Biochemical and Biophysical Research Communications 458 (2015) 70–76

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Heterogeneous expansion of CD4+ tumor-infiltrating T-lymphocytes in clear cell renal cell carcinomas

Qian Zhang [a, b], Qingzhu Jia [c], Tianxing Deng [a], Bo Song [b, **, 1], Longkun Li [a, *, 1]

[a] Department of Urology, XinQiao Hospital, Third Military Medical University, Chongqing, China
[b] Department of Urology, Southwest Hospital, Third Military Medical University, Chongqing, China
[c] Biomedical Analysis Center, Third Military Medical University, Chongqing, China

ARTICLE INFO

Article history:
Received 27 December 2014
Available online 28 January 2015

Keywords:
Renal cell carcinoma
Repertoire sequencing
TCRB
Tumor-infiltrating lymphocyte

ABSTRACT

Aberrant expression of tumor-associated antigens (TAAs) mediates the effective mounting of adaptive immunity in human solid tumors. The foundations of this tumor-host interaction strongly depend on specific recognition via TAA-cognate-receptors in T-cell repertoires. Previous studies focused on the phenotypic and functional properties of CD4+/CD8+ tumor-infiltrating T-lymphocytes (TILs), but the detailed composition of T-cell repertoires of these fundamental subsets remains largely unknown. This study recruited 10 clear cell renal cell carcinoma (ccRCC) patients and obtained samples from various tissues, including tumors, adjacent healthy renal tissue and peripheral blood. We utilized deep sequencing of T-cell receptor beta chains (TCRB), which serve as a unique identifier for each T clonotype, to characterize the CD4+/CD8+ TIL repertoire in ccRCC patients, assess the diversity and clonality of infiltrated T-cells in distinct tissues from patients and depict the clonal expansion events that occur in anti-tumor immune responses. We found that the CD4+ TIL repertoire exhibited signatures of heterogeneous T-cell expansion, which were characterized by divergent TRBV/J usage and an enrichment of expanded dominant clones. Taken together, our findings provide additional evidence of CD4+ T-cell-mediated anti-tumor immunity. The identification of the underlying molecular mechanisms of this process may provide novel avenues for targeted immunotherapeutic interventions.

1. Introduction

Development of effective adjuvant immunotherapies to ccRCC is becoming a focusing area because of the relative resistance of ccRCC to radiation therapy and chemotherapy [1]. The somatic mutations that accumulate within tumor cells lead to the presentation of aberrant matrices of TAAs on the cell surface, which is a consequence of pathological genetic alterations. TILs can recognize these TAAs to provoke anti-tumor adaptive immunity. Tumor cells derived from the same histological origin may exhibit largely diverse signatures of neo-epitope presentation. These diversely presented neo-epitopes were paralleled with an apparently heterogeneous T-cell infiltration, which was characterized by variable infiltration magnitude, different T-cell subsets compositions and uneven spatial distribution in the tumor microenvironment [2].

CD8+ TILs mediate direct TAA-recognizing and cytotoxic activity, and the favorable clinical significance of these cells has been established in several tumor types [3]. However, the situation is much more ambiguous for CD4+ compartments as suggested by their manifold subpopulations and convertible biological characteristics in tumor niches [4]. The helper function of autologous CD4+ TILs might enhance and maintain the full efficacy of TAA-reactive CD8+ T-cells [5]. Furthermore, CD4+ TILs eliminate tumor cells in the absence of CD8+ TILs via PRF1/GZMB [6] or Th1/M1-like macrophage-dependent [7] mechanism in MHC-II-positive or negative conditions, respectively. Therefore, an understanding of which fraction of intra-tumoral T-cells are activated and expanded subsequent to tumor challenge and the factors that determine

Abbreviations: TCR, T-cell receptor; TCRB, beta chain of T-cell repertoire; CDR3, complementary determining region 3; ccRCC, clear cell renal cell carcinoma; TIL, tumor-infiltrating lymphocyte; HEC, highly expanded clone; ANDEC, antigen-driven expanded clone.

* Corresponding author. Department of Urology, XinQiao Hospital, Third Military Medical University, XinQiao St 83, Chongqing, China.
** Corresponding author. Department of Urology, Southwest Hospital, Third Military Medical University, GaoTanYan St 30, Chongqing, China.
E-mail addresses: songb-mr@vip.sina.com (B. Song), lilongk@hotmail.com (L. Li).
[1] Equal contribution as co-corresponding authors.

http://dx.doi.org/10.1016/j.bbrc.2015.01.069
0006-291X/

antigen-specificities are of obvious interest for the advancement of personalized immunotherapy.

The effective recognition of numerous antigens by adaptive immunity is critically dependent on the extremely diverse repertoire expressed by TCRs, which is formed by somatic recombination of TCRA and TCRB loci during early development in the thymus [8]. The CDR3 regions within TCRs determine their specificities and binding strengths, which allow the receptors to recognize a wide variety of antigens and primarily contribute to the diversity of the repertoire [9]. The effective activation and subsequent expansion of specific T-cell clones should lead to the over-representation of T-cells that bear a particular TCR in a population. Therefore, the quantification of expanded TCR clonotypes has sensitive and robust implications for the underlying antigen-specific T-cell responses. Recently, a large-scale analysis of immune repertoires using high-throughput sequencing approach provided a more elaborate overview of the adaptive immune response [10]. We performed a multiplex PCR assay to amplify and sequence the CDR3 regions of rearranged TCRB molecules from TILs, T-cells in the adjacent healthy renal tissue and peripheral blood of 10 ccRCC patients to establish the links between tumor microenvironments and TIL repertoires in this disease.

