Tumor-specific draining lymph node CD8 T cells orchestrate an anti-tumor response to neoadjuvant PD-1 immune checkpoint blockade

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Abstract

PD-1 immune checkpoint blockade (ICB) with platinum chemotherapy (ChemoIO) has transformed treatment for resectable non-small cell lung cancer (NSCLC). PD-1 ICB was established and created to reinvigorate tumor T cells and reduce T cell exhaustion. However, the impact of PD-1 blockade on the induction and maintenance of tumor-relevant tdLN T cells and functional T cell memory in the context of surgical resection is not fully understood. We hypothesized that T cells activated in the tumor draining lymph node (tdLN) also respond to PD-I ICB. We assessed clonal dynamics of tumor-specific tdLN T cell clones by using single-cell TCR barcoding integrated with transcriptional profiling (scRNA sequencing) by using machine learning (ML) and bioinformatic approaches. We displayed that within the tumor tdLN-paired clones were less exhausted than clones not clonally linked to the tdLN. We established that there are two distinct LN-paired anti-tumor clonal populations: those that directly link from the tdLN and tumor (tdLN-tumor paired) with tumor killing capacity, and clones that circulate within the blood with cytotoxic memory surveillance potential. Furthermore, the transcriptional distinction of these two LN-paired subsets was set in the earliest memory state of expanded tumor-specific tdLN T cells and reflected in the tumor as similarly, more differentiated, effector subsets. ChemoIO was also found to directly impact tdLN-paired clonal diversity and memory phenotypic differentiation. Finally, our findings demonstrate that ChemoIO is enhancing the cytotoxic function of circulating LN-paired clones in the tdLN and subsequently also altering circulating blood LN-paired clonal phenotype. Together our results established that tumor-relevant T cells are transcriptionally altered by PD-1 ICB locally in the tdLN and provide evidence that lymph node derived T cells are a primary source of anti-tumor immunity in surgically resectable NSCLC.

Introduction

Complete oncologic resection of solid tumors typically includes removal of regional lymph nodes, both to ensure accurate staging and to eliminate sites of microscopic spread. However, these lymph nodes may serve as critical reservoirs for treatment-responsive T cells. In melanoma models, PD-1 blockade facilitates priming of naïve T cells by dendritic cells within the tumor draining lymph nodes (tdLNs).[20] In addition, previous reports show that tdLN maintain a population of pre-formed stem-like CD8 T cells with distinct phenotypic and functional properties that are responsive to PD-1 blockade.[21] When immune checkpoint blockade (ICB) immunotherapy is administered only after surgery, the opportunity to engage these lymph node–derived precursors may be diminished, potentially reducing systemic efficacy.

In non-small cell lung cancer (NSCLC), the contribution of the benign tdLNs to the anti-tumor response is poorly defined. Tumor-specific antigens are typically unknown in patients, making it challenging to identify tumor-reactive T cells within the tdLN. Prior studies using T cell receptor (TCR) lineage (Pauken et al) have demonstrated that tumor matched T cells in the blood are more activated than unmatched clones, providing a strategy to track tumor-relevant T cells across tissues.[3] Pai et al applied this method to metastatic lung cancer patients' tdLNs and tumors, where they determined that tumor TCR-linked T cells within the tdLN have a subset of progenitor exhausted T cells. However, the role of benign tdLN-paired clones in early-stage, surgically resectable NSCLC is unknown. Moreover, the functional distinctions between tdLN-paired and tumor-restricted T cell populations remain unclear.

In conclusion, the impact of PD-1 blockade on the induction and maintenance of tumor-specific, functional T cell memory in the context of surgical resection is not fully understood.[16]

Whether tdLN tumor-specific CD8 T cells respond to PD-1 ICB has yet to be delineated, particularly in the context of surgically resected NSCLC. Here we define the contribution of tdLN-derived CD8 T cells in resectable NSCLC patients, map their clonal relationships across tumor, blood and lymph node compartments, and demonstrate that PD-1 blockade modulates memory-like T cell populations within the tdLN. These findings demonstrate that the regional tdLNs are a primary source for systemic anti-tumor response and provide a strong rationale for both lymph node preservation and initiating immunotherapy prior to lymph node resection.

Results

Tumor-specific clones in early-stage NSCLC are found in the tdLN, blood, and tumor have different tissue lineage.

To assess the CD8 T cell response of neoadjuvant chemotherapy and PD-1 ICB in early-stage NSCLC within the tumor, tdLN (defined as regional intralobar N1 lymph nodes closest to the tumor) and circulating blood, tissues samples were collected from clinically stage-matched treated and treatment-naive early-stage NSCLC patients at tumor surgical resection. Tumor, tdLN, blood samples were collected from eight patients who received neoadjuvant PD-1 ICB (Nivolumab or Pembrolizumab) and platinum-based chemotherapy and from six patients who were treatment naive.[2] Samples were then sorted for CD45+ CD3+ CD8 T cells and sequenced for paired single cell (sc)RNA and scTCR sequencing (Figure 1a). A T cell clone was defined by a collection of T cells with the same unique combination of all TCR alpha and TCR beta cdr3 amino acid regions. [3,4,5] From the 672,886 CD8 T cells sequenced, 390,375 CD8 T cells had both TCR alpha and TCR beta regions sequenced and RNA sequencing (Figure 1b).

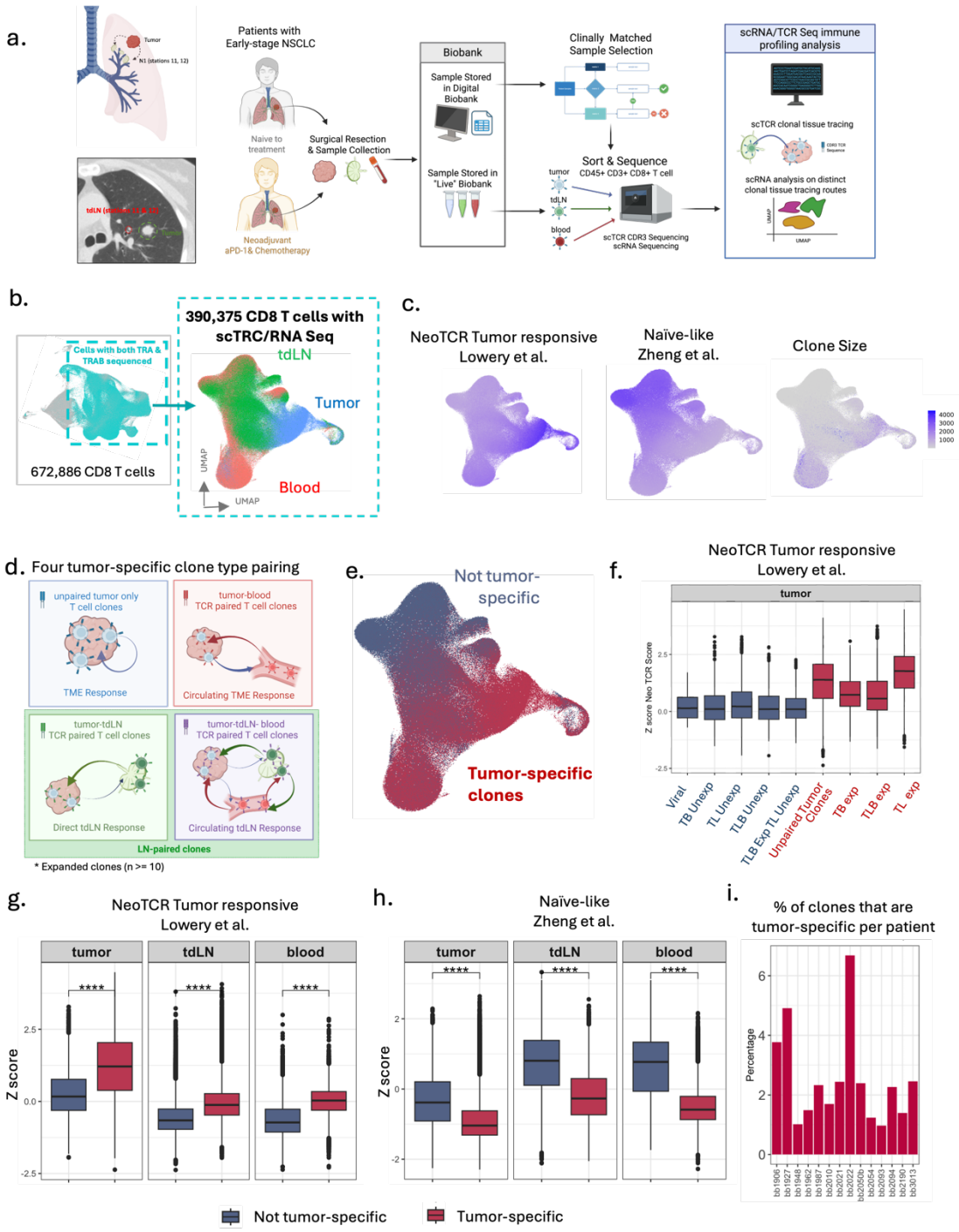

Figure 1: Defining and validating tumor-specific T cell clones in early-stage non-small cell lung cancer.

a. Schematic of patient sample collection sorting, sequencing and analysis pipeline. b. Selection of sequenced CD8 T cells (672,886 total) which have both scRNA and alpha and beta chains TCR sequenced (390,375 total) followed by dimensional UMAP dimensional reduction. c. Feature plots of NeoTCR tumor responsive signature, naïve-like signature and clone size. d. Schematic of tumor-specific clone-type pairings based on TCR tissue lineage tracing. e. UMAP labeling T cell tumor-specificity based TCR tissue lineage tracing described in d. f. Validation of tumor-specific clone type pairing categorization. Clonal average z-score of tumor T cell NeoTCR tumor responsive signature by TCR tissue lineage tracing, expansion and if the TCR was found as a viral epitope on the VDJ database. g. NeoTCR tumor responsive signature (Lowery et al.) clonal average z-score applied to each tissue by clonal tumor-specificity based on TCR lineage tracing described in d. (t-test, $p < 0.0001$). h. Naïve-like signature (Zheng et al.) clonal average z-score applied to each tissue by clonal tumor-specificity based on TCR lineage tracing described in d. (t-test, $p < 0.0001$). i. Percent of paired sc RNA/TCR clones that are tumor-specific per patient.

