The M Cell-Targeting Ligand Promotes Antigen Delivery and Induces Antigen-Specific Immune Responses in Mucosal Vaccination

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The M Cell-Targeting Ligand Promotes Antigen Delivery and Induces Antigen-Specific Immune Responses in Mucosal Vaccination

Sae-Hae Kim,*† Ki-Weon Seo,*† Ju Kim,*†,1 Kyung-Yeol Lee,‡,§ and Yong-Suk Jang*†

Oral mucosal immunization can induce protective immunity in both systemic compartments and the mucosa. Successful mucosal immunization depends on Ag delivery to the mucosal immune induction site. The high transepithelial activity of M cells within the mucosa makes these cells attractive targets for mucosal Ag delivery, although it remains unclear whether delivery of Ag to M cells only can guarantee the induction of effective immune responses. In this study, we evaluated the ability of an M cell-targeting ligand with adjuvant activity to induce immunity against ligand-fused Ag. We selected M cell-targeting ligands through biopanning of a phage display library against differentiated in vitro M-like cells and produced the recombinant Ags fused to the selected ligands using the model Ag. One of the selected peptide ligands, Co1, promoted the binding of ligand-fused Ag to mouse Peyer’s patch M cells and human M-like cells that had been defined by binding with the M cell-specific and anti-GP2 Abs. In addition, Co1 ligand enhanced the uptake of fused Ag by immunogenic tissue in an ex vivo loop assay and in vivo oral administration experiments. After oral administration, the ligand-fused Ag enhanced immune responses against the fused Ag compared with those of the control Ag without ligand. In addition, this use of the ligand supported a skewed Th2-type immune response against the fused Ag. Collectively, these results suggest that the ligand selected through biopanning against cultured M-like cells could be used as an adjuvant for targeted Ag delivery into the mucosal immune system to enhance immune induction. The Journal of Immunology, 2010, 185: 000–000.

The mucosal surface is exposed to a diverse and very large number of microorganisms and is simultaneously the site of infection and the site of protection against diverse pathogens (e.g., influenza virus, Vibrio cholerae, Type I reovirus, and rhinovirus) (1). Consequently, it is conceivable that vaccination via a mucosal entry route may enhance the protective response against pathogens. Vaccine application via the oral mucosal route may also offer advantages, such as convenience of vaccine administration and cost-effectiveness due to needle-free delivery (2). Nevertheless, only a few oral mucosal vaccines are currently available; most current vaccines are administered through systemic routes and induce protective immune responses dominantly in systemic compartments (3, 4). One of the reasons why few oral mucosal vaccines are available is the difficulty of delivery of Ags into the mucosa; another issue is poor immune response induction due to the tendency of tolerance induction of the oral mucosal immune system (5, 6). However, numerous efforts are being directed toward developing oral vaccines due to the outstanding merits of the vaccination strategy (7).

One key consideration in oral vaccine development is that the introduced Ag needs to pass through the gut wall. The thin layer of epithelial cells lining the gut is tightly regulated to protect the host from pathogenic invasion. Ag sampling occurs by transcytosis in membranous or microfold cells, also known as M cells, as well as by dendrite extension by dendritic cells into the lumen (8). In particular, M cells transcytose the internalized Ag without degradation and are thought to play an important role in initiating Ag-specific mucosal immune responses through inducing the production of secretory IgA, although the mechanism of Ag uptake by M cells is not completely understood (9, 10). The importance of M cells in mucosal immune induction is supported by the finding that Peyer’s patches (PPs) with M cells need to protect against Salmonella spp. invasion and can induce tolerance to commensal bacteria (11–13). Furthermore, recent studies of effective mucosal immune induction through targeting of Ags using the M cell-specific Ab NKM 16-2-4 and the M cell-specific expression molecule GP2 have suggested that M cells are key to eliciting the mucosal immune system by taking up Ags from the lumen (14, 15). Therefore, the M cell-mediated Ag sampling procedure is believed to be an essential and critical step for establishing a successful mucosal immune response.

Although M cells appear to be ideal for eliciting Ag-specific immune responses via oral mucosal vaccines, there are challenges to using this cell as a target for Ag delivery (16, 17). Identification of M cells is problematic because M cells occupy only 10% of the intestinal lymphoid follicle surface area in humans and mice and their specific markers are not yet completely understood (18). This may explain why much of the effort to improve suboptimal vaccines has focused on developing mucosal delivery and adjuvant systems (19, 20). The development of an in vitro M cell coculture system and its modified models greatly advanced the study of M cells and has been used effectively to identify mucosal targeting drugs and adjuvants for mucosal vaccine development (21–23). For example,
it was confirmed using an in vitro M cell coculture model that macrophage inhibitory factor regulates M cell-mediated transport (24). Furthermore, specific expression of TLR4, α5β1 integrin, galectin-9, and caveolin-1 and transcytosis of mucosal pathogens (*Salmonella* and Gram-negative bacteria) by these molecules were identified using the same in vitro M cell culture model (25–27).

