CD39 identifies a specific CD8+T cell population in EGFR-driven lung adenocarcinoma related metastatic pleural effusion.

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Abstract

Malignant pleural effusions (MPE) are common in lung cancer, which were a complex microenvironment containing a plethora of immune and tumor signals. Gene alterations such as driver gene mutations were considered to affect the components in the TIME of NSCLC. Here, we demonstrated that pleural CD39+CD8+T cells were selectively elevated in firstly-diagnosed lung adenocarcinoma with wild-type EGFR compared to that in mutant EGFR, while abnormally more represented in MPE with epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) acquired resistance. Analysis showed that pleural CD39+CD8+T cells display exhausted phenotype and potential cytolytic function, together with skewed usages of T cell receptor (TCR)-Vβ repertoire in comparison with CD39-CD8+T cells, which constituted common feature of lung adenocarcinoma related MPE. Further study revealed TCR-Vβ diversity tended to be more enhanced in pleural CD39+CD8+T cell from MPE coupled with EGFR-TKI acquired resistance. Taken together, we have identified a subset of CD8+T cells expressing CD39 in MPE, whom proposed as the potential tumor-reactive CD8+T cells, and further provided a new understanding of dynamic immune composition of EGFR-mutant tumor microenvironment.
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Conflict of interest

The authors have declared that no commercial or financial conflict of interest exists.

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Abstract

Malignant pleural effusions (MPE) are common in lung cancer, which were a complex microenvironment containing a plethora of immune and tumor signals. Gene alterations such as driver gene mutations were considered to affect the components in the TIME of NSCLC. Here, we demonstrated that pleural CD39+CD8+T cells were selectively elevated in firstly-diagnosed lung adenocarcinoma with wild-type EGFR compared to that in mutant EGFR, while abnormally more represented in MPE with epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) acquired resistance. Analysis showed that pleural CD39+CD8+T cells display exhausted phenotype and potential cytolytic function, together with skewed usages of T cell receptor (TCR)-Vβ repertoire in comparison with CD39-CD8+T cells, which constituted common feature of lung adenocarcinoma related MPE. Further study revealed TCR-Vβ diversity tended to be more enhanced in pleural CD39+CD8+T cell from MPE coupled with EGFR-TKI acquired resistance. Taken together, we have identified a subset of CD8+T cells expressing CD39 in MPE, whom proposed as the potential tumor-reactive CD8+T cells, and further provided a new understanding of dynamic immune composition of EGFR-mutant tumor microenvironment.

Keywords: CD39; CD8; Malignant pleural effusion; EGFR; Lung cancer
1. Introduction

Malignant pleural effusion (MPE), caused by tumor metastasis to the pleural space, is a common complication of advanced lung cancer\(^1\). An accumulation of lymphocytes frequently occurs in MPE might represent alternative microenvironment of TME\(^2\). Nevertheless, an important concern is whether a cancer therapy will have the same immune-modulated effects in both MPE and the primary sites. Further, the role of suppressive immune cells related to tumor driver gene in NSCLC patients has not been fully established\(^3\). Improved understanding of the pathogenesis of MPE may be helpful for development of more effective therapeutic options.

CD39 is an ectonucleotidase expressed by B cells, innate cells, regulatory T cells as well as activated CD4+ and CD8+T cells, which can result in local production of adenosine leading to an immunosuppressive environment\(^4\)\(^-\)\(^7\). Recent evidences proposed the expression of CD39 on CD8+T cells as a marker of exhausted tumor-infiltrating lymphocytes (TILs)\(^4\),\(^8\). Thus, a recent study demonstrated the co-expression of CD39 with the marker of resident memory CD8+T cells, suggesting a protective role for these cells in cancer survival\(^9\),\(^10\). Moreover, CD39 has also been reported as a useful marker to discriminate antigen-specific from nonspecific bystander CD8+T cells in a tumor environment, which provide an approach to identify tumor-reactive CD8 T cells and have important ramifications for developing future therapeutic strategies\(^11\)\(^-\)\(^13\). However, the functional characteristics by which CD39+CD8+T cell infiltrate into lung cancer related MPE is unknown so far.

