Retrospective analysis of the efficacy of adjuvant cytokine-induced killer cell immunotherapy combined with chemotherapy in colorectal cancer patients after surgery

Xiao Li 1,2,3†, Haodong Zhou 1,2†, Wenwen Huang 1,2†, Xuejuan Wang 1,2,3, Mingyao Meng 1,2,3, Zongliu Hou 1,2,3, Liwei Liao 1,2, Weiwei Tang 1,2,3, Yanhua Xie 1,2,3, Ruotian Wang 1,2, Haidong Yu 1,2, Lijiong Wang 1,2, Huirong Zhu 1,2, Wenju Wang 1,2,3, Jing Tan 1,2 & Ruhong Li 1,2,3

1Yan’an Hospital Affiliated to Kunming Medical University, Kunming, China
2Key Laboratory of Tumor Immunological Prevention and Treatment of Yunnan Province, Kunming, China
3Key Laboratory of Biotherapy of Kunming City, Kunming, China

Correspondence
W Wang, J Tan or R Li, Yan’an Hospital Affiliated to Kunming Medical University, No. 245 East of Renmin Road, Kunming 650051, China.
E-mails: wangwenju_vip@hotmail.com; kmtjing@sina.com or lrh272@126.com

†Equal contributors.

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Abstract

Objectives. Even though postoperative chemotherapy can eliminate residual tumor cells in patients with colorectal cancer (CRC), severe adversity, weakened immunity and drug resistance are still problems. Adjuvant cytokine-induced killer (CIK) cell therapy is an alternative to CRC patients after surgery. The present study investigated the efficacy of adjuvant CIK cell therapy combined with chemotherapy in postoperative CRC patients. Methods. This retrospective analysis included 137 postoperative CRC patients, including 71 who received adjuvant chemotherapy alone (control group) and 66 who received adjuvant immunotherapy based on CIK cells combined with chemotherapy (CIT group). Results. Long-term follow-up study indicated that overall survival (OS) and progression-free survival (PFS) were significantly longer in the CIT group than in the control group. Subgroup analyses showed that CIT treatment significantly improved OS and PFS of CRC patients classified as stage II and N0 stage and in patients with primary tumors in the rectum. Increasing the number of CIK infusions resulted in better prognosis. CRC patients aged < 65 years were found to benefit more from CIT-based therapy than patients aged ≥ 65 years. A retrospective case-control study indicated that the primary tumor expression of signalling lymphocytes activating molecule family 7 (SLAMF7) was associated with increased efficacy of CIT treatment. Conclusions. Adjuvant CIT therapy was an effective therapeutic strategy for postoperative CRC patients prolonging OS and PFS. Patient age, tumor stage and expression of SLAMF7 may be potential indicators of the efficacy of CIT therapy.

Keywords: adjuvant immunotherapy, colorectal cancer, cytokine-induced killer cells, prognosis, signalling lymphocytes activating molecule family 7
INTRODUCTION

Colorectal cancer (CRC) is a common digestive tract carcinoma, ranking third in incidence and second in mortality among 36 cancers in worldwide. Although the 5-year survival rates of CRC patients with early stage have increased to 90% owing to advances in detection and treatment, the prognosis of patients with distant metastases remains poor. Endoscopic/surgical resection, chemotherapy and radiotherapy are the main approaches to CRC treatment, but these methods have limited long-term efficacy in patients with advanced stage primary tumors and tiny metastases that cannot be completely removed surgically. Adjuvant chemotherapy and radiotherapy can effectively eliminate tumor cells and residual lesions, thereby prolonging survival, but they can cause serious side effects, including gastrointestinal reactions, damage to the immune system and even bone marrow suppression. Effective adjuvant therapy with fewer and less intense side effects is needed for patients with CRC.

Immunotherapy has shown promising clinical effects in patients with various types of tumors. Tumor immunotherapy includes monoclonal antibodies, tumor vaccines, immune checkpoint inhibitors, adoptive cell therapy and cytokine therapy. Adoptive cell therapies entail the transfer of activated or educated immune cells, including lymphokine-activated killer (LAK) cells, natural killer (NK) cells, tumor-infiltrating lymphocytes (TIL), chimeric antigen receptor (CAR) T cells and cytokine-induced killer (CIK) cells. These treatments have been found to result in strong and durable antitumor responses with minimal adverse events. CIK cells, which are derived from peripheral blood mononuclear cells (PBMCs), are heterogeneous cell populations, consisting of mixtures of CD3+CD56- , CD3+CD56+ and CD3-CD56+ T cells. CIK cells secrete various cytokines, including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-2 (IL-2), which simulate the cytotoxic activity of macrophages, NK cells and CD8+ T cells, modulating the immune system to indirectly promote the tumor cytotoxicity. CIK cells can recognise tumor cells through the binding of the receptor NKG2D on CIK cells to MIC-A/B or ULBP1, 2, 3 on tumor cells.