Biopanning with a phage display library is one of the most effective techniques currently available to select target-specific peptide ligands. For example, a peptide against sialylgalactose of influenza virus, which was selected from a phage library, inhibited virus infection markedly (28). In addition, ligands targeting human papillomavirus, which were selected through biopanning against human papillomavirus 16-transformed SiHa cells, could deliver cargo molecules to the target cells (29). Moreover, ligands against PPs, the gut epithelium, or polymeric IgRs for vaccine or drug delivery into the mucosal area were isolated using a biopanning approach, although the efficiency of Ag delivery was tested only in an in vitro cell culture system (30–32).

In a previous study, we showed that oral vaccination using the GM1 ganglioside-targeting ligand, which was selected from panning of a phage display library, induced increased mucosal and systemic immune responses against a ligand-fused Ag (33).

In this study, we used an in vitro M cell coculture system to search for M cell-specific peptide ligands with adjuvant functions. We performed biopanning of a phage display library against the M cell model system, which was confirmed by M cell-specific Ab NKM 16-2-4 and by evaluation of the expression of GP2, and then selected M cell-specific peptide ligands. The function of the selected ligand in mucosal immune induction was assessed by evaluating the delivery of a ligand-fused Ag into PPs in vivo and the induction of specific immune responses against a ligand-fused Ag after oral administration.

**Materials and Methods**

**Experimental materials**

All of the chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Transwell polycarbonate filter inserts (12 wells, pore diameter of 3 μm) were purchased from Corning Costar (New York, NY), and rhodamine-conjugated *Ulex europaeus* agglutinin 1 (UEA-1) and marine blue-conjugated wheat germ agglutinin (WGA) were obtained from Vector Laboratories (Burlingame, CA). Either FITC- or biotin-conjugated M cell-specific mAb NKM 16-2-4 were provided kindly by Drs. H. Kyono (University of Tokyo, Tokyo, Japan) and M.-N. Kweon (International Vaccine Institute, Seoul, South Korea) (26).

**In vitro M cell coculture model**

A human colon carcinoma cell line, Caco-2, and a human Burkitt’s lymphoma cell line, Raji, were purchased from the American Type Culture Collection (Manassas, VA). The Caco-2 cells were cultured in DMEM (25 mM glucose) with 10% (v/v) FBS (Hyclone Laboratories, Logan, UT), and the Raji cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS (Hyclone Laboratories, Logan, UT), and the Raji cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS (Hyclone Laboratories, Logan, UT). The Caco-2 cells (5 × 10^5) then were spread to the apical side of the transwell. After incubation for 3 h, the medium at the apical side was changed, and the upper medium was changed every other day for the next 14 d of culture. Raji cells (5 × 10^5) suspended in RPMI 1640/DMEM (1:2) mixture then were added to the basolateral chamber of the transwell, and the coculture was maintained for 2 d. Caco-2 cells were cultured alone by this procedure, without the Raji cells, served as a control.

**Immunohistochemistry and immunofluorescent staining analyses**

To characterize M cells, cells were stained with standard lectin, the M cell-specific Ab NKM 16-2-4 and an anti-GP2 Ab (IMGENEX, San Diego, CA) (14, 15, 34). Cocultured Caco-2 and Raji cells or Caco-2 cells only were fixed in transwells by incubation with 4% paraformaldehyde for 30 min, and then blocking solution (0.1% glycine and 1% FBS in PBS) was added. After the blocking solution was washed off, rhodamine-labeled UEA-1, marine blue-labeled WGA, either biotin- or FITC-labeled NKM 16-2-4 and anti-GP2 Abs, enhanced GFP (EGFP), and EGFP-Co1 were added to the fixed cells in the transwells, followed by incubation for 2 h. The transwells then were washed with TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] and examined using confocal laser scanning microscopy for 2 d (CLSM; LSM 510 META; Carl Zeiss, Thornwood, NY). Alkaline phosphatase activity and ICAM-1 expression in transwell cultures were measured as described previously (22).

Frozen sections (10–15 μm in thickness) of PPs from BALB/c mice, which had been obtained using a cryomicrotome (Thermo Fisher Scientific, Waltham, MA), were fixed with 4% paraformaldehyde, blocked with 10% (v/v) PBS/PBS, and stained with rhodamine-labeled UEA-1, biotin-conjugated NKM 16-2-4 Ab and anti-GP2 Ab, and either EGFP or EGFP-Co1. Finally, the sections were counterstained with DAPI (Invitrogen, Carlsbad, CA) and analyzed by CLSM.

**Biopanning**

The Ph.D. phage display library (New England Biolabs, Boston, MA), consisting of random dodecapeptides fused to the N terminus of the pIII (fusions of the filamentous phage M13KE, was used to select peptides with a high affinity to M-like cells as described in our previous study (33) and as recommended by the manufacturer. Briefly, transwells containing either Caco-2 cells cocultured with Raji cells or Caco-2 cells alone were blocked with blocking solution [0.1 M NaHCO3 (pH 8.6), 5 mg/ml BSA, and 0.02% NaN3 in DMEM] for 2 h at 37°C, and then 1.5 × 10^11 PFU phage was added to the transwell for incubation for 2 h at 4°C. After washing three times with DMEM, the bound phage was eluted by adding general buffer [0.2 M glycine-HCl (pH 2.2) and 1 mg/ml BSA], and the eluant was neutralized with 1 M Tris-HCl (pH 9.1). The eluted phage particles were titrated and used for further screening cycles to concentrate phage particles displaying specific peptides to M-like cells. Finally, collected phage clones were amplified and precipitated with polyethylene glycol (PEG) to obtain phage DNA. The amino acid sequences of the selected ligands were deduced from the nucleotide sequences.