Our previous studies have demonstrated that an increased frequency of CD8+T cells in MPE compared to peripheral blood (PB) among lung cancer patients, whom contained more the effector memory subset (Tem) and central memory subset (Tcm). In addition, MPE-derived Tem and Tcm subsets expressed more CD39 together with higher cytokine production\(^14\). In the present study, we further investigated the accumulation of CD39+CD8+T cell
in MPE, and explored the possibility of the co-occurring genetic alterations as its determinant, especially EGFR. Our findings would help to characterize MPE-derived CD39+CD8+T cells as a potential target for immunotherapy and figure out the relationship between targeted therapy and immunotherapy in NSCLC.

2. Materials and methods

2.1 Patients

The 125 subjects studied, classified according to diverse criteria, are shown in Table 1. These patients were subjected to routine laboratory diagnosis, and the samples were analyzed using conventional cytology. Effusions were considered malignant if the cytological examination was evaluated as being positive for malignant cells. A diagnosis of tuberculosis was established by positive culture findings of the Mycobacterium tuberculosis (pleural fluid or other biological material) or by proof of granulomatous lesions in pleural biopsy specimens. Exudative pleural effusion was diagnosed based on the level of protein, LDH, cell number of pleural effusion.

Subjects with autoimmune diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus), chronic infections (e.g. human immunodeficiency virus infection) and those who had received immunotherapy were excluded. The study was approved by ethics committee of the First Affiliated Hospital of Soochow University.

2.2 Pleural effusion preparation

Mononuclear cells were isolated from pleural effusions using Histopaque gradient centrifugation. The mononuclear cell band was carefully transferred into a conical centrifuge tube. Phosphate buffer saline (PBS) was added for the later use.

2.3 Flow cytometry analysis
In total, 100 μL aliquots were transferred to polypropylene test tubes and conjugated mAb or isotype controls was added to each tube. Multiparametric flow cytometry was performed to determine cell marker expression. The following fluorochrome-conjugated mAb were used: CD45 (APC-conjugated anti-CD45, Biolegend), CD8 (APC-Cyanine7-conjugated anti-CD8, Biolegend), CD39 (PE-Cyanine7-conjugated anti-CD39, Biolegend), PD-1 (APC-conjugated anti-PD-1, Biolegend) and Tim-3 (PE-conjugated anti-Tim-3, Biolegend).

2.4 Intracellular staining

For ex vivo intracellular IFN-γ and TNF-α detection, freshly isolated single-cell suspension was cultured in complete RPMI 1640 medium containing PMA (50 ng/ml) and ionomycin (500 ng/ml) for 3 hours, following, it was analyzed for by intracellular staining with FITC-conjugated anti-IFN-γ mAb (eBioscience) and PE-conjugated anti-TNF-α mAb (Biolegend).

2.5 Analysis of the TCR-Vβ repertoire

We evaluated the usages of the TCR-Vβ family of CD39+CD8+T cell subsets through flow cytometric analysis (IOTest Beta Mark TCR Repertoire Kit®, Beckman Coulter, Marseille, France). This kit consists of mAb designed to identify 24 distinct TCR-Vβ families. Each set consisted of three different reserved anti-Vβ family-specific mAb labeled with FITC, PE, or both. Furthermore, we calculated the means and standard deviations (SD) of TCR-Vβ family usage to compare difference between CD39+ and CD39-CD8+ T cell. We created heat maps of TCR-Vβ usages of CD39+CD8+ T cell and CD39-CD8+ T cell. In heat maps, squares from green to red respectively represent values exceeding the lower (<-1 SD) to upper (>+6 SD) limits of the CD39+ subset value of the TCR-Vβ family respectively.

2.6 Statistics

Statistical analysis was performed with GraphPad Prism 5 (GraphPad, La Jolla,
Data were analyzed using paired t-test for matched samples. Two-sided t-tests were used for continuous variables. Receiver operating characteristic (ROC) curves were calculated to select the cut-off level of ratio of pleural CD39+CD8+T cell indicating occurrence of EGFR mutation in adenocarcinoma. All tests were two sided with a P-value of less than 0.05 being considered statistically significant.