CIK cell-based therapies are extensively used in the postoperative adjuvant treatment of patients with various solid tumors, including ovarian cancer, non-small cell lung cancer, liver cancer, breast cancer and gastric cancer. A 10-year study of 106 international clinical trials of CIK cells found that the combination of CIK cell therapy with chemotherapy could significantly prolong progression-free survival (PFS) and overall survival (OS) in patients with cancers of the respiratory, digestive, nervous, urinary and gynaecological systems compared with chemotherapy alone. The combination of adjuvant CIK cell treatment and chemotherapy was also found to reduce recurrence rate, prolong survival and decrease the expression of serum tumor markers, with fewer side effects, compared with chemotherapy alone, in postoperative CRC patients. In addition, this combination was associated with fewer side effects, such as fever, fatigue and myalgia. Although several clinical studies have assessed the efficacy of CIK cell treatments, less is known about the clinical indications for these treatments or factors, especially the molecular biomarkers, predictive of their long-term efficacy.

The process of CIK cell induction involves the different expression of various receptors. Our previous sequencing results showed several activating receptors were found to be expressed on CIK cells, including killer Ig-like receptors 2DL4 (KIR2DL4) and 2DS4 (KIR2DS4) and signalling lymphocytes activating molecule family member 7 (SLAMF7). SLAMF7 is a type I transmembrane glycoprotein receptor expressed by activated B cells, mature DC cells and NK cells that interact with its natural ligand. This interaction results in the activation of SLAMF7, resulting in signal transduction through the EAT-2/PLC-γ pathway and enhancing the cytotoxic activity of NK cells. The monoclonal antibody elotuzumab was shown to specifically target SLAMF7, which is highly expressed on multiple myeloma (MM) cells, recruiting NK cells and resulting in an antitumor effect through antibody-dependent cell-mediated cytotoxicity (ADCC). Importantly, the expression of SLAMF7 was detected in specimens from CRC patients. We hypothesised that the level of expression of SLAMF7 on tumors from CRC patients could influence the efficacy of CIK cell-based therapy. The present study therefore
retrospectively investigated the clinical efficacy of CIK cells combined with chemotherapy (CIT) in postoperative CRC patients as well as assessing the association between SLAMF7 expression in CRC tissues and the efficacy of postoperative CIT in CRC patients. Our study will present clinical evidence to support the efficacy of CIT against CRC and provide biomarkers for clinicians to identify CRC patients who will benefit from CIT.

RESULTS

Demographic and clinical characteristics of CRC patients

The present study included 137 patients who underwent radical surgery for CRC, including 71 who received postoperative chemotherapy alone (control group), and 66 who received postoperative CIK cell-based therapy combined chemotherapy (CIT group). Their demographic and clinical characteristics are shown in Table 1. There were no significant differences between the CIT and control groups in patient gender, age, primary tumor location, TNM stage, carcinoembryonic antigen (CEA) concentration and degree of histological differentiation (Table 1).

Phenotypic analysis and quality control of CIK cells

CIK cells were harvested for infusion on days 14, 15 and 16. Before each infusion, the cells were sampled for quality control and safety tests. Flow cytometry determination of the phenotypes of these immune cells result showed that the average percentages of CD3^+CD56^- CIK cells and CD3^+CD8^- cells were > 40% and > 70% respectively (Supplementary figure 1). Each injection consisted of 2.0–5.0 × 10^9 cells, with > 90% of these cells being viable. The CIK cell preparations were free of bacteria, fungi and mycoplasma.

Adverse reactions

None of the patients in the CIT group experienced disease progression or relapse after chemotherapy and before administration of CIK cells, with only one patient in this group showing postoperative colonic leakage. During the process of chemotherapy before CIK treatment, 22 (33.33%) of the 66 patients in the CIT group experienced nausea, vomiting and other digestive symptoms; seven (10.61%) showed decreases in white blood cells counts, and five (7.58%) patients experienced pulmonary infections, but there were no severe adverse reactions. During the process of CIK cell treatment, eight (12.12%) of these patients developed fever; however, their body temperature was below 38.3°C, and all recovered naturally in 24 h with or without treatment of symptoms, which may be considered as self-limited fever. In addition, two (3.57%) patients experienced fatigue, recovering naturally without medical treatment. None of the patients in the

