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ORIGINAL ARTICLE

Heterologous expression of carcinoembryonic antigen in *Lactococcus lactis* via LcsB-mediated surface displaying system for oral vaccine development

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S-layer

Background/Purpose: Carcinoembryonic antigen (CEA) is an attractive target for immunotherapy because it is expressed minimally in normal tissue, but is overexpressed in a wide variety of malignant epithelial tissues. Lactic acid bacteria (LABs), widely used in food processes, are attractive candidates for oral vaccination. Thus, we examined whether LABs could be used as a live vaccine vector to deliver CEA antigen.
Methods: CEA was cloned into an *Escherichia coli/Lactococcus lactis* shuttle vector pSEC:LEISS under the control of a nisin promoter. For displaying the CEA on the cell surface of the *L. lactis* strain, the anchor motif LcsB from the S-layer protein of *Lactobacillus crispatus* was fused with CEA. Intracellular and cell surface expression of the CEA–LcsB fusion was confirmed by western blot analysis.
Results: Significantly higher levels of CEA-specific secretory immunoglobulin A in the sera of mice were observed upon oral administration of strain cultures containing the CEA–LcsB fused protein. In addition, the CEA–LcsB antigen group showed a higher spleen index compared to the CEA antigen alone or negative control, demonstrating that surface-displayed CEA antigen could induce a higher immune response.

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Conclusion: These results provided the first evidence for displaying CEA antigen on the cell surfaces of LABs as oral vaccines against cancer or infectious diseases.

Introduction

Carcinoembryonic antigen (CEA), which was first identified in human colon cancer in 1963, is a 180-kDa glycoprotein. Normally, CEA can be expressed in limited areas of the adult human body, but it is overexpressed in a high percentage of adenocarcinomas, particularly in those of the colon, pancreas, breast, lungs, rectum, and stomach. Hence, CEA is used as a tumor-related marker at clinic diagnosis.[1,2] Previous studies have proved that CEA has high immunogenicity and can elicit strong T-cell and humoral immune responses, which are helpful in inhibiting the growth of malignant tumors *in vivo*.[3] Apparently, development of a CEA-based vaccine to induce potent T-cell immunity will be significant in the immunotherapy of tumors.

Many strategies for development of CEA-based vaccines have been reported, including recombinant live vector vaccines (viral and/or bacterial vector vaccines), nucleic acid vaccines (DNA and/or RNA replicon vaccines), and protein or peptide vaccines.[4–8] Viral vectors or DNA vaccines offer the ability to deliver antigen into the cytosol, and therefore into the conventional major histocompatibility complex (MHC) class I processing pathway. However, concern about the disadvantages, including the limited DNA carrying capacity, toxicity, immunogenicity, the possibility of random integration of the vector DNA into the host genome, and their high coat, may limit the application of viral and DNA vaccines.[9,10] Thus, to circumvent these issues, the use of live, food-grade, noninvasive, nonpathogenic bacteria as antigen vehicles is a promising strategy.

Lactic acid bacteria (LABs) are generally regarded as safe microorganisms, and some of them are able to stimulate the immune system of the host as adjuvants due to their probiotic properties and their immunomodulation capacity, which makes them advantageous live vaccines.[11,12] Among LABs, *Lactococcus lactis* is, by far, the most extensively studied with respect to its physiology, metabolic pathways, and regulatory mechanisms, and its genome was the first LAB genome to be completely sequenced and annotated.[13] Because of its ability to pass through the intestinal tract without colonization, the use of *L. lactis* as a vaccine vector is emerging as one of the most advanced prototypes of a possible new class of bacterial vaccines derived from noninvasive, nonpathogenic Gram-positive bacteria.[11,14,15] Immunization with *L. lactis*-delivered vaccines elicits immune responses specific to heterologous antigens.[16,17] To date, many expression systems have been developed to produce recombinant proteins for various biotechnological applications in LABs. The best-characterized controllable expression system is based on the use of the *nisin biosysthesis* promoter p*nisA*. This system is versatile and can be used to produce large quantities of prokaryotic and eukaryotic proteins because there is a linear dependency between the amount of nisin added to the culture medium and the promoter activity.[18] Several antigens (e.g., L7/L12, TTFC, HA, and capsid protein of PCV) have been successfully expressed using the NICE system (nisin-controlled gene expression) or its derivatives.[16,17,19,20] Note that a protective immune response depends not only on the antigen and the delivery vehicle, but also on the location of the antigen.[19] Various genetic engineering tools have been developed to express antigens and therapeutic molecules efficiently at different cellular localizations (i.e., cytoplasm, cell wall, or extracellular medium).[19,21] To display antigen on the cell surface of *L. lactis* cells, various anchoring motifs, such as LysM and LcsB, as well as M6 protein, have also been explored for their efficiency in attaching hybrid protein to the cell membrane or cell wall of LABs.[19,22–24]