**Production of the ligand-fused recombinant Ags**

EGFP was used as a model Ag in this study. To produce ligand-fused EGFP recombinant proteins, the EGFP gene was amplified by PCR from pEFGP-1 (Clontech, Mountain View, CA) using forward and reverse primers containing the nucleotide sequences of the targeting ligand and restriction site. The forward primer sequence for EGFP alone and ligand-fused EGFPs is 5'-GCT TGA GCT CCT ACT TGT ACA GCT CGT CCA-T-3', 5'-AGC TGA GCT CCT ACC GGA GAC GCG ACC GCG CCA GCT GAT GAA ACC GCT TGT ACA GCT CGT CCA T-3', 5'-AGC TGA GCT CCT ACC GGA GAC GCG ACC GCG CCA GCT GAT GAA ACC GCT TGT ACA GCT CGT CCA T-3', and 5'-AGC TGA GCT CCT ACC GGA GAC GCG ACC GCG CCA GCT GAT GAA ACC GCT TGT ACA GCT CGT CCA T-3', respectively, where underlined letters represent the BamHI restriction site. The reverse primer sequences to amplify genes for EGFP alone and Co1-, Co2-, and Co3-fused ligands are 5'-AGC TGA GCT CCT ACC GGA GAC GCG ACC GCG CCA GCT GAT GAA ACC GCT TGT ACA GCT CGT CCA T-3', 5'-AGC TGA GCT CCT ACC GGA GAC GCG ACC GCG CCA GCT GAT GAA ACC GCT TGT ACA GCT CGT CCA T-3', and 5'-AGC TGA GCT CCT ACC GGA GAC GCG ACC GCG CCA GCT GAT GAA ACC GCT TGT ACA GCT CGT CCA T-3', respectively, where underlined and italic letters represent the SacII restriction site and corresponding peptide ligand, respectively. Amplified gene products were digested with BamHI and SacI and cloned into the pQE31 expression vector (Qiagen, Hilden, Germany), Recombinant proteins were expressed using M15 and S1300 expression hosts and purified as described in our previous study (33) and as suggested by the manufacturer.

**Ex vivo and in vivo Ag uptake assays**

A gut loop containing PPs was prepared, and an ex vivo Ag uptake assay was performed as described previously (27–29). Briefly, either EGFP alone or ligand-fused EGFP protein (0.2 mg/ml) was added to 2- to 3-cm-long ligated gut loops prepared from a male BALB/c mouse (Charles River Technol., Boston, MA). The mesentery of the gut loop was ligated by 5-0 Prolene (Ethicon, Somerville, MA) that had been fastened overnight. After incubation for 1 h at 37°C, the gut loops were washed with HBSS, the tissue was fixed with 4% paraformaldehyde, and the preparations were washed and dehydrated with serially increasing concentrations of ethanol alcohol. The tissues then were washed with chloroform and impregnated with paraffin. The embedded tissue was sectioned at intervals of 7 μm using a Leica RM 2235 microtome (Leica Microsystems, Wetzlar, Germany) and analyzed with a Carl Zeiss Axioskop 2 fluorescence microscope.

Five, 10, and 20 min after oral administration of either EGFP or EGFP-Co1 to mice, the mice were sacrificed and PPs were excised from the small intestine at the ileo-caecal valve and sectioned for light microscopy.
intestine followed by vigorous washing with ice-cold PBS. The frozen sections of each PP were prepared and stained with either UEA-1 or NKM 16-2-4 and analyzed under a CLSM after counterstaining with DAPI. For whole-mount staining, PPs were fixed with 4% paraformaldehyde, stained with UEA-1 and NKM 16-2-4 without permeabilization, embedded in a 30% solution of glycerol in PBS, and then analyzed with a CLSM (14).

Immunochemistry with a ligand-fused Ag
A total of 100 μg of experimental recombinant Ag was administrated by oral gavage without anesthesia once every week for 6 wk to 4- to 6-wk-old male BALB/c mice, with five mice assigned to each group. If required, systemic immunization was performed by s.c. injection of the recombinant Ag 24 h after the last immunization, sera and fecal extracts were prepared and analyzed for Ag-specific IgA and IgG by ELISA as described previously (35). Ab titers were expressed as the reciprocal log₂ of the highest sample dilutions that gave an OD₅₄₀ of 0.08, which was the value of the PBS blank.

Lymphocyte preparation
Lymphocytes of immunized mice were isolated from the spleen, lamina propria of the small intestine, and PPs at 6 d after final immunization as described previously (36, 37). Briefly, PPs and lamina propria were minced and digested twice with 300 U/ml Clostridium histolyticum collagenase D (Roche, Mannheim, Germany) for 30 min at 37°C. The digested mixture was passed through a nylon mesh to remove undigested tissue and subject to Percoll (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation. Cells at the interface between 40 and 75% Percoll were collected as mononuclear cells. In some experiments, CD4⁺ T lymphocytes were isolated by positive selection using mouse CD4 Dynabeads (Invitrogen).

