Disruption of Nasal Bacteria Enhances Protective Immune Responses to Influenza A Virus and SARS-CoV-2 Infection

Minami Nagai
University of Tokyo
Miyu Moriyama
Yale University School of Medicine
Takeshi Ichinohe (ichinohe@ims.u-tokyo.ac.jp)
University of Tokyo Institute of Medical Science: Tokyo Daigaku Ikagaku Kenkyujo
https://orcid.org/0000-0002-6376-2841

Research

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Disruption of nasal bacteria enhances protective immune responses to influenza A virus and SARS-CoV-2 infection

Minami Nagai[1], Miyu Moriyama[1], and Takeshi Ichinohe[1, *]

[1]Division of Viral Infection, Department of Infectious Disease Control, International Research Center for Infectious Diseases, Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

*Corresponding author. Mailing address: Division of Viral Infection, Department of Infectious Disease Control, International Research Center for Infectious Diseases, Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan. Phone: +81-3-6409-2125, Fax: +81-3-6409-2134 ,

E-mail: ichinohe@ims.u-tokyo.ac.jp

Abstract

Background: Gut microbiota and these microbial-derived products play a critical role in the induction of adaptive immune responses to influenza virus infection. However, the role of nasal bacteria in the induction of the virus-specific adaptive immunity is less clear. Here, we examine whether nasal bacteria critically regulates the generation of influenza virus specific adaptive immune response after infection or intranasal vaccination.

Results: We demonstrated that disruption of nasal bacteria by topical mucosal application of antibiotic enhances the virus-specific antibody responses to influenza virus infection. Although intranasal administration of hemagglutinin (HA) vaccine alone was insufficient to induce the HA-specific antibody responses, disruption of nasal bacteria by lysozyme or addition of culturable oral bacteria from a healthy human volunteer rescued inability of the nasal bacteria to generate antibody responses to intranasally administered split-virus vaccines. Myd88-depdnent signaling in the hematopoietic compartment was required for adjuvant activity of intranasally administered oral bacteria. In addition, we found that the oral bacteria-combined intranasal vaccine induced protective antibody response to influenza virus and SARS-CoV-2 infection.

Conclusion: We show for the first time that disruption of nasal bacteria enhances protective immune responses to influenza virus and SARS-CoV-2 infection. Our findings here have identified a previously unappreciated role for nasal bacteria in the induction of the virus-specific adaptive immune responses.

Keywords: mucosal immunity, intranasal vaccine, adjuvant, SARS-CoV-2

Background

Respiratory infectious diseases such as influenza and coronavirus disease 2019 (COVID-19) cause substantial morbidity and mortality. Influenza A virus is responsible for annual epidemics that cause severe morbidity and mortality involving 3 to 5 million people worldwide. In addition, the constant pandemic potential of newly emerging viruses remains a serious threat to public health, the economy and society as illustrated by the recent COVID-19 global pandemic. Therefore, there is an urgent need to develop effective vaccines against not only seasonal influenza viruses but also against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Since it is difficult to predict which strain of influenza virus or coronavirus cause a pandemic, it is advantageous to produce vaccines that induce cross-protective immunity against variants of the particular virus strain. Mucosal immunity induced by natural infection of influenza virus is more effective and cross-protective against heterologous virus infection than systemic immunity induced by parenteral vaccines (1). It is believed that the virus-specific IgA in upper respiratory tract is more cross-protective against heterologous influenza viruses compared with the virus-specific IgG in the serum due to its dimeric or tetrameric forms (higher avidity) and location (2, 3). Indeed, polymeric immunoglobulin receptor-knockout mice failed to secrete nasal IgA and protect against

61 heterologous virus challenge (4). Therefore, induction of the virus-specific secretory
62 IgA in the upper respiratory tract by intranasal vaccination has a great advantage in
63 conferring protection against an unpredictable pandemic of viral pathogens such as the
64 swine-origin H1N1 and avian-origin H7N9 influenza A viruses, or zoonotic origin of
65 SARS-CoV-2 (5, 6). In the effort to develop effective intranasal vaccines, several
66 adjuvants such as cholera toxin (7), synthetic double-stranded RNA poly(I:C) (8),
67 synthetic toll-like receptor 4 agonist (9), zymosan (10), flagellin (11), immune
68 stimulating complexes (ISCOMs) (12), or type-I interferons (13) have been developed
69 to enhance the vaccine-specific nasal IgA response. While upper respiratory tract
70 contains commensal bacteria (14, 15), intranasal administration of split vaccine alone
71 was insufficient to induce the vaccine-specific nasal IgA response (8, 16), suggesting
72 that the amounts of commensal bacteria in upper respiratory tract are insufficient to
73 stimulate the vaccine-specific nasal IgA response.
74 A recent study has demonstrated that nasal mucosa-derived *Staphylococcus*
75 *epidermidis*, one of the most abundant colonizers of healthy human skin and mucosal
76 surface, suppressed influenza virus replication by stimulating IFN-λ production (17). In
77 addition, influenza virus-infected mice lacking both toll-like receptor 7 (TLR7) and
78 mitochondrial antiviral signaling (MAVS) had elevated nasal bacterial burdens, which

79 resulted in death from pneumonia caused by secondary bacterial infections (18). In
80 contrast to the role of nasal bacteria in innate antiviral resistance to influenza virus
81 infection or severity of the disease (17, 18), it remains unclear whether nasal bacteria
82 critically regulates the generation of influenza virus-specific adaptive immune responses
83 after infection or intranasal vaccination. Here, we show that depletion of nasal bacteria
84 by intranasal administration of antibiotics enhanced the virus-specific nasal IgA and
85 serum IgG response following influenza virus infection. In addition, we found that
86 lysozyme-induced disruption of nasal bacteria or culturable oral bacteria from a healthy
87 volunteer significantly enhanced the vaccine-specific nasal IgA and serum IgG
88 responses. Myd88-depdnent signaling in the hematopoietic compartment was required
89 for adjuvant activity of intranasally administered oral bacteria. Our findings here have
90 identified a previously unappreciated role for nasal bacteria in the induction of the
91 virus-specific adaptive immune responses.
92

Methods

Mice

Age- and sex-matched Balb/c mice obtained from Japan SLC, Inc. were used as WT controls. MyD88-deficient Balb/c mice were a gift from T. Taniguchi. All animal experiments were performed in accordance with the University of Tokyo's Regulations for Animal Care and Use, which were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo (approval number PA17–69).

Cells

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (E-MEM; Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). VeroE6 cells stably expressing transmembrane protease serine 2 (VeroE6/TMPRSS2; JCRB Cell Bank 1819) were maintained in Dulbecco's modified Eagle's medium (DMEM) low glucose (Cat#08456-65; Nacalai Tesque) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and G418 (1mg/ml) (19).

Depletion of nasal bacteria *in vivo*

The antibiotic cocktail consisted of ampicillin sodium salt (1 g/L), neomycin sulfate (1 g/L), metronidazole (1 g/L), vancomycin hydrochloride (0.5 g/L), gentamicin (10 mg/L), penicillin (100 U/ml), streptomycin (100 U/ml), and amphotericin B (0.25 mg/L) (20). For intranasal treatment, mice were anaesthetized and 5 µl of antibiotic was administered dropwise into each nostril using a pipette tip. All antibiotics with the exception of vancomycin hydrochloride were obtained from Nacalai Tesque. Vancomycin hydrochloride was obtained from Duchefa Biochemie.

Virus infection

WT A/Puerto Rico/8/34 (A/PR8) and A/Narita/1/09 (pdm09) influenza viruses were grown in allantoic cavities of 10-d-old fertile chicken egg at 35°C for 2 d (21). Viral titer was quantified by a standard plaque assay using MDCK cells and viral stock was stored at −80°C (22). For intranasal infection, mice were fully anesthetized by i.p. injection of pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) and then infected by intranasal application of 30 µl of virus suspension (1,000 pfu of A/PR8 or pdm09 in PBS). This procedure leads to upper and lower respiratory tract infection (20).

129 SARS-CoV-2 (a gift from Y. Kawaoka) was amplified on VeroE6/TMPRSS2 cells
130 and stored at −80°C until use. The infectious titer was determined by a standard plaque
131 assay using VeroE6/TMPRSS2 cells, as described previously (23). For intranasal
132 infection, one-month-old female Syrian hamsters (Japan SLC Inc.) were fully
133 anesthetized by i.p. injection of pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku
134 Co., Ltd., Tokyo, Japan) and then infected intranasally with 2×10^6 pfu (in 100 μL) of
135 SARS-CoV-2.
136
137 **Vaccination**
138 For intranasal infection, mice were fully anesthetized by i.p. injection of pentobarbital
139 sodium (Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) and then infected
140 intranasally by dropping 2 μl of PBS containing 1,000 pfu of A/PR8 into the nostril.
141 The quadrivalent inactivated influenza vaccine (split-product virus vaccines,
142 hemagglutinin [HA] vaccine) prepared for the 2015–2016 season and including
143 A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2),
144 B/Phuket/3073/2013, and B/Texas/2/2013 were purchased from Kaketsuken
145 (Kumamoto, Japan). Mice were immunized by intranasal administration of the
146 quadrivalent HA vaccine containing 150 ng of each HA with or without 5 μg of

147 lipopolysaccharide (LPS; InvivoGen), 5 μg of poly(I:C) (InvivoGen), 250 μg of
148 lysozyme (Thermo Fisher Scientific), or 1 mg of culturable oral bacteria from a healthy
149 volunteer.

150 SARS-CoV-2 spike S1+S2 ECD-His recombinant protein was purchased from Sino
151 Biological Inc. (Cat# 40589-V08B1). Hamsters were immunized subcutaneously or
152 intranasally with 1 μg of the recombinant spike protein with or without 1 mg of
153 culturable oral bacteria from a healthy volunteer.

154

155 **Clinical specimens**

156 Oral and nasal washes were collected from a healthy volunteer by rinsing the mouth
157 with 50 ml of saline or washing the nasal cavity with 50 ml of saline using a syringe.
158 For preparation of oral bacteria adjuvant, oral wash samples were grown in brain heart
159 infusion broth (BD 237500) at 37°C overnight, washed repeatedly, and resuspended in
160 PBS (200 μg/ml).

161

162 **Bacterial recovery and identification**

163 Oral and nasal washes were collected from a healthy volunteer as described above.
164 Aliquots of 100μl of serial 10-fold dilution of the oral and nasal wash were inoculated

165 into brain heart infusion agar plates (BD 252109). After incubation at 37°C overnight
166 under the aerobic conditions, the bacterial colonies were grown in brain heart infusion
167 broth (BD 237500) at 37°C overnight. Bacterial DNA was isolated as described
168 previously (20). A 300-bp portion of the 16S rRNA was amplified by PCR using
169 specific primer pairs of 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R
170 (5'-GGACTACHVGGGTWTCTAAT-3'), purified (Qiagen), sequenced, and the
171 sequence compared by Blast analysis to known bacterial sequences.
172
173 **Bone marrow chimera**
174 Bone marrow chimeras were generated as described (24). WT and MyD88-deficient
175 mice were γ-irradiated with 6 Gy, then were reconstituted with 5×10^6 bone marrow
176 cells of the appropriate genotype via i.v. injection and allowed to recover for 8 weeks
177 before vaccination.
178
179 **Measurement of virus titers**
180 For measurement of influenza virus titer, bronchoalveolar (BAL) fluid was collected
181 by washing the trachea and lungs of mice twice by injecting a total of 2 ml PBS
182 containing 0.1% bovine serum albumin (BSA). The virus titer was measured as follows:

aliquots of 200 μl of serial 10-fold dilution of the BAL fluid by PBS containing 0.1% BSA were inoculated into MDCK cells in 6-well plates. After 1 hour of incubation, cells were washed with PBS thoroughly and overlaid with 2 ml of agar medium.

For measurement of SARS-CoV-2 titer, BAL fluid was collected by washing the trachea and lungs of hamsters twice by injecting a total of 2 ml DMEM containing 5% FBS. The virus titer was measured as follows: aliquots of 200 μl of serial 10-fold dilution of the BAL fluid by DMEM containing 5% FBS were inoculated into VeroE6/TMPRSS2 cells in 6-well plates. After 1 hour of incubation, cells were washed with PBS thoroughly and overlaid with 2 ml of agar medium. The number of plaques in each well was counted 2 days after inoculation.

Enzyme-linked immunosorbent assay (ELISA)

Serum and nasal wash were collected from the immunized mice for measurement of the PR8- or HA-specific nasal IgA and serum IgG antibodies. Nasal wash was collected by washing the nasopharynx three times by injecting a total of 1 ml PBS containing 0.1% BSA. The levels of the PR8- or HA-specific nasal IgA and serum IgG antibodies were determined by ELISA as described previously (20). Standards for PR8- or HA-reactive IgA and IgG antibody titration were prepared from the nasal wash or serum

of the virus-infected or vaccinated mice, and expressed as the same arbitrary units (160-unit). The antibody titers of unknown specimens were determined from the standard regression curve constructed by two fold serial dilution of the 160-unit standard for each assay.

Quantification and statistical analysis

Statistical significance was tested by one-way ANOVA followed by Tukey test or unpaired t tests with PRISM software (Version 5; GraphPad software). Data are presented as mean ± SEM. Statistical details can be found directly in the figure legends. P values of less than 0.05 were considered statistically significant.