| Clinicopathological variables | Control group (n = 71) | CIT group (n = 66) | P      |
|-------------------------------|-----------------------|-------------------|--------|
| Gender                        | Male                  | 46                | 37     | 0.296  |
|                               | Female                | 25                | 29     |        |
| Age                           | < 65                  | 44                | 32     | 0.112  |
|                               | ≥ 65                  | 27                | 34     |        |
| Tumor Location                | Rectum               | 34                | 24     | 0.173  |
|                               | Colon                | 37                | 42     |        |
| Tumor                         | T1/T2                | 8                 | 8      | 0.876  |
|                               | T3/T4                | 63                | 58     |        |
| Lymph Node Metastasis         | N0                    | 35                | 39     | 0.250  |
|                               | N1/N2                | 36                | 27     |        |
| TNM Stage                     | II                    | 35                | 39     | 0.250  |
|                               | III/IV               | 36                | 27     |        |
| CEA (ng mL^-1)                | 2.62 (1.30, 4.80)     | 1.69 (0.73, 6.90) | 0.666  |
| Histological differentiation  | Poor                  | 5                 | 3      | 0.398  |
|                               | Moderate              | 56                | 54     |        |
|                               | Well                  | 3                 | 6      |        |
|                               | Missing data          | 7                 | 3      |        |
CIT group experienced severe adverse reactions, such as anaphylactic shock.

**Survival analysis of CRC patients**

Overall, the median OS during the follow-up period was 70.16 months (range 1.02–147.12 months) and the median PFS was 53.98 months (range 1.02–147.02 months). The OS rates of the CIT and control groups were 46.97% (31/66) and 32.39% (23/71), respectively, and their median OS was 101.3 months (range 1.02–147.12 months) and 53.98 months (range 3.02–123.12 months) respectively. The 1-, 3-, and 5-year OS rates were 93.94%, 78.79%, and 66.67%, respectively, in the CIT group and 97.18%, 66.20%, and 45.07%, respectively, in the control group. Median PFS was significantly longer in the CIT group (82.62 months, range 1.02–147.12 months) than in the control group (35.67 months, range 3.02–123.12 months). The 1-, 3-, and 5-year PFS rates were 83.33%, 65.15%, and 56.06%, respectively, in the CIT group, and 83.10%, 49.30%, and 39.44%, respectively, in the control group. Kaplan–Meier analysis indicated that the OS \((P = 0.0128)\) and PFS \((P = 0.0212)\) was significantly longer in the CIT group than in the control group (Figure 1, Supplementary table 1).

**Demographic and clinical characteristics associated with clinical outcome**

Cox proportional hazard regression model analysis was employed to assess the significance of risk factors based on multivariable and univariable adjustment. Univariate analyses demonstrated that early T stage, overall TNM stage, a higher degree of histological differentiation, the absence of lymph node metastases and CIT treatment were associated with longer OS and PFS (Table 2). Moreover, multivariable analyses indicated that CIT treatment was independently prognostic for longer OS and that a higher degree of histological differentiation and overall TNM stage were independently prognostic for both improved OS and PFS (Table 2).

**Subgroup analysis**

To further identify the clinical parameters affecting efficacy of CIT treatment, subgroup analysis was performed based on overall TNM stage, T stage, lymph node metastasis, age, numbers of CIK cell infusions, grade of differentiation and primary tumor location (Figures 2 and 3). CIT treatment was found to significantly improve OS and PFS compared with chemotherapy alone in patients with stage II CRC but did not affect OS or PFS of CRC in patients with later stage (stage III/IV). CIT treatment also resulted in better OS and PFS than chemotherapy alone in patients with advanced T stage (T3 and T4) but not in those with early T stage CRC. Moreover, CIT treatment significantly increased OS and PFS in CRC patients without lymph node metastasis (N0 group), but had no effect in patients with lymph node metastases (N1/N2). To evaluate the effects of number of CIK cell

![Figure 1. Kaplan–Meier analyses of overall survival and progression-free survival in postoperative CRC patients treated with CIT or chemotherapy alone. Overall survival (OS) and progression-free survival (PFS) curves were calculated for all CRC patients \((n = 137)\) and for those treated postoperatively with CIT \((n = 66)\) or chemotherapy alone \((n = 71)\). *\(P < 0.05\) by the stratified log-rank test.](image-url)
Table 2. Univariate and multivariate analysis of overall survival and progress-free survival in patients with CRC

| Variables                        | Overall survival |                      | Progress-free survival |                      |
|---------------------------------|-----------------|----------------------|------------------------|----------------------|
|                                 | Univariate      | Multivariate         | Univariate             | Multivariate         |
|                                 | analysis        | analysis             | analysis               | analysis             |
|                                 | HR (95% CI)     | P^                   | HR (95% CI)            | P^                   |
| Age (< 65 vs. ≥ 65)             | 1.529 (0.992–2.355) | 0.054               | NA                     | NA                   |
| Gender (Male vs. Female)        | 0.870 (0.556–1.362) | 0.543               | NA                     | NA                   |
| Primary Tumor Location (Rectum vs. Colon) | 0.926 (0.600–1.427) | 0.726               | NA                     | NA                   |
| Histological Differentiation (Poor vs. Moderate vs. High) | 0.402 (0.209–0.776) | 0.007**              | 0.431 (0.212–0.879) | 0.021*              |
| T Stage (T1/T2 vs. T3/T4)       | 2.587 (1.126–5.949) | 0.025*              | 2.519 (0.901–7.046) | 0.078               |
| N Stage (N0 vs. N1/N2)          | 2.572 (1.655–3.996) | 0.00002***          | NA                     | NA                   |
| TNM Stage (III vs. IV)          | 2.572 (1.655–3.996) | 0.00002***          | 2.224 (1.384–3.572) | 0.001**             |
| Treatment (CIT vs. Control)     | 1.754 (1.121–2.744) | 0.014*              | 1.634 (1.017–2.626) | 0.042*              |

