sites, such as in PPs, are needed to elucidate the role of the carbohydrate moiety containing α(1,2) fucose in the mucosal immune system.

In summary, we established a novel M cell–specific mAb (NKM 16–2–4; rat IgG2c) that selectively recognizes M cells, but not goblet cells or epithelial cells, and we characterized the M cell–specific carbohydrate moiety containing α(1,2) fucose. Our strategy for M cell–targeted vaccination with NKM 16–2–4 is attractive for the development of mucosal vaccines.

MATERIALS AND METHODS

Animals. Female BALB/c mice, Crlj: CD1-*Foxn1*^{nu} mice, and SD rats between 6 and 8 wk old were obtained from CREA and Charles River Laboratories. All of them were maintained in the experimental animal facility at the Institute of Medical Science, the University of Tokyo, and experiments were performed according to the guidelines provided by the Animal Care and Use Committee of the University of Tokyo.

Establishment of an M cell–specific mAb. The M cell–enriched fraction was prepared from murine PPs as previously described, with some modification, by using UEA-1 (4). In brief, cells isolated from murine PPs were fixed in 4% paraformaldehyde (Wako) and stained with 500 ng/ml PE-conjugated UEA-1 (Biogenesis). UEA-1–positive cells were sorted by a FACSAria cell sorter (Becton Dickinson) and injected into the footpads of SD rats (10^6 cells/rat) 4 times at 2-wk intervals, with TiterMax Gold (TiterMax) as an adjuvant. 4 d after the final immunization, lymphocytes isolated from the spleen and inguinal lymph nodes of the immunized rats were fused with P3X63-AG8.653 myeloma cells (CRL-1580; American Type Culture Collection) in the presence of 50% (wt/vol) polyethylene glycol 1500 (Roche). Established hybridomas were injected into Crlj: CD1-*Foxn1*^{nu} mice, and mAbs were then purified from ascitic fluids by using protein G–Sepharose (GE Healthcare) and labeled with EZ-Link Sulfo-NHS-LC-biotin (Thermo Fisher Scientific), FITC (Sigma-Aldrich), or Alexa Fluor 647 (Invitrogen).

Immunohistochemical analysis. One monoclonal antibody (NKM 16–2–4; rat IgG2c) was selected on the basis of the initial screening and its specificity to M cells determined by immunohistochemical and whole-mount staining analyses, as described previously, with some modification (4). In brief, after a blocking step with 1% BSA, 7-μm fixed frozen sections or fixed tissues containing PPs were stained with 5 μg/ml FITC-conjugated NKM 16–2–4 or FITC-conjugated isotype control (FITC-conjugated rat IgG2c; MBL International) and 1 μg/ml tetrarhodamine isothiocyanate–conjugated UEA-1 (Vector Laboratories). The sections were then counterstained with 400 ng/ml DAPI (Sigma-Aldrich) for histochemical analysis and analyzed under a confocal laser-scanning microscope (TCS SP2; Leica). For electron-microscopic analysis, ultrathin sections (100 nm) were incubated with 1 μg/ml purified NKM 16–2–4 after blocking with 1% BSA, followed by 18-nm gold particle–conjugated goat anti–rat IgG (Jackson Immunoresearch Laboratories) diluted 1:10. Finally, the sections were stained with 4% uranyl acetate and analyzed under a transmission electron microscope (JEM100S; JEOL).

Uptake of NKM 16–2–4 by M cells. After the mice were anesthetized with 2 mg ketamine (Sigma-Aldrich), we injected 100 μg of FITC-conjugated NKM 16–2–4 or FITC-conjugated control rat IgG (Sigma-Aldrich) into intestinal loops containing PPs, in accordance with our previous study (4). The mice were killed 10 or 30 min, or 4 h, after the inoculation, and frozen sections (7 μm) of intestinal loop were prepared and analyzed under a confocal laser-scanning microscope after counterstaining with DAPI.

M cell–targeted vaccination. TT (provided by the Research Foundation for Microbial Diseases, Osaka University, Osaka, Japan) and type A BT (prepared according to a previous study; reference [27]) were first treated with EZ-Link Sulfo-NHS-LC-biotin. Next, biotinylated TT or BT at 1 mg/ml was incubated with the same volume of avidin (1 mg/ml; Sigma-Aldrich). The complexes were then incubated with twice the volume of biotinylated NKM 16–2–4, biotinylated control rat IgG (Sigma-Aldrich), or biotinylated UEA-1 (Vector Laboratories; each 1 mg/ml). Mice were orally immunized with the complexes (in total, each 200 μg contained 50 μg TT or BT per mouse), noncoupled TT (50 or 500 μg per mouse), or PBS alone 3 times (once a week), together with 10 μg CT (List Biological Laboratories) as a mucosal adjuvant. 7 d after the final immunization, serum and fecal extracts were collected and analyzed for TT- or type A botulinum toxin–specific serum IgG and fecal IgA responses by ELISA, as previously described (5, 27). To examine the protective immunity, the mice were challenged via the i.p. route with 200 ng type A botulinum toxin (10,000× LD_{50} i.p.) diluted in 100 μl of 0.2% gelatin/PBS (27). To confirm the universality of M cell–targeted mucosal vaccine with NKM 16–2–4, OVA (Sigma-Aldrich) was conjugated with NKM 16–2–4 or control rat IgG and orally immunized together with 10 μg CT. In addition, intestinal loop assay was performed by using M cell–targeted OVA composed of Alexa Fluor 647-conjugated OVA (Invitrogen), FITC-conjugated avidin (Sigma-Aldrich), and NKM 16–2–4 or control rat IgG. Conjugation of NKM 16–2–4 or control rat IgG and the protein antigen was confirmed by sandwich ELISA (unpublished data).

Analysis of antigen recognized by NKM 16–2–4. To identify the antigen recognized by NKM 16–2–4, we performed an immunoprecipitation assay with NKM 16–2–4 followed by an LC-MS/MS analysis. In brief, a lysate of M cells was incubated with 10 μg/ml NKM 16–2–4 or an isotype control antibody (rat IgG2c; BD Biosciences) followed by protein G–Sepharose (GE Healthcare). Immune complexes were analyzed by SDS-PAGE and Western or lectin blot with 5 μg/ml biotinylated NKM 16–2–4, 5 μg/ml biotinylated isotype control antibody (biotin-conjugated rat IgG2c; BD Biosciences), or 5 μg/ml biotinylated UEA-1 (Vector Laboratories) and ABC–AP complex (Vector Laboratories). To identify the precipitated antigen, LC-MS/MS analysis was performed after digestion with 50 nM trypsin gold (Promega).

Transfection of cells. mFUT1 and mFUT2 genes were synthesized from mRNAs from intestinal tissue, including PPs, using specific primers (mFUT1: sense, 5′-TACTAAGCTAGCATGTGGACTCCCAGCCGGAGGCAG-3′, antisense, 5′-GCTAGCGGATCCTCAGACCAATCTAAAAAGACTGTC-3′; mFUT2: sense, 5′-ATCTAAGCTAGCATGGCGAGTGCCCAGGTAC-CTTTC-3′, antisense, 5′-TGCAGCGAATTCTTAGTGCTTAAGGAGT-GGGGACAG-3′; NheI and BamHI [mFUT1] and NheI and EcoRI [mFUT2] restriction enzyme sites are shown by underlining) by RT-PCR and inserted into pIRES2-EGFP vector (BD Biosciences). These plasmids were then transformed into CHO-K1 cells (CCL-61; American Type Culture Collection) and three CHO-cell–derived mutant lines (Lec1, CRL-1735 [reference 25]; Lec2, CRL-1736 [reference 24]; and Lec8, CRL-1737 [reference 26]). 2 d after transfection, the cells were stained with 500 ng/ml Alexa Fluor 647–conjugated NKM 16–2–4 and 500 ng/ml PE-conjugated UEA-1, followed by the application of 10 μl/test VIA-PROVE (BD Biosciences). They were then analyzed by flow cytometry with FACSCalibur (Becton Dickinson). For blocking analysis, 500 ng/ml Alexa Fluor 647–conjugated NKM 16–2–4 or 500 ng/ml PE-conjugated UEA-1 was first pretreated with 0.5 M α-L-fucose (Wako).

Data analysis. Data are expressed as the mean ± the SD. All analyses for statistically significant differences were performed by Tukey's *t* test, with $P < 0.01$ considered significant (denoted in the figures with an asterisk).

Online supplemental material. Fig. S1 shows the specificity of NKM 16–2–4 to isolated UEA-1–positive M cells. Fig. S2 shows that NKM 16–2–4 specifically reacts with M cells in NALT, similar to its reaction with PP-associated M cells. Fig. S3 shows the expression of maltase glucoamylase and alanyl aminopeptidase mRNAs in PPs. Fig. S4 shows that NKM 16–2–4 reacts with different form of UEA-1–reactive portion of α(1,2) fucose. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070607/DC1.

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Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination

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Capable of inducing antigen-specific immune responses in both systemic and mucosal compartments without the use of syringe and needle, mucosal vaccination is considered ideal for the global control of infectious diseases. In this study, we developed a rice-based oral vaccine expressing cholera toxin B subunit (CTB) under the control of the endosperm-specific expression promoter 2.3-kb glutelin *GluB-1* with codon usage optimization for expression in rice seed. An average of 30 μg of CTB per seed was stored in the protein bodies, which are storage organelles in rice. When mucosally fed, rice seeds expressing CTB were taken up by the M cells covering the Peyer's patches and induced CTB-specific serum IgG and mucosal IgA antibodies with neutralizing activity. When expressed in rice, CTB was protected from pepsin digestion *in vitro*. Rice-expressed CTB also remained stable and thus maintained immunogenicity at room temperature for >1.5 years, meaning that antigen-specific mucosal immune responses were induced at much lower doses than were necessary with purified recombinant CTB. Because they require neither refrigeration (cold-chain management) nor a needle, these rice-based mucosal vaccines offer a highly practical and cost-effective strategy for orally vaccinating large populations against mucosal infections, including those that may result from an act of bioterrorism.

mucosal immunity | protein body | oral vaccine | IgA | cholera toxin B subunit

The majority of emerging and reemerging infectious pathogens, including *Vibrio cholerae*, *Escherichia coli*, HIV, influenza virus, or coronavirus causing severe acute respiratory syndrome, invade and infect the host via the mucosal surfaces of the gastrointestinal, respiratory, and/or genitourinary tracts (1–3). Mucosal immunity forms a first line of defense by means of secretory IgA and cytotoxic T cells against epithelium-transmitted pathogens, and so it would seem important to develop vaccines that induce effective immune responses at mucosal barriers. Most current vaccines are administered by needle and syringe, generating effective antibody and cell-mediated responses in the systemic compartment, but not in mucosal sites (4). In contrast, mucosal vaccines administered either orally or nasally have been shown to be effective in inducing antigen-specific immune responses in both systemic and mucosal compartments (5–8). Because it elicits this two-layered protective immunity, mucosal vaccination is thought to be an ideal strategy for combating both emerging and reemerging infectious diseases (5–8). In fact, the Bill and Melinda Gates Foundation and the National Institutes of Health have proposed that mucosal vaccines be a focus of future vaccine development (9), a vision underlying the foundation of the Gates' research initiative, "Grand Challenges in Global Health" (9). Most traditional vaccines are not cost-effective because they cannot be stored at room temperature (RT), instead requiring that the "cold chain" be preserved en route from vaccine manufacturer to the field of vaccination (i.e., that no gap be allowed in the refrigeration) (10). The cost of preserving that cold chain for currently used vaccines is estimated at between $200 and $300 million a year (10). Further, if inappropriately processed or disposed of, the needles and syringes used for the vaccination can pose the threats of environmental contamination and second-hand spread of infectious disease. Producing vaccine antigens in plants would offer many practical advantages (11, 12). First, it would be less expensive to produce vaccine antigens in plants than via industrial fermentation. Second, there is no need to take elaborate means to purify the vaccine if it is expressed in plant tissue. Third, the plant expression system minimizes risks arising from contamination. Collectively, these advantages make a plant-based subunit vaccine not only attractive, but also practical for the propagation of mucosal vaccine on the global scale (12).

As early as 1990, Curtiss and Cardineau expressed *Streptcoccus mutans* surface protein antigen, the causative epitope for dental caries, in tobacco as a first step toward a potential plant-based mucosal vaccine (13). Since then, many vaccine antigen candidates, including bacterial diarrhea antigens, hepatitis B antigen, Norwalk virus antigen, and respiratory syncitial virus antigen, have been expressed in tobaccos or potatoes to demonstrate the feasibility of edible plant-based vaccines (14–21). However, these plant-based vaccines have remained a function of sophisticated bench-driven experiments and have not yet advanced to practical application. If such a vaccine is to be practicable for global immunization, it must be storable at RT for long periods, be protected from the harsh environment of the gastrointestinal tract, and target mucosal inductive tissues, including Peyer's patches (PPs) (8, 22).

We here introduce a rice-based oral vaccine possessing many practical advantages over most traditional or other plant-based oral vaccines. The rice-based oral vaccine is stable at RT for several years and is protected from digestive enzymes. When ingested, this vaccine induced antigen-specific antibodies with neutralizing activities. These results show that the rice-based oral vaccine offers a highly practical global strategy for cold-chain- and needle-free vaccination against infection.

Author contributions: T.N. and H.T. contributed equally to this work; T.N., Y.Y., and H.K. designed research; T.N., H.T., Y.Y., L.Y., T.M., M.M., U.N., A.M., A.U., T.H., S.M., K.T., and F.T. performed research; T.N., H.T., and Y.Y. analyzed data; and T.N., Y.Y., and H.K. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: CHO, Chinese hamster ovary; CT, cholera toxin; CTB, cholera toxin B subunit; LTB, heat labile enterotoxin B subunit; PPs, Peyer's patches; PB, protein body; RT, room temperature.

See Commentary on page 10757.

