Early clonal expansion of tumor-infiltrating lymphocytes predicts response to immune checkpoint therapy

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Article

Keywords:

Posted Date: May 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1613398/v1

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Title: Early clonal expansion of tumor-infiltrating lymphocytes predicts response to immune checkpoint therapy

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Abstract: Immune checkpoint therapy (ICT) causes durable tumor responses in a subgroup of patients. Profiling T cell receptor beta (TCRβ) repertoire structure in ICT responders and non-responders provides mechanistic insight into what constitutes an effective anti-tumor response, and could result in the development of predictive biomarkers of response to identify and stratify patients for ICT. To examine how the TCRβ repertoire dynamics contribute to ICT response, we utilized an established murine model that excludes variation in host genetics, environmental factors and tumor mutation burden, limiting variation between animals to naturally diverse TCRβ repertoires. Oligoclonal expansion of TCRβ clonotypes that corresponded with a low TCRβ diversity was observed in responding tumors prior to ICT. We modeled TCRβ cluster dynamics during ICT and found that select clonotypes expanded slower in responders compared to non-responders. Clonally expanded CD8⁺ tumor infiltrating T cells in non-responders exhibited a T cell exhaustion phenotype. We conclude that an early burst of clonal expansion followed by a contraction during ICT is associated with response.

One Sentence Summary: Early reduction in tumor TCRβ repertoire diversity predicts responders from non-responders to cancer immunotherapy.

INTRODUCTION
Drugs that target immune checkpoint receptors on T cells, such as programmed death (PD)-1 and cytotoxic T lymphocyte-associated antigen (CTLA)-4, are approved for the treatment of metastatic melanoma, non-small cell lung cancer, mesothelioma, renal cell cancer, hepatocellular carcinoma and colorectal cancer, with durable responses observed in a subset of patients (1, 2). However, not all patients treated with immune checkpoint therapy (ICT) benefit. Tumors with high PD-L1 expression, mutational burden and increased lymphocytic infiltration are more likely to respond to ICT (3-6). However, there is no single accurate biomarker of ICT response that can be applied across all cancers. Because of the financial costs and toxicities associated with ICT, there is an urgent need to develop accurate predictors of response (7). As ICT primarily acts upon adaptive immunity, in-depth profiling of T cell phenotype and specificity might improve development of predictive biomarkers for response (8-10).

High throughput sequencing of TCRα/β chains (TCRseq) has been widely used to profile the distribution of TCR clonotypes within tumor and peripheral blood samples (11). For example, anti-PD-1 antibody therapy drives clonal expansion of antigen-specific T cells, reflected by more clonal TCRβ repertoires of tumor infiltrating lymphocytes (TILs) (12-14). Measurements of tumor and peripheral blood TCR repertoire diversity or clonality at a single time-point (before or after ICT) correlate with clinical response in some studies, but not others (12, 15, 16). Other advances in the field include sequence-based clustering of TCRs to identify common motifs predictive of specificity to the same antigen (17, 18). Despite these advances, the value of TCR repertoire profiling as a prognostic or treatment biomarker is limited in the context of ICT. This may be because clinical studies are confounded by variability in host genetics, tumor heterogeneity and environmental factors. Furthermore, frequent serial tumor biopsies are not feasible in clinical studies, making it difficult to assess dynamics within the tumor microenvironment.

To study if TCR repertoire dynamics are predictive of response, we leveraged murine models with limited genetic, antigenic and environmental variation, allowing us to examine tumors and blood before and after ICT (19, 20). By applying bulk TCRβ sequencing and single cell sequencing, we interrogated TCRβ diversity in pre and post ICT samples. Dynamics in overall tumor TCRβ diversity, and different rates of clonal bursts delineated ICT responders from non-responders.

RESULTS

Early changes in blood TCRβ repertoire diversity do not correlate with ICT outcome

It is not clear whether there is a relationship between the available peripheral blood T cell repertoire and an ability to respond to ICT. Clinical TCRβ sequencing studies are limited by heterogeneity in tumor antigen expression, patient HLA diversity, differences in prior infections and thus anti-viral TCRβ repertoire, all of which could affect the interpretation of TCRβ diversity and clonality. We used a murine model to exclude variation in tumor antigen load (tumors were derived from clonal cancer cell line AB1-HA), host genetics and MHC haplotype (inbred BALB/c strain of one gender), environmental factors (mice were kept under highly controlled conditions) and treatment schedule (all mice were treated identically). As reported before, despite this homogenous background, mice still separated into responders and non-responders (19-21). An important reason for this dichotomy in response could be the variability in TCR repertoire between individual mice. We therefore interrogated their blood TCRβ repertoire...
following treatment with antibodies targeting CTLA4 and PD-L1. We performed bulk TCRβ sequencing on sequential blood samples from a cohort of tumor bearing animals 1 day prior to, 3 and 6 days after the start of ICT (Figure 1A). We chose these early time points because key changes in peripheral blood T cells have been noted early after the commencement of immunotherapy (22-24). Importantly, tumor sizes were identical at these time points, regardless of eventual response (Figure 1B). We found that the distribution of TCRβ clones in peripheral blood, as represented by Shannon’s diversity index, was high (0.97 ± 0.1), suggesting that TCRβ clones were mostly evenly distributed within mice, with no particular clone dominating any repertoire (Figure 1C). The number of total and unique TCRβ sequences were likewise similar between responders and non-responders at all time points (Supp Figure 1A, B). There were also no patterns of change in TCRβ repertoire diversity associated with response, as individual animals maintained high diversity after ICT (Figure 1C, D). To determine if ICT resulted in an increase of TCRβ clones that persisted in peripheral blood, we enumerated abundant TCRβ clones that were present at all timepoints for each animal (Figure 1E). Although ICT treated animals had more persistent TCRβ clones compared to PBS treated animals, there were no differences between responders and non-responders (Figure 1F). Endogenous TCRβs (Clone 4, CL4) specific for the model antigen haemagglutinin (HA), which was expressed by the tumor cells, were present at low frequencies in the blood (0.0% - 0.4%), and changes did not associate with ICT response (Supp Figure 1C). We hypothesized that transferring a fixed number of naïve, GL4 TCR transgenic CD8+ T cells in a parallel experimental setup would increase the precursor frequency of tumor antigen-specific T cells and would enable detection of any changes in the frequency of these cells (Supp Figure 1D, E). In this setting, ICT responders displayed an increase in the frequency of HA-specific CD8+ T cells earlier in time than non-responders (Figure 1G, H), indicating that the timing of increase in tumor antigen-specific CD8+ T cells in blood is predictive of ICT response. Together, these data suggest that dynamics in tumor antigen specific TCRβ clones, but not overall TCRβ diversity, differentiate responders from non-responders and are detectable in blood.

