

## Original Article

# LAP+CD4+T cells regulate the anti-tumor role of CIK cells in colorectal cancer through IL-10 and TGF- $\beta$

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**Abstract:** The rate of colorectal cancer (CRC) is increasing. Adoptive immune cell therapy (ACT) is a research hotspot in CRC treatment, and the common adoptive cells are cytokine-induced killer cells (CIK). The problem of ACT is that some regulatory T cells (Treg) will affect the efficacy. Latent associated polypeptide (LAP)+CD4+T is a new Treg, and its immunosuppressive effect is much higher than that of traditional Tregs. This research mainly explored the influence of LAP+CD4+T cells on anti-tumor lethality of CIK cells, so as to fill this gap. The LAP+CD4+T CIK cells and LAP-CD4+T CIK cells were sorted by immunomagnetic beads. LAP+CD4+T cells were expanded *in vitro*, and high expression cytokine genes were screened by RT-qPCR. LAP+CD4+T and LAP-CD4+T CIK cells were co-cultured to test cyto-activity. Transplanted tumor models of CRC were established in nude mice, which were randomized into a control group (CG), CIK group, LAP (-) group, LAP (+) group, IL-10 siRNA group, and TGF-siRNA group, and the tumor growth in each group was observed. The research results revealed that interleukin-10 (IL-10) and transforming growth factor- $\beta$  (TGF- $\beta$ ) were highly expressed in LAP+CD4+T cells. LAP+CD4+T could effectively suppress CIK cell proliferation and activity. LAP-CD4+T could suppress IL-10 and TGF- $\beta$ , and inhibit CIK cell apoptosis, proliferation, and tumor growth, thus improving their anti-tumor lethality. LAP+CD4+T cells regulate the anti-tumor role of CIK cells in CRC through IL-10 and TGF- $\beta$ .

**Keywords:** Colorectal cancer, LAP+CD4+T cells, CIK cells, IL-10, TGF- $\beta$

## Introduction

Colorectal cancer (CRC) may be a fatal malignancy, and its treatment strategy has been constantly improving [1]. Among them, adoptive immune cell therapy (ACT) is a topic of current research. The autoimmune cells are activated and expanded *in vitro*, and then re-injected into patients to exert their cytotoxicity, thus achieving better efficacy [2, 3]. Since the immune cells used for ACT treatment come from patients themselves, they exert higher biosafety and stability compared to radiotherapy and chemotherapy [4]. Thus, cytokine-induced killer cells (CIK) are widely used in ACT, which have high anti-tumor activity, wide anti-tumor spectrum, low toxicity to normal tissues, and high amplification *in vitro* [5, 6]. CIK has been widely used in treatment of hepatocellular carcinoma

(HCC), cervical cancer and CRC resulting in not only enhanced immunity but also longer survival time [7-9].

There are still some difficulties with ACT, mainly because regulatory T cells (Treg) exist in the body. Those cells help tumor cells develop immune tolerance through different pathways, escape immune monitoring, and then affect the treatment efficacy [10-12]. Latent associated polypeptide (LAP)+CD4+T is a new lymphocyte subset, and its immunosuppressive effect is far superior to that of traditional lymphocytes (CD4+CD25+T) [13]. It is known that (LAP)+CD4+T cells are highly expressed in CRC tumor tissues and can secrete immunosuppressive factors such as IL-10 and TGF- $\beta$ , which negatively hinders the normal function of immune cells [14, 15]. A recent research has

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shown that LAP+CD4+T immunosuppression can be partially reversed by the intervention of IL-10 and TGF- $\beta$  monoclonal antibodies, suggesting that its function may be mediated by IL-10 and TGF- $\beta$  [16]. The relationship between LAP+CD4+T cells and lethality of CIK cells is unknown. It is confirmed that traditional immune cells CD4+CD25+T can depend on TGF- $\beta$  and further downregulate the expression of NK cell activating receptor (NKG2D) to suppress the anti-tumor lethality of CIK cells [17]. In the current study, high-purity and high-activity LAP+CD4+T cells and LAP-CD4+T CIK cells were sorted by the immunomagnetic bead sorting method. By analyzing the expression of cytokines in LAP+CD4+T cells, the related inhibitory plasmids were constructed and transfected, and it was determined whether the inhibitory plasmids with high expression of cytokines could reverse the immunosuppression of CIK cells. We also verified whether LAP-CD4+T or blocking some of its functions could enhance the anti-tumor lethality of CIK cells through further tumor cell co-culture and animal model ACT experiments. This will provide new theoretical support for future ACT.

### Materials and methods

#### *CIK cell preparation*

Peripheral blood (50 ml) from 50 CRC patients was collected. Peripheral blood mononuclear cell (PBMC) were obtained by density gradient centrifugation and cultivated 2 h in a 5% CO<sub>2</sub> incubator at 37 °C to make it adhere to the wall. Suspended cells were collected, concentration was adjusted to  $1 \times 10^6$ /ml, and IFN- $\gamma$  (Fusheng Industrial Co., Ltd., Shanghai, China) (1000 U/ml) was added and cultivated for 24 h. Anti-Human CD3 (Yihui Biological Technology Co., Ltd., Shanghai, China, 05121-25-500) (50 ng/ml) and IL-2 (300 U/ml) were added, and the liquid was changed or the bottle was expanded every 3 days. Afterwards, IL-2 (Baiao Laibo Technology Co., Ltd., Beijing, China, GS4703-OQT) was supplemented, and CIK cells were harvested on the 7th day.

#### *Immunomagnetic bead sorting method*

CD4+T cells were selected negatively: The concentration of prepared CIK cells was adjusted appropriately. After non-CD4+T cells that were labeled by Biotin-Antibody Cocktail (ZZBIO

Biotech Co., Ltd., Shanghai, China, ZGT-GTX76-130) were added, they were cultivated 10 min at 4 °C and washed with PBS. Then Anti-Biotin Microbeads (Huzhen Industrial Co., Ltd., Shanghai, China, hzMMI-104) were added and cultivated 15 min at 4 °C. When the MS separation column was added, there were non-CD4+ cells in the column, and the eluted cells were CD4+ cells. Non-CD4+ cells were obtained by positive sorting in an MS separation column.

