

# A novel mucosal vaccine targeting Peyer's patch M cells induces protective antigen-specific IgA responses

Hideaki Shima<sup>1,2</sup>, Takashi Watanabe<sup>3</sup>, Shinji Fukuda<sup>2,4</sup>, Shin-Ichi Fukuoka<sup>5</sup>, Osamu Ohara<sup>3</sup>  
and Hiroshi Ohno<sup>1,2</sup>

<sup>1</sup>Division of Immunobiology, Graduate School of Supramolecular Biology, Yokohama City University, Yokohama, Kanagawa 230-0045, Japan

<sup>2</sup>Laboratory for Intestinal Ecosystem, RIKEN Center for Integrative Medical Sciences, Kanagawa 230-0045, Japan

<sup>3</sup>Laboratory for Immunogenomics, RIKEN Center for Integrative Medical Sciences, Kanagawa 230-0045, Japan

<sup>4</sup>Gut Environmental Systems Biology, Integrated Omics, Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0035, Japan

<sup>5</sup>Department of Chemistry and Biological Science, College of Science and Engineering, Aoyama Gakuin University, Kanagawa, 229-8558, Japan

Correspondence to: H. Ohno; E-mail: ohno@rci.riken.jp

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## Abstract

**Mucosal vaccines can induce mucosal immunity, including antigen-specific secretory IgA production, to protect from invasion by pathogens and to neutralize toxins at mucosal surfaces. We established an effective antigen-delivering fusion protein, anti-GP2-SA, as a mucosal vaccine. The anti-GP2-SA consists of streptavidin (SA) fused to the antigen-binding fragment region from a mAb against glycoprotein 2 (GP2), an antigen-uptake receptor specifically expressed on M cells. Anti-GP2-SA targets antigen-sampling M cells in the follicle-associated epithelium covering Peyer's patches. Immunofluorescence showed that anti-GP2-SA specifically bound to M cells. Orally administered biotinylated ovalbumin peptide (bOVA) conjugated with anti-GP2-SA more efficiently induced OVA-specific fecal IgA secretion compared with bOVA alone or bOVA conjugated with SA. Furthermore, mice immunized by oral administration of the biotinylated *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) lysate conjugated with anti-GP2-SA were significantly better protected from subsequent infection by virulent *S. Typhimurium* than mice treated with the bacterial lysate alone or conjugated with SA. These results suggest that anti-GP2-SA-based M-cell-targeting vaccines are a novel strategy for inducing efficient mucosal immunity.**

**Keywords:** IgA, M cell, mucosal vaccine, vaccine delivery

## Introduction

Mucosal vaccines given *via* the oral route can induce mucosal immunity, such as antigen-specific secretory IgA (SIgA) production, as well as systemic immunity (1). By contrast, systemic vaccination, for example mumps vaccine, *via* the cutaneous route, will induce only systemic immunity with poor mucosal immunity (2). Accordingly, effective mucosal vaccines can protect from pathogens before their invasion across mucosal barriers and neutralize toxins, ingested or generated in the mucosal space by pathogens (3). On the other hand, mucosal vaccines have some disadvantages; for example, oral vaccines need to resist harsh pH conditions, such as pH 1–2 in gastric acid in the stomach, and multiple digestive enzymes to reach mucosal immune tissues such

as Peyer's patches (PPs) in sufficient amounts. To overcome the problem, live attenuated pathogens can be used as oral vaccines since they can propagate in the intestine, providing adequate immunogenicity even if only a small portion of the administered antigen reaches the immune tissue. However, attenuated pathogens can give rise to virulent revertants, causing adverse side-effects (4). Several studies are underway to circumvent the problem of revertants. Safer adjuvants are being created by removing harmful structures from pathogenic antigens (5) on the basis of findings that adjuvants such as cholera toxin (CT) can induce efficient mucosal immune responses to bystander antigens when they are coadministered, even when the antigens by themselves cannot induce

good immune responses (6). However, in clinical trials it has been reported that CT can also induce adverse side-effects (7). In addition to the mucosal adjuvants, efforts have been made to establish more efficient antigen delivery systems for effective mucosal vaccines, such as enteric-coated capsules (8), nanoparticles (9) and M-cell-targeting molecules (10).

M cells are located in the follicle-associated epithelium overlaying the PP and take up luminal antigens to initiate antigen-specific mucosal immune responses (11). Recently, several molecules important for M-cell differentiation and function have been identified (12–14). In particular, glycoprotein 2 (GP2) is specifically expressed by M cells among the intestinal epithelium and is essential for uptake of type I-piliated bacteria for induction of effective mucosal as well as systemic antigen-specific immune responses. These findings led us to consider GP2 as a suitable target molecule for efficient mucosal vaccine delivery. In support of this idea, a previous mucosal vaccine study of M-cell-targeting molecules suggested that development of novel M-cell-targeting mucosal vaccines is a realistic approach (10).

