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Jennifer Carlisle

Emory University

Caroline Jansen

Emory University

Maria Cardenas

Emory University

Ewelina Soierajska

Emory University

Adriana Reyes Moon

Emory University

Rachel Greenwald

Emory University

Luke delBalzo

Emory University

Nataliya Prokhnevskaya

Emory University

Omer Kucuk

Emory University

Bradley Carthon

Emory University

Patrick Mullane

Emory University

Adeboye Osunkoya

Emory University

Deborah Baumgarten

Emory University

Fares Hosseinzadeh

Emory University

Scott Wilkinson

National Cancer Institute <https://orcid.org/0000-0002-2613-8425>

Ross Lake

Laboratory of Genitourinary Cancer Pathogenesis, NCI, Bethesda, MD 20892

Adam Sowalsky

National Cancer Institute <https://orcid.org/0000-0003-2760-1853>

Yuan Liu

Emory University

Viraj Master

Emory University

Mehmet Bilen

Emory University

Haydn Kissick (✉ haydn.kissick@emory.edu)

Emory University <https://orcid.org/0000-0001-7624-5598>

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Clinical and immunological response to checkpoint therapy in renal cell carcinoma is associated with TCF1+ CD8 T-cells in the tumor

Jennifer Carlisle^{1,2}, Caroline S. Jansen^{1,3,4}, Maria A. Cardenas^{3,4}, Ewelina Sobierajska^{1,3,4}, Adriana ReyesMoon³, Rachel Greenwald³, Luke del Balzo³, Nataliya Prokhnevskaya^{3,4}, Omer Kucuk^{1,2}, Bradley Carthon^{1,2}, Patrick Mullane^{1,5}, Adeboye O. Osunkoya^{1,5}, Deborah Baumgarten^{1,6}, Fares Hosseinzadeh³, Scott Wilkinson⁷, Ross Lake⁷, Adam G. Sowalsky⁷, Yuan Liu^{1,8}, Viraj Master^{1,3,*}, Mehmet Asim Bilen^{1,2,*}, Haydn Kissick^{1,3,4,*}

1: Winship Cancer Institute of Emory University, Atlanta, GA, USA

2: Department of Hematology and Medical Oncology, Emory University School of Medicine, Atlanta, GA, USA

3: Department of Urology, Emory University School of Medicine, Atlanta, GA, USA

4: Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA, USA

5: Department of Pathology, Emory University School of Medicine, Atlanta, GA, USA

6: Department of Radiology, Emory University School of Medicine, Atlanta, GA, USA

7: Laboratory of Genitourinary Cancer Pathogenesis, National Cancer Institute, Bethesda, MD, USA

8: Rollins School of Public Health, Emory University, Atlanta, GA, USA

*Corresponding Authors

Haydn Kissick, PhD

Department of Urology
Emory University School of Medicine
1462 Clifton Rd, Room 420
Atlanta, Georgia, 30322
haydn.kissick@emory.edu

Mehmet Asim Bilen, MD

Department of Hematology and Medical Oncology
Emory University School of Medicine
1365B Clifton Rd NE
Suite B400, Office 4212
Atlanta, GA 30322
mehmet.a.bilen@emory.edu

Viraj Master, MD PhD

Department of Urology
Emory University School of Medicine
1365 Clifton Rd NE, Building B
Atlanta, Georgia, 30322
vmaster@emory.edu

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Abstract: We investigated dynamic T cell responses in the peripheral blood of patients with advanced renal cell carcinoma (RCC) after receiving immunotherapy. We found that a small proportion of both CD4 and CD8 cells activate and express the proliferation marker Ki67 and the activation markers HLA-DR and CD38. Patients who had the highest increase in these HLA-DR+CD38+ CD8 T cells after treatment had the best anti-tumor response. We studied these newly activated cells in more detail using flow cytometry and RNAseq and found that while these cells expanded in most patients, their phenotype did not drastically change during treatment. However, when we analyzed the TCR repertoire of these HLA-DR+CD38+CD8+ T cells, we found only patients who responded to the treatment had a burst of new clonotypes enter this pool of activated cells. Finally, we investigated how the T-cell response in the resected tumor months or years before receiving checkpoint therapy predicted later response to checkpoint therapy. Together, these data suggest that having a strong pre-existing immune response and immediate T cell response to checkpoint therapy is a predictor of anti-tumor response in patients with RCC.

Introduction

Renal cell carcinoma (RCC) has been identified as an immunogenic tumor and patients with RCC have been treated since the mid-1980s (1). High dose IL-2 remains an option, albeit for only select patients given its substantial toxicity. Objective responses are seen in around 20% of patients, of whom a subset experience complete response and substantial(2). More recently, nearly all patients with advanced clear cell RCC are treated with checkpoint (3-5). The latest clinical trials in clear cell RCC have led to FDA approval or breakthrough therapy designations for various frontline tyrosine kinase inhibitor + checkpoint inhibitor (6-9). Moreover, ongoing trials are investigating both neoadjuvant and adjuvant immunotherapy for resectable RCC at high risk of disease recurrence. While early data show both safety and efficacy signals, checkpoint inhibition is not without risk of significant immune mediated adverse events, and many patients still do not respond to these treatments. Unfortunately, common biomarkers of response to immunotherapy that have been useful in other solid tumors such as PD-L1 expression and tumor mutation burden, have not shown a clear role in predicting response to immunotherapy (10) despite usefulness (11). Thus, it is crucial to better understand the immunologic mechanisms that underlie an effective immune response in order to advance treatment options for patients with high risk localized or advanced RCC.

In the first 10 years of treating patients with immunotherapy we have learnt a great deal about what immune factors correlate with therapeutic response. Most studies have found that features of CD8 T-cells are predictive of how a patient will respond to therapy, such as increased expression of Ki67 by CD8 cells in the blood. This marker has been reported to be associated with a better response in melanoma and lung cancer patients (12-20). Several studies investigating the TCR repertoire of CD8 cells in the blood of have found that new clonotypes enter the blood after treatment and increase in clonotypes is associated with better survival in many cancers (21-23) . These studies seem to suggest that these proliferating CD8 cells may be newly activated or re-activated clones that were previously dormant (24). In addition to these responses in the blood, CD8 T cell infiltration into the tumor seems to be an important correlate of patient survival and response to therapy (25-30). This was first shown in melanoma where patients with higher CD8 infiltration into their tumor at the time of therapy were much more likely to respond to treatment, and similar findings have since been reported in other cancers (27-29, 31-33). More recent studies have found that a subset of CD8 tumor infiltrating T-cells, TCF1+ CD8 T-cells, are mechanistically important for the response to PD1 and that TCF1+ CD8 T cell numbers in tumors correlated with response in melanoma (31, 32, 34-36). These studies together highlight a common feature of the immune response to cancer—some patients seem to generate a strong T-cell response to their cancer either before or during checkpoint therapy, and others do not. Based on these studies, we aimed to analyze how the T cell response in patients with RCC was affected by immunotherapy, and how pre-existing immune response to that cancer might correlate with a later capacity to respond to checkpoint therapy.