P < 0.05; **P < 0.01; ***P < 0.001.
CI, confidence interval; HR, hazard ratio; NA, not analysed.
^Cox regression.
infusions, patients in the CIT group were divided based on the mean number of CIK cell infusions (3.17 ± 1.63), with OS and PFS being significantly longer in patients receiving ≥ 3 than < 3 infusions. CIT treatment prolonged the OS and PFS in CRC patients aged < 65 years, but not in patients aged ≥ 65 years. CIT treatment also significantly improved OS and PFS in patients having CRC tumors with a moderate grade of differentiation group. In addition, CIT treatment significantly prolonged OS and PFS in patients with rectal cancer, but not in those with colon cancer.

Upregulated SLAMF7 may mediate the tumor cytotoxicity of CIK cells

To identify receptors and immune checkpoints expressed on CIK cells, deep sequencing was performed on CIK cells sampled on sequential days of entire generation process, including on day 0, day 7 and day 14. The expression of genes encoding receptors and immune checkpoints reported to be expressed on NK cells and T cells was extracted from sequencing data for CIK cells, with the expression patterns displayed using a

Figure 2. Kaplan–Meier analyses of the effects of TNM stage on overall survival and progression-free survival in CRC patients treated postoperatively with CIT or chemotherapy alone. (a) OS and PFS of stage II CRC patients treated with CIT (n = 39) or chemotherapy alone (n = 35); (b) OS and PFS of stage III/IV CRC patients treated with CIT (n = 27) or chemotherapy alone (n = 36). (c) OS and PFS of T1/T2 CRC patients treated with CIT (n = 8) or chemotherapy alone (n = 8); (d) OS and PFS of T3/T4 CRC patients treated with CIT (n = 58) or chemotherapy alone (n = 63). (e) OS and PFS of N0 CRC patients treated with CIT (n = 39) or chemotherapy alone (n = 35); (f) OS and PFS of N1/N2 CRC patients treated with CIT (n = 27) or chemotherapy alone (n = 36). (g) OS and PFS of N0 (n = 39) and N1/N2 (n = 27) CRC patients treated with CIT. *P < 0.05, **P < 0.01, ***P < 0.001 by stratified log-rank tests.
Of the activating receptors, SLAMF7 was found to be increasingly upregulated in response to cytokines stimulation, with the expression patterns of SLAMF7 validated by qRT-PCR, flow cytometry and Western blotting (Figure 4b–d). The expression profile of SLAMF7 was consistent with the deep sequencing data. Because of the self-ligand nature of SLAMF7, the presence of SLAMF7 on CRC cells is likely the key to activating the tumor cytotoxicity of CIK cells. Western blots showed the self-ligand of SLAMF7 was the expression on the human CRC cell lines HCT-116 and HT-29 (Figure 4e), with CIK cells exhibiting potent and dose-dependent cytotoxicity against HCT-116 and HT-29 cells (Figure 5a). To determine whether SLAMF7 participates in tumor recognition, CIK cells with high and low expression of SLAMF7 were sorted by fluorescent-activated cell sorting (FACS) (Figure 5b) and the antitumor activities of these two CIK cells populations were assessed against HCT-116 and HT-29 cells. The cytotoxicity of two
CIK cell populations, when tested at an effect-to-target ratio of 16:1, was found to differ, with the results indicating that the antitumor activity of CIK cells with high expression of SLAMF7 was significantly higher than CIK cells with low expression of SLAMF7 (Figure 5c).

**Associations between SLAMF7 expression and prognosis of CRC patients receiving CIT treatment**

To explore the relationship between SLAMF7 and the efficacy of CIT, the expression of SLAMF7 in primary tumor specimens was analysed by immunohistochemistry, and the correlation between SLAMF7 expression and patient prognosis tested. Specimens from 56 CRC patients had been archived for evaluation, with both paracancer and tumor tissue analysed in specimens from 39 patients, and tumor tissue alone analysed in specimens from 17 patients. The expression of SLAMF7 in each sample was scored based on the intensity and area of the staining (e.g. Figure 6). Statistical analyses indicated that SLAMF7 expression did not differ significantly in paracancer and tumor tissue samples and that SLAMF7 expression did not correlate with TNM stage (Figure 7).
To investigate the correlation between SLAMF7 expression and immune cell infiltration, the densities of CD8⁺ T cells and NK cells in tumor tissue were compared in CIT-treated CRC patients with high and low SLAMF7 expression (e.g. Figure 8). Overall, 112 slides were enrolled, 56 slides were evaluated for CD8⁺ T-cell infiltration, and 56 slides were collected for NK cell infiltration analysis. Statistical analyses showed that the expression of SLAMF7 did not correlate with either CD8⁺ T cell or NK cell infiltration (Figure 9a).