One strategy in efforts to establish efficient targeting vehicles for vaccines has been to fuse recombinant antigen-binding antibody domains, such as an antigen-binding fragment (Fab), with other proteins (15). There are essentially two types of recombinant Fab. One is the single-chain variable fragment (scFv), composed of the variable fragments of heavy chains and light chains connected by a flexible peptide linker. The other is disulfide-stabilized Fvs (dsFv), in which the variable fragments of heavy and light chains are bound to each other by disulfide bonds, as exist within the intact Fab. The advantages of dsFv over scFv are the biological half-life, stability to denaturants and temperature (16), and the slow unfolding rate that may confer resistance to a short period of adverse conditions (17).

Here, we report the construction of a fusion protein of streptavidin (SA) with dsFv originating from an anti-GP2 mAb. The resulting dsFv-anti-GP2-SA can bind both biotinylated antigen and GP2 and, as a consequence, serve as an efficient mucosal vaccine by delivering the antigen to M cells. The remarkable effectiveness of the antigen-conjugated anti-GP2-SA was demonstrated in mice, which were able to respond with antigen-specific SIgA even without adjuvant.

## Methods

### Animals

Male BALB/cA mice (6–8 weeks old) and C57BL/6 mice (5–7 weeks old) were obtained from CLEA Japan. C57BL/6 back-crossed *GP2*<sup>-/-</sup> mice (18) were maintained in the RIKEN Yokohama Animal Facility. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in RIKEN and Institutional Animal Care and Use Committee of Yokohama City University Graduate School of Supramolecular Biology and Medical Life Science.

### Antibodies and reagents

The following antibodies were used: rabbit anti-streptavidin (Abcam), anti-rat HRP (Invitrogen), anti-rabbit HRP

(Invitrogen), Alexa Fluor 488 anti-rat (Invitrogen), Alexa Fluor 488 anti-rabbit (Invitrogen) biotinylated rabbit IgG (Abcam), Alexa Fluor 633 phalloidin (Invitrogen), rhodamine-labeled UEA-1 (Vector Laboratories), VectorShield containing DAPI (Vector Laboratories). The anti-GP2 mAb (2F11-C3) was obtained as described elsewhere (13).

### Complementary DNA cloning and plasmid vector construction

Anti-GP2 antibody cDNAs were obtained from total RNA extracted from a hybridoma producing anti-GP2 antibody using the SMARTer PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions. Variable regions of Ig $\gamma$  (VH) and Ig $\kappa$  (VL) in the cDNA were amplified and sequenced, and then the native signal peptides in the amplicons were replaced with the human IL-6 signal peptide for secretion. The VH was connected to a mouse IgG2a CH1 with its hinge sequence and the SA core sequence (15). The VL was connected to a mouse Ig $\kappa$  C $\kappa$  sequence. Both sequences contained a His Tag embedded in the plasmid vector (VH-CH1-SA and VL-C $\kappa$ ).

### Fusion protein expression and purification

A Ni-NTA column was used to purify anti-GP2-SA from Hyclone SFM4HEK293 without Biotin (Thermo Scientific) culture supernatants of 293F cells transfected with the mCH1-SA and mlg $\kappa$ -C $\kappa$ .

### Structural assessment of anti-GP2-SA and immunostaining

Purified anti-GP2-SA, rat IgG and SA were subjected to 10 or 7.5% SDS-PAGE. After electrophoresis, gels were directly stained with Coomassie Brilliant Blue (CBB) or blotted onto an Immobilon-P membrane (Millipore). CBB stained gels were decolorized by soaking in reverse osmosis water. Blotted membranes were incubated overnight in 2% BSA/skimmed milk in PBS for blocking, and then with anti-rat IgG HRP or anti-SA antibody. Anti-SA antibody was detected with an anti-rabbit HRP secondary antibody. Chemical luminescence was detected by LAS-3000 (FUJIFILM).

