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SHORT COMMUNICATION

In vivo targeted delivery of CD40 shRNA to mouse intestinal dendritic cells by oral administration of recombinant *Sacchromyces cerevisiae*

L Zhang, T Zhang, L Wang, S Shao, Z Chen and Z Zhang

Short hairpin RNA (shRNA)-mediated gene regulation is a commonly used technique for gene manipulation. An efficient and safe delivery system is indispensable when shRNA is delivered into living organisms for gene therapy. Previous studies have proved that DNA and protein can be delivered into dendritic cells (DCs) by non-pathogenic *Saccharomyces cerevisiae* without being degraded. CD40 is closely related to apoptosis of tumor cells and some immune mechanisms. In this study, we demonstrated that recombinant yeast *S. cerevisiae* efficiently delivered the shRNA of immune-associated gene (*CD40*) into mouse intestinal DCs *via* oral administration. Western blot analysis of isolated intestinal DCs indicated that the inhibition of *CD40* gene expression reached up to 56–91%. The secretion of cytokines such as interleukin-2 (IL-2), IL-6, IL-10, IL-12, tumor necrosis factor-α and interferon-γ in intestinal DCs had varying degrees of changes. In conclusion, we found that orally administered recombinant yeast can be used as an efficient shRNA delivery system for intestinal DC-specific gene silencing and immunomodulation *in vivo*.

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INTRODUCTION

RNA interference (RNAi) is a natural process in eukaryotic cell,[1] which knocks down the expression of target gene with high specificity at the transcriptional level.[2–4] This kind of gene-silencing mechanism by RNAi ordinarily uses small interfering RNAs and short hairpin RNAs (shRNAs).[5]

shRNA is the precursor of small interfering RNA[6] and is synthesized in the nucleus. Previous studies have shown that the U6 promoter along with a U6 small nuclear ribonucleic acid (snRNA) leader sequence gives the best and most successful repression on target gene.[7–9] On the basis of this result, the strong U6 snRNA promoter was used in our shRNA expression vectors. Effective suppression could be enhanced by inserting the shRNA targeting sequence into an endogenous miRNA, miR30.[7,10]

Dendritic cells (DCs) are immune cells, which function as professional antigen-presenting cells both in innate and adaptive immune responses.[11–14] CD40 is an activation receptor[15] that has an important role in the maturation of DCs.[16] Previous studies had shown that blockade of CD40 provided effective immunotherapy for allergy,[17] autoimmune renal disease[18] and inhibition of arthritic disease.[19] Therefore, we surmise that knocking down the expression of CD40 in DCs may have some impacts on immune responses.[3,20–23] Extensive studies have shown that proteins can be successfully delivered into DCs by *Saccharomyces cerevisiae*[24–27] without being degraded in the digestive environment.[28]

Therefore, we hypothesize that we can regulate the expression of CD40 by orally delivering CD40 sequence-specific shRNA in recombinant *S. cerevisiae*. To test this hypothesis, we designed miR30-based shRNA expression vectors with interference sequences flanked by ~ 120 bases 5' and 3' of the miR30 sequence to target the *CD40* gene with the U6 promoter along with a U6 snRNA leader sequence driving the shRNA expression. The shRNA action principle in this study is diagrammatically shown in Figure 1.

RESULTS

Expression of shRNA in 293T cells *in vitro*

To verify our shRNA expression vectors functioned in mammalian cells, the shRNA vectors were transfected into 293T cells with the reporter vector JMB84-CMV-CD40-GFP-polyA that contained GFP driven by the CMV promoter. If the expression of CD40 was knocked down, then the GFP expression will be reduced. Compared with the control (JMB84-hU6-miR30 without shRNA segment), the intensity of green fluorescence (Figure 2) in the experimental cells (JMB84-hU6-shRNA-miR30) was weakened, especially for cells transfected with plasmid JMB84-hU6-CD40-shRNA1656-miR30.

Also, total RNA was extracted, reverse transcribed and polymerase chain reaction (PCR) amplified with forward primer DF and reverse primer DR (Table 1) revealing the expression of shRNA in 293T cells as shown in Figure 3.