Here, we developed a sequencing approach to characterize the CD4+/CD8+ TIL repertoires and compare them with paired compartments from the vicinity and circulation. This comparison, addressed (1) the general characterization of T-cell subset repertoire in ccRCC, (2) whether oligo-expansion of T-cells occurred in tumor sites, (3) the antigen-specification of these expanded clones.

2. Materials and methods

2.1. Specimen collection

Tumors (2 g), adjacent non-invasive renal tissue (2 g) and peripheral blood (5 ml) were obtained from 10 ccRCC patients at the time of primary laparoscopic nephrectomy. The American Joint Committee on Cancer (AJCC) pathological stage was confirmed. None of patients had received anti-cancer treatment before the samples were taken. Patients in infectious states, with autoimmune diseases and who had taken medicine that could perturb the peripheral hemogram were excluded. All subjects were provided written informed consent for the use of their tissue prior to the sampling. Detailed information about the cohort is listed in Supplemental Table 1. HLA types were provided in Supplemental Table 2.

2.2. Isolation of single T-cells from samples

Single cell isolations from the solid tissues were performed according to previously published protocol. PBMCs from patients were isolated by density gradient centrifugation using Lymphoprep according to standard protocol. Isolating products were subjected to subset sorting.

2.3. Sorting for T-cell subset

T-cell isolations were performed using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) coated with monoclonal antibodies specific for the CD4+/CD8+ T-cell subset. First, monocytes were removed from the isolated PBMCs using anti-CD14 microbeads. Next, CD4+ and CD8+ T-cells were isolated using positive selection with anti-CD4 and anti-CD8 microbeads, respectively. All isolated CD4+ and CD8+ T-cell samples containing more than 2000 cells were stored immediately in Trizol reagent at −80 °C until analysis.

2.4. Sequencing of the TCRB

Total RNA from sorted cells was extracted according to the Trizol manufacturer's protocol. Complementary DNA was prepared using 200 ng total RNA template and TRBC-specific reverse transcriptional primers (5′-ATCTCTGCTTCTGATGGCTCA-3′). One set of primers with incorporated sequencing adaptors/barcodes was introduced to perform multiplex PCR. The following PCR conditions were used: 94 °C, 5 min; (94 °C, 30 s; 62 °C, 1 min; 72 °C, 90 s)*35 cycles, 72 °C, 5 min; and 16 °C, hold. Amplicon of CDR3-region in TCRB was purified and sequenced using the Ion Torrent PGM platform.

2.5. Sequence alignment and statistics

Raw sequences with recognizable barcode identities were converted to a FASTQ format and imported into MATLAB 2014a software (MathWorks). A MATLAB script was introduced to assemble each raw read into a functional TCRB molecular structure. The CDR3 region in TCRB was identified according to the definitions of the ImMunoGeneTics (IMGT) collaboration [11]. Further analyses were performed using MATLAB 2014a. P values below 0.05 were considered significant in all tests.

3. Results

3.1. TRBV/J usage of the TIL repertoire

To investigate any ccRCC-associated repertoire reformations in the TRBV/J usage patterns, we evaluated deviations in TRBV/J usage frequency of TILs between patients and compared these results with the inter-renal and inter-blood repertoires as controls. The Kolmogorov–Smirnov test (KS test) was introduced to evaluate the difference of TRBV/J usage between two patients. Resulting p-values (pKS) were used to estimate whether the usage patterns were significant difference. TRBV usage of the CD4+ TILs repertoire in this analysis exhibited significantly divergent patterns compared to the counterparts from renal and blood repertoires (Fig. 1A). Despite falling short of statistical significance, we still observed higher divergent propensity for TRBJ segment usage (Fig. 1C). Surprisingly, we failed to identify any divergent usage pattern for CD8+ TIL subsets (Fig. 1B, D). Therefore, although it is believed that CD8+ TILs should recognized TAAs and expand subsequently in the tumor site, we found that the CD4+ TIL repertoire exhibited a significantly divergent TRBV/J usage pattern in TIL repertoire, indicating a robust repertoire reforming process.

3.2. Enrichment of highly expanded clones within the tumor microenvironment

Given our findings of divergent CD4+ TIL TRBV/J usage in ccRCC patients, we postulated that this phenomenon is caused by the selective expansion of TAA-reactive clones in anti-tumor responses. Here, we defined T-cell populations that contained identical amino acid sequences in the CDR3 region as CDR3 AA clonotypes [12]. We selected highly expanded clones (HECs, defined by CDR3 AA clonal abundances exceeding certain threshold) from the TIL, renal and blood repertoires for each patient and assessed their cumulative proportions to determine whether HECs were enriched in the tumor site. We assigned this threshold across a rationale range from 0.1% to 5% and performed the statistical test under each threshold. This measure can help reflect the lurking tendencies towards clonal