The 56 CRC patients were divided into two groups based on their SLAMF7 expression scores. The OS rate was higher in patients with higher than low SLAMF7 expression (58.82% vs. 36.36%; Supplementary table 2). Survival analysis indicated that high expression of SLAMF7 in primary CRC specimens was a prediction of better prognosis in patients receiving CIT treatment (Figure 9b).

**DISCUSSION**

Although surgery is still the first-line treatment for patients with CRC, various adjuvant and neoadjuvant treatments, including chemotherapy, radiotherapy, targeted therapy, immunotherapy and their combinations, can significantly eliminate...
residual disease and improve patient prognosis.\textsuperscript{33} Chemotherapy, however, has both advantages and disadvantages, as it not only inhibits the tumor cell growth, but also weakens the immune system, by reducing white blood cell counts. Nevertheless, systemic chemotherapy has been reported to enhance the immunogenicity of tumor cells and eradicate immunosuppressive cells in the tumor microenvironment including MDSC, M2 macrophages and Tregs.\textsuperscript{5,34,35} CIK cells have shown potent antitumor activity against many tumor types, reducing tumor recurrence and metastasis, while being associated with fewer and less severe adverse effects. The cytotoxic function of CIK cells may be significantly impaired by the expression of immune checkpoints, including PD-L1, LAG-3 and TIM-3.\textsuperscript{36,37} The present study hypothesised that the combination of CIK cells and chemotherapeutic agents would be more effective in treating CRC than chemotherapy alone. Results consistently showed that CIT treatment improved OS and PFS compared with chemotherapy alone. Univariate and multivariate analyses further indicated that CIK cell combined with chemotherapy was an independent prognostic factor for OS and PFS. These findings, showing that postoperative CIT treatment could significantly prolong OS and PFS and reduce the rate of metastasis and recurrence in CRC patients, were consistent with results in patients with ovarian cancer, non-small lung cancer, triple-negative breast cancer, gastric cancer and liver cancer, as well as in patients with CRC metastases.\textsuperscript{20–26}

Subgroup analyses showed that, compared with chemotherapy alone, CIT improved the OS and PFS in CRC patients with stage II, but not stage III/IV. Moreover, CIT treatment was more effective than chemotherapy alone in CRC patients at advanced T (T3/T4) and earlier N (N0) stage. The
limited efficacy of CIT therapy in patients with advanced stages may be because of the further progression of malignancy in these tumors, as this treatment has been shown ineffective with highly malignant CRC. Interestingly, the present study found that prognosis was better in patients receiving three or more infusions of adjuvant CIK cells than in those receiving fewer than three infusions. This finding was in agreement with the results of adjuvant CIK cell treatment in gastric cancer patients. CIK cells are a key determinant of the efficacy of CIT treatment, with more courses of infusion resulting in a better prognosis. Age is a major risk factor during tumorigenesis, with susceptibility to tumors increasing with age because of the accumulation of somatic mutations. The present study found that, compared with chemotherapy alone, CIT significantly improved OS and PFS in patients aged < 65 years, but not in older patients aged ≥ 65 years. The reduced activity of immune cells from elderly patients may have reduced the cytotoxicity of CIK cells. CIT therapy was also associated with better OS and PFS than chemotherapy alone in patients with rectal but not colon cancer. Patients with rectal cancer were reported to have a better long-term prognosis than patients with colon cancer. The different outcomes of CIT treatment in rectal and colon cancer may be because of the properties of the tumors.

This study also found that CIT therapy was more effective than chemotherapy alone in CRC patients with T3/T4 stage, but not T1/T2 stage. This result was surprising, as it conflicts with common knowledge in clinical practice, but may be ascribed to relatively few samples of T1/T2 stage CRC included in this study. OS and PFS were also longer in patients with moderate tumor differentiation receiving CIT than chemotherapy alone, whereas there were no significant differences in patients with poorly or well differentiated. However, the numbers of patients in this study with poorly and well differentiated
tumors were small, indicating a need for comparisons in larger numbers of samples.