Bilateral tumors from the same animal exhibit highly similar TCRβ repertoires

Since the overall TCRβ diversity in peripheral blood did not correlate with response to ICT, we queried whether that was due to the compartment examined. Therefore, we examined changes in TCRβ repertoires of TILs from responding and non-responding mice. We utilized a previously established bilateral tumor model, whereby tumors on contralateral flanks of an animal respond symmetrically following the administration of ICT, allowing us to surgically excise one tumor to examine the TCRβ repertoire and leave the remaining tumor as a readout for response. We first examined if TCRβ repertoires in symmetrical, bilateral tumors have similar distributions of identical TCRβ clones. We performed bulk TCRβ sequencing on sorted CD4+ and CD8+ TILs from bilateral AB1-HA tumors treated with combination ICT with anti-CTLA4 and anti-OX40 (25)(Figure 2A, B, C). Tumors were harvested when sizes between ICT and control groups were similar for both flanks (44.8 ± 9.7 mm²; left versus right; untreated: p = 0.31, ICT: p = 0.16) (Figure 2B, Supp Figure 2A). The number of total and unique TCRβ sequences from CD4+ and CD8+ TILs were similar between control and ICT treated animals, and between both flanks of the same animal (Figure 2D, Supp Figure 2B, 2C). Next, we compared the TCRβ repertoires of bilateral tumors within the same animal, and between different animals, using the Morisita-Horn overlap index, which accounts for the number of identical TCRβ
clonotypes and their distribution across each paired comparison. Regardless of treatment, CD4+ and CD8+ TCRβ repertoires in bilateral tumors were highly related within animals (0.64 ± 0.20), but not between animals (0.09 ± 0.19) (Figure 2E). Interestingly, although a few tumors from different animals had high TCRβ overlap before treatment, this rarely occurred after ICT (Figure 2E), suggesting that clonal expansion after ICT is unpredictable. Importantly, these data demonstrate that within an individual mouse the T cell repertoire of one flank tumor is highly representative of the other flank tumor, giving us a reliable and valid readout of intra-tumoral TCRβ repertoire changes in relation to ICT outcomes.

**ICT responding tumors exhibit early clonal expansion of unique TCRβ clones**

To characterize tumor TCRβ repertoires over time in relation to ICT outcomes, we excised one flank tumor before (day 0), or 2, 4 or 6 days after anti-CTLA4 and anti-PDL1 treatment for bulk TCRβ sequencing in two mouse models, AB1 mesothelioma and RENCA renal cancer (Figure 3A). All tumors yielded between $10^3$ – $10^5$ total TCRβ clones and between $10^2$ – $10^4$ unique TCRβ clones (Figure 3B, Supp Figure 3A). The total number of TCRβ clones increased over time in both models, suggesting that ICT increased the number of TILs. At most timepoints, responding tumors had a greater number of total TCRβ clones compared to non-responding tumors, while the number of unique TCRβ clones remained similar between responders and non-responders at most time-points (Figure 3B).

We next examined how ICT changed the diversity and clonality of T cells within each tumor. We measured TCRβ diversity and clonality with Rényi entropy and Shannon’s index, which summarizes the proportional effect of each clone on the repertoire (Supp Figure 3B). The bulk of the pre-treatment tumor T cells were not highly expanded clones, with the top 10 most abundant clones occupying 18.9% ± 8.2% of repertoires (Figure 3C). Diversity, as represented by Shannon’s index, decreased over time following ICT, with the top 10 most abundant clones occupying 49.6% ± 9.0% of the repertoires and the lowest diversity indices measured 6 days after treatment (Figure 3C, D). ICT treatment restricted the TCRβ repertoires in both responders and non-responders, with the number of expanded clones (that occupied more than 0.5% of the whole repertoire) remaining stable after ICT (Figure 3E).

Importantly however, ICT responding tumors had significantly lower TCRβ diversity at all timepoints measured (Figure 3D). In both models, tumors from responding animals displayed TCRβ clonal expansion earlier in time, as the number of expanded clones was significantly higher in responders prior to treatment (Figure 3E). Concordantly, responding tumors displayed clonally expanded TCRβ repertoires earlier than non-responders (Figure 3C, 3D, Supp Figure 3C).

By making pairwise comparisons across all animals, we found low Morisita-Horn’s overlap indices (ranging from 0.0003 – 0.16), indicating that tumor TCRβ repertoires from individual animals consisted of clones that were mostly unique, and were rarely shared with other animals (Supp Figure 3D). There were minimal differences in the number of public tumor clones between responders and non-responders (Supp Figure 3E), suggesting that there is no single clone that is shared between all tumors. The overlap in the Morisita-Horn indices also reduced regardless of response, suggesting that ICT drives clonal expansion of unique TIL TCRβ clones for each animal. Non-responding tumors had a significantly higher overlap index than responding tumors, pre to post-ICT (Figure 3F). Taken together, our data suggests that ICT
responding tumors have more unique T cell clones and that these clones expanded earlier after treatment compared to non-responders.

Large TCR clusters with slow growth predict ICT response

There was limited clonal sharing between tumors from different animals (Supp. Figure 3E), which prevented us from tracking identical TCRβs between mice. Antigen-specific T cell responses are often associated with the presence of TCR clusters with similar CDR3 peptide binding sequences(17, 18). We assumed that TCRβs from different mice harboring the same tumor would be composed of similar CDR3 residue sequences because of specificity against shared tumor-antigens. We investigated if TCRβ cluster dynamics could predict ICT response in our models. We therefore applied an unsupervised deep learning method (DeepTCR) that clusters TCRβs based on patterns such as CDR3 amino acid motifs and V/D/J gene usage (Figure 4A). DeepTCR is a neural network learning tool that previously identified patterns in TCRβ sequences shared by T cell populations specific for the same antigen(26).

