Degree of Malignancy of T-Cell Acute Lymphocytic Leukemia Related to Autofluorescence in an EL4-Based Model

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Abstract

T cell acute lymphocytic leukemia (T-ALL) is an aggressive hematologic malignancy in terms of its pathology and populations. It is important to understand the phenomenon of autofluorescence in living cells because normal and cancer cells can be distinguished by this feature. However, the autofluorescence link to T-ALL is poorly understood. We provide direct evidence that EL4-based T-ALL model expresses both high autofluorescence (FL1⁺) and low autofluorescence (FL1⁻) in the current study. The FL1⁺ phenotype is not as stable as the FL1⁻ phenotype. Interestingly, CD25⁺ cells of FL1⁺ can transform to CD25⁺ cells when injected into mice. Autofluorescence associated with differential expression of CD25 is related to the malignant degree of disease, which is presented by the tissue infiltration and growth speed of EL4 cells. Of note, the phenotypes of autofluorescence and CD25 expression of EL4 cells are related to the sensitivity to NK cells killing and the survival time of EL4-treated leukemic mice in vivo. Thus, autofluorescence monitoring might provide as new insight for investigating the malignant degree and prognosis index of T-ALL.

Keywords: Autofluorescence; T-ALL; CD25; Survival Term

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) accounts for ~20% of acute lymphoma leukemias [1]. Patients usually exhibit hepatomegaly, splenomegaly and lymphadenectomy because of leukemic cell infiltration. In a number of non-hematopoietic and non-lymphatic tissues, such as endothelial cancers, including lung, breast, esophageal, skin, cervical and colorectal cancers, autofluorescence is abnormal in these neoplastic tissues; its imaging has also been used for the detection of precancerous lesions [2-5]. It is well known that autofluorescence is produced by living tissues or cells. As a safe, noninvasive, and rapid diagnostic strategy, autofluorescence detection is accepted by some institutions to research the function and phenotype of living cells [6]. Therefore, the purpose of our study was to research the characteristics of autofluorescence in T-ALL to improve strategies of management for this disease. Autofluorescence can distinguish between normal or malignant tissues; the same malignant tissue may differ according to its macroscopic type [7]. Fluorescence spectroscopy can detect the morphology and biochemistry of living cells, which are related to the metabolic activity, cellular proliferation and death of cells undergoing malignant changes. In neoplastic tissues, several factors result in reduced autofluorescence, such as decreased cytoplasmic ratio, loss of the submucosal collagen and increased hemoglobin concentration during neovascularization [8]. As a single-cell level, tumor endothelial cells present differing fluorescence compared with normal cells [9]. Impaired mitochondrial metabolism can change autofluorescence by mitochondrial NADH and FAD in cancer cells [10]. Excitation of mitochondrial NADH and FAD can result in cytoplasmic autofluorescence [11,12]. Dysplastic changes of cells can increase cytoplasmic autofluorescence by changing NADH and FAD levels [13]. Changes in cell physiology and pathology can alter the concentration of NADH [14]; NADH has been regarded as a biomarker for mitochondrial anomalies associated with cancer [15,16]. Relatively few studies on autofluorescence imaging and its mechanisms have been carried out; however, analyses of malignant hematopoietic and lymphatic tissues have been carried out more frequently than with endothelial cancers. It has been reported that leukocytes present autofluorescence [17]. In a lymphoma cell line, Chiaretti S et al. used autofluorescence to distinguish between normal and malignant cells [2]. Thus, it is necessary to further elucidate whether autofluorescence exists in T cell acute lymphocytic leukemia and lymphoma. We used EL4 cells, a T lymphoma leukemia cell line of mice, which can perfectly mimic the process
of T-ALL pathogenesis *in vivo* and represents a valuable cell culture model to study the biological characteristics of T-ALL. Firstly, we found that EL4 cells express FL1+ and FL1− when injected into mice or cultured *in vitro*. Also, the FL1+ phenotype is not as stable as that of FL1− cells. The expression of interleukin (IL)-2 receptor α chain (CD25) is strongly induced at the transcriptional level after T cell activation [18]. CD25 is a marker of activation of T cells; as EL4 cells are a T lymphoma leukemia cell line, it is needed to explore CD25 expression within such cells. Interestingly, CD25+FL1− cells cultured *in vitro* transform to CD25+ cells after the inoculation of mice; although FL1+ and FL1− cells cannot transform into each other. We demonstrated that the differing growth speeds among different EL4 cell groups may be related to their expression of autofluorescence and CD25 (interleukin-2 receptor α; IL-2Ra). Furthermore, the EL4 groups with rapid growth speeds *in vitro* result in the short-term survival of mice when injected *in vivo*. These results may provide a cell biology-based insight into autofluorescence and its associated characteristics of T-ALL.

