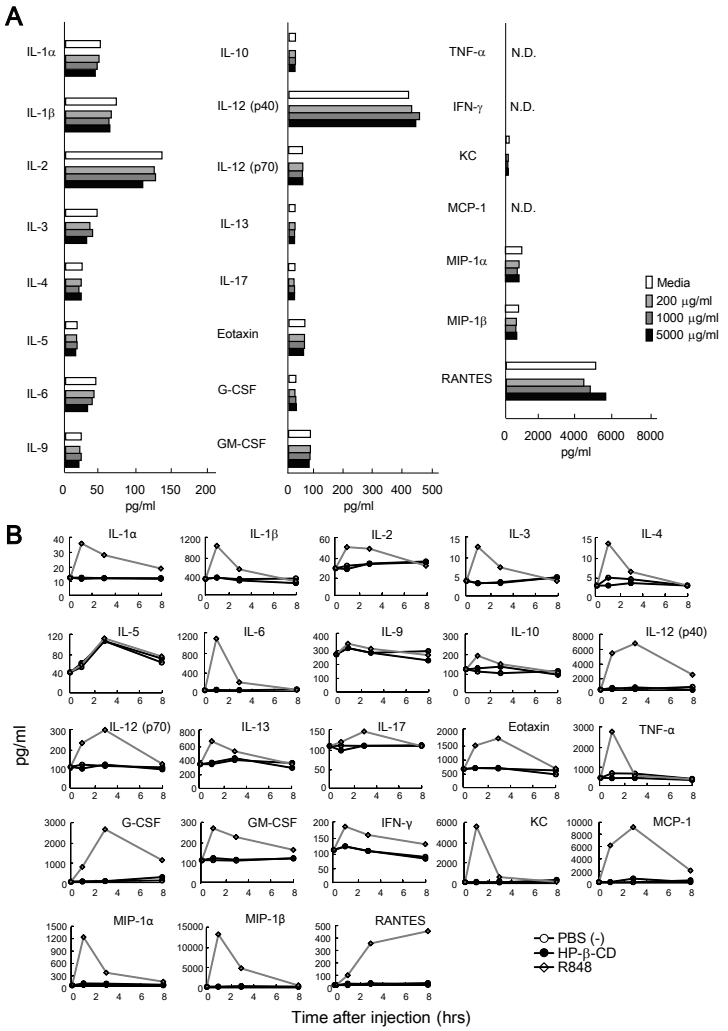
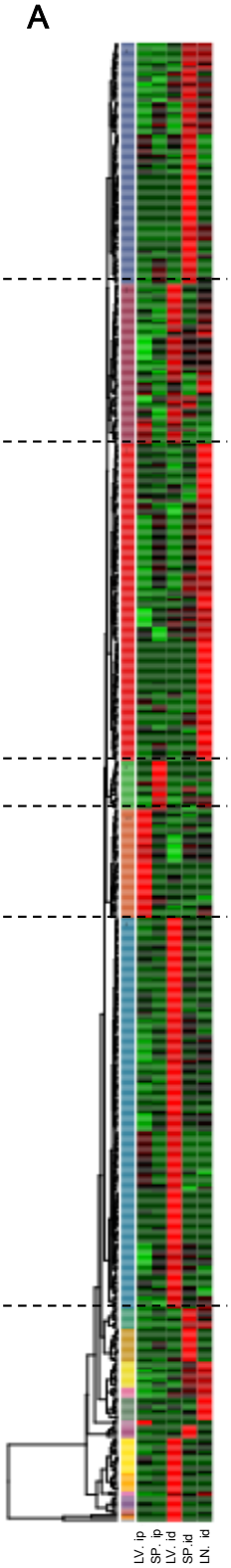
Supplemental figures.