We followed this with clonal tissue lineage tracing, using the TCR CDR3 region as a clonal barcode across tissues. Clones were then categorized by whether the TCR was listed as a viral epitope in a TCR VDJ database, by T cell tissue presence within the clone, and whether the clone was expanded (at least 10 T cells) or unexpanded (less than 10 T cells). Previous work has described clonal expansion within the tumor relating to tumor-reactive T cells. [3,4,7] To determine tumor relevance, we defined clonal tumor-specificity as expanded clones with T cells present in the tumor. Potential "bystander" CD8 T cells defined by TCRs listed as viral epitope species in a TCR VDJ database were not included as tumor-specific clones (Figure 1d).[3,4,5,6]

Tumor-specific clone categories included unpaired tumor only clones with expanded TCRs only found in the tumor, LN-paired expanded clones (clones with TCR lineage with the tdLN), and tumor-blood clones with TCR only present in tumor and blood. LN-paired clones were subdivided into two categories: circulating LN-paired clones found in tumor and tdLN and circulating in peripheral blood, and direct LN-paired clones that had TCR lineage only between tdLN directly to tumor (Figure 1d). Of the 112,553 unique TCR clones found across

all three tissues, 4% of clones were tumor-specific, on average 2.5% of clones per patient (Figure 1i).

This clonal labeling was then supported by comparing non-tumor-specific clones (viral, unexpanded, not clonally linked to the tumor) and tumor-specific T cell clonal NeoTCR CD8 T cell tumor-reactivity score from Lowery et al. and Naive-like score from Zheng et al.[7,8] Within the tumor, tdLN and blood, tumor-specific clone categories were found to increase in tumor-reactivity scoring and decrease in Naive-like scoring relative to non-tumor-specific clone categories. Interestingly, viral clones had low Neo TCR tumor-reactivity but were less naive than the unexpanded clones (Figure 1f-h).

Circulating LN-paired clones that were primarily found in the blood and had less than 10 tumors and tdLN had low NeoTCR score and higher Naive score and potentially represented another subset of bystander T cells. Therefore, circulating LN-paired clones were only considered tumor-specific if there were at least 10 cells found within the tumor and tdLN. (Figure 1f).

Tumor-specific clonal function across tdLN, blood, and tumor

Prior lineage training scTCR/RNA analysis involving the tdLN have focused on late-stage metastatic disease. Thus, we set out to explore how tumor-specific T cells phenotype in the benign tdLN of early stage NSCLC patients may be altered by the tissue environment. To do this, we compared functional scoring and gene expression of tumor-specific clones within blood, tdLN, and tumor. Tumor T cells were the most effector-like, terminally exhausted and had high expression of CD39+CD69+ terminal CD8 T cells signatures from previous

literature.[8,9] On the other hand, tdLN and blood T cells had higher expression of Naive/CM-like and memory-like scores than tumor T cells. (**Supplement Figure 1**)[8,9] Blood circulating T cell clones also had in increased expression of TEMRA and cytotoxic signatures relative to tdLN, as was found in previous literature.[8] Tumor T cells also had increased expression of TEMRA signature expression compared to tdLN.[8] TdLN T cells also displayed unique expression of the lymph node homing memory marker IL7R, while blood T cells had increased expression of migration marker CX3CR1 and memory marker KLF2. (**Supplement Figure 1**) Similar patterns between tissue functions were found within direct LN-paired clones and circulating LN-paired clones. Within both direct LN-paired clones and circulating LN-paired clones, lymph node T cells were increased in Naive-like scores, while the same clones in the tumor were increased in tissue resident memory function, and dysfunction.[8,9] (**Supplement Figure 1**) These results show that T cells go from a quiescent, less dysfunctional, state in the tdLN to a more dysfunctional state in the tumor and lose plasticity. We hypothesize that without chronic antigen and immune suppression T cells within the tdLN and blood can expand without dysfunction and that these clones become dysfunctional within the tumor.

Tumor-draining lymph nodes' clonal lineage impacts tumor T cell function

We aimed to determine if lineage with tdLN impacted the tumor T cell landscape. To determine whether tumor T cell function is influenced by the clonal contribution and lineage of the tdLN, we explored the tumor T cell response of all tumor-specific clones (Unpaired tumor only clones, direct tumor-tdLN clones, circulating LN-paired clones, tumor-blood). Unpaired tumor-only clones represented the impact of the tumor microenvironment (TME), without

contribution of the tdLN. On the other hand, circulating LN-paired clones and direct LN-paired clones represent the contribution of the tdLN in the tumor T cell response. Finally, tumor-blood clones represent clones that we hypothesize originate from the tumor that also circulate the blood.

We first explored the tdLN clonal contribution within the tumor by comparing the scTCR sequencing based clonal function of tumor-specific clones. The relative abundance of tdLN TCR linked cells within the tumor was higher than clones only found in the tumor. TdLN-linked cells contributed on average 75% of tumor T cells per patient, while unpaired tumor only clones contained 22% of tumor T cells and tumor-blood clones were only 3% of cells. (Figure 2b) Tumor-specific clones paired to the LN were more clonally diverse (number of unique TCRs) per 5000 cells than unpaired tumor only clones. There were on average 73.6 tumor-specific clones with LN clonal lineage compared to 22.9 unpaired tumor only clones per 5000 cells. (Figure 2a) Previous work hypothesizes that clonal diversity may relate to increased ability for T cells to find tumor antigens and thus a beneficial tumor response. These results indicate that the tdLN has a large contribution towards the clonal landscape of T cells within the tumor.

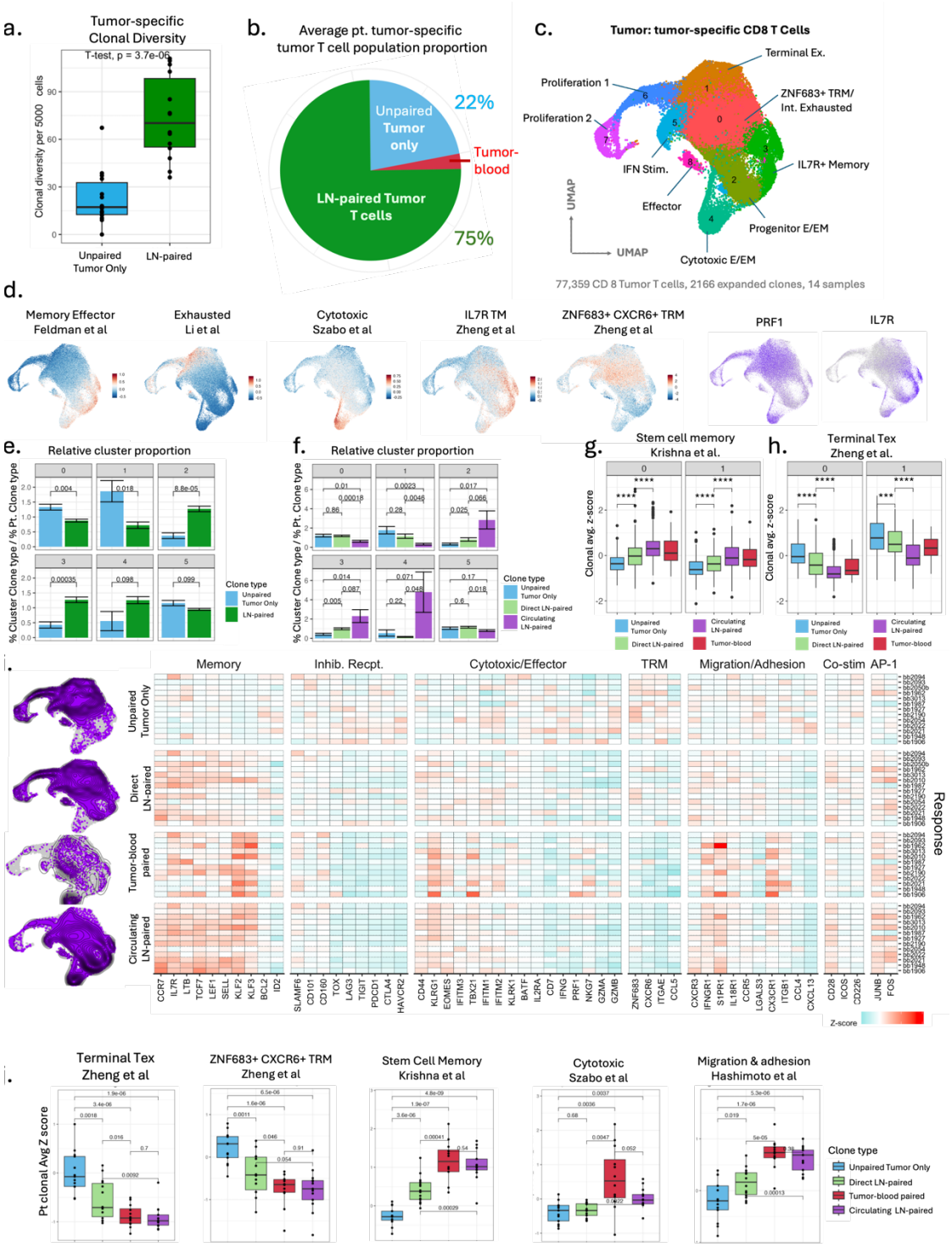

Figure 2:LN-paired T cells within the tumor are distinct from unpaired tumor T cells.