In this work, we constructed genetically modified *L. lactis* that produced recombinant CEA antigen by using the NICE system. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting showed that CEA was displayed on the cell surface of *L. lactis* under the aid of the anchoring motif LcsB. Immunization analysis demonstrated that the surface-displayed CEA induced an immune response according to activation of the spleen and secretion of immunoglobulin A (IgA).

Methods

Bacterial strains and growth conditions

All of the strains, plasmids, and oligonucleotide primers used in this study are described in Table 1. *L. lactis* NZ9000 was grown in M17 broth (Oxoid, Basingstoke, Hants, UK) supplemented with 0.5% (w/v) glucose (GM17) at 30°C or 42°C anaerobically, respectively. *Escherichia coli* DH5α was grown aerobically in Luria–Bertani broth at 37°C. Chloramphenicol was used at 10 μg/mL and 5 μg/mL for *E. coli* and *L. lactis*, respectively.

BALB/c mice (6–8 weeks old; Medical Experimental Center of Shandong University, China) were housed in a pathogen-free isolator under sterile conditions in the animal facilities of Jinan Health Science Exchange and Service Center (China). All experiments were performed according to protocols in accordance with institutional guidelines.

Plasmids and DNA manipulations

Standard recombinant DNA techniques were performed as described previously.[23] Electroporation of *L. lactis* was performed using a Gene Pulser (2500 V, 200 Ω, 25 μF, 5 ms,

Table 1 Strains, plasmids and primers used in this study

Strain, plasmid, or primer	Relevant characteristics [a]	Refs
Strains		
E. coli DH5α	*supE44ΔlacU169φ80lacZΔM15hsdR17recA1endA1gyrA96thi-1relA1*	Novagen
L. lactis NZ9000	MG1363 *pepN*::*nisRK*. Most commonly used host of the NICE system	20
Plasmids		
pSEC:LEISS	GK:Cmr; mature Nuc fused at 5′ end with sequence of propeptide LEISSTCDA	20
pMD18-T-S	pMD18-T containing *slpB* of *Lactobacillus crispatus* K2-4-3	26
pMD18-T-cea	pMD18-T containing *cea* of HT-29 cells	This study
pSEC:LEISS-Cea	pSec:Leiss:Nuc(*Nsi*I/*Hin*dII::*cea*)	This study
pSEC:LEISS-Cea2	pSec:Leiss:Nuc(*Nsi*I/*Eco*RI::*cea2*)	This study
pSEC:LEISS-Cea-LcsB	pSec:Leiss:Nuc(*Eco*RI/*Hin*dIII::*cea2*-lcsB)	This study
Primers		
CEA-NsiI	5′-GCTATGCATCCATGGAGTCTCCCTCGGCCCCT-3′	This study
CEA-ClaI	5′-CCAATCGATCTATATCAGAGCAACCCCAACCAG-3′	This study
CEA-NsiI&NcoI	5′-GAGATATCGTCGACTTGTGATGCAT-3′	This study
CEA-EcoRI	5′-CAG AATTCTATCAGAGCAACCCCAACCA-3′	This study
LcsB-EcoRI	5′-GAGAATTCAATTGGTTCGTTGCACCTAAG-3′	This study
LcsB-HindIII:	5′-GCGAAGCTTTTAAAAGTTTGAAGCCTTTACG-3′	This study

Bio-Rad Laboratories, USA) as described previously.[25] Polymerase chain reaction (PCR) products were generated using rTaq polymerase (TaKaRa, Tokyo, Japan).