Results

Activation of CD8 T-cells in blood after the first treatment predicts response to checkpoint therapy.

We enrolled 36 patients with metastatic renal cell carcinoma who were about to undergo checkpoint therapy (Fig. 1A and Extended data Fig 1A). These patients received either nivolumab, ipilimumab + nivolumab, bempegaldesleukin + nivolumab (37). Twenty-seven of the patients had clear cell RCC, and the remainder had other, non-clear cell RCC histologies (Extended data Fig 1B). Of these patients, 3 had a complete response (CR) to therapy, 4 had a partial response (PR), 14 had stable disease (SD), and 15 had progressive disease (PD). A total of 21 of 36 (58.3%) patients received clinical benefit from this treatment and 7/36 (19.4%) achieved an objective response (Fig 1B). Blood was collected from patients immediately before initiating immunotherapy (baseline) and following treatment cycles in order to examine how changes in the circulating T cell immune response might correlate with response to therapy. CD4 and CD8 cells (as a percent of total PBMCs) were unchanged over the course of the disease (Extended data 2A and B). Similarly, the breakdown of naïve, T_{em}, T_{cm}, and T_{emra} CD8+ T-cell subsets (as defined by CD45RA and CCR7 expression) were not significantly different at any time point during treatment (Extended data 2C). The proportion of CD4 or CD8 cells expressing Granzyme B did not differ following treatment (Extended data 2D).

In comparison to these general T cell parameters, we found increased expression of several markers indicating T-cell activation. The most striking change following therapy was an expansion of HLA-DR+ CD38+ CD8 T-cells (recently activated CD8 T cells) in the blood with at least a 2.68-fold increase compared to baseline after cycle 1 (Fig. 1C&D). This increase over baseline was also found at cycle 2, but to a somewhat lesser extent (2.2-fold). These changes occurred within the first few weeks of immunotherapy initiation, as cycles ranged 2-4 weeks depending on the regimen, then returned to near baseline levels. A similar pattern was found in the dynamics of HLA-DR+CD38+ CD4 T cells—there was a large increase in these cells after 1 cycle of treatment that diminished in later treatment cycles (Extended data 2F&G). There was also a significant increase in the proportion of both CD4 and CD8 cells expressing Ki67+ after treatment (Extended data 2E, I, J). Interestingly, patients with the largest increase in HLA-DR+ CD38+ CD8+ T-cells also had expansion of HLA-DR+CD38+CD4 T-cells (Extended data Fig 2H). In addition, patients who had large increases in these Ki67+ CD8 T cells had a comparable increase in the Ki67+ CD4 cells in their blood suggesting general activation of both CD4 and CD8 T cells in the blood of most patients after treatment with immunotherapy (Fig 1E).

We were next interested in how the magnitude of this response might correlate with a change in tumor size. Each patient's change in tumor area was assessed using RECIST v1.1 criteria (38), and we correlated the change in the target lesion size with the magnitude of HLA-DR+CD38+ T cell expansion. Patients with the largest fold increase in HLA-DR+CD38+ CD8 T-cells in their blood had the most significant reductions in tumor size (Fig 1E) ($\rho = -0.39$, $p < 0.05$). Importantly, patients with a greater than 2.7-fold increase in HLA-DR+CD38+ CD8 cells were far more likely to have a complete or partial response to therapy than those with smaller expansion of these cells (Fig. 1G). Patients with a large burst of new HLA-DR+CD38+ CD8+ T cells generally had an anti-tumor effect that occurred after the first scan that continued for several treatment cycles. (Fig. 1H). Together, these data show that a strong T-cell response measured in the blood immediately after the first cycle of checkpoint therapy is a strong predictor of anti-tumor effect.

2. HLA-DR+CD38+ CD8 T-cells are phenotypically and transcriptionally stable throughout the treatment course

Given that the expansion of peripheral HLA-DR+CD38+ CD8+ T-cells had such a strong correlation with response to checkpoint therapy, we were interested in how the phenotype of these cells might change throughout the treatment. Compared to the total CD8 T-cell population, the HLA-DR+CD38+ cells had the highest levels of the proliferation marker Ki67, the cytotoxic molecule GZMB, and the migration marker CXCR3, indicating that these cells had acquired 3 key traits of effector CD8 T-cells; ongoing division, cytotoxicity, and the ability to migrate to areas of inflammation (Fig. 2A). Expression of these key functional markers remained stable in HLA-DR+CD38+ cells at all time points during the treatment (Fig 2A). Furthermore, after cycle 1, there was no difference between patients who had a clinical benefit from therapy compared to those with progressive disease (Fig. 2B). We were interested if more subtle changes might occur in this population that might not be detected by measuring these limited markers of T-cell function by flow cytometry, so we performed RNAseq on the HLA-DR+CD38+ CD8 T-cells from 28 patients at baseline, cycle 1, and cycle 2 after treatment. Similar to our findings by flow cytometry, the HLA-DR+CD38+ cells expressed genes associated with T-cell activation, showed high expression of effector molecules (GZMB, PRF1, IFNg), and showed reduced expression of genes typically expressed by naïve cells, such as CCR7 or IL7R (Extended data Fig 2A). We performed gene set enrichment analysis comparing baseline samples to cycle 1 or cycle 2. Of the top 100 pathways turned on after the first treatment, 77 were related to the cell cycle (Fig. 2C). The pathways most significantly downregulated after treatment were pathways such as TNF signaling, TLR signaling, and the inflammasome. Similar results were found when comparing gene expression at cycle 2 to expression at baseline (Fig C). Interestingly, there were few additional pathways among the most differentially expressed pathways, and very few of these were pathways related to immune signaling.

