

# Tim-3ScFv-Transforming *Lactobacillus* Inhibits Transplanted Tumour of Renal Cell Carcinoma

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## Research Article

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# Abstract

## Background

T cell immunoglobulin-3(Tim-3) is an immune checkpoint molecule; Tim-3 antibody is suitable for treating malignant renal tumours. However, Tim-3 antibody drugs are expensive, which limits their application. To overcome the disadvantages of expensive immunotherapeutic drugs, *Lactococcus lactis* was used as the host bacteria to express Tim-3 single-chain antibody in the intestine, and its promotion of the mouse immune system and inhibitory effects on the transplanted tumour of kidney cancer in mice were tested.

## Methods

Molecular cloning technology was used to construct plasmids pLAN-CTB-Tim3scFv and pLAN-Tim3scFv, which were transformed into *Lactococcus lactis*. The expression of the transformed bacteria was analysed using western blotting, and the immune activity of secreted proteins of the transformed bacteria was detected using ELISA *in vitro*. A subcutaneous transplanted tumour model of renal adenocarcinoma was constructed in RAG mice, and the promoting effect of transforming bacteria on the activation of mouse spleen lymphocytes, and its inhibitory effect on transplanted tumours in mice was analysed.

## Results

(1) Transformed *Lactococcus lactis* NZ -CTB-Tim3scFv and NZ -Tim3scFv, which secrete CTB-Tim3scFv and Tim3scFv single-chain antibodies, were successfully constructed. (2) CTB-Tim3scFv secreted by NZ-CTB-Tim3scFv transformed bacteria showed immunological activity. (3) Compared with the NZ-Tim3scFv and NZ-Vector groups, the characteristics of the subgroups of splenic lymphocytes in the NZ-CTB-Tim3scFv group had a higher proportion of CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8a<sup>+</sup>, and CD3<sup>+</sup>CD69<sup>+</sup> cells. Ki67 and CD31 expression in the NZ-CTB-Tim3scFv group was significantly reduced. The tumour volume of the NZ-CTB-Tim3scFv group increased the least, and was statistically different from that of the other two groups.

## Conclusions

The Tim-3 single-chain antibody gene was successfully constructed and transformed in *Lactococcus lactis*. After feeding mice NZ-CTB-Tim3scFv transforming *Lactobacillus*, the CTB-Tim3scFv secreted by the transforming *Lactobacillus* promoted the proliferation and activation of spleen lymphocytes and inhibited volume growth, cell proliferation, and angiogenesis of the tumour in mice. In summary, apply transgenic lactobacillus secreting CTB-Tim-3scFv, to perform its role in anti-tumor immunotherapy

through oral approach, is low cost and convenient. It is expected to become a new way of immunotherapy for renal cell carcinoma.

## 1. Background

Immunotherapy is one of the most promising methods for cancer treatment. Malignant tumours can escape immune surveillance owing to the ability of immune and tumour cells to express the coinhibitory molecules (CIMs) and their corresponding receptors, referred to as checkpoints, leading to immunosuppression.<sup>1</sup> T cell immunoglobulin-3 (Tim-3) is an immune checkpoint molecule, and is mainly expressed in activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells; its receptor is galectin-9. Tumour cells or tumour antigen-presenting cells express galectin-9 and bind to Tim-3 of lymphocytes, which leads to a decrease in T cell consumption and affects the tumour microenvironment.<sup>2-4</sup>

Research has shown a high expression of Tim-3 in both clear cell renal cell carcinoma (CCRCC) and papillary renal cell carcinoma (PRCC), which suggests the dysfunction of tumour-infiltrating lymphocytes.<sup>5</sup> Tim-3 is not only expressed on immune cells, but also on some tumour cells; immunotherapy targeting Tim-3 not only reduces immunosuppression and improves anti-tumour immunity, but also directly affects the tumour itself, therefore making Tim-3 antibody suitable for the treatment of renal cell carcinoma. However, immunotherapy drugs are expensive, which limits their application. Biological drugs are produced via traditional processes, which attribute to the exorbitant cost of immunotherapy drugs; therefore, there is a need to change the traditional method of production for cost reduction.

*Lactobacillus* and *Bifidobacterium* are the safest and the most suitable host bacteria for intestinal bioreactors, which are suitable for recombinant protein expression and administration in the intestinal tract.<sup>6</sup> Although the intestinal administration of macromolecular polypeptide is limited by the mucosal barrier, the fusion expression with cholera toxin B subunit (CTB) can effectively allow the macromolecular polypeptide to enter the blood circulation through the intestinal mucosal barrier.<sup>7</sup>

In this study, a CTB-Tim-3ScFv-transforming *Lactococcus lactis* was constructed and its promoting effects on the immune system of mice and inhibitory effects on transplanted tumours of renal cell carcinoma in mice were evaluated.

## 2. Methods

### 2.1. Reagents

*Lactobacillus* strain NZ-3900 was kindly provided by the Nestle Research Centre (Lausanne, Switzerland). The RAG mouse renal adenocarcinoma line was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The polymerase, restriction endonucleases, and T4 DNA ligase were purchased from MBI Fermentas Inc. (Vilnius, Lithuania). CTB-Tim-3scFv DNA was synthesised by Wuhan Genecreate

Biological Engineering Technology and Services Ltd. (Wuhan, China). L-arabinose was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Tim-3 monoclonal antibodies were purchased from Proteintech Inc. (Chicago, IL, USA). Chemiluminescent substrate was purchased from ThermoFisher Inc. (Waltham, MA, USA). Tim-3 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Adlitteram Diagnostic Laboratories Inc. (Fremont, CA, USA). The Mouse cytokine FlowCytomix (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8a<sup>+</sup>, CD69<sup>+</sup>, Ki67, and CD31) Simple Kits and Mouse FlowCytomix Basic Kit were purchased from Bender MedSystems GmbH (Vienna, Austria). Lymphocyte separation medium was purchased from Dakewe Ltd. (Beijing, China). Fluorescence-conjugated antibodies (Alexa Fluor 700-anti-CD4, PerCP-Cyanine5.5-anti-CD8a, PE anti-CD69, and APC-anti-CD3) were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

## 2.2. Plasmid construction, transformation, and identification of *Lactococcus*

To allow for the recombinant protein to pass through the intestinal mucosal barrier, Tim-3scFv and cholera toxin B subunit (CTB) were fused together when designing the gene. The CTB-Tim-3scFv gene and its control gene, Tim-3scFv, were synthesised via DNA chemical synthesis and inserted into the *NcoI* and *XbaI* cloning sites, downstream of the secretory signal peptide of the shuttle vector pLAN to form a fusion protein with the signal peptide. To facilitate detection and to distinguish the gene from the endogenous Tim-3 antibody, a FLAG tag was introduced in the hinge region of the CTB-Tim-3scFv gene and the furin-specific amino acid recognition sequence (Arg- Ala-Arg-Arg), which facilitates the endocytosis and enzymolysis of the fusion protein by intestinal mucosal cells, releasing free Tim-3scFv.

The empty control vector (pLAN-0) was constructed by cutting off the GFP DNA fragment from the plasmid pLAN-GFP at the two *Sall* sites. The human CTB-Tim3scFv and Tim3scFv expressing vector pLAN-CTB-Tim3scFv and pLAN-Tim3scFv were constructed by inserting the DNA fragment of human CTB-Tim3scFv or Tim3scFv at the restriction sites of *NcoI* and *XbaI* of the plasmid (Fig. 1). Both recombinant plasmids were verified using DNA sequencing (Takara, Japan). *Lactococcus* was prepared by electroporation with plasmids pLAN-0, pLAN-CTB-Tim3scFv, and pLAN-Tim3scFv, respectively. The transformed *Lactococcus* were selected with selective EM agar plates supplemented with 0.5% lactose and 0.04% bromocresol purple. Positive colonies containing the plasmid with the Lac F gene were picked and inoculated in EM containing lactose but not glucose. Using PCR to identify genetically transformed *Lactococcus*.