a. Patient tumor-specific clonal diversity (number of unique TCRs per 5,000 cells) of unpaired tumor only clones versus LN-paired clones (paired t-test, $p < 0.0001$). b. Average patient tumor-specific tumor T cell population proportion based on clonal lineage with the LN (clone

type pairing described in 2.1 d.). c. UMAP dimensional reduction of tumor-specific CD8 T cells, including 77,359 T cells and 2,166 expanded clones, 14 patient tumor samples. Nine T cell cluster subsets were generated. d. Feature plot of signatures relating to T cell function normalized using z-score (mean = 0, STD = 1) and of gene expressions *PRF1* and *IL7R*. e. Relative clone type cluster proportion of unpaired tumor only clones and LN-paired clones (percent of clone type T cells within cluster for each patient/percent of clone type T cells for each patient). A value of 1 represents a cluster with clone type population proportions the same as the patient tumor clone type population proportions. f. Relative clone type cluster proportion of unpaired tumor only clones, direct LN-paired clones, and circulating LN-paired (percent of clone type T cells within cluster for each patient/percent of clone type T cells for each patient). A value of 1 represents a cluster with clone type population proportions the same as the patient tumor clone type population proportions. g-h. The clonal average z-score of stem cell-like (g.) and terminal exhaustion (h.) signatures within ZNF683+ TRM/Int Exhausted and Terminal Ex. clusters by clone type pairing. i. Heatmap of average z-score clonal expression levels per patient of genes representing a range of T cell functions (memory, inhibitor receptors, cytotoxic/effector, etc.) for each tumor-specific clonal type pairing category (unpaired tumor only clones, direct tumor-tdLN clones, circulating LN-paired clones, tumor-blood). The left of the heatmap are the density plots of cells per clone type pairing. j. Patient clonal average z-score of signatures from literature relating to T cell function by clone type within the tumor (t-test, ANOVA, all $p < 0.0001$, except ZNF683+ CXCR6+ TRM $p < 0.01$).

For phenotypic analysis we then explored the tumor T cell tumor-specific cellular landscape by unsupervised dimensionality reduction of all tumor-specific tumor T cells. In total 77,349 CD8 T cells and 2,166 expanded clones from 14 patients were dimensionally reduced to 9 distinct clusters and T cell subtypes. (**Figure 2c**) Clusters ranged from memory state to dysfunctional and exhausted. Three clusters were more memory-like (IL7R+ Memory, Progenitor E/EM, and cytotoxic E/EM), defined by increase in memory markers *IL7R*, *TCF7*, *LEF1*, and *SELL.* These clusters were also enriched the Memory Effector signature by Feldman et al and Responsive Stem Cell Memory (CD69-CD39-) signature by Krishna et al.[7,9] IL7R+ Memory was defined by increased expression of *IL7R* and low expression of activated, cytotoxic, and effector genes, while the Progenitor E/EM and Cytotoxic E/EM clusters did display signs of effector activation. Progenitor E/EM and cytotoxic E/EM memory functions differed in progenitor capacity (*TOX, EOMES, GZMK, CD44*), cytotoxic and migration (*KLF2,*

KLF3, CXCR1). Four clusters represented a variety of differentiated T cells (Effector, ZNF683+ Tissue Resident Memory Intermediate Exhausted, IFN stimulated, and Terminally Exhausted), defined by upregulation of effector (*PRF1*, *CD44*, *GZMB*, etc.) and inhibitory receptors (*HAVCR2, TIGIT, CTLA4*) but low expression of memory markers. While all of these clusters have some expression of tissue resident memory (TRM) genes, the intermediate exhausted cluster had highest expression of *CXCR6* and *ZNF683* and highest z-score of Zheng et al's ZNF683+ CXCR6+ TRM signature.[8] On the other hand, the terminally exhausted cluster displayed the highest expression of inhibitory markers and was highly expression in signatures relating to exhausted (Li et al), terminal exhaustion (Zheng et al) and Exhausted TRM (Pauken et al.).[8,10] IFN-stimulated T cells had increased gene relating to T cells responding to IFN stimulation (*STAT1, STAT2, MX1,* etc) and had increased z-scores of IFN stimulation from Pauken et al and Szabo et al.[3,7,8,9,10,11] Lastly, we determine two clusters of proliferation, defined by the expression of *MKI67* (Figure 2d, Supplement Figure 2).[3,4,11]

We aimed to understand the phenotypic influence of tdLN clonal lineage (LN-paired clones) within the tumor. We compared unpaired tumor-only clones using UMAP cluster contributions. To establish the tdLN contribution in tumor T cell cluster subtyping, we quantified whether a cluster contained a higher-than-average LN-paired or unpaired tumor-only clone proportion per patient. If LN-paired clones and unpaired tumor-only clones have the same proportion across all clusters, it would indicate that the tumor T cell subtype is independent of LN contribution. To measure this, we divided the clonal pairing proportions within each cluster per patient by the patient's global clonal pairing proportions. If the contribution of LN-paired clones was equal to unpaired tumor-only clones, they would each have a relative proportion ratio of 1, while a value larger than 1 would indicate a larger

contribution of LN-paired clones within the cluster. Our results concluded that not only did tdLN clonal lineage favor the memory-like clusters IL7R+ Memory and Progenitor Exhausted, but that unpaired tumor only clones whose response is directly from the TME had a larger contribution to the most exhausted clusters (Int exhausted TRM and Terminally Exhausted). These results display that the tdLN heavily contributes to effector memory and memory function within the tumor (**Figure 2e**).

Two distinct LN-paired clones are found within the tumor: directly LN-paired (tumor-tdLN) and circulating LN-paired (tumor-tdLN-blood).

We investigated whether there were differences between the direct LN-paired and circulating LN-paired clones within the tumor, and whether the additional contribution of the circulating blood tumor-specific T cells further altered tumor cell state.

We compared the relative cluster proportions of direct LN-paired clones and circulating LN-paired clones. The direct LN-paired clones had an increased proportion in the terminally exhausted subset and intermediate TRM cluster subset, while the circulating LN-paired clones had high contribution on the cytotoxic E/EM cluster. This displays that T cells directly coming from the tdLN have effector function more like unpaired tumor only clones compared to circulating tdLN paired clones, which have more cytotoxic function (Figure 2f).[8,9,10]

Tumor-blood T cells in the blood represented fewer than 4% of cells within all clusters except the cytotoxic cluster, where they represented 8.5% of cells. Together, these results display that the majority of clones paired to the blood within the tumor are LN-paired and that blood paired clones favor the cytotoxic E/EM cluster (Supplement Figure 2).

We followed our analysis via a more granular comparison of average clonal expression levels per patient of genes representing a variety of T cell functions for each tumor-specific clonal pairing category (unpaired tumor only clones, direct tumor-tdLN clones, circulating LN-paired clones, tumor-blood). Unpaired tumor only clones displayed low relative average expression of memory-like genes except for *BCL2* and *ID2*, while paired (direct tumor-tdLN clones, circulating LN-paired clones, tumor-blood) clones had heightened average expression of memory genes (*CCR7, IL7R, TF7, SELL*, etc).[3,4,7,8,9,10,11] Tumor-blood and circulating LN-paired clones specifically had an increase in *KLF2* and *KLF3*, which was not found in direct LN-paired clones. Interestingly, *KLF2* has been found to be increased in migratory T cells.[12] Unpaired tumor only clones show increased expression of inhibitor receptors/dysfunctional genes, such as *TOX* and *LAG3,* which were relatively decreased in paired clones.[3,13] All four clone pairings had cytotoxic/effector function. This shows that paired clones average as differentiated, as expected, into effector/effector memory subsets, rather than early naive stage within the tumor. Furthermore, cytotoxic/effector genes were specific to certain clonal pairings, indicating potentially different effector functions within the tumor. Tumor-blood clones had heightened expression of *KLRG1, NKG7* and *TBX21* (T-bet).[14] Direct LN-paired clones and circulating LN-paired clones had an increased expression of *CD44* and *EOMES*. Unpaired tumor-only clones had high expression TRM genes (*CXCR6, ZNF683, ITGAE*, etc.)[8], while circulating LN-paired clones and tumor-blood clones had low expression. Migration and adhesion markers *IFNGR1* and *S1PR1* were highly expressed in paired clones, with strongest relative expression in tumor-blood and circulating LN-paired clones.[3] Tumor-blood clones uniquely displayed an increase in *CXCR1.* Lastly, AP-1 activation markers *JUNB* and *FOS* were increased in paired clones and decreased in unpaired tumor only clones. These results

indicate that although all clones within the tumor are exposed to the same TME for each patient, clonal lineage with the tdLN and circulating blood impact clonal function and anti-tumor T cell response within the tumor (**Figure 2i**).