Results

Depletion of nasal bacteria enhanced antibodies response to influenza virus infection

Gut commensal microbiota play a key role in innate and adaptive immune defense against influenza virus infection (25-30). However, the role of oral or nasal bacteria in the induction of mucosal immune responses following influenza virus infection remains unknown. To assess the effects of oral or nasal bacteria in the induction of mucosal immune responses to influenza virus infection, we treated mice intranasally with an antibiotic cocktail for five consecutive days before influenza virus infection. This treatment resulted in significant reduction in the numbers of culturable oral and nasal bacteria (**Supplementary Fig. 1**). Antibiotic-treated mice were then infected intranasally with a mouse-adapted influenza A virus strain A/Puerto Rico/8/1934 (PR8). Surprisingly, influenza virus-specific nasal IgA and serum IgG levels were significantly elevated in the antibiotic-treated group (**Fig. 1**). This led us to consider the possibility that depletion of commensal bacteria in upper respiratory tract enhances influenza virus replication, resulting in enhancement of the virus-specific antibody responses. However, depletion of commensal bacteria in upper respiratory tract significantly reduced influenza virus replication at 2 days post infection (**Supplementary Fig. 2A**). This is

231 consistent with a previous report showing that antibiotic treatment significantly reduce
232 influenza virus replication at early time point (31). In addition, the viral replication in
233 upper respiratory tract became comparable between antibiotics-treated and control
234 groups at 3 and 5 days post infection (**Supplementary Fig. 2B, C**). These data indicated
235 that the levels of influenza virus replication in upper respiratory tract is unlikely to
236 account for increased the virus-specific antibody responses in antibiotic-treated animals.
237
238 **Lysozyme-induced disruption of nasal bacteria enhances antibody responses**
239 **induced by intranasal vaccination**
240 Thus, we next examined the possibility that antibiotic-induced disruption of nasal
241 bacteria releases pathogen-associated molecular patterns, which may act as adjuvants to
242 enhance the virus-specific antibody responses. To assess the possibility that disruption
243 of nasal bacteria acts as adjuvant for intranasal influenza vaccine, we immunized mice
244 intranasally with influenza virus hemagglutinin (HA) protein and lysozyme to disrupt
245 nasal bacteria. We used poly(I:C) adjuvant as a positive control (8). Strikingly, we
246 found that intranasal immunization with HA and lysozyme significantly enhanced the
247 HA-specific nasal IgA and serum IgG responses (**Fig. 2**). While upper respiratory tract
248 contains commensal bacteria (14, 15), intranasal administration of hemagglutinin (HA)

vaccine alone was insufficient to induce the HA-specific antibody responses (**Fig. 2**). Taken together, these results suggest that disruption of nasal bacteria by intranasal administration of antibiotics or lysozyme acts as adjuvant for intranasal influenza vaccine.

Oral bacteria act as adjuvant for intranasal vaccine

While upper respiratory tract contains commensal bacteria (14, 15), we found that relative amounts of 16S rRNA and culturable bacteria in nasal mucosal surface were significantly lower than that in the oral cavity (**Supplementary Fig. 3**). Thus, we next examine whether oral bacteria act as adjuvant for intranasal vaccine. Intranasal vaccination with HA and culturable oral bacteria from mice or a healthy volunteer significantly enhanced the HA-specific nasal IgA and serum IgG responses (**Fig. 3A, B**). In addition, the oral bacteria from a healthy volunteer stimulated the HA-specific nasal IgA and serum IgG responses in a dose-dependent manner (**Fig. 3C, D**). Next, we compared the ability of isolated bacterial strains from oral wash sample of a healthy volunteer to stimulate the HA-specific antibody responses. To this end, we immunized mice intranasally with HA and *streptococcus salivarius* (*S. salivarius*), *streptococcus parasanguinis* (*S. parasanguinis*), or *streptococcus infantis* (*S. infantis*). Mice

267 immunized with HA and each isolated bacterial strain induced comparable levels of the
268 HA-specific nasal IgA and serum IgG responses (**Fig. 4**), suggesting that adjuvant
269 activity of the oral bacteria is unlikely to account for strain specific.
270
271 **Myd88-depdnent signaling in the hematopoietic compartment is required for**
272 **adjuvant activity of intranasally administered oral bacteria**
273 Next, we wished to determine the innate immune signaling through
274 pattern-recognition receptors required for adjuvant activity of the oral bacteria. To this
275 end, we immunized WT and MyD88-deficient mice intranasally with HA and culturable
276 oral bacteria from a healthy volunteer and measured the HA-specific nasal IgA and
277 serum IgG responses. The HA-specific nasal IgA and serum IgG responses were found
278 to be completely dependent on MyD88 (**Fig. 5A, B**). In addition, lysozyme-induced
279 disruption of nasal bacteria stimulated the HA-specific nasal IgA and serum IgG
280 responses in a MyD88-dependent manner (**Fig. 5C, D**). To determine the cellular
281 compartment responsible for adjuvant activity of oral bacteria, we generated bone
282 marrow (BM) chimeric mice in which only the hematopoietic (WT→MyD88$^{-/-}$) or the
283 stromal cells (MyD88$^{-/-}$→WT) expressed MyD88. After intranasal vaccination with HA
284 and oral bacteria, the HA-specific nasal IgA and serum IgG responses were significantly

285 reduced in MyD88$^{-/-}$→WT BM chimeric mice compared to WT→MyD88$^{-/-}$ BM
286 chimeric mice (**Fig. 6**). These data indicate that MyD88-dependent signaling in the
287 hematopoietic, but not stromal, compartment is required for adjuvant activity of
288 intranasally administered oral bacteria.
289
290 **Oral bacteria-combined intranasal vaccine protects from influenza virus and**
291 **SARS-CoV-2 infection**
292 Finally, we examined protective effects of intranasal vaccination with oral
293 bacteria-adjuvanted vaccine against influenza virus and SARS-CoV-2 infection. To this
294 end, we immunized mice intranasally with quadrivalent influenza HA vaccine
295 containing A/California/7/2009 HA together with culturable oral bacteria or lysozyme.
296 Two weeks after the second vaccination, we challenged vaccinated mice intranasally
297 with a heterologous A/Narita/1/2009 (pdm09) strain (**Fig. 7**). Mice immunized with HA
298 vaccine adjuvanted with oral bacteria or lysozyme significantly reduced the virus titer
299 compared to control mice immunized with the HA vaccine alone (**Fig. 7**). We next
300 assessed protective effects of intranasal vaccination with oral bacteria-adjuvanted
301 SARS-CoV-2 spike protein against SARS-CoV-2 infection in Syrian hamsters. To this
302 end, we immunized hamsters intranasally with a recombinant SARS-CoV-2 spike

303 protein and culturable oral bacteria from a healthy volunteer. We immunized hamsters
304 subcutaneously with the spike protein alone as a control. We
305 Both the spike- and the virus-specific serum IgG levels were significantly elevated in
306 immunized hamsters (**Fig. 8A, B**). In addition, immunized hamsters significantly
307 reduced the virus titer compared to naïve animals following high-dose (2×10^6 pfu of
308 SARS-CoV-2) challenge (**Fig. 8C**). These data collectively indicated that disruption of
309 nasal bacteria or intranasal administration of oral bacteria compensate inability of nasal
310 bacteria to generate protective adaptive immunity to intranasally administered split
311 vaccines.
312
313

Discussion

The innate immune system, the first line of defense against pathogens, utilizes pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs). The recognition of influenza virus by PRRs plays a key role not only in limiting virus replication at early stages of infection, but also in initiating the virus-specific adaptive immune responses. In addition, previous studies have demonstrated that gut commensal microbiota play a key role in innate and adaptive immune defense against influenza virus infection (25-30). Further, recent studies have indicated the roles of nasal bacteria in innate antiviral resistance to influenza virus infection or severity of the diseases (17, 18). However, it remains unclear whether nasal bacteria critically regulates the generation of influenza virus-specific adaptive immune responses after influenza virus infection. In this study, we demonstrated that depletion of commensal bacteria in upper respiratory tract by intranasal administration of antibiotics enhanced the virus-specific antibodies response following influenza virus infection. Surprisingly, depletion of nasal bacteria by intranasal administration of antibiotics before influenza virus infection significantly reduced the virus titer at 2 days post infection. This is consistent with a previous report showing that antibiotic treatment significantly reduce influenza virus replication at 6 hours post infection (31). Intranasal application of antibiotics suppressed

influenza virus replication through at least two possible mechanisms. First, intranasal administration of antibiotics enhances host resistance to influenza virus infection in a microbiota-independent manner (31). Second, disruption of nasal bacteria by intranasal antibiotic treatment may release PAMPs from the antibiotic-killed bacteria, which stimulate innate antiviral immune responses to suppress influenza virus replication (32). After 3 and 5 days post infection, the viral replication in upper respiratory tract became comparable between antibiotic-treated and control groups, indicating that the levels of influenza virus replication in upper respiratory tract is unlikely to account for increased levels of the virus-specific antibodies response in antibiotic-treated mice.

Since the primary targets of influenza virus are the nasal epithelial cells in upper respiratory tract, it is beneficial to induce the virus-specific nasal IgA antibody at the nasal mucosal epithelium. However, intranasal vaccination with split-virus vaccine alone is often insufficient to elicit proper immune responses at the upper respiratory tract. Therefore, adjuvants are required for a given vaccine to induce the vaccine-specific nasal IgA response. In developing intranasal vaccines, cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) have been used as adjuvant to enhance nasal immune response (33). Although CT and LT are effective adjuvants to enhance mucosal immune responses including secretory IgA responses, they have some side

350 effects in humans, including Bell's palsy and nasal discharge (34). Therefore, several
351 adjuvants that are as effective as CT or LT and are also safe for human use have been
352 developed for clinical application with intranasal influenza vaccine (8-13). In this study,
353 we show that intranasal vaccination with influenza virus HA vaccine and culturable oral
354 bacteria from a healthy human volunteer induced significant levels of the
355 vaccine-specific nasal IgA and serum IgG responses in a dose-dependent manner. All
356 commensal bacterial strains tested, including *S. salivarius*, *S. parasanguinis*, or *S.*
357 *infantis*, induced comparable levels of the HA-specific nasal IgA and serum IgG
358 responses, suggesting that adjuvant activity of the oral bacteria is unlikely to account for
359 strain specific. In addition to culturable oral bacteria from a healthy human volunteer,
360 we demonstrated that disruption of nasal bacteria by lysozyme induced significant
361 levels of the vaccine-specific antibodies response. Although relative amounts of nasal
362 bacteria were significantly lower than that in the oral cavity, disruption of nasal bacteria
363 by lysozyme could rescue the inability of nasal bacteria to generate the vaccine-specific
364 antibodies response. In mice, nasal commensal microbiota are predominantly composed
365 of gram-positive bacteria including *Lactobacillus spp*., *Bacillus spp*., *Staphylococcus*
366 *spp*., and *Streptococcus spp*. (25). In addition, *Lactobacillus spp*. were found to contain
367 higher amounts of double-stranded RNA than the pathogenic bacteria (35). Since

368 activation of TLRs by different PAMPs such as poly(I:C) and zymosan synergistically
369 enhanced the nasal IgA response to intranasally administered influenza virus HA
370 vaccine (10), disruption of nasal bacteria could stimulate different TLRs to enhance the
371 vaccine-specific antibodies response. Most TLRs signal though the adaptor protein
372 MyD88 (36, 37). Although nasal epithelial cells express various TLRs (38, 39),
373 deficiency of MyD88 in stromal compartment did not significantly affect the levels of
374 nasal IgA and serum IgG responses following intranasal vaccination with influenza
375 virus HA and culturable oral bacteria. Instead, MyD88-dependent signaling in the
376 hematopoietic cells were required for adjuvant activity of intranasally administered oral
377 bacteria. These data are consistent with previous studies showing that both
378 TLR-induced dendritic cell maturation and B-cell activation are required for optimal
379 antibody responses to T-dependent antigens (40, 41).
380

381 **Conclusion**

382 Our study demonstrated the effects of commensal microbiota in upper respiratory
383 tract in the induction of the virus-specific adaptive immune responses after influenza
384 virus infection or intranasal vaccination. Our data indicated that disruption of nasal
385 bacteria by lysozyme or supplementation of oral bacteria from a healthy volunteer

386 enhanced nasal IgA and serum IgG antibodies response to intranasally administered
387 influenza virus HA or SARS-CoV-2 S proteins. Although the vaccinated animals
388 significantly reduced the virus titer compared to unadjuvanted group or naïve animals
389 following high-dose of influenza virus or SARS-CoV-2 challenge, further studies are
390 needed to establish the safety and efficacy of this vaccination method in an additional
391 animal model such as nonhuman primate.
392
393

Abbreviations

Abx: Antibiotic cocktail

BAL: Bronchoalveolar

BM: Bone marrow

BSA: Bovine serum albumin

COVID-19: Coronavirus disease 2019

CT: Cholera toxin

DMEM: Dulbecco's modified Eagle's medium

ELISA: Enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

HA: Hemagglutinin

ISCOMs: Immune stimulating complexes

LT: Escherichia coli heat-labile toxin

MAVS: Mitochondrial antiviral signaling

MDCK: Madin-Darby canine kidney

MyD88: Myeloid differentiation primary response 88

PAMPs: Pathogen-associated molecular patterns

PFU: Plaque-forming unit

Pdm09: A/Narita/1/2009

Poly(I:C):

PR8: A/Puerto Rico/8/1934

PRRs: pattern recognition receptors

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

S. salivarius: *Streptococcus salivarius*

S. parasanguinis: *Streptococcus parasanguinis*

S. infantis: *Streptococcus infantis*

TLR7: Toll-like receptor 7

TMPRSS2: Transmembrane protease serine 2

WT: Wild type

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Author's contributions

M. N., M.M., and T.I. performed experiments and analyzed data; T.I. designed research and wrote the paper.

Corresponding authors

Correspondence to Takeshi Ichinohe

Ethics approval and consent to participate

Informed consent was obtained for all participants. The research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science, the University of Tokyo (approval number 2019-42-1121). All experiments with SARS-CoV-2 were performed in enhanced biosafety level 3 (BSL-3) containment laboratories at the University of Tokyo, in accordance with the institutional biosafety operating procedures.

Availability of data and material

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Figure legends

Figure 1. Disruption of nasal bacteria enhances the virus-specific antibody responses following influenza virus infection.

(**A and B**) Mice were inoculated intranasally with an antibiotic cocktail (Abx) for 5 consecutive days. Two days later, mice were intranasally infected with 1,000 pfu of A/PR8 virus. The nasal wash and serum were collected at 4 weeks p.i., and the virus-specific nasal IgA and serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from three independent experiments (mean ± SEM). $*P < 0.05$ and $***P < 0.001$; (one-way ANOVA and Tukey's test).

Figure 2. Disruption of nasal bacteria induces the HA-specific antibody responses after intranasal vaccination.

(**A and B**) Mice were immunized intranasally with quadrivalent HA vaccine with or without poly(I:C) or lysozyme twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from three independent experiments (mean ± SEM). $**P < 0.01$ and $***P < 0.001$; (one-way ANOVA and Tukey's test).