### Ligated ileal loop experiment and whole-mount staining of PPs

Mice were anesthetized with isoflurane. Ten micrograms of anti-GP2 antibody or anti-GP2-SA with biotinylated rabbit-IgG or SA with biotinylated rabbit-IgG were injected into the ligated ileal loop containing a single PP. After incubation for 10–15 min, the mice were sacrificed and PPs were excised from the intestine. Specimens were washed gently and then fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences). Anti-GP2 antibody-treated specimens were incubated with Alexa Fluor 488 anti-rat IgG. The specimens treated with anti-GP2-SA or SA plus biotinylated rabbit IgG were incubated with Alexa Fluor 488 anti-rabbit IgG. All PP specimens were stained with rhodamine-labeled UEA-1 and Alexa Fluor 633 phalloidin to detect F-actin. The specimens were embedded in a 30% solution of glycerol in PBS and analyzed with a confocal laser scanning microscope (Leica Microsystems).

### Immunization studies

Ovalbumin peptide (OVA) was biotinylated with Biotin-(AC5)2 Sulfo-OSu (DOJINDO) according to the manufacturer's instructions. Biotinylated OVA (bOVA) was incubated for 30 min with SA or anti-GP2-SA to form complexes just before administration. Mice were orally immunized three times every other day with bOVA (50  $\mu$ g) or the complexes (50  $\mu$ g of bOVA with 30  $\mu$ g of SA or anti-GP2-SA in PBS) or PBS alone. One, two and three weeks after the final immunization, feces were collected, freeze dried and crushed. The crushed feces were re-suspended (1 g ml<sup>-1</sup>) in proteinase inhibitor-containing PBS and extracted by pestle homogenization and diluted: 2<sup>6</sup>-fold (Fig. 4) and 2<sup>3</sup>-fold (Supplementary Figure 1, available at *International Immunology Online*). OVA-specific IgA in the fecal extracts was analyzed by ELISA using plates coated with 1 mg ml<sup>-1</sup> OVA. Optical density (OD) was normalized to the anti-OVA ELISA titers in fecal extracts from each mouse prior to immunization.

*Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) carrying a nalidixic acid resistance gene ( $\chi$ 3306) was a gift from H. Matsui (19) and the *fimH* deletion mutant of *S. Typhimurium* (*fimH*<sup>-</sup> *S. Typhimurium*) was established elsewhere (13) from toxC-harboring *S. Typhimurium* (a gift from Dr Gordon Dougan). An insoluble protein fraction of *fimH*<sup>-</sup> *S. Typhimurium* was prepared by repeating freeze-thaw cycles three times and extensive washing with PBS. The resulting insoluble protein fraction was biotinylated and used for complex formation with SA or anti-GP2-SA. Mice were separated into four groups, and mice in each group were orally administered with

solutions containing the biotinylated insoluble protein fraction from  $3 \times 10^7$  colony forming unit (CFU) of *FimH*<sup>-</sup> *S. Typhimurium* alone, or with SA or anti-GP2-SA, or PBS three times every other day. Two weeks after the final immunization, feces were collected and fecal IgA content in each mouse was examined by ELISA using plates coated with the insoluble protein fraction of *FimH*<sup>-</sup> *S. Typhimurium*. The mice were then fed with  $1 \times 10^7$  CFU of *S. Typhimurium*  $\chi$ 3306 and survival of treated mice was observed at a fixed time each day.

