

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

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13 **Abstract**

14 Gut microbiota plays a critical role in the induction of adaptive immune responses to
15 influenza virus infection. However, the role of nasal bacteria in the induction of the
16 virus-specific adaptive immunity is less clear. Here we demonstrate that while intranasal
17 administration of influenza virus hemagglutinin vaccine alone was insufficient to induce
18 the vaccine-specific antibody responses, disruption of nasal bacteria by lysozyme or
19 addition of culturable oral bacteria from a healthy human volunteer rescued inability of
20 the nasal bacteria to generate antibody responses to intranasally administered the
21 split-virus vaccine. Myd88-dependent signaling in the hematopoietic compartment was
22 required for adjuvant activity of intranasally administered oral bacteria. In addition, we
23 found that the oral bacteria-combined intranasal vaccine induced protective antibody
24 response to influenza virus and SARS-CoV-2 infection. Our findings here have
25 identified a previously unappreciated role for nasal bacteria in the induction of the
26 virus-specific adaptive immune responses.

27

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

29 **Introduction**

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

38 Since it is difficult to predict which strain of influenza virus or coronavirus cause a
39 pandemic, it is advantageous to produce vaccines that induce cross-protective immunity
40 against variants of the particular virus strain. Mucosal immunity induced by natural
41 infection of influenza virus is more effective and cross-protective against heterologous
42 virus infection than systemic immunity induced by parenteral vaccines (S. Tamura &
43 Kurata, 2004). It is believed that the virus-specific IgA in upper respiratory tract is more
44 cross-protective against heterologous influenza viruses compared with the virus-specific
45 IgG in the serum due to its dimeric or tetrameric forms (higher avidity) and location
46 (Liew, Russell, Appleyard, Brand, & Beale, 1984; Suzuki et al., 2015). Indeed,

47 polymeric immunoglobulin receptor-knockout mice failed to secrete nasal IgA and
48 protect against heterologous virus challenge (Asahi et al., 2002). Therefore, induction of
49 the virus-specific secretory IgA in the upper respiratory tract by intranasal vaccination
50 has a great advantage in conferring protection against an unpredictable pandemic of
51 viral pathogens such as the swine-origin H1N1 and avian-origin H7N9 influenza A
52 viruses, or zoonotic origin of SARS-CoV-2 (Gao et al., 2013; Neumann, Noda, &
53 Kawaoka, 2009). In the effort to develop effective intranasal vaccines, several adjuvants
54 such as cholera toxin (Watanabe et al., 2002), synthetic double-stranded RNA poly(I:C)
55 (Ichinohe et al., 2005), synthetic toll-like receptor 4 agonist (Spinner et al., 2015),
56 zymosan (Ainai et al., 2010), flagellin (