Supplementary information

Nod2-mediated recognition of the microbiota is critical for mucosal adjuvant activity of cholera toxin

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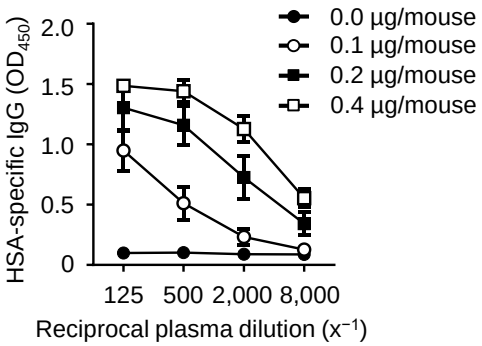
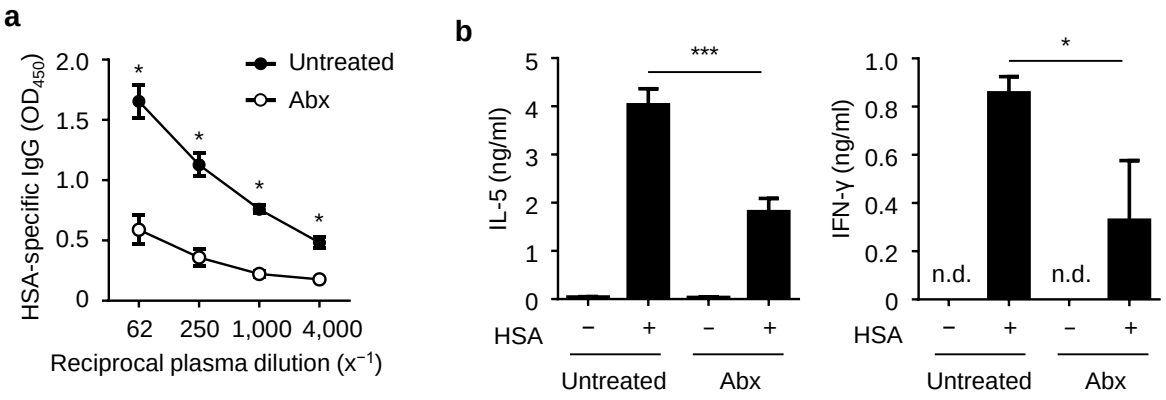
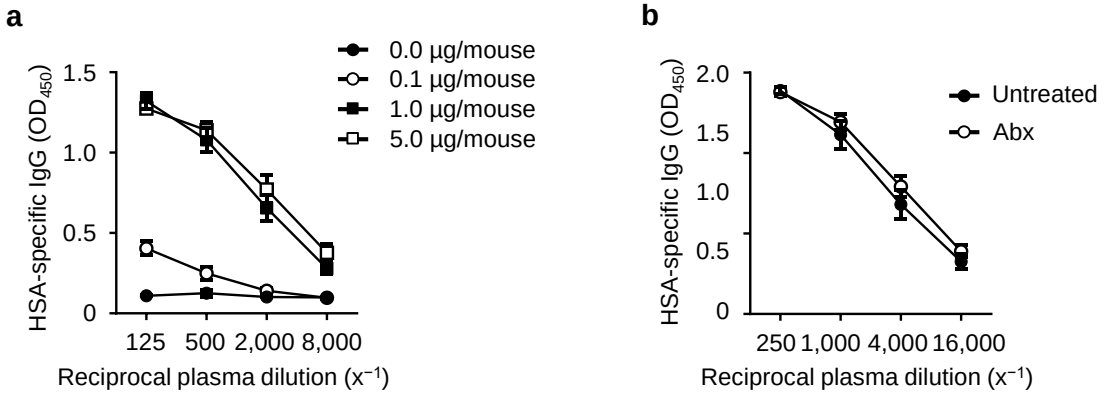