We next compared pathway enrichment between patients who responded to treatment or those who did not. Cell cycle pathways were upregulated in most patients, but were not significantly different between responding and non-responding patients and were generally more highly enriched in all patients after treatment, when compared to baseline (Fig 2D). We then aimed to specifically identify pathways that were up or downregulated following treatment. (Fig 2E). Of these, the most significantly upregulated pathway in responding patients was 'cytosolic sensing of DNA', a pathway related to type I interferon signaling (Fig 2F). We also compared individual genes that were significantly upregulated in responding patients vs. non-responders (Extended Data Fig 3B). Many of the genes that were upregulated in the non-responders included checkpoint molecules like PD1 and TIGIT, in addition to transcription factors such as TOX and EOMES. In comparison, genes upregulated in responders included IRF7 and IRF9, as well as many canonical interferon stimulated genes like MX1, MX2, OAS1, and OAS2 (Extended Data Fig 3C). Similarly, when we compared the size of the HLA-DR+ CD38+ burst following treatment, patients with the largest increase in these cells had the largest increase in many of the type I interferon related genes like IRF-9 and MX2 (Extended data Fig 3D & E). These data indicate that there are some differences in the HLA-DR+ CD38+ cells between responding and non-responding patients, mostly related to IFN signaling. However, the most consistent and clear response to PD-1 therapy is the induction of a proliferative transcriptional program.

3. Responding patients have an influx of new TCR clonotypes to the HLA-DR+CD38+ pool after checkpoint blockade

We were next interested in how the TCR repertoire of the HLA-DR+CD38+ population might change during treatment. Among all the patients sampled, HLA-DR+ CD38+ T cells were dominated by a relatively small number of TCR clones, where the top 50% of the TCR repertoire was determined by an average of 40 TCR clones at baseline and after checkpoint therapy, regardless of response to therapy (Extended Data Fig 4A). The overall diversity of the TCR repertoire was also unchanged after checkpoint therapy, as measured by the Shannon entropy index (Extended Data Fig 4B). Reflective of the clonality within this population, the top clonotype accounted for around 20% of the cells at baseline, cycle 1 and cycle 2 (Fig. 3A). For most patients, the top 5 clonotypes made up around 30% of the repertoire, but for some patients this number was as high as 80%, suggesting that this population of newly activated effector cells was expanded against a small range of antigens (Fig. 3B).

While overall diversity is unchanged, there was an expansion of TCR clones in HLA-DR+ CD38+ CD8 T cells in patients with clinical benefit suggesting that tumor responsive effector T cells are within this population. For three patients, we were able to sort infiltrating PD1+ CD8 T cells from the resected tumor prior to undergoing immunotherapy (Extended Data Fig 4C). In all three of these patients, we found TCR overlap (1-30%) between the circulating HLA-DR+ CD38+ CD8 T cell population at the time of therapy and the PD1+ CD8 TILs at the time of surgery (Fig 3 C and Extended Data Fig 4D), suggesting that these HLA-DR+ CD38+ effector cells in the periphery can migrate to and carry out their effector functions in the tumor.

We were next interested in how the TCR repertoire changed during checkpoint blockade therapy. Interestingly, in responding patients we found significant changes in the TCR repertoire, but in non-responding patients the repertoire was mostly unchanged. Figure 3D shows the top 20 clonotypes ordered by TCR clone frequency for an example patient who had a strong anti-tumor response to therapy. In this representative plot, 16 out of the top 20 clonotypes lose their dominance in the repertoire after one cycle of treatment (gray dots) and 16 new TCRs now occupy these top spots (green dots). In comparison, in the non-responding patient, only 4 new clonotypes enter the top 20 dominant clones after treatment, while most TCR clones kept exactly the same rank as baseline (Fig. 3D). Given the TCR dominance within this expanded population, the top 20 clones account for the majority of the responding TCRs in the repertoire. Thus, the changes of the top 20 TCR clonotypes are reflective of changes in the overall TCR repertoire, suggesting that only in patients with clinical response to therapy is there a dynamic shift in TCR dominance after immunotherapy. We used the Morisita-Horn index (MH index), a measurement of the similarity of TCR clonotypes, to quantify this change in TCR repertoire between baseline and post-treatment samples. We found a significantly lower MH index in patients with clinical benefit (MH = 0.36) when compared to patients who had worsening disease (MH = 0.63), showing lower similarity (higher variability) between the baseline and post-treatment clonotypes, supporting the notion that clinical benefit is associated with a dynamic TCR repertoire in the effector HLA-DR+ CD38+ T cell population (Fig. 3E).

These data, together with those from previous figures, suggest a model of CD8 T cell activation and response to checkpoint therapy where the pre-existing effector CD8 T cells do not undergo large transcriptional changes

to improve their proliferation or killing ability in response to PD1 therapy. Rather, it suggests that the burst of the HLA-DR+ CD38+ CD8 T cell population after checkpoint blockade is due to activation and expansion of new or previously dormant TCR clones that played a minor or low dominance role in the initial anti-tumor response. Although the mechanisms remain to be defined, the clinical benefit of a dynamic CD8 T cell TCR repertoire suggests that recently expanded TCR clones, now accounting for the majority of the TCR repertoire, have functional effector capacity and can positively contribute to the anti-tumor response.

4. Pre-existing tumor immunity is an important predictor of later immunologic response to checkpoint blockade

Activation of new TCR clonotypes and a large burst of new HLA-DR+CD38+ T-cells following checkpoint blockade raises the question of why some patients have this immunological response to treatment while others don't. Previous work has found that a TCF1+ CD8+ T-cell is the cell that proliferates in response to checkpoint blockade, and that having these cells present in tumors is an important predictor of later response to checkpoint therapy (31, 32). Our previous work found that kidney tumors harbor TCF1+ CD8 T-cells in regions of dense antigen presenting cells, and that having these in a tumor predicts the magnitude of T-cell response and patient survival (39). Based on these data, and in keeping with previous reports in other tumor types (27, 33, 40), we reasoned that having a strong immune response at the time of surgery might predict features of a patient's later response to immunotherapy.