Measurement of Ag-specific immune responses
To determine the level of cytokine secretion after Ag stimulation, cytokine-specific ELISAs were performed with culture supernatants of lymphocytes stimulated with the Ag for 24 h. Briefly, MaxiSorp 96-well plates (Nunc; Roskilde, Denmark) were coated with each cytokine-specific capture Ab (BD Biosciences, Franklin Lakes, NJ). After either the culture supernatants or the recombinant cytokine was added in the appropriate subwells, bound cytokines were detected by sequential incubations with the appropriate biotin-conjugated cytokine-specific developing Ab followed by incubation with streptavidin-HRP for 30 min. Finally, tetramethylbenzidine substrate solution was added, and enzyme activity was measured using an ELISA reader (Packard Instrument, Meriden, CT). Cytokine concentrations were expressed as mean ± SE (ng/ml) of each group as calculated from the plotted standard curves of serial dilutions of the recombinant cytokine.

To measure the number of Ag-specific Ab- and cytokine-expressing cells, ELISPOT assays were performed with lymphocytes isolated from various lymphoid tissues. PPs were added to mixtures of lulose ester membrane-lined microtiter plates (MultiScreen-HA; Millipore, Billerica, MA) precoated with EGFP or capture Ab specific to the cytokine of interest and stimulated with the Ag, 30 nM EGFP, for 36 h at 37°C. Cells then were washed, and Abs specific to IgA, IgG, IFN-γ, IL-4, IL-5, or IL-6 were added, followed by incubation for 2 h at 37°C. Finally, the wells were washed and developed with 100 μl of either 3-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium or 3-amino-9-ethylcarbazole substrate solution. The spots were counted under a microscope, and the mean number of spots from the triplicates at each dilution was calculated.

Ag-specific lymphocyte stimulation was determined using a thymidine incorporation assay. Briefly, either splenocytes or purified CD4⁺ T lymphocytes were isolated 6 d after the final immunization and stimulated as described previously (30). Cells were distributed to 96-well culture plates at 5 × 10⁵ cells per well and stimulated with the appropriate Ag for 3 d followed by a pulse with 0.5 μCi [³H]thymidine deoxyribose (Amer- sham Life Science, Buckinghamshire, U.K.) per well for an additional 18 h. Cells then were harvested with a 96-well cell harvester (Inotech, Dottikon, Switzerland), and incorporated tritium was determined using a liquid scintillation counter (Packard Instrument, Meriden, CT). Stimulation indices were calculated by dividing the tritium incorporation (cpm) in cells treated with EGFP by incorporation in control cells treated with PBS.

Statistical analysis
Calculations were performed using SigmaPlot (Systat Software, Chicago, IL). Results are presented as the mean ± SE. An unpaired Student t test was used to compare groups, and a p value <0.05 was considered significant.

Results
Characterization of in vitro cultured M-like cells by M cell-specific Ab binding and GP2 expression
To select peptide ligands that could be used as mucosal adjuvants, we screened a phage display library against M cells using a bio-panning procedure. Due to the limited availability of M cells, we initially assembled an M-like cell culture model system as described in the Materials and Methods (22, 23) and confirmed differentiation of M-like cells using various criteria (Fig. 1). Although M cell-specific markers are not yet defined clearly, mouse M cells of PPs have well-defined characteristics, such as the specific binding of lectin UEA-1 but not WGA (15, 38). In addition, recent studies have reported that the M cell-specific Ab NKM 16-2-4 and the anti-GP2 Ab interact specifically with the M cells of both mice and humans (14, 15).

Monitoring of M-like cells that were established using an in vitro coculture system with WGA, UEA-1, and NKM 16-2-4 (Fig. 1A, upper panel) and with NKM 16-2-4, anti-GP2 Ab, and UEA-1 (Fig. 1A, lower panel) revealed overlapping binding of UEA-1, NKM 16-2-4, and anti-GP2 Ab, confirming the presence of M-like cells. In addition, the observation that the binding areas of UEA-1, NKM 16-2-4, and anti-GP2 Ab occurred at a lower depth than others in the Z-stack images, which were created by using the DepthCod option of the CLSM software, suggested that UEA-1, NKM 16-2-4, and anti-GP2 Ab bound to M-like cells (data not shown). However, when we evaluated the same interaction using monocultured Caco-2 cells, we detected no specific binding of UEA-1, NKM 16-2-4, or anti-GP2 Ab in the merged staining images (Fig. 1B). These results suggest that the M-like cells that were recognized by NKM 16-2-4 and that expressed GP2 could be differentiated correctly from Caco-2 cells in our in vitro M cell coculture system.

We then calculated the relative area of binding from these image data using CLSM software; a differentiation ratio of ~7.5% of M-like cells was calculated for transwells in which Caco-2 and Raji cells were cocultured (Fig. 1C). We then measured alkaline phosphatase activity and the expression levels of the cellular adhesion molecule ICAM-1 from the apical side of the transwell (23, 34, 39) (Fig. 1D). Similar to the previous studies, in which cultured M-like cells showed reduced alkaline phosphatase activity and a higher expression level of adhesion molecules, such as VCAM and ICAM-1, than those of monocultured Caco-2 cells, we found a 33% lower level of alkaline phosphatase activity and a 23% higher level of ICAM-1 expression in the cocultured Caco-2 cells compared with those of the monocultured Caco-2 cells. These results are consistent with those reported in previous studies and collectively suggest that some of the Caco-2 cells cocultured with Raji cells in our in vitro coculture system differentiated to cells with the characteristics of M cells.

