Clinical & Translational Immunology

Australian and New Zealand Society for Immunology Inc.

Clinical & Translational Immunology 2020; e1113. doi: 10.1002/cti2.1113
www.wileyonlinelibrary.com/journal/cti

ORIGINAL ARTICLE

Retrospective analysis of the efficacy of cytokine-induced killer cell immunotherapy combined with first-line chemotherapy in patients with metastatic colorectal cancer

Qiu-Zhong Pan[1,2†], Jia-Mei Gu[1,2†], Jing-Jing Zhao[1,2†], Yan Tang[1,2], Qi-Jing Wang[2], Qian Zhu[1], Meng-Jia Song[1], Yong-Qiang Li[2], Jia He[2], Shi-Ping Chen[2], De-Sheng Weng[1,2] & Jian-Chuan Xia[1,2]

[1]State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-Sen University Cancer Center, Guangzhou, China
[2]Department of Biotherapy, Sun Yat-Sen University Cancer Center, Guangzhou, China

Correspondence
J-C Xia or D-S Weng, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine and Department of Biotherapy, Sun Yat-Sen University Cancer Center, Guangzhou 510060, China.
E-mails: xiajch@mail.sysu.edu.cn or wengds@sysucc.org.cn

[†]Equal contributors.

Received 13 October 2019;
Revised 3 February 2020;
Accepted 3 February 2020

doi: 10.1002/cti2.1113

Clinical & Translational Immunology
2020; **9**: e1113

Abstract

Objectives. Fluoropyrimidine-based chemotherapy regimens are the current first-line treatment for metastatic colorectal cancer (mCRC); however, the outcome is often unsatisfactory. The present study aimed to determine the effect of combined cytokine-induced killer (CIK) cell immunotherapy and first-line chemotherapy in patients with mCRC. **Methods.** This retrospective study included 252 patients with mCRC treated with first-line chemotherapy. Among them, 126 patients received first-line chemotherapy only (control group), while the other 126 patients, with similar demographic and clinical characteristics, received CIK cell immunotherapy combined with first-line chemotherapy (CIK group). Overall survival (OS) and progression-free survival (PFS) were compared between the two groups using the Kaplan–Meier method. **Results.** The median OS for the CIK group was 54.7 versus 24.1 months for the controls, and the median PFS for the CIK group was 25.7 versus 14.6 months for the controls. Univariate and multivariate analyses indicated that CIK cell treatment was an independent prognostic factor for patients' OS and PFS. Subgroup analyses showed that CIK cell treatment significantly improved the OS and PFS of patients with metastatic colon cancer, but not those with metastatic rectal cancer. Additionally, the change in $CD3^+CD56^+$ subsets after the fourth treatment cycle might be an indicator of successful CIK cell treatment: Patients with increased $CD3^+CD56^+$ subsets had better survival than those with decreased $CD3^+CD56^+$ subsets. **Conclusion.** Cytokine-induced killer cell immunotherapy combined with first-line chemotherapy could significantly improve the OS and PFS of patients with mCRC, particularly for patients with metastatic colon cancer.

Keywords: chemotherapy, cytokine-induced killer cells, immunotherapy, metastatic colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide, with approximately 1.8 million new cases and approximately 861 663 deaths per year.[1] About 40% of patients with CRC present with distant metastasis (mCRC) at the time of diagnosis, and half of all recurrences are in the form of metastatic disease.[2] Doublet chemotherapy regimens, consisting of fluoropyrimidines and oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) or capecitabine and oxaliplatin (XELOX), are the current standard-of-care options for the first-line treatment of mCRC.[3] Adding monoclonal antibodies such as cetuximab, bevacizumab or panitumumab provide a modest increase in overall survival (OS).[4] However, OS is poor, with an approximate expected survival of only 30 months.[5] In addition, approximately 86% of mCRC patients die within 5 years of diagnosis.[6] Thus, there is an unmet need for more effective and novel therapies for patients with mCRC.

The recent accelerated development of cancer immunotherapy has revolutionised the landscape for the management of many solid tumors. In some situations, cell-based immunotherapy offers a valuable and more effective alternative to conventional cytotoxic therapy.[4,7] A series of studies have demonstrated the efficacy of adoptive cell immunotherapy, including tumor-infiltrating lymphocytes, antigen-specific T lymphocytes, cytokine-induced killer (CIK) cells and chimeric antigen receptor (CAR) T cells.[8–11] Among them, CIK cells have characteristics of rapid proliferation, strong antitumor activity, broad spectrum of antitumor activity and minimal toxicity and have been demonstrated as effective in many tumors,[12] including CRC.[11,13–15] Moreover, our previous studies demonstrated the feasibility and low toxicity of CIK cell treatment for several kinds of cancer, including hepatocellular carcinoma,[16] breast cancer,[17,18] epithelial ovarian cancer,[19] non-small-cell lung cancer[20] and nasopharyngeal carcinoma.[21] Other studies reported the effectiveness of dendritic cell (DC)–CIK/CIK immunotherapy for colorectal cancer.[11,13–15] However, the therapeutic efficacy of CIK cell treatment for patients with mCRC is poorly understood and needs further confirmation.

The aim of the present study was to evaluate the clinical effect of CIK cell therapy in patients with mCRC. We assessed retrospectively the clinical efficacy of CIK cells combined with first-line chemotherapy for patients with unresectable mCRC. Our data provide evidence as to whether the combination of CIK cell immunotherapy and first-line chemotherapy could improve the clinical outcomes in patients with mCRC.

RESULTS

Patients' characteristics

The baseline data for the population are described in Supplementary table 1. After one-to-one propensity score matching, 126 pairs of patients with mCRC from the CIK group and the control group were matched (Table 1). There were no statistically significant differences in demographic or clinical characteristics such as patients' year of diagnosis, sex, age, primary tumor location, histology, metastatic site, number of metastatic sites, prior adjuvant therapy and chemotherapy regimen (Table 1). All the patients in the CIK group received at least one cycle of CIK cell treatment; the median cycle of CIK cell treatment was 8.5 cycles (range, 1–54 cycles). Sixty-three (50%) patients received more than eight cycles of CIK cell treatment (Supplementary table 2).

Characteristics of final CIK cells

In all cycles of CIK cell treatment, there were only three cases whose CIK cells failed to be produced because of low lymphocyte count after chemotherapy. The median count of CIK cells after 14 days of expansion reached 1.2×10^{10} (range, 9.5×10^{9}–1.7×10^{10}) for all cycles of CIK cell treatment. The median percentage of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD56^+$ and $CD3^+CD56^+$ population in the cultured CIK cells was 97.6% (range, 68.9–99.4%), 29.9% (range, 8.9–69.7%), 64.4% (range, 24.6–90.7%), 2.1% (range, 0.3–30.9%) and 16.8% (range, 3.4–48.3%), respectively (Supplementary figure 1). The viable CIK cells were found to exceed 95% on the basis of trypan blue staining. The cells were without any bacterial, fungal or mycoplasma contamination. The result of the endotoxin test was < 5 EU. Following quality testing, all numbers of harvested autologous CIK cells were infused back to patients at one time.

