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Construction and characterization of C-C motif chemokine ligand 20 (CCL20) secreting recombinant *Lactococcus lactis* IL1403 as a mucosal adjuvant

점막면역백신 어쥬번트로 활용하기 위한 CCL20 분비 재조합 *Lactococcus lactis* IL1403의 구축 및 특성화

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By
Dong Suk Park

Department of Agricultural Biotechnology
Graduate School, Seoul National University

농 학 석 사 학 위 논 문

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지도교수 최 윤 재
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Abstract

Construction and characterization of C-C motif chemokine ligand 20 (CCL20) secreting recombinant *Lactococcus lactis* IL1403 as a mucosal adjuvant

Dongsuk Park
Department of Agricultural Biotechnology
The Graduate School
Seoul National University

In livestock industry, it is necessary to protect livestock from disease such as porcine epidemic diarrhea (PED). Vaccination has been broadly used for disease control. Oral vaccination is a less stressful and safe method compared to other vaccination. Although, efficiency of oral vaccination is lower than other vaccination method because of internal and external barriers such as gastric acid and enzymes. Vaccine adjuvant has been used for enhancing oral vaccine efficiency. Lactic acid bacteria has been also used as oral vaccine delivery vehicle for enhancing oral vaccine efficiency because lactic acid bacteria easily endure acidic condition and is safe for animals.

Oral vaccination stimulates mucosal immune system and induces the secretion of immunoglobulin A. M cells mainly contribute to activating oral vaccine. M cells are antigen sampling cells in Peyer's patch of gut-associated lymphoid tissue (GALT).

CCL20 is C-C motif chemokine ligand 20 and is secreted

beneath follicle-associated epithelium (FAE) of GALT. The basic function of the CCL20 is to chemoattract immune cells including dendritic cells, B lymphocytes and macrophages. Recently, it was reported that CCL20 can induce M cell differentiation.

In this study, we hypothesized that recombinant CCL20 can be a potential vaccine adjuvant for enhancing oral vaccine efficiency.

CCL20 has antimicrobial activity against gram negative bacteria. Minimal inhibition concentration (MIC) test was conducted for confirming whether CCL20 has antimicrobial activity against recombinant host, *L. lactis*. It is validated that CCL20 has no antimicrobial activity against *L. lactis*.

The peptide sequence of mouse CCL20, USP 45 and M cell targeting peptide was obtained from National Center for Biotechnology Information (NCBI). And then, recombinant DNAs (UC, UCM, UMC) were codon-optimized for *L. lactis* IL1403 and synthesized. Recombinant DNAs and mother plasmid (pIL.Ptuf) were enzyme-digested with restriction enzymes, Nde I and Xho I and cloned with DNA ligase. As a result, three recombinant CCL20 vectors (pIL.Ptuf.UC, pIL.Ptuf.UCM, pIL.Ptuf.UMC) were constructed.

To validate whether recombinant DNAs were correctly inserted into backbone vector, antibiotic resistance selection, colony PCR with specific primer and DNA sequencing with M17 primer was conducted. All of constructed vectors correctly contained each recombinant CCL20 DNAs (UC, UCM, UMC). After validation, recombinant CCL20 vectors were electroporated into *L. lactis* IL1403 competent cells.

SDS-PAGE and western blot assay were conducted to validate whether recombinant CCL20 proteins were expressed from constructed CCL20 transformants. Recombinant CCL20 peptides (UC, UCM, UMC) were correctly expressed from transformants.

In vitro chemotaxis assay with JAWS II cells was also conducted to validate biological activity of recombinant CCL20. JAWS II cells were correctly migrated to lower well of transwell chamber containing recombinant CCL20 expressed from CCL20 transformants (UC, UCM, UMC).

To validate whether recombinant *L. lactis* IL1403 can be used as oral vaccine adjuvant, *in vivo* functional assay was conducted in Balb/c mouse with a Brachyspira membrane protein B (BmpB) as model antigen. Recombinant CCL20 transformants were administrated into mouse for 1 weeks and BmpB was administrated 3 times for 3 weeks. After challenging BmpB, IgA from feces and IgG from serum was increased in UC, UCM expressing group.

In conclusion, BmpB specific mucosal immune response was increased in UC, UCM group indicating that recombinant CCL20 transformants could be an oral vaccine adjuvant.

Key words : C-C motif chemokine ligand 20, Lactic acid bacteria, M cell, Oral vaccine adjuvant
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List of Abbreviations

BLAST : Basic local alignment search tool
BmpB : Brachyspira membrane protein B
CCL20 : C-C motif chemokine ligand 20
CCR6 : C-C motif chemokine receptor 6
DC : Dendritic cell
DNA : Deoxyribonucleic acid
D.W. : Distilled water
E.coli : *Escherichia coli*
ECL : Enhanced chemiluminescence
ELISA : Enzyme-linked immunosorbent assay
FAE : Follicle associated epithelium
FBS : Fetal bovine serum
GALT : Gut-associated lymphoid tissue
GRAS : Generally recognized as safe
HRP : Horse radish peroxidase
IACUC : Institute animal care and use committees
IgA : Immunoglobulin A
IL-1α : Interleukin-1 alpha
kDa : kilo dalton
LAB : Lactic acid bacteria
L. lactis : *Lactococcus lactis*
M : Marker
M cell : Microfold cell
MIP-3α : Macrophage inflammatory protein-3 alpha
MTP : M cell targeting peptide
NCBI : National Center for Biotechnology Information
NICEM : National Instrumental Center for Environmental

Management

OD : Optical density

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

PED : Porcine epidemic diarrhea

PPs : Peyer' patches

RankL : Receptor activator of NF-κB ligand

SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SED : Sub-epithelial dome

TCA : Trichloroacetic acid

TNF-α : Tumor necrosis factor-alpha

UC : USP45-CCL20

UCM : USP45-CCL20-MTP

UMC : USP45-MTP-CCL20

USP45 : Ubiquitin specific peptidase 45

I. Introduction

Digestive tract from mouth to rectum in animal and human has a tubular structure and is in close contact with gastrointestinal environment including pathogen. There is several specific immune system in gut called as a mucosal immune system to protect animal's body from pathogens. In livestock industry, one of the waterborne diseases such as porcine epidemic diarrhea (PED) is closely associated with mucosal immune system. Therefore, protection of the waterborne disease by inducing mucosal immune response should be conducted to prevent livestock industry from financial loss.

M cells as speciality of antigen sampling cells have been used for enhancing mucosal immune response. M cells are a kind of intestinal epithelial cells located in Peyer's patch of gut associated lymphoid tissue (GALT). Also, M cells have specific structures without microvilli on apical side of M cells and intraepithelial pocket. This structures make pathogens and organisms pass through M cells, and mucosal immune response such as production of immunoglobulin A (IgA) is rapidly enhanced.

The differentiation of M cells is still controversial and there are several factors such as RANKL and CCL20 (C-C motif chemokine ligand 20) for M cell differentiation. CCL20 is chemokine to attract CCR6 expressing dendritic cells, B lymphocytes and macrophages. In addition, CCL20 induces M cell differentiation by chemoattracting B lymphocytes toward follicle associated epithelium (FAE).

In this study, we designed recombinant CCL20 expressing *L. lactis*. And then, we studied whether recombinant CCL20 expressing *L. lactis* can be used as mucosal vaccine adjuvant.

Ⅱ. Review of Literature

1. Microfold (M) cell

1) Gut-associated lymphoid tissue (GALT)

The immune system of digestive tract is essential because gastrointestinal tract is directly adjacent to gastric environment including pathogens. Gut-associated lymphoid tissues (GALT) as the mucosa-associated lymphoid tissue lining the gastrointestinal tract plays a major role for immune response in gut although digestive tract can prevent pathogens by gastric acid, antimicrobial peptides and enzymes (Kunisawa et al., 2008). GALT contains a lot of lymphoid follicles and around 70% of immune cells (Heel et al., 1997).

2) Peyer' patch (PP)

Peyer's patch (PP) as a kind of secondary lymphoid organ constitutes a essential part of gut-associated lymphoid tissue (GALT) (Heel et al., 1997). PPs are composed of sub-epithelial lymphoid follicle containing dendritic cells and macrophages, T lymphocytes and B lymphocytes follicle called as a germinal center, and specialized follicle-associated epithelium (FAE) containing Microfold (M) cells (Figure 1) (Fagarasan and Honjo, 2003).

The populations of PPs vary with species. There are approximately 8~10 PPs in small intestine of mice, 2~10 PPs in small intestine of rabbits and ~300 PPs in small intestine of

humans (Heel et al., 1997) (Kunisawa et al., 2012). The structure of PPs is dome-like shape because of the lymphoid follicles. Futhermore, the size of PPs is approximately 25mm length in human and varies with the number follicles within them (Heel et al., 1997).

PPs are a major site for sampling intestinal antigen and inducing an immunological response in gut. In other words, PPs help immune cells quickly interact with luminal antigen. The covering layer of the PPs is called as a follicle-associated epithelium (FAE). FAE consists of specialized epithelial cells like M cells. M cells play a major role for antigen uptaking from gut lumen to sub-epithelial dome (SED) inside of epithelial cells (Heel et al., 1997).

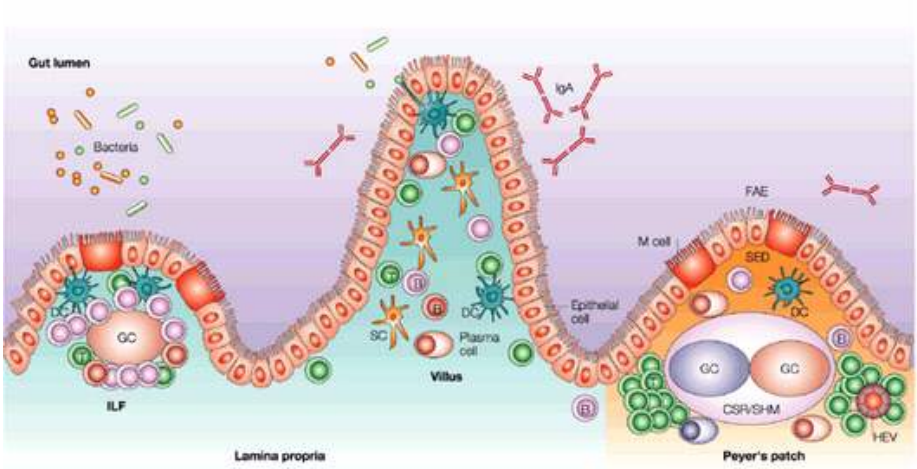

Figure 1. Schematic picture of GALT. There are several differences between intestinal villi and Peyer's patches(PPs). PPs constitute germinal center containing B lymphocytes and T lymphocyte follicles. In addition, M cells are located within FAE. DC, dendritic cell; CSR, class-switch recombination; FAE, follicle associated epithelium; GC, germinal center; HEV, high endothelial venules; ILF, isolated lymphoid follicle; SC, stromal cell; SHM, somatic hypermutation (Fagarasan and Honjo, 2003).

3) M cells

(1) Structure and function

The special structure of M cells contributes to transepithelial transporting foreign antigen. Apical surface of M cells has few brush border and reduced microvilli (Mabbott et al., 2013). In addition, M cells have a thin glycocalyx because it does not secrete mucus (Mabbott et al., 2013). These structures help luminal antigen easily pass through apical side of M cells. Basolateral membrane of M cells is largely folded and contains an intraepithelial pocket (Kraehenbuhl and Neutra, 2000). B lymphocyte, T lymphocyte and dendritic cells locate within this pocket and quickly interact with pathogens or gut luminal macromolecules (Figure 2) (Mabbott et al., 2013). Consequently, M cells are functionally and structurally specialized for sampling antigen and inducing immune response.

