 2, only present at week 0, and present in both week 0 and week 2 were calculated. The read depth as far as RNA molecules was largely similar between IRAE and non_IRAE groups as well as week 0 and week 2 samples ($P = 0.412$ for week 0 vs. week 2; 0.9712 for IRAE vs. non_IRAE at week 0) and did not vary in a way that would account for the presence of new clones in the AE group at week 2. In addition the amount of RNA input was not significantly correlated either with IRAE status, or with clonality of the entire cohort or IRAE/non_IRAE groups (data not shown). To examine the change in TCR sequence frequency from week 0 to week 2, the fold change (FC) was defined as the sequence frequency at the week 2 divided by the sequence frequency at week 0. Each sequence was categorized as "increased" if fold change (FC) was ≥ 4, as "decreased" if FC was ≤ 0.25, and as "unchanged" if $0.25 < FC < 4$. All TCR sequences detectable at week 0 or 2 were included in the analysis. For clones with nonmeasurable frequency counts at only one timepoint (and measurable at the other timepoint), the number of reads at the nonmeasurable timepoint was arbitrarily set to 1, and then FC was calculated as above. For each subject, the percentage of TCR sequences falling into each change category was computed. The comparison of the proportions between IRAE versus non_IRAE patients was done by two-sample Wilcoxon test.

T-cell sorting

PBMCs from weeks 2 and 6 were FACS sorted (FACSAria, BD Biosciences) into four populations: Treg ($CD4^+$ $CD25^{hi}$ $CD127^{lo}$), helper T ($CD4^+$ $CD25^{lo}$ $CD127^+$), naïve CTL ($CD8^+$ $CD27^+$ $CD45RA^+$), and nonnaïve CTL ($CD8^+$ $CD27^-$ or $CD27^+$ but $CD45RA^{-/lo}$), and then the clonotypes present in these subpopulations were identified as above. Each of these clonotypes

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from sorted cells was used to mark this clonotype as arising from a particular T-cell subset when found in bulk PBMCs from the same patient at all timepoints for further analysis.

Results

T-cell repertoire changes occur early with treatment

To characterize the functional effects of ipilimumab and GM-CSF on T-cell phenotype, we first looked at changes in T-cell activation markers in the peripheral blood of mCRPC patients who received both of these agents. Treatment with ipilimumab and GM-CSF induced proliferation in both circulating $CD4^+$ $FOXP3^-$ and $CD3^+$ $CD4^-$ T cells (which contain $CD8^+$ T cells as well as a mixture of other populations) as measured by increases in either absolute numbers or percentages of $Ki67^+$ lymphocytes (relative percentages shown for illustration in Fig. 1A). Proliferation was maximal at 2 weeks after the first dose (Supplementary Fig. S1A and S1C; for week 0 versus week 2 comparisons of $Ki67^+$ $CD4^+$ $FOXP3^-$ cells, $P < 0.001$ for absolute numbers or percentages; for $Ki67^+$ $CD3^+$ $CD4^-$ cells, $P = 0.001$ for absolute numbers and $P < 0.001$ for percentages; $n = 12$ patients with available Ki67 data from weeks 0 and 2). These changes, however, were not significantly associated with IRAEs at any timepoint (Supplementary Fig. S1B and S1D; $n = 21$ patients across all timepoints).

Given this finding of robust activation across the study cohort, we assessed how this is reflected in changes in the TCR repertoire. Using next-generation sequencing of TCRs from peripheral blood across time, we calculated the clonality index for each available sample ($n = 35$ patients with available sequence at any timepoint), which is inversely proportional to diversity (i.e., a lower clonality denotes a more diverse TCR repertoire) and is normalized to the number of unique clonotypes. A majority of treated patients experienced an immediate decline in clonality within 2 weeks of starting ipilimumab with GM-CSF (Supplementary Fig. S2A); similar results are found with other diversity indices such as Hill numbers (16). Declining clonality continued through the first 6 weeks of treatment (Supplementary Fig. S2B), which indicates that there is successive TCR repertoire diversification with repetitive anti-CTLA-4 administration, which in our cohort of mCRPC patients, occurs within the first several doses.

IRAEs are associated with TCR diversification following treatment

Given that we initially observed robust T-cell activation following ipilimumab and GM-CSF, our initial hypothesis was that IRAEs would be correlated with the emergence of an oligoclonal response (i.e., characterized by a narrowing of the repertoire toward fewer TCR clones) following checkpoint inhibition, consistent with clonal expansion driven by a dominant autoantigen-driven process. To evaluate this, we examined the frequency distribution of unique clonotypes in ipilimumab-treated patients who either experienced an IRAE or did not (non_IRAE). Contrary to our initial expectation of an induced oligoclonal response, treatment triggered a broad expansion in lower-frequency clonotypes in IRAE patients (representative patient shown in Fig. 1B).