## 2.3. Gene expression induction *in vitro*

Transformed bacteria were cultured in EM medium broth containing 0.5% lactose until the absorption of the bacterial suspension at OD<sub>600nm</sub> reached approximately 0.6, then Nisin was added to 1.0 ng/ml (final concentration) to induce target gene expression. The culture supernatants and pellets were collected at 3 h, 6 h, 9 h, 12 h, 24 h, and 36 h after induction and stored at -70°C.

## 2.4. Animal experiment protocol

Six-week-old male BALB/c mice that weighed 18–20 g were purchased from the Laboratory Animal Centre of Southern Medical University (PR China). Mice were kept at room temperature ( $22\pm 1^\circ\text{C}$ ) in a room with controlled 12 h light/dark cycle, and free access to standard rodent chow. The mice (total 21) were randomly assigned to 3 groups and treated as follows: the CTB-Tim3scFv group ( $n=7$ ) was treated with 0.2 ml ( $6\times 10^9$  cells  $\text{ml}^{-1}$ ) pLAN-CTB-Tim3scFv transformed *Lactococcus* (referred to as NZ-CTB-Tim3scFv), which was induced with 1 ng/ml Nisin (final concentration) for 9 h, by intragastric administration on alternate days; the Tim3scFv group ( $n=7$ ) was treated with 0.2 ml ( $6\times 10^9$  cells  $\text{ml}^{-1}$ ) pLAN-Tim3scFv transformed *Lactococcus* (referred to as NZ-Tim3scFv) used as the negative control; the Vector group ( $n=7$ ) was treated with 0.2 ml ( $6\times 10^9$  cells  $\text{ml}^{-1}$ ) pLAN-0 transformed *Lactococcus* (referred to as NZ-Vector) used as the blank control. The animals were sacrificed 4 weeks later, and the spleens were obtained. Lymphocytes were separated from spleens using a lymphocyte separation medium. To investigate the effect of CTB-Tim3scFv on transplanted tumours of renal cell carcinoma in mice, a subcutaneous transplanted tumour model of PRCC-bearing mice with RAG cells was constructed. Fifteen tumour-bearing mice were divided into three groups and administered orally 0.2 ml ( $6\times 10^9$  cells  $\text{ml}^{-1}$ ) pre-induced NZ-CTB-Tim3scFv with 1 ng/ml (final concentration) Nisin for 9 h every other day for 4 weeks. NZ-Tim3scFv and NZ-Vector were used as the negative and blank control, respectively. The size of subcutaneous transplanted tumours in mice was surveyed after being sacrificed. Tissues were immediately fixed with 4% phosphate-buffered paraformaldehyde and tissue sections were used for immunofluorescence detection of mouse tumour cell proliferation (Ki67) and tumour tissue microangiogenesis (CD31).

## 2.5. Enzyme-linked immunosorbent assay

The CTB-Tim3scFv and Tim3scFv levels in the culture supernatants were detected using ELISA kits. Test procedures were performed according to the manufacturer's protocol.

## 2.6. Chemiluminescence-enhanced western blot analysis

Take 50  $\mu\text{l}$  of the supernatant and 50  $\mu\text{l}$  of the sonicated cell, add an equal volume of 2 $\times$  protein loading buffer, boil for 10 min to denature, centrifuge at 10 000 r/min for 20 min, take 30  $\mu\text{l}$  of supernatant and load the sample. Proteins were separated by SDS-PAGE 15% (w/v) and transferred electrophoretically to nitrocellulose membranes (Whatman, UK). The Flag expressions in supernatants and pellets were detected using the western blot method with rabbit anti-Flag IgG and monitored with enhanced chemiluminescence.

## 2.7. Cytokine flow cytometry assay

CD3<sup>+</sup>, CD4<sup>+</sup>, CD8a<sup>+</sup>, and CD69<sup>+</sup> levels in spleen were detected by flow cytometry using the Mouse cytokine FlowCytomix Simple Kits according to the manufacturer's instructions.

## 2.8. Statistical analysis

All data were expressed as mean  $\pm$  SD and analysed using SPSS 20.0 One-way ANOVA was performed for multiple comparisons, followed by the least significant difference (LSD) test method. When the equal

variance test failed to produce results, the Dunnett's T3 test was used. Difference with a  $p$  value less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Identification of transformed *Lactococcus*

The CTB-Tim3scFv transformed *Lactococcus* clones were identified by DNA sequencing using the plasmids extracted from the selected *Lactococcus* as templates. Plasmids from NZ-CTB-Tim3scFv-transformed *Lactococcus* contained a fragment of the synthesised human CTB-Tim3scFv DNA sequence, and the NZ-Tim3scFv-transformed *Lactococcus* contained the Tim3scFv fragment. As expected, the empty vector pLAN-0-transformed *Lactococcus* did not contain the CTB-Tim3scFv or Tim3scFv fragments.

### 3.2. Detection of CTB-Tim3scFv expression in transformed *Lactococcus in vitro*

The detection of CTB-Tim3scFv and Tim3scFv peptides in culture pellets was performed using western blot analysis. CTB-Tim3scFv or Tim3scFv could be detected in culture pellet lysate after being induced with 1 ng/ml nisin. The target bands (approximately 53 kDa or 33 kDa) were observed in the induced culture pellet lysate of pLAN-CTB-Tim3scFv or pLAN-Tim3scFv transformed *Lactococcus* by using western blotting; no band was observed in the uninduced culture pellet lysate or the pLAN-0 transformed *Lactococcus* after induction. The molecular weight of the detected bands was concordant with the 53 kDa or 33 kDa theoretical molecular weight of mature CTB-Tim3scFv or Tim3scFv peptide, respectively (Fig. 2a). The level of recombinant CTB-Tim3scFv in culture pellets of transformed *Lactococcus* reached a maximum at 9 h of induction and during induction the optimal concentration of nisin was 1 ng/ml (Fig. 2b and Fig. 2c).

### 3.3. Detection of the immune activity of the secreted protein of transformed bacteria *in vitro*

Western blotting revealed that mouse renal adenocarcinoma cells (RAG cells) express Tim3 (Fig. 3a). The double antibody sandwich method was used to identify the immune activity of the proteins secreted by the transformed bacteria. As the concentration of RAG cells decreased, the OD gradually decreased, and the two showed a linear relationship, indicating that the single-chain antibody secreted by the transformed bacteria could bind to the Tim3 antigen. (Table 1) Similarly, as the dilution factor of the coated antibody increased, the OD also showed a downward trend, and the relationship between the two was also linear, indicating that the target antibody could bind to the Tim3 antigen. By drawing a line graph, the standard curve equation for each group was obtained (Table 2). The  $R^2$  value was greater than 0.95, suggesting that as the concentration of RAG cells (Tim3 antigen) increased, the binding of the Tim3 single-chain antibody secreted by the two transformed bacteria to the antigen increased; indicating that

after induction, the transformed bacteria could secrete the target antibody and interact with the Tim3 antigen binding of RAG cells.

