

Fig 4. The analyses of A β and GA β in the brains of normal and DM-affected monkeys. (A) A β level in young monkey, normal adult monkey, DM-affected monkey, and normal aged monkey brains were assessed with sandwich ELISA. A β level was significantly increased in normal aged monkey brains. In DM-affected monkey brains, A β level seemed unchanged. Data obtained from young monkey brains were set as standards. Y-axes show the mean values of the quantified data. Values are means \pm SD. *P < 0.02. (B) Image of temporal lobe sections from a 19-year-old normal adult monkey and an 18-year-old cynomolgus monkey with DM. Sections were immunostained with the anti-GA β -specific antibody 4396C and counterstained with hematoxylin. In the brain of the normal adult monkey, we observed little, if any, immunoreactivity for anti-GA β antibody. By contrast, in the brain of the DM-affected adult monkey, we observed several neurons immunopositive for anti-GA β antibody. Scale bars, 100 μ m. (C) Quantitative image analysis of GA β -immunopositive area in the sections obtained from female normal adult monkey and DM-affected adult monkey brains. Data obtained from normal adult monkey brains were set as standards. Y-axes show the mean values of the quantified data. Values are means \pm SD. *P < 0.02. (D) Dot blots showing the amount of GA β generated in brains of cynomolgus monkeys of different ages. The blot samples were adjusted to 1 μ g, 2.5 μ g, or 5 μ g of total protein. Dot blot analyses showed that GA β generation increased in an age-dependent manner. (E) Dot blots showing the amount of GA β generated in the brains of normal adult monkeys and DM-affected adult monkeys. The blot samples were adjusted to 1 μ g, 2.5 μ g, or 5 μ g of total protein. The amount of GA β in brains samples from DM-affected monkeys was significantly increased compared to those from normal adult monkeys. CT, normal adult monkeys; DM, DM-affected adult monkeys.

doi:10.1371/journal.pone.0117362.g004

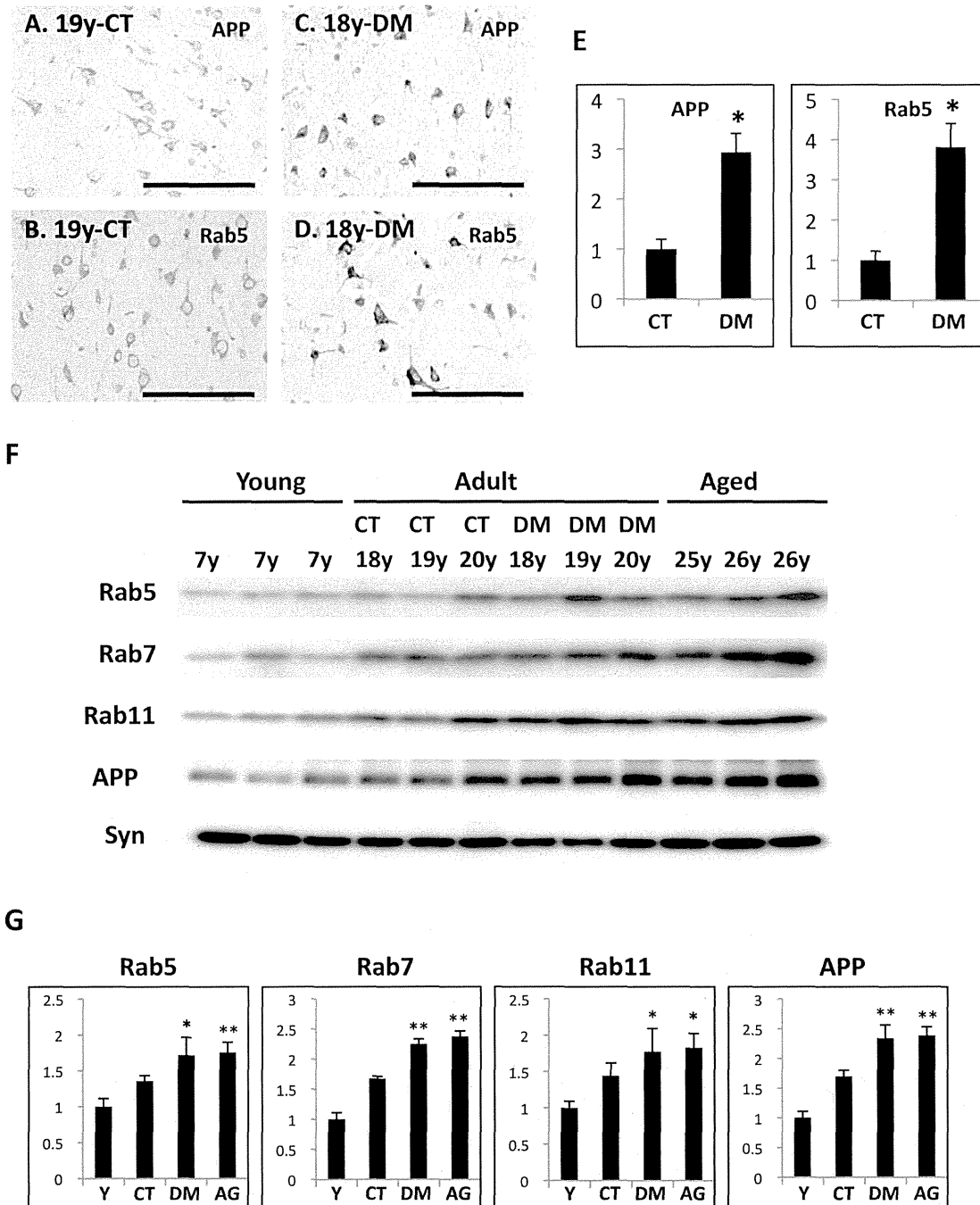


Fig 5. Immunohistochemistry and Western blot analyses of Rab5, Rab7, Rab11, and APP in the brains of normal and DM-affected monkeys. Images of temporal lobe sections from a 19-year-old normal cynomolgus monkey (A, B) and an 18-year-old cynomolgus monkey with DM (C, D). Sections were immunostained with anti-APP antibody (A, C) or anti-Rab5 antibody (B, D), and then counterstained with hematoxylin. Scale bars, 100 μ m. In the brains of normal adult monkeys, APP and Rab5 were observed as small granules in neurons (A, B). By contrast in the brains of DM-affected adult monkeys, APP- and Rab5-immunopositive granules were enlarged, and APP and Rab5 immunoreactivity was significantly more robust (C, D). (E) Quantitative image analysis of APP or Rab5-immunopositive area in the sections obtained from female normal adult monkey and DM-affected adult monkey brains. Data obtained from normal adult monkey brains were set as standards respectively. Y-axes show the mean values of the quantified data. Values are means \pm SD. * $P < 0.01$. (F) Western blots showing the amounts of APP, Rab5, Rab7, Rab11, and synaptophysin in the brains of normal monkeys and DM-affected monkeys of different ages. Western blot analyses showed that APP and Rab GTPases were significantly increased in both DM-affected adult and aged monkey brains. In the brains of DM-affected adult monkeys, APP and Rab GTPases levels were obviously increased compared to those of normal adult monkeys. Lanes contained microsomal fractions derived from the brains of young monkeys, normal adult monkeys, DM-affected adult monkeys, and aged

monkeys. CT, normal adult monkeys; DM, DM-affected adult monkeys. (G) Age-related and DM-related changes in APP, Rab5, Rab7, and Rab11 in cynomolgus monkey brains. Data obtained from young monkey brains were set as standards; * $P < 0.05$ ** $P < 0.01$. Y-axes show the mean values of the quantified data. Y, young monkeys; CT, normal adult monkeys; DM, DM-affected adult monkeys; AG, normal aged monkeys.

doi:10.1371/journal.pone.0117362.g005

studies are needed, these findings suggest that DM can induce not only parenchymal A β pathology but also vascular A β pathology in an age-dependent manner.

To clarify the mechanism of how DM enhances A β pathology in the brain, we also assessed the amount of A β and GA β , a seed molecule for A β aggregation [32]. Intriguingly, A β level was not so much increased in DM-affected adult monkey brains, in contrast to aged monkey brains (Fig. 4A). In DM-affected adult monkey brains, SP depositions were quite small quantities (Fig. 2I), and a couple of more years can induce age-dependent SP depositions in normal adult monkey brains [26, 36]. That may be why we could not find the significant increase of A β level between DM-affected adult monkey and normal adult monkey brains. On the other hand, both immunohistochemical and dot blot analyses demonstrated that the amount of GA β was clearly increased in the brains of DM-affected adult monkeys compared to control adult monkey brains (Fig. 4B-E). These findings strongly suggest that the acceleration of GA β generation might be responsible for the early deposition of SPs in the brains of DM-affected adult

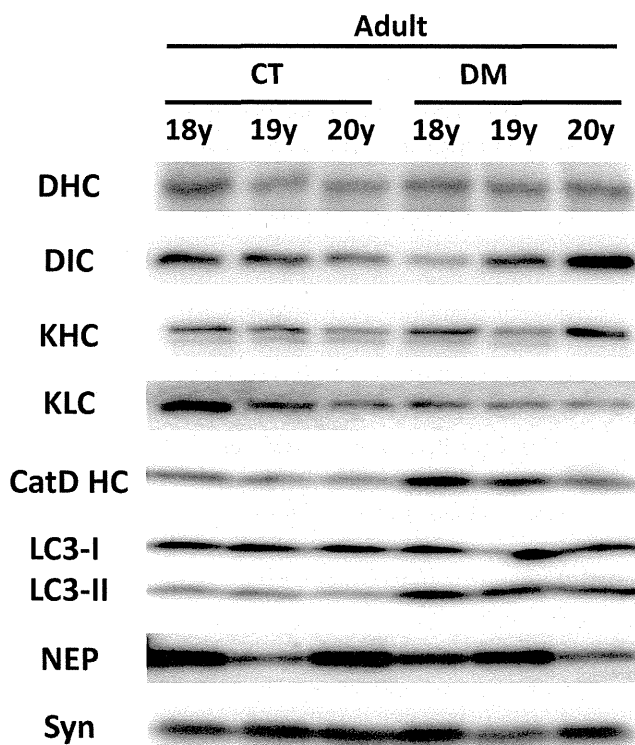


Fig 6. Western blot analyses of axonal motor proteins, cathepsin D heavy chain, autophagosome marker LC3, and neprilysin in the brains of normal and DM-affected adult monkeys. Western blots showing the amounts of axonal motor proteins, cathepsin D heavy chain (CatD HC), autophagosome marker LC3, and neprilysin (NEP) in the brains of normal and DM-affected adult monkeys. Western blot analyses showed that the level of axonal motor proteins such as dynein heavy chain (DHC), dynein intermediate chain (DIC), kinesin heavy chain (KHC), and kinesin light chain (KLC) unchanged. The level of CatD HC increased in DM-affected monkey brains, and LC3-II showed significant increase in DM-affected adult monkeys. We did not observe DM-related changes in LC3-I and neprilysin (NEP) level. CT, normal adult monkeys; DM, DM-affected adult monkeys.

