Oligoclonal expansion of TCR Vδ T cells may be a potential immune biomarker for clinical outcome of acute myeloid leukemia

Zhenyi Jin†, Qiang Luo†, Shuai Lu3, Xinyu Wang1, Zifan He1, Jing Lai1,2, Shaohua Chen1, Lijian Yang1, Xiuli Wu1* and Yangqiu Li1,2,3*

Abstract

Background: Recent data have shown that γδ T cells can act as mediators for immune defense against tumors. Our previous study has demonstrated that persisting clonally expanded TRDV4 T cells might be relatively beneficial for the outcome of patients with T cell acute lymphoblastic leukemia after hematopoietic stem cell transplantation (HSCT). However, little is known about the distribution and clonality of the TRDV repertoire in T cell receptor (TCR) of γδ T cells and their effects on the clinical outcome of patients with acute myeloid leukemia (AML). The aim of this study was to assess whether the oligoclonal expansion of TCR Vδ T cells could be used as an immune biomarker for AML outcome.

Findings: γδ T cells were sorted from the peripheral blood of 30 patients with untreated AML and 12 healthy donors. The complementarity-determining region 3 (CDR3) sizes of eight TCR Vδ subfamily genes (TRDV1 to TRDV8) were analyzed in sorted γδ T cells using RT-PCR and GeneScan. The most frequently expressed TRDV subfamilies in the AML patients were TRDV8 (86.67%) and TRDV2 (83.33%), and the frequencies for TRDV1, TRDV3, TRDV4, and TRDV6 were significantly lower than those in healthy individuals. The most frequent clonally expanded TRDV subfamilies in the AML patients included TRDV8 (56.67%) and TRDV4 (40%). The clonal expansion frequencies of the TRDV2 and TRDV4 T cells were significantly higher than those in healthy individuals, whereas a significantly lower TRDV1 clonal expansion frequency was observed in those with AML. Moreover, the oligoclones of TRDV4 and TRDV8 were independent protective factors for complete remission. Furthermore, the oligoclonal expansion frequencies of TRDV5 and TRDV6 in patients with relapse were significantly higher than those in non-recurrent cases.

Conclusions: To the best of our knowledge, we characterized for the first time a significant alteration in the distribution and clonality of the TRDV subfamily members in γδ T cells sorted from AML patients. Clonally expanded TRDV4 and TRDV8 T cells might contribute to the immune response directed against AML, while oligoclonal TRDV5 and TRDV6 might occur in patients who undergo relapse. While the function of such γδ T cell clones requires further investigation, TRDV γδ T cell clones might be potential immune biomarkers for AML outcome.

Keywords: Acute myeloid leukemia, γδ T cells, T cell receptor, Clonality
Introduction
Acute myeloid leukemia (AML) is a fast-growing malignant hematological disease that occurs in large, immature white blood cells [1]. The immune systems of patients with AML become uncontrolled, leading to leukemia that cannot develop normal-functioning blood cells. Although treatments for curing AML, such as chemotherapy and hematopoietic stem cell transplantation (HSCT), have appeared in recent years, the outcome of some patients who are unable to undergo intensive chemotherapy and HSCT remains dismal with a poor survival of only 5 to 10 months [2, 3]. Therefore, novel strategies such as cellular immunotherapy have been proposed and increasingly investigated.

In the past decade, there have been numerous efforts toward developing specific T cell-based immunotherapies to manage cancer [4–6]. γδ T cells are a T cell subset that comprise approximately 5–10% of all peripheral T cells in healthy individuals [7]. Due to the antitumor function of γδ T cells, they have been proposed to have therapeutic potential for cancer treatment [8–10]. Several in vivo and in vitro data have demonstrated that γδ T cells are excellent candidates for further improving immunotherapy efficacy because of their intrinsic characteristics and function [11, 12]. Accumulating evidence supports a particular antitumor cytotoxicity value for γδ T cells in the development of immunotherapy-based approaches for hematological malignancies such as myelodysplastic syndromes (MDS), multiple myeloma (MM), and chronic myeloid leukemia (CML) [13–15]. Despite encouraging preclinical studies of some hematological malignancies, γδ T cell-based immunotherapy for AML patients remains in its infancy, and the immune characteristics of γδ T cells in AML require further elucidation.