We validated clone pairing differences by applying modular functional scores from the literature across each clone, averaged per patient. Signatures of terminal exhaustion and tissue residents were highest in unpaired tumor only clones followed by direct LN-paired clones. Migration and adhesion scores were highest in clones paired to the blood (tumor-blood and circulating LN-paired clones).[15] Direct LN-paired clones had a higher migration and adhesion score compared to unpaired tumor only clones.[15] These results display that paired clones within the tumor have imprints of migration from other tissues. Paired clones were also more memory-like than unpaired tumor only clones. Including an increase in responsive CD39-CD69- DN stem-cell like function, specifically highest within tumor-blood and circulating LN-paired clones.[8,9] Tumor-blood clones lastly demonstrated high cytotoxicity vs dysfunction expression, which were also increased in circulating LN-paired clones and direct LN-paired clones relative to unpaired tumor only clones.[10] These results demonstrate that there are two distinct LN-paired anti-tumor clonal responses within the tumor: a cytotoxic response from circulating LN-paired clones, and a more activated TRM response from direct LN-paired clones.

We further examined signature differences between direct LN-paired clones and unpaired clones within the terminally exhausted cluster to determine if LN clonal lineage contributed to differences within the most exhausted T cells. Terminally exhausted unpaired tumor only clones displayed higher expression T cell dysfunction (Li et al) while direct LN-paired clones had relatively higher expression of effector memory function (Figure 2g, Figure

2h).[10] We hypothesize that this difference in memory plasticity may be instrumental in clonal replacement of exhausted unpaired tumor only clones (Figure 2j, Supplement Figure 2).

The clear differences between unpaired tumor-only clones (T cells found exclusively within tumors) and paired clones (T cells clonally related to lymph nodes or blood) demonstrate that sc TCR sequencing from lymph nodes and blood effectively distinguishes these two populations. Notably, the circulating LN-paired clones and tumor-blood clones (found only in tumor and blood) show distinct cytotoxic functional profiles, suggesting that circulating through the blood modifies their characteristics and behavior within tumors. Although direct tumor-tdLN clones (T cells likely migrating directly from lymph nodes into tumors) differ from unpaired tumor-only clones, they do share some similar functional traits, such as increased activation and exhaustion. Together, this indicates that direct LN-paired clones and circulating LN-paired clones represent functionally distinct subsets within tumors, each likely playing different roles in shaping anti-tumor immunity.

TdLN clonal function: clonal differentiation of LN-paired clones begins in the tdLN and reflects the subtypes seen in the tumor.

TdLNs have been previously characterized as a potential reservoir of tumor-specific T cells.[20] To determine whether the tdLN was a site for early memory differentiation of these tumor-specific T cells, we characterized 51,249 tumor-specific tdLN CD8 T cells that belonged to 1,689 expanded clones. Unsupervised dimensionality reduction revealed 10 distinct memory and effector memory clusters. (Figure 3e). Each of the tdLN CD8 T cell clusters had high expression of memory genes (*TCF7, LEF1, IL7R*, etc.)[3,4,8] (Figure 3f, Supplement Figure 3).

This is supported by previous work from Pai et al demonstrated that tumor-specific tdLN T cells are generally memory-like.[4] These cluster functions ranged from more quiescent Naive/central memory (CM)-like, early TRM, cytotoxic memory, progenitor exhausted, and to more differentiated effector/ effector memory function.

Although circulating LN-paired clones comprised the majority (81.5%) of LN-linked tumor-specific T cells, the clonal diversity between direct clones and circulating LN-paired clones was comparable on a per patient basis. (Figure 3a, Figure 3 B) Direct LN-paired clones had balanced tissue distribution of T cells across tdLN and tumor tissues per patient, whereas circulating LN-paired clones contained a higher proportion of T cells within the tdLN than the blood and tumor. Despite these differences in distribution, the number of circulating and direct LN-paired T cells within the tumor was similar in each patient. (Supplement Figure 1) These data suggest that circulating LN-paired clones undergo preferential expansion within the tdLN, distinct from their direct counterpart clones, highlighting a differential amplification pattern that is specific to the lymph node compartment.

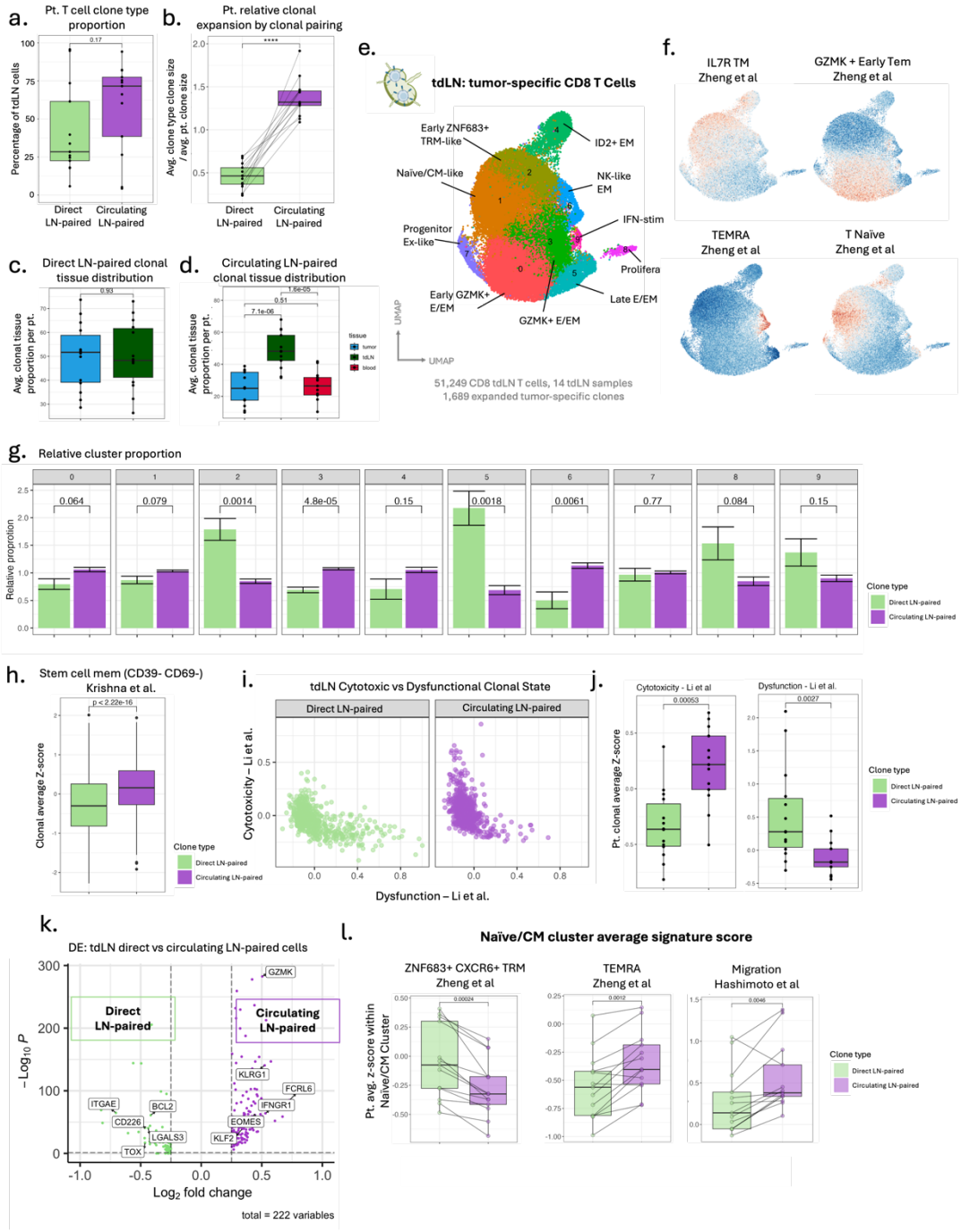

Figure 3: Clonal differentiation of LN-paired clones begins in the tdLN and reflects the subtypes seen in the tumor.

a. Patient T cell clone type proportion within tdLN (paired t-test, $p = 0.17$). b. Clone type relative clonal expansion (average clone size) per patient (paired t-test, $p < 0.0001$). c-d. Average tissue proportion per clone per patient for direct LN-paired clones (c., paired t-test, $p=0.93$) and circulating LN-paired clones (d., paired t-test, ANOVA $p < 0.0001$). e.

Dimensional reduction using UMAP of 51,249 tumor-specific CD8 tdLN T cells, including 1,689 expanded LN-paired tumor-specific clones from 14 patient samples. f. Feature plots of memory-like T cell signatures described by Zheng et al. g. Relative clone type cluster proportion of direct and circulating LN-paired T cells (percent of clone type T cells within cluster for each patient/percent of clone type T cells for each patient). A value of 1 represents a cluster with clone type population proportions the same as the patient tdLN clone type population proportions. h. Stem cell memory CD8 T cell average clonal signature z-score by clone type. i. Average clonal dysfunction versus average clonal cytotoxicity by Li et al. to distinguish cytotoxic and dysfunctional clones. j. Patient clonal average z-score of Li et al.'s cytotoxicity and dysfunction signatures. k. Differential expression between tdLN direct versus circulating LN-paired cells. l. Patient clonal average z-score signature expressions of T cells found within tdLN Naïve/CM cluster.