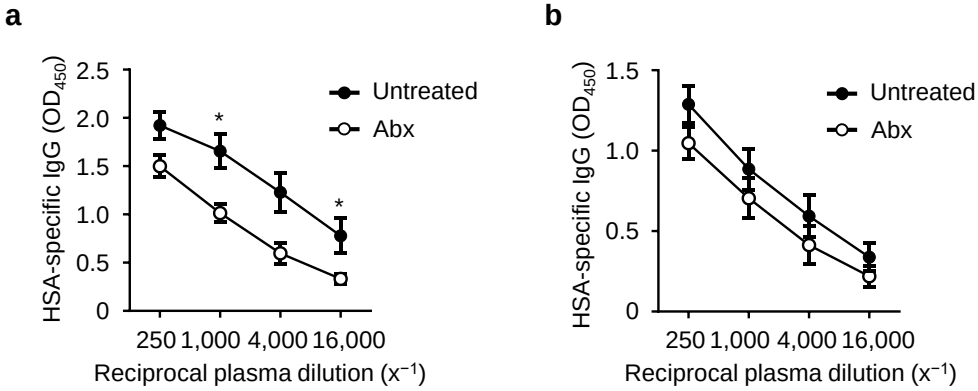
Supplementary Figure 1. Antigen-specific IgG production induced by various amounts of cholera toxin. Mice were intranasally immunized with 30 µg of HSA and various amounts of CT. The amounts of HSA-specific IgG were analyzed in plasma on day 14 post-immunization. Data are shown as mean ± s.e.m.



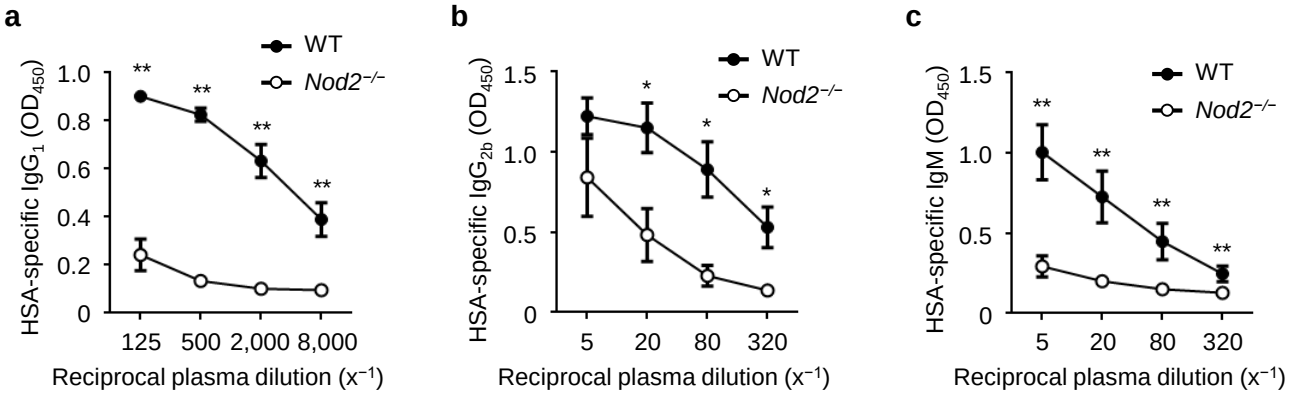
Supplementary Figure 2. Symbiotic bacteria are critical for oral immunization with antigen and cholera toxin. (**a**) The amounts of HSA-specific IgG were analyzed in plasma obtained from antibiotic (Abx)-treated mice (n = 5) and untreated control mice (n = 4) on day 14 after oral immunization with 10 mg of HSA and 10 μg of CT. (**b**) Splenocytes were isolated from Abx-treated mice and untreated mice on day 14 post-immunization, and then restimulated with or without 500 μg/ml of HSA in triplicate cultures for 4 d. The production of IFN-γ and IL-5 was examined in supernatants of restimulated splenocytes. The results are representative of at least two independent experiments. Values represent mean ± s.e.m. (**a**) or mean of three technical replicates ± s.d. (**b**). *P < 0.05 and ***P < 0.001 by Mann-Whitney test (**a**) and by Student's t-test (**b**). n.d., not detected.