DeepTCR classified ~5x10⁵ unique TCRβs from both models into 87 final clusters, with each cluster having a median of 5710 unique TCRβ sequences. Samples primarily grouped by tumor models (AB1 vs RENCA) based on the expression of these TCRβ clusters (Figure 4B). This suggested that TCRβ clusters successfully delineated tumor models, and possibly antigen specificity. Consistent with previous results, pre-treatment samples grouped closely together on the Multi-Dimensional Scaling (MDS) plot, as post-treatment tumors TCRβs were mostly unique to each animal (Figure 4C).

To model how clusters changed over time, we applied a generalized linear model that represented key features as a numerical function (Δ1, Δ2, Δ3) (Supp Figure 4). For each cluster, Δ1 measured the initial pre-treatment difference in TCRβs between responders and non-responders; Δ2 indicated if there was an increase or decrease of TCRβs over time, and Δ3 indicated how the pattern of change was different in responders versus non-responders. By applying the generalized linear model, we could map all possible dynamic trajectories of each cluster, in responders versus non-responders (Supp Figure 4). We identified 22 AB1 clusters and 11 RENCA clusters with an 80% confidence interval that did not cross 0 for all Δ1, Δ2, Δ3 values (Figure 4D, 4E). All identified clusters followed the same pattern of change: Prior to treatment, responding tumors had larger numbers of total TCR counts than non-responders, but after treatment they increased at a slower rate compared to non-responders (Figure 4F, G, H). Even though these cluster counts were increasing at a slower rate in responders, the differences prior to treatment were sufficient to result in more and bigger clonal bursts in responders than non-responders at all timepoints (Figure 2D). Because of the numerous unique TCRβ clusters that displayed this pattern of change, it also suggests that the rate of clonal expansion of TILs specific for a broad range of antigens were different in responders compared to non-responders.

Taken together, our analysis identifies differential clonal dynamics for response in distinct TCRβ clusters between 2 tumor models.

Expanded CD8⁺ T cell clones display an exhausted phenotype in non-responders:

To determine the phenotype of clonally expanded TILs in responders and non-responders, we next characterized the transcriptome of clonally expanded T cells from AB1 tumors 6 days post ICT using 5’ single cell RNA and TCR sequencing on sorted CD45⁺ cells.
Cell clusters were assigned to cell types including CD8\(^+\), CD4\(^+\) T cells, Foxp3\(^+\) regulatory T cells, monocytes, NK cells and macrophages, showing no significant difference between responders and non-responders in overall cell proportions (Figure 5B, C). We focused our analysis on cells with annotated TCR\(\alpha\beta\)s. Large clones, defined as cells with identical TCR sequences \(\geq 1.0\%\) of the total number of TCR expressing cells, were almost exclusively CD8\(^+\) T cells (Figure 5D). We compared the transcriptome of these CD8\(^+\) large clones with the other CD8\(^+\) clones in responders and non-responders (Figure 5E). CD8\(^+\) large clones significantly upregulated a gene signature of dysfunctional and exhausted CD8\(^+\) TILs\(^{27}\), compared to other CD8\(^+\) clones (Figure 5F, Supp Figure 5A). When we compared the gene expression between all 4 groups, CD8\(^+\) large clones in non-responders significantly upregulated genes associated with stress (Hspa1a, Hspa1b) and T cell exhaustion (Havcr2, Tox, Nr4a2, Pdcd1). CD8\(^+\) large clones from responders upregulated Gzmf, whilst other CD8\(^+\) clones in responding tumors upregulated genes associated with T cell memory and IFNa signaling (Ly6a, Ly6c2 CD7, Isg15) (Figure 5G, Supp Figure 5B). Together, these data indicate that expanded CD8\(^+\) TILs have an exhausted phenotype in non-responding tumors compared to responding ones.

*ICT responders exhibit early clonal expansion of tumor-antigen specific CD8\(^+\) T cells*

Since expansion of tumor associated antigen-specific TCR\(\beta\) clones predicted ICT response, we searched public TCR databases for H-2K\(d\), H-2L\(d\) and H-2D\(d\) restricted antigen specific TCR\(\beta\) clonotypes (Supp Table 1). The murine leukemia virus envelope glycoprotein gp70 is a tumor-associated self-antigen expressed in BALB/c-derived tumor cell lines including AB1. We tracked 40 unique and public TCR\(\beta\) sequences derived from sorted H2-Ld restricted gp70\(_{423-431}\) (gp70-AH1) specific CD8\(^+\) TILs\(^{28, 29}\). In our bulk TCR\(\beta\) sequencing data set, gp70-AH1 clones significantly increased over time in AB1 tumors and were dominant in some tumors, making up approximately 10 - 30\% of the TCR\(\beta\) repertoire. They were expressed at lower levels in RENCA tumors except for one sample (Figure 6B). Importantly, responding AB1 tumors had more gp70-AH1 TCR\(\beta\) clonotypes before and 6 days after treatment than non-responding tumors, suggesting that expansion of tumor associated antigen gp70-AH1 specific T cell clones contributed to the difference in TCR\(\beta\) repertoire diversity between responders and non-responders in the AB1 model (Figure 6A). Unique individual gp70-AH1 TCR\(\beta\) clones were distributed randomly between mice (Figure 6C). We also found 224 cells expressing gp70-AH1 associated TCR\(\beta\) genes in our TIL single cell data set, and \(> 90\%\) of these gp70-AH1 cells were large clones (Figure 6D), suggesting that they were likely to be exhausted within the non-responding AB1 tumor microenvironments.