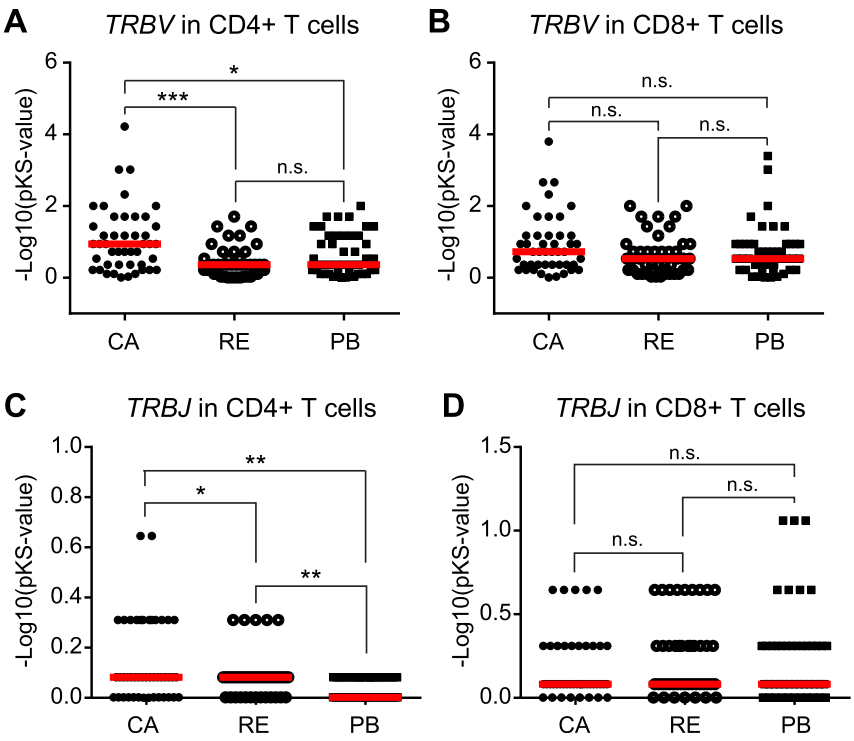

Fig. 1. Divergent usage in the CD4+ T-cell repertoires. Overall usage of the rearranged TRBV/J segments within the tumor (CA), adjacent healthy renal tissue (RE) and the peripheral blood (PB) repertoires for each T-cell subset in ccRCC patients. Kolmogorov–Smirnov test was utilized to evaluate differences in the usage frequencies between every pair of repertoires from a common tissue origin. P-values of Kolmogorov–Smirnov test (pKS) were chosen as indicators for this evaluation. (A, C) Significantly divergent usage patterns observed in the CA repertoire compared to the RE/PB repertoires for CD4+ T-cells. (B, D) Indistinguishable usage frequencies between the observed CD8+ T-cell repertoires. Higher −1og10 (pKS) value, significant divergent usage; lower −log10 (pKS) value, undistinguished usage.

expansion because of possible insufficient statistical significance yielding in some HEC cutoff settings. These observations identified a general trend toward oligo-clonal expansion of the CD4+ TIL compared to the renal and blood repertoires (Fig. 2A). In contrast, no such trends were found at any cutoff values for the CD8+ TILs (Fig. 2B).

Based on our observations that over-expanded CD4+ T-cell populations accumulated in tumor sites, we reasoned that these TIL repertoires should exhibit relatively restricted and contracted repertoire compositions. Therefore, we calculated the normalized Shannon diversity entropy (NSDE) [13] and Simpson diversity index (SDI) [14] for each sample to quantify the expansion extent of repertoires. Expanded-experienced repertoire expected to show a lower diversity value than paired control repertoire. As expected, the CD4+ TIL showed a much lower diversity index according to both the NSDE and SDI (Fig. 2C, E). In contrast, the diversity index observed for the CD8+ TIL repertoires of distinct tissue origins were indistinguishable by the NSDE and SDI indicators (Fig. 2D, F), albeit the general diversity of the CD8+ TILs was much lower than the CD4+ counterparts (Supplemental Fig. 1).

3.3. Signatures of selective-expansion in CD4+ TILs

Based on previous observations, we next focused our analysis on the CD4+ TIL subset. To confirm the TAA-specific T-cell expansion during tumorigenesis, we introduced the concept of 'antigen-driven expanded clones' (ANDECs) into our analysis. An ANDEC clone should meet three independent criteria, i.e., high clonal abundance in its repertoire, complex coding degeneration of the CDR3 AA sequence and complex TRBV usage. First, it is reasonable to assume that reactive clones should be expanded in the response. Second, facing the driving force from TAA-TCR encountering, it's reasonable to expect that TILs containing certain TAA-specific CDR3 region would prime and proliferate regardless of their TCRB nucleotide coding strategy (certain CDR3 AA could translate from several coding degenerated 3-nt codon in the entire TCRB locus). Third, if the selective TAA-specific expansion is primarily based on selective pressure on the CDR3 AA sequence, it is conceivable that a complex panel of TRBV segments should contribute to ANDECs if they share common CDR3 regions. If T-cell expansion occurs in an antigen-independent manner without CDR3-specificity, e.g., only as a consequence of the prolonged infiltration in inflammatory mellitus or pro-inflammatory cytokine exposure, complex coding degeneration and diverse TRBV usage would not be observed compared with a control repertoire.