Table 1  
 Detection and data analysis of samples with different concentrations

Anti	Sample	OD1	OD2	Mean $\pm$ SD	P/N
Tim3-100	976.42 $\mu$ g/ml RAG	0.5298	0.5044	0.52 $\pm$ 0.02	24.22
	488.20 $\mu$ g/ml RAG	0.2429	0.2382	0.24 $\pm$ 0	11.27
	244.10 $\mu$ g/ml RAG	0.1055	0.1318	0.12 $\pm$ 0.02	5.56
	122.05 $\mu$ g/ml RAG	0.0696	0.0811	0.08 $\pm$ 0.01	3.53
	61.0 $\mu$ g/ml RAG	0.0478	0.0629	0.06 $\pm$ 0.01	2.59
	30.51 $\mu$ g/ml RAG	0.0416	0.047	0.04 $\pm$ 0	2.07
	KB	0.0176	0.0251	0.02 $\pm$ 0.01	1.00
Tim3-500	976.42 $\mu$ g/ml RAG	0.5603	0.5688	0.56 $\pm$ 0.01	20.79
	488.20 $\mu$ g/ml RAG	0.242	0.2294	0.24 $\pm$ 0.01	8.68
	244.10 $\mu$ g/ml RAG	0.1378	0.1585	0.15 $\pm$ 0.01	5.46
	122.05 $\mu$ g/ml RAG	0.0505	0.1077	0.08 $\pm$ 0.04	2.91
	61.0 $\mu$ g/ml RAG	0.0482	0.0448	0.05 $\pm$ 0	1.71
	30.51 $\mu$ g/ml RAG	0.0429	0.0432	0.04 $\pm$ 0	1.59
	KB	0.0235	0.0308	0.03 $\pm$ 0.01	1.00
Tim3-CTB-100	976.42 $\mu$ g/ml RAG	0.8734	0.6389	0.76 $\pm$ 0.17	16.24
	488.20 $\mu$ g/ml RAG	0.5754	0.2785	0.43 $\pm$ 0.21	9.17
	244.10 $\mu$ g/ml RAG	0.1303	0.1151	0.12 $\pm$ 0.01	2.64
	122.05 $\mu$ g/ml RAG	0.0796	0.0772	0.08 $\pm$ 0	1.68
	61.0 $\mu$ g/ml RAG	0.0629	0.0742	0.07 $\pm$ 0.01	1.47
	30.51 $\mu$ g/ml RAG	0.0604	0.0596	0.06 $\pm$ 0	1.29
	KB	0.0487	0.0444	0.05 $\pm$ 0	1.00
Tim3-CTB-500	976.42 $\mu$ g/ml RAG	0.5159	0.5688	0.54 $\pm$ 0.04	13.89
	488.20 $\mu$ g/ml RAG	0.2823	0.2503	0.27 $\pm$ 0.02	6.82
	244.10 $\mu$ g/ml RAG	0.1438	0.1303	0.14 $\pm$ 0.01	3.51
	122.05 $\mu$ g/ml RAG	0.0666	0.0702	0.07 $\pm$ 0	1.75
	61.0 $\mu$ g/ml RAG	0.0619	0.0543	0.06 $\pm$ 0.01	1.49
	30.51 $\mu$ g/ml RAG	0.0545	0.0486	0.05 $\pm$ 0	1.32

Anti	Sample	OD1	OD2	Mean $\pm$ SD	P/N
	KB	0.0376	0.0405	0.04 $\pm$ 0	1.00

Table 2  
Analysis of single chain antibody titre by RAG cells

RAG	a	b	c	R2	Protein amount of 1 OD sample( $\mu$ g/ml)
Tim3-100	1.00E-07	0.0004	0.0282	0.9993	1703.78
Tim3-500	1.00E-07	0.0004	0.0311	0.9971	1417.54
Tim3-CTB-100	1.00E-07	0.0007	0.0234	0.9783	1192.12
Tim3-CTB-500	1.00E-07	0.0004	0.0333	0.9982	1696.89

### 3.4. Analysis of the inhibitory effect of proteins secreted by transformed bacteria on the proliferation of RAG cells *in vitro*

Proteins CTB-Tim3scFv, Tim3scFv, and Vector secreted by transformed bacteria NZ-CTB-Tim3scFv, NZ-Tim3scFv, and NZ-Vector, after pH adjustment, filtration, and sterilisation, respectively, acted on mouse renal adenocarcinoma RAG. CCK-8 solutions were added and the OD450 value of each group was measured. Results showed that the OD value of RAG cells in the CTB-Tim3scFv group was the lowest, suggesting that the protein secreted by the transformed bacteria in the CTB-Tim3scFv group could significantly inhibit the proliferation of RAG cells. (Fig. 3b)

### 3.5. Effect of proteins secreted by transformed bacteria on lymphocyte subpopulation in the spleen

The CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8a<sup>+</sup>, and CD3<sup>+</sup>CD69<sup>+</sup> cell percentages in the spleen were detected with Alexa Fluor 700-conjugated anti-CD4 antibody, PerCP-Cyanine5.5 anti-CD8a antibody, PE-conjugated anti-CD69 and APC-conjugated anti-CD3 monoantibody. The percentages of spleen lymphocyte subtypes in mice in each group after treatment are shown in Table 3.

Table 3  
Percentage of lymphocyte subtypes in spleen of three groups of mice after transformed *Lactococcus lactis* treatment.

Group	N	CD3 <sup>+</sup> CD4 <sup>+</sup>	CD3 <sup>+</sup> CD8a <sup>+</sup>	CD3 <sup>+</sup> CD69 <sup>+</sup>
NZ-CTB-Tim3scFv	7	11.57 $\pm$ 1.41%	26.06 $\pm$ 6.16%	4.74 $\pm$ 2.57%
NZ- Tim3scFv	7	8.01 $\pm$ 1.77%	18.52 $\pm$ 6.14%	2.46 $\pm$ 1.09%
NZ-Vector	7	6.29 $\pm$ 1.43%	13.68 $\pm$ 4.41%	1.55 $\pm$ 0.85%

The results showed that the characteristics of the subgroups of splenic lymphocytes in the NZ-CTB-Tim3scFv group included a higher proportion of CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8a<sup>+</sup>, and CD3<sup>+</sup>CD69<sup>+</sup> cells. Figure 4 shows that the percentage of CD3<sup>+</sup>CD4<sup>+</sup> cells in the NZ-CTB-Tim3scFv group was 11.57±1.41%, which was significantly higher than that in the NZ-Tim3scFv group (8.01±1.77%) and NZ-Vector (6.29±1.43%) (P<0.05). Figure 5 shows that the percentage of CD3<sup>+</sup>CD8a<sup>+</sup> cells in NZ-CTB-Tim3scFv group was 26.06±6.16%, which was also significantly higher than that of NZ-Tim3scFv group (18.52±6.14%) and NZ-Vector group (13.68±4.41%) (P<0.05). Figure 6 shows that the percentage of CD3<sup>+</sup> CD69<sup>+</sup> cells in the NZ-CTB-Tim3scFv group was 4.74% ±2.57%, which was significantly higher than that of the NZ-Vector group (1.55±0.85%) (p<0.05), and higher than that of the NZ-Tim3scFv group (2.46±1.09%), though it is not statistically significant (P=0.16); this may be related to the damage of throat mucosa, poor anorexia, and decreased nutritional status after oral administration of some mice.

### 3.6. Effect of proteins secreted by transformed bacteria on subcutaneous tumour grow

Tumour size was measured after treatment, and results are shown in Fig. 7.

The tumour volumes of each group before and after treatment are shown in Table 4.

Table 4  
The tumour volume of mice in each group before and after treatment(mm<sup>3</sup>)

Group	Treatment time	NO.1	NO.2	NO.3	NO.4	NO.5
NZ-CTB-Tim3scFv	Before	172.102	91.133	178.324	260.372	154.126
	after	1694.120	566.009	1348.132	1548.972	1631.142
NZ- Tim3scFv	Before	191.230	19.236	266.250	172.526	142.150
	after	2325.624	2152.019	2836.546	1945.632	2018.566
NZ-Vector	Before	167.240	142.540	133.421	132.540	288.216
	after	2824.136	3324.102	3752.625	2423.324	5109.528

Results showed that the tumour volume of the NZ-CTB-Tim3scFv group increased the least, and was statistically different from that of the other two groups (Fig. 7b).

### 3.7. Analysis of transformed bacteria inhibiting the proliferation of transplanted tumours in mice

Ki67 is mainly expressed in cells in the proliferation phase, whereas cells in the quiescent phase, i.e., cells in the G0 phase, are not expressed. It is closely related to the cell cycle and is a reliable marker that reflects the activity of cell proliferation. The results of immunofluorescence staining for Ki67 are shown in

Fig. 8a. Results showed that the expression of Ki67 in the NZ-CTB-Tim3scFv group was significantly lower than that in the other two groups.