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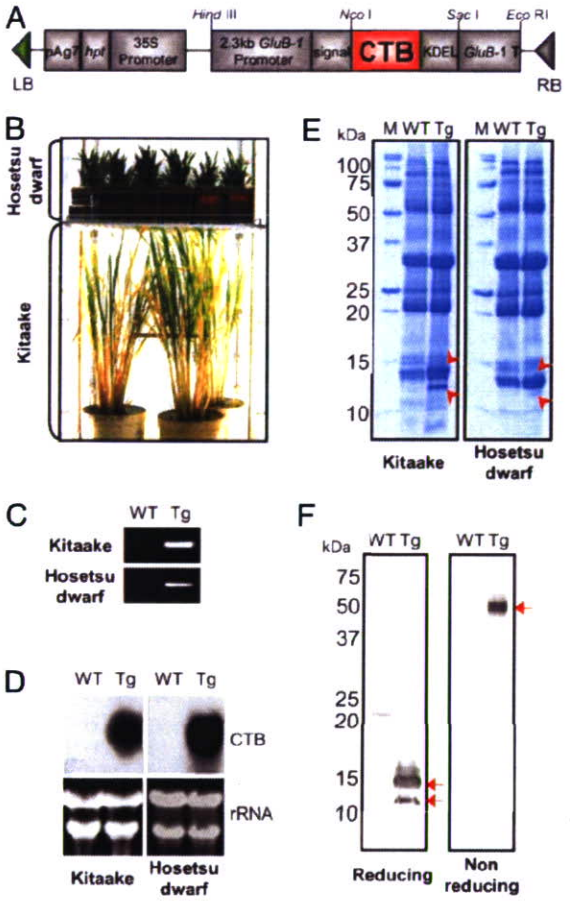

Fig. 1. Expression of CTB in transgenic rice. (*A*) T-DNA plasmid-inserted, codon-optimized CTB gene for rice seed, controlled by the rice seed storage protein glutelin 2.3-kb *GluB-1* promoter. The signal sequence of *GluB-1* and the retention signal to the endoplasmic reticulum coding KDEL are located at the N- and C-terminal regions, respectively. (*B*) The Kitaake and Hosetsu dwarf type rice strains expressed CTB in a closed chamber. (*C*) Integration of the CTB gene into the genomic DNA was confirmed by PCR. WT, Wild-type nontransgenic rice; Tg, CTB-expressed transgenic rice. (*D*) Northern blot analysis was performed for the confirmation of CTB mRNA expression. (*E* and *F*) SDS/PAGE and Western blot analysis revealed that high levels of CTB protein were expressed in rice. Arrowheads indicate 12- and 15-kDa forms of CTB (*E*). The CTB protein, composed of two fragments, forms a 55- to 65-kDa pentamer structure under nonreducing conditions. Arrows indicate monomeric (under reducing condition) and pentamer (under nonreducing condition) forms of CTB (*F*).

Results

Development of Rice-Based Mucosal Vaccine Expressing Cholera Toxin B Subunit (CTB) in Seeds. We purposely chose CTB as a prototype antigen to demonstrate both the capacity of the rice-based mucosal vaccine to induce systemic as well as mucosal immunity and to showcase the practicality of using the rice transgenic expression system. Once generated with binary vector (pGPTV-35S-HPT) (23), as described in Fig. 1*A*, codon-optimized CTB genes for rice seed were transfected into the rice plant [*Oryza sativa* L. cv Kitaake, a normal-sized rice (24); and Hosetsu, a dwarf type rice (25, 26), shown in Fig. 1*B*] by using *Agrobacterium tumefaciens*-mediated transformation (27). Hosetsu dwarf type rice, a naturally occurring gene mutant on the gibberellin biosynthesis pathway (25), is 20 cm in height and has a short life cycle (≈3 months). Genomic PCR analysis revealed that the CTB gene was integrated into the genomic DNA of both lines of rice plants (Fig. 1*C*). In addition, high levels of CTB-specific mRNA in the seeds of both lines of transgenic but not nontransgenic wild-type (WT) rice were expressed at 15 days after flowering (Fig. 1*D*). When we examined the accumulation of CTB protein in the transgenic rice seed by SDS/PAGE and Western blot analysis with anti-CTB polyclonal antibody, two bands (12 kDa and 15 kDa) were detected under denatured conditions (Fig. 1 *E* and *F*). Using densitometry analysis with rCTB as a standard, we found that expression levels of CTB reached an average of 30 μg per seed in the Kitaake strain, representing 2.1% of the total seed protein (0.15% of seed weight). The expression level of CTB in Hosetsu dwarf type rice was lower (average of 5 μg per seed) than that in Kitaake rice. Furthermore, Western blot analysis under nonreducing conditions revealed that CTB expressed in rice formed a pentamer with 55 to 65 kDa (Fig. 1*F*), indicating that most of the CTB expressed in rice seed is considered to be a functionally native form possessing the ability to bind to the GM1 ganglioside, known to be expressed at the apical surface of the intestinal epithelium and to be a receptor for CTB (28).

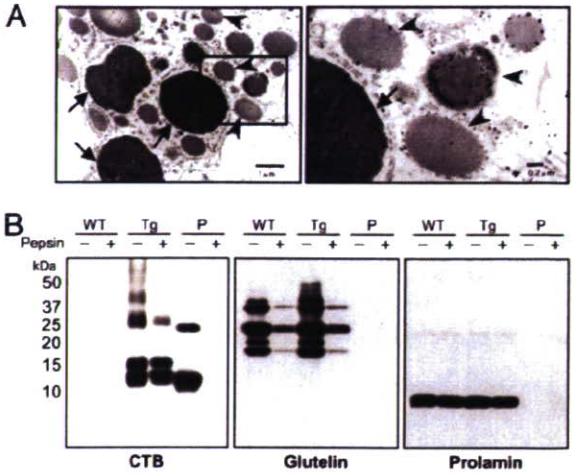

Fig. 2. Localization and digestive enzyme resistivity of rice-expressed CTB in PBs. (*A*) Data obtained through immunoelectron microscopic analysis with anti-CTB antibody. A positive signal was obtained with 20 nm of gold particles. CTB expressed in rice are stored in PB-I (arrowheads) and PB-II (arrows). (*B*) Pepsin digestion was carried out under the conditions described in *Materials and Methods*. Approximately 90% of glutelins but not 13k prolamins were digested *in vitro*, whereas ≈75% of rice-based CTB but not purified rCTB remained intact.

CTB Expressed in Protein Bodies (PBs) of Rice Seed Is Resistant to Gastrointestinal Harsh Environment. In addition to being easy to produce and administer, an effective oral vaccine would also have to have a built-in safeguard against digestion, particularly against the harsh acidic environments found in the stomach. The starchy endosperm in rice contains two types of protein storage organs, PB-I and PB-II, which are distinguished by their shape, density, and protein composition (29). The main storage proteins for PB-I are the alcohol-soluble prolamins (e.g., 13k prolamin) and the water-soluble glutelins (e.g., glutelin B1) (29, 30). Because they are water-soluble, the glutelins (PB-II) are more vulnerable to digestion in the gastrointestinal tract than are prolamins (PB-I). Immunoelectron microscopic analysis reveals that CTB is localized not only on the surface of PB-I, but also on the inside of PB-II (Fig. 2*A*). To examine the ability of the CTB accumulated in the rice PB to withstand protease digestion in the stomach, total seed proteins were subjected to pepsin treatment. Western blot analysis (Fig. 2*B*) revealed that the signal intensity of the 13k prolamin was not significantly changed by the pepsin treatment, whereas ≈90% of the glutelins were digested by pepsin under these conditions. In addition, ≈75% of the CTB accumulated in rice seed remained intact after pepsin treatment (Fig. 2*B*). These findings suggest that most

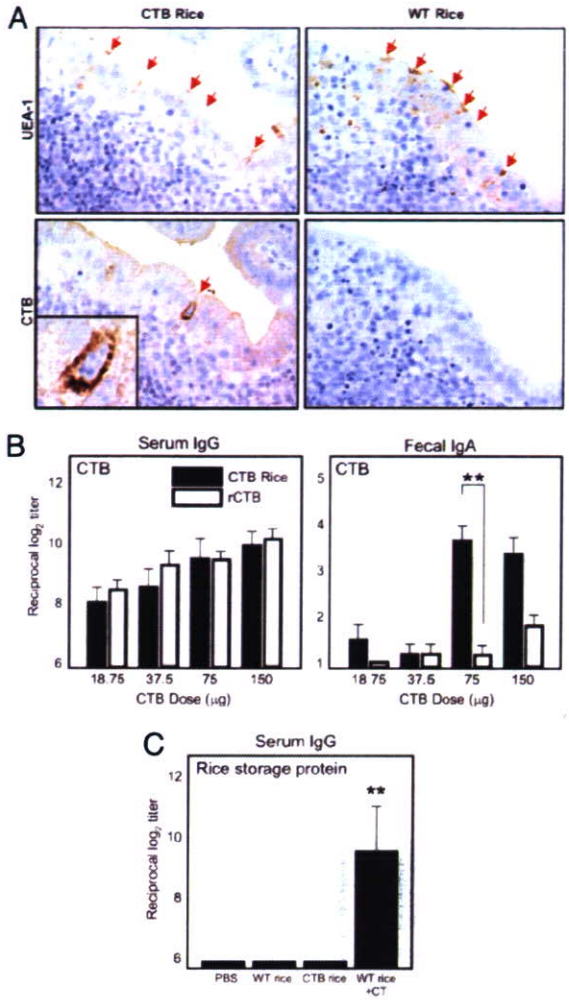

Fig. 3. Effective uptake of rice-expressed CTB by M cells for the induction of antigen-specific immune responses. (*A*) Rice-expressed CTB was administered into an intestinal loop containing PPs. Thirty minutes after the inoculation, the brisk CTBs were taken up by UEA-1-positive M cells (arrow), but not columnar epithelial cells. (*B*) When mice were orally immunized with rice-expressed CTB, purified rCTB, or nontransgenic rice dissolved in water or water alone as controls, equal levels of CTB-specific serum IgG responses were induced in mice immunized with rice-expressed CTB or purified rCTB, but not in mice receiving WT rice or water alone. In contrast, CTB-specific fecal IgA responses were also induced in mice immunized with a small amount (50 mg of seed powder containing 75 μg of CTB) of rice-expressed CTB, but not with an identical dose of purified rCTB. **, $P < 0.01$, CTB rice vs. rCTB. (*C*) Rice-expressed CTB did not induce rice storage protein-specific serum IgG responses. **, $P < 0.01$, WT rice plus CT vs. CTB rice.

of the CTB expressed in transgenic rice seed can be protected from the harsh conditions of the gastrointestinal tract. To characterize the mucosal immunogenicity of the rice-based oral vaccine in more detail, we opted to use the Kitaake CTB system for the remainder of the study.

Rice-Expressed CTB Is Effectively Taken Up by Antigen-Sampling M Cells for the Induction of Antigen-Specific Immune Responses. To confirm the M cell uptake of rice-expressed CTB, a suspension of rice-expressed CTB or nontransgenic WT rice was administered into the ligated small intestinal loops, including the PPs of naive mice. Histological analysis with *Ulex europaeus* agglutinin (UEA-1), which is a well known marker of murine M cells (31), demonstrated a strong presence of CTB antigen in UEA-1$^+$ M cells (Fig. 3*A*). We

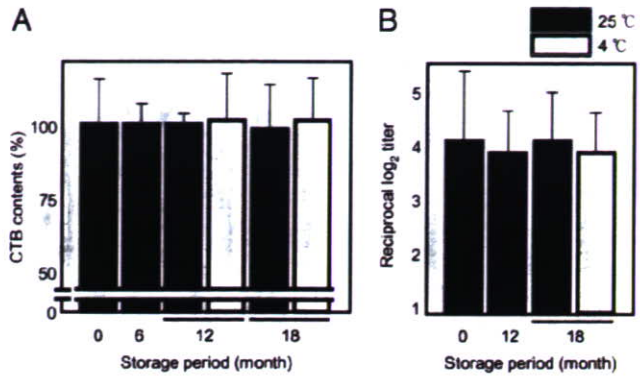

Fig. 4. Temperature stability of rice-expressed CTB. (*A*) One thousand rice seeds expressing CTB were preserved in a 500-ml sealed bottle for >1.5 years at 4°C as well as at RT (25°C). The content of CTB in preserved rice was not changed compared to that in freshly harvested rice (29 ± 4 μg per seed). (*B*) Mice were orally immunized with preserved rice-expressed CTB (50 mg of seed powder containing 75 μg of CTB) as described in Fig. 3. The preserved rice induced CTB-specific mucosal IgA responses that were comparable to those observed for freshly harvested rice.

next orally immunized mice with the seed powder of rice-expressed CTB or purified rCTB. Rice-expressed CTB induced CTB-specific serum IgG and fecal IgA antibodies (Fig. 3*B*). CTB-specific fecal IgA responses were also induced in mice immunized with a low dose of rice-expressed CTB (e.g., 50 mg of rice powder containing 75 μg CTB), whereas the same dose of purified rCTB induced no or very low levels of antigen-specific IgA responses (Fig. 3*B*). Furthermore, it should be emphasized that rice-expressed CTB induced no rice storage protein-specific immune responses (Fig. 3*C*). These findings demonstrated that the rice-based mucosal vaccine is an effective delivery vehicle for the induction of antigen-specific mucosal IgA responses.

Rice-Based Mucosal Vaccine Maintained Immunogenicity for More than 1.5 Years at RT. Inasmuch as our results provide supportive evidence for the protective advantage of rice-based mucosal vaccine, which includes stability in the harsh condition of the gastrointestinal tract (Fig. 2*B*), it was logical to examine whether the rice-based vaccine preserved at RT (25°C) for an extended period maintained its stability and mucosal immunogenicity. To this end, rice-based mucosal vaccine was preserved for 0.5, 1.0, or 1.5 years at either RT (25°C) or 4°C. Densitometry analysis revealed that the antigen in rice seed remained stable at RT for >1.5 years (Fig. 4*A*). Furthermore, the rice preserved at RT for 1.5 years induced the same level of CTB-specific fecal IgA responses as freshly harvested rice (Fig. 4*B*). These data suggest that the rice-based mucosal vaccine is more stable than the purified antigen of the subunit vaccine, as well as more effective for induction of IgA-committed mucosal immune responses.