doi:10.1371/journal.pone.0117362.g006

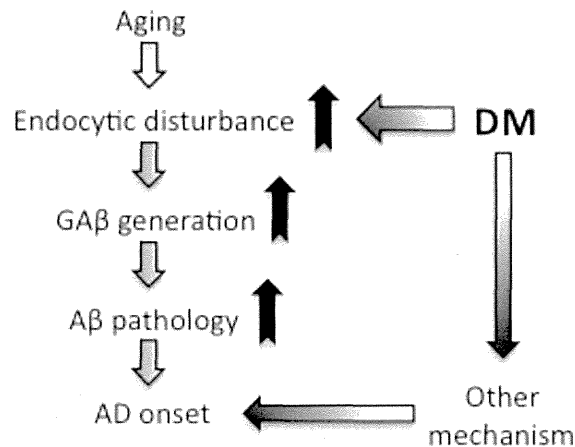


Fig 7. Hypothetical schema of DM-induced A β pathology leading to AD onset. From the results of this study, we propose that DM induces GA β generation by exacerbating age-dependent endocytic disturbance, resulting in enhanced A β pathology in the brain. Although additional studies are needed to clarify the whole mechanisms underlying DM-associated pathology, we hypothesize that, at the very least, enhanced A β pathology accompanied by endocytic disturbance might be involved in the development of AD.

doi:10.1371/journal.pone.0117362.g007

monkeys. Moreover, the result of this study also suggests that enhanced A β aggregation could induce SP deposition without significant changes in total A β level. Relevant to proposed AD pathophysiological mechanisms, we also observed apparent endocytic pathology, including enlarged early endosomes and APP accumulation in neurons of DM-affected adult monkeys (Fig. 5A-E). Western blot analyses confirmed a significant increase of Rab GTPases in these brains at nearly the same level as in aged monkey brains (Fig. 5F, G). Our previous studies showed that an increase in Rab GTPases is a good indicator for alterations in intracellular endosome trafficking associated with a particular Rab GTPase [45, 46]. Indeed, increased Rab GTPase levels are strongly associated with endocytic disturbance [45, 46]. The observation that experimentally induced disorders of the endocytic pathway cause GA β -dependent A β pathology [34, 52] supports the premise that endocytic disturbance is likely responsible for enhanced GA β generation. Along these lines, we surmise that intracellular endosome trafficking would be altered in the brains of DM-affected adult monkeys, resulting in severe endocytic disturbance, as observed in aged monkey brains. This might be why GA β generation was enhanced, thereby inducing SP deposition (Fig. 2). Moreover, the results of this study strongly support the idea that endocytic disturbance is essentially involved in the development of AD pathology [33, 34, 42–45].

A recent study showed that the expression of axonal transport motor proteins was altered in experimentally DM-induced rodent model, and axonal transport motor proteins are indeed required for endosome trafficking [48, 49]. However, in the present study, we did not find any changes in axonal motor protein levels, suggesting that the mechanism underlying endocytic disturbance in the brains of DM-affected adult monkeys would be independent of axonal motor protein levels. Previous finding showed that the breakdown in lysosomal degradation also induces endocytic disturbance [50]. In DM-affected adult monkey brains, the level of CatD heavy chain increased in DM-affected adult monkey brains, indicating that the endosomal-lysosomal system is activated as such in AD patient brains (Fig. 6) [53]. This finding suggests that DM really enhances AD pathology. On the other hand, we observed the significant increase in autophagosome marker LC3-II level in DM-affected adult monkey brains (Fig. 6). Since LC3-I level was unchanged, the induction of autophagy was not altered, but lysosomal-

autophagosome clearance was likely disturbed in DM-affected adult monkey brains (Fig. 6). The defective lysosomal-autophagosome clearance is associated with AD pathology [50, 54–56], and the result of this study is also consistent with a previous finding that the aberrant lysosomal-autophagic turnover is associated with the accumulation of GA β in rodent brain [57].

Given that CatD heavy chain level was increased, i.e. lysosomal degradation was induced (Fig. 6), the disturbance in the fusion of autophagosome and lysosome might be responsible for impaired lysosomal-autophagosome clearance in DM-affected adult monkey brains. The fusion step is indispensable for lysosomal-autophagosome clearance [58, 59] and mediated by Rab7 [60]. In DM-affected adult monkey brains, Rab7 level was obviously increased as compared to normal adult monkey brains, indicating that Rab7-mediated transport was really disturbed. Growing evidences suggest that membrane-bound phosphoinositides regulate Rab-mediated endosome trafficking [61, 62], and the metabolism of phosphoinositides was affected by the disruption of insulin signaling [63–65]. Recent studies also showed that Rab activity is affected by insulin signaling and that PI3K inhibition causes upregulation of Rab5 [66, 67]. In the present study, we observed amyloid deposition in the pancreatic islets of all adult monkeys with DM. The remaining islet cells were severely degenerated and few in number, all characteristics of DM pathology in humans. These pancreatic pathologies suggest that insulin signaling also would be greatly impaired in the brains of DM-affected adult monkeys (Fig. 1A–D). Thus, although additional investigations are needed, impaired insulin signaling would exacerbate age-related endocytic disturbances via such alteration in the metabolism of phosphoinositides and/or Rab GTPases, inducing GA β generation and ultimately resulting in enhanced A β pathology. It is reasonable idea because of the fact that insulin resistance is the core defect in DM [68]. In the brains of DM-affected adult monkeys, NEP levels were not affected (Fig. 6), suggesting that the enhanced SP deposition we observed is not due to disturbances in A β degradation by NEP.

In conclusion, we provide evidence that DM induces GA β generation and accelerates A β pathology *in vivo* in cynomolgus monkey brains. Since the amino acid sequence of cynomolgus monkey A β corresponds completely with that of human A β , it is reasonable that the enhanced A β pathology we observed in monkeys with DM should also occur in humans with DM. Moreover, our present study showed that DM could also exacerbate endocytic disturbance. Although additional studies are needed to determine more precisely the mechanisms responsible for enhanced A β pathology in the brains of DM-affected monkeys, our findings suggest that DM may exacerbate age-dependent endocytic disturbance, leading to enhanced GA β generation and A β fibril formation (Fig. 7). Importantly, several studies showed that A β impairs insulin signaling itself [69–71], and then it may lead to aggravate the insulin resistance-related AD pathology [11–13]. Thus enhanced A β pathology would contribute to DM-induced AD pathogenesis with such other mechanism (Fig. 7). Moreover, DM may also alter neuronal activity by exacerbating endocytic disturbance as we previously reported [46]. Hence, a reasonable therapeutic strategy to prevent the development of AD pathology is to treat or prevent DM.

Supporting Information

S1 ARRIVE Checklist. The ARRIVE Guidelines Checklist.
(PDF)

S1 Table. Clinical Background of monkeys analyzed in the present study.
(XLSX)

Acknowledgments

The authors thank Dr. Fumiko Ono for autopsy of cynomolgus monkeys. The authors also thank Dr. Naoto Oikawa for technical advice in dot blot analyses.

Author Contributions

Conceived and designed the experiments: NK. Performed the experiments: SO NK. Analyzed the data: SO NS YY KY NK. Contributed reagents/materials/analysis tools: NS YY. Wrote the paper: NK.

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Hydroxypropyl- β -Cyclodextrin Spikes Local Inflammation That Induces Th2 Cell and T Follicular Helper Cell Responses to the Coadministered Antigen

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Cyclodextrins are commonly used as a safe excipient to enhance the solubility and bioavailability of hydrophobic pharmaceutical agents. Their efficacies and mechanisms as drug-delivery systems have been investigated for decades, but their immunological properties have not been examined. In this study, we reprofiled hydroxypropyl- β -cyclodextrin (HP- β -CD) as a vaccine adjuvant and found that it acts as a potent and unique adjuvant. HP- β -CD triggered the innate immune response at the injection site, was trapped by MARCO⁺ macrophages, increased Ag uptake by dendritic cells, and facilitated the generation of T follicular helper cells in the draining lymph nodes. It significantly enhanced Ag-specific Th2 and IgG Ab responses as potently as did the conventional adjuvant, aluminum salt (alum), whereas its ability to induce Ag-specific IgE was less than that of alum. At the injection site, HP- β -CD induced the temporary release of host dsDNA, a damage-associated molecular pattern. DNase-treated mice, MyD88-deficient mice, and TBK1-deficient mice showed significantly reduced Ab responses after immunization with this adjuvant. Finally, we demonstrated that HP- β -CD–adjuvanted influenza hemagglutinin split vaccine protected against a lethal challenge with a clinically isolated pandemic H1N1 influenza virus, and the adjuvant effect of HP- β -CD was demonstrated in cynomolgus macaques. Our results suggest that HP- β -CD acts as a potent MyD88- and TBK1-dependent T follicular helper cell adjuvant and is readily applicable to various vaccines. *The Journal of Immunology*, 2015, 194: 000–000.

The term “adjuvant” has its origin in the Latin “*adjuvare*” meaning “to help,” because adjuvants enhance the effects of vaccines. Many substances can act as adjuvants, and their modes of action vary widely (1, 2). Recent studies showed that conventional adjuvants, such as alum, act via the “Ag-depot” mechanism, as well as induce multiple innate immune pathways, including the activation of inflammasomes, the production of

PGE₂, and the release of damage-associated molecular patterns (DAMPs), such as DNA (3–5). In addition to enhancing the immunogenicity of vaccines, adjuvants can be responsible for their adverse effects, including local swelling, systemic fever, and a theoretical risk for autoimmunity (6). Even alum and MF59, widely used adjuvants for human vaccines, induce local and systemic adverse effects (6–8). Therefore, the high safety profile of an

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Received for publication August 11, 2014. Accepted for publication December 3, 2014.

This work was supported by a Health and Labour Sciences Research Grant “Adjuvant Database Project” of the Japanese Ministry of Health, Labour and Welfare, the Regional Innovation Strategy Support Program, and Shionogi & Co., Ltd.

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M. Kobayashi, and A.S. designed and performed the murine influenza infectious model research and analyzed the data. E.W., Y.I., N.N., H.Y., A.V., D.M.S., and T.A. designed and performed the bioinformatics research and analyzed the data. E.K., K.K., Y.K., and Y.Y. designed and performed the cynomolgus macaque model research and analyzed the data. M.O., C.C., T.A., and K.J.I. wrote the manuscript.

