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Notch signaling pathway suppresses CD8$^+$ T cells activity in patients with lung adenocarcinoma

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ABSTRACT

Evolution and progression of cancer always leads to CD8$^+$ T cells dysfunction/exhaustion. Controversy remains as to the role of Notch signaling pathway in CD8$^+$ T cells regulation in tumorigenesis. Thus, the aim of this study was to investigate the immunomodulatory activity of Notch signaling pathway to peripheral and lung-resident CD8$^+$ T cells in patients with lung adenocarcinoma. Forty-eight lung adenocarcinoma patients and twenty healthy individuals were enrolled in the current study, and CD8$^+$ T cells were purified from both peripheral bloods and bronchoalveolar lavage fluids. Notch receptor mRNA expression was semi-quantified by real-time PCR. Cytolytic and noncytolytic activity of CD8$^+$ T cells evaluated in direct and indirect contact co-culture with A549 cells in response to Notch signaling inhibition by measuring of lactate dehydrogenase release and cytokines production. Expression of Fas ligand (FasL), perforin, and granzyme B were also assessed by flow cytometry. Notch2 mRNA expression was elevated in both peripheral and lung-resident CD8$^+$ T cells in lung adenocarcinoma patients, however, did not correlated with tumor stages or epidermal growth factor receptor mutation. Peripheral CD8$^+$ T cells from healthy individuals exhibited stronger cytotoxicity in direct contact co-culture system, which was not influenced by Notch signaling inhibition. Moreover, suppression of Notch signaling augmented cytotoxicity of peripheral and lung-resident CD8$^+$ T cells from lung adenocarcinoma patients in direct contact co-culture system, and promoted interferon-γ production in both systems. This process was accompanied by increased expression of FasL and perforin within CD8$^+$ T cells. The current data revealed a potential immunosuppressive property of Notch signaling pathway to CD8$^+$ T cells probably *via* inhibition of FasL and perforin in lung adenocarcinoma patients.

1. Introduction

Non-small cell lung cancer (NSCLC), which mainly comprises adenocarcinoma, squamous cell carcinoma, and large-cell lung cancer, accounts for approximate 85% of lung cancer [1]. Administration of molecularly targeted therapy to epidermal growth factor receptor (EGFR) and immune check point inhibitor [antibodies against programmed death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)] have achieved promoting therapeutic management of lung cancer even in advanced stages [2,3]. However, lung cancer remains the most common cause of cancer-related deaths worldwide due to invasion, migration, and acquired resistance to anti-cancer agents [4]. Thus, it is still important to better understand the mechanism for lung cancer progression and clinical relevant insight for disease managements.

It is well accepted that CD8$^+$ T cells play pivotal role in controlling metastasis and prognosis of cancers. However, continuous exposure to cancer antigens always results in insufficient or dysfunctional CD8$^+$ T cell response, leading to immunological tolerance and hyporesponsiveness of host immunity [5]. Thus, the current therapeutic approaches are mainly focused on restoration of CD8$^+$ T cells function, especially for tumor-infiltrating or tissue-resident CD8$^+$ T cells, which are critical for tumoral immune attack in tumor microenviroments [6]. Notch signaling pathway is a highly conserved signaling system present in most multicellular organisms, and plays a major role in the regulation of cellular development [7]. Notch signaling also drives critical decision in mature T lymphocytes development, activation, proliferation, and differentiation [8,9], and has important physiological roles in CD8$^+$ T cell functions, especially in the production of effector molecules [10]. However, Controversy remains as to the role of Notch signaling pathway in CD8$^+$ T cells regulation in tumorigenesis. Cho et al. revealed that Notch signaling activity mediates CD8$^+$ T cells function through direct modulation of perforin and granzyme B [11]. In contrast, inhibition of Notch signaling enhanced effector memory CD8$^+$ T cell-

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mediated airway hyperresponsiveness and inflammation *in vivo* [12], and decreased PD-1 expression by activated $CD8^+$ T cells [13]. A more recent study by Yu et al. demonstrated an immunosuppressive activity of Notch signaling to tumor-infiltrating $CD8^+$ T cells *via* PD-1 pathway in colorectal carcinoma [14]. Notch receptors were also showed to be differential expressed in lung cancer [15]. Thus, we hypothesized that Notch signaling contributes to $CD8^+$ T cells dysfunction in the pathogenesis of lung adenocarcinoma. To test this possibility, we investigated cytolytic and noncytolytic activity of peripheral and lung-resident $CD8^+$ T cells purified from patients with lung adenocarcinoma in direct and indirect contact co-culture system targeting A549 cells *in vitro*.