To more directly characterize the diversity of the TCR repertoire at each timepoint, we assessed both the number of unique TCR clonotypes and the clonality index in patients. Total number of unique clonotypes increased in the overall population (Fig. 1C), with a concomitant decrease in clonality during the first 2 weeks of treatment (Fig. 1D, $P = 0.0016$) indicating significant diversification of the T-cell repertoire. Comparing the patients with IRAE (Fig. 1E and F) versus non_IRAE (Fig. 1G and H), we found that the IRAE patients had the significant declines in clonality ($P = 0.023$), while non_IRAE patients did not ($P = 0.057$). Similar results supporting a greater increase in diversity in IRAE patients are found using other diversity indices such as Hill numbers (16). Hence, early T-cell diversification after ipilimumab plus GM-CSF is related to the development of IRAEs.

Clonotype expansion and mobilization of newly detected T-cell clones are associated with IRAE development

To assess the nature of these changes in the repertoire, we classified each unique clone as found only after checkpoint blockade at week 2, only at week 0, or at both timepoints. Most clones were newly detected at week 2 (>50%), while slightly less than half of clones were only present at baseline; and a small fraction of clones (<10%) were present at both timepoints (Fig. 2A; new clones from week 2 at left; clones present only at baseline at week 0 at right). Although both IRAE and non_IRAE patients have a large fraction of clones newly detected at week 2, IRAE patients had a significantly greater fraction of newly detected clones compared with non_IRAE patients ($P = 0.042$), as well as a smaller fraction of clones that were present only at baseline ($P = 0.049$; Fig. 2B). Hence, the development of IRAEs is associated with an increased number of TCR clones newly detected in the periphery following ipilimumab plus GM-CSF.

To assess the dynamics of T-cell clones, we classified clonotypes into those whose frequency increased >4-fold, decreased >4-fold, or remained the same over time (change in frequency <4-fold) from week 0 to week 2 (data for all patients are shown in Fig. 2C). IRAE patients had a higher proportion of clones that increased with treatment compared with non_IRAE patients ($P = 0.028$; Fig. 2D). Thus, IRAEs are also associated with increases in preexisting clonotypes in addition to newly detected TCR clones in the blood.

Initial changes in clonality precede toxicity and response

To assess the kinetics of changes in clonality in IRAE and non_IRAE patients, we calculated the relative clonality for each group at each timepoint relative to the immediately preceding timepoint, and found significantly greater declines in clonality at week 2 relative to week 0 in the IRAE versus non_IRAE population ($P = 0.045$; Fig. 3A), but not at later timepoints. This confirms that changes in diversity in the IRAE group are concentrated at early timepoints after ipilimumab and GM-CSF. This early change was evident in one representative patient (Fig. 3B), who had an initial drop in clonality at the 2-week timepoint, preceding the development of an IRAE, panhypopituitarism, and clinical response. This patient also appeared to demonstrate a declining clonality in the several timepoints preceding onset of the actual IRAE. However, when we assessed all 8 IRAE patients with data at weeks 0 and 2 for changes in clonality from the timepoint immediately preceding IRAE onset to the timepoint immediately after onset, any changes in clonality at the time of IRAE onset were not significant ($P = 0.07813$ by Wilcoxon test). In our 42-patient cohort, there was correlation between toxicity and response in a subset of patients: 12 patients had IRAEs and 5 patients had clinical responses (≥50% PSA decline), and 4 of 5 responders also developed IRAEs. As with IRAEs, clinical responses were also significantly associated with an early decline in clonality at 2 weeks after starting ipilimumab and GM-CSF (Fig. 3C, left), while nonresponders lack this correlation (Fig. 3C, right, $P = 0.01$ and