Cea gene was PCR amplified by primers CEA-NsiI/CEA-ClaI using the cDNA of HT-29 cells as template.[26] The PCR products were ligated into pMD18-T, resulting in the pMD18-T-cea vector. Plasmids were extracted using Plasmid Mini Mix (Omega, GA, USA) and sent to the Beijing Genomics Institute (BGI, China) for sequencing.

The different CEA expression vectors are described in Fig. 1. The *cea* fragments were digested from pMD18-T-cea vector with *Nsi*I and *Hin*dIII, and ligated into the *Nsi*I/*Hin*dIII digested plasmid pSEC:LEISS, generating the pSEC:LEISS-Cea vector. *Cea* was PCR amplified with primers CEA-NsiI or NcoI and CEA-EcoRI using the pSEC:LEISS-Cea as templates. The PCR products were digested with *Nsi*I/*Eco*RI and cloned into *Nsi*I/*Eco*RI digested pSEC:LEISS vector, yielding plasmid pSEC:LEISS-Cea2. The LcsB fragments were PCR amplified using primers LcsB-EcoRI and LcsB-HindIII from pMD18-S. The resulting PCR products were digested with *Eco*RI/*Hin*dIII and cloned into the corresponding site of pSEC:LEISS-Cea2, resulting in the recombinant plasmid pSEC:LEISS-Cea-LcsB. Transformation of *L. lactis* by electroporation was performed as described previously,[22] resulting in the recombinants, *L. lactis*/pSEC:LEISS, *L. lactis*/pSEC:LEISS-Cea, and *L. lactis*/pSEC:LEISS-Cea-LcsB, respectively.

Expression and extraction of nisin-induced proteins in *L. lactis*

Gene expression was carried out as described previously.[23] *L. lactis* NZ9000 harboring the recombinant plasmids constructed above were grown overnight at 37°C in GM17 medium containing 5 μg/mL chloramphenicol. Subsequently, the overnight cultures were 100-fold diluted in 1 L fresh GM17 medium with 5 μg/mL chloramphenicol, and incubated until the cell density reached 0.4 (at 600 nm). Then, nisin (Sigma, St Louis, MO, USA) was added to cultures at a final concentration of 10 ng/mL. Growth was continued for 7 hours at 18°C to induce protein expression.

The extraction of proteins from *L. lactis* was performed as described by Hu et al.[23] The supernatant fraction was obtained according to the trichloroacetic acid/acetone method. Briefly, 100 μL 100% trichloroacetic acid was mixed with 1 mL supernatant for protein precipitation. Subsequently, the mixtures were vortexed for 15 seconds and placed on ice for 15 minutes. The pellets of protein were obtained by centrifugation at 14,000 *g* for 10 minutes and washed twice with 100 μL acetone. After drying in air, the pellets were dissolved in 50 μlL 1 × SDS loading buffer.

Membrane and cell wall fractions were isolated from 10-mL cultures of *L. lactis* induced by nisin at 18°C for 6 hours as follows. The cells from 10 cultures were harvested and resuspended in 10 mL phosphate-buffered saline (PBS). After disrupting the cells by sonication on ice at 400 W for three cycles (one cycle consisted of 50 times sonication for 3 seconds, with 8-second intermissions), the resuspended cells were centrifuged for 10 minutes at 10,000 *g* to remove cell debris. The resulting supernatants were centrifuged for 30 minutes at 100,000 *g* and the membrane and cell wall fractions were recovered in the pellet, which were resuspended in 50 μL PBS and mixed with 1 × SDS-PAGE buffer. The sample was incubated at 37°C for 5 minutes before electrophoresis in standard 12% SDS-PAGE.

