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A Thesis
for the Degree of Master of Science

Soluble RANKL expression in *Lactococcus lactis*
and investigation for its potential
as an oral vaccine adjuvant

February, 2014

By
Jeong In Kim

Department of Agricultural Biotechnology
Graduate School, Seoul National University
Soluble RANKL expression in *Lactococcus lactis* and investigation for its potential as an oral vaccine adjuvant

수용성 RANKL을 발현하는 유산균 경구백신 면역증강제 개발

지도교수 최 윤 재
이 논문은 농학 석사학위논문으로 제출함

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김 정 인

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2014년 2월

위원 장 _____________ (인)

부위원장 _____________ (인)

위원 _____________ (인)
Summary

Oral vaccines have various advantages against other types of vaccines, such as reduced stress. When mucosal immune system is stimulated by oral vaccine, secretory IgA are produced which can neutralize pathogens at intestinal lumen, suggesting that mucosal immune response can protect against pathogens and inhibit pathogens to enter the body. However, major disadvantage of oral vaccines is low efficiency. Therefore developing adjuvant for enhancing the efficiency of oral vaccine is very important.

To initiate mucosal immune responses, antigens in the intestinal lumen should be transcytosed through epithelial cells. M cells are specialized cells in that it can transport antigens from the intestinal lumen to peyer’s patches. This study starts from the hypothesis that if we can improve the activity of M cells, the efficiency of mucosal immune response can be enhanced.

Receptor activator of NF-κB ligand (RANKL), one of the Tumor Necrosis Factor cytokine, has been known as an important factor for activation and survival of osteoclast cells. Recently it has been shown that RANKL is not only essential factor for peyer’s patches (PPs) development but critical factor for M cell differentiation.

In this study, we designed two recombinant L. lactis, which express and/or secret sRANKL, and investigated their potential possibility as an oral vaccine adjuvant.

We optimized the sequence of sRANKL for L. lactis and constructed sRANKL expression vector using PCR and other
DNA works. We inserted the plasmid into *L. lactis* by electroporation and these are confirmed by colony PCR and sequencing.

Expression of sRANKL was confirmed through western blot using sRANKL specific antibody. Biological activity of recombinant protein was validated by *in vitro* assay with RAW 264.7 cells. RAW 264.7 cells, mouse macrophage cell line, can be differentiated to osteoclast–like cells when M–CSF and RANKL are added to medium. By observing RAW 264.7 cells differentiation to osteoclast–like cells after treatment with sRANKL from recombinant *L. lactis*, recombinant sRANKL was recognized having functional activity similar with commercial sRANKL.

We also performed *in vivo* experiment with BALB/c mice to test the potential of recombinant *L. lactis* as an oral vaccine adjuvant. Mice were administered recombinant *L. lactis* every day for a week. Then mice were immunized with M–BmpB, which is subunit vaccine of *Brachyspira* conjugated with M cell targeting peptide. After 3 and 4 weeks after the priming, serum and feces were collected and analyzed for IgA and IgG level.

The groups which were administered recombinant *L. lactis* showed high level of M–BmpB specific fecal IgA compared to the other control groups, indicating that mucosal immune responses were enhanced by recombinant *L. lactis*.

In conclusion, the IgA level was increased at the group of recombinant *L. lactis* compared to the control group, indicating that recombinant *L. lactis* could be an oral vaccine adjuvant.

Key words: M cell, soluble RANKL, *Lactococcus lactis*, oral vaccine adjuvant.
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List of Abbreviations

ANOVA: Analysis of variance
BSA: Bovine serum albumin
DAPI: 4',6-Diamino-2-phenylindole dihydrochloride
DC: Dendritic cell
FAE: Follicle-associated epithelium
ELISA: Enzyme-linked immunosorbent assay
GALT: Gut-associated lymphoid tissue
GP-2: Glycoprotein-2
GRAS: Generally recognized as safe
HRP: Horseradish peroxidase
IHC: Immunohistochemistry
LAB: Lactic acid bacteria
*L. lactis*: *Lactococcus lactis*
DMEM: Dulbecco’s Modified eagle media
M cell: Microfold cell
M-CSF: Macrophage colony stimulating factor
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NFATc1: Nuclear factor of activated T cells
OPG: Osteoprotegerin
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PED: Porcine epidermic diarrhea
PFA: Paraformaldehyde
PP: Peyer’s patch
RANKL: Receptor activator of NF-κB ligand
SD: Swine dysentery
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SED: Subepithelial dome
SEM: Standard error of the mean
TMB: 3,3',5,5’-tetramethylbenzidine
TNF: Tumor necrosis factor
TRAP: Tartrate resistant acid phosphatase
Traf 6: TNF receptor associated factor 6
UEA-1 lectin: Ulex europaeus agglutinin-1 lectin
I. Introduction

In livestock industry, the prevention of diseases is very important in that it connects directly to the economics. Generally, the immune system of animals can be divided into systemic immune system and mucosal immune system. Systemic immune response is defense system against pathogens entered through wound. After activation of helper T and B cells by antigen presenting cells, blood immunoglobulin G is produced. For preventing waterborne diseases, inducing mucosal immune responses is more efficient than inducing systemic immune responses. After inducing mucosal immune responses, secretory immunoglobulin A (IgA) are produced by plasma cells. IgA block infection by inhibiting pathogens from adhering to mucosa site. In other words, by inducing mucosal immune responses, we can protect waterborne diseases at the first time. Therefore oral vaccine is essential to prevent digestive and respiratory diseases such as porcine epidermic diarrhea(PED) and swine dysentery(SD).

M cells are known as portals of pathogens and antigens in small intestine, which make them enter through epithelial cells. Therefore M cells have been issued for its importance in induction of mucosal immune response. My study began with the idea that if the function of M cells is enhanced, more antigens could be entered through epithelial cells and consequently mucosal immune responses could be induced more effectively.

In this study, we designed recombinant *L.lactis* which express and secret soluble RANKL. Also we investigate recombinant *L.lactis* whether it could be a potential oral vaccine adjuvant.
II. Review of Literature

1. Gut-associated lymphoid tissue (GALT) and M cell

1) Mucosal immune system

It has been documented that the mucosal immune system has three functions. First, it protects the mucous membranes against invasion and colonization and invasion of potentially harmful microbes that may be encountered. Second, it inhibits uptake of undergraded antigens including non-self proteins derived from ingested food, airborne materials and commensal microbes. Third, it prevents the stimulation of potentially harmful immune responses to the antigens when they reach the interior of body (Jan Holmgren, 2005).

2) Peyer’s patches (PPs)

The mucosal surfaces of animal’s body represent an enormous surface area that covered by specific epithelial barriers. Because mucosal tissues encounter the outside world, they are exceptionally immunologically active. (Bradtzaeg 1999).

PPs are thought to be one of the largest organized lymphoid tissues in the gastrointestinal immune system. There are commonly 8 to 10 PPs in the small intestine of mice. Each PP has several B cell–rich follicles surrounded by a mesh–like structure containing T cells. Although PPs have several common
immunological and microarchitectural features with other lymphoid organs, they have special features as the mucosa-associated lymphoid tissue (J.Kunisawa 2008). For example, PPs doesn’t contain afferent lymphatics. To compensate, follicle-associated epithelium (FAE), specialized epithelial region, covers the PPs. In FAE, specialized antigen-sampling M cells are exist (Figure 1). The M cells are short microvilli with a thin mucus layer. M cells can transcytosis efficiently, allowing transferring antigens from the intestinal lumen into PPs (A. Foussat 2001).
Figure 1. Schematic picture of the lymphoid elements of the intestinal immune system. While MLNs (mesenteric lymph nodes) and organized tissues of the PPs are related to the induction of immune response or immune tolerance, the effector sites are scattered around the lamina propria and mucosal epithelium. SED, subepithelial dome; TDA, thymus-dependent area (Allan McI. Mowat. 2003).
3) Microfold cell (M cell)

(1) Discovery of M cell

M cells are specialized epithelial cells of the follicular associated epithelium (FAE) which are characterized by short microvilli and thin layers, covering the lymphoid follicles such as peyer’s patches (PPs) (Owen RL et al., 1999). M cells constitute a minor proportion (in human and mice, less than 10%) but play role of antigen-transporting (Kato T et al., 2005).