**Materials and Methods**

**Cell Line and Cytotoxicity Assays**

EL4 cell line (C57BL/6 origin) was obtained from the lab of the Translational Medicine Institution in Jilin University and was thawed from a frozen stock. Then was cultured on standard tissue culture plastic (12-well round-bottom plates) at an original concentration of 5×10⁴ cells/mL and 1 mL/well in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 100 U/mL penicillin (Thermo Fisher Scientific), and 50 µmol/L 2-mercaptoethanol (Thermo Fisher Scientific), at 37 °C with 5% CO2. 1×10⁶ cells/mL EL4 cells were cultured with or without 100 µl/mL IL-2 in 12-well-plates. We harvested EL4 cells and renovated complete medium every three days. On day 12, EL4 cells in each well were removed to culture on standard tissue culture plastic (6-well round-bottom plates) and 3 mL/well in the same complete medium. EL4 cells were harvested and counted on day 14. EL4 cells were cultured with NK cells in a 4-hour chromium release assay as reported previously [19].

**Mice**

Wild-type (WT) C57BL/6 (B6) or C57BL/6(LY 5.2) mice were bred in pathogen-free conditions of the animal center affiliated to the First Bethune Hospital.

**Flow Cytometry**

Subsets of EL4 cells were stained with rat anti-mouse CD25-Allophycocyanin (APC). Propidium iodine (PI) staining was used to exclude dead cells. Rat anti-mouse FcγR mAb 2.4G2 was used to block non-specific FcγR binding. Antibodies were from BD Biosciences (San Diego, CA). Aliquots of 1×10⁶ EL4 cells labeled with rat anti-mouse CD25-APC antibody were placed on ice for half an hour. After washing, we tested CD25 expression on FACS Canto™ II (BD Biosciences). In some experiments, FL1−, FL1+CD25−, CD25− EL4 cells were prepared by using the MACS system according to the manufacturer’s instructions (Miltenyi Biotec) and cell sorting prior to injection into mice or culture *in vitro*. The purify (>99%) was tested by FACS.

**Ethical Clearance**

This research was performed according to the Care and Use of Laboratory Animals of the National Institutes of Health. The Ethical Review Boards of First Bethune Hospital of Jilin University approved this study (Protocol Number: 2017-004).

**Statistical Analysis**

Survival rate were analyzed using a Kaplan-Meier curve+logrank test with GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA). One-way ANOVA for multiple comparisons and unpaired student’s t-test for two independent comparisons were used. Flow cytometric analyses were analyzed using FlowJo 10.0.7 software (BD FACSDivaTM software, New Jersey, USA). The statistical analysis was conducted using JMP13.0. Values indicate mean±standard deviation (SD). The data are presented as mean±SD. P<0.05 statistically significant.