Efficient inhibition of CD40 protein in intestinal DCs

Protein from intestinal DCs was isolated for western blot 50 days after feeding the mice with recombinant yeast. Compared with the control (yeast containing JMB84-hU6-miR30), the expression of CD40 protein in experimental mice (treated with yeast containing JMB84-hU6-shRNA-miR30) had varying degrees of

College of Animal Science and Technology, Shaan'xi Key Laboratory of Molecular Biology for Agriculture, Northwest A&F University, YangLing, Shaan'xi, People's Republic of China. Correspondence: Professor Z Zhang, College of Animal Science and Technology, Shaan'xi Key Laboratory of Molecular Biology for Agriculture, Northwest A&F University, No. 22 Xinong Road, YangLing, Shaan'xi 712100, People's Republic of China.
E-mail: zzhang888@gmail.com
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inhibition (Table 2 and Figure 4), with CD40 shRNA1656 having the highest efficiency in gene silencing. This verified that shRNA driven by the U6 promoter along with a U6 snRNA leader sequence was delivered into intestinal DCs via orally administered yeast and effectively repressed the target gene (*CD40*) *in vivo*.

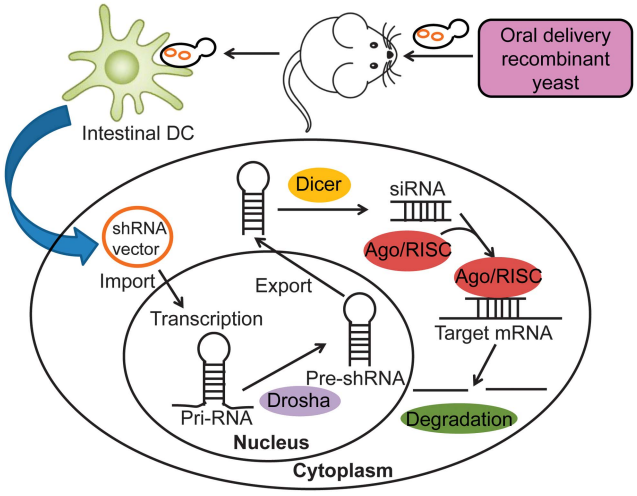

Figure 1. Schematic representation of yeast target intestinal DCs gene-silencing system. After orally administering recombinant yeasts, the shRNA expression vector is delivered to the intestine of the mouse. The *S. cerevisiae* yeast will be recognized and swallowed by DCs, which function as professional antigen-presenting cells (APCs) both in innate and adaptive immune responses. The shRNA expression vector is transported into the nucleus and transcribed into pri-shRNA, which is further processed into pre-shRNA by the RNase III enzyme Drosha. With the help of Exportin-5, pre-shRNA is transported to the cytoplasm and cleaved into a mature shRNA by Dicer. After adhering onto the relationship between insulin sensitivity and cardiovascular disease associate with Ago2 protein, it has the function of mRNA cleavage and degradation.

Analysis of cytokines by ELISA

To determine whether cytokine secretion was affected by the inhibition of CD40 expression, serum samples were collected by tail venipuncture, and interleukin-2 (IL-2), IL-6, IL-10, IL-12, tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) expression were measured by enzyme-linked immunosorbent assay (ELISA). In the experimental mice (treated with yeast with JMB84-hU6-shRNA-miR30), the secretion of IL-12 was downregulated, while the other cytokines were upregulated (Figure 5) with the CD40 shRNA1656, which had the greatest efficiency. Three shRNAs had significantly changed in IL-6, IL-10, IL-12 and TNF-α compared with control group ($P < 0.01$). And, the inhibition of *CD40* gene by CD40 shRNA1656 induced INF-γ increased expression ($P < 0.05$). No significant difference was observed in CD40 shRNA309 for IL-2 and CD40 shRNA367 for INF-γ.

DISCUSSION

shRNA-mediated RNAi in eukaryotic cells has been shown to knockdown the expression of target genes with high specificity at the transcriptional level,[1,5,9] but depends on the availability of an efficient delivery system for gene therapy applications.[29] We used a fast and effective three-step PCR method (Figure 6) for the preparation of miR30-based shRNA expression vectors driven by the U6 promoter along with a U6 snRNA leader sequence. Our *in vitro* experiment had indicated the shRNA constructed by this method was effective. While bacteria and virus are commonly used shRNA delivery vehicles, their use has been limited owing to safety concerns.[30–32] Moreover, viruses have limited packaging capacity[6] and bacteria have low survival in the stomach and small intestine.[33] Recently, *S. cerevisiae* has been shown to avoid digestion in the stomach and small intestine,[34] and thus could be safe and effective for gene delivery. Previous study showed

