Expression of Lymphocyte-Activation Gene 3 (LAG-3) Immune Checkpoint Receptor Identifies a Tumor-Reactive T Cell Population in the Peripheral Blood of Patients with Colorectal Cancer

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Background: The use of adoptive T cell therapy has proven to be effective in some advanced malignancies. This study aimed to investigate the effects of lymphocyte-activation gene 3 (LAG-3) immune checkpoint receptor in the enrichment of tumor antigen-specific CD8+ T lymphocytes derived from peripheral blood mononuclear cells (PBMCs) in patients with colorectal cancer.

Material/Methods: Peripheral blood samples were obtained from 20 patients with colorectal cancer and apheresis was performed with enrichment and cell sorting to obtain CD8+LAG-3+ T cells, which were expanded using high-dose interleukin-2 (IL-2). T cell subsets were detected using fluorescence-activated cell sorting (FACS), and T cell receptor (TCR) sequencing was used to determine specific clone types. Interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) and cell counting kit-8 (CCK-8) assays were used to measure cell avidity and cytotoxicity.

Results: The cultured cells increased in number over time and had the greatest proliferative activity at 15 days, at which time the percentage of CD3+, CD3+CD8+, and CD8+CD28+ reached maximal levels. High purity CD8+LAG-3+ T cells were isolated by FACS and at 15 days. TCR sequencing showed that CD8+LAG-3+ T cells were oligoclonal, ELISpot identified increased production of tumor-specific IFN-γ, and the CCK-8 assay showed increased cytotoxicity when compared with pre-cultured CD8+LAG-3+ T cells.

Conclusions: In patients with colorectal cancer, CD8+LAG-3+ T cells showed more specific anti-tumor activity following cell culture in vitro, which supported the potential role for the LAG-3 immune checkpoint receptor in enriching tumor-specific T cells in patients with cancer.

MeSH Keywords: Blood Cells • Costimulatory and Inhibitory T-Cell Receptors • Cytokine-Induced Killer Cells

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Background

Immunotherapy for cancer has developed rapidly during the past decade and includes adoptive T cell transfer and the use of immune checkpoint inhibitors [1,2]. Studies have provided evidence to support the effective use of combined immunotherapy for advanced cancer, including adoptive T cell immunotherapy, and transfer of activated cells cultured in vitro, including natural killer (NK) cells, cytokine-induced killer (CIK) cells [3], and tumor-infiltrating lymphocytes (TILs). However, cultured T cells from the peripheral blood of patients with cancer are a heterogeneous population and an effective therapeutic targeted population of tumor antigen-specific CD8+ T lymphocytes requires cell isolation. The hallmarks of activated T cells following cell culture include the expression of CD28 and 4-1BB (CD137), which are co-stimulatory receptors [4].

The lymphocyte-activation gene 3 (LAG-3) immune checkpoint receptor protein, also known as CD223, is inducible on several types of immune cells, including CD8+ and CD4+ T cells, and has been shown to be a major inhibitory T cell receptor (TCR) [4,5]. Interaction between LAG-3 and its corresponding ligand, major histocompatibility complex (MHC) class II, can induce immune tolerance, resulting in cancer progression. When exposed to an antigen-rich microenvironment, TILs express high levels of LAG-3 and are mostly functionally impaired in response to antigenic failure to produce cytokines [6,7]. However, there is a remaining paradox in that adoptive T cell therapy using in vitro cultured TILs or CIKs has been shown to be a clinically effective treatment. Rosenberg et al. found that functionally impaired CD8+ TILs expressing negative regulatory molecules could recover higher reactivity after expanding the cells in high-dose interleukin-2 (IL-2) in vitro [4]. This expansion of CD8+ TILs resulted in increased release of tumor-specific interferon-γ (IFN-γ) following stimulation with autologous or HLA-matched tumor cells compared with CD8+LAG-3+ T cells, which was more effective than culture with costimulatory molecules, such as 4-1BB+ T cells [4].