**Results**

**Distribution and Phenotype of EL4 Cells**

To identify the biological characteristics of EL4 cells, including the distribution and their phenotype *in vivo*, original EL4 cells were recovered from liquid nitrogen and cultured for 7 days *in vitro*. WT B6 mice received EL4 cells and were then sacrificed when moribund. Vβ12 is the marker of EL4 cells and 100% EL4 cells were determined as Vβ12 positive (Vβ12+) cells. As shown in Figure 1A, all EL4 cells expressing Vβ12+ were distinguished from normal cells (Vβ12−) by flow cytometry [20]. A total of 1×10⁶ EL4 cells were injected into naïve B6 mice-induced T-ALL mice. Vβ12+ EL4 cells infiltrated every tissue, such as the spleen, bone marrow (BM), lymph nodes (LN), peripheral blood (PBMC), thymus, ovary, liver, lung (21.45±13.98%), and the kidney. The percentage of EL4 cells in every tissue was significantly different as determined by one-way ANOVA for multiple comparisons (*P<0.05). Especially in the liver, almost 57% EL4 cells were detected due to its percentage of nucleated
cells. Thus, EL4 cells harvested from the liver were used in the following experiments as the source of cells. We compared the phenotype of EL4 cells harvested in vivo and cultured in vitro. The same phenotype of CD3 on the surfaces of EL4 cells was seen in vivo and in vitro. As shown in Figure 1B, FL1+ and FL1 EL4 cells were detected; EL4 cells expressed CD3 both in vivo and in vitro. Interestingly, there was a different phenotype on the surfaces of EL4 cells. Although the expression of CD25 could be detected within FL1+ EL4 cells in vitro, no FL1 CD25+ cells were noted in vivo (Figure 1C). It is well known that CD25 expression can change during T cell activation. EL4 cells, as a T cell acute lymphoma leukemia cell line, were expected to show altered CD25 expression due to some unknown mechanisms. Thus, it was necessary to explore how CD25+ cells change when injected in vivo. From the results above, we concluded that some phenotypes of FL1+ EL4 cells were not as stable as that of FL1 EL4 cells, particularly CD25 expression. To determine the associated property of autofluorescence on EL4 cells, CD25 was employed as an important factor for investigation.

**Changes of Phenotype on EL4 Cells**

To elucidate the phenotypic changes of CD25+ cells in vivo, original EL4 cells were recovered from liquid nitrogen and cultured for 7 days in vitro. Cells were then separated into FL1 CD25+, FL1 CD25-, and FL1+ cells by a MACS separation system. Then A20 C57BL/6(LY 5.2) mice received 1x10⁶ A20 FL1 CD25+, FL1 CD25-, and FL1+ EL4 subsets respectively. Two weeks later, mice were sacrificed when moribund. As normal cells were FL1 in B6 mice, we harvested A20 cells to observe changes in EL4 cells. The process of phenotype change was investigated when injected in vivo (Figure 2). FL1 CD25+ EL4 cells harvested from the livers of mice may originate from FL1 CD25+ cells in vitro as there were no FL1 CD25+ cells in vivo (Figure 2A).

**Figure 1:** Distribution and Phenotype of EL4 cells. A. Left panel showed that all EL4 cells with Vβ12 were distinguished from normal cells with Vβ12 by flow cytometry. Naïve B6 mice (n=6) were injected i.v. with 1x10⁶ EL4 cells. Right panel showed percentages (mean±SD, n=8 per group) of Vβ12 EL4 cells in total neucleared cells in the indicated tissues when mice were moribund and assessed by flow cytometry two weeks later as following: spleen (2.58±1.32%), BM (1.84±0.66%), LN (2.56±1.77%), PBMC (0.41±0.22%), thymus (14.56±9.48%), ovary (28.62±12.88%), liver (56.77±8.29%), and kidney (23.47±12.81%). (P<0.05). B and C. The normal cells types in the various tissues were Vβ12, while EL4 cells were Vβ12+. Representative flow cytometry profiles showed the expression of CD3 and CD25 on original Vβ12 EL4 cells in vitro and in vivo. C. CD25 EL4 cells existed in original FL1 EL4 cells in vitro (left). No CD25 EL4 cells existed in FL1 EL4 cells in vivo (right).

