

A high density of tertiary lymphoid structure B cells in lung tumors is associated with increased CD4⁺ T cell receptor repertoire clonality

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Abbreviations: ADC, adenocarcinoma; BCR, B cell receptor; CDR3, complementarity-determining region 3; DC, dendritic cell; FOLL, B cell, follicular B cell; IgH, immunoglobulin heavy chain; LCC, large-cell carcinoma; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; NT, non-tumoral distant tissue; P, peripheral blood or draining lymph node; SCC, squamous cell carcinoma; TFH, follicular helper T cell; TRM, tissue resident-memory T cell; TCR, T cell receptor; TLS, tertiary lymphoid structure.

T and B cell receptor (TCR and BCR, respectively) V β or immunoglobulin heavy chain complementarity-determining region 3 sequencing allows monitoring of repertoire changes through recognition, clonal expansion, affinity maturation, and T or B cell activation in response to antigen. TCR and BCR repertoire analysis can advance understanding of antitumor immune responses in the tumor microenvironment. TCR and BCR repertoires of sorted CD4⁺, CD8⁺ or CD19⁺ cells in tumor, non-tumoral distant tissue (NT), and peripheral compartments (blood/draining lymph node [P]) from 47 non-small cell lung cancer (NSCLC) patients (age_{median} = 68 y) were sequenced. The clonotype spectra were assessed among different tissues and correlated with clinical and immunological parameters. In all tissues, CD4⁺ and CD8⁺ TCR repertoires had greater clonality relative to CD19⁺ BCR. CD4⁺ T cells exhibited greater clonality in NT compared to tumor ($p = 0.002$) and P ($p < 0.001$), concentrated among older patients (age > 68). Younger patients exhibited greater CD4⁺ T cell diversity in P compared to older patients ($p = 0.05$), and greater CD4⁺ T cell clonality in tumor relative to P ($p < 0.001$), with fewer shared clonotypes between tumor and P than older patients ($p = 0.04$). More interestingly, greater CD4⁺ and CD8⁺ T cell clonality in tumor and P, respectively (both $p = 0.05$), correlated with high density of tumor-associated tertiary lymphoid structure (TLS) B cells, a biomarker of higher overall survival in NSCLC. Results indicate distinct adaptive immune responses in NSCLC, where peripheral T cell diversity is modulated by age, and tumor T cell clonal expansion is favored by the presence of TLSs in the tumor microenvironment.

Introduction

Methods to obtain both the identity and classification of tumor antigens have become fundamental to our understanding of the host-triggered immune response in multiple cancers. When exposed to either tumor-specific or tumor-associated antigens, the adaptive immune system reacts with a collection of antigen-specific receptors expressed by populations of B and T lymphocytes.¹ This large repertoire of T or B cell receptors

(TCRs or BCRs, respectively), generated by random rearrangements of the variable (V), diversity (D), and joining (J) segments during haematopoiesis, is intentionally diverse for generalized targeting of a broad range of potential antigens.¹⁻³ Following binding of the TCR or BCR to its cognate antigen, naïve T or B lymphocytes become activated, proliferate and may undergo somatic mutations of the V regions to improve the binding affinity of the TCR and BCR to antigen as well as class switch recombination.^{4,5} Clonal expansion (i.e., high clonality), or the large

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increase in T or B cells from a single cell, can be interpreted as the opposite of diversity – the latter of which indicates a variety of different clones with few dominant clones at high frequencies. The expansion of clones with the highest affinity TCRs and BCRs for antigen may reduce the diversity (increase the clonality) of the responding lymphocyte populations.² As humans age, immune responses become impaired, characterized by (1) reduced lymphopoiesis of new naïve B and T cells, with novel BCRs⁶ and TCRs,⁷ respectively, (2) reduced dendritic cell (DC) function resulting in poor responses to inflammatory signals and lowered capacity to present antigen and to activate adaptive immune responses,⁸ (3) poor germinal center formation, site of B cell expansion and selection,⁹ and (4) decreased potential for T cell expansion, expression of activation markers, production of cytokines, diversity of TCR repertoire and delayed T cell responses to antigen.^{7,10} Thus, these changes during aging help to explain the increased susceptibility to infection and reduced response to vaccinations in the elderly population.^{3,11,12}

Previous work has characterized the presence of immune cells organized as ectopic lymphoid structures, referred as tertiary lymphoid structures (TLS), in the tumor of non-small cell lung cancer (NSCLC) patients.^{13,14} These TLS contain a large density of DC-Lamp⁺ mature DCs and CD20⁺ follicular B (Foll-B) cells, both of which home selectively into TLS. The densities of these mature DCs or Foll-B cells have been shown to associate with improved prognosis in NSCLC patients,^{13,15} antitumor humoral immune response and cytotoxic and Th1 T cell differentiation.¹⁶ However, detailed studies to understand the role of TLS in the generation and expansion of intra-tumoral immune cells (T and B cell subsets) and shaping of TCR and BCR repertoire are lacking.

With the advancement of next-generation sequencing (NGS), it is now possible to fully characterize TCR and BCR repertoires, to accurately represent the clonal diversity in humans.¹⁷⁻²⁰ Primers specific for the V, D, J, and constant (C) gene segments of the complementarity-determining region 3 (CDR3) of TCRs and BCRs are used to generate millions of sequencing reads and thousands of unique clonotypes, with distributional properties that can be monitored over time or compared between the tumor or normal tissue compartments within a cancer patient. The CDR3 region of the receptor is the primary CDR that contributes most to the antigen recognition by the interaction between the TCR and its cognate peptide antigen located within the major histocompatibility complex groove, or BCR with its cognate polypeptide antigen,^{2,21} and is the principle component of antigen recognition specificity in both TCRs and BCRs.²² TCR and BCR repertoire technology can provide a robust characterization of the global receptor repertoire of particular lymphocyte populations, though within both the peripheral and disease-affected compartments, there is a mixed population of T cells that can differ by clonotype contribution, cell subtypes, and sensitivities to therapies.^{2,3,23} Further, the frequencies of monoclonal and oligoclonal TCR V β families have been shown to significantly differ between CD4⁺ and CD8⁺ T cell subsets.²⁴ Because it represents a challenge to discern the cellular composition within the population of tumor-infiltrating lymphocytes, sorting

of cell subsets is important to further accurately distinguish contribution between the CD4⁺ and CD8⁺ adaptive response.

