The phenotype of ex vivo generated cytokine-induced killer cells is associated with overall survival in patients with cancer

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Abstract Cytokine-induced killer (CIK) cells are ex vivo generated heterogeneous NK-like T lymphocytes. It is not very clear whether the phenotype of CIK cells is associated with their therapeutic efficacy to cancer patients. Thus, in this study, the association of phenotype of CIK cells and the overall survival of 121 patients with hepatocellular carcinoma (HCC), 74 patients with lung cancer and 42 patients with colorectal cancer, all of whom underwent surgical resection and received autogenous CIK cell therapy, was analyzed. We found that high ratio of the CD3+CD4+ subset was associated with poorer overall survival in colorectal cancer, but not HCC or lung cancer. A high ratio of the CD3+CD8+ subset was associated with improved overall survival in all three types of cancer. A high ratio of the CD3+CD56+ NK-like subset was associated with improved overall survival in lung and colorectal cancer, but not HCC. A high ratio of the CD3-CD56+ NK subset was associated with poorer overall survival in lung and colorectal cancer, but not HCC. In conclusion, the CD3+CD8+ and CD3+CD56+ subsets, especially the CD3+CD8+ subset, may be the major phenotypes responsible for anti-tumor immunity in vivo after autogenous CIK cell therapy.

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Introduction

Cytokine-induced killer (CIK) cells are a unique population of anti-tumor immuno-effector cells which are generated by ex vivo expansion of peripheral blood lymphocytes in the presence of interferon (IFN)-γ, anti-CD3 antibody, and interleukin (IL)-2 [1]. CIK cells present a mixed define (T-NK) phenotype and exhibit potent, non-define (MHC)-restricted cytolytic activities against a variety of tumor cells, even tumor cells which are resistant to chemotherapeutic agents [2–4]. Compared to other immuno-effector cells, CIK cells have a number of favorable characteristics, such as the ability to undergo rapid expansion ex vivo, non-MHC-restricted tumor kill, and reduced alloreactivity [5, 6]. Recently, several phase I/II clinical trials have preliminarily demonstrated that treatment with CIK cells demonstrated an encouraging efficacy in patients with cancer, including hematological malignancies and solid tumors [7–9]. Thus, CIK cells may serve as an alternative adoptive cellular immunotherapy for the treatment of cancer.

In bulk cell culture, populations of CIK cells have heterogeneous phenotypic characteristics, containing CD3+CD4+, CD3+CD8+, CD3+CD56+, and CD3-CD56+ subsets. The ratio of each subset varies between patients [10–12]. Some in vitro experimental results indicated that the CD3+CD56+ subset might possess the strongest antitumor activity [2, 3, 13]. However, the precise identity of the subsets of ex vivo generated CIKs which actually exert an anti-tumor effect in vivo remains unclear. No studies have yet used clinical analysis to clarify whether the CD3+CD56+ NK-like T cell subset is the most important anti-tumor cell population. Therefore, in this study, we performed a retrospective analysis of the

overall survival of a number of patients with different types of cancer who underwent surgical resection and received CIK infusion therapy, to evaluate the association between the phenotype of ex vivo generated CIK cells and their therapeutic efficacy. This study provides valuable evidence to indicate which subsets of CIK cells are most important to play anti-tumor immunity in vivo.

Patients and methods

Patient selection

This study of CIK cell-based immunotherapy was approved by the institutional ethics committee of Sun Yet-sen University Cancer Center, and written consent was obtained from each patient. In total, 121 hepatocellular carcinoma (HCC) patients (treated between 2002 and 2010), 74 lung cancer patients (treated between 2005 and 2010), and 42 colorectal cancer patients (treated between 2007 and 2011) were included in this study. All of the patients underwent surgical resection and received CIK cell infusion therapy at Sun Yat-sen University Cancer Center.

