

## Observational Study

# Prolonged overall survival in gastric cancer patients after adoptive immunotherapy

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## Abstract

**AIM:** To assess the efficacy of immunotherapy with expanded activated autologous lymphocytes (EAALs) in gastric cancer.

**METHODS:** An observational study was designed to retrospectively analyze the clinical data of 84 gastric cancer patients, of whom 42 were treated by EAAL immunotherapy plus conventional treatment and another 42 only received conventional treatment (control group). EAALs were obtained by proliferation of peripheral blood mononuclear cells from patients followed by phenotype determination. Clinical data including age, gender, clinical stage, chemotherapeutic regimens, hospitalization, surgical, radiotherapy, and survival data were collected along with EAAL therapy details and side effects. Patients were followed and the relationship between treatment and overall survival (OS) data obtained for the immunotherapy and control groups were compared retrospectively. The safety of EAAL immunotherapy was also evaluated.

**RESULTS:** After *in vitro* culture and proliferation, the percentages of CD3+, CD3+CD8+, CD8+CD27+, CD8+CD28+, and CD3+CD16+/CD56+ cells increased remarkably ( $P < 0.05$ ), while the percentages of CD3+CD4+, CD4+CD25+, and CD3+CD16+/CD56+ (natural killer cells) were overtly decreased ( $P < 0.05$ ); no significant change was observed in CD4+CD25+CD127- cells ( $P = 0.448$ ). Interestingly, OS in the immunotherapy group was significantly higher than that in the control group, with 27.0 and 13.9 mo obtained for the two groups, respectively ( $P = 0.028$ , HR = 0.573, 95%CI: 0.347-0.945). These findings indicated a 42.7% decrease in the risk of death. In addition, we found that clinical stage and application of EAAL immunotherapy were

independent prognostic factors for gastric cancer patients. Indeed, the OS in stage IIIc and IV patients that had received surgery was prolonged after EAAL immunotherapy ( $P < 0.05$ ). Importantly, *in vitro* induction and proliferation of EAAL were easy and biologically safe.

**CONCLUSION:** Overall, EAAL adoptive immunotherapy might prolong the OS in gastric cancer patients.

**Key words:** Adoptive immunotherapy; Gastric cancer; Expanded activated autologous lymphocytes; Lymphocytes; Observational study

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**Core tip:** We undertook a retrospective analysis of patients with gastric cancer to perform an observational study on whether expanded activated autologous lymphocytes (EAALs) improved treatment outcomes. The results provide the first evidence for EAALs being an effective treatment regimen in gastric cancer. The therapy was straightforward and showed good safety, and overall survival may be improved with EAAL treatment in gastric cancer patients.

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## INTRODUCTION

Adoptive cellular immunotherapy has been considered an important antitumor treatment for many years. In this process the patient's own peripheral immune cells are collected and proliferated *in vitro* to produce larger quantities by the thousands with enhanced antitumor ability. The cells are then infused back into the patient, in this way the patient's active or passive immunity is strengthened and tumor cells are killed. Adoptive cellular immunotherapy is suitable for immunocompromised patients, such as those who have received high-dose chemotherapy, radiotherapy, and/or bone marrow transplantation<sup>[1,2]</sup>.

Interestingly, various *in vitro* proliferated effector cells, such as lymphokine-activated killer (LAK) cells<sup>[3,4]</sup>, anti-CD3 induced activated killer (CD3AK) cells<sup>[5]</sup>, activated natural killer (NK) cells<sup>[6-9]</sup>, dendritic cells (DCs)<sup>[10,11]</sup>, tumor-infiltrating lymphocytes (TILs)<sup>[12,13]</sup>, and cytokine-induced killer (CIK) cells<sup>[14-16]</sup>, have shown some anti-tumor effects. However, subsequent large scale clinical trials have failed to demonstrate any advantage in the use of LAK cells in combination with large IL-2 doses over IL-2 monotherapy for the treatment of melanoma and renal cell carcinoma<sup>[17,18]</sup>.

Although TILs have been shown to improve IL-2 therapeutic responses in about one third of patients with metastatic melanoma<sup>[19]</sup>, the rather complex preparation process of autologous TILs limits their application in routine clinical practice. It is known that CIK cells exhibit potent cytotoxicity against a variety of tumor cells, including autologous and allogeneic acute myeloid leukemic (AML) targets<sup>[20]</sup> as well as various types of solid cancers<sup>[21]</sup>.