T cells that upregulate LAG-3 are not always functionally impaired. In patients with advanced melanoma, LAG-3 has been shown to be transiently upregulated sequentially by neotumor-specific CD8+ T cells following T cell activation by in vitro exposure to common gamma-chain cytokines, including IL-2 [7]. TCR sequencing data has shown that tumor antigen-specific phenotypes of the cell clones were preferentially expanded in the coinhibitory positive TIL population, such as PD-1+ T cells, which is consistent with TCR stimulation simultaneously driving upregulation of both co-inhibitory and co-stimulatory receptors [8].

However, TILs must be cultured from fresh tumor tissue, and it is difficult to obtain sustainable TILs for immunotherapy. Therefore, because there was a remaining question regarding whether effective immunoreactive cells that can target tumor cells could be obtained from peripheral blood mononuclear cells (PBMCs), our previously reported study confirmed that dendritic cell (DC) combined with CIK cell immunotherapy from PBMCs could activate the cellular immune response and improved clinical outcome in patients with pancreatic carcinoma [9]. Therefore, this study aimed to investigate the effects of LAG-3 immune checkpoint receptor in the enrichment of tumor antigen-specific CD8+ T lymphocytes derived from PBMCs in patients with colorectal cancer.

Material and Methods

Patient characteristics, peripheral blood mononuclear cells (PBMCs), and colorectal cancer cell lines

The study was approved by the Regional Ethical Review Board of Capital Medical University. Twenty patients with colorectal cancer were recruited at Beijing Shijitan Hospital Cancer Center, Beijing, China. All study participants signed informed consent to participate in the study. Patients were included in the study if they were between 20–75 years of age and had an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2, and histologically or cytologically confirmed colorectal cancer. The patient demographic and clinical characteristics are described in Table 1.

Venous blood samples were obtained from the study participants. Peripheral blood mononuclear cells (PBMCs) were stimulated until the mononuclear cell number reached 1.5×10^9/L by daily treatment with granulocyte-macrophage-colony-stimulating factor (GM-CSF). Then, PBMCs prior to treatment were separated using a COBE Spectra cell separator (COBE BCT Inc., Lakewood, CO, USA) and cryopreserved at –80°C until analysis and recovery, as previously described [9].

The colorectal cancer cell lines were established from each patient’s tumor specimens, after surgery or biopsy. Briefly, resected or biopsied tumor fragments were enzymatically digested and then cultured in RPMI 1640, containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin (Life Technologies, Carlsbad, CA, USA) [10].

Flow cytometry and fluorescence-activated cell sorting (FACS)

Cell subpopulations of PBMCs were detected prior to culture, and cultured cytotoxic T lymphocytes (CTLs) were characterized by flow cytometry and FACS analysis, as described previously [9]. Cultured CTLs and PBMCs were stained with antibodies conjugated to the fluorochromes, PerCP-Cy5.5, fluorescein...
isothiocyanate (FITC), and phycoerythrin (PE). Fluorochrome-conjugated antibodies included CD3 PerCP-Cy5.5, CD4 FITC, CD8 FITC, CD28 PE, (Beckman Coulter, Brea, CA, USA), LAG-3 PE, and 4-1BB PE (Biolegend San Diego, CA, USA), which were incubated with the cells at 4°C for 30 min in the dark. Then, the stained cells were centrifuged for 10 min at 1,500 rpm at room temperature and washed twice before acquisition. Flow cytometry analysis detected cell subset phenotypes using a Cytomix™ FC 500 benchtop flow cytometer (Beckman Coulter, Brea, CA, USA), and data were analyzed using the CXP analysis software package (Beckman Coulter, Brea, CA, USA), as previously described [9].

CD8+LAG-3+ T cell sorting was performed using the MoFlo Astrios EQ cell sorter (Beckman Coulter, Brea, CA, USA). First, CD8+ cells were enriched from PBMCs using CD8 microbeads (Biolegend San Diego, CA, USA). For FACS sorting, the enriched T cells were incubated with FITC-conjugated-CD8, PCS.5-conjugated-CD3, and PE-conjugated-LAG-3, as previously described [8]. Cell sorting was based on the gating strategy of setting thresholds on the measurement parameters, and filtering out cells to leave the cell population of interest (PI+, CD3+, CD8+, and LAG-3+). The same protocol was used to sort CD8+4-1BB+ cells.