Ex vivo generation of CIK cells

CIK cells were prepared using a standard method [12]. Briefly, 50 mL heparinized peripheral blood was obtained from each patient. Peripheral blood mononuclear cells were separated by Ficoll-Hypaque density centrifugation, resuspended at 2×10^6 cells/ml in fresh serum-free X-VIVO 15 (Lonza, Shanghai, China) medium containing 1,000 U/mL recombinant human IFN-γ (rhIFN-γ; (ShangClone, Shanghai, China), and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for 24 h. Then, 100 ng/ml mouse-anti-human CD3 monoclonal antibody (OKT3, R&D Systems, Shanghai, China), 100 U/mL recombinant human IL-1 (rhIL-1, Life Technologies, Guangzhou, China), and 1,000 U/mL recombinant human IL-2 (rhIL-2, Beijing Sihuan, Beijing, China) were added to the media. Fresh IL-2 and fresh medium were added every 2 days, and the cell density was maintained at 2×10^6 cells/ml. CIK cells were harvested on day 14. A fraction of harvested CIK cells were taken for phenotype analysis, and the most of fresh CIK cells were infused to patients immediately after harvesting.

CIK cell phenotype analysis

The phenotype of the autologous CIK cells after culturing at day 14 from each patient was characterized by flow cytometry (Beckman, FC500) using four-color fluorescence. The following mAbs were used: anti-CD3-PE-Cy5, anti-CD4-PE-Cy7, anti-CD8-PE, anti-CD56-FITC (all from BD Bioscience).

The ratio of each subgroup (CD3+CD4+, CD3+CD8+, CD3+CD56+, and CD3-CD56+) was calculated according to the cell density of each gate. Then, according to the median of ratio value of each subgroup of CIK cells, the patients were divided into low ratio group and high ratio group. The survival of the two groups of patients was compared.

CIK cell infusion therapy

One month after surgical resection, patients start to receive i.v. infusion of autogenous CIK cells. After culturing in day 14 and before administration, the CIK cells were assessed for viability using the dye-exclusion test and checked twice for possible contamination by bacteria, fungi, or endotoxins. These fresh CIK cells were washed at least three times with normal saline, resuspended in 100 mL normal saline containing 1 % human serum albumin, and transfused into the patients within 4 h. The number of ex vivo generated CIK cells is various from different patients, as well as different batch of culture. The range of the number of CIK cells for infusion is 1.0×10^{10} to 1.5×10^{10}. After culturing each cycle, all number of CIK cells was used for infusing to patients. Patients received 4 to 8 cycles of CIK cell transfusion with 2-week interval.

Follow-up

All patients were followed up every 3 months for the first 2years, every 6 months for the next 3 years, and yearly thereafter. At each follow-up visit, serum biomarkers such as AFP and CEA, abdominal ultrasonography or computed tomography, and chest radiography were performed. Patients were followed up until they were lost to follow-up or died, or until December 31, 2012.

Statistical analysis

The overall survival was estimated by Kaplan–Meier curve. The difference of survival time between each subgroup was assessed by log-rank test. All statistic analysis was performed by SPSS Statistical Software (SPSS Inc. 18.0, Chicago, IL, USA). $P < 0.05$ was considered significant.

Results

Phenotypic characteristics of ex vivo generated CIK cells

Similarly to previous studies [10–12], there are great differences in the phenotype of ex vivo generated CIK cells among the patients (Table 1). In the 121 HCC patients, the ratio of the CD3+CD4+ subset ranged from 2.22 to 63.39 % with a median of 24.94 %; the ratio of the CD3+CD8+ subset ranged

Table 1 Phenotypic characteristics of ex vivo generated CIK cells

		CD3+CD4+%	CD3+CD8+%	CD3+CD56+%	CD3-CD56+%
Hepatocellular carcinoma ($n=121$)	Range	2.22–63.39	16.88–88.2	1.42–44.06	0.1–64.74
	Median	24.94	60.98	10.9	3.87
Lung cancer ($n=74$)	Range	1.01–55.38	13.7–88.2	3.31–34.5	0.27–63.84
	Median	27.35	64.02	9.86	2.55
Colorectal cancer ($n=42$)	Range	12.3–66.81	21.8–78.9	2.05–31.8	0.07–33.5
	Median	27.36	60.53	10.63	3.76

from 16.88 to 88.2 % with a median of 60.98 %; the ratio of the CD3+CD56+ subset ranged from 1.42 to 44.06 % with a median of 10.9 %; and the ratio of the CD3-CD56+ subset ranged from 0.1 to 64.74 % with a median of 3.87 %.