Screening of a phage display library against M-like cells
To select M cell-specific ligands for mucosal Ag delivery, we screened a phage display library against the cultured M-like cells using the biopanning protocol described in the Materials and Methods (Table I). In round 1 of panning, we recovered 2.28 × 10⁹ PFU that bound to the M-like cells from the 1.50 × 10¹³ PFU initially added, giving a recovery rate of 1.52 × 10⁻⁶%. In the next round, we performed negative panning to remove the phage particles displaying peptides capable of binding to the monocultured Caco-2 cells; the amplified phage particles recovered from round 1 of panning were added to the apical side of the transwell holding the monocultured Caco-2 cells, and the phage particles contained in the washed fraction were recovered. After three washes, 2.85 × 10⁻⁵% of particles from the 1.56 × 10¹¹ PFU initially added were recovered from the round 2 negative
2 cells were analyzed to determine the nucleotide sequences of the peptides contained 7 × 10^3 phage particles collected from the first panning of round 2 as bound to monocultured Caco-2 cells. In parallel, to obtain ligands, we constructed recombinant EGFP genes by PCR using primers containing the ligand-specific nucleotide sequences and produced recombinant EGFPs either with or without fused ligands using an Escherichia coli expression system. We first tested the ability of the EGFP fusion proteins to bind to the UEA-1^+^WGA^−^ M-like cells established in our in vitro coculture system using fluorescence microscopy. When we incubated Co1-, Co2-, and Co3-fused EGFP or EGFP alone with M-like cells, all of the ligand-fused EGFPs but not EGFP alone interacted well with M-like cells (data not shown). In particular, the binding of EGFP-Co1 overlapped with that of UEA-1^+^WGA^−^ more closely than that of the other ligand-fused EGFPs (data not shown).

M cell-mediated transcytosis of luminal Ags into PPs is essential for the induction of effective mucosal immune responses. To this end, we used an ex vivo ligated loop assay to determine whether ligand-fused EGFP was transcytosed (Fig. 2). After incubation for 1 h of either ligand-fused EGFP or EGFP alone within ligated intestinal segments containing one or two PPs, sequential histological analyses of the loop confirmed the presence of EGFP in the PPs in varying amounts. When we compared the fluorescence emission from each of the ligand-fused EGFP proteins and EGFP alone, we found that the Co1-fused EGFP transferred its fluorescence into the PPs most efficiently (Fig. 2A). These results indicate that ligands selected through biopanning against M-like cells promote M cell-specific binding and transcytosis of ligand-fused proteins into PPs.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Sequence</th>
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<tr>
<td>Co1</td>
<td>S F H Q L P A R S P L P</td>
</tr>
<tr>
<td>Co2</td>
<td>G S T Q A W M S P P L A</td>
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<td>Co3</td>
<td>L L A D T H S H H P W T</td>
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Results were calculated as the reciprocal of the geometric mean log2 titer in the sera after oral administration of ligand-fused EGFP or EGFP alone.

The results from light and fluorescence microscopy, respectively (original magnification ×10), B, Levels of EGFP-specific serum IgG and fecal IgA (upper panel) and EGFP-specific IgGs of different subclasses (lower panel) in the sera after oral administration of ligand-fused EGFP or EGFP alone. Results were calculated as the reciprocal of the geometric mean log2 titer. C, EGFP-specific splenocyte proliferation was determined using a thymidine incorporation assay, and results are expressed as stimulation indices, as described in the Materials and Methods. *p < 0.05; **p < 0.01; ***p < 0.001 indicate significant differences between the values compared.

**FIGURE 2.** Targeting of ligand-fused EGFP into PPs and induction of EGFP-specific mucosal and systemic immune responses after oral administration of the ligand-fused Ags. A, Immunohistochemical analysis of mouse gut loops incubated with (a) EGFP-Co1, (b) EGFP-Co2, (c) EGFP-Co3, and (d) EGFP (green) alone using the ex vivo loop assay described in the Materials and Methods. Left and right columns of each panel represent the results from light and fluorescence microscopy, respectively. (Original magnification ×400). B, Levels of EGFP-specific serum IgG and fecal IgA (upper panel) and EGFP-specific IgGs of different subclasses (lower panel) in the sera after oral administration of ligand-fused EGFP or EGFP alone. Results were calculated as the reciprocal of the geometric mean log2 titer. C, EGFP-specific splenocyte proliferation was determined using a thymidine incorporation assay, and results are expressed as stimulation indices, as described in the Materials and Methods. *p < 0.05; **p < 0.01; ***p < 0.001 indicate significant differences between the values compared.