In this study, we used NGS to evaluate the spectra of the V β of TCR or immunoglobulin heavy (IgH) chain of BCR repertoires in CD4⁺, CD8⁺, or CD19⁺ cells from tumor (T), non-tumoral distant tissue (NT), or peripheral (peripheral blood or draining lymph node, P) compartments of NSCLC patients. We revealed the differences in immunodiversity in CD4⁺ and CD8⁺ TCR repertoires compared to CD19⁺ BCR repertoire, the most recurrent differences in clonality between the three compartments in CD4⁺ T cells across patients, as well as age-specific differences in TCR/BCR repertoires. Additionally, intratumoral or peripheral CD4⁺ or CD8⁺ TCR repertoires, respectively, were correlated with TLS B cell density, a previously characterized biomarker of higher overall survival in NSCLC patients.¹⁵ These findings further our understanding of the adaptive response to tumor antigens and help to characterize the TCR and BCR diversity in the tumor microenvironment in NSCLC.

Results

Global differences in clonality index between NSCLC patients and healthy donors

In this study, non-productive or out-of-frame rearrangement TCR V β or BCR IgH CDR3 sequences were not included in the analysis. Among all productive clones, the TCR and BCR repertoire clonality was evaluated among the 47 NSCLC patients across four tissue compartments: tumor, non-tumoral (normal) distant tissue, peripheral blood, or draining lymph node (LN). Few public clones were shared between patients, as expected from a previous report²⁵. In both CD4⁺ and CD8⁺ TCR and CD19⁺ BCR repertoires, the clonality in the peripheral blood and to a lesser extent the lymph node of NSCLC patients was generally higher than that observed in a cohort of 11 normal healthy donor blood specimens (10 BCR and 9 TCR; **Fig. 1A**). Within both the NSCLC patient and healthy donor cohorts, the TCR repertoire had higher clonality than the BCR repertoire in the blood. As expected, in both cohorts, the clonality of the TCR and BCR repertoires was positively correlated with the maximum single clonotype frequency (**Fig. 1B**).

Both cell counts and nucleic acid extraction yields for all samples (**Table S1**) were compared against the various measures of immunodiversity and no correlations were identified (**Figs. S1–S4**).

Differences in CD4⁺ TCR repertoire clonal expansion between the different tissue compartments of NSCLC patients

Across all four different tissue compartments, there was a general increase in average clonality index from CD19⁺ to CD4⁺ to CD8⁺ cells (**Fig. 2**). Specifically, regardless of the anatomical site, the average CD8⁺ TCR clonality is much higher than both the CD4⁺ T and B cells. This pattern is most consistent in the tumor, NT, and peripheral blood compartments and less consistent in CD4⁺ cells in the draining lymph node (data not shown). Subjects had either matched peripheral blood or draining lymph node specimens, but not both. To

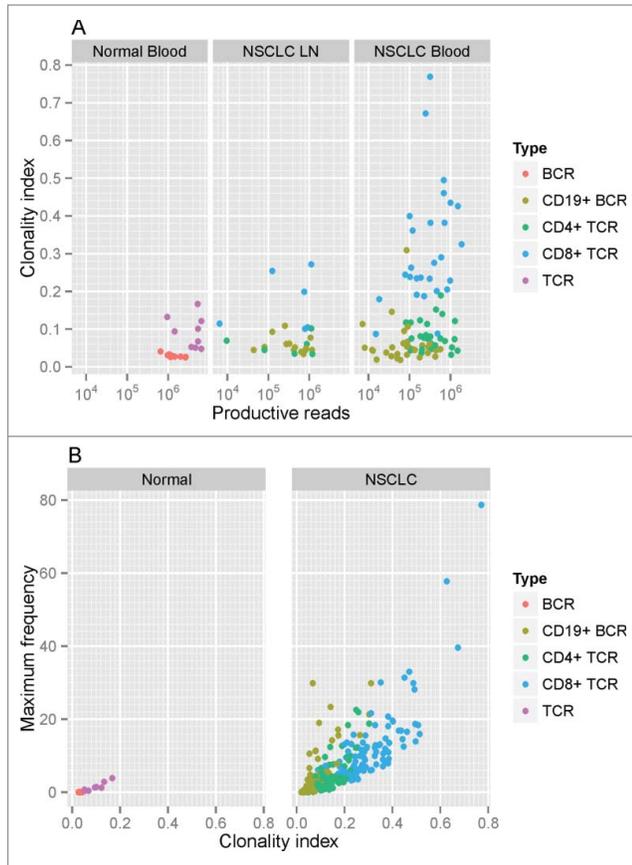


Figure 1. Distributional properties of clonality index, productive read count, and maximum clonotype frequency in peripheral compartments of NSCLC patients as compared to peripheral blood from normal donors. **(A)** No bias in clonality index as a result of productive read count was observed in peripheral blood from normal donors (left), draining lymph node from NSCLC patients (middle), or peripheral blood from NSCLC patients (right), and **(B)** correlation observed between the maximum clonotype frequency and clonality index in peripheral blood from normal donors (left) and peripheral blood from NSCLC patients (right). LN = draining lymph node.

maximize the number of patient samples with matched tumor and matched NT, peripheral blood or draining lymph node specimens, the two peripheral compartments were combined into a single measurement (peripheral blood/draining lymph node). Then the clonality was compared between the tumor (T), non-tumoral distant tissue (NT) and peripheral blood/draining lymph node (P) for each patient within the CD19⁺, CD4⁺, or CD8⁺ cell populations. Within the CD4⁺ T cells specifically, the NT specimens had a significantly higher average TCR repertoire clonality index than both the peripheral compartment ($p < 0.001$) and the tumor ($p = 0.002$), while the tumor had a significantly higher average clonality index compared to the peripheral compartment ($p < 0.001$; Fig. 3). No significant differences in clonality were observed between the tumor, the NT, or the peripheral compartments in either the CD19⁺ or CD8⁺ cell populations (Fig. 3).