In the 74 lung cancer patients, the ratio of the CD3+CD4+ subset ranged from 1.01 to 55.38 % with a median of 27.35 %; the ratio of the CD3+CD8+ subset ranged from 13.7 to 88.2 % with a median of 64.02 %; the ratio of the CD3+CD56+ subset ranged from 3.31 to 34.5 % with a median of 9.86 %; and the ratio of the CD3-CD56+ subset ranged from 0.27 to 63.84 % with a median of 2.55 %.

In the 42 colorectal cancer patients, the ratio of the CD3+CD4+ subset ranged from 12.3 to 66.81 % with a median of 27.36 %; the ratio of the CD3+CD8+ subset ranged from 21.8 to 78.9 % with a median of 60.53 %; the ratio of the CD3+CD56+ subset ranged from 2.05 to 31.8 % with a median of 10.63 %; and the ratio of CD3-CD56+ subset ranged from 0.07 to 33.5 % with a median of 3.76 %. Based on the data above, we observed variation in the range and median ratios for each subset in patients with different types of cancer.

Relationship between the phenotype of ex vivo generated CIK cells and overall survival after autogenous CIK cell immunotherapy in patients with cancer

According to the median ratio for each subset, the patients were divided into two groups for each subset (high ratio group vs. low ratio group). The overall survival of these groups of patients was analyzed and compared for each type of cancer.

In patients with HCC (Fig. 1), there was no significant association between overall survival and the ratio of the CD3+CD4+ subset (Fig. 1a), CD3+CD56+ subset (Fig. 1c), or CD3-CD56+ subset (Fig. 1d). However, the high CD3+CD8+ ratio group of patients with HCC demonstrated a trend towards improved overall survival compared to the low ratio group (Fig. 1b), though this difference was not statistically significant ($p=0.068$).

For patients with lung cancer (Fig. 2), there was no significant association between overall survival and the ratio of the CD3+CD4+ subset (Fig. 2a); however, a high ratio of the CD3+CD8+ subset was significantly associated with improved

overall survival (Fig. 2b, $p=0.036$). In contrast to HCC, patients with lung cancer and a high ratio of the CD3+CD56+ subset had significantly improved overall survival compared to the low ratio CD3+CD56+ subset group (Fig. 2c, $p=0.015$). Additionally, a high ratio of the CD3-CD56+ subset was associated with poorer overall survival in patients with LC, though this difference was not significant (Fig. 2d, $p=0.271$).

In patients with colorectal cancer (Fig. 3), none of the subsets were significantly associated with overall survival, as the survival curves of the high ratio groups and low ratio groups overlapped before 30 months. However, in patients who survived for more than 30 months, we observed that high ratios of the CD3+CD4+ subset and CD3-CD56+ subset displayed trends towards poorer overall survival (Fig. 3a, d). In contrast, high ratios of the CD3+CD8+ subset and CD3+CD56+ subset displayed trends towards improved overall survival in patients with colorectal cancer (Fig. 3b, c).

Discussion

For the first time, this study investigated the association between the phenotype of ex vivo generated CIK cells and their therapeutic efficacy through a retrospective analysis of the overall survival of patients with three different types of cancer. In line with previous studies [10–12], we observed that the ratios of the CD3+CD4+, CD3+CD8+, CD3+CD56+, and CD3-CD56+ subsets varied among patients with different types of cancer. However, the ranges of the ratios of each subset were similar among patients with different types of cancer.

In overall survival analysis, we observed that high ratios of the CD3+CD4+ subset and CD3-CD56+ subset had no significant association with overall survival in HCC, lung cancer, or colorectal cancer, and even demonstrated trends towards poorer overall survival, indicating that these subsets may play a less important role in the anti-tumor activity of CIKs.