On the basis of the observation that the selected ligands promoted the delivery of ligand-fused EGFP into PPs, we hypothesized that the ligands similarly could deliver Ag into mucosal immunogenic sites and enhance the induction of systemic and mucosal immune responses. To test this hypothesis, we administered the ligand-fused EGFPs to mice orally for 6 wk and monitored the induction of EGFP-specific mucosal and systemic immune responses (Fig. 2B). Using a serum IgG ELISA, we found that immunization with the ligand-fused EGFP increased the induction of EGFP-specific serum IgG compared with that with EGFP alone. In particular, conjugation of EGFP to Co1 enhanced the induction of EGFP-specific IgG more efficiently than fusion to other ligands and increased the level of EGFP-specific serum IgG by >3-fold compared with that of the other ligand-fused EGFPs. More importantly, mice immunized with Co1-fused EGFP induced EGFP-specific fecal IgA levels ∼2-fold higher than those in mice immunized with other ligand-fused EGFPs or with EGFP alone (Fig. 2B, upper panel).

To clearly characterize the enhancement in immune response achieved with the targeting ligand-fused EGFP, we analyzed IgG subclasses in the sera using an isotype-specific ELISA system (Fig. 2B, lower panel). As shown in the figure, induction of EGFP-specific IgG1 was enhanced by >2-fold, although no significant increase in the level of Ag-specific IgG2a was detected even with the Co1 ligand-fused Ag (Fig. 2B). In addition, oral administration of Co1 ligand-fused EGFP significantly enhanced the priming of EGFP-specific lymphocytes (Fig. 2C). Collectively, these results indicate that Co1 ligand-fused Ag, administered orally, was able to enhance the induction of T cell proliferation and induce a predominately Th2-type immune response against the fused Ag.

Co1 ligand-mediated delivery of Ag to M cells in PPs and M-like cells in an in vitro coculture system

To understand the characteristics of the Co1 ligand-mediated enhancement of fused Ag-specific systemic and mucosal immune response induction, we further analyzed the delivery of Co1-fused EGFP to mucosal immune induction sites after oral administration of the Ag. We initially monitored the fluorescence of fixed-frozen sections of the PPs of mice after binding with EGFP-Co1 and either UEA-1 or NK6 16-2-4 (Fig. 3A). We found that M cells that interacted with either UEA-1 or NK6 16-2-4 in the follicle-associated epithelium of PPs costained with EGFP-Co1 (within the dotted box of the far left column of Fig. 3A, upper panel). In addition, the same costaining was observed in the villus (second right column of Fig. 3A, lower panel). However, that costaining pattern was not detected in PPs incubated with EGFP alone (far right columns of Fig. 3A, upper and lower panels).

Next, we confirmed the transport of the EGFP Ag that had been targeted to M cells through Co1 ligand conjugation using CLSM (Fig. 3B). When we prepared whole-mount PPs from mice 5 min after oral administration of EGFP-Co1, we detected EGFP fluorescence below the area stained with UEA-1 and NK6 16-2-4 (Fig. 3B, upper panel). To follow up the transport of the orally introduced Ag, PPs excised at 5, 10, and 20 min after oral administration of either EGFP-Co1 or EGFP alone were counterstained with UEA-1 and DAPI (Fig. 3B, lower panel). Five minutes after introduction of EGFP-Co1 through oral gavage, EGFP fluorescence was detected under the dome area of PP and decreased with time while the fluorescence was transferred deeper into the PP area. However, EGFP fluorescence was barely detected at the dome area of PPs even 20 min after oral administration of EGFP alone and was not detected throughout the time periods tested (far right column of Fig. 3B, lower panel). Taken together, these results confirm that Co1-fused EGFP not only binds to M cells but also is transported effectively into the mucosal immune induction site.

We then confirmed the binding of EGFP-Co1 to M cells using our in vitro human M-like cell culture system (Fig. 3C). When we add 100 µg EGFP-Co1 to the in vitro coculture system, EGFP-Co1 attached specifically to the apical areas of the M-like cells that were stained by both NK6 16-2-4 and anti-GFP2 Ab (left four columns of Fig. 3C, upper panel). However, similar binding was not detected when EGFP was added (far right column of Fig. 3C, upper panel). When only 1 µg EGFP-Co1 was added, we could still detect the area stained both with NK6 16-2-4 and EGFP-Co1 (left four
prepared through cryosections and stained as indicated in each column. Human M-like cells. A