Rice-Expressed CTB Induces Protective Immunity Against Cholera Toxin (CT). Finally, to examine the biological activities of antibodies induced by oral administration of rice-expressed CTB, CT-neutralizing activities were investigated by using a GM1-binding inhibition assay with GM1-ELISA (17) and an elongation assay with Chinese hamster ovary (CHO) cells (17, 32). When serum samples from mice orally immunized with rice-expressed CTB or WT rice were subjected to GM1-ELISA, the binding of CT to the coated GM1 ganglioside was blocked in the former but not the latter group of samples (Fig. 5*A*). The elongation assay also revealed no morphological changes in CHO cells cocultured with CT that had been pretreated with serum from mice orally vaccinated with rice-expressed CTB. In contrast, CT pretreated with

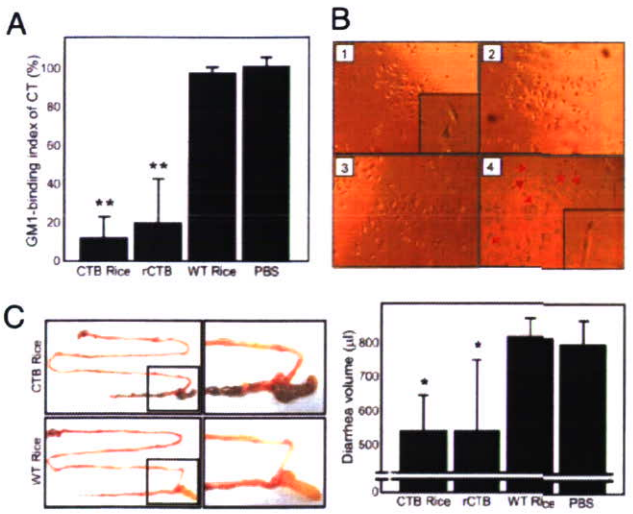

Fig. 5. Induction of protective immunity against CT by rice-expressed CTB. (*A*) The neutralizing index calculated with OD_{450} obtained by GM1-ELISA. The serum of mice immunized with rice-expressed CTB or purified rCTB, but not with nontransgenic rice or PBS, completely blocked the binding of CT to coated GM1-ganglioside. **, $P < 0.01$, CTB rice or rCTB vs. PBS. (*B*) The elongation assay with CHO cells revealed a morphology similar to normal cells (1) when the cells were stimulated with CT pretreated with serum from mice immunized with rice-expressed CTB (2) or purified rCTB (3), but a marked elongation when stimulated by CT pretreated with the serum from mice immunized with nontransgenic rice, PBS, or nontreated CT (4) as shown by the arrows. (*C*) In contrast to mice receiving WT rice or PBS, mice orally vaccinated with rice-expressed CTB showed no symptoms of diarrhea and a low level of intestinal water. *, $P < 0.05$, CTB rice or rCTB vs. PBS.

serum of mice immunized with WT rice showed a massive elongation of CHO cells (Fig. 5*B*) similar to that induced by the native form of CT. Most important, when orally challenged with CT, the mice vaccinated with rice-expressed CTB showed no clinical sign of diarrhea (Fig. 5*C*), whereas those fed the WT rice or PBS developed severe diarrhea. However, some mice immunized with purified rCTB suffered from diarrhea (Fig. 5*C*). Consistent with these findings, the volume of intestinal water in mice immunized with rice-expressed CTB was significantly lower after challenge with CT than in mice receiving WT rice or PBS (Fig. 5*C*). These data directly demonstrate that oral vaccination with rice-expressed CTB could offer a high degree of protection against CT challenge.

Discussion

In this study, we have developed a physically and chemically stable and immunologically effective vaccine antigen-expressing transgenic rice seed that can withstand the harsh environment of the gastrointestinal tract and induce protective immunity against mucosal infections. The use of transgenic rice for vaccine production offers several benefits over other plants for vaccine production. For the implementation of global vaccination strategy, a well designed oral vaccine system should satisfy the following criteria: (*i*) produce sufficient quantities of inserted antigen for the immunization (33), (*ii*) preserve the expressed antigen for a long time at RT (9, 34), (*iii*) induce protective immunity (8, 34), (*iv*) protect from enzymatic digestion in gastrointestinal tract (8), and (*v*) effectively deliver the inserted antigen to mucosal inductive tissues, including antigen-sampling M cells (8, 35). Although several plants have currently been used for the creation of an "edible vaccine," seed crops such as soybean, maize, wheat, or rice seem to be the most suitable plants for fulfillment of the previous requirements. It was recently shown that a soybean-based oral vaccine expressing heat-labile toxin B subunit (LTB) of *E. coli* induced antigen-specific IgG and IgA responses (36). Although maize also has been used for the expression of LTB (20), a biological nature of long-distance pollen scattering is the major environmental concern (37). Further, the difficulty of transforming the inserted gene by use of the wheat vector system unfortunately disqualified its suitability for the oral vaccine development. In contrast, rice self-fertilizes, and thus its pollen is considered to fry within only 10 m (37). In addition, rice plants have unique features in the storage of protein using two systems of PB-I and PB-II (29), which are suitable for accumulation of vaccine antigen. Furthermore, rice is the only crop that full of genome sequences was elucidated, and thus it easily applied the genetic information for the creation of gene-manipulated product (38). It is expected that this 430-Mb genome information contributes to the development of useful transgenic rice (38).

To show the unique features and feasibility of rice-based mucosal vaccine, we purposely used CTB as a vaccine antigen because CTB has been immunologically well characterized and extensively used for the analysis of antigen-specific immune response in both mucosal and systemic compartments. One of the major limitations of plant-based vaccines is the achievement of a high expression of inserted vaccine antigen that is sufficient to induce protective immunity (33). To achieve high expression and accumulation of inserted vaccine antigen in rice seed, an endosperm-specific expression promoter gene, 2.3-kb *GluB-1*, followed by an endoplasmic reticulum retention signal peptide, KDEL, was used for the expression of CTB (Fig. 1*A*). By optimizing the codon usage of CTB for expression in rice, the accumulation level of CTB was achieved at ≈2.1% of total seed protein (0.15% of seed weight) (Fig. 1 *E* and *F*). Although CTB has been expressed in the potato, the level of expression was ≈0.3% of total protein (0.002% of fresh weight) in potato tubers (17); ≈4% of total leave protein (0.5% of leave weight) was achieved in tobacco leaves by using a chloroplast expression system (35). However, the tobacco leaf is not applicable in the practical sense for oral vaccination. Thus, the use of the rice transgenic system allowed the efficient expression of inserted vaccine antigen, although we cannot directly compare the level of the inserted antigen expression to other previously published plant-based vaccine systems.

The SDS/PAGE and Western blot analyses with anti-CTB polyclonal antibody showed two protein species with 12 kDa, which was almost the same as that of authentic CTB (39), and 15 kDa were detected (Fig. 1 *E* and *F*), suggesting that part of CTB expressed in rice seed might contain a full or partial *GluB-1* signal peptide at the N terminus. The SDS/PAGE under nonreducing conditions and subsequent Western blot analyses showed that the molecular mass of two protein species was shifted to 55–65 kDa (Fig. 1*F*). Because these two protein species were recognized by anti-CTB polyclonal antibody and possessed a molecular weight comparable to a pentameric structure under natural condition, most rice-expressed CTBs were considered to be a functionally native pentameric form for the induction of an antigen-specific immune response.

The tolerableness of inserted vaccine antigen in rice seed against the harsh digestive tract environment was also attributed by the site of protein accumulation in the rice seed. In general, rice starchy endosperm cells contain two types of protein storage organelles (PB-I and PB-II) with a different shape, density, and protein composition (29). PB-I and PB-II mainly contain prolamins (e.g., 13k prolamin) and glutelins (e.g., glutelin B1) as storage proteins, which are defined as alcohol- and water-soluble proteins, respectively (29, 30). Thus, glutelins (PB-II) are considered to be more digestible and sensitive than prolamins (PB-I) in the gastrointestinal tract. The immunoelectron microscopic analysis showed that CTB accumulation occurred in PB-I and PB-II of the endosperm cells of the rice seed (Fig. 2*A*). Thus, the direction of inserted protein expression (e.g., CTB) in PB-I seems to be responsible for the tract of resistance against digestive enzyme activity, and thus allows for

the effectiveness in the induction of antigen-specific immune response by minimum dose of oral antigen. To support this view, an *in vitro* pepsin digestion study showed that most of prolamin and ≈75% of the CTB accumulated in the rice seed were protected from the pepsin treatment, whereas most of the glutelin in the rice seed and all purified rCTB were digested (Fig. 2*B*). In contrast, LTB expressed in maize kernel seems to be less resistant to peptic degradation when compared to CTB in the rice seed because LTB protein was accumulated mainly in the starch granules of transgenic maize kernels (40). These finding indicated that accumulation of vaccine antigen in PB-I would provide physicochemical stability against digestive enzymatic effects. Taken together, the rice-based vaccine was stable and more effective than the purified subunit vaccine, as well as other plant-based vaccines, in the harsh environment of the gastrointestinal tract for the induction of protective immunity.

As described above, in the gastrointestinal tract, the antigens ingested from the luminal site are taken up by PPs via antigen-sampling cells known as M cells for initiation of antigen-specific T helper cells and IgA-committed B cells (41). Targeting vaccine antigen delivery to M cells should be a goal in mucosal vaccine development. Intestinal loop assay with rice-expressed CTB demonstrated that CTB were taken up by M cells (Fig. 3*A*). According to many biodegradable microsphere studies, the <10-μm microspheres have been shown to be efficient delivery vehicles into antigen-sampling M cells in PPs (42). The diameters of rice PB-I and PB-II range in size between 1 to 2 μm and 3 to 4 μm, respectively (43). In addition, PB-I, but not PB-II, accumulated most of its CTB at the surface, perhaps further enhancing antigen uptake by M cells. Taken together, these results demonstrated that rice-expressed CTBs were not only effectively taken up by M cells located in the follicle-associated epithelium of PPs, but also could serve as effective carriers of mucosal vaccines to intestinal inductive tissues such as the PPs.

Oral immunization with rice-expressed CTB induced CTB-specific serum IgG and mucosal IgA responses (Fig. 3*B*). A low dose of rice-expressed CTB (e.g., 50 mg of rice powder containing 75 μg CTB) sufficiently induced CTB-specific mucosal IgA responses, whereas the same contents of purified rCTB induced no or low levels of antigen-specific IgA responses (Fig. 3*B*). The differences in required dosage may be because of the physicochemical stability exhibited by rice-based mucosal vaccines and their ability to withstand digestive effects. Although oral immunization with rice-expressed CTB can induce CTB-specific immune responses, it did not induce any rice storage protein-specific immune responses (Fig. 3*C*), suggesting that rice-expressed CTB did not show adjuvant activity for rice protein. Furthermore, Southern blot analysis confirmed the genetic stability of the CTB-transgenic rice (data not shown); CTB expressed in the rice seed could be preserved for >1.5 years not only at 4°C, but also at RT, without any degradation (Fig. 4*A*), and the rice preserved for 1.5 years at RT induced an equal level of CTB-specific fecal IgA responses as freshly harvested rice (Fig. 4*B*). Our results provided further evidence for a significant potential benefit of rice-based mucosal vaccine for the development of stable vaccine with immunogenicity. Thus, a rice-based mucosal vaccine can be introduced as a first cold-chain-free vaccine.

Finally, we showed the protective effect induced by oral immunization with a rice-based mucosal vaccine. CT binds its receptor GM1-ganglioside, which is ubiquitously expressed in intestinal epithelium, and causes severe diarrhea (39). Our results demonstrated that the serum from mice immunized with rice-expressed CTB completely blocked the binding of CT to GM1-ganglioside (Fig. 5*A*) and also inhibited the elongation of CHO cells caused by CT (Fig. 5*B*). Most important, mice immunized with rice-expressed CTB showed no clinical sign of diarrhea after orally challenged with CT, whereas some mice immunized with purified rCTB suffered from diarrhea (Fig. 5*C*) perhaps because the level of antigen-specific mucosal IgA was lower in the purified rCTB-fed group than in the group receiving rice-expressed CTB. Therefore, we conclude that the rice-based mucosal vaccine would be effective for the induction of protective immunity compared to other types of mucosal vaccine.

In summary, we have developed a rice-based oral vaccine that offers significant advantages over currently available vaccines. In the rice-based vaccine, the vaccine antigen, CTB, accumulated in the PBs of starchy endosperm cells, from which they were taken up by M cells for the induction of antigen-specific mucosal immune responses with neutralizing activity. In addition, the rice-based CTB vaccine remained stable and maintained immunogenicity at RT for >1.5 years and was protected from pepsin digestion *in vitro*. Taken together, these findings suggest that a rice-based oral vaccine would be a most effective and highly practical vaccine regimen against infectious diseases, whether naturally occurring or stemming from acts of bioterrorism. Given its cost effectiveness and ease of administration, it would be a vaccine whose benefits could be fully enjoyed in developing countries, where the need is often the greatest.

Materials and Methods

DNA Constructions and Transformation of Rice Plants. The CTB gene of *V. cholerae* was modified to a suitable form for rice seed and inserted into a binary vector (pGPTV-35S-HPT) (23). The resulting plasmid (Fig. 1*A*) was transformed in two lines of rice plants, *Oryza sativa* L. cv Kitaake (24) and Hosetsu (25, 26), using an *Agrobacterium*-mediated method described previously (27).

DNA and RNA Analyses. Using the cetyltrimethylammonium bromide extraction method, we extracted genomic DNA from the leaf tissues of transgenic rice and analyzed the integration of the CTB gene in genomic DNA using PCR (23). The expression of mRNA encoding CTB in the rice seed was analyzed by Northern blot as previously described (23). Briefly, total RNA (30 μg) extracted from the developing seeds of rice plants using the phenol/chloroform extraction method was separated on a 1.0% (wt/vol) formaldehyde/agarose gel and transferred to Hybond N^+ membranes (GE Healthcare, Piscataway, NJ). The amplified CTB gene was used as a hybridization probe after labeling with [α-^{32}P] dCTP (GE Healthcare).