Table 1. Demographics and clinical characteristics after 1:1 propensity score matching

Variables	Total	Control	CIK	*P*
No. of patients	252	126	126	
Median follow-up time	55.2	50.4	60.8	–
Range (months)	3.3–154.4	3.3–128.6	5.4–154.4	
Year of diagnosis				
2002–2009	43	19	24	0.402
2010–2018	209	107	102	
Sex				
Male	155	78	77	0.897
Female	97	48	49	
Age (years)				
≥ 60	86	43	43	1.000
< 60	166	83	83	
Primary tumor				
Rectum	92	42	50	0.426
Left-sided colon	81	45	36	
Right-sided colon	79	39	40	
Histology				
Well differentiated	14	8	6	0.609
Moderate differentiated	208	101	107	
Poorly differentiated	30	17	13	
Metastatic site				
Liver	154	75	79	0.276
Lung	120	52	68	
Lymph nodes or others	127	68	59	
No. of metastatic sites				
1	112	59	53	0.547
2	78	35	43	
≥ 3	62	32	30	
Prior adjuvant therapy				
Yes	126	57	69	0.131
No	126	69	57	
Chemotherapy regimen				
FOLFIRI	71	31	40	0.436
FOLFOX	97	50	47	
CAPOX	84	45	39	

CAPOX, capecitabine + oxaliplatin; CIK, cytokine-induced killer; FOLFIRI, irinotecan + 5-fluorouracil; FOLFOX, oxaliplatin + 5-fluorouracil.

Side effects of CIK cell treatment

No severe side effects (grade 3 or 4) were observed in the patients who received CIK cell therapy. In our study, only 14 patients who were treated with CIK cells experienced adverse events, including 10 cases of fever (38–39 °C), two cases of transient hypertension and two cases of fatigue and anorexia. All the adverse events were mild, and the patients recovered spontaneously without any medical treatment.

OS and PFS analysis

All 252 patients with mCRC included in this study were first assessed for OS. Over a median follow-up of 55.2 months (range, 3.3–154.4 months), 56.0% (141/252) of the patients died, providing a median OS of 35.9 months (range, 1.9–101.4 months). The median follow-up time for the CIK group and the control group was 60.8 months (5.4–154.4 months) and 50.4 months (3.3–128.6 months), respectively. The 1-, 3- and 5-year OS rates were 97.6%, 63.0% and 50.0%, respectively, in the CIK group, and 76.6%, 33.1% and 26.3%, respectively, in the control group. The median OS in the CIK group (54.7 months; 95% CI, 38.56–70.8 months) was significantly higher than in the control group (24.1 months; 95% CI, 21.3–26.9 months; $P < 0.0001$; Figure 1a). The PFS rates at 1, 3 and 5 years were 84.8%, 36.1% and 26.1% for the CIK group and 55.3%, 19.5% and

16.9% for the control group, respectively. The median PFS in the CIK group (25.7 months; 95% CI, 22.4–29.1 months) was significantly superior to that in the control group (14.6 months; 95% CI, 15.3–17.9 months; $P < 0.0001$; Figure 1b). Thus, the combination of CIK cell treatment and first-line chemotherapy could significantly improve the OS and PFS of patients with mCRC.

Univariate and multivariate analyses

The effects of CIK cell treatment on the prognosis of patients with mCRC were further assessed using univariate and multivariate Cox proportional hazards regression analysis. Primary tumor in the rectum, only one metastatic site and CIK cell treatment were significantly associated with improved PFS and OS in the univariate analysis (Tables 2 and 3). Further multivariate survival analysis indicated that primary tumor in the rectum, only one metastatic site and CIK cell treatment remained associated with improved PFS and OS (Tables 2 and 3).

Subgroup analysis

The primary tumor location and number of metastatic sites have been associated with the prognosis of patients with mCRC; therefore, we assessed which subgroup of patients with mCRC, classified according to these clinical parameters, could benefit most from CIK cell treatment. Analysis of these subgroups revealed that patients with metastatic rectal cancer might derive some survival benefit from CIK cell immunotherapy; however, this benefit was not statistically significant (Figure 2a; $P = 0.3663$ for PFS; $P = 0.1101$ for OS). By contrast, patients with metastatic colon cancer in the CIK group exhibited significantly better PFS and OS than those in the control group (Figure 2b; $P < 0.0001$ for PFS and OS). Moreover, for patients with only one metastatic site, CIK cell immunotherapy significantly prolonged the PFS and OS in comparison with the control group (Figure 3a; $P = 0.0003$ for PFS; $P = 0.001$ for OS). However, CIK treatment was not significantly associated with improved PFS of patients with ≥ 2 metastatic sites (Figure 3b and c, right); however, the OS for these patients was enhanced significantly (Figure 3b and c, left). In addition, cancer-specific survival was also significantly longer in the CIK group irrespective of the number of metastatic sites (Supplementary figure 2, $P = 0.0005$ for one metastatic site, $P = 0.0044$ for two metastatic sites, $P = 0.0454$ for ≥ 3 metastatic sites).

Effect of CIK cell phenotypes on patient survival

The phenotypes of CIK cells harvested after 14 days of expansion showed variation among different patients or different treatment cycles in the same patient (Supplementary figure 1 and Figure 4); therefore, we sought to assess the relationship between the phenotype of CIK cells and survival benefit after autogenous CIK cell immunotherapy in patients with mCRC. First, we

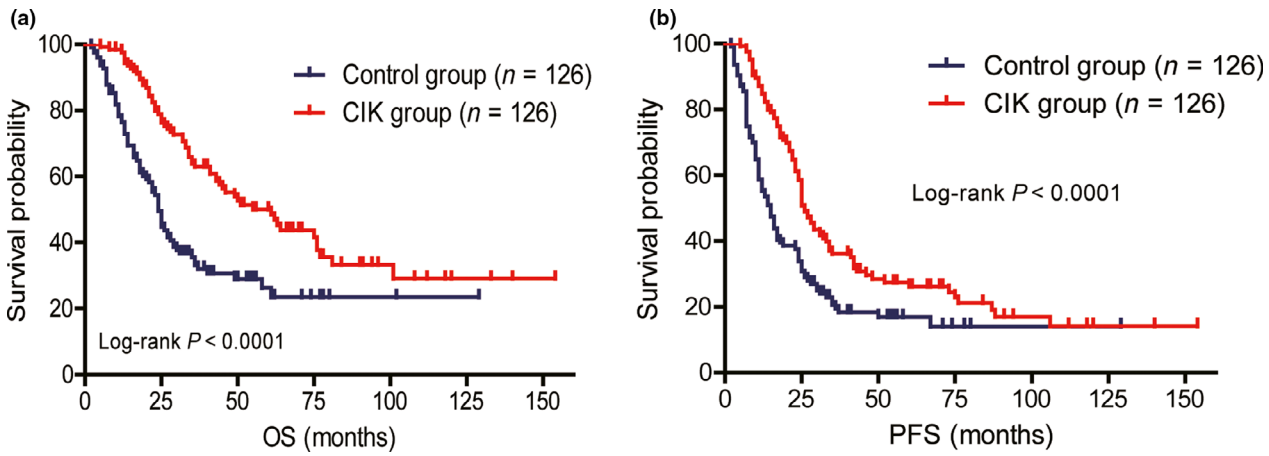

Figure 1. Kaplan–Meier estimates of overall survival (OS) **(a)** and progression-free survival (PFS) **(b)** of patients with mCRC by treatment group. Significantly improved OS and PFS were observed in the CIK group ($n = 126$) versus the control group ($n = 126$). CIK, cytokine-induced killer; mCRC, metastatic colorectal cancer; *n*, number of patients.