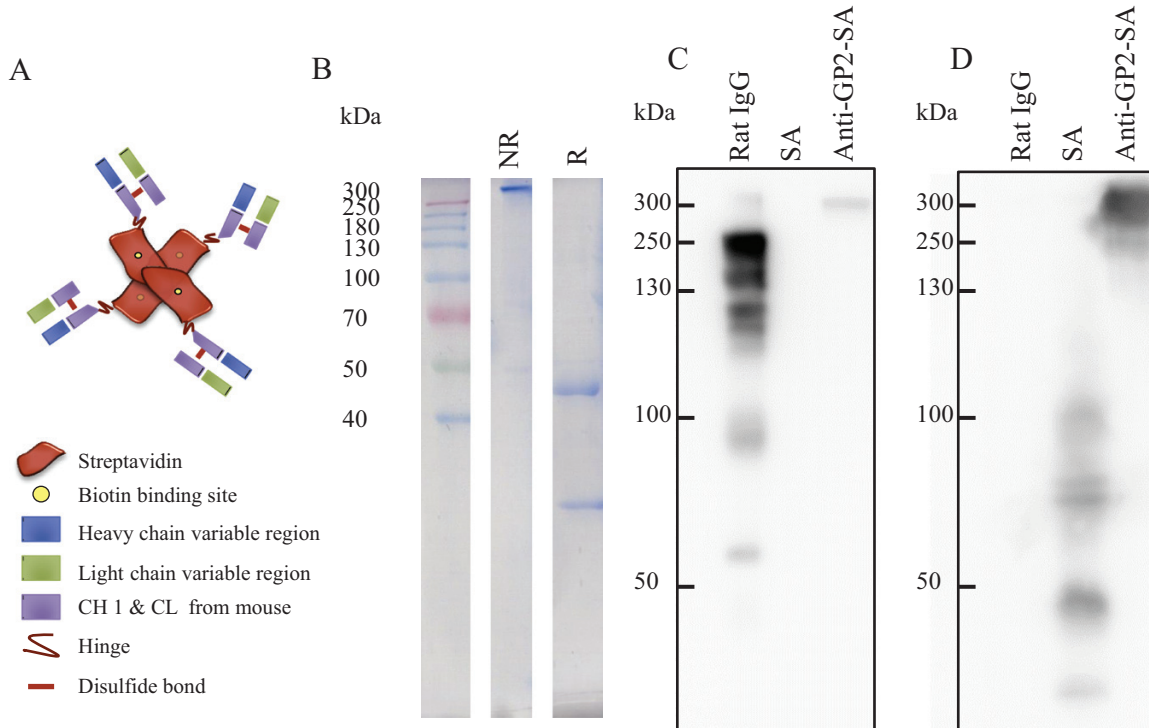
### Data analysis

Data are expressed as the mean  $\pm$  SEM. Statistical significance was determined by analysis of variance (Fig. 3A, Supplementary Figure 1, available at *International Immunology Online*) or the Holm method (Figs 3B and 4A) or the Student's *t*-test (Fig. 3C) or log-rank test (Fig. 4B) or Pearson's product-moment correlation coefficient (Fig. 4C).

## Results

### Establishment of anti-GP2-SA

To establish a mucosal vaccine with novel features, we first constructed a recombinant anti-GP2-SA plasmid vector and transfected it into 293-F cells. Expressed anti-GP2-SA was expected to form a tetramer because SA normally forms a tetramer, as shown in Fig. 1(A). Thus, we tested whether anti-GP2-SA formed a tetramer possessing the IgG variable regions from the parental anti-GP2 antibody and the SA



**Fig. 1.** Characterization of the recombinant anti-GP2-SA. (A) Schematic model of anti-GP2-SA. (B) CBB staining of anti-GP2-SA. Anti-GP2-SA was analyzed by 10% SDS-PAGE under non-reducing (NR) and reducing (R) conditions (2-ME plus boiling). (C and D) Western blotting was performed after 7.5% SDS-PAGE under NR conditions as described in Methods. The anti-GP2-SA was detected by anti-rat IgG antibody (C) or anti-SA antibody (D). Data are representative of experiments performed at least three times. Rat IgG, purified serum IgG.



core by CBB staining and western blotting (Fig. 1B–D). The theoretical molecular weight of VH-CH1-SA is ~49000Da, whereas VL-C $\kappa$  is ~30000Da. CBB staining showed a band with the expected molecular weight for the whole anti-GP2-SA tetramer, about 320000Da, under non-reducing conditions, as well as VH-CH1-SA and VL-C $\kappa$  monomers when the samples were reduced with 2-mercaptoethanol (Fig. 1B). In addition, western blotting showed that the anti-GP2-SA tetramer contains the rat IgG components and SA core proteins (Fig. 1C and D). These observations confirm that the generated anti-GP2-SA had the expected structure.