We analyzed surgically resected tumors from 15 patients of the original 36 analyzed in figures 1-3 by immunofluorescence. In the patients who clinically benefitted following therapy, there were significantly more total CD8 T cells, more TCF1+ CD8 T cells, and MHC-II+ cells in the tumors of these patients at the time of surgery. Figures 4A and 4B highlight respective examples of patients who did or did not respond to checkpoint therapy, respectively. The patient in Fig 4A was diagnosed with stage IV (pT3N0M1) clear cell renal carcinoma. They underwent a radical nephroureterectomy with adrenalectomy, IVC tumor thrombectomy, partial IVC resection, resection of psoas and diaphragm crus, retroperitoneal lymph node dissection, and omentoplasty. The tumor invaded the perinephric and renal sinus fat and angiolymphatics, and showed eosinophilic features, WHO/ISUP nuclear grade 3, with a multifocal growth pattern. By immunofluorescence (IF) of this surgically resected tumor, we found extensive CD8 T cell and MHC-II+ cell infiltration and that TCF1+ CD8 T-cells were predominantly resident in dense antigen presenting niches (Fig 4A). By flow cytometry, this patient had 1.2% CD8 T-cell infiltration, which is near the mean for all RCC patients we have previously analyzed (Extended data Fig 5K). Importantly, we also found many TCF1+ stem-like CD8 T-cells in the tumor by flow cytometry, and we observed a sizeable population of CD39+ terminally differentiated effector T cells in the tumor. Five months after surgery, the patient's previously suspicious but non-diagnostic pulmonary nodules increased in size, indicating growing metastases. This patient then received checkpoint immunotherapy, and measurement of the peripheral blood immune response revealed a large expansion of the HLA-DR+CD38+ cells in the blood when compared to baseline levels. The patient had a complete response to therapy, with resolution of all lung metastasis at the 3-month follow-up scan, and remained disease free at our censoring date more than 800 days after starting immunotherapy.

In comparison, Figure 4B shows a patient with similar advanced disease who did not respond to checkpoint immunotherapy. This patient was also diagnosed with stage IV disease (pT3N1M1) clear cell renal carcinoma and underwent radical nephrectomy, adrenalectomy, resection of the psoas, lymph node dissection, and IVC resection with tumor thrombectomy. The tumor invaded the perinephric and renal sinus fat, angiolymphatic invasion was present, several paraaortic and retroperitoneal lymph nodes were positive, and extracapsular extension was noted. The tumor showed eosinophilic and rhabdoid features and WHO/ISUP nuclear grade 4. Extremely few immune cells were found in this patient's tumor at the time of surgery by IF. By flow cytometry this patient had less than 0.1% of the tumor as CD8⁺ T cells, which places this patient in the bottom 10% of all patients. When they received immunotherapy approximately one month later, there was no appreciable immunological response in the blood and their disease progressed rapidly.

This trend of strong immune infiltration in the tumor and later systemic response to checkpoint therapy was found in 15 total patients analyzed by immunofluorescence. A representative patient, demonstrating an intermediate amount of immune infiltration, is shown in Extended data Fig 5 highlighting the location of the tumor and immune infiltrate (Extended data Fig. 5A&B). Whole slide immunofluorescence data was quantified to discern the xy location of each cell as well as the intensity of staining for each marker in each cell. The immunomaps shown in Supp Fig 5C demonstrate the xy location of all CD8 T cells (red, top) and of TCF1⁺ CD8 T cells (cyan, top), as well as the xy location of each MHC-II⁺ cell (green, middle). Importantly, we are also able to define areas that contain both TCF1⁺ CD8⁺ T cells and MHC-II⁺ cells, which we term "immune niches" (orange, bottom, defined as 100µm x 100µm areas with ≥1 TCF1⁺ CD8 T cells and ≥1 MHC-II⁺ cells). In line with our previous studies, in this cohort of patients we found the number of T cells correlates strongly with the amount of MHC-II⁺ (Supp Fig 5D), and TCF1⁺ CD8 T cells in the tissue (Supp Fig 5E). We then examined if these immunologic features of the tumor microenvironment correlated with patients' later response to immunotherapy. In this group of 15 patients, those who had clinical benefit following checkpoint blockade had significantly higher numbers of total CD8 T-cells in their tumor (Fig 4C), and a higher trend towards more TCF1⁺ CD8s ($p=0.055$, Extended Data Fig 5H). These patients also had more areas of MHC-II⁺ cell density (where TCF1⁺ cells are usually found) (Fig 4D), and more areas of defined as immune niches. Having both more MHC-II dense regions and immunological niches was strongly correlated with the size of the HLA-DR⁺CD38⁺ burst in CD8 T-cells after later checkpoint therapy (Extended data fig 5I and J). Amongst these 15 patients, those with an above median increase in HLA-DR⁺CD38⁺ cells had significantly more of these immune niches in their tumors at the time of surgery (Fig. 5F).

To extend this analysis, we also had data available from 36 additional patients for whom we had flow cytometry analysis quantitating immune infiltration in their primary tumors that later went on to receive checkpoint blockade treatments. In these patients, the overall T cell infiltration in this cohort spans the range we have typically seen and reported in RCC (Supp Fig 5K), and importantly, we were able to identify terminally differentiated and stem-like CD8 T cells, as we have previously extensively characterized (Extended data Fig 5L) (Jansen et al). In this cohort of 36 patients, those that had >2.2% of the total cells in the tumor as CD8⁺ T cells had significantly longer overall survival (Fig. 4G), and significantly longer time to tumor progression after initiating checkpoint blockade therapy (Fig. 4H). Together, these two cohorts highlight that a patient's ability to mount an immune response against their tumor is a feature that extends and endures across the course of their disease. When patients have

a strong intra-tumoral immune response at the time of surgery, characterized by infiltration of TCF1+ CD8 T-cells and dense regions of antigen presenting cells, the clinical and immunological response to later checkpoint therapy is much stronger and patient have improved outcomes.

Discussion

In this study we investigated how the T-cell response in the blood of RCC patients was altered after checkpoint blockade. We found a rapid expansion in HLA-DR⁺ CD38⁺ CD4 and CD8 T cells immediately after the first cycle of immunotherapy. These cells expressed the proliferation marker Ki67, important effector molecules like Granzyme B and CXCR3, and were strongly enriched for cell cycle genes. Patients with a better clinical response typically had a much larger increase in these cells, in line with previous reports (12-14). Our data also found that in patients that responded to therapy, the most dominant clonotypes in the TCR repertoire of these HLA-DR⁺CD38⁺ T-cells before treatment were overtaken by new clones. In comparison, patients who did not respond to treatment had a stable TCR repertoire where the most frequent clones before treatment maintained their dominance after therapy. Similarly, several studies have previously reported that response to checkpoint therapy is associated with new clones entering the total CD8 or the activated PD1⁺CD8 T-cell pool (22, 41). Finally, we find that the T cell response in a patient's originally resected tumor (with surgery occurring months to years before receiving immunotherapy) predicts later response to checkpoint therapy, where patients with more CD8 T cells in their tumor exhibit improved responses to therapy. Many of these individual observations have been made alone in other cancers, but here we have been able to show them all together and illustrate how they relate to each other. In future work, larger prospective and randomized studies are needed to confirm the clinical use of these early transient predictors of response to immunotherapy, with the hope of informing selection of optimal treatment. However, by having all these parameters together in the same patients, we believe it reveals potential mechanisms of how checkpoint therapy functions in patients.