To test if an early increase in clonal populations of tumor-antigen specific CD8\(^+\) TILs improved ICT responses, we utilized a model of Adoptive Cell Therapy (ACT) with our model (HA) antigen system. ACT of activated, effector CL4 T cells resulted in rapid migration of these cells into HA-expressing tumors, reducing the tumor TCR\(\beta\) diversity (Figure 6E)\(^{30, 31}\). Adoptive transfer of activated CL4 T cells prior to ICT delayed tumor growth and significantly increased overall survival, compared to transfer of activated wildtype T cells with ICT (Figure 6F, 6G). This difference was observed in ACT groups without ICT, suggesting an increase in tumor-infiltrating antigen specific CD8\(^+\) TILs improved anti-tumor immunity and responses to ICT.

Taken together, these data show that change in tumor TCR\(\beta\) repertoires in ICT are distinct between responders and non-responders. Responding animals displayed oligoclonal expansion of tumor-specific TCR\(\beta\) clonotypes and a decrease in overall TCR\(\beta\) diversity earlier in time than
non-responding animals, while infiltrates in non-responders were dominated by large CD8+ T cell clones with an exhausted phenotype.

**DISCUSSION**

ICT increases T cell infiltration into tumors, and the clonal composition of TIL repertoires can be measured by TCRβ sequencing. Oligoclonal expansion of TILs post ICT is often indicative of antigen-specific T cell activation and proliferation, which is reflected by a decrease in tumor TCRβ diversity (28, 32, 33). Differences in TCRβ diversity of responding and non-responding tumors were previously found in some clinical studies (12, 34, 35), but not in others (14, 36, 37). However, most studies were limited to a single measurement of TCRβ diversity because multiple serial tumor biopsies are not feasible in most cancers. Our group and others have highlighted the utility of bilateral tumor models to track anti-tumor responses because of the high fidelity of T cell repertoires within the same animal (38, 39). For these reasons, we studied mouse models that allow tumor sampling at multiple time points during the response to ICT.

ICT responding murine tumors were characterized by a decrease in TCRβ diversity and an increase in clonality earlier in time than non-responding tumors. This supports findings that reduction of TIL TCRβ diversity in paired biopsies before and after ICT correlated with increased overall survival or responses to ICT (12-14, 34, 36). Although changes in peripheral blood TCRβ diversity were not detectable in our models, an increase in peripheral blood TCRβ clonality from pre- to 3 weeks post-ICT was associated with a positive outcome in clinical trials (22, 24). Our results highlight the importance of understanding TCRβ repertoire dynamics early, after the first few cycles of ICT, as these changes could inform the development of a T cell-based biomarker of response.

Clustering highly similar TCRβ sequences has been applied to understand how ICT changes TCRβ repertoire structure (the number and size of such clusters) (40, 41). TCRβ clusters can be linked to TIL phenotype (42, 43), and possibly tumor antigen-specificity. Our study adds to the field by modelling novel dynamics in TCRβ clusters that were significantly different between response groups (44). We found that the increase in select clusters was slower in responders compared to non-responders. Responding tumors had higher initial cluster sizes, which was reflected by increased clonality earlier in time. The specificity and phenotype of clones within these clusters that display dynamics associated with response are of great interest.

Decrease in TCRβ diversity associated with ICT response was partly attributed to clonal expansion of tumor-antigen specific T cells. Modelling of TCRβ cluster dynamics identified multiple TCRβ clusters that correlated with response, suggesting that TIL responses are likely directed against multiple antigens, some of which could be tumor-associated self-antigens. Oligoclonal expansion of gp70-AH1 specific T cells in AB1 and CT26 murine cancer models were similarly observed after different therapies including anti-CTLA-4 and anti-PD-L1 (28, 29, 45-47). Gp70-AH1 associated TCRβs were preferentially expressed in some but not all responding animals. This supports our previous work that an increased T cell response against a single tumor antigen favors, but is not necessary for ICT response. In addition to the nature of the cognate antigen, dynamics of antigen specific TCRβs will be informative of ICT outcomes (48, 49).
Oligoclonal expansion of TILs independently of clinical response to ICT might predict that the differentiation status of TILs, such as T cell stemness or exhaustion would be different in responders versus non-responders. Indeed, clonally expanded CD8+ TILs upregulated multiple genes associated with T cell exhaustion and dysfunction in non-responding tumors. Based on the dynamic modeling of TCRβ clusters, we speculate that treatment with ICT results in CD8+ T cell activation and proliferation, with CD8+ TILs becoming terminally differentiated faster in non-responding animals. Genes associated with T cell memory and stemness were significantly upregulated in non-expanded CD8+ TILs from responding tumors, and these non-expanded TILs could be a reservoir of cells that can differentiate into anti-tumor effector CTLs(8, 50). Further studies are required to elucidate the dynamics in TIL clone differentiation in relation to ICT outcomes.

Mapping TCRβ repertoire dynamics during other oncological therapies is of great interest, especially if they are administered in combination with ICT. Immunogenic chemotherapies that improve tumor-antigen cross presentation could increase tumor clonal expansion and reduce tumor TCRβ diversity, favoring ICT responses(51). In addition to the expression of T cell antigenic targets, T cell phenotype, and strength of T cell responses, we provide a strong rationale for mapping the dynamics in T cell repertoires as an important way forward for understanding and improving the response to ICT.

MATERIALS AND METHODS

Mice:

BALB/c mice were bred and maintained at the Animal Resource Centre (ARC; Murdoch, WA Australia) or Harry Perkins Medical Research Institute (Nedlands, WA, Australia). Clone 4 (CL4xThy1.1) TCR transgenic mice express a TCR that recognizes a MHC class I-restricted influenza A/PR/8 hemagglutinin (HA533-541) epitope. T cells expressed allelic marker Thy1.1. CL4xThy1.1 mice were bred at the Animal Resource Centre (ARC)(52). Female mice aged between 8 and 10 weeks of age were used for experiments. All mice were maintained under standard specific pathogen free housing conditions at the Harry Perkins Bioresources Facility (Nedlands, WA, Australia). All animal experiments were carried out in accordance with approved Harry Perkins Institute of Medical Research Animal Ethics guidelines and protocols.