The datasets presented in this article have been submitted to the National Center for Biotechnology Information’s Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE63332.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DAMP, damage-associated molecular pattern; DC, dendritic cell; GC, germinal center; GO, Gene Ontology; HA, hemagglutinin; HP- β -CD, hydroxypropyl- β -cyclodextrin; i.d., intradermal(ly); IFNAR, IFN- α β receptor; KO, knockout; LN, lymph node; PA, presence/absence; SV, influenza trivalent split vaccine; Tfh, T follicular helper; TPRC, Tsukuba Primate Research Center; WT, wild-type.

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adjuvant, based on scientific evidence, must be demonstrated before its application to clinical practice. One approach to identifying ideal adjuvants is the selection of candidate substances from among approved drugs or excipients with very high safety profiles.

Cyclodextrin is a bucket-shaped oligosaccharide derived from starch, with a hydrophobic cavity and hydrophilic exterior (9). Natural cyclodextrins contain six (α -CD), seven (β -CD), eight (γ -CD), or more (α -1,4)-linked α -D-glucopyranose units, and many chemically modified cyclodextrins have been developed, including hydroxypropyl- β -cyclodextrin (HP- β -CD) and sulfobutyl ether- β -cyclodextrin (9). Because of their molecular structure and shape, the cyclodextrins can form an inclusion complex with hydrophobic molecules, improving the solubility of hydrophobic drugs (9), stabilizing proteins (10), and controlling the release of drugs (11). For these reasons, cyclodextrins are widely used excipients for pharmaceutical agents (9). Recent studies showed another application of cyclodextrins in the treatment of Niemann–Pick type C1 disease; they reduced the lysosomal accumulation of cholesterol and glycosphingolipids (12). However, only a few studies have investigated the usefulness of cyclodextrins as vaccine adjuvants. Mucosal vaccination with dimethyl- β -cyclodextrin was shown to enhance the immunogenicity of anti-diphtheria and anti-tetanus toxoid total IgG titers in mice (13). Sulfolipo-cyclodextrin showed improved adjuvant activity when combined with squalene for use in cattle (14). The high safety profiles of the cyclodextrins and their potential adjuvant activities demonstrated in these studies are very valuable contributions to the development of new and safer adjuvants. However, research into cyclodextrins as vaccine adjuvants has not been thorough, and the mechanism of action of HP- β -CD as a vaccine adjuvant and its immunological characteristics are not well understood. In this study, we focused on HP- β -CD, one of the cyclodextrins most commonly used in pharmaceutical preparations, because its solubility is higher and its safety profile is better than those of other cyclodextrins (9). We demonstrate its activity as an immune adjuvant and the mechanisms involved. Our results indicate that HP- β -CD is a potent and promising T follicular help (T_{fh}) cell adjuvant for purified protein-based vaccines.

Materials and Methods

Ags and adjuvants

The following were used as Ags: extremely pure LPS-free OVA protein ([Seikagaku, Tokyo, Japan]; LPS contamination was <0.1 EU/mg, as measured with the *Limulus* Color KY test [Wako Pure Chemical Industries, Osaka, Japan], according to the manufacturer's protocol); influenza trivalent split vaccine (SV), containing influenza virus hemagglutinin (HA) surface Ags from three viral strains—A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008; and monovalent SV, containing influenza virus HA surface Ag from New Caledonia/20/1999 (H1N1) (The Research Foundation for Microbial Diseases of Osaka University). SV was manufactured with egg-based technology (15). HP- β -CD (ISP Technologies, Assonet, MA), Good Manufacturing Practice grade K3 CpG-ODN (Gene Design, Osaka, Japan), and alum (InvivoGen, San Diego, CA) were used as adjuvants.

Mice and immunizations

Mice deficient for *Tlr1*, *Tlr2*, *Tlr3*, *Tlr4*, *Tlr5*, *Tlr6*, *Tlr7*, *Tlr9*, *Tlr7/9*, *Tlr2/4/9*, *Il1R*, *Il18R*, *St-2*, *Myd88*, *Tbk1*, *Irf3/7*, *Ifnar2*, *Asc*, and *Caspase 1* were generated and used for experiments previously (16). *Tlr*- or *Myd88*-knockout (KO) mice were purchased from Oriental BioService (Kyoto, Japan). *Irf3/7* double-KO mice were generated by cross-breeding with *Irf3*-KO mice and *Irf7*-KO mice (17). *Irf7*-KO mice were provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (Ibaraki, Japan). *Bcl6^{lox/+} Cd4-cre (Bcl6^{+/+})* and *Bcl6^{lox/lox} Cd4-cre (Bcl6^{0/0})* mice were used for experiments previously (18). The other KO mice were housed and maintained at the National Institute of Biomedical Innovation. Wild-type (WT) C57BL/6 mice were purchased from CLEA Japan. The mice were immunized s.c. twice with 100 μ g OVA mixed with PBS (–),

30 μ g K3 CpG-ODN, 1 mg alum, or 3–30% HP- β -CD [total volume: 200 μ l in PBS (–)/mouse] on day 0 and day 10 after the first immunization. Sera were collected at 10, 17, 24, and 31 d after the first immunization. To evaluate the cellular immune responses, the mice were sacrificed and their spleens were collected 10 d after the second immunization. All experiments were performed under the appropriate laws and guidelines and after approval was obtained from the National Institute of Biomedical Innovation, the Animal Research Committee of the Research Institute for Microbial Diseases of Osaka University, and the Shionogi Animal Care and Use Committee.

Measurement of Ab titers

To measure the OVA- or HA-specific Ab titers, 96-well plates were coated with 100 μ g/ml OVA solution or 1 μ g/ml SV solution overnight at 4°C. The plates were washed and incubated for 1 h with blocking buffer [PBS (–) containing 1% BSA and 0.05% Tween 20]. After blocking, the plates were washed and incubated with 3-fold serially diluted serum for 2 h. To detect the bound Ab, the plates were washed and incubated for 1 h with HRP-conjugated anti-mouse total IgG, IgG1, or IgG2c Ab (Southern Biotech, Birmingham, AL) or anti-monkey total IgG Ab (Sigma, St. Louis, MO). After the plates were washed, TMB substrate solution was added to each well to initiate the color reaction. The reaction was stopped by the addition of 2 N H₂SO₄, and the OD was measured at a wavelength of 450 nm (OD₄₅₀). The Ab titer was defined as the highest serum dilution that yielded an OD₄₅₀ > OD₄₅₀ of the negative-control serum. A DS Mouse IgE ELISA (OVA) kit (DS Pharma Biomedical, Osaka, Japan) was used to measure the concentration of anti-OVA IgE in the sera.

Evaluation of cellular immune responses

To evaluate the cellular immune responses, splenocytes (2×10^6 cell/well) were prepared and incubated with complete RPMI 1640 medium, containing 20 μ g/ml OVA class I peptide, 20 μ g/ml OVA class II peptide, or 20 μ g/ml OVA Ag for 48 h at 37°C under 5% CO₂. The concentrations of IFN- γ , IL-5, and IL-13 in the cell culture supernatants were measured with the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN).

Two-photon imaging of draining lymph nodes

OVA–Alexa Fluor 647 (Invitrogen, Carlsbad, CA) and HP- β -CD–FITC (Nanodex, Kanagawa, Japan) were used to visualize the distributions of Ag and HP- β -CD in the inguinal lymph nodes (LNs). The mice were injected intradermally (i.d.) at the tail base with 100 μ g OVA–Alexa Fluor 647 mixed with 3% HP- β -CD–FITC. After 30 min, 10 μ l rat anti-MARCO Ab (clone ED31; Serotec, Kidlington, U.K.) was injected i.d. The inguinal LNs were excised 30 min later and visualized with two-photon excitation microscopy (FV1000MPE; Olympus, Tokyo, Japan). The imaging data were analyzed with Volocity 3D Image Analysis Software (PerkinElmer, Waltham, MA). Pearson's correlation was calculated using Volocity colocalization analysis (PerkinElmer).

Evaluation of OVA uptake in draining LNs

To evaluate Ag uptake, we used DQ–OVA (Invitrogen), a self-quenching conjugate of OVA that displays bright green fluorescence upon proteolytic degradation. The mice were injected s.c. with PBS (–), 100 μ g DQ–OVA, or 100 μ g DQ–OVA mixed with 30% HP- β -CD (total volume, 200 μ l/mouse), and the LNs were excised 24 h later. The LNs were cut and incubated in CO₂-independent medium (Life Technologies, CA) containing 1 mg/ml collagenase D and 2 μ g/ml DNase I (both from Roche, Penzberg, Germany) for 30 min at 37°C. The cells (2×10^6 cell/sample) were prepared in FACS buffer [PBS (–) containing 1% FCS and 0.1% NaN₃]. Fc γ R was blocked with anti-mouse CD16/32 Ab (clone 93; eBioscience, San Diego, CA). Dead cells were stained with 7-aminoactinomycin D (eBioscience) and excluded from the analysis. To detect cells, Pacific Blue–conjugated anti-CD11c Ab (clone N418; BioLegend, San Diego, CA) was used. OVA uptake was assessed with a FACS LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ). The data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Gene expression analysis

Mice ($n = 3$) were injected i.d. or i.p. with a total volume of 200 μ l 30% HP- β -CD in PBS (–). The vehicle-control mice ($n = 3$) were injected with the same volume of PBS (–) without adjuvant. After 6 h, the inguinal LNs, liver, and spleen were removed and stored in RNAlater RNA Stabilization Reagent (Life Technologies). Each sample in RNAlater was homogenized in Buffer RLT (QIAGEN, Hilden, Germany) by adding a 5-mm-diameter zirconium bead (AS ONE, Osaka, Japan) and shaking with a Mixer Mill 300 (QIAGEN) at 20 Hz for 5 min. The total RNA was isolated and

purified from the sample homogenates with an RNeasy Mini Kit (QIAGEN), according to the manufacturer's instructions. The gene expression profiles were determined using the 3' IVT Express Kit and the GeneChip Mouse Genome 430 2.0 Array (both from Affymetrix, Santa Clara, CA), according to the manufacturer's instructions. The expression values were normalized to the median value of each GeneChip readout. The resulting digital image files were preprocessed with the Affymetrix Microarray Suite version 5.0 algorithm (MAS5.0). The fold-change values were calculated as the ratio of the mean value for the treated samples/mean value for the vehicle-control samples. The presence/absence (PA) call in MAS5.0 was customized as described below. When the ratio was >1 , the PA call was dependent on the treated samples, and when the ratio was ≤ 1 , the call was dependent on the vehicle-control samples. Dominant calls (over half) were applied to a set of samples as the customized PA call, "P1" and "A0" (e.g., when the ratio < 1 and the PA call of control samples were "P," "P," and "A," the customized PA call of the set was "P1"). The MAS5.0 data and PA calls were analyzed with the Bioconductor Affy package for R (<http://www.bioconductor.org>). The p values indicating the significance of differentially expressed genes were calculated using a t test when comparing the normalized treated samples with the normalized vehicle-control samples. For our subsequent analysis, we only included probes for which the fold change between the control and the stimulated samples was >2 . We excluded a probe when it was flagged "absent" (i.e., the PA call was "A0"). The fold change of the probes with PA call "A0" were replaced with 0. Finally, we used Z-score scaling for all of the probes to generate Fig. 3. All datasets were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE63332.