Interestingly, a method was developed to isolate T lymphocytes from cancer patients with immobilized anti-CD3 monoclonal antibodies; the resulting expanded activated autologous lymphocytes (EAALs, all CD3+ and HLA-DR+) were shown to be a heterogeneous cell population containing about 30% of CD4+ and 60% of CD8+ cells<sup>[22]</sup>. Importantly, the same research group demonstrated that EAALs can reduce postsurgical recurrence rates of hepatocellular carcinoma (HCC) in a randomized clinical trial, indicating that EAAL adoptive immunotherapy is a safe and feasible treatment that can improve outcomes for HCC after surgery<sup>[23]</sup>. These findings demonstrated the superiority of EAALs over other immune cells used in adoptive immunotherapy: in addition to decreasing the frequency of recurrence by 18% compared with controls, EAALs showed a mean expansion index of 1560-fold<sup>[22,23]</sup>.

The potential benefits of EAALs in gastric cancer have not been explored. Therefore, this study aimed to further define EAAL phenotypic traits and examine the clinical effects of EAALs in a case-control observational study where overall survival time of gastric cancer patients was assessed retrospectively.

## MATERIALS AND METHODS

### Generation of EAALs

Activated lymphocytes were generated using an anti-CD3 monoclonal antibody (OKT3) and IL-2 as described previously<sup>[24]</sup>. Briefly, 50 to 100 mL of peripheral blood was collected from each patient, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation. The isolated PBMCs were washed and resuspended in RPMI-1640 culture medium (Gibco, Grand Island, United States) containing 10% serum from human blood group AB plasma supplemented with 700 U/mL of IL-2. The PBMC suspension was then placed in a flask coated with immobilized anti-CD3 antibody and incubated for one week. Afterwards, the lymphocyte suspension was transferred to a gas permeable bag for two more weeks. The resulting activated lymphocytes were harvested, passed through a 100  $\mu$ m filter membrane and resuspended in 100 mL normal saline containing 1% human serum albumin for intravenous and/or intrapleural infusion.

### Peripheral blood lymphocytes and EAAL phenotype determination

Fasting venous blood samples (2-3 mL) were drawn

in EDTA-Na<sub>2</sub> anticoagulant tubes, during the morning, from gastric cancer patients before EAAL generation. After red blood cell hemolysis, blood samples were mixed with 10  $\mu$ L fluorescence labeled antibodies, including CD3-FIT, CD8-APC, CD4-PerCP-Cy5.5, CD27-PerCP-Cy5.5, CD28-PE, CD25-PE, CD127-Alexa Fluor<sup>®</sup> 647, CD16-PE, CD56-APC and their isotype negative controls (BD Biosciences, United States), respectively, and incubated in the dark at room temperature for 20 min. Samples were analyzed by flow cytometry on a BD Accuri C6 (BD Biosciences, United States). A total of  $5 \times 10^5$  EAALs were washed twice with PBS, resuspended in 100  $\mu$ L PBS, and labeled with 10  $\mu$ L fluorescence antibodies as described above. After two more washes, EAAL samples were resuspended in 1 mL PBS for flow cytometry.

### **Cell survival and proliferation assessment**

Cell survival was calculated by estimating the number of live cells. A cell suspension was stained with trypan blue solution, and undyed cells were regarded as live. Cell survival rate was calculated as undyed cells/total cells  $\times 100\%$ .

The proliferation of the cells was estimated as proliferation multiplicity. Isolated PBMCs from the patients were appropriately diluted and cultured. Before and after culture, a cell suspension was counted using a counting chamber under a microscope. The total cell number was calculated by the cell concentration multiplied by the volume, and the cell proliferation multiplicity was calculated as the ratio of cell numbers before and after culture.

### **Bio-safety of EAAL assessment**

According to the safety requirement of an important cellular immunotherapy product, PROVENGE<sup>®</sup> (Dendreon Corporation, Seattle, WA, United States), which was approved by United States Food and Drug Administration in April, 2010, and China's pharmacopeia, the sterility test for bacteria and fungi was performed during the culturing of EAALs. Upon the day when the EAALs were harvested and ready to be delivered back the cancer patients, Gram staining, sterility and bacterial endotoxin tests were performed. Negative for sterility test and endotoxin concentration  $< 2.5$  EU/mL in EAAL cells were regarded as the criteria for EAAL treatment.