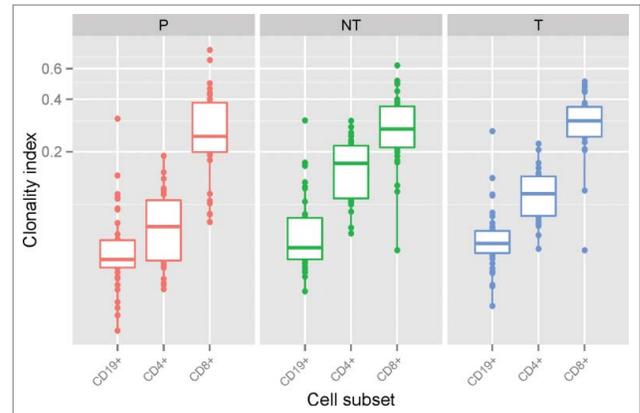


Figure 2. Increase in clonal expansion of clonotypes from CD19⁺ cells to CD4⁺ cells to CD8⁺ cells in all four tissue compartments of NSCLC patients. P = peripheral compartments (peripheral blood/draining lymph node); NT = non-tumoral distant tissue; T = tumor.

Younger NSCLC patients have higher CD4⁺ clonal diversity in the peripheral compartment and older NSCLC patients have highest clonality in the non-tumoral distant tissue

The patient population was divided into two age groups at the median of 68 y. Within the CD4⁺ cell population, the average clonality index was significantly higher in the patients age > 68 compared to patients age ≤ 68 in the peripheral compartment ($p = 0.05$) or stated alternatively, younger patients (age ≤ 68) exhibited higher immunodiversity in the peripheral compartment (Table 1 and Fig. 4A). The same trend (though not statistically significant) was also observed for CD4⁺ T cells in non-tumoral distant lung. The younger patient cohort had a significantly higher average clonality index in the tumor compared to the peripheral compartment ($p < 0.001$; Fig. 4B), while the older

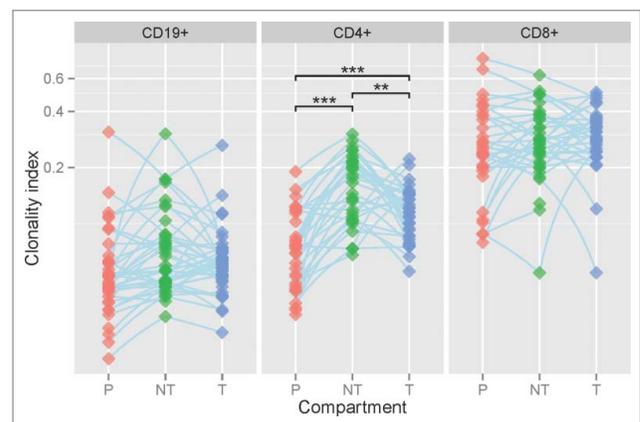


Figure 3. Significantly higher clonal expansion observed in the non-tumoral distant tissue compared to both tumor and peripheral compartments in CD4⁺ TCR repertoire from NSCLC patients. NT = non-tumoral distant tissue; P = peripheral blood/draining lymph node; T = tumor; ** = $p < 0.01$; *** = $p < 0.001$; all p -values are adjusted for multiple comparisons.

Table 1. NSCLC patient baseline variables evaluated for significant differences in clonality between levels using adjusted *p*-values

Variable	No. (%)	NT CD19 ⁺	NT CD4 ⁺	NT CD8 ⁺	P CD19 ⁺	P CD4 ⁺	P CD8 ⁺	T CD19 ⁺	T CD4 ⁺	T CD8 ⁺
Gender		0.20	0.05	0.57	0.63	0.35	0.57	0.57	0.63	0.49
Male	21 (44.7%)									
Female	26 (55.3%)									
Age at Surgery		0.54	0.09	0.19	0.48	0.05	0.25	0.41	0.31	0.57
< 68	22 (46.8%)									
≥ 68	25 (53.2%)									
Histological Type		0.16	0.19	0.61	0.31	0.31	0.63	0.43	0.31	0.43
ADC+neuroendocrine	1 (2.13%)									
ADC	23 (48.93%)									
ADC+SCC	1 (2.13%)									
LCC	1 (2.13%)									
neuroendocrine	1 (2.13%)									
SCC	20 (42.55%)									
Smoking Status		NE	NE	NE	NE	NE	NE	NE	NE	NE
Smoked/Current smoker	36 (76.6%)									
Never Smoked	6 (12.8%)									
NA	5 (10.6%)									
Dendritic cell Group		NE	NE	NE	NE	NE	NE	NE	NE	NE
High	39 (83.0%)									
Low	8 (17.0%)									
CD8⁺ T cell Group		0.35	NE	0.09	0.51	NE	NE	0.63	0.09	NE
High	36 (76.6%)									
Low	11 (23.4%)									
Follicular B cell Group		0.27	0.35	0.19	0.35	0.19	0.05	0.35	0.05	0.61
High	30 (63.8%)									
Low	17 (36.2%)									
T Stage		0.48	0.43	0.26	0.46	0.16	0.31	0.09	0.63	0.16
≤ T2a	33 (70.2%)									
≥ T2b	14 (29.8%)									
N Stage		0.16	0.25	0.31	0.35	0.61	0.31	0.35	0.35	0.63
N0	29 (61.7%)									
N1/2	18 (38.3%)									

Footnote: calculations were conducted with a Welch's two-group test, cutting each variable at the two most prevalent levels for each variable. All *p*-values reported are adjusted for 62 different comparisons using the *q*value method and rounded to two decimal places; ADC = adenocarcinoma; LCC = large-cell carcinoma; NE = not evaluated (due to highly unbalanced sample sizes between variable levels [variables with < 20% in one group were not included]); NT = normal distant tissue; P = peripheral compartment (peripheral blood/drainage lymph node); SCC = squamous cell carcinoma; T = tumor.