LAP+CD4+T cells were selected positively: Anti-Human LAP-PE (Fubo Biotechnology Co., Ltd., Beijing, China, 12-9829-42) was added into eluted CD4+ cells and cultivated at 4 °C for 10 min; then, they were cleaned by Phosphate Buffer Solution (PBS) (Yaji Biotechnology Co., Ltd., Shanghai, China, YS-1050). After that, Anti-PE Microbeads (Univ-bio Co., Ltd., Shanghai, China, 130-048-801) was added and cultivated 15 min at 4 °C, then washed with PBS. The positive sorted cells in the column were LAP+CD4+T cells (spare), and the eluted ones were negative sorted LAP-corresponding cells. The LAP-corresponding cells were mixed with non-CD4+ corresponding cells; that is, the LAP-CD4+T CIK cells (spare). The survival rate and purity of sorted cells were measured by trypan blue staining and flow cytometry, respectively.

#### *Trypan blue staining*

First, a single cell suspension ( $10^6$  cells/ml) was prepared, and then it was mixed with 0.4% trypan blue solution (Reanta Biotechnology Co., Ltd., Beijing, China, K940-100ML) at 9:1 for staining. Then, within 3 min, live and dead cells were counted by counting plate. Cell survival rate was the percentage of living cells.

#### *Flow cytometry*

Cell purity and phenotype were evaluated. First of all, sample monodisperse cell suspension ( $10^6$  cells/mL) was prepared with PBS, and then 10  $\mu$ L fluorescein isothiocyanate (FITC) (Beiyu Biotechnology Co., Ltd., Nanjing, China, BYAS-60659-FITC) was added to a 100  $\mu$ L sample to label monoclonal antibody. Antibodies were: latent associated polypeptide (LAP), CD4, forkhead (Fox) p3, cytotoxic T lymphocyte associated protein (CTLA)-4, chemokine CC receptor (CCR) 4, CCR5, CCR7, CD45-RA (Hengfei Biotechnology Co., Ltd., Shanghai,

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China), C/EBP $\beta$  (LAP) Antibody, (130-109-451, bs-10211R-1, bs-1179R-1, bs-1168R-1, A00-061, bs-1305R-1, 130-109-776). Next, this was swirled gently to ensure full mixing, and cultivated 30 min at room temperature under dark conditions. After cell staining, the samples were stored at 4°C away from light before computer analysis, and then the cell purity and phenotype were determined by NovoCyt flow cytometry (Ranger Instrument & Equipment Co., Ltd., Shanghai, China) and FlowJo within 1 h.

The apoptosis level was evaluated. The cells were inoculated in a six-hole plate at  $1 \times 10^6$  cells/well. They were evaluated by annexin V-FITC and propidium iodide (PI) staining kit (Baiao Laibo technology Co., Ltd., Beijing, China, GL0322). Lastly, quantitative analysis was performed by flow cytometry.

### *CIK cells were cultured separately*

CIK and deleted LAP+CD4+T CIK cells were inoculated into 96-hole plates and cultured separately. Cell concentration in each hole was  $1 \times 10^5$ /ml, and the cells were cultivated in RPMI 1640 medium (Yaji Biotechnology Co., Ltd., Shanghai, China, P004-1) comprising 10% FBS at 37°C, 5% CO<sub>2</sub>. The culture system contained L-glutamine 2 mmol/L, penicillin 100 U/mL and streptomycin 100 U/L. IL-2 200 U/mL and anti-CD3/CD28 mAb (Ziqi Biotechnology Co., Ltd., Shanghai, China, MA112097) were added. The number of cells was counted, the growth curve was drawn, and the cell proliferation was tested by CCK-8 method.

### *Cell counting kit 8 (CCK-8) method*

Cells transfected for 24 h were collected and inoculated into a 96-hole plate ( $2 \times 10^3$  cells/hole). CCK-8 solution (10  $\mu$ l) (Fusheng Industrial Co., Ltd., Shanghai, China, FS-79071) was added to each hole and cultivated 4 h at 37°C. The absorbance at 450 nm was measured by microplate reader (Yanhui Biotechnology Co., Ltd., Shanghai, China, HBS-1096A).

### *LAP+CD4+T in vitro culture, amplification, phenotype and cytokine detection*

The selected LAP+CD4+T cells, with a concentration of  $1 \times 10^5$ /ml, were inoculated into 96-hole plates and cultivated in RPMI 1640

medium comprising 10% calf serum at 37°C and 5% CO<sub>2</sub>. In the culture system, there were L-glutamine 2 mmol/L, penicillin 100 U/mL and streptomycin 100 U/L. IL-2 200 U/mL and anti-CD3/CD28 mAb coated with magnetic beads were supplemented. The culture supernatant was collected at the logarithmic growth phase of cells, and the levels of cytokines (IL-2, IL-4, IL-9, IL-10, IL-17, IL-22, IFN- $\gamma$ , TGF- $\beta$ ) were assessed by liquid phase chip analysis system (Image Trading Co., Ltd., Beijing, China, DLK0004210). The phenotypes of LAP+CD4+T (expression of Foxp3, CTLA-4, CCR4, CCR5, CCR7, CD45RA) were tested by flow cytometry, and cytokines with high expression were screened out.

### *Co-culture of LAP+CD4+T and CIK cells after amplification*

CIK cells were inoculated into a culture plate with  $1 \times 10^5$ /ml per hole and cultivated for 24 h. Soon afterwards, the amplified LAP+CD4+T cells were added based on an effect to target ratio of 1:1 and 1:2. Meanwhile, a control group (CG) and a blank CG were set up and cultivated for 24 h. Cell proliferation was tested by CCK-8 method, and whether LAP+CD4+T still retained immunosuppression after amplification was verified.

### *siRNA interference experiment*

A siRNA plasmid targeting the cytokines with high expression of LAP+CD4+T was constructed, and the expression of IL-10 and TGF- $\beta$  was silenced. si-IL-10: 5'-GAC TTTCTTTGTGAGTATG-ATTC-3'; si-TGF- $\beta$ : 5'-GCCCATCTAGGTTATTTCCGTGG3'. LAP+CD4+T was transfected by using Lipofectamine 2000 (Invitrogen, MA, USA), and the level of cytokines was analyzed by real-time polymerase chain reaction (RT-PCR) to test the transfection efficiency. Afterwards, siRNA was transfected into LAP+CD4+T and then co-cultured with CIK cells to verify whether the siRNA transfection could reverse the immunosuppressive effect of LAP+CD4+T cells.