### **3.8. Analysis of transformed bacteria inhibiting the vascular growth of transplanted tumours in mice**

CD31 is used to mark the density of microvessels in tumour tissues, detect the presence of endothelial cell tissues, and is often used to evaluate microangiogenesis in tumour tissues and to judge the rapid growth of tumours. The results of immunofluorescence detection of CD31 are shown in Fig. 8b. Results showed that the expression of CD31 in the NZ-CTB-Tim3scFv group was weaker than that in the other two groups, indicating that the growth of tumour blood vessels was inhibited.

## **4. Discussion**

T cell immunoglobulin-3 (Tim-3) is one of the immune checkpoint molecules. It is expressed in cells that secrete IFN- $\gamma$ , including Th1 cells, dendritic cells, monocytes, CD8+ T cells and other lymphocytes.<sup>2,3</sup> The corresponding ligand of Tim-3 is Galectin-9. Tumour cells or tumour antigen-presenting cells continuously express the antigen Galectin-9 and bind to the Tim-3 of lymphocytes, resulting in the consumption of T cells in the tumour microenvironment, thus resulting in a decline in immune tolerance. One of the immune checkpoint inhibitors, Tim-3 antibody, can effectively prevent the binding of Tim-3 and Galectin-9, promote the proliferation and activation of T cells, and improve the apoptotic effect of T cells on tumour cells.<sup>4,8</sup> According to the expression of Tim-3 and the clinicopathological correlation of renal cancer, the polymorphism of Tim-3 is related to the prognosis of renal cancer.<sup>9</sup> We therefore chose to determine the role of Tim-3 single-chain antibody in renal tumour immunotherapy.

Currently, the first-line treatment for advanced renal cancer is a combination of two immune checkpoint inhibitors, ipilimumab (CTLA-4 antibody) and nivolumab (PD-1 antibody), or immune checkpoint inhibitors combined with targeted drugs.<sup>10</sup> However, the treatment costs of these immune checkpoint inhibitors or target drugs are very high, thus limiting their use. In this study, the transformed lactic acid bacteria were used to express Tim-3scFv, and immunotherapy of advanced renal tumours was carried out via the oral route.

The gastrointestinal tract is the simplest and the most economical bioreactor for the generation and transformation of biological drugs, and probiotics such as lactic acid bacteria are good carriers for use in oral administration of immunotherapeutic drugs. In recent years, research on recombinant protein expression, gene therapy, and oral vaccines with lactic acid bacteria and bifidobacteria as host bacteria has been highly valued by scientific researchers worldwide.<sup>11-14</sup> A study has managed to transform the endostatin gene in bifidobacteria to produce an endostatin transgenic bifidobacteria that yielded better anti-tumour effects.<sup>15</sup> Research by Japanese scholars has also shown that the immunologically active interleukin 6 can be expressed by transforming lactic acid bacteria.<sup>16</sup> We used the food-grade lactic acid bacteria intestinal controllable expression vector pLAN to express the CTB-Tim3scFv fusion gene,

*Lactococcus lactis* lacking the LacF gene as the host bacteria, the auxotrophic complementation method, and lactose as the sole carbon source to select the CTB-Tim3scFv fusion gene-transformed bacteria. Positive monoclonal bacteria were picked backward, the culture was expanded, the corresponding plasmid was extracted, separated using 1.2% agarose gel electrophoresis and observed under the gel imager, and gene expression was determined through PCR. Results showed that the plasmid size and PCR band size of pLAN-0, pLAN-Tim3scFv, and pLAN-CTB-Tim3scFv were consistent with the known sequences.

To identify whether the transformed bacteria constructed in the previous experiment could correctly express the CTB-Tim3scFv fusion protein, we used nisin to induce the transformed bacteria NZ-CTB-Tim3scFv and NZ-Tim3scFv *in vitro*, with the empty vector bacteria NZ-Vector as a control, and performed immunoblotting identification. Results showed that the induced expression of the fusion protein CTB-Tim3scFv had a molecular weight of 53 KD, similar to the protein molecular weight estimated by the known amino acid sequence. The transformed bacteria that did not undergo induction did not express the protein. Western blot analysis indicated that both RAG and CACO<sub>2</sub> cells expressed Tim3. Using these two cells as antigens, the immunological activity of the secreted protein of transformed bacteria was detected by ELISA, and results showed that the Nisin-induced and secreted Tim-3scFv and CTB-Tim-3scFv of transformed bacteria had immunological activity. *In vitro* analysis of proteins expressed by the three transformed bacteria that caused RAG cell apoptosis detection showed that the transformed bacteria in the NZ-CTB-Tim3scFv group could significantly inhibit the proliferation of RAG cells.

We determined how macromolecular substances could enter the blood circulation through the intestinal mucosal barrier to exert systemic functions. We aimed to allow Tim3scFv and cholera toxin B subunit (CTB) to be fused and expressed in lactic acid bacteria, and to add a furin-specific enzyme cleavage sequence between the target functional gene and CTB and a Flag tag. With the help of receptor-mediated endocytosis, the target protein was absorbed through the intestinal epithelium, and the enzyme interpretation released free Tim3scFv into the blood circulation to exert a systemic therapeutic effect. *In vivo* analysis of the effect of Tim3 antibody-transformed lactic acid bacteria on the treatment of transplanted tumours in mice showed that the NZ-CTB-Tim3scFv group had a better effect than that did the NZ-Tim3scFv and NZ-Vector groups. This indicated that CTB plays an important role in the passage of macromolecular substances through the intestinal mucosal barrier.

Results of mouse spleen lymphocyte subtypes detection using flow cytometry showed that the splenic CD3<sup>+</sup>, CD4<sup>+</sup> cells, CD3<sup>+</sup>, and CD8a<sup>+</sup> cells in the NZ-CTB-Tim3scFv group increased significantly compared with those in the NZ-Tim3scFv and NZ-Vector groups, suggesting that the transformed bacteria can promote the proliferation of splenic lymphocytes and the generation of Th cells in the spleen to enhance Th-mediated cellular immunity. The expression of CD69 is often used as an indicator of T-cell activation. The proportion of CD3<sup>+</sup> and CD69<sup>+</sup> cells in the NZ-CTB-Tim3scFv group was significantly higher than that in the NZ-Vector group, indicating that feeding CTB-Tim-3ScFv-transforming *Lactococcus lactis* promotes the activation of mouse spleen lymphocytes.

Tumour size measurements, immunofluorescence detection of Ki67 to analyse the proliferation of mouse tumour cells, and CD31 detection to analyse the micro-angiogenesis of mouse tumours indicated that the CTB-Tim3scFv transformed bacteria have obvious therapeutic effects and can inhibit the increase in tumour volume, tumour cell proliferation, and inhibits tumour angiogenesis.

## 5. Conclusions

In summary, the Tim-3 single-chain antibody gene was successfully constructed to transform *Lactococcus lactis*. After feeding mice with the NZ-CTB-Tim3scFv transforming lactobacillus, the CTB-Tim3scFv secreted by the transforming lactobacillus could promote the proliferation and activation of spleen lymphocytes in mice, and inhibit the increase in volume, cell proliferation, and angiogenesis of the tumour in mice. Using transgenic lactobacillus secreting CTB-Tim-3scFv for anti-tumour immunotherapy with an oral approach is economical and convenient, and is expected to become a new method of immunotherapy for renal cell carcinoma.

## Abbreviations

CCRCC: Clear cell renal cell carcinoma, CTB: Cholera toxin B subunit, PCR: polymerase chain reaction, PRCC: Papillary renal cell carcinoma, ScFv: Single chain antibody variable region gene fragment, Tim-3: T cell immunoglobulin-3.