Recent insights into the structure of the γδ T cell receptor (TCR) and its ligands strongly indicate that γδ T cells possess unique functions for defending hosts against an extensive range of infections and stresses [7, 10, 16]. A growing body of evidence demonstrates that γδ T cells can act as functional agents for immune defense against tumors or pathogenic invaders in inflammatory reactions; they perform different functions based on their tissue distribution, antigen–receptor structure, and local microenvironment [17]. Recently, it has been reported that the phenotype and distribution of γδ T cells in human breast cancer might serve as a prognostic factor predicting clinical outcome [18]. Our previous study reported that clonally expanded TRDV4 T cells might lead to relatively better outcome for patients diagnosed with T-cell acute lymphoblastic leukemia (T-ALL) after HSCT [19]. However, little is known about the correlation between γδ T cells and AML outcome. In this study, we analyze the distribution and clonality of TRDV subfamilies in γδ T cells sorted from the peripheral blood (PB) and discuss the clinical relevance of γδ T cell subfamilies in AML patients.

Results
Expression frequency and clonality of TCR Vδ T cells in AML
In this study, the complementarity-determining region 3 (CDR3) sizes of eight TRDV subfamily genes were analyzed in γδ T cells sorted from peripheral blood mononuclear cells (PBMCs) from 30 patients with AML and 12 healthy individuals using RT-PCR and GeneScan (Fig. 1). Approximately, 25–75% of the TRDV subfamilies were expressed in 30 different AML patients. The mean value of the number of expressed TRDV subfamilies was 4.40 ± 1.07, which was significantly lower than that in healthy individuals (6.67 ± 1.23, \(P = 0.000\)). The most frequently expressed subfamilies in the AML patients were TRDV8 (26/30; 86.67%) and TRDV2 (25/30; 83.33%). TRDV6 was detected in only 11 patients (11/30; 36.67%), and the frequencies of TRDV1, TRDV3, TRDV4, and TRDV6 were significantly lower than those in healthy individuals (\(P = 0.000, 0.031, 0.037,\) and 0.015, respectively) (Fig. 2a).

The majority of the TRDV subfamilies in the γδ T cells displayed polyclonal expansion with a Gaussian distribution of CDR3 lengths (multi-peaks) corresponding to a polyclonal rearrangement pattern. PCR product analysis produced a single dominant peak or double peaks, which demonstrate a skewed spectratype profile termed “oligoclonality” or “biclinality”, respectively. “Oligoclonality trending” is a classification with a profile between that of polyclonality and oligoclonality [19]. Clonal expansion was detected for all eight TRDV subfamilies in the γδ T cells. Greater than two TRDV subfamilies demonstrated oligoclonality, biclonality, or oligoclonality trending in all of the AML samples. In addition, the oligoclonally expanded γδ T cells were distributed in almost all of the TRDV subfamilies in the AML patients with the exception of TRDV1 (6.67%, 2/30), and the most frequently oligoclonally expanded TRDV subfamilies were TRDV8 (17/30, 56.67%) and TRDV4 (12/30, 40%). The clonal expansion frequencies of the TRDV2 and TRDV4 subfamilies were significantly higher than those in healthy individuals (\(P = 0.012\) and \(P = 0.009\)); however, a significantly lower clonal expansion frequency for TRDV1 was observed in the AML patients (\(P = 0.046\)) (Fig. 2b).

Clinical relevance of the oligoclonal expansion of TCR Vδ T cells in AML
The association between AML outcome, the clonality of TRDV subfamilies in γδ T cells, age, WBCs, blast cell percentage in PB, and the absolute number of γδ T cells in PB was analyzed by multivariate non-conditional logistic regression analysis and multivariate stepwise regression analysis. The results demonstrated that oligoclonal
expansion of the TRDV4 and TRDV8 subfamilies are independent protective factors (odds ratio (OR) = 0.137, 95% confidence interval (CI) 0.015–1.210; OR = 0.067, 95% CI 0.005–0.843), and the percentage of blast cells in PB was an independent risk factor for complete remission (CR) (OR = 1.047, 95% CI 1.009–1.087).