FIGURE 3. Transport of EGFP-Co1 into PPs through mouse M cells and human M-like cells. A, Immunohistochemical analyses of PPs and villi prepared through cryosections and stained as indicated in each column. Left and right columns of the upper panel show the merged images of DAPI (blue) and UEA-1 (red) staining together with the image of green fluorescence after applying EGFP-Co1 and EGFP, respectively. Images in the middle columns of the upper panel show expanded images of the dotted area in the left column and individual staining images as indicated in each column. The left and far right columns of the lower panel show merged images of DAPI (blue) and NKM 16-2-4 (red) staining together with green fluorescence images obtained after applying EGFP-Co1 and EGFP alone, respectively. Images in the middle column of the lower panel are magnifications of the dotted area in the left column and the individual staining images indicated in each column. The second right column of the lower panel shows an image of villi stained with DAPI and NKM 16-2-4 merged with the green fluorescence image obtained after applying EGFP-Co1. B, Transport of Ag into PPs after oral administration of 100 μg EGFP-Co1. The left and middle columns of the upper panel show whole-mount staining of PPs prepared 5 min after oral administration of the Ag and a magnified image of the dotted area, respectively. Images in the upper panel were created using slices from Z-stacks projected into one slice along the x (left and middle columns) or z (right column)-turning axes. The lower panel shows merged images of DAPI (blue), UEA-1 (red), and green fluorescence images of PPs prepared at the indicated times after oral feeding of either EGFP-Co1 or EGFP alone, as indicated in each column. All of the images in the lower panel were prepared through cryosections in comparison with the whole-mount staining of PPs shown in the upper panel. C, Individual and merged CLSM images after staining of M-like cells with anti-GP2 Ab (red) and NKM 16-2-4 (blue) together with green fluorescence images (four columns from the left of the upper panel) and after staining of the M-like cells with DAPI (blue) and NKM 16-2-4 (red) together with green fluorescence images (four columns from the left of the lower panel) after applying EGFP-Co1. Merged CLSM image of monocultured Caco-2 cells stained with anti-GP2 Ab and NKM 16-2-4 together with green fluorescence after applying EGFP alone also is shown in the far right column of the upper panel.

Columns of Fig. 3C, lower panel) in contrast to the monocultured Caco-2 cells, in which staining was not detected (far right column of Fig. 3C, lower panel). Collectively, we interpret these results to mean that the Co1–M cell interaction is specific.

Mucosal adjuvant activity of the M cell-targeting Co1 ligand

To confirm the adjuvant activity of the Co1 ligand, we characterized the Co1 ligand-mediated enhancement of immune responses against ligand-fused Ag after oral administration. In addition, patterns of immune response induction mediated by Co1 ligand conjugation were compared with those induced by cholera toxin (CT), a well-known adjuvant for mucosal immunization (Fig. 4). When we measured the levels of EGFP-specific Abs in systemic and mucosal compartments after oral administration of the Ag, a significantly higher level of EGFP-specific serum IgG was detected, even after 3 wk of oral administration of EGFP-Co1, than EGFP alone, and Co1-mediated enhancement of immune response induction was detected both in serum IgG and fecal IgA after 6 wk of oral administration of EGFP-Co1 (Fig. 4A). However, the adjuvant activity of Co1 was inferior to that of CT, and oral coadministration of CT with EGFP induced higher levels of both EGFP-specific serum IgG and fecal IgA than those induced by oral administration of EGFP-Co1.

Next, we evaluated the priming activity of oral administration of EGFP-Co1 by measuring the levels of EGFP-specific Ab and EGFP-specific proliferation of CD4+ T lymphocytes prepared from spleen after systemic injection of mice that had been immunized orally with corresponding Ag once every week for 6 wk with EGFP (100 μg) (Fig. 4B). The mice primed with EGFP-Co1 were able to secrete higher levels of EGFP-specific serum IgG and fecal IgA than EGFP-primed mice after systemic boost immunization. In addition, the lymphocyte proliferation assay revealed the presence of more EGFP-specific precursor CD4+ T cells after oral administration of EGFP-Co1 than EGFP alone. Moreover, the pattern of cytokine secretion after in vitro stimulation of lymphocytes from spleen and PPs revealed that immunization with EGFP-Co1 predominantly stimulated the production of IL-6, which is able to modify the polarization of Th2-type cells (Fig. 4C). In any case, coadministration of CT together with EGFP was able to induce more efficient systemic and mucosal immune responses against the EGFP Ag than administration of EGFP-Co1. Our results indicate that conjugation of an Ag with the Co1 ligand could induce enhanced systemic and mucosal immune responses against the fused Ag through T cell-mediated Ag specific IgG and IgA induction. In addition, we were able to rule out possible tolerance induction by oral administration of Co1-fused EGFP.

Discussion

Application of vaccines through an oral mucosal route is an ideal vaccination strategy because it induces effective protective immunity through eliciting Ag-specific IgA Ab production in mucosal tissue, a major site of pathogen infection, and simultaneously through assisting the induction of Ag-specific systemic immune responses (40, 41). Because a single epithelial layer of mucosa protects the host from pathogenic invasion through tight regulation of luminal access and surveillance and because the mucosal environment induces tolerogenic immunity against the introduced