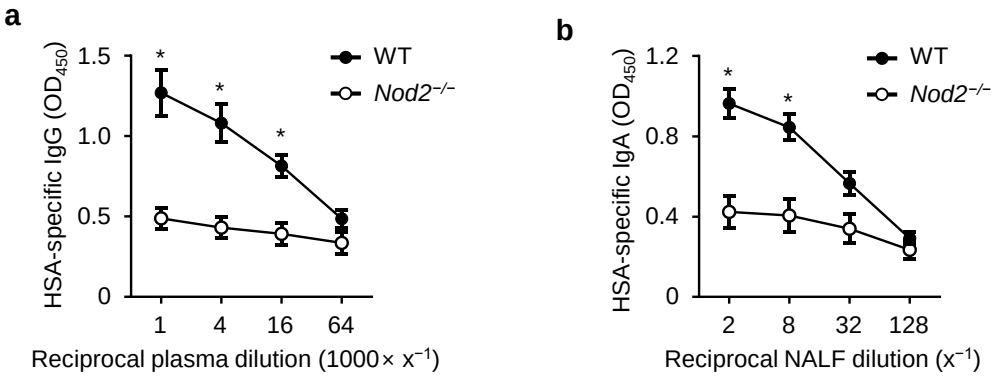
Supplementary Figure 3. Symbiotic bacteria are dispensable for antigen-specific IgG response induced by intraperitoneal immunization with antigen and cholera toxin. Mice were intraperitoneally immunized with 100 μl of PBS containing 100 μg of HSA and various amounts of CT (**a**) or 1 μg of CT (**b**). (**a, b**) The amounts of HSA-specific IgG were analyzed in plasma on day 14 post-immunization. (**b**) Antibiotic-treated mice (n = 5) were given antibiotic cocktail in the drinking water ad libitum from 2 weeks prior to immunization while control animals (n = 5) were provided with normal water. Values represent mean ± s.e.m.



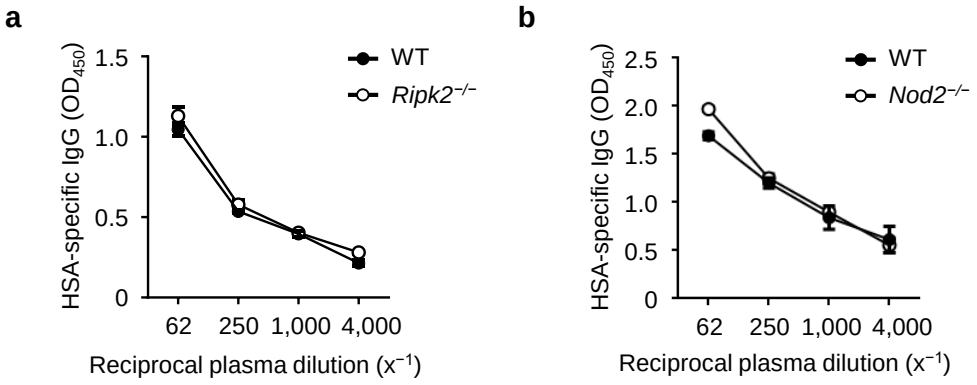
Supplementary Figure 4. Symbiotic bacteria enhance antigen-specific IgG production after nasal immunization with CpG, but not MALP-2. The amounts of HSA-specific IgG in plasma were analyzed in Abx-treated and untreated mice on day 14 after intranasal immunization with 30 μg of HSA and 10 μg of CpG (*n* = 4 or 5 per group) (**a**) or 0.5 μg of MALP-2 (*n* = 5 or 7 per group) (**b**). Values represent mean ± s.e.m. **P* < 0.05 by Mann-Whitney test.



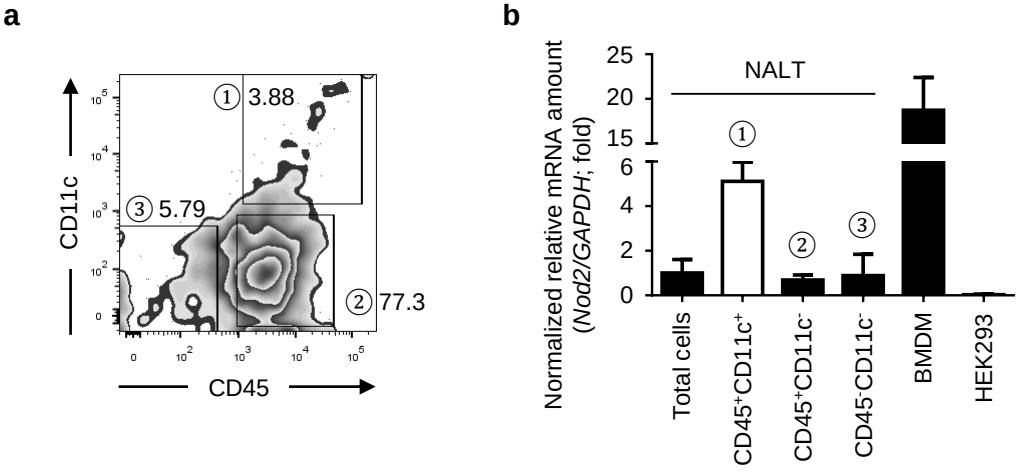
Supplementary Figure 5. Antigen-specific IgG_1, IgG_{2b}, and IgM induced by nasal immunization with HSA and cholera toxin are decreased in *Nod2* deficient mice. The amounts of HSA-specific IgG_1, IgG_{2b}, and IgM were measured in plasma from *Nod2*$^{-/-}$ and WT mice (n = 5 per group) on day 14 after intranasal immunization. Data shown represent mean ± s.e.m. *P < 0.05 and **P < 0.01 by Mann-Whitney test.