Table 2. Univariate and multivariate analyses of progression-free survival in patients with mCRC

	Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex (male *vs*. female)	1.195 (0.886–1.613)	0.243		
Age (≥ 60 *vs*. < 60)	0.878 (0.644–1.196)	0.410		
Primary tumor (right- *vs*. left-sided colon *vs*. rectum)	1.217 (1.019–1.454)	0.030*	1.229 (1.025–1.474)	0.026*
Histology (poorly *vs*. well/moderate)	1.049 (0.665–1.653)	0.838		
No. of metastatic sites (≥ 3 *vs*. 2 *vs*. 1)	1.412 (1.184–1.684)	< 0.001*	1.437 (1.203–1.715)	< 0.001*
Prior adjuvant therapy (yes *vs*. no)	0.743 (0.556–0.993)	0.045*	0.787 (0.587–1.056)	0.110
Chemotherapy regimen (CAPOX/FOLFOX *vs*. FOLFIRI)	0.857 (0.628–1.169)	0.331		
Treatment (CIK *vs*. control)	0.556 (0.416–0.745)	< 0.001*	0.551 (0.410–0.739)	< 0.001*

CI, confidence interval; CIK, cytokine-induced killer; HR, hazard ratio; mCRC, metastatic colorectal cancer.
**P*-value < 0.05.

Table 3. Univariate and multivariate analyses of overall survival in patients with mCRC

	Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex (male *vs*. female)	1.117 (0.793–1.573)	0.528		
Age (≥ 60 *vs*. < 60)	0.996 (0.702–1.414)	0.982		
Primary tumor (right- *vs*. left-sided colon vs. rectum)	1.261 (1.029–1.547)	0.026*	1.291 (1.050–1.587)	0.015*
Histology (poorly *vs*. well/moderate)	1.272 (0.775–2.088)	0.342		
No. of metastatic sites (≥ 3 *vs*. 2 *vs*. 1)	1.270 (1.036–1.557)	0.021*	1.302 (1.065–1.594)	0.010*
Prior adjuvant therapy (yes *vs*. no)	0.812 (0.583–1.132)	0.219		
Chemotherapy regimen (CAPOX/FOLFOX *vs*. FOLFIRI)	0.837 (0.588–1.191)	0.322		
Treatment (CIK vs. control)	0.455 (0.324–0.637)	< 0.001*	0.439 (0.313–0.616)	< 0.001*

CI, confidence interval; HR, hazard ratio; mCRC, metastatic colorectal cancer.
**P*-value < 0.05.

determined the phenotypic composition of CIK cells from the first treatment cycle to the fourth treatment cycle in 65 patients and found that the percentage of $CD3^+CD4^+$ subsets decreased and $CD3^+CD8^+$ subsets increased significantly after the fourth treatment cycle (Figure 4; $P = 0.0339$ and $P = 0.0115$, respectively); however, these changes were not statistically significant after the second or third treatment cycles. Besides, there was no significant difference in the percentage of $CD3^-CD56^+$ subsets during the four treatments, while the percentage of $CD3^+CD56^+$ subsets present at the second, third and fourth cycles showed significant incremental increases with treatment (Figure 4; $P = 0.0456$, $P < 0.0001$ and $P = 0.0163$, respectively). The impact of different first-line chemotherapy regimens on $CD3^+CD56^+$ subsets was also investigated. No significant difference in the distribution of the percentage change in the $CD3^+CD56^+$ subset after the fourth CIK cell treatment cycle among different chemotherapy regimens was observed (Supplementary table 3; $P = 0.526$). Furthermore, first-line chemotherapy regimens were not associated with patients' prognosis (Supplementary figure 3; $P = 0.3759$ for PFS; $P = 0.2748$ for OS).

The $CD3^+CD56^+$ population is considered to be the main antitumor immuno-effector cells,[22,23] and their proportion increased after the first treatment cycle; therefore, we next investigated the effect of $CD3^+CD56^+$ subsets on patient prognosis. Survival analysis revealed that there was no significant improvement in OS or PFS when a higher percentage of the $CD3^+CD56^+$ subset was present in the first CIK cell treatment cycle conducted for patients with mCRC as compared with that for patients with a low percentage of the subset (Figure 5a; $P = 0.4105$ for PFS; $P = 0.3524$ for OS). However, according to the percentage change in the $CD3^+CD56^+$ subset after the fourth CIK cell treatment cycle, the survival of patients with increased amounts of the $CD3^+CD56^+$ subset was significantly better than

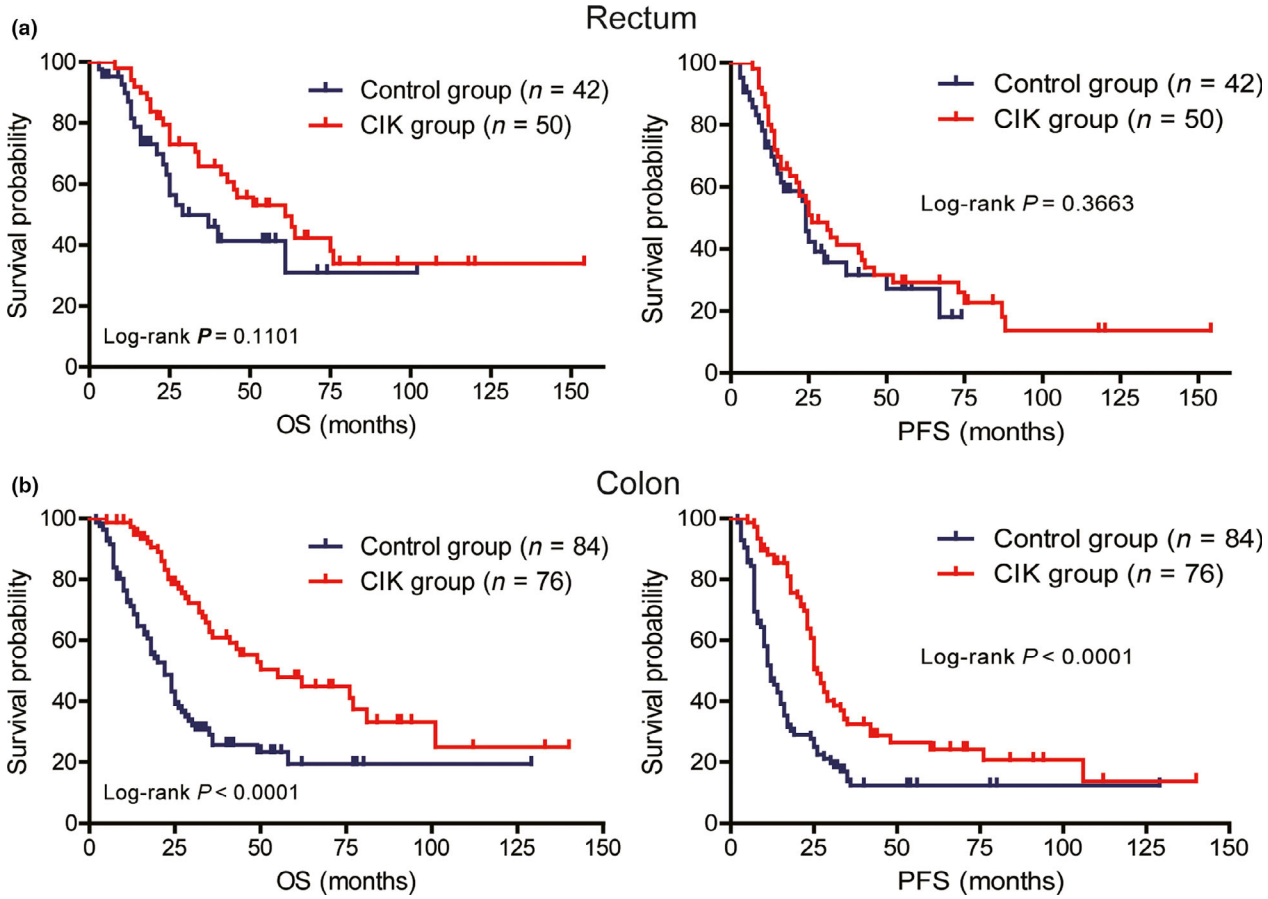

Figure 2. Subgroup analysis to estimate the survival benefits from CIK cell immunotherapy according to the primary tumor location. **(a)** CIK cell immunotherapy did not significantly affect the overall survival (OS) and progression-free survival (PFS) of patients with metastatic rectal cancer. **(b)** CIK cell immunotherapy significantly improved the OS and PFS of patients with metastatic colon cancer. CIK, cytokine-induced killer; *n*, number of patients.

