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Systemic administration of RANKL overcomes the bottleneck of oral vaccine delivery through microfold cells in ileum

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A successful delivery of antigen through oral route requires to overcome several barriers, such as enzymatic barrier of gastrointestinal tract and epithelial barrier that constitutes of microfold cells (M cells) for antigen uptake. Although each barrier represents a critical step in determining the final efficiency of antigen delivery, the transcytosis of antigen by M cells in the follicle-associated epithelium (FAE) to Peyer's patches appears to be a major bottleneck. Considering the systemic administration of receptor activator of nuclear factor (NF)-κB ligand (RANKL) induces differentiation of receptor activator of nuclear factor (NF)-κB (RANK)-expressing enterocytes into M cells, here, we illustrated a promising approach of antigen delivery using full length transmembrane RANKL (mRANKL). The results showed that the intraperitoneal injection of mRANKL increased the population of dendritic cells and macrophages in mesenteric lymph nodes and spleen. Subsequently, systemic administration of mRANKL resulted in significantly higher number of functional $GP2^+$ M cells leading higher transcytosis of fluorescent beads through them. To corroborate the effect of mRANKL in antigen delivery through M cells, we orally delivered microparticulate antigen to mice treated with mRANKL. Oral immunization induced strong protective IgA and systemic IgG antibody responses against orally delivered antigen in mRANKL-treated mice. The higher antibody responses are attributed to the higher transcytosis of antigens through M cells. Ultimately, the higher memory B cells and effector memory CD4 T cells after oral immunization in RANKL-treated mice confirmed potency of RANKL-mediated antigen delivery. To the best of our knowledge, this is the first study to demonstrate significant induction of mucosal and humoral immune responses to M cell targeted oral vaccines after the systemic administration of RANKL.

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1. Introduction

Most human pathogens enter the body through a mucosal surface such as intestine. Therefore, strong immune responses are required to protect this physiologically important tissue and to provide long-term protection against such invading pathogens [1].

The organized lymphoid tissues of the intestine serve as the principal mucosal inductive sites where luminal antigens are being ferried and immune responses are initiated [2]. The follicle-associated epithelium (FAE) overlying the gut-associated lymphoid tissue such as Peyer's patches (PPs) and isolated lymphoid follicles possess microfold cells (M cells) that are specialized for phagocytosis and transcytosis of luminal antigens and pathogens. Following their uptake and transcytosis by M cells, antigens exit into the intraepithelial pocket beneath the basolateral membrane where they are subsequently processed by underlying cells of the lymphoid tissues, especially antigen-presenting cells

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such as dendritic cells (DCs) or macrophages. Antigens are then presented to T cells that support B cell activation, which ultimately generates an appropriate mucosal immune response [3,4]. The ability of M cells to efficiently transport antigens from the lumen to organized lymphoid tissues within the mucosa has been extensively documented in the small intestine [5,6]. Studies have shown that in the absence of M cells, antigen sampling or antigen-specific T-cell responses in the PPs of mice are significantly reduced [7–9]. Thus, the transcytosis of antigens across the FAE of PPs by M cells is a prerequisite step for the induction of efficient immune responses to mucosal antigens.

M cells are a unique subset of epithelial cells derived from intestinal epithelial stem cells and are restricted to the FAE. Under stead-state conditions, approximately 10% of the intestinal epithelial cells within the FAE are M cells [10,11]. Moreover, it has been elucidated that the cells of the immune system underneath the FAE play a crucial role in M cell differentiation [8]. In fact, receptor activator of nuclear factor-κB ligand (RANKL) produced by the subepithelial stromal cells beneath the FAE is a critical factor that stimulates the differentiation of RANK-expressing enterocytes into M cells [12]. In addition, RANKL-deficient mice have markedly fewer M cells, and systemic administration of extracellular soluble RANKL in these mice restored the number of functional M cells [13].

RANKL is particularly expressed on osteoblasts, stromal cells, and activated T cells, and binds to the signaling receptor RANK and the decoy receptor osteoprotegerin [14–17]. RANKL/RANK system also serves an important role in the development and regulation of the immune system, including lymph-node organogenesis, lymphocyte differentiation, DC survival and T-cell activation, and promotes antigen-presenting cell function [15]. It is also suggested that RANKL is important for the regulation of early T- and B-lymphocyte development [17]. Notably, the interaction of RANKL with RANK, which is highly expressed on DCs, increases DC survival and enhances induction of T-cell responses [15].

Till date, most of the studies on the potential role of RANKL have been performed with extracellular soluble RANKL (RANKL-Ex), probably due to the difficulty of attaining high level expression of functional full-length transmembrane RANKL (mRANKL). Nevertheless, we successfully achieved high level expression of soluble mRANKL which had cell differentiation function in vitro [18].