patient cohort (age > 68) had a significantly lower average clonality index in the tumor compared to the NT ($p < 0.001$; Fig. 4B). This age stratification demonstrates the bias in the older patient cohort for driving the observation reported previously in this study regarding higher average CD4⁺ TCR clonality index observed in the NT compared to the two other compartments. No significant difference in clonality was observed between the peripheral compartment and tumor in the older patient cohort or between the NT and tumor in the younger patient cohort. The NT in both the younger and older patient cohorts had higher average CD4⁺ TCR clonality indices compared to the peripheral compartment ($p < 0.001$; Fig. 4B). Though statistical significance was not observed, this similar trend of increased average clonality in older patients in the NT and peripheral compartment was observed for the CD8⁺ TCR repertoire, noting that the CD8⁺ T cells showed the highest overall clonality index (Fig. 2).

When evaluating the prevalence of shared clonotypes (Horn index) between pairs of each of the three tissue compartments for a patient, there was a higher average prevalence of shared CD4⁺ clonotypes between the peripheral compartment and tumor in

the older patient cohort compared to the younger patient cohort ($p = 0.04$; Fig. 5). A similar trend in shared CD4⁺ clonotypes was also observed between the NT and peripheral compartment, though not statistically significant ($p = 0.08$). No difference in prevalence of shared clonotypes between the tumor and NT was observed between the age groups.

Also within CD4⁺ cells, female NSCLC patients exhibited a higher average clonality index in the NT compared to males ($p = 0.05$; Table 1), though across tissue compartments, there was no significant association observed with gender, in contrast to that observed with age. No significant difference in comparisons of clonotypes with either the highest frequency or richness value (R) between age groups was observed (Fig. S5).

CD4⁺ TCR repertoire clonal expansion correlates with high TLS B cell density in the tumor

A previous study has demonstrated a correlation between TLS CD20⁺ Foll-B cell density and patient survival in NSCLC, thus connecting TLS to a protective B cell-mediated immunity (Fig. S6).¹⁵ Among the NSCLC patients, 30 were determined to be Foll-B cell high and 17 were Foll-B cell low (Table 1). Using

this population of 47 patients, the CD4⁺ and CD8⁺ TCR repertoires had higher average clonality indices in the Foll-B cell high group ($p = 0.05$) compared to the Foll-B cell low group in the tumor (CD4⁺) and in the peripheral (CD8⁺) compartment, respectively (Fig. 6). In contrast, neither CD8⁺ nor CD19⁺ cells demonstrated a significant difference between Foll-B cell high or low groups in the tumor and no difference between Foll-B cell high or low groups was correlated with average clonality for CD8⁺, CD4⁺, or CD19⁺ cells in the non-tumoral distant lung.

The prevalence of shared clonotypes between pairs of each of the three compartments for a patient was evaluated between the Foll-B cell high or low groups and no significant difference was observed for any compartment pairing or cell population (data not shown). Similarly, no difference in richness was observed between Foll-B cell high or low groups (data not shown).

Discussion

Here we characterized the TCR and BCR repertoires in NSCLC patients in sorted CD4⁺, CD8⁺, or CD19⁺ cell subsets to understand the clonotype spectra differences between the tumor, NT, and peripheral compartments. The greatest average clonality was observed in CD4⁺ and CD8⁺ TCRs, with CD4⁺ TCRs having greatest average clonal expansion in the NT, though this was shown to be driven by the clonal expansion in the older patient cohort. The tumor exhibited greatest average clonotype expansion compared to peripheral compartments, but only in the younger patient cohort. High immunodiversity was observed in CD4⁺ TCRs within the peripheral compartment in the young patient cohort relative to old patient cohort. Few public clonotypes were shared between patients, though the highest clonotype commonality occurred between compartments within the same patient. Specifically, among common clonotypes for a patient between tissue compartments, the older patient cohort had significantly more CD4⁺ clonotypes shared between the tumor and peripheral compartment as compared to the younger patient cohort; a similar trend with age groups was observed between the peripheral compartment and NT. Further, among variables with at least 20% patients in one group, there was no correlation observed in any compartment or cell type between