In early 1900s, Dr. Kenzaburo Kumagai, discovered that FAE took up the Mycobacterium tuberculosis and then it appeared in GALT follicles (Kumagai K et al., 1922). 50 years later, Bockman and Cooper discovered unique cells in the FAE which ingest ferritin and india ink vigorously at appendix of rabbit and bursa of Fabricus of chicken (Bockman DE et al., 1973). Owen and Jones identified the counterparts in PP FAE of human and they were named as M cells (Owen RL et al., 1974).

(2) M cell marker

More than three decades after M cells were discovered, M cells had been limited to morphological analysis because there was not sufficient amount of M cells for molecular or biochemical analysis. At first, there was no M cell specific markers.

Ulex europaeus agglutinin-1 (UEA-1) which is a plant lectin has been used for M cell markers, but there were some drawbacks of using UEA-1 as M cell markers. Because, first, UEA-1 recognizes α(1–2)-fucose, but this reactivity is restricted
to mouse M cells. In addition, other studies have shown that UEA-1 didn’t play role as M cell markers in animals and humans. Second, UEA-1 recognizes goblet and Paneth cells among intestinal epithelial cells in mice (Figure 2). Nowadays, glycoprotein 2 (GP2) has been identified as M cell marker (Hase K et al., 2009). Original function of GP2 is receptor for bacterial uptaking. GP2 recognizes FimH, which is major outer membrane components of gram-negative enterobacilli such as E. coli and Salmonella enterica (Yu S et al., 2009).
Figure 2. Confocal laser microscopy image of whole-mount staining of mouse PP. GP2 (green, left) and UEA-1 (red, middle) mostly colocalize (merge, right). However, GP2 signal is restricted to the FAE (in the middle of the picture, an oval area) whereas UEA-1 reacts to mucin-secreting goblet cells in the villi. Scale bars, 100 µm (Hiroshi Ohno and Koji Hase, 2010).
2. Receptor activator of NF-κB ligand (RANKL)

1) The crucial role of RANKL in bone

RANKL is a type II membrane protein which belongs to the TNF (Tumor Necrosis Factor) superfamily. RANKL possesses a transmembrane domain and C-terminal receptor-binding domain. RANKL is a ligand against specific receptor named RANK which is a type I membrane protein and OPG (decoy receptor osteoprotegerin) inhibits the binding of RANKL to RANK (Simonet, W. S. et al., 1997). In bone, osteoclastogenesis-supporting cells such as osteoblasts express RANKL in response to osteoclastogenic factors, including parathyroid hormone, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and prostaglandin E₂ (PGE₂). Genetically modified mice has shown that RANK–RANKL signaling is essential for osteoclastogenesis (Theill, L. E. et al., 2002).

When RANKL binds to RANK, TRAF6, which activates NF-κB and JNK/c–Jun, is recruited. RANK–RANKL signal also stimulates c–Fos induction, which is a component of AP-1, through NF-κB and CaMK. NF-κB and c–Fos robust NFATc1 induction, which is the master regulator of osteoclastogenesis. When NFATc is activated by calcineurin, immunoglobulin-like receptors such as OSACR, PIR–A and TREM–2, positive feedback loop is activated, and the level of NFATc1 increases by autoamplification. NFATc1 co-operates with other transcription factors and induces numerous osteoclast-specific genes in the nucleus (Figure 3).
Figure 3. Schematic diagram of osteoclastogenesis in osteoclast precursor cells. RANK–RANKL signaling is a trigger of osteoclast–specific genes expression (Hiroshi Takayanagi, 2009).
2) Immunological functions of RANKL.

RANKL\(^{-/-}\) mice lack lymph nodes and have abnormality in the spleen (Y.Y. Kong. et al., 1999). These defects in secondary lymphoid organs development provide the first genetic evidence for the important role of RANKL in both immune systems.

*In vitro*, T cells which express RANKL play critical role in DC activation and survival (Iwasaki, A. and Kelsall, B., 2001), indicating that the RANK–RANKL signal regulates DC function through the interactions between T cell and DC. However, RANKL\(^{-/-}\) mice have normal DC development and function (Izadpanah, A. et al., 2001).

Although the role of RANKL in the immune system needs to be identified in more detail, it is obvious that RANKL is important molecule that lucidly link the bone and immune systems.

3) Relationship between RANKL and M cell differentiation

In PP domes, subepithelial stromal cells selectively express RANKL. And throughout the small intestine, epithelial cells express RANK. RANKL\(^{-/-}\) mice have less than 2% of wild-type levels of M cells on PP and uptake ability of intestinal epithelial cells is markedly diminished. Furthermore, after treating RANKL specific antibodies in adult wild-type mice, most PP M cells were also eliminated. Interestingly, the M cell deficit in RANKL\(^{-/-}\) mice could be corrected by exogenous RANKL systemic administered (Figure 4), indicating that RANKL is the critical factor for controlling the M cell differentiation from RANK–expressing precursor cells of intestinal epithelia(Kathryn A. Knoop, 2009).
Figure 4. Relationship between RANKL and M cell differentiation. (a) PP of RANKL\(^{-/-}\) mice contain very few M cells. (b) Treatment of RANKL specific RANKL antibody to wild-type mice leads to loss of PP M cells. (c) Administration of rRANKL to RANKL\(^{-/-}\) mice restores PP M cells(Kathryn A. Knoop, 2009).
3. Lactic acid bacteria as adjuvant vehicle

1) Lactic acid bacteria

LAB are a group of Gram-positive, non-sporulating bacteria. For long times, dietary LAB have been used in food processing and food preservation. They have been involved in the manufacturing of diary products, bread, ensilage, fermented meat and so on. Among LAB, some species are important members of the endogenous microbiota which are associated with mucosal compartments of the body. Lactobacilli and streptococci are highly exist in the human ileum and jejunum \(10^3 - 10^5\) organisms/g of luminal contents) In colon, complex microbiota comprises about \(10^{11}\) bacteria/g such as streptococci and lactobacilli (Hayashi et al., 2005).

2) LAB as delivery vehicles

LAB possess numerous properties that make them attractive candidates for oral delivery vehicles. LAB have been used in the fermentation and food preservation for centuries, and are thought to be 'safe' organisms with a GRAS (Generally Recognized As Safe) status even though other live vaccine carriers such as Salmonella, Escherichia coli, Vaccinia etc., cannot be classified as 'safe'. In contrast to the latter type of carriers, LAB themselves are not hight immunogenic (Peter H Pouwels et al., 1998).