**Figure 2:** Changes of phenotype on EL4 cells. Representative flow cytometry profiles showed the expression of CD25 on EL4 cells in vitro and in vivo. Original EL4 cells were recovered from liquid nitrogen and cultured 7 days in vitro, then these cells were separated into FL1 CD25+, FL1 CD25- and FL1+ cells with anti-CD25 microbeads followed by positive or negative selection using the MACS separation system. The 1x10⁶ EL4 cells were injected into C57BL/6(LY 5.2) mice expressing A20+ and A20- EL4 cells for the sake of discrimination FL1 EL4 cells and normal cells. Mice with EL4 cells were sacrificed when moribund two weeks later. A. CD25 EL4 cells transformed to CD25 EL4 cells in vivo. B and C. No phenotype change of CD25 EL4 blasts in vivo. D. Comparison of percentages of CD25 expression with IL-2. EL4 cells without IL-2 (left) and EL4 cells with IL-2 (right) were prepared as above and were cultured with or without 100 µl/mL IL-2 at an original concentration of 1x10⁶ cells/mL in 12-well-plates. Cultures were set up in three replicates each. Shown are percentages (mean±SD) of CD25+ cells in EL4 cells on day 7 or day 14 by flow cytometry. Data represented the average of triplicate samples. There were no significant differences between groups with or without IL-2.
addition, no FL1 CD25+ cells in vitro transformed into FL1 CD25+ cells in vivo (Figure 2B). Furthermore, FL1+ cells in vitro could not transform into FL1+ cells; CD25+ cells in vitro could not transform into CD25+ cells in vivo (Figure 2C). These results indicated that FL1+ and FL1 EL4 cells cannot transform into each other; however, CD25+ cells were found to transform into CD25- cells. Therefore, no CD25+ EL4 cells were seen in vivo. It was suggested that the expression of CD25 is in proportion to the FL1 phenotype of EL4 cells. We also aimed to study the factors that affect the expression of CD25. IL-2 is known to induce CD25 expression on activated T cells. In our study, however, no changes in CD25 expression were seen when EL4 cells were cultured with or without IL-2 at day 7 or 14 (Figure 2D). Therefore, it is possible that there are other factors that regulate the expression of CD25 on EL4 cells. Although mechanisms remain unclear, our study shows that changes in CD25 expression may independent of IL-2.

Characteristics of Different Phenotypes of EL4 Cells

To further test whether there were differences in the biological characteristics of CD25+ or CD25- EL4 cells, WT B6 mice were injected the same number of CD25+ or CD25- EL4 cells (1x10^6), and were sacrificed 2 weeks later. We found that there were differences in the sizes of liver infiltrated by EL4 cells. Hepatomegaly was markedly severe in mice injected with CD25+ EL4 cells than those injected with CD25- EL4 cells (Figure 3A). The average liver weight between CD25+ and CD25- EL4-treated mice is significantly different (1.763±0.072 g vs. 1.458±0.076 g) (n=5 per group, mean ± SD, P<0.05). Therefore, it is necessary to further demonstrate whether there is a disparity of survival term between these two groups of mice due to the notable degree of tissue infiltration of EL4 cells. As shown in Figure 3B, WT B6 mice received the same number of CD25+ or CD25- EL4 cells (5x10^5); the survival term was significantly reduced in mice injected with CD25+ EL4 cells than those injected with CD25- EL4 cells (P<0.01). These results demonstrated that CD25+ EL4 cells induced notably severe hepatomegaly and short survival term compared with CD25- EL4 cells. Therefore, it is likely that CD25+ EL4 cells are more malignant, leading to rapid disease progression. Furthermore, an NK cell cytotoxicity assay was performed with CD25+ and CD25- EL4 cells. The result showed that CD25+ EL4 cells were more sensitive to NK cells (Figure 3C). Therefore, it is possible that the decreased malignancy of CD25+ EL4 cells may be attributed to NK cell killing. Another possibility is that the transformation of CD25+ EL4 cells may affect the degree of malignancy. As

**Figure 3:** Characteristics of CD25 expression on EL4 cells. A-C. CD25+ and CD25- EL4 cells were prepared as above. A. Mice with CD25+ and CD25- EL4 cells were moribund and sacrificed two weeks later. Representative Hepatomegaly was observed much more severe in mice injected with CD25+ EL4 cells than mice with CD25- EL4 cells (Figure 3A). The average liver weight between CD25+ and CD25- EL4-treated mice was statistically significant (p=0.01) (n=7 per group). B: WT B6 mice were injected with 5x10^4 CD25+ (☐) or CD25- (■) EL4 cells respectively. The difference of survival curves of CD25+ or CD25- recipients was statistically significant (p=0.01) (n=7 per group). C. CD25+ EL4 cells were more sensitive to NK cells cytotoxicity (n=5, p<0.05)