## Declarations

### Ethics approval and consent to participate

All animal procedures were performed in accordance with the experimental animal administration measures of the South Medical University (Guangzhou, China).

### Consent for publication

Not applicable

### Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

CZR was mainly responsible for the design of the work, the acquisition and analysis of data and manuscript writing. HJL and ZHB carried out the acquisition of data. JYD, HZH and CZF participated in the analysis of data. ZWS revised the manuscript. All authors read and approved the final manuscript.

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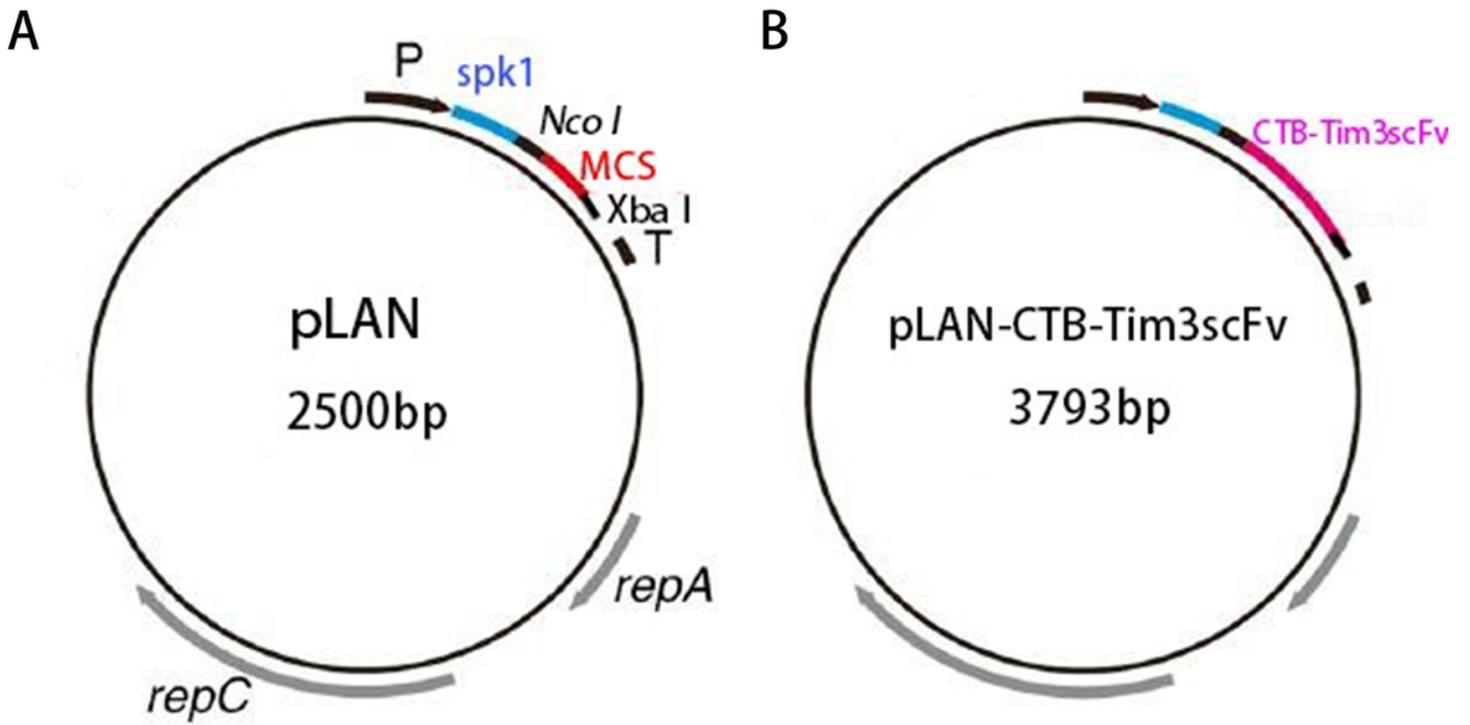
Not applicable

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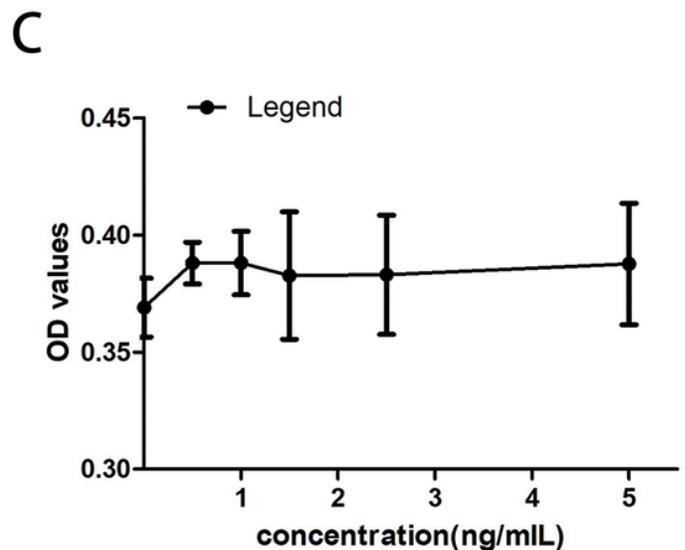
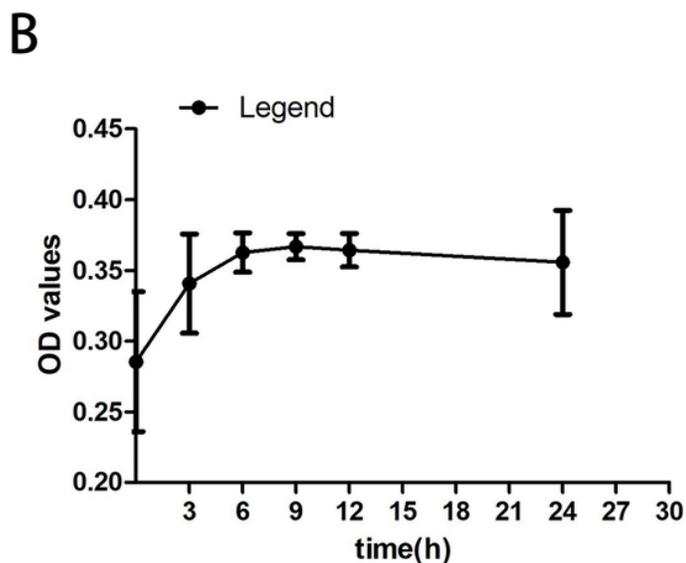
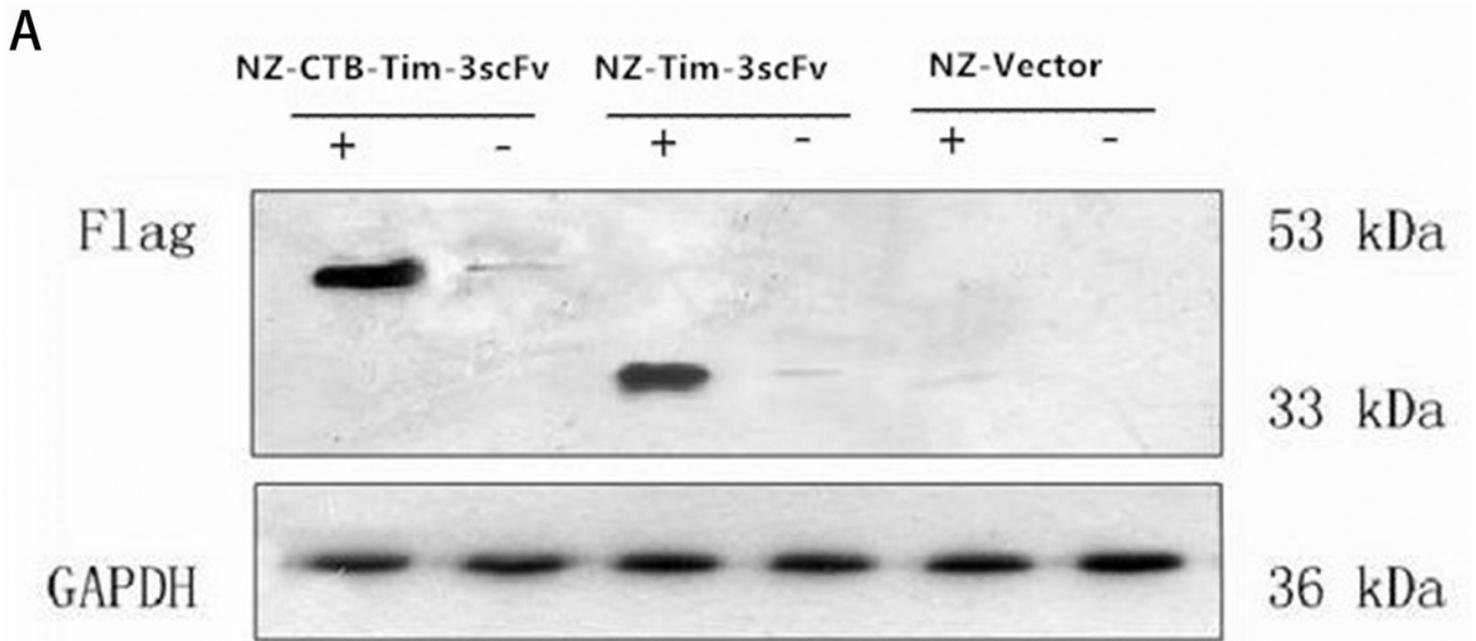
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## Figures