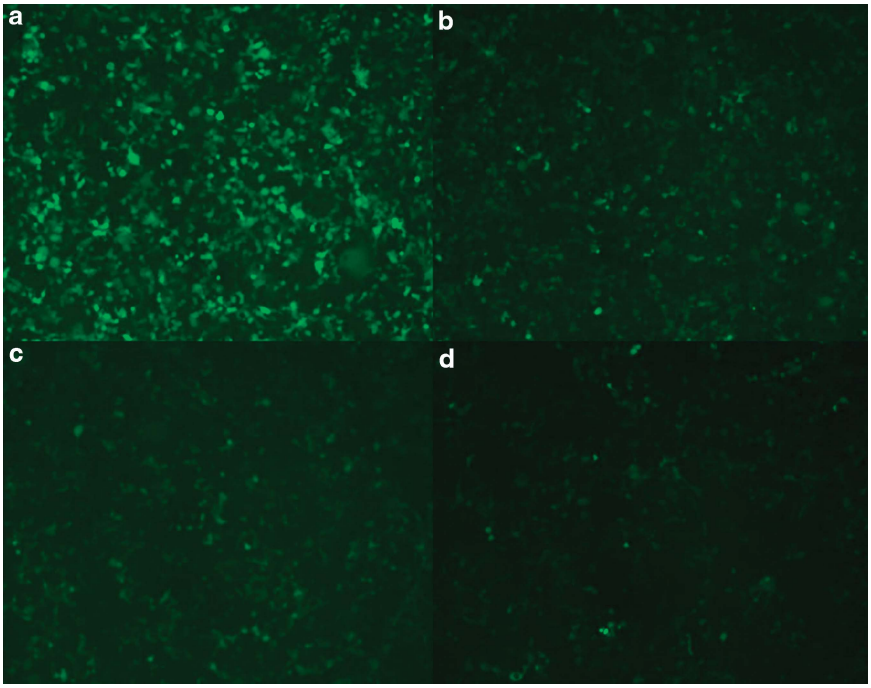

Figure 2. *In vitro* detection of the function of the shRNA in 293T cells by the expression of GFP. (**a**) RP with JMB84-hU6-miR30 (control), (**b**) RP with JMB84-hU6-CD40-shRNA309-miR30, (**c**) RP with JMB84-hU6-CD40-shRNA367-miR30 and (**d**) RP with JMB84-hU6-CD40-shRNA1656-miR30. RP was the reporter vector JMB84-CMV-CD40-GFP-polyA.

that the efficient delivery of yeast avoids the undesired gene integration and potential disease such as cancer when nucleic acid was delivered into antigen-presenting cells such as DCs.[35] However, whether shRNA could be delivered into DCs via yeast has never been proven before. In this study, the shRNA expression construct was designed based on the backbone of human miR30 sequence. In the control group, the construct expresses only miR30 backbone sequence without specific target sequence of CD40. In the experimental groups, we chose three targets on *CD40* gene to determine which site could show the highest knockdown efficiency. Compared with control group, three target shRNA showed different knockdown efficiency. The highest target site is CD40 shRNA1656, which reached up to 91% knockdown efficiency of CD40 protein, whereas CD40 shRNA309 site has only 56% knockdown efficiency. Three different target site designs with different knockdown efficiency suggested its sequence specificity to some degree.

Here, we have demonstrated for the first time that shRNA could be successfully delivered into intestinal DCs via oral administration of the recombinant yeast carrying shRNA expression vectors.