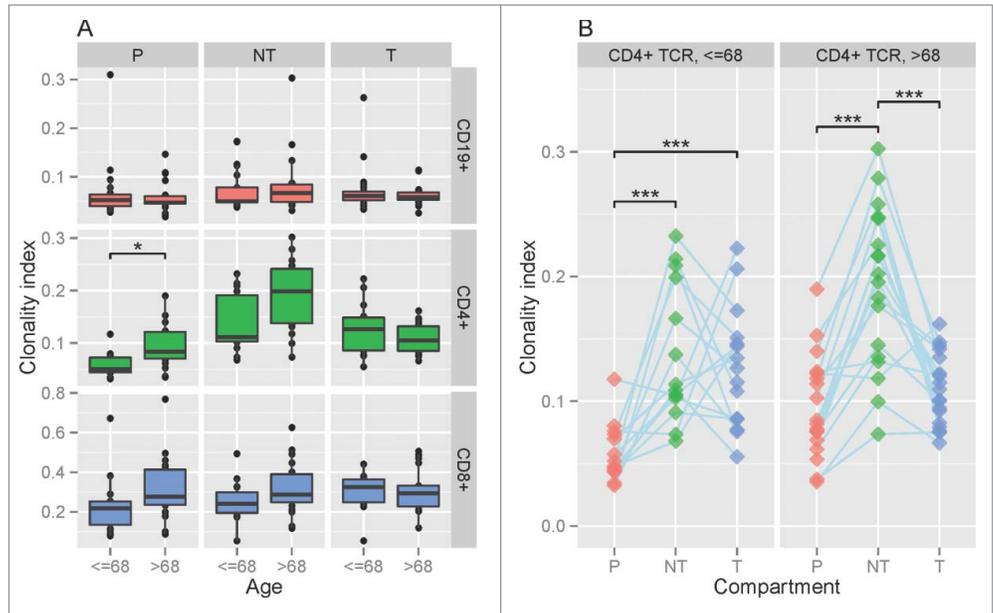


Figure 4. Clonal expansion in the tumor and non-tumoral distant tissue/peripheral compartments correlates with age in the CD4⁺ TCR repertoire. (A) Significantly increased diversity (decreased clonality) in the peripheral compartments of younger NSCLC patients (age ≤ 68) compared to older NSCLC patients (age > 68) in CD4⁺ cells. (B) Significantly increased clonality in the tumor compared to the peripheral compartments of young NSCLC patients in CD4⁺ cells, and significantly increased clonality in the non-tumoral distant tissue compared to the tumor of older NSCLC patients in CD4⁺ cells. In both age groups, the non-tumoral distant tissue had higher clonality CD4⁺ cells compared to the peripheral compartments. NT = non-tumoral distant tissue; P = peripheral blood/draining lymph node; T = tumor; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; all p -values are adjusted for multiple comparisons.

clonality index and T stage, N stage, histological type, or density of CD8⁺. Smoking status was highly skewed toward smokers in this study (77% smokers vs. 13% non-smokers), thus no association with clonality was calculated with this variable.

Intrinsic and extrinsic factors that define the diversity of different T cell subsets within the tumor have been previously documented,²⁶ in addition to the correlation between the intratumoral prevalence of certain subsets such as CD8⁺ or regulatory T cells (Tregs) and clinical prognosis in specific cancers.²⁷⁻²⁹ Among the different T cell subsets, both the intratumoral and inter-compartmental heterogeneity of the TCR repertoire has been reported in studies in many types of cancer including colorectal, renal cell, and ovarian cancer.^{23,30,31} Even within CD8⁺ T cells, subsets with expression of different surface markers have revealed differences in oligoclonal expansion in TCRβ clonotypes.³² These results underlie the importance of appropriate characterization of the activation and infiltration status within tumor or normal compartments with a mixed population of T cell subsets that differ in complexity and diversity.^{3,32,33} In this study, we address this issue with sorted CD4⁺ or CD8⁺ T cells and CD19⁺ B cell subsets within the tumor, NT, or peripheral compartments of a large population of NSCLC patients.

This type of cell-specific TCR or BCR sequencing from multiple tissue compartments requires sufficient specimen and yields following cell sorting and DNA extraction. Limitations in substantial material can greatly bias sequenced read counts, which

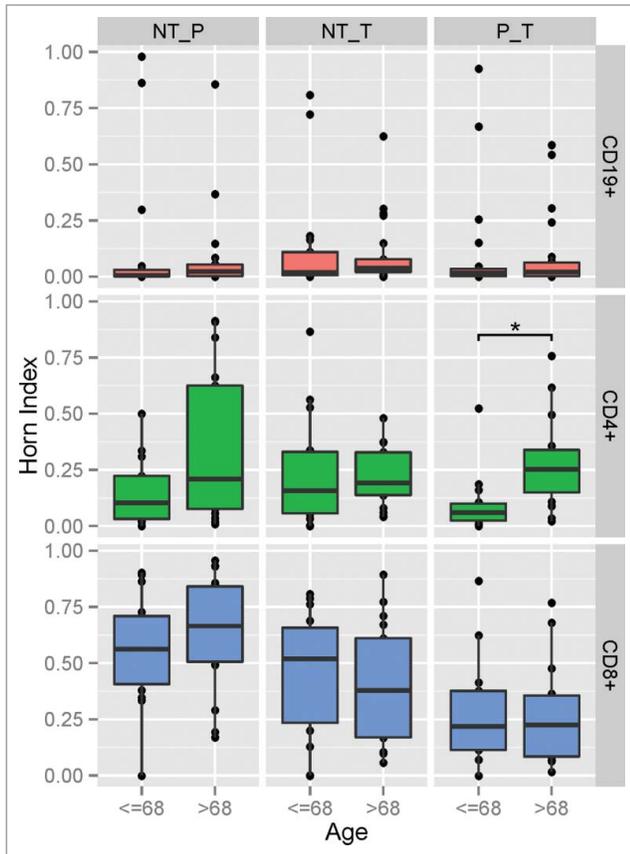


Figure 5. Prevalence of shared clonotypes (Horn index, **H**) between the tumor and peripheral compartments correlates with age in the CD4⁺ TCR repertoire. Significantly increased prevalence of shared clonotypes between the tumor and peripheral compartments in the older NSCLC patients (age > 68) compared to younger NSCLC patients (age ≤ 68) in CD4⁺ cells. NT_P = shared between non-tumoral distant tissue and peripheral blood/draining lymph node; NT_T=shared between non-tumoral distant tissue and tumor; P_T = shared between peripheral blood/draining lymph node and tumor; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; all p -values are adjusted for multiple comparisons.

influences normalization and measures of diversity/clonality, richness, and shared clonotypes. In cases where certain specimens had insufficient read counts, these samples were removed from the analysis. Similarly, both cell counts and nucleic extraction yields for all samples were compared against the various measures of immunodiversity and no correlations were identified, thus reducing any potential technical biases in findings. Nonetheless, future studies may benefit from previous methods developed to estimate patient CD4⁺ or CD8⁺ TCR contribution from peripheral blood mononuclear cell populations, rather than relying on cell sorting and potentially compromising sufficient sample yields for TCR repertoire characterization.³⁴

Across all NSCLC patients, the lowest average CD4⁺ clonality (thus the highest immunodiversity) was observed in the peripheral compartment (blood/lymph nodes) compared to both non-tumoral and tumoral lung of patients. In the peripheral compartment, naïve T cells are typically primed within the