Figure 3. Oral bacteria acts as adjuvant for intranasal vaccine.

(**A and B**) Mice were immunized intranasally with quadrivalent HA vaccine with or without LPS, poly(I:C), or culturable oral bacteria from mice or a healthy volunteer twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and serum IgG titers were determined by ELISA. (**C and D**) Mice were immunized intranasally with quadrivalent HA vaccine with or without indicated amounts of oral bacteria from a healthy volunteer twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from two independent experiments (mean ± SEM). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$; (one-way ANOVA and Tukey's test).

Figure 4. Adjuvant activity of *S. salivarius*, *S. parasanguinis*, and *S. infantis* for intranasal vaccine.

(**A and B**) Mice were immunized intranasally with quadrivalent HA vaccine with or without *S. salivarius*, *S. parasanguinis*, or *S. infantis* twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and

serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from two independent experiments (mean ± SEM). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$; (one-way ANOVA and Tukey's test).

Figure 5. Oral bacteria acts as adjuvant for intranasal vaccine.

(**A-D**) WT and MyD88-deficient mice were immunized intranasally with quadrivalent HA vaccine with or without culturable oral bacteria from a healthy volunteer (**A and B**) or lysozyme (**C and D**) twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from two independent experiments (mean ± SEM). ***$P < 0.001$; (one-way ANOVA and Tukey's test).

Figure 6. Oral bacteria acts as adjuvant for intranasal vaccine.

(**A and B**) WT→MyD88 KO and MyD88 KO→WT BM chimeric mice were immunized intranasally with quadrivalent HA vaccine with or without culturable oral bacteria from a healthy volunteer twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and serum IgG titers

were determined by ELISA. Open circles indicate values for individual mice. The data are from two independent experiments (mean ± SEM). ***$P < 0.001$; (one-way ANOVA and Tukey's test).

Figure 7. Protective effects of oral bacteria-adjuvanted intranasal vaccine against influenza virus infection.

Mice were immunized intranasally with quadrivalent HA vaccine with or without culturable oral bacteria from a healthy volunteer or lysozyme twice in a 3-week interval. Two weeks after the last vaccination, mice were challenged with 1,000 pfu of A/PR8 virus. The nasal wash of influenza virus-infected mice was collected at 3 days post infection, and viral titers were determined by plaque assay. Open circles indicate values for individual mice. The dashed line indicates the limit of virus detection. The data are from two independent experiments (mean ± SEM). ***$P < 0.001$; (one-way ANOVA and Tukey's test).

Figure 8. Protective effects of oral bacteria-adjuvanted intranasal vaccine against SARS-CoV-2 infection.

(**A-C**) Hamsters were immunized subcutaneously or intranasally with the spike protein of SARS-CoV-2 with or without culturable oral bacteria from a healthy volunteer twice in a 3-week interval. Two weeks after the last vaccination, hamsters were challenged with 2×10^6 pfu of SARS-CoV-2. (**A and B**) Serum were collected at 3 days post infection. The spike protein- (**A**) or SARS-CoV-2- (**B**) specific serum IgG antibody titers were determined by ELISA. (**C**) The lung wash of SARS-CoV-2-infected hamsters was collected at 3 days post infection, and viral titers were determined by plaque assay. Open circles indicate values for individual hamsters. The data are from two independent experiments (mean ± SEM). $*P < 0.05$ and $**P < 0.01$; (one-way ANOVA and Tukey's test).

Figure 1

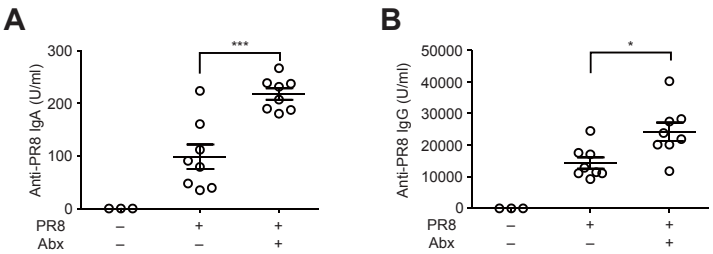

A
B
Anti-PR8 IgA (U/ml)
Anti-PR8 IgG (U/ml)
300
200
100
0
50000
40000
30000
20000
10000
***
*
PR8
Abx
–
+

Figure 2

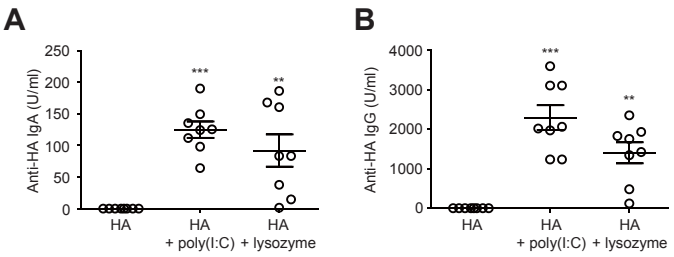

A
B
Anti-HA IgA (U/ml)
Anti-HA IgG (U/ml)
250
200
150
100
50
0
4000
3000
2000
1000
0
***
**
HA
HA
+ poly(I:C)
HA
+ lysozyme

Figure 3

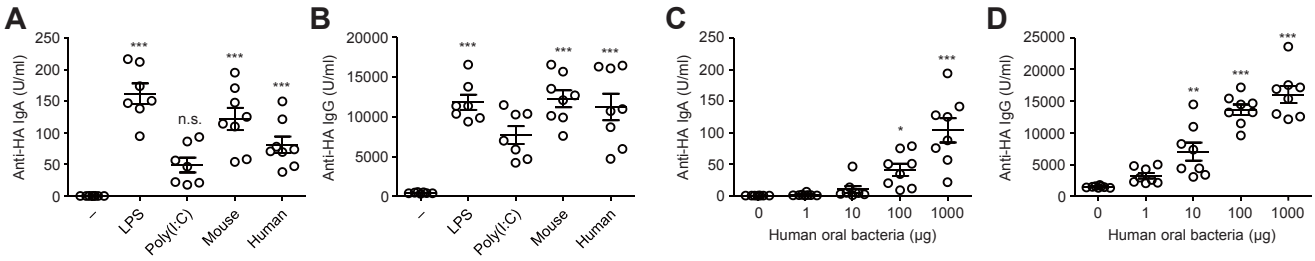

A
Anti-HA IgA (U/ml)
250
200
150
100
50
0
***
n.s.
***
***
–
LPS
Poly(I:C)
Mouse
Human
B
Anti-HA IgG (U/ml)
20000
15000
10000
5000
0
***
***
***
–
LPS
Poly(I:C)
Mouse
Human
C
Anti-HA IgA (U/ml)
250
200
150
100
50
0
*
***
0
1
10
100
1000
Human oral bacteria (μg)
D
Anti-HA IgG (U/ml)
25000
20000
15000
10000
5000
0
**
***
***
0
1
10
100
1000
Human oral bacteria (μg)

Figure 4

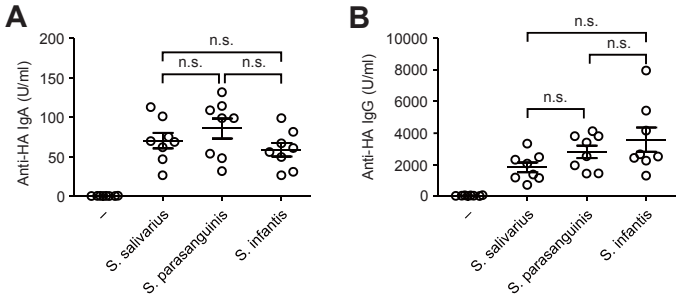

A
Anti-HA IgA (U/ml)
200
150
100
50
0
n.s.
n.s.
n.s.
–
S. salivarius
S. parasanguinis
S. infantis
B
Anti-HA IgG (U/ml)
10000
8000
6000
4000
2000
0
n.s.
n.s.
n.s.
–
S. salivarius
S. parasanguinis
S. infantis

Figure 5

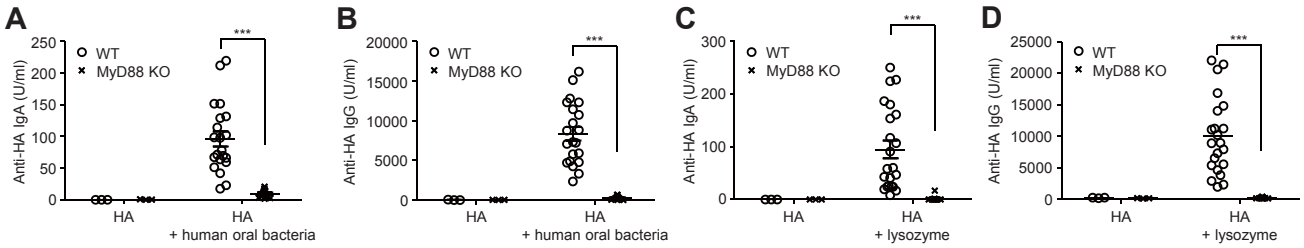

A
B
C
D
WT
MyD88 KO
Anti-HA IgA (U/ml)
Anti-HA IgG (U/ml)
***
HA
HA
+ human oral bacteria
+ lysozyme

Figure 6

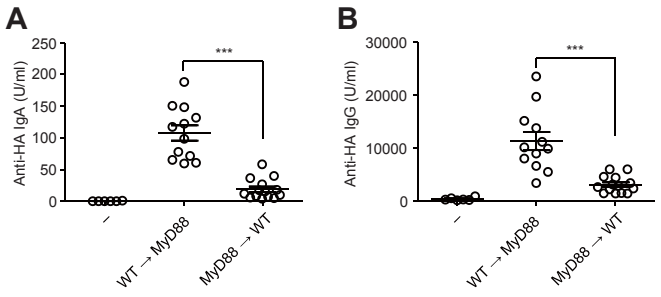

A
Anti-HA IgA (U/ml)
250
200
150
100
50
0
***
–
WT → MyD88
MyD88 → WT
B
Anti-HA IgG (U/ml)
30000
20000
10000
0
***
–
WT → MyD88
MyD88 → WT

Figure 7

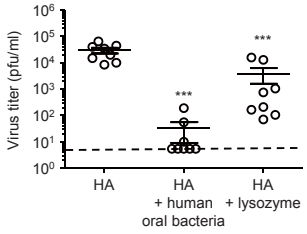
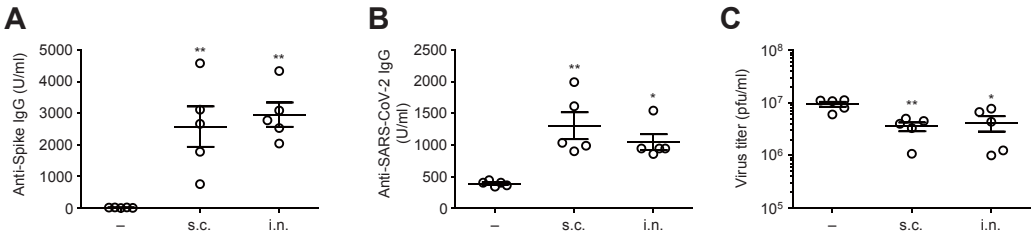

Virus titer (pfu/ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
***
***
HA
HA
+ human
oral bacteria
HA
+ lysozyme

Figure 8


A
Anti-Spike IgG (U/ml)
5000
4000
3000
2000
1000
0
**
**
–
s.c.
i.n.
B
Anti-SARS-CoV-2 IgG (U/ml)
2500
2000
1500
1000
500
0
**
*
–
s.c.
i.n.
C
Virus titer (pfu/ml)
10^8
10^7
10^6
10^5
**
*
–
s.c.
i.n.

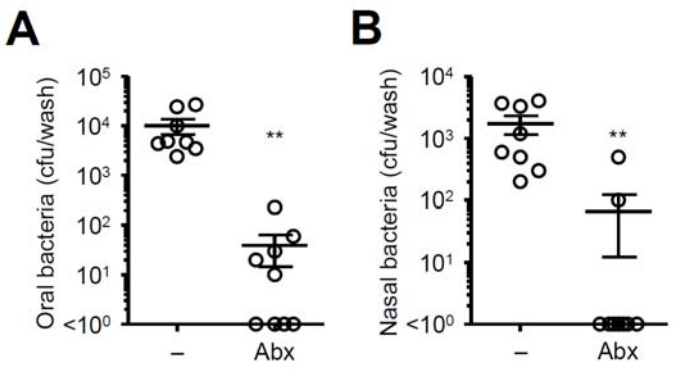


Figure S1. Effect of intranasal antibiotic treatment on oral and nasal bacterial load. Mice were inoculated intranasally with an antibiotic cocktail (Abx) for 5 consecutive days. Two days later, tongue (A) and nasal wash (B) were collected by washing the nasopharynx three times by injecting a total of 1 ml of brain heart infusion broth. Bacterial load in the tongue (A) and nasal wash (B) were measured. **P < 0.01; (one-way ANOVA and Tukey's test).

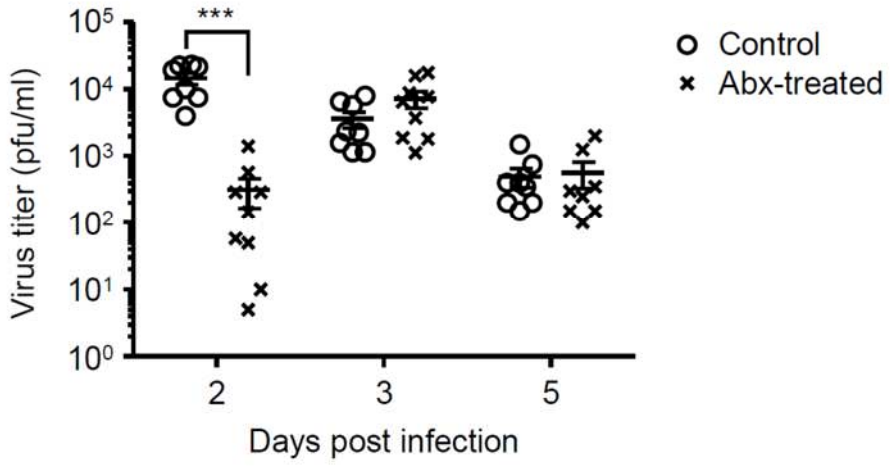


Figure S2. Effect of intranasal antibiotic treatment on influenza virus replication. Mice were inoculated intranasally with an antibiotic cocktail (Abx) for 5 consecutive days. Two days later, mice were intranasally infected with 1,000 pfu of A/PR8 virus. The nasal wash was collected at indicated time points, and viral titers were determined by plaque assay. ***$P < 0.001$; (one-way ANOVA and Tukey's test).