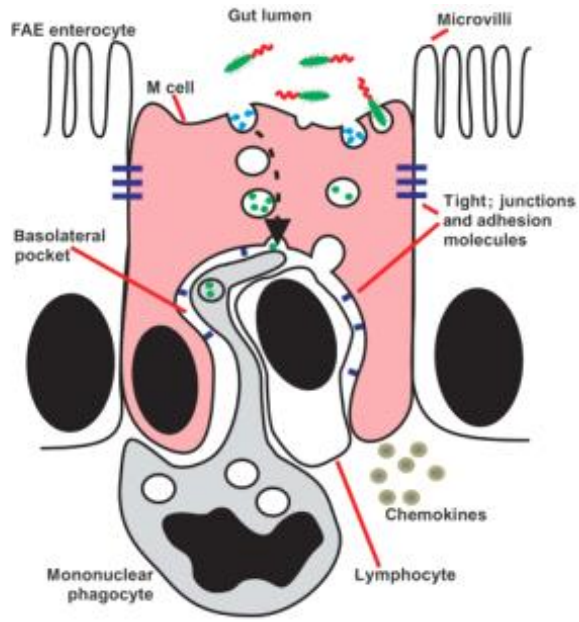

Figure 2. Schematic view of M cell morphology. There is less microvilli and basolateral pocket containing lymphocyte and mononuclear phagocyte in M cells. Antigens from gut lumen could be rapidly delivered due to specialized M cell structure. FAE; follicle-associated epithelium (Mabbott et al., 2013).

(2) Origin of M cells

The origin of M cells is still controversial. There is two main hypothesis of the M cell origin; stem cell hypothesis and trans-differentiation hypothesis. First, M cells are originated from Lgr5+ stem cells located in crypt of intestinal epithelial cells (de Lau et al., 2012). Receptor activator of NF-κB ligand(RankL) contributes to control the M cell differentiation from Lgr5+ stem cells (Knoop et al., 2012). Second, M cells are derived from trans-differentiation of intestinal epithelial cells. C-C motif chemokine ligand 20 (CCL20) and its receptor CCR6 expressing cells induce trans-differentiation of other epithelial cells to M cells (Ebisawa et al., 2011).

2. C-C motif chemokine ligand 20 (CCL20)

1) CCL20 and antimicrobial function

There is a report that many chemokines have similar structure to antimicrobial peptides and antimicrobial activity (Hoover et al., 2002). CCL20 structure is also similar to β-defensin as an antimicrobial peptide (Yang et al, 2003). High number of positive charged residues are in the C-terminal of CCL20 as a defensin has cationic residues (Hoover et al., 2002). Therefore, CCL20 has antimicrobial activity on gram-negative bacteria like *Escherichia coli (E.coli), Staphylococcus aureus* and *Streptococcus pyogenes* although salt concentration has an effect on the efficiency of antimicrobial activity (Yang et al, 2003).

2) Chemotaxis

Chemotaxis is directional migration of cells and organisms as a response to an external chemical stimulus (Bagorda and Parent, 2008). Strategies of chemotaxis vary across the cell types and the environmental conditions. For example, bacterial chemotaxis is exhibited by using flagella as a locomotion organelle (Macnab and Koshland, 1972). The movement of bacteria such as *E. coli* is conducted by alternating swim and tumble phases (Khan et al., 2004). And then, bacteria determine their direction based on chemical concentration. Unlike bacterial chemotaxis, the movement of eukaryotic cells such as neutrophils and lymphocytes, is conducted by a dynamic distribution of chemokine receptor (Bagorda and Parent, 2008). When the high concentration of chemoattractants interacts with the receptors located on the cell membrane, cell protrusions are formed in high concentration side of cells (Junger, 2011). And then, cell protrusions lead cell contraction to restore the instantly modified cell structure (Junger, 2011). Therefore, the cells are attracted toward high concentration side. (Figure 3)

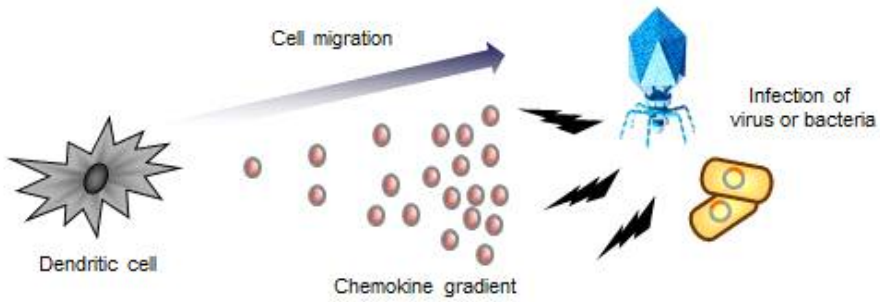

Figure 3. Schematic view of the movement of dendritic cell based on chemokine gradient. Infection of virus and bacteria make chemokine gradient. Dendritic cells migrate toward high concentration of chemoattractants. (Condliffe and Hawkins, 2000).

3) Immunological function of CCL20

The C-C motif chemokine ligand 20 (CCL20; MIP-3α, macrophage inflammatory protein-3α) is a 5-20 kDa chemokine, 8 kDa in mice and 9 kDa in human (Hoover et al., 2002). CCL20 is mainly expressed beneath the FAE of PPs in small intestine. C-C motif chemokine receptor 6 (CCR6) is the sole chemokine receptor of its ligand CCL20 (Williams, 2006). CCR6 is expressed in most B lymphocytes, T lymphocytes, dendritic cells and natural killer cells in the gut mucosal immune system. Therefore, CCL20 expressed beneath the FAE recruits CCR6 expressing cells to the SED of PPs (Finke and Kraehenbuhl, 2001).

Recruitment of CCR6 expressing natural killer cells and memory T-cells induces innate immune response by activating complement system, secreting pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-α), and so on (Arash et al., 2001). In addition, adaptive immune response is exhibited by attracting CCR6 expressing immature dendritic cells and B cells. As a result, CCL20 controls both innate immune response and adaptive immune response (Hoover et al., 2002). Several hypothesis insists that the recruitment of CCR6 expressing B cells induces differentiation of intestinal epithelial cells to M-like cells (Williams, 2006).

It is obvious that CCL20 is important for enhancing immune response.

4) Effects of CCL20 on M cell origin

Trans-differentiation group insisting differentiation of M cells is induced by relation between FAE and PPs although differentiation of M cells is still controversial as mentioned above. The

interaction of CCL20 expressed beneath FAE and CCR6 expressing PP cells is deeply involved with this hypothesis. For example, $CCR6^{-/-}$ mice have approximately 40% of the wild type levels of M cells on PPs (Ebisawa et al., 2010). $CD11c^{int}$ B-cell subset among CCR6 expressing cells contributes to M cell differentiation. $CCR6^{hi}CD11c^{int}$ B cell deficiency caused reduction of M cell differentiation and restored the M cell differentiation decrement when $CCR6^{hi}CD11c^{int}$ B cell is adoptive transferred (Ebisawa et al., 2010). As a result, CCL20-CCR6 system plays a crucial role in M cell differentiation although it is not a necessary and sufficient condition (Figure 4.).

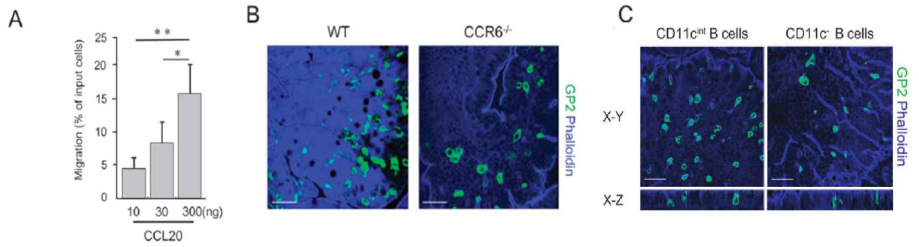

Figure 4. The effects of B cells on M cell differentiation. (a) CCR6 expressing cells attract toward CCL20. More CCL20 can attract more CCR6 cells. (b) GP2 is a M cell marker indicating M cells. $CCR6^{-/-}$ cells contain few $GP2^{+}$ cells and M cells in PPs. (c) $CD11c^{-}$ B cells contain very few M cells. WT, wild type (Ebisawa et al., 2010).

3. Mucosal vaccine and LAB

1) Mucosal vaccine and oral vaccine

As mentioned above, mucosal surface is adjacent to external environment including pathogens, nutrient and so on. Therefore, the mucosal immune system is important to protect animal's body from infectious pathogens. The researches about mucosal vaccine have been studied for enhancing mucosal immune response by various ways. The mucosal vaccine category includes oral, genital , rectal, nasal, inhalation and sublingual vaccinations (Ali Azizi et al., 2010).

Oral vaccination provides several advantages compared to other vaccinations. First, it is safe and less-stressful method because oral vaccination do not need needle (Kunisawa et al., 2012). Second, it is comfortable and easy method. Third, it is significant that oral vaccination induces both mucosal immune response and systemic immune response (Ali Azizi et al., 2010). However, oral vaccine also has several disadvantages such as large dose of antigen and tolerance (Table 1).

Table 1. Pros and cons of mucosal vaccine immunization.

Route of immunization	Advantages	Disadvantages
Genital delivery	Enhancement of specific mucosal and systemic antibody responses	Immunological properties of the female reproductive tract alter during the menstrual cycle
Rectal delivery	Specific antibodies and cytotoxic T lymphocyte response	Modest levels of local IgG and IgA titers; difficulty in the route of inoculation
Nasal delivery	Enhancement of both humoral and cellular immune responses in systemic and mucosal sites; easy to administer, no needles are needed	Lack of strong adjuvants; serious side effects
Inhalation delivery	Enhancement of both humoral and cellular immune responses in systemic and mucosal sites; administered in both dry powder or liquid formulations	A device is required; risk of exacerbation of respiratory infections; dose delivery issue
Sublingual delivery	Antigens are absorbed quickly; no needles are needed	Dose delivery issues; lack of strong adjuvants
Oral delivery	Enhancement of immune responses in systemic and mucosal sites; safe; easy to administer; easy to scale up	Induction of tolerance; requires large dose of antigens

(Ali Azizi et al., 2010)

2) Oral vaccine and M cell targeting

Oral vaccine delivery systems have a purpose of delivering specific antigens into mucosal immune system such as gut-associated lymphoid tissue(GALT). M cells are the major starting point of inducing mucosal immune response by taking up specific antigens or microorganisms from the intestinal lumen to the SED of PPs (Mabbott et al., 2013). The population of M cells is approximately 10% in mice and 5% in humans (Kunisawa et al., 2012). The efficiency of oral vaccine is not sufficient due to low number of M cells. M cell targeting strategy increases the oral vaccine efficiency by helping target antigen effectively interact with immune cells within M cell pocket (Ali Azizi et al., 2010). In other words, targeting M cells may increase the target antigen sampling and consequently enhancing the immune response (Ali Azizi et al., 2010).

3) Lactic acid bacteria as a mucosal vaccine vehicle

Lactic acid bacteria (LAB) is gram-positive bacteria. LAB has been used in dietary products such as dairy products. LAB has been recently studied for using mucosal vaccine vehicle because LAB has wide interaction with mucosal surface of body (Wells et al., 1996).

LAB possess several attractive points as mucosal vaccine delivery vehicles. First, LAB is GRAS (Generally Recognized As Safe) organisms that confirms safety of using LAB for mucosal vaccine delivery vehicle. Second, LAB itself can stimulate the mucosal immune response like probiotics and secrete antimicrobial peptide such as bacteriocin (Wells et al., 1996). Third, LAB can be conveniently engineered. Fourth, LAB secretes lactic acid that

make LAB endure acidic condition of gastric condition and kill pathogens.

