Application of an M-cell-targeting ligand for oral vaccination induces efficient systemic and mucosal immune responses against a viral antigen

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Abstract

Oral mucosal vaccination is an alternative method to overcome the pitfalls of current injection-based vaccines, such as pain and high cost of vaccination. It is a feasible and economic vaccine application, especially in developing countries. However, achieving effective antigen delivery into mucosal lymphoid organs and efficient immune stimulation are prerequisites to successful oral mucosal vaccination. One promising approach for oral mucosal vaccine development is exploring the potential of M cells via M-cell-targeting ligands that have the potential to deliver ligand-conjugated antigens into mucosal lymphoid organs and evoke conjugated-antigen-specific systemic and mucosal immune responses. Here, we investigated the M-cell-targeting ligand, Co1, in inducing specific immune responses against a pathogenic viral antigen, envelope domain III (EDIII) of dengue virus, to provide the foundation for oral mucosal vaccine development against the pathogen. After oral administration of Co1-conjugated EDIII antigens, we observed efficient antigen delivery into Peyer’s patches. We also report the elicitation of EDIII-specific immunity in systemic and mucosal compartments by Co1 ligand (located in the C-terminus of EDIII). Furthermore, the antibodies induced by the ligand-conjugated EDIII antigen showed effective virus-neutralizing activity. The results of this study suggest that the M-cell-targeting strategy using Co1 ligand as a mucosal adjuvant may be applicable for developing oral vaccine candidates against pathogenic viral antigen.

Keywords: adjuvant, dengue virus, ligand, mucosal immunity, vaccine

Introduction

Mucosal vaccines offer excellent possibilities for the induction of antigen-specific immune responses in mucosal compartments including small intestine, ascending colon and mammary and salivary glands, which is not currently feasible through injection-based vaccines, together with systemic immune response induction (1–3). Among mucosal vaccination technologies, oral vaccines offer great advantages as they are safe, easy to administer and do not require trained medical personnel for vaccine delivery. This last point is especially relevant in developing countries with insufficient public health infrastructure (4, 5). However, oral mucosal vaccine technologies are not currently applied to commercial vaccines due to serious pitfalls including possible oral tolerance induction and low efficiency in antigen delivery to the mucosal lymphoid organs (6, 7). Consequently, the development of successful mucosal immunization technologies using the strategy to enhance the efficiency of antigen delivery to mucosal lymphoid tissue and to avoid possible oral tolerance induction is of great interest (7).
A ligand for oral vaccination of viral antigen

To overcome obstacles to administering oral mucosal vaccines, many previous studies including our own indicate that the elucidation of a mucosal adjuvant and carrier system are critical factors for successful application (8, 9). These efforts include using TLR ligands and bacterial toxins as mucosal adjuvants and using polymers, chitin and chitosan as mucosal vaccine carriers (8). The most promising development in mucosal vaccine adjuvant and carrier systems is the utilization of M cells, which have been identified as ideal targets for mucosal vaccine delivery. This is based on the ability of M cells to take up antigen and microorganisms from the luminal cavity and to initiate antigen-specific immune responses, despite low numbers in the intestinal tract (10–12). Therefore, efforts are being directed toward developing mucosal vaccine adjuvants through M-cell targeting of the antigen using the M-cell-specific binding lectin, M-cell-specific antibody, NKM 16-2-4 and/or identifying the specific receptors expressed in M cells such as TLR-4, platelet-activating factor receptor and glycoprotein 2 (GP2) (13–16). In previous studies, we also suggested that an M-cell-targeting ligand, Co1, selected from phage display library panning against the in vitro M-cell co-culture system, not only delivers the ligand-conjugated model antigen into M cells through interaction with complement C5a receptor but also provokes antigen-specific immune response both systemically and in mucosal compartments (17, 18).