that of patients with decreased amounts of the $CD3^+CD56^+$ subset (Figure 5b; $P = 0.0376$ for PFS; $P = 0.0414$ for OS). Interestingly, a higher percentage of the $CD3^+CD56^+$ subset in the second, third and fourth CIK cell treatment cycle correlated significantly with improved patient OS and PFS compared with that of patients with a low percentage of the subset, and this correlation increased as the number of CIK treatment cycles increased, as suggested by the decreased *P*-value (Supplementary figure 4a, b and c). Moreover, we also evaluated the correlation between the mean number of the total infused CIK cells or $CD3^+CD56^+$ CIK cells and patients' prognosis. Based on the median of mean number of total infused CIK cells, patients were divided into high-dose group and low-dose group. We found that neither the mean number of total CIK cells nor that of $CD3^+CD56^+$ CIK cells infused was significantly correlated with patients' OS and PFS, although there was a trend towards superior OS in the high-dose $CD3^+CD56^+$ CIK group compared with that in the low-dose $CD3^+CD56^+$ CIK group (Supplementary figure 5a and b).

DISCUSSION

Metastatic colorectal cancer remains an incurable condition, and only modest progress in the treatment of mCRC has been made to improve patient survival. The standard doublet chemotherapeutic regimens, FOLFOX, XELOX and FOLFIRI, have similar efficacy and have been used for several decades.[5,24] New biological agents such as cetuximab, bevacizumab, panitumumab, regorafenib and aflibercept have expanded the treatment options for mCRC.[5,25,26] Immunotherapy, as the fourth treatment modality

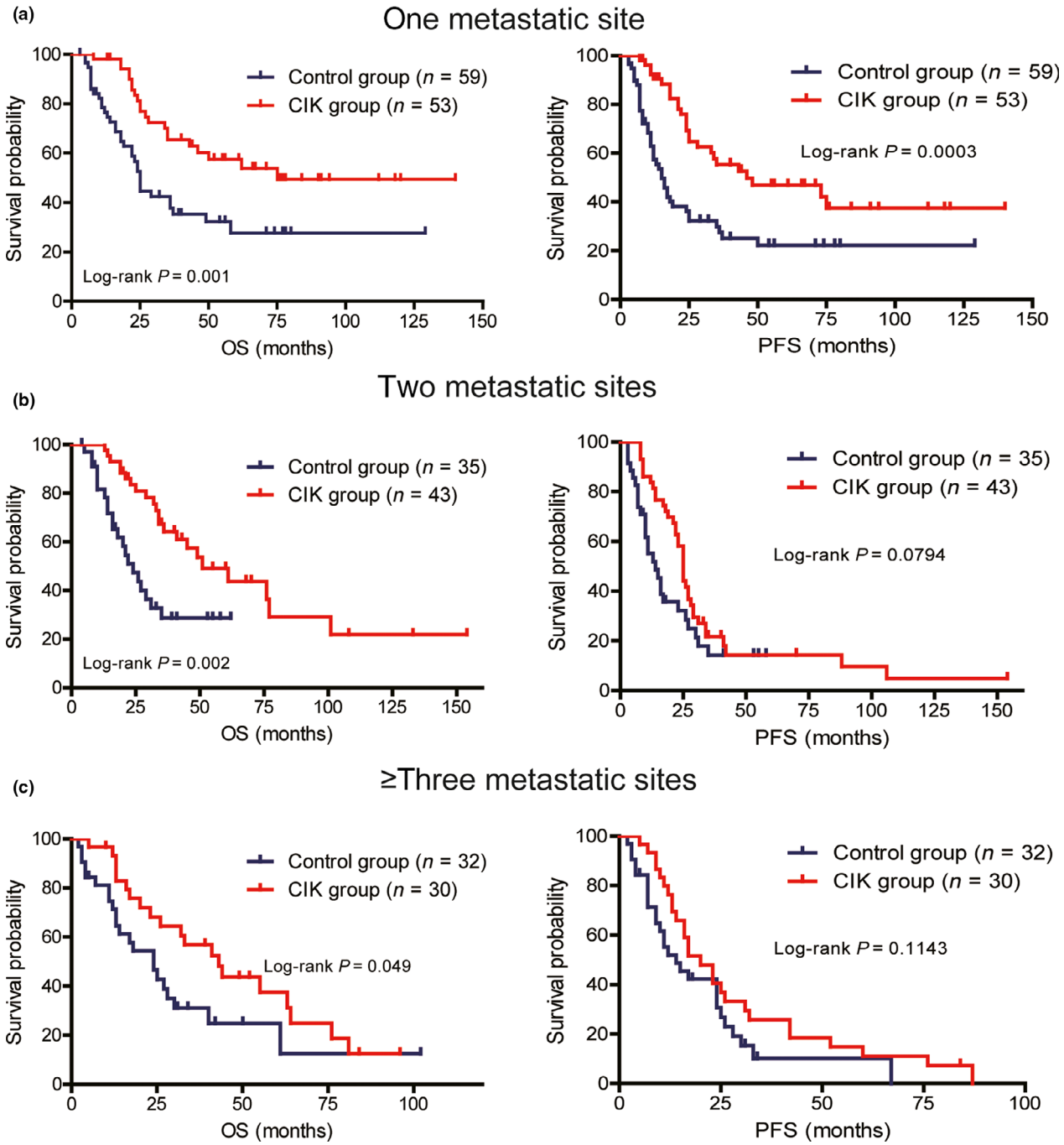

Figure 3. Subgroup analysis to estimate the survival benefits from CIK cell immunotherapy according to the number of metastatic sites. **(a)** CIK cell immunotherapy significantly prolonged the overall survival (OS) and progression-free survival (PFS) of mCRC patients with only one metastatic site. **(b, c)** CIK cell immunotherapy significantly prolonged the OS, but not the PFS, of mCRC patients with two **(b)** or ≥ 3 **(c)** metastatic sites. CIK, cytokine-induced killer; mCRC, metastatic colorectal cancer; *n*, number of patients.

for malignant tumors, has shown potential efficacy in tumor growth control and patient survival.[27] Previous studies have shown that chemotherapy combined with CIK cell immunotherapy can prolong the survival of patients with CRC.[14,15] However, those results were limited by a small sample size. In the present study, we sought to validate the efficacy of CIK cell immunotherapy combined with first-line chemotherapy for patients with unresectable