### **Patients, treatments and trial design**

The treatment regimen and the retrospective case-control study were both approved by the Medical Ethics Committee of PLA General Hospital, China. All of the included patients signed written informed consent. The patients enrolled in the present study were admitted to our hospital from October 2006 to December 2009. The inclusion criteria for the EAAL treatment group were as follows; the patients received EAAL therapy according to the cell therapy records accessed through the China PLA General Hospital electronic medical

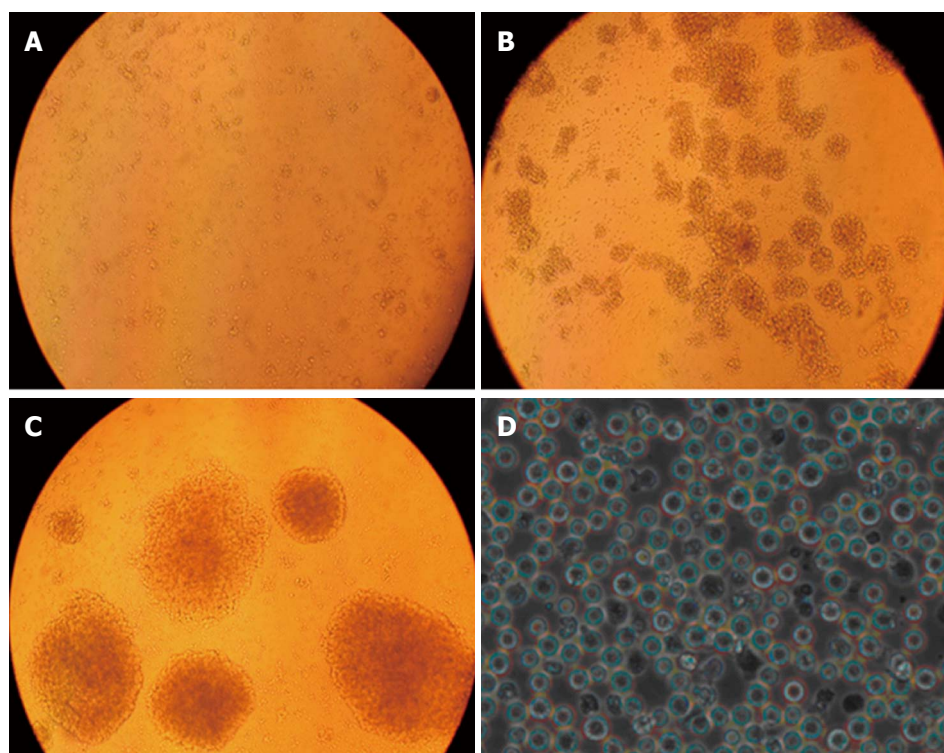
reviewing system, and the patients were histologically or cytologically diagnosed with gastric cancer and had a life expectancy  $> 12$  wk with an Eastern Cooperative Oncology Group (ECOG) performance status score of 0-2. Patients with an ECOG score  $> 2$ , an incomplete medical history, or who were lost to follow-up were excluded. All patients agreed to their treatment regimen and signed informed consent. Forty-two of these patients had undergone adoptive EAAL cellular immunotherapy and constituted the EAAL group. Clinical data including age, gender, clinical stage, chemotherapeutic regimens, and hospitalization, surgical, radiotherapy, and survival data were collected along with EAAL therapy details and side effects by medical record review. In parallel, 42 patients were selected for the control group from the China PLA General Hospital electronic medical reviewing system as histologically confirmed gastric cancer patients who were admitted to the same hospital in the same first admission month as the EAAL patients, and had experienced surgery, chemotherapy or radiotherapy. Patients with a history of cell therapy and ECOG  $> 2$  were excluded. The control candidates were grouped and numbered according to their clinical cancer stages, and were randomly selected to match the number of the patients as EAAL patients using the Statistical Package for Social Science (SPSS) 17.0 (SPSS Inc., Chicago, IL, United States). Clinical data were collected in the same fashion and those with incomplete medical history or lost to follow-up were substituted.

Patients were followed and the relationship between treatment and overall survival (OS), from the diagnosis (nearly identical to the start of chemotherapy because the patients underwent the treatment soon after the diagnosis) until death or last follow-up, of gastric cancer patients was retrospectively analyzed in the EAAL and control groups. In addition, the safety of EAAL immunotherapy was evaluated.