Protein Analyses. Total seed protein was extracted from seeds as described previously (23). Briefly, seeds of rice plants were ground to a fine powder using a Multibeads shocker (Yasui Kikai, Osaka, Japan) and extracted in 2% (wt/vol) SDS, 8 M urea, 5% (wt/vol) β-mercaptoethanol, 50 mM Tris·HCl (pH 6.8), and 20% (wt/vol) glycerol before being separated by SDS/PAGE with a 15% to 25% gradient polyacrylamide gel (Daiichi Pure Chemical, Tokyo, Japan). The gel was subsequently transferred to Hybond-P PVDF membranes (GE Healthcare) for Western blot analysis with 5 μg/ml rabbit anti-CTB antibody prepared in our laboratory. Accumulation levels of CTB were determined by densitometry analysis of Western blot against a standard curve generated with the use of rCTB expressed in *Bacillus brevis* and purified by using immobilized galactose (Pierce, Rockford, IL) in our laboratory (44). Using immunoelectron microscopic analysis, the distribution of CTB expressed in rice seed was analyzed. Briefly, rice seeds (at 12–15 days after flowering) were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) and 0.1% glutaraldehyde (Nacalai Tesque, Kyoto, Japan) and embedded in LR White (London Resin, Hampshire, U.K.). Ultrathin sections (150 nm) were stained with 5 μg/ml rabbit anti-CTB antibody, followed by gold particle (20 nm)-conjugated goat anti-rabbit IgG (E.Y. Labs, San Mateo, CA) diluted to 1:10. The sections were then stained with 4% uranyl acetate and examined by using a transmission electron microscope (JEM100S; JEOL, Tokyo, Japan).

Pepsin Treatment. Seed powder (10 mg containing 15 μg of CTB) and purified rCTB (15 μg) were incubated with 0.5 mg/ml pepsin (2,500–3,500 units per mg protein; Sigma–Aldrich, St Louis, MO) in 0.1 ml of 0.1 M sodium acetate buffer (pH 1.7) with gentle rocking for 1 h at 37°C. After neutralization, the degradation of CTB, glutelin, or prolamin was analyzed by Western blot with 5 μg/ml rabbit anti-CTB, anti-glutelin GluB-1, or anti-13k prolamin antibodies prepared in our laboratory, respectively.

Uptake of CTB by M cells. A rice-expressed CTB or a nontransgenic rice was administered into an intestinal loop ($\approx$1 cm) containing PPs of mice anesthetized by using 2 mg of ketamine (Sigma–Aldrich) per mouse. Thirty minutes after inoculation, the tissues were removed and fixed in tissue fixative (Genostaff, Tokyo, Japan) overnight at 4°C and then embedded in paraffin. Several mirror sections (5 μm) were subjected to immunohistochemistry with 5 μg/ml anti-CTB antibody and biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and to lectin histochemistry with 20 μg/ml biotinylated UEA-1 (Vector Laboratories). Both sections were finally incubated with SAB-PO (Nichirei, Tokyo, Japan) and visualized for the distribution of CTB and the localization of M cells by 3,3′-diaminobenzidine tetrahydrochloride (Dojin Laboratories, Kumamoto, Japan).

Oral Immunization. One immunization study was carried out by using 6-week-old C57BL/6J and BALB/c mice (CLEA, Tokyo, Japan). On six occasions at 2-week intervals, mice (six mice per group) were orally immunized with 12.5, 25, 50, or 100 mg of CTB-transgenic rice, with a corresponding dose of purified rCTB (18.75, 37.5, 75, or 150 μg) or with either 100 mg of nontransgenic rice dissolved in water or water alone as controls. For examination of the adjuvant effect of rice-expressed CTB, 100 mg of CTB-transgenic or WT rice was orally immunized with or without 10 μg of CT (List Biological Laboratories, Campbell, CA). One week after the final immunization, serum and fecal extracts were collected, and CTB or rice storage protein-specific Ig responses were measured by ELISA with 5 μg/ml rCTB or 20 μg/ml rice-storage protein extracted with 0.01% Triton X-100 as described previously (45).

Neutralizing Assay. Serial-diluted serum collected from immunized mice were treated with 50 ng/ml of CT and subjected to GM1-ELISA as previously described with some modifications (17). Briefly, 5 μg/ml of monosialoganglioside GM1 (Sigma–Aldrich)-coated 96-well plates (Thermo, Milford, MA) were incubated with CT that had been treated first with serum from immunized mice and then with an HRP-conjugated anti-CTB antibody prepared in our laboratory. The color was developed with the addition of TMB substrate (Moss, Pasadena, MD), and absorbance was measured at 450 nm. In addition, a CHO cell (ATCC, CCL-61) assay (32) was performed by using serum treated with 50 ng/ml CT. After 14 h of stimulation in 5% CO_2 in a humidified incubator at 37°C, morphological changes were observed under a microscope. In addition, we performed an *in vivo* challenge experiment with CT. The vaccinated mice were orally challenged with 20 μg of CT. After 14 h, clinical symptoms of diarrhea were observed, and the volume of intestinal water was measured.

Data Analysis. Data are expressed as the mean $\pm$ SD. All analyses for statistically significant differences were performed with Tukey's t test, with P values of <0.01 and <0.05 considered significant.

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Role of Peyer's patches in the induction of *Helicobacter pylori*-induced gastritis

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***Helicobacter pylori* is a Gram-negative spiral bacterium that causes gastritis and peptic ulcer and has been implicated in the pathogenesis of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. Although Th1 immunity is involved in gastritis and the accumulation of *H. pylori*-specific CD4$^+$ T cells in the *H. pylori*-infected gastric mucosa in human patients, how T cells are primed with *H. pylori* antigens is unknown because no apparent lymphoid tissues are present in the stomach. We demonstrate here that Peyer's patches (PPs) in the small intestine play critical roles in *H. pylori*-induced gastritis; no gastritis is induced in *H. pylori*-infected mice lacking PPs. We also observed that the coccoid form of *H. pylori* is phagocytosed by dendritic cells in PPs. We propose that *H. pylori* converts to the coccoid form in the anaerobic small intestine and stimulates the host immune system through PPs.**

CD4 T cells | coccoid form | dendritic cells | gastric epithelial cells | inflammation

Helicobacter pylori is a Gram-negative microaerophilic bacterium that infects human gastric epithelial cell (gEC) surfaces and the overlying gastric mucin. More than 50% of the world's population is infected by *H. pylori*, although most patients have no remarkable symptoms (1). However, in some of patients, *H. pylori* infection leads to active chronic gastritis or peptic ulcer (2). In addition, *H. pylori* has also been implicated in the pathogenesis of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (3). When *H. pylori* colonizes gastric mucosa, effector molecules are injected into gastric epithelial cells or the submucosal area through the type IV secretion system (1, 4). For example, the CagA effector is phosphorylated in the target cells and activates a signaling pathway to elicit growth factor-like responses. Another effector molecule, VacA, causes the massive vacuolar degradation of epithelial cells, thus disrupting the gastric epithelial barrier. VacA also interferes with the activation and proliferation of T lymphocytes within the gastric lamina propria (gLP) (5).

It was originally proposed that effector molecules, including CagA, trigger the secretion of chemokines such as IL-8 and RANTES from gECs, which attract neutrophils and mononuclear cells into the gLP (4). However, it was later shown that *H. pylori* did not induce gastritis in lymphopenic SCID mice, although gastritis was induced after adoptive transfer of naive CD4$^+$ T cells (6). The importance of CD4$^+$ T cells was underscored by the fact that *H. pylori* is not eliminated from gastric mucosa in MHC class II-deficient mice (7).

Gastritis is more severe in Th1-prone mice than Th2-prone mice on infection with the mouse-adapted *H. pylori* strain SS1 (8). Furthermore, the accumulation of *H. pylori*-specific CD4$^+$ T cells in the *H. pylori*-infected gastric mucosa in human patients (9) suggests that CD4$^+$ T cell-mediated Th1 immune responses play a critical role in *H. pylori*-induced gastritis. However, how CD4$^+$ T cells are primed by *H. pylori* antigens in the stomach where no apparent lymphoid tissues are present and how the *H. pylori*-induced chronic inflammation is maintained by T cells is unknown.

Although *H. pylori* forms an actively dividing, spiral-shaped morphology in the stomach, it is able to convert to a nonculturable, but viable, coccoid form under unfavorable conditions such as an anaerobic environment, increased oxygen tension, and long-term culture (10, 11). The coccoid form is thought to be important for transmission to new hosts by an oral–oral or oral–feces route because this form is more resistant to environmental stresses. Although the coccoid form is not culturable *in vitro*, transcription and translation actively take place in the coccoid form (12, 13). However, it is unknown whether the coccoid form is involved in the pathogenesis of *H. pylori*-induced gastritis.

In this study, we demonstrate that *H. pylori* antigen-specific CD4$^+$ T cells are necessary and sufficient for the induction of gastritis by *H. pylori*. We also demonstrate that CD4$^+$ T cells are likely primed with *H. pylori* antigens captured in the small intestine, where the coccoid form of *H. pylori* is taken up by dendritic cells (DCs) in Peyer's patches (PPs).

Results

Adoptive Transfer of Naive CD4$^+$T Cells Induces Gastritis in *H. pylori*-Infected Rag2$^{-/-}$ Mice. The *H. pylori* SS1 strain induces more severe gastritis in Th1-prone C57BL/6 than Th2-prone BALB/c mice as demonstrated by the infiltration of neutrophils and lymphocytes into the gLP and the submucosal area (Fig. 1*a* and data not shown). In contrast, when C57BL/6-Rag2$^{-/-}$ mice lacking T and B cells were infected with *H. pylori*, no gastritis was observed (Fig. 1*b*), as previously shown with SCID mice (6). The clearance of bacteria in Rag2$^{-/-}$ mice was impaired because $>10^7$ cfu/g tissues of *H. pylori* colonized the gastric mucosa (Table 1), and the colonization of *H. pylori* was readily detected by anti-*H. pylori* antibody staining (Fig. 1*c*). However, adoptive transfer of naive splenic CD4$^+$ T cells into Rag2$^{-/-}$ mice 2 months after *H. pylori* infection induced severe gastritis, with massive infiltration of neutrophils and lymphocytes into the gLP and the submucosal area (Fig. 1*d*). This massive infiltration resulted in the exclusion of colonized *H. pylori* from gastric mucosa (Table 1).

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Abbreviations: DKO, double knockout; LP, lamina propria; gLP, gastric LP; gEC, gastric epithelial cell; DC, dendritic cell; PPs, Peyer's patches; OVA, ovalbumin; NK, natural killer; APC, antigen-presenting cell; β-Rag DKO, IL-2 receptor β chain (IL-2Rβ)$^{-/-}$ Rag2$^{-/-}$ DKO; γ_c-Rag DKO, cytokine receptor common γ chain (γ_c)$^{-/-}$ Rag2$^{-/-}$ DKO; GALT, gut associated lymphoid tissue; ILF, isolated lymphoid follicle; BMDC, bone marrow derived cell; SED, subepithelial dome; mLN, mesenteric lymph node.

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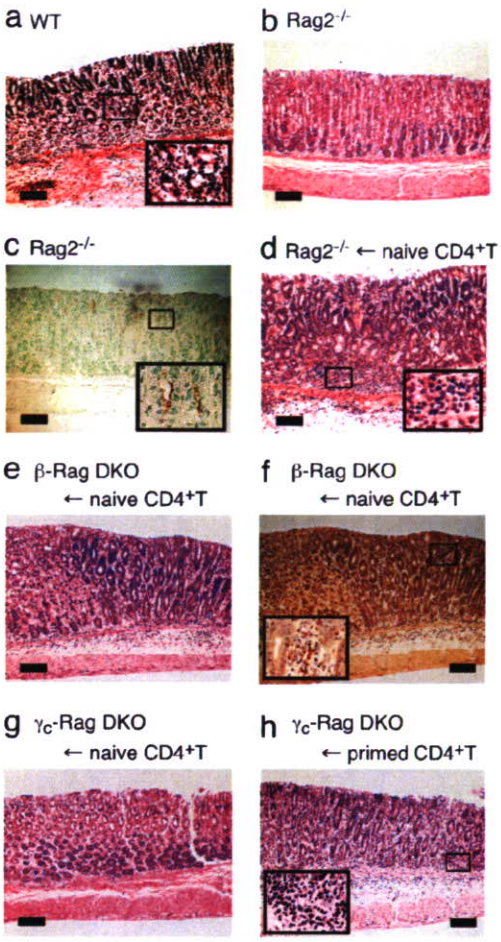

Fig. 1. Naive CD4$^+$ T cells did not induce gastritis in *H. pylori*-infected γ_c-Rag double knockout (DKO) mice. (*a–c*) Wild-type (*a*) or Rag2$^{-/-}$ (*b* and *c*) mice were infected with *H. pylori*. Two months after the infection, gastric specimens were prepared. (*d–h*) Rag2$^{-/-}$ (*d*), β-Rag DKO (*e* and *f*), and γ_c-Rag DKO (*g* and *h*) mice were infected with *H. pylori*. Two months after the infection, naive (*d–g*) or primed (*h*) splenic CD4$^+$ T cells were transferred. Two months after the cell transfer, gastric specimens were prepared. Specimens were stained with H&E (*a*, *b*, *d*, *e*, *g*, and *h*), anti-*H. pylori* antisera (brown) (*c*), or chloroacetate esterase (red) for infiltrated neutrophils and mast cells (*f*). (Scale bars: 200 μm.)