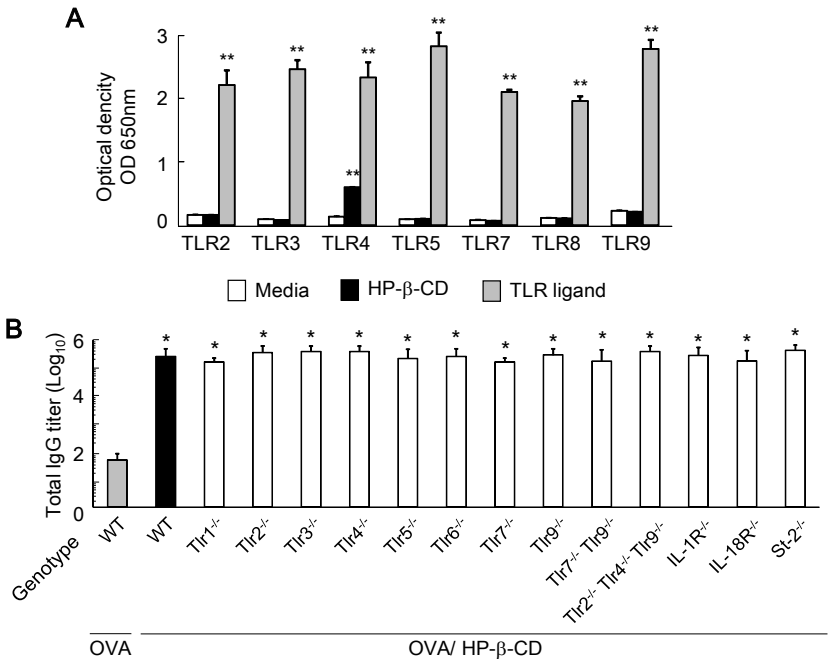
Supplemental Fig. 1. The potential of HP-β-CD to induce proinflammatory cytokine responses *in vitro* and *in vivo*. (**A**) Evaluation of the cytokine responses in splenocytes stimulated with HP-β-CD. Splenocytes were stimulated with HP-β-CD and the supernatant was collected 24 h after stimulation. The concentration of each cytokine in the supernatant was measured with the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad Laboratories, Inc.). Data are the averages of two independent experiments. (**B**) Evaluation of the cytokine responses in mice injected with HP-β-CD. Mice were s.c. injected with 30% HP-β-CD or 100 μg of R848 (n = 3). The sera were collected 1, 3, and 8 h after injection, and those from three mice treated identically were pooled. Each cytokine concentration was measured with the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay.



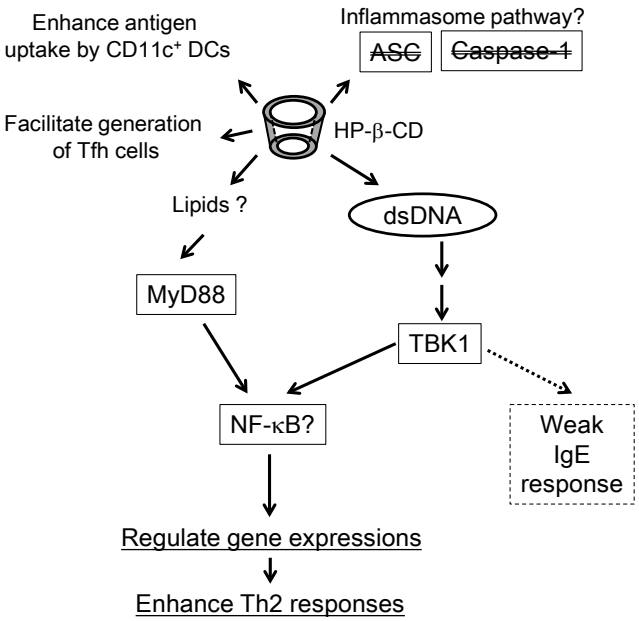
B

GO_term	Genes
Group3: SP_id	
biological_process (1)	Btbd1,Ifitm6,4930435H24Rik,Pm20d1,Ms4a6d,Mpped2,AA467197,Fam19a3,Stfa2l1,Gm10590,Daf2,Styx1
metabolic process (2)	Ganab,Enpp6,Pm20d1,Mgam,Hmgcs2,Acly
signal transduction (13)	Fpr2,Gpr85,Trpm1,C5ar1,Cxcr2,Il1rap
lipid metabolic process (7)	Enpp6,Sult1a1,Hrasls,Hmgcs2,Acly
positive regulation of cell proliferation (11)	Pdgfra,Osmr,Shmt2,Pdgfc,Cxcr2
proteolysis (7)	Prss2,Pcsk5,Mmp9,Pm20d1,Asprv1
regulation of transcription, DNA-templated (1)	Dmrt3,Pax8,Fosl2,Cebpd,Nfil3
transcription, DNA-templated (29)	Dmrt3,Pax8,Fosl2,Cebpd,Nfil3
chemotaxis (7)	Pdgfra,Fpr2,Cxcl2,Cxcr2
G-protein coupled receptor signaling pathway (15)	Fpr2,Gpr85,C5ar1,Cxcr2
multicellular organismal development (7)	Dmrt3,Pdgfra,Pax8,Pdgfc
neutrophil chemotaxis (25)	Cxcl2,Csf3r,C5ar1,Cxcr2
Group2: LN&LV_id	
transport (4)	Slc16a6,Atp2a2,Lyve1,Lbp,Slc41a1,Slc10a6,Scnn1a,Slc39a14,Syt11
regulation of transcription, DNA-templated (1)	Sbno2,Ell,Arntl,Jun,Zfat,Ctnnd1,Arid5a,Tnip2
transcription, DNA-templated (29)	Sbno2,Ell,Arntl,Jun,Zfat,Ctnnd1,Arid5a,Tnip2
biological_process (1)	Slc16a6,Cpne8,Syngr4,Slc41a1,Acnat2,G530011O06Rik
positive regulation of transcription from RNA polymerase II promoter (62)	Arntl,Jun,Myd88,Zfat,Arid5a,Tnip2
angiogenesis (17)	Hmox1,Jun,Nos3,Nrp1,Efna1
ion transport (7)	Atp2a2,Slc41a1,Slc10a6,Slc39a14,Scnn1a
negative regulation of cell proliferation (11)	Hmox1,Cdh5,Jun,Nos3,Ptpn2
oxidation-reduction process (5)	Hmox1,Cyp2e1,Cdo1,Nos3,Acoxl
cell adhesion (6)	Cdh5,Lyve1,Ctnnd1,Nrp1
positive regulation of I-kappaB kinase/NF-kappaB signaling (88)	Hmox1,Adipoq,Myd88,Tnip2