2. Patients, materials, and methods

2.1. Enrolled subjects

The study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University, and written informed consent was obtained from each individual. A total of 48 pathologically diagnosed lung adenocarcinoma patients were enrolled in this study. All patients were hospitalized in Department of Thoracic Surgery, The First Affiliated Hospital of Xi'an Jiaotong University from March 2016 to March 2017. Patients who were afflicted with pneumonia, autoimmune diseases, or chronic viral infections were excluded from the study. No patients received surgery, chemotherapy, radiotherapy, or immunoregulatory therapy before baseline sampling. The tumor-node-metastasis (TNM) stage was evaluated following the American Joint Committee on Cancer/Union for International Cancer Control TNM classification (7th ed.). Sixteen patients (4 in stage I and 12 in stage II) were tested for epidermal growth factor receptor (EGFR) mutation. Six were EGFR mutated, while other ten patients were non-EGFR mutated. For normal controls (NC), 20 sex- and age-matched healthy individuals were also enrolled. The clinical characteristics of all enrolled subjects were shown in Table 1.

2.2. Peripheral blood mononuclear cells (PBMC) isolation

20 ml of EDTA-anticoagulant peripheral bloods were obtained from each enrolled subjects. PBMC was isolated using Ficoll-Hypaque (Sigma, St. Louis, MO, USA) density gradient centrifugation.

2.3. Bronchoalveolar lavage fluid (BALF) preparation

The top of bronchofiberoscope was closely wedged into opening of subsegmental bronchus. 50 ml of sterilized saline was rapidly injected through biopsy hole, and the lavage fluid was immediately recovered with 100 mmHg negative pressure with a recovery rate between 40% and 60%. This process was repeated five times. BALF was filtered, and was centrifuged at 1200 $\times g$ for 10 min at 4 °C. Cellular precipitations were washed twice and cultured in RPMI 1640 supplemented with 10% of fetal bovine serum (FBS).

2.4. $CD8^+$ T cells purification

$CD8^+$ T cells were purified from PBMC or BALF using human $CD8^+$ T Cells Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) following manufacturer's instruction. The purity of enriched cells was > 95% by flow cytometry determination.

Table 1
Clinical characteristics of enrolled subjects.

	NC	Lung adenocarcinoma
Case (n)	20	48
Gender (male/female)	13/7	37/11
Age (years)	44.8 ± 9.1	50.9 ± 11.7
Smoking history (n)	9	39
Stage (I/II/III/IV)	N.A.	8/17/14/9

2.5. Cell culture

10^6 of $CD8^+$ T cells were seeded into 24-well plates, and were incubated in RPMI 1640 supplemented with 10% of FBS at 37 °C under 5% CO_2 condition. Cells were stimulated with anti-CD3/CD28 (eBioscience, Thermo Fisher, San Diego, CA, USA; final concentration: 1 μg/ml) in the presence or absence of Notch signaling inhibitor, γ-secretase inhibitor (GSI) LY-411575 (Adooq, Irvine, CA, USA; final concentration: 1 μM) for 12 h. In certain experiments, 10^6 of GSI-stimulated $CD8^+$ T cells from HLA-A2 restricted lung adenocarcinoma patients were co-cultured in direct or indirect contact with 5×10^6 of pcDNA3.1-HLA-A*0201 stably transfected lung adenocarcinoma cell line A549 cells for 48 h in the presence of anti-CD3/CD28 as previously described [14,16]. Cells and supernatants were harvested for further analyses.

2.6. Real-time PCR

Total RNA was purified from $CD8^+$ T cells using RNeasy Minikit (Qiagen, Hilden, Germany) following manufacturer's instruction. First strand cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa, Dalian, Liaoning Province, China) with random hexamers. Real-time PCR was performed using SYBR Premix Ex *Taq* (TaKaRa). Relative gene expression was semi-quantified by $2^{-\Delta\Delta CT}$ method using Applied Biosystems 7500 System Sequence Detection Software (Applied Biosystems, Foster, CA, USA). The primer sequences were previously described [17].

2.7. Cytotoxicity assay

The cytotoxicity of $CD8^+$ T cells was determined by measuring lactate dehydrogenase (LDH) level in the supernatants of co-culture systems. LDH expression was measured using LDH Cytotoxicity Assay Kit (Beyotime, Wuhan, Hubei Province, China) following manufacturer's instruction. LDH expression in stably transfected A549 cells was determined as low-level control, while LDH expression in Triton X-100-treated, stably transfected A549 cells was determined as high-level control. The percentage of cell death was calculated by the following equation: (experimental value − low-level control)/(high-level control − low-level control) × 100% [14,16].

2.8. Enzyme-linked immunosorbent assay (ELISA)

Expressions of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were measured using commercial ELISA kits (CUSABIO, Wuhan, Hubei Province, China) following manufacturer's instruction.

2.9. Flow cytometry

$CD8^+$ T cells were cultured with anti-CD3/CD28 and monensin (eBioscience, final concentration: 10 μg/ml) in the presence or absence of GSI. Cells were then stained with anti-CD8-APC (eBioscience) along with anti-Fas ligand (FasL)-PE (eBioscience; for surface staining), anti-perforin-PE (eBioscience; for intracellular staining), or anti-granzyme B-PE (eBioscience; for intracellular staining), respectively. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and were analyzed using FlowJo software version 8.6.1 (Tree Star Inc., Ashland, OR, USA).

2.10. Statistical analysis

All data were analyzed using SPSS 21.0 Version for Windows (SPSS, Chicago, IL, USA). Student's *t*-test was used for comparison between two