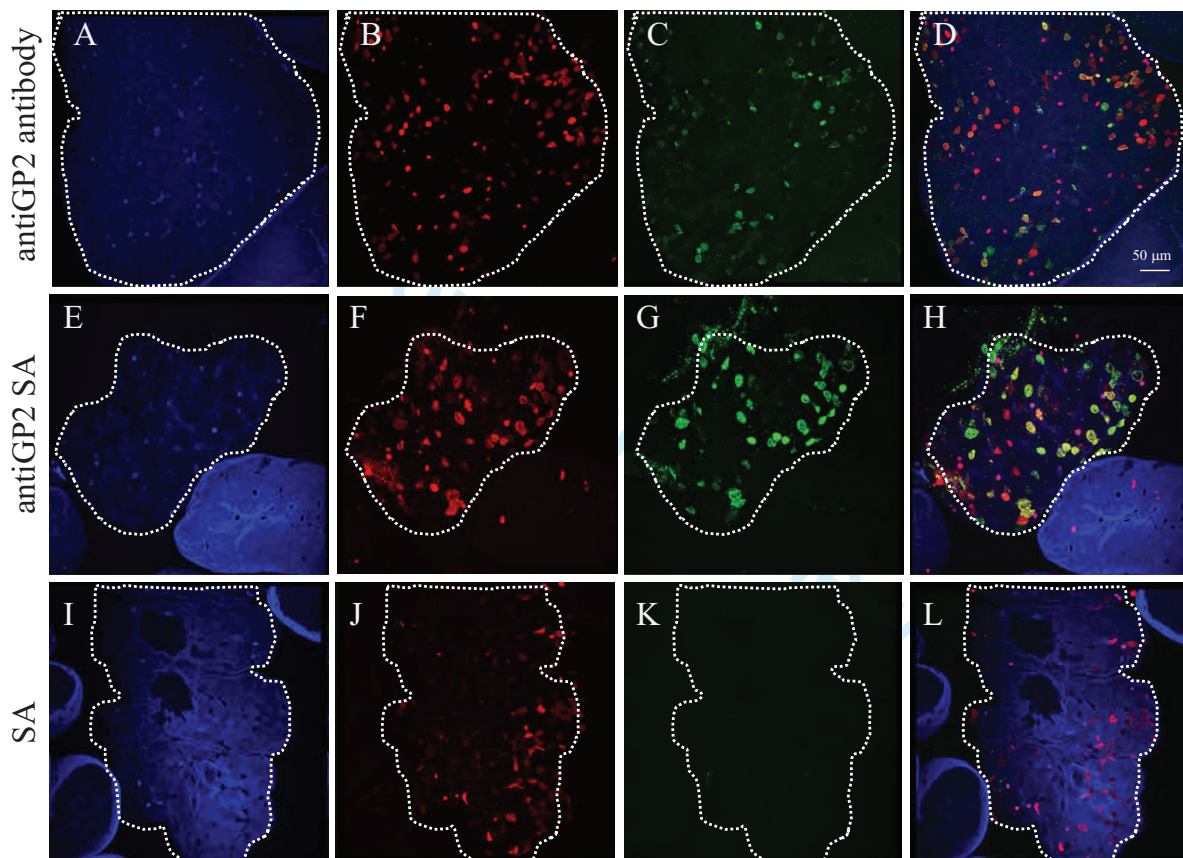
#### *Anti-GP2-SA delivers biotinylated molecules to PP M cells in mice*

We next asked whether anti-GP2-SA maintained the capacity to bind GP2 on M cells by using a ligated ileal loop assay and whole-mount immunostaining. Anti-GP2-SA conjugated with biotinylated rabbit IgG, or SA as a negative control, was injected into the lumen of a ligated ileal loop. As shown in Fig. 2, anti-rabbit IgG was found to localize on UEA-1<sup>+</sup> M cells in anti-GP2-SA-injected, but not SA-injected, samples (Fig. 2G and K). The fact that anti-GP2-SA administered into the intestinal lumen could deliver conjugated rabbit IgG to

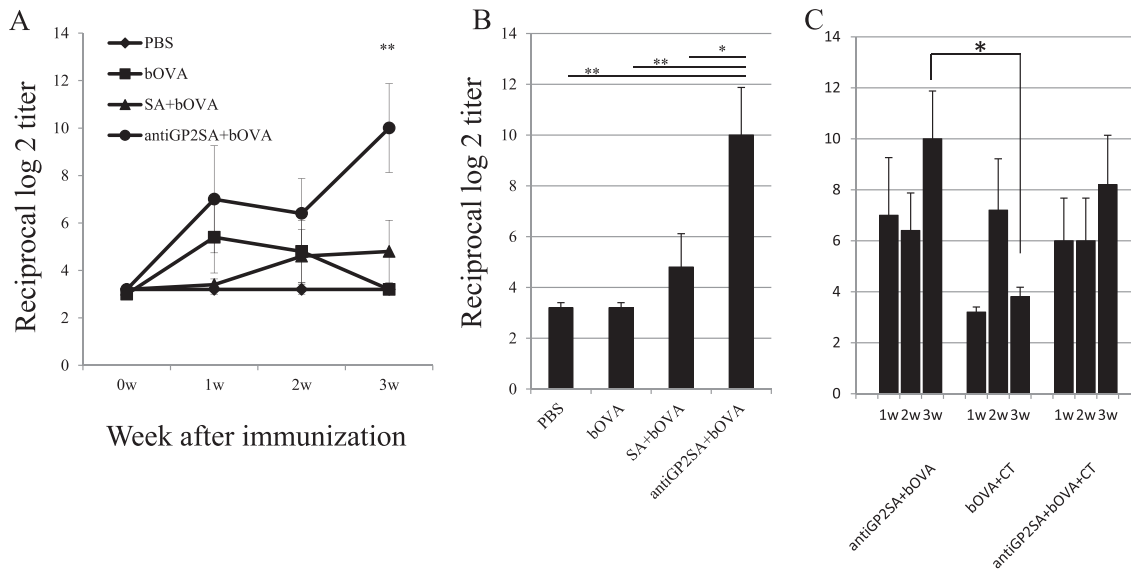
PP M cells raised the possibility that this molecule would be a useful vehicle for generating efficient mucosal immune responses to biotinylated oral vaccine antigens.