### **Statistical analysis**

Statistical analyses were performed using SPSS 17.0. Summary statistics were given for patient characteristics and treatment administration. Frequencies were reported as number and percentage. Phenotypes of lymphocyte cells in peripheral blood and harvested EAALs were expressed as means  $\pm$  SD, and comparisons between groups were made by self-paired *t* tests. Comparisons of basic clinical characteristics between the immunotherapy and control groups were carried out by the Pearson  $\chi^2$  test. OS was analyzed by the Kaplan-Meier method and the differences in the distributions were compared by the log-rank test. Factors that might affect patients' OS were analyzed by the COX multivariate regression method. Subgroup analysis was used to analyze the OS in different subgroups of patients who had received EAAL immunotherapy. A *P* value  $< 0.05$  was considered statistically significant.





**Figure 1** Morphology of expanded activated autologous lymphocytes at different proliferation times. Peripheral blood lymphocytes from gastric cancer patients were cultured *in vitro* and stimulated. A: Day 2,  $\times 10$  magnification; B: Day 3,  $\times 10$  magnification; C: Day 5,  $\times 10$  magnification; D: Day 14,  $\times 40$  magnification.

**Table 1** Expanded activated autologous lymphocyte cell properties

Property	Value
Proliferation time (d)	$13.55 \pm 1.25$
Number of cells before proliferation ( $10^6/L$ )	$7.65 \pm 1.52$
Number of cells after proliferation ( $10^9/L$ )	$8.76 \pm 1.82$
Proliferation multiplicity	$1156.57 \pm 167.88$
Survival rate of cells	$97.57\% \pm 0.94\%$

## RESULTS

### Proliferation of EAALs

A representative example of T cell proliferation from a patient in the EAAL cohort is shown in Figure 1. Initially, cells were attached to sidewalls of the culture flask, gradually becoming larger and round, and forming colonies. On about day 14, adherent cells and colonies fell off, forming a cell suspension (Figure 1).

After  $13.55 \pm 1.25$  d of culture, the total cell number went from about  $7.65 \times 10^6 \pm 1.52 \times 10^6$  to  $8.76 \times 10^9 \pm 1.82 \times 10^9$ , and the proliferation multiplicity obtained by calculating the difference in cell number before and after culture was  $1156.57 \pm 167.88$ . The survival rate of effector cells was  $97.57\% \pm 0.94\%$  (Table 1).

After *in vitro* culture and proliferation, the percentages of  $CD3^+$ ,  $CD3^+CD8^+$ ,  $CD8^+CD27^+$ ,  $CD8^+CD28^+$ , and  $CD3^+CD16^+/CD56^+$  cells increased remarkably ( $P < 0.05$ ), while those of  $CD3^+CD4^+$ ,  $CD4^+CD25^+$ ,  $CD3^+CD16^+/CD56^+$  (NK cells) were overtly decreased ( $P < 0.05$ ); no significant change was observed in  $CD4^+CD25^+CD127^+$

cells ( $P = 0.448$ , Table 2).

### Baseline clinical characteristics of the patients

Eighty-four patients were enrolled in the study (aged 40–85 years). Among the 58 screened patients who had undergone EAAL therapy from October 2009 to December 2012, 42 were included in the EAAL group. The cellular immunotherapy ranged from 2–24 treatments, total immunotherapy times were 242, and median immunotherapy times were 5. Based on clinical stage at the beginning of the study the EAAL group was further subdivided: 10 patients with stage I and II disease formed EAAL group 1; 12 individuals with stage IIIa and IIIb disease constituted EAAL group 2; 20 patients with stage IIIc and IV disease were included in EAAL group 3. Control subgroups with corresponding number of patients were obtained after random selection from 246 patients fulfilling the inclusion criteria for the control group.

The EAAL group was composed of 34 males and 8 females, while 33 males and 9 females constituted the control group. The patient age in the EAAL and control groups was  $57.54 \pm 11.93$  and  $58.98 \pm 11.17$  years, respectively, indicating that this parameter was similar in both groups ( $P = 0.740$ ). Patients were further subdivided into  $< 60$  and  $\geq 60$  years.