Expansion of T cells in vitro

The initial PBMCs and sorted populations were activated and expanded with irradiated (5,000 rad) allogeneic feeder cells pooled from three donors using T cell medium consisting of AIM-V serum-free lymphocyte-activation medium, 10% heat-inactivated human AB serum, and cytokines including IL-2 (2,000 U/mL) (Boehringer, Mannheim, Germany), IFN-γ (1,000 U/mL) (Boehringer, Mannheim, Germany) and the CD3 antibody (OKT3) (1.7 mL/mL) (Boehringer, Mannheim, Germany). After initiation, half of the culture medium was replaced with fresh AIM V medium containing 10% human type AB serum and IL-2 (2000 IU/ml) every other day [11]. After approximately 15 days, the autologous cultured T cells were harvested to measure the recognition of target antigens.

Assessment of tumor recognition and cytolytic assays

An enzyme-linked immunospot (ELISpot) IFN-γ release assay (Cellular Technology Limited, Shaker Heights, OH, USA) [8] was used to assess specific T cell avidity and reactivity. Cultured T cells were washed and cocultured, either alone or with autologous tumor cell lines, as established above. In the ELISpot assays, effector cells (E) (1 x 10⁶) were added to target cell lines (T) (1 x 10⁶) at an E: T ratio of 10:1 per well in a 96-well plate and incubated for 48 h, according to the manufacturer’s instructions (Cellular Technology Limited, Shaker Heights, OH, USA). The raw data were analyzed and plotted using Immunospot software (Cellular Technology Limited, Shaker Heights, OH, USA). More than 40 spots and twice background was defined as positive T cell reactivity.

The cell counting kit-8 (CCK-8) assay was used to detect cytolyis. Target cells were plated per well with effector cells at varying effector to target ratios (1.8:1, 5.5:1, 16.6:1, and 50:1) in 96-well plates for 24 hours in an incubator. The supernatants were obtained for absorbance measurement at OD 450 nm. The percentage of lysed cells were calculated according to the formula: control absorbance–experimental absorbance / control absorbance × 100%.

T cell receptor (TCR) determination and analysis

TCR determination was performed by DNA sequencing, as previously described [11]. Briefly, total DNA was extracted from sorted T cells without cytokine stimulation (Qiagen, Hilden, Germany), followed by agarose gel electrophoresis (Bioewest, Nauliillé, France). Multiplex polymerase chain reaction (PCR) was performed according to the manufacturer’s protocol using a Multiplex PCR Kit (Qiagen, Hilden, Germany), with purified PCR products with a length of 100–200 base pair (bp) were selected using QiAquick gel extraction kit, according to the manufacturer’s instructions (Qiagen, Hilden, Germany).

DNA library construction and paired-end sequencing were performed using the Illumina platform (Illumina, San Diego, CA, USA), and followed the same workflow as previously described [9,11]. Finally, the raw data of the DNA sequences were analyzed to identify V and J genes by MiXCR software for analysis of T cell receptor repertoire sequencing data and focused on evaluating the sequencing data of the CDR3 regions of the TCR β chain, especially the most frequent sequences.

Statistical analysis

Continuous variables were reported as the mean ± standard error from the mean (SEM), mean ± standard deviation (SD), or median, as specified and were compared with a two-tailed unpaired Student’s t test. Categorical variables were compared using the chi-squared (χ²) test or Fisher’s analysis. The Mann-Whitney or Wilcoxon signed rank tests were used to determine the statistical significance of the data. All statistical evaluations were performed with GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered to be statistically significant.

Results

Patient characteristics

From August 2015 to July 2017, 20 patients with colorectal cancer who had available tumor tissue were enrolled in this study at Beijing Shijitan Hospital Cancer Center. Peripheral
blood mononuclear cells (PBMCs) and the cell-sorted T cell population (CD8\(^+\)LAG-3\(^-\); CD8\(^+\)LAG-3\(^+\)) was expanded for 21 days in vitro with a cocktail of high-dose interleukin-2 (IL-2). The matched autologous tumor cell lines established from tumor specimens were cultured to approximately 80–90% confluence and proliferated beyond the tenth passage. The characteristics of all patients are detailed in Table 1. There were no statistically significant differences in relevant baseline characteristics between the treatment groups.