Evaluation of the generation of Tfh cells

Bcl6^{fl/+} or *Bcl6^{fl/fl}* mice were immunized s.c. twice with 100 μ g OVA mixed with 30% HP- β -CD [total volume: 200 μ l in PBS (-)/mouse] on day 0 and 10 d after the first immunization. Inguinal LNs were collected 7 d after the second immunization. To detect Tfh cells, PE anti-mouse CD4 (clone RM4-5; BioLegend), allophycocyanin anti-mouse CD279 (PD-1; clone 29F.1A12; BioLegend), and FITC anti-mouse CD185 (CXCR5; clone L138D7; BioLegend) were used. Fc γ R was blocked with anti-mouse CD16/32 Ab (clone 93; eBioscience). Tfh cell generation was assessed with a BD LSRFortessa (BD Biosciences). The gating strategy is shown in Fig. 4A. The data were analyzed with FlowJo software (Tree Star).

Cytotoxicity assay

Cell viability after exposure to HP- β -CD was assessed in HEK293T cells (5×10^4 cell/well). Cytotoxicity was examined with a water-soluble tetrazolium salt-8 assay using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan), according to the manufacturer's instructions.

Measurement of dsDNA concentrations at injection sites

The mice were injected s.c. with 200 μ l PBS (-) or 30% HP- β -CD. After 0.25, 0.5, 1, 3, 6, and 24 h, 400 μ l PBS (-) was injected into the injection site, and the liquid in the pouch was collected. The liquid volume was adjusted to 1000 μ l with PBS (-), and the acellular fraction was collected by centrifugation at $400 \times g$ for 5 min at 4°C. The concentration of dsDNA in the acellular fraction was measured with a Qubit dsDNA HS Assay Kit (Invitrogen), according to the manufacturer's protocol.

Adjuvant activity of HP- β -CD in a murine influenza virus infection model

Mice were immunized s.c. twice with 3 μ g SV mixed with PBS (-) or 3–30% HP- β -CD (total volume: 200 μ l/mouse) on day 0 and 14 d after the first immunization. Sera were collected 14 and 21 d after the first immunization. Eight days after the second immunization, the mice were infected intranasally with 4×10^4 50% tissue culture infective dose (TCID₅₀) clinically isolated A/Osaka/129/2009 [A(H1N1)pdm09] influenza virus (19), which was obtained from Tetsuo Kase (Osaka Prefectural Institute of Public Health, Osaka, Japan). The body weights and survival rates of the mice were monitored for 14 d postinfection.

Adjuvant activity of HP- β -CD in a cynomolgus macaque model

Twelve male 3–5-y-old cynomolgus macaques (*Macaca fascicularis*) were obtained from the Tsukuba Primate Research Center (TPRC) of the National Institute of Biomedical Innovation and randomly assigned to four groups ($n = 3$). The macaques were immunized s.c. with saline or 5 μ g New Caledonia/20/1999 (H1N1) SV mixed with PBS (-), 3% HP- β -CD, or 30% HP- β -CD (total volume: 500 μ l/macaque) on day 0 and 14 d after the

first immunization. Sera were collected at -2, 2, 4, 6, and 8 wk after the first immunization, and HA-specific IgG titers were measured with ELISAs. The experimental protocol was approved by the Animal Welfare and Animal Care Committee of the TPRC. All macaques were housed and handled by veterinarians in accordance with the Guidelines for Laboratory Animals of the TPRC.

Statistical analysis

The statistical significance of differences between groups was determined with the Student t test. The survival curves postinfection were compared with a Kaplan–Meier analysis (log-rank test and Wilcoxon test) using the statistical analysis software SAS for Windows (version 9.2; SAS Institute).

Results

HP- β -CD induces Th2 responses in a mouse model

To examine the activity of HP- β -CD as a vaccine adjuvant, we immunized C57BL/6 mice s.c. with LPS-free OVA, with or without different doses of HP- β -CD (3, 10, or 30%). Then the OVA-specific total IgG, IgG1, and IgG2c titers in the sera were determined with ELISAs. After the first immunization (day 10), the anti-OVA total IgG and IgG1 Ab titers were enhanced by all of the doses of HP- β -CD tested (Fig. 1A). In particular, the anti-OVA total IgG and IgG1 titers in the OVA/30% HP- β -CD group were significantly higher than those in the group treated with OVA alone. After boosting, the IgG2c response was also weakly induced, even by 3% HP- β -CD. Thus, all of the Ab titers in the OVA/30% HP- β -CD group were significantly increased on day 31 (21 d after boosting) relative to those in the group treated with OVA alone (Fig. 1A).

The T cell responses also were examined with cytokine ELISAs. Splenocytes from immunized mice were stimulated with OVA Ag or OVA-derived MHC class I- and MHC class II-restricted peptides. After 48 h, the production of IFN- γ , IL-5, and IL-13 was compared with that in splenocytes immunized with K3 CpG-ODN (a typical Th1-type adjuvant) or alum (a typical Th2-type adjuvant). Although HP- β -CD did not enhance IFN- γ production compared with K3 CpG-ODN, OVA/HP- β -CD immunization significantly increased IL-5 and IL-13 production, and the levels of these Th2-type cytokines were similar to those induced with alum (Fig. 1B). These results indicate that HP- β -CD elicits a Th2-type cell-mediated immune response when it is coadministered s.c. with OVA protein.

We then compared the IgE response induced by OVA/HP- β -CD immunization with that induced by alum. Alum is known to induce a high IgE response in animals and is commonly used to generate allergy models in mice (20). Mice were immunized s.c. twice with either OVA/30% HP- β -CD or OVA/alum. We also included K3 CpG-ODN, which is known to suppress Ag-specific IgE (21), as the negative control. Seven days after the booster immunization, the serum anti-OVA IgE concentrations were measured by ELISA. HP- β -CD induced a lower OVA-specific IgE response than did alum, whereas K3 induced virtually no IgE response (Fig. 1C). Interestingly, although HP- β -CD induced typical Th2-type cytokine (IL-5 and IL-13) responses (Fig. 1B), HP- β -CD also significantly increased the IgG2c titer, an indicator of the Th1-type immune response, in mice relative to that induced with OVA alone, although the levels of IgG2c were lower than those induced with K3. Taken together, these results suggest that HP- β -CD is a potent Th2-inducible adjuvant for OVA Ag, but it induces IgE less potently than does alum.

Additionally, we examined the adjuvant activity of HP- β -CD via several routes. When OVA/HP- β -CD was injected via the i.p. route, weaker adjuvant activity was observed compared with the s.c. route. With i.d. injection, Ab titer was elevated as seen with the s.c. route; however, skin inflammation was observed at the local injection site. No adjuvant activity of HP- β -CD was observed when

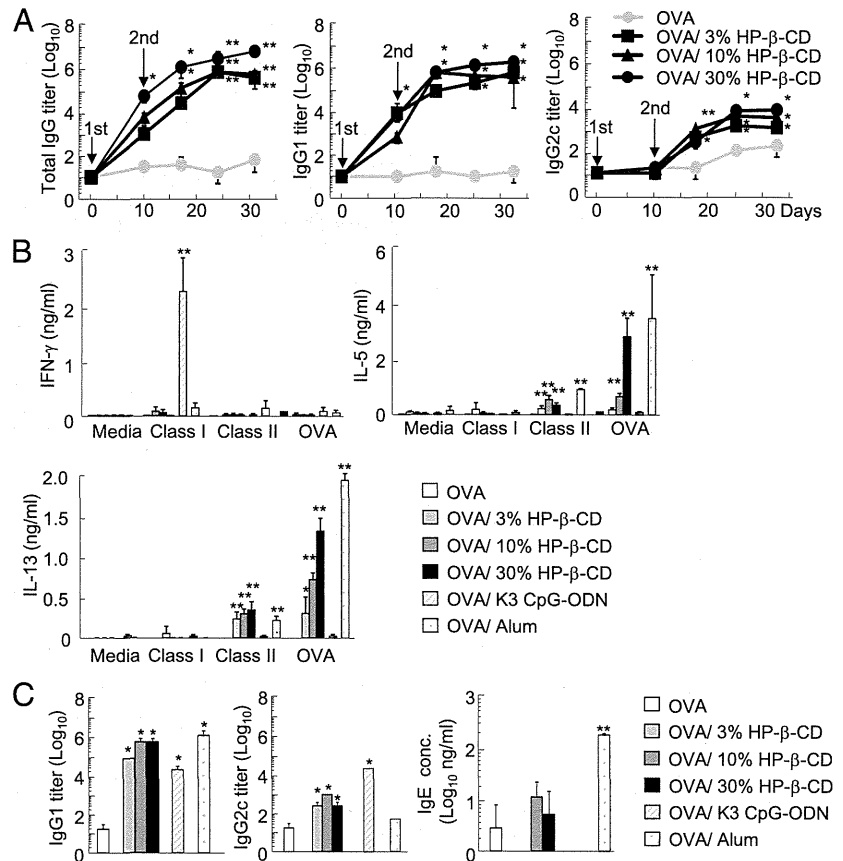


FIGURE 1. Humoral and cellular immune responses in mice after s.c. immunization with OVA/HP- β -CD. **(A)** Anti-OVA total IgG, IgG1, and IgG2c titers were measured with ELISAs at 10, 17, 24, and 31 d after the first immunization with OVA or OVA/HP- β -CD (each group, $n = 3$). **(B)** Splenocytes were collected from mice immunized with OVA, OVA/HP- β -CD, OVA/K3 CpG-ODN, or OVA/alum (each group, $n = 3$) at 10 d after the second immunization and stimulated with class I peptide (OVA_{257–264}), class II peptide (OVA_{323–339}), or OVA Ag. After incubation for 48 h, IFN- γ , IL-5, and IL-13 were measured in the supernatants with ELISAs. **(C)** IgG1 and IgG2c titers and IgE concentrations were compared 7 d after the second immunization (each group, $n = 3$). Data are representative of two independent experiments; error bars denote SD. * $p < 0.05$, ** $p < 0.01$ versus OVA group on Student t test.

given via i.m. injection. These data suggest that the s.c. route results in adjuvanticity without inducing skin inflammation (data not shown).