Dengue virus (DENV), a positive-sense single-stranded RNA virus of the genus Flavivirus, is the causative agent of dengue and dengue hemorrhagic fever, which is fatal in as many as 5% of infected people (19, 20). Dengue, one of the world’s most important emerging infectious human diseases, infects 50 million people annually worldwide, mostly in tropical and subtropical regions (21, 22). Technical problems associated with dengue vaccines, such as immunological priming among different serotypes and the requirement for tetravalent vaccines, still need to be resolved. However, one of the greatest challenges in developing a vaccine for dengue is the cost of vaccine application in developing and underdeveloped countries because of insufficient health infrastructure (23, 24). In this context, oral mucosal vaccination against DENV antigen is an excellent option, because it induces protective immunity in systemic compartments and can be applied relatively inexpensively. In this study, we applied the Co1 ligand to the envelope domain III (EDIII) of DENV type 2 (DENV-2) and analyzed the sequential and directional specificity of Co1 ligand using recombinant proteins containing the ligand conjugated to either the N- or C-terminus of EDIII antigen in either forward or reverse direction. After the oral administration of each ligand-conjugated or non-conjugated (control) EDIII antigen, we found that EDIII-Co1A was dominantly transported into C5aR on M cells of the Peyer’s patch (PP) and evoked an EDIII-specific immune response both systemically and in the mucosal compartment.

Methods

Experimental materials

All reagents used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise specified.

Virus and cell line

DENV was propagated in the C6/36 mosquito cell line that had been obtained from the ATCC (Manassas, VA, USA) and maintained in minimal essential medium supplemented with 5% (v/v) fetal bovine serum (PAA, Etobicoke, Ontario, Canada), with CO₂ incubation at 28°C.

Production of ligand-conjugated recombinant antigens

EDIII of DENV-2 was used as an immunogen in this study. The EDIII gene was obtained from DENV-2-infected C6/36 cells and reconstructed by PCR using primers containing nucleotide sequences for corresponding forward (Co1A; N-SFHQLPASPLP-C) and reverse (Co1B; N-PLPSRPLQHFS-C) directions of Co1 ligand to the C- or N-terminus of the antigen in a pQE 90 or pRSET A expression vector (17). Detailed primer sequences for PCR amplification of the antigen gene are summarized in Table 1. Each recombinant protein was expressed by Escherichia coli clones harboring the gene for EDIII-Co1A, EDIII-Co1B, Co1A-EDIII or Co1B-EDIII and purified by Ni-NTA affinity chromatography. His-tag amino acids present in front of Co1A-EDIII and Co1B-EDIII were removed by using enterokinase to expose the conjugated ligand.

Table 1. Primer sequences used to amplify antigen genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDIII</td>
<td>Forward</td>
<td>5′-GGA TCC ATG TCA TAC TCT ATG TGT-3′</td>
</tr>
<tr>
<td>EDIII</td>
<td>Reverse</td>
<td>5′-GGT ACC TTT CTT GAA CCA GTT-3′</td>
</tr>
<tr>
<td>EDIII-Co1A/B</td>
<td>Forward</td>
<td>5′-GGA TCC ATG TCA TAC TCT ATG TGT-3′</td>
</tr>
<tr>
<td>EDIII-Co1A</td>
<td>Reverse</td>
<td>5′-GGT ACC CTA CGG CAG AGG CGA CCG CGC CCG CAG CTG ATG AAA CQA TTT CTT GTA CCA GGT GAG-3′</td>
</tr>
<tr>
<td>EDIII-Co1B</td>
<td>Reverse</td>
<td>5′-GGT ACC CTA CGA AAA ATG CTG CAG CGG CGC CCG CAG AGG CAG CCG TTT CTT GTA CCA GGT GAG-3′</td>
</tr>
<tr>
<td>Co1A-EDIII</td>
<td>Forward</td>
<td>5′-GGA TCC ATG GGA TCA ATG GAC GAT GAC GAT AAG TCG TTT CAT CAG CTG CCG GCC CGG TCG CCT CTG CCG ATG TCA TAC TCT ATG-3′</td>
</tr>
<tr>
<td>Co1B-EDIII</td>
<td>Forward</td>
<td>5′-GGA TCC ATG GGA TCA ATG GAC GAT GAC GAT AAG CCG CTT TCG CCG GCC CCG CTG CAT TTT TCG ATG TCA TAC TCT ATG-3′</td>
</tr>
<tr>
<td>Co1A/EDIII</td>
<td>Reverse</td>
<td>5′-GGT ACC CTA TTT CTT GTA CCA GGT GAG-3′</td>
</tr>
</tbody>
</table>