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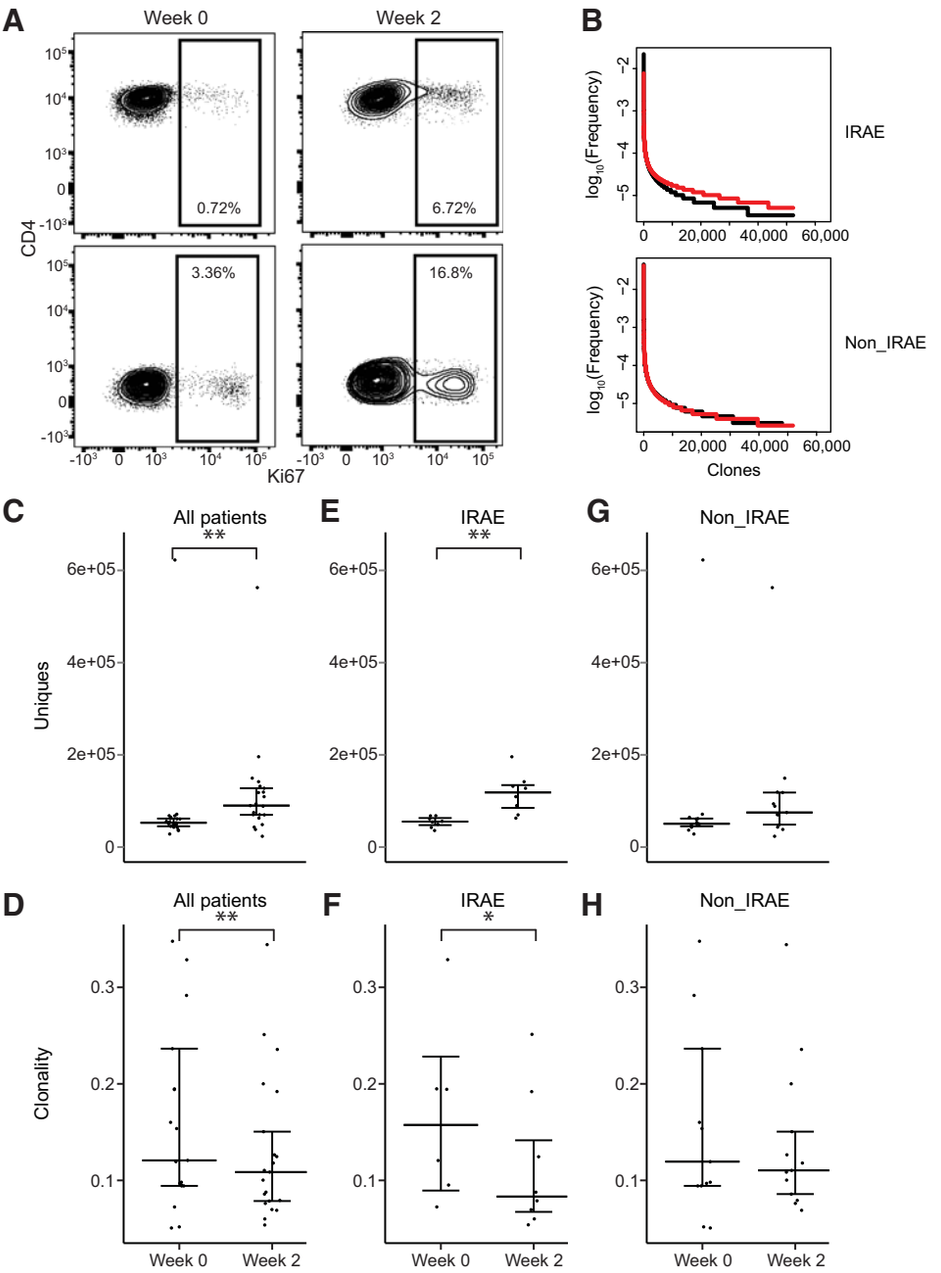

Figure 1.

Changes in the T-cell repertoire in patients with or without IRAE following treatment with ipilimumab plus GM-CSF. **A,** Flow cytometry was used to measure the percentage of Ki67$^+$ expression in CD4$^+$ CD3$^+$ FOXP3$^-$ lymphocytes (top row) or from CD4$^-$ CD3$^+$ lymphocytes (bottom row), either before (week 0, left column) or after treatment (week 2, right column). A representative mCRPC patient who received ipilimumab plus GM-CSF is shown. Statistical analysis was done both on percentages and absolute numbers of Ki67$^+$ lymphocytes. **B,** The frequency distribution of unique TCR clonotypes is shown for one representative patient who developed an IRAE (IRAE; top plot) and one representative patient who did not develop an IRAE (non_IRAE; bottom plot). The *x*-axis represents each unique clonotype in descending order of frequency, and the $\log_{10}$ of the frequency for each clonotype at week 0 (black) and week 2 (red) on the *y*-axis. **C–H,** The number of unique clones (**C**, **E**, and **G**) and clonality (**D**, **F**, and **H**) of TCR from PBMCs either before (week 0) or 2 weeks after treatment with ipilimumab (week 2) was calculated for all patients (**C** and **D**), IRAE patients (**E** and **F**), or non_IRAE patients (**G** and **H**). The median and interquartiles are shown. *, $P < 0.05$; **, $P < 0.01$ by paired Wilcoxon test. IRAE patients exhibit a significant decrease in clonality with treatment. Only data from patients who had samples at both timepoints are shown ($n = 21$ total patients, 8 IRAE, 13 non_IRAE).