In the past few years, we have learned many details of the cellular mechanisms that control the T-cell response to PD-1 blockade. Most notably, the TCF1⁺ CD8 T-cells we have previously described in these patient's tumors are likely the same cells that proliferate in response to PD-1 blockade (34, 42, 43). Upon treatment with anti-PD1, these stem-like CD8 T-cells proliferate, but more importantly, give rise to cytotoxic daughter cells that are responsible for clearing virally-infected or tumor cells. Based on these studies and our data here, we hypothesize that there is a pool of tumor specific TCF1⁺ stem-like CD8 T-cells that are not proliferating or generating anti-tumor effector cells but that are the biologic reservoir that is set free by checkpoint blockade. Currently it is unclear if these dormant TCF1⁺ cells are the ones in the tumor we identified here or are in other locations outside the tumor such as the tumor draining lymph nodes, and this is important to identify in future studies. Based on this hypothesis, we propose that patients who have this dormant TCF1⁺ stem-like T cell pool are those who can generate a large new repertoire of T cells after checkpoint blockade. Evidence of this process occurring is given by the large burst of HLA-DR⁺CD38⁺ CD8 T cells seen in the blood following immune checkpoint blockade and is likely why measuring this burst in activated cells is good biomarker of clinical response (Extended data figure 6).

In this proposed model, several possible explanations can be imagined for patients who don't respond to treatment. It is possible that these non-responding patients do not have sufficient tumor specific TCF1⁺ cells, and therefore lack the underlying cellular resources to respond to anti-PD1 blockade. This is somewhat in line with our observations in the tumors of non-responding patients, where there are typically fewer antigen presenting niches and TCF1⁺ cells needed to respond to checkpoint therapy. However, our data does not

preclude a situation where anti-PD1 alone might not be enough to cause activation of dormant TCF1+ T cells. For example, other studies have found CD28 signaling is necessary for CD8 T-cells to proliferate in response to anti-PD1 treatment, and it is conceivable that non-responding patients may have some TCF1+ stem-like cells, but lack sufficient activating signals (e.g. insufficient levels of CD28) or enough antigen presenting cells to cause their differentiation, even when PD1 is blocked (24, 44). Identifying which of these situations is the cause for a poor T-cell response to checkpoint immunotherapy, and thus a poor clinical response is critical to improving the success rate of immunotherapy.

Methods

Sample Collection, Preparation, and Storage

Patients with stage IV Renal Cell Carcinoma, either treated with SOC immunotherapy with nivolumab, or nivolumab + ipilimumab, or on clinical trial with nivolumab + bempedegaldesleukin formulation were recruited (37). Patients consented for blood collection under the Emory University Urological Satellite Specimen Bank in accordance with the Institutional Review Board (IRB00055316). To minimize impact on patient schedules, the collection time points were cycle-dependent, coinciding with scheduled phlebotomy for standard laboratory analysis. This practical approach, as well as approval of extended nivolumab dosing (45), led to collection intervals that ranged from 2-4 weeks. Peripheral blood was obtained in cell preparation tubes at baseline and study specific timepoints and processed to cryopreserve PBMCs and plasma.

Patient tumour samples were collected immediately after undergoing partial or radical nephrectomy. Tumor samples for flow cytometric analysis were harvested in Hank's Balanced Salt Solution, cut into small pieces, digested using Liberase enzyme cocktail (Roche), and homogenized using a MACS Dissociator. Single cell suspensions were obtained, RBC ACK lysed, and stored at -80°C in freezing media for batch analysis. Samples for immunofluorescence analysis were formaldehyde fixed and embedded in paraffin blocks by Emory Pathology. Unstained and haematoxylin/eosin-stained sections of FFPE blocks were obtained from Emory Pathology.

Assessment of Therapeutic Response

Clinical benefit (CB) was defined as a best response of complete response (CR), partial response (PR), or stable disease (SD). Objective response to treatment was determined by using Response Evaluation Criteria in Solid Tumor version 1.1 (38) by a board-certified radiologist. Re-staging radiograph interval varied among patients on standard of care (SOC) and clinical trial treatments, and in the SOC group modalities may have switched between CT and MRI depending on other clinical factors.

Flow Cytometry & Fluorescence Activated Cell Sorting

Single cell suspensions from human tumors and human peripheral blood were stained with antibodies listed below. Live/dead discrimination was performed using fixable Aqua or Near-IR Dead Cell Stain Kit (Invitrogen). Samples were acquired with a Becton Dickinson LSRII or sorted with a Becton Dickinson FACS Aria II and analysed using FlowJo software. For intracellular staining, cells were fixed and permeabilized using the FOXP3 Transcription Factor Staining Buffer Set (eBioscience). For cell sorting, cryopreserved samples were thawed, stained, and sorted as detailed above. For RNA and TCR sequencing experiments, total CD8 T cells and naïve CD8 T cells were sorted, using a dump/dead-CD3+CD8+ gating strategy. For TCR sequencing experiments, CD8 T cells were gated as shown, and CD38+HLA-DR+ were sorted.

RNA & TCR Sequencing

RNA was isolated from sorted CD8 populations using a Qiagen AllPrep DNA/RNA Micro Isolation Kit according to manufacturers' instructions. RNA was sequenced using the Clontech smartSeq kit following manufacturers instruction. RNA was sequenced on an Illumina HiSeq 3000 at the Yerkes Genomic. For some samples, RNA processing to obtain complete T cell receptor V(D)J clonotypes of TCR transcripts was done using the SMARTer Human TCR a/b Profiling Kit (Takara Biosciences) following manufacturer's user manual. TCR sequencing analysis was performed using the immunarch R package (v 0.6.5) and custom R scripts.