Firstly, we defined ANDEC clones as frequencies greater than 1% with no less than five coding strategies in the CDR3-region and at least two TRBV usages. All CDR3 AA clonotypes were analyzed in three tissue compartments from ten patients (Fig. 3A–C). Correlations among all three parameters were observed, but an overwhelming uptrend in the distribution of clones for the TIL repertoire was found, which represented an expansion pattern. Furthermore, we applied this analysis to the repertoire of each individual, and the total percentages of ANDECs in different repertoire types were evaluated (Fig. 3D). We observed remarkably higher proportions of ANDECs in the TIL repertoires. Additionally, we also assessed the clonotype abundances (Fig. 3E), number of coding strategies (Fig. 3F) and TRBV usage (Fig. 3G) of ANDECs in each tissue. These analysis confirmed our speculation that the ANDECs in the TIL repertoires exhibited significant antigen-driven associated signatures: high clonal abundance, complex coding strategies and diverse TRBV segment composition.

Notably, the definitions of ANDEC populations were an empirical selection in the above analysis. Therefore, we performed a

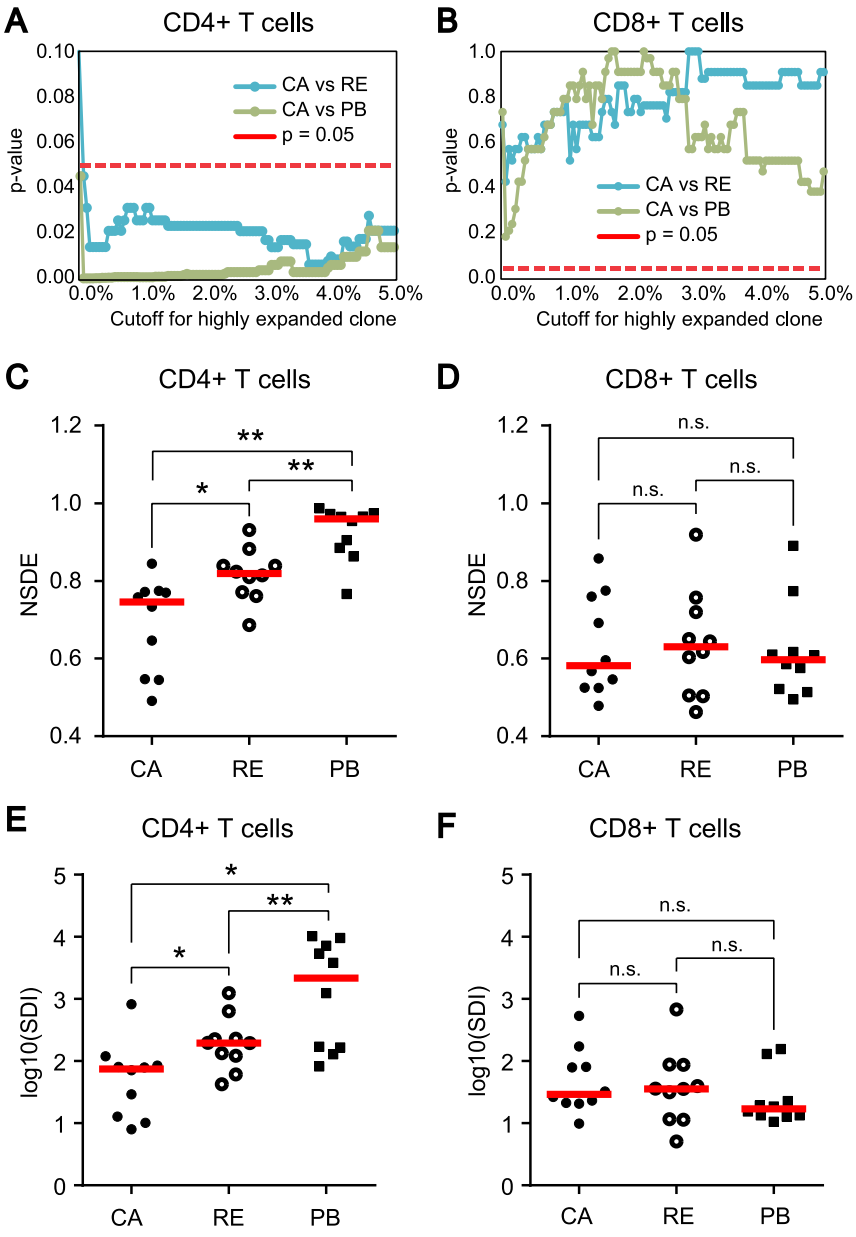

Fig. 2. Enrichment of expanded CD4+ clones in tumor niches. Enrichment of highly expanded clones in tumors. (A, B) Proportions of HECs were determined according to certain threshold values for each patient. Solid line indicates the statistical significance (p-value, transformed to −log10 based on two-tailed Mann–Whitney *U*-tests) against the threshold values that defined the HEC populations for CD4+ (A) and CD8+ (B) T-cells. (C–F) Comparisons of the normalized Shannon diversity entropy (NSDE) and Simpson diversity index (SDI) for the CDR3 clonotypes of CD4+ and CD8+ T-cells. Higher value, flat repertoire; lower value, expanded repertoire. Horizontal bars, median value.

criteria screening to test the robustness and reproducibility of our findings in TIL ANDECs. Surprisingly, we observed an accumulation of ANDECs in the TIL repertoire in a wide range of settings, with the exception of several extremes, which demonstrated the credibility of our findings (Supplemental Fig. 2).