**Figure 4:** A: Comparison of growth curves of different phenotypes of EL4 cells. FL1+ EL4 cells (☐), FL1+CD25- EL4 cells (■), or FL1+CD25 EL4 cells (●) were prepared by MACS as above. Every group was cultured in 12-well plate at an original concentration of 5x10^3 cells/mL for 14 days and every line represented every group. Absolute number of EL4 cells was indicated by growth curve. In comparison with FL1+CD25- EL4 cells, FL1+ and FL1+CD25 EL4 cells showed significantly quick growth speed (n=7 per group, p=0.013, p=0.004 respectively). The similar growth speed was observed between FL1+CD25- and FL1+CD25+ EL4 cells. Data represented the average of triplicate samples and results were expressed as Mean±SDs. B: Comparison of survival term of different phenotypes of EL4 cells. The same number of 5x10^5 EL4 cells in every phenotype were injected into WT B6 mice including FL1+ EL4 cells (☐), FL1+CD25+ EL4 cells (■), or FL1+CD25- EL4 cells (●); and survival term was shown. In comparison with FL1+CD25- EL4 mice, FL1+ and FL1+CD25 EL4 cells showed significantly short survival term (p=0.040, p=0.037 respectively) (n=7 per group). The similar survival term was observed between FL1+ and FL1+CD25- EL4 cells. The median time to death was (☐) 32days, (■) 31days and (●) 41days. Results shown were representative of three independent experiments

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there were differing degrees of disease progression induced by EL4 cells according to their expression of CD25, we investigated whether there were differences in growth speed between CD25+ and CD25− EL4 cells. Since no expression of CD25 in FL1 EL4 cells was detected, EL4 cells were separated into FL1−CD25−, FL1−CD25+ and FL1+ populations by a MACS separation system. We found that FL1+CD25+ EL4 cells grew significantly slower than FL1−CD25− EL4 (P=0.004) and FL1+ EL4 cells (P=0.013) (Figure 4A). Similar growth speeds were seen between FL1−CD25− and FL1+ cells. Then, we continued to test the hypothesis that the mortality of mice treated with various EL4 cell groups differs according to their phenotype in vivo. So, 5×10^6 FL1−, FL1+CD25− and FL1+CD25+ EL4 cells were respectively injected into naïve B6 mice. The survival time of FL1−CD25− EL4 treated mice was longer than that of FL1−CD25− EL4 and FL1+ EL4-treated mice. As shown in Figure 4B, the difference in mortality of mice was statistically significant for the FL1−CD25− EL4-treated mice versus the FL1+ EL4-treated mice (P=0.04), and the FL1+CD25− EL4-treated mice versus FL1+CD25+ EL4-treated mice (P=0.037). Taken together, these results demonstrated that FL1+CD25− EL4 cells induced slow progression of disease, which was presented as slow growth speed in vitro and long survival term in vivo.

**Discussion**

The illumination of unstained cells that naturally emit light is termed as autofluorescence. It is important to understand phenomenon of autofluorescence in living cells as this distinguishes normal and cancer cells. For many endothelial tissues, dysplastic changes result in increased contents of mitochondrial NADH and FAD, which leads to changes in autofluorescence in cancer [13]. For diseases of malignant hematopoietic and lymphatic tissues, a previous report showed that autofluorescence can distinguish between a type of malignant B cell and normal lymphocytes [2]; however, the characteristics of autofluorescence in T cells lymphoma leukemia remains unknown. T cell acute lymphoma leukemia is characterized as a heterogeneous disease with poor prognosis [21]. By using an EL4-based T-ALL model, to the best of our knowledge, we first reported that these cells express FL1− and FL1+ both in vivo and in vitro. CD25+ EL4 cells only persisted in FL1+ populations in vitro as they are observed to transform to CD25− cells following injection into mice. It is possible that the transformation may require time as CD25+ cells induce notably longer terms of survival compared with CD25− cells in vivo. Another possibility is that CD25+ EL4 cells are more sensitive to NK cell cytotoxicity. Furthermore, notable severe tissue infiltration, such as hepatomegaly, appears in mice with CD25+ cells when the same numbers of CD25− or CD25+ EL4 cells are injected at the same time. These results indicate that the expression of CD25 in FL1− might be related to the degree of malignancy of T-ALL in an EL4 model. This was further determined by separating EL4 cells into FL1−CD25+, FL1+CD25− and FL1−CD25− populations. We observed that FL1+CD25+ cells grow more slowly than FL1−CD25+ and FL1− cells, which was statistically significant as determined with growth curves. Additionally, FL1+CD25+ cells result in statistically significant long-term survival compared with FL1−CD25+ and FL1+ cells. The survival term of FL1−CD25+ and FL1− cells is similar without statistical significance. Autofluorescence was associated with expression of CD25 and may therefore be related to tissue infiltration, speed of growth and survival term in the EL4-based T-ALL model in the current study.