Lactococcus lactis (*L. lactis*) is a kind of LAB and widely used for mucosal vaccine delivery vehicle. It is a non-colonising bacteria and non-invasive bacteria (Norton et al., 1994).

Ⅲ. Materials and Methods

1. Bacterial culture conditions

1) Bacteria and culture medium

E.coli DH5α strains were used in this study and cultured with LB broth and agar (Difco, BD). Ampicillin (Amp, 100 μg/ml) was supplemented to LB broth and agar for selection of Amp resistance gene containing *E.coli*. *L. lactis* IL1403 strains were used in this study and cultured in M17 broth and agar (Difco, BD) with 0.5% (w/v) glucose (M17G). Erythromycin (5 μg/ml) was supplemented to M17 broth and agar for selection of erythromycin resistance gene containing *L. lactis*.

2) Culture condition

E.coli in LB broth was incubated at 37℃ with shaking. *E.coli* in LB agar was incubated at 37℃ without shaking. *L. lactis* in M17G broth and agar was incubated at 30℃ without shaking.

2. Minimum inhibition concentration (MIC) test

1) Cultivation of *L. lactis* for MIC test

Streaking of *L. lactis* on M17G agar plate was conducted and incubated for overnight at 30℃. After incubation, single colony of *L. lactis* was inoculated into M17G broth without antibiotics and

cultivated for 24 hours at 30℃ without shaking. 0.1% (v/v) of the cultured *L. lactis* was inoculated into M17G broth without antibiotics and cultivated to OD_{600nm} 0.5.

2) MIC test

Four different concentrations of CCL20 (0.08 μg/ml, 0.4 μg/ml, 2 μg/ml and 10 μg/ml) containing M17G broth were prepared for MIC test. OD_{600nm} 0.5 of cultured *L. lactis* was diluted to $O.D_{600nm}$ 0.2 with fresh M17G medium. 100 μl of OD_{600nm} 0.2 *L. lactis* was mixed with 100 μl M17G containing each concentration of CCL20 (0.16 μg/ml, 0.8 μg/ml, 4 μg/ml or 20 μg/ml). The final cultured *L. lactis* with CCL20 was incubated for 10 hours at 30℃ without shaking. Optical density was calculated after incubation.

3. Recombinant *L. lactis* construction

1) CCL20 sequence and recombinant CCL20 synthesis

Recombinant CCL20 consists of the combination of *L. lactis* signal peptide Ubiquitin Specific Peptidase 45 (USP45, 29 a.a.), murine CCL20 (70 a.a.) and M cell targeting peptide (MTP, 9 a.a.) (Table 2.). There are three experimental groups for this study. One group is M cell targeting peptide-free group, USP45-CCL20 (UC). The other two groups have different M cell targeting peptide position: USP45-MTP-CCL20 (UMC) has M cell targeting peptide at N-terminal and USP45-CCL20-MTP (UCM) has M cell targeting peptide at C-terminal. Enzyme site Nde I and Xho I were inserted into each recombinant CCL20 DNAs

(deoxyribonucleic acid).

Table 2. Peptide sequences for recombinant CCL20 synthesis

Peptide	Sequence
USP45	MKKKIISAIL MSTVILSAAA PLSGVYADT
murine CCL20	ASNYDCCLSY IQTPLPSRAI VGFTRQMADE ACDINAIIFH TKKRKSVCAD PKQNWVKRAV NLLSLRVKKM
MTP	CKSTHPLSC

L. lactis was used as a recombinant CCL20 expression host. Therefore, codon optimization of recombinant protein for *L. lactis* is fundamental step to produce ideal recombinant CCL20 peptides. After then, recombinant CCL20 DNAs were requested to synthesize from a professional company, M.biotech (Gyeongi, Korea). This recombinant CCL20 DNAs were cloned into pIDTSmart vector including ampicilin resistance gene. pIDTSmart vectors cloned with recombinant CCL20 DNAs (pIDT.CCL20) were transformed into *E.coli* DH5α competent cells by heat shock transformation at 42℃ for 90 seconds.

2) Construction of recombinant CCL20 expression vector

pIL.Ptuf.Mb researched from previous study in this lab (Kim et al., 2009) was used as a backbone vector for cloning recombinant CCL20 DNAs in this study. pIL.Ptuf.Mb including erythoromycin resistance gene for selection and restriction enzyme site, Nde I and Xho I was cloned into *L. lactis* IL1403 strains.

pIDT.CCL20 from *E.coli* DH5α and pIL.Ptuf.Mb from *L. lactis* IL1403 were isolated with the plasmid purification kit. Restriction enzymes (Nde I and Xho I) were used for extracting recombinant CCL20 DNAs from pIDT.CCL20 and pIL.Ptuf as a backbone vector from pIL.Ptuf.Mb. Enzyme digetion was exhibited at 37℃ for overnight. Each recombinant CCL20 DNAs (UC, UCM and UMC) and pIL.Ptuf backbone vectors were gained by gel elution. Recombinant CCL20 DNAs and pIL.Ptuf backbone vectors were ligated with 1:4 molar ratio by using T4 ligase. The mixture for lagation was incubated at 4℃ for overnight.

The constructed recombinant CCL20 vectors were named as pIL.Ptuf.UC, pIL.Ptuf.UCM and pIL.Ptuf.UMC.

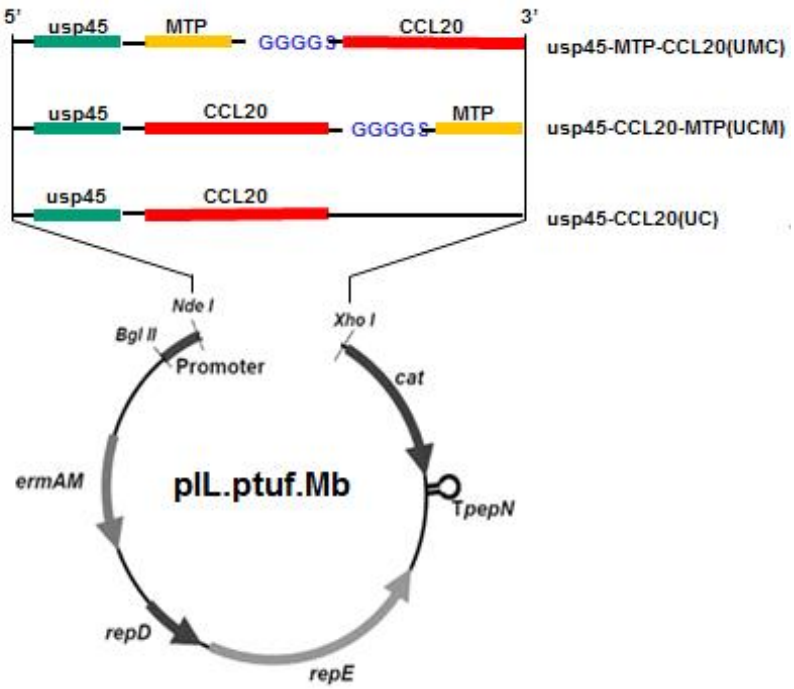

Figure 5. Schematic view of the recombinant CCL20 vector.

3) Preparation of *L. lactis* IL1403 competent cells and transformation

Competent cells for storing constructed CCL20 expressing vectors should be prepared. *L. lactis* IL1403 wild type was cultured at 30℃ for overnight. 500 μl of precultured wild type *L. lactis* IL1403 was inoculated into 50 ml M17G medium without antibiotics and cultured to OD_{600nm} 0.5. Cultured wild type *L. lactis* IL1403 was harvested by centrifugation at 4℃ and 4,500 rpm for 10 minutes and washed with triple distilled water (D.W.). 10 mM $MgCl_2$ solution and 0.5 M sucrose/10% glycerol solutions were used for making *L. lactis* IL1403 competent cells. The harvested cell pellet was resuspended with 30 ml of 10 mM $MgCl_2$ solution and centrifugation was conducted at 4℃ and 4,000 rpm for 10 minutes. This step was repeated twice. And then, the harvested cell pellet was resuspended with 20 ml of 0.5 M sucrose/10% glycerol solution and centrifugation was conducted at 4℃ and 4,000 rpm for 10 minutes. After last centrifugation, harvested pellets were resuspended with 1 ml of 0.5 M sucrose/10% glycerol solution.

Pellet painting was conducted to wash salts from constructed recombinant CCL20 vectors. Pellet painted recombinant CCL20 vectors were transformed into prepared *L. lactis* IL1403 competent cells by electroporation at 2.5 kV, 10 μF and 300 ohm with Gene Pulser Xcell System (Bio-Rad, USA). Transformed *L. lactis* IL1403 were inoculated into 1 ml of M17G medium without antibiotics and cultured at 30℃ for 2 hours without shaking. After then, transformed *L. lactis* IL1403 were cultured with M17G agar with erythromycin.

4) Selection for transformants

Selection using erythromycin resistance was conducted at first. Remained single colonies were inoculated into M17G medium with erythromycin and stored at －80℃.

Colony PCR (polymerase chain reaction) selection using specific primer was conducted after antibiotic resistance selection. pIL.Ptuf.UC was verified by conducting colony PCR with 10 pmole of UC specific primer, ID.UC-forward and reverse. pIL.Ptuf.UCM was verified by conducting colony PCR with 10 pmole of UCM specific primer, ID.UCM-forward and reverse. pIL.Ptuf.UMC was verified by conducting colony PCR with 10 pmole of UMC specific primer, ID.UMC-forward and reverse (Table 3). Each primers were synthesized by Bioneer (Daejeon, Korea).

Table 3. Primer used for colony PCR.

Primer names (Length)	Sequence
ID.UC-F (20)	5'-ATGAAGAAGAAAATCATTTC-3'
ID.UC-R (20)	5'-TTACATTTTTTTTACTCGAA-3'
ID.UCM-F (20)	5'-ATGAAGAAGAAGATTATCTC-3'
ID.UCM-R (20)	5'-TTATCCACATGAAAGTGGAT-3'
ID.UMC-F (20)	5'-ATGAAGAAGAAGATCATCAG-3'

ID.UMC-R (20)	5'-TCACATCCTTCTTGACACGCA-3'

After colony PCR with specific primer, recombinant CCL20 vectors containing pIL.ptuf.UC, pIL.ptuf.UCM and pIL.ptuf.UMC respectively were sequenced with pIL252 vector sequencing primer (Table 4) at National Instrumental Center for Environmental Management (NICEM). Basic local alignment search tool (BLAST) was used for validating each sequences.

Table 4. Primers used for vector sequencing.

Primer names (Length)	Sequence
Seq.pIL252-F (24)	5′-CTCTTTTCTCTTCCAATTGTCTAA-3′
Seq.pIL252-R (23)	5′-CGGTAGTTTGCTTTATGCAGACT-3′

4. Recombinant CCL20 expression in *L. lactis*

1) Protein preparation from recombinant transformants

The protein preparation from transformants was conducted for confirming the expression of recombinant CCL20 transformants via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Recombinant CCL20 transformants and wild type *L. lactis* IL1403 were inoculated into M17G medium with erythromycin and cultured at 30℃ for 22 hours without shaking. And then, 7 ml of cultured transformants

and wild type *L. lactis* IL1403 were used for collecting cytoplasmic proteins and 2 ml of cultured cells were used for collecting secreted proteins. Cytoplasmic proteins of recombinant CCL20 transformants and wild type *L. lactis* IL1403 were extracted by bead beating. 7 ml of cultured transformants were harvested by centrifugation at 13,000 rpm for 1 minute. Cell pellets of recombinant CCL20 transformants were washed with D.W. and repeated twice. After then, cells were smashed by bead beater with 0.1 mm and 0.5 mm of glass beads. Secreted proteins of recombinant CCL20 transformants were extracted by trichloroacetic acid (TCA) solution precipitation. 2 ml of cultured transformants was harvested by centrifugation at 13,000 rpm for 1 minute. 1.6 ml of supernatant was mixed with 400 μl of TCA solution and incubated for 30 minutes in ice. After incubation, centrifugation at 4℃ and 13,000 rpm for 10 minutes was conducted. Protein pellets were washed with acetone and centrifugation was repeated at same condition. After centrifugation, protein pellets were resuspended in pH 8.0 of Tris-HCl buffer.