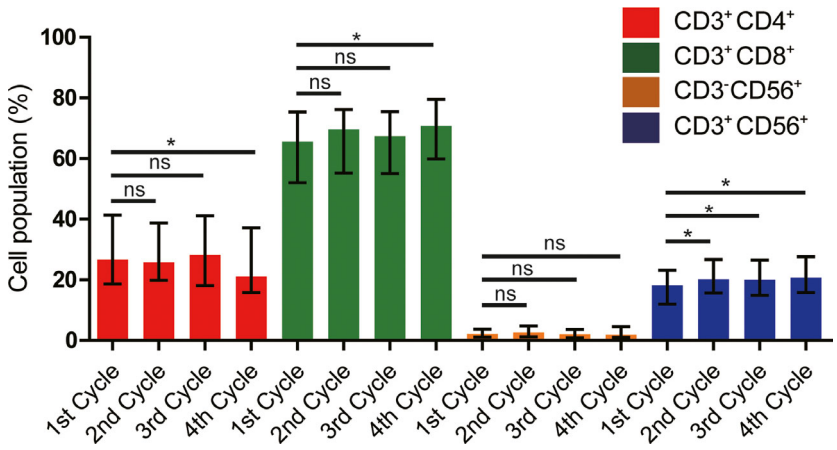

Figure 4. The phenotypic composition of CIK cells after culture for different cycles. The percentage of $CD3^+CD4^+$ subsets decreased and $CD3^+CD8^+$ subsets increased significantly after the fourth treatment cycle. The percentage of $CD3^-CD56^+$ subsets was unaffected during the four treatments. The percentage of $CD3^+CD56^+$ subsets increased significantly as the number of CIK treatment cycles increased. The results are from 65 patients and are represented as median with interquartile range. CIK, cytokine-induced killer; ns, not significant. $^*P < 0.05$.

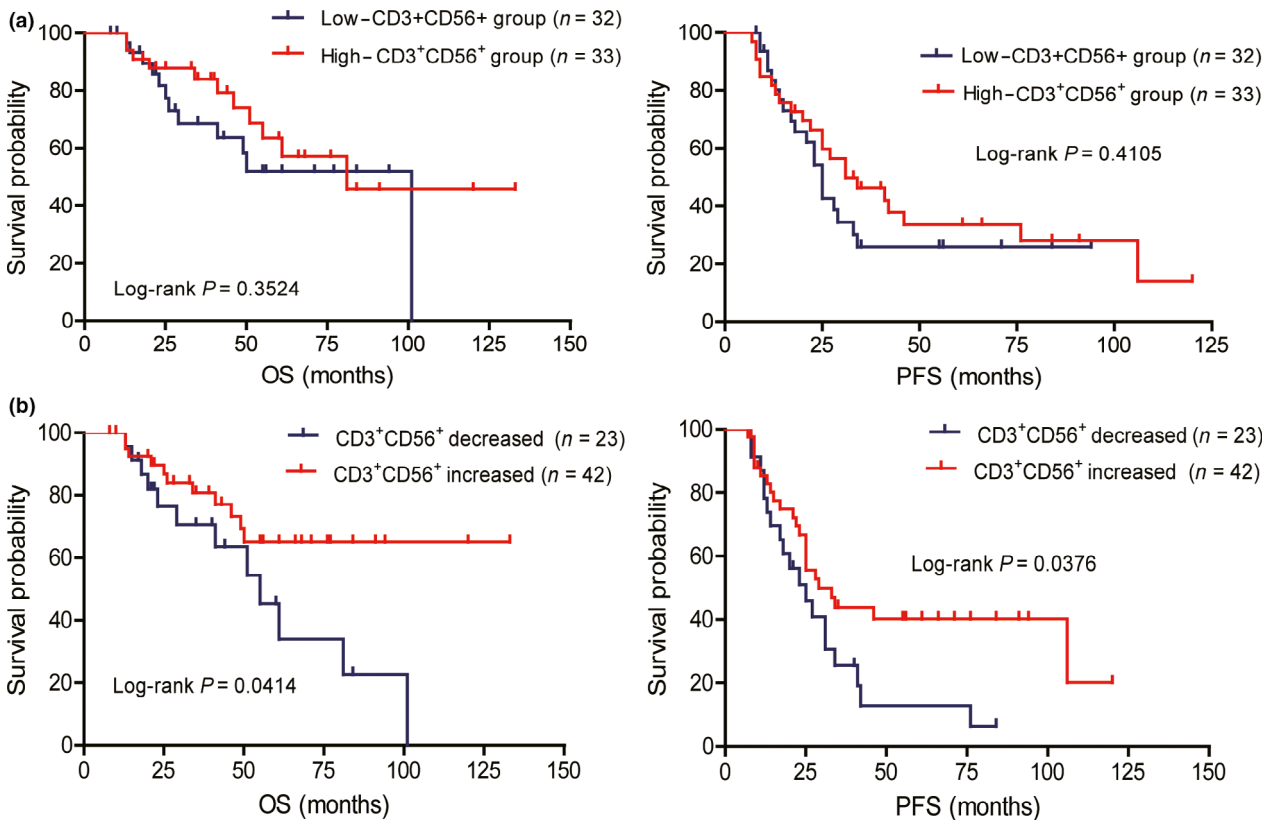

Figure 5. Effects of CIK cell phenotype on patient survival. **(a)** Association of the percentage of $CD3^+CD56^+$ subsets in the first CIK cell treatment cycle and the patients' overall survival (OS) and progression-free survival (PFS). The median percentage of $CD3^+CD56^+$ subsets was chosen as the cut-off point to separate the low and high groups. **(b)** Association of the percentage change in $CD3^+CD56^+$ subsets after the fourth CIK cell treatment cycle and the patients' OS and PFS. If the percentage of $CD3^+CD56^+$ subset within the CIK cell product increased between the first and the fourth treatment cycle, then it was defined as increased. Otherwise, it was defined as decreased. CIK, cytokine-induced killer; *n*, number of patients.

mCRC in a relatively larger sample size of 252 cases.

Cytokine-induced killer cells are a group of heterogeneous immune-active host effector cells, generated by *in vitro* expansion of peripheral blood lymphocytes using anti-CD3 antibodies, IL-2, and IL-1. These cells have dual-functional capability that a T-cell subset which acquires NK cells function and reserves TCR-mediated specific cytotoxicity.[28] In the present study, we found that patients with unresectable mCRC who received the combination of CIK cell immunotherapy and first-line chemotherapy demonstrated significantly improved OS and prolonged PFS in comparison with patients in the control group, who received first-line chemotherapy alone. Previously, Zhao *et al.*[14] showed that CIK cell immunotherapy in combination with chemotherapy improved the OS of patients with mCRC and demonstrated a trend towards contributing to patient PFS. The difference in effect of CIK cell on OS and PFS of patients with mCRC in our and Zhao's study might be a consequence of the different patients' condition, chemotherapeutic regimes and sample size. Nonetheless, these results collectively indicate that combining CIK cell immunotherapy with first-line chemotherapy improves the OS and PFS of patients with mCRC.

Several mechanisms may explain the synergistic effects of CIK cell treatment combined with the chemotherapy. First, CIK cells can reduce the incidence of bone marrow suppression of chemotherapy and improve the immunological status of patients with mCRC.[29] Meanwhile, chemotherapeutic cytotoxic drugs can attack tumor cells, resulting in the release of tumor antigens from tumor tissues, which would increase the susceptibility of malignant cells to the cytotoxic activity of immune effector cells.[30] Second, chemotherapy can remove immune suppressor factors, such as regulatory T cells and myeloid-derived suppressor cells (MDSCs), and reshape the vascularisation, which creates a microenvironment suitable for CIK cell proliferation and infiltration into tumor cells.[31] In addition, CIK cell infusion can reverse the neutrophil-to-lymphocyte ratio (NLR), a poor prognostic indicator, resulting in a restored immune equilibrium to reduce tumor recurrence and metastasis.[32] Thus, these findings indicate the theoretical rational for CIK cell immunotherapy combined with chemotherapy to gain improved therapeutic efficacy in patients with cancer, including those with mCRC.