Flow cytometry and cell sorting of PBMCs

The proportion of PBMC subgroups and T lymphocytes were examined to include the expression of the lymphocyte-activation gene 3 (LAG-3) immune checkpoint receptor and 4-1BB on CD8\(^+\) T cells (Figure 1A). The expression of each subgroup of PBMCs is shown in detail in Figure 2B. Compared with the previously reported expression levels on tumor-infiltrating lymphocytes (TILs), PBMCs contained a mean of 1.8% CD8\(^+\)LAG-3\(^-\), and 2.0% CD8\(^+\)LAG-3\(^+\) T cells (Figure 1A). Therefore, in the present study, cell sorting from PBMCs required that CD8\(^+\) T cells were enriched using CD8 microbeads. Then, according to the gating strategy described above, up to 93% of CD8\(^+\)LAG-3\(^-\) and CD8\(^+\)4-1BB\(^+\) T cells were obtained (Figure 1B–1E), and the sorted cell yields ranged from approximately 5×10\(^3\) to 5×10\(^5\).

Proliferation and differentiation of cultured cells

The cultured cells from unsorted PBMCs, enriched CD8\(^+\)T cells, and the cell-sorted populations (CD8\(^+\)LAG-3\(^-\); and CD8\(^+\)4-1BB\(^+\) cells) gradually increased during in vitro expansion and showed the highest cell proliferation rate when cultured for 15 days (Figure 2A). The percentage of CD3\(^+\), CD3\(^+\)CD4\(^+\), CD3\(^+\)CD8\(^+\), and CD8\(^+\)CD28\(^+\) reached the maximal levels (Figure 2B). In contrast, the T cell subsets that had a negative regulatory role, such as regulatory T cells (Tregs) (CD4\(^+\)CD25\(^+\)CD127\(^-\)), were reduced by 15 days. Based on these findings, we selected day 15 to harvest cultured cells from the cell-sorted population for further analysis. The expression levels of 4-1BB and LAG-3 from the unsorted cells showed significant upregulation during in vitro expansion at day 15, as shown in Figure 3.

Evaluation of target cell recognition and cytololytic assays

To investigate whether CD8\(^+\)LAG-3\(^-\); CD8\(^+\)4-1BB\(^+\) T cells represented the tumor-reactive population, expanded T cells were cocultured as effector cells with autologous tumor cells. CD8\(^+\)LAG-3\(^-\); CD8\(^+\)4-1BB\(^+\) T cells showed increased tumor-specific IFN-γ release detected by ELISpot (Figure 4A) and stronger cytotoxicity detected by the cell counting kit-8 (CCK-8) cytolytic assay (Figure 4B, 4C) compared with the CD8\(^+\)LAG-3\(^+\); CD8\(^+\)4-1BB\(^-\) T cells and unsorted bulk CD8\(^+\) cells.

Table 1. Patient demographics and baseline clinical characteristics.

| Characteristic          | Total (%) |
|------------------------|-----------|
| Total patients         | 20        |
| Mean age (years)       | 61.2      |
| ≤50                    | 7 (37)    |
| 50–65                  | 11 (57)   |
| >65                    | 6 (32)    |
| Gender                 |           |
| Male                   | 13 (65)   |
| Female                 | 7 (35)    |
| ECOG score             |           |
| 0–1                    | 16 (80)   |
| 2                      | 4 (20)    |
| TNM                    |           |
| I–II                   | 13 (65)   |
| III–IV                 | 6 (30)    |
| Unknown                | 1 (5)     |
| Prior treatment        |           |
| No treatment           | 3 (15)    |
| Surgery                | 13 (65)   |
| Chemotherapy           | 5 (25)    |
| Radiotherapy           | 3 (15)    |
| Immunotherapy          | 6 (30)    |
| Any 2 or more          | 7 (35)    |
| Specimens              |           |
| Tumor                  | 15 (75)   |
| Biopsy                 | 4 (20)    |
| Ascites                | 1 (5)     |

ECOG – Eastern Cooperative Oncology Group performance status; TNM – tumor, mode, metastasis.