HP- β -CD did not induce a systemic cytokine response

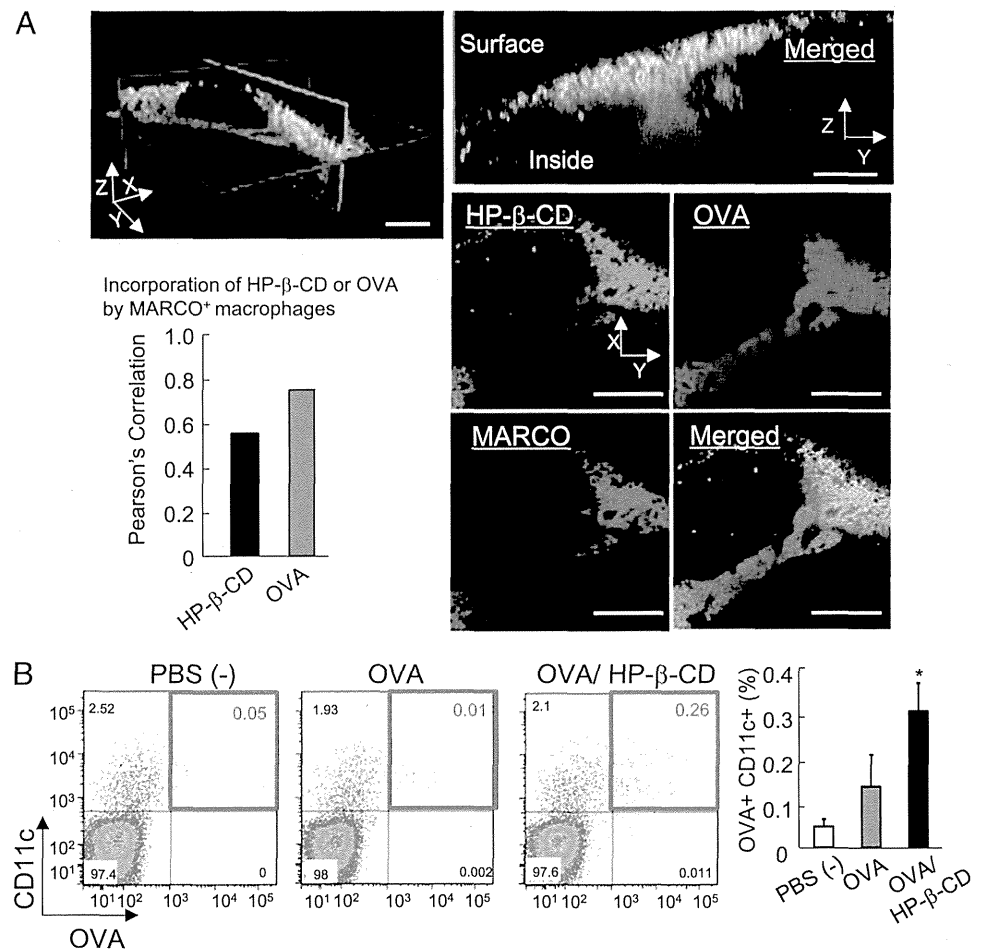
To further clarify the effects of HP- β -CD on the immune system, we investigated HP- β -CD-induced cytokine responses in naive mouse splenocytes *in vitro*. Splenocytes were stimulated with 200–5000 μ g/ml (equivalent to 0.02–0.5%) HP- β -CD, and the cytokine concentrations in the supernatant were measured 24 h later using a multiplex assay. There was no significant increase in the cytokines (Supplemental Fig. 1A). Importantly, we could not stimulate the splenocytes with >0.5% HP- β -CD because higher concentrations appeared to cause immediate cell death *in vitro*. Therefore, we also investigated the cytokine responses *in vivo*. Mice were injected s.c. with 30% HP- β -CD, and their sera were collected 1, 3, and 8 h after injection. R848 also was injected into a separate group of mice as the positive control for systemic cytokine responses. Consistent with the *in vitro* result, we did not observe any detectable cytokine response in the sera at any time point examined when 30% HP- β -CD was injected, whereas injection of R848 induced significant responses in many cytokines (Supplemental Fig. 1B). These results indicate that HP- β -CD does not induce detectable levels of systemic cytokines either *in vitro* or *in vivo* (Supplemental Fig. 1).

Distribution of HP- β -CD and OVA in draining LNs

Without inducing proinflammatory responses *in vitro* and *in vivo*, HP- β -CD enhanced humoral responses after immunization. This result prompted us to investigate which cells incorporate HP- β -CD and play crucial roles in the adjuvanticity. We first used flow cytometry to investigate the cells in draining inguinal LNs after immunization; however, we could not identify the cells at a dose

of 3% (the maximum dose of FITC-labeled HP- β -CD applicable to cells as a result of the HP- β -CD-FITC stock concentration limitation; data not shown). Therefore, we next performed two-photon microscopic analysis, which allows imaging of tissue to a substantial depth. We focused first on Siglec-1⁺ (also called MOMA-1) macrophages, because inactivated influenza virus is captured by Siglec-1⁺ macrophages, which induces humoral immune responses (22). The mice were injected with OVA-Alexa Fluor 647/3% HP- β -CD-FITC, and the explanted inguinal LNs were analyzed 1 h later. HP- β -CD was not incorporated into Siglec-1⁺ macrophages (data not shown), but the majority of HP- β -CD was incorporated into macrophages carrying the macrophage receptor with collagenous structure (MARCO⁺ macrophages) (23), and some HP- β -CD was found in the B cell follicle covering the CD169⁺ subcapsular sinus macrophage area (Fig. 2A, Supplemental Video 1). When the distribution patterns of HP- β -CD and OVA were compared using Velocity's colocalization analysis, injected OVA was distributed more selectively in MARCO⁺ macrophages than was HP- β -CD (Pearson correlation: HP- β -CD, +0.548, OVA, +0.756) (Fig. 2A, Supplemental Video 1), which is consistent with our recent observation (24). The recent study indicated that, although OVA and CpG (K3-SPG) were incorporated primarily into MARCO⁺ macrophages, the macrophages are dispensable for inducing adaptive immune responses by CpG (K3-SPG). In addition, the study indicated the involvement of dendritic cells (DCs) in the adjuvanticity of CpG (K3-SPG). Based on these data, we next focused on DCs in LNs. To further clarify the uptake of Ag by DCs in LNs, mice were injected s.c. with DQ-OVA or DQ-OVA/30% HP- β -CD, and the uptake of OVA Ag by CD11c⁺ cells was analyzed 24 h later using flow cytometry. Although the overall uptake was very small, OVA uptake by CD11c⁺ DCs was enhanced significantly by HP- β -CD (Fig. 2B), suggesting that HP- β -CD acts as an Ag-delivery system

FIGURE 2. Distribution of HP- β -CD and OVA in LNs after injection. **(A)** Mice were injected i.d. with OVA–Alexa Fluor 647/HP- β -CD–FITC; anti-MARCO Ab was injected i.d. 30 min later. LNs were collected 30 min after the Ab injection (i.d.). Two-photon images of the LN, HP- β -CD (green), OVA (blue), MARCO⁺ macrophages (red), and merged image. The distribution patterns of HP- β -CD and OVA were measured by Velocity’s colocalization analysis. Scale bar, 100 μ m. **(B)** Mice were injected s.c. with PBS (–), DQ-OVA, or DQ-OVA/30% HP- β -CD, and the LNs were collected after 24 h ($n = 3$). OVA uptake was assessed by flow cytometry. OVA⁺CD11c⁺ cells increased after DQ-OVA/30% HP- β -CD treatment. The bar graph shows the percentages of CD11c⁺/OVA⁺ cells. Data are representative of two independent experiments; error bars denote SD. * $p < 0.05$, versus PBS (–)-injected mice, Student t test.



to CD11c⁺ cells. We also examined DC maturation after HP- β -CD injection in vivo but did not detect any upregulation of CD40 in the DCs (data not shown). It was difficult to investigate the function of MARCO⁺ macrophages by flow cytometry (25). Collectively, we found a novel immunological mechanism of HP- β -CD that enhances Ag uptake by DCs.

HP- β -CD induced immune-related gene expression after local administration

To further clarify the mode of action of HP- β -CD, we performed a comprehensive mRNA GeneChip analysis. In our preliminary experiments, we noted that the adjuvant activity of HP- β -CD was weak after i.p. injection compared with that after s.c. or i.d. injection. Therefore, we compared gene expression in the draining inguinal LNs and other organs, such as the spleen and liver, 6 h after i.d. injection of HP- β -CD into the tail base (local) or i.p. injection (systemic). After local i.d. injection of HP- β -CD, the expression of substantial numbers of genes was induced >2-fold in the draining LNs, spleen, and liver (Fig. 3A). In contrast, i.p. injection of the same amount of HP- β -CD induced only limited changes in gene expression in the spleen and liver (Fig. 3A). We also performed a cluster analysis and categorized the induced genes into seven major groups (Supplemental Fig. 2A). Each group of genes was examined using Gene Ontology (GO) term analysis (Supplemental Fig. 2B). This analysis showed that HP- β -CD induced the expression of genes related to the innate immune response and the inflammatory response in the LNs, spleen, and liver after i.d. injection but not after i.p. injection (Fig. 3B, Supplemental Fig. 2, group 7), consistent with its route-dependent adjuvant activity. The GO term “defense response to virus” also

was called in the group 1 cluster (Supplemental Fig. 2, group 1); correspondingly, the IFN response genes also were induced in the LNs after i.d. injection of HP- β -CD (Fig. 3C). The analysis of individual genes in Supplemental Fig. 2 revealed that those associated with the Th2-type immune response, including *Ptgs2* (26), *Nfil3* (27), and *Il33* (28), also were upregulated after i.d. injection of HP- β -CD (Fig. 3D). The induction of *Il1 β* and *Myd88* genes (Fig. 3D) also suggested that inflammasomes could be activated in vivo by HP- β -CD. These data suggest that, despite the lack of detectable levels of cytokine production in the serum (Supplemental Fig. 1B), our GeneChip analysis demonstrated that HP- β -CD induced the expression of inflammatory and immune-related genes, including type I IFNs and *IL1 β* , after i.d. injection but not after i.p. injection. These data are consistent with the route-dependent adjuvant activity of HP- β -CD.