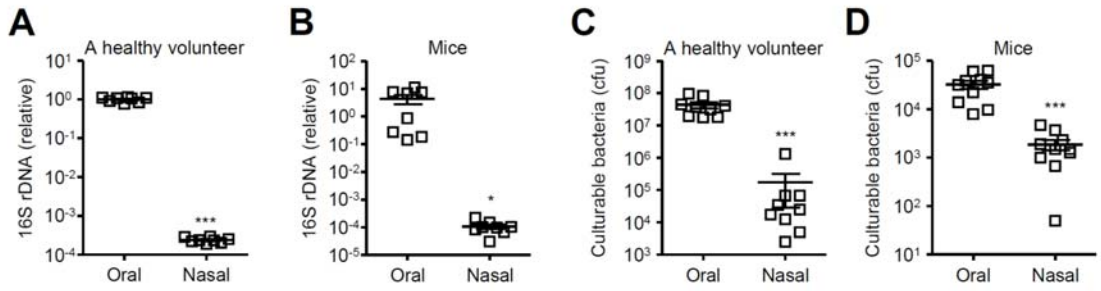


Figure S3. Relative gene copies of 16S rDNA and bacterial load in in the tongue and nasal wash. Relative gene copies of 16S rDNA isolated from tongue (A) and nasal wash (B) were quantified by qPCR. Culturable bacterial load in the tongue (C) and nasal wash (D) were measured. **P* < 0.05 and ****P* < 0.001; (one-way ANOVA and Tukey's test).

Figures

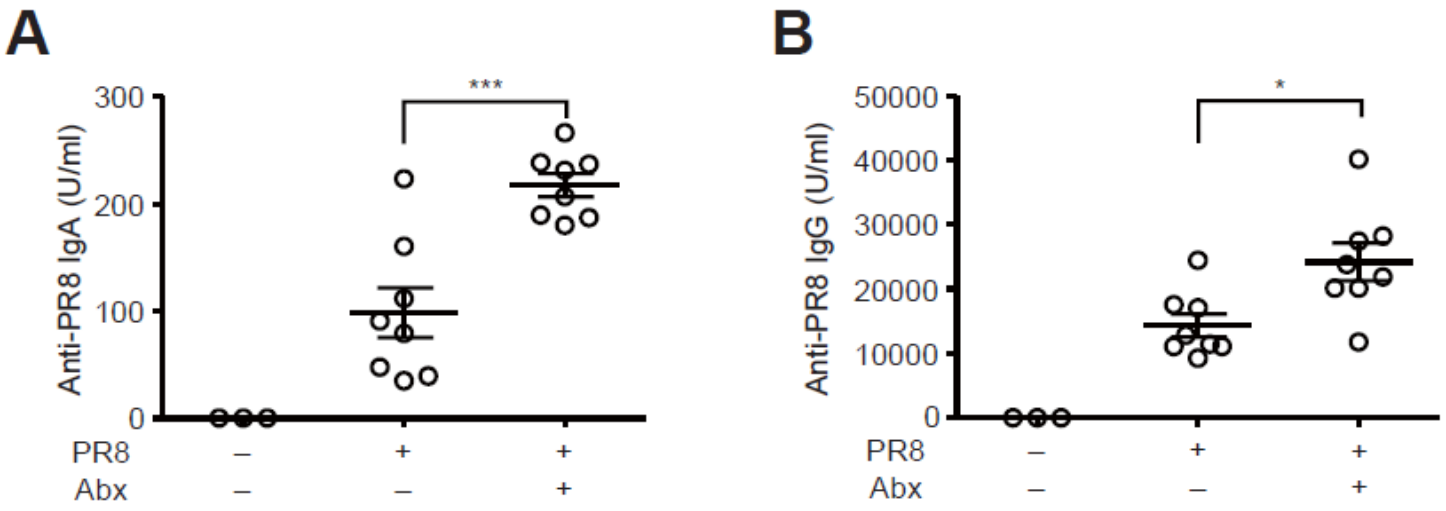
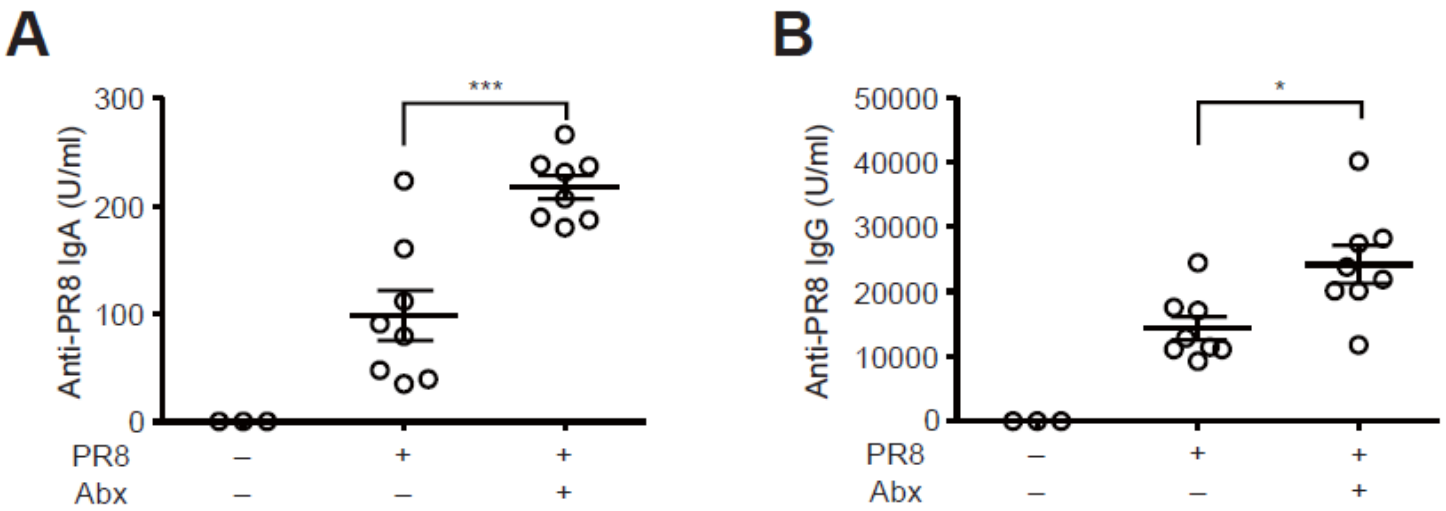


Figure 1

Disruption of nasal bacteria enhances the virus-specific antibody responses following influenza virus infection. (A and B) Mice were inoculated intranasally with an antibiotic cocktail (Abx) for 5 consecutive days. Two days later, mice were intranasally infected with 1,000 pfu of A/PR8 virus. The nasal wash and serum were collected at 4 weeks p.i., and the virus-specific nasal IgA and serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from three independent experiments (mean ± SEM). *P < 0.05 and ***P < 0.001; (one-way ANOVA and Tukey's test).



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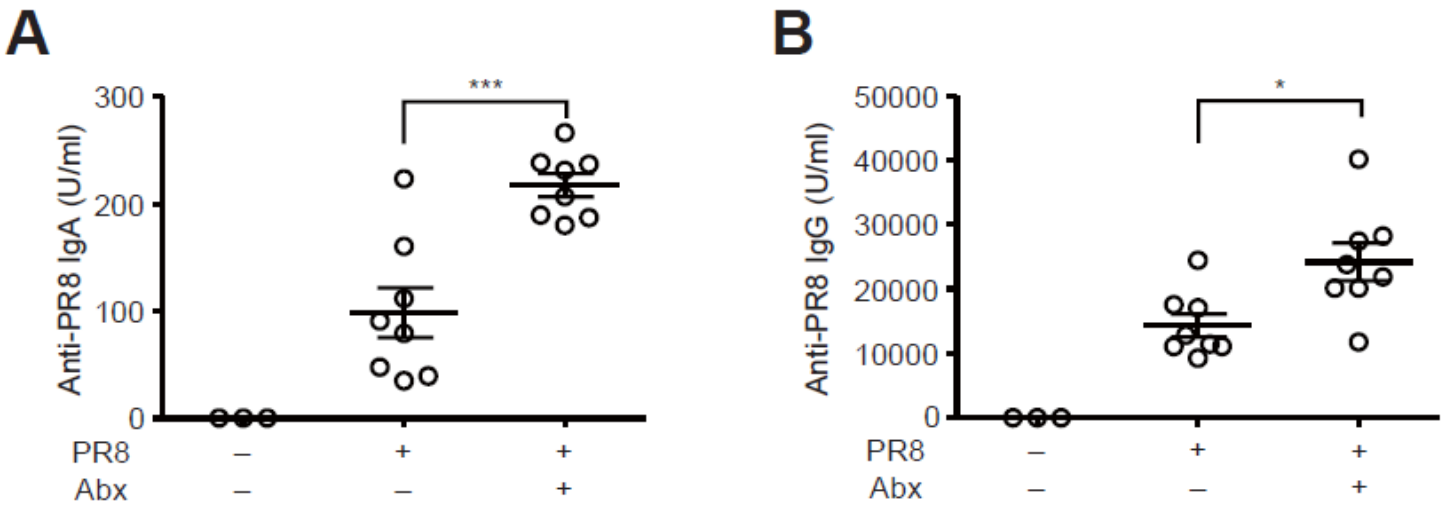

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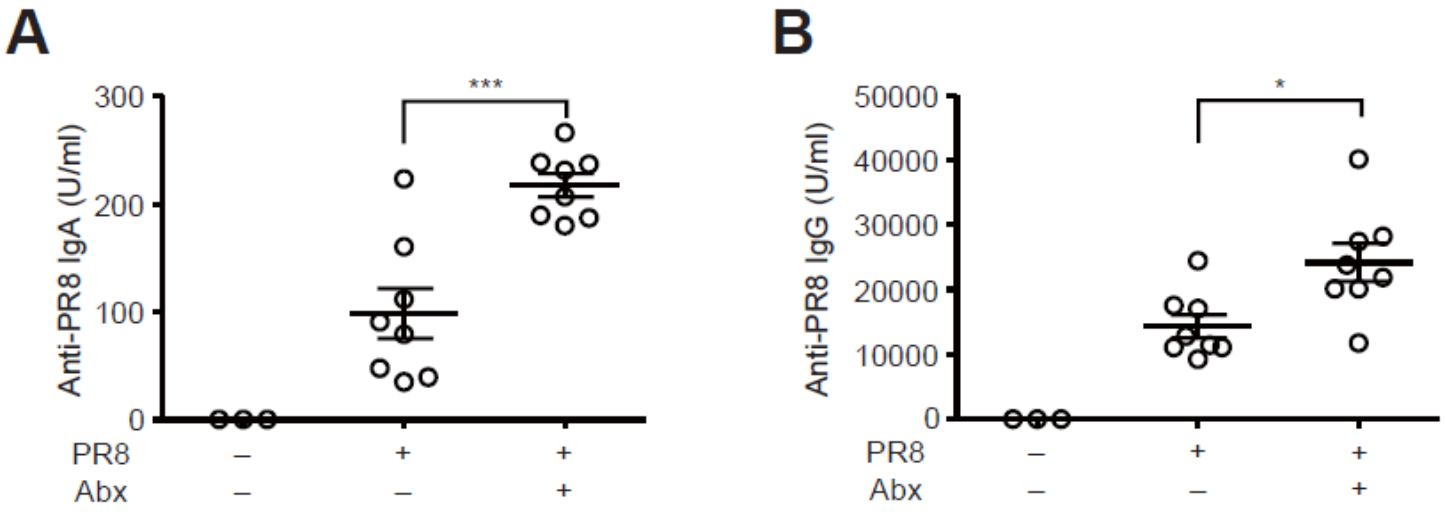