One of the most important advantage of LAB used as delivery vehicles for vaccine is the potential of eliciting antigen-specific
immunoglobulin A responses at mucosal sites. Because mucosal vaccines can elicit both secretory IgA and systemic immune responses, oral vaccines using LAB as delivery vehicles have advantages over many existing vaccines (Marian R. Neutra and Pamela A. Kozlowski, 2006). Another advantage of using LAB is their convenience of genetic engineering. Therefore they can express foreign proteins and other materials effectively (Table 1).
<table>
<thead>
<tr>
<th>Vaccine target</th>
<th>Vehicle</th>
<th>Antigen (mode)</th>
<th>Model (route)</th>
<th>Immune responses*</th>
<th>Protection model (outcome)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helicobacter pylori</td>
<td>Lactobacillus plantarum and L. plantarum air</td>
<td>Unosra B (cytoplastic)</td>
<td>Intragastric</td>
<td>Serum antibody</td>
<td>Colonization level (partial protection)</td>
</tr>
<tr>
<td>H. pylori</td>
<td>Lactobacillus lactis</td>
<td>Unosra B (cytoplastic)</td>
<td>Intragastric</td>
<td>None</td>
<td>Colonization level (full protection)</td>
</tr>
<tr>
<td>Tetracina</td>
<td>L. lactis</td>
<td>TFC (cytoplastic)</td>
<td>Intragastric, intranasal and intramuscular</td>
<td>Serum antibody, fimbrial IgA, T cells and ELISPOT</td>
<td>Survival after tetracina toxin challenge (protection)</td>
</tr>
<tr>
<td>Tetracina</td>
<td>L. plantarum, L. lactis and L. plantarum air</td>
<td>TFC (cytoplastic)</td>
<td>Intragastric, intranasal and intramuscular</td>
<td>Serum antibody, T cells and neutralizing antibody</td>
<td>Survival after tetracina toxin challenge (protection)</td>
</tr>
<tr>
<td>Strep. pneumoniae</td>
<td>L. lactis</td>
<td>PapA</td>
<td>Intranasal</td>
<td>Serum antibody and IBAF antibody</td>
<td>Infectious lethal challenge (intraperitoneally and intranasally (increased survival))</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>Lactobacillus and Lactobacillus lactis</td>
<td>PapA</td>
<td>Intranasal</td>
<td>Antibody in serum, BALF and nasal washes</td>
<td>Nasal colonization (reduction in pneumococcal load)</td>
</tr>
<tr>
<td>Strep. pyogenes</td>
<td>L. lactis</td>
<td>Cross region of M proteins</td>
<td>Intramuscular and subcutaneous</td>
<td>Salivary IgA and serum antibody</td>
<td>Pharyngeal infection (intranasal route; protective)</td>
</tr>
<tr>
<td>HPV-1</td>
<td>L. lactis</td>
<td>VZV-420 (cyto)</td>
<td>Intranasal</td>
<td>Serum antibody, fimbrial IgA, IBAF, T cells and ELISPOT</td>
<td>Intraperitoneal challenge with HPV-1 (longer-increasing vaccine intraperitoneal load reduced)</td>
</tr>
<tr>
<td>E. coli</td>
<td>L. lactis</td>
<td>SPaK (cyto/secreted)</td>
<td>Intranasal</td>
<td>Serum antibody and fimbrial IgA</td>
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</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>Lactobacillus acidophilus</td>
<td>E. coli (cyto)</td>
<td>Intranasal</td>
<td>Not applicable</td>
<td>Inhibition of E. coli adhesion in presence intestinal barrier</td>
</tr>
<tr>
<td>SARS-CoV-2-associated coagulopathy</td>
<td>Lactobacillus casei</td>
<td>Spike antigen segments</td>
<td>Intranasal</td>
<td>Serum antibody and mucosal IgA</td>
<td>Viral neutralizing antibody elicited</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>L. lactis</td>
<td>VLP (cytoplastic, cell wall associated and secreted)</td>
<td>Intranasal</td>
<td>Serum antibody</td>
<td>Virus neutralization assay (neutralizing antibody demonstrated for VLP, cell wall associated vaccine)</td>
</tr>
<tr>
<td>IBDV</td>
<td>L. lactis</td>
<td>VP1 and VP3</td>
<td>Oral</td>
<td>None</td>
<td>None performed, as no immune response was detectable</td>
</tr>
<tr>
<td>Group B Streptococcus</td>
<td>L. lactis</td>
<td>Porin (Lac) (cytoplastic)</td>
<td>Intranasal</td>
<td>Serum antibody and antibodies in nasal and vaginal washes</td>
<td>Survival of offspring from vaccinated mothers after infection challenge</td>
</tr>
<tr>
<td>Brucella abortus</td>
<td>L. lactis</td>
<td>E2 or E3 (cytoplastic)</td>
<td>Intranasal</td>
<td>IgA</td>
<td>Partial protection against intraperitoneal inoculation of virulent B. abortus</td>
</tr>
<tr>
<td>HPV16-induced tumours</td>
<td>L. lactis</td>
<td>E7 and E5 (secreted)</td>
<td>Intranasal</td>
<td>CTL and ELISPOT</td>
<td>Protection demonstrated against infection of E7 expressing tumour cell line</td>
</tr>
<tr>
<td>HPV16-induced tumours</td>
<td>L. casei</td>
<td>E7</td>
<td>Intragastric</td>
<td>Serum antibody, mucosal IgA and ELISPOT</td>
<td>Protection demonstrated against infection of E7 expressing tumour cell line</td>
</tr>
<tr>
<td>Plasmodium yoelii</td>
<td>L. lactis</td>
<td>MSP1 (cytoplastic)</td>
<td>Intranasal</td>
<td>None</td>
<td>Challenge with P. yoelii parasites (reduced patent amastigote)</td>
</tr>
</tbody>
</table>

Table 1. Lactic acid bacteria used as delivery vehicles of mucosal vaccine. air, alanine racemase mutant; BALF, bronchoalveolar lavage fluid; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunospot; HPV, human papillomavirus; IBDV, infectious bursal disease virus; ICCS, intracellular cytokine staining; SARS, severe acute respiratory syndrome; TTFC, tetanus toxin fragment C (Jerry M. Wells and Annick Mercenier, 2008).
III. Materials and Methods

1. Microorganism strains and growth conditions

*E. coli* and *L. lactis* IL1403 were used in this study. *E. coli* DH5α strains were grown in LB medium (Difco, BD). Recombinant *E. coli* which have plasmid with Amp resistance gene were grown in LB medium with 100㎍/ml ampicillin. *E. coli* were incubated at 37°C, 250rpm shaking incubator. Wild type *L. lactis* were grown in M17 medium (Difco, BD) supplemented with 5% glucose (M17G) without antibiotics and recombinant *L. lactis* were grown in M17G medium with 5㎍/ml erythromycin at 30°C.

2. Recombinant *L. lactis* construction

1) sRANKL sequence

Murine RANKL consists of extracellular domain (247 a.a.), transmembrane domain (21 a.a.) and cytoplasmic domain (48 a.a.). RANKL protein can be cleaved to a soluble form by extracellular proteases such as ADAM10, matrix metalloproteases. There are several soluble RANKL sequence candidates, which has slightly different length. In this study, the 181 a.a. soluble RANKL sequence was used according to the paper by Knoop et al.(2009).

2) Codon optimization and synthesis of sRANKL

In this study, *L. lactis* were used as recombinant protein
expression host. Because codon usage is different depending on host, codon optimization for *L. lactis* is essential. After codon optimization, signal sequence usp45 and sRANKL sequence was cloned into pGEM-B1 vector (bioneer) including 2 different restriction enzyme, Nde1 and Xho1 at each side.

By PCR-amplification with the specific primers, sRANKL sequence was ligated directly into T–vector. Schematic diagrams of the sRANKL expression vectors are shown on figure 5.

![Schematic diagrams of the sRANKL expression vectors.](image)

**Figure 5.** Schematic diagrams of the sRANKL expression vectors.

3) **Construction of sRANKL expression vector for *L. lactis***

Because p–GEM–B1 vector and T–vector have the origin for E.coli, they are not proper for *L. lactis* expression vector. For inserting into pIL252 expression vector, which is commonly used
as *L. lactis* expression vector, restriction enzyme Nde1 and Xho1 were used. After enzyme digestion of both pGEM-B1 and T-vector two different inserts were gained by gel elution. At the same time, pIL252 vector backbone was gained by gel elution. For ligation, vector backbone and inserts were mixed with 1:4 molar ratio and T4 ligase and 10x ligase buffer was added. The mixture was incubated at 4°C for overnight.

4) **Transformation and selection for transformants**

After pellet painting the ligation products, constructed vectors were transformed into *L. lactis* by electroporation method. (2.5kV, 10μF, 300Ω) After electroporation, *L. lactis* were pre-incubated at 30°C for 2h. Cells were spun down by centrifugation at Max speed for 10 sec, resuspended with 100μl of supernatant and spreaded on M17G agar plate. After 24h incubation at 30°C, colony PCR was conducted with the sRANKL specific primers (Tabel 2).