In contrast, a high ratio of the CD3+CD8+ subset was associated with significantly improved overall survival in lung cancer, and trended towards associations with improved
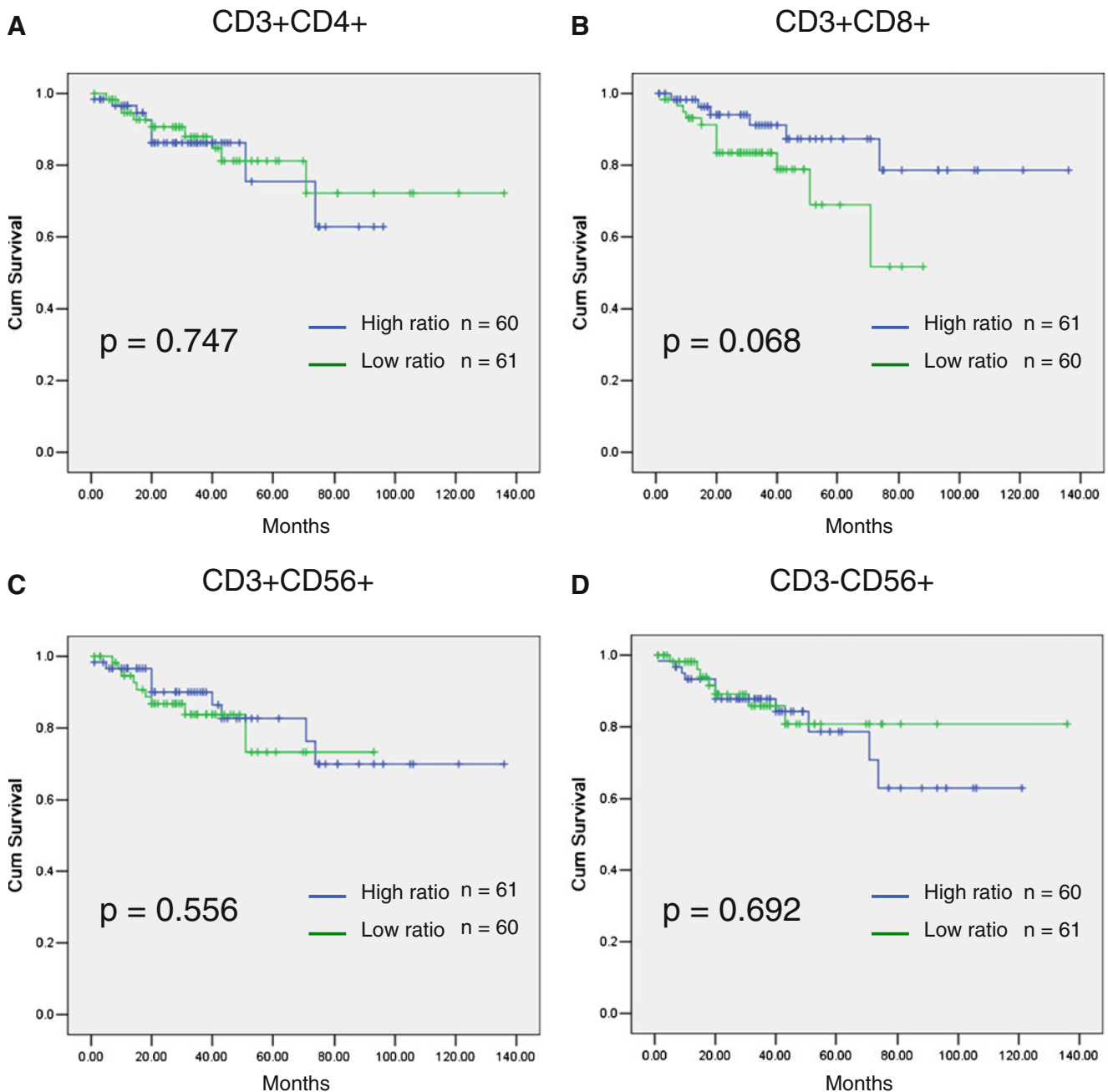
Fig. 1 Kaplan–Meier overall survival curves for patients with HCC stratified into groups with high and low ratios of different subsets of ex vivo generated CIK cells. **a** CD3+CD4+ subset, **b** CD3+CD8+ subset, **c** CD3+CD56+ subset, and **d** CD3-CD56+ subset