2) SDS-PAGE and western blot

Cytoplasmic and secreted proteins extracted from recombinant CCL20 transformants and wild type *L. lactis* IL1403 were mixed with 5X SDS-PAGE loading buffer and incubated at 95℃ for 5 minutes. After incubation, mixed solution was loaded into 4-20% (w/v) Tris-Glycine SDS-PAGE Pre-cast gel (Komabiotech, Korea). Electrophoresis was conducted at 120 V for 10 minutes and 150 V for 120 minutes. The gel was transferred onto two

overlapped 0.2 μm nitrocellulose membrane (Whatman, USA) by using transfer buffer containing 20% (v/v) methanol. The transferred membrane was blocked with TBST containing 5% (w/v) skim milk at RT for 1 hour. The blocked membrane was washed with fresh TBST. After washing, the membrane was incubated with 1 μg/ml of rat anti-mouse CCL20/MIP-3α monoclonal antibody (R&D, USA) as a primary antibody at 4℃ overnight and the membrane was washed 3 times with TBST for 15 minutes each. After then, the membrane was incubated with goat anti-rat IgG-HRP (Santa Cruz biotechnology, USA) as a secondary antibody at RT for 1 hour and the membrane was washed 3 times with TBST for 15 minutes. At last, the membrane was mixed with 200 μl of enhanced chemiluminescence (ECL) solution and verified by Chemidoc for reading horse radish peroxidase (HRP) signals.

3) Enzyme-linked immunosorbent assay (ELISA)

ELISA is a quantitative assay for validating the expression of proteins. Recombinant CCL20 transformants and wild type *L. lactis* IL1403 were inoculated into M17G broth with erythromycin and cultured at 30℃ for 22 hours without shaking. The cultured cells were harvested by centrifugation at 4℃ and 13,000 rpm for 1 minute. Supernatant from each recombinant CCL20 transformants was used for ELISA. ELISA was conducted with ELISA kit, Quantikine mouse CCL20/MIP-3α ELISA kit (R&D, USA). ELISA for time curve was conducted in 3 hour, 6 hour, 9 hour, 12 hour, 24 hour and 48 hour cultured supernatant.

4) Physiological test of recombinant CCL20 transformants

Physiological test was conducted to validate growth and acidity compared to wild type *L. lactis* IL1403. Recombinant CCL20 transformants were inoculated into M17G medium with erythromycin and wild type *L. lactis* IL1403 were inoculated into M17G medium without antibiotics. Pre-cultured cells were inoculated into 50 ml of each M17G medium and measured at OD_{600nm} and pH of 2, 4, 6, 8, 10, 12, 24 and 48 hour cultured cells.

5. *In vitro* functional activity assay

1) JAWS Ⅱ cell culture conditions

The growth medium for JAWS Ⅱ cells, mouse immature dendritic cells, was α-MEM containing ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 ng/ml murine GM-CSF, 20% fetal bovine serum (FBS) and 100 U/ml of penicillin/streptomycin. JAWS Ⅱ cells were incubated in 100 mm cell culture dish with 10 ml of specific growth medium at 37℃. JAWS Ⅱ cells were sub-cultured with 0.25% trypsin-EDTA and centrifuged at 1,000 rpm for 10 minutes. After centrifugation, harvested cells were dissolved in 10 ml of growth medium and incubated at same condition.

2) *In vitro* chemotaxis assay

5 μm pore - 24 well transwell chambers (corning, USA) were

used for *in vitro* chemotaxis assay. The lower wells of a transwell chamber was filled with 600 μl of medium containing each concentration of CCL20 (0.01 ng/ml, 0.1 ng/ml, 0.5 ng/ml, 1 ng/ml and 5 ng/ml). Supernatant of recombinant CCL20 transformants was used for adjusting each CCL20 concentration. Therefore, growth medium mixed with the supernatant of wild type *L. lactis* IL1403 as much as each experimental group used in lower well, was used as negative control of this study. The upper wells of a transwell chamber was filled with 100 μl of growth medium containing $5x10^5$ JAWS Ⅱ cells. The upper chamber and lower chamber were assembled and incubated at 37℃ for 90 minutes. After incubation, chemoattracted cells in lower chamber were calculated with hemocytometer.

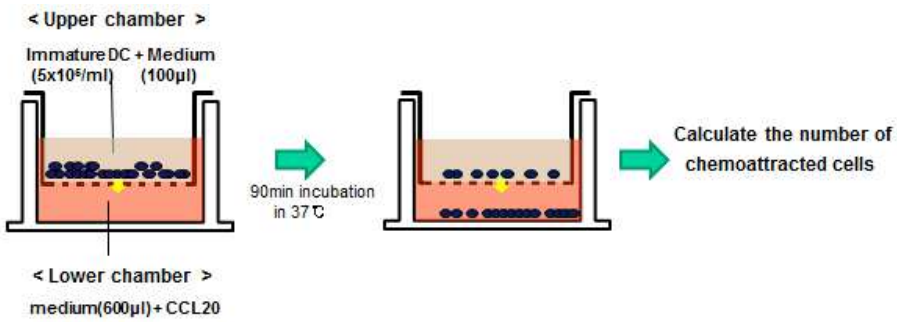

Figure 6. Schematic image of *in vitro* chemotaxis assay.

6. *In vivo* functional activity assay

1) Preparation of BmpB

Brachyspira membrane protein B (BmpB) was used as model

antigen of *in vivo* functional assay. The first step of preparing BmpB is to extract BmpB from *E.coli*. *E.coli* expressing BmpB was inoculated into 5 ml of LB broth with ampicillin and incubated at 37℃ overnight. Two 2 L flasks were used for IPTG induction. 500 μl of pre-cultured cells were inoculated into 500 ml of LB broth with ampicillin and cultured to OD_{600nm} 0.8 at 37℃ in each 2 L flask. After then, 0.4 mM of IPTG was supplemented and cultured at 26℃ for 4 hours. The cultured cells were harvested by centrifugation at 6,000 rpm for 10 minutes. Cell pellets were washed with Phosphate buffered saline (PBS) and repeated 3 times. After washing, cell pellets were resuspended with binding buffer and sonicated at a 200 watt of power and cycles repeated with 9 seconds of pulse and 4 seconds of pause for 14 minutes. After sonication, centrifugation was conducted at 12,000 rpm at 4℃ for 15 minutes and supernatant was obtained.

The next step of preparing BmpB is to purify extracted BmpB. For purifying BmpB, column chromatography using nickel affinity was conducted. 7ml of His-binding resin was inserted into chromatography column (BioRad, USA) and settled overnight. After then, chromatography column was washed with 3 volume (1 volume is half of His-binding resin used for chromatography) of binding buffer at first and charged with 5 volume of charging buffer. The charged column was equilibrated with 3 volume of binding buffer. The extracted BmpB was loaded in equilibrated column. His-binding resin bound with BmpB was washed with 10 volume of binding buffer and 5 volume of washing buffer. After washing, purified BmpB was eluted with 3 volume of elution buffer. Dialysis of eluted BmpB with elution buffer was conducted at 4℃ for 48 hours.

2) Oral administration for *in vivo* functional assay

Before animal experiment, the plan for animal experiment was permitted from institute animal care and use committees (IACUC) in Seoul national university. BALB/c mice for experiment were bought from Samtako (Gyeonggi, Korea) and stabilized for 1 week in mouse room. Recombinant CCL20 transformants were administrated for 1 week to induce the differentiation of M cell. 500 μl of gastric antacid agent with 1.5% of NaH_2CO_3 was administrated always before oral administration. 200 μl of recombinant CCL20 transformants with 10^9 cells were administrated 30 minutes after neutralizing. After administrating recombinant CCL20, purified BmpB was administrated in the same way above for 3 days. Boosting was conducted 1 week and 2 weeks after priming. Serum and fecal samples was obtained 3 weeks and 4 weeks after priming.

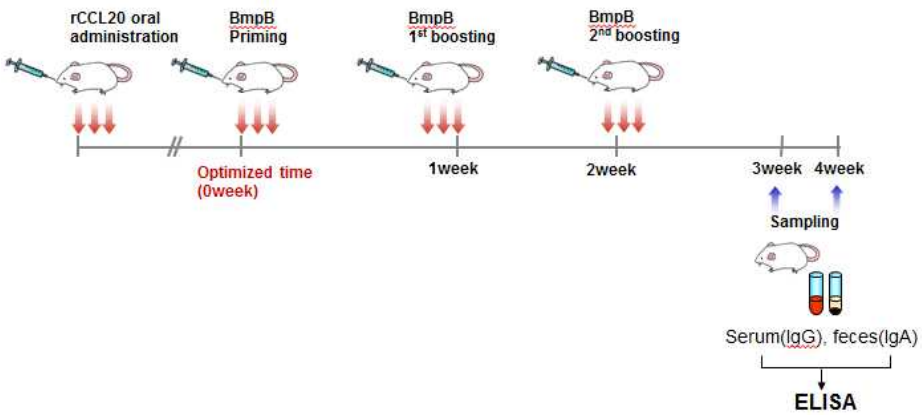

Figure 7. Schedule for *in vivo* functional assay.

3) Sample collection

Serum samples were used for detecting the change of IgG. The blood of mouse was collected from tail vein by using Capillary tube. Collected blood samples were centrifuged at 8,000 rpm for 10 minutes. Only serum was collected from centrifuged blood and stored at -70℃.

Fecal samples were used for detecting the level of IgA. The feces of mouse was collected and added with PBS. Fecal sample with PBS was vortexed vigorously and incubated at 4℃ overnight. After incubation, fecal samples were vortexed again and conducted centrifugation at 14,000 rpm for 10 minutes. The supernatant was used for ELISA.

4) ELISA for detecting anti-BmpB immunoglobulin

Indirect ELISA method was used for detecting anti-BmpB immunoglobulins (IgG and IgA) in this study. BmpB was coated on non-coated 96 well plate at first. To make BmpB coating solution, 25 μg/ml of BmpB was dissolved in coating buffer. 100 μl of BmpB coating solution was loaded on each well of 96 well plate and incubated at 4℃ overnight. After incubating, 200 μl/well of PBST (500 μl tween in 1L PBS) was used for washing each well and washing step was repeated 3 times. BmpB coated plate was blocked with 200 μl/well blocking solution (1% BSA in PBS) at RT for 2 hours and washed with PBST. Collected serum was diluted in 1:10000 ratio with dilution buffer to make diluted serum. Collected feces was diluted in 1:10 ratio with dilution

buffer to make diluted fecal sample. 100 μl of each diluted serum and fecal samples were loaded on each well of 96 well plate and incubated at RT for 2 hours. After incubation, the plate was washed with PBST. HRP-conjugated goat anti-mouse IgG was diluted in 1:5000 ratio. HRP-conjugated goat anti-mouse IgA was diluted in 1:2000 ratio. 100 μl of antibody was loaded on each well and incubated at RT for 1 hour. Washing step was conducted after incubation. 100 μl of TMB solution was loaded on each well and incubate at RT in dark room for 30 minutes. After then, 100 μl of stop solution (2 N H_2SO_4) was added on each plate. Signals from each wells on the plate was read with microplate reader.

7. Statistical analysis

One-way ANOVA followed by Tukey's test was conducted to analyze differences among each two groups. Statistical analysis was conducted with Graph Prism 5 (Graph Pad Software incorporated).