### *RT-PCR*

The cells were digested by Trizol reagent (Wenren Biotechnology Co., Ltd., Shanghai, China, Invitrogen) to obtain total RNA. Next, cDNA was prepared by TaqMan miRNA reverse

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**Table 1.** Primer sequences

Gene	Upstream primer (5'-3')	Downstream primer (5'-3')
GAPDH	CGCTCTCTGCTCCTCTGTTC	ATCCGTTGACTCCGACCTTCAC
IL-10	GCGGTCTTTTTGATGCCCTTT	TGCGTTCTCTCTCGTGCTGA
TGF- $\beta$	GACCTTGTAACCGCCGACC	GCTGTCAGCTCCAAACTCCT-

transcription kit (Lianqiao Biotechnology Co., Ltd., Shanghai, China, 4366596), and then analyzed by RT-PCR with SYBR Green Master Mix (Hengfei Biotechnology Co., Ltd., Shanghai, China, A25742). PCR reaction system: 2X SYBR qPCR Mix 25  $\mu$ L, DNA Template 2  $\mu$ L, upstream and downstream primers 1  $\mu$ L, and ddH<sub>2</sub>O was added to 50  $\mu$ L. PCR reaction conditions: pre-denaturation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 10 s, annealing at 65°C for 15 s and elongation at 72°C for 30 s. The target RNA level standardized to GAPDH was quantified using the  $2^{-\Delta\Delta CT}$  method. The primer sequences are listed in **Table 1**.

### Co-culture experiment of tumor cells

Frozen HCT-116 cells (Yingwan Biotechnology Co., Ltd., Shanghai, China, C1125) were resuscitated, cultivated in RPMI 1640 medium to logarithmic growth stage, digested by pancreatin (Jizhi Biotechnology Co., Ltd., Shanghai, China, P31340-25g), cleaned, adjusted to  $1 \times 10^6$ /ml, and inoculated into 48-hole cell culture plates and cultivated overnight. The concentration of immune effector cells was adjusted to  $1 \times 10^6$ /ml and added to the adherent tumor cells. The grouping scheme was as follows: (1) HCT-116 group: HCT-116 cells were cultivated alone; (2) CIK group: CIK and HCT-116 cells were co-cultured; (3) LAP (-) group: LAP-CD4+T CIK and HCT-116 cells were co-cultured; (4) LAP (+) group: LAP+CD4+T CIK and HCT-116 cells were co-cultured; (5) siRNA group: X-siRNA transfected LAP+CD4+T CIK and HCT-116 cells were co-cultured. Each group was set up within 3 multiple holes, including for the cell CG and blank CG. The experiment was repeated 3 times with incubation time of 24 h. The lethality of effector cells to tumor cells was tested by lactate dehydrogenase (LDH) cytotoxicity assay kit (Yujin Biotechnology Co., Ltd., Shanghai, China, C2LD-100).

### Animal adoptive immunotherapy experiment

The resuscitated HCT-116 cells were sub-cultured, digested in the logarithmic growth pha-

se, centrifuged and counted, and the cell concentration was adjusted to  $5 \times 10^6$ /ml. After the axillary skin of BALB/c nude mice (4-5 weeks old, magnetic) (Junke Bioengineering Co., Ltd., Nanjing, China) was sterilized, 0.2 mL HCT-116 cell suspension was

inoculated subcutaneously, and the tumor formation at the injection site was observed. The tumor bearing mice were randomly divided into 5 groups with 5 rats in each group. The cell concentration of immune cells in each group was adjusted to  $1 \times 10^6$ /mL, and immune effector cells were injected into the tail vein of mice. The specific scheme was as follows: (1) CG: normal saline was injected; (2) CIK group: CIK cells were injected; (3) LAP (-) group: LAP+CD4+T CIK cells were injected; (4) LAP (+) group: LAP+CD4+T CIK cells were injected; (5) siRNA group: X-siRNA transfected LAP+CD4+T CIK cells were injected.

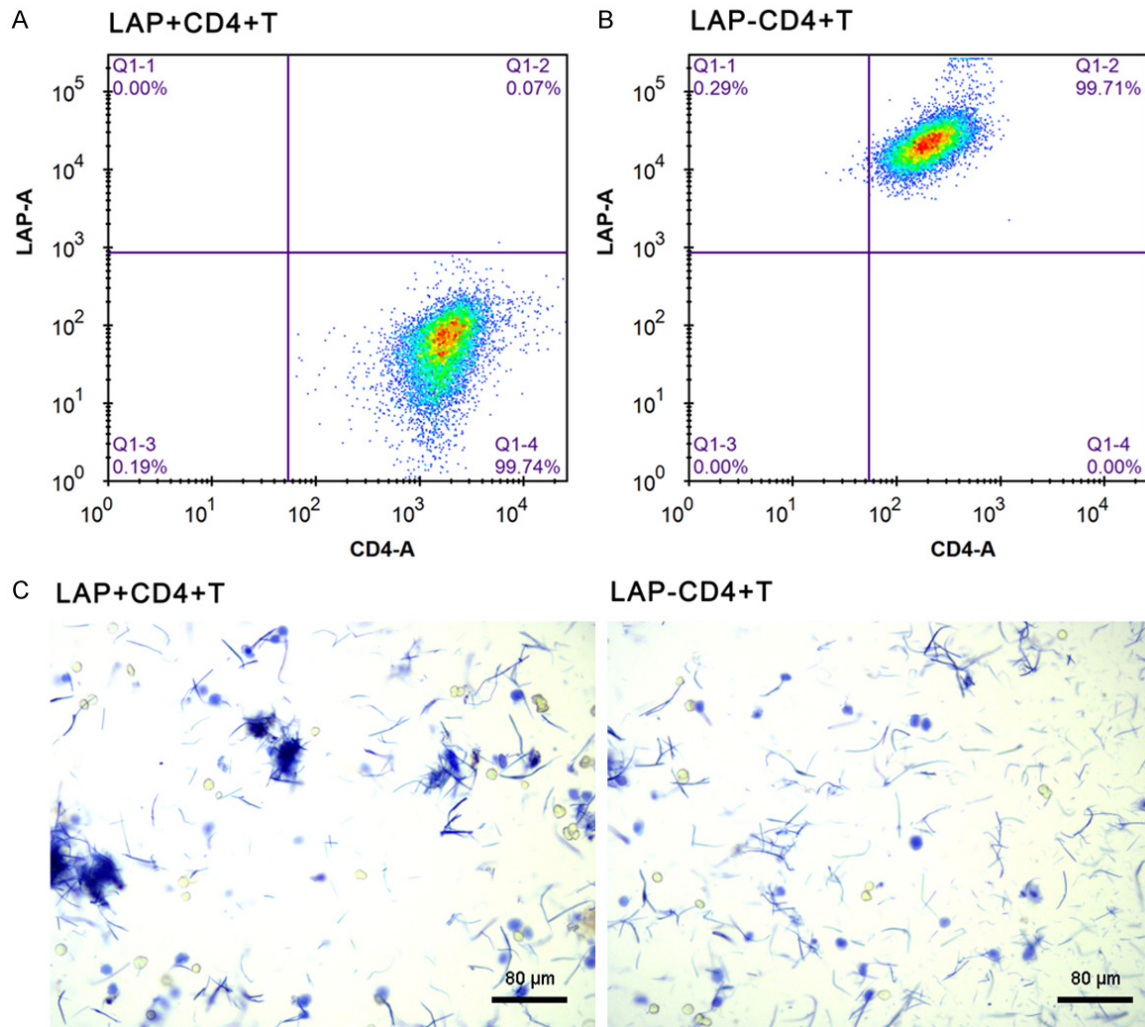
The tumor growth in mice was observed every day. Ten days later, mice were killed, and the tumor was harvested, weighed, and measured. The volume and tumor inhibition rate were counted, and the formula was as follows:

$$\text{Tumor volume} = \text{length} \times \text{width}^2/2.$$

$$\text{Tumor inhibition rate} = (\text{average tumor volume of CG} - \text{average tumor volume of treatment group})/\text{average tumor volume of CG} \times 100\%.$$

The harvested tumor was divided into two equal parts, and the LAP, CD3, and CD56 expression was tested by Histostain<sup>TM</sup>-Plus Kits (Huzheng Biotechnology Co., Ltd., Shanghai, China, HZ00236). The staining intensity and range were scored on the semi-quantitative scale. The staining intensity scores were 0 (no staining), 1 (weakly positive), 2 (moderately positive) and 3 (strongly positive). The degree of staining was based on the percentage of positive staining tumor cells: 0 (negative), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (76%-100%). The apoptosis level of tumor tissues in each group was assessed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) test kit (Baiao Laibo Technology Co., Ltd., Beijing, China, BTN81026-FGS). The nuclei of live cells were stained blue and the nuclei of apoptotic cells were brown. Five cancer regions were ran-

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**Figure 1.** Similar abundance of LAP+CD4+T and LAP-CD4+T cells. A, B. LAP+CD4+T and LAP-CD4+T cells were sorted by immunomagnetic beads, and then the enrichment of LAP+CD4+T and LAP-CD4+T cells was detected by flow cytometry. C. The survival rate of the two cells was evaluated by trypan blue staining ( $\times 400$ ).

domly selected under  $\times 400$  high power field (HP) light microscope, and the number of apoptotic cells per HP field was counted.

### Statistical analysis

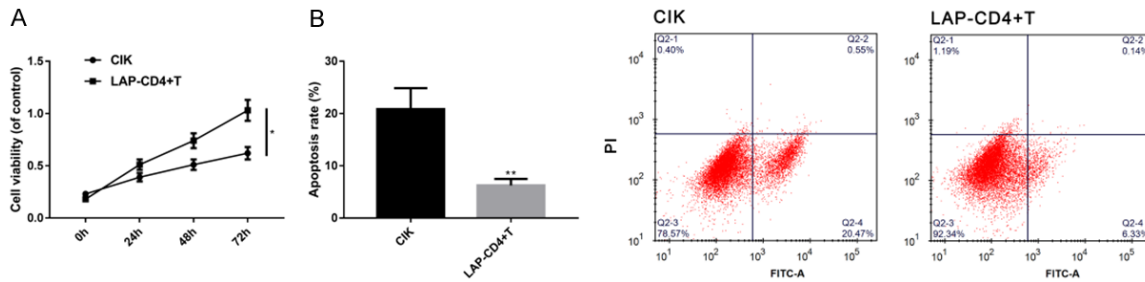
SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) was applied for statistical analysis. The normally distributed data were expressed as mean  $\pm$  standard deviation (means  $\pm$  SD). The measured data were compared between two groups by independent sample t-test, and paired t-test was used for intra-group comparison. One-way ANOVA was used to compare the mean among multiple groups, and LSD-t test was used for the post hoc test.  $P < 0.05$  was considered significant.

### Results

*LAP+CD4+T and LAP-CD4+T cells were acquired by immunomagnetic bead sorting method*

To verify the influence of LAP on the anti-tumor lethality of CIK cells in CRC and the underlying mechanism, we first collected the peripheral blood of 50 CRC patients and 45 healthy volunteers, extracted the PBMCs, and prepare CIK cells. LAP+CD4+T and LAP-CD4+T cells were separated by immunomagnetic bead sorting, and then the cell purity was tested by flow cytometry. This showed that the purity of LAP+CD4+T cells was  $95.63\% \pm 2.38\%$  (Figure 1A), and that of LAP-CD4+T cells was

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**Figure 2.** Comparison of proliferation between CIK and LAP-CD4+T cells. A. CCK-8 method was used to compare the proliferation of CIK cells and LAP-CD4+T cells. B. Their apoptosis with LAP-CD4+T cells was examined by flow cytometry. Note: compared to CIK, \*P<0.05, \*\*P<0.01.

95.11%±2.26% (**Figure 1B**). The purity of the two cell types was similar. Trypan blue staining revealed that the cell survival rates of LAP-CD4+T and LAP+CD4+T cells were 93.12%±3.05% and 90.31%±4.10%, respectively.

### Comparison between proliferation of CIK and LAP-CD4+T cells

We also compared the viability of CIK cells with LAP-CD4+T cells. According to CCK-8 data, the proliferation of LAP-CD4+T cells was higher than that of CIK cells (P<0.05, **Figure 2A**). Also, the apoptosis rate of LAP-CD4+T cells was dramatically lower than that of CIK cells (P<0.05, **Figure 2B**). The results indicated that LAP depletion helped to improve the proliferation of LAP-CD4+T cells.