Also, CD8\(^+\)LAG-3\(^-\) T cells showed the strongest activity and cytotoxicity compared to CD8\(^+\)4-1BB\(^+\) T cells from the same patients.

T cell receptor (TCR) sequencing analysis

Based on previous reports that the T cell receptor (TCR) phenotype of the cell clones could transform due to cytokine stimulation, T cells were sorted prior to expansion in vitro for TCR sequencing. DNA was extracted from the highly purified CD8\(^+\)LAG-3\(^-\) and CD8\(^+\)LAG-3\(^+\) T cell population according to the procedure described above for TCR sequencing. The relative frequencies of the most frequent TCRβ phenotypes are shown in Figure 4D, where red represents the most frequent phenotypes of the TCRβ gene and blue represents the least frequent.
the cell clones, which also were understood to be unique CDR3β amino acid sequences, and yellow, green and blue reflected the second most frequent, third most frequent, and fourth to 20th most frequent phenotypes, respectively. The relative proportion of the top 20 frequent TCRβ phenotypes of LAG-3+ T cell subsets was significantly higher than the LAG-3− T cell population. The most frequent phenotypes of the cell clones were formed and amplified due to long-term stimulation from tumor antigens in the tumor microenvironment and were assumed to be highly specific for tumor antigens in colorectal cancer.

**Discussion**

Poor outcomes for advanced colorectal cancer remain a clinical challenge, despite the development of new treatments,
Figure 3. The cultured cells from unsorted peripheral blood mononuclear cells (PBMCs). (A, B) The expression levels of CD8\(^{+}\)LAG-3\(^{+}\) and CD8\(^{+}\)4-1BB\(^{+}\) cells from the unsorted peripheral blood mononuclear cells (PBMCs) were significantly upregulated during in vitro expansion.

Figure 4. CD8\(^{+}\)LAG-3\(^{+}\) T cells were identified as tumor-reactive CD8 T cells. CD8\(^{+}\)LAG-3\(^{+}\), CD8\(^{+}\)4-1BB\(^{+}\), and CD8\(^{+}\) T cells were co-cultured with autologous tumor cell lines, and reactivity was assessed by measuring IFN-\(\gamma\) secretion (A) and lysis of target cells (B, C). (D) Diversity of the T cell receptor \(\beta\) (TCR\(\beta\)) repertoire within the bulk CD8\(^{+}\)LAG-3\(^{+}\), and CD8\(^{+}\)LAG-3\(^{+}\) T cells populations. The relative frequencies of the most frequent TCR\(\beta\) phenotype of the cell clone, the second most frequent, the 3\(^{rd}\) most frequent, the 4\(^{th}\) to the 20\(^{th}\) most frequent, and the rest of the phenotypes of the cell clones are shown.
including targeted therapy. However, adoptive immunotherapy, including T cell transfer might be an effective therapeutic approach. We previously observed improved outcome in patients with advanced pancreatic carcinoma who received a combination of 5-FU-based chemotherapy (tegafur/gimeracil/oteracil) with dendritic cell (DC) and cytokine-induced killer (CIK) cell immunotherapy [9]. However, DC-CIKs were a heterogeneous population of cells, some of which have anti-tumor activity but some of which could be potentially immunosuppressive [9]. Therefore, an effective biomarker that could specifically identify the repertoire of tumor-reactive and tumor neoantigen-specific CD8+ T lymphocytes might enhance the clinical efficacy and extend this treatment approach for other malignancies. The findings of the present study showed that the lymphocyte-activation gene 3 (LAG-3) immune checkpoint receptor could be a potential biomarker to better select tumor-reactive T cells within the heterogeneous T cell population.