According to the number of chemotherapy cycles patients were divided into subgroups with  $\leq 6$  and  $> 6$  cycles. Finally, surgery and radiotherapy status of patients allowed the formation of Yes and No subgroups. Detailed basic clinical characteristics of

**Table 2** Phenotypes of lymphocytes before and after *in vitro* culture

Phenotype	Before (%)	After (%)	<i>P</i> value <sup>a</sup>
CD3 <sup>+</sup>	67.39 ± 7.55	96.71 ± 3.85	< 0.001
CD3 <sup>+</sup> CD4 <sup>+</sup>	33.22 ± 11.57	10.17 ± 8.83	< 0.001
CD3 <sup>+</sup> CD8 <sup>+</sup>	27.85 ± 8.51	79.33 ± 13.58	< 0.001
CD8 <sup>+</sup> CD28 <sup>+</sup>	13.19 ± 6.08	51.01 ± 15.34	< 0.001
CD3 <sup>+</sup> CD16 <sup>+</sup> /CD56 <sup>+</sup>	28.12 ± 12.10	5.56 ± 6.48	< 0.001
CD3 <sup>+</sup> CD16 <sup>+</sup> /CD56 <sup>+</sup>	9.19 ± 6.42	12.73 ± 7.65	0.019
CD45RA <sup>+</sup>	60.84 ± 8.26	9.74 ± 5.87	< 0.001
CD45RO <sup>+</sup>	46.53 ± 10.01	94.32 ± 4.57	< 0.001
CD4 <sup>+</sup> CD25 <sup>+</sup>	5.61 ± 4.19	3.17 ± 2.15	0.002
CD4 <sup>+</sup> CD25 <sup>+</sup> CD127 <sup>-</sup>	1.00 ± 0.46	0.94 ± 1.50	0.448
CD29 <sup>+</sup>	62.15 ± 8.77	93.04 ± 3.98	< 0.001
CD4 <sup>+</sup> CD29 <sup>+</sup>	20.11 ± 5.97	12.36 ± 7.55	< 0.001

<sup>a</sup>*P* < 0.05 vs the phenotype before *in vitro* culture.**Table 3** Basic clinical characteristics of patients treated with or without expanded activated autologous lymphocyte adoptive immunotherapy

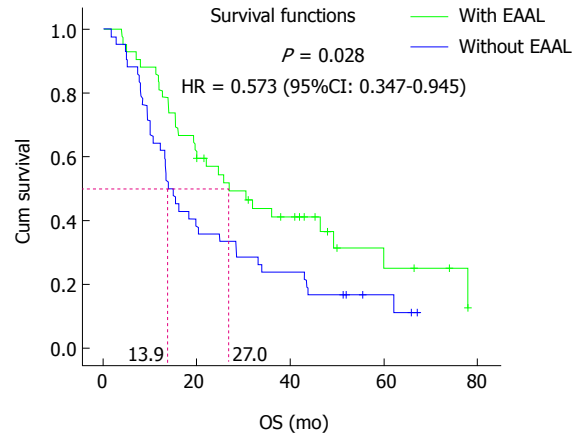
Characteristic	Patients treated with EAALs ( <i>n</i> )	Patients treated without EAALs ( <i>n</i> )	<i>P</i> value
Patient number	42	42	
Age (yr)			
< 60	24	22	0.661
≥ 60	18	20	
Sex			
Male	34	33	0.786
Female	8	9	
Stage			
I and II	10	9	0.909
IIIa and IIIb	12	11	
IIIc and IV	20	22	
Surgery			
Yes	33	37	0.242
No	9	5	
Radiotherapy			
Yes	8	4	0.212
No	34	38	
Chemotherapy			
≤ 6 cycles	19	24	0.275
> 6 cycles	23	18	

EAALs: Expanded activated autologous lymphocytes.

the enrolled patients are summarized in Table 3 and statistical analysis showed that there were no significant differences between the two groups in these parameters (*P* > 0.05).

### Chemotherapeutic features

Patients in the EAAL group received a total 288 cycles of chemotherapeutic regimens with a median of 7 cycles per patient, while 2 patients received no chemotherapy. Seven patients received post-surgery adjuvant chemotherapy alone and EAAL treatment afterwards; 13 individuals used first-line chemotherapeutic regimens in combination with EAAL treatment and 20 patients received second-line or combined chemotherapeutic regimens. EAAL cell therapy was administered alone in 2 patients because

**Figure 2** Comparison of overall survival between the expanded activated autologous lymphocyte and control groups. EAAL: Expanded activated autologous lymphocyte.

they were radically resected stage I patients, with post-surgery adjuvant chemotherapy in 13 patients, with first-line chemotherapeutic regimens in 16 patients, with second-line or combined chemotherapeutic regimens in 16 patients, and with both first-line and second-line regimens in 7 patients.