### Cytokine and phenotypic analysis of LAP+CD4+T cells cultured and expanded in vitro

We compared cytokines and phenotypes of LAP+CD4+T and LAP-CD4+T cells *in vitro*. The results manifested that the IL-10 and TGF-β levels were the highest in LAP+CD4+T cells, whereas IL-2 was the highest in LAP-CD4+T cells, and the IL-10 (**Figure 3A**) and TGF-β (**Figure 3B**) levels were inhibited in LAP-CD4+T cells. Compared to LAP-CD4+T cells, LAP+CD4+T cells have lower Foxp3, but higher CTLA-4, CCR4, CCR5, CCR7, and CD45RA levels (all P<0.05, **Figure 3C, 3D**).

### Proliferation of LAP+CD4+T cells co-cultured with CIK cells

The proliferation of LAP+CD4+T cells co-cultured with CIK cells (LAP+ group) was verified by CCK-8 method, and si-IL-10 and si-TGF-β were transfected into LAP+CD4+T cells to verify

whether they had the ability to reverse the immunosuppressive effect of the LAP+ group. At first, the transfection efficiency of si-IL-10 and si-TGF-β was analyzed by RT-PCR. Statistics revealed that the IL-10 and TGF-β levels decreased after si-IL-10 and si-TGF-β transfection in LAP+CD4+T cells (all P<0.01 **Figure 4A**). On the other hand, compared to the CIK group, the OD value of LAP+ group decreased, but it increased under the intervention with si-IL-10 or si-TGF-β (**Figure 4B**, P<0.05). This showed that LAP+CD4+T cells exhibited immunosuppression after amplification, and si-IL-10 and si-TGF-β could reverse this function.

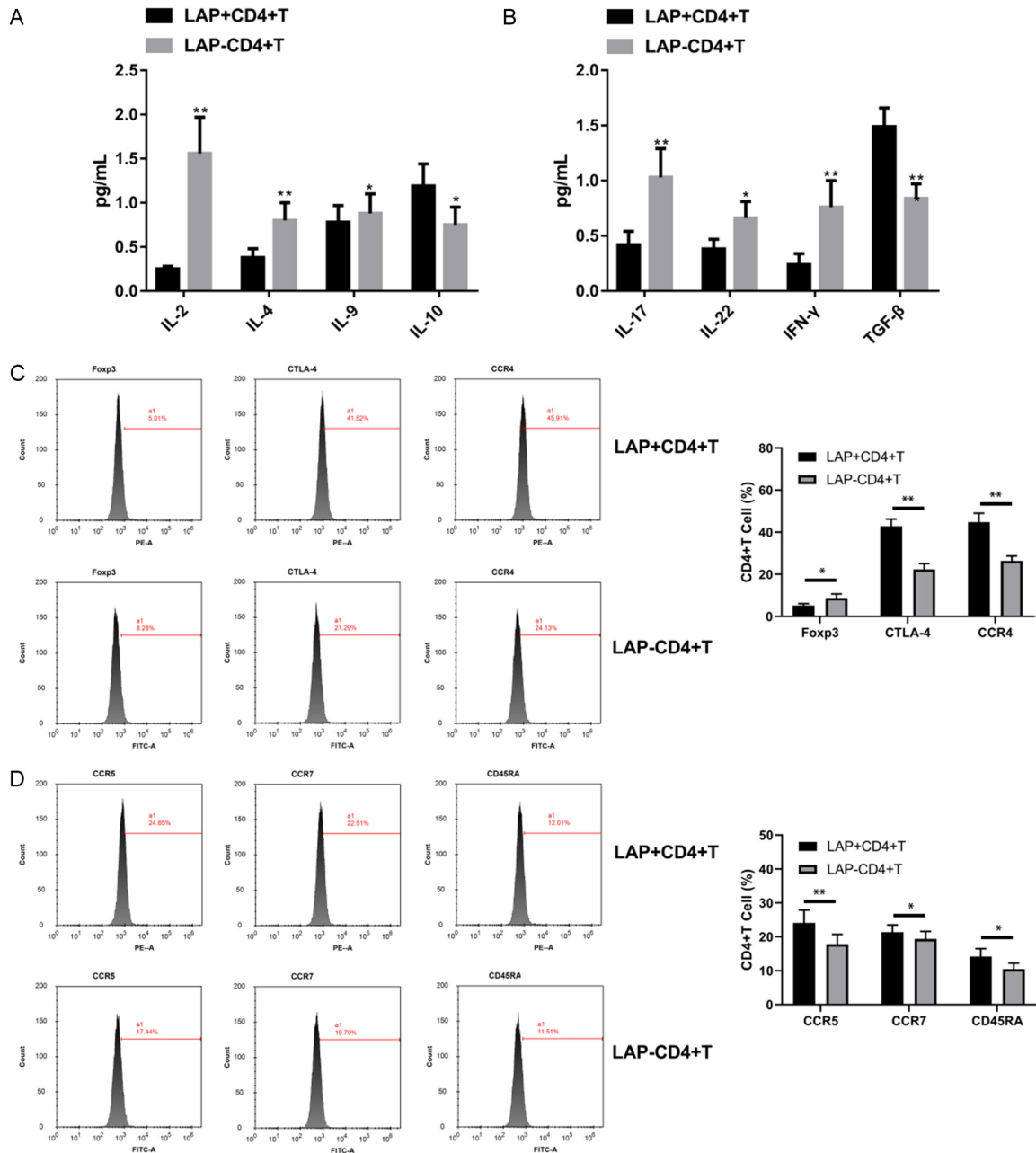
### Cytotoxicity of each group of cells in co-culture with tumor cells

HCT-116 cells were cultured separately (HCT-116 group) and co-cultured with CIK (CIK group), LAP-CD4+T CIK (LAP- group), and LAP+CD4+T CIK cells (LAP+ group). On the basis of the LAP+ group, we also transfected si-IL-10 and si-TGF-β into LAP+CD4+T cells as si-IL-10 and si-TGF-β groups, respectively. The anti-tumor lethality of cells in each group was tested by the LDH method. As shown in **Figure 5**, compared to the HCT-116 group, the cytotoxicity of the LAP- group increased (P<0.05), while the cytotoxicity of the LAP+ group had no obvious difference (P>0.05). The cytotoxicity of si-IL-10 and si-TGF-β groups also increased (P<0.05). The above data indicate that LAP-CD4+T cells or si-IL-10 and si-TGF-β can enhance cytotoxicity of CIK cells.