**Table 2.** Primer used for colony PCR

<table>
<thead>
<tr>
<th>Primer names (Length)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID.sRANKL-F (24)</td>
<td>5'-GCCGCAAGTATCCCCATCAGGATCA-3'</td>
</tr>
<tr>
<td>ID.sRANKL-R (24)</td>
<td>5'-CACGTAATTAAAAACCCTCCAA-3'</td>
</tr>
</tbody>
</table>
The transformants candidates were verified by DNA sequencing from National Instrumental Center for Environmental Management (NICEM) with the primer set for sequencing pIL252 vector (Table 3).

**Table 3.** Primers used for sequencing.

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seq.pIL252-F (24)</td>
<td>5’-CTCTTTTCTCTTCCAATTGTCTAA-3’</td>
</tr>
<tr>
<td>Seq.pIL252-R (23)</td>
<td>5’-CGGTAGTTTGCTTTATGCAGACT-3’</td>
</tr>
</tbody>
</table>

3. sRANKL expression in *L. lactis*

1) Protein preparation from *L. lactis*

Expression of sRANKL from *L. lactis* was confirmed by a SDS–PAGE and western blot assay. For SDS–PAGE and western blot, transformed *L. lactis* were cultured at M17G medium supplemented with erythromycin at 30°C for overnight. For preparation cytoplasmic proteins, cells from 7㎖ media were collected by centrifugation at 13,000 rpm for 1min. After washing twice with D.W., cell walls were broken by bead beater with glass beads of 0.1mm and 0.5mm size. Secreted proteins from usp45–sRANKL expression vector were collected by TCA precipitation method. After overnight culture of *L. lactis*, 1.6㎖ supernatants were added with 400㎕ of 80% TCA. After
centrifugation at 12,000rpm for 10 min, sinked proteins were washed with ethanol, and centrifuged again. At last, proteins were dissolved at Tris–HCl buffer.

2) SDS–PAGE and western blot assay

The sample proteins were mixed with 5X buffer containing DTT and SDS and incubated for 10min at 95°C. The sample proteins were separated by SDS–PAGE using 10~20 %(w/v) polyacrylamide gel. After separating by protein size, the gel was transferred onto a 0.2㎛ Nitrocellulose membrane (Whatman, USA). The membrane was blocked with blocking buffer containing 5%(w/v) skim milk of TBST at RT for 1h. After blocking, the membrane was incubated with anti–RANKL antibody (R&D systems) at 0.1㎍/㎖ concentration at 4°C for overnight. After washing the membrane with TBST 3times for 10min each, the membrane was incubated with Goat IgG horseradish peroxidase–conjugated antibody at 1:1000 dilution at RT for 1h. After washing with TBST 3 times, ECL was added to membrane and the signals were readed by Chemidoc.

3) ELISA assay

Quantification of sRANKL expression was done with ELISA kit from R&D systems. For ELISA assay, cytoplasmic proteins were prepared from the method described above. ELISA assay was performed according to the manufacturer’s instructions.
4) Physiological characterization of transformed *L. lactis*

Two different recombinant *L. lactis* which have usp45-sRANKL and sRANKL vector respectively, and wild type *L. lactis* were pre-incubated for overnight at 30. After pre-incubation, 10㎕ of *L. lactis* were inoculated into 50㎖ of M17G broth supplemented with erythromycin and M17G broth. OD600nm and pH were measured every 2h until 12 h and at 24 h.

4. *In vitro* functional activity assay

1) RAW 264.7 cells and growth conditions

Mouse macrophage cell line, RAW 264.7 cells were from Korean Cell Line Bank(KCLB). RAW 264.7 cells were grown at DMEM high glucose medium with 10% FBS and 1% P/S.

2) *In vitro* functional activity assay

2×10⁵ RAW 264.7 cells were plated at 100mm petri dish with DMEM medium with 10% FBS and 1% P/S. After 4 h, when most cells were adhered at the plate bottom, DMEM medium with 10% , 1% P/S, 30ng/㎖ of M-CSF and sRANKL.

Preliminary experiment was conducted for elucidating of commercial sRANKL effect on RAW 264.7 cells differentiation into osteoclast–like cells. 5 groups of cells were treated with 0, 20, 30, 50, 100 ng/㎖ respectively. At day 3, medium was changed again and day 6, RNA from the RAW 264.7 cells were
collected (Figure 6).

For elucidating biological activity of sRANKL from recombinant \textit{L.lactis}, cell extracts from recombinant \textit{L.lactis} and wild type \textit{L.lactis} were collected by bead beating method described above. Cell extracts were added to the medium. As preliminary experiment, medium was changed at day 3, and cell RNA was collected at day 6.

\textbf{Figure 6}. Schematic diagram of \textit{in vitro} assay step.

\textbf{3) MTT assay}

MTT assay was performed for identifying the viability of RAW 264.7 cells after treatment of commercial sRANKL and \textit{L.lactis} cell extracts. Cells were cultured in the medium of DMEM high glucose with 10% FBS, 1% P/S and 50ng/㎖ of commercial sRANKL or \textit{L.lactis} cell extracts. At day 2, day 4 and day 6 cells were used for MTT assay. At day 3, cell culture media were changed.
4) RT-PCR

At day6, culture medium were discarded and cells were washed with PBS. 750μl of Trizol was added to each well and incubated at RT for 10 min. Trizol mixture was transferred to 1.5ml tube and 150μl of chloroform was added. Shake the tube vigorously for 15 sec and samples were incubated at RT for 10 min. After centrifugation at 12,000g for 15 min, transparent part was transferred to 1.5ml tube. 500μl of isopropanol was added and samples were incubated at -20°C for overnight. The samples were centrifuged at 16,000rpm for 30min and supernatant was aspirated. After adding 1ml of 70% ethanol, the samples were centrifuged again at 16,000rpm and supernatant was aspirated again. The samples were dissolved at 10μl of DEPC treated water and stored at -70°C until analyzed.

cDNA synthesis was performed with RT (dT20) kit (Bioneer, USA). 1μg of RNA was added to each tube and DEPC treated water was added. After synthesis of cDNA, qRT-PCR was performed with the specific primers (Table 4).
Table 4. Primers used for RT-PCR.

<table>
<thead>
<tr>
<th>Primer names (Length)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFATc1-F (24)</td>
<td>5'-TCCTGCTCCTCCTCCTGCTGCTCG-3'</td>
</tr>
<tr>
<td>NFATc1-R (24)</td>
<td>5'-GCTGCTGGCAAGGCAGAGTGTGCT-3'</td>
</tr>
<tr>
<td>Traf6-F (24)</td>
<td>5'-GCCTGCATCATCAAATCCATAAGG-3'</td>
</tr>
<tr>
<td>Traf6-F (27)</td>
<td>5'-AATTCACAATGTACTTGATGATCCTCG-3'</td>
</tr>
<tr>
<td>TRAP-F (24)</td>
<td>5'-GCGACCATTGTTAGCCACATACGG-3'</td>
</tr>
<tr>
<td>TRAP-R (24)</td>
<td>5'-CGCCCAGGGGAGTCTCAGATCCCAT-3'</td>
</tr>
<tr>
<td>GAPDH (24)</td>
<td>5'-AAGGCCATGCCAGTGAGCTTC-3'</td>
</tr>
<tr>
<td>GAPDH (21)</td>
<td>5'-AAGGCCATGCCAGTGAGCTTC-3'</td>
</tr>
</tbody>
</table>

5. *In vivo* immunization assay

1) Oral administration in BALB/c mice

All animal experiments were approved by Institute of Laboratory Animal Resources Seoul National University (SNU-130506-3). 6 weeks old female BALB/c mice were maintained in filter top cage. Before PBS or *L. lactis* oral administration, 500μl of 1.5% NaH₂CO₃ buffer, which acts as neutralizing agent, was administered orally. 30 min after the oral administration of neutralizing agent, 200μl of *L. lactis* or PBS was administered by
oral zonde needle. Control groups of five mice were administered 200\(\mu\)l of PBS every day for 7 days. And 3 groups of five mice were administered with suspension of WT and recombinant *L. lactis* prepared as follow. 10\(\mu\)l of *L. lactis* stock were inoculated into 50\(\text{m}\)l of M17G broth and incubated at 30°C for overnight. When OD\text{600nm} reaches to 2.0, cell pellets were harvested by centrifugation at 4,500rpm for 10min. Cell pellets were resuspended in 1\(\text{m}\)l of M17G broth at a concentration of 10\(^9\) CFU/\(\text{m}\)l and incubated another 1 h at 30°C.