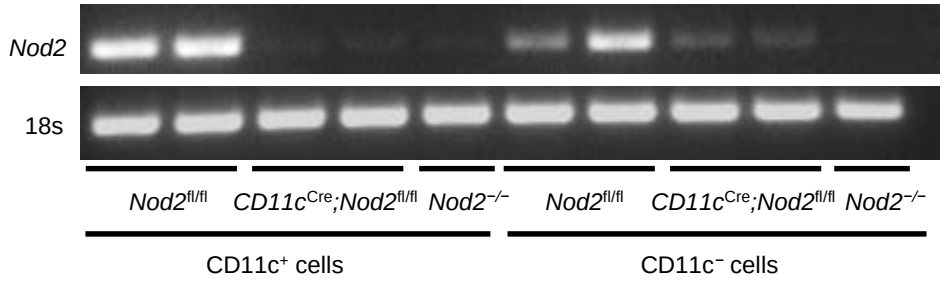
Supplementary Figure 6. Nod2 is important for systemic production of IgG and local production of IgA induced by nasal immunization with high dose of cholera toxin. WT and *Nod2*$^{-/-}$ mice (n = 4 per group) were intranasally immunized with 100 μg of HSA and 1 μg of CT. HSA-specific IgG in plasma (**a**) and HSA-specific IgA in NALF (**b**) were measured on day 14 post-immunization. Data represent mean ± s.e.m (**a**, **b**) *P < 0.05 by Mann-Whitney test.



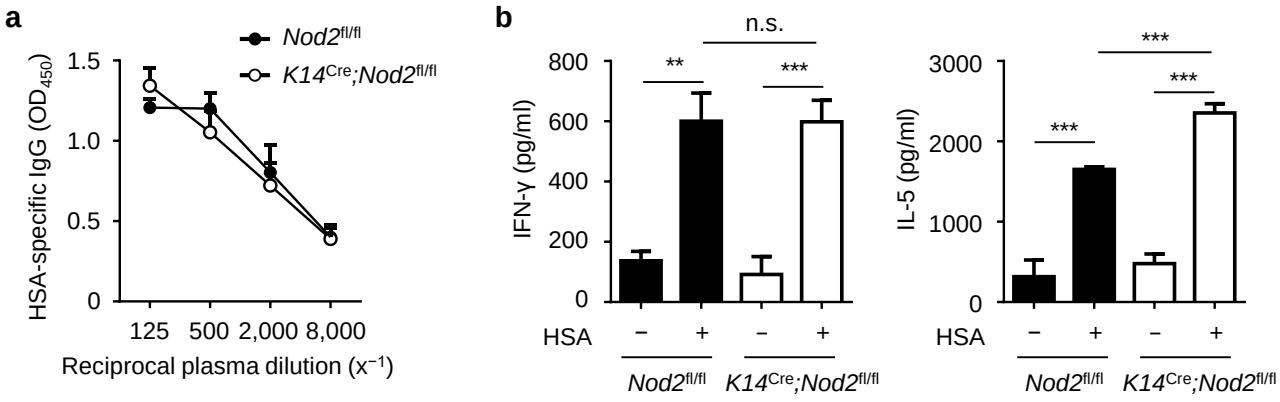
Supplementary Figure 7. Ripk2 and Nod2 are dispensable for intraperitoneal immunization with cholera toxin or alum. WT and *Ripk2*$^{-/-}$ (**a**) or *Nod2*$^{-/-}$ (**b**) mice (*n* = 3 - 5 per group) were intraperitoneally immunized with 100 µl of PBS containing 100 µg of HSA and 1 µg of CT (**a**) or 30 µg of HSA and 100 µl of alum (**b**), respectively. HSA-specific IgG was measured in plasma on day 14 post-immunization. Data represent mean ± s.e.m.