Primary rectal and colon cancers require different staging procedures and therapeutic strategies because of their different embryological origin, anatomy and metastatic patterns.[33] However, the divergent treatment for localised rectal and colon cancers is not exhibited in the metastatic setting, and metastasised rectal and colon cancers are commonly treated in the same way.[33] In the subgroup analyses, CIK cell immunotherapy was found to be significantly associated with an improved OS and PFS in patients with metastatic colon cancer, but this association was absent in patients with metastatic rectal cancer. This improvement, or lack thereof, might be explained by the fact that the prognosis and immune microenvironment are different between rectal cancer and colon cancer.[34–36] Patients with rectal cancer have significantly better prognosis than those with colon cancer, especially for those with stage IV disease,[34] which was also observed in our study. Thus, patients with metastatic rectal cancer might derive some benefit from CIK cell treatment, but this benefit would not be statistically significant. Meanwhile, patients with metastatic colon cancer exhibited worse survival, and CIK cell treatment could significantly improve the prognosis of this subset of patients. Moreover, lymphocytic infiltration was reported to correlate with OS in cases of colon cancer but not in rectal cancer,[37] indicating that immunotherapy might not be as effective in rectal cancer as it is in colon cancer. Our results revealed that CIK cell treatment achieved favorable clinical outcomes for patients with metastatic colon cancer, suggesting its effectiveness as a treatment for this subset of patients.

Cytokine-induced killer cell treatment significantly improved the PFS rate of patients with only one metastatic site, but not those with two or more metastatic sites. However, the 1-year disease progression rate was significantly reduced after CIK cell treatment for patients with one, two or more metastatic sites, which was 9.4%, 16.3% and 20%, respectively, for the CIK group as compared with 39%, 42.9% and 46.9%, respectively, for the control group ($P < 0.001$ for one metastatic site; $P = 0.009$ for two metastatic sites; $P = 0.025$ for ≥ 3 metastatic sites). Moreover, the OS and cancer-specific survival were also significantly improved irrespective of the number of metastatic sites. CIK cell immunotherapy can stimulate and restore the body's natural

immunity.[23] Thus, patients can achieve a sustained antitumor immune response and exhibited improved OS after CIK cell immunotherapy combined with chemotherapy. Therefore, our data suggest that patients with mCRC are recommended to take the combination therapy regardless of their number of metastatic sites.

As observed above, not all patients with mCRC who receive CIK cell treatment exhibit improved outcomes; some patients were nonresponsive. Except for the patients' disease condition, identifying other biomarkers that can differentiate between responders and nonresponders is warranted for personalised treatment. CIK cell treatment can activate and enhance the body's immune system to improve its antitumor reaction, which is intrinsically a type of immune regulation.[23] In turn, the activation of infused CIK cells will also be affected by the immune microenvironment *in vivo*.[38,39] CIK cells are a heterogeneous cell population, containing $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD56^+$ and $CD3^-CD56^+$ cell subsets; therefore, we explored whether the CIK cell phenotype could serve as a predictor of the efficacy of the combination of CIK cell immunotherapy and first-line chemotherapy among patients with mCRC. Our previous study found that none of these four cell subsets were associated with survival benefit in patients with colorectal cancer who received adjuvant CIK cell treatment.[40] In the present study, we found that a significant increase in the percentage of the $CD3^+CD56^+$ subset arose after one treatment cycle. Therefore, the $CD3^+CD56^+$ subset, representing the main antitumor immuno-effector cells,[22,23] was further investigated as a prognostic biomarker for CIK cell treatment among patients with mCRC. Consistent with our previous study,[40] there was no significant association between survival benefit and the percentage of the $CD3^+CD56^+$ subset in the first cultured CIK cells. However, increased amounts of the $CD3^+CD56^+$ subset correlated with better OS and PFS compared with patients with decreased amounts of the $CD3^+CD56^+$ subset after the fourth CIK cell treatment cycle. The ever-increasing percentage of $CD3^+CD56^+$ subsets after CIK cell treatment might be caused by a significant improvement in the reactivity of immune cells to exogenous stimuli. It is therefore logical to assume that a high percentage of the $CD3^+CD56^+$ subset delivered via CIK cell infusion might induce potent antitumor effects and better prognosis in patients. Thus, the change in the percentage of the $CD3^+CD56^+$ subset after the fourth treatment cycle could be used as an indicator of effective CIK cell treatment, although these analyses are still somewhat preliminary and warrant further investigation.

Some survival benefits have been observed in patients with unresectable mCRC; however, the results should be interpreted carefully and more studies are required. The present study has certain limitations, for example its retrospective nature, treatment selection bias, uniformity of patients between the two groups and the lower frequency of follow-up for the control group, which might have affected the results. In addition, a well-designed prospective study should be carried out to examine these results further. However, our study demonstrated that the combination of CIK cell treatment and first-line chemotherapy is a safe and potential therapeutic modality for patients with mCRC.

In conclusion, this single-centre retrospective study showed that the combination of CIK cell immunotherapy and first-line chemotherapy could improve the OS and PFS of patients with mCRC. Patients with metastatic colon cancer, but not metastatic rectal cancer, could benefit more from CIK cell immunotherapy. Moreover, our data provided the first clinical evidence that a change in the $CD3^+CD56^+$ subset after the fourth treatment cycle might be an indicator of effective CIK cell treatment, and patients with increased amounts of the $CD3^+CD56^+$ subset experienced better survival than those with decreased amounts of the $CD3^+CD56^+$ subset. External validation and prospective randomised studies are warranted to further confirm the present results.

METHODS

Study population

The study protocol was designed in accordance with the guidelines outlined in the Declaration of Helsinki and was approved by the Institutional Review Board of the Sun Yat-sen University Cancer Center. A retrospective analysis was carried out on the medical records of patients with colorectal cancer in our hospital from 14 June 2002 to 21 April 2018. Patients with histologically confirmed, unresectable mCRC at first diagnosis and relapsed or metastatic colorectal cancer after surgery were eligible. No prior systemic therapies for metastatic disease were allowed, except for adjuvant chemotherapy completed more than 180 days before relapse. Patients were required to have adequate haematology and clotting and adequate

hepatic and renal function and were free of cardiac disease. Patients were excluded if they had a concurrent malignancy other than CRC, or a serious, uncontrollable medical condition. A total of 3743 patients with mCRC who received first-line chemotherapy with or without CIK cell treatment were included, of which 3617 patients received only first-line chemotherapy (control group) and 126 patients received first-line chemotherapy combined with the CIK cell treatment (CIK group). To reduce selection bias between the two groups, we derived one-to-one paired cohorts of the CIK group and the control group using propensity score matching. The preliminary selected variables entered into the propensity model included year of diagnosis, sex, age and primary tumor location. Finally, a total of 126 pairs of patients were included in the final analysis (Supplementary figure 6).