Patients in the control group received a total 264 cycles of chemotherapeutic regimens with a median of 6 cycles per patient, while 3 patients received no chemotherapy. Six patients were administered post-surgery adjuvant chemotherapy alone; 16 individuals used first-line chemotherapeutic regimens, and 17 patients were treated with second-line or combined chemotherapeutic regimens.

Chemotherapeutic regimens utilized in the study population included mFLOFOX6 (oxaliplatin, fluorouracil, leukovorin), mDCF (taxotere, cisplatin, fluorouracil), DF (taxotere, fluorouracil), mECF (epirubicin, cisplatin, fluorouracil), XELOX (oxaliplatin, capecitabine), SOX (oxaliplatin, tegafur/gimeracil/oteracil capsule), FOLFIRI (CPT-11, fluorouracil, leukovorin), capecitabine alone, and tegafur/gimeracil/oteracil capsule alone.

Patients were further subdivided according to the number of chemotherapy cycles received (≤ 6 and > 6 cycles), their status of surgery, and radiotherapy (negative or positive). Clinical characteristics are detailed in Table 3 and no statistical significance was found between the two groups (*P* > 0.05 for all).

### Kaplan-Meier survival analysis

At the last follow-up on December 31, 2012, 28 (28/42, 66.7%) patients had died in the EAAL immunotherapy group, indicating a median OS of 27.0 mo. In the control group, 36 (36/42, 85.71%) patients had died and the median OS was 13.9 mo. All deaths were associated with tumor progression. Further analysis showed that OS time in the EAAL group was significantly higher than that obtained for the control group (*P* = 0.028, HR = 0.573, 95%CI: 0.347-0.945, Figure 2). The 1- to 5-year survival rates were 80.95%, 54.35%, 41.10%,

**Table 4** Comparison of survival rates in gastric cancer patients with or without expanded activated autologous lymphocyte immunotherapy

Group	<i>n</i>	1 yr survival rate (%) (95%CI)	2-yr survival rate (%) (95%CI)	3-yr survival rate (%) (95%CI)	4-yr survival rate (%) (95%CI)	5-yr survival rate (%) (95%CI)
Immunotherapy group	42	80.95 (69.08-92.83)	54.35 (39.16-69.54)	41.1 (25.79-56.41)	36.54 (20.52-52.55)	25.05 (7.78-42.33)
Control group	42	61.9 (47.22-76.59)	33.33 (19.08-47.59)	16.67 (5.40-27.94)	11.11 (0-22.75)	11.11 (0-22.75)
<i>P</i> value		> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

**Table 5** Subgroup analysis

Subgroup	Median OS (mo)		RR (95%CI)	<i>P</i> value
	Immunotherapy group	Control group		
Age (yr)				
< 60	25.8	13.4	0.65 (0.333-1.272)	0.209
≥ 60	46.5	13.9	0.502 (0.233-1.079)	0.078
Gender				
Male	20.0	15.0	0.439 (0.146-1.32)	0.143
Female	30.5	13.9	0.625 (0.355-1.099)	0.103
Clinical stage				
I and II	60.0	62.2	1.212 (0.293-5.013)	0.791
IIIa and IIIb	49.3	28.4	0.358 (0.128-1.003)	0.051
IIIc and IV	14.0	10.0	0.484 (0.251-0.933)	0.03 <sup>a</sup>
Surgery				
Yes	36.0	15.4	0.561 (0.319-0.986)	0.045 <sup>a</sup>
No	19.7	9.4	0.265 (0.069-1.015)	0.053
Radiotherapy				
Yes	60.0	43.5	0.382 (0.094-1.55)	0.178
No	22.0	13.4	0.624 (0.364-1.069)	0.086
Chemotherapy cycles				
≤ 6 cycles	27.0	10.0	0.544 (0.255-1.159)	0.115
> 6 cycles	30.5	18.4	0.554 (0.28-1.093)	0.089

<sup>a</sup>*P* < 0.05 was considered statistically significant.

36.54% and 25.05%, respectively, in the EAAL group, and 61.90%, 33.33%, 16.67%, 11.11% and 11.11%, respectively, in controls. These data suggest a slightly better 1- to 5-year patient survival in the immunotherapy group compared with the control group, although the differences were not statistically significant (*P* > 0.05 for all, Table 4).