Over the past few decades, autofluorescence analysis is regarded as a promising way to distinguish between normal and cancer cells. The diagnostic applications of this method has notably increased [22]. The characteristics of autofluorescence in tissues are determined by the morphological and biochemical states of cells [23]. Malignant tissues and the corresponding normal tissues can be distinguished by autofluorescence [24]. Regarding the autofluorescence of malignant lymphatic tissues, a study reported that the fluorescence of lymphoma is significantly lower than that of lymphoid hyperplasia and normal tissues. It has also been shown that the accuracy is 91.5% by the visual classification of autofluorescence imaging (AFI). Thus, AFI may be regarded as a useful tool to indicate the disease stage and appropriate therapy in the certain types of lymphoma [25]. Furthermore, autofluorescence has been observed in leukocytes, such as macrophages, eosinophils and neutrophils [26]. Our data indicates that autofluorescence appears in an EL4-based T-ALL model; investigation of its characteristics might have clinical value for patients with T-ALL. CD25 is termed as the interleukin-2 receptor α chain, which is expressed on the surfaces of activated T cells and a type of T cell lymphoma leukemia, known as adult T-cell leukemia [27]. In our study, we reported that EL4 cells, as a model of T-ALL, partially express CD25 in vitro. We also demonstrated that CD25+ cells are not detected in vivo because they transform into CD25− cells. Since both the expression and transformation of CD25 appear with high autofluorescence of EL4 cells, these data raise a possibility that high autofluorescence might not be as stable as low autofluorescence. Furthermore, different phenotypes associated with autofluorescence result in varying extents of tissues infiltration, growth speed and survival term. This may be consistent with the findings of the current study in which cell autofluorescence was related to the degree of cell differentiation. It has been identified that autofluorescence decreased when cells differentiate [28]. Until recently, the biological knowledge of T-ALL was limited [29]. Thus, autofluorescence monitoring might provide new insight into the processes of differentiation, which may further contribute to investigations into the malignant degree and prognosis index of T-ALL. However, a limitation of the present study is that we did not analyze clinical samples of T-ALL patients; we lacked clinical data to further identify our results. In the future, we will collect peripheral blood samples of T-ALL patients to investigate the expression CD25 and in relation to autofluorescence. In conclusion this new finding that autofluorescence exists in EL4-based T-ALL cells provides an insight into hematologic malignancies and lymphoma disease. Although cells with different autofluorescence intensities cannot transform into each other from in vitro to in vivo, its stability is not coincident such as different expression of CD3, CD25, and TCRαβ only existed in FL1− EL4 cells. The expression of CD25 can transform...
in FL1+ cells as seen in vitro and in vivo, and is related to growth speed and survival term. Although no exact mechanisms have been clarified, our study shows that T-ALL, at least in the EL4 model, may present varying intensities of autofluorescence. This application of this approach showed that the expression of CD25 in FL1+ cells is related to the degree of malignancy of T-ALL, which may facilitate future mechanistic studies. Therefore, autofluorescence combined with CD25 expression may be considered as a useful adjunctive diagnostic tool and prognosis index in EL4-based T-ALL. Future studies of autofluorescence and the expression of CD25 should be broadened to research the underlying mechanism of the changes, regulations and biological characteristics of CD25 expression, in which we can collect extensive information to address the clinical application of potential treatments for T-ALL.

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Contributions of Authors

Zhonghua Du: designed and performed experiments, analyzed data, and drafted the manuscript; Lixia Wang: performed experiments and analyzed data; Yongguang Yang: contributed to the development of the project and edited the manuscript; Yanping Yang: conceived the research project, designed experiments, analyzed data, and wrote the paper.

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