Cytokines have significant roles in many diseases, signaling pathways[23,36–40] and immune responses.[37,41] Previous studies have indicated that the *CD40* gene regulates immune responses in DCs,[3,23–26] and CD40 signaling induced the production of cytokines, including IL-2, IL-6, IL-10, IL-12, IFN-γ and TNF-α.[42–45] Here, we have demonstrated a method to manipulate the immune response in these cells. When the level of CD40 protein in the intestinal DCs was reduced by our shRNAs, the immune response was modified with cytokine expression changed. Cytokines IL-2, IL-6, IL-10, TNF-α and INF-γ were upregulated and IL-12 was downregulated. IL-2 is necessary for the differentiation of T cells to become effector T cells. IL-6 is secreted by T cells and macrophages to stimulate immune response. IL-10 has pleiotropic effects in immunoregulation and inflammation. DCs have been shown to increase IL-10 production, resulting in increased tolerance by modulating the expression of CD40 in DCs.[46] It also enhances B-cell survival and antibody production. Previous studies demonstrated that IL-10 production can stimulate IL-6 expression,[47,48] and IL-12 was downregulated when CD40 was blocked,[3] which exactly was consistent with our observations. Primary role of TNF-α is in the regulation of immune cells. INF-γ is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for antitumor effect. Most of the serum level cytokines changed as expected, except TNF-α. The mechanism of serum TNF-α increase by knockdown of *CD40* gene in DC remains to be elucidated. Compared with control group, all shRNAs had significant effect on IL-6, IL-10, IL-12 and TNF-α ($P < 0.01$). And, the inhibition of *CD40* gene by CD40

Table 1. Primers for the construction of CD40 shRNA expression vectors

Primer name	*Sequence*
hU6-F	5'-TTGGGTACCCCCGAGTCCAACACCCGTGGG-3'
hU6-R	5'-CTAGTCGACTAGTATATGTGCTGCCGAAGCG-3'
miR30PCREcoRIR	5'-CTTGAATTCCGAGGCAGTAGGCA-3'
CD40shRNA309R1	5'-TACATCTGTGGCTTCACTAGATTGGGTTCACA GTGTCTGTTCGCTCACTGTCAACAGCA-3'
CD40shRNA309R2	5'-TTCCGAGGCAGTAGGCACACAGACACTGTGA ACCCAATCTACATCTGTGGCTTCACTA-3'
CD40shRNA367R1	5'-TACATCTGTGGCTTCACTATTACAGGTACAGAC AGTGTCTTCGCTCACTGTCAACAGCA-3'
CD40shRNA367R2	5'-TTCCGAGGCAGTAGGCACAGACACTGTCTGTA CCTGTAATACATCTGTGGCTTCACTA-3'
CD40shRNA1656R1	5'-TACATCTGTGGCTTCACTAGTAAATATAAAGGT TGAGTGTTCGCTCACTGTCAACAGCA-3'
CD40shRNA1656R2	5'-TTCCGAGGCAGTAGGCACACACTCAACCTTTA TATTTACTACATCTGTGGCTTCACTA-3'
DF	5′-AGAATCGTTGCCTGCACATC-3′
DR	5′-GAGATAGCAAGGTATTCAG-3′

Abbreviations: F, forward, R, reverse; shRNA, short hairpin RNA.

Table 2. The inhibition ratio of shRNA for CD40 protein

shRNA name	*Inhibition ratio (%)*
CD40 shRNA309	56
CD40 shRNA367	72
CD40 shRNA1656	91

Abbreviation: shRNA, short hairpin RNA.

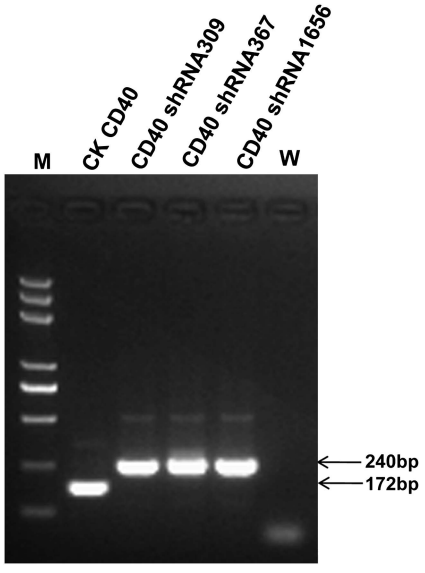

Figure 3. Detection of shRNA expression. shRNAs were amplified from total cDNA. The size of PCR product in the experimental groups was 68 bp longer than that in the control group (CK CD40). W (water as template) was the negative control and M was the DNA maker (2K plus).