3.4. Heterogeneity of ANDEC antigen specificity in CD4+ TILs

To further investigate whether there were public TCRs that responded to ccRCC-associated antigens, we examined the overlap of the CD4+ ANDEC TILs among patients (Supplemental Table 3). We observed 68 unique ANDECs in TIL repertoires that contained 42 different TRBV segment families. Only five ANDECs (5.88%, 5/68) appeared in more than half of the patients, and the majority of them (86.76%, 59/68) were simultaneously detected in no more than three patients. Additionally, clone 'CASQGYTEAF' was observed across the entire cohort with only two exceptions, but the extremely low clonal abundance makes it difficult to judge whether there was a public ANDEC TILs that is specific to a common TAA that has not yet been determined.

Following these findings, we speculate that the lack of a detectable consensus CDR3 AA sequence within the CD4+ TIL repertoires might have resulted from the highly diverse and individual-dependent TAA-encountering experiences during tumorigenesis. Kidera factors capture uncorrelated physicochemical properties of peptide chains, which has been shown to delineate the structural properties of proteins that are encoded by distinct AA compositions [15]. Completely different CDR3 AA

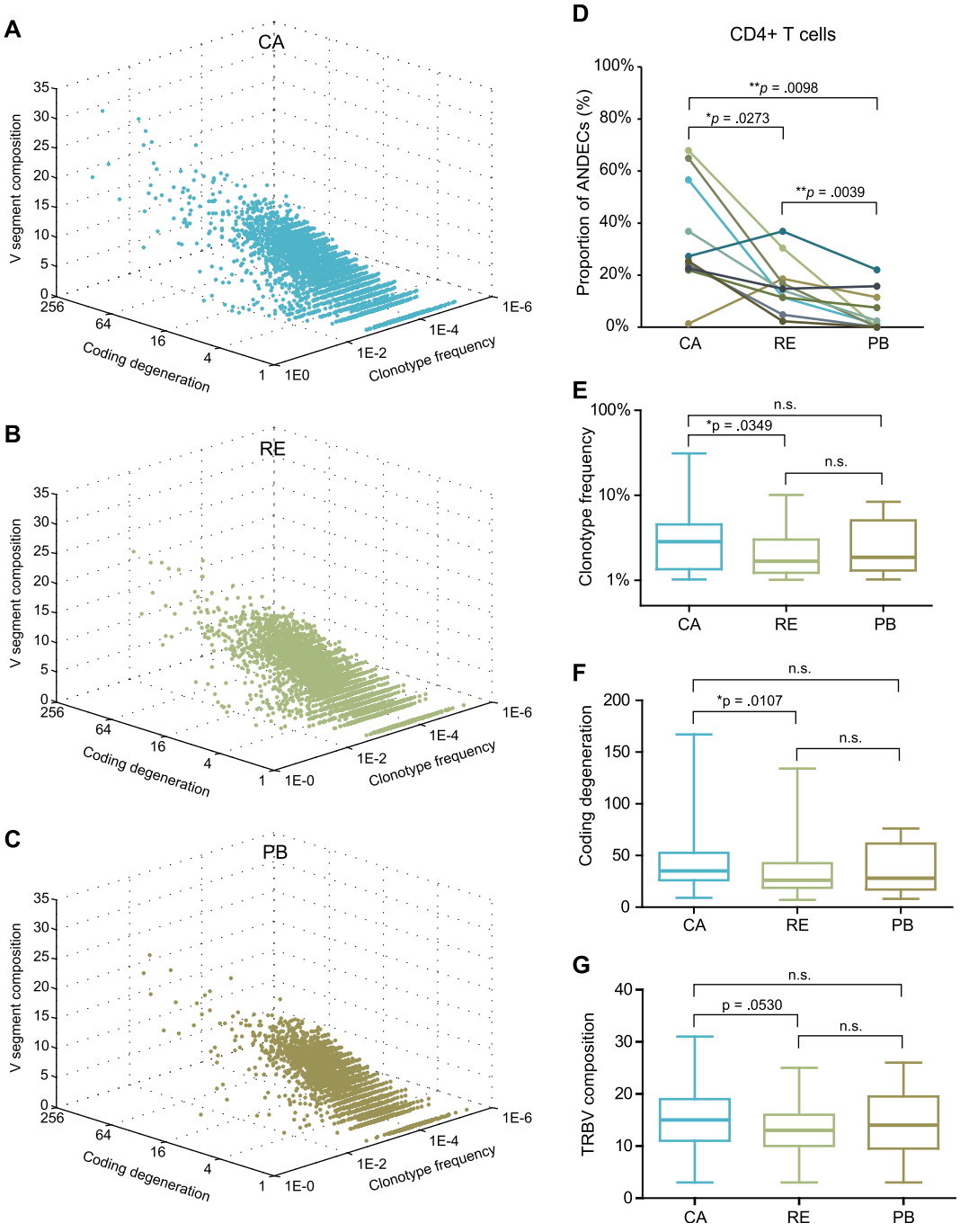

Fig. 3. Signatures of antigen-driven clonal expansion. (A–C) For the CD4+ T-cells, the clonal abundance, TRBV compositions (i.e., the numbers of different TRBV segments that contributed to each clonotype) and level of coding degenerations (i.e., the numbers of unique TCRB nucleotide sequences bearing a common CDR3 amino acid sequence) for each CDR3 clone were determined for each tissue type. (D) Comparison of the accumulated proportions of ANDECs in each tissue type. Tested with a two-tailed Wilcoxon matched test. (E–G) Comparison of the properties of each of the CD4+ clones between different tissue types. The ANDECs in the tumor group exhibited antigen-driven signatures that were characterized by higher clonal abundance, more complex CDR3 coding strategies and more TRBV segment usages. Test on two-tailed Mann–Whitney U-tests.