SLAMF7, which acts as an activating receptor against cancer, is highly expressed on NK cells.\textsuperscript{43} SLAMF7 on NK cells is thought to be involved in the identification and killing ability of multiple myeloma cells.\textsuperscript{44,45} To date, few studies have assessed the expression of SLAMF7 on CIK cells. The present study found that the expression of SLAMF7 gradually increased during the induction of CIK cells, suggesting that SLAMF7 expression may correlate with the gain of antitumor function of CIK cells. Interestingly, although the self-ligand of SLAMF7 was reported to be rarely expressed on solid tumor cells,\textsuperscript{27,30,32} the present study found that SLAMF7 was strongly expressed on the human CRC lines HCT-116 and HT-29, as well as on tumor specimens from patients with CRC. Subsequent tumor cytotoxic assay showed that SLAMF7\textsuperscript{High} CIK cells exhibited stronger tumor-killing ability than SLAMF7\textsuperscript{Low} CIK cells, suggesting that SLAMF7 may be involved in

\begin{figure}[h]
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\caption{Immunofluorescence staining for infiltrated immune cell of CRC surgical specimens. Immunofluorescence staining for CD3, CD8 and CD56 of CRC surgical specimens in the CIT group.}
\end{figure}
triggering the tumor-killing effect of CIK cells against CRC cells. Although SLAMF7 was expressed on both tumor and paracancer tissues, its expression did not differ significantly, nor did SLAMF7 expression correlate with tumor TNM stage. SLAMF7 expression, however, was reported to be significantly lower in stage IV than in early-stage CRC, which could correlate with lymphatic, vascular, peripheral nerve and/or resection margin invasion, leading to CRC progression and metastasis.\textsuperscript{32}

High-density infiltration into tumors of immune cells, including CD8\textsuperscript{+} T cells, NK cells, macrophages and regulatory T cells, may be associated with better prognosis in patients with various cancers, including CRC.\textsuperscript{46} Meanwhile, many immune cells are positive for SLAMF7 expression.\textsuperscript{27} To determine whether tumor-infiltrating immune cells that express high level of SLAMF7 are associated with better patient prognosis, immunofluorescence assays were performed to assess the relationships between tumor-infiltrating CD8\textsuperscript{+} T cells and NK cells and the expression of SLAMF7 in cancer tissues of CRC patients treated with CIT. Our finding, that SLAMF7 expression did not correlate with the infiltration of either CD8\textsuperscript{+} T cells or NK cells, suggested that these immune cells did not express higher levels of SLAMF7. Further investigation of the associations between SLAMF7 expression in CRC specimens and the prognosis of patients receiving CIT treatment showed that the OS ($P = 0.0512$) and PFS ($P = 0.0552$) were tended to be longer in the SLAMF7\textsuperscript{High} than in the SLAMF7\textsuperscript{Low}. These findings suggested that SLAMF7 may be a potential indicator of the efficacy of CIT treatment in patients with CRC.

In conclusion, the present study showed that, compared with adjuvant chemotherapy alone, the combination of postoperative CIK cell treatment and chemotherapy could significantly prolong OS and PFS in postoperative patients with CRC. Subgroup analyses showed that CIT treatment could particularly benefit CRC patients aged...
< 65 years old, patients without lymph node metastasis and patients with rectal cancer. Furthermore, an increased number of CIK cell infusions during CIT treatment were positively associated with improved prognosis. In addition, the expression of SLAMF7 in malignant tissues from CRC patients could be a potential molecular indicator of the efficacy of postoperative CIT treatment.

METHODS

Patients – inclusion and demographics

This study and the protocol of CIK cell-based immunotherapy were reviewed and approved by the Bioethics Committee of Yan’an Affiliated Hospital of Kunming Medical University. The investigation was performed in accordance with the Helsinki declaration and national and international guidelines. Written informed consent was provided by all patients or their legal guardians.

The medical records of CRC patients who underwent radical tumor resection from February 2008 to July 2014 were reviewed. Patients were included if they were aged between 18 and 80 years; if the resected tumors had been pathologically confirmed as primary colorectal adenocarcinomas; and if the patients did not receive preoperative chemotherapy or radiotherapy. Patients diagnosed with concurrent cancer except for CRC; CRC patients with severe cardiovascular, liver, kidney or autoimmune disease, or with syphilis or HIV infection; and women who were pregnant or lactating were excluded. The study included 137 patients, including 71 who received chemotherapy alone and 66 who received CIK cell treatment combined with chemotherapy. Patient treatment was determined by a multidisciplinary team, which included oncologists, surgeons, immunologists and pathologists. The demographic and clinicopathological characteristics of the included patients are shown in Table 1.

Preparation of CIK cell

Whole blood obtained from individual patients was treated with red blood cell lysis buffer, and the resulting PBMCs were suspended at a density of $1 \times 10^6$ mL$^{-1}$ in CIK cell culture medium. CIK cells were generated using a standard protocol, which included priming with 1000 U mL$^{-1}$ IFN-γ (Miltenyi Biotec, Bergisch Gladbach, Germany) on Day 0 and stimulation with 100 ng mL$^{-1}$ OKT-3 (Miltenyi Biotec) and 500 U mL$^{-1}$ IL-2 (Miltenyi Biotec) each on the following 4 or 5 days, and the resulting CIK cells were harvested at days 14, 15 and 16 depending on the quantities. Samples of each cell preparation were withdrawn for quality control and safety assays. Each batch of cells was subjected to analyses of viability, the expression of a series of phenotypic markers and contamination with bacteria, fungi and mycoplasma.