Immunofluorescence

Sections were deparaffinized in successive incubations with xylene and decreasing concentrations (100, 95, 75, 50, 0%) of EtOH. Antigen retrieval utilized Abcam 100x TrisEDTA Antigen Retrieval Buffer (pH = 9) heated under high pressure and washed in PBS + 0.1% Tween20. Sections were blocked for 30 minutes with 10% goat serum in 1x PBS + 0.1% Tween20 before staining with primary and secondary antibodies. Primary antibodies were used at a concentration of 1:100 (MHC-II) or 1:150 (CD8, TCF1) and incubated for 1 hour at room temperature. Secondary antibodies were used at a concentration of 1:250 (A488, A568) or 1:500 (A647) and incubated for 30 minutes at room temperature. Detailed information about antibodies used is listed below. Sections were counterstained with DAPI according to manufacturer instructions (Thermo-Fisher). Immunofluorescence images were collected using a Zeiss Z.1 Slide Scanner equipped with a Colibri 7 Flexible Light Source, and Zeiss ZenBlue software was used for post-acquisition image processing. CellProfiler (46, 47) and custom R and python scripts were used for image analysis, as previously described(39), to determine the xy coordinates of cells within tissue slices, measure fluorescence intensity within each cell, calculate cellular density, and create spatial maps of cell locations and features within the tissue.

Patient survival analysis

Survival analysis of patients was performed using the log-rank test from the R package, Survminer.

Flow Cytometry Antibodies

Target	Clone	Fluorochrome	Source
CD3	UCHT1	FITC	Biolegend
CD4	OKT4	PerCP	Biolegend
CD28	CD28.2	PE/Cy7	eBioscience
CD39	A1	BV421	Biolegend
CD38	HIT2	BV510	Biolegend
CD38	HIT2	BV711	Biolegend
CD8	RPA-T8	BV605	Biolegend
HLA-DR	L243	BV711	Biolegend
HLA-DR	L243	APC/Cy7	Biolegend
PD-1	29F.1A12	BV786	Biolegend
CD45RA	HI100	BV785	Biolegend
CD25	M-A251	APC	Biolegend
CCR7	G043HI	PE-TR	Biolegend
CD19	HIB19	700	Biolegend
CD14	HCD14	700	Biolegend
Tbet	4B10	421	Biolegend
FOXP3	PCH101	PE	Invitrogen
Ki67	B56	APC	BD Biosciences
GZMB	GB11	700	BD Biosciences

Immunofluorescence Antibodies

Target	Antibody Type	Clone	Concentration	Secondary	Concentration
MHC-II (HLA-DR, DP, DQ)	Mouse IgG2a	Tu39	1:100	Goat anti-mouse IgG2a A488	1:250
TCF1	Rabbit	C63D9	1:150	Goat anti-rabbit A568	1:250
CD8	Mouse IgG1	C8/144B	1:150	Goat anti-mouse IgG1 A647	1:500

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A **B** **C** **D** **E** **F** **G** **H**

Figure 1 – Expansion of HLA-DR+ CD38+ CD8 T cells after checkpoint immunotherapy (IO) is predictive of response in RCC patients. **A) Study Design.** Patients with metastatic RCC receiving checkpoint immunotherapy were recruited. Blood was collected at baseline and at each treatment interval. Blood was assessed for various immune parameters over the course of treatment. **B) Clinical response.** Patients were assessed for clinical response using RECIST criteria. Data shown indicates outcomes for the cohort of 36 patients. **C) HLA-DR and CD38 expression by CD8 T cells during treatment.** Blood was analyzed by flow cytometry. Flow plots shown are gated on CD8+ T cells and show the expression of HLA-DR and CD38 by these cells. **D) Fold change in HLA-DR+CD38+ CD8 T cells after treatment.** Baseline was set as the untreated level for each patient, and fold change in these cells expressed vs. this timepoint. **E) Correlation of various T cell parameters in blood.** Heatmap shows correlation of each parameter measured, clustered by Euclidean distance. **F) Correlation with burst of HLA-DR+CD38+ T-cells and change in tumor size.** Patient in orange are those with a clinical response as defined by at least a 30% reduction in the target lesion. **G) Waterfall plot showing deepest response.** Target lesions were assessed by a pathologist and waterfall plots show the greatest change at any timepoint during the treatment. Patients with a >2.7-fold increase in HLA-DR+CD38+ cells are highlighted in orange. **H) Spider plot showing change in major lesion size over treatment course.** Patients underwent scans at 3 to 6 month intervals. Target lesion was assessed by a pathologist for changes in size during the treatment course. Patients with a >2.7-fold increase in HLA-DR+CD38+ cells are highlighted in orange.

A **B**

	Total Number (n=36)
Median age, years (range)	58 (33-78)
Male	24 (67%)
Race: Caucasian	24 (89%)
African American	6 (17%)
Histology: pure clear cell	27 (75%)
+ sarcomatoid features	5 (14%)
Papillary + clear cell	1 (3%)
Chromophobe + clear cell	1 (3%)
Collecting duct	1 (3%)
Unclassified	1 (3%)
Former or current smoker	24 (67%)
Lung metastasis at baseline either > 1 cm or biopsy proven	20 (56%)

Extended data figure 1: A) Inclusion details of patients in the study. Flow chart showing details of patients recruited for this study **B) Patient characteristics**

Extended data figure 2: Flow cytometry characterization of T-cells after PD1 blockade:

A & B) CD4 and CD8 T-cells in blood after PD1 blockade. FACS plots shows cells gated on CD3, Live, singlets. The percent of total PBMCs that were CD4+ or CD8+ was determined from this gate at baseline and all timepoints during treatment. Shown in B is the fold change over baseline for matched patient samples throughout the treatment course. **C) Canonical CD8 memory subsets over treatment course.** Cells were gated from panel A on CD8 and analyzed for expression of CD45RA and CCR7. Subsets were mostly stable over the course of treatment with no significant changes at any time points. **D & E) CD8 expression of Gzmb.** CD8 cells from panel A were gated and expression of GzmB and Ki67 analyzed. Adjacent plot shows summary data from patients over their treatment time course. **F &G) Expression of HLA-DR and CD38 by CD4 and CD8 cells following checkpoint blockade.** Plots in F show HLA-DR and CD38 expression by CD4 and CD8 cells in a representative single patient over a treatment time course. The fold change in these cells from baseline is in all patients is shown in G. **H) Correlation of CD4 and CD8 response.** Plot shows the fold change in the HLA-DR+CD38+ cells for CD4 T cells on the x-axis and CD8 T cells on the y-axis. **I & J) HLA-DR+ CD38+ T cells express Ki67.** Flow plots show CD8 and CD4 cells gated from panel A and their expression of HLA-DR and CD38. Overlayed in blue are CD8 and CD4 cells that were gated to be positive for Ki67 (See E) to show their relative position in the HLA-DR x CD38 plots. Summary data shows the correlation in expansion of the HLA-DR+CD38+ cells and increased expression of Ki67 after checkpoint blockade.