After 7 days, mice were immunized with purified M–BmpB by oral route 2 times in every three weeks. One group was administered with PBS.

Small intestine tissues for immunohistochemistry (IHC) assay were prepared at day 8. And 3 and 4 weeks after the first immunization, serum and feces were collected for analyzing anti M–BmpB immunoglobulins (Figure 7).

**Figure 7.** *In vivo* immunization and sampling schedule.
2) Whole mount IHC assay

After one week oral administration of L. lactis or PBS, mice were euthanized for removing PPs of small intestine. Small intestine tissues were washed with PBST (0.05% Tween 20) and PPs were detached from the tissues. Detached tissues were incubated with PBST supplemented with DNase (PBST 3㎖ + DNase 3㎕ (1,500 unit)) at 37°C for 20min. After washing with cold PBST 3times, the tissues were fixed with 4% (v/v) paraformaldehyde for 2 h at 4°C. After 3 times washing, the tissues were blocked with 3% goat serum at RT for 1 h. Blocked tissues were incubated with 400 diluted anti GP2 monoclonal antibody at 4°C for overnight. After washing 3 times, 1㎎/㎖ DAPI were treated for 10 min. The tissues were washed for 3times and mounted on coverglass bottom dish (SPL Lifesciences, Korea) with 30㎕ of mounting solution. The fluorescence signals were detected with Confocal Laser Scanning Microscope (NICEM).

3) Preparation of M-BmpB as model antigen

In this study M-BmpB was used as model antigen for immunization assay (Kim.S.H. 2008). Recombinant E.coli with pET-M-BmpB plasmid was grown in LB medium containing 100 µg/㎖ ampicillin at 37°C. When OD600 reaches at 1.0, isopropyl-thiogalactopyranoside (IPTG) was added at a final concentration of 0.4mM. Recombinant E.coli were cultured another 4h at 37°C and the cell pellets were harvested by centrifugation at 15,000rpm for 10 min, washed with PBS and resuspended with
After resuspension, bacterial cells were sonicated at pulse for 9 sec and pause for 4 sec cycles for 15 min. After centrifugation at 14,000 rpm at 4°C for 20 min, the supernatants were prepared. M-BmpB proteins were purified using nickel affinity chromatography. A chromatography column (BioRad, USA) was packed with 8 ml of His binding resin (Novagen, USA). After washing with D.W., column was charged with charging buffer and equilibrated with binding buffer. After equilibration, crude proteins from recombinant E.coli was loaded and washed with binding buffer. Finally 15 ml of elution buffer was added and eluted protein was dialysis for 48 h (Table 3). After dialysis, the sample was freeze-dried and stored at −70°C until used.

Table 5. General composition of purification buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X Binding buffer</td>
<td>5mM imidazole</td>
</tr>
<tr>
<td></td>
<td>0.5M NaCl</td>
</tr>
<tr>
<td></td>
<td>20mM Tris–Cl (pH 7.9)</td>
</tr>
<tr>
<td>1X Washing buffer</td>
<td>60mM imidazole</td>
</tr>
<tr>
<td></td>
<td>0.5M NaCl</td>
</tr>
<tr>
<td></td>
<td>20mM Tris–Cl (pH 7.9)</td>
</tr>
<tr>
<td>1X Charging buffer</td>
<td>50mM NiSO₄</td>
</tr>
<tr>
<td>1X Elute buffer</td>
<td>1M imidazole</td>
</tr>
<tr>
<td></td>
<td>0.5M NaCl</td>
</tr>
<tr>
<td></td>
<td>20mM Tris–Cl (pH 7.9)</td>
</tr>
<tr>
<td>1X Strip buffer</td>
<td>100mM EDTA</td>
</tr>
<tr>
<td></td>
<td>0.5M NaCl</td>
</tr>
<tr>
<td></td>
<td>20mM Tris–Cl (pH 7.9)</td>
</tr>
</tbody>
</table>
4) Sample collection

(1) Serum sample

After immunization, mouse blood was collected for monitoring of M–BmpB specific serum IgG. 3 weeks after first immunization, mouse blood was taken from tail vein using Capillary tube treated with heparin. After a week, mouse blood was taken from orbital venous/petrosal sinus using Capillary tube treated with heparin. Blood was centrifuged at 12,000 rpm for 10 min, and serum was collected and used for detecting anti M–BmpB immunoglobulin.

(2) Fecal sample

Feces were collected for monitoring of M–BmpB specific fecal IgA. 3 and 4 weeks after the first immunization. 5~8 pieces of fresh fecal pellets were collected and added with PBS. After vortex extensively, soluble fecal samples were incubated at 4°C for overnight. Soluble fecal samples were vortexed severely again and centrifuged at 14,000 rpm for 10 min. The supernatants were collected and used for detecting anti M–BmpB IgA.
(3) Intestinal lavage sample

Before collecting intestinal lavage from mice, lavage solution (25 mM NaCl, 40 mM Na2SO4, 10 mM KCl, 20 mM NaHCO3, and 48.5 mM (162g/l) polyethylene glycol (PEG, Mw 3350) and pilocarpine were prepared. Mice were orally administered 0.5 ml of the lavage solution four times with 15-min intervals using a blunt zonde. 30 min after the last administration of lavage solution, 0.1mg pilocarpine was given to the mice intraperitoneally. Over the next 20 min, intestinal contents (up to 0.5 ml) discharged were collected for monitoring anti M-BmpB IgA.

5) Detection of anti M-BmpB immunoglobulins by ELISA

Before monitoring M-BmpB specific IgG and IgA from serum or fecal samples, purified M-BmpB was coated at 96 well immuno-plates. Carbonated–Bicarbonated buffer (Sigma, USA) containing M-BmpB (10㎍/㎖) was coated at 96 well at 4℃ for overnight. After washing 3 times with PBST (0.05% Tween 20) 96 well plates were blocked with blocking buffer (1% BSA of PBS). After 3 times of wash with PBST, 100㎕ of diluted serum and fecal samples were loaded and incubated at RT for 2h. Diluted HRP–conjugated goat anti–mouse IgG, IgG1, IgG2a or IgA (Santa cruz, USA) was treated for 1 h after 3 times of washing. After incubation, the plate was washed 3 times with PBST, and 100㎕ of TMB substrate buffer (Santa cruz, USA) was added to each well for developing HRP signal. After treating 100㎕ of stop solution, the signal was detected by microplate
reader at 450µm (Infinite M200, TECAN).

6. **Statistical analysis**

Standard error of the mean (SEM) was expressed at results unless it is described. For significance tests, ANOVA (analysis of variance) and t-test were used. Statistic analysis was performed using OriginPro 9.0 software (OriginLab Corp.).
IV. Results and Discussion

1. Recombinant *L. lactis* construction

LAB play role as delivery vehicles such as oral vaccine or adjuvant. LAB protect delivery materials from harsh condition of gastro-intestinal such as gastric acid and bile juice. However, LAB are not perfect delivery vehicles because most of LAB were digested before reaching to the small intestine. For these reason, two vectors were designed; sRANKL and usp45-sRANKL. Recombinant *L. lactis* which has sRANKL vector expresses sRANKL as cytoplasmic protein, whereas recombinant *L. lactis* which has usp45-sRANKL secretes sRANKL.