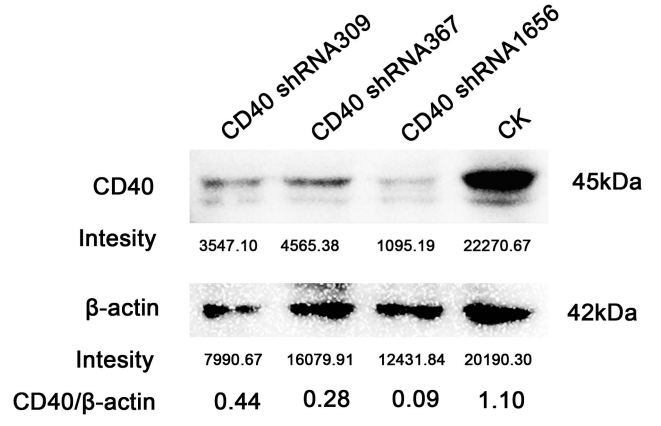

Figure 4. The detection of CD40 protein expression from intestinal DCs. After 50 days of orally administered *S. cerevisiae* yeast, the intestinal DCs were isolated from mice for western blot analysis. Compared with the control group (CK), shRNA in each experimental group had an effective repression on target protein (CD40 protein) *in vivo*. The data were quantified by quantity one (Bio-Rad, Hercules, CA, USA).

shRNA1656-induced INF-γ increased the expression ($P < 0.05$). Thus, the inhibition of CD40 via orally administered recombinant yeast may be used in immunotherapy.

Maturation of DCs is vital to initiate immune responses, because antigen-bearing immature DCs could induce immune tolerance.[49] However, previous studies had proved that CD40/CD40L pathway is important in DC maturation and antigen presentation of DCs to T cells.[16] Blockade of CD40 expression can prevent or treat many autoimmune diseases, vascular disease, transplant rejection without systemic immunosuppression and associated thrombosis.[18,50]

In conclusion, we created a novel and efficient shRNA delivery system via orally administered recombinant yeast for intestinal DC-specific gene silencing and immunomodulation *in vivo* (in mice) for the first time. It has a potential application in many diseases for gene therapy.

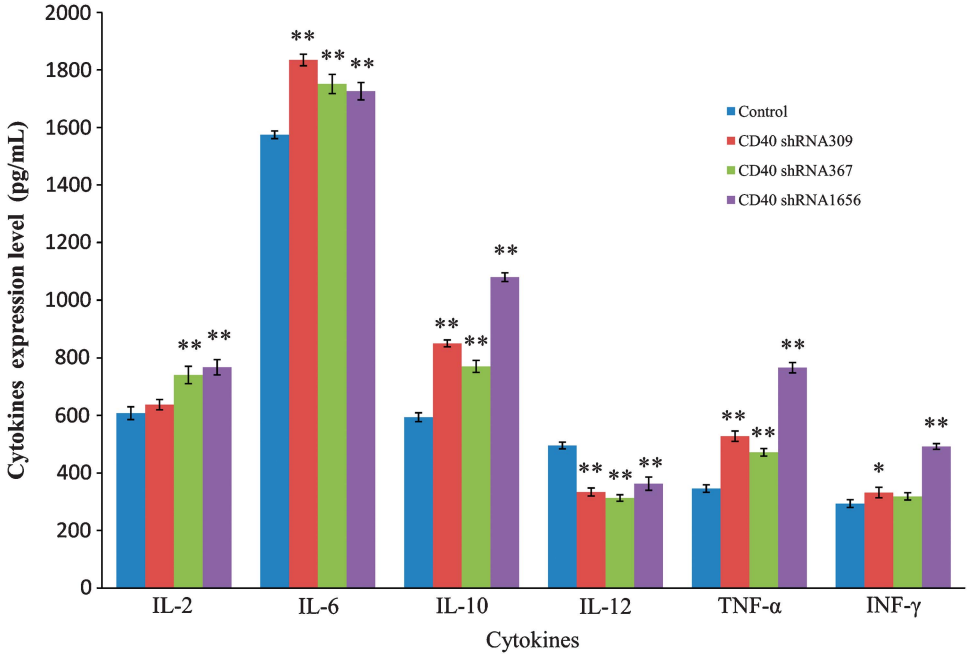

Figure 5. Detection of serum cytokines. Serum cytokines of IL-2, IL-6, IL-10, IL-12, TNF-α and INF-γ were analyzed. There were three repeats in each cytokine detection. Data are expressed as mean ± s.e.m. of three experiments. *$P <$ 0.05, **$P <$ 0.01 vs control shRNA group.