overall survival in HCC and colorectal cancer. Additionally, a high ratio of the CD3+CD56+ subset was associated with significantly improved overall survival in lung cancer and trended towards an association with improved overall survival in colorectal cancer, but not in HCC. Previous in vitro studies demonstrated that, after bulk culture, the CD3+CD56+ NK-like subset displayed the highest tumor cell-killing ability; therefore, this subset is considered to be the main population of anti-tumor immuno-effector cells [2, 3, 13]. However, our study indicates that the CD3+CD8+ subset might play a more

important role in anti-tumor activity in vivo than the CD3+ CD56+ subset, as a high ratio of the CD3+CD8+ subset was associated with improved overall survival in HCC, lung cancer, and colorectal cancer. One reason for this observation may be the fact that the CD3+CD8+ subset of CIK cells are early effector T cells. This subset was less cytotoxic but possessed a higher proliferative potential than the CD3+CD56+ subset [14]. Recently, some studies demonstrated that after adoptive transfer, these early-stage T cells can potentially persist in vivo for a long time and thereby establish long-term anti-tumor
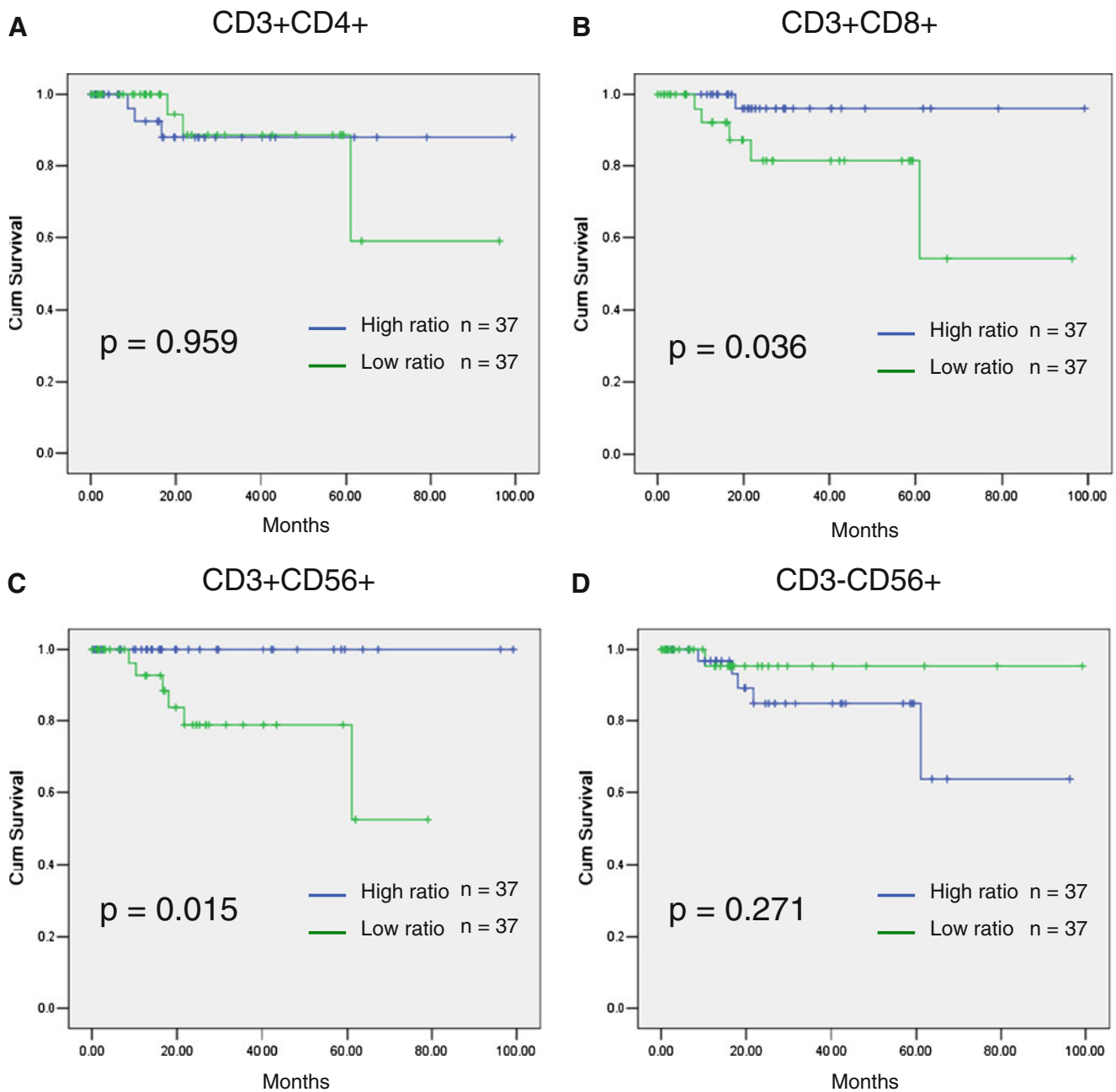
Fig. 2 Kaplan–Meier overall survival curves for patients with lung cancer stratified into groups with high and low ratios of different subsets of ex vivo generated CIK cells. **a** CD3+CD4+ subset, **b** CD3+CD8+ subset, **c** CD3+CD56+ subset, and **d** CD3-CD56+ subset