We also observed that seven patients underwent relapse after achieving CR. In addition, we compared differences in the oligoclonal expansion of TRDV subfamilies between those with recurrence and those with non-recurrence. Interestingly, the oligoclonal expansion frequencies of TRDV5 and TRDV6 in the recurrence group were significantly higher than those in the non-recurrence group (P = 0.031 and P = 0.007) (Figs. 3 and 4).

Logistic regression analysis demonstrated that oligoclonal expansion of TRDV5 and TRDV6 was an independent risk factor for AML recurrence (OR = 21.822, 95% CI 1.426–333.877; OR = 44.603, 95% CI 2.169–917.358, respectively).

Discussion

Although treatments for curing AML have appeared in recent years, the clinical outcomes of some AML patients have not been positive. Recent studies have suggested that there were restricted distribution and clonality for the TRDV subfamilies in different diseases including immune thrombocytopenic purpura, B cell non-Hodgkin lymphoma, allergic rhinitis, MDS, CML, and graft versus host disease (GVHD) [20–25]. Understanding the mechanisms...
underlying the γδ T cell immune response in patients with leukemia is vital for developing strategies for leukemia immunotherapy [26–28]. To investigate the immune characteristics of γδ T cells in patients with AML, we first sorted the γδ T cells from the PB of AML patients and analyzed their TCR Vδ repertoire. We then attempted to characterize the correlation between oligoclonal expansion of TCR Vδ repertoire and clinical outcome.

In PB T cells from healthy individuals, the TRDV repertoire expression pattern is unrestricted. In contrast, we found significantly restricted TRDV subfamily expression in the γδ T cells from patients with AML. Such an alteration in the TRDV repertoire distribution in AML appeared to be different for different diseases, e.g., the most frequently expressed TRDV genes were TRDV1 and TRDV2 followed by TRDV8 and TRDV3 in MDS patients [22]. This observation suggests that different subfamilies of γδ T cells might be preferentially active in different diseases and different immune statuses for patients with the same disease.

In immunodeficient patients with leukemia, it is difficult to distinguish the role of oligoclonal T cells, which may serve as reactive T cell clones directed against leukemia. In contrast, there may be clonal absence because T cell proliferation is suppressed by different factors in leukemia. For example, TRDV2 T cells are reduced and dysfunctional in some MDS patients [15, 19, 29]. To further investigate the role of oligoclonal TRDV T cells in AML patients, we first analyzed the correlation between clonally expanded TRDV T cells and clinical outcome. We found that different oligoclonal TRDV subfamily T cells might have unique functions. We found that the clonal expansion patterns of TRDV4 and TRDV8 T cells might be independent protective factors for CR, which is consistent with our previous findings in which we found that clonally expanded TRDV4 T cells might be related to better outcome for a T-ALL patient [19]. We suggested that such expanded TRDV4 and TRDV8 T cell clones might be reactive T cell clones directed against leukemia that serve as biomarkers for the therapeutic efficacy of AML patients. However, a higher frequency of clonally expanded TRDV8 was also found in MDS patients who developed AML [22]. Thus, further investigation is needed to characterize the function of TRDV8 T cell clones in vitro and in vivo. Interestingly, we also found that TRDV5 and TRDV6 T cells might be related to AML recurrence. These oligoclonal TRDV5 and TRDV6 T cells might be indicators of minimal residual disease in AML patients. However, this hypothesis requires confirmation with a larger cohort.