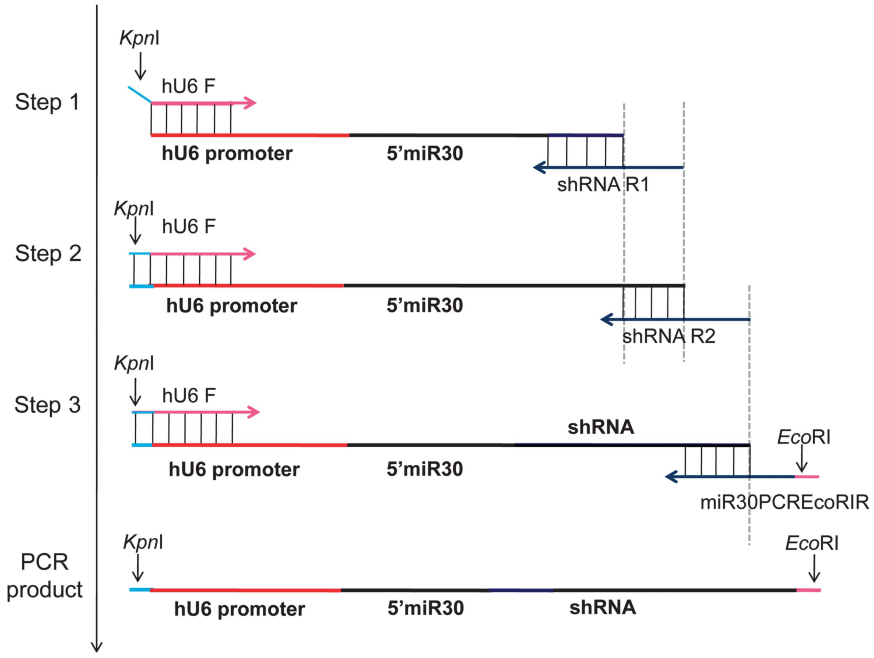

Figure 6. The flow chart of three sequential PCR steps for construction of miR30 expression cassette. In the first-step PCR, plasmid JMB84-hU6-miR30 was used as template. The templates used both in the second and third steps were produced in the preceding step. And, the primers used in each step were: hU6F/shRNA R1, hU6-F/shRNA R2 and hU6-F/miR30PCREcoRIR, respectively.

MATERIALS AND METHODS

Construction of CD40 shRNA expression vectors

The shRNA sequences for CD40 were designed following the website (http://cancan.cshl.edu/RNAi_central/main2.cgi). Three such short hairpin sequences of CD40 are shown in Table 3. The yeast cloning vector JMB84 has multiple cloning sites: *Kpn*I–*Xho*I–*Sal*I–*Cla*I–*Eco*RI–*Sac*I. The human U6 promoter along with 27 nucleotides of U6 snRNA leader sequence was amplified with a forward primer (hU6-F) and a reverse primer (hU6-R), and directionally cloned into plasmid JMB84 between *Kpn*I/*Sal*I sites, resulting in JMB84-hU6. To generate JMB86-hU6-miR30, miR30 sequence that contained an *Eco*RI site was synthesized (Genscript, Nanjing, China) and inserted into *Sal*I/*Sac*I. To introduce the shRNA sequence into the vector and construct JMB84-hU6-shRNA-miR30, three sequential PCR steps were required (Figure 6). For the first-step PCR, we used JMB84-hU6-miR30 as template and hU6-F/shRNA R1 as primers. The template used for amplification in both the second and third steps was produced in the preceding cycle. The primers separately used in the second and third PCR steps were hU6-F/shRNA R2 and hU6-F/miR30PCREcoRIR. Then, the third PCR product was digested and cloned into *Kpn*I/*Eco*RI sites to generate JMB84-hU6-shRNA-miR30. Three different shRNA vectors were constructed with different target sequences of CD40. The primers were shown in Table 1.

Yeast transformation

The yeast strain used in this study was JMY1 (*MATa, his3-△1 trp1-289 rad1-△ ura3-52)*. Plasmids JMB84-hU6-shRNA-miR30 and JMB84-hU6-miR30 (control vector without shRNA expression cassette) were transformed into JMY1 by LiAc method as described by Gietz *et al.*[51] Then, single yeast clones were picked and cultured in the liquid-selective medium until reaching a density of OD600 1.0. Later, the cells were harvested, re-suspended in phosphate-buffered saline (PBS)[52] and stored at − 20 °C until oral administration.

Mice and diets

A total of 16 female C57BL/6 mice aged 10 weeks were purchased from the Animal Breeding and Research Center of Xi'an Jiaotong University, China. They were housed under standard conditions of room temperature and dark-light cycles with plenty of food and water. All mice were treated according to the policy and regulations for care and use of laboratory animals.