To identify whether expression vector was transformed into *L. lactis*, colony PCR was conducted with sRANKL specific primers (Figure 8).

![Figure 8. Colony PCR for selection of recombinant L. lactis. Lane 1~4 : usp45-sRANKL colonies; M, molecular marker; lane 5~7, sRANKL colonies](image)
Colony 2,3,4,5 and 6 were inoculated into M17G broth and incubation for overnight. After plasmid preparation, plasmids were sequenced from NICEM and the sequences were aligned with reference sequences (Figure 9).
Figure 9. Sequence alignment of plasmid from *L. lactis* against references. (a) usp45–sRANKL. (b) sRANKL.
2. Confirmation of sRANKL expression

1) Western blot and ELISA assay

To confirm whether recombinant *L. lactis* express sRANKL, western blot assay was performed. Commercial sRANKL was used as a positive control. As shown in figure 10, sRANKL proteins were expressed by both recombinant *L. lactis*. And recombinant *L. lactis* which has usp45-sRANKL vector secretes sRANKL protein. The band of commercial sRANKL is located above, because molecular mass of commercial sRANKL is 28kDa whereas that from recombinant *L. lactis* is 21kDa. And because of the signal sequence, the band of usp45-sRANKL is located slightly above compared with the band of sRANKL. When sRANKL is secreted from *L. lactis*, the signal sequence is detached from the protein. Therefore the band of lane 1 in panel (b) is located at the same line with lane 2 in panel (a).

Through western blot image, quantitative analysis can be possible compared with the commercial sRANKL. There are 150ng of sRANKL in lane C. Compared the thickness of band, in lane 2, there might be 50ng of sRANKL. Because 20㎕ of samples were loaded, the concentration of the sample might be 2.5ng/㎕. When preparing proteins from 7㎖ of cultured *L. lactis*, cytoplasmic proteins were dissolved with 500㎕ of D.W. Therefore recombinant *L. lactis* might express about 180ng/㎖ of sRANKL. In a similar way, recombinant *L. lactis* might secret about 60ng/㎖ of sRANKL.
Figure 10. Confirmation of sRANKL expression. (a) SDS–PAGE and western blot image of cytoplasmic proteins. (b) SDS–PAGE and western blot image of secreted proteins. Protein band of 66kDa is BSA protein which acts as a carrier protein. M, marker; C, commercial sRANKL; lane 1, usp–sRANKL; lane 2, sRANKL; lane 3 wild-type

For quantification of sRANKL expression level, ELISA assay was performed. usp45–sRANKL express 193ng/㎖, and sRANKL express 141ng/㎖ of sRANKL (figure 11).
Figure 11. Quantification of sRANKL expression by ELISA.

It is similar amount compared with the quantification analysis estimates of western blot,

2) Physiological characterization of transformed *L. lactis*

To validate physiological characteristic under the influence of foreign protein production, sRANKL, wild type and recombinant *L. lactis* growth patterns were analyzed. The patterns of growth were monitored by OD$_{600nm}$ and pH value in the culture media. Because LAB produce lactic acid, changes of pH can be indirect measure of growth. The growth rates of the recombinant *L. lactis* were slightly delayed compared with that of wild type *L. lactis*. However there was no significant difference among the groups (Figure 12).

Those growth curve and pH curve during 24 hours cultivation indicate recombinant *L. lactis* transformed with sRANKL-encoding plasmids maintain normal condition.
Figure 12. Physiological characterization of recombinant *L.lactis*. (a) Growth curve of WT and recombinant *L.lactis*. (b) pH curve of WT and recombinant *L.lactis*. Growth rates and pH values were monitored in every 2 hours during 12h-cultivation.
3. *In vitro* functional activity assay

1) MTT assay

After treating crude proteins of *L. lactis*, the morphology of RAW 264.7 cells was changed dramatically. The cell size was increased, but the cell number was decreased. We conducted MTT assay for monitoring the cell viability after treating commercial RANKL as a positive control and crude proteins from wild type and recombinant *L. lactis* (Figure 13).

![Figure 13](image)

**Figure 13.** Cell viability assay after treating cell extract of WT and recombinant *L. lactis* and commercial sRANKL (50nm/ml). At day 3, cell culture media were changed.

As shown in Figure 13, cell viability of positive control group and negative control group was increased. In the contrary, the groups treated with *L. lactis* crude proteins showed low cell
viability. Compared with day 2, cell viability at day 6 seemed slightly decreased.

There are lots of other proteins in crude extracts of *L. lactis* such as cell walls, organelles. Therefore, when crude proteins were treated in the medium, there are plenty amount of proteins other than sRANKL. Some of them might be toxic to the cells and consequently the cell viability was decreased dramatically.

Because RAW 264.7 cells are mouse macrophages cell line, they might phagocytosed other materials, and that signal might affect to morphology of the cells.

2) RT-PCR

To confirm whether the recombinant sRANKL protein expressed from the *L. lactis* has biological activity, RT-PCR was performed. Preliminary experiment was conducted with commercial sRANKL. As shown in Figure 14, NFATc1, Traf6, TRAP genes were highly expressed normalized with GAPDH gene, depending on the sRANKL concentration.

When crude proteins containing sRANKL (final concentration, 20ng/ml) were added to the medium, three genes, which are related to osteoclastogenesis, were increased compared with the wild type group. The group treated with usp45-sRANKL showed significantly difference in NAFTc1, whereas Traf6 and TRAP gene expression didn’t show significant difference compared with the wild type group (Figure 14). In cytoplasm, sRANKL is conjugated with the signal sequence, usp45. Therefore the biological function of the sRANKL could be inhibited by the sequence.
The result of three gene expression level showed that sRANKL from the recombinant *L. lactis* has similar biological activity with the commercial sRANKL. And it can be assumed that N-terminus of sRANKL might play critical role, because the functional activity of usp45-sRANKL, which has signal sequence at N-terminus, was lower than that of sRANKL.
Figure 14. Osteoclastogenesis-related gene expression level after treating commercial RANKL (20ng/ml–100ng/ml) or crude proteins from WT and recombinant *L.lactis*. Concentration of sRANKL derived from recombinant *L.lactis* is 20ng/ml. (a) Relative TRAP gene expression. (b) Relative Traf6 gene expression. (c) Relative TRAP gene expression. Gene expression levels were normalized with the GAPDH gene expression.

4. *In vivo* immunization assay

1) Whole mount IHC of PPs

To validate the effect of recombinant sRANKL from *L.lactis*, we performed IHC of PPs after administered PBS, WT *L.lactis* and recombinant *L.lactis* for 7 days.
Figure 15. Whole mount immunohisto-chemistry. (a) The step of whole mount IHC. (b) IHC image of BALB/c mouse small intestine PPs after oral administration of WT and recombinant *L. lactis*. DAPI: Nucleus (Blue), Anti-GP2: GP2 from M cells (Green). Scale bar = 100μm.
In figure 15, GP-2 green signals represent M cells. Therefore, the intensity of green signals are thought to be indication of M cell activity. Compared with the PBS and WT group, the mice administered recombinant \textit{L.lactis} seemed to show high GP-2 signals, indicating recombinant sRANKL from the \textit{L.lactis} induce M cell differentiation.

\textbf{2) Preparation of M–BmpB as model antigen}

For immunization assay, recombinant M–BmpB protein was purified by nickel affinity chromatography. In figure 16, M–BmpB protein was detected at lane O (Original sample). After passing through the column which has His binding resins, the band of M–BmpB protein was dimmed (lane F1~F3), which means M–BmpB protein was attached to the resins. After washing, M–BmpB protein was purified and enriched with the elution buffer (Figure 16).