3 Results

3.1 Baseline characteristics of patients

A total of 125 eligible patients were enrolled. Baseline characteristics of the subjects were listed in Table 1. The patients included 91 male and 34 female and the median age was 67 years (range, 17-90 years), 63 with lung adenocarcinoma, 13 with small cell lung cancer, 8 with lung squamous cancer, 14 with others malignant disease (3 with mesothelioma, 5 with adenosquamous, 6 with metastatic carcinoma), 11 with tuberculosis and 16 with exudative pleural effusion. Additional, patients with lung adenocarcinoma were subtyped into EGFR mutation (31 cases) and wild-type (28 cases) groups, 4 unknown EGFR status.

3.2 CD39+CD8+T cell was accumulated in MPE

Firstly, we used multiparametric flow cytometry to identify the CD39+CD8+T cells in all type of PE (Figure 1A). By calculating the levels of CD39 expression as CD39<sup>pos</sup> and CD39<sup>neg</sup>, we found that percentages of CD39+CD8+T cells represented the higher values in MPE (15.97±1.72%) in comparison with those in tuberculosis (3.74±0.80%, P=0.03) and exudative pleural effusion (4.47±0.66%, P=0.02). In addition, we also analyzed this cell population present in MPE with different pathologic type (Figure 1B). It was indicated that CD39+CD8+T cells were detected at relatively high frequencies in adenocarcinoma-related MPE (15.29±2.14%) compared to small cell lung cancer (12.40±2.66%, P=0.94) and squamous cancer (10.30±1.73%, P=0.86).
These data revealed that double positive CD39+CD8+T cells were accumulated in MPE, which would participate in regional tumor immune response.

3.3 EGFR status driven accumulation of CD39+CD8+T cells in lung adenocarcinoma related MPE

As EGFR gene mutations were considered to affect the components in the TIME of NSCLC. We then examined whether the accumulation of pleural CD39+CD8+T cell was associated with the status of EGFR in lung adenocarcinoma related MPE. Strikingly, as shown in Figure 2A, it was found that pleural CD39+CD8+T cells were selectively elevated in firstly-diagnosed lung adenocarcinoma with EGFR$^{\text{wt}}$ compared to that in EGFR$^{\text{mu}}$ (18.85±3.53% vs 4.24±2.39%, $P=0.04$). Notably, it was visualized a substantial accumulation of pleural CD39+CD8+T cell in EGFR mutant-MPE upon acquired resistance to EGFR-TKI (19.43±4.26%, $P=0.04$). These data suggested that the EGFR gene can shape CD39 expressed by CD8+T cell, and mark a specific subset of CD8+T cell in lung adenocarcinoma related MPE.

Furthermore, the frequency of pleural CD39+CD8+T cell indicating occurrence of EGFR mutation was analyzed among lung adenocarcinoma first diagnosed. As showed in Figure 2B, the area under curve was 0.78 ($P<0.01$) with sensitivity and specificity of 83.33% and 71.40%, respectively, when the critical value was 4.94%. It was suggested that the determination of pleural CD39+CD8+ T cell alone can show good results for the prediction of occurrence of EGFR mutation in lung adenocarcinoma.

3.4 Pleural CD39+CD8+T cell exhibit an inherent phenotype associated with exhaustion

The expression of PD-1 and Tim-3 has been described as hallmark of CD8+T cell exhaustion in cancer and chronic infections. To further increase our knowledge on the functionality of MPE associated CD39+CD8+T cell, we
performed a staining for PD-1 and Tim-3 on CD39+CD8+T cell. As shown in Figure 3A-B, CD39+CD8+T cells contained more ratio of PD-1+ population (31.95±4.74% vs 10.07±1.62%, \( P<0.01 \)) and Tim-3+ population (35.10±3.56% vs 10.28±1.93%, \( P<0.01 \)) compared to their counterparts.

To determine the impact of tumor environment on the phenotypic changes of CD39+CD8+T cells, we comparatively analyzed the expression of PD-1 and Tim-3 on pleural CD39+CD8+T cells obtained from firstly-diagnosed lung adenocarcinoma with EGFR\(^{wt}\) or EGFR\(^{mu}\) as well as MPE with acquired resistance to EGFR-TKI (Figure 3C). It was found that CD39+CD8+T cells from various MPE display comparable PD-1 and Tim-3 expression, which mean these cells are inherently exhausted and not TME specific.