Underlined letters represent the sequences for the BamHI or KpnI site.
In vitro and in vivo antigen-uptake assays

Frozen sections (10–15 µm in thickness) of PPs from normal or orally immunized BALB/c mice were obtained using a cryomicrotome (Thermo Fisher Scientific, Waltham, MA, USA), fixed with 4% paraformaldehyde and blocked with 2.5% BSA and 0.1% glycine in PBS. The sections were then stained with rhodamine-labeled *Ulex europaeus* agglutinin 1 (UEA-1, Vector Laboratories, Burlingame, CA, USA) or anti-GP2 antibody (IMGENEX, San Diego, CA, USA) followed by allophycocyanin-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA), anti-EDIII antibody (Serotec, Oxford, UK) followed by FITC-conjugated anti-mouse IgG (BD Bioscience, Franklinton Lakes, NJ, USA) and analyzed by confocal laser scanning microscopy (CLSM; LSM 510 META; Carl Zeiss, Thornwood, NY, USA) after counterstaining with 4′,6-diamidino-2-phenylindole (Invitrogen) (17).

In order to monitor in vivo antigen uptake, mice were sacrificed at 10 and 20 min after oral administration of ligand-conjugated, or non-conjugated, EDIII to BALB/c and PPs were excised from the small intestine. The PPs were washed with ice-cold PBS and whole mounted using 4% paraformaldehyde. After blocking with 2.5% BSA and 0.1% glycine in PBS, specimens were stained with anti-EDIII antibody followed by FITC-conjugated anti-mouse IgG and rhodamine-conjugated UEA-1. In some experiments, specimens were stained with anti-EDIII antibody followed by FITC-conjugated anti-rat IgG (Invitrogen), efluor 405-conjugated wheat germ agglutinin (Invitrogen), and PE-conjugated anti-mouse C5aR (BioLegend, San Diego, CA, USA). The specimens were then analyzed by CLSM (25).

Measurement of antigen-specific immune responses

A total of 100 µg experimental recombinant antigen was administrated by oral gavage to 4-week-old female BALB/c mice, five mice per group, without anesthesia once per week for 6 weeks followed by monitoring for up to 6 weeks (17, 26). Then, splenic lymphocytes (SPLs) and PP lymphocytes (PPLs) were subjected to characterization of the immune response induction. In order to test the oral priming effect, orally immunized mice were boosted systematically at 20 days after the last immunization by intra-peritoneal injection of recombinant antigen (100 µg) without ligand, and sera and fecal extracts were prepared 5 days after the boost immunization to measure levels of antigen-specific IgG and IgA by ELISA, as described previously (17, 26). Antibody titers were expressed as the reciprocal log2 titer of the highest sample dilution that gave an OD492 of 0.08, the value of the PBS blank. In addition, cells were prepared from the spleen and PPs 10 days after the boost immunization and subjected to analysis of frequency of antigen-specific IgG, IgA+, and cytokine-secreting cells. Numbers of antigen-specific antibody- and IL-6-secreting cells were measured by ELISPOT assay using lymphocytes isolated from various lymphoid tissues, as described previously (17). To analyze cytokine secretion by T cells after antigen stimulation, CD4+ T cells were prepared from SPLs by a CD4+ T cell isolation kit II (Miltenyi Biotec, Auburn, CA, USA) from each immunized mouse and in vitro stimulated with EDIII antigen for 48 h and then the supernatant was analyzed with a T5/1/T2/T17 array kit (BD Bioscience).