We then aimed to further investigate if the memory-like tdLN environment formed transcriptionally distinct direct and circulating LN-paired clones. Within the tdLN UMAP clustering, direct LN-paired clone T cells were relatively more expanded within the Early TRM and Late E/EM clusters. On the other hand, circulating LN-paired clones were expanded within the GZMK+ E/EM cluster and the cytotoxic NK-like memory cluster. (Figure 3g) Furthermore, direct LN-paired clones had higher expression of *CXCR6,* a gene relating to tissue residency and long survival in other cancers[21]. On the other hand, circulating LN-paired clones were increased in *S1PR1* expression, useful in peripheral migration and adhesion.[3]

Further differential expression between direct and circulating LN-paired tdLN T cells reveals direct LN-paired clone enrichment in TRM, tumor infiltrating, progenitor exhausted, and activation and progenitor exhausted-associated genes (*ITGAE, S100A11, CXCR4, PDCD1, TOX*)[4,16] and cytotoxic/NK-like (*NKG7, KLRG1, FCRL6, KLF2*)[14,16] gene enrichment on circulating LN-paired clones. (Figure 3e) These differences were validated by comparing functional signature scoring from the literature. Direct tdLN-tumor clones also had an increase in ZNF683+ CXCR6+ TRM scoring and activation (Figure 3d, Supplement Figure 3).[8,10] TdLN circulating LN-paired clones were more cytotoxic, had increased migration and adhesion

signature expression, and increased CD39-CD69- stem cell memory (Figure 3h, Figure 3d, Supplement Figure 3).[9,10,11] These results display a transcriptional imprint in tissue clonal lineage within the tdLN that is carried forward into differentiated differences within the tumor.

Moreover, these anti-tumor clonal response differences in the tdLN were set in an early state of differentiation and memory. The cytotoxic markers that were found to be enriched in the circulating LN-paired clones had increased expression in Naive/CM-like compared to direct LN-paired clones. On the other hand, direct LN-paired clones were increased in expression of *ZNF684, CXCR6,* and *ITGAE* within the Naive/CM-like cluster, genes that are found in TRM tumor subsets, resulting in an increased expression of ZNF683+ CXCR6+ TRM T cell signature from Zheng et al.[8] This displays a differentiated separation of direct LN-paired clones and circulating LN-paired clones starting at the earliest memory stage of expanded tumor-specific tdLN T cells that reflects the continued TRM and cytotoxic differences within effector tumor LN-paired clones (Figure e, Figure 3f).

To assess functional clonal divergence between circulating and direct LN-paired clones, we quantified the average clonal expression of cytotoxic and dysfunctional gene signatures defined by Li et al.[10] These two signatures were generated to describe the two independent T cell differentiation trajectories. Based on these scores, each clone was categorized as cytotoxic (high cytotoxicity, low dysfunction), dysfunctional (high dysfunction, low cytotoxicity), or transitional—a pre-dysfunctional state along the exhaustion trajectory as previously described Direct LN-paired clones were predominantly classified within the transitional state, whereas approximately half of circulating LN-paired clones exhibited a cytotoxic profile.[10] Overall, circulating LN-paired clones demonstrated elevated cytotoxicity and reduced dysfunction

scores relative to direct LN-paired clones, indicating distinct differentiation trajectories shaped by lineage origin (**Figure i, j; Supplement Figure 3**).

Interestingly, direct LN-paired clones within the tdLN resembled memory-like versions of direct LN-paired and tumor-unpaired tumor-only clones, suggesting early priming towards a tumor-homing effector state. In contrast, circulating LN-paired clones within the tdLN mirrored the transcriptional features of circulating blood-derived tumor-specific clones, even in their earliest memory-like state. These findings indicate that the tdLN supports the priming of at least two distinct tumor-specific $CD8^+$ T cell programs: one favoring tissue-resident effector differentiation (direct LN-paired clones), and another favoring migratory, cytotoxic phenotypes (circulating LN-paired clones).

Tumor-specific Blood T cells linked to the tdLN are more memory-like

To investigate the influence of tdLN clonal lineage on circulating blood tumor-specific CD8 T cells, performed dimensionality reduction on 62,863 tumor-specific T cells, encompassing 1,156 expanded tumor-specific clones in peripheral blood. (Figure 4 a) This analysis identified seven transcriptionally distinct clusters, with the majority of cells partitioned into two dominant groups: memory (Naive/CM and *GZMK*+ CM), and cytolytic TEMRA (cytotoxic E/EM, Super KILR E/EM, and effector E/EM). Memory clusters were enriched for canonical stem-like and homeostatic memory genes, including *TCF7, LEF1, IL7R, SELL*[3,4,8]. Cytolytic clusters had lower expression of memory-like genes and higher expression of genes associated with NK-like cytotoxicity (*NKG7, IFITM1, IFITM2, IFITM3, FCGR3A,* etc.).[16,17,18] Other clusters included a non-cytolytic CCR7-CD27+ CXCR1- population and a proliferative T

cell cluster.[18] Most clusters displayed expression of *TOX* and *TIGIT,* but low expression of *CTLA4* and *PDCD1* (**Figure 4 a, Figure 4 b, Supplement Figure 4**).

Remarkably, an average of 95% of circulating blood tumor-specific CD8 T cells per patient were clonally linked to the tdLN. (**Figure 4c**) While both circulating LN-paired clones and tumor-blood clones displayed cytotoxicity, circulating LN-paired clones were consistently less differentiated (Figure 4d). To further resolve phenotypic differences, we compared transcriptional signatures, and we compared signature differences within the Cytolytic E/EM and GZMK+ CM clusters, the largest memory and cytolytic clusters. Within the cytotoxic E/EM cluster, tumor-blood clones had increased expression of cytotoxicity and TEMRA-associated signatures. Similarly, within the GZMK+ CM cluster, tumor-blood clones had increased expression of the cytotoxic signatures, exhibited elevated memory-associated programs, including Naive-like and IL7R memory signatures (Naïve-like, IL7R memory, etc.) (**Figure 4e, Figure 4f, Supplement Figure 4**)[8,9]. These findings indicate that circulating tumor-specific T cells clonally linked to the tdLN are less differentiated and retain a more memory-like transcriptional profile than those not linked to the tdLN. This suggests that tdLN lineage may support the generation of circulating T cells capable of persistence.

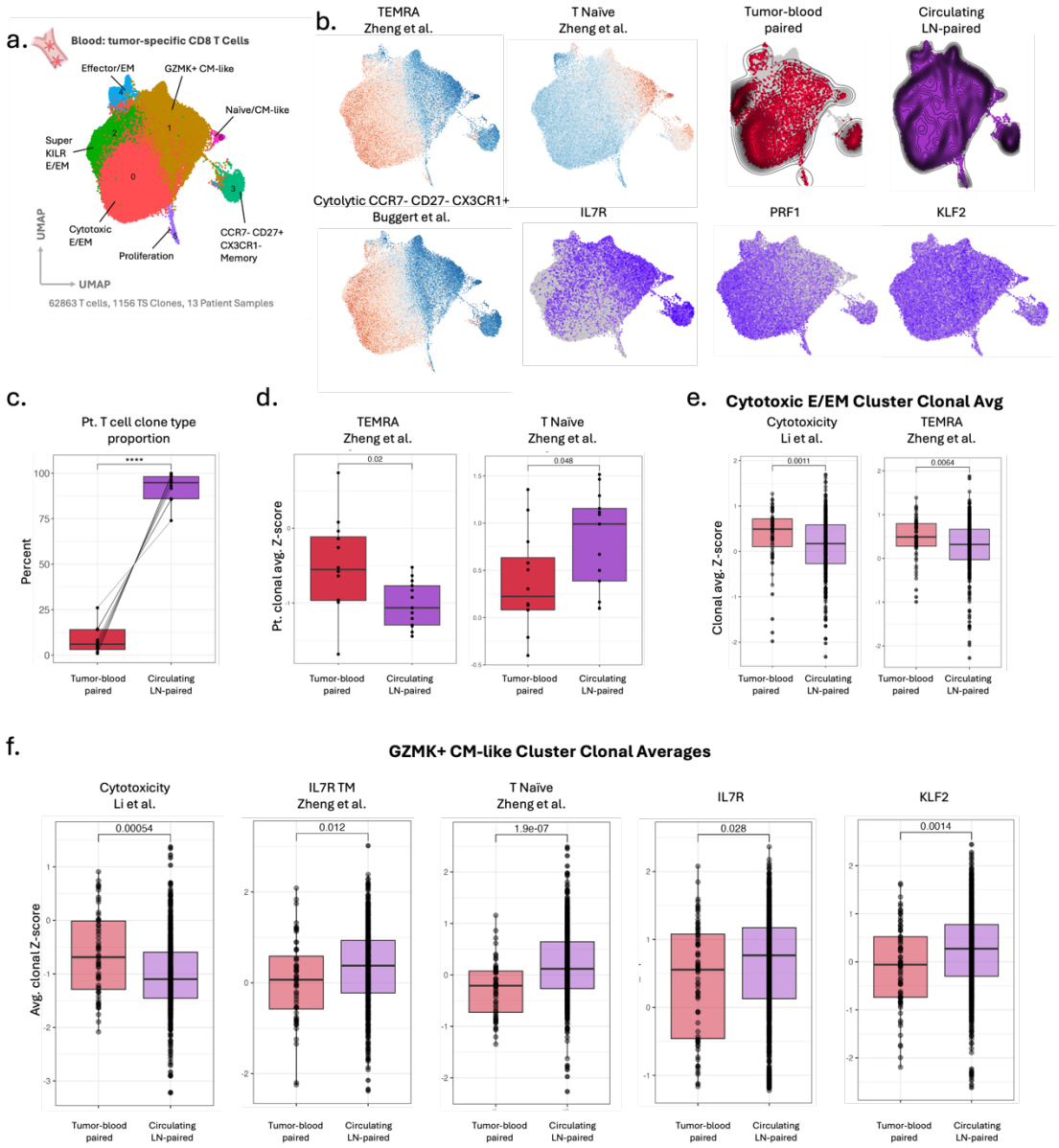

Figure 4: Contribution of LN-paired clones in circulating blood tumor-specific T cells.