Ⅳ. Results and Discussion

1. MIC test

CCL20 has antimicrobial activity mainly against gram negative bacteria. Therefore, if CCL20 has antimicrobial activity to oral vaccine delivery host *L. lactis* although *L. lactis* is gram positive bacteria, recombinant CCL20 transformants would not be used as a vaccine adjuvant for enhancing mucosal immune response. For this reason, MIC test was conducted for identifying MIC value of CCL20 against *L. lactis* (Figure 8).

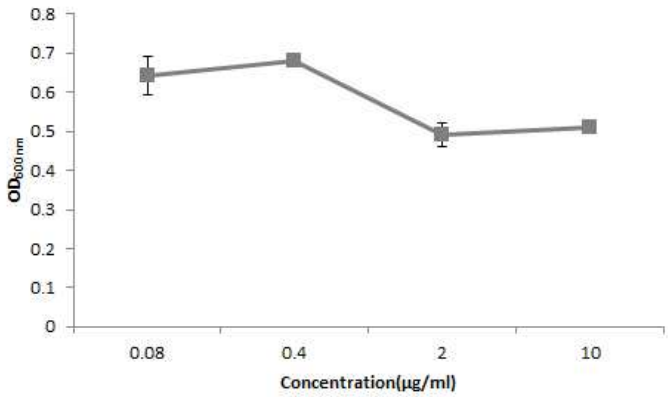

Figure 8. MIC test of *L. lactis* with commercial CCL20. 0.08, 0.4, 2 and 10 μg/ml of CCL20 was used for MIC test.

In normal state, CCL20 has its biological function in the pg/ml range. However, MIC value of CCL20 against *L. lactis* is more than 10 μg/ml. Therefore, CCL20 did not have antimicrobial

activity against *L. lactis* in normal state. The results indicated that recombinant CCL20 did not affect its host, *L. lactis*. In conclusion, *L. lactis* could be used as recombinant host.

2. Construction of recombinant CCL20 expressing *L. lactis* IL1403

L. lactis is a outstanding candidate as an oral vaccine delivery vehicle because *L. lactis* can endure more acidic condition than other bacteria and has not any harm to animals. Therefore, constructed recombinant CCL20 vectors (pIL.Ptuf,UC, pIL.Ptuf.UCM and pIL.Ptuf.UMC) were transformed into *L. lactis*.

To validate whether recombinant CCL20 vertors were correctly transformed into *L. lactis*, colony PCR of constructed recombinant CCL20 transformants was conducted with each specific primers (Figure 9).

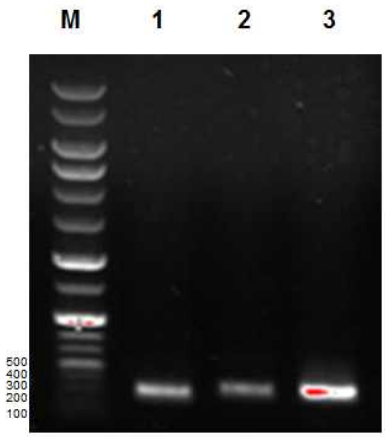

Figure 9. Colony PCR for identifying correctly transformed

recombinant *L. lactis*. UC, UCM and UMC recombinant CCL20 vectors were correctly transformed into *L. lactis*. M, molecular marker; lane 1 : UC colony PCR with UC primer ; lane 2 : UCM colony PCR with UCM primer; lane 3 : UMC colony PCR with UMC primer. UC, USP45-CCL20; UCM, USP45-CCL20-MTP; UMC, USP45-MTP-CCL20.

DNA length of UC is 309 bp, UCM is 357 bp and UMC is 354 bp. Each bands of recombinant CCL20 transformants were vivid and located in their own position. The results indicated that recombinant CCL20 transformants were correctly constructed.

To validate whether recombinant CCL20 vectors in constructed transformants have correct synthesized recombinant CCL20 DNAs (UC, UCM and UMC), each recombinant vectors were sequenced at NICEM (Figure 10).

(a)