Tfh cells are required for the adjuvanticity of HP- β -CD

Our GeneChip analysis found the induction of immune-related gene expression in LNs after HP- β -CD injection. A recent study indicated that Tfh cells, a Th cell subset present in germinal centers (GCs) in LNs, facilitate the generation of long-lived plasma cells and memory B cells, which are required for long-term Ab responses (29). To investigate the involvement of Tfh cells in the adjuvanticity of HP- β -CD, we evaluated their generation in LNs from *Bcl6*^{fl/fl} or *Bcl6*^{fl/fl} mice immunized with OVA or OVA/30% HP- β -CD. After the second immunization, the generation of Tfh cells was significantly increased by HP- β -CD in *Bcl6*^{fl/fl} mice ($p < 0.05$), and it was significantly decreased in *Bcl6*^{fl/fl} mice ($p < 0.0001$) (Fig. 4B). We also investigated the humoral responses between *Bcl6*^{fl/fl} and *Bcl6*^{fl/fl} mice after OVA/30% HP- β -CD

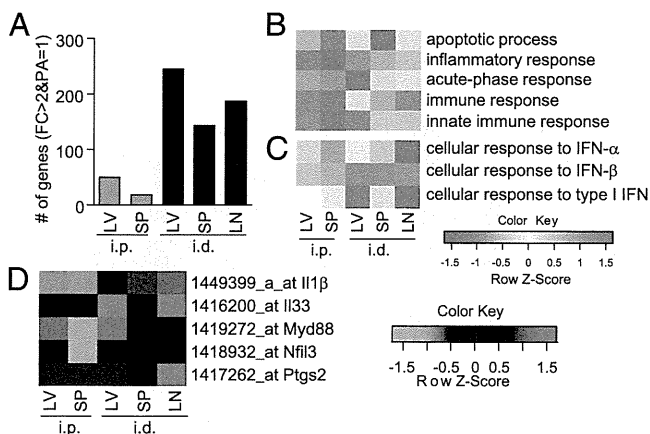


FIGURE 3. HP- β -CD induced substantial expression of immune-related genes after its local (i.d.) injection but not after its systemic (i.p.) injection. **(A)** The number of genes for which the fold change was >2 between HP- β -CD ($n = 3$) and PBS ($n = 3$) treatments and for which the customized PA call = 1 after 6 h when examined with a GeneChip array. **(B and C)** The expression levels of a selected immune-related set of genes categorized by GO terms are shown in the form of Z-scored fold-change values. The fold-change values were averaged and normalized using all of the genes in the corresponding GO term. The GO terms were derived and evoked from Supplemental Fig. 2. **(D)** Selected feature genes from Supplemental Fig. 2, presented by their Z-scores. The data are representative of two independent experiments with similar results. LV, liver; SP, spleen.

immunization. Consistent with the result of flow cytometric analysis, the anti-OVA IgG1 titer of *Bcl6*^{fl/fl} mice was significantly lower than that of *Bcl6*^{+/+} mice after the second immunization ($p < 0.01$) (Fig. 4C). However, the IgG1 titer of *Bcl6*^{fl/fl} mice immunized with OVA/30% HP- β -CD was not decreased to the level of that of *Bcl6*^{fl/fl} mice immunized with OVA alone, suggesting that the GC-independent pathway contributes, in part, to the induction of humoral responses after OVA/HP- β -CD immunization (30). These results suggested that the generation of Tfh cells is facilitated by the administration of HP- β -CD and that Tfh cells are required for the high adjuvanticity of HP- β -CD.

IL-21 secreted by Tfh cells can act in an autocrine manner to maintain Tfh cells at various stages of differentiation (29). Meanwhile, IL-21 was reported to prevent Ag-specific IgE response in mice (31). In this study, although HP- β -CD induced Th2 responses, it did not strongly induce an IgE response after immunization (Fig. 1C). To explain the weak IgE response after OVA/HP- β -CD immunization, we investigated IL-21 responses after stimulation of splenocytes from immunized *Bcl6*^{+/+} mice; IL-21 responses were not observed from stimulated splenocytes (data not shown). Further research is necessary to explain why HP- β -CD does not induce strong IgE responses after immunization.

Inflammasome pathway does not contribute to the adjuvanticity of HP- β -CD

Our GeneChip analysis suggested that inflammasomes can be activated by HP- β -CD in vivo (Fig. 3B). Th2-inducible adjuvants, such as alum and silica, also were shown to activate inflammasomes in macrophages, whereas the role of inflammasomes in their adjuvant activity has been questioned (5). To clarify the immunological mechanisms and pathways essential for the adjuvant activity of HP- β -CD, we examined whether it activates inflammasomes in macrophages in vitro. Peritoneal macrophages were primed with LPS and stimulated with HP- β -CD 18 h later. Compared with alum, which induced high amounts of IL-1 β , 5000 μ g/ml (0.5%) HP- β -CD did not enhance the production of IL-1 β by macrophages (data not shown). Because HP- β -CD is cytotoxic (see

Fig. 5C), we could not examine the activation of inflammasomes at a higher concentration of HP- β -CD (equivalent to the maximum dose used in the immunization experiments performed in vivo) (Fig. 1). Therefore, we examined the humoral responses in *Asc*^{-/-} mice and *Caspase 1*^{-/-} mice, which are known to be deficient in inflammasome activation (32). The humoral response after s.c. immunization with OVA/HP- β -CD was not reduced in either gene-KO mouse strain (data not shown). Although these data do not exclude the possibility that the inflammasomes were activated in vivo after HP- β -CD injection, inflammasome activation was not required for the adjuvant activity of HP- β -CD.

MyD88 is involved in the adjuvant activity of HP- β -CD

To identify the pathway essential for the adjuvant activity of HP- β -CD, we focused on MyD88, a critical adapter protein of TLRs and the IL-1R superfamily, which are involved in the downstream activation of NF- κ B and MAPK (33). TLRs are also key players in the activation of innate immunity and recognize various adjuvants and pathogen-associated molecular patterns. Mice lacking *Myd88* were immunized s.c. twice with OVA/HP- β -CD. After the second immunization, the anti-OVA IgG and IgG1 titers of *Myd88*^{-/-} mice were significantly lower than those of *Myd88*^{+/+} mice (Fig. 5A). Interestingly, *Myd88*^{-/-} mice immunized with OVA/HP- β -CD showed significantly higher total IgG and IgG1 titers than did WT mice immunized with OVA alone. These results suggest that both MyD88-dependent and -independent signaling pathways are involved in the adjuvant activity of HP- β -CD. A TLR-screening assay was performed in vitro to identify the upstream receptor of MyD88-dependent signaling. The TLR4-signaling pathway was activated slightly by HP- β -CD (Supplemental Fig. 3A), so we examined whether TLR4 is involved in the adjuvant activity of HP- β -CD in vivo; however, the humoral response was not reduced in *Tlr4*^{-/-} mice (Supplemental Fig. 3B). We then investigated other TLRs or members of the IL-1R superfamily by s.c. immunizing other *Tlrs*, *Il1R*, *Il18R*, and *St-2* KO mice. ST-2 is a receptor that forms a complex with IL-1R accessory protein; it

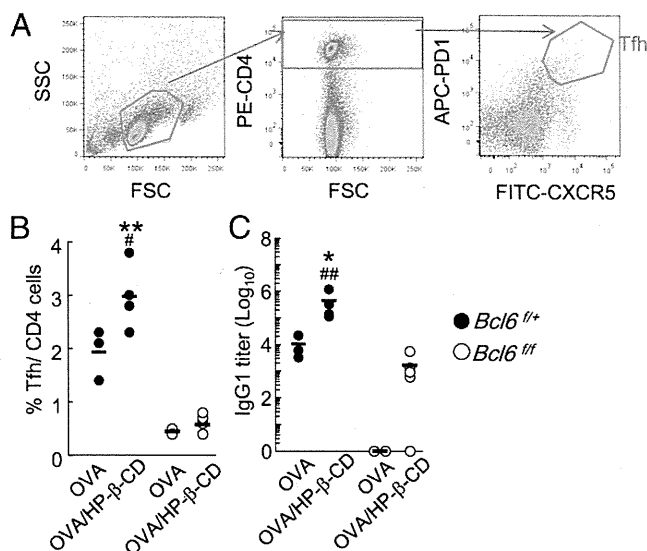
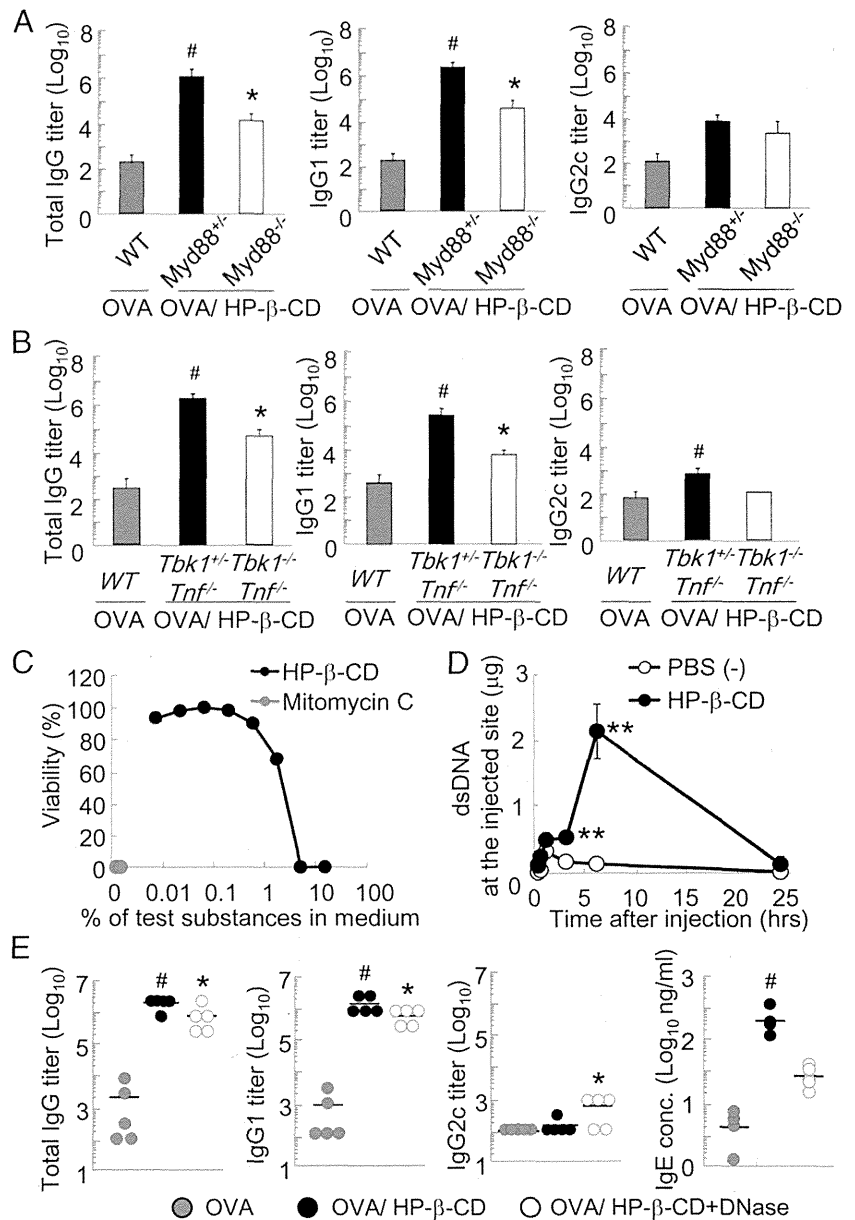