***H. pylori* Antigen-Specific CD4$^+$ T Cells Are Indispensable for Induction of Gastritis.** Primary gECs secrete MIP-2, a functional homolog of IL-8, on *H. pylori* infection *in vitro*, and the amount produced by Rag2$^{-/-}$ gECs was comparable to that produced by wild-type gECs [supporting information (SI) Fig. 5*a*]. CD4$^+$ T cells isolated from the gLP of *H. pylori*-infected mice were able to produce larger amounts of MIP-2 than splenic CD4$^+$ T cells from the same mice in response to *H. pylori* antigens (SI Fig. 5*b*). Moreover, the amounts of MIP-2 produced by gLP CD4$^+$ T cells were much larger than those produced by gECs (compare SI Fig. 5 *a* and *b*). The importance of CD4$^+$ T cells for neutrophil infiltration on *H. pylori* infection was further confirmed by the depletion of CD4$^+$ T cells from wild-type mice that had already developed gastritis by *H. pylori* infection. After depleting CD4$^+$ T cells by the i.v. injection of anti-CD4 mAb, the gastritis became milder (Table 1 and SI Fig. 6 *a* and *b*), and the number of bacteria in the gastric mucosa increased (Table 1). These results indicate the critical role of CD4$^+$ T cells for both triggering and maintaining gastritis. When CD4$^+$ T cells from OT-II transgenic mice on a Rag2$^{-/-}$ background (OT-II-Rag mice), specific for an ovalbumin ($OVA_{323-339}$ peptide on an MHC class II molecule I-A^b), were transferred into *H. pylori*-infected Rag2$^{-/-}$ mice, no gastritis was induced (SI Fig. 6*c*). Similarly, when OT-II-Rag mice were infected with *H. pylori*, no gastritis was induced despite the presence of CD4$^+$ T cells (Table 1 and SI Fig. 6*d*). Furthermore, when OVA protein or $OVA_{323-339}$ peptide was administered into *H. pylori*-infected OT-II-Rag mice, no inflammation was observed, although CD4$^+$ T cells were activated in these mice (Table 1, SI Fig. 6*e*, and data not shown). These results collectively indicate the importance of *H. pylori* antigen recognition by CD4$^+$ T cells in the induction of gastritis.

CD4$^+$ T Cells Are Not Primed with *H. pylori* Antigen in γ_c-Rag DKO Mice. IFNγ, a key cytokine for Th1 immune responses, is important for the pathogenesis of *H. pylori*-induced gastritis (14). Natural killer (NK) cells and antigen-presenting cells (APCs) including DCs are able to produce IFNγ to prevent bacterial infection (15). The interaction between DCs and NK cells enhances the production of IFNγ during *H. pylori* infection (16, 17). To test the importance of DC–NK interaction in the *H. pylori*-induced inflammatory response, we transferred splenic CD4$^+$ T cells into *H. pylori*-infected IL-2 receptor β chain (IL-2Rβ)$^{-/-}$Rag2$^{-/-}$ (β-Rag DKO) mice and cytokine receptor common γ chain (γ_c)$^{-/-}$Rag2$^{-/-}$ DKO (γ_c-Rag DKO) mice. These mice lack NK cells because of impaired IL-15 signaling, which is critical for NK cell development (18, 19). In addition, the production of IL-12 and IFNγ by APCs from these mice is impaired (20). As shown in Fig. 1 *e* and *f*, gastritis was induced in *H. pylori*-infected β-Rag DKO mice when naive CD4$^+$ T cells were transferred. Clearance of bacteria was also achieved by the naive CD4$^+$ T cell transfer (Table 1), indicating that NK cells and NK–DC interaction are dispensable for the induction of gastritis by *H. pylori* infection.

Surprisingly, there was no gastritis induced in γ_c-Rag DKO mice even after the transfer of naive CD4$^+$ T cells (Fig. 1*g*), NK cells (SI Fig. 7*a*), or NK cells with naive CD4$^+$T cells (SI Fig. 7*b*), suggesting that γ_c-Rag DKO mice have additional defects compared with β-Rag DKO mice. Interestingly, when splenic CD4$^+$ T cells isolated from *H. pylori*-infected wild-type mice were transferred, gastritis was induced in *H. pylori*-infected γ_c-Rag DKO mice (Fig. 1*h*), and the clearance of bacteria was evident (Table 1). These results suggest that CD4$^+$ T cells were not primed in γ_c-Rag DKO mice. In fact, splenocytes from these mice did not respond to DCs preincubated with *H. pylori* lysate, whereas splenocytes from wild-type mice infected with *H. pylori* strongly responded and produced IFNγ in response to the same DC preparation (data not shown). It should be noted that there were no apparent defects in DCs from γ_c-Rag DKO mice compared with those from wild-type mice with regard to their ability to induce T cell activation and present antigen as examined by the induction of CD69 expression and IFNγ production by splenic CD4$^+$ T cells (SI Fig. 8).

PPs Are a Critical Tissue for Priming CD4$^+$T Cells with *H. pylori* Antigen. One difference between β-Rag DKO and γ_c-Rag DKO mice is that the latter lack gut-associated lymphoid tissues (GALT) such as PPs and isolated lymphoid follicles (ILFs) due to impaired IL-7 signaling (21) (Fig. 2 *a–d*). Thus, we hypothesized that CD4$^+$ T cells are primed in GALT such as PPs or ILFs. To test this possibility, we generated PP-null mice by administration of anti-IL-7Rα mAb *in utero* (22) (Fig. 2 *e* and *f*). As observed in γ_c-Rag DKO mice, no gastritis was induced in PP-null mice 2 months after *H. pylori* infection, and a large number of *H. pylori* was detected in the gastric mucosa (Fig. 2 *h* and *i* and Table 1). We also generated PP-null mice on a Rag2$^{-/-}$ background (PP-null-Rag2$^{-/-}$ mice) (Fig. 2*g*). The adoptive transfer of CD4$^+$ T cells from *H. pylori*-infected wild-type mice, but not naive CD4$^+$ T cells, induced strong inflammation in PP-null-Rag2$^{-/-}$

Table 1. PP-dependent bacterial clearance in *H. pylori* infection

Mouse*	*n*	Cells transferred†	Bacterial colonization,‡ cfu/g tissue × 10^{-6}	Neutrophils, average (range)	Active inflammation, average (range)	GAIS,§ average (range)
Wild type	7	None	2.2 ± 1.3	1.6 (0–3)	1.4 (0–3)	13.6 (0–34)
Wild type	4	$CD4^+$ T cell-depleted	25 ± 7	0 (0)	1.0 (0–2)	0 (0)
$Rag2^{-/-}$	4	None	14 ± 4	0 (0)	0 (0)	0 (0)
$Rag2^{-/-}$	5	Naive $CD4^+$ T	0.15 ± 0.17	2.0 (2)	2.0 (2)	5.0 (2–8)
$Rag2^{-/-}$	5	OT-II-Rag $CD4^+$ T	34 ± 11	0 (0)	0 (0)	0 (0)
β-Rag DKO	4	Naive $CD4^+$ T	0.67 ± 0.39	1.5 (1–2)	1.3 (1–2)	9.2 (3–18)
γ_c-Rag DKO	6	Naive $CD4^+$ T	18 ± 11	0 (0)	0 (0)	0 (0)
γ_c-Rag DKO	6	Primed $CD4^+$ T	0.22 ± 0.39	0.66 (0–1)	1.5 (0–2)	1.6 (0–5)
γ_c-Rag DKO	3	Primed $CD4^+$ T from PPs	<0.01	1.0 (1)	2.0 (2)	1.0 (0–2)
γ_c-Rag DKO	3	Primed $CD4^+$ T from mLN	0.88 ± 0.78	0.5 (0–1)	1.5 (1–2)	4.0 (3–5)
γ_c-Rag DKO	3	Primed $CD4^+$ T by coccoid form	1.8 ± 1.6	0.33 (0–1)	0.66 (0–1)	2.0 (0–6)
PP-null-wild type	8	None	16 ± 9	0 (0)	0.37 (0–1)	0 (0)
PP-null-$Rag2^{-/-}$	3	Naive $CD4^+$ T	5.3 ± 4.0	1.0 (0–2)	1.0 (0–2)	4 (0–7)
PP-null-$Rag2^{-/-}$	3	Primed $CD4^+$ T	0.53 ± 0.28	2.6 (2–3)	2.6 (2–3)	21 (13–33)

*All mice were on a C57BL/6 background. Although not shown, the degrees of bacterial colonization in β-Rag DKO, γ_c-Rag DKO, and PP-null-$Rag2^{-/-}$ mice without $CD4^+$ T cell transfer were similar to those of $Rag2^{-/-}$ mice.

†Splenocytes were used for cell transfer unless otherwise stated. Five million cells were transferred except for the transfer of PP-derived cells, where 5 × 10^5 cells were used.

‡Mean ± SD.

§Gland active inflammatory score. See *Materials and Methods*.

mice just as in the γ_c-Rag DKO mice (Fig. 2 *j* and *k* and Table 1). These results strongly suggest that PPs are critical for priming $CD4^+$ T cells in *H. pylori* infection, but dispensable for the effector phase.

The Coccoid, but Not Helical, Form of *H. pylori* Is Phagocytosed by DCs in PP. Although *H. pylori* is helical in the stomach, it transforms to the coccoid form under anaerobic conditions, such as in the small intestine (23). Interestingly, the coccoid form of *H. pylori* induced higher IL-12 production from bone marrow-derived cells (BMDCs) than the helical form (SI Fig. 9). There is a possibility that *H. pylori* transforms to the coccoid form in the intestine and is then captured by DCs present in PPs to induce a Th1 response. To test this possibility, the coccoid and helical forms of *H. pylori* were inoculated into ligated small intestinal loops. As shown in Fig. 3*a*, immunofluorescence staining detected *H. pylori* in the subepithelial dome (SED) region of PPs in a time-dependent manner, and the number of bacteria in the SED region inoculated with the coccoid form was larger than that inoculated with the helical form of *H. pylori*. In addition, double immunofluorescence staining with anti-*H. pylori* antibody and anti-CD11c mAb demonstrated that the bacteria were captured by $CD11c^+$ DCs in the SED region (Fig. 3*b*). Although the helical form of *H. pylori* kept the rod shape 1.5 h after inoculation (SI Fig. 10 *a* and *b*), the bacteria phagocytosed by $CD11c^+$ DCs in PPs were round (SI Fig. 10 *c* and *d*). These results suggest that the coccoid, but not the helical, form of *H. pylori* is captured by DCs in PPs and activates immune responses by generating *H. pylori*-specific pathogenic $CD4^+$ T cells. Consistent with this observation, $CD4^+$ T cells from the PPs as well as mesenteric lymph node (mLN) of *H. pylori*-infected wild-type mice were also able to eliminate the bacteria in γ_c-Rag DKO mice infected with *H. pylori* (Table 1).

When wild-type mice were infected with the coccoid form of *H. pylori*, gastritis was not induced because the coccoid form of *H. pylori* was unable to colonize in the stomach (Fig. 4*a* and data not shown). However, $CD4^+$ T cells from these mice induced gastritis in γ_c-Rag DKO mice infected with the helical form of *H. pylori* (Fig. 4 *b* and *c* and Table 1). These results indicate that $CD4^+$ T cells primed with the coccoid form of *H. pylori* in the intestine are sufficient to induce inflammation in the stomach infected with the helical form of *H. pylori*.

Discussion

We showed here that *H. pylori* antigen-specific $CD4^+$ T cells are required to induce and maintain gastritis on infection with *H. pylori*. Because *H. pylori* interacts with and injects pathological molecules into gECs, it is generally thought that neutrophils infiltrating the gLP are attracted by chemokines produced by gECs. However, neutrophil infiltration was not observed in $Rag2^{-/-}$ mice, although gECs of $Rag2^{-/-}$ mice were able to secret MIP-2 on *H. pylori* infection. Thus, the secretion of chemokines by gECs seems insufficient for the induction of gastritis. In addition, adoptive transfer of $CD4^+$ T cells recognizing *H. pylori*-independent antigens did not induce gastritis, suggesting that *H. pylori*-specific $CD4^+$ T cells directly or indirectly regulate production of chemokines that attract neutrophils. In fact, a large amount of MIP-2 was produced by activated $CD4^+$ T cells derived from the gLP of *H. pylori*-infected mice. In addition, infiltrated neutrophils were located around $CD4^+$ T cells in the gLP of *H. pylori*-infected mice (data not shown). It is known that another keratinocyte-derived chemokine is able to recruit neutrophils. However, the amounts of keratinocyte-derived chemokine produced by both gECs and CD4 T cells were much lower than those of MIP-2 (data not shown).