a. Dimensional reduction using UMAP of 62,863 tumor-specific blood T cells including 1,156 clones and 13 patient samples. b. Feature plots of T cell signatures from the literature and gene expression signatures (IL7R, PRF1, and KLF2). Top right are Dimplots highlighting tumor-blood paired and circulating LN-paired T cell UMAP locations overlaid by a density plot. c. Patient T cell clone type proportion within blood (paired t-test, $p < 0.001$). d. Patient average clonal z-score of TEMRA (t-test, $p=0.02$) and T Naïve (t-test, $p=0.048$) signatures by Zheng et al. e. Patient clonal average z-score signature expressions of T cells found within the blood Cytotoxic E/EM cluster (t-test, $p < 0.01$). f. Patient clonal average z-score signature expressions of T cells found within the blood GZMK+ CM-like cluster (t-test, $p < 0.05$).

Treatment makes unpaired tumor only clones less dysfunctional

To determine the impact of treatment on tumor-specific T cells across all tissues we compared the differences between patients treated with ChemoIO (n=8) and treatment-naïve patients (n=6). In the cohort of treated patients there is an even distribution of non-responders (residual viable tumor < 10%, n=4) and major pathological responders (residual viable tumor < 10%, n=3), and one partial responder (residual viable tumor = 30%, n=1) (Figure 5a). Clonal diversity of unpaired tumor-only CD8 T cell clones did not differ significantly between ChemoIO-treated and treatment-naive patients (**Figure 5b**). In unpaired tumor only clones, ChemoIO reduced signature expression of dysfunction, terminal differentiation, terminal exhaustion, IFN stimulation and gene expression of proinflammatory cytokines, inline prior literature describing TIL ChemoIO response (**Supplementary Figure 7**).[2,3,8,10,11] Despite their differentiated status, these clones in treated patients retained greater memory potential, as evidenced by increased IL7R^{+} tissue-memory (TM) signature expression and elevated *IL7R* transcript levels (**Supplementary Figure 7**).

To assess how treatment influenced clonal differentiation trajectory, we applied the cytotoxic versus dysfunctional classification framework described by Li et al. (**Figure 5d**). Compared to treatment-naive tumors, fewer ChemoIO treated T cell clones were categorized as dysfunctional and more were categorized as transitional clones as compared to treatment-naïve clones. As transitional clones represent a pre-dysfunctional state along the exhaustion trajectory, these findings suggest that neoadjuvant ChemoIO may limit the acquisition of terminal dysfunction and/or partially reinvigorate dysfunctional tumor-infiltrating clones (**Figure 5d; Supplementary Figure 6**).

T cell clones within tdLN and with tdLN lineage respond to ChemoIO

LN-paired clonal diversity was significantly increased by 46.4% in patients who received neoadjuvant ChemoIO compared to untreated patients (58.2 clones/5000k cells in untreated patients to 85.2 clones/5000k cells in neoadjuvant ChemoIO). (**Figure 5b**) LN-paired clones were proportionally more expanded within the tumor following ChemoIO treatment. Although a similar trend was observed in treatment-naive patients, the difference did not reach statistical significance. (**Figure 5b**). Across nearly all the subtype cluster in both tdLN T cell and tumor T cell UMAPS ChemoIO treatment was associated with increased cytotoxicity (Li et al.) and stem cell memory CD39-CD69- (Krishna et al) programs in LN-paired clones (**Supplementary Figure 5, 2.6**). Throughout all tissue and clone types, treatment consistently increased PRDM1, a marker relating to T cell differentiation, highlighting a global treatment-induced shift in T cell fate (**Supplementary Figure 7**).

Clonal trajectory-based analysis further revealed that ChemoIO increased the proportion of tumor LN-paired clones classified as cytotoxic and transitional, suggesting enhanced functional differentiation and potential resistance to terminal exhaustion following treatment (Figure 5d; Supplementary Figure 6).

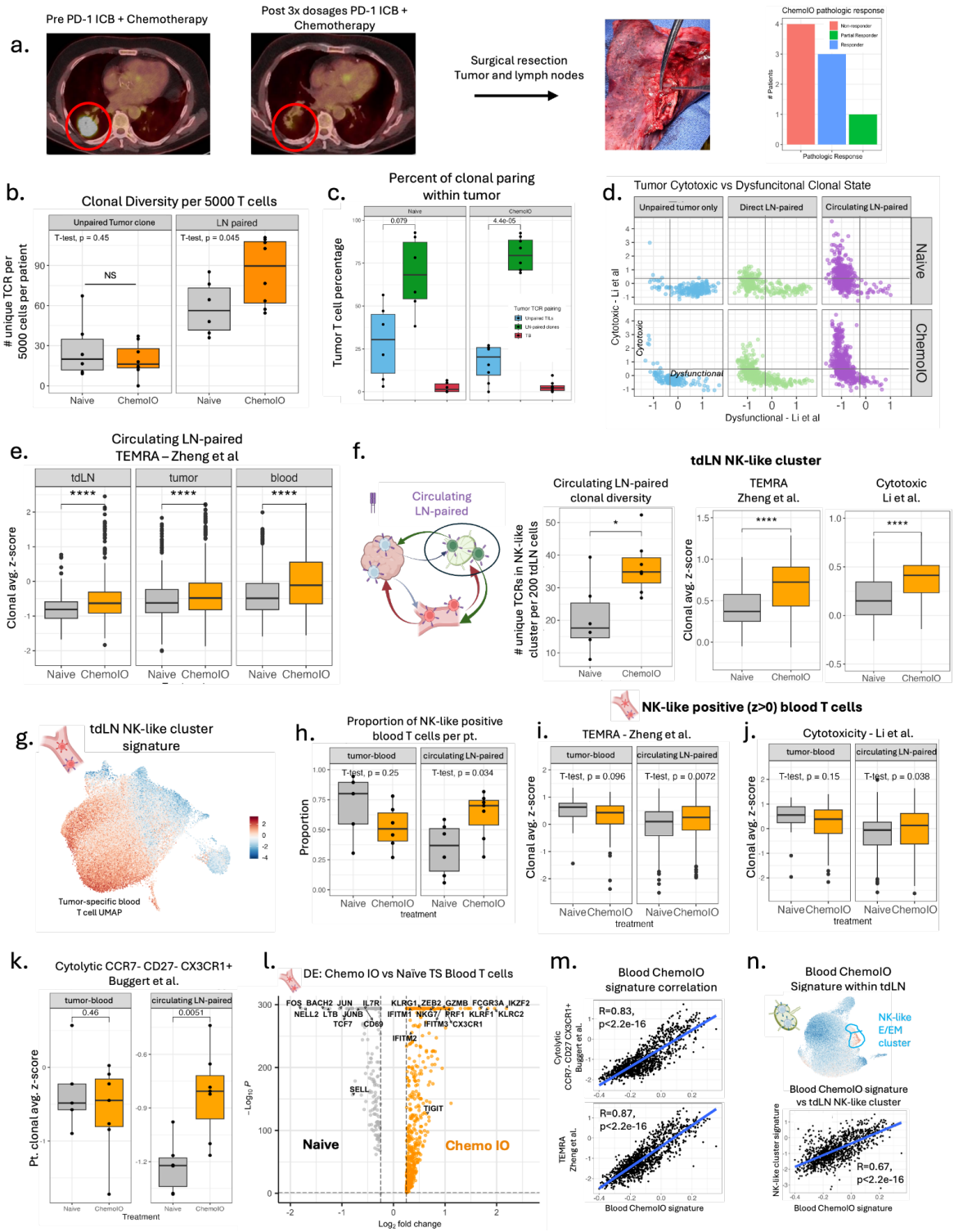

Figure 5: ChemoIO tumor-specific LN-paired response.