Range 1: 1 to 309 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
571 bits(309)	7e-168	309/309(100%)	0/309(0%)	Plus/Plus

```
Query  1    CATATGAAGAAGAAAAATCATTTCAGCCATTTTGATGTCTACCGTTATCTTATCAGCTGCA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CATATGAAGAAGAAAAATCATTTCAGCCATTTTGATGTCTACCGTTATCTTATCAGCTGCA  60

Query  61   GCACCTTTAAGTGGAGTATATGCAGATACGGCAAGTAATTATGACTGTTGTTTATCATAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GCACCTTTAAGTGGAGTATATGCAGATACGGCAAGTAATTATGACTGTTGTTTATCATAT  120

Query  121  ATTCAAACTCCTCTTCCATCACGTGCTATTGTAGGTTTTACACGTCAAATGGCTGATGAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  ATTCAAACTCCTCTTCCATCACGTGCTATTGTAGGTTTTACACGTCAAATGGCTGATGAA  180

Query  181  GCATGCGATATTAATGCAATCATTTTCCACACAAAGAAACGTAAGAGTGTTTGCGCAGAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCATGCGATATTAATGCAATCATTTTCCACACAAAGAAACGTAAGAGTGTTTGCGCAGAT  240

Query  241  CCTAAGCAAAATTGGGTGAAACGAGCGGTTAATCTTTTGTCACTTCGAGTaaaaaaaaTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CCTAAGCAAAATTGGGTGAAACGAGCGGTTAATCTTTTGTCACTTCGAGTAAAAAAAATG  300

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Sbjct  301  TAACTCGAG  309
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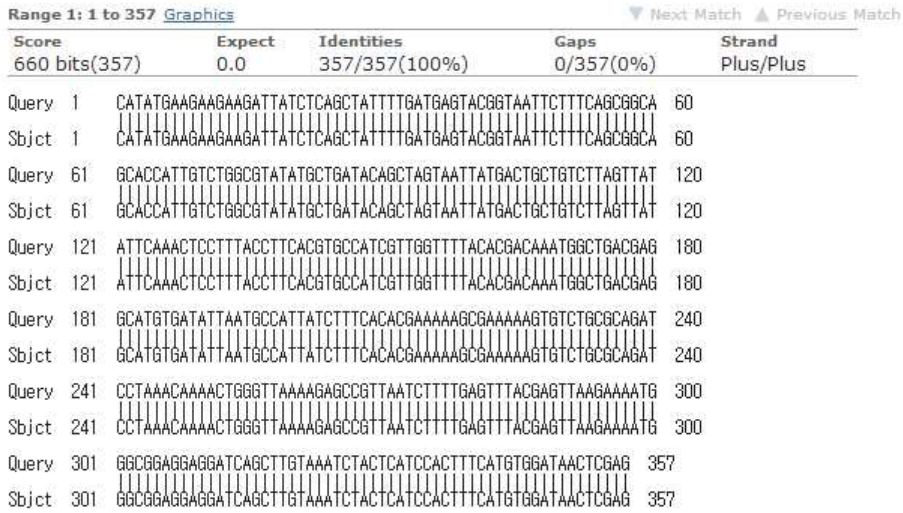
(b)



(c)

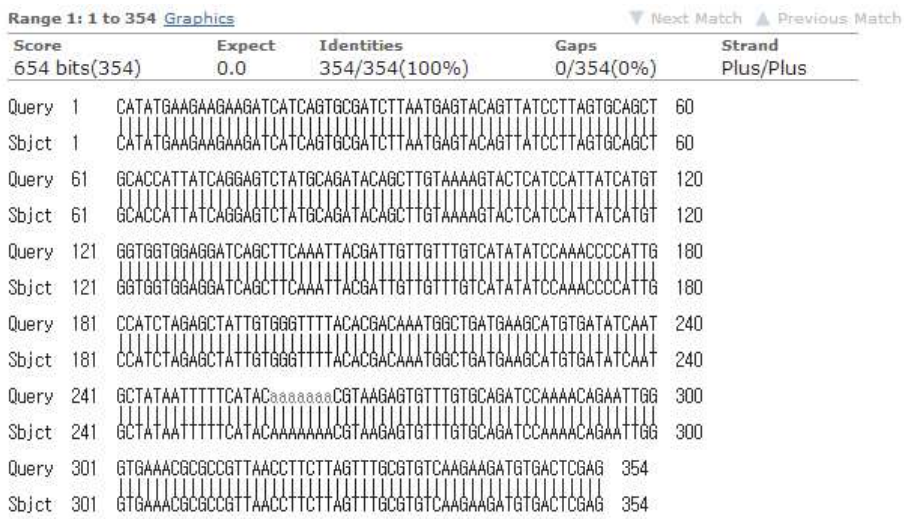


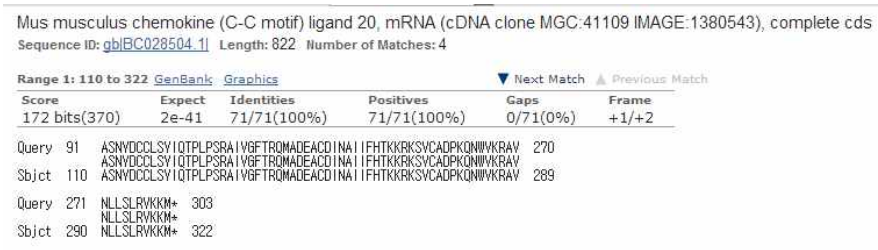
Figure 10. Sequencing results of recombinant CCL20 vectors. UC, UCM and UMC DNAs were correctly inserted into pIL.Ptuf

backbone vector. (a) pIL.Ptuf.UC containing UC DNA. (b) pIL.Ptuf.UCM containing UCM DNA. (c) pIL.Ptuf.UMC containing UMC DNA.

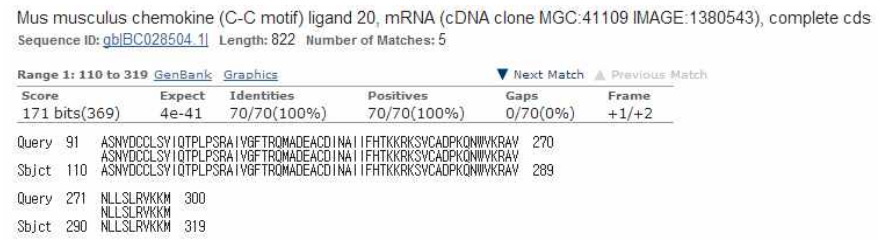
The sequencing results were exactly corresponded with the sequence of synthesized recombinant CCL20 DNAs.

To validate whether codon optimization was correctly conducted, BLAST search of each recombinant CCL20 DNAs was exhibited from NCBI (Figure 11).

(a)



(b)



(c)

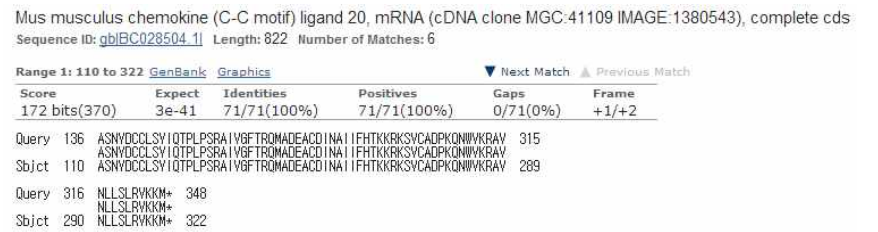


Figure 11. BLAST results of recombinant CCL20 vectors. recombinant CCL20 DNAs were correctly codon-optimized. (a) pIL.Ptuf.UC containing UC. (b) pIL.Ptuf.UCM containing UCM. (c) pIL.Ptuf.UMC containing UMC.

The BLAST results were exactly corresponded with the peptide sequence of murine CCL20. As a result, it is identified that constructed transformants have a potential to secrete correct recombinant CCL20.

In conclusion, UC, UCM and UMC expressing recombinant *L. lactis* was constructed and confirmed that they exactly have recombinant CCL20 DNAs (UC, UCM and UMC).

3. Validation of CCL20 expression

1) SDS-PAGE and western blot

To validate whether recombinant CCL20 transformants correctly express recombinant CCL20, SDS-PAGE and western blot were conducted for qualitative analysis. Recombinants CCL20 might be expressed in both cytoplasmic condition and secreted condition. Therefore, cytoplasmic proteins were extracted from recombinants CCL20 transformants by performing bead beating and secreted proteins were extracted by performing TCA precipitation. Both cytoplasmic proteins and secreted proteins were identified with SDS-PAGE and western blot.

200 ng of commercial CCL20 was used as a positive control. The molecular weight of commercial CCL20 is 7.9 kDa. *L. lactis*

IL1403 containing pIL252 vector was used as a negative control because pIL252 vector has same genome except Ptuf.UC, Ptuf.UCM and Ptuf.UMC. USP 45 signal peptide or M cell targeting were conjugated with murine CCL20 in recombinant CCL20. The molecular weight of USP45 and MTP is 3.0 kDa and 1.4 kDa, respectively. Therefore, recombinant CCL20 peptides were heavier than commercial CCL20 and MTP containing recombinant CCL20 peptides were heavier than UC. The molecular weight of UC is approximately 10.9 kDa and the molecular weight of both UCM and UMC is approximately 12.4 kDa. SDS-PAGE and western blot analysis were conducted based on the informations described above (Figure 12).

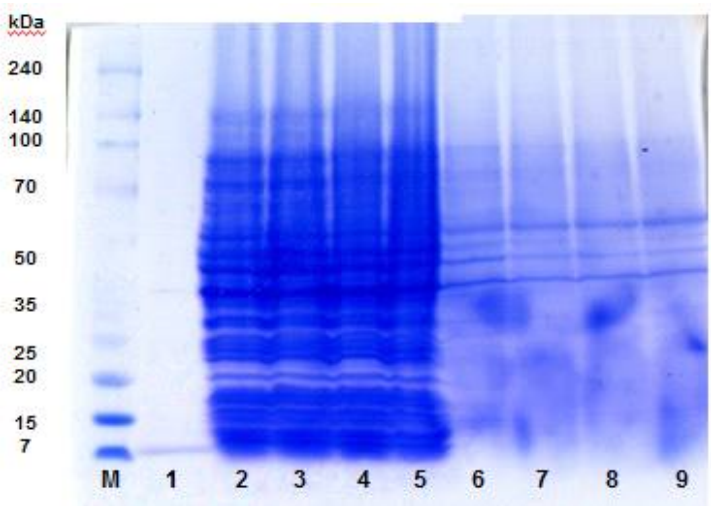


Figure 12. SDS-PAGE image for identifying recombinant CCL20 expression. Molecular weight of murine CCL20 is 7.9 kDa. UC is 10.9 kDa. UCM and UMC are 12.4 kDa. Recombinant CCL20 proteins were not detected in SDS-PAGE image. M, marker; lane

1, commercial CCL20; lane 2, pIL252 cytoplasmic protein; lane 3, pIL.Ptuf.UC cytoplasmic protein; lane 4, pIL.Ptuf.UCM cytoplasmic protein; lane 5, pIL.Ptuf.UMC cytoplasmic protein; lane 6, pIL252 secreted protein; lane 7, pIL.Ptuf.UC secreted protein; lane 8, pIL.Ptuf.UCM secreted protein; lane 9, pIL.Ptuf.UMC secreted protein.

The band of recombinant CCL20 was not well detected because the peptide recognition sensitivity of SDS-PAGE is considerably low. Therefore, western blot assay with anti-CCL20 antibody was conducted to validate the expression of recombinant CCL20 (Figure 13).

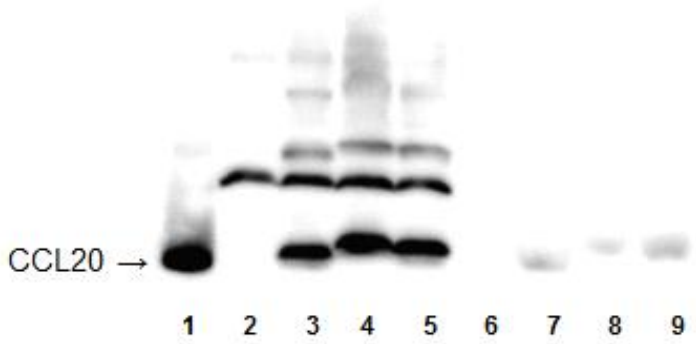


Figure 13. Western blot image for identifying recombinant CCL20 expression. Recombinant CCL20 proteins (UC, UCM and UMC) were detected in western blot image. M, marker; lane 1, commercial CCL20 (100 ng CCL20 in 20 μl TBST) ; lane 2, pIL252 cytoplasmic protein; lane 3, pIL.Ptuf.UC cytoplasmic protein; lane 4, pIL.Ptuf.UCM cytoplasmic protein; lane 5, pIL.Ptuf.UMC cytoplasmic protein; lane 6, pIL252 secreted protein;

lane 7, pIL.Ptuf.UC secreted protein; lane 8, pIL.Ptuf.UCM secreted protein; lane 9, pIL.Ptuf.UMC secreted protein.

In western blot image, background bands were detected in cytoplasmic proteins because *L. lactis* IL1403 secretes a lot of proteins for its survive. In addition, M17G broth has plenty of proteins to supply nutrients for *L. lactis* IL1403. Therefore, background bands were formed in cytoplasmic protein fraction. Resultingly, when background bands were disregarded, the bands of cytoplasmic proteins (UC, UCM and UMC) were correctly located as their protein sizes. The bands of secreted protein were correctly located as their protein size although their intensities are slightly weak compared to cytoplasmic proteins. It is thought that secreted protein precipitation was not enoughly conducted although the expression of secreted protein was confirmed.