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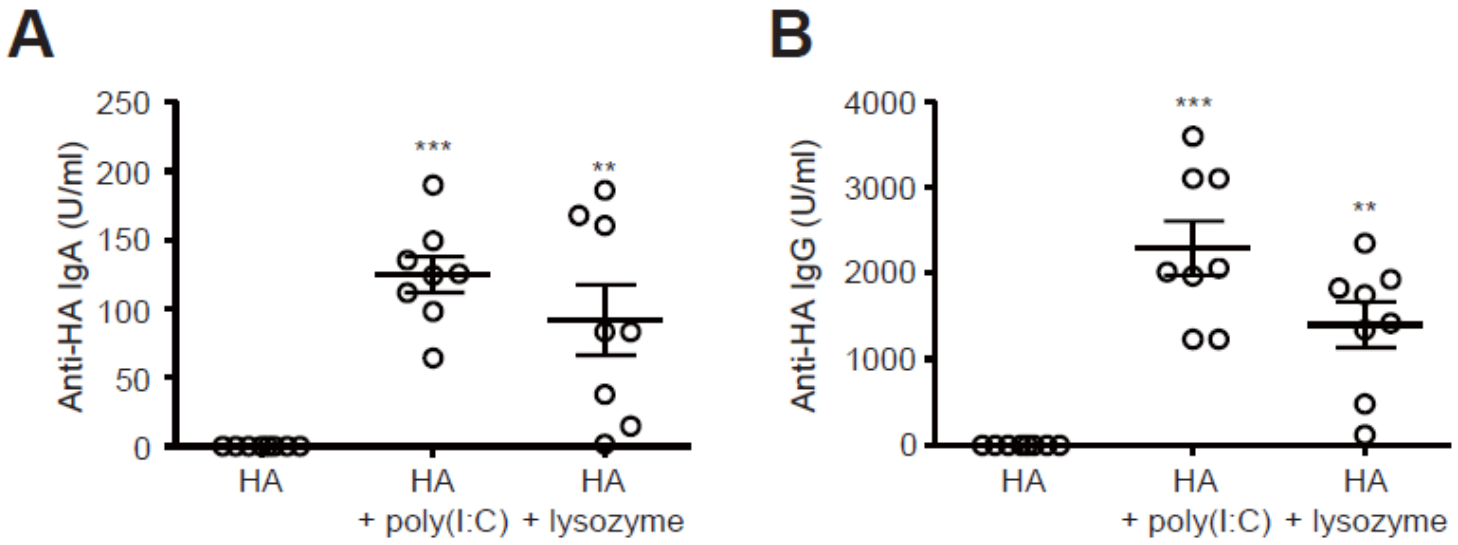
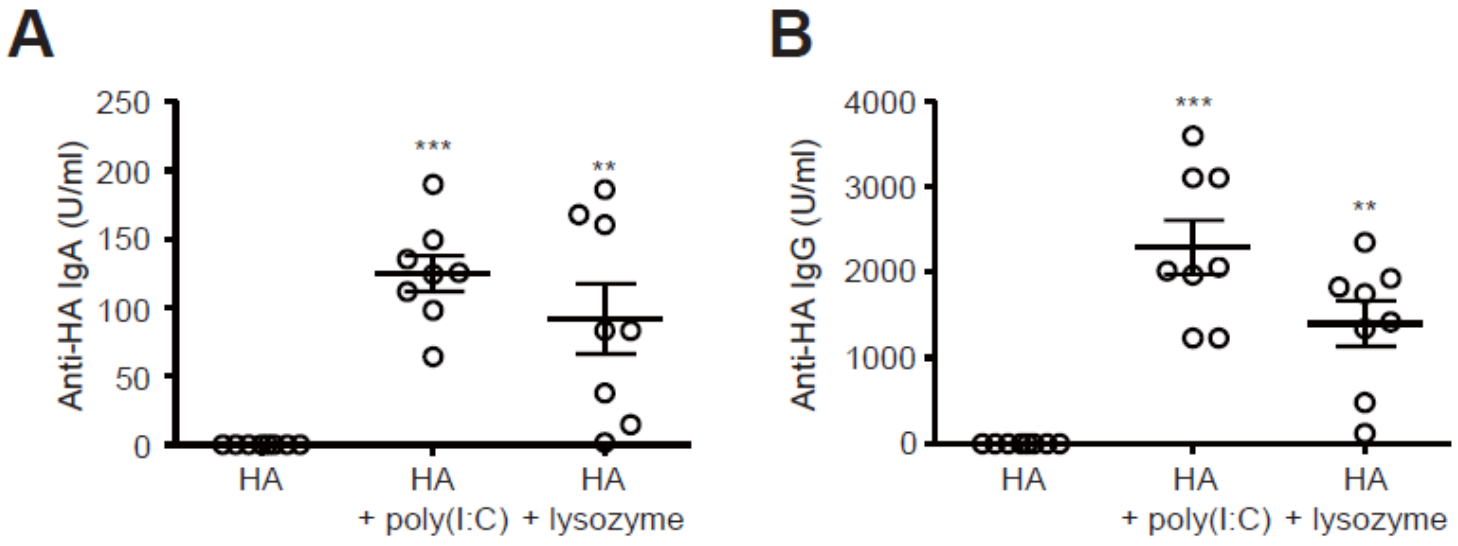


Figure 2

Figure 2. Disruption of nasal bacteria induces the HA-specific antibody responses after intranasal vaccination. (A and B) Mice were immunized intranasally with quadrivalent HA vaccine with or without poly(I:C) or lysozyme twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from three independent experiments (mean ± SEM). **P < 0.01 and ***P < 0.001; (one-way ANOVA and Tukey's test).



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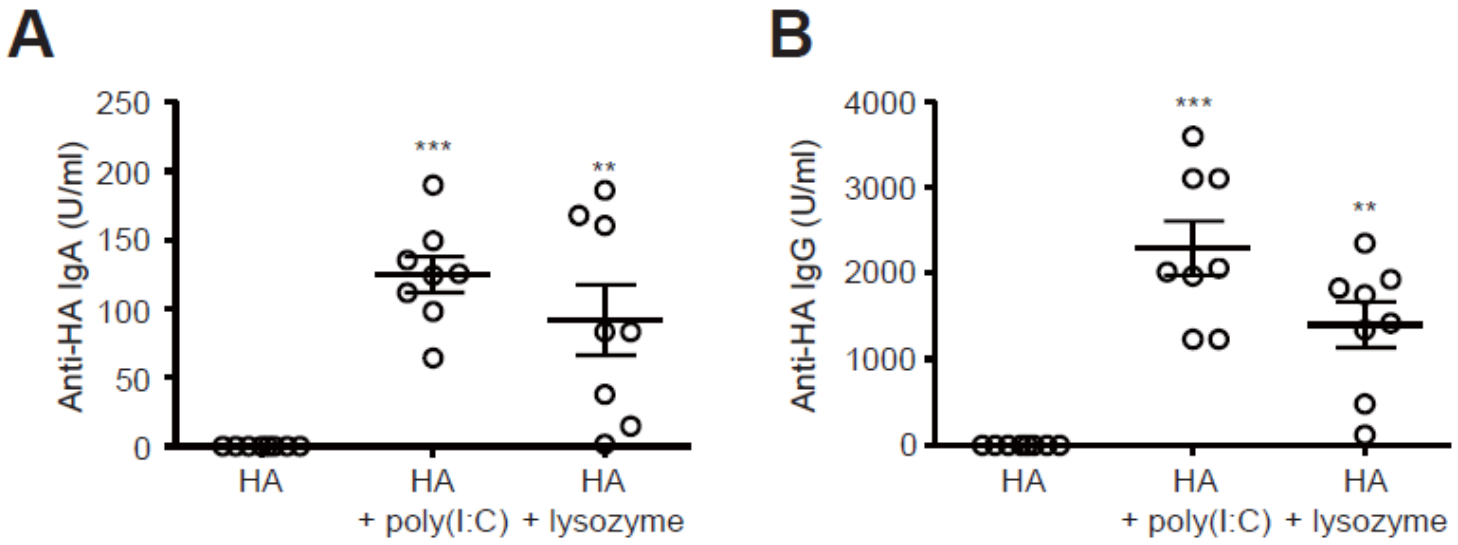

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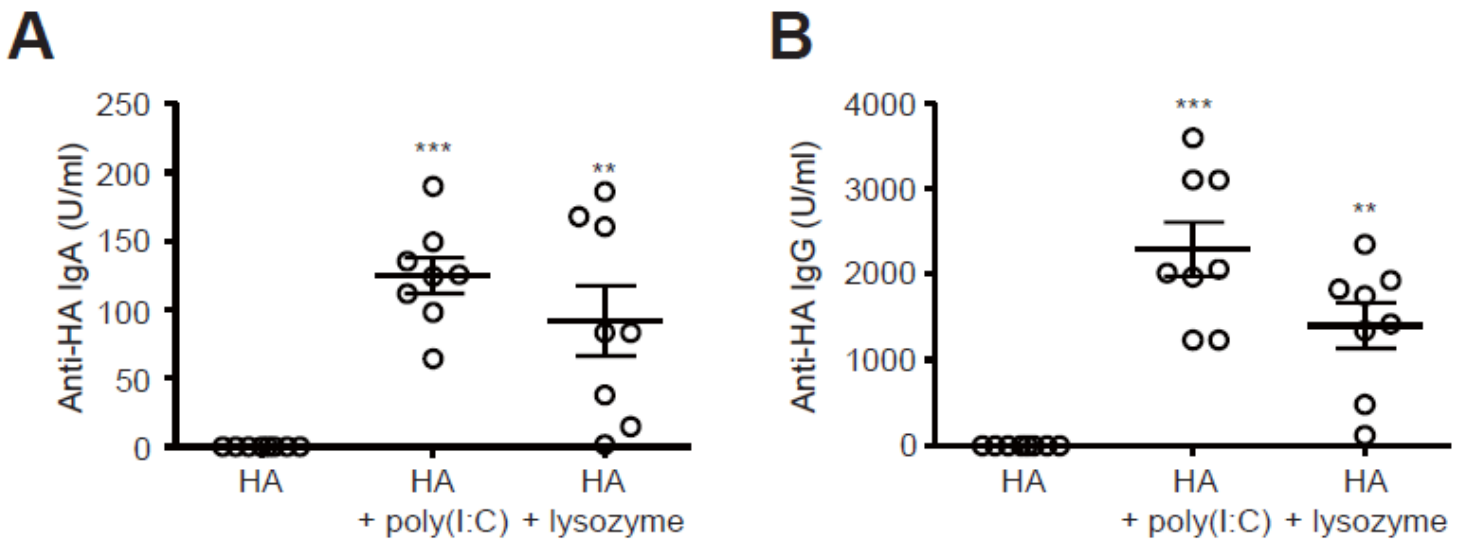

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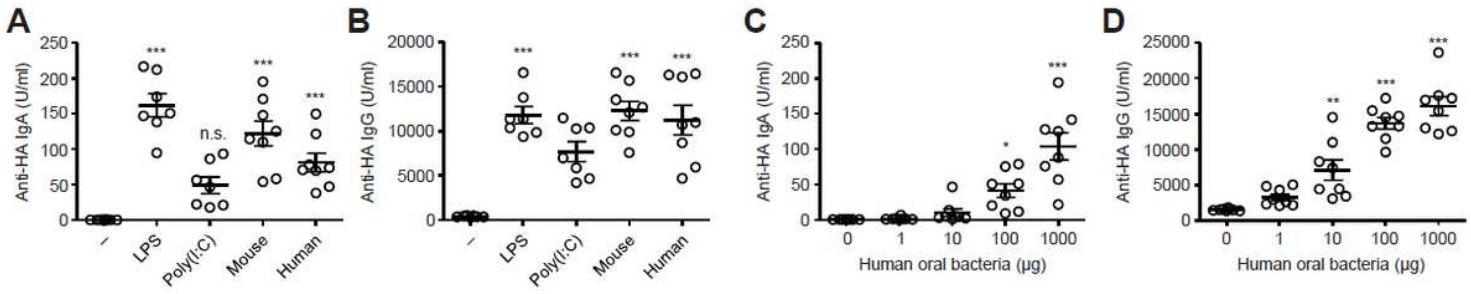

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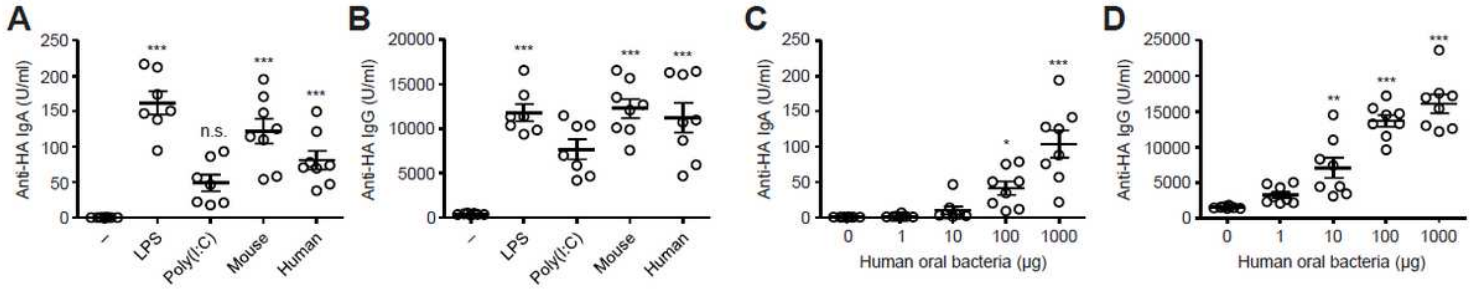

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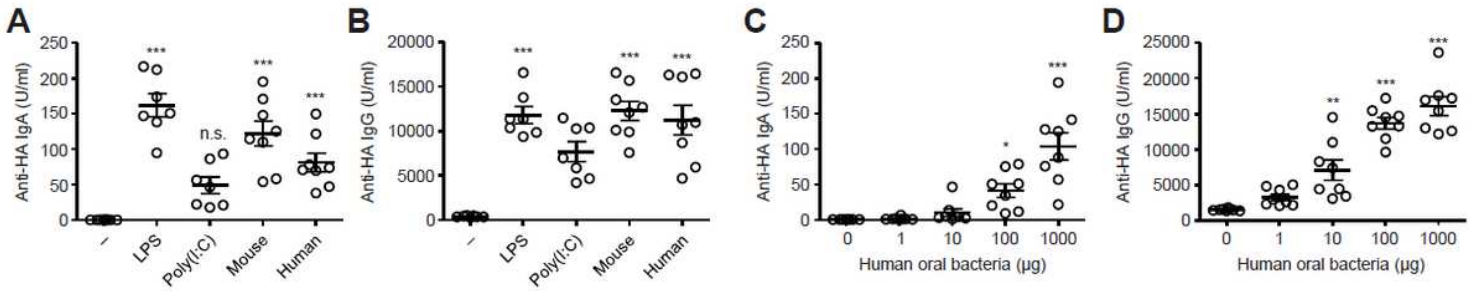

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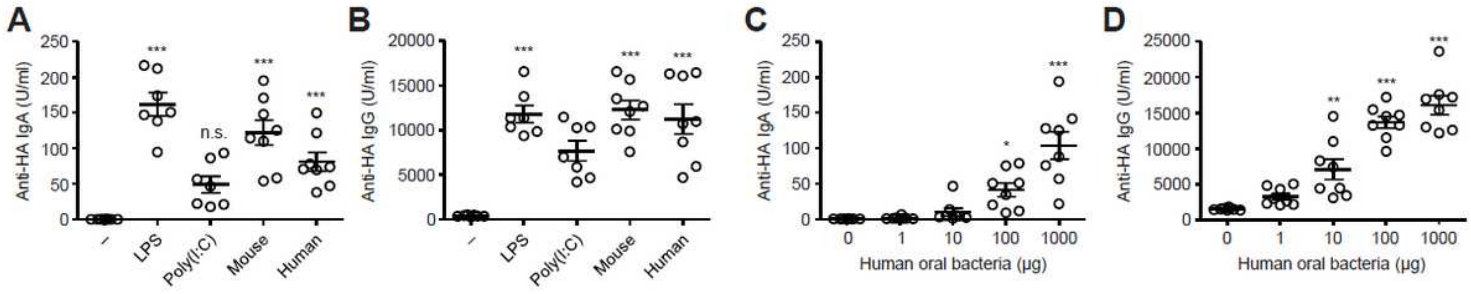