sequence with similar structural properties may respond to a common epitope. We adopted this scoring system in combination with the PCA method [16], which was used to facilitate visualization, to uncover the hidden and important notions regarding the antigen-specificity of CD4+ ANDEC TILs. Coefficient matrixes for the first three components were listed in Table 1. Notably, there were no measurable differences in the clonal distributions between the ANDECs and their CD4+ TIL backgrounds according to PCA analyses (Fig. 4A, B). These results suggest that ANDEC TILs might have been randomly sampled from background T-cell pools according to their histories of TAA-exposure and diverse driving potentials.

4. Discussion

Beginning with the first understanding that TAAs could be recognized by the adaptive immune system, progress has emerged from the identification and characterization of TAAs for therapeutic

Table 1
Composition of principal component.

No	Properties	1st Component	2nd Component	3rd Component
1	Helix/bend preference	0.045	0.571	0.150
2	Side-chain size	0.414	−0.195	−0.341
3	Extended structure preference	0.099	0.429	−0.359
4	Hydrophobicity	0.435	0.161	−0.363
5	Double-bend preference	0.187	0.248	0.570
6	Partial specific volume	0.455	0.251	−0.070
7	Flat extended preference	−0.265	−0.016	−0.172
8	Occurrence in alpha region	−0.360	0.129	−0.138
9	pK-C	0.286	−0.528	0.023
10	Surrounding hydrophobicity	−0.322	0.078	−0.470

purposes. The identification of autologous TAAs and corresponding generation of spontaneous anti-tumor T-cell responses in vivo are of critical importance for reinforcing our target selection in the design of T-cell-based immunotherapies. These urgent needs promote the development of comprehensive screening strategies to discover antitumor-potential T-cell clones [17].

Facilitate by the analytical power of repertoire sequencing, we defined compositions of CD4+ and CD8+ TIL TCRB repertoires for ccRCC patients and investigated the reformation of these repertoires. To the best of our knowledge, this study is the first general characterization of the CD4+/CD8+ subsets of T-cell repertoires in ccRCC patients. We found that the divergent signatures of the CD4+ TIL repertoire were likely a consequence of TAA-driven expansion

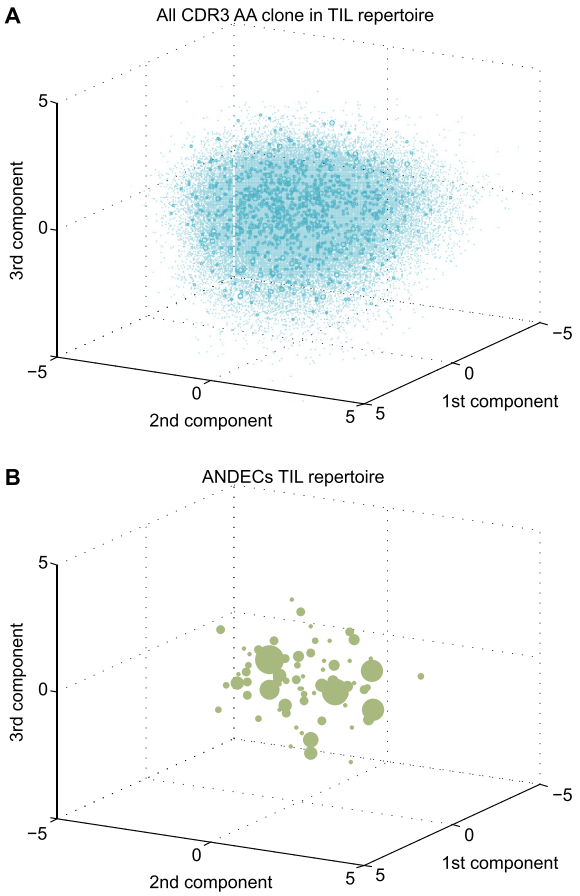

Fig. 4. Heterogeneous properties of CDR3-region for CD4+ ANDEC TILs. PCA analysis was used to visualize the Kidera vectors and show the overall structural information of the CDR3 AA clonotypes. (A) The first three principle components for the CDR3 AA clones for a combined sequencing data from all patients' TIL repertoire. (B) The properties of the ANDECs as shown in (A).

with heterogeneous antigen-specificity. Since the TCR molecule formed as a TCRA/TCRB heterodimer, we cannot perform screening assay now only based on a TCRB candidate list of ANDECs. Thus, further TCRA/B combing sequencing and experimental identification is needed to validate these observations. Conclusively, these observations suggest the possibility that inter-patient heterogeneity hinders the efficacy of adoptive anti-tumor treatments, which might provide novel avenues for personalized therapeutic interventions.