Antigen-specific lymphocyte stimulation was determined using a thymidine incorporation assay, as described previously (17). Briefly, SPLs were prepared from mice 10 days after the last oral immunization and stimulated in vitro with cognate antigens for 3 days followed by a pulse with 0.5 µCi of [3H]-thymidine deoxyribose (TdR) (Amersham Life Science, Buckinghamshire, UK) per well, for an additional 18 h. Using a 96-well cell harvester (Innotech, Dottikon, Switzerland), the cells were collected and assessed for tritium incorporation using a liquid scintillation counter (Packard Instrument, Meriden, CT, USA). Stimulation indices were calculated by dividing the tritium incorporation (counts per minute) in cells treated with cognate antigens by the incorporation in control cells treated with PBS.

Plaque reduction neutralization test

The level of neutralization antibody against each DENV serotype was measured using the microneutralization plaque reduction neutralization test (PRNT) assay in C6/36 cells or Vero cells in 96-well ELISpot plates, as described previously (27). Briefly, after heat inactivation of final diluted sera at 56°C for 30 min, the samples were incubated at 37°C with 34 plaque-forming units DENV per well for 60 min, absorbed to a host cell monolayer at 37°C for 90 min, followed by PBS washing and then overlaid with 1.4% methylcellulose. Virus plaques were stained with anti-EDIII monoclonal antibody followed by alkaline phosphate-conjugated anti-mouse IgG at 3 days post-infection of DENV and then monitored for development of spots by BCIP/NBT alkaline phosphatase substrate solution.

Statistical analysis

Statistical analyses were performed using SigmaPlot™ (Systat Software, Chicago, IL, USA). Results are presented as the mean ± SE. An unpaired Student’s t-test was used to compare groups and P < 0.05 was considered significant.

Results

In vitro and in vivo M-cell targeting of Co1-conjugated pathogenic EDIII antigen

We previously showed that ligand Co1 (Co1A; N-SFHQLPARSPLP-C) mediated the targeting and transport of ligand-conjugated enhanced GFP antigen into mouse M cells (17). Consequently, we hypothesized that EDIII antigen of DENV could also be targeted to M cells through ligand conjugation. To this end, we produced recombinant EDIII antigens conjugated with Co1 ligand in forward and reverse directions (Co1A or Co1B) and in both the N- and C-terminus (Co1A/B-EDIII or EDIII-Co1A/B). Constructs were monitored by in vitro interaction between the antigens and M cells of mouse PPs (Fig. 1A–C). In tissue slices containing follicle-associated epithelium (FAE) of normal mouse PPs, recombinant EDIII-Co1A
Enhancement of EDIII-specific systemic and mucosal immune response induction by oral administration of Co1-conjugated EDIII

In order to test the possible practical application of the M-cell-targeting ligand, Co1, in oral mucosal vaccination against DENV infection, we measured the EDIII-specific immune response induction in systemic and mucosal compartments using ELISA and ELIspot after oral administration of EDIII or Co1-conjugated EDIII proteins weekly for 6 weeks (Fig. 2). When we measured the level of EDIII-specific serum IgG, a significantly higher level (P < 0.001) of EDIII-specific IgG was detected in mice orally immunized with EDIII-Co1A compared with that of EDIII alone or other EDIII proteins conjugated with ligands with different positions and directions (Fig. 2A). This EDIII-Co1A-mediated efficient systemic immune response induction was confirmed by more than a 5-fold increase in EDIII-specific IgG-secreting cells in SPLs from EDIII-Co1A-immunized mice compared with that of the EDIII alone (Fig. 2C). In addition, Co1A-mediated enhancement of EDIII-specific immune response induction was also detected in the mucosal compartment. Oral immunization with EDIII-Co1A not only enhanced the induction of EDIII-specific fecal IgA but also significantly increased (P < 0.01) the number of EDIII-specific IgA-secreting cells in PPs, compared with induction after oral immunization with EDIII alone (Fig. 2B and D). Again, this Co1A-mediated enhancement in EDIII-specific mucosal immune response induction was not prominent in other EDIII proteins conjugated with ligands in different positions and directions.