a. CT scan of patient pre- and post-3 dosages of PD-1 ICB + chemotherapy followed by surgical resection of tumor and LNs. To the right is the pathologic response distribution of the

eight patients treated by ChemoIO. b. Patient tumor-specific clonal diversity (number of unique TCRs per 5,000 cells) between treatment Naïve and ChemoIO treated patients for unpaired tumor only clones (t-test, $p = 0.45$) and LN-paired clones (t-test, $p = 0.045$). c. Tumor-specific tumor T cell population proportion based on clonal lineage with the LN (clone type pairing described in 2.1 d.) per patient (paired t-test between unpaired tumor only clones and LN-paired clones) by treatment. d. Average clonal dysfunction versus average clonal cytotoxicity by Li et al. to distinguish cytotoxic and dysfunctional clones separated by treatment and clone type pairing. Gating Cytotoxic clones to have > 0.5 average cytotoxic signature and < -0.25 average dysfunctional signature and Dysfunctional clones < 0.5 average cytotoxic signature and > -0.25 average dysfunctional signature. e. Treatment differences of average clonal z-score of TEMRA expression within circulating LN-paired clones within the tdLN, tumor and blood (t-test, $p < 0.0001$). f. Treatment differences in circulating LN-paired clones found within tdLN NK-like cluster: clonal diversity per 200 cells (t-test, $p=0.05$), clonal average TEMRA signature expression (t-test, $p < 0.0001$), and clonal average cytotoxicity signature expression (t-test, $p < 0.0001$). g. Feature plot on tumor-specific blood T cell UMAP of tdLN NK-like cluster signature (z-score normalized). h-j. Comparison of treatment response of blood T cells with tdLN NK-like positive (NK-like cluster signature high, $z>0$) within tumor-blood clones and circulating and LN-paired clones. h. Proportion of T cells per patient with tdLN NK-like positive (t-test, tumor/blood clones $p=0.25$, circulating LN-paired $p=0.034$). i-j. NK-like positive blood T cell average clonal z-score of TEMRA and cytotoxicity signature. k-n. Treatment differences between tumor-blood (t-test, $p=0.46$) and circulating LN-paired (t-test, $p=0.0051$) patient clonal average of cytolytic CCR7- CD27- CX3CR1+ signature by Buggert et al. l. Differential expression between ChemoIO versus Naïve tumor-specific blood T cells. m. Feature plot of tdLN UMAP of blood ChemoIO high signature (differentially expressed genes from l. with average log full change > 0.5 and p adjusted value < 0.05). Linear regression of tumor-specific blood T cell clonal averages of blood ChemoIO high signature versus CCR7- CD27- CX3CR1+ signature by Buggert et al. ($R=0.83$, $p<0.001$), blood ChemoIO high signature versus TEMRA signature by Zheng et al. ($R=0.87$, $p<0.0001$), and blood ChemoIO high signature versus tdLN NK-like cluster signature ($R=0.67$, $p<0.0001$).

Circulating LN-paired blood T cell clones have increased cytotoxicity and NK-like phenotype

The peripheral blood T cell response to ICB therapy is critical in the context of early-stage NSCLC, as these T cells may play a role in surveilling for micro-metastatic disease, potentially even after the tumor is resected.[22] Given the inherently cytotoxic profile of circulating LN-paired clones, we next examined how ChemoIO treatment further modulates already cytotoxic subsets across tissues. Circulating LN-paired clones from patients treated

with ChemoIO exhibited increased TEMRA (Zheng et al.) signature expression in the tdLN, tumor, and blood (**Figure 5e**).

Within the tdLN's NK-like cluster, ChemoIO-treated patients' circulating LN-paired clones exhibited increased cytotoxic and TEMRA functional scores relative to naive patients (**Figure 5f**).[8,10] This cluster also had a greater circulating LN-paired clonal diversity in treated patients compared to both untreated patients and treated direct LN-paired clones (**Figure 5f**). This means that ChemoIO increased the number of unique clones that were composed of NK-like T cells within the tdLN. Notably, expression of *KLF2*, *KLF3*, and *NKG7* was increased, even within Naive/CM-like tdLN clusters, suggesting a treatment-induced cytotoxic shift across differentiation states (**Supplementary Figure 8**).

To determine whether this tdLN cytotoxic program response extended to the circulation, we generated a tdLN-based NK-like signature from differential expression of the NK-like memory cluster in the tdLN and applied it to tumor-specific peripheral blood (**Figure 5g**). Circulating tumor-specific T cells from ChemoIO-treated patients demonstrated a significant proportional expansion of tdLN NK-like memory positive T cells (defined by tdLN NK-like memory T cell signature z-score > 0) (**Figure 5h**). These NK-like memory positive cells from ChemoIO-treated patients exhibited increased cytotoxic and TEMRA signature expression, consistent with observations in the tdLN (**Figure 5i, j**).[8,10] Importantly, this treatment-enhanced NK-like signature was also enriched within tumor-infiltrating circulating LN-paired clones in ChemoIO-treated patients compared to treatment-naïve patients (**Supplementary Figure 6**).

We then aimed to establish a more granular treatment response of tumor-specific blood T cells, and to see if it was related to the enhanced NK-like tdLN derived response. Differential expression analysis revealed that circulating LN-paired clones in the blood of untreated patients

were enriched in memory-associated transcription factors such as *TCF7, IL7R, SELL,* and *NELL2* (**Figure 5l**).[8,18] On the other hand, ChemoIO patients' circulating LN-paired clones T cells were enriched in NK-like, TEMRA and effector related genes *NKG7, FCGR3A, PRF1, IFITM1, IFITM2, IFITM3, GZMB*, etc. (Figure 5l)[8,14,16] These treatment differences were also validated with signatures from the literature (**Figure 5k, Supplementary Figure 8**).

To determine if these genes were biased by any clusters, we defined a ChemoIO circulating blood response signature (log fold change > 0.25, adjusted p value <0.05) and computed the patient clonal average score within each cluster. In every cluster, ranging from Naive/CM-like to effector/EM, ChemoIO treated patients displayed a statistical increase in the score. We compared how the average clonal ChemoIO clones blood score related to other cytolytic scores (**Supplementary Figure 6**).[10,18] Blood T cell clonal average treatment response score was strongly correlated with independent cytotoxicity signatures, including TEMRA (R=0.87) and cytolytic CCR7-CD27-CX3CR1 score (R=0.83) (Figure 5m).[8,18] Interestingly, the treatment score also was moderately correlated our tdLN NK-like memory signature with a R value of 0.67 (**Figure 5n**).

In contrast, tumor-blood clones—presumed to originate within the tumor and not linked to the tdLN—did not show enhanced cytotoxic, NK-like signature, or TEMRA-like features following ChemoIO (**Supplementary Figure 6, 2.8**).[8,10,18] Moreover, while circulating LN-paired clones were also relatively more clonally expanded after ChemoIO per patient compared to tumor-blood clones, Naïve treated patients' circulating LN-paired clones and tumor-blood clones had similar clonal expansion (Supplementary Figure 8). Furthermore, unlike circulating LN-paired clones, tumor-blood NK-like positive populations in the blood were unchanged between the treatment cohorts (ex. in proportion, cytotoxicity, TEMRA) (**Figure 5 h-j**). These

data reveal a distinct, tdLN-specific effect of ChemoIO that promotes the expansion and functional activation of circulating tumor-specific CD8 T cells, with no analogous effect on tumor-intrinsic tumor-blood clones.

In conclusion, ChemoIO treatment consistently enhanced cytotoxicity and promoted a stem-cell–like activated phenotype in memory-associated tissues, including tdLN and circulating blood. Within the tumor itself, treatment reduced markers of exhaustion and dysfunction while preserving certain memory-like characteristics. Additionally, ChemoIO selectively increased the cytotoxic potential of circulating tumor-specific T cells clonally derived from the tdLN. Overall, clonal lineage across tissues similarly reflected increased cytotoxic function and reduced exhaustion following treatment. Importantly, these results newly demonstrate that ICB treatment alters memory-like differentiation, and does so locally in the tdLN of NSCLC patients.

Discussion

We aimed to determine the baseline role of tumor specific tdLN T cell clones in the anti-tumor response and whether these T cell clones respond to ChemoIO. We showed that compared to tumor tdLN-paired clones, tdLN T cells were more memory-like. Furthermore, we characterized two distinct tdLN-paired clones: those that are directly linked to the tumor (tdLN-tumor TCR paired clones) and those that also circulate within the blood (tdLN-tumor-blood TCR paired clones). Interestingly, the phenotypes of these clones within the tdLN mirrored the memory-like state they adopted within the tumor. Direct tdLN-tumor paired clones functioned as tissue resident and progenitor exhausted T cells within the tdLN and differentiated into tissue resident exhausted T cells within the tumor. On the other hand, circulating LN-paired tdLN T

cells held migration and cytotoxic potential within their tdLN memory function. Circulating T cells within the tumor were cytotoxic E/EM. These results indicate that LN-paired T cell clones were likely activated within the tdLN and subsequently migrated towards the tumor, where they further differentiated toward an effector phenotype. Furthermore, they display that the tdLN's role in the anti-tumor response is to differentiate which clones have circulation and migration potential and which clones directly go to the tumor to kill cancer.

Future work should determine which tdLN T cells persist in the blood in the long term. It would be important to determine if persisting tumor-specific LN-paired clones mainly originated from circulating or direct LN-paired clones. Given the increase in migration and adhesion and increase in memory-like state of circulating clones, we hypothesize that the majority of clones that persist will be the circulating clones at time of resection. The distinction of direct effector and surveilling circulating LN-paired T cell clones is critical for future research on therapeutic options. Depending on the cancer biology and stage, it may be beneficial to either improve primary tumor killing or metastatic surveillance function. For example, in early-stage NSCLC, where the primary tumor is surgically removed, the importance of metastatic surveillance function may be essential.