Consistent with previous findings that TIL repertoires exhibit a lower diversity index [18], we found a significantly modified TIL repertoire, and focused further on CD4+ subset for ccRCC. It is known that HLA II molecule expressed on ccRCC tumor cells abundantly for the majority of patients [19]. These expression characteristics of HLA II approved for the effective anti-tumor helper T-cell anti-tumor response [20] and aid in the prediction of treatment response [21]. Due to the highly diverse population and dual characteristics (i.e., immune effective and regulatory) within the CD4+ T-cell, it is presently difficult to provide a definitive answer of which phenotype or functional orientation of T-cells contributes most to our observations. However, previous studies may provide valuable evidence on the properties of CD4+ TILs in ccRCC. First, Wang et al. demonstrated that approximately 65% of CD4+ TILs were the effector memory phenotype (TEM: CD62L-CD45RO+), and these expanded TEM clones could mediated MHC II-restricted anti-tumor reactivity [22]. Second, Siddiqui demonstrated that, increased proportion of CD4+CD25+Foxp3-helper T-cells correlated with poor prognosis, suggesting a reliable tumor-helper TIL interaction in ccRCC [23]. In conclusion, it is reasonable to consider that, at least for ccRCC patients, CD4+ TILs exhibit TAA-specific expansion in response to tumor challenge.

Some patients generate effective T-cell responses spontaneously, but the majority do not. Further, the quantities and qualities of such anti-tumor responses seem to be highly heterogeneous and mounted in individual-dependent fashions [24]. Our analysis using a combination of the Kidera scoring system [15] and PCA visualization revealed that, in terms of the TCRB CDR3 structural properties, the random and unbiased ANDEC distribution fully overlapped with the background repertoire. This heterogeneous antigen specificity certainly agrees with previous observations of the heterogeneous expression of ccRCC-associated antigens for different patients, and it is consistent with the study that the majority driver mutation was highly individual-dependent. Notably, we defined strict criteria for antigen-driven event which could lead to the loss of sensitivity (too less clones involved) of this ANDEC list. Further effort would be needed to burden the molecular identification of ccRCC-reactive clones for therapeutic purpose.

Taken together, our findings strongly suggested that TAA-driven CD4+ TILs form a heterogeneous population. Although the detailed mechanisms remain unclear, these observations demonstrated a heterogeneous intra-tumoral T-cell response in tumor sites, and provides a novel understanding for the enhancement of personalized anti-tumor treatments.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgment

We would like to thank the National Natural Science Foundation of China for funding (Grant 81230017, 81170705).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.069.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.01.069.

References

[1] E.A. Singer, G.N. Gupta, D. Marchalik, R. Srinivasan, Evolving therapeutic targets in renal cell carcinoma, Curr. Opin. Oncol. 25 (2013) 273–280 doi: 210.1097/CCO.1090b1013e32835fc32857.

[2] W.H. Fridman, F. Pages, C. Sautes-Fridman, J. Galon, The immune contexture in human tumours: impact on clinical outcome, Nat. Rev. Cancer 12 (2012) 298–306 doi: 210.1038/nrc3245.

[3] M.J. Gooden, G.H. de Bock, N. Leffers, T. Daemen, H.W. Nijman, The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis, Br. J. Cancer 105 (2011) 93–103 doi: 110.1038/bjc.2011.1189. Epub 2011 May 1031.

[4] M. Vukmanovic-Stejic, Y. Zhang, J.E. Cook, J.M. Fletcher, A. McQuaid, J.E. Masters, M.H. Rustin, L.S. Taams, P.C. Beverley, D.C. Macallan, A.N. Akbar, Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo, J. Clin. Invest. 116 (2006) 2423–2433.

[5] E.M. Janssen, E.E. Lemmens, T. Wolfe, U. Christen, M.G. von Herrath, S.P. Schoenberger, CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes, Nature 421 (2003) 852–856. Epub 2003 Feb 2009.

[6] S.A. Quezada, T.R. Simpson, K.S. Peggs, T. Merghoub, J. Vider, X. Fan, R. Blasberg, H. Yagita, P. Muranski, P.A. Antony, N.P. Restifo, J.P. Allison, Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts, J. Exp. Med. 207 (2010) 637–650 doi: 610.1084/jem.20091918. Epub 20092010 Feb 20091915.

[7] O.A. Haabeth, K.B. Lorvik, C. Hammarstrom, I.M. Donaldson, G. Haraldsen, B. Bogen, A. Corthay, Inflammation driven by tumour-specific Th1 cells protects against B-cell cancer, Nat. Commun. 2 (240) (2011), http://dx.doi.org/10.1038/ncomms1239.

[8] D.G. Schatz, Y. Ji, Recombination centres and the orchestration of V(D)J recombination, Nat. Rev. Immunol. 11 (2011) 251–263.

[9] T.P. Arstila, A. Casrouge, V. Baron, J. Even, J. Kanellopoulos, P. Kourilsky, A direct estimate of the human alphabeta T cell receptor diversity, Science 286 (1999) 958–961 (New York, N.Y.).

[10] H. Robins, Immunosequencing: applications of immune repertoire deep sequencing, Curr. Opin. Immunol. 25 (2013) 646–652.