3.5 Pleural CD39+CD8+T cell exhibit an unaffected effector cytokine production

In order to evaluate whether exhausted phenotype was related with cytotoxicity-related cytokines, we checked the capacity of CD39+CD8+T cells to produce IFN-\(\gamma\) and TNF-\(\alpha\). As shown in Figure 4, the percentage of IFN-\(\gamma\)+ cells (37.02±5.53% vs 38.22±7.66%, \( P=0.75 \)) and TNF-\(\alpha\)+ cells (21.92±6.13% vs 23.41±4.97%, \( P=0.51 \)) elicited in response to phorbol 12-myristate 13-acetate-ionomycin (PMA-IONO) stimulation were comparable between CD39+ and CD39-CD8+T cells, suggesting exhausted feature and potential cytolytic function were simultaneously hold by CD39+CD8+T cells.

3.6 Skewed TCR-V\(\beta\) repertoire usage in pleural CD39+CD8+T cell

As reported, CD39 has been reported as a useful marker to identify tumor-reactive CD8+T cells. Then, the usages of 24 TCR-V\(\beta\) families in pleural CD8+T cell were individually analyzed (Figure 5A). As shown in Figure 5B-C, it was observed that skewed usage was prominently in pleural CD39+CD8+T cell compared with those recorded in CD39-CD8+T cell. In detail, 19 of 24 TCR-V\(\beta\) families were significantly enriched in CD39+CD8+T cell as compared
to CD39- counterpart. Our data thus far indicated that CD39+CD8+ T cell display a signature of TCR diversity that suggests they are programmed as tumor-reactive CD8 T cells.

3.7 EGFR-TKI acquired resistance enhanced diversity of TCR-Vβ repertoire in pleural CD39+CD8+ T cell

Thus far, our data suggest that CD39+CD8+ T cell may be enriched for cells recognizing antigens within the tumor. We then investigated whether EGFR-TKI acquired resistance could influence the TCR-Vβ diversity in pleural CD39+CD8+ T cell. As shown in Figure 6, it was found that TCR-Vβ repertoire usage tended to be more enhanced in pleural CD39+CD8+ T cell from patients with EGFR-TKI acquired resistance than in those with firstly-diagnosed lung adenocarcinoma with EGFRwt or EGFRmut, supporting the hypothesis that CD39+CD8+ T cells may harbor a tumor reactive role during cancer progression.

4 Discussion

Given MPE represents the tumor microenvironment involving of changes of immune cells and offers an alternative source as a non-invasive specimen[2], in this regard, we discovered a specific subset of tumor infiltrating CD8+ T cells in MPE. It was indicated that pleural CD39+CD8+ T cells were selectively elevated in firstly-diagnosed lung adenocarcinoma with wild-type EGFR compared to that in mutant EGFR, while abnormally more represented in MPE with EGFR-TKI acquired resistance. Analysis showed that pleural CD39+CD8+ T cells display exhausted phenotype and potential cytolytic function, together with skewed usages of T cell receptor (TCR)-Vβ repertoire compared to CD39-CD8+ T cells. Especially, our study revealed TCR-Vβ diversity tended to be more enhanced in pleural CD39+CD8+ T cell from MPE with EGFR-TKI acquired resistance.

The molecular mechanisms underlying the generation and regulation of
CD39+CD8+ T cells in MPE remain unknown. Previous studies reported that expression of CD39 is both increased by hypoxic conditions and TGF-β\[^8, 15\]. Others reported that CD39 expression is detected on CD8+T cells with hallmarks of chronic antigenic stimulation at the tumor site\[^11\]. Here, we hypothesized that a low TMB presented by EGFR mutated NSCLC results in a lack of immunogenic neo-antigens, in turn decreases the numbers of CD39+CD8+ T cells in MPE. This result was in consistent with finds in melanoma and MSI high colon cancer, in which possessed high mutational burden\[^16-18\]. Over all, these variances in CD39 expression could be explained by the genetic background of tumors, suggesting that EGFR mutation integrate immunological, metabolic and environmental signals to regulate the immune response\[^19, 20\].