### Effect of animal adoptive immunotherapy on growth of transplanted tumors in nude mice model

HCT-116 cells were inoculated under the armpit of BALB/c nude mice, and normal saline, CIK,

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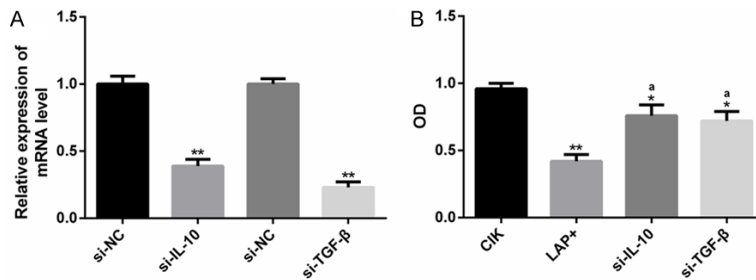


**Figure 3.** Phenotypes and cytokine analysis of LAP+CD4+T cells cultivated and amplified *in vitro*. A, B. The expression of cytokines (IL-2, IL-4, IL-9, IL-10, IL-17, IL-22, interferon- $\gamma$ , transforming growth factor- $\beta$ ) in LAP+CD4+T cells and LAP-CD4+T cells was detected by liquid phase chip technology. C, D. The phenotypes of LAP+CD4+T cells (Foxp3, CTLA-4, CCR4, CCR5, CCR7, CD45RA expression) were detected by flow cytometry to screen for high levels of cytokines. Note: compared to LAP-CD4+T cells, \* $P < 0.05$ , \*\* $P < 0.01$ .

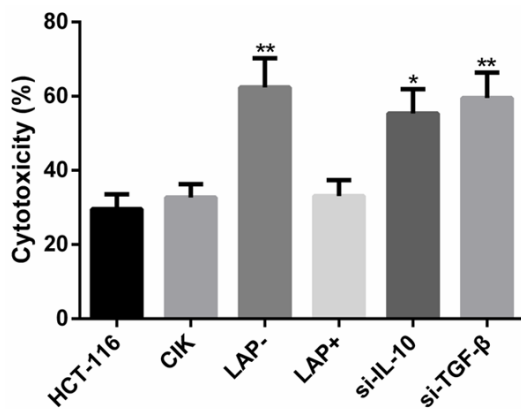
LAP-CD4+T CIK and LAP+CD4+T CIK cells were injected into the tail vein as groups CG, CIK, LAP- and LAP+, respectively. In view of the LAP- and LAP+ groups, si-IL-10 and si-TGF- $\beta$  were transfected into LAP-CD4+T cells as si-IL-10 and si-TGF- $\beta$  groups respectively. Our research manifested that compared to the CG, the tumor

sizes of groups CIK, LAP-, si-IL-10 and si-TGF- $\beta$  was markedly smaller (Figure 6A, 6B), and the tumor inhibition rate increased (Figure 6C). The tumor inhibition rates of groups LAP-, si-IL-10 and si-TGF- $\beta$  were dramatically higher than that of group CIK (Figure 6C). Compared to groups CG or CIK, the LAP level of groups LAP-, si-IL-10

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**Figure 4.** Proliferation of LAP+CD4+T cells co-cultured with CIK cells. A. The transfection efficiency of si-IL-10 and si-TGF-β was detected by RT-PCR. B. The proliferation of LAP+CD4+T and CIK cells in co-culture and the intervention effects of si-IL-10 and si-TGF-β were detected. Note: compared to CIK, \*P<0.05, \*\*P<0.01; compared to LAP+ group, \*P<0.05.



**Figure 5.** Anti-tumor lethality of each group of cells detected by LDH test. Note: compared to HCT-116, \*P<0.05, \*\*P<0.01.

and si-TGF-β decreased, while the CD3 and CD56 levels increased (**Figure 7**). Compared to the CG, the apoptosis levels of groups CIK, LAP-, si-IL-10, and si-TGF-β were dramatically higher in TUNEL results, and the levels of groups LAP-, si-IL-10, si-TGF-β were higher (all P<0.05, **Figures 6D, 8**). This signified that deleting LAP or down-regulating IL-10 and TGF-β could enhance the adoptive immunotherapy effect of CIK cells in a tumor-bearing nude mice model.

### Discussion

ACT, as a tumor immunotherapy, is safe and effective in colorectal cancer (CRC) [18]. CIK cells are commonly used in ACT therapy of CRC, mainly composed of CD3+CD56+ subsets. They can be induced by IFN-γ and IL-2, and their anti-tumor lethality is mainly based on direct contact and stimulation by these

cytokines [19, 20]. The purpose of this research was to probe into the tumor inhibition mechanism of CIK cells in CRC, so as to provide new data for ACT treatment.

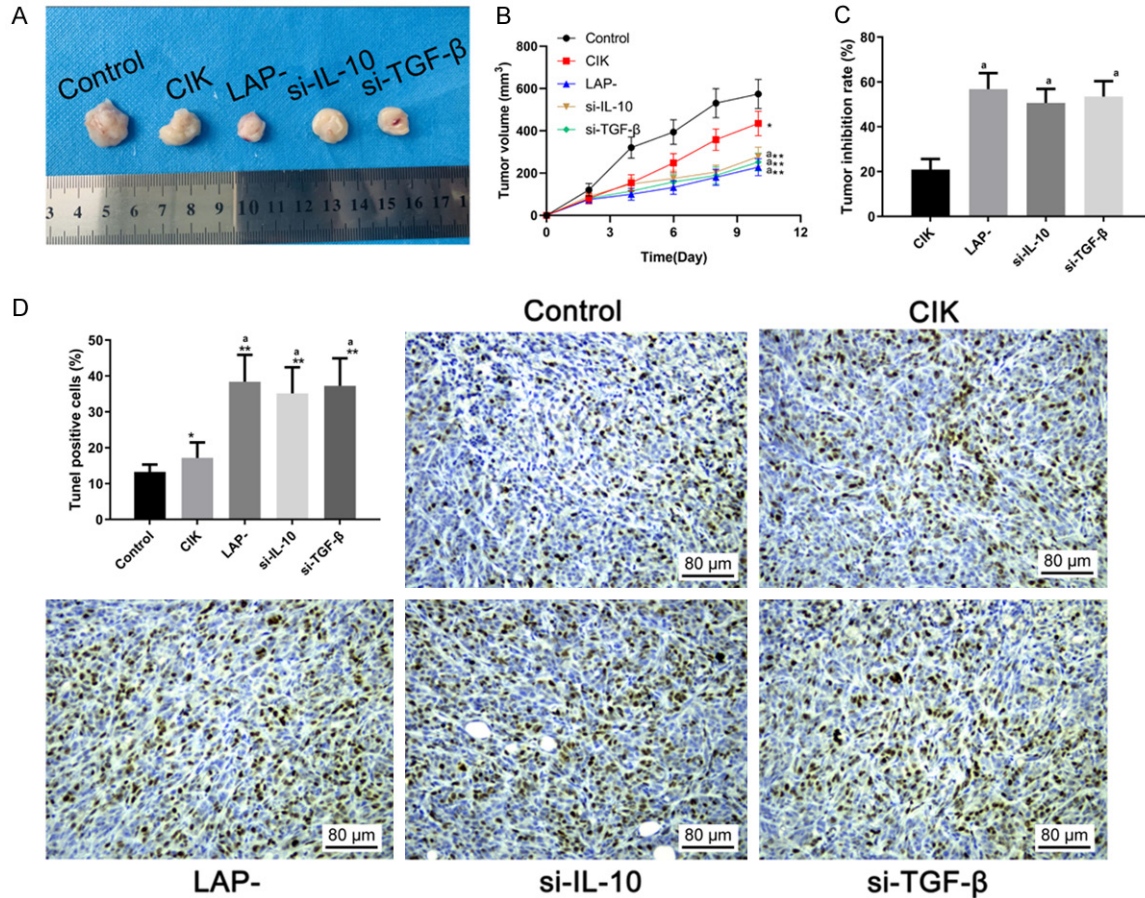
Many researchers have analyzed the molecular mechanism of CIK cells in CRC. For example, Shi *et al.* [21] found that the anti-CRC activity of CIK cells was related to its regulation of the AMPK/Akt/mTOR pathway, and the proliferation and invasion were