### Subgroup analysis

Results of subgroup analyses are displayed in Table 5. For the subgroup composed of clinical stage IIIb and IV patients, the median OS was longer in the EAAL group than in the controls (14.0 mo vs 10.0 mo, *P* = 0.03). In addition, for patients who underwent surgery, the median OS was longer in EAAL treated individuals than the control group (36.0 mo vs 15.4 mo, *P* = 0.045). However, other subgroups showed no significant differences in median OS between the EAAL and control groups (*P* > 0.05).

### COX multivariate regression analysis

COX multivariate regression analysis showed that gender, age, cancer stage, surgery, radiotherapy, chemotherapy, and EAAL immunotherapy were independent risk factors for OS in gastric cancer patients (Table 6).

### Safety assessment of EAAL immunotherapy

Fifty grade 1 or 2 and self-limiting adverse events developed in 242 EAAL transfers (Table 7). No patient showed pulmonary or renal symptoms, sign of infection, hepatic function deterioration, or autoimmune disorder. There was no treatment-related death recorded.

## DISCUSSION

Tumor cells adopt diverse mechanisms to escape tumor-specific immunity in the neoplastic process. The pathological interactions between cancer cells and host immune cells create an immunosuppressive network, not only in the tumor microenvironment, but also systemically<sup>[25,26]</sup>. Transfusion of an adequate quantity of lymphocytes, which are capable of recognizing and lysing tumor cells, is the basis for successful adoptive cell therapy<sup>[27,28]</sup>. Previous reports have suggested that T-cells from non-tumor-bearing hosts can boost anti-tumor immunity to break the morbid equilibrium formed between tumor cells and the host<sup>[29,30]</sup>. Indeed, cell transfer therapy for cancer has been recognized as the fourth anticancer modality after operation, chemotherapy, and radiotherapy<sup>[31]</sup>. However, the use

**Table 6 COX multivariate regression analysis of gastric cancer patients**

Factor	Wald	P value	HR	95%CI
Gender	2.527	0.112	1.723	0.881-3.369
Age	1.799	0.180	0.659	0.359-1.212
Clinical stage	41.852	< 0.001		
Clinical stage (1)	37.267	< 0.001 <sup>a</sup>	0.050	0.019-0.131
Clinical stage (2)	22.155	< 0.001 <sup>a</sup>	0.172	0.083-0.358
Surgery	0.474	0.491	0.777	0.378-1.595
Chemotherapy cycles	0.069	0.793	1.083	0.597-1.966
Radiotherapy	0.581	0.446	1.386	0.599-3.209
Application of EAALs	7.819	0.005 <sup>a</sup>	2.249	1.274-3.969

<sup>a</sup>*P* < 0.05 was considered statistically significant. EAALs: Expanded activated autologous lymphocytes.

**Table 7 Adverse events *n* (%)**

Event	Immunotherapy patients ( <i>n</i> = 42) <sup>1</sup>	Number of EAAL cellular transfers ( <i>n</i> = 242)
Fever	8 (19.05)	26 (10.74)
Chill	4 (9.52)	13 (5.37)
Headache	3 (7.14)	5 (2.07)
Nausea	2 (4.76)	2 (0.83)
Itching	1 (2.38)	1 (0.41)
Rash	1 (2.38)	1 (0.41)
Tachycardia	1 (2.38)	1 (0.41)
Diarrhea	1 (2.38)	1 (0.41)

<sup>1</sup>Five patients showed more than one type of event attributed to immunotherapy. EAAL: Expanded activated autologous lymphocyte.

of several immune cell types has been hampered by serious drawbacks including the poor efficacy and/or the complexity of cell propagation<sup>[17-19]</sup>. Interestingly, these shortcomings can be overcome through infusion of a large number of EAALs, as demonstrated in HCC<sup>[23]</sup>. An additional advantage of EAALs is that their use presents no risk of violating medical ethics since the effector cells originate from the patient's PBMCs.

Herein, we assessed a variety of molecular markers to further characterize EAAL phenotypes. We found that CD3<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes represented more than 95% and 80% of total EAALs, respectively, while the proportions of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD16<sup>+</sup>/CD56<sup>+</sup> NK cells were relatively low. CD8<sup>+</sup>CD27<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cytotoxic T lymphocytes (CTLs) and CD3<sup>+</sup>CD16<sup>+</sup>/CD56<sup>+</sup> T lymphocytes are essential effector cells, which play an important role in anti-tumor immunity<sup>[32-34]</sup>. Therefore, the high contents described above for these cell types in EAALs may result in increased anti-tumor immunity.