#### *Anti-GP2-SA efficiently induces antigen-specific fecal SIgA responses*

To test the hypothesis that anti-GP2-SA can effectively deliver biotinylated antigen to M cells for efficient induction of mucosal immune response, we measured fecal SIgA specific for various forms of orally administered bOVA. Orally administered OVA by itself does not normally induce mucosal immune responses (20). As expected, orally administered bOVA alone or conjugated with SA did not evoke efficient secretion of OVA-specific fecal SIgA (Fig. 3A). By contrast, when orally administered as a conjugate with anti-GP2-SA, bOVA efficiently induced OVA-specific fecal SIgA secretion (Fig. 3A) and the titer at week 3 was significantly higher compared with the groups immunized with SA-bOVA or bOVA alone (Fig. 3B). In addition, we confirmed whether the effect of anti-GP2-SA seen above evoked through its GP2 binding by utilizing *GP2*<sup>-/-</sup> mice. As expected, administration of bOVA-anti-GP2-SA to *GP2*<sup>-/-</sup> mice barely induced OVA-specific fecal SIgA (Supplementary Figure 1, available



**Fig. 2.** Binding of anti-GP2-SA to PP M cells in a ligated ileal loop assay. Ligated ileal loops were injected with anti-GP2 antibody as a positive control (A–D), complexes of anti-GP2-SA and biotinylated rabbit IgG (E–H) or complexes of SA and biotinylated rabbit IgG as a negative control (I–L). Alexa Fluor 633 phalloidin (blue) was used to stain F-actin (A,E,I). M cells are stained with UEA-1 (red) (B,F,J). Anti-GP2 antibody was detected with Alexa Fluor 488 anti-rat IgG (green) (C). Rabbit-IgG was detected with Alexa Fluor 488 anti-rabbit IgG (green) (G,K). The right-most panels (D,H,L) are the merged images of the left three panels. Note that UEA-1 also reacts with goblet cells so the co-localization is incomplete. Scale bar, 50  $\mu$ m. Results represent at least three specimens in each group.



**Fig. 3.** Induction of OVA-specific fecal SIgA upon oral immunization of mice with anti-GP2-SA-conjugated bOVA. Mice were orally administered with PBS, bOVA, SA-conjugated bOVA or anti-GP2-SA-conjugated bOVA. Fecal SIgA was determined by ELISA as described in Methods. (A) OVA-specific SIgA titers. The titers were normalized to the OVA-specific SIgA titer in feces from each mouse before treatment (0 week). (B) OVA-specific fecal SIgA titers in each group at week 3. (C) OVA-specific fecal SIgA titers in mice treated with anti-GP2-SA conjugated to bOVA in the presence or absence of CT and mixture of bOVA with CT. Data are expressed as the mean  $\pm$  SEM from five mice per each group. \* $P < 0.05$ , \*\* $P < 0.01$ .

at *International Immunology Online*), indicating that anti-GP2-SA indeed delivered bOVA in a GP2-dependent manner. Interestingly, the OVA-specific fecal SIgA titer induced by oral administration of bOVA-anti-GP2-SA was comparable in the presence and absence of CT as a mucosal adjuvant (Fig. 3C). By contrast, bOVA-anti-GP2-SA without CT group showed a higher SIgA titer than the bOVA and CT-treated group after 3 weeks (Fig. 3C). These results suggest that anti-GP2-SA can induce an efficient SIgA response by delivering biotinylated antigens to M cells, and that the addition of mucosal adjuvant may be dispensable for the anti-GP2-SA-mediated SIgA response.