signal transduction (13)	Arntl,Myd88,Lyve1,Nos3
Group1: LN_id	
biological_process (1)	Urgcp,Gm7694,4930408O17Rik,AI607873,Gm10309,Tmem167b,Fam181b,A930009A15Rik,1700080O16Rik,Sh2d5,2500004C02Rik,Ifi44,4930553M12Rik,B430306N03Rik
proteolysis (7)	Adamts9,Adamts4,Gzmb,Mmp13,Phex,Plat,F10,Adamts1,Cfd
transcription, DNA-templated (29)	Tfec,Sbno2,Adnp2,Glis3,Cebpb,Il33,Nfatc1,Kdm2a
cell adhesion (6)	Emilin2,Cdh17,Selp,Tnfaip6,Spon1,Klra2,Sele
innate immune response (7)	Ifit3,Oas2,Vnn1,Lbp,Ifit1,Cfd,Rsad2
regulation of transcription, DNA-templated (1)	Tfec,Sbno2,Adnp2,Glis3,Cebpb,Nfatc1,Kdm2a
signal transduction (13)	Ank3,Niacr1,Gpr85,Rasd1,Ednrb,Gpr1,Sele
apoptotic process (8)	Acvr1c,Plscr1,Niacr1,Gzmb,Xaf1,Sema3a
G-protein coupled receptor signaling pathway (15)	Rgs16,Niacr1,Gpr85,Rgs4,Ednrb,Gpr1
positive regulation of transcription from RNA polymerase II promoter (62)	Plscr1,Glis3,Cebpb,Il33,Nfatc1,Il10
defense response to virus (12)	Ifit3,Oas2,Plscr1,Ifit1,Rsad2
immune response (4)	Oas2,Plscr1,Ccl2,Ccl9,Il10
inflammatory response (6)	Vnn1,Selp,Ccl2,Il10,Sele
cell differentiation (8)	Acvr1c,Cebpb,Fgf23,Sema3a
oxidation-reduction process (5)	Cyp7b1,Aldh1a1,Kdm2a,Fmo2
positive regulation of apoptotic process (19)	Adcy10,Acvr1c,Plscr1,Aldh1a1
positive regulation of transcription, DNA-templated (1)	Cebpb,Fgf23,Nfatc1,Il10
transcription from RNA polymerase II promoter (30)	Plscr1,Glis3,Cebpb,Nfatc1
transport (4)	Cdh17,Lbp,Got2,Hook2
Group4: SP_ip	
transport (4)	Hbb-y,Fabp12,Chrna3,Atp6v1e1
biological_process (1)	Gm7969,Fabp12,1600023N17Rik,Gm7173,Asb17
Group5: LV_ip	
regulation of transcription, DNA-templated (1)	Sp5,Zfp354c,Brd3,Dmtf1,Crem,Nr0b2
transcription, DNA-templated (29)	Sp5,Zfp354c,Brd3,Dmtf1,Crem,Nr0b2
biological_process (1)	Slain1,8430408G22Rik,Ces2b,Dock6
Group6: LV_id	
biological_process (1)	Osgin2,Cpne8,Tspan31,Slc37a1,Trmt61a,Tmem87b,9530077C05Rik,Gm13446,2010003K11Rik,Lpgat1,Kbtbd8,1110018N20Rik,Ankhd1,Zfp9,Tlpal,Arhgef26,Izumo2,Ddhd1,Zc3h13
regulation of transcription, DNA-templated (1)	Gata6,Foxq1,Btg2,Bcl3,Grhl1,Cidec,E2f8,Foxr2,Junb,Prdm1,Litaf,Onecut1,Zbtb16
transcription, DNA-templated (29)	Foxq1,Btg2,Bcl3,Junb,Onecut1,Id3,Gata6,Grhl1,Cidec,E2f8,Foxn2,Prdm1,Litaf
transport (4)	Mfsd2a,Slc38a10,Apoa4,Steap4,Rab2a,Slc22a5,Slc41a2,Gabra1,Tlpal,Slc39a14,Slc25a30,Snx10
multicellular organismal development (7)	Gadd45g,Themis,Grhl1,Id2,Ifi81,Prdm1,Id3
ion transport (7)	Slc38a10,Steap4,Slc22a5,Slc41a2,Gabra1,Slc39a14
negative regulation of transcription from RNA polymerase II promoter (63)	Foxq1,Id2,Id3,Gata6,E2f8,Prdm1