immunity [15–18]. Thus, these early less differentiated T cells may prime superior anti-tumor immunity in vivo.

The CD3+CD4+ and CD3-CD56+ subsets of ex vivo generated CIK cells have rarely been investigated in detail. In vitro experiments have only compared the tumor cell-killing activity of the CD3+CD56+ subset and CD3+CD56- (including CD4+ and CD8+ cells) subset or CD3+CD8+ subset [2, 3, 13]. There are little reports to evaluate the association of the phenotype of CIK cells and their anti-tumor immunity in vivo. Thus, our results provide the first in vivo evidence to clarify

which subsets of CIK cells play a more important in anti-tumor immunity. The results of this study may help to design more rational and effective CIK cell immunotherapy strategies for the clinic.

In conclusion, through a retrospective analysis of patients with three different types of cancer, we found that the CD3+CD8+ and CD3+CD56+ subsets of ex vivo generated CIK cells, especially the CD3+CD8+ subset, are the main populations which exert anti-tumor activity in vivo; the CD3+CD4+ and CD3-CD56+ subsets might play a less important role in
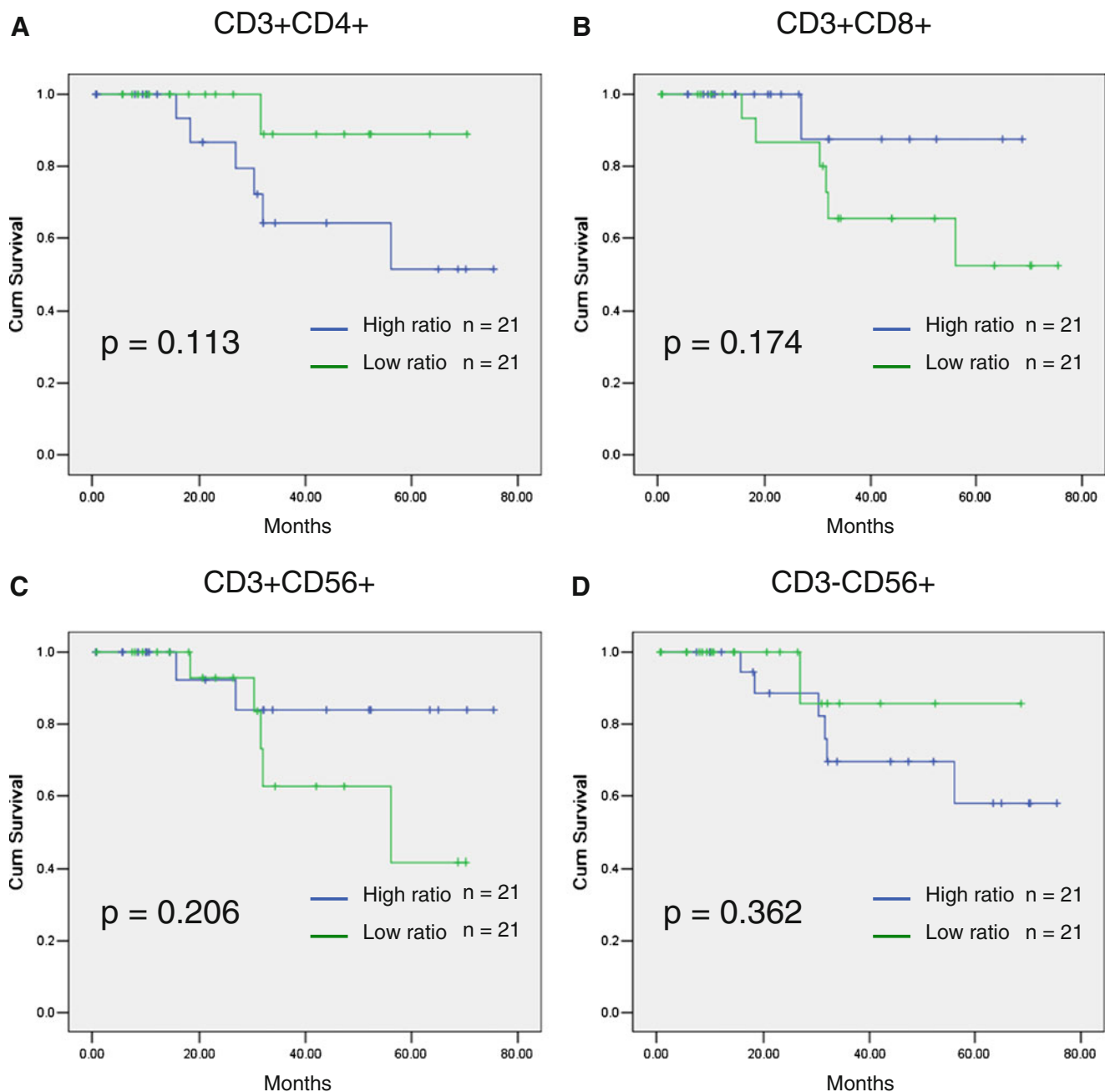
Fig. 3 Kaplan–Meier overall survival curves for patients with colorectal cancer stratified into groups with high and low ratios of different subsets of ex vivo generated CIK cells. **a** CD3+CD4+ subset, **b** CD3+CD8+ subset, **c** CD3+CD56+ subset, and **d** CD3-CD56+ subset

anti-tumor immunity. However, although we found there is the tendency that high ratio of CD3+CD8+ CIK cells is associated with better overall survival of HCC and CRC patients, and high ratio of CD3+CD56+ CIK cells is associated with better overall CRC patients, the statistical difference is not significant. We think the main reason is due to small sample size, especially for CRC patients (only 42 cases). Furthermore, insufficient follow-up time may be another reason. In CRC patients, we found that after 30 months, the survival difference in CD3+CD8+ and CD3+CD56+ subgroup analysis shows more and more significant trend. Thus, if follow-up time was extended, the statistical difference of two groups' survival might become significant. These are the limitations of this study. Thus, prospective clinical analyses with larger number size of patients and sufficient follow-up time are required to confirm the findings of this study. Furthermore, detailed study of in vivo mouse models may provide stronger evidence to verify our results and investigate the mechanisms by which the phenotype of ex vivo generated CIK cells determines their therapeutic efficacy.

Conflicts of interest None

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