**Figure 1**

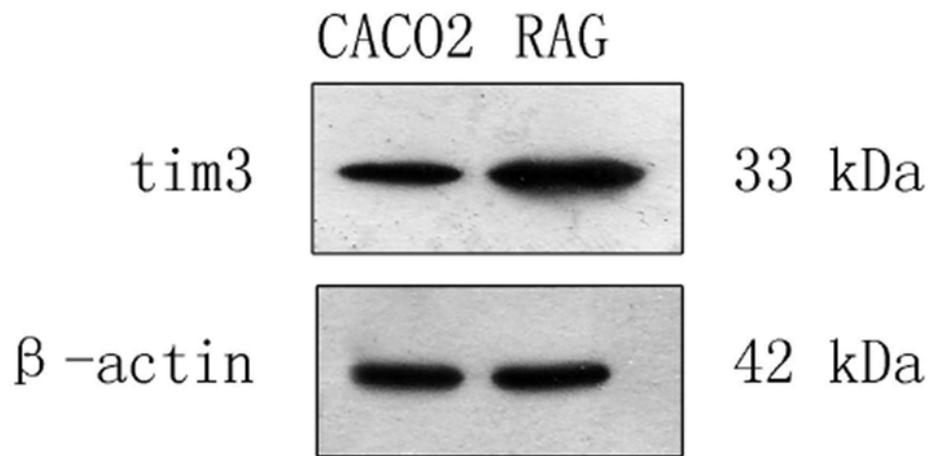
Schematic of lactococcal gene expression vectors. (a) A lactococcal secretion plasmid, pLAN, P nisin promoter, SPK1 sequence of the signal peptide, MCS multiple cloning site, *Nco*I and *Xba*I restriction sites, T terminator, *rep* replication gene. (b) Insert the DNA fragment encoding the CTB-Tim3scFv gene into the MCS of the plasmid to obtain the CTB-Tim3scFv secretion vector pLAN-CTB-Tim3scFv.



**Figure 2**

Identification of NZ-CTB-Tim-3scFv, NZ-Tim3scFv, and NZ-Vector by using western blotting. Gene expression was induced in the transformed bacteria NZ-CTB-Tim-3scFv, NZ-Tim3scFv, and NZ-Vector with (+) or without (-) nisin (a). Changes to CTB-Tim3scFv cell expression after different induction times under the same inducer concentration (b). Changes to CTB-Tim3scFv cell expression after different induction concentrations under the same induction times (c).

A



B

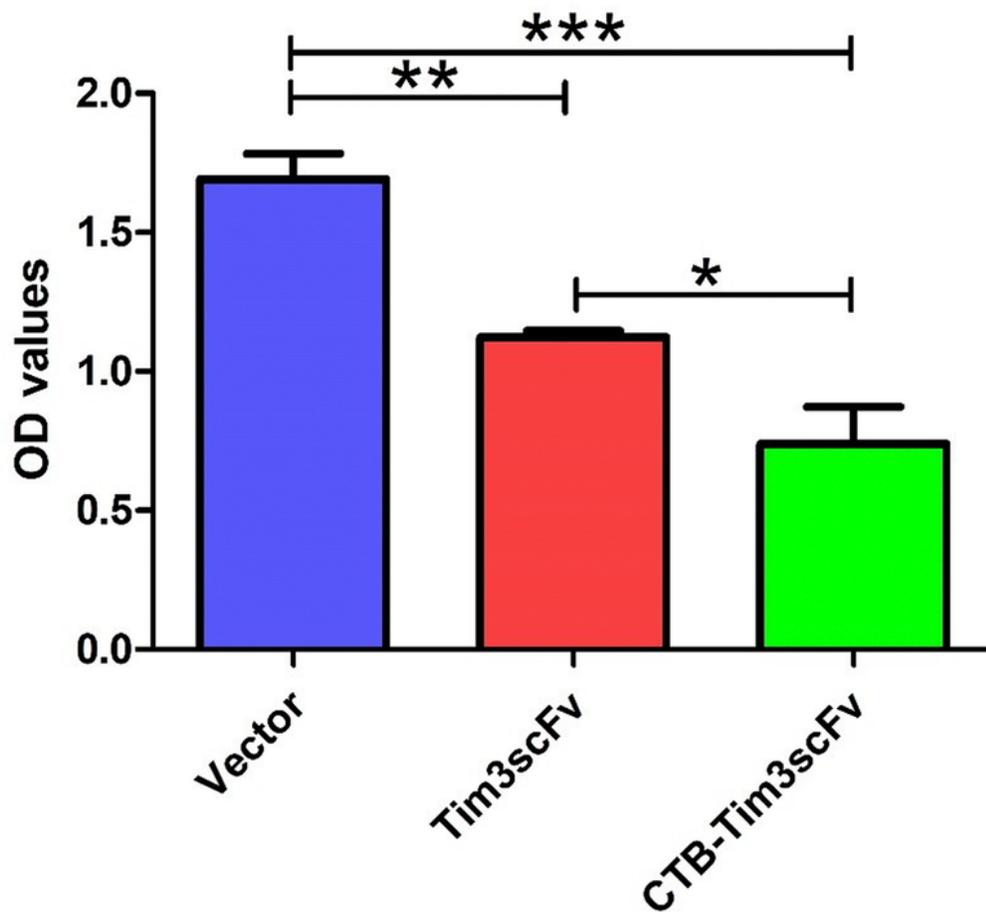
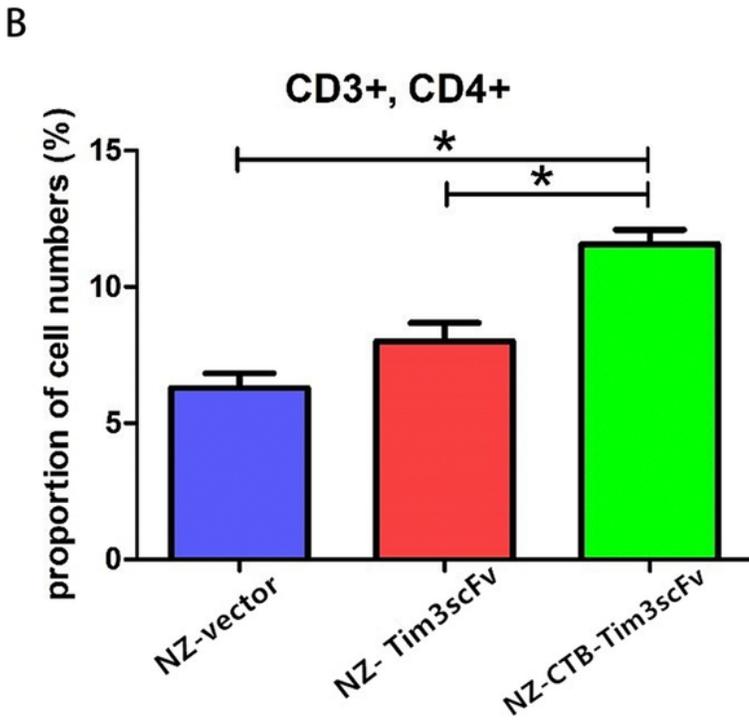
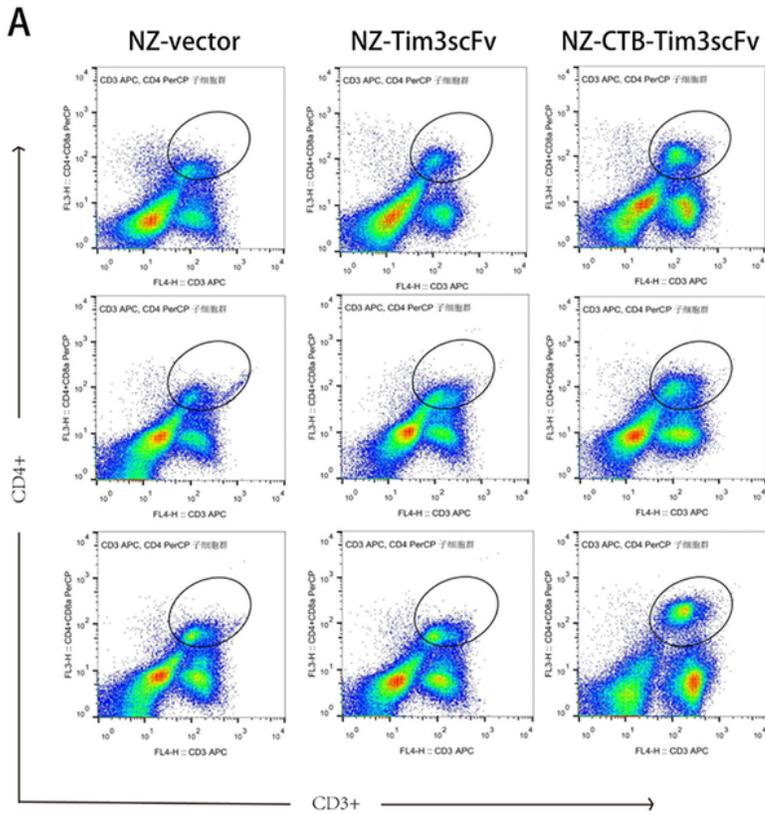