Treatment scheme

The 137 patients who underwent radical resection were treated with 2-6 cycles of FOLFOX chemotherapy, consisting of intravenous (i.v.) oxaliplatin 85 mg m$^{-2}$, i.v. leucovorin 400 mg m$^{-2}$ and i.v. 5-fluorouracil (5-FU) 400 mg m$^{-2}$ for 2 h each on Day 1, followed by continuous i.v. 5-FU 1200 mg m$^{-2}$ per day for 2 days, for a total of 2400 mg m$^{-2}$ dripping for 46-48 h, with cycles repeated at intervals of 3–4 weeks. Patients in the CIT group were started on adjuvant CIK cell infusion beginning during the fourth week after the last chemotherapy cycle. CIK cells were intravenously infused at a dosage of $2 \times 10^9$ cells per day, with each cycle consisting of three sequential injections. Patients in the CIT group were treated with 1-8 cycles of immunotherapy at intervals of 4 weeks.

Tumor cell lines and culture

The human CRC cell lines HCT-116 and HT-29 were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China) and cultured in DMEM/F12 medium (Thermo Fisher Scientific/Invitrogen, South San Francisco, CA, USA), supplemented with 10% foetal bovine serum (FBS) (Beit Haemek Ltd, Beit Haemek, Israel) and 1% penicillin-streptomycin solution (Beit Haemek Ltd).

Western blotting

Total proteins were extracted from CIK, HCT-116 and HT-29 cells with radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China), supplemented with PMSF and Complete EDTA-free protease inhibitor cocktail tablets (Roche, Basel, Switzerland). Total protein concentrations were measured with bicinchoninic acid (BCA) protein assay kits (Meilunbio, Dalian, China). Protein samples were separated on 10% SDS-PAGE gels and electrotransferred to PVDF membranes (Immobilon, Merck, Darmstadt, Germany). The membranes were blocked by incubation with 5% skimmed milk powder in TBST for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against SLAMF7 (CRACC/SLAMF7/CD319 (E5C4M) Rabbit mAb, Cell Signaling Technology (CST), Denver, MA, USA) and β-actin (Beta Actin Monoclonal Antibody, Proteintech, Rosemont, IL, USA). After washing three times with TBS, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and washed four times with TBS, and the bands were visualised using a chemiluminescence kit (Affinity Biosciences, Cincinnati, OH, USA).

Flow cytometry analysis and sorting

CIK cells were harvested on days 0, 7 and 14, and suspended at a density of $1 \times 10^5$ mL$^{-1}$. Aliquots of 100 μL containing $1 \times 10^4$ cells in suspension were transferred to a flow tube and incubated with PE-conjugated anti-mouse CD319/SLAMF7 antibody (Biolegend, San Diego, CA, USA) and its isotype control (PE-conjugated Rat IgG1, λ Isotype Ctrl Antibody (Biolegend)) in the dark for 30 min. A 1 mL
a aliquot of PBS was added to each tube, and the tubes were centrifuged for 5 min at 300 g and the cells washed twice. Finally, each CIK pellet was resuspended in 200 µL PBS and subjected to flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA and Cytomixs FC500, Beckman Coulter, Indianapolis, IN, USA) analyses. CIK cells expressing different amounts of SLAMF7 were sorted using an Inflinx cell sorter (Becton Dickinson). The antibodies used for phenotyping of CIK cells, including antibodies to CD3, CD4, CD8, CD56 and NK2D and their isotype controls, were purchased from Becton Dickinson and BioLegend.

Tumor cytotoxicity assay

HCT-116 and HT-29 cells were plated in 96-well cell culture plate 24 h before the assay, with the number of cells in each well determined by cell counting. The sorted CIK cells expressing high or low levels of SLAMF7 were added to each well at different ratios. After incubation for 24 h, the cells in each well were washed three times with PBS, followed by the addition of fresh growth medium supplemented with 10 µL of CCK-8 solution (Proteintech). After incubation for 2-4 h, cell viabilities were determined by measuring the absorbance of each well at 450 nm using a spectrophotometer.

Immunohistochemical analysis and evaluation

Of the 66 patients in CIT group, 10 were excluded from this analysis because no tumor sample could be retrieved. Of the remaining 56 CRC patients, 39 had available paracancer and cancer tissues, with the order 17 having only cancer tissue available. The tissue samples were blocked with paraffin, sectioned and dewaxed, followed by antigen retrieval. The sections were treated with 3% hydrogen peroxide and blocked by incubation with goat serum for 10 min, followed by overnight incubation at 4°C with rabbit monoclonal anti-SLAMF7 primary antibody (Cell Signaling Technology). The sections were washed three times with PBS and incubated with HRP conjugated secondary antibody for 45 min at 37°C. The sections were again washed three times with PBS, and the colour was developed by adding the chromagen 3’3’-diaminobenzidine tetrahydrochloride (DAB), followed by counterstaining with haematoxylin. Each slide was sequentially evaluated by two experienced pathologists blinded to sample identification at haematoxylin. Each slide was sequentially evaluated by two experienced pathologists blinded to sample identification at haematoxylin.