Treatments

A multidisciplinary team of doctors from different departments, including surgeons, physicians, radiation oncologists, interventional oncologists and immunologists, made the treatment decisions. CIK cell-based treatment is an observational clinical immunotherapy in our hospital. The use of CIK cell immunotherapy was approved by the Institutional Review Board of the Sun Yat-sen University Cancer Center. Written consent from each patient was obtained before the initiation of CIK cell treatment. Whether to combine CIK cell treatment with first-line chemotherapy depended on the patients' preference after complete communication and understanding of each possible accessible therapeutic option provided by the multidisciplinary team.

All patients in the control and CIK groups received first-line chemotherapy comprising the FOLFOX regimen (oxaliplatin, leucovorin and 5-fluorouracil), the FOLFIRI regimen (irinotecan, leucovorin and 5-fluorouracil) or the XELOX regimen (oxaliplatin and capecitabine), with or without bevacizumab or cetuximab. Dose reductions or treatment delays were calculated according to the nonhaematological toxicity or myelosuppression. Treatment was continued until disease progression (PD) or for 6 months. Patients who completed the 6-month first-line chemotherapy without PD entered the maintenance treatment phase and continued treatment until PD. Patients whose tumors became suitable for local treatments, such as surgery, radiotherapy or interventional therapy, could receive these local therapies based on the advice of the multidisciplinary team. Patients in the CIK group were subject to CIK cell treatment between the intervals of first-line chemotherapy or in the maintenance treatment phase. In general, patients would receive CIK cell infusion with 2- or 3-week intervals between each cycle. In the every-2-week therapy, 50 mL of blood was collected for CIK cell preparation 1 week before the first chemotherapy cycle or every CIK cell treatment. In the every-3-week therapy, blood was collected before each chemotherapy cycle. About 2 weeks later, autologous CIK cells were infused intravenously. The detailed treatment protocol is shown in Supplementary figure 7. The patients were eligible for CIK cell maintenance treatment at an interval of 1–3 months if they had stable disease.

CIK cell preparation

Cytokine-induced killer cells were prepared as described in our previous studies.[17,20] Briefly, 50 mL peripheral blood was obtained from each patient in the CIK group. Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Hypaque density centrifugation to induce CIK in a good manufacturing practice-compliant facility. To generate autologous CIK cells, PBMCs were cultured in X-VIVO 15 serum-free medium (Lonza, Visp, Switzerland) supplemented with 1000 U mL^{-1} recombinant human IFN-γ (ShangClone, Shanghai, China) for the first 24 h. Then, the following cytokines were added: 100 ng mL^{-1} mouse anti-human CD3 monoclonal antibody (R&D Systems, Minneapolis, MN, USA), 1000 U mL^{-1} IL-2 (Beijing Sihuan Pharm, Beijing, China) and 100 U mL^{-1} IL-1α (Life Technologies, San Francisco, CA, USA). Fresh medium containing IL-2 was added periodically, and the CIK cells were harvested at 14 days. A fraction of the harvested CIK cells was collected to evaluate the number, viability and possible contamination by bacteria, fungi or endotoxins; the majority of the fresh CIK cells were transfused intravenously (iv) into the patients within 30 min after cell harvest. Before and after CIK cell transfusion, vital signs such as respiratory rate, pulse, blood pressure and temperature were monitored and recorded.

Phenotypic analysis of CIK cells

The phenotypes of the autologous CIK cells from the first cycle of 79 patients were characterised using flow cytometry (FC500; Beckman Coulter, Brea, CA, USA). The following anti-human antibodies were used: anti-CD3-phycoerythrin (PE)-cyanine (Cy)5, anti-CD4-PE-Cy7, anti-CD8-PE and anti-CD56-fluorescein isothiocyanate (FITC; all from BD Bioscience, Franklin Lakes, NJ, USA). Corresponding isotype antibodies were used to stain the cells of the negative control. After washing three times, the cells were analysed using a Cytomics™ FC500 Flow Cytometer (Beckman Coulter), and data analysis was performed using CXP Analysis software (Beckman Coulter). The phenotypes of the CIK cells from the first four cycles of 65 patients were also determined. Based on the median percentage of $CD3^+CD56^+$ subset, patients were divided into a high-$CD3^+CD56^+$ group and a low-$CD3^+CD56^+$ group. If the percentage of $CD3^+CD56^+$ subset within the CIK cell product increased between the first and the fourth treatment cycle, then it was defined as increased. Otherwise, it was defined as decreased.

Follow-up

All the patients with mCRC were followed up regularly after discharge, including clinic or telephone contact once every 2 months during the first year, every 3 months for the next 2 years and every 6 months thereafter. Follow-up included physical examination, routine blood examination, carcinoembryonic antigen (CEA) levels and chest/abdominal/pelvis computed tomography (CT) scans every 6–12 months. Progression-free survival (PFS) and overall survival (OS) were defined according to the National Cancer Institute's

Response Evaluation Criteria in Solid Tumors (RECIST).[41] PFS was defined from the date of initial treatment to the date of first disease progression or the date of last follow-up. OS was defined from the time of initial treatment until death from any cause or the end of follow-up, and cancer-specific survival was defined as the time between initial treatment and death because of mCRC. If disease progression was confirmed during follow-up, remedial treatments, including second- or third-line systemic therapy, palliative surgery or palliative radiotherapy, were recommended by our multidisciplinary team. Supportive treatment was provided for patients who were intolerant of any systemic and local treatment. All toxicities were graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events version 4.0.

Statistical analysis

Pearson χ^2 test or Fisher's exact test was employed to compare the differences in demographic and clinical variables between the two groups of patients with mCRC. The student's *t*-test was used to compare CIK cell phenotype. One-to-one propensity score matching using the nearest-neighbour matching method was utilised to assemble two comparable groups. Survival curves were constructed according to the Kaplan–Meier method and compared using the log-rank test. The Cox proportional hazards regression model was used for univariate and multivariate analyses. Results were reported as hazard ratios (HR) and their 95% confidence intervals (CI). All statistical evaluations were performed using SPSS software (Statistical Package for the Social Sciences, version 22.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA). All statistical tests were two-sided, with the threshold of significance set at $P < 0.05$.

ACKNOWLEDGMENTS

This work was primarily supported by a grant from the National Key Research and Development Program of China (No. 2018YFC1313400), the National Natural Science Foundation of China (Nos. 81803079, 81402560, 81572865 and 81472387), the Guangdong Natural Science Foundation (No. 2018A030310237) and the Guangdong Province Science and Technology Plan Project (No. 2017A020215029).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