0.055, respectively). However, when we dichotomized the population into those patients who lived longer or shorter than the median overall survival on study of 23.6 months, there was no specific correlation between decline in clonality and longer overall survival (data not shown).

Changes in clonality can be used to reclassify temporal arteritis as a putative IRAE

We examined whether other observed AEs might be similarly classified as IRAEs based on their effect on clonality. Given the small number of patients with any given AE, we added patients with various AEs to the IRAE category to see whether this addition would increase the significance of the IRAE versus non_IRAE comparison. Including temporal arteritis with IRAEs as a revised AE classifier increased the significance of the comparison between patients with and without AE (Table 1, $P = 0.029$ compared with $P = 0.045$ for consensus IRAEs alone); this is one example of an AE that may be plausibly reclassified as an IRAE. Other AEs did not have this effect: in particular, other thrombotic AEs (including deep venous thrombosis/pulmonary embolism), cardiac AEs (including troponin leak, arrhythmias), all grade 3+ AEs, and fatigue (a common AE with checkpoint inhibition) did not result in a decreased *P* value when added to the IRAE group (Table 1 and data not shown).

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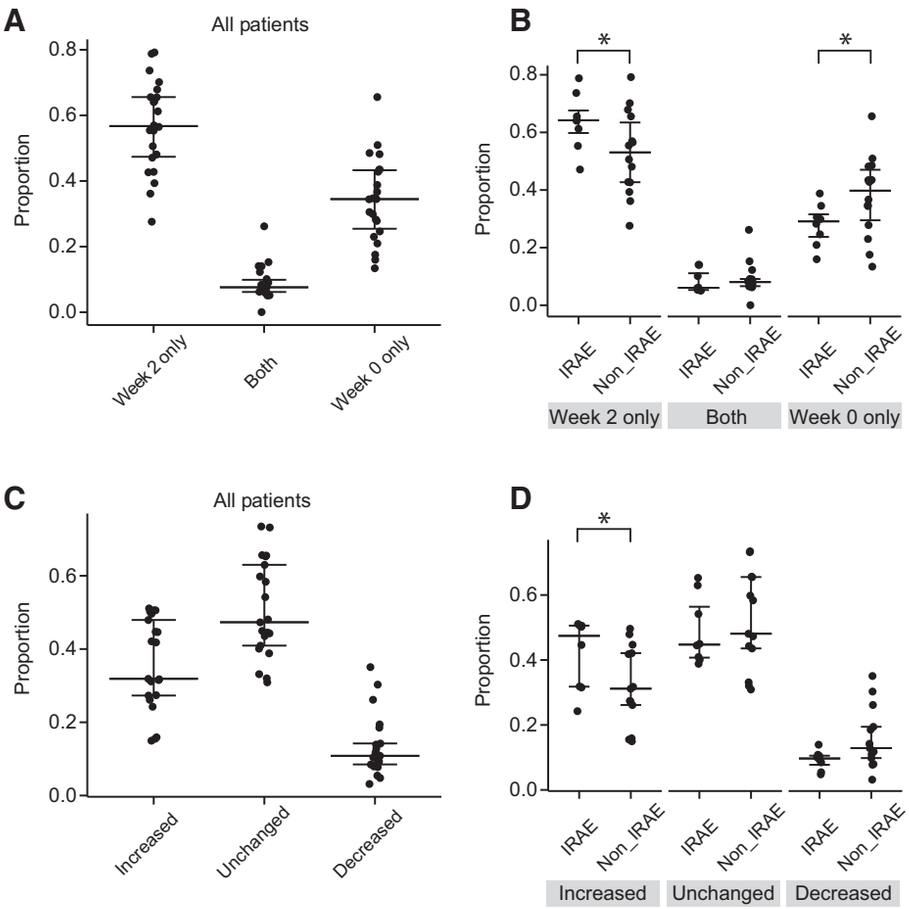