A **C** **D** **E** **F**

Figure 2 – Transcriptional and phenotypic characteristics of HLA-DR+ CD38+ CD8 T cells during immunotherapy:

A) Flow cytometry analysis of molecules associated with T-cell function. HLA-DR+CD38+ cells expression of Ki67, Gzmb, and CXCR3 at baseline, cycle 1 and cycle 2 of immunotherapy. Total CD8 cells are shown as a control. Summary plots show MFI of these markers for each patient.

B) HLA-DR+CD38+ CD8s are phenotypically similar in responding and non-responding patients: HLA-DR+CD38+ cells expression of Ki67, Gzmb, and CXCR3 were measured at cycle 1 in patients that had clinical benefit or not. Plots show MFI of these parameters (n=21).

C) Pathways altered in HLA-DR+ CD38+ CD8 T-cells after PD-1 blockade. Pathway analysis comparing baseline to either cycle 1 or cycle 2 cells was performed for each patient. The average of all patients for each pathway is plotted in order of highest pathways upregulated post-treatment. Highlighted in red are pathways within the top 100 related to cell cycle and blue pathways related to innate inflammation. Selected pathways are listed in plots.

D) Cell cycle response after checkpoint blockade. GSEA plots show the enrichment scores of the cell cycle pathway comparing baseline to either cycle 1 or cycle 2 after checkpoint blockade. Summary plots show most patients had increased cell-cycle activity compared to baseline and there was no significant difference between patients with different immunologic outcomes.

E) Pathway analysis comparing responders and non-responders. GSEA for all REACTOME pathways was performed on all samples comparing HLA-DR+CD38+ cells from baseline to either cycle 1 or cycle 2 cells. The enrichment scores for each pathway were then compared between patients who responded or not. Volcano plot shows the pathways most significantly activated or de-activated in responding patients..

F) Cytosolic sensing of DNA after checkpoint blockade. GSEA plots show the enrichment scores of the Cytosolic sensing of DNA pathway comparing baseline to either cycle 1 or cycle 2 after checkpoint blockade. This pathway is highly related to IFN signaling and includes many molecules related to this process. This pathway is significantly upregulated in the HLA-DR+CD38+ cells of responding patients after checkpoint blockade.

A **B** **C** **D** **E**

Extended data figure 3: Extended RNAseq analysis of CD8+ HLA-DR+ CD38+ T cells:

A) Canonical effector signature expressed by HLA-DR+ CD38+ CD8 cells. Expression of key markers associated with effector function were compared to previously collected naïve CD8 T-cells (Akondy et al., Nature 2017).

B) Genes different between responders non responders. Differentially expressed genes between patients with a RECIST defined response were compared to patients with either stable or progressive disease. Volcano plots show the fold change between responders and other patients vs. significance value. Data points highlighted in orange have an adjusted p-value less than 0.05.

C) Type-I Interferon genes significantly up-regulated in responding patients. Heatmap shows z-scored expression values for each of the genes noted. Genes selected are all significantly higher in the HLA-DR+CD38+ CD8 T cells from responding patients.

D) Correlation of genes with burst size. Plot shows genes in order of correlation with the size of the HLA-DR+CD38+ response to checkpoint blockade. Top genes were analyzed for what pathways they belong too. Top pathways included RNA Processing, Histone Modification, and Interferon Signaling. Example genes are highlighted.

F) Example genes that correlate with the size of CD8 burst. Plots show size of burst vs change in expression for genes listed. Correlation and p-values are calculated using Spearman correlation.

A **C** **D** **E**

Figure 3 – Patients with clinical response have a burst of new TCR clonotypes: **A) HLA-DR CD38+ CD8 T-cells are clonally expanded.** Plot shows the frequency distribution of the dominant TCR clone of circulating activated CD8 T cells (HLA-DR+ CD38+) at baseline and after each CB therapy cycles (n=26 patients). **B) Clonotype distribution of HLA-DR+CD38+ CD8 T-cells.** Example circle diagrams from patients with average (left, ~20%) and highest (right) TCR clonotype dominance among their activated circulating CD8 T cells. Shown as different shades of each color are the top 5 TCR clonotypes in the same patient at each timepoint. **C) Frequency of TCR repertoire overlap between circulating CD8 T cells before/after CB therapy and tumor infiltrating CD8 T cells at the time of surgery.** Representative proportion of the detected TCR repertoire in circulating CD8 T cells that is unique (gray) or overlapping (orange) with tumor infiltrating CD8 T cells at the time of surgery. Summary of TCR repertoire overlap with primary tumors at the time of surgery for 3 patients at 3 timepoints. **D) TCR clones were ranked based on Representative responding and non-responding patients are shown.** **E) Morisita-Horn index was used to quantify the similarity between the different CB therapy cycles within each individual patient. Mean and standard deviation (SD) are shown. *, $P < 0.05$ determined by unpaired Welch's t-test.**

A **B** **C** **D**

Extended data figure 4: TCR diversity and sorting strategy for tumor CD8 TILS. A) Top 50% clonotype distribution. Number of TCR clonotypes that account for 50% of the repertoire for each patient at baseline and after checkpoint therapy. Mean +/- sd. are shown for each timepoint. **B) Shannon's Entropy index of TCR repertoire.** HLADR+ CD38+ CD8 T cells from each patient before and after checkpoint therapy. **C) Sorting strategy for tumor infiltrating PD1+ CD45RA- CD8 TILS from a representative patient at the time of resection prior to undergoing checkpoint therapy.** The frequency (%) of PD1+ CD45RA- CD8 TILS for all three patients is represented in the bar graph. **D) Representative TCR overlap between blood HLADR+ CD38+ CD8 TILs and tumor infiltrating CD8 TILS.** The proportion of detected TCR repertoire in each cycle that is unique (gray) or shared (orange) with the tumor is shown.