Due to their key role in the regulation of T-cell responses, PD-1 and Tim-3 are known as immune checkpoint molecules\[^21\]. Here, most CD39+CD8+T cells in MPE displayed the exhausted phenotype of T cells, indicative of expressing of PD-1, Tim-3. However, we also noted that these cells exhibited unimpaired production of IFN-γ and TNF-α, all features of a Tc1-like phenotype, suggesting that CD39+CD8+T cell were different from the other known exhausted T cell subsets, and arguing for CD39 as activation rather than exhaustion marker. As supported, a recent study has reported that T cell dysfunction is epigenetically imprinted and that two dysfunctional states exist, a “plastic” state from which T cells can be rescued and a “fixed” state, in which T cells are resistant to reprogramming\[^22, 23\]. Hence, considering the immunotherapeutic strategies, it provides a new understanding of overcoming the dysfunction or exhaustion of T cells in MPE, with implications for targeting of CD39+CD8+T cell represents a promising therapeutic approach to improve immune responses against tumors\[^24, 25\].

As identifying tumor antigen-specific T cells from cancer patients has important implications for immunotherapy, in present study, T cell receptor (TCR)-Vβ
repertoire analysis was used for studying selective T-cell responses against tumor antigens. Our findings were in agreement with observations made by Ting Liu et al. who described high-affinity neoantigens trigger anti-tumor activity by activating tumor-reactive CD39+CD8+ T cells\(^{26}\). Thomas Duhen also demonstrated that CD103+CD39+CD8+ TILs have a distinct T-cell receptor (TCR) repertoire, can be identified as tumor-reactive CD8+ T cells in human solid tumors\(^{27}\). Again, Simoni and colleagues found that CD8+ T cells specific for tumor neoantigens show high CD39 expression\(^{28}\). A phase III clinical trial of NSCLC indicated that a gene signature of CD39+CD8+ T cells predicted benefit from ICB, but not chemotherapy. As improved identification of anti-tumor T cells is needed to advance cancer immunotherapies, the expected efficacy of pleural CD39+CD8+ T cell-based immunotherapy could be based on both the cytolytic activity together with tumor-reactive\(^{29}\).

Despite the relevant antitumor efficacy of immunotherapy in advanced NSCLC, the results in patients who harbors activating EGFR mutations are disappointing. The biological mechanisms underlying both unresponsiveness and resistance to immunotherapy in EGFR-mutant NSCLC patients have been partially investigated. EGFR mutated NSCLC lacked immunogenic neo-antigens, thereby displayed “lymphocyte depletion” phenotype of TME, it was hypothesized that generation and evolution of TCR repertoire was impacted in this T cell population. Here, we highlighted CD39+CD8+ T cell as a proxy of tumor-reactive CD8+ T cells in human lung cancer, and functions as a key modulator immunotherapy. We also found the number of pleural CD39+CD8+ T cell was associated with the acquired resistance to TKI in adenocarcinoma. Theoretically, targeted therapy may lead to neoantigens release through tumor cell death, resulting in immunomodulation that can potentiate immune responses\(^{30}\). The increases of CD39+CD8+ T cell in tumor microenvironment was suggested as a potential mechanism involved in the immunotherapy in EGFR-mutant NSCLC patients with 3\(^{rd}\)-EGFR-TKI acquired
resistance. So, it was rationale for planning strategies of immunotherapy to overcome immune escape and 3rd-EGFR-TKI acquired resistance in NSCLC.

In summary, our results suggest that the EGFR mutation in the MPE induces an altered immune regulatory CD8+T cell phenotype that is associated with CD39 expression. This provides a new understanding of immune regulation in MPE, with implications for defining a biomarker of CD8+T cell subset and a target for immunotherapeutic intervention. The defined state of CD8+T cells may be the key to figure out the relationship between targeted therapy and immunotherapy in MPE. Furthermore, of the multiple predictive biomarkers explored for immunotherapy, it would be of interest to examine lung cancer patients that respond to anti-PD-1 therapies for changes in the frequency and/or activation status of the CD39+CD8+TILs.
Table 1 Subject characteristics