In conclusion, to the best of our knowledge, this is the first attempt to analyze the distribution and clonality of the TRDV repertoire in γδ T cells in AML patients. Alterations in the peripheral TRDV gene repertoire are an important characteristic of γδ T cells in AML patients, which may be related to the immune response, antileukemia effects, and patient outcome. These findings might provide new data regarding the characteristics of cellular immunity in AML patients. The oligoclonal expansion of TCR Vδ T cells may serve not only as an
immune biomarker for clinical outcome but also as an antileukemia immune status indicator in AML patients. Based on this study, we will further investigate the function of the TCR Vδ T cells subfamilies in co-culture models and mouse xenograft model.

Materials and methods

Samples
After obtaining patient consent, PBMCs from 30 AML patients (17 males and 13 females, median age 33 years, range 17–67 years) was collected. The diagnosis of AML was based on the French–American–British (FAB) criteria: 6 patients were classified as M0, 1 patient was M1, 5 patients were M2, 10 patients were M3, 3 patients were M4, and 5 patients were M5. Twelve healthy individuals (5 males and 7 females, median age 41 years, range 29–62 years) served as the control group. The clinical data of the patients are listed in Additional file 1: Table S1. This study was approved by the Ethics Committee of the Medical School of Jinan University of Guangdong Province in China, and all procedures were conducted according to the guidelines of the Medical Ethics Committees of the Health Bureau of the Guangdong Province of China.

γδ T cell sorting
The γδ T cells in the PB from 30 AML patients and 12 healthy individuals were sorted by using γδ monoclonal antibodies and the MACS magnetic cell sorting technique (Miltenyi Biotec, Bergisch Gladbach, Germany) [30].

RNA isolation and cDNA synthesis
RNA was extracted from the sorted γδ T cells using TRIzol RNA extraction buffer according to the manufacturer's
protocol (Invitrogen, Carlsbad, CA, USA). The quality of the RNA was analyzed in a 1.5 % agarose gel stained with ethidium bromide. Two micrograms of RNA was reverse transcribed into first-strand complementary DNA (cDNA) with random hexamer primers using the reverse transcriptase of the SuperScript II Kit (Gibco, Gaithersburg, MD, USA). The cDNA quality was confirmed by RT-PCR of the β2 microglobulin (β2M) gene [31].

**TRDV subfamily expression analysis by RT-PCR**

Eight sense **TRDV** sense primers and a single **TRDC** reverse primer were used in unlabeled PCR to amplify the **TRDV** subfamilies. Subsequently, runoff PCR was performed with fluorescent primers labeled at the 5’ end with a FAM fluorophore (**Cδ-FAM**), which was purchased from TIB MOLBIOL GmbH, Berlin, Germany. The sequences of the primers are listed in Additional file 2: Table S2. PCR was performed as previously described [22]. The cDNA aliquots (1 μl) were amplified in 20 μl reactions using one of the eight **Vδ** primers and a **Cδ** primer. The final reaction mixture contained 0.5 μM sense and antisense primers, 0.1 mM dNTPs, 1.5 mM MgCl2, 1 × PCR buffer, and 1.25 U Taq polymerase (Promega, Foster City, CA, USA). Amplification was performed with a thermal cycler (BioMetra, Germany). After a 3-min denaturation at 94 °C, 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min were performed followed by a final 6-min elongation at 72 °C. The products were then stored at 4 °C [32].

**TRDV subfamily clonality identification by GeneScan analysis**

Aliquots of unlabeled PCR products (2 μl) were subjected to a runoff reaction cycle using a fluorophore-labeled Cδ-FAM primer. The labeled runoff PCR products (2 μl) were heat-denatured at 94 °C for 4 min with 9.5 μl formamide (Hi-Di Formamide, ABI, USA) and 0.5 μl size standards (GENESCAN™-500-LIZ™, Perkin Elmer, ABI). The samples were then loaded in a 3100 POP-4™ gel (Performance Optimized Polymer-4, ABI, USA) and resolved by electrophoresis with a 3100 DNA sequencer (ABI, PerkinElmer) for size and fluorescence intensity determination using GeneScan software [33–35].