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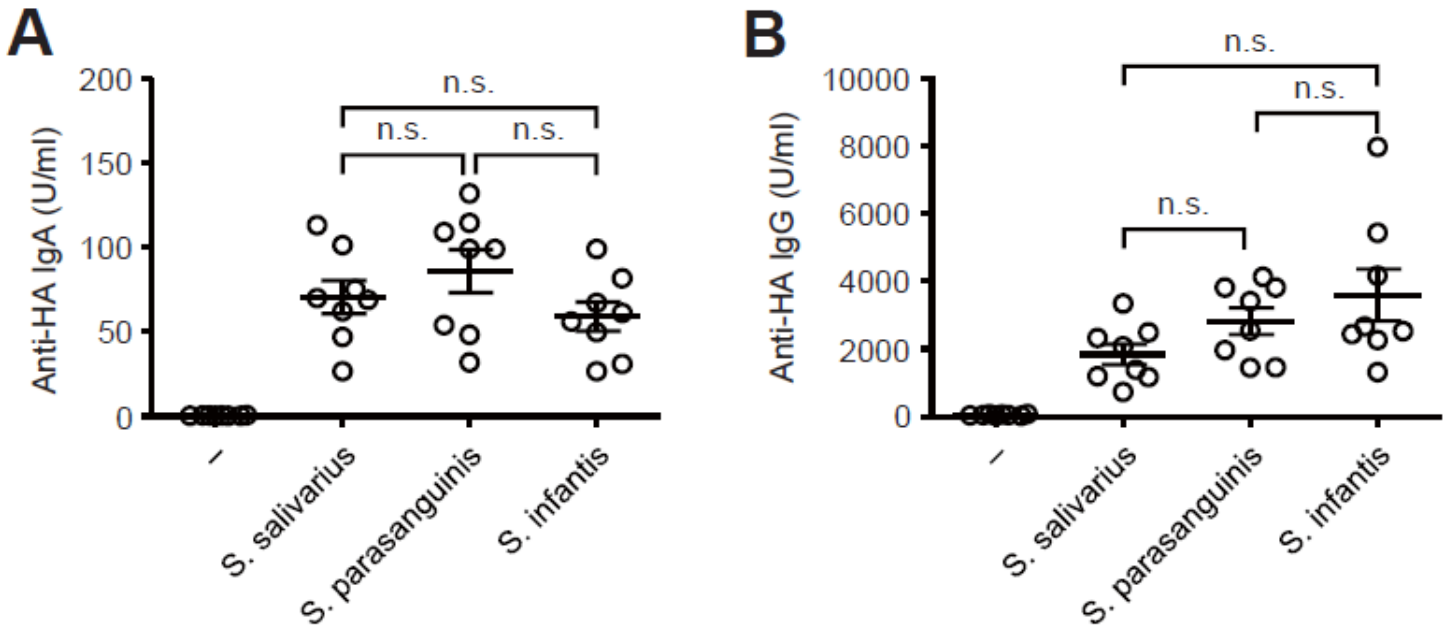

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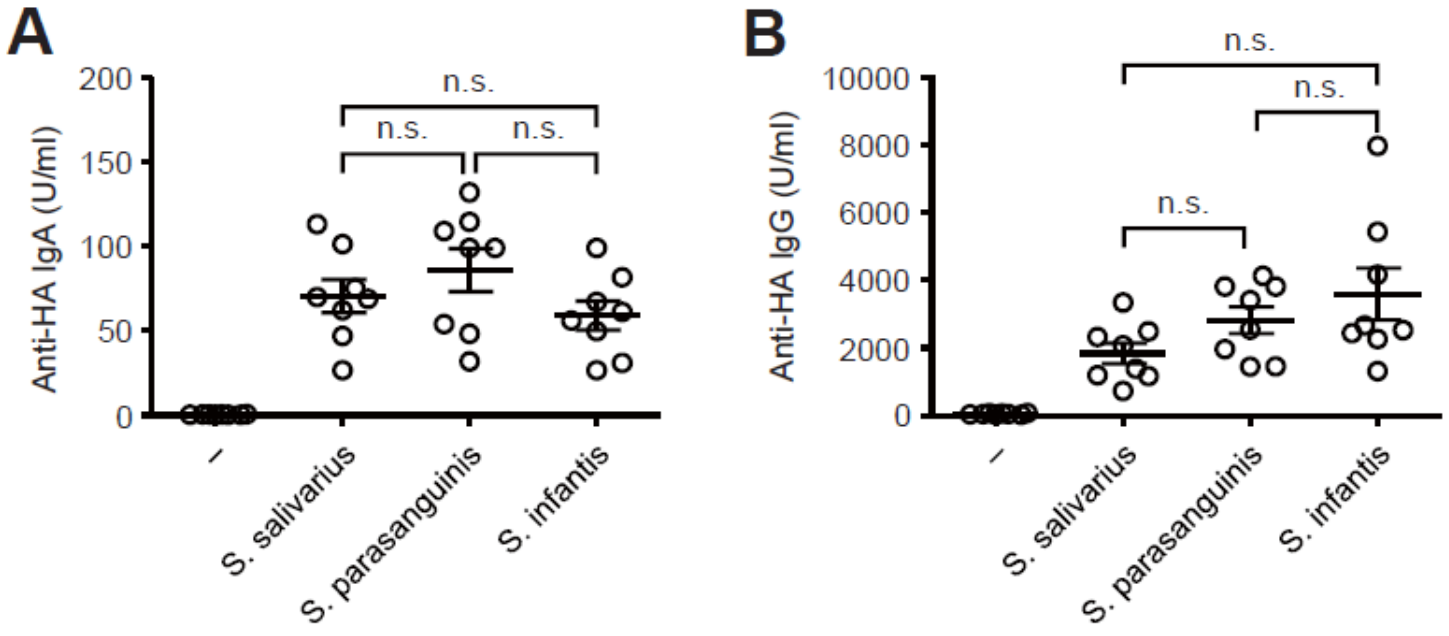
Adjuvant activity of S. salivarius, S. parasanguinis, and S. infantis for intranasal vaccine. (A and B) Mice were immunized intranasally with quadrivalent HA vaccine with or without S. salivarius, S. parasanguinis, or S. infantis twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and 35 serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from two independent experiments (mean ± SEM). *P < 0.05, **P < 0.01 and ***P < 0.001; (one-way ANOVA and Tukey's test).



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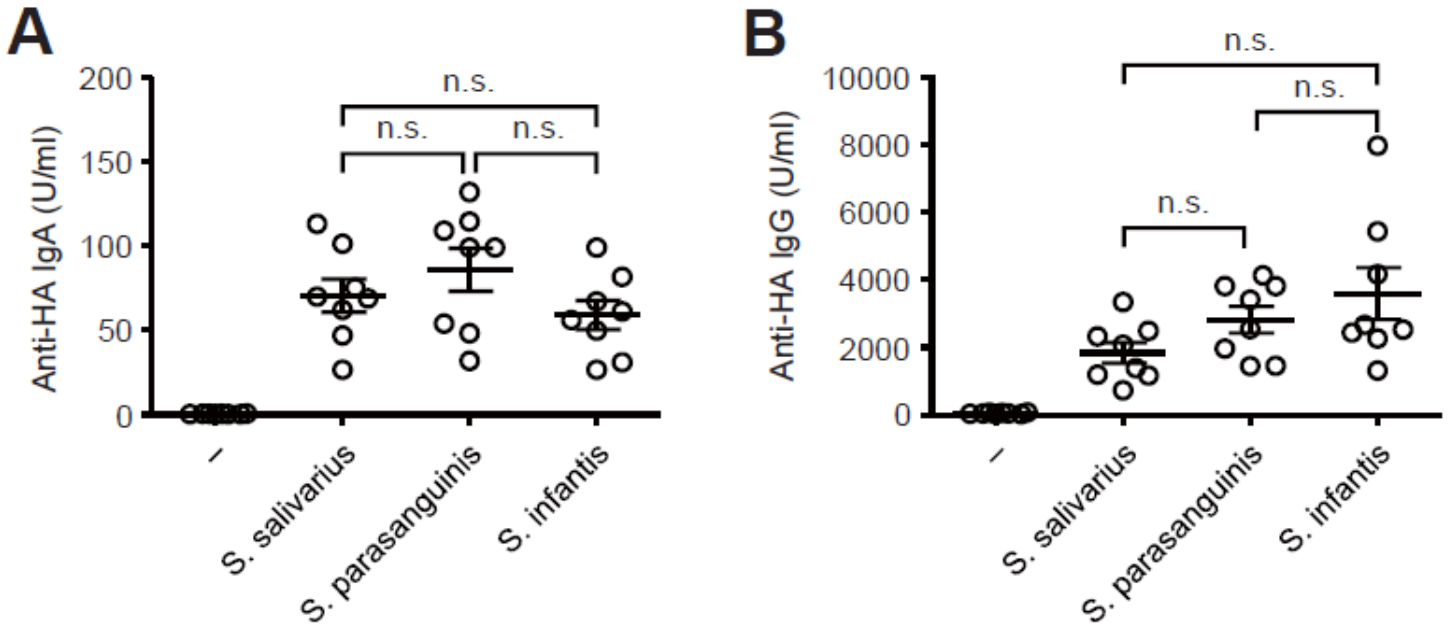

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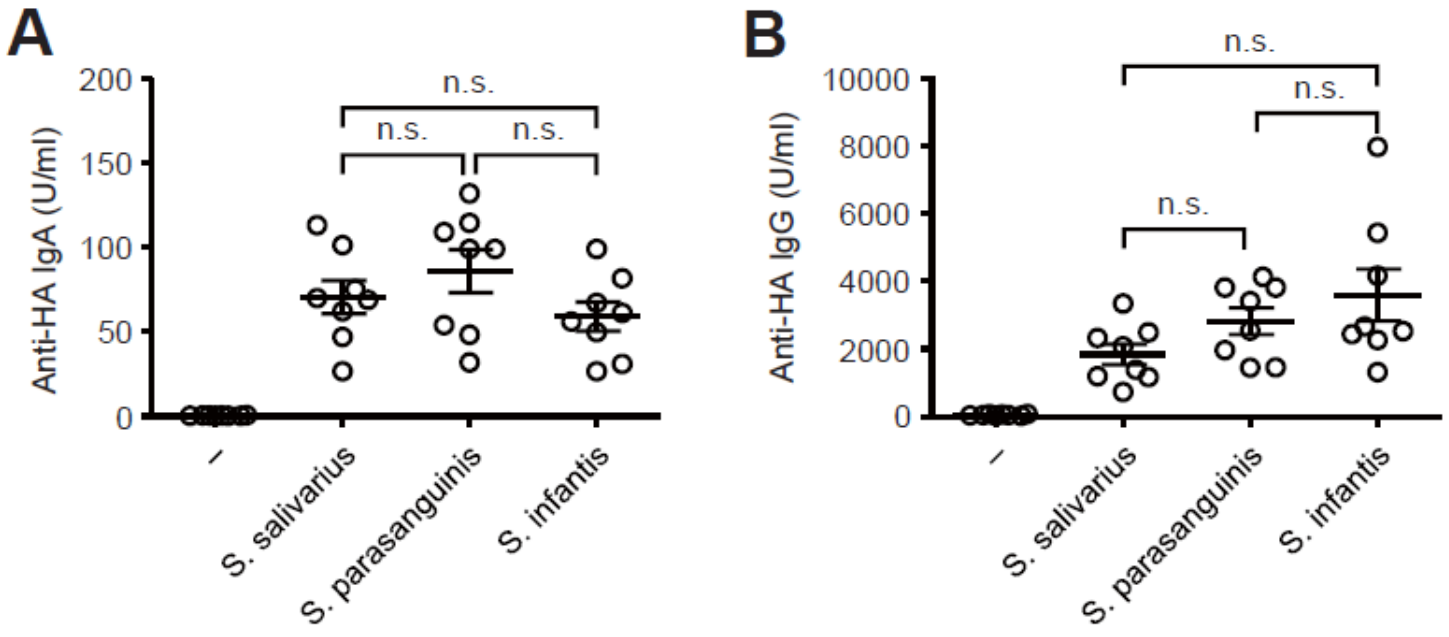

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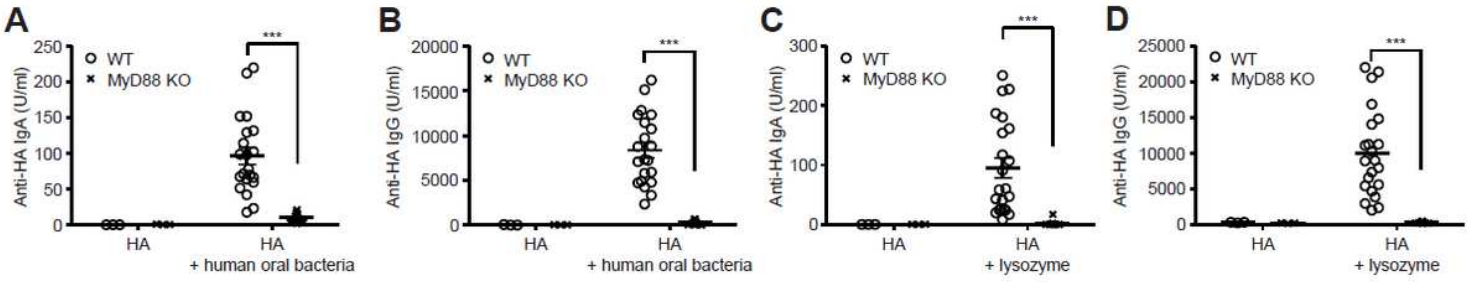