Figure 2.
T-cell clonotype expansion and mobilization of newly detected T-cell clones following treatment with ipilimumab plus GM-CSF. Top, fraction of clones, which were found only at week 2 (Week 2 only), only at week 0 (Week 0 only), or at both timepoints (Both) for all patients (**A**), and separated by IRAE and non_IRAE patients (**B**). Compared with non_IRAE patients, IRAE patients had a significantly higher fraction of clones that were newly detected at week 2, and a lower fraction of clones that were only found at week 0 (baseline). **C** and **D,** T-cell clonotype changes from week 0 to week 2 were binned by fold change in clonal frequency into "increased" (greater than 4-fold change), "decreased" (less than 0.25-fold change), or "unchanged" (between 0.25- and 4-fold change). The analysis is shown for all patients (**C**) and separated by IRAE and non_IRAE patients (**D**). The median and interquartiles are shown. IRAE patients also showed a significant increase in the fraction of clones whose frequencies increased with treatment with ipilimumab plus GM-CSF compared with non_IRAE (*, $P < 0.05$). There was also a borderline significant reduction in the fraction of clones, which decreased with treatment in the IRAE group ($P = 0.05$, bar not shown). All P values were calculated by two-sample Wilcoxon test.

Changes in CD4$^+$ and CD8$^+$ T-cell subsets after treatment with ipilimumab plus GM-CSF

Using FACS to isolate CD4$^+$ and CD8$^+$ T cells that were then sequenced, we followed the evolution of the repertoire after ipilimumab and GM-CSF in these distinct T-cell subsets. We sorted these subsets from 2 IRAE patients and 1 non_IRAE patient at 2 posttreatment timepoints (weeks 2 and 6), and used the identified clonotypes coming from CD4$^+$ or CD8$^+$ T cells to track these populations in all available bulk PBMC populations over time. Compared with one non_IRAE patient, the two available IRAE patients appeared to have lower CD4$^+$ T-cell clonality (Fig. 4A, top plot), and lower CD8$^+$ clonality (Fig. 4A, bottom plot), across all timepoints. Despite these trends, we did not see a significant difference in the numbers of Tregs between IRAE and non_IRAE patients after ipilimumab treatment (Fig. 4B–D). Using a similar clonotype analysis as before, we found that IRAE patients also demonstrated a trend in CD8$^+$ T cells toward higher frequencies of newly detected clones (Supplementary Fig. S3A), as well as clones with increased frequencies (Supplementary Fig. S3B), compared with CD4$^+$ T cells.

Discussion

Despite assessing circulating T cells serially over the first 12 weeks of treatment, we found that the most relevant change in T-cell repertoire occurred at 2 weeks. Early T-cell diversification after checkpoint inhibitors may therefore prove useful as an indicator of patients at risk for later IRAE development, and could serve as a new adjunct to current clinical management algorithms for IRAEs, which rely upon early recognition and intervention (17). This is especially pertinent as CTLA-4 blockade is being combined with anti-PD1 therapy (18) with significantly improved clinical outcomes, but where the vast majority of patients develop IRAEs with combination therapy (19). Also this may be relevant to the timing or sequencing of CTLA-4 blockade when given in combination with radiotherapy and how this may affect the types of repertoire changes leading to either toxicity or response (20). The directionality of changes in diversity we observe are likely related to the specific modality of immunotherapy used (e.g., checkpoint inhibition in this study); in other work, we have recently shown that treatment of mCRPC patients with the immunotherapy sipuleucel-T, which is thought to be a cancer vaccine, results in a decline in circulating T-cell repertoire diversity, which is the opposite of what we see here (21).

The specific changes in the TCR repertoire we observe in IRAE patients place some constraints on possible mechanisms by which IRAEs develop. First of all, although we demonstrate an early increase in diversity in IRAE patients, the actual toxicities do not typically manifest until later, with variable kinetics depending on the affected organ system: the skin is typically involved earliest, followed by colitis (after 1–3 doses), then hepatitis and endocrinopathies last (5). Particularly for later-onset IRAEs, this lag