FIGURE 4. Tfh cell development and humoral responses after OVA/HP- β -CD immunization. *Bcl6*^{+/+} or *Bcl6*^{fl/fl} mice were immunized s.c. with OVA or OVA/30% HP- β -CD ($n = 2-5$). **(A)** Flow cytometric analysis was performed to assess the generation of Tfh cells. **(B)** Seven days after the second immunization, Tfh cell generation was measured. **(C)** Anti-OVA IgG1 titers were measured with ELISAs 7 d after the second immunization. Error bars denote SD. # $p < 0.05$, ## $p < 0.01$ versus *Bcl6*^{+/+} mice immunized with OVA alone, * $p < 0.01$, ** $p < 0.0001$ versus *Bcl6*^{fl/fl} mice immunized with OVA/30% HP- β -CD, Student t test.



can bind IL-33 directly, and it mediates Th2 responses (28). All of the examined KO mice, including TLR7/9 double-deficient and TLR2/4/9 triple-deficient mice, showed anti-OVA total IgG titers that were similar to those of WT mice 7 d after the booster immunization (Supplemental Fig. 3B). Notably, after stimulation with HP-β-CD, no significant increases in proinflammatory cytokines, chemokines, or growth factors were detected in vitro (Supplemental Fig. 1A). Taken together, these results suggest that HP-β-CD itself may activate TLR4 in vitro. However, in vivo, the adjuvant activity of HP-β-CD is independent of any single TLR, TLR7/9, TLR2/4/9, or the IL-1R superfamily, although the adjuvant activity of HP-β-CD is partially dependent on MyD88 signaling.

HP-β-CD induces dsDNA release, and TBK1 contributes to its adjuvant activity

The results described above suggest that a MyD88-independent pathway is involved in the adjuvant activity of HP-β-CD. Our GeneChip analysis suggested that HP-β-CD induces type I IFN-related gene responses in the draining LNs (Fig. 3). Therefore, we examined the involvement of IFN-αβ receptor (IFNAR), IRF3/7, and TBK1 by using mice in which the representative gene is

knocked out. *Tbk1*^{-/-} mice on a TNF-deficient (*Tnf*^{-/-}) background were used because *Tbk1*^{-/-} mice die in utero, and this lethal effect can be rescued in the absence of TNF (34). The humoral responses in IFNAR2 and IRF3/7 double-KO mice were similar to those in WT mice (data not shown). However, the anti-OVA IgG titer in *Tbk1*^{-/-} mice was significantly lower than that in *Tnf*^{-/-} mice (Fig. 5B), suggesting that TBK1 also contributes to the adjuvant activity of HP-β-CD but that its activity is independent of type I IFN responses.

We showed that alum induces the release of dsDNA from host cells, resulting in TBK1/IRF3-dependent, but IFNAR2-independent, Th2 responses (16). The results described above indicating TBK1 involvement prompted us to examine the release of dsDNA from host cells after the local injection of HP-β-CD. We first examined the cytotoxicity of HP-β-CD against HEK293T cells in vitro. HP-β-CD started killing HEK293 cells at a concentration of 0.5%; at 1.5%, almost all of the cells were killed. However, the overall cytotoxicity of HP-β-CD was 1,000,000 times lower than that of mitomycin C, which damages cells by DNA cross-linking (35) (Fig. 5C). In an in vivo study, we observed the adjuvant activity of 3% HP-β-CD in mice (Fig. 1A). These data suggest that HP-β-CD

potentially causes the release of dsDNA from host cells *in vivo*, similar to alum. To examine this possibility, we tested whether HP- β -CD induced the release of host DNA *in vivo*. HP- β -CD was injected s.c. into mice, and the concentration of host DNA at the injection site, which formed a liquid pouch under the skin, was measured. Increasing amounts of dsDNA, triggered by HP- β -CD, were detected until 6 h, after which they decreased and returned to background levels at 24 h after injection (Fig. 5D). This result suggests that HP- β -CD triggers temporary host dsDNA release at its injection site. Furthermore, DNase I treatment at the time of immunization significantly reduced the total IgG, IgG1, and IgE titers but enhanced the IgG2c response (Fig. 5E). This result strongly suggests that HP- β -CD triggers dsDNA release, which acts as a DAMP, triggering a signal through the TBK1-dependent pathway and resulting in an enhanced Th2-type immune response. However, at the same time, HP- β -CD induces a MyD88-dependent response, which may modulate the IgE response upregulated by the dsDNA/TBK1 axis. Further study is required to identify the critical upstream/downstream pathways of MyD88 and TBK1 induced by the local administration of HP- β -CD.

HP- β -CD shows potent adjuvant activity with SV

Although HP- β -CD temporarily triggered the release of dsDNA (Fig. 5D), it did not elicit strong IgE responses (Fig. 1C), and it induced almost no systemic cytokine responses in mice (Supplemental Fig. 1B). This suggests that HP- β -CD is a suitable adjuvant for a current split influenza vaccine. Most influenza vaccines are used to immunize healthy individuals, so minimizing the toxicity of the vaccine is an absolute requirement for the adjuvant. We evaluated the adjuvant activity of HP- β -CD using an influenza SV immunization model in mice and macaques. Mice were immunized s.c. twice with SV only or with SV/3–30% HP- β -CD, and their total IgG titers were measured with an ELISA. After the booster immunization, the anti-HA total IgG titer was significantly enhanced by all doses of HP- β -CD tested (Fig. 6A). After the immunized mice were challenged with a lethal dose of A/Osaka/129/2009 [A(H1N1)pdm09] influenza virus, their body weights

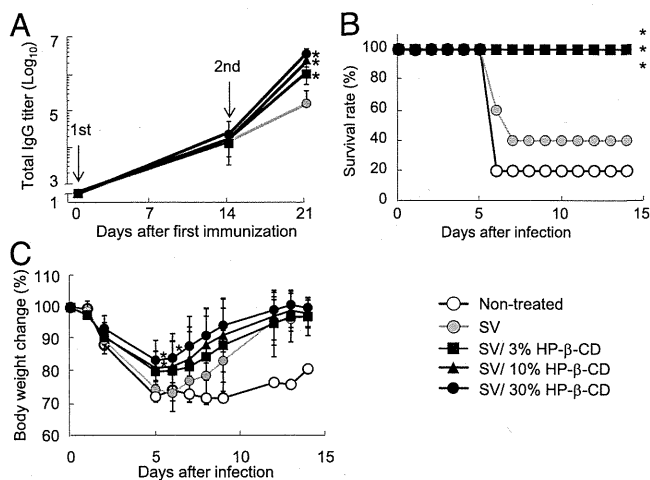


FIGURE 6. Efficacy of SV/HP- β -CD against influenza virus infection in mice. (A) Mice were immunized s.c. twice with SV or SV/HP- β -CD on day 0 and 14 d after the first immunization, and the total anti-HA IgG titers were measured with ELISA at 14 and 21 d ($n = 5$). (B and C) Mice were challenged with 4×10^4 50% TCID₅₀ A/Osaka/129/2009 [A(H1N1)pdm09] influenza virus 21 d after the first immunization. Their body weight changes and survivals were monitored. Data are representative of two independent experiments. Error bars denote SD. * $p < 0.05$, versus SV group, Student t test (A and C) or Kaplan–Meier analysis (log-rank test and Wilcoxon test) (B).

and survival rates were determined for 14 d. The survival and body weight parameters of the SV/HP- β -CD-immunized groups were significantly better than were those of the group immunized with SV only (Fig. 6B, 6C).

To further estimate the adjuvant activity of HP- β -CD in humans, cynomolgus macaques were immunized s.c. twice with saline, SV, SV/3% HP- β -CD, or SV/30% HP- β -CD, and their anti-HA IgG titers were evaluated every 2 wk. The anti-HA IgG titers of all macaques in the SV/30% HP- β -CD group were increased 2 wk after the first immunization (Fig. 7A). A boosting effect of HP- β -CD also was observed in this group (Fig. 7A). The anti-HA IgG titers of the SV group varied greatly among individuals (Fig. 7A). When we compared the geometric mean titer of the anti-HA IgG titers of the SV/30% HP- β -CD group and the SV group, the anti-HA IgG titer of the SV/30% HP- β -CD group was 3-fold higher than that of the SV group 2 wk after the first immunization, and it was 2-fold higher than that of the SV group 6 wk after the second immunization (Fig. 7B). Unlike the results for mice, the anti-HA IgG titers in the SV/3% HP- β -CD group were not better than those in the SV group (Fig. 7). These results demonstrate that 30% HP- β -CD displays adjuvant activity with the influenza SV vaccine in both mice and cynomolgus macaques.