Adoptive transfer of TCR Transgenic Splenocytes:

Spleens from CL4xThy1.1 mice were manually dissociated through 40 µm strainers with phosphate-buffered saline (PBS) supplemented with 2% Newborn Calf Serum (NCS; Life Technologies). Red blood cells were lysed with Pharm Lyse (BD Biosciences) and splenocytes were washed twice with PBS. CL4 T cells were activated with 0.5 µg anti-CD3 clone 17A2 and anti-CD28 clone 37.52 antibodies (Thermofisher), supplemented with 100 U/ml IL-2 (Peprotech) for 5 days prior to adoptive cell therapy. Mice were intravenously injected with 1 x 10^6 naïve or 2 x 10^6 activated cells where indicated.

Tumor cell lines and inoculation:

The murine malignant mesothelioma cell line AB1, and AB1 cell lines transfected with the influenza hemagglutinin (HA) from PR8/24/H1N1 strain (AB1-HA) were generated as previously described(52). Murine renal cell carcinoma RENCA was obtained from ATCC. Tumor cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 20 mM HEPES
(Gibco), 0.05 mM 2-mercaptoethanol (Sigma Aldrich), 100 units/ml benzylpenicillin (CSL), 50 µg/ml gentamicin (David Bull Labs), 10% NCS (Life Technologies) and 50 mg/ml of geneticin for AB1-HA only (G418; Life Technologies). Cells were grown to 80% confluence, and passed three times prior to inoculation. For all bilateral tumor experiments, the shaved right and left flanks of mice were inoculated subcutaneously with 5 x 10^5 tumor cells in each flank(20). The same number of cells was inoculated only into the shaved right hand flanks for single flank experiments.

**Immune checkpoint blockade (ICT) therapy:**

All immune checkpoint antibodies were administered by intraperitoneal injection. Combination anti-CTLA-4 clone 9H10 (100 µg) and anti-PD-L1 clone MIH5 (100 µg) was administered when tumors were 10-15 mm^2, with two additional doses of anti-PD-L1 (100 µg) every two days. All treatment was randomized and blinded. Mice were defined as responders when their tumor completely regressed and remained tumor-free for at least 4 weeks after treatment. Mice were designated as non-responders if their tumors grew to > 100 mm^2 within 4 weeks after the start of therapy. Mice that had a delay in tumor growth or partial regression before reaching >100 mm^2 were designated as partial responders. Where indicated, three doses of combination anti-CTLA-4 (100 µg) clone 9H10 (Bioxcell) and anti-OX-40 clone OX-86 (Bioxcell) (200 µg) were administered three days apart, starting when tumors were 20 - 30 mm^2 (25).

**Tumor debulking surgery and blood collection:**

Complete tumor debulking of the right flank was performed on the day prior to anti-CTLA-4 and anti-PD-L1 (pre-treatment), 2 days (+2), 4 days (+4) or 6 days (+6) post the first dose of treatment (28). Briefly, whole tumors were surgically excised when mice were under anesthesia (4% isoflurane in 100% oxygen at a flow rate of 2 liters/min). Surgical wounds were closed using Reflex wound clips (CellPoint Scientific). Mice received 0.1 mg/kg of buprenorphine, 1 hour prior to, 6 and 24 hours after surgery for pain relief. In a different series of experiments, serial peripheral blood samples from animals were collected one day prior to anti-CTLA-4 and anti-PD-L1 (pre-treatment), 3 days (+3) and 6 days (+6) post first dose of treatment via the retro-orbital sinus.

**Tissue Processing:**

Surgically excised or harvested tumors were dissociated using gentleMACS (Miltenyi Biotec) as per manufacturer’s instructions. Briefly, whole tumors were minced into 1 mm^3 pieces using a scalpel and added to 2.35 mL RPMI 1640, 100 µL enzyme D, 50 µL enzyme R and 12.5 µL enzyme GentleMACS protocol 37C_m_TDK_1 was used to dissociate the tumor tissue. Dissociated tumor tissue was then strained through a 70 µm strainer and stored in Qiagen RNAprotect Cell Reagent (Qiagen #76526) prior to RNA extraction, or flow cytometry. Heparinized peripheral blood was incubated with BD red blood cell (RBC) lysis buffer for 5 minutes at room temperature. Samples were washed twice in PBS + 2% NCS and stored in Qiagen RNA cell protect prior to RNA extraction.

**Fluorescence Activated Cell sorting:**

Single cell suspensions were stained for 30 minutes with CD4[BV421] (Biolegend clone GK1.5), CD45[APC] (Biolegend clone 30-F11), CD8[APC-eFlour780] (Invitrogen clone 53-6.7), CD3[PE-Cy7] (Biolegend, clone 17A2) antibodies, and a viability dye[efluor450]
Live CD45^+CD3^+CD4^+ or Live CD45^+CD3^+CD8^+ cells were sorted on a BD FACS Melody cell sorter. Sorted cells were collected directly into tubes containing RNAProtect.

**Bulk TCRβ sequencing:**

RNA extracted was performed using Qiagen RNeasy Plus Micro kits. TCRβ amplicon libraries were generated as previously described(31). 1 μg of sorted T cell or bulk tumor RNA was reverse transcribed to cDNA using SMARTerIIA-PID 5’RACE and TCRβ constant region primers, Invitrogen SuperScript IV reverse transcriptase (18090050), Invitrogen RNaseOUT ribonuclease inhibitor (10777-019) and dNTP mix (Qiagen 1039394, 1039395, 1039396, 1039397). Primer IDentifiers (PIIDs) account for sequencing error and amplification bias in downstream PCRs by creating a unique identifier for each RNA transcript that can be deconvoluted TCRseq pre-processing. The 5’ Illumina sequencing adaptor was incorporated in a PCR using Roche KAPA HiFi HotStart ReadyMix (KK2602). An instep TCRβ constant region and outstep priming site 3’ primer was used for the 3’ cDNA end. The 5’ primer also contained an outstep priming site to preserve the adaptor sequence. The 3’ Illumina sequencing adaptor was incorporated in a second PCR reaction using the compliment primers to the priming sites incorporated in the previous PCR. cDNA was purified between reactions using Beckman Coulter AMPure XP magnetic beads (A63882). 300bp paired end sequencing was performed on an Illumina HiSeq at the Institute for Immunology and Infectious Diseases (IIID) (Murdoch, WA, Australia).