Secreted proteins were extracted by TCA precipitation method. Recently, it is insisted that acetone precipitation method is much effective than TCA precipitation when precipitated proteins are below 15 kDa. Therefore, comparison of precipitation efficiency between TCA and acetone precipitation help the precipitation of secreted proteins be effective in next study.

2) ELISA

Protein detection from SDS-PAGE and western blot is a qualitative analysis. However, equal concentration of CCL20 should be used for *in vitro* chemotaxis assay because the difference of CCL20 concentration could make misunderstandings. Therefore, standarization of CCL20 concentration should be

conducted before *in vitro* chemotaxis assay. For this reason, ELISA assay was performed to quantify the recombinant CCL20 expression level (Figure 14). Supernatant of each recombinant CCL20 transformants was used for ELISA.

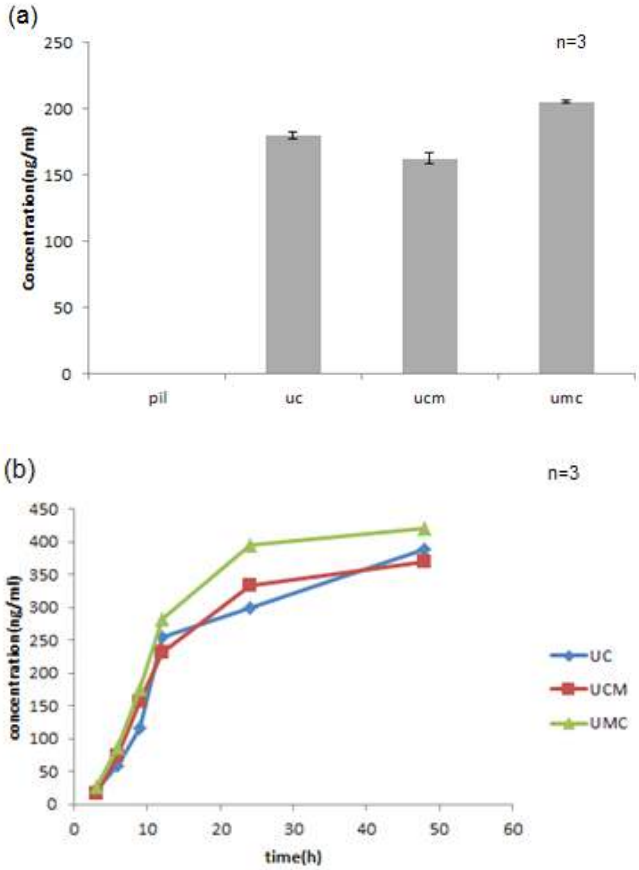


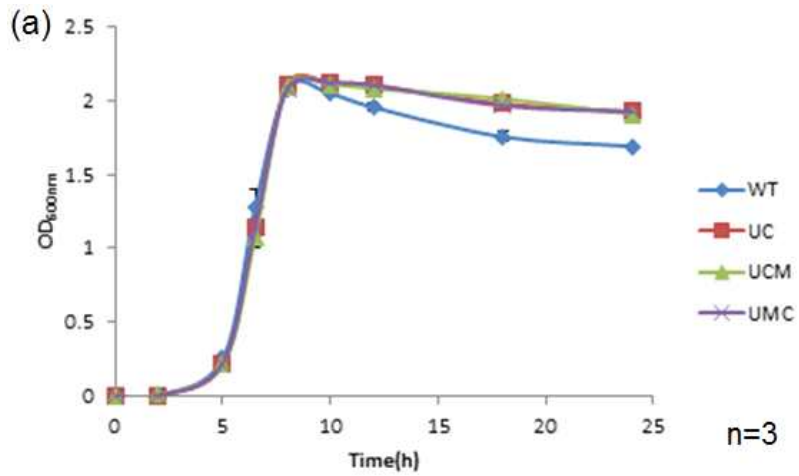
Figure 14. Quantification of recombinant CCL20 expression by ELISA. Recombinant CCL20 proteins were detected in ELISA. (a) Supernatant of recombinant CCL20 transformants after culturing for 22 hours. (b) Supernatant of recombinant CCL20 transformants after culturing for 3, 6, 9, 12, 24 and 48 hours.

180 ng/ml of UC, 163 ng/ml of UCM and 105 ng/ml of UMC proteins were secreted after 22 hours culture. Secreted proteins were accumulated until 48 hours.

In conclusion, recombinant CCL20 proteins (UC, UCM and UMC) were correctly expressed from each transformants.

3) Physiological test of recombinant CCL20 transformants

To validate whether the expression of recombinant CCL20 affects the physiology of *L. lactis* IL1403, physiological test was conducted. OD_{600nm} and pH were measured to check growth rate of transformants and pH value because LAB secrete lactic acid. Physiological test was performed based on the information described above (Figure 15).



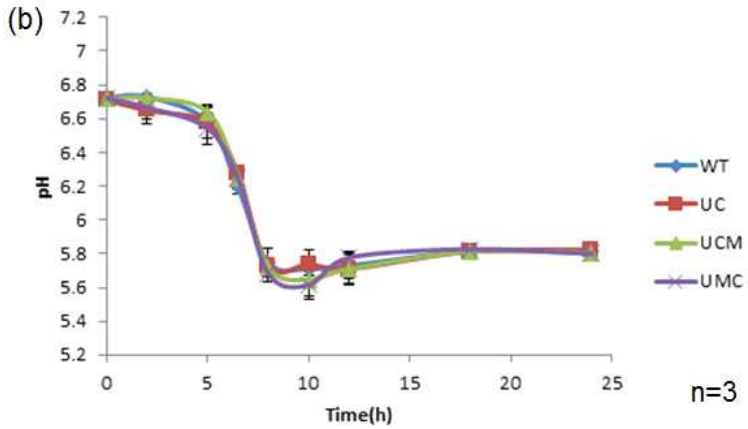


Figure 15. Physiological test of recombinant CCL20 transformants. OD value means the growth rate of transformants and pH stands for secretion of lactic acid from transformants. (a) OD value of WT and recombinant CCL20 transformants. (b) pH value of WT and recombinant CCL20 transformants.

The growth rate and pH of the recombinant CCL20 transformant were not much changed compared to wild type *L. lactis*, suggesting that recombinant CCL20 does not affect physiology of *L. lactis* IL1403.

4. *In vitro* chemotaxis assay

In vitro chemotaxis assay was conducted to validate whether recombinant CCL20 has biological activity. Transwell chamber is an appropriate tool for identifying chemotaxis of chemokines such as CCL20 because chemoattracted cells can pass through the pore between two wells of transwell chamber.

The growth medium without supplemented material was used as

a negative control. And then, the growth medium mixed with the supernatant of *L. lactis* IL1403 containing pIL252 vector (mixed medium) was used as another negative control because the supernatants of recombinant CCL20 transformants were used as a experimental group. 10 pg/ml, 100 pg/ml, 500 pg/ml, 1 ng/ml and 5 ng/ml concentrations of CCL20 were used for this assay. The supernatant of recombinant CCL20 transformants was mixed with growth medium to adjust target concentration. Therefore, the supernatant of *L. lactis* IL1403 containing pIL252 vector was mixed with growth medium as same ratio as each concentration of experimental group for negative control of each group. *In vitro* chemotaxis assay was performed based on the information described above (Figure 16).

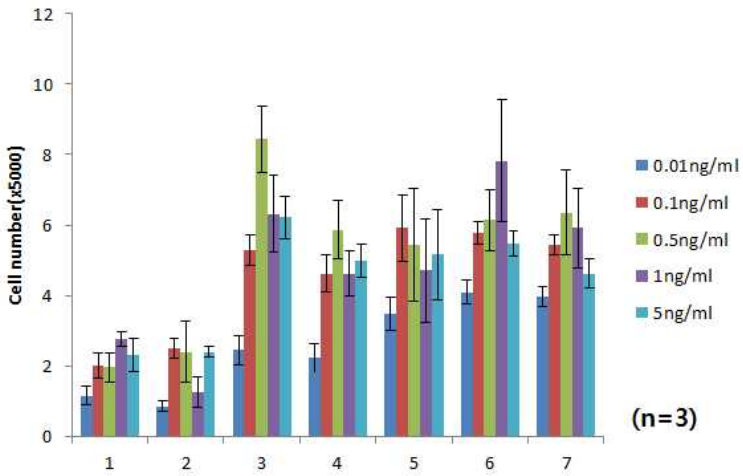


Figure 16. *In vitro* chemotaxis assay with JAWS II cells. 0.01, 0.1, 0.5, 1 and 5 ng/ml of CCL20 was used for chemotaxis assay. The number of JAWS II cells migrated to lower well of transwell was calculated with hemocytometer. (1) growth medium; (2)

mixed medium; (3) commercial CCL20 in growth medium; (4) commercial CCL20 in mixed medium; (5) UC with mixed medium; (6) UCM with mixed medium and (7) UMC with mixed medium.

There were significant differences between CCL20 containing group and negative controls. In addition, chemoattracted cells were migrated to lower chamber within the concentration of from 10 pg/ml to 5 ng/ml having appropriate concentration of from 0.5 to 1 ng/ml.

4. *In vivo* functional assay

1) Purification of BmpB

Recombinant BmpB was used as model antigen for mouse oral immunization. BmpB expressed from *E.coli* was purified by His-tag affinity chromatography. BmpB was mainly detected on lane O, lane W2 and lane E1. Lane O is the lane of original sample. The BmpB from original sample means BmpB was well extracted. BmpB band signal was weak in Lane S (sample flow through) and Lane B (binding fraction for washing) that means BmpB was well bound to resin. BmpB was detected in Lane E (elution fraction) that means BmpB was correctly obtained from His-binding resin (Figure 17).

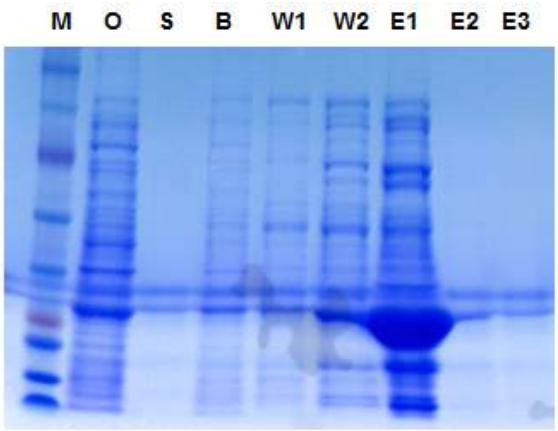


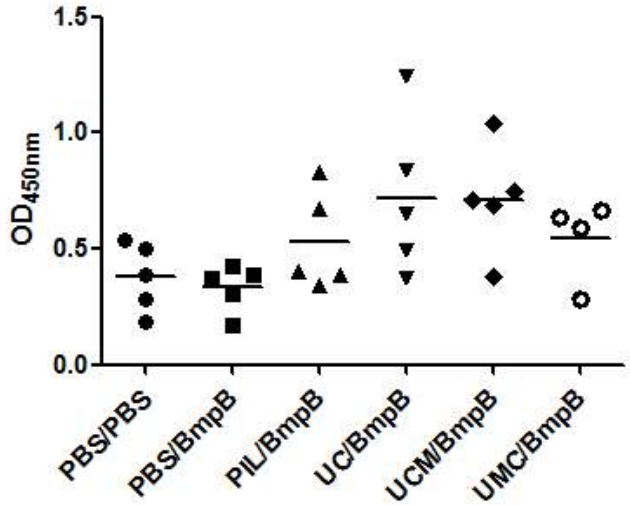
Figure 17. SDS-PAGE image of BmpB purification. BmpB is 29.7 kDa. M, marker; O, original sample; S, sample flow through; B, binding fraction; W1~2, washing fraction; E1~3, elution fraction.

In conclusion, BmpB was purified by affinity chromatography and used for *in vivo* functional assay.

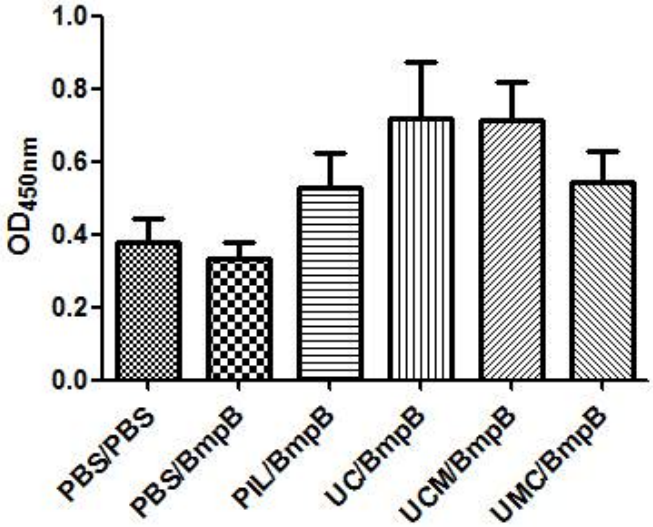
2) Detection anti-BmpB serum immunoglobulin G

ELISA detecting anti-BmpB IgG was conducted for validating whether recombinant CCL20 enhances immune response in animals. 6 groups (PBS/PBS, PBS/BmpB, PIL/BmpB, UC/BmpB, UCM/BmpB and UMC/BmpB) were used for *in vivo* functional assay. The words before slash means recombinant CCL20 transformants or PBS to validate whether they could enhance M cell differentiation and words after slash means model antigen for immunization. Mice administrated PBS and PIL were negative controls.

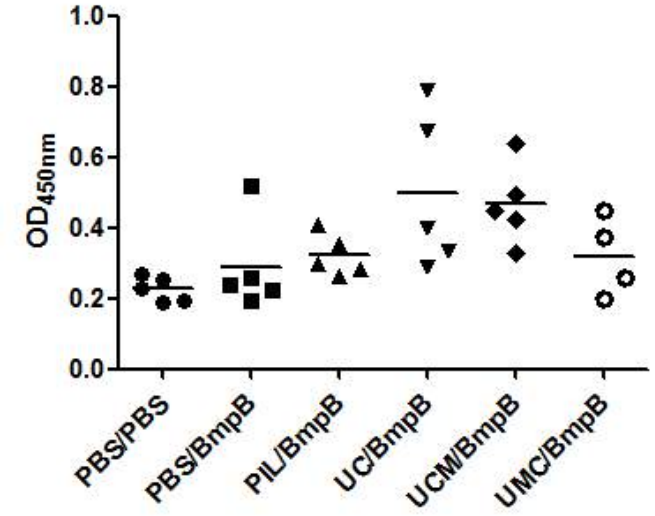
(a)


1.5
1.0
0.5
0.0
OD450nm
PBS/PBS
PBS/BmpB
PIL/BmpB
UC/BmpB
UCM/BmpB
UMC/BmpB

(b)


1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
PBS/PBS
PBS/BmpB
PIL/BmpB
UC/BmpB
UCM/BmpB
UMC/BmpB

(c)



(d)

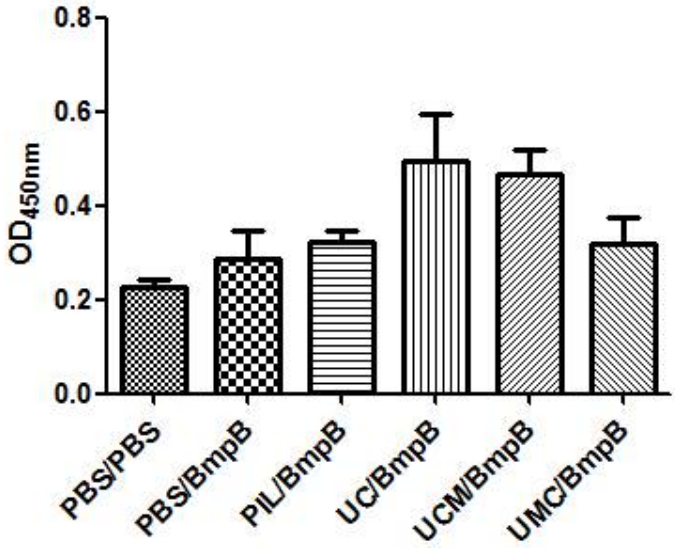