inhibited by down-regulating FOXM1 and mTOR. Zou *et al.* [22] found that CIK cells could be stimulated by the chemokine receptor (CXCR3) axis to improve the transport capacity of targeted tumors *in vitro*, thus enhancing their anti-CRC tumor activity. All the above studies confirmed that CIK cells can resist tumor progression through different molecular mechanisms in CRC. It is known that the anti-tumor ability of CIK cells is limited by the immunosuppression of Tregs, and an abnormal increase of Tregs in the tumor microenvironment is related to the poor prognosis of patients [23]. Tregs are essentially a CD4+CD25+T cell subset, which is characterized by high expression of Forkhead box protein-3 (Foxp3) [24]. Previous studies have shown that down-regulating Foxp3 can suppress the level of this cell subset, thus reducing their interference in the tumor toxicity of CIK cells and further improving the lethality against leukemia cells [25]. In this research, LAP+CD4+T is a new Treg, and we speculated that LAP-CD4+T cells also have a positive effect on the anti-CRC effect of CIK cells. We first prepared LAP+CD4+T and LAP-CD4+T cells with high purity and activity. The purities were 95.63%±2.38% and 95.11%±2.26%, and the activities were 90.31%±4.10% and 93.12%±3.05%, respectively. The proliferation of LAP-CD4+T cells was higher but its apoptosis was lower than that of CIK cells, suggesting that the cytotoxicity of LAP-CD4+T cells was far superior to that of CIK cells.

Then, we expanded LAP+CD4+T cells *in vitro*, and found that the IL-10 and TGF-β levels were higher than those in LAP-CD4+T cells, while the IL-2 expression was lower. By phenotype, com-