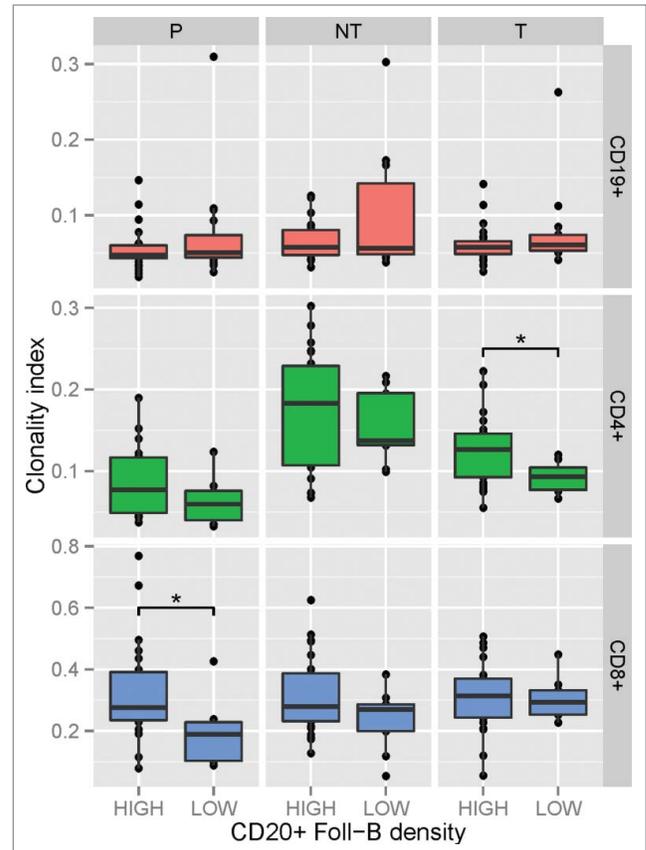


Figure 6. Clonal expansion of CD8⁺ T cells in the peripheral compartment and of CD4⁺ T cells in the tumor is associated with increased Foll-B cell density. NT = non-tumoral distant tissue; P = peripheral blood/draining lymph node; T = tumor; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; all p -values are adjusted for multiple comparisons.

draining lymph node, antigen-specific T cells are activated and undergo clonal expansion, and then migrate to tumor sites (and distant normal); a greater number of specific T cell clones would be expected in inflammatory sites/tumor tissues. We previously reported that lung tumor associated-TLS are mainly composed of CD4⁺ T cell subsets among the T cell compartment.¹⁶ Even rare, tumor-infiltrating naïve T cells are selectively detected in contact with mature DC in TLS suggesting that TLS may represent an active site for the priming and the proliferation of selected CD4⁺ T cell clones and thus may explain why the CD4⁺ T cell clonality is higher in tumor *versus* the peripheral compartment. As the immune infiltrate, and in particular TLS are mainly detected in the invasive margin of the tumor (i.e., areas at the interface between the non-tumoral and the tumoral tissues), TLS-derived effector T cells may also migrate to the NT, an argument in favor of a high clonality index of CD4⁺ T cells also in the non-tumoral distant lung.

This pattern was exemplified in the younger patient cohort in CD4⁺ T cells, where the tumor exhibited higher clonality compared to the peripheral compartment, as opposed to older NSCLC patients who displayed a high CD4⁺ T cell clonality in the periphery. A recent study showed a similar pattern in the

peripheral compartment of younger healthy subjects, with increased TCR repertoire diversity in the blood of younger subjects and significant decrease with age.³⁵ Using ultra-deep sequencing, the authors revealed that diversity of T cell clonotypes decreased almost linearly with age. In addition, there was a significant negative correlation of age with the percentage of naïve T cells in peripheral blood, though this trend was most pronounced up to subjects age 70.³⁵ Therefore age-related immunosenescence may be the major mechanism responsible for the difference of CD4⁺ T cell clonality between younger and older patients. The underlying mechanisms of immunosenescence of T cells can be traced back to thymic involution with aging.³⁶ However, the immune system may also selectively purge certain T cell clonotypes while keeping others. In a recent study, it was demonstrated that in addition to repertoire richness contraction, age was associated with inequality of clonal sizes, namely the presence of large naïve T cell clones in the repertoire of elderly individuals, reflecting uneven homeostatic proliferation; this hypothesis needs further investigation.³

Also relevant in the older patient cohort, is the reduced clonality within the tumor relative to the NT potentially as a result of an immunosuppressive tumor microenvironment, the magnitude of which may further increase due to age-related immunosenescence.³⁷⁻⁴⁰ The promotion of a tolerant microenvironment by tumors through mechanisms of antigen-presentation impairment, defects in TCR signaling, and introduction of immunosuppressive factors has been well characterized.⁴¹⁻⁴⁷ Within antigen-specific CD4⁺ T cells specifically, both *in vivo* and *in vitro* studies have demonstrated that they become tolerant during tumor growth.⁴² T cells are not primed and rendered unresponsive during tumor progression, a mechanism found in both solid and hematologic tumors.^{41,42,48,49} Tumor-induced tolerance in CD8⁺ T cells also exists, though both the definition of tolerance in the CD8⁺ subset as well as the downstream mechanistic effect is not as clearly known as that reported in CD4⁺ T cells.⁴² Induced defects in TCR signaling is another strategy exhibited by the tumor to promote growth and progression. Studies have shown a reduction in components that drive the effector phase of T cells to induce antitumor responses, such as decreased expression of CD3⁺ ζ chain and reduced activity of the TCR-associated tyrosine kinases p56(Lck) and p59(Fyn).^{50,51}