Figure 5

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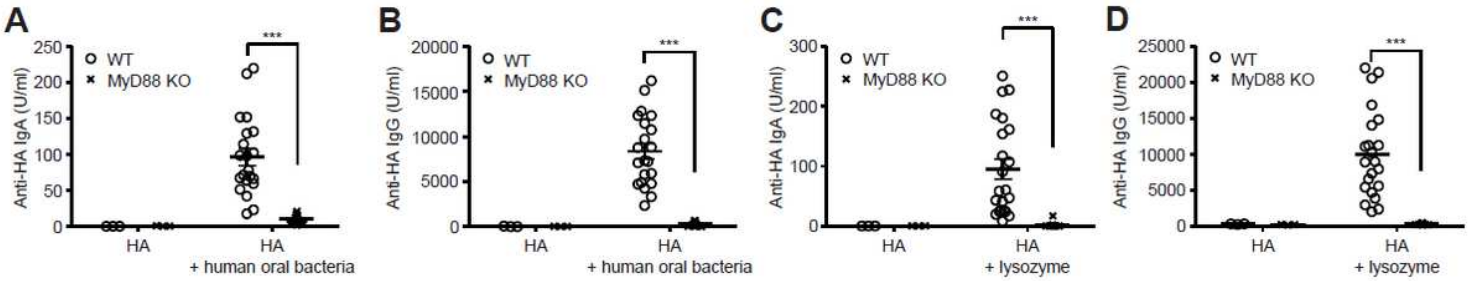

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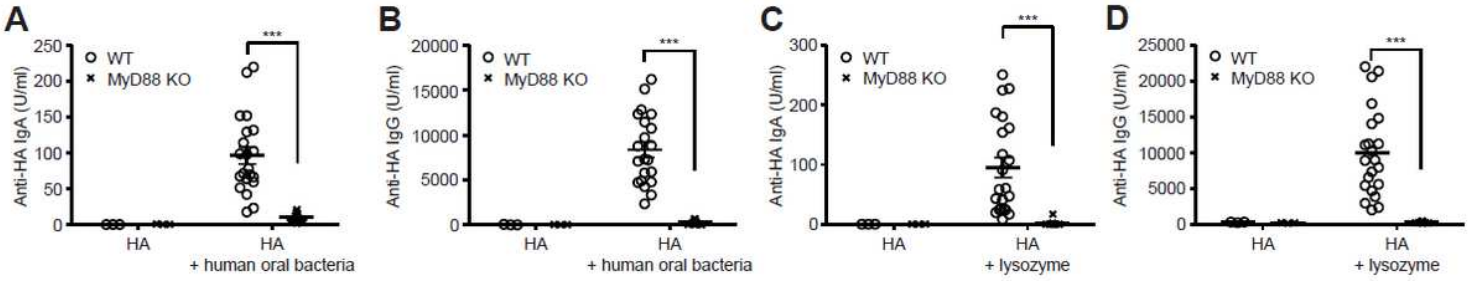

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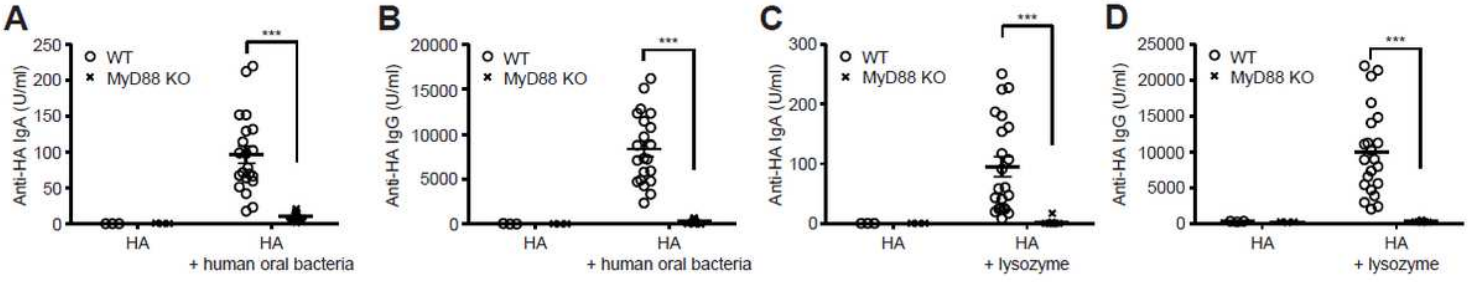

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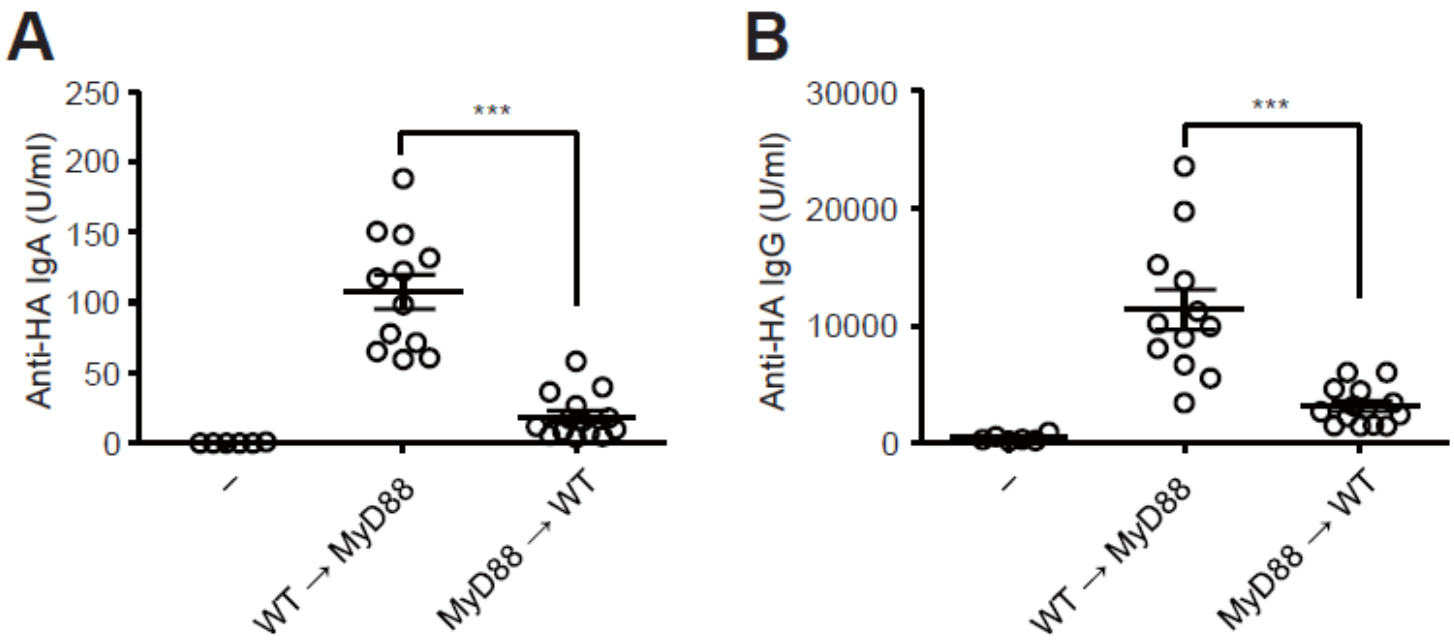

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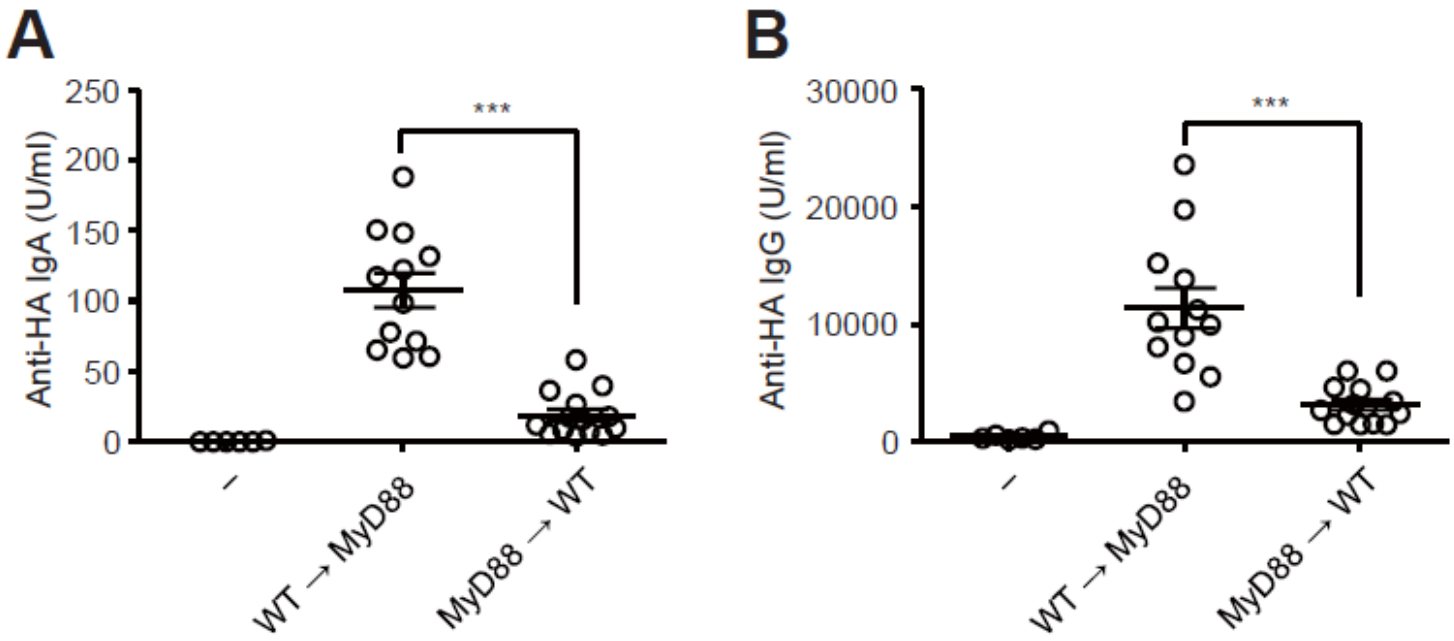
Oral bacteria acts as adjuvant for intranasal vaccine. (A and B) WT⟶MyD88 KO and MyD88 KO⟶WT BM chimeric mice were immunized intranasally with quadrivalent HA vaccine with or without culturable oral bacteria from a healthy volunteer twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected and the HA-specific nasal IgA and serum IgG titers were determined by ELISA. Open circles indicate values for individual mice. The data are from two independent experiments (mean ± SEM). ***P < 0.001; (one-way ANOVA and Tukey's test).



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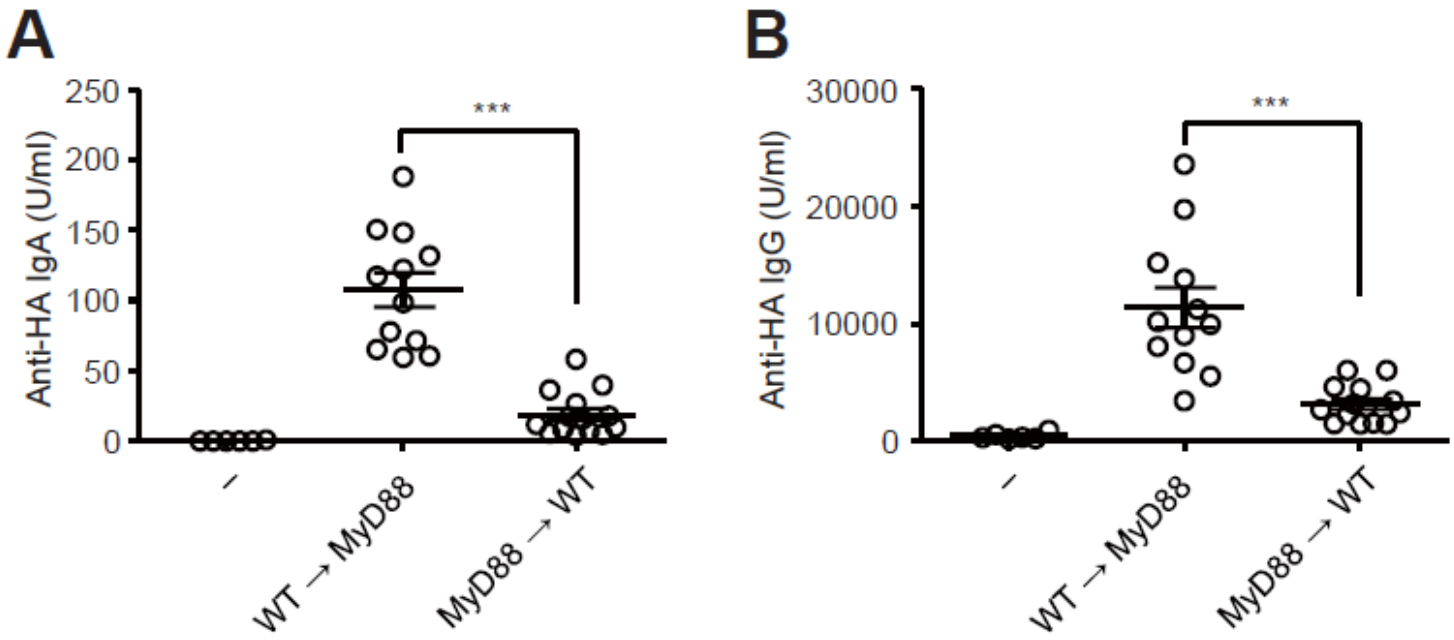