**TCRβ sequencing data analysis:**

Data processing, aggregation of PIIDs and alignment of CDR3 sequences to the IMGT/V-QUEST reference genome were performed using repertoire analysis software based on MIGEC and MiXCR pipelines(53, 54). Only sequences with UMIs were aligned. TCRβ CDR3s containing fewer than 8 or more than 20 amino acid residues were excluded from analysis, consistent with approximately 3 standard deviations away from the average CDR3 length. Clonal T cells are defined by distinct TCRβ CDR3 amino acid regions. TCRβ CDR3 sequence for the CL4 clone (CASGETGTNERLFF) was previously determined by bulk TCRβ sequencing of sorted CD8^+ splenocytes from CL4xThy1.1 mice Repertoires within an experiment were down sampled to the smallest sample to ensure comparability. All diversity and dissimilarity indexes were computed using the R package vegan. Shannon’s diversity transforms each clone’s proportion of a repertoire into a weighting and sums them all together, producing a unique numerical value for each unique distribution of clones in a repertoire. Shannon’s diversity

\[ H = -\sum_{i=1}^{n} p_i \log(p_i) \]

was normalized by dividing by the natural log of the number of unique clones in the repertoire

\[ H_{\text{norm}} = \frac{-\sum_{i=1}^{n} p_i \log(p_i)}{\log(n)} \]

producing a value between 0 and 1. p represents the proportion of each clone of the whole repertoire. Higher sums indicate a more diverse repertoire and lower sums indicate a more clonally expanded repertoire. Renyi diversity
\[ qH = \frac{1}{1-q} \ln \left( \sum_{i=1}^{n} p_i^q \right) \]

is a parametrization of Shannon’s diversity that uniquely defines the distribution of clones in a repertoire. Morisita-Horn index

\[ C_H = \frac{2 \sum_{i=1}^{S} x_i y_i}{\sum_{i=1}^{S} x_i^2 + \sum_{i=1}^{S} y_i^2} \]

was used to compare TCRseq libraries. The Morisita-Horn index has a maximum value of 1 where both TCRseq libraries are identical and 0 where they have no clones in common.

**Neural network based TCRβ clustering:**

To group TCRβ sequences with similar features, we applied a convolutional neural network (CNN) with a variational auto-encoder 1 hot encoding was used to prepare TCR sequences for the CNN(26). Briefly, each location in a CDR3 sequence was transformed into a vector length 20 (labelled for each genetically coded amino acid). For the residue present in that location, the vector position labelled with that residue was set to 1 and all other values set to 0. This transformed CDR3 sequence was then passed to the (CNN). Each node in the first layer of the CNN was trained to recognise a specific residue or VJ gene. Training was setup with sparsity 1.0, explained variance 0.99 and to only use sequence information. Hidden layers of the CNN were trained to recognise features of TCRs that minimised dispersion in the final output. The variational auto-encoder removes residue sequence combinations that are possible, but never arise in the sampled TCRβ CDR3 sequences (e.g., long sequences of a repeated residue). Output from the CNN was clustered using phenograph with a sample size of 40000. These clusters were subsequently used to link TCRs between mice.

**Multi-Dimensional Scaling:**

TCRseq data contains count values for each T cell clone by proportional detection of TCR mRNA. Counts are not continuous so characterisation by mean or standard deviation do not apply. By extension, PCA is not appropriate to draw similarities between samples by comparing the counts of TCRs. Multi-dimensional scaling is a dimensionality reduction technique that does not assume linear relationships or continuous variables. 2 dimensions were chosen to represent TCR repertoires in lower dimensional space. Random locations were selected for the initialization of MDS. The Morisita-Horn metric was chosen to calculate distances between samples. Non-parametric regression was chosen to predict distances in 2 dimensions from the original data. Kruskal’s stress was used to compare stress between predictions and original data. Gradient descent was used to minimize stress and converged after 20 iterations.

**Modelling dynamics of individual TCRβ clusters:**

We developed a mathematical model based on 1) differences in the mean of TCRβs between the two groups (responders and non-responders), 2) increase/decrease in TCRβs with time, and if there were differences in the changes, was this increase/decrease different between
the two response groups. We first constructed two (X) features. X₁ is a binary variable where 1 represents responders, and 0 represents non-responders. X₂ was a discrete variable representing time during the treatment schedule TCRβ clusters were measured. For each cluster, we modelled the relation between these features and the response variables: TCRβ clusters (Y).

As the response variable are TCRβs from individual animals, we could not consider our response variable as continuous random variables. We therefore used a generalised linear model where the response variable was given by Poisson distribution, with parameter \( \lambda \). For the linear part of the model, we considered the log-link function and the interaction between the features:

\[
Y \sim \text{Poisson}(\lambda),
\]

\[
\log(\lambda) = \Delta_0 + \Delta_1 X_1 + \Delta_2 X_2 + \Delta_3 X_1 X_2.
\]

Since \( E(Y) = \lambda \) (the expectation of the response variable equals \( \lambda \)), we considered that \( E(Y) = \exp\{\Delta_0 + \Delta_1 X_1 + \Delta_2 X_2 + \Delta_3 X_1 X_2\} \). That is, the log-expectation equaled the linear regression part of the model ([55]).

To verify the significance of \( \Delta \)s, we used a bootstrap method ([56]) to compute the 95% confidence interval for parameters \( \Delta_1 \), \( \Delta_2 \), and \( \Delta_3 \). Then, we selected the clusters where the three confidence intervals do not contain the value zero simultaneously. That is, we rejected (marginally) each hypothesis \( H : \delta = 0 \), for \( i = 1, 2, 3 \) at the 5% significance level.

**Single cell sequencing:**

For single cell analysis, surgical excision of flank tumors was performed on ICT treated animals. Warm tissue dissociation was performed as described above. 50,000 to 100,000 CD45⁺ cells from each tumors were sorted. Sorted cells underwent methanol fixation, as previously described ([57]), prior to library preparation. All single-cell libraries were constructed using the 10x Chromium 5’ workflow as per the manufacturers’ directions. All libraries were quantified with qPCR using the NEBnext Library Quant Kit for Illumina and checked for fragment size using the TapeStation D1000 kit (Agilent). The libraries were pooled in equimolar concentration for a total pooled concentration of 2 nM. 10x single-cell libraries were sequenced using the Illumina NovaSeq 6000 and S2 flow cells (100 cycle kit).