LAG-3 has been previously identified as a negative regulatory molecular marker expressed on activated T cells and dendritic cells [12]. The interaction between LAG-3 with its corresponding ligands, including FGL1, is associated with immune dysfunction and decreased IFN-γ release through reducing TCR/CD28 signaling and inhibiting T cell activation. Inhibition of LAG-3 can reactivate exhausted T cells and can restore anti-tumor immune responses. Therefore, LAG-3 and other inhibitory molecules, such as PD-1, TIM-3, CTLA-4, and BTLA, have been identified as hallmarks of exhausted T cells in the tumor microenvironment [13–16]. However, T cells that upregulate LAG-3 are not always functionally impaired. The adoptive cell therapy of tumor-infiltrating lymphocytes (TILs) with a high level of LAG-3 can mediate the regression of advanced tumors. This phenomenon has resulted in interest regarding CD8+LAG-3+ T cells and with the possibility that exhausted T cells could be reinvigorated.

The findings of the present study showed that CD8+LAG-3+ T cells expanded in high-dose interleukin-2 (IL-2) were capable of secreting high levels of interferon-γ (IFN-γ) and lyzing tumor cells in coculture with autologous tumor cell lines. These findings support that impaired T cell function associated with upregulation of inhibitory receptors can be effectively rescued and may enable the reproducible enrichment of tumor-reactive cells for developing personalized therapies [8]. In this study, LAG-3 expression by T cells was gradually upregulated after expansion, and 4-1BB was also upregulated [10]. There were no differences in the level of LAG-3 or 4-1BB after expansion in further analysis. Consistent with previous reports, expansion with IL-2 and CD3 microbead stimulation upregulated the expression of immunosuppressive receptors and reduced the potential value of co-inhibitory molecules to select tumor-reactive cells [4,17,18]. Therefore, tumor-reactive CD8+LAG-3+ sub-populations were identified from the primary peripheral blood mononuclear cells (PBMCs) before culture to exclude the interference of upregulation resulting from expansion.

We also observed that the relative frequencies of the 20 most frequent T cell receptor β (TCRβ) phenotypes of the cell clones were significantly increased in LAG-3+ T cell subsets than the LAG-3- T cell subpopulation. This finding supports that TCR stimulation drives the expression of inhibitory molecules on T cells and suggests that LAG-3+ T cells are the population being directly exposed to tumor antigens in the tumor microenvironment [19]. Therefore, if the exhausted T cells were reinvigorated [16], LAG-3 might be a more promising biomarker for enriching tumor-reactive T cells. Other co-inhibitory molecules, such as PD-1, TIM-3, and CTLA-4, could play the same role. The 4-1BB receptor as a co-stimulatory molecule was previously described as a biomarker for activated cells after stimulation or expansion [4,20].

The LAG-3 protein has been reported to amplify the inhibitory function of regulatory T cells (Tregs) [21]. Therefore, LAG-3 might also be a more potent checkpoint inhibitor when compared with CTLA-4 or PD-1. LAG-3 antibody not only reverses CD8+ T effector cells but also inhibits the suppressor activity of Tregs, as shown by studies using monoclonal antibodies or combination immunotherapies, such as adoptive cell therapy plus LAG-3 antibody, which are currently ongoing in preclinical studies [22,23].

This study had several limitations. This study included a small patient sample size, and so the findings require validation in larger controlled studies. Further studies are needed to investigate the molecular or genetic mechanisms underlying the effects of the LAG-3 immune checkpoint receptor. However, although matched human leukocyte antigen (HLA) cell lines have previously been widely used to assess tumor reactivity, the present study has shown that the use of autologous tumor cell lines can avoid the limitations of matched HLA cell lines when targeting tumor-associated neoantigens.

Conclusions

This study aimed to investigate the effects of lymphocyte-activation gene 3 (LAG-3) immune checkpoint receptor in the enrichment of tumor antigen-specific peripheral blood CD8+ T lymphocytes in patients with colorectal cancer. In patients with colorectal cancer, CD8+LAG-3+ T cells showed more specific anti-tumor activity following cell culture in vitro, which supported the potential role for the LAG-3 immune checkpoint receptor in enriching tumor-specific T cells in patients with cancer. The isolation tumor-reactive T cells from peripheral blood, which are more sustainable than tumor-infiltrating lymphocytes (TILs) for immunotherapy might lead to the development of a novel noninvasive approach to personalized immunotherapy.
Conflict of interest

None.

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