[11] S. Li, M.P. Lefranc, J.J. Miles, E. Alamyar, V. Giudicelli, P. Duroux, J.D. Freeman, V.D. Corbin, J.P. Scheerlinck, M.A. Frohman, P.U. Cameron, M. Plebanski, B. Loveland, S.R. Burrows, A.T. Papenfuss, E.J. Gowans, IMGT/HighV QUEST paradigm for T cell receptor IMGT clonotype diversity and next generation repertoire immunoprofiling, Nat. Commun. 4 (2333) (2013), http://dx.doi.org/10.1038/ncomms3333.

[12] J.W. van Heijst, I. Ceberio, L.B. Lipuma, D.W. Samilo, G.D. Wasilewski, A.M. Gonzales, J.L. Nieves, M.R. van den Brink, M.A. Perales, E.G. Pamer, Quantitative assessment of T cell repertoire recovery after hematopoietic stem cell transplantation, Nat. Med. (2013) 372–377.

[13] J.J. Stewart, C.Y. Lee, S. Ibrahim, P. Watts, M. Shlomchik, M. Weigert, S. Litwin, A Shannon entropy analysis of immunoglobulin and T cell receptor, Mol. Immunol. 34 (1997) 1067–1082.

[14] V. Venturi, K. Kedzierska, S.J. Turner, P.C. Doherty, M.P. Davenport, Methods for comparing the diversity of samples of the T cell receptor repertoire, J. Immunol. Methods 321 (2007) 182–195.

[15] M. Epstein, M. Barenco, N. Klein, M. Hubank, R.E. Callard, Revealing individual signatures of human T cell CDR3 sequence repertoires with Kidera factors, PLoS One. 9 (2014) e86986 doi: 86910.81371/journal.pone.0086986. eCollection 0082014.

[16] S. Rackovsky, Sequence physical properties encode the global organization of protein structure space, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 14345–14348 doi: 14310.11073/pnas.0903433106. Epub 0903432009 Aug 0903433112.

[17] H. Matsushita, M.D. Vesely, D.C. Koboldt, C.G. Rickert, R. Uppaluri, V.J. Magrini, C.D. Arthur, J.M. White, Y.S. Chen, L.K. Shea, J. Hundal, M.C. Wendl, R. Demeter, T. Wylie, J.P. Allison, M.J. Smyth, L.J. Old, E.R. Mardis, R.D. Schreiber, Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting, Nature 482 (2012) 400–404 doi: 410.1038/nature10755.

[18] A.M. Sherwood, R.O. Emerson, D. Scherer, N. Habermann, K. Buck, J. Staffa, C. Desmarais, N. Halama, D. Jaeger, P. Schirmacher, E. Herpel, M. Kloor, A. Ulrich, M. Schneider, C.M. Ulrich, H. Robins, Tumor-infiltrating lymphocytes in colorectal tumors display a diversity of T cell receptor sequences that differ from the T cells in adjacent mucosal tissue, Cancer Immunol. Immunother. 62 (2013) 1453–1461 doi: 1410.1007/s00262-00013-01446-00262. Epub 02013 Jun 00216.

[19] J. Dengjel, M.D. Nastke, C. Gouttefangeas, G. Gitsioudis, O. Schoor, F. Altenberend, M. Muller, B. Kramer, A. Missiou, M. Sauter, J. Hennenlotter, D. Wernet, A. Stenzl, H.G. Rammensee, K. Klingel, S. Stevanovic, Unexpected abundance of HLA class II presented peptides in primary renal cell carcinomas, Clin. Cancer Res.: Official J. Am. Assoc. Cancer Res. 12 (2006) 4163–4170.

[20] M.J. Stassar, L. Raddrizzani, J. Hammer, M. Zoller, T-helper cell-response to MHC class II-binding peptides of the renal cell carcinoma-associated antigen RAGE-1, Immunobiology 203 (2001) 743–755.

[21] S. Bierer, I. Hoffmeister, J. Gerss, E. Herrmann, C. Wulfing, W. Sibrowski, L. Hertle, R. Kelsch, Combined immunochemotherapy in selected patients with metastatic renal cell carcinoma: HLA class II genotype can help to predict response to therapy, J. Immunother. 34 (2011) 196–201 (Hagerstown, Md.: 1997).

[22] Q.J. Wang, K. Hanada, P.F. Robbins, Y.F. Li, J.C. Yang, Distinctive features of the differentiated phenotype and infiltration of tumor-reactive lymphocytes in clear cell renal cell carcinoma, Cancer Res. 72 (2012) 6119–6129.

[23] S.A. Siddiqui, X. Frigola, S. Bonne-Annee, M. Mercader, S.M. Kuntz, A.E. Krambeck, S. Sengupta, H. Dong, J.C. Cheville, C.M. Lohse, C.J. Krco, W.S. Webster, B.C. Leibovich, M.L. Blute, K.L. Knutson, E.D. Kwon, Tumor-infiltrating Foxp3-CD4+CD25+ T cells predict poor survival in renal cell carcinoma, Clin. Cancer Res.: Official J. Am. Assoc. Cancer Res. 13 (2007) 2075–2081.

[24] S.A. Rosenberg, N.P. Restifo, J.C. Yang, R.A. Morgan, M.E. Dudley, Adoptive cell transfer: a clinical path to effective cancer immunotherapy, Nat. Rev. Cancer. 8 (2008) 299–308 doi: 210.1038/nrc2355.