**scRNA-seq data processing:**

Cell ranger version 4.0.0 (10x Genomics) was used to process the data from the 10X-5’ single cell RNA-seq and TCR-seq experiments using mouse reference mm10. Gene counts were normalized with Seurat v3.1 using SCtransform and scale-factor transform methods. Low quality cells that had either greater than 10% mitochondria content or less than 500 UMIs were filtered. Seurat’s CellCycleScoring function was used for determining cell-cycle phases. Identification of clusters of single cells was performed by dimensional reduction using PCA and by applying graph clustering algorithms to the reduced components. Visualization of the results was performed with uniform manifold approximation and projection (UMAP) and t-distributed stochastic neighbor embedding (t-SNE). The clusters were annotated by cell types derived using ScMatch and SingleR with the help of FANTOM5 reference datasets. Joint analysis of merged...
samples was performed using R package Harmony. Sub clustering and gene expression were visualized with Loupe Cell Browser v.5.0.0 (10X Genomics)

Statistics:

All statistical tests were performed in R or in GraphPad Prism. Statistics on comparisons between flanks of animals were calculated by paired students t-tests. Diversity statistics between responders and non-responders were compared using students t-tests. Hierarchical clustering was used to group similar gene activity in single cell populations. Morisita-Horn index was used to compare TCRseq libraries. Random subset permutations of mice were used to compare shared clones. Figures were made with BioRender.com.

Supplementary Materials

References and Notes


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Acknowledgments: The authors acknowledge the facilities, and the scientific and technical assistance of the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments. The authors also acknowledge Perkins Institute Bioresources Staff for their assistance with animal husbandry.

Funding: All work was supported by grants from the iCare Dust Diseases Board, Cancer Council Western Australia CCGS, and the University of Western Australia Raine Foundation. JK received a scholarship from the iCare Dust Diseases Board. JC was supported by Cancer Council Western Australia Fellowship. WJL was supported by fellowships from NHMRC and the Simon Lee foundation. This work was supported in part by a collaborative cancer research grant from the Cancer Research Trust “Enabling advanced single-cell cancer genomics in Western Australia” and an enabling grant from the Cancer Council of Western Australia. Genomic data was generated at the Australian Cancer Research Foundation Centre for Advanced Cancer Genomics.

Author contributions:
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Funding acquisition: AN, RL, WJL, JC
Project administration: JC
Supervision: MW, RL, WJL, JC
Writing – original draft: JK, JC
Writing – review & editing: All authors

Competing interests:
LB is employed by the company JJP Biologics. WJL received research funding from Douglas Pharmaceuticals, AstraZeneca, ENA therapeutics, consultancy for Douglas Pharmaceuticals and MSD. AN is on the advisory board of Boehringer Ingelheim, Bayer, Roche, BMS; and received research funding from AstraZeneca. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data and materials availability: TCRseq data and single cell 5’VDJ RNAseq and TCRseq data will be made available on a public repository upon acceptance.

Figures:

Fig. 1. Dynamics in blood TCRβ repertoires were similar between ICT responders and non-responders. (A) Experimental timeline when blood samples were acquired, and (B) AB1-HA tumor growth curves of PBS and ICT (anti-CTLA-4 + anti-PD-L1) treated animals. PBS (black) treated, ICT responding (blue) and non-responding (red) animals represented. Dotted lines on growth curves represent when ICT was administered. (C, D) Change in TCRβ repertoire diversity in sequential blood samples from individual animals, represented by Shannon’s Diversity Indices. (E) Circos plots represent the distribution of most abundant clones across 3 timepoints. Each colored trine represents one time point, and connecting bands represent individual TCRβ clones. The width of each band connecting the trines depicts the frequency of that particular clone at both time points. (F) Number of persistent and abundant TCRβ clones, defined as any TCRβ clones that ranked top 100 in abundance at more than 1 timepoint. (G, H) Sequential change in the frequency of HA-specific CD8+ T cells in different groups. Each dot represents one animal, mean ± SEM represented in dot plots. One-way ANOVA with Kruskal-Wallis tests were used to compare between groups at each time-point. One-way ANOVA with Friedman tests were used to compare TCRβ repertoires in individual animals over time. *p < 0.05.

Fig. 2. Bilateral tumors have highly similar TCRβ repertoires. (A) Experimental timeline, and (B) tumor growth of PBS (black) and ICT (anti-CTLA-4 + anti-OX-40, red) treated AB1-HA bearing animals. Animals were euthanized so that tumors were size matched (n=5/group). (C) Survival curves of a different cohort of AB1-HA bearing animals undergoing the same ICT (n=10/group). (D) Number of total and unique clones derived from bulk TCRβ sequencing of sorted CD4+ and CD8+ TILs. (E) Morisita-Horn Overlap index values comparing TCRβ repertoires of bilateral tumors within animals, or tumors
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**Fig. 3.** Checkpoint blockade responders display clonal expansion of unique tumor TCRβs earlier than non-responding mice. (A) Mice with bilateral AB1 or RENCA murine tumors were administered anti-CTLA-4 and anti-PD-L1 ICT. Each group had right flank tumors surgically excised before ICT, or every two days after ICT 6-8 mice for each response group at each time point in each model. Table depicts the number of tumors per group sequenced (B) Number of total and unique TCRβ clones in tumors from responding (blue) and non-responding (red) mice across 4 timepoints. (C) TCRβ clones were ranked on their abundance within each tumor repertoire, and proportions of ranked TCRβs were plotted in relation to time and response. (D) Tumor TCRβ diversity represented by a normalized Shannon’s Index in responding or non-responding mice. Higher indices indicate a more diverse repertoire and lower values correspond to more clonal repertoires. (E) The number of large TCRβ clones (occupying > 0.5% of each repertoire) compared between ICT response, and tumor models across time. (F) Morisita-Horn Overlap index values of pairwise comparisons of TCRβ repertoires between tumors from the same group within animals, or tumors between different animals. CD4+ and CD8+ TILs, ICT treatment plotted. All box plots depict mean ± SEM of individual animals, n=6-8 mice/group. Mann-Whitney test used to compare diversity index between responders vs non-responders. *p < 0.05, ***p < 0.005, ****p < 0.001