In order to confirm the characteristics of Co1A-mediated enhancement of EDIII-specific systemic and mucosal immune responses, we monitored the expression patterns of IL-6 in SPLs and PPLs, since IL-6 is a maturation factor for IgA-class-switched B cells and has been identified previously as a critical cytokine in the activity of Co1 ligand (17, 28). As shown in Fig. 3, the number of IL-6-secreting cells was increased in mice immunized with EDIII-Co1A compared with EDIII alone (up to 2- and 3-fold) in SPLs and PPLs, respectively. In addition, the T-cell-mediated immune response against EDIII was significantly evoked by Co1
ligand conjugation, such that more than a 2-fold increase in lymphocyte proliferation was detected in SPLs (Fig. 3B). Collectively, these results suggest that EDIII-Co1A, administered orally, was able to enhance the EDIII-specific immune response in systemic and mucosal compartments through T-cell-mediated immune stimulation.

**Non-tolerogenic oral priming of Co1-conjugated EDIII**

A critical point to be cleared before achieving practical application of an oral mucosal vaccine is to ensure that the administered antigen does not induce tolerance. In order to validate that oral administration of EDIII-Co1A primed EDIII-specific immune responses, rather than EDIII-specific immune tolerance, we analyzed the EDIII-specific immune response after systemic challenge of EDIII in orally immunized mice, as described above (Fig. 4). When we measured levels of EDIII-specific serum IgG and numbers of EDIII-specific IgG- and IgA-secreting cells from SPLs, significantly higher levels of EDIII-specific immune responses were detected in the systemic immune compartment after the systemic challenge (Fig. 4A and B). Notably, the greater ability of EDIII-Co1A compared with other ligand-conjugated EDIII antigens in inducing EDIII-specific systemic immune responses was maintained even after systemic challenge. The priming effect, not tolerogenic effect, of C-terminal Co1A ligand-conjugated EDIII was also detected in mucosal immune responses, such that significantly higher levels of EDIII-specific fecal IgA and EDIII-specific IgG- and IgA-secreting cells in PPLs (compared with EDIII-alone-immunized mice) were detected after systemic challenge of EDIII in orally immunized mice (Fig. 4C and D).

To explore the relevance of the Co1-mediated adjuvant effect, we next characterized the pattern of IL-6-secreting cells in SPLs and PPLs prepared from mice that were orally immunized and systemically challenged (Fig. 5). As shown in the oral priming response, significantly higher levels of IL-6-secreting cells in SPLs and PPLs (Fig. 5A and B) and in vitro EDIII-specific proliferation (Fig. 5C) were detected from the mice immunized orally with EDIII-Co1A and systemically challenged with EDIII compared with those immunized and challenged with EDIII alone. Interestingly, this also showed that oral immunization of EDIII alone induced some level of
tolerogenic response since the level of EDIII-specific serum IgG was almost the same as that of the PBS control (Fig. 4A). This speculation was supported by the observation that SPLs prepared from EDIII-alone-immunized and -challenged mice did not proliferate in vitro in response to EDIII antigen and the level was >30-fold lower than that from the mice immunized with EDIII-Co1A and systemically challenged with EDIII (Fig. 5C). In addition, we found that the enhanced systemic immune response was closely related with antigen-specific Th2/Th17-skewed immune response induction because CD4+ T cells in SPLs from the mice immunized with EDIII-Co1A dominantly secreted IL-2, IL-4, IL-6 and IL-17 after in vitro EDIII stimulation (Fig. 6). Collectively, our results indicate that Co1-mediated M-cell targeting, especially Co1A in the C-terminus, could efficiently induce systemic and mucosal immune responses, not a tolerogenic response, against EDIII of DENV-2 through T-cell-mediated immune responses.