\textbf{Figure 16.} SDS-PAGE of M–BmpB purification steps. M, Molecular marker; O, original sample; F1~3, sample flow through; W1~3, washing fraction; E, elution fraction
3) Detection of anti M-BmpB immunoglobulins by ELISA

(1) Detection of M-BmpB specific serum IgG

To investigate recombinant *L. lactis* enhance the M-BmpB specific systemic immune response, anti M-BmpB IgG was monitored by ELISA. The tested serum samples were diluted 100 fold with 1% (w/v) BSA blocking buffer.

As shown in Figure 17, 3 weeks after the first immunization, the level of serum IgG in two groups which were administered recombinant *L. lactis* before immunization with M-BmpB (s.LAB/M-BmpB, u.LAB / M-BmpB) seemed slightly higher compared with the PBS/PBS, PBS/M-BmpB and WT/M-BmpB even though there was no significant difference. Because lactic acid bacteria could play role as an adjuvant by themselves, the IgG level of WT/M-BmpB group also seemed high tendency compared with PBS/PBS or PBS/M-BmpB.

4 weeks after the first immunization, anti M-BmpB IgG levels were no significantly difference among the groups. However, As shown in pannel (c), some mice in group s.LAB/M-BmpB, u.LAB/M-BmpB seemed to be enhanced immune response compared with the other groups.
Figure 17. Serum M-BmpB specific IgG level of BALB/c mouse after immunization with M-BmpB. (a) Serum IgG level of 3 weeks after priming (dot plot), (b) bar graph, (c) Serum IgG level of 4 weeks after priming (dot plot), (d) bar graph.
(2) Detection of M–BmpB specific IgA

To test the induction of mucosal immune response after M–BmpB immunization, levels of IgA from the mice were analyzed by indirect ELISA. Soluble fecal samples were diluted 2 fold with 1% BSA blocking buffer, and intestinal lavage samples were diluted 3 fold with the same buffer.

3 weeks after the first immunization, M–BmpB specific IgA level was significantly increased in group administered recombinant *L. lactis* compared with the group administered wild type *L. lactis*. Compared with the s.LAB group, u.LAB group seemed higher level of M–BmpB IgA level. 4 weeks after the priming, IgA level of the group administered s.LAB/M–BmpB was significantly increased compared with that of the WT/M–BmpB group, whereas there’s no significant difference between WT/M–BmpB group and u.LAB/M–BmpB group. However, the groups administered recombinant *L. lactis* have tendency of immunizing efficiently compared with the other group (Figure 18, 19).
Figure 18. Fecal M-BmpB specific IgA level of BALB/c mouse after immunization with M-BmpB. (a) Fecal IgA level of 3 weeks after priming (dot plot), (b) bar graph, (c) Fecal IgA level of 4 weeks after priming (dot plot), (d) bar graph.
Figure 19. Intestinal lavage M-BmpB specific IgA level of BALB/c mouse after immunization with M-BmpB. (a) Intestinal IgA level of 4 weeks after priming (dot plot), (b) bar graph.
V. Literature Cited


Owen RL, Jones AL. (1973). "Epithelial cell specialization within human Peyer’s patches: an ultrastructural study of intestinal
lymphoid follicles.” Gastroenterology. 66:189 - 203.


경구백신은 주사로 인한 스트레스가 적다는 장점 뿐 아니라 병원균을 초도에 방어할 수 있다는 장점이 있다. 경구백신을 통해 접막면역반응이 활성화 되면, 분비형 항체인 IgA가 소장 내각에서 병원균을 무력화시키기 때문에, 병원균이 소장 상피세포를 통과하여 체내로 들어오지 못하게 막아주기 때문이다. 그러나 경구백신의 경우 주사형 백신에 비해 효율성이 매우 약하다는 단점이 있다. 따라서 경구백신의 효율성을 증진시키기 위한 면역증강제를 개발하는 것은 매우 중요하다고 할 수 있다.

소장에 유입된 항원이 체 내의 dendritic cell을 만나기 위해서는 소장 상피세포를 통과해야 한다. 이 때 외부 항원을 통과시키는 역할을 하는 세포가 M cell이다. 따라서 본 연구는 M cell의 기능을 활성화 시킬 수 있다면 경구백신의 효율성을 증진시킬 수 있을 것이라는 가설에서 시작되었다.

RANKL은 TNF cytokine 중 하나로 파골세포의 기능을 활성화 시키는 데 매우 중요한 역할을 한다고 알려져 있다. 최근 들어 RANKL은 peyer’s patch 발달에 꼭 필요할 뿐 아니라 M cell 분화를 유도하는 인자라는 연구가 보고된 바 있다. 따라서 본 연구는 soluble RANKL을 분비 및 발현하는 L.lactis를 구축하고 제조합 유산균이 경구백신의 면역증강제로서의 가능성을 검증하였다.

우선 mouse sRANKL의 서열을 발현 숙주인 L.lactis에 맞춰 코돈 최적화를 진행하였다. PCR과 제한효소 Nde1, Xho1을 이용하여 pIL252 벡터에 sRANKL 유전자를 삽입하고 electroporation을 통해 유산균에 벡터를 도입한 후, colony PCR과 sequencing을 통해 제조합 유산균을 선택하였다.

Western blot을 통해 제조합 유산균에서 sRANKL이 발현된다는 것을 확인할 수 있었다. 또한 쥐의 대식세포주인 RAW 264.7 cell이 RANKL과 M-CSF가 존재하는 환경에서 osteoclast-like cell로 분화
한다는 특성을 이용하여 제조합 유산균에서 발현된 sRANKL의 생물학적 활성을 검정하였다.
제조합유산균의 경구 백신 면역증강제로서의 가능성을 검정하기 위해 BALB/c mouse를 이용하여 in vivo 면역실험을 실시하였다. 1주일간 Mouse에 PBS 혹은 제조합 유산균주를 매일 한번씩 경구투여하고, 실험실에서 보유하고 있는 돼지적리균의 막 단백질 아단위 백신에 M cell 표적 펩타이드가 conjugation한 형태의 M-BmpB를 경구투여하였다. M-BmpB 2차 boosting 한주 후 2주일이 지났을 때 mouse의 혈액과 분을 채취하여 M-BmpB 특이적인 IgG와 IgA의 양을 살펴보았다. 결과적으로 대조군에 비해 제조합유산균을 경구투여한 그룹에서 M-BmpB 특이적인 IgG와 IgA가 증가하는 양상을 보였기에 제조합유산균을 경구백신의 면역증강제로서의 가능성을 확인하였다.
Ⅷ. Acknowledgement

최윤재 교수님께서는 “대학원생시절이 인생의 황금기다.” 라는 말씀을 자주 하셨습니다. 제 인생의 황금기를 다른 곳이 아닌 동물세포공학연구실에서 보낼 수 있던 것을 매우 감사하게 생각합니다.

가장 먼저 존경하는 최윤재 교수님께 감사의 말씀을 드리고 싶습니다. 지난 2년간 교수님께 학문적인 가르침 뿐 아니라, 인생을 살아가는 자세에 대해 배울 수 있었습니다. 학기 시작 전 모든 강의자료를 몇 번씩 수정하실 뿐 아니라, 매일 매일 꼼꼼히 수업준비를 하시는 모습을 보면서 앞으로 제가 맡는 일에 대해 항상 최선을 다하고 열정을 다해야겠다는 다짐을 하게 되었습니다. 또한 교수님께서는 나 혼자만을 위해 사는 삶이 아니라, 남들에게 도움을 주는 삶을 사는 것이 얼마나 귀한 것인지에 대해 알려주셨습니다. 앞으로도 교수님께 부끄럽지 않은 제자가 되도록 노력하겠습니까.

항상 늦은 시간까지 연구하시며 과학자의 마음을 알려주신 조종수 교수님과 논문에 귀한 조언을 해주신 백명기 교수님께도 감사의 말씀을 드리고 싶습니다. 특히 멋진 목소리로 모든 어려운 일이 해법을 알려주시는 강상기 교수님, 덕분에 2년을 무사히 지냈습니다. 강상기 교수님, 지우개로 지워지는 볼펜은 정말 인상적이었습니다. 그리고 날카로운 시각을 가지신 실험실의 연예인, 복진덕 박사님과 웃는 모습이 매력적인 이윤석 박사님, 그리고 학위논문을 몇 시간만에 검수해주신 능력자 문현석 박사님께도 진심으로 감사드립니다.