Oral or intra-PP immunization with *H. pylori* antigens was effective in enhancing *H. pylori*-specific $CD4^+$ T cell responses and reducing *H. pylori* colonization in the stomach (24, 25). These reports are consistent with our current observation that PPs play critical roles in priming $CD4^+$ T cells, and *H. pylori* is indeed captured by DCs in PPs. *H. pylori* antigen-specific $CD4^+$ T cells would be primed by DCs in PPs or mLNs where DCs migrate after capturing antigens. Interestingly, $CD4^+$ T cells cannot be primed by DCs in the gLP or gEC, both of which are capable of expressing class II MHC and presenting antigens. The lack of antigen presentation is partly due to the fact that the helical form of *H. pylori* is resistant to phagocytosis in a type IV secretion system-dependent manner, although the molecular mechanisms of antiphagocytic activity remain to be determined (26). It should be noted that the transformation of the helical to the coccoid form is accompanied by changes in the composition

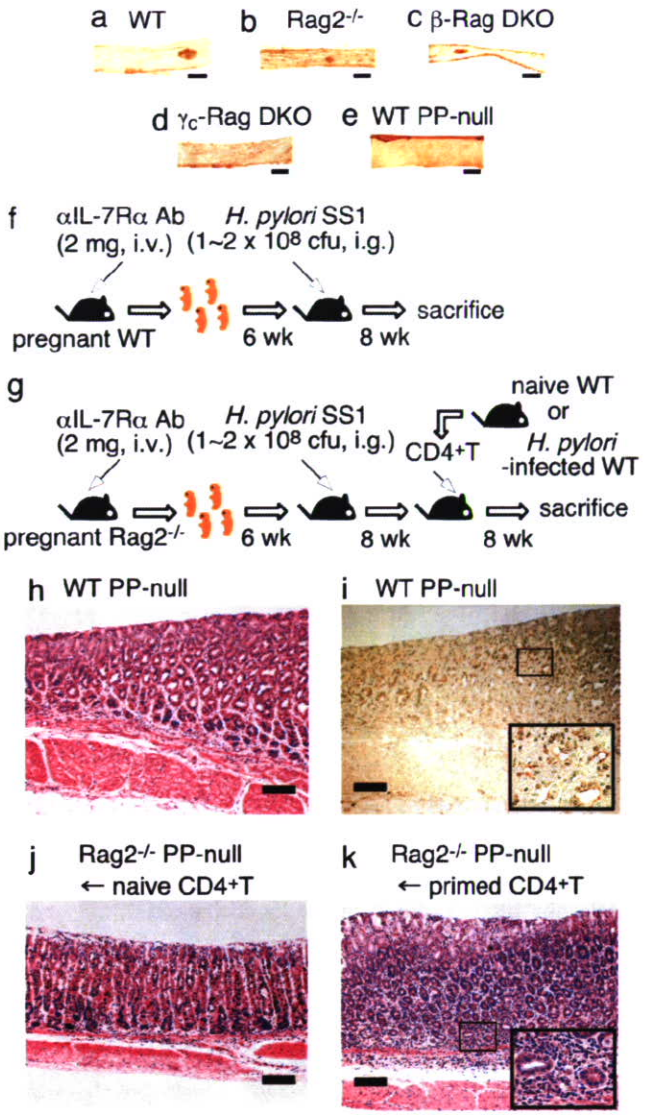

Fig. 2. PPs are critical for the priming of CD4$^+$ T cells in *H. pylori* infection. (*a–e*) Small intestines from wild-type (WT) (*a*) and PP-null (*e*) mice were stained with anti-B220 mAb, and small intestines from Rag2$^{-/-}$ (*b*), β-Rag DKO (*c*), and γc-Rag DKO (*d*) mice were stained with anti-CD45 mAb. (*f* and *g*) Schemes of the generation of PP-null WT (*f*) or PP-null-Rag2$^{-/-}$ (*g*) mice. (*h* and *i*) PP-null WT mice were infected with *H. pylori*. Two months after the infection, gastric specimens were prepared. (*j* and *k*) PP-null-Rag2$^{-/-}$ mice were infected with *H. pylori*. Two months after the infection, naive (*j*) or primed (*k*) splenic CD4$^+$ T cells were transferred. Two months after the cell transfer, gastric specimens were prepared. Specimens were stained with H&E (*h*, *j*, and *k*) or anti-*H. pylori* antisera (brown) (*i*). (Scale bars: 200 μm.)

of surface proteins and/or carbohydrates, which may make the bacteria susceptible to phagocytosis (27, 28), a subject worthy of further studies. It has been shown that mast cells are able to present *H. pylori* antigens to *H. pylori*-specific CD4$^+$ T cells, which in turn activate mast cells to degranulate (29). When we infected W/W^v and Sl/Sl^d mice lacking mast cells with *H. pylori*, gastritis was readily induced in both strains of mice on infection (S.N., T.Y., Y.B., and S.K., unpublished data), indicating that mast cells are not essential in priming CD4$^+$ T cells. In an *in vitro* experiment, BMDCs infected with the coccoid form of *H. pylori* produced larger amounts of IL-12 than those infected with the helical form of *H. pylori*, suggesting that the coccoid form of

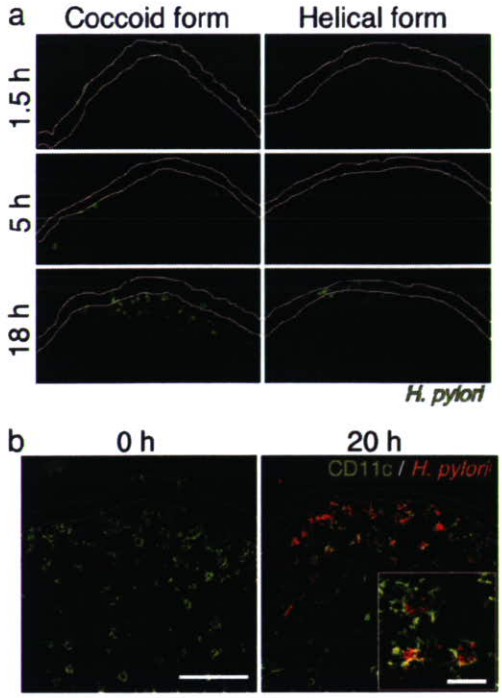

Fig. 3. The coccoid form of *H. pylori* is captured by DCs in PPs. The coccoid or helical form of *H. pylori* was inoculated into the ligated small intestines of wild-type mice. (*a*) After the indicated incubation times, PPs were stained with anti-*H. pylori* antibody. (*b*) Twenty hours after inoculation of the coccoid form, PPs were stained with anti-CD11c mAb (green) and anti-*H. pylori* antibody (red). (Scale bars: 0 h, 100 μm; 20 h, 20 μm.)

H. pylori easily induces Th1 immune responses on *H. pylori* infection.

Importantly, CD4$^+$ T cells primed with the coccoid form of *H. pylori* were able to induce gastritis in *H. pylori*-infected GALT-null γc-Rag DKO mice where CD4$^+$ T cells are not

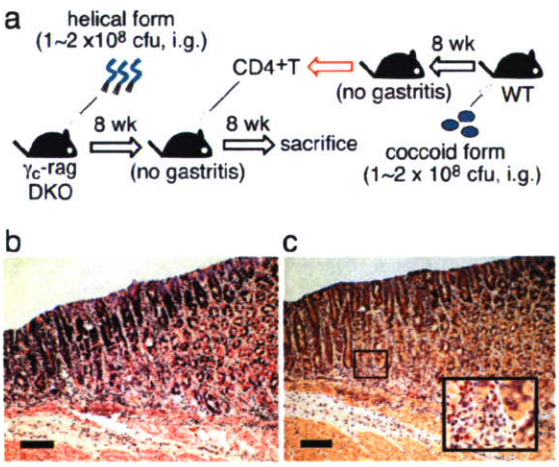

Fig. 4. Gastritis is induced by CD4$^+$ T cells primed by the coccoid form of *H. pylori*. Two months after infection of γc-Rag DKO mice with the helical form of *H. pylori*, splenic CD4$^+$ T cells from wild-type (WT) mice orally infected by the coccoid form of *H. pylori* were transferred to the infected γc-Rag DKO mice (*a*). Two months after the cell transfer, gastric specimens were prepared. Specimens were stained with H&E (*b*) or chloroacetate esterase (red) for infiltrated neutrophils and mast cells (*c*). (Scale bars: 200 μm.)

primed with *H. pylori* antigen at all. Thus, the following scenario emerges from our results: *H. pylori* transforms to the coccoid form when entering the intestinal tract and is captured by DCs in PPs. *H. pylori* antigens presented by DCs are recognized by $CD4^+$ T cells in PPs or mLN, and activated T cells migrate to the gastric mucosa to induce and maintain inflammatory responses. We noted that PP-null-$Rag2^{-/-}$ mice exhibited modest inflammation in the stomach after naive $CD4^+$ T cell transfer, compared with γ_c-Rag DKO mice, which showed no sign of inflammation. Because treatment by anti-IL-7Rα mAb *in utero* suppresses PP development, but leaves ILFs and recently discovered villous M cells (30) intact, ILFs and/or villous M cells may also participate in the capture of *H. pylori* in the intestine. Although luminal antigens can be taken up by M cells located over the follicular epithelia of ILF, the tissue is predominantly occupied by B cells (31). Furthermore, in a separate study, we have shown that no apparent organized lymphoid structure is developed under villous M cells (data not shown).

H. pylori is also implicated in the cause of other diseases such as idiopathic thrombocytopenic purpura (32) and Sjögren syndrome (33). Indeed, it has been shown that T cells migrate from the intestine to the salivary gland in Sjögren syndrome patients (34). It will be of interest to examine the functional relationship between these diseases and the coccoid form of *H. pylori* captured via PPs.

Materials and Methods

Mice. All mice used in this study were on a C57BL/6 background and were maintained at Taconic (Germantown, NY) or in our animal facility under specific pathogen-free conditions. Wild-type C57BL/6 mice were purchased from Sankyo Labo Service (Shizuoka, Japan) and CLEA Japan (Tokyo, Japan). $Rag2^{-/-}$ mice, γ_c-Rag DKO mice, and OT-II-Rag mice were obtained from Taconic. IL-$2R\beta^{-/-}$ mice (35) were generously provided by T. W. Mak (Ontario Cancer Institute, Toronto, ON, Canada). β-Rag DKO mice were obtained by crossing IL-$2R\beta^{-/-}$ with $Rag2^{-/-}$ mice (20). All experiments were approved by the Animal Care and Use Committee of the Keio University School of Medicine and were performed in accordance with institutional guidelines.

Antibodies. Fluorescein-conjugated antibodies for flow-cytometric analysis and biotin anti-mouse $CD2_c$ (HL3) were purchased from BD Bioscience (San Jose, NJ). Anti-*H. pylori* antibodies were purchased from Biomeda (Foster City, CA) or DAKO (Glostrup, Denmark).

Bacteria. *H. pylori* strain SS1, a mouse-adapted human isolate, was used for all experiments. To prepare the helical form of *H. pylori*, SS1 was grown on 5% sheep blood agar plates for 2 days. Before inoculating into mice, bacteria were grown in *Brucella* broth with 5% FCS overnight at 37°C under microaerobic conditions with gentle agitation. To prepare the coccoid form, SS1 was grown on 5% sheep blood agar plates under microaerobic conditions for 3 days at 37°C and then cultured under anaerobic conditions for 7 days at 37°C.

***In Vivo* Infection of Mice.** Bacteria were prepared from logarithmic phase cultures. Mice were intragastrically infected with $1–2 \times 10^8$ cfu *H. pylori* in 0.15 ml of broth. After the indicated time period, mice were killed and the stomach was aseptically removed. The stomach was then bisected along the greater and lesser curvatures. Half of the stomach was homogenized for the determination of bacterial colonization by a plate-dilution method. The rest of the stomach was sectioned transversely into two strips for frozen and paraffin-embedded sections.

***In Situ* Infection of Intestinal Loop.** Wild-type mice (6-week-old females) were anesthetized by an i.m. injection of 2 mg of ketamine hydrochloride (Sankyo, Tokyo, Japan) and 0.1 mg of Xylazine per mouse. An ≈4-cm-long piece of the small intestine containing one or two PPs was ligated at both ends with surgical thread. *H. pylori* (1×10^9) suspended in 0.2 ml of saline was inoculated into the loop. After the indicated time periods, PPs were removed and extensively washed with PBS. After fixation in 4% paraformaldehyde in PBS, specimens were processed for histopathological examination.

Generation of PP-Null Mice. PPs were depleted from a small intestine as previously described (22). Briefly, 14.5 days postcoitum pregnant wild-type or $Rag2^{-/-}$ mice were i.v. injected with 2 mg of anti-IL-7Rα mAb (A7R34; kindly provided by S.-I. Nishikawa, RIKEN CDB, Kobe, Japan). To confirm the depletion of PPs, a dissected small intestine from one of the offspring was fixed with acetone and stained with anti-B220 or anti-pan CD45 mAb (BD Biosciences).

Adoptive Transfer of $CD4^+$ T Lymphocytes. Naive or *H. pylori* antigen-primed $CD4^+$ T cells were purified from splenocytes, mLN, and PPs by using anti-mouse CD4-microbeads and AutoMACS (Miltenyi Biotech, Sunnyvale, CA) according to the manufacturer's instruction. The purity of isolated cells was >95%. Isolated cells (5×10^6 per mouse for splenocytes and mLN, 5×10^5 per mouse for PP-derived cells) were injected i.v. into recipient mice infected with *H. pylori* for 8 weeks. Eight weeks after the transfer, mice were killed for the indicated analyses.

Histological Analysis. An excised stomach was fixed in a neutral-buffered 10% formalin solution and cut into four strips. Samples were processed by standard methods, embedded in paraffin, and sectioned at 4 to 5 μm. Specimens were stained with H&E or used for cytochemical and immunohistochemical studies. The Leder method was used to assess naphtol-AS-D-chloroacetate esterase detection (36). Immunohistochemical analysis was performed with formalin-fixed and paraffin-embedded tissue sections by using heat-induced epitope retrieval and the ABC (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) method. Anti-*H. pylori* serum from DAKO was used for *H. pylori* staining. In some cases, frozen sections (7 μm) were prepared, fixed with 4% paraformaldehyde in PBS, and blocked with 2% BSA-PBS, and immunofluorescence was performed using the tyramide amplication method (TSA-Plus Fluorescein System; PerkinElmer Life and Analytical Sciences, Boston, MA) and then incubated with anti-*H. pylori* antibody from Biomeda, followed by Cy5 (GE Healthcare Bioscience AB, Uppsala, Sweden) or TRITC-linked rabbit IgG (Sigma–Aldrich, St. Louis, MO). The specimens were mounted with Vectashield (Vector Laboratories) and examined with a confocal laser-scanning microscope LSM510 by using version 3.2 software (Carl Zeiss, Thornwood, NY). The zymogenic zone of middle corpus ≈3 mm from the FS/Z transition zone was examined in each sample.

Histological Score. For assessment of gastric histopathology, blinded sections stained with H&E were examined by light microscopy. Neutrophil infiltration was assessed by the presence of neutrophils in the gastric mucosa. Active inflammation was assessed by the degree and area of damages of mucosal tissue and muscular layers because of infiltrations of neutrophils, lymphocytes, and/or macrophages. The scoring was graded as 0 (no), 1 (mild), 2 (moderate), or 3 (severe). The total number of glands with neutrophil infiltration in the crypt and lumen was also counted to produce a gland active inflammatory score.