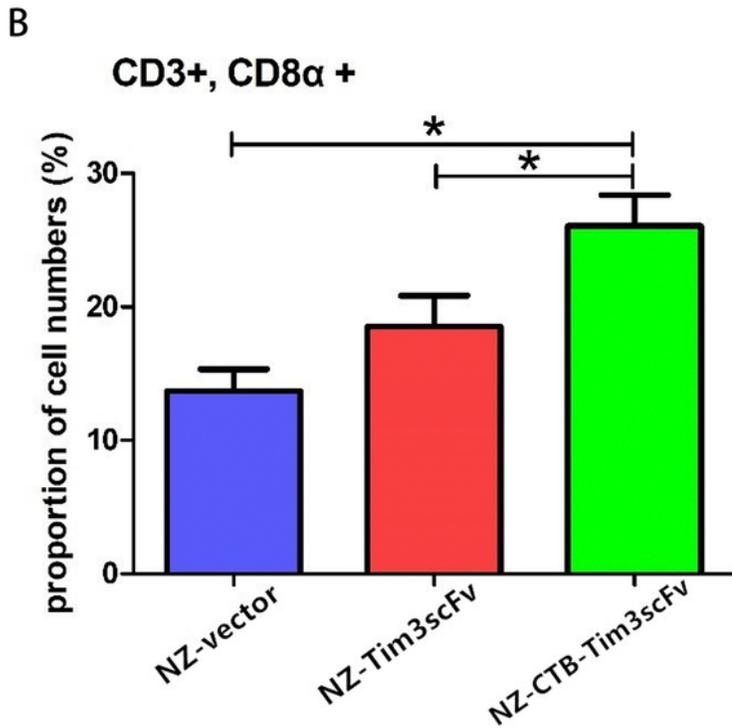
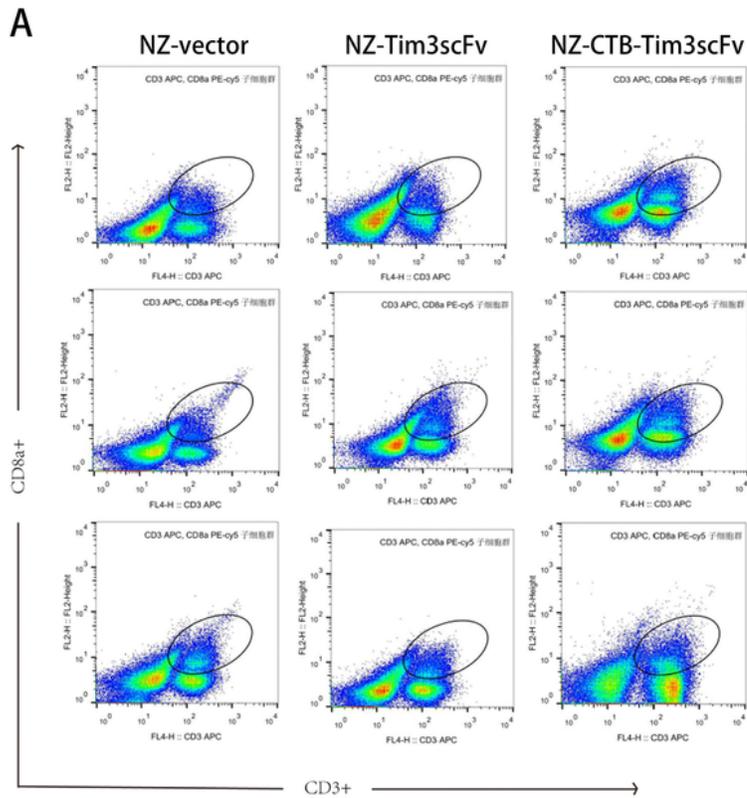
Figure 3

Identification of RAG and CACO2 using western blotting (a). Proteins expressed by transformants inhibit the proliferation of RAG cells (b).