Furthermore, a merged image of monocultured Caco-2 cells stained with DAPI and NKM 16-2-4 together with green fluorescence after applying EGFP-Co1 is shown in the far right column of the lower panel. The upper and lower panels of the images were taken after binding of 100 and 1 μg Ag, respectively. Scale bars, 50 μm.
animal models, have limited application because of side-effects. Terotoxin, the most well-known and effective mucosal adjuvants in well characterized (42, 43). Even CT and heat-labile bacterial toxins (e.g., CT and heat-labile E. coli), saponins (e.g., imiquimod, LPS, and flagellin), and lectins, like sialylactose or anti-lectin Ab and M cell-specific Ab NKM 16-2-4 using CLSM (Figs. 2, 3). Although efforts have been made to use lectins, ligands, Abs, and pathogens with binding specificities to M cells as adjuvants (49, 50). For example, NKM 16-2-4, an M cell-specific mAb established after sorting murine M cells according to their UEA-1 binding characteristics, was shown to mediate the uptake of an Ab-fused botulinum toxoid or OVA Ag, resulting in the induction of high levels of Ag-specific serum IgG and fecal IgA after oral administration of the Ab-linked Ag together with the mucosal adjuvant, CT, in mice (15). In addition, oral administration of killed whole bacteria, together with UEA-1 as an M cell-targeting ligand, induced a protective immune response even without the use of an immunostimulatory adjuvant, although questions were raised about the safety and binding specificity of UEA-1 (51). However, it is not clear whether Ag delivery through M cells induces mucosal immunity or mucosal tolerance, although it has been suggested that mucosal tolerance induction is dependent on the amount or nature of the delivered Ag (17). For example, reovirus protein σ1, a well-known M cell-binding protein, elicited Ag-specific IgG, secretory IgA, and CTL responses when it was applied as a DNA vaccine by intranasal immunization for 3 wk. However, when 1 mg OVA was intubated together with 15 μg CT once per week for 3 wk after oral administration of recombinant σ1-OVA Ag, systemic and mucosal tolerance was induced, as demonstrated by a reduction in Ag-specific CD4+ T cell stimulation and an increase in the number of TGF-β1- and IL-10-producing CD25+CD4+ regulatory T cells. These opposite outcomes indicate that oral vaccines require adjuvants to induce Ag-specific immunity and that M cell targeting only does not guarantee mucosal immune induction but not mucosal immune tolerance (52–54).

Phage display libraries are powerful tools to obtain specific ligands against small molecules, cells, and even tissues (31, 55–58). Previously, this method has been used to identify M cell-targeting ligands using rat PPs, although the identified peptides showed high binding affinity only to mucosa and Caco-2 cells (46). In the current study, however, we identified peptide ligands capable of binding to both mouse M cells and human M-like cells from an in vitro culture model that were characterized by anti-GP2 Ab and M cell-specific Ab NKM 16-2-4 using CLSM (Figs. 2, 3). Moreover, among the selected peptide ligands, the Co1 ligand showed promising characteristics with respect to the targeting of fused Ag and immune induction against fused Ag. For example, the Co1 ligand increased specific binding of fused Ag to M-like cells, which were stained with both anti-GP2 and NKM 16-2-4, compared with that of monocultured Caco-2 cells, such that EGFP binding to M cells was higher when 1 μg EGFP-Co1 was used than when 100 μg EGFP not fused to Co1 ligand was used (Fig. 3C). The superior targeting of Ag conjugated to Co1 to M cells and the increase in the transcytotic ability of M cells were confirmed by oral administration experiments; EGFP fluorescence was identified below the M cells of mouse PPs only 5 min after oral administration. Transcytosis of Co1-fused Ag into M cells was observed time-dependently in sequential sections; this rapid transcytosis of Ag is consistent with a previous study that reported rapid transport of Ag (within 15 min) through M cells (59). Therefore, one of the functions of the Co1 ligand appears to be the
effective delivery of Co1-fused Ag to mucosal immune inductive sites through M cell targeting.

M cell targeting only is not sufficient for a molecule to function as an oral mucosal adjuvant; therefore, we evaluated the capacity of selected ligands to stimulate the mucosal immune system after oral administration of ligand-fused EGFPs (59). Interestingly, we found that the EGFP-Co1 protein induced Ag-specific serum IgG and fecal IgA responses more efficiently than EGFP alone or other ligand-fused EGFP proteins without any adjuvant (Figs. 2, 4). Furthermore, we could detect higher levels of lymphocyte proliferation and secretion of IFN-γ and IL-6 after stimulation of splenocytes with EGFP from mice orally immunized with EGFP-Co1 compared with those from mice orally immunized with EGFP alone (Fig. 4). In particular, lymphocytes from PPs, a mucosal inductive site, were found to secrete IL-4, IL-5, and IL-6; these cytokines are known to be associated with isotype switching to secretory IgA through an autocrine TGF-β1-dependent loop and with overcoming oral tolerance environments (40, 60). It is possible that by targeting M cells immune tolerance rather than immune stimulation may be induced. However, the Co1 ligand identified in this study did not induce oral tolerance because an EGFP-specific recall immune response was induced successfully by s.c. challenge immunization with EGFP in mice orally immunized with EGFP-Co1 (Fig. 4B). Collectively, our results suggest that the Co1 ligand has mucosal adjuvant ability; conjugation of Co1 to an Ag results in delivery of the Ag to M cells as well as the induction of Th2-type cytokines.

To obtain a more detailed understanding of Co1-mediated adjuvant activity, we initially tested the ligand with the reverse sequence of Co1 conjugated to the C terminus of EGFP; no marked enhancement of EGFP-specific immune response induction was observed after oral administration of this conjugated Ag (data not shown). Consequently, we investigated the possible involvement of Co1 in signal transduction rather than just binding through searching for homologous proteins in protein databases. The outer membrane protein H (OmpH) of Yersinia enterocolitica, an M cell pathogen recently used as an oral carrier, shares 66 and 64% amino acid homology with Co1 helix of Skp, whose sequence is homologous to that of the Co1 helix, plays a pivotal role in C5a receptor activation via M cell targeting.

A novel M cell-specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific regulatory T cells.

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Disclosures
The authors have no financial conflicts of interest.

References