Follow-up

All included CRC patients were followed up by phone calls once every 3 months during the first 2 years after surgery and every 6 months from 3 to 5 years. Follow-up included physical examination, laboratory tests, colonoscopy, chest and abdominal CT scans and brain MRI. The start of follow-up was the day of surgery, with follow-up continued until 1 September 2020. OS was calculated from the date of surgery to the date of death or last follow-up, and PFS was calculated from the date of surgery to the date of disease progression (tumor recurrence or metastasis) or last follow-up. Patients with clinical evidence of recurrence or metastasis during follow-up were provided individualised treatments to prolong survival and improve patient quality of life.

Immunofluorescence analysis

Two slides were sectioned from each of the 56 tissue samples in the CIT group, as described above. All sections were dewaxed in xylene twice for 15 min each, rehydrated in a gradient ethanol series (absolute, 85% and 75% ethanol for 5 min each), immersed in retrieval buffer (EDTA antigen retrieval buffer pH 8.0, Servicebio, Wuhan, China) and incubated in a microwave oven for 15 min to retrieve antigens. After cooling, the slides were washed three times with PBS and incubated in 3% H2O2 at room temperature in the dark for 25 min to block endogenous peroxidases. After three washes with PBS, the slides were blocked by incubation in 3% bovine serum albumin (BSA, Servicebio) for 30 min and incubated overnight at 4°C with rabbit monoclonal anti-CD3 primary antibody (Servicebio). The slides were again washed three times with PBS and incubated in the dark for 1 h at room temperature with FITC-conjugated goat anti-Rabbit IgG (H+L) secondary antibodies (Servicebio). The slides were washed three times with TBST for 5 min each. After antigen retrieval in a microwave oven for 15 min, the slides were incubated overnight at 4°C with rabbit polyclonal anti-CD8 beta antibody (Servicebio) or mouse monoclonal anti-NCAM1 (CD56) antibody (Servicebio). The slides were again washed three times with PBS and incubated for 1 h at room temperature in the dark condition with Cy3-conjugated goat anti-rabbit IgG (H+L) (Servicebio) or Cy3-conjugated donkey anti-Mouse IgG (H+L) (Servicebio) secondary antibody. After washing three times with PBS, the slides were dried and incubated with DAPI reagents (DAPI solution, Servicebio) for 10 min in the dark. The slides were again washed three times with PBS, sealed with anti-fluorescence quencher (Servicebio), digitised with a fluorescence pannoramic slide scanner (PANNORAMIC DESK/ MIDI/250/1000, 3DHISTECH, Budapest, Hungary) and analysed with appropriate analytic software (Halo v3.0.311.314, Indica labs, Albuquerque, NM, USA). Tissue area, total number of cells and the number of CD3-positive, CD8-positive, CD3-positive/CD8-positive (CD8+ T cell), CD56-negative and CD3-positive/CD56-negative (NK cell) cells were measured to calculate positive or co-localised cell density.

Statistical analysis

Continuous variables were compared by the Student’s t-tests, and categorical variables by Pearson’s χ2 test or Fisher’s exact test. To analyse demographics, clinical
characteristics of the patients, tumor cytotoxicity assay, the expression of SLAMF7 in clinical samples and to assess the correlations between SLAMF7 expression and immune cell infiltration. OS and PFS were determined by the Kaplan–Meier methods and compared by the log-rank test. All statistical analyses were performed using SPSS (Statistical Package for the Social Science, version 22.0, IBM Corp., Armonk, NY, USA) and GraphPad Prism 8 (Version 8.0.2, GraphPad Software, Inc. San Diego, CA, USA) software, with \( P < 0.05 \) defined as statistically significant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Xiao Li: Data curation; formal analysis; investigation; visualization; writing – original draft. Haodong Zhou: Data curation; investigation; software; validation; visualization. Wenwen Huang: Data curation; investigation; methodology; software; visualization. Xuejuan Wang: Formal analysis; investigation; software; validation. Mingyao Meng: Methodology; resources; supervision. Zongliu Hou: Supervision. Liwei Liao: Supervision. Weiwei Tang: Formal analysis; methodology; Resources. Yanhua Xie: Methodology; resources. Ruotian Wang: Methodology. Haidong Yu: Methodology. Liqiong Wang: Methodology. Huirong Zhu: Methodology. Wenju Wang: Conceptualization; funding acquisition; project administration; validation; Writing – review and editing. Ruotian Wang: Conceptualization; funding acquisition; project administration; writing – review and editing. Haodong Zhou: Conceptualization; funding acquisition; project administration; writing – review and editing.

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Supporting Information

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