Western blotting

The proteins separated by 12% SDS-PAGE were electrically transferred onto a polyvinylidene fluoride membrane at 200 mA for 1.5 hours in an ice-water bath. The membrane was washed with TBST buffer [Tris Buffered Saline with Tween, 20mM Tris—HCl (pH 7.4), 500mM NaCl, 0.01% Tween 20) three times for 15 minutes each in an ice bath and blocked in blocking buffer (TBST buffer with 5% nonfat milk) for 1 hour at room temperature. The membrane was

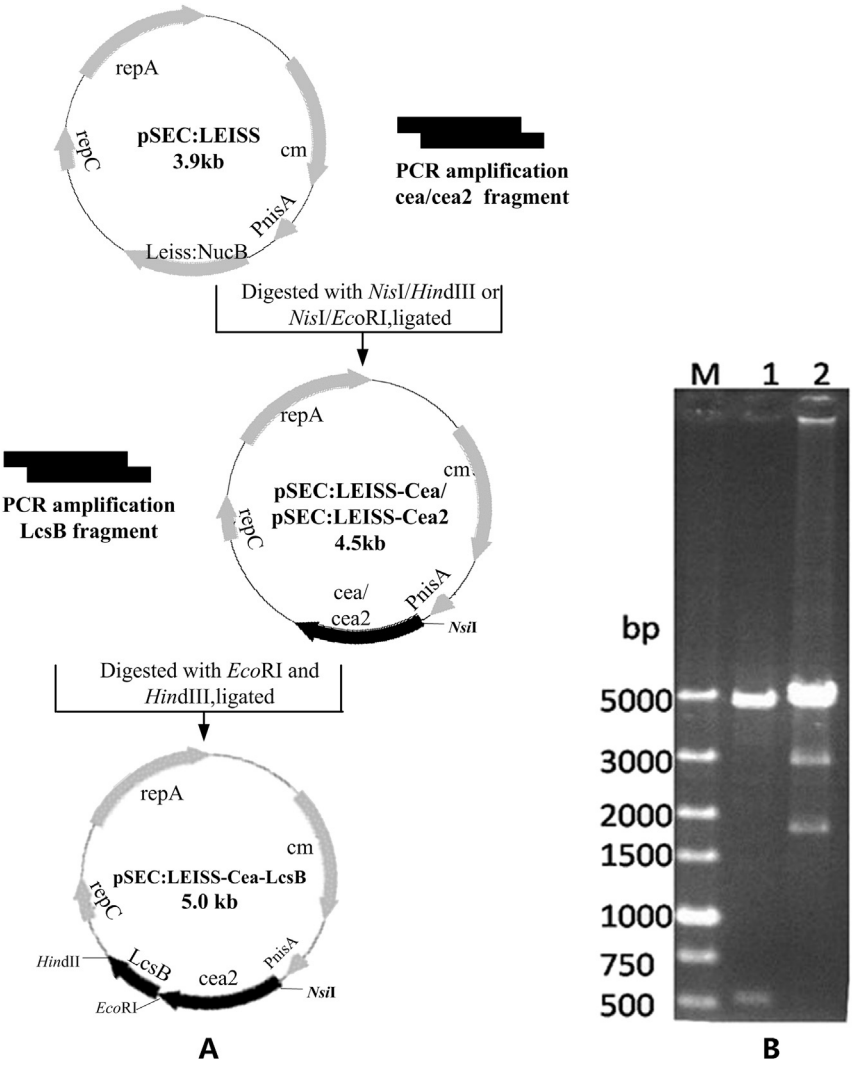

Figure 1. (A) A schematic construction of the expression vectors pSEC:LEISS-Cea, pSEC:LEISS-Cea2, and pSEC:LEISS-Cea-LcsB. (B) PCR products of CEA and CEA—LcsB gene fragments. Lane M, DNA markers; Lane 1, PCR products of CEA gene; Lane 2, the CEA-LcsB gene. CEA = carcinoembryonic antigen; PCR = polymerase chain reaction.

washed three times with TBST buffer again and then incubated overnight with the primary anti-CEA polyclonal antibody (eBioscience, California, USA) at a dilution of 1:600. Subsequently, the membrane was washed three times in TBST, and incubated with horseradish peroxidase (HRP)-coupled secondary antibody (Solarbio, Beijing, China) at a dilution of 1:5000 with TBST buffer containing 5% nonfat milk powder for 1 hour. The membrane was washed as above. The chromogenic reaction was terminated with distilled water after 1 minute using DAB (3,3-diaminobenzidine)/$NiCl_2$ as the chromogenic agent.