Additional support for the increased clonality in the non-tumor relative to the tumor compartment is based on recent studies describing the presence of tissue resident-memory T (T_{RM}) cells in peripheral non-lymphoid tissues. T_{RM} cells have an important role in protective responses against infection and in the recruitment of immune cells to tissues in cancer.⁵²⁻⁵⁹ These clonally expanded memory T cells, located in peripheral tissues, can launch a rapid response to antigen following an initial antigen exposure.⁵³ CD4⁺ T_{RM} cells reside in non-lymphoid tissues such as skin, lung, and mucosal compartments.⁵⁴⁻⁵⁹ Within non-inflamed normal lung tissue, distant from localized tumor in patients with various types of malignancies (localized to specific segments of the lung), Vβ segments of TCRs from CD4⁺ and CD8⁺ were measured and proliferation of these cells in response to influenza exposure was compared between lung, skin, and

blood.⁵⁹ Results indicated that functional T_{RM} cells were much more prevalent in the lung compared to the other two compartments and enriched for T cells specific for influenza antigens. A similar finding was identified in mice recipients of spleen- or lung-derived memory CD4⁺ T cells, which were compared following challenge with influenza virus. The mice recipients of lung-derived memory T cells showed a much faster and robust protection against the virus compared to spleen-derived memory T cells, suggesting that functional T_{RM} cells have certain tissue compartmentalization properties, such that those retained in lung tissue provide increased protection to respiratory virus infection.^{56,59} These mechanisms support our observation of increased clonotype expansion in the NTs for CD4⁺ T cells relative to the tumor (as well as the periphery) of the NSCLC patients – a pattern driven by the older patient population.

Also in this older patient cohort, the prevalence of shared CD4⁺ clonotypes between the tumor and peripheral compartment is significantly higher than the younger patients – a trend that is apparent in the peripheral compartment and NT as well. This implies that more unique CD4⁺ clonotypes are identified in the peripheral compartment in the younger patient cohort compared to the older patient counterpart, which might be explained by age-related immune responses (i.e., reduced lymphopoiesis of new naïve CD4⁺ T cells in older patients). This concept is also supported by the lack of difference in shared clonotypes between the NT and tumor when comparing the young and old patient cohorts. Previous reports have reinforced the concept of age-related shifts of the TCR repertoire in the peripheral compartment toward memory T cells in both mouse models and humans, with naïve T cells maintained in part by homeostatic proliferation.⁶⁰⁻⁶² This ratio of naïve to memory T cells is reduced with age, as is TCR repertoire diversity in the naïve T cells, consistent with the CD4⁺ results shown here in the peripheral compartment, as well as more shared CD4⁺ clonotypes between the peripheral and tumor compartments. These findings, along with the aforementioned study by Qi et al.,³ suggest further investigation into the relative abundance and clonotype profiles of naïve and memory CD4⁺ T cells and association with age.

A smaller study in 15 colorectal cancer patients has shown increased oligoclonality in tumors compared to mucosal tissue samples from the same patient,³⁰ which agrees with what is reported in this study for patients less than the median age (≤ 68). For NSCLC patients older than the median age, a shift in adaptive response occurs, with the NT having higher oligoclonality than the tumor. However, it is important to note that the clonality indices of CD4⁺, CD8⁺ and CD19⁺ cells did not differ between younger and older NSCLC patients in the tumor suggesting that an active process is involved in the selection and maintenance of specific T and B cell clones in the tumor, even in the presence of immunosenescence. One explanation would be the continuous expression of cognate tumor antigens in the tumor microenvironment over time in order to maintain clonal expansion of specific clones within the tumor relative to distant sites.

We also observed higher average clonality in female NSCLC patients, compared to males within the NT in CD4⁺ T cells.

This pattern was not associated with the age-related differences in clonality reported here, which dismisses any confounding associations with the age-specific findings (Fisher's exact test $p =$ not significant). Studies by Murray et al. have demonstrated gender-specific V(D)J recombinase-mediated TCR β gene usage and coding joint processing in human development that demonstrate direct effects on TCR diversity, though these studies were conducted on healthy children at a maximum age of 12.5 y.^{63,64} In this study, these gender-specific differences in clonality did not occur across compartments – an observation that did occur with age-related associations, thus the strength of this gender-specific observation in NSCLC needs to be further evaluated in subsequent studies.

In previous work, we reported the presence of TLS consisting of Foll-B cells surrounding clusters of DC-Lamp⁺ mature DCs and T cells in NSCLC tumors, and that a high density of mature DCs was correlated with improved patient prognosis.¹³ Similarly, we later showed that a high density of TLS Foll-B cells was correlated with improved survival in NSCLC patients, and that the combination of both mature DC and TLS B cell densities further improved this survival benefit.¹⁶ Here we observe a significant correlation between high TLS Foll-B cell density and high CD4⁺ TCR repertoire clonality in the tumor, as well as high CD8⁺ TCR repertoire clonality in the periphery. A similar trend was also shown for CD4⁺ T cells in the non-tumoral lung and the peripheral sites suggesting that selective CD4⁺ T cell clones may spread throughout the whole body.

It is known that B cells can act as powerful antigen-presenting cells in many diseases. In particular, Bouaziz et al. demonstrated in mouse models of autoimmune disorder that B cell depletion selectively impaired the activation and clonal expansion of autoreactive CD4⁺ T cells but not of CD8⁺ T cells.⁶⁵ As TLS represents a privileged site for the initiation of adaptive immune responses, it is tempting to speculate that TLS B cells may play a key role in the priming and the proliferation of selected intratumoral CD4⁺ T cell clones. In particular, among the CD4⁺ T cell subsets, follicular helper T cells (T_{FH}) are selectively detected within the B cell follicle of secondary lymphoid organs as well as TLS.⁶⁶ T_{FH} specialize in providing cognate help to B cells in follicles, and are fundamental for germinal center formation, affinity maturation and the differentiation of naïve B cells into memory B cells and plasma cells secreting high-affinity antibodies. Thus, it is tempting to speculate that the high clonality of CD4⁺ T cells observed in TLS Foll-B cell high group may be the result of an active cross-talk between T_{FH} and Foll-B cells leading to the proliferation of selective T_{FH} clones. This hypothesis is in accordance with the favorable prognostic value of T_{FH} reported in breast cancer patients.⁶⁶