**Statistical analysis**

All data analyses, including statistical calculations and graphical displays, were performed using SPSS 13.0 and GraphPad software. Univariate analysis was performed using the Mann–Whitney test to compare the means of the expression of the clonally expanded **TRDV** subfamilies between AML patients and healthy individuals. Different frequencies of **TRDV** subfamilies were compared using Fisher’s exact test. Oligoclonal **TRDV** expansion differences between the recurrence and non-recurrence groups were measured using the Fisher’s exact test. Binary logistic regression analysis was performed to determine associations between the clonal expansion of γδ T cells and the outcome of the AML patients. All analyses included the following variables: γδ T cell clonal expansion, age, WBC count, percentage of blast cells in PB, absolute number of γδ T cells in PB, and clinical status. Odds ratios and 95 % confidence intervals were also calculated. Only values with *P* < 0.05 were considered statistically significant.

## Additional files

**Additional file 1: Table S1.** AML patient characteristics. (DOCX 15 kb)

**Additional file 2: Table S2.** List of primer sequences used for the **TRDV** subfamilies. (DOCX 13 kb)

**Abbreviations**

- AML: Acute myeloid leukemia
- CDR3: Complementarity-determining region
- CI: Confidence intervals
- CML: Chronic myeloid leukemia
- CR: Complete remission
- FAB: French–American–British
- GVHD: Graft versus host disease
- HSCT: Hematopoietic stem cell transplantation
- MDS: Myelodysplastic syndromes
- MM: Multiple myeloma
- OR: Odds ratio
- PB: Peripheral blood
- PBMCs: Peripheral blood mononuclear cells
- T-ALL: T cell acute lymphoblastic leukemia
- TCR: T cell receptor
- β2M: β2 microglobulin

**Acknowledgements**

Not applicable.

**Funding**

This study was supported by grants from the Natural Science Foundation of China (No. 81200388), the Natural Science Foundation of Guangdong Province (No. 2014A030313380), the Guangdong Provincial Basic Research Program (No. 2015B020227003), the Project of the Zhujiang Science & Technology Star of Guangzhou City (No. 20131200046), the Guangzhou Science and Technology Project Foundation (20151010211), and the ‘Challenge Cup’ National Undergraduate Curricular Academic Science and Technology Works (No. 16112027).

**Availability of data and materials**

The data supporting our findings can be found in the supplementary data.

**Authors’ contributions**

XLW and YQL contributed to the concept development and study design. SL, ZFH, XXW, SHC, and LJY performed the laboratory studies. ZYJ, QL, and JL collected the clinical data. ZYJ and QL participated in the figure preparation. XLW and YQL coordinated the study and helped draft the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

This study was approved by the Ethics Committee of the Medical School of Jinan University, Guangzhou, China.

**Author details**

1Institute of Hematology, Medical College, Jinan University, Guangzhou 510632, China.
2Department of Hematology, First Affiliated Hospital, Jinan University, Guangzhou 510632, China.
3Key Laboratory for Regenerative Medicine of Ministry of Education, Jinan University, Guangzhou 510632, China.
References

1. Döhner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. New Engl J Med. 2015;373(1):1136–52.

2. Lu S, Wang J. Homoharringtonine and omacetaxine for myeloid hematological malignancies. J Hematol Oncol. 2014;7:2.

3. Loghavi S, Zuo Z, Ravandi F, Kantarjian HM, Bueno-Ramos C, Zhang L, et al. Clinical features of de novo acute myeloid leukemia with concurrent DNMT3A, FLT3 and NPM1 mutations. J Hematol Oncol. 2014;7:74.

4. Cheng HH, Soeue C, Yu EY. Improved disease markers suggest dual response in a patient with metastatic castration resistant prostate cancer and chronic lymphocytic leukemia following active cellular immunotherapy. J Hematol Oncol. 2015;8:51.

5. Chen S, Zha X, Shi L, Zhou L, Yang L, Li B, et al. Upregulated TCR zeta increases cytokine secretion in T cells from patients with AML. J Hematol Oncol. 2015;8:72.

6. Xu L, Zhang Y, Luo G, Li Y. The roles of stem cell memory T cells in hematological malignancies. J Hematol Oncol. 2015;8:113.