Mice were randomly allocated to four immunization groups, each group had four mice. One group was treated with yeast containing JMB84-hU6-miR30 without shRNA interference segment as a negative control. And, three experimental groups were treated with yeast cells harboring vector JMB84-hU6-shRNA-miR30. They were all given 5×10^8 yeast cells every 3 days. After 50 days, serum samples were collected from the tail veins for cytokine analysis via ELISA and intestinal DCs were isolated for the detection of CD40 protein expression by western blot.

Detection of shRNA expression

The shRNA expression vectors were transfected into 293T cells with the reporter vector JMB84-CMV-CD40-GFP-polyA that contained a GFP expression cassette driven by CMV promoter. The intensity of green fluorescence was detected by the fluorescent microscope Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). Total RNA from the 293T cells was extracted using TransZol UP (TransGen, Beijing, China), followed by chloroform extraction and isopropanol precipitation according to the operating manual. Subsequently, cDNA was synthesized with PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). The expression of shRNA in mammalian cells (293T cells) was investigated by PCR with a forward primer (DF) and a reverse primer (DR) (Table 1), respectively, binding onto the 5' and 3' miR30.

Table 3. CD40 shRNA target sequences

Name	*shRNA target sequence*
CD40 shRNA309	5'-CACAGACACTGTGAACCCAATC-3'
CD40 shRNA367	5'-CAGACACTGTCTGTACCTGTAA-3'
CD40 shRNA1656	5'-CACACTCAACCTTTATATTTAC-3'

Abbreviation: shRNA, short hairpin RNA.

Isolation of intestinal DCs

The intestinum tenues and colons without mesenterium and fat in each group were separated, which were operated in the precooling PBS. Before cutting the samples into tiny tissue, the intestinum tenues and colons were washed with PBS containing 10% fetal bovine serum. Then, adding 20 ml digestive juice I (PBS with 1 mM EDTA, 1 mM dithiothreitol, 10% fetal bovine serum) for each group to digest the samples. After 40 min incubation at 37 °C, 155 r.p.m., digested fragments were obtained by centrifugation at 1500*g* for 5 min. Further digestion was achieved by adding 20 ml digestive juice II (PBS with 100 U l^{-1} collagenase IV, 10% fetal bovine serum) and incubation at 37 °C, 155 r.p.m. for 40 min after two times washing with PBS. The digested cells were filtered through a stainless-steel sieve, and cell suspensions were washed two times with PBS. The supernatant was completely discarded and added 400 μl of buffer per 10^8 cells (PBS with 0.5% bovine serum albumin, 2 mM EDTA) and 100 μl of anti-CD11c-conjugated magnetic cell sorting microbeads per 10^8 cells (Miltenyi Biotec, Bergisch, Germany). After incubation for 30 min at 4 °C, intestinal DCs were purified from the mix cells by magnetic cell sorting with MACS Separation Columns (Miltenyi Biotec).

Western blot

Total lysates with intracellular proteins were treated with loading buffer in boiling water for 10 min. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis acrylamide gels and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). Then, the membrane was blocked with 5% skim milk and washed six times with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% (v v^{-1}) Tween-20). The membrane was incubated with primary rabbit anti-mouse CD40 polyclonal antibodies (cat. no. ab13545; Abcam, Hong Kong, China). The membrane was washed again and then reacted with the second antibody, which was horseradish peroxidase-conjugated goat anti-rabbit antibody (cat. no. sc2004; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After removal of the nonspecific binding antibodies by washing the membrane with TBST, the signal was detected by MicroChemi (Bio-Imaging Systems, DNR, Jerusalem, Israel) with chemiluminescent substrates.

ELISA assay

CD40 immunization treatment mice contained three vaccinated groups and a control group as mentioned previously. Sera from four mice in each experimental group were mixed for ELISA using PeproTech's Full ELISA Kits (PeproTech, Rocky Hill, NJ, USA) to estimate IL-2, IL-6, IL-10, IL-12, TNF-α and INF-γ according to the manufacturer's instructions. Three repeats detection of each cytokine were preformed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

LZ, TZ, LW and ZC performed research; TZ, LW, SS, ZC analyzed data; LZ and ZZ wrote the paper; and ZZ designed experiments.

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