Induction of neutralizing antibodies against DENV infection by oral immunization of the EDIII antigens

We next examined whether EDIII-specific antibodies, induced by oral immunization with recombinant EDIII antigens of DENV-2, can recognize all four serotypes of DENV. We measured by ELISpot assay the number of DENV-specific IgG-secreting cells in SPLs of the orally immunized mice, by coating wells with each serotype of DENV. Interestingly, immunization with EDIII-Co1A could evoke the induction of cross-reactive antibodies recognizing all four serotypes of DENV (data not shown). To identify the neutralizing ability of the antibodies against DENV-2 infection, we performed a microneutralization PRNT assay against DENV-2 in C6/36 cells (Fig. 7). We demonstrate that oral immunization of mice with both EDIII and ligand-conjugated EDIII was capable of inducing neutralizing antibodies against DENV-2 infection. More importantly, immune sera from the mice immunized with EDIII-Co1A or EDIII-Co1B showed the most efficient neutralizing activity against DENV-2 infection, such that the geometric mean PRNT50 titer was well over 128 and was 4-fold higher than the level obtained from EDIII-alone-immunized mice. In addition, the sera from EDIII-Co1A-immunized mice showed more efficient neutralizing activity than that from EDIII-Co1B-immunized mice. To further analyze the neutralizing activity of immune sera against each DENV serotype, we monitored spot reduction by using Vero cells (see Fig. 8). As shown, immune sera from EDIII-Co1A-immunized group showed neutralizing activity against all DENV serotypes and the activity was superior to those from other groups, although the difference was not dramatic against certain DENV serotypes. Collectively, these results suggest that Co1-mediated M-cell targeting of EDIII, especially EDIII-Co1A, effectively induced DENV-specific antibodies with cross-reactivity against all four serotypes of DENV.

Discussion

The mucosal surface is the front line of defense against various pathogens invading the body (29, 30). A single layer of mucosal epithelial cells is protected from infectious agents that are recognized by the mucosal immune system, which consists of innate and adaptive immune components (31). In this context, mucosal vaccination that could establish mucosal immune induction, together with systemic responses, is an ideal strategy to deliver an effective vaccine. In addition, a mucosal vaccine, especially a mucosal oral vaccine, offers great advantages, such as convenience, safety and cost effectiveness, over conventional injection-based vaccines (32). However, there are obstacles to be overcome for efficient induction of oral mucosal immunization such as the harsh environment imposed by low pH and proteases, inefficient antigen delivery to immune inductive site and possible oral tolerance induction (32). In order to enhance the efficiency, therefore, many efforts have been tried using various antigen...
delivery systems and immunostimulators such as emulsions, microparticles, immune stimulating complexes, TLR ligands (e.g. CpG DNA, lipopolysaccharide and flagellin) and bacterial toxins (e.g. cholera toxin and heat-labile E. coli enterotoxin). However, their efficacy and action mechanisms are not clearly identified in the mucosal environment and some of them even displayed obvious side-effects such as antigen targeting into neurons in the central nervous system. In this regard, we previously tried to identify the M-cell-targeting peptide having the immunostimulatory activity (17).