Given that the transcytosis of antigens across the gut epithelium by M cells to PPs is critical for the induction of efficient immune responses to mucosal antigens, here, we presented a novel strategy for enhancing oral vaccine efficacy by synchronous induction of supraphysiological numbers of M cells and immune cells by systemic administration of mRANKL, and subsequent delivery of M cell targeted antigen by mucoadhesive and pH sensitive oral delivery system.

2. Materials and methods

2.1. Materials

Alpha-modified minimum essential medium (α-MEM), RPMI medium and fetal bovine serum (FBS) were purchased from Thermo Scientific HyClone (Waltham, MA, USA). BD Difco™ Luria–Bertani (LB) broth was obtained from Becton, Dickinson and Company (New Jersey, USA). Hydroxypropyl methyl cellulose phthalate-55 (HPMCP) was obtained from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), L-cysteine hydrochloride monohydrate, dimethyl sulfoxide (DMSO), poly(vinyl alcohol) (PVA), Pluronic® F-127, dichloromethane, 4′,6-diamidino-2-phenyindole dilactate (DAPI), carbonate-bicarbonate buffer capsule, and fluorescein isothiocyanate (FITC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tris–glycine–PAG pre-cast SDS gel was purchased from Komabiotech (Seoul, Korea). His•Bind® Resin was purchased from Novagen Inc. (California, USA) and HiTrap Q FF prepacked columns (5 ml) were obtained from GE Healthcare Life Sciences (New Jersey, USA). Detoxi-Gel™ endotoxin removing columns and bicinchoninic acid (BCA) protein assay reagents (A and B) were purchased from Thermo Scientific Pierce (Illinois, USA). BD OptEIA reagent kit was purchased from BD Biosciences (California, USA).

2.2. Antibodies and lectin

Alexa fluor 488-conjugated anti-mouse glycoprotein 2 (GP2) monoclonal antibody (mAB) was obtained from MBL International (USA) and rhodamine-conjugated *Ulex europaeus* agglutinin I lectin was purchased from Vector Laboratories (USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA and IgG antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.3. Mice

Female BALB/c mice (6 weeks old), purchased from Samtako, Co. Ltd. (Osan, Korea), were used throughout this study. Mice were housed and maintained under specific pathogen free conditions in accordance with the guidelines for the care and use of laboratory animals (Seoul National University). All mice from each group received ad libitum access to identical diet and water.

2.4. Expression and purification of RANKL

The soluble mRANKL (mouse RANKL, 316 aa) was expressed, purified and quantified as described previously [18]. Briefly, single colony of SHuffle® Express *E. coli* harboring gene encoding mRANKL was inoculated in 4 ml LB medium supplemented with 100 μg/ml of ampicillin and incubated overnight with shaking at 37 °C. 500 μl of overnight culture was used to inoculate 800 ml of the same medium and incubated overnight with shaking at 37 °C. Expression of mRANKL was induced with 7.5 mM lactose when the culture reached OD_{600} of 0.6 and re-incubated at 30 °C for 5 h. The soluble maltose binding protein (MBP)-tagged mRANKL (MBP-mRANKL) was extracted and purified by amylose affinity chromatography. The purified MBP-mRANKL was dialyzed against 20 mM Tris–HCl (pH 7.4), 25 mM NaCl buffer at 4 °C and endotoxins were removed by Detoxi-Gel™ endotoxin removing columns. Cleavage of mRANKL from its MBP fusion partner was accomplished with Factor Xa (Amersham Biosciences) followed by removal of uncleaved fusion protein as well as MBP using HiTrap Q FF prepacked columns according to the manufacturer's instructions. The purity of the protein was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and concentration of purified mRANKL was determined at 280 nm using a Nanophotometer (Implen GmbH, Germany). Finally, purified mRANKL was lyophilized and stored at −70 °C until use. Similarly, extracellular soluble RANKL (RANKL-Ex) comprising 137–316 aa of mRANKL was expressed in SHuffle® Express *E. coli* and purified as described above.

2.5. Flow cytometry analysis of immune cells

The purified mRANKL or RANKL-Ex (10, 100 or 200 μg/day) was administered through intraperitoneal (IP) injection to mice for 4 consecutive days. On day 5, mesenteric lymph nodes (MLNs) and spleen were harvested aseptically from each mice and mashed well with the plunger of a syringe in 60 mm dish containing 1.5 ml of medium (RPMI-1640 supplemented with 5% heat inactivated FBS).

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