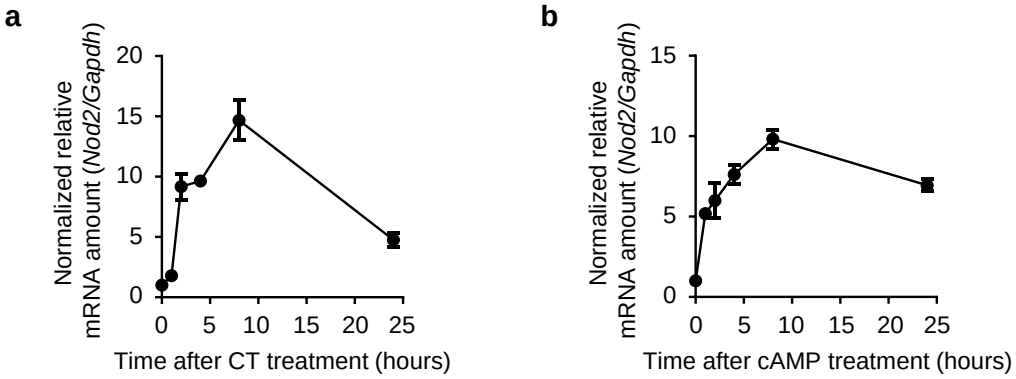
Supplementary Figure 8. *Nod2* expression in NALT cell populations. (**a**) Cells isolated from NALT were sorted by flow cytometry as indicated. (**b**) Analysis of *Nod2* expression in mRNA from total cells and sorted NALT cells was performed by real-time qPCR. Bone marrow-derived macrophages (BMDM) and HEK293 cells are shown as positive and negative controls, respectively. *Gapdh* gene expression was used for data normalization.



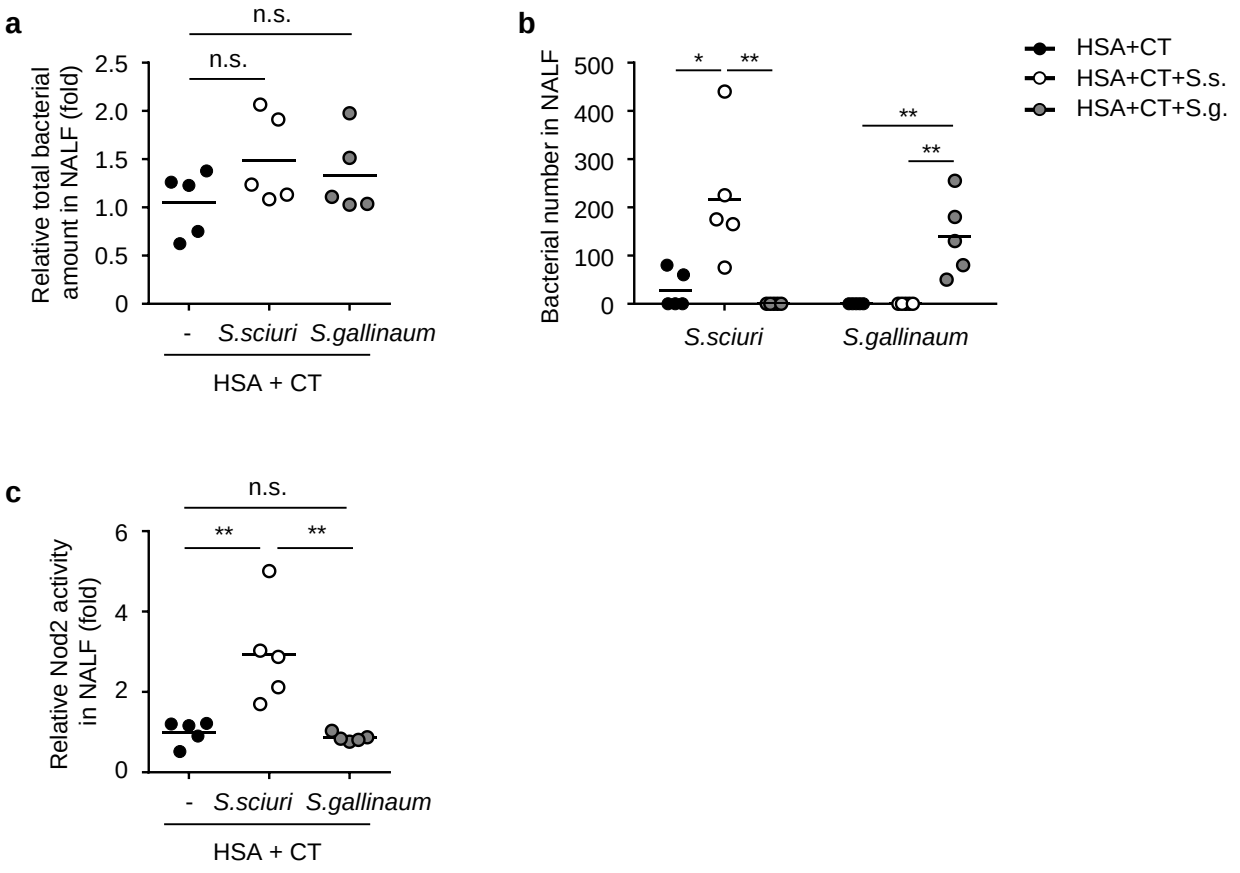
Supplementary Figure 9. Verification of CD11c positive cell-specific knockout of *Nod2*. Total splenocytes were isolated from *CD11c*^{Cre}*;Nod2*$^{fl/fl}$, their littermate *Nod2*$^{fl/fl}$ and whole-body *Nod2*$^{-/-}$ mice. CD11c-positive and CD11c-negative cells were isolated using a magnetic sorting kit. RT-PCR was performed on total RNA isolated from purified cells using primers spanning exons 2 and 3 of the *Nod2* transcript.