M cells located in FAE of PPs and in isolated lymphoid follicle are characterized by short and irregular microvilli, a flattened apical surface and a thin layer of glycocalyx. M cells are specialized epithelial cells, which function to take up antigens from the intestinal lumen and deliver them to the underlying follicle (33, 34). In that sense, one of the effective strategies to develop successful mucosal vaccines is to search for mucosal vaccine adjuvants that exploit M cells (33). However, M cells only populate 5–10% of the intestinal tract, thereby limiting the efficient delivery of antigens to mucosal lymphoid organs in oral vaccination (11, 35). Recently, many efforts have been concentrated to identify and use M-cell-specific markers such as UEA-1-binding α(1,2)-fucose residue, M-cell-specific NKM 16-2-4 antibody and GP2 protein to enhance M-cell-targeting efficiency of the antigen in oral vaccination (12, 14, 15). For example, specific expression of GP2 in M cells was anticipated from microarray results and its expression was confirmed in mouse and human M cells (36). GP2 was also revealed to be associated with specific uptake of FimH⁺ bacteria in M

Fig. 4. Maintenance of non-tolerogenic systemic and mucosal immune responses against EDIII was confirmed through a systemic boost immunization in mice orally immunized with ligand-conjugated EDIII of DENV-2. Level of serum IgG (A) and numbers of IgG- and IgA-secreting cells per 10⁶ cells (B) specific for EDIII were determined through ELISA and ELIspot, respectively, from SPLs prepared at 10 days after a systemic challenge that was done 20 days after the last oral administration of the antigen, as described in Methods. Level of fecal IgA (C) and numbers of IgG- and IgA-secreting cells per 10⁶ cells (D) specific for EDIII were determined through ELISA and ELIspot, respectively, from PPLs prepared at 10 days after a systemic challenge that was done 20 days after the last oral administration of the antigen, as described in Methods. *P < 0.05 and **P < 0.01 indicate significant differences compared with the control EDIII-immunized group.
cells and induction of antigen-specific immune responses. Consequently, anti-GP2 antibody was suggested as a new approach for an ‘M-cell-targeted’ vaccination protocol (15). In our previous study, we also suggested that an M-cell-targeting ligand, Co1, selected by biopanning the phage display library against human M-like cells, could be used as a mucosal adjuvant through effective delivery of the ligand-conjugated model antigen, enhanced GFP, into the mucosal lymphoid organs for elicitation of immune responses against the conjugated model antigen (17). Moreover, we suggested
serotype (Figs 7 and 8) but also a T_{h}2/T_{h}17-skewed immune response (Fig. 6). Especially, SPLs prepared after systemic challenge of the antigen from EDIII-Co1A-immunized mice proliferated efficiently against in vitro antigen stimulation (Fig. 5C). This result suggests that oral immunization with EDIII-Co1A did not induce oral tolerance. Interestingly, we found that both Co1B-EDIII and EDIII-Co1A interacted well with mouse M cells in in vitro binding (Fig. 1A–C). However, in comparison with Co1B-EDIII whose efficient in vivo M-cell targeting was not detected, EDIII-Co1A in which the same ligand was conjugated in the C-terminus of EDIII promoted in vivo targeting of EDIII to C5aR on M cells and transport of EDIII into mouse PP M cells (Fig. 1D and E). When we consider that Co1 ligand in Co1B-EDIII and EDIII-Co1A was exposed as the same sequence direction with the homologous domain in C5aR ligand, the observed inability of Co1B-EDIII in M-cell targeting was unexpected. We assume that the inability of Co1B-EDIII-mediated in vivo M-cell targeting was closely related with protein instability, since it is known that anchoring of a protein in its C-terminus generally increases protein stability through reducing flexibility of the protein (37). We believe that induction of EDIII-specific immune response with neutralizing activity by EDIII-Co1B may support our assumption although the level was not as strong as EDIII-Co1A. Therefore, we conclude that the highly enhanced EDIII-specific immune response induced by oral administration of EDIII-Co1A resulted from effective antigen delivery through C5aR-mediated M-cell targeting by Co1 ligand and increased protein stability in vivo because of the sequence and location of Co1A ligand. Collectively, we suggest that the M-cell-targeting ligand, Co1A, conjugated in the C-terminus of the antigen could act as an effective mucosal vaccine adjuvant through delivery of antigen into the mucosal immune compartment and induction of efficient mucosal and systemic immune responses against the ligand-conjugated antigen.

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A ligand for oral vaccination of viral antigen