Vaccination with CEA

Cultures of the strain *L. lactis*/pSEC:LEISS, *L. lactis*/pSEC:LEISS-Cea, and *L. lactis*/pSEC:LEISS-Cea-LcsB were grown and induced as described above. Cells from 10-mL cultures were harvested and resuspended in PBS to a final concentration of 10^9 colony-forming units (CFU)/mL for *L. lactis*/pSEC:LEISS and *L. lactis*/pSEC:LEISS-Cea. For *L. lactis*/pSEC:LEISS-Cea-LcsB, the cell densities were adjusted to a series of concentrations of 10^8 CFU/mL, 10^9 CFU/mL and 10^{10} CFU/mL, respectively.

Immunization of mice

BALB/c mice (6—8 weeks old) were obtained from the Medical Experimental Centre of Shandong University and divided into six groups (PBS, *L. lactis*/pSEC:LEISS, *L. lactis*/pSEC:LEISS-Cea, 10^8 CFU/mL *L. lactis*/pSEC:LEISS-Cea-LcsB, 10^9 CFU/mL *L. lactis*/pSEC:LEISS-Cea-LcsB, and 10^{10} CFU/mL *L. lactis*/pSEC:LEISS-Cea-LcsB). Each group contained 12 mice. Doses of 0.5 mL were administered orally to each group on Days 1, 4, 7, 14, 21, 28, and 35. One week after the last administration, thymus and spleen were collected and weighed to compare the thymus index and spleen index for those groups. Sera and intestinal washings were collected to evaluate the immunogenicity with enzyme-linked immunosorbent assay (ELISA). The small intestine was collected and cut into 1-cm pieces. The intestines were suspended in 1 mL

PBS and vortexed for 30 seconds. After centrifugation at 12,000 *g* for 20 minutes, supernatants were collected and stored at −80°C.

Analysis of antibody response

ELISA was used to determine serum immunoglobulin G (IgG) and fecal secretory IgA (sIgA) antibody responses. Flat transparent 96-well microtiter plates (Costar, Maine, USA) were coated overnight at 4°C with 80 μL purified CEA protein per well at a concentration of 50 ng/mL in three parallel groups. After washing three times with PBST (PBS containing 0.05 % Tween 20), the wells were blocked with 100 μL PBST containing 5% non-fat milk powder at 4°C for 8 hours. Twenty-five-fold dilutions of sera were applied and incubated at 37°C for 1 hour after being washed three times with PBST. The wells were washed again and 80 μL HRP-conjugated antimouse IgG antibodies was added (γ-chain specific; Sigma—Aldrich, Steinheim, Germany) at 1:2000 dilutions, and incubated for 1 hour at 37°C. The wells were washed again and incubated with 100 μL 3,3′,5,5′-tetramethylbenzidine substrate (Sigma—Aldrich) for 10 minutes at room temperature avoiding light. Color reaction was terminated by the addition of 50 μL 2M H_2SO_4 and absorbances were read at 450 nm.

ELISA was performed essentially as described for serum samples, with minor modifications as follows. Purified CEA protein at a concentration of 5 μg/mL, and 100-fold dilution of intestinal washings were applied and HRP-conjugated antimouse IgA antibodies (α-chain specific; Sigma—Aldrich) at 1:2000 dilution were used instead of antimouse IgG antibodies.

Statistical analysis

One-way analysis of variance (SAS, version 6.03) was performed to find significant differences among various parameters. A significance level of $p < 0.05$ was used.

Results

Cloning and characterization of CEA gene

The whole *cea* gene was obtained from the cDNA of HT-29 cells, which was extracted previously in our laboratory.[26] The *cea* consisted of 1872 bp that encoded a polypeptide of 623 amino acid residues, which shared 99% identity with CEA-related cell adhesion molecule 5.