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**Fig. 5.** Clonally expanded CD8+ TILs have a gene expression profile associated with T cell dysfunction and exhaustion. (A) AB1 tumor growth curves of ICT (anti-CTLA-4 + anti-PD-L1) treated animals (n = 3/group). Dotted lines on growth curves represent when ICT was administered. CD45+ T cells from tumors excised 6 days post ICT sent for single cell analysis. (B) Proportion of different annotated cell types based on transcriptome of individual cells in responding and non-responding animals. (C) tSNE clustering of all 18029 cells, labelled by color and cell type. (D) Cells with clonal TCRβ sequences that are > 1% of the total TCRβ+ cells (labelled purple), < 1% other clones labelled brown. (E) Sub-clustering of CD8+ T cells with annotated TCRs. CD8+TCR+
cells that were large clones in responders (n = 569), non-responders (n = 1731), other
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Heatmap of differentially expressed genes associated with T cell dysfunction in
individual CD8\(^+\)TCR\(^{\beta}\) cells that were large clones (>1%) and all other CD8\(^+\)TCR\(^{\beta}\) cells,
regardless of response. (G) Heatmap of differentially expressed genes between CD8\(^+\)
large, other clones in responding versus non-responding(n = 1947) tumors. Log2fold
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**Fig. 6. Tumor antigen associated TCR\(^{\beta}\) clonotypes increase earlier in time in ICT
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responding and non-responding (A) AB1 and (B) RENCA tumors across time. (C) The
distribution of each gp70-AH1 associated TCR\(^{\beta}\) clone across individual animals
represented as a heatmap. Each rectangle on the heatmap represents an individual animal
at a particular time points, and ICT response. (D) The number of cells with gp70-AH1
antigen associated TCR\(^{\beta}\) clonotypes in CD8\(^+\) large clones, or other clones derived from
single cell data. (E) Experimental outline, (F) mean tumor growth and (G) overall
survival of adoptive cell therapy (ACT) of activated HA-specific CTLs into AB1-HA
bearing animals in combination with (anti-CTLA-4 + anti-PD-L1) ICT. All box plots
depict mean ± SEM of individual animals. Mann-Whitney test used to compare the
number of TCR\(^{\beta}\) clonotypes between responders vs non-responders. Mantel-Cox Log
Rank test used to compare survival between groups *p < 0.05, **p < 0.005, ****p <
0.001

**Fig. S1. Blood TCR\(^{\beta}\) repertoire metrics.** (A) Total, (B) unique number of TCR\(^{\beta}\) sequences, and
(C) number of endogenous CL4 clones from sequential blood samples of PBS treated,
ICT non-responding, and responding animals. (D) Timeline and (E) tumor growth curves
of experiment setup to track TCR transgenic tumor-antigen specific T cells in ICT treated
animal. Each dot represents one animal, mean ± SEM represented in dot plots. Dots
connected by line represents sequential samples from an individual animal. One-way
ANOVA with Kruskal-Wallis tests were used to compare between groups at each time-
point.

**Fig. S2. TCR\(^{\beta}\) repertoire features in bilateral tumors.** (A) Tumor sizes, total and unique
number of TCR\(^{\beta}\) clonotypes in ICT (anti-CTLA-4 + anti-OX-40) and PBS treated
animals, plotted by (B) cell type and (C) flanks. Each dot represents one sample, mean ±
SEM represented in dot plots. Dots connected by line represents bilateral, paired samples
from an individual animal. Wilcoxon-matched-pairs signed rank test used to compare
paired samples.

**Fig. S3. Dynamic tumor TCR\(^{\beta}\) repertoire features in ICT responders and non-responders.**
(A) Total and unique number of TCR\(^{\beta}\) clonotypes, (B) Renyi entropy curves representing
tumor TCR\(^{\beta}\) diversity. (C) TCR\(^{\beta}\) diversity of responding and non-responding tumors
depicted over time. Wilcoxon’s ranked test was used to compare the mean diversity index
in each group against the global mean *p < 0.05, **p < 0.005, ****p< 0.001. (D)
Heatmap comparing TCR\(^{\beta}\) repertoire overlap in all samples. Morisita-Horn Overlap
index between all tumors for both models, ICB response and all time-points represented.
(E) Mean number of clones shared by the number of different animals in responding and non-responding groups.

**Fig. S4. Generalized linear model of dynamic change.** Dynamic changes of individual TCRβ clusters were modelled based on coefficients that measured different dynamic features. Graphs represent some patterns of change associated with different combinations of coefficient values.

**Fig. S5. Differentially expressed gene expression of individual CD8\(^+\) TILs.** (A) Heatmap of significant differentially expressed genes and (B) T cell exhaustion geneset in CD8\(^+\) TILs from the large clones, other clones, ICT responders and non-responder groups.
Figures

Figure 1

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Figure 6

Tumor antigen associated TCRβ clonotypes increase earlier in time in ICT responders. The number, and proportion of gp70-AH1 associated TCRβ clones in ICT responding and non-responding (A) AB1 and (B) RENCA tumors across time. (C) The distribution of each gp70-AH1 associated TCRβ clone across individual animals represented as a heatmap. Each rectangle on the heatmap represents an individual animal at a particular time points, and ICT response. (D) The number of cells with gp70-AH1 antigen associated TCRβ clonotypes in CD8+ large clones, or other clones derived from single cell data. (E) Experimental outline, (F) mean tumor growth and (G) overall survival of adoptive cell therapy (ACT) of activated HA-specific CTLs into AB1-HA bearing animals in combination with (anti-CTLA-4 + anti-PD-L1) ICT. All box plots depict mean ± SEM of individual animals. Mann-Whitney test used to compare the number of TCRβ clonotypes between responders vs non-responders. Mantel-Cox Log Rank test used to compare survival between groups *p < 0.05, ***p < 0.005, ****p < 0.001

Supplementary Files

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- SuppFigswithLegendNatComm.pdf
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