QZP, JMG and JJZ collected, assembled and analysed the data. JMG, YT, QJW, QZ, MJS, YQL, JH and SPC carried out cell generation and data analysis and interpretation. DSW and JCX designed and directed the overall project. QZP, JJZ, DSW and JCX wrote the manuscript and gave final approval of the manuscript. All authors read and approved the manuscript.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394–424.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87–108.
3. Benson AB 3rd, Venook AP, Cederquist L *et al*. Colon cancer, version 1.2017, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw* 2017; **15**: 370–398.
4. Bashir B, Snook AE. Immunotherapy regimens for metastatic colorectal carcinomas. *Hum Vaccin Immunother* 2018; **14**: 250–254.
5. Yamazaki K, Nagase M, Tamagawa H *et al*. Randomized phase III study of bevacizumab plus FOLFIRI and bevacizumab plus mFOLFOX6 as first-line treatment for patients with metastatic colorectal cancer (WJOG4407G). *Ann Oncol* 2016; **27**: 1539–1546.
6. Siegel RL, Miller KD, Fedewa SA *et al*. Colorectal cancer statistics, 2017. *CA Cancer J Clin* 2017; **67**: 177–193.
7. Wrobel P, Ahmed S. Current status of immunotherapy in metastatic colorectal cancer. *Int J Colorectal Dis* 2019; **34**: 13–25.
8. Xiao L, Cen D, Gan H *et al*. Adoptive transfer of NKG2D CAR mRNA-engineered natural killer cells in colorectal cancer patients. *Mol Ther* 2019; **27**: 1114–1125.
9. Kawamura J, Sugiura F, Sukegawa Y *et al*. Cytotoxic T lymphocyte response to peptide vaccination predicts survival in stage III colorectal cancer. *Cancer Sci* 2018; **109**: 1545–1551.
10. Gardini A, Ercolani G, Riccobon A *et al*. Adjuvant, adoptive immunotherapy with tumor infiltrating lymphocytes plus interleukin-2 after radical hepatic resection for colorectal liver metastases: 5-year analysis. *J Surg Oncol* 2004; **87**: 46–52.
11. Liu Y, Zheng Z, Zhang Q, Zhou X, Feng Y, Yan A. FOLFOX regimen plus dendritic cells-cytokine-induced killer cells immunotherapy for the treatment of colorectal cancer: a meta-analysis. *Onco Targets Ther* 2017; **10**: 2621–2633.
12. Mata-Molanes JJ, Sureda Gonzalez M, Valenzuela Jimenez B, Martinez Navarro EM, Brugarolas Masllorens A. Cancer immunotherapy with cytokine-induced killer cells. *Target Oncol* 2017; **12**: 289–299.
13. Zhou X, Mo X, Qiu J *et al*. Chemotherapy combined with dendritic cell vaccine and cytokine-induced killer cells in the treatment of colorectal carcinoma: a meta-analysis. *Cancer Manag Res* 2018; **10**: 5363–5372.
14. Zhao H, Wang Y, Yu J *et al*. Autologous cytokine-induced killer cells improves overall survival of metastatic colorectal cancer patients: results from a phase II clinical trial. *Clin Colorectal Cancer* 2016; **15**: 228–235.
15. Zhu Y, Zhang H, Li Y *et al*. Efficacy of postoperative adjuvant transfusion of cytokine-induced killer cells combined with chemotherapy in patients with colorectal cancer. *Cancer Immunol Immunother* 2013; **62**: 1629–1635.
16. Pan K, Li YQ, Wang W *et al*. The efficacy of cytokine-induced killer cell infusion as an adjuvant therapy for postoperative hepatocellular carcinoma patients. *Ann Surg Oncol* 2013; **20**: 4305–4311.

17. Pan K, Guan XX, Li YQ *et al*. Clinical activity of adjuvant cytokine-induced killer cell immunotherapy in patients with post-mastectomy triple-negative breast cancer. *Clin Cancer Res* 2014; **20**: 3003–3011.
18. Zhou ZQ, Zhao JJ, Pan QZ *et al*. PD-L1 expression is a predictive biomarker for CIK cell-based immunotherapy in postoperative patients with breast cancer. *J Immunother Cancer* 2019; **7**: 228.
19. Zhou Y, Chen CL, Jiang SW *et al*. Retrospective analysis of the efficacy of adjuvant CIK cell therapy in epithelial ovarian cancer patients who received postoperative chemotherapy. *Oncoimmunology* 2019; **8**: e1528411.
20. Pan QZ, Tang Y, Wang QJ *et al*. Adjuvant cellular immunotherapy in patients with resected primary non-small cell lung cancer. *Oncoimmunology* 2015; **4**: e1038017.
21. Li JJ, Gu MF, Pan K *et al*. Autologous cytokine-induced killer cell transfusion in combination with gemcitabine plus cisplatin regimen chemotherapy for metastatic nasopharyngeal carcinoma. *J Immunother* 2012; **35**: 189–195.
22. Schmidt-Wolf IG, Lefterova P, Mehta BA *et al*. Phenotypic characterization and identification of effector cells involved in tumor cell recognition of cytokine-induced killer cells. *Exp Hematol* 1993; **21**: 1673–1679.
23. Lu PH, Negrin RS. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent *in vivo* antitumor activity in mice with severe combined immunodeficiency. *J Immunol* 1994; **153**: 1687–1696.
24. Cassidy J, Clarke S, Diaz-Rubio E *et al*. Randomized phase III study of capecitabine plus oxaliplatin compared with fluorouracil/folinic acid plus oxaliplatin as first-line therapy for metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 2006–2012.
25. Douillard JY, Siena S, Cassidy J *et al*. Final results from PRIME: randomized phase III study of panitumumab with FOLFOX4 for first-line treatment of metastatic colorectal cancer. *Ann Oncol* 2014; **25**: 1346–1355.
26. Van Cutsem E, Tabernero J, Lakomy R *et al*. Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. *J Clin Oncol* 2012; **30**: 3499–3506.
27. Kantoff PW, Higano CS, Shore ND *et al*. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010; **363**: 411–422.
28. Pievani A, Borleri G, Pende D *et al*. Dual-functional capability of $CD3^+CD56^+$ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. *Blood* 2011; **118**: 3301–3310.
29. Lin T, Song C, Chuo DY, Zhang H, Zhao J. Clinical effects of autologous dendritic cells combined with cytokine-induced killer cells followed by chemotherapy in treating patients with advanced colorectal cancer: a prospective study. *Tumour Biol* 2016; **37**: 4367–4372.
30. Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G. Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. *Immunity* 2013; **39**: 74–88.
31. Herber DL, Nagaraj S, Djeu JY, Gabrilovich DI. Mechanism and therapeutic reversal of immune suppression in cancer. *Cancer Res* 2007; **67**: 5067–5069.
32. Templeton AJ, McNamara MG, Seruga B *et al*. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst* 2014; **106**: dju124.
33. Tamas K, Walenkamp AM, de Vries EG *et al*. Rectal and colon cancer: not just a different anatomic site. *Cancer Treat Rev* 2015; **41**: 671–679.
34. Buchwald P, Hall C, Davidson C *et al*. Improved survival for rectal cancer compared to colon cancer: the four cohort study. *ANZ J Surg* 2018; **88**: E114–E117.
35. Perez-Ruiz E, Berraondo P. Immunological landscape and clinical management of rectal cancer. *Front Immunol* 2016; **7**: 61.
36. Di J, Zhuang M, Yang H, Jiang B, Wang Z, Su X. Clinical significance of circulating immune cells in left- and right-sided colon cancer. *PeerJ* 2017; **5**: e4153.
37. Deschoolmeester V, Baay M, Van Marck E *et al*. Tumor infiltrating lymphocytes: an intriguing player in the survival of colorectal cancer patients. *BMC Immunol* 2010; **11**: 19.
38. Gammaitoni L, Giraudo L, Macagno M *et al*. Cytokine-induced killer cells kill chemo-surviving melanoma cancer stem cells. *Clin Cancer Res* 2017; **23**: 2277–2288.
39. Gu Y, Lv H, Zhao J *et al*. Influence of the number and interval of treatment cycles on cytokine-induced killer cells and their adjuvant therapeutic effects in advanced non-small-cell lung cancer (NSCLC). *Int Immunopharmacol* 2017; **50**: 263–269.
40. Pan K, Wang QJ, Liu Q *et al*. The phenotype of *ex vivo* generated cytokine-induced killer cells is associated with overall survival in patients with cancer. *Tumour Biol* 2014; **35**: 701–707.
41. Tsuchida Y, Therasse P. Response evaluation criteria in solid tumors (RECIST): new guidelines. *Med Pediatr Oncol* 2001; **37**: 1–3.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.