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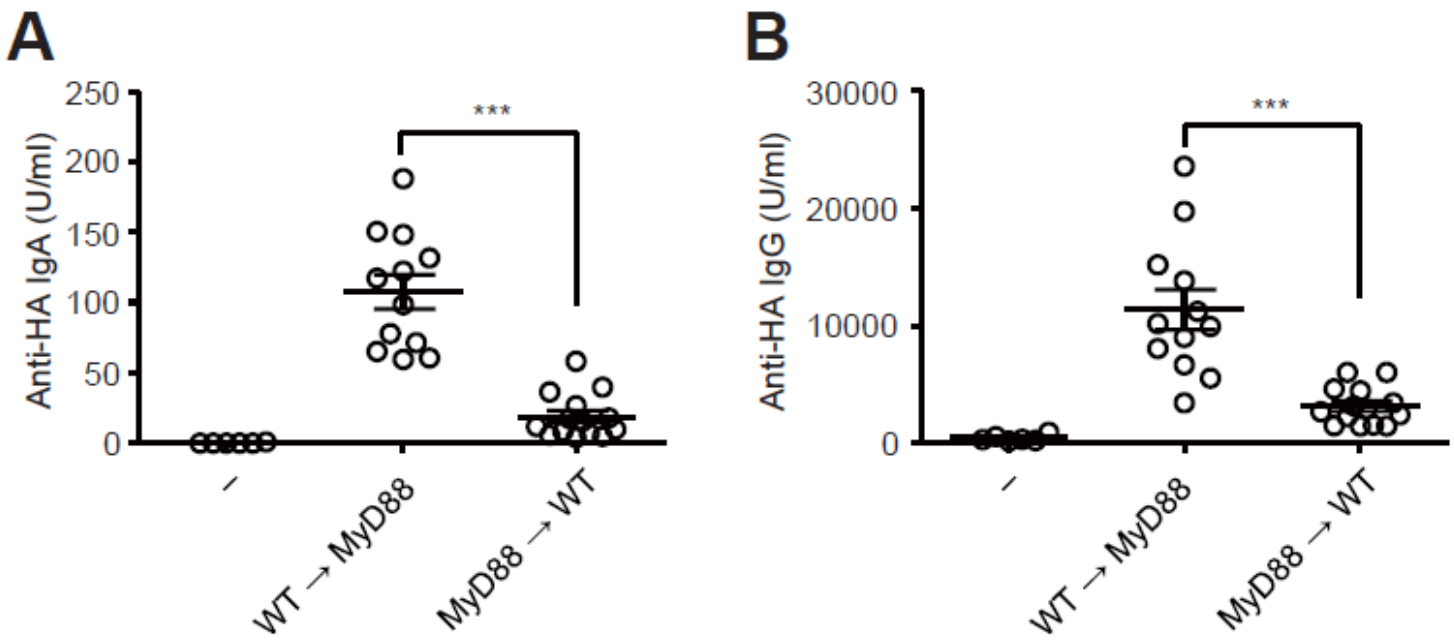

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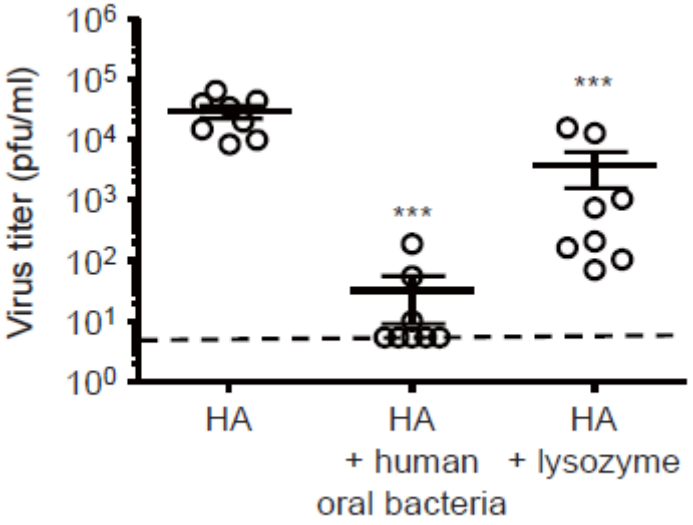
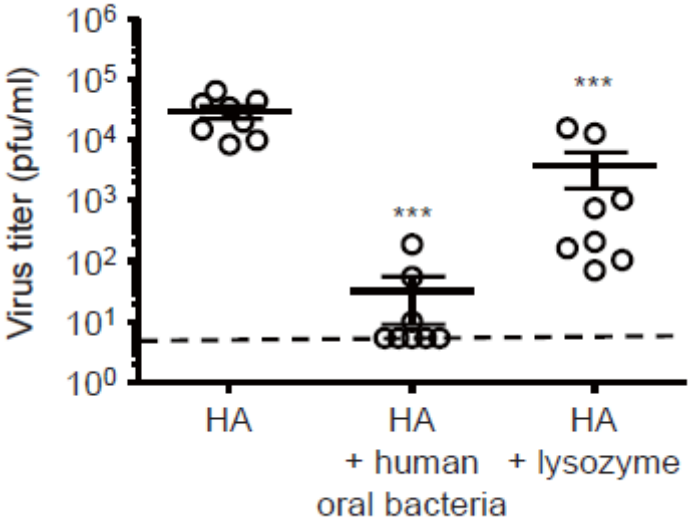


Figure 7

Protective effects of oral bacteria-adjuvanted intranasal vaccine against influenza virus infection. Mice were immunized intranasally with quadrivalent HA vaccine with or without culturable oral bacteria from a healthy volunteer or lysozyme twice in a 3-week interval. Two weeks after the last vaccination, mice were challenged with 1,000 pfu of A/PR8 virus. The nasal wash of influenza virus-infected mice was collected at 3 days post infection, and viral titers were determined by plaque assay. Open circles indicate values for individual mice. The dashed line indicates the limit of virus detection. The data are from two independent experiments (mean ± SEM). ***P < 0.001; (one-way ANOVA and Tukey's test).



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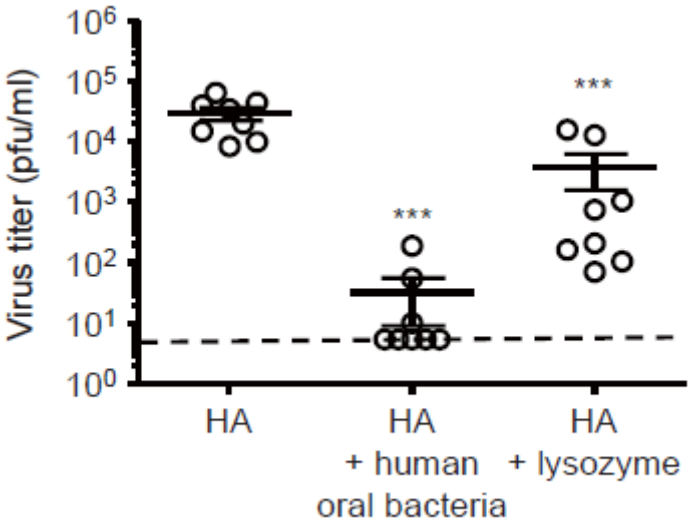

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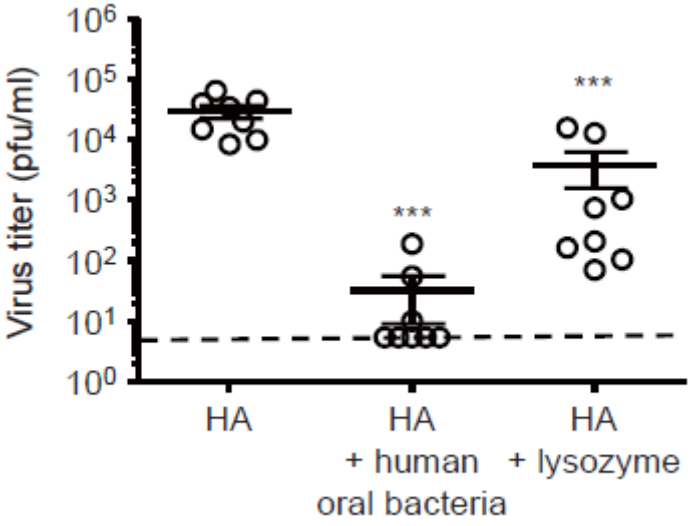

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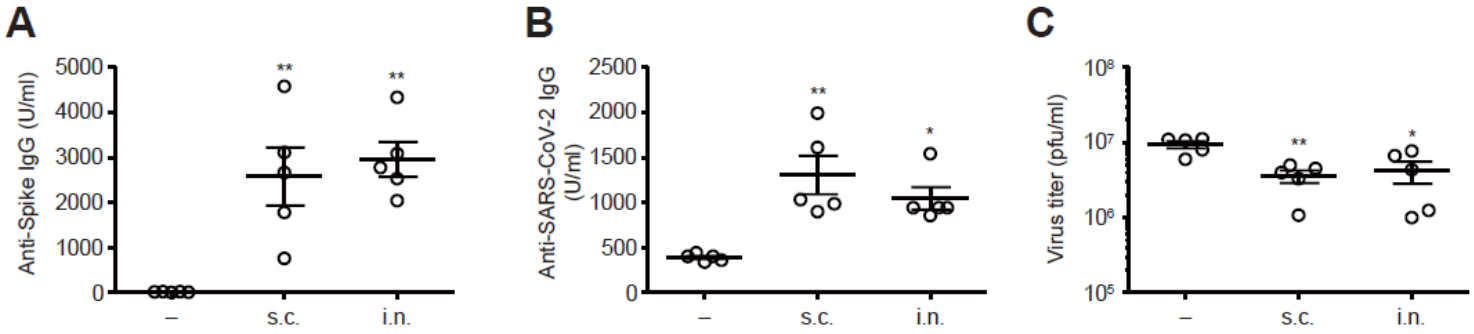
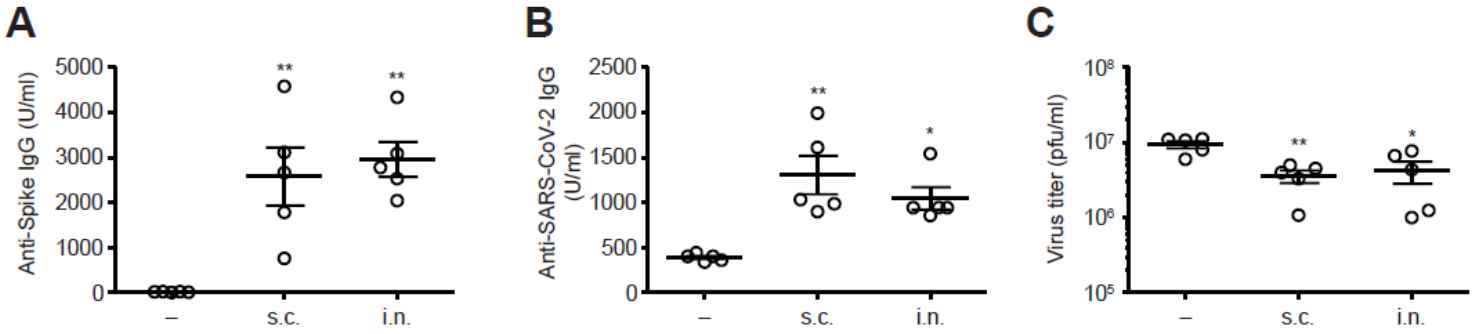


Figure 8

Protective effects of oral bacteria-adjuvanted intranasal vaccine against SARS-CoV-2 infection. (A-C) Hamsters were immunized subcutaneously or intranasally with the spike protein of SARS-CoV-2 with or without culturable oral bacteria from a healthy volunteer twice in a 3-week interval. Two weeks after the last vaccination, hamsters were challenged with 2×106 pfu of SARS-CoV-2. (A and B) Serum were collected at 3 days post infection. The spike protein- (A) or SARS-CoV-2- (B) specific serum IgG antibody titers were determined by ELISA. (C) The lung wash of SARS-CoV-2-infected hamsters was collected at 3 days post infection, and viral titers were determined by plaque assay. Open circles indicate values for individual hamsters. The data are from two independent experiments (mean ± SEM). *P < 0.05 and **P < 0.01; (one-way ANOVA and Tukey's test).



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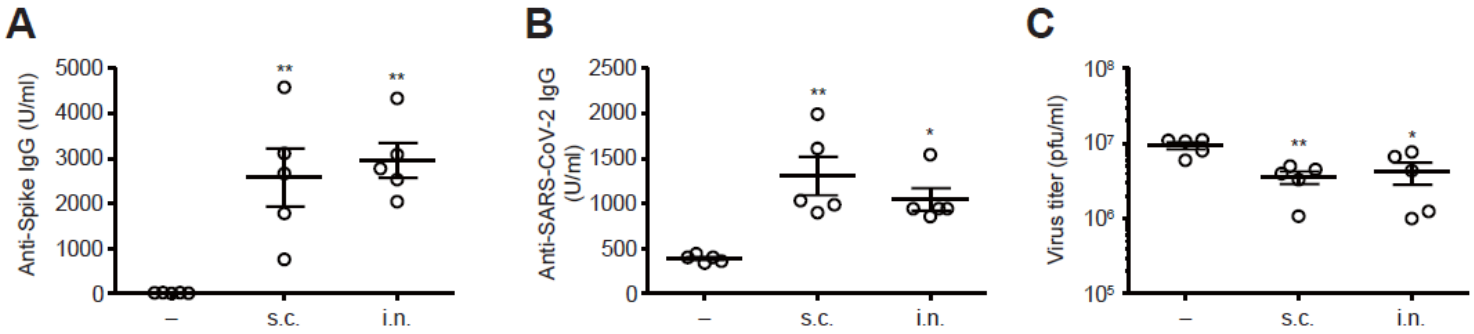

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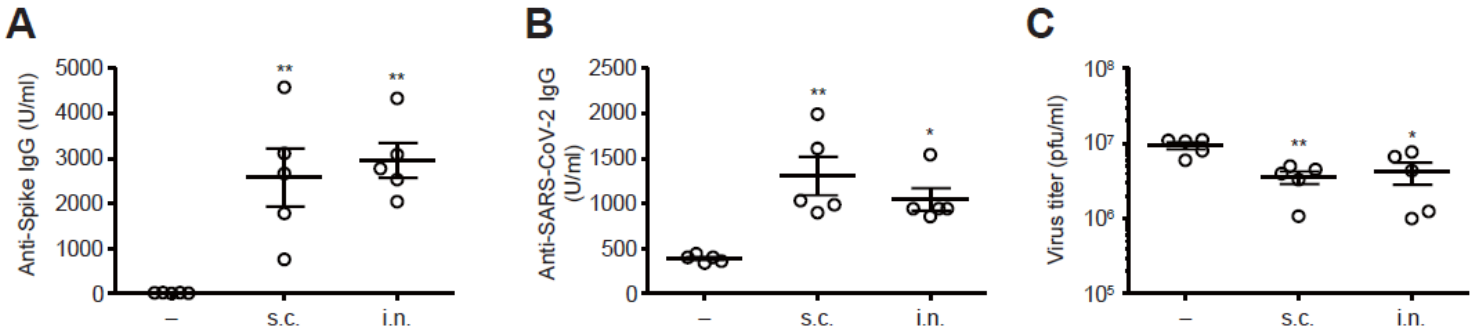

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1S2andS3.pdf