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Figure 1 Analysis of CD39+CD8+ T cell derived from pleural effusion by FCM. A, representative dot plots were shown. B, CD39+CD8+ T cells were significantly more represented in MPE (15.97±1.72%) compared to tuberculosis (3.74±0.80%, P=0.03) and exudative (4.47±0.66%, P=0.02) and comparable among MPE with different pathological type.
Figure 2 EGFR mutation and infiltration of CD39+CD8+ T cell population in MPE of lung adenocarcinoma. A, the accumulation of CD39+CD8+ T cells in MPE was selectively elevated in firstly-diagnosed lung adenocarcinomic MPE with EGFR\textsuperscript{mu} (18.85±3.53%), EGFR\textsuperscript{wt} (4.24±2.39%, \(P=0.04\)) and MPE with EGFR-TKI acquired resistance (AR-EGFR-TKI) (19.43±4.26%, \(P=0.04\)). B, prediction of the frequency of pleural CD39+CD8+ T cell indicating occurrence of EGFR mutation in lung adenocarcinomic MPE. The AUC was 0.78 (\(P<0.01\)) with sensitivity and specificity of 83.33% and 71.40%, when the cut-off value was 4.94%.
Figure 3 Exhaust phenotype of CD39+CD8+ T cell was analyzed. A, representative dot plots of staining for PD-1 and Tim-3 on CD39+CD8+ T cell. B, CD39+CD8+ T cells expressed the higher level of PD-1 (31.95±4.74% vs 10.07±1.62%, P<0.01) and Tim-3 (35.10±3.56% vs 10.28±1.93%, P<0.01), compared to their systemic counterparts. C, CD39+CD8+ T cells from various MPE displayed comparable PD-1 and Tim-3 expression.
Figure 4 Cytolytic markers of MPE infiltrated CD39+CD8+ T cell. A, representative dot plots of staining for IFN-γ and TNF-α in CD39+CD8+ T cell. B, it was observed that CD39+CD8+ T cells are able to produce comparable IFN-γ (37.02±5.53% vs 38.22±7.66%, P=0.75) and (21.92±6.13% vs 23.41±4.97%, P=0.51) compared to CD39-CD8+ T cells.
Figure 5 The usage of TCR-Vβ families in CD39+/CD39-CD8+ T cells. A, representative dot plots are shown. Each set consisted of three different anti-Vβ family-specific mAb labeled with FITC, PE, or both. B, the numbers on the left side of heatmaps represent participants, the short phrases on the top represent the usages of TCR-Vβ families, the redder color represents the higher value of the TCR-Vβ family. C, individual usage of TCR-Vβ families in CD39+/CD39-CD8+ T cells were analyzed by Paired T test.
Figure 6 The usage of TCR-Vβ families of pleural CD39+CD8+T cells in EGFR-TKI acquired resistance. It was found that TCR-Vβ repertoire usage tended to be more enhanced in pleural CD39+CD8+T cell from patients with EGFR-TKI acquired resistance than in those with firstly-diagnosed lung adenocarcinoma with EGFR<sup>wt</sup> or EGFR<sup>mu</sup>.
Reference


[17] CHEN J, SONG Y W, LIANG G Z, et al. A Novel m7G-Related Gene Signature Predicts the
Prognosis of Colon Cancer [J]. Cancers (Basel), 2022, 14(22).


Figure A: Comparison of CD39+CD8+CD8+T cell percentages in EGFR
mut_ vs. EGFR_exp and AR-EGFR-TKI groups. Statistically significant difference: *p<0.05*, non-significant: ns.

Figure B: Receiver Operating Characteristic (ROC) curve showing AUC = 0.7842.
**A**

- **PD-1**
  - CD39+CD8+T vs. CD39-CD8+T: P < 0.01
  - CD39+CD8+T vs. CD39-CD8+T: P < 0.01

- **Tim-3**
  - CD39+CD8+T vs. CD39-CD8+T: NS
  - CD39+CD8+T vs. CD39-CD8+T: NS

**B**

- **PD-1**
  - EGFR<sup>+</sup> vs. AR-EGFR-TKI
  - EGFR<sup>-</sup> vs. AR-EGFR-TKI

- **Tim-3**
  - EGFR<sup>+</sup> vs. AR-EGFR-TKI
  - EGFR<sup>-</sup> vs. AR-EGFR-TKI