귀찮은 부탁 드려도 웃으면서 도와주신 장도오빠, 감작 놀랄 때 “Oops”가 아닌 “아이고!”를 외치시는 Singh 박사님, 실험 하면서 많은 논의를 하고, 방향성을 알려 주신 Sushila 박사님께도 감사의 인사 드리고 싶습니다.
힘들다는 대학원 시절이 제게 아름다운 추억으로 남을 수 있었던 이유는 모두 세포공학연구실 식구들 덕분입니다. 모두가 따뜻한 마음으로 서로를 위해주었기에 실험이 항상 화기애애하고 즐거웠습니다. 우선 제 연구가 시작할 수 있는 계기를 만들어주신 대천 오빠, 모르는 부분 꼼꼼하고 자세히 가르쳐 주셔서 너무 감사드립니다. 실험을 하면서 가장 많이 도움을 받은 혜선언니, 항상 웃는 모습이 보기 좋았습니다. 수영장에서의 언니 모습, 평생 기억에 남을 것 같습니다. 실험실의 절대 동안 창윤오빠, 앞으로도 오빠의 작품 사진 기대하겠습니다. 농생대 수에 태은언니, 제가 학부생때부터 ‘워너비 태은’이란 말했었나요? 항상 우아하고 멋진 모습 보여주셔서 감사드립니다. 세포공학실 아이들 준영오빠, 공과사가 뚜렷한 모습이 엿있었고, 오빠의 ppt는 항상 제 마음에 쏙 들었어요. 세포공학 실험실 실장 수나언니, 언니의 머릿결도, 긍정적인 성격도, 남치는 애교도 정말 매력적이어요. 웃는 얼굴이 매력적인 건구오빠, 오빠와 지녀는 시간이 길어질수록 오빠의 따뜻한 마음씨를 많이 느껴졌습니다. 수나언니와 건구오빠와 함께한 ‘갈바탈님’ 잊지 않을게요. 원석 오빠도 유학준비 잘하시고, 외국에서도 힘내시기 바랍니다. 요즘 실험을 정말 열심히 하는 호빈오빠, 우리나라에서 더 이상 FMD가 발생하지 않게 해주세요. 오빠는 06학번이라기 보단 제2의 08학번이에요. 이제 윤정이란 이름보다 허찌가 더 익숙한 윤정이, 지난 1년간 내가 없었다면 어떻게 삶이 안갈 정도로 붉어있었던 것 같아. 항상 즐거워. 도운오빠도 학위주제 잘 정하시고, 열심히 실험해서 좋은 결과가 있길 바랄게요.

대학원으로서의 제가 축복 받았다고 생각하는 가장 큰 이유는 저에게 소중한 동기들이 있었기 때문입니다. 학위주제를 정하면서도, 실험발표를 준비하면서도, 논문을 쓰면서도 많은 고민을 공유했던 인선언니, 언니가 가끔 가졌던 투썸 디저트 타임은 제 대학원 시절
중 가장 소중했던 시간이에요. 언제 어디서나 셔플 댄스를 출 수 있는 실험실 춤꾼 성현오빠, 나중에 스노우보드 좀 가르쳐주세요. 내년 겨울, 스키장에서 봐요. 학부 동기, 대학원 동기 동석오빠, 항상 긍정적으로 생각하는 마음 너무 보기 좋았어요. 실험 마무리 잘하고, 건강 잘 챙겼으면 좋겠어요.

졸업논문을 쓸데라 밤을 샌 후 아침 7시에 인선언니, 성현오빠와 눈길을 뜯고 가서 먹었던 완산정의 콩나물국밥을 평생 잊을 수 없을 깊습니다. ‘동기’라는 말이 지금 보다 시간이 지날수록 빛날 것임을 알고 있습니다. 10년 후에도 20년 후에도 연락하고 자주 보는 세포공학연구실 12학번 동기들이 되었으면 좋겠습니다.

동자과 08학번 동기들, 송도에서 얼굴을 보게 될 유림이, 유진이, 명은이, 대학원생 보람이, 세라, 윤정이, 그리고 사진 적을 때 타인을 배려해주라는 우경이를 비롯한 모든 동기들, 덕분에 감사했습니다. 특히 같은 대학원생의 길을 걸었던 동자과 08학번 윤철이, 종남이, 기현오빠, 재학이에게도 같은 말을 하고 싶습니다. 20년 후에도 연락하고 자주 보는 사이가 되자. 열심히 하라고 다독여주시고, 잘하고 있다고 격려해주시는 재현오빠와 동현오빠, 정말 감사드립니다. 바로 한층 위에 있는데도 2년간 많이 보지는 못했던 상윤오빠, 오빠는 영원히 저의 단장님입니다. 자주 연락드릴게요.

내 인생의 보물, 소중한 31 동기 범수, 원준이, 민희, 대섭이, 구희, 너희와 함께한 많은 추억 평생 잊지 않을게. 바다아저씨 슈, 유학생 비 잘하고, 항상 지금처럼 든든한 에이스로 남아줘. 갖 민간인 된 죄, 남은 학기 잘 마무리하고 지금 하려고 하는 일 모두 이루길 바랄게. 미나상, 의전가서도 잘 할거라 믿고, 지금처럼 자주 연락하고 방학마다 만나서 맞잡담방도 하자. 대학원가는 섬이, 이번 학기에 너의 따뜻한 마음을 많이 느꼈어. 석사생활 잘하고, 힘내. 항상 현실적인 조언을 해주는 예비 PD 정구희, 의도하지 않은 수렴청정 미안
했던 그indices 우리는 대장을 존중했어요. 대장, ‘그 약속’은 잊지 말아주셔. 31기, 너희가 있어 나의 대학교시절은 참 행복했어요.

같은 시기 대학원에 와서 수영 수업도 함께 듣고 많은 추억 쌓은 우리의 난잔니 난주언니, 어려운 일 상담할 때마다 진정성 있고 조리 있는 조언을 해준 멋진 재훈오빠, 섬이의 소울메이트이자 재미있는 성훈오빠를 비롯한 수중탐사대 동아리원, 자주 얼굴 보면서 즐거운 모임 이어갑니다. 세상에서 가장 잘생긴 수환오빠, 임사에서 유명한 신입사원이 되고, 항상 지금처럼 잘할거라 믿어. 고마웠고, 나이가 들어서 2011년 1월 1일보다 2015년 1월 1일을 더 추억하는, 지난날을 여유롭게 돌아볼 수 있는 사이가 되었으면 좋겠어요.

마지막으로 항상 든든한 우리 가족. 엄마, 아빠, 언니, 형부, 그리고 곧 태어날 나의 조카 단이에게 감사의 인사를 드립니다. 항상 미안한 마음이 앞서는 아빠, 앞으로 더 잘하는 딸이 되겠습니다. 두 딸이 원하는 것이라면 뭐든 들어주고 하시는 세상에서 가장 멋진 엄마, 항상 고맙습니다. 앞으로도 지금처럼 쿨하고 잘지게 지내주세요. 어렸을 때는 정말 많이 싸웠던 언니, 나이가 들수록 언니가 참소중하단 생각이 들어. 앞으로도 잘 부탁해요. 아직 어색한 형부, 앞으로 더 친해져요. 언니 없어도 얘기할 수 있을 만큼요. 아직 태어나지 않았지만, 태어나면 엄청 예뻐해 줄 내 조카 단이, 건강하게만 자라남. 가족들에게 부끄럽지 않은 사람이 되겠습니다. 앞으로도 지금처럼 제 곁에 있어주십시오.

2014년 1월 1일
모든 분들에게 희망차고 아름다운 한해가 되기를 기원하며,
김 정인