Figure 18. Serum anti-BmpB IgG level. (a) Serum anti-BmpB IgG level from each mice 3 weeks after priming (dot plot), (b) Serum anti-BmpB IgG level from each mice 3 weeks after

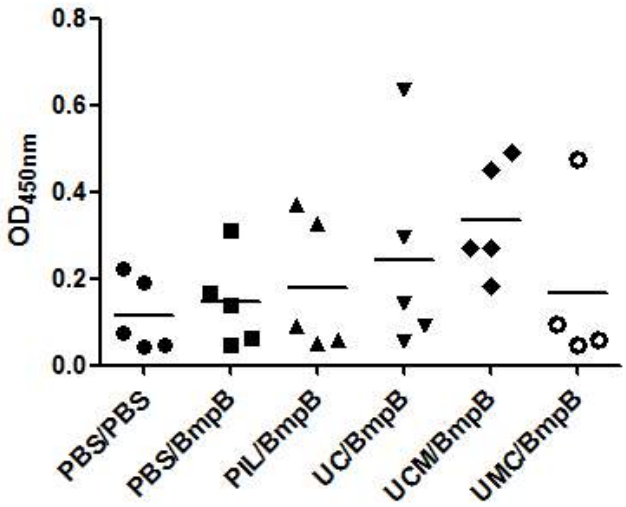
priming (bar graph). (c) Serum anti-BmpB IgG level from each mice 4 weeks after priming (dot plot), (d) Serum anti-BmpB IgG level from each mice 4 weeks after priming (bar graph). values are mean ± SEM. No significance were shown from one-way ANOVA test.

More serum IgG was detected from Mice administrated with recombinant CCL20 transformants (UC/BmpB, UCM/BmpB) than negative control group. Interestingly, UMC/BmpB group was similar with PIL/BmpB group. It could be understood that LAB could be an adjuvant themselves, although UMC did not have a biological functional activity (Figure 18). As a result, UC and UCM transformants had biological activity for enhancing serum IgG.

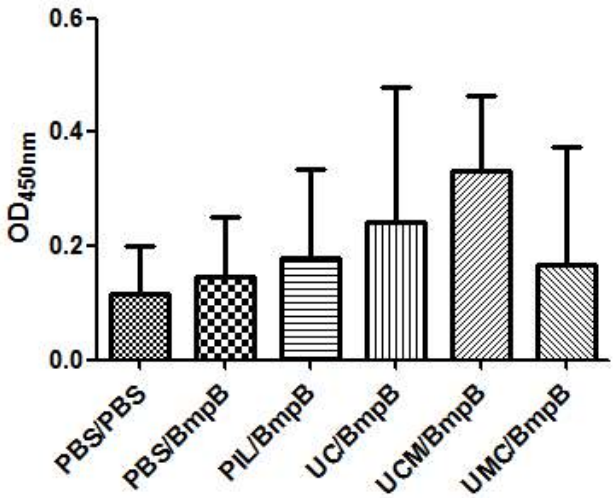
3) Detection anti-BmpB fecal immunoglobulins A

Contrary to serum IgG level generally means systemic immune response, fecal IgA level stands for mucosal immune response. To confirm enhancement of mucosal immune response, ELISA detecting anti-BmpB IgA was conducted. 3 weeks after BmpB priming, anti-BmpB IgA was increased in UC and UCM group. It indicates that recombinant CCL20 (UC and UCM) enhances mucosal immune response (Figure 19).

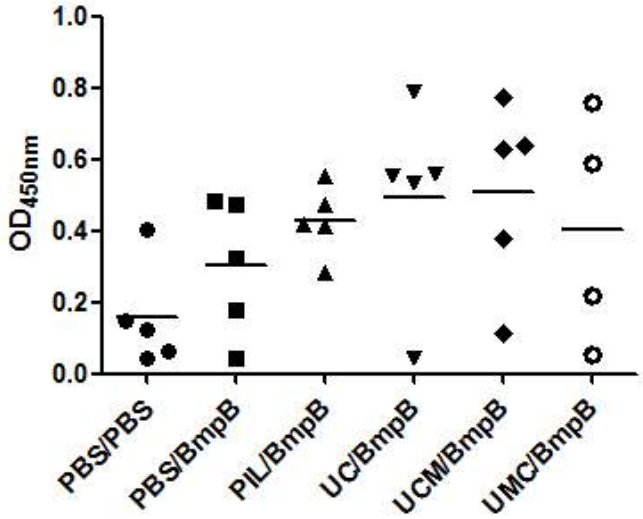
(a)


0.8
0.6
0.4
0.2
0.0
OD450nm
PBS/PBS
PBS/BmpB
PIL/BmpB
UC/BmpB
UCM/BmpB
UMC/BmpB

(b)


0.6
0.4
0.2
0.0
OD450nm
PBS/PBS
PBS/BmpB
PIL/BmpB
UC/BmpB
UCM/BmpB
UMC/BmpB

(c)



(d)

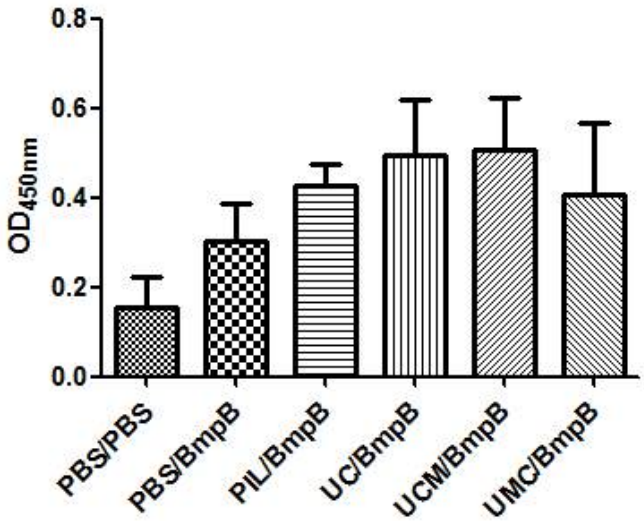


Figure 19. Fecal anti-BmpB IgA level. (a) Fecal anti-BmpB IgA level from each mice 3 weeks after priming (dot plot), (b) Fecal anti-BmpB IgA level from each mice 3 weeks after

priming (bar graph). (c) Fecal anti-BmpB IgA level from each mice 4 weeks after priming (dot plot), (d) Fecal anti-BmpB IgA level from each mice 4 weeks after priming (bar graph). values are mean ± SEM. No significance were showed from one-way ANOVA test.

In conclusion, UC and UCM, not UMC, transformants enhanced both systemic and mucosal immune response. It indicates that recombinant CCL20 transformants could be used as oral vaccine adjuvant. In further study, recombinant CCL20 transformants could be one of adjuvant candidate for enhancing the efficiency of oral vaccine against critical disease to livestock industry including PED and FMD.

PPs are important to the differentiation of M cells because PPs have a lot of lymphocytes within germinal center and T cell dependent area. Therefore, the number change of PPs after administrating recombinant CCL20 transformants was also checked. There was no significant differences between PBS administrated group and recombinant CCL20 administrated group ; data is not shown. It indicates recombinant CCL20 enhances differentiation of M cells, not development of Peyer's patch.

In this study, M cell targeting peptide was used for mimicking the tendency of CCL20 secretion in normal state because CCL20 is generally secreted to SED, beneath of FAE. However, function of M cell targeting peptide was uncertain because UMC did not enhance immune response at *in vivo* functional assay. Therefore, immunohistochemisty detecting M cell targeting peptide within PPs after administration of recombinant CCL20 transformatns could help evaluate the functional activity of M cell targeting

peptide.

It is validated that CCL20 could enhance mucosal immune response with model antigen by enhancing M cell differentiation. However, the increase number of M cell could have other unexpectable effects to mucosal immune response. For example, IFN-γ as pro-inflammatory cytokine could be enhanced when CCL20 attracts the CCR6 expressing lymphocytes. Therefore, cytokines level about pro-inflammation and anti-inflammation should be checked to validate whether recombinant CCL20 has adverse effects on immune system of body in next study.

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Ⅵ. Abstract in korean (초록)

축산업에서 경제성과 생산성을 증진시키기 위해서는 여러 가지 질병으로 가축을 보호할 필요가 있다. 이를 위해 축산업에서는 백신을 주로 이용해 왔다. 이러한 백신을 투여하는 방법에는 몇 가지가 알려져 있지만, 그 중 경구투여 방법이 가축에게 가장 스트레스를 적게 주며 안전한 방법이다. 하지만, 위산과 단백질분해 효소 등에 의해 경구백신의 전달효율은 현저하게 떨어진다. 이런 문제를 해결하기 위해 백신의 효율을 증진시킬 수 있는 어쥬번트를 함께 투여하는 것이 좋다. 뿐만 아니라 유산균과 같이 낮은 pH에도 잘 견디며 인체에 해가없는 백신전달체를 이용해 전달효율을 높여줄 수 있다.

경구투여 된 백신은 점막면역을 자극하여 IgA의 분비를 유도하는 방식으로 면역작용의 상승을 유도한다. 이러한 작용에 있어서 소장상피세포에 존재하는 M 세포가 중요한 역할을 한다. M 세포는 소장상피세포 근처의 항원을 포집하는 세포로서 점막면역을 유도하는데 대표적인 역할을 수행한다. 이번 연구에서는 M 세포의 분화를 유도할 수 있는 요인들 중에 CCL20를 연구해보고자 한다.

CCL20는 C-C motif chemokine ligand 20으로서 Peyer' patch의 상피세포인 FAE층의 하부로 분비가 된다. 기본적인 CCL20의 기능은 수지상세포, B 림프구, macrophage와 같은 면역세포들을 끌어들이는 역할을 하고 있다. 하지만 최근 CCL20가 M 세포의 분화를 증진시키는데 영향을 준다는 논문이 발표되고 있다. 따라서 본 연구는 재조합된 CCL20를 통해 M 세포의 분화를 유도, 점막면역을 증강시킬 수 있다는 가설에서부터 시작되었으며 그에 따라 이를 백신 어쥬번트로 활용하고자 했다.

CCL20는 주로 그람음성균에 대한 항균능력을 가지고 있다. 따라서 CCL20가 숙주인 *L. lactis* IL1403에 항균능력을 지니는지 조사하기 위해 MIC test를 시행했다. 그 결과 CCL20가 *L. lactis* IL1403에 항균능력을 지니지 않는 것을 확인하였고, 재조합 벡터의 숙주로 사용할 수 있음을 확인하였다.

Mouse CCL20, USP 45, M cell targeting peptide의 서열을 조사하여 발현 숙주인 *L. lactis*에 맞추어 코돈최적화를 진행한 후 유전자를 합성하였다. 합성된 유전자는 제한효소 Nde Ⅰ과 Xho Ⅰ 그리고 DNA ligase를 이용하여 pIL.Ptuf 벡터에 삽입되었다. 이에 따라 CCL20를 분비하는 3가지의 재조합 플라스미드 벡터 (pIL.Ptuf.UC, pIL.Ptuf.UCM, pIL.Ptuf.UMC)가 구축되었다.

항생제저항성 그리고 colony PCR과 DNA sequencing을 통해 원하는 DNA가 삽입되었는지 확인하였다. 이후 구축된 벡터는 *L. lactis* IL1403 competent cell에 electroporation되어 재조합 유산균이 구축되었다. 그 결과 세가지의 벡터 모두 원하는 recombinant CCL20 DNAs (UC, UCM, UMC)가 삽입되어 있음을 확인할 수 있었다.

다음으로 재조합 유산균에서 CCL20가 제대로 발현되는지를 CCL20 특이적 항체를 통한 western blot과 SDS PAGE로 확인하였다. 그 결과 UC, UCM, UMC에서 모두 재조합 단백질이 잘 발현되고 있음을 확인할 수 있었다. 그리고 JAWSⅡ 세포를 이용한 *in vitro* chemotaxis assay를 통해서 재조합된 단백질의 생물학적 활성도를 확인하였다. 그 결과 UC, UCM, UMC군에서 모두 JAWSⅡ 세포가 이동한 것을 볼 수 있었다.

마지막으로 Balb/c 쥐에서 *in vivo* functional assay를 수행함으로써 재조합된 유산균이 백신 어쥬번트로서 이용할 수 있는지 확인해보았다. 우선 일주일간 재조합 유산균을 매일 투여하여 M cell의 분화를 유도한다. 이후 BmpB를 이용하여 priming, 일주일 후와 이주일후에 boosting을 수행한다. priming 3주와 4주에 분과 혈청 sample을 채취한 후에 IgA와 IgG 수준을 측정했다. 그 결과 UC, UCM군에 IgA와 IgG가 증가한 것을 볼 수 있었다.

이러한 결과들을 종합하여, 구축된 재조합 유산균 UC, UCM을 경구백신 어쥬번트로 사용될 수 있음을 확인할 수 있었다.

주요어 : C-C motif chemokine ligand 20 (CCL20), 유산균, M 세포, 경구백신 어쥬번트
학번 : 2012-21161