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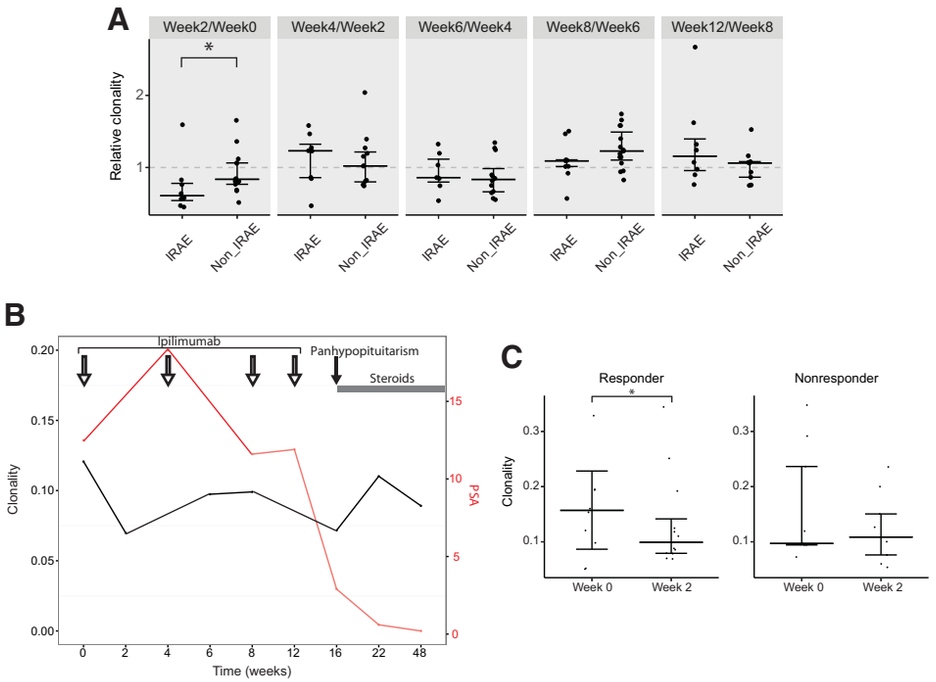

Figure 3.

Timing of changes in clonality and the development of toxicity or clinical response. **A,** The relative clonality (i.e., the ratio of clonality at each posttreatment timepoint to the clonality at the immediately preceding timepoint) is shown for IRAE and non_IRAE patients for posttreatment weeks 2, 4, 6, 8, and 12. The median and interquartiles are shown. A dotted line at a ratio of 1.0 indicates the relative clonality at which the earlier and later clonality indices were identical, that is, there was no difference in diversity between successive timepoints. Significant decreases in the relative clonality were seen in IRAE patients compared with non_IRAE patients at week 2 (*P* value for relative clonality of IRAE vs. non_IRAE: 0.045 for week 2/week 0 by two-sample Wilcoxon test). **B,** Clonality over time is shown for a patient who was treated with four planned doses of ipilimumab (open arrows) plus scheduled GM-CSF, and then later developed an IRAE (panhypopituitarism, onset indicated with filled arrow), which was treated with steroids (gray bar), but also had an exceptional clinical response (PSA decline >90%) to therapy. Clonality (black) is plotted against serum PSA levels (red). The early increase in diversity (i.e., drop in clonality) preceded the subsequent development of AEs and clinical response, and there was no marked change in clonality at the time of IRAE development or PSA decline. **C,** Clonality at weeks 0 and 2 for PSA responders (>50% decline in PSA, left) versus nonresponders (right) are shown. *, $P < 0.05$ by two-sample Wilcoxon test. PSA responders specifically show a significant decline in clonality with treatment. The median and interquartiles are shown.

between early repertoire changes and toxicity implies that if repertoire diversification is part of the pathogenesis of IRAEs, this may represent a necessary step but is clearly not sufficient by itself—other steps may occur at subsequent times prior to the clinical manifestation of toxicity. We cannot exclude the possibility of additional repertoire changes that are more proximal in time to the actual onset of toxicity; however, our findings speak to early repertoire diversity immediately following initiation of ipilimumab as a possible means of predicting IRAE development, and also a possible first step in the pathogenic mechanism underlying toxicity.