Expression of CEA in L. lactis

The pSEC:LEISS vector, which contained the Usp45 signal peptide (Usp45 is the major secreted protein of *L. lactis*) and synthetic oligopeptide LEISSTCDA (called Leiss), was used in this study. To express and target CEA on the surface of *L. lactis*, the LcsB anchor motif, the C-terminal region of SlpB of *Lactobacillus crispatus* K2-4-3 that served as a functional scaffold to target the heterologous proteins to the cell surfaces of LABs *in vitro* and *in vivo*, was fused to CEA.[23] Expression of CEA and CEA—LcsB was confirmed by SDS-PAGE and western blot analysis using the CEA-specific polyclonal antibody. As shown in Fig. 2, the obvious bands of CEA (69 kDa, Lane 1) and CEA—LcsB (89 kDa, Lane 3) were observed at the estimated size, suggesting that CEA was successfully expressed in *L. lactis*. Moreover, an obvious band, representing the CEA—LcsB fusions, was clearly visible in the membrane and cell wall fractions (Fig. 2B, Lane 4), indicating that CEA was displayed on the cell walls of *L. lactis* with the aid of LcsB motif.

Oral immunization, thymus index and spleen index

After immunizing mice six times over a 5-week period, thymus and spleen were collected and weighed to determine the thymus index and spleen index for the six groups.

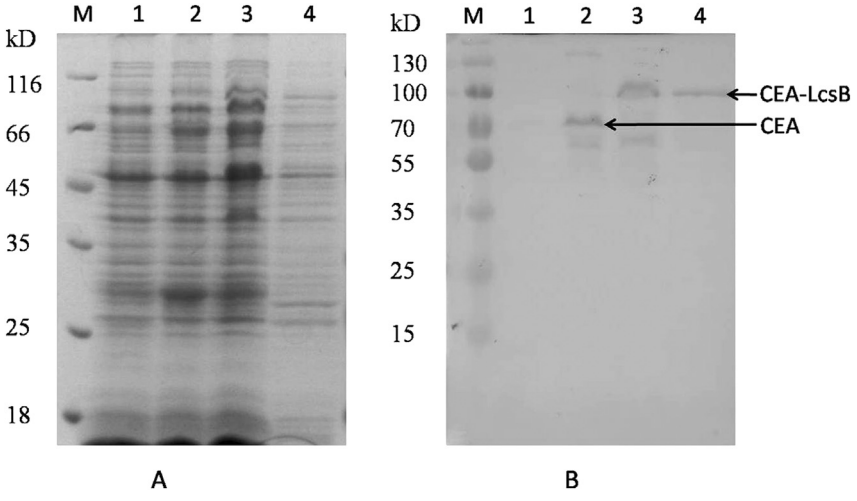

Figure 2. Expression and fractionation of recombinant CEA proteins synthesized from pSEC:LEISS-Cea and pSEC:LEISS-Cea-LcsB in *Lactococcus lactis* NZ9000 by (A) sodium dodecyl sulfate polyacrylamide gel electrophoresis and (B) western blot analysis. M, Protein marker; Lane 1, total proteins in *L. lactis* harboring pSEC:LEISS; Lane 2, total proteins in *L. lactis* harboring pSEC:LEISS-Cea; Lane 3, total proteins in *L. lactis* harboring pSEC:LEISS-Cea-LcsB; Lane 4, surface proteins in *L. lactis* harboring pSEC:LEISS-Cea-LcsB. CEA = carcinoembryonic antigen.