Whole-Mount Immunohistochemistry. Small intestines were removed and stained with antibodies as described previously (37). Briefly, small intestines were washed, incubated twice in HBSS containing 5 mM EDTA at 37°C for 20 min, and fixed with ice-cold formalin

for 1 h. After blocking, specimens were incubated with biotin-conjugated anti-B220 mAb for wild-type mice or anti-CD45 mAb for mice on a Rag2$^{-/-}$ background in Solution A containing 0.6% Triton X-100 and 0.1% BSA for 1 h, incubated with ABC reagent (Vector Laboratories) at room temperature for 2 h, and reacted with diaminobenzidene.

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Neuroregulator RET Initiates Peyer's-Patch Tissue Genesis

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The tyrosine kinase receptor RET regulates the intestinal nervous system. A recent paper by Veiga-Fernandes et al. (2007) demonstrates that RET is also involved in the intestinal immune system through the initiation of Peyer's-patch tissue genesis.

The development of a Peyer's patch (PP) is controlled by a tissue-genesis program that is mediated by a group of the tissue-genesis-associated cytokines, chemokines, and transcriptional factors (Mebius, 2003; Nishikawa et al., 2003). $CD3^-CD4^+CD45^+$IL-7 receptor (R) α^+ cells (PP inducer [PPi]) and lymphotoxin (LT) βR^+ stromal cells (PP organizer [PPo]) have been shown to be centers for the initiation of PP genesis; they use tissue genesis-associated cytokines (e.g., LT and IL-7) and lymphoid chemokines (CXCL13, CCL19, and CCL21) as reciprocal stimulator and attractant molecules, respectively (Mebius, 2003). The central role of PPi- and PPo-mediated tissue genesis has now been further enriched by the recent elegant study by Veiga-Fernandes et al. (2007). Their study has identified a new $CD45^+CD3^+CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ hematopoietic cell population that produced high amounts of LTβ in the PP anlagen of the E15.5 intestine. The $CD3^-CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ cells expressed the tyrosine kinase receptor RET, which is known to regulate the development of the enteric nervous system (Barlow et al., 2003). Veiga-Fernandes et al. (2007) demonstrated that *Ret*$^{-/-}$ mouse embryonic intestines lack PP formation despite having a normal number of the newly identified $CD3^-CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ cells and PPi. One of the RET ligands, artemin (ARTN), was shown to be produced by PPo. This ligand was found to have a role in the recruitment of $RET^+CD3^-CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ cells at the site of the PP anlagen. However, RET signaling did not influence the differentiation of the hematopoietic cell population or the maturation and maintenance of PP structure. Therefore, formation of the initial cellular cluster under the control of RET (produced by $CD3^-CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ cells) and ARTN (produced by PPo) is the essential step for the initiation of PP genesis for subsequent recruitment of PPi into the PP anlagen (Figure 1).

The outcome of these exciting findings by Veiga-Fernandes et al. (2007) presents us with at least two new facts important for our understanding of the mechanism of lymphoid tissue development. The first, identification of a new subset of hematopoietic cells (the $RET^+CD3^-CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ cells, which produce LTβ) and their crucial contribution to the initiation of PP genesis, should be recognized. LTβ-producing PPi were thought to be the sole hematopoietic cell population that initiated PP genesis. However, we now have to consider that the newly identified $CD3^-CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ cells could, together with PPo, be initiator cells that function one step earlier than PPi at the site of PP genesis. On the basis of the results presented by Veiga-Fernandes et al. (2007), it is interesting to postulate that $CD3^-CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ cells could be considered a new category of tissue-genesis-associated cells involved in the determination of the site for tissue genesis together with PPo before the recruitment of PPi; therefore, they could be called PP initiator cells (PPin). Thus, the initial interaction between PPin and PPo via RET and ARTN is the first step in the PP tissue genesis and is followed by the recruitment of PPi at the site of PP tissue genesis (Figure 1). For the testing of this possibility, *Rorc*$^{-/-}$ mice and *Id2*$^{-/-}$ mice should be helpful, because these mutant mice genetically lack PPi, and thus lack PP (Mebius, 2003). It would be interesting to examine whether PPin develop in these PP-tissue-genesis-associated, transcriptional-factor-deficient mice. If the development of PPin is independent of RORγ and Id2, then this will further suggest the presence of at least two initiation steps whereby the interaction of PPin and PPo may decide the site of PP development and subsequent mobilization of PPi, but their own cellular lineage and development might be regulated in an independent manner.

Another intriguing question is whether $CD3^-CD4^-$IL-$7R\alpha^-$c-kit^+CD11c^+ cells (or PPin cells) are involved in the genesis of the other gut-associated lymphoid tissues such as isolated lymphoid follicles (ILF), which develop postnatally. $CD3^-CD4^+CD45^+$IL-$7R\alpha^+$ cells producing $LT\alpha1\beta2$ (or lymphoid tissue inducer [LTi]) are essential for the tissue genesis of ILF because *Rorc*$^{-/-}$ mice lack ILF formation as a result of the failure of LTi differentiation (Newberry and Lorenz, 2005). However, $B220^+$ B cells that produce $LT\alpha1\beta2$ can reconstitute ILF, but not PP, formation in *Lta*$^{-/-}$ mice (Newberry and Lorenz, 2005). Thus, LTi is involved in the tissue genesis of ILF, but might not be essential for postnatal development of ILF. It would be interesting to elucidate

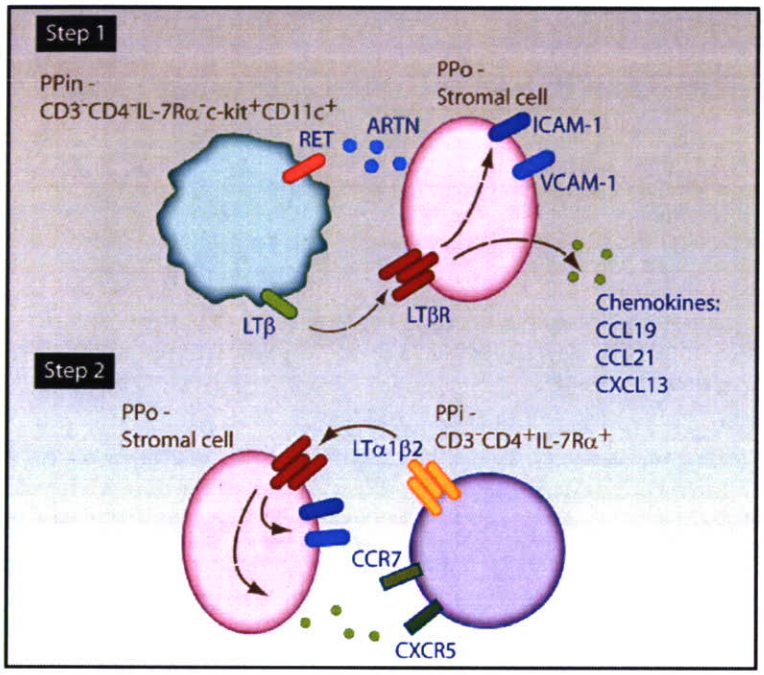

Figure 1. Neuroregulator RET Adds a New Layer in the Initiation Stage of Peyer's-Patch Genesis

As the first step of Peyer's patch (PP) genesis, PP organizers (PPo) produce ARTN, the ligand of RET. ARTN induces the recruitment and the interaction of RET⁺CD3⁻CD4⁻IL-7Rα⁻c-kit⁺CD11c⁺ cells (PP initiatior cells [PPin]) with PPo. This cell-to-cell interaction may determine the site of PP anlagen. PPin produce lymphotoxin (LT) β, which can enhance the production of adhesion molecules (e.g., ICAM-1 and VCAM-1) and lymphoid chemokines (e.g., CXCL13, CCL19, CCL21) from LTβR-expressing PPo. This first step will create the tissue-genesis environment for the recruitment of PPi at the site of PP anlagen as the initiation of the second step. PPi migrate into the PP anlagen and interact with PPo in a CXCL13- and CXCR5-dependent manner. The cell-to-cell interaction of LTβR-expressing PPo and LTα1β2-expressing PPi then leads to the recruitment of necessary lymphocytes to form PP.

whether RET⁺CD3⁻CD4⁻IL-7Rα⁻c-kit⁺CD11c⁺ cells (or PPin-equivalent initiator cells) are involved in the LTi-independent postnatal development of ILF genesis. Furthermore, in some cases lymphoid tissue development does not require LTi. In *Id2*$^{-/-}$ mice, tertiary lymphoid tissue in the thyroid was induced by mature CD4⁺ T cells with transgenic expression of CCL21 in the thyroid (Marinkovic et al., 2006). This finding further indicates that LTi may be not essential for the postnatal development of lymphoid tissue in adult mice. Therefore, it is possible that the role of LTi differs between the initial steps of lymphoid tissue development in the embryonic and postnatal periods.

In the respiratory organs, nasopharynx-associated lymphoid tissue (NALT) possesses a unique mechanism of lymphoid tissue genesis (Kiyono and Fukuyama, 2004). NALT inducer cells share some features of LTi and PPi (CD3⁻CD4⁺CD45⁺ cells), but are independent of RORγ, LTα1β2 and LTβR, and IL-7R requirements for the induction of postnatal development of tissue. Furthermore, the genesis of bronchus-associated lymphoid tissue (BALT), especially inducible BALT, is also independent of LTβR signaling (Moyron-Quiroz et al., 2004). Taken together, these data indicate that there should be an organ-specific mechanism of lymphoid tissue genesis among mucosa-associated lymphoid tissues (e.g., ILF, NALT, and inducible BALT) in addition to the common tissue-genesis program shared with other secondary lymphoid tissues (e.g., PP). We can speculate on the involvement of an identical or as-yet-unidentified hematopoietic cell population as the tissue initiator in the initial step of postnatal NALT and BALT geneses, in the same way that RET⁺CD3⁻CD4⁻IL-7Rα⁻c-kit⁺CD11c⁺ cells (or PPin) are involved in PP genesis.

The second major contribution of the study by Veiga-Fernandes et al. (2007) is the demonstration that RET, an essential molecule in the formation of the mammalian enteric nervous system, is critically involved in the development of the core lymphoid tissue in the intestinal immune system. The nervous system is well known to be essential for the maintenance of gut physiological and anatomical development and homeostasis (Sharkey and Mawe, 2002). RET plays a key role in organizing enteric ganglia, and thus disruption of the *RET* gene in humans results in the development of Hirschsprung's disease, marked by the lack of enteric ganglia (Fitze et al., 2002). Although the contribution of RET in the nervous system of the gastrointestinal tract has been extensively investigated, this appears to be the first demonstration of the role of RET in the development of the immune system, and especially in the tissue genesis of PP.

It is important to note that the partial removal of RET⁺CD3⁻CD4⁻IL-7Rα⁻c-kit⁺CD11c⁺ cells (or PPin) during E14.5-E17.5 did not result in the complete lack of PP development, and 75% of the tissues developed. Veiga-Fernandes et al. (2007) suggested that the development of normal numbers of PP during the tissue-genesis program requires a full complement of RET⁺CD3⁻CD4⁻IL-7Rα⁻c-kit⁺CD11c⁺ cells (or PPin). Alternatively, the finding may suggest that several subsets of PPin might exist and migrate to different sites of PP genesis. Because the initiation of PP genesis occurs chronologically and sequentially from the pylorus to the ileum in the small intestine (Nishikawa et al., 2003), it is important to investigate whether the PPin or their subtypes accumulate chronologically at the site of the PP anlagen under this anatomical and sequential rule. The other curious aspect of the newly identified hematopoietic cells (or PPin) is

their expression of surface markers associated with professional antigen-sampling cells including CD11c, CD11b, and MHC class II. Furthermore, the cell subset also expresses the cell markers associated with innate immunity, such as Gr-1 and NK 1.1. On the basis of the unique surface-marker expression, it is important and interesting to further elucidate the nature and fate of $RET^+CD3^-CD4^-IL\text{-}7R\alpha^-c\text{-}kit^+CD11c^+$ (or PPin) cells as the part of intestinal immune system.

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Gut lymphocyte migration: we are halfway 'home'

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The gastrointestinal immune system consists of immune cells in organized gut-associated lymphoreticular tissues (GALT) and diffuse lamina propria, which give rise to mucosal secretory IgA antibody responses. A recent study showed that the retinoic acid produced by GALT dendritic cells (DCs) imprints B cells for gut homing. Surprisingly, GALT DCs, together with interleukin-5 (IL-5) and IL-6, also provided a milieu for both B cell switching to IgA and IgA synthesis.

Introduction to gut lymphocyte homing

The mucosal immune system protects the host from environmental antigens (Ags) and microbial infections, in part by the production of secretory immunoglobulin A (S-IgA) antibodies (Abs). Its largest component protects the gastrointestinal (GI) tract from the stomach to the colon and comprises Peyer's patches (PPs) and other organized GALT, which contain all the necessary immunocompetent cells, where initial mucosal immune responses are induced [1]. The more diffuse effector sites, for example the gut lamina propria, consist of Ag-presenting cells including DCs, and T cell subsets [both $CD4^+$ T helper (Th) and $CD8^+$ cytotoxic T lymphocytes (CTLs)], in addition to B cells and plasma cells (PCs), of which >90% produce IgA. Polymeric (usually dimeric) IgA molecules contain a joining (J) chain and bind to the polymeric Ig receptor (pIgR) at the basolateral surface of epithelial cells (Figure 1). They are then transported across the epithelium and released as S-IgA, which is exceptionally well adapted to the harsh gut environment [1]. Recent studies have shown that GALT DCs, but not DCs from other central lymphoid tissues such as the spleen or peripheral lymph nodes (PLNs), produce retinoic acid (RA) [2]. Imprinted by this RA with an upregulated expression of the chemokine receptor CCR9 and the integrin $\alpha_4\beta_7$, $CD8^+$ precursors, Ag-specific CTLs [3–5] and $CD4^+$ Th cells [2] migrate exclusively to the gut epithelium in response to thymus-expressed chemokine (TECK; also known as CCL25) selectively produced by the gut epithelial cells and to the mucosal addressin cell adhesion molecule (MAdCAM)-1 expressed by the gut-associated high endothelial venules (HEVs) [6,7]. Mora *et al.* [8] now significantly extend this to B lymphocytes for IgA Ab responses.