Our findings of increased diversity would support a mechanism whereby autoreactivity to multiple antigens is induced by ipilimumab, in part by mobilization of newly detected clones. The specific clones mediating IRAE may be enriched within the population of activated T cells we see after ipilimumab, although for technical reasons it would not be possible to look specifically at clones enriched in $Ki67^+$ T cells as Ki67 staining requires fixation. It remains unclear whether additional ipilimumab doses result in further enrichment of these clones, although at a population level, there does not appear to be further diversification after the first dose in IRAE patients based on relative clonality (e.g., Fig. 3B). This would represent a distinct mechanism of pathogenesis from other tissue-specific immunopathologies, which are thought to be driven by an oligoclonal response; for instance, celiac sprue is thought to be driven by distinct gluten-derived antigens presented by HLA-DQ (22), and in acute gastrointestinal graft-versus-host disease, patients with steroid-refractory disease exhibit similar TCRβ repertoires at different sites in the gastrointestinal tract as well as oligoclonal expansion postdiagnosis of the TCR clones found most frequently in the gut, suggesting a pathogenic mechanism driven by relatively few antigens (23). Formal testing of this hypothesis would require additional extensive efforts including confirmation of increased TCR diversity in IRAE-affected tissue, further FACS profiling of the surface phenotype of tissue-infiltrating lymphocytes including checkpoint and adhesion receptor expression, pairing of TCRα with β chains using single-cell approaches to provide a more complete picture of repertoire specificity, and mapping of specific antigens that may be recognized by identified TCRα/β heterodimers. These efforts may be hampered by the difficulty in obtaining tissue involved by IRAEs as well as the potential difficulty of identifying specific autoantigen-reactive clones given that numerous low-frequency clonotypes are generated by ipilimumab. However, pending confirmation, our results may suggest an alternate scenario

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Table 1. Impact of other AEs on clonality

		Week 0				Week 2				
	AE	***N***	**25th percentile**	**Median**	**75th percentile**	***N***	**25th percentile**	**Median**	**75th percentile**	***P*[a]**
IRAE	AE	8	0.089468	0.157429	0.228236	8	0.067392	0.083255	0.141532	0.045
	Non-AE	13	0.094322	0.119431	0.236446	13	0.085831	0.110382	0.150491	
IRAE + Thrombotic (TA)	AE	10	0.094503	0.140441	0.19463	10	0.071419	0.083255	0.121108	0.029
	Non-AE	11	0.095658	0.119431	0.264041	11	0.093072	0.11799	0.175283	
IRAE + Thrombotic (all other)	AE	9	0.072503	0.120721	0.194795	9	0.069039	0.078629	0.124683	0.169
	Non-AE	12	0.096326	0.136566	0.250244	12	0.096692	0.114186	0.162887	
IRAE + Thrombotic (all)	AE	11	0.083399	0.120721	0.194466	11	0.069432	0.078629	0.117532	0.114
	Non-AE	10	0.097281	0.136566	0.277838	10	0.102383	0.122248	0.18768	
IRAE + Cardiac (all)	AE	9	0.095123	0.194137	0.32856	9	0.069825	0.087881	0.19208	0.082
	Non-AE	12	0.094316	0.108787	0.179232	12	0.084206	0.109488	0.132502	
IRAE + Grade 3–4	AE	12	0.089468	0.156931	0.228236	12	0.069628	0.099132	0.141532	0.148
	Non-AE	9	0.094322	0.098143	0.236446	9	0.085831	0.108594	0.150491	
IRAE + Fatigue	AE	10	0.057035	0.107922	0.19463	10	0.069235	0.08223	0.115483	0.512
	Non-AE	11	0.097568	0.153701	0.264041	11	0.104453	0.11799	0.175283	

NOTE: Shown are median values with interquartile percentages, as well as *P* values for the comparison of relative clonality (week 2/week 0) for patients with or without AEs (two-sample Wilcoxon test) when specific AEs are added to the IRAE category as a test of the magnitude of their effect on clonality. The addition of temporal arteritis to the IRAE category increases the significance of difference in clonality between patients with or without AEs.

[a]*P* value was calculated for the comparison of relative clonality (week 2 divided by week 0) for AE and non-AE groupings listed using two-sample Wilcoxon test. Underline indicates the degree of significance.

where the emergence of TCR diversity in IRAE patients reflects the polyclonal expansion of numerous T-cell clones, which contribute to the development of end-organ toxicity, but the actual recognition of specific autoantigens may in fact be less critical in the pathogenesis of IRAEs. This is reminiscent of the phenotype of CTLA-4 knockout mice, which develop a fulminant multi-organ lymphoproliferative disorder characterized by tissue infiltration by polyclonal-activated T-cell blasts, which appears to be more autoinflammatory rather than autoimmune in nature (24–26). Of note, we did not find evidence for a quantitative difference in Tregs correlating with IRAE development.