## Influence of LAP+CD4+T cells on tumor lethality of CIK cells in CRC

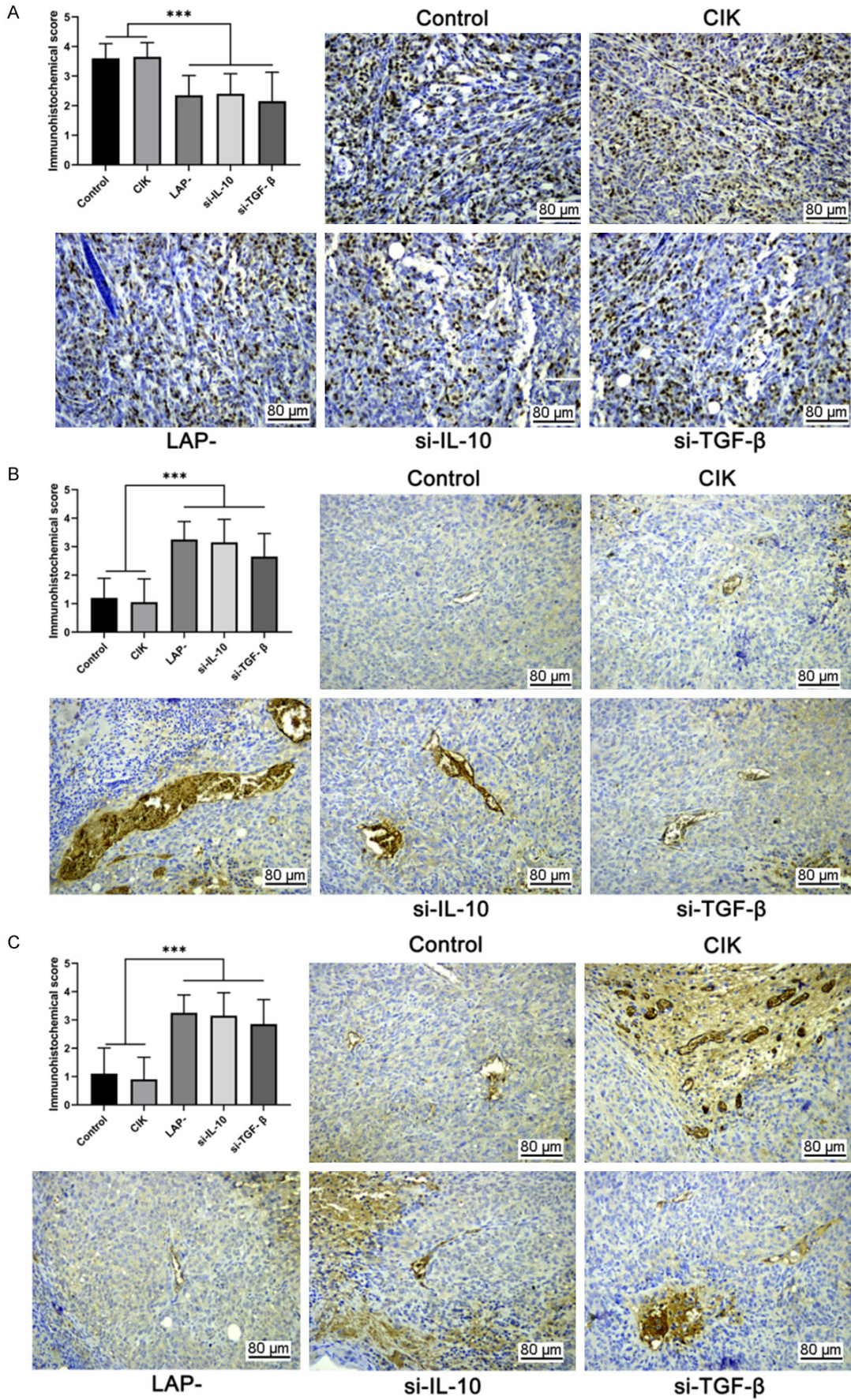


**Figure 6.** Animal adoptive immunotherapy effect on growth of transplanted tumors in nude mice. A. The tumor size (n=5) of each group of BALB/c nude mice was recorded. B. Tumor growth curve (n=5). C. Tumor inhibition rate of each group (n=5). D. The level of apoptosis was detected by TUNEL method (n=5, × 400). Note: compared to the CG, \*P<0.05, \*\*P<0.01; compared to CIK group, <sup>a</sup>P<0.05.

pared to LAP-CD4+T cells, CTLA-4, CCR4, CCR5, CCR7 and CD45RA were higher in LAP+CD4+T cells, while Foxp3 was lower. It is known that the CTLA-4 A49G polymorphism is tied to increased CRC risk in the Chinese Han population [26]. CCR4 and CCR5 are abnormally up-regulated in the tumor microenvironment, where they may act as pro-inflammatory cytokine receptors [27, 28]. CCR4 has been reported to be related to an increased risk of tumor recurrence in gastric cancer patients, while CCR5 is relevant to angiogenesis and tumor metastasis [29, 30]. CCR7 positive cells were correlated with a lower survival time of CRC patients [31]. Furthermore, we also found that LAP+CD4+T cells had an immunosuppressive effect after amplification, and this could be reversed by si-IL-10 and si-TGF-β. This revealed that the immunosuppression mechanism of

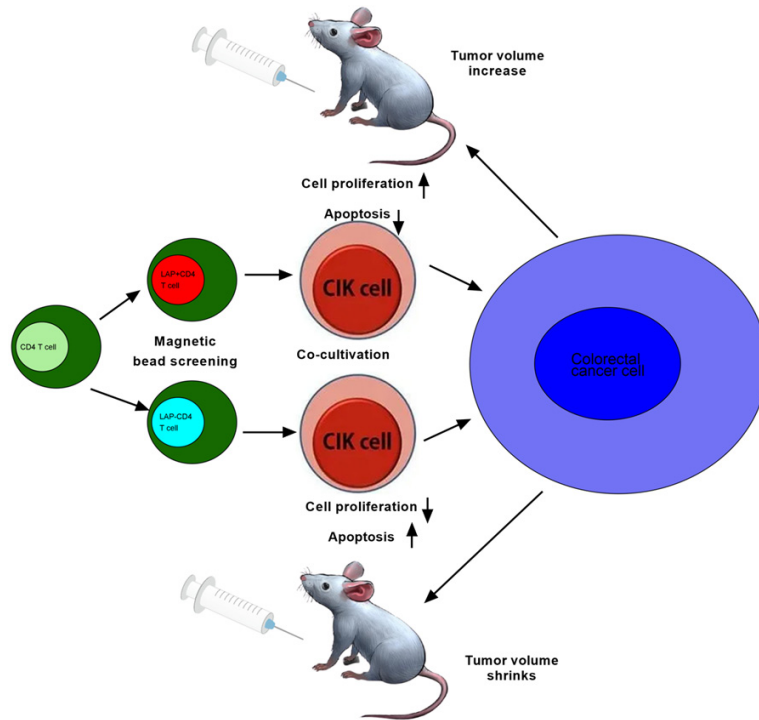
LAP+CD4+T cells on CIK cells could be mediated by IL-10 and TGF-β, which are also the key breakthrough points to enhance the anti-tumor lethality of CIK cells. As important factors of the immune tolerance mechanism, IL-10 and TGF-β can maintain the steady state of number of Treg cells, while the latter can cooperate with mesenchymal stem cells with stronger immunosuppression to exert effects [32-34]. Our cytotoxicity research revealed that LAP-CD4+T cells or si-IL-10 and si-TGF-β could enhance the anti-tumor lethality of CIK cells. In addition, in the CRC nude mice model, the tumor inhibition rates of group LAP-, si-IL-10 and si-TGF-β were outstanding, and these three groups were accompanied by lower LAP level and higher CD3 and CD56 levels. TUNEL data manifested that group LAP-, si-IL-10, si-TGF-β had higher apoptosis rate. Based on

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**Figure 7.** The expression of LAP, CD3 and CD56 in transplanted tumors in nude mice of each group. A. The LAP levels in the tissues of nude mice in each group were detected by immunohistochemistry (n=5, × 400). B. The CD3 levels in the tissues of nude mice in each group were detected by immunohistochemistry (n=5, × 400). C. The CD56 levels in the tissues of nude mice in each group were detected by immunohistochemistry (n=5, × 400).



**Figure 8.** LAP-CD4 T cells can inhibit the growth and apoptosis of colorectal cancer and reduce tumor growth in nude mice.

the above data, deleting LAP or down-regulating IL-10 and TGF- $\beta$  was helpful to enhance the efficacy of CIK cells in nude mice with transplanted tumor.

The novelty of this research is that we found for the first time that LAP-CD4+T cells can promote the anti-tumor lethality of CIK cells in CRC, and transfection using si-IL-10 or si-TGF- $\beta$  can also enhance the cancer-killing toxicity of LAP+CD4+T cells. Nevertheless, there is still room for improvement. For one thing, we can conduct a screening for miRNAs or lncRNAs and explore the possible upstream mechanism of LAP+CD4+T cells on CIK anti-tumor activity. For another, we can also identify whether multiple molecular pathways mediate the regulation of IL-10 or TGF- $\beta$  on immunosuppression of LAP+CD4+T cells.

In short, LAP+CD4+T cells regulate the cancer inhibition ability of CIK cells in CRC, mediated through IL-10 and TGF- $\beta$ .

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### Disclosure of conflict of interest

None.

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