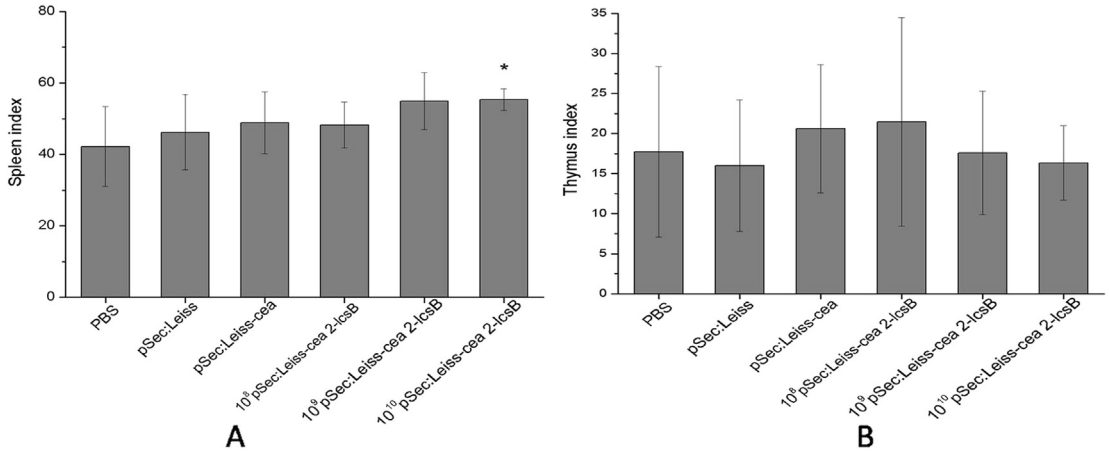

Figure 3. (A) Spleen index and (B) thymus index of mice orally immunized with phosphate-buffered saline, *Lactococcus lactis* CEA, or CEA—LcsB. CEA = carcinoembryonic antigen.

The spleen index of mice increased with the increase of the antigen concentration by gavage (Fig. 3). The spleen index in Group 6, which was orally administered 10^{10} CFU/mL *L. lactis*/pSEC:LEISS-Cea-LcsB cells, was significantly higher than in the control group ($p < 0.05$). Meanwhile, the thymus index of each group of mice did not obviously differ among the groups. These results indicated that oral gavage did not significantly affect the mouse thymus index.

Immune response in mice against CEA

To examine the immunogenicity of CEA, sera and intestinal washings were collected for ELISA analysis to determine sera IgG and intestinal washings sIgA antibody responses. As shown in Fig. 4, *L. lactis*/pSEC:LEISS-Cea-LcsB with different cell densities showed a significant sIgA response, while no significant sIgA response was found in *L. lactis*/pSEC:LEISS and *L. lactis*/pSEC:LEISS-Cea (Fig. 4A), suggesting that only the surface-displayed CEA induced hosts to produce specific anti-CEA antibody of sIgA. However, no obvious difference in the value of sera IgG was found, which may have been because of the lack of immune time or fewer immune times.

Discussion

LABs possess a number of properties that make them attractive candidates for oral vaccination. These bacteria have been used for centuries in the fermentation and preservation of food, and are considered to be safe organisms with a GRAS (Generally Recognized As Safe) status.[11] *L. lactis*, a model LAB, is a potential candidate for the production of biologically useful proteins.[21] Protein production in *L. lactis* for oral vaccines offers several advantages: *L. lactis* produces low amounts of native exoproteins and it does not colonize the digestive tract of humans or animals, but can survive passage through the gut.[14,15] Thus, a transient presentation of the antigen to the mucosal immune system by noncommensal bacteria may avoid the risk of immunotolerance. Furthermore, *L. lactis* is approximately the same size as biodegradable