7. Vantourout P, Hayday A. Six-of-the-best: unique contributions of gamma delta T cells to immunity. Nat Rev Immunol. 2013;13(2):88–100.

8. Paul S, Lag T. Regulatory and effector functions of gamma-delta (gamma delta) T cells and their therapeutic potential in adoptive cellular therapy for cancer. Int J Cancer. 2016;139(5):976–85.

9. Saito A, Narita M, Watanabe N, Tochiki N, Satoh N, Yano T, et al. Anti-tumor cytotoxicity of gamma delta T cells expanded from blood cells of myeloma and leukemia patients against self tumor cells—enhancement of the anti-tumor cytotoxicity by type IIIFN, dendritic cells, and activated alpha beta T cells. Blood. 2007;101(11):2648.

10. Lafont V, Pannetier C, Jotereau F, Favrot M, Kourilsky P. Oligoclonality of tumor-infiltrating lymphocytes from human melanomas. J Immunol. 2007;178(2):1007–15.

11. Mariani S, Muraro M, Pantaleoni F, Fiore F, Nuschak B, Peola S, et al. Effector gamma delta T lymphocytes bearing T cell receptor gamma/delta are phenotypically distinct from gamma delta T lymphocytes in allergic rhinitis patients. J Med Biochem. 2012;31(2):94–101.

12. Xu L, Weng J, Huang X, Zeng C, Chen S, Geng S, et al. Persistent donor derived gamma delta T cell response to acute leukemia. Cancer Immunol Immunother. 2006;55(9):1072–80.

13. Siegers GM, Felizardo TC, Matheson AM, Kosaka Y, Wang Y, Medin JA, et al. Anti-leukemia activity of in vitro-expanded human gamma delta T cells in a xenogeneic Ph + leukemia model. Plos ONE. 2011;6(2):e16700.

14. D’Asaro M, La Mendola C, Orlando V, Todaro M, Spina M, et al. Human gamma delta T cell clones may improve survival for recurrent T cell acute lymphoblastic leukemia. Cell. 2013;154(4):941–9.

15. Jin Z, Wu X, Chen S, Yang L, Liu Q, Li Y. Distribution and clonality of the V alpha and V beta T-cell receptor repertoire of regulatory T cells in leukemia patients with and without graft versus host disease. DNA Cell Biol. 2014;33(3):182–8.

16. Puisieux I, Even J, Pannetier C, Jotereau F, Favrot M, Kourilsky P. Oligoclonality of tumor-infiltrating lymphocytes from human melanomas. J Immunol. 1994;153(3):2807–18.

17. Li Y, Chen S, Yang L, Yin Q, Geng S, Wu X, et al. Restricted TRBV repertoire and clonality expansion from peripheral blood T cells in twenty-three patients with breast cancer. J Biol Chem. 2010;285(13):10143–50.

18. Li Y, Chen S, Yang L, Wu X, et al. The T-cell receptor V beta gene repertoire and clonal expansion from peripheral blood T cells in breast-Wei-exposed workers in China. Hematol Oncol. 2010;28(4):223–30.

19. Jin Z, Wu X, Chen S, Yang L, Liu Q, Li Y. Distribution and clonality of the V alpha and V beta T-cell receptor repertoire of regulatory T cells in leukemia patients with and without graft versus host disease. DNA Cell Biol. 2014;33(3):182–8.

20. Puisieux I, Even J, Pannetier C, Jotereau F, Favrot M, Kourilsky P. Oligoclonality of tumor-infiltrating lymphocytes from human melanomas. J Immunol. 1994;153(3):2807–18.

21. Li Y, Chen S, Yang L, Yin Q, Geng S, Wu X, et al. Restricted TRBV repertoire and clonality expansion from peripheral blood T cells in twenty-three patients with breast cancer. J Biol Chem. 2010;285(13):10143–50.

22. Li Y, Chen S, Yang L, Wu X, et al. The T-cell receptor V beta gene repertoire and clonal expansion from peripheral blood T cells in breast-Wei-exposed workers in China. Hematol Oncol. 2010;28(4):223–30.