Figure 4 – Long-term immunological status is correlated with response to immunotherapy: A and B) Examples of longitudinal immune analysis in a responding and non-responding patient: Plots show the timeline of a patient and immunologic status at each point. This timeline includes when they were diagnosed and received surgery, details of disease progression, timepoint where immunotherapy was started, subsequent scans after immunotherapy. Below the timeline are the immunological parameters collected showing H&E with tumor areas outlined in yellow, immunofluorescence of tumor at the time of surgery, flow cytometry of the tumor at time of surgery showing total CD3 infiltration and the phenotype of CD8 cells, and the CD8 response in the blood after receiving IO. **C and D) Quantitative analysis of CD8 T-cells and MHC-II+ cells in responding and non-responding patients.** Immunofluorescence images for responding and non-responding patients were analyzed from 15 patients for infiltration by total, TCF1+, and TCF1- CD8 T-cells (C), and MHC-II+ cells (D). Spatial plots show where each of these subsets are found in tumor tissue and summary plots show the proportion of these cells in tumors of patients who had clinical benefit (CB) vs. those who did not (no CB) (n=15). **E) Patients who respond to checkpoint therapy have a higher proportion of immunologic niches in their tumors.** Immune niches were defined as regions with ≥ 1 MHC-II+ cells and ≥ 1 TCF1+ CD8 T cells in the same local neighborhood ($100 \mu\text{m} \times 100 \mu\text{m}$) and proportions were calculated out of the total number of $100 \mu\text{m} \times 100 \mu\text{m}$ neighborhoods contained within the whole tissue slice. Spatial plots demonstrate these immune niches in a responding (top) and non-responding patient (bottom), and a summary plot demonstrates the proportion of immune niches in tumors of patients who had clinical benefit (CB) vs. those who did not (no CB). **F) Overall survival after initiation of IO for patients with high or low CD8 infiltration into tumors measured by flow-cytometry.** Patients were stratified into CD8 high ($>2.2\%$ CD8 infiltration into tumor) (n=8) or CD8 low (n=28) groups. Initiation of immunotherapy (IO) was set as starting point for survival analysis. **G) Progression free survival after initiation of IO for patients with high or low CD8 infiltration into tumors.** Patients were stratified in the same manner as F) and the time until progression as defined by RECIST criteria was measured.

Extended data figure 5: Quantitative Imaging for a representative patient with an intermediate anti-tumor immune response (A-C). **A.** Hematoxylin and eosin whole slide for a representative patient with an intermediate anti-tumor immune response. Yellow outlines highlight the location of tumor tissue within the whole slide scan. **B.** Immunofluorescence whole slide scan for the patient shown in A, with DAPI (nuclei) shown in blue, CD8 in red, and MHC-II in green. Below, an area of interest niches from the whole slide scan shown above, illustrating strong immune cell infiltration and the presence of antigen presenting niches, with DAPI (nuclei) shown in blue, CD8 in red, MHC-II in green, and TCF1 in cyan. **C.** Immunomap quantifications of the whole slide immunofluorescence shown in B, illustrating from top to bottom, (1) the xy location of all CD8 T cells in red and of TCF1+ CD8 T cells in cyan, (2) the xy location of all MHC-II+ cells in green, and (3) the location of immune niches, defined as those 100µm x 100µm areas containing ≥ 1 TCF1+ CD8 T cells and ≥ 1 MHC-II+ cells, in orange. **(D-G). Summary data for quantitative analysis of whole slide immunofluorescence imaging.** **D.** CD8 T cell infiltration (CD8+ cells per mm²) correlates with MHC-II+ cell infiltration (MHC-II+ cells per mm²). **E.** CD8 T cell infiltration (CD8+ cells per mm²) correlates with the proportion of stem-like CD8 T cell infiltration (TCF1+ CD8 T cells as a proportion of all DAPI+ cells). **F.** MHC-II+ cell infiltration (MHC-II+ cells per mm²) correlates with the proportion of tissue containing immune niches (as defined in **C**). **G.** Stem-like CD8 T cell infiltration (TCF1+ CD8 T cells as a proportion of all DAPI+ cells) correlates with the proportion of tissue containing immune niches (as defined in **C**). **H.** Summary data for quantitative immunofluorescence comparing the proportion of TCF1+ cells (as % of all DAPI+ cells) in patients with and without clinical benefit following therapy (CB, no CB, respectively). **H) Relationship of the intra-tumoral immune response at the time of surgery with the later, peripheral response to immunotherapy.** **I.** Correlation of the burst in HLA-DR+CD38+ CD8 T cells in the blood following immunotherapy with the average MHC-II+ cellular density in tumor tissue at the time of surgery as measured by quantitative immunofluorescence. **J.** Correlation of the burst in HLA-DR+CD38+ CD8 T cells in the blood following immunotherapy with the proportion of tumor tissue occupied by immune niches (as defined in **C**). **L) Flow cytometric analysis of tumor samples:** Comparison of selected cohort with previously published unselected RCC patient cohort (Jansen *et al* Nature 2019). Proportion of CD8 T cells in kidney tumors by flow cytometry at the time of surgery shown as percent of total cells in patients from Jansen *et al* (black, n=68), non-progressors (NP, orange, n=9), and progressors (P, gray, n=28). **M.** Sample gating strategy for a representative patient to examine terminally differentiated (PD1+CD39+) and stem-like (PD1+CD39-CD28+) CD8 T cells. Leftmost plot is gated on live, single cells and shows selection of CD3+ cells. Moving rightward, CD8 (CD4-) T cells are selected for further examination, and terminally differentiated and stem-like cells are identified based on PD1, CD39 and CD28 expression.

A**RESPONDERS** **B****NON-RESPONDERS**

Extended data figure 6: A) Proposed model regulating the CD8 response to checkpoint therapy in RCC patients. Based on extensive mechanistic mouse studies, the burst of new HLA-DR+CD38+ cells that express high levels of effector molecules must come from a precursor TCF1+ CD8 T-cell. A new influx of TCRs in the HLA-DR+CD38+ population implies that there must be dormant TCF1+ CD8 T-cells that re-activate in response to the checkpoint blockade. **B) Proposed situations for why checkpoint blockade may not induce an expansion of CD8 T-cells.** In situation i) we envision a situation where PD-1 blockade is not sufficient to cause the activation of TCF1+ CD8 T-cells either due to lack of other signals like co-stimulation or presence of various suppressive factors. In situation ii), patients may have very low numbers of TCF1+ CD8 T-cells and are therefore unable to provide a burst of many new effector cells after checkpoint therapy. This model fits the findings in figure 4, where patients who do not respond to therapy had very few TCF1+ CD8 T-cells in their tumors.