#### *Anti-GP2-SA-mediated vaccine delivery can protect against S. Typhimurium infection*

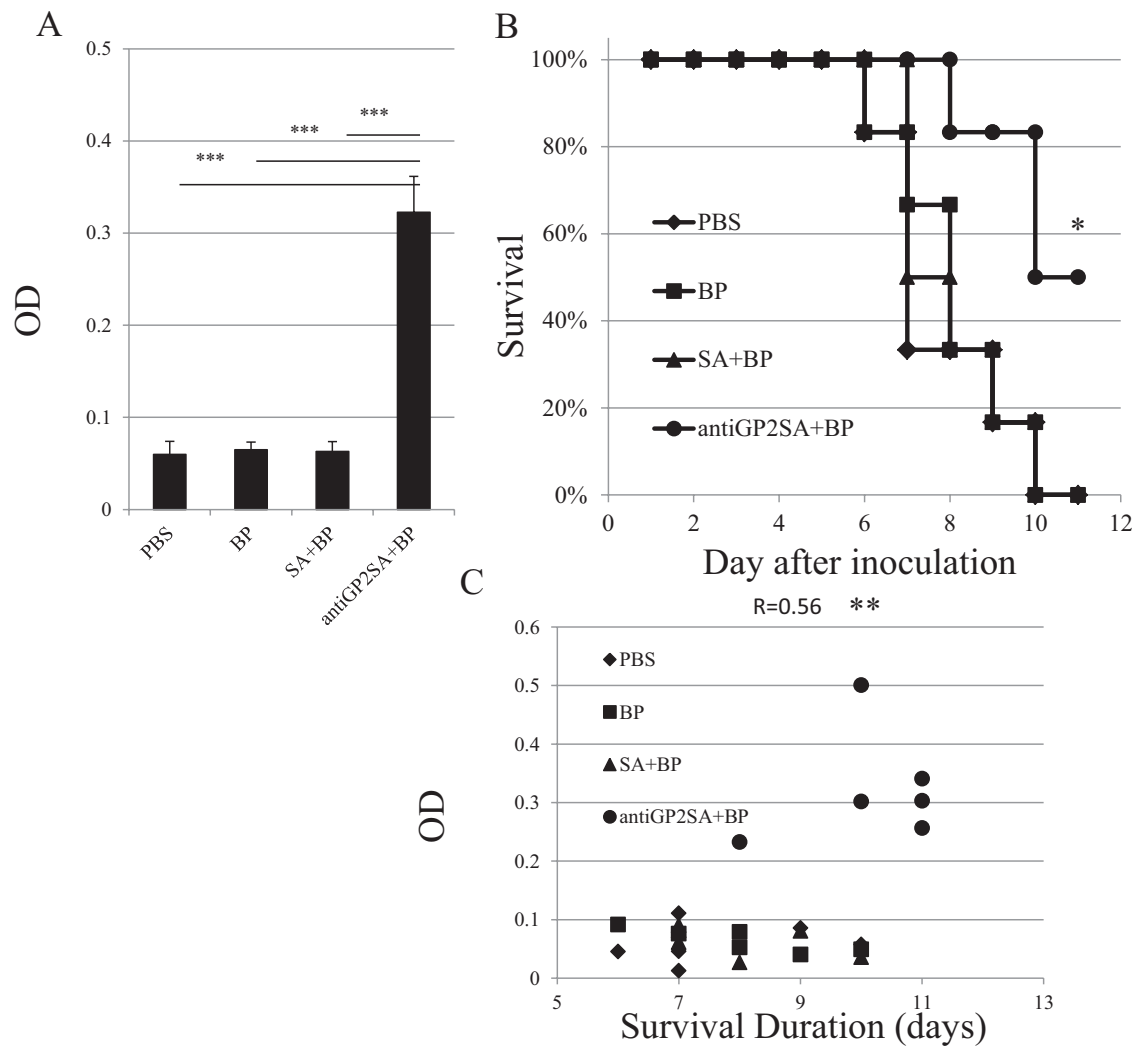
Since delivery of bOVA to M cells by anti-GP2-SA led to induction of an efficient OVA-specific fecal SIgA response, we hypothesized that anti-GP2-SA could be a useful mucosal vaccine delivery tool. To test the possibility, we asked if anti-GP2-SA-mediated M-cell delivery of *S. Typhimurium* vaccine antigens could protect mice from subsequent *S. Typhimurium* infection. We used a lysate of *fimH*<sup>+</sup> *S. Typhimurium* as the source of vaccine antigens to avoid autonomous delivery of antigens to GP2-expressing M cells, since the FimH<sup>+</sup> type 1 pilus is a GP2 ligand (13).

Mice were orally immunized with a biotinylated lysate of *fimH*<sup>+</sup> *S. Typhimurium* alone, or conjugated with anti-GP2-SA or SA, or PBS as a negative control and challenged with virulent *S. Typhimurium*. Two weeks after the final immunization, a significant *S. Typhimurium*-specific fecal IgA response was induced only in mice immunized with the biotinylated

bacterial lysate with anti-GP2-SA (Fig. 4A). Consistently, mice vaccinated by this regimen survived significantly longer upon subsequent oral challenge with virulent *S. Typhimurium* than mice from the other three groups, where all mice died within 10 days after infection (Fig. 4B). Furthermore, the fecal SIgA content and mouse survival showed a significant positive correlation (Fig. 4C,  $R = 0.56$ ). Taken together, these results confirmed that an M-cell-targeting vaccine is a useful tool for efficient mucosal vaccination, and that anti-GP2-SA should be very useful for this purpose.