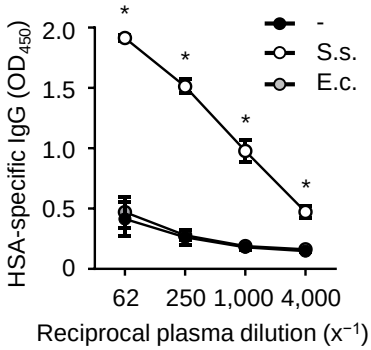
Supplementary Figure 10. Nod2 in epithelial cells is dispensable for adjuvant activity of cholera toxin. (**a**) HSA-specific IgG was measured in plasma from immunized *K14*^{Cre}*;Nod2*$^{fl/fl}$ and their littermate *Nod2*$^{fl/fl}$ mice (*n* = 4 per group) on day 14 post-immunization.(**b**) Splenocytes were isolated from *K14*^{Cre}*;Nod2*$^{fl/fl}$ and *Nod2*$^{fl/fl}$ mice on day 14 post-immunization, and then restimulated with or without HSA for 4 d. Concentrations of IFN-γ and IL-5 were determined in the supernatants of restimulated splenocytes . Results are representative of at least two independent experiments. Data are shown as mean ± s.e.m. (**a**) or ± s.d. (**b**). ***P* < 0.01 and ****P* < 0.001 by Mann-Whitney test (**a**) and by Student's *t*-test (**b**) n.s.; not significant.



Supplementary Figure 11. Cholera toxin and cAMP induce the expression of *Nod2* mRNA in DCs. BMDCs were treated with 0.5 μg/ml of CT (**a**) and 100 μM of cAMP (**b**) in triplicate cultures and total RNA was prepared at various time-points, followed by real-time qPCR analysis using specific primer sets for *Nod2* and *Gapdh*. *Gapdh* was used for data normalization. Results are representative of two independent experiments. Data shown represent mean ± s.d.



Supplementary Figure 12. Bacterial colonization and Nod2-stimulatory activity in the nasal cavity of mice stimulated intranasally with HSA, cholera toxin and indicated bacteria. SPF mice (each group *n* = 5) were intranasally immunized with HSA and CT together with 5×10^5 c.f.u./mouse of live *S. sciuri* or *S. gallinarum* and, 2 weeks later, NALFs were collected from each mouse. (**a**) Total bacterial colonization was measured by real-time qPCR analysis using total DNA obtained from NALF and a set of universal 16S rDNA sequence primers (**b**) The numbers of *S. sciuri* and *S. gallinarum* in the NALF were calculated based on distinct colony morphology after culturing NALF bacteria on BHI agar plates. The identity of selected colonies was verified by 16S rDNA sequencing of V3-V4 regions. (**c**) Nod2-stimulatory activity in NALF from SPF, *S. sciuri*-, or *S. gallinarum*-treated mice. Each dot represents an individual mouse and the mean value is displayed by a line. **P* < 0.05 and ***P* < 0.01 by Mann-Whitney test. n.s.; not significant.



Supplementary Figure 13. Antigen-specific IgG responses in germ-free mice stimulated with UV-inactivated bacteria. GF mice (*n* = 3 or 4 per group) were intranasally immunized with HSA and CT together with 5×10^3 c.f.u./mouse of UV-inactivated *S. sciuri* or *E. coli*. The amounts of HSA-specific IgG were analyzed in plasma on day 14 post-immunization. Data are shown as mean ± s.e.m. **P* < 0.05 by Mann-Whitney.