apoptotic process (8)	Chac1,Gadd45g,Ctsc,Cidec,Litaf
cellular response to DNA damage stimulus (9)	Fbxo31,Btg2,Bcl3,Recql5,Gadd45a
lipid metabolic process (7)	Insig2,Acox1,Agpat9,Ddhd1,Lpgat1
negative regulation of transcription, DNA-templated (1)	Bcl3,Id2,Id3,Zbtb16,Gata6
positive regulation of transcription from RNA polymerase II promoter (62)	Btg2,Bcl3,Onecut1,Gata6,E2f8
signal transduction (13)	Olfr78,Gabra1,Gnrhr,Lepr,Litaf
transcription from RNA polymerase II promoter (30)	Foxq1,Btg2,Junb,Onecut1,Gata6
cell cycle (5)	Gadd45a,Fbxo31,E2f8,Recql5
cell differentiation (8)	Srrm4,Onecut1,Gadd45g,Ifi81
metabolic process (2)	Lpgat1,Acox1,Agpat9,Lyz1
methylation (3)	Trmt61a,Nnmt,Prdm1,Fbll1
negative regulation of apoptotic process (19)	Btg2,Bcl3,Gata6,Fn1
negative regulation of cell proliferation (11)	Btg2,B4galt1,Zbtb16,Gnrhr
positive regulation of apoptotic process (19)	B4galt1,Id3,Zbtb16,Gata6
positive regulation of transcription, DNA-templated (1)	Gata6,Bcl3,Id2,Zbtb16
protein transport (9)	Rab2a,Rab30,Snx10,Rab27a
regulation of cell cycle (9)	Junb,Gadd45a,Id3,Gadd45g
tRNA processing (22)	Trmt61a,Clp1,Pus7l,Fbll1
Group7: LN_SP,LV_id	
apoptotic process (8)	S100a9,S100a8,Lcn2,Bnip2
inflammatory response (6)	Irg1,Il1f9,S100a9,Cxcl3,Cd7,S100a8,Cxcl1,Ccr1,Il1b,Ptgs2
acute-phase response (8)	Serpina3n,Orm2,Saa1,Hp,Saa2,Saa3
biological_process (1)	Orm3,Wdr37,Farsa,Tmem2,Mfsd2b,Slfn4
chemotaxis (7)	S100a8,Prok2,S100a9,Cxd3,Ccr1,Ccl7
immune response (4)	Cxcl3,Ccl7,Cxcl1,Clec4e,Ccr1,Il1b
innate immune response (7)	S100a8,Irg1,S100a9,Clec4d,Lcn2
negative regulation of apoptotic process (19)	Ier3,Cdkn1a,Prok2,Sycp2
neutrophil chemotaxis (25)	S100a8,Cxcl1,S100a9,Cxcl3,Il1b
positive regulation of cytosolic calcium ion concentration (22)	Cxcl3,Cxcl1,Ccr1,Il1b
transport (4)	Mfsd2b,Scara5,Orm2,Lcn2

Supplemental Fig. 2. Group clustering using mRNA expression levels and GO analysis of HP-β-CD -induced genes in different organs after its i.d. or i.p. administration. (**A**) The expression levels were normalized using the Z-scores for the fold-change values. Upregulated genes were selected, under the condition of a fold change > 2 and a customized PA call = 1 in at least one sample, and clustered into seven groups using complete-linkage hierarchical clustering. (**B**) For each group, the clustered genes were annotated by their functional roles using the AMIGO gene ontology database, and representative GO terms are indicated. The number in parentheses next to the GO term in the column indicates its rank in the GO hierarchy, and relates to the number of ancestors of the given term (i.e., biological process (1) indicates the root of the GO hierarchy tree). The gene list in the column shows the GO-categorized genes found in each grouped cluster.