**Figure 4**

CD3 + and CD4 + lymphocyte percentage in the spleen of three groups of mice after transformed *Lactococcus lactis* treatment. (a: CD3+CD4+FACS graph, b: statistical histogram)



**Figure 5**

CD3 + and CD8a + lymphocyte percentage in the spleen of three groups of mice after transformed Lactococcus lactis treatment. (a: CD3+CD8a+FACS graph, b: statistical histogram)

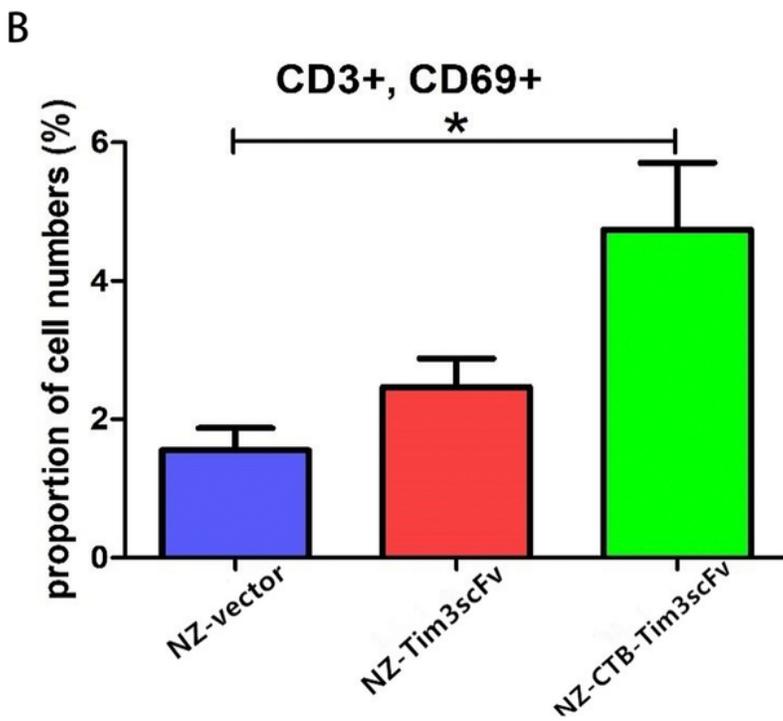
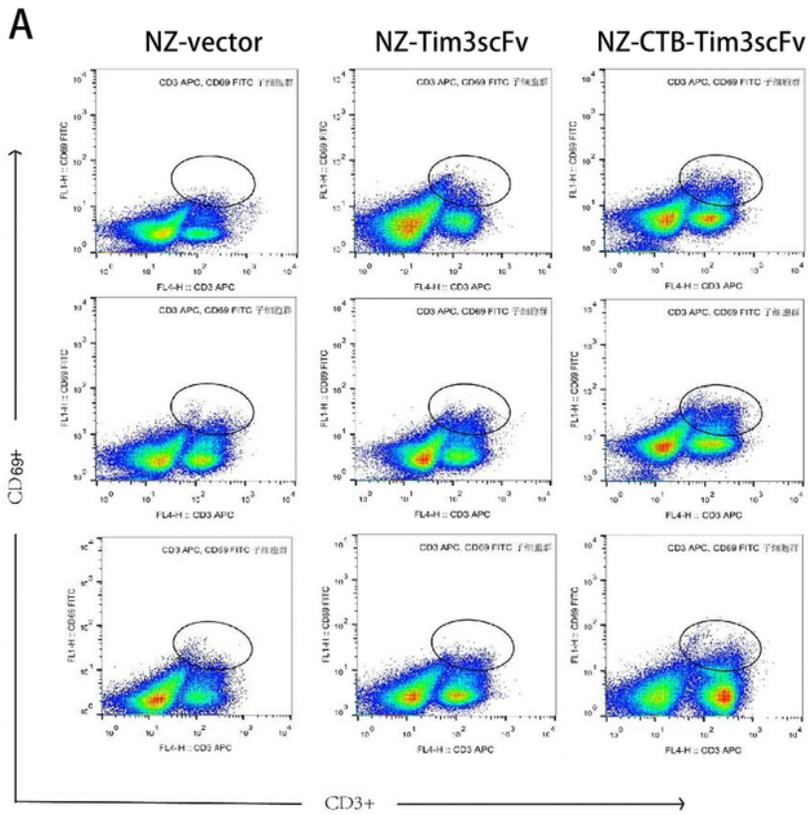
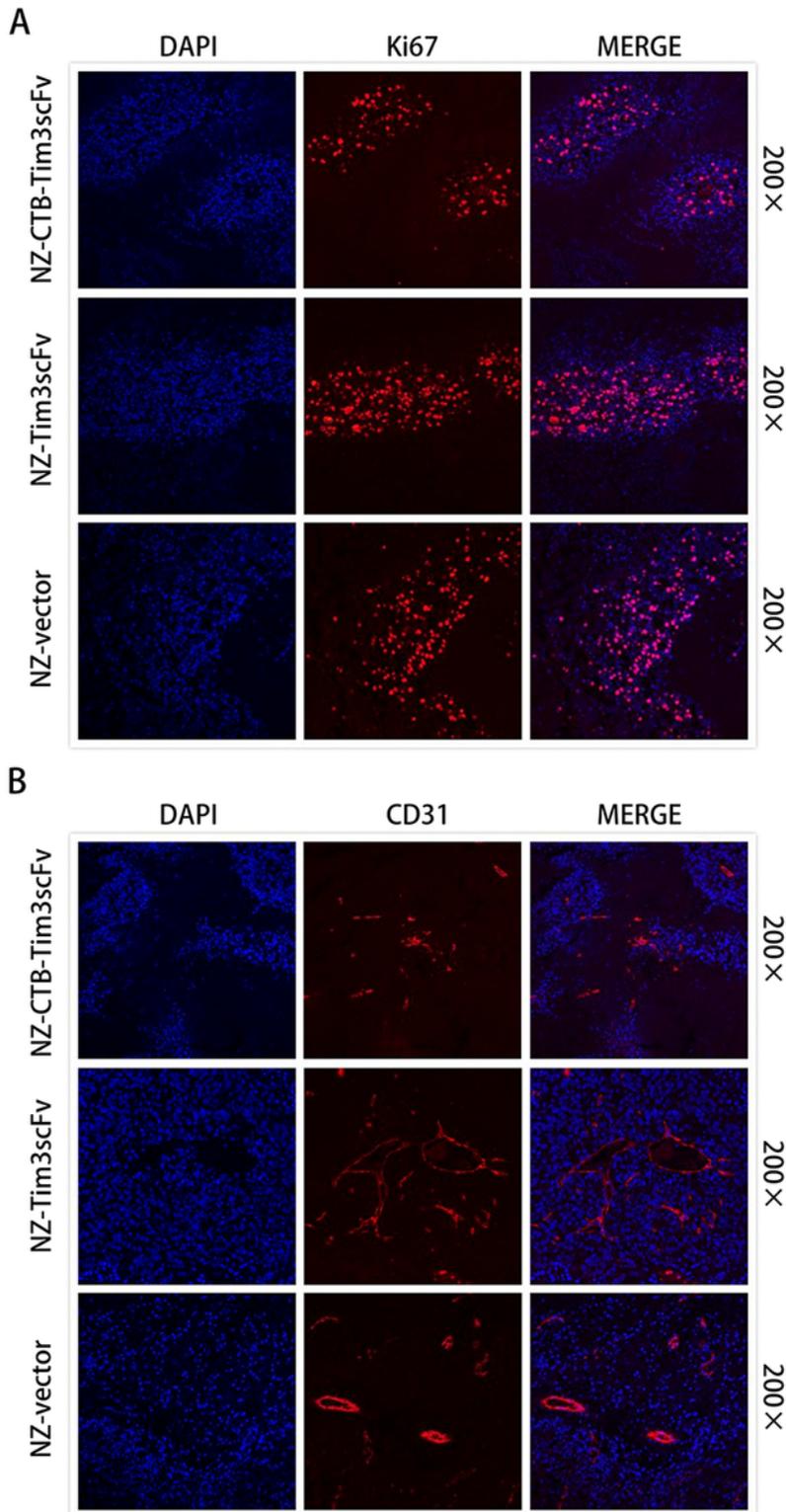


Figure 6

CD3 + and CD69 + lymphocyte percentage in the spleen of three groups of mice after transformed Lactococcus lactis treatment. (a: CD3+CD69+FACS graph, b: statistical histogram)

Figure 7

Tumour volume comparison of three groups of mice after treatment (a). Comparison of mice tumour volume in each group, before and after treatment. D-value: the value of tumour volume increase, \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , N=5 (b).



**Figure 8**

Ki67 expression in renal cell carcinoma transplanted tumour of mice in each group (a). CD31 expression in renal cell carcinoma transplanted tumour of mice in each group (b).

## Supplementary Files

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