The expression of regulatory T cell (T<sub>reg</sub>) specific transcription factors such as Foxp3<sup>[35,36]</sup> was not assessed in this study. However, the rather low percentage (0.80% ± 1.59%) of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> T cells, which were considered CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells<sup>[37]</sup>, implied the extremely low proportion of T<sub>reg</sub> cells in EAALs. These findings indicate that EAAL would not suppress immunity in patients.

As a result, although not all lymphocytes are tumor-

specific, the high expression of CD3, CD8, CD27, CD28, CD56 and CD16 in EAALs implies that a large number of EAALs have the potential to exert or improve anti-tumor effects.

In order to further characterize the clinical effect of EAALs, we adopted a case-control study to retrospectively analyze whether EAALs could prolong the OS of gastric cancer patients. We demonstrated that the baseline clinical features were similar and comparable in the EAAL and control groups. Interestingly, the Kaplan-Meier survival analysis showed that median OS time was significantly longer in EAAL treated individuals than in the control group (*P* = 0.028, HR = 0.573, 95%CI: 0.347-0.945), indicating a 42.7% decrease in the risk of death in gastric cancer patients after treatment with EAALs. In addition, EAAL immunotherapy seemed to improve 1- to 5-year survival rates in gastric cancer patients, although the differences did not reach statistical significance.

We demonstrated by COX multivariate regression analysis that clinical stage and EAAL immunotherapy were independent risk factors for OS in gastric cancer patients. The hazard ratios for clinical stages I and II were 0.050 (95%CI: 0.019-0.131) and 0.172 (95%CI: 0.083-0.358), respectively, suggesting that stage I and II patients might live longer than patients with more advanced stage disease. These data corroborated previous reports and clinical observations<sup>[38,39]</sup>. The hazard ratio for application of EAAL immunotherapy was 2.249 (1.274-3.969), which suggested that patients receiving EAAL immunotherapy could live 2.249 times longer than those in the control group.

In the subgroup analysis, the OS in clinical stages IIIc and IV patients who had received surgery could be prolonged by EAAL immunotherapy (*P* < 0.05). The OS was also improved by EAAL immunotherapy in other subgroups, but the difference was not significant (*P* > 0.05). These findings indicate that advanced or locally advanced gastric cancer patients might benefit more from EAAL immunotherapy.

As for safety of EAAL immunotherapy, the most common adverse reactions were fever (10.74%) and chill (5.37%). Other adverse reactions included headache, nausea, itching, rash, tachycardia and diarrhea, with low incidence rates of no more than 3%. Importantly, all the adverse reactions were of grade 1 or 2 and self-limiting, suggesting a good safety profile for EAAL immunotherapy.

In conclusion, the *in vitro* induction and proliferation method described in this study was easy and highly efficient, with good repeatability and biological safety. Our data suggest that EAAL immunotherapy might prolong the OS in gastric cancer patients. Meanwhile, a good level of safety was obtained for EAAL cellular immunotherapy with only mild adverse reactions. However, this study was a retrospective observational study. Prospective cohort clinical studies with larger sample sizes are required for confirmation



of these findings.

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## COMMENTS

### Background

Adoptive cellular immunotherapy is a process by which the patient's own peripheral immune cells are collected and their numbers are increased by growing them *in vitro*. This then allows the cells to be delivered back to the patient's blood stream and results in increased anti-tumor immunity. Expanded activated autologous lymphocytes (EAALs) have been found to be particularly successful in this therapy in hepatocellular carcinoma.

### Research frontiers

Many different *in vitro* proliferated effector cells have been tested for use as adaptive immunotherapy. These include lymphokine-activated killer cells, anti-CD3 induced activated killer cells, activated natural killer cells, dendritic cells, tumor-infiltrating lymphocytes, and cytokine-induced killer cells. The current research hotspot is to find the most effective method for this immunotherapy.

### Innovations and breakthroughs

Previously EAALs have shown promise as the most successful adaptive immunotherapy cell type in improving cancer patient outcomes. This is the first investigation into their use in gastric cancer patients.

### Applications

The study results suggest that EAAL immunotherapy may improve overall survival of gastric cancer patients.

### Terminology

Effector cells are immune cells that become active in order to defend the body in an immune response. EAALs are the patient's white blood cells (lymphocytes), which have been activated and grown outside the body in a culture dish.

### Peer-review

The manuscript entitled: "Prolonged survival in gastric cancer patients after adoptive immunotherapy" is an interesting study. The novelty of the immunotherapy should be acknowledged.

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