Furthermore, we propose that the tdLN responds to treatment and impacts the differentiation of tdLN-paired clones. Generally speaking, tdLN-paired clones had increased clonal diversity compared to unpaired tumor clones per patient. The addition of ChemoIO increased the clonal diversity of only LN-paired clones, while unpaired tumor T cell clonal diversity was unchanged. This displays the how the tdLN creates a reservoir of tumor-specific TCRs and that treatment will impact the function of the reservoir. The chemotherapy in ChemoIO likely may increase the exposure to new tumor antigens, while blocking the PD-

1/PD-L1 interaction of DCs and T cells promotes the activation of more TCR combinations. The introduction of ChemoIO also increases memory-function of tdLN-paired clones within the tdLN and tumor. Direct tumor-tdLN paired clones had an increase in stem-cell memory function, which increases regeneration and proliferation capacity. Circulating tumor-paired clones displayed an increased clonal diversity in the cytotoxic memory NK-like/TEMRA cluster within the tdLN and an increased expression TEMRA function throughout the tdLN. This function was carried over in the blood. Interestingly, ChemoIO only increased TEMRA function in LN-paired blood T cells, while the TEMRA function of tumor-blood clones was not changed. These results display that ChemoIO is particularly increasing LN-paired TEMRA function. This increase may be beneficial for killing micro-metastatic disease. Together, these results indicate the Chemo /IO is not only affecting function within the tumor of LN-paired clones but altering differentiation trajectories in the tdLN memory-state.

However, within the blood, patients naïve to treatment had more memory-like and less activated circulating tumor-specific T cells. Further work should determine the balance between cytotoxic function and memory function of circulating clones for long term response. For example, are clones that are more memory-like at resection more likely to persist 3 to 5 years after initial treatment?

We demonstrated that ChemoIO is impacts tumor-specific tdLN clonal phenotypes and differentiation. However, ChemoIO only improves the long-term survival of early-stage NSCLC patients by 10%.[23,24] Therefore, although the tdLNs are demonstrably impacted and responsive to treatment, the characteristics of a “favorable” tdLN remain undefined. Consequently, future research should aim to understand the differences of tumor-specific tdLN-paired clones between treatment responders and non-responders. Such investigations could

reveal key insights on which tdLN functions should be targeted with future immunotherapy strategies.

Our results also give insight to T cell clonal lineage. Unpaired tumor T cell clones had distinct phenotypes from LN-paired and tumor-blood paired clones, therefore we hypothesize that these T cells originate in the tumor and were activated within the tumor. Phenotypically they have the highest resident memory signatures and are more exhausted, which also supports this hypothesis. The tdLN phenotype of LN-paired clones are more naïve-like and memory-like than tumor T cells. T cells are lineage restricted, and therefore dedifferentiation from effector states to naïve-like memory state is very unlikely.[19] Furthermore, the tumor T cell function of LN-paired clones is reflected as a memory-like state in the tdLN, indicating initial clonal trajectories start in the tdLN and then migrate to the tumor.

Circulating LN-paired clones have two tissues where naïve-like states are present: tdLN and blood. However, T cells are rarely activated within circulating blood and thus a majority of these T cell clones most likely originated in the tdLN and then circulated to the blood. However, it should be noted these results cannot indicate if circulating LN-paired clones migrate in and out of tdLN, blood, and tumor tissues after initial activation. In addition, given the transcriptional signature in these clones, we hypothesize that these clones are responsible for migration to non-draining lymph nodes. The comparison of tumor-blood clones and circulating LN-paired clones further supports the notion of tdLN as a key activation site. The heighted activation of tumor-blood clones, relative to their circulating LN-paired counterparts, suggests tdLN involvement imprints a memory-like state on blood T cells.

Overall, our results indicate that intratumoral ICB response is not the sole determinant of anti-tumor immunity. Moreover, the distinct functional implications of clonal pairing (unpaired tumor T cell clones, direct LN-paired clones, circulating LN-paired) warrant individual consideration and dedicated investigation when designing treatment and combination therapies.

Methods

Clinical samples, imaging, and pathology evaluation

Tumor, tumor-draining lymph node, and peripheral blood samples were collected under an Institutional Review Board–approved protocol (#23-04025976) at Weill Cornell Medicine, under the study titled Mechanism of T-cell memory differentiation in a lung cancer model and the role of PD-1 blockade on the tumor draining lymph nodes of NSCLC patients. Samples were obtained prospectively through the Thoracic Surgery Biobank, with informed consent. Pre-treatment and post-treatment CT imaging was reviewed independently by board-certified pulmonary radiologists to assess radiographic features and treatment response. Resected tumor and lymph node specimens were processed and reviewed by a board-certified pulmonary pathologist to confirm histologic diagnosis and treatment-associated changes.

Flow cytometry and cell sorting

Prior to sequencing, immune cells in single cell suspension were thawed and stained for sorting and were sorted using the flow cytometry for CD45 CD3 and CD8.

scRNASeq and scTCRSeq and demultiplexing and read processing

To align raw single cell RNA Seqeuncing and single cell V(D)J sequencing data we used Cell Ranger version 8.0 usng the *multi* program. The GEX GRCh38-2024-A human reference genome was used for transcriptional reference along with VDJ GRCh38-2024-A, human reference for V(D)J. Aligned transcriptional data is the processed using Read10X using R version 4.4.1. Seurat version 5 was used to create a seurat object with the following parameters: min.cells set to 3 and min.features parameter set to 200. Patient's where the overall number of features were less than 200 per cell were removed. TCR V(D)J data was then processed by combining the filtered_contig_annotations.csv and clonotypes.csv dataset to get the cdr3 alpha and beta amino acid sequence for each cell, which was then added as meta data to the Seurat object. If T cells were not sequenced for both TCR and RNA sequencing, they were removed from the dataset. T cells that did not have both beta and alpha chains of the TCR sequenced were removed.

Defining T cell TCR clones and tumor-specific clones

T cells were in the same clone if they shared the same TCR alpha and beta genes. T cell clone size was defined as the number of cells within the given shared clone. Clonal diversity referred to the number of unique TCRs in a given population (ex: patient, or cluster per patient). Tumor specific clones were expanded (clone size >=10), non-viral T cell clones that have TCR linage with the tumor are tumor specific. Viral clones were defined as clones with

TRC alpha and beta chains found on the human viral VDJ database (https://vdjdb.cdr3.net/), which lists TCR CDR3 amino acid sequences that relate to known viral antigens.

scRNA-seq data integration and clustering

Our T cell single RNA sequencing database required thoughtful integration due to the large number of patient sample cells sequence for one cell type (CD8 T cells). Due to the different tissue types, treatment, and treatment response, we aimed to minimize over-correction. We tested 4 different integration methods (no integration, RPCA, CCA, and harmony) as well as different anchor values for RPCA and CCA methodology. We integrated based on patient ID. When no integration method was applied, each cluster was predominately defined by patient ID, displaying the need for integration. RPCA integration, using anchor value of 15, ultimately was the best integration method. Patients with the same treatment (either Naïve to treatment or ChemoIO) were clustered in a similar pattern. In addition, T cells clustered into biologically relevant T cell subsets. Each UMAP subset (ex: tumor-specific tumor T cells, tumor-specific tdLN T cells, etc.) was re-normalized and re-integrated. Each UMAP had its own PCA analysis, which determine the number of PCAs required for dimensional reduction. Dimensional reductions were performed as instructed by Seurat. T cell cluster subsets were then defined by the up or down regulation of memory markers (ex. *SELL, LEF1, TCF7*, etc.), effector-related genes (ex. *CD44, PRF1, GZMB, EOMES*, etc.), dysfunctional/inhibitory receptor genes (ex. *TOX, CTLA4, HAVCR2*, etc.), and proliferation markers (ex, *MKI67*). In addition, the *FindAllMarkers* function was used to determine which genes were differentially expression within each cluster. These results were used to determine

both T cell cluster function, and if further dimensional reduction were required. Cluster subset labels were then validated using modular scores of T cell signatures, which received z-score normalization, found in the literature.

Gene signature scoring analysis and statistical tests

To compare phenotypic differences between groups of T cells (ex: between clusters, between treatments, between tissues, etc), modular score was made of signatures from the literature. These scores were then normalized within a UMAP via z-score normalization (mean of zero, standard deviation of one). Three forms of analysis were made: comparing individual T cell scores, averaging the score across all cells within the same clone, and averaging each clonal average per patient. While comparing T cell score averages give single cell level insight, averaging across clones provide information regarding a clonal state. In addition, due to the large number of T cells, a small score difference can lead to a statistical difference. Therefore, using clonal averages decreases the total number of points on the statistical test, making a statical significance more powerful. When comparing two groups, a test was performed. A paired T test was performed if changes were compared within the same group (i.e. the same clone in different states, or same patient in different state or clonal pairing). When comparing multiple t test within the same dataset, an Anova test was also performed to reduce type 1 error.

Relative clonal diversity and clone pairing expansion analysis

The number of T cell sequenced for each patient sample ranged from a few thousand to 20,000 cells. Because of this, the absolute number of tumor specific T cells for each tissue sample also ranges from a few thousand to 13,000. Therefore, to compare the clonal diversity between patients, we normalized the number of clones found by the number of cells within the clonal pairing. We followed this by multiplying the normalized clonal diversity by 5000 to get the number of unique clones per 5000 tumor-specific cells.

To compare the relative proportion of clonal pairing (ex: unpaired tumor only versus LN-paired clones) per cluster we calculated the proportion of paired clones within a given cluster and divided it by the patient's specific overall clonal pairing portion. Therefore, a relative proportion 1 for each clonal pairing represented equal proportions within a cluster as the patient's proportion.

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Author contributions

J.V.V and O.E. supervised this study. R.H. and J.V.V designed the experiments. R.H. performed the computational analysis and wrote the manuscript. T.D.C, S.T. performed clinical sample acquisition. R.H., S.T. and O.D. performed biobanking. L.V. provided methodological and computational guidance. J.V.V. edited the manuscript. G.E., M.M., N.K.A. gave suggestions. A.S. coordinated the clinical sample acquisition.