Discussion

In addition to their efficacy, one of the most important characteristics of vaccine adjuvants is their safety, because many vaccines are intended for use in healthy individuals. HP- β -CD has been used safely as an excipient for pharmaceutical agents for decades (9). However, no extensive immunological characterization of

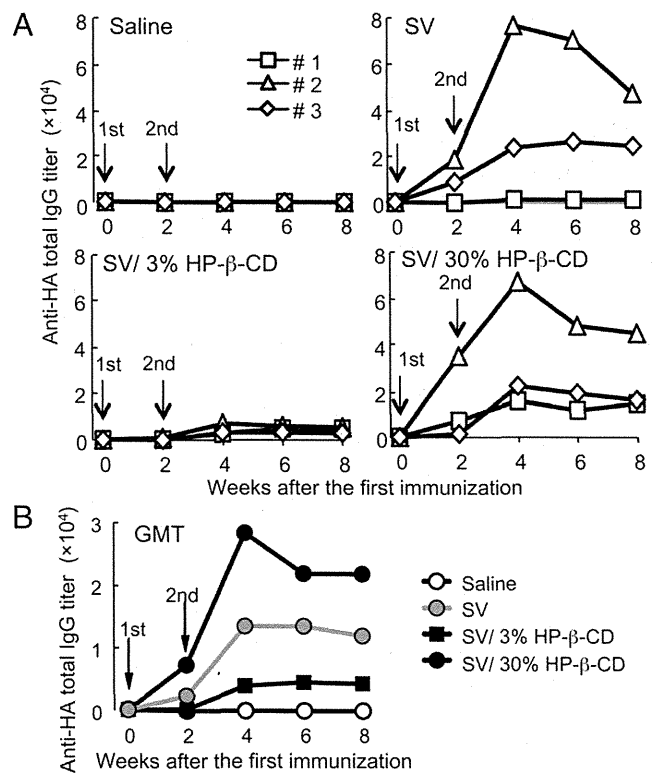


FIGURE 7. Immunogenicity of SV/HP- β -CD in cynomolgus macaques. Cynomolgus macaques were immunized twice with saline, SV, SV/3% HP- β -CD, or SV/30% HP- β -CD on day 0 and 14 d after the first immunization ($n = 3$). The sera were collected every 2 wk, and anti-HA total IgG titers were measured using ELISA. (A) Individual results. (B) Geometric mean titers (GMT) of the groups ($n = 3$).

cyclodextrins as adjuvants has been conducted, so the molecular and cellular mechanisms of their actions are largely unknown. Our characterization of HP- β -CD as a vaccine adjuvant revealed that it induces unique Th2 responses, enhancing Ag-specific IgG titers, including IgG1 and IgG2c titers, by s.c. injection. Furthermore, the adjuvanticity is largely dependent on Tfh cells. Unlike alum, a commonly used adjuvant for many human vaccines throughout the world, HP- β -CD induced little IgE production (Fig. 1A, 1C). A T cell analysis clearly indicated that HP- β -CD preferentially activates Th2 cells over Th1 cells (Fig. 1B). Traditionally, it has been considered that the Th2 and IgE responses are sequential, but our current data indicate that Th2 induction does not always induce strong IgE production. The ability of an adjuvant to induce IgE may be a crucial risk factor affecting the allergenic potential of vaccines. Therefore, HP- β -CD may be a safer adjuvant than alum because it entails less risk for inducing potentially allergic IgE responses. Injection of HP- β -CD obviously induced some acute inflammatory, type I IFN-related and Th2-associated gene responses in LNs. However, no systemic proinflammatory cytokine responses were observed after its injection into mice (Supplemental Fig. 1B), suggesting that the gene expression induced in LNs does not induce systemic proinflammatory cytokine responses, which can be detrimental to the host. Without inducing systemic proinflammatory cytokine responses, HP- β -CD exhibited enough adjuvanticity to improve the immunogenicity and protective efficacy of SV in mice and cynomolgus macaques. Hence, we conclude that HP- β -CD is a well-balanced adjuvant possessing high adjuvant activity and little risk for inducing allergic potential IgE responses and systemic inflammation.

The activation of immune responses in LNs is a major event to establish adaptive immunity (29). Our cellular analysis found enhanced Ag uptake by DCs and generation of Tfh cells in LNs after OVA/HP- β -CD injection (Figs. 2B, 4B). In addition, Tfh cells were required for the high adjuvanticity of HP- β -CD (Fig. 4C). DCs provide signals that upregulate CXCR5 and downregulate CCR7 on naive CD4⁺ T cells, allowing them to migrate to B cell follicles and, consequently, facilitate the generation of Tfh cells (29). Tfh cells are recognized as the key player required for the formation of GCs and the generation of long-lived serological memory (30). Therefore, these results explained why humoral responses were enhanced and maintained by HP- β -CD.

The biological effects of HP- β -CD are reported to include the binding and sequestration of plasma membrane cholesterol and, consequently, the dispersal of lipid rafts, which include lipids and associated plasma membrane-spanning proteins, such as sphingolipids (36). Such treatments are considered to affect the lateral diffusion, aggregation, and function of receptor complexes (37). Bioactive lipids, such as ceramides and sphingosine 1-phosphate, are known to regulate inflammation in cells and to activate several signaling pathways (38). Therefore, it is reasonable to infer that HP- β -CD activates the innate immune response and exerts its adjuvant activity by modifying these lipid-dependent biological processes. Methyl- β -cyclodextrin is reported to disperse lipid rafts, thereby stimulating MyD88-dependent NF- κ B activation in immature B cells, and it partially stimulates the TLR4-signaling pathway in vitro (39). In this study, the results of an in vitro TLR-screening assay supported those findings (Supplemental Fig. 3A). However, analysis of gene-KO mice indicated that MyD88, but not TLR4, contributes, in part, to the adjuvant activity of HP- β -CD (Supplemental Fig. 3B), suggesting that signaling pathways other than TLR pathways, such as lipid raft dispersal/MyD88/NF- κ B activation, contribute to the MyD88-dependent mechanism underlying the adjuvant activity of HP- β -CD (Supplemental Fig. 4). This is an important issue for future research.

We also demonstrated that TBK1 contributes, in part, to the adjuvant activity of HP- β -CD (Fig. 5B). HP- β -CD causes the release of several factors, including lipids and dsDNA, that may function as DAMPs to activate the innate immune response. In this study, HP- β -CD induced the temporary release of dsDNA at its injection site. These facts strongly suggest that the adjuvant activity of HP- β -CD is also mediated by host-derived factors, such as DAMPs. Therefore, it is reasonable that the adjuvant activity of HP- β -CD was not diminished in single *Myd88*- or *Tbk1*-deficient mice. Our findings indicate that the activation of several signaling pathways as a result of HP- β -CD injection contributes to its adjuvant activity (Supplemental Fig. 4).

It is important to exploit the safety profile of HP- β -CD for the development of adjuvanted vaccines. Vaccination is the primary strategy used to prevent influenza infection. The efficacy of influenza vaccines in young and healthy adults is estimated to be 70–90%, but it is <17–53% in the elderly (40). The use of an adjuvant to improve the efficacy of a vaccine has been investigated, but minimizing adjuvant toxicity remains one of the major challenges in adjuvant research (41). To exploit the safety profile of HP- β -CD, we evaluated the combined effects of HP- β -CD and SV and demonstrated that HP- β -CD enhanced the protective efficacy of SV against influenza virus infection in mice (Fig. 6). Its adjuvant activity also was examined in cynomolgus macaques (Fig. 7). In the macaque model, the anti-HA IgG titers were not strongly elevated in any of macaques in the SV group. In contrast, immunization with SV/30% HP- β -CD strongly enhanced the anti-HA IgG titers of all of the macaques in the group. To improve the efficacy of SV, it is important to overcome the poor immunogenicity and enhance the humoral responses of weak responders with an adjuvant. In contrast to the results with s.c. injection, we did not find adjuvanticity for HP- β -CD via the i.m. route. The i.m. route is a major route for human vaccines; however, all seasonal influenza SVs are injected s.c. in Japan. Outside of Japan, some influenza vaccines, such as Fluvax (42) and VAXIGRIP (43), are acceptable to inject s.c. Therefore, the lack of adjuvanticity of HP- β -CD via i.m. injection is not a major concern. Collectively, our results suggest that HP- β -CD is a promising adjuvant for enhancing the immunogenicity of s.c. SV injections in humans.

This study has important implications, demonstrating that HP- β -CD is safe and can be used effectively as an adjuvant for SV in humans. It also contributes novel immunological findings that advance cyclodextrin research and promote the innovation of a rationally designed safer adjuvant.

Acknowledgments

We thank Tetsuo Kase for providing the A/Osaka/129/2009 virus. We also thank Akiko Okabe, Mariko Nakamura, Yuko Fujita, Aki Konishi, Kousaku Murase, and Yasunari Haseda for excellent technical assistance with animal husbandry and genotyping, as well as the members of K.J.L.'s and C.C.'s laboratories for valuable comments and help.

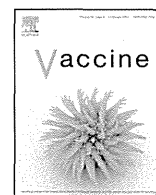
Disclosures

The authors have no financial conflicts of interest.

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Recombinant Ag85B vaccine by taking advantage of characteristics of human parainfluenza type 2 virus vector showed *Mycobacteria*-specific immune responses by intranasal immunization



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ARTICLE INFO

Article history:

Received 24 May 2013

Received in revised form

25 November 2013

Accepted 29 November 2013

Available online 29 January 2014

Keywords:

Human parainfluenza virus

Ag85B

Tuberculosis

Mucosal immunity

ABSTRACT

Viral vectors are promising vaccine candidates for eliciting suitable Ag-specific immune response. Since *Mycobacterium tuberculosis* (Mtb) normally enters hosts via the mucosal surface of the lung, the best defense against Mtb is mucosal vaccines that are capable of inducing both systemic and mucosal immunity. Although *Mycobacterium bovis* bacille Calmette–Guérin is the only licensed tuberculosis (TB) vaccine, its efficacy against adult pulmonary forms of TB is variable. In this study, we assessed the effectiveness of a novel mucosal TB vaccine using recombinant human parainfluenza type 2 virus (rhPIV2) as a vaccine vector in BALB/c mice. Replication-incompetent rhPIV2 (M gene-eliminated) expressing Ag85B (rhPIV2–Ag85B) was constructed by reverse genetics technology. Intranasal administration of rhPIV2–Ag85B induced Mtb-specific immune responses, and the vaccinated mice showed a substantial reduction in the number of CFU of Mtb in lungs and spleens. Unlike other viral vaccine vectors, the immune responses against Ag85B induced by rhPIV2–Ag85B immunization had an advantage over that against the viral vector. In addition, it was revealed that rhPIV2–Ag85B in itself has an adjuvant activity through the retinoic acid-inducible gene I receptor. These findings provide further evidence for the possibility of rhPIV2–Ag85B as a novel TB vaccine.

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Abbreviations: BAL, bronchoalveolar lavage; BCG, *Mycobacterium bovis* bacille Calmette–Guérin; BEAS cells, bronchial epithelial cells; hPIV2, human parainfluenza type 2 virus; pLN, pulmonary lymph node; Mtb, *Mycobacterium tuberculosis*; NHBE, normal human bronchial epithelial; rhPIV2–Ag85B, recombinant hPIV2 expressing Ag85B; TB, tuberculosis.

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1. Introduction

Recombinant viral vector vaccines have several advantages for preventing infection with pathogens [1]. The vaccines induce a full spectrum of immune responses including humoral and cellular immune responses. These immune responses can be initially induced at the viral vector infection site such as mucosal immune responses [2]. Moreover, the viral vector itself has adjuvant activities through the innate immune systems [3]. Pre-existing or post-priming immune responses against the vaccine vector itself, however, could be an obstacle to effective immune responses to recombinant Ag [4]. Negligible immune responses against vector viruses compared with recombinant vaccine Ags after immunization is considered most desirable for recombinant viral vaccines.