In summary, advancements in deep sequencing have paved the way for measures of the antitumor immune response at the detail of the TCR and BCR involvement. This study is the first large patient study ($n = 47$) to our knowledge to examine the TCR and BCR repertoires in sorted CD4⁺, CD8⁺, and CD19⁺ cell subsets across four tissue compartments in NSCLC patients, with stringent tests of association. Here we notably present distinctive CD4⁺ T cell involvement in the adaptive immune

response for patients with NSCLC, with a shift in age from high clonal expansion in the periphery to reduced clonal expansion in the tumor, potentially due to systemic immunosenescence and/or putative immunosuppression in tumors. Recently, clinical results for immune-mediated therapies for cancers (IMT-C) have shown encouraging results for treatment of cancers such as lung and melanoma.⁶⁷ As these therapies generally activate T cells as a primary mechanism against tumor cells, the reduction in T cell diversity in older cancer patients may require unique personal healthcare strategies to consider potential T cell replenishment to maximize effectiveness of IMT-C in older patients. We also correlate tumoral CD4⁺ TCR repertoire clonality to high Foll-B cell density in the tumor microenvironment – a previously identified prognostic biomarker in NSCLC. Better understanding the mechanisms of the locally developed adaptive immune response may have important implications for the development of new drug interventions based on TCR repertoire within this cancer indication.

Materials and Methods

NSCLC patients

A prospective study was performed on fresh NSCLC samples, comprised of 47 untreated patients with NSCLC (Table 1). Tumor tissue (T), non-tumoral distant tissue (NT), peripheral blood (Bl), or draining lymph node (LN) were obtained from patients who underwent a complete surgical resection of their lung tumors at Hotel Dieu hospital, Cochin hospital or Institut Mutualiste Montsouris (Paris, France). Patients who received neoadjuvant chemotherapy or radiotherapy were ineligible. A written informed consent was obtained from patients before inclusion in the prospective study. The protocol was approved by the local ethics committee (n°2012-0612) in application with the article L.1121.1 of French law.

Cell sorting

Fresh tissues were mechanically (manual) dissociated and digested in the presence of DNase I (Sigma Aldrich) and collagenase A (Roche) for 1 h at 37°C. The cell suspension was then filtered through a 70- μ m filter (BD Biosciences), and mononuclear cells were isolated by centrifugation over a Ficoll Hypaque density gradient. CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells as well as CD19⁺ B cells were sorted using a FACS Aria III cell sorter (BD Biosciences) among alive cells (gated DAPI⁻), non-epithelial cells (gated CD227⁻, epithelial antigen⁻, pan-cytokeratins⁻), and haematopoietic cells (gated CD45⁺) (antibodies used are listed in Table S2). Reanalysis of the sorted populations showed a purity > 98%.

Immunohistochemistry

Serial sections of paraffin-embedded NSCLC tumors were stained as previously described (De Chaisemartin, Cancer Res 2011),¹⁶ using antigen retrieval conditions, antibodies and reagents listed in Table S2.

Method for immune cell quantification

Tumor-infiltrating CD8⁺ T cells were enumerated in the tumor stroma of the whole tumor sections. DC-Lamp⁺ mature DCs and CD8⁺ T cells were quantified with Calopix software (TribvN, Paris, France), and expressed as an absolute number of positive cells per mm² of tumor. Follicular CD20⁺ B cell quantification was performed with Calopix, and expressed as the surface of CD20⁺ B-cell follicles (mm²) per mm² of tumor. Both immunostaining and quantification were reviewed by two independent observers (CG, SK, PD, and/or MCDN).

TCR or BCR repertoire sequencing

DNA was isolated from sorted cell populations utilizing the Qiagen QIAamp DNA micro kit, according to the manufacturer's suggested protocol. Resulting DNA concentrations were assessed by Qubit[®] fluorometric quantitation. TCR and BCR sequencing was then performed by Adaptive Biotechnologies via the immunoSEQ service.^{19,25} Due to the yields obtained from the relatively low counts of sorted cells, survey level depth sequencing was performed for all samples, but sufficient number of sequenced reads were required for final inclusion in the study: specimens with read counts < 5,000 were removed from all analysis.

Statistical analysis

The proportion of U productive, or in-frame rearrangement unique TCR or BCR clonotypes i is defined as

$$p_i = \frac{n_i}{\sum_{i=1}^U n_i},$$

where n_i is the count of the productive reads of the clonotype $i = 1, 2, \dots, U$. Diversity D is calculated by the normalized Shannon entropy given by^{19,68}

$$D = - \sum_{i=1}^U \frac{p_i \log_2 p_i}{\log_2(U)}.$$

Then the clonality index (CI) can be defined as the complement of diversity, $CI = (1 - D)$. So as CI approaches 0, this indicates a diverse TCR or BCR repertoire, or a preponderance of unique clones within the repertoire. In contrast, as CI approaches 1, this indicates a clonal TCR or BCR repertoire, or increased expansion of certain clones in the repertoire. A measure of relative clonotype richness R was also calculated as the average unique clonotypes per cell (assuming one unique clonotype per cell), given by³⁵

$$R = \frac{U}{\sum_{i=1}^U n_i}.$$

Finally, the prevalence of shared clonotypes between tissue compartments within the same patient was calculated using the Horn

index, defined as⁶⁹

$$H_{NT,T} = \frac{2 \sum_{i=1}^U p_i^{NT} p_i^T}{\sum_{i=1}^U (p_i^{NT})^2 + \sum_{i=1}^U (p_i^T)^2},$$

where T and NT are the tumor and non-tumoral distant tissue compartments, respectively. H can range in value from 0 to 1 with values that approach 1 indicating more shared clonotypes between tissue compartments for a patient. The same calculation was conducted between the tumor and peripheral compartment as well as the NT and peripheral compartment.

All unpaired two sample tests were conducted with a Welch's modified t-test, while paired comparisons were calculated with a Student's paired t-test. All p -values are adjusted for multiple testing comparisons using the qvalue package (62 comparisons for within-compartment analyses [Table 1] and 9 or 6 comparisons in across-compartment analyses) and rounded to two decimal places, so values reported at $p = 0.05$ are in fact < 0.05 prior to rounding. To avoid biased comparisons, only variables with at least 20% of patients in one of the two groups were used in correlation analysis with clonality (those < 20% denoted with NE in Table 1). All analysis was conducted in the R statistical programming environment (R Development Core Team 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org>).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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