GALT-DCs and RA imprint gut homing for mouse and human B cells

Gut homing receptors, including $\alpha_4\beta_7$ and CCR9 on B cells, are upregulated both by murine GALT-DCs (PP-DCs), and exogenous RA. In addition, human mesenteric lymph node

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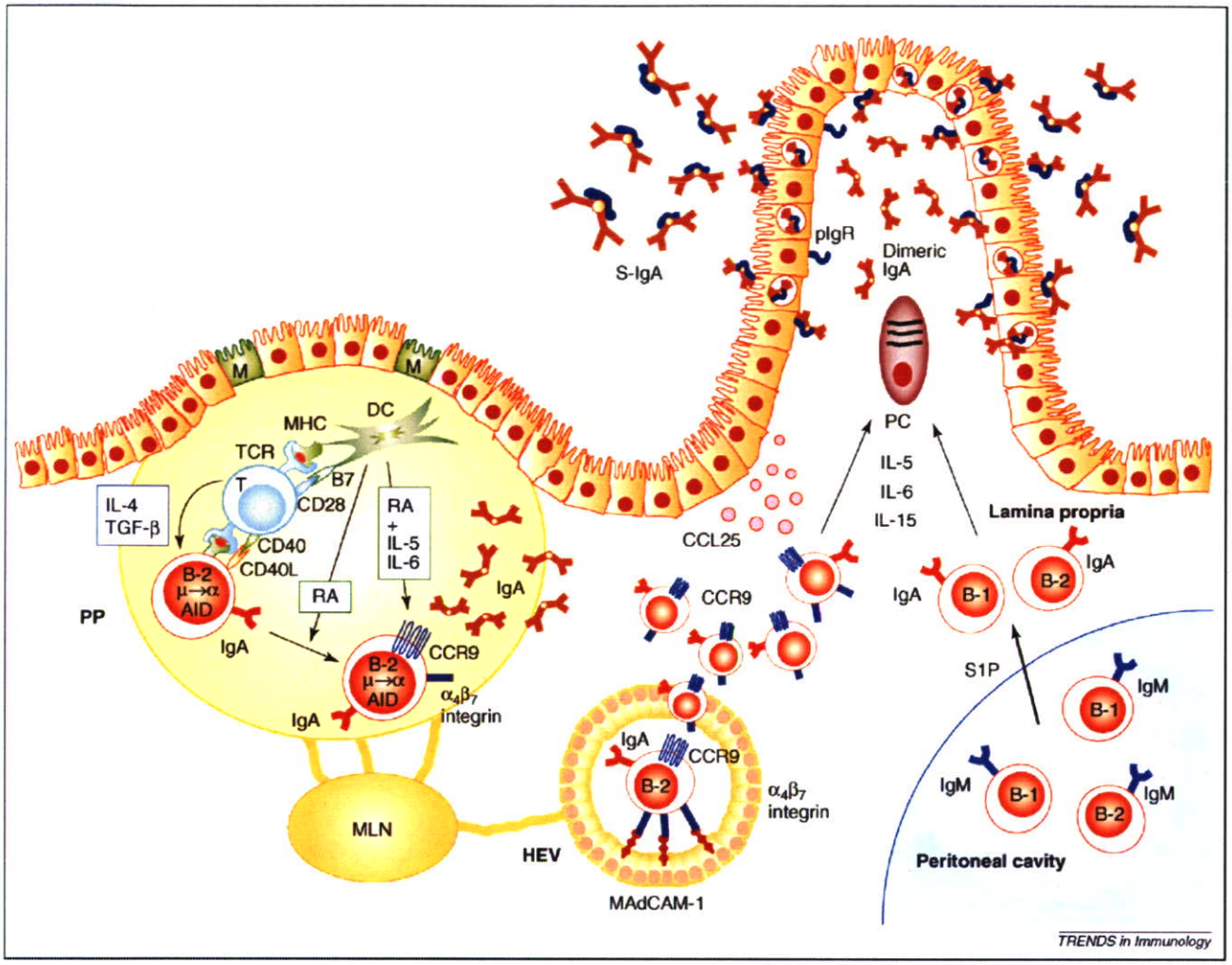

Figure 1. Classical and novel pathways in the regulation of B cell responses for the production of intestinal S-IgA Abs. In the first pathway for S-IgA Ab responses, DCs take up luminal Ag through M cells, and then process and present the peptide to $CD4^+$ Th cells in PPs. The Ag-primed Th cells produce IL-4 and TGF-β, rendering B cells to undergo μ→α switching under the regulation of AID. Simultaneously, RA produced by DCs increases gut homing receptors ($\alpha_4\beta_7$ integrin and CCR9) on Ag-primed IgA-committed B cells, which directs them into the lamina propria in response to MAdCAM-1 expressed on HEVs and to the CCL25 produced by gut epithelial cells. In the lamina propria, $CD4^+$ Th2 cell-derived IL-5 and IL-6 induce terminal differentiation of IgA^+ B cells to IgA-producing PCs secreting dimeric (or polymeric) forms of IgA. The dimeric IgA binds to the pIgR expressed on the basolateral surface of epithelial cells, which transports the molecule into the lumen as S-IgA. In the second pathway, DCs produce IL-5 and IL-6, together with RA, in PPs, providing a milieu to induce both B cell switching to IgA and IgA production without any help provided by Th cells. In the third pathway, peritoneal B cells (mainly B-1 B cells) migrate into the lamina propria in a sphingosine 1-phosphate (S1P)-dependent manner, where they differentiate into IgA-producing PCs in the presence of IL-15. Abbreviation: AID, activation-induced cytidine deaminase.

(MLN)-DCs also imprinted gut trophism. Such imprinting could be blocked by LE540, which inhibits RA binding to the RA receptor (RAR). CCR10, like CCR9, is important to gut tropism, with IgA^+ B cells that express both CCR9 and CCR10 homing to the small intestine and those that express only CCR10 homing to the large intestine [9]. Thus, by imprinting $\alpha_4\beta_7$ and CCR9, PP-DC-derived RA directs B and T lymphocytes to migrate selectively into the small intestine (Figure 1).

How do GALT-DCs and RA regulate IgA Ab responses?

The most intriguing results of the Mora *et al.* study [8] involve evidence that GALT-(PP)-DCs and RA (PP-DC–RA) induce anti-μ-treated splenic B cells to switch to the IgA isotype and to secrete IgA. Transforming growth factor (TGF)-β1 is known to induce activated mouse [10,11] or human [12,13] B cells to undergo μ→α switching (Box 1; Figure 1). A subset of $CD4^+$ T cells has also been reported to induce μ→α switching [14]. By contrast, cytokines such as IL-5 and, in particular, IL-6 induce IgA-committed B cells to differentiate into IgA-producing PCs [15–17] (Figure 1). Mora *et al.* [8] show that PP-DCs, but not PLN-DCs, induce maximum IgA synthesis in the presence of the cytokines IL-5 and IL-6, an effect blocked by LE540. Maximum IgA responses were also noted with human MLN-DCs and anti-μ-activated B cells when both RA and IL-6 were present. Mora *et al.* found no evidence that TGF-β1 affected μ→α switching in the PP-DC–RA system ([8]; also see the online Supplementary data for Ref. [8]). However, as PP-DCs form 'clusters' with T cells, creating a productive milieu for IgA synthesis [18], and produce IL-6, which, together with $CD4^+$ T cells, supports IgA B cell responses [19], great care must be taken when using partially purified DCs to exclude the possibility

Box 1. Mechanisms for B cell switching to the IgA isotype

B cell commitment to IgA (through $\mu \rightarrow \alpha$ isotype class switching) is thought to occur in GALT and NALT, which contain distinct B cell zones with germinal centers and are well organized into follicles with follicular DCs and adjacent interfollicular T cell zones. Isotype switching itself involves recombination between tandem repetitive DNA sequences [known as switch (S) regions] located 5′ of the respective constant region heavy chain (C_H) genes. Switching is an irreversible DNA deletional event in which recombination between upstream and downstream S regions forms a DNA circle containing the deleted intervening C_H genes. Several tangible events, including the demethylation of 5′ flanking region DNA, deoxyribonuclease hypersensitivity and the transcription of unrearranged C_H genes, precede cytokine-induced switching. The germline transcripts correspond to the Ig isotype to which the B cell will switch; switching is initiated 5′ of the targeted C_H gene and upstream of so-called I-region exons. These exons contain stop codons in all translational reading frames, rendering the resulting transcripts 'sterile'. TGF-β is thought to be the major cytokine for B cell switching to IgA, as shown by definitive experiments in which the addition of TGF-β to LPS-triggered murine splenic B cell cultures resulted in switching to IgA, and in which IL-2 or IL-5 enhanced IgA synthesis markedly [10,11]. TGF-β also induces sterile Cα germline transcripts, an event that clearly precedes actual switching to IgA. Subsequent studies showed that TGF-β induced human B cells to switch to either IgA1 or IgA2, an event clearly shown to be preceded by the formation of Cα1 and Cα2 germline transcripts [12,13]. Deletion of the TGF-β1 gene would be expected to result in IgA deficiency in mice. Though such mice live for only 3–5 wks and die of a generalized lymphoproliferative syndrome, they show low levels of S-IgA. In separate studies, conditional mutagenesis to knock out the TGF-β1 receptor resulted in mice characterized by expanded peritoneal B-1 B cells, PP B cell hyperplasia and an absence of serum IgA.

that contaminating cells such as $CD4^+$ T cells are skewing the results. More studies are needed to rule out such contamination.

A definitive set of experiments in vitamin A-deficient (RA-negative) mice showed that gut homing receptors and IgA responses are relevant to RA induction *in vivo*. These studies showed lower numbers of $\alpha_4\beta_7^+$ B cells in PP, MLN and spleen, in addition to significantly fewer IgA^+ PCs in the lamina propria of RA-negative mice [8]. However, the finding of low, but significant, numbers of IgA-producing PCs does suggest that other mechanisms can lead to some $\mu \rightarrow \alpha$ switching in GALT with subsequent migration into mucosal effector sites. In addition, vitamin A deficiency might not have been complete in RA-negative mice, which would also result in a partial IgA deficiency. Additional studies will be required to distinguish between these two broad possibilities.

Questions raised by the Mora *et al.* study

Because nasal-associated lymphoreticular tissues (NALT) exhibit features similar to GALT, the findings by Mora *et al.* [8] also have implications for NALT research. For example, NALT, like GALT, consists of follicle-associated epithelium (FAE) containing microfold (M) cells that are responsible for Ag uptake [20,21]. Furthermore, as in PPs, lymphocytes traffic into NALT through HEVs and exhibit distinct T and B cell zones. More importantly, as in oral delivery, using mucosal adjuvants with nasal delivery of Ags results in mucosal S-IgA Ab responses in distant effector sites such as salivary glands, the vaginal mucosa and the upper respiratory tract [1,21]. By showing that GALT-DCs offer a productive milieu for gut S-IgA Ab responses, the Mora *et al.* study [8] raises questions about whether NALT-DC subsets perform a similar function in the upper airways and other mucosal effector sites, and whether RA or similar molecules can help imprint 'mucosal homing'. The common mucosal immune system (CMIS), in which NALT-imprinted or GALT-imprinted lymphocytes repopulate several distant mucosal effector sites, is still the subject of debate, as some argue that differences in NALT-based versus GALT-based mucosal immunity make its existence improbable. RA involvement in mucosal homing could provide the means for resolving this dispute. By developing *in vitro* systems with NALT-DCs as Mora *et al.* [8] have done with GALT-DCs, researchers should be able to map RA-like pathways and to determine whether they are a common link in NALT-based and GALT-based mucosal immunity or whether they distinguish them, thereby providing evidence for or against the existence of a CMIS.

There is evidence that Ig isotype switches to IgA by classical B-2 B cells only occur in GALT or NALT and not in mucosal effector sites such as the gut lamina propria. A study showed that $\mu^+\alpha^-B220^+$ B cells with circular DNA transcripts (Iα-Cμ), which are in the process of switching to IgA, are only seen in PPs (GALT) and NALT [22]. No evidence for Iα-Cμ circular transcripts was found in gut lamina propria or in nasal passages, strongly suggesting that most $\mu \rightarrow \alpha$ switches in classical B-2 B cells occur at mucosal inductive sites. Of importance, recent studies have now shown the presence of Iα-Cμ transcripts in human GALT but not in the diffuse lamina propria tissues [23]. By contrast, it has been suggested that $\mu \rightarrow \alpha$ switches are induced in diffuse gut lamina propria in the absence of an organized lymphoid structure [24]. It has yet to be shown that NALT-DC-derived RA-like molecules serve a similar function in NALT.

A still-unexplained source of gut-derived IgA is B-1 B cells. Residing primarily in the peritoneal and pleural cavities, these B cells arise early during ontogeny, undergo self renewal and can produce 'natural' Abs in a T cell-independent manner. We now know that about half of the IgA PCs in the small intestinal lamina propria originate from peritoneal B-1 B cells (Figure 1); however, little is known about how these B cells reach the gut or where the $\mu \rightarrow \alpha$ switch occurs. We do know that the commensal bacterial flora induces an IgA Ab response to the bacterial Ags themselves, which prevents subsequent penetration of commensal Ags into the bloodstream for the induction of systemic Ab responses [25]. It is tempting to speculate that gut DCs might be involved in this process and that $\mu \rightarrow \alpha$ B-1 B cell switches could emanate through CD40–CD40L-independent mechanisms [26].

In summary, we are halfway home to understanding mucosal imprinting. We understand how classical B-2 B cells, which switch to the IgA isotype, become programmed to migrate to the gut, but not how B-1 B cells contribute to the innate gut IgA response, how the two B cell systems work together to engender host mucosal immunity, or whether similar homing mechanisms are at work in NALT and GALT, which would suggest that they form a CMIS.