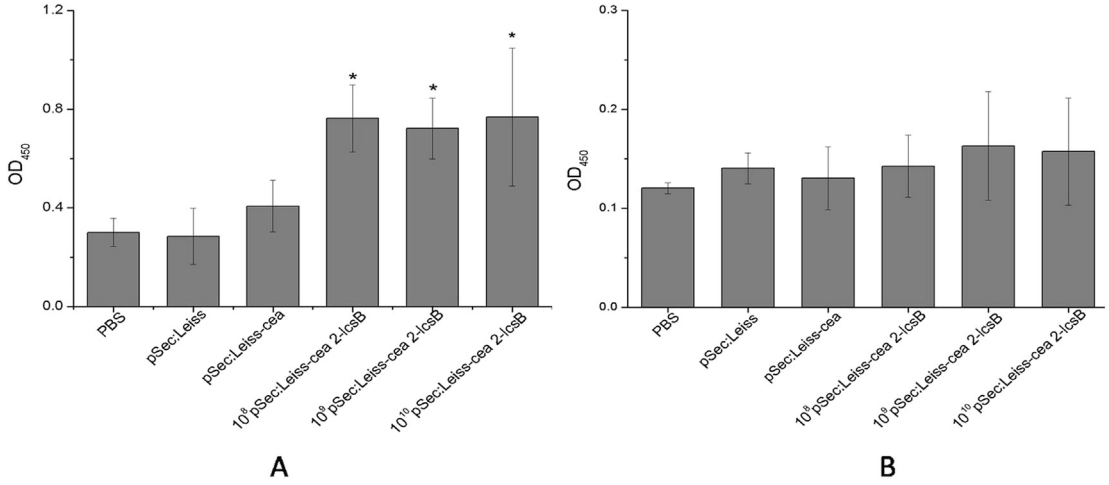

Figure 4. (A) CEA-specific mucosal secretory IgA and (B) serum IgG detected by enzyme-linked immunosorbent assay from mice orally immunized with phosphate-buffered saline or *Lactococcus lactis* CEA or CEA—LcsB strains. CEA = carcinoembryonic antigen.

microparticles that are taken up by M cells and can act as effective oral vaccine vehicles.[27,28] In this study, we first developed a CEA vaccine based on live recombinant *L. lactis* that produced CEA on the surface of *L. lactis* cells.

The production of a desired antigen by *L. lactis* can occur in three locations: intracellular, extracellular, and cell wall anchored. Extracellular antigen results in direct interaction with the environment. Cell-wall anchored antigen, not only interacts directly with the environment, but also can avoid proteolytic degradation.[21] In this study, only the surface-displayed CEA induced the gut to produce specific anti-CEA antibody of sIgA, confirming that the surface-displayed antigens on the *L. lactis* cells were more resistant to proteolysis and easily taken up by the epithelial M cells of the small intestine.[16,29] To date, many anchor motifs were exploited to target the heterologous proteins to the cell surface of LABs, such as LPXTG (conserved Leu-Pro-X-Thr-Gly)-containing proteins, transmembrane protein PgsA, LysM motif-containing proteins, and S-layer proteins.[11,24] However, most surface display systems for vaccine applications are limited in the size of foreign antigens they can display. A previous study has shown that LcsB has the ability to locate the heterologous proteins (green fluorescent protein 27 kDa, β-galactosidase 66 kDa) on the cell surface, indicating that LcsB can carry amino acid insertions of up to 500 residues to the cell surface.[23] Therefore, LcsB was used as the anchor motif to target the CEA on the surface of the *L. lactis* cells. The results here showed that LcsB displayed the CEA protein on the surface of *L. lactis* cells, resulting in an obvious band in the membrane and cell wall fractions (Fig. 2B, Lane 4). This was also the first evidence to display CEA on the cell wall of *L. lactis* with the aid of LcsB.

CEA is an attractive target for immunotherapy because it is expressed minimally in normal tissue, but is overexpressed in a wide variety of malignant epithelial tissues.[1,2] This study demonstrated for the first time that the surface-displayed CEA in *L. lactis* can induce immune responses. The spleen, which contains a large number of lymphocytes and macrophages, is the largest immune organ, and exerts an antitumor effect through a variety of mechanisms. As shown in Fig. 3A, the mouse spleen index in Group 6, which was orally administered 10^{10} CFU/mL *L. lactis*/pSEC:LEISS-Cea-LcsB cells, was significantly higher than in the control group ($p < 0.05$), indicating that the surface-displayed CEA activated the spleen. Moreover, recombinant CEA improved secretion of specific sIgA according to activated lymphoid tissue of intestinal mucosa and increased immune function of gastrointestinal mucosa, thus exerting significant effects on antiallergic, anti-inflammatory and antitumor activity. Unfortunately, the recombinant CEA in *L. lactis* did not induce serum IgG (Fig. 4B). This might have been because serum IgG was produced slowly or serum IgG had not yet reached a detectable titer.

In conclusion, the surface-displayed CEA constructed here effectively induced the immune response, providing the possibility of the use of *L. lactis* CEA in vaccination protocols against cancer or infectious diseases.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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