#### Discussion

Mucosal vaccination is theoretically superior to systemic vaccination since the former can evoke both mucosal and systemic immunity whereas the latter can only induce systemic responses (2). However, mucosal vaccination has not been widely used because of the difficulty in designing practical vaccines. Mucosal vaccines have to survive harsh conditions such as the highly acidic gastric environment and intestinal digestive enzymes to reach the mucosal immune system. To overcome the insufficient immunogenicity because of the small amount of residual vaccine surviving the gastrointestinal environment, the existing mucosal vaccines utilize attenuated live pathogens, which can propagate in the mucosal tissue, or supplementation of the vaccine with adjuvants to enhance immunogenicity. However, these strategies have intrinsic disadvantages for clinical use due to, for example diarrhea, the emergence of virulent revertants and adverse side-effects of the adjuvants (4). An efficient adjuvant-free vaccine delivery vehicle for the mucosal immune system could overcome these problems and would be a valuable addition to the vaccine armamentarium.



**Fig. 4.** Induction of protective immunity against *S. Typhimurium* infection by oral immunization with biotinylated *S. Typhimurium* lysate conjugated to anti-GP2-SA. (A) Fecal *S. Typhimurium*-specific IgA. Mice were orally administered with solutions containing the biotinylated insoluble protein fraction of *fimH*- *S. Typhimurium* (BP) alone, or with SA or anti-GP2-SA, or PBS three times as described in Methods. Feces were collected 2 weeks after the final immunization and fecal IgA specific to the insoluble protein fraction of *fimH*- *S. Typhimurium* for each mouse was determined by ELISA as described in Methods. Results are derived from six mice per each group and shown as mean  $\pm$  SEM. (B) Survival curves of *S. Typhimurium*-infected mice. Mice immunized in A were fed  $1 \times 10^7$  CFU of *S. Typhimurium*  $\chi$ 3306 and the survival of treated mice was observed at a fixed time every day. (C) Correlation of fecal SIgA concentration (OD) in A and survival (days) in B of each mouse. Kaplan-Meier survival curves were constructed and analyzed by a log-rank test. Data were analyzed by the Holm method (B) or Pearson's product-moment correlation coefficient (C). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

To tackle this issue, we chose GP2 as the target for vaccine delivery to the mucosal immune system. GP2 is one of the most highly expressed apical surface molecules specific to PP M cells and serves as a receptor for antigen uptake and delivery to the mucosal immune system (13). Moreover, a previous report had shown that M cells are a good target for induction of antigen-specific mucosal immune responses (10).

To design the GP2-targeting vehicle, we took advantage of a GP2-specific mAb generated in our laboratory (13). In this report, we constructed an anti-GP2-SA fusion protein, in which SA, as a receptor for biotinylated antigens, was appended to a dsFv from the anti-GP2 antibody. SA was chosen because of its remarkably high affinity for biotin ( $K_d \approx 10^{-14}$ ) and, more

importantly, since it is very stable under adverse conditions such as those causing denaturation (16), making it suitable for gastrointestinal administration. In fact, anti-GP2-SA was able to survive the gastrointestinal luminal environment to efficiently deliver antigens, bOVA and the biotinylated insoluble protein fraction of *fimH*- *S. Typhimurium* to PP M cells and to induce antigen-specific SIgA responses. These results also indicate that the affinity for GP2 of the dsFv in the anti-GP2-SA is sufficient to bind to, and be taken up into, PPs *via* M cells.

Intriguingly, the fecal OVA-specific SIgA titer induced by oral bOVA-anti-GP2-SA was comparable in the presence or absence of CT as an adjuvant. Although we currently have no experimental evidence to explain this observation, one possibility is that SA, a bacterial component, could possess

some degree of adjuvant activity. Alternatively, commensal microbes taken up in parallel with anti-GP2-SA by M cells may serve as natural adjuvants to enhance immune responses against bOVA. Further studies will be required to clarify the point.

In this study, we introduce anti-GP2-SA as a novel adjuvant-free mucosal vaccine delivery tool that can efficiently induce mucosal SIgA responses. Our results also indicate that M-cell-targeting vaccination should be an effective and safe strategy, in that inactivated vaccines can be delivered in sufficient amounts to evoke protective immunity against pathogens.

### Supplementary data

Supplementary data are available at *International Immunology* Online.

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