Our findings raise a larger question of whether IRAEs and clinical response after treatment with ipilimumab are driven by shared versus unique processes. TCR diversity can be associated with both beneficial and detrimental outcomes,

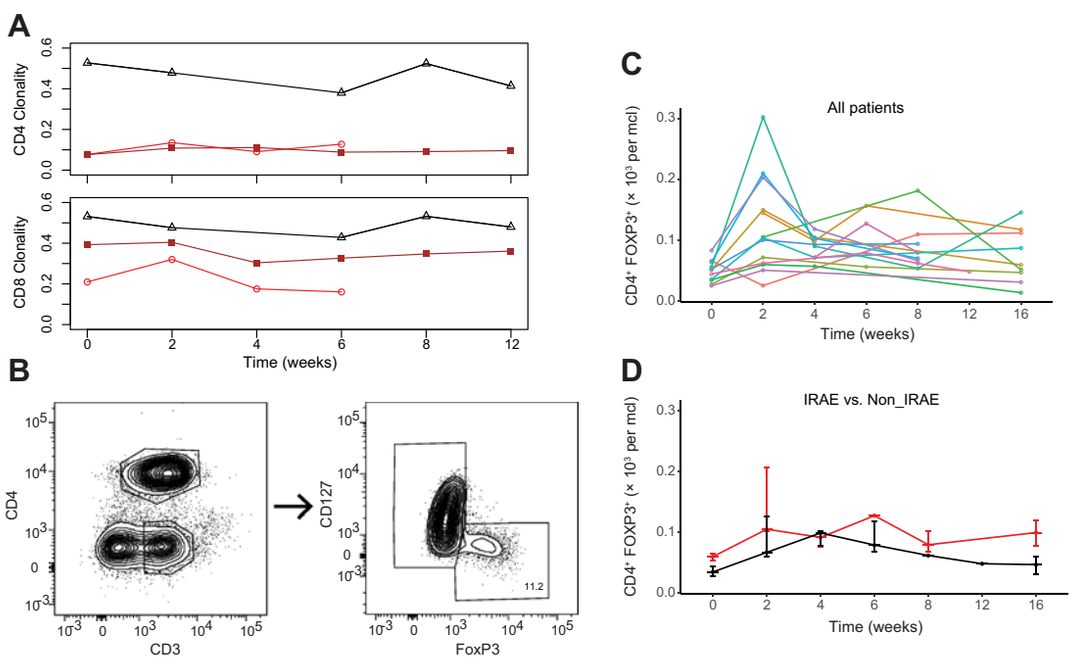

Figure 4.

Changes in $CD4^+$ and $CD8^+$ T cells after ipilimumab plus GM-CSF. **A,** Clonality over time is shown for specific TCR clones, which were identified in sorted $CD4^+$ and $CD8^+$ cells from individual patients and then mapped to bulk PBMCs from all available timepoints in the same patients. Data from available patients who had baseline week 0 data is shown (two IRAE patients, shown in red; one non_IRAE patient, shown in black). **B,** Flow cytometry was used to quantitate $CD4^+$ $FOXP3^+$ Treg cells. Data including gating from a representative timepoint are shown. Absolute numbers of Tregs are shown for all patients (**C**) and separated by IRAE (red) and non_IRAE (black; **D**).

consistent with clinical associations that have been previously reported (27, 28). The observation that almost all of our clinical responders had IRAEs, and that increased diversity is correlated with PSA response as well as IRAE development, suggests that in a subset of patients who experience both toxicity and response, there may be some common underlying mechanisms that may involve diversification of the TCR repertoire. At the same time, our observation that most of our patients with IRAEs did not develop PSA responses, and that longer overall survival was not correlated with increased diversity, also indicates that for most patients, toxicity and clinical benefit are likely mediated by divergent pathways. If initial diversification of the T-cell repertoire represents a common initial pathway in the subset of patients experiencing both IRAEs and response, it is unlikely that these distinct outcomes are driven by shared or common antigens. Whether these initial changes are stochastic or reflect other factors (including tumor-intrinsic features such as neoantigen load, or host-intrinsic polymorphisms) will require further investigation.

Disclosure of Potential Conflicts of Interest

L. Fong reports receiving a research grant from Bristol Myers Squibb, Dendreon, Genentech, Amgen, Merck, and Abbvie. M. Faham has ownership interest (including patents) from Adaptive Biotechnologies. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Fong, D.Y. Oh, J. Cham, L. Zhang, G. Fong, M. Klinger
Writing, review, and/or revision of the manuscript: L. Fong, D.Y. Oh, J. Cham, L. Zhang
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Study supervision: L. Fong

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Immune Toxicities Elicted by CTLA-4 Blockade in Cancer Patients Are Associated with Early Diversification of the T-cell Repertoire

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