Supplemental Fig. 3. Involvement of TLRs and IL-1R superfamily in the adjuvanticity of HP-β-CD (**A**) TLR ligand screening with HEK-Blue™ TLR cells. HEK-Blue TLR cells (Invivogen) are NF-κB-secreting alkaline phosphatase reporter cells that stably express mouse TLR genes (*Tlr*2, 3, 4, 5, 7, 8, or 9). TLR stimulation was tested by assessing NF-κB activation in HEK293 cells expressing a given TLR. After incubation for 16–20 h with the test substance, the OD was measured at 650 nm on a Molecular Devices Spectra Max 340PC Absorbance Detector. HP-β-CD was tested at 5000 μg/ml. The following substances were used as positive controls: for TLR2: heat-killed *Listeria monocytogenes* (HKLM) at 10^8 cell/ml; for TLR3: poly(I:C) at 1 μg/ml; for TLR4: *E. coli* K12 LPS at 100 ng/ml; for TLR5: *Salmonella enterica* subspecies I, serovar *typhimurium* flagellin at 100 ng/ml; for TLR7: CL097 at 1 μg/ml; for TLR8: CL075 at 10 μg/ml + poly(dT) at 10 μM; and for TLR9: CpG ODN 1826 at 1 μg/ml. An assay was performed according to the manufacturer's instructions. Data are representative of two independent experiments. **P < 0.01 v.s. media on Student's *t* test. (**B**) Evaluation of the humoral responses in KO mice lacking TLRs or IL-1R superfamily genes. Mice were s.c. immunized twice with OVA/30% HP-β-CD (n = 4–6). The total anti-OVA antibody titers were measured with an ELISA 7 days after the second immunization. Error bars denote SD. *P < 0.05 v.s. WT mice immunized with OVA alone on Student's *t* test.



Supplemental Fig. 4. Biological activities of locally injected HP-β-CD and the proposed immunological mechanisms/signaling pathways involved in the adjuvant activity of HP-β-CD. 1) Antigen uptake: HP-β-CD enhances antigen uptake by $CD11c^+$ DCs; 2) Facilitates the generation of Tfh cells; 3) MyD88-dependent signaling pathway; 4) TBK1-dependent signaling pathway: the temporary release of dsDNA at the injection site activates the TBK1-dependent signaling pathway.

Legend for Movie

Movie S1. 3D reconstruction of the two-photon images of the LN. HP-β-CD (green), OVA (blue), MARCO$^+$ macrophages (red) . Mice were i.d. injected with OVA–Alexa Fluor 647/ 3% HP-β-CD -FITC, and after 30 min, anti-MARCO antibody was i.d. injected. LNs were collected 30 min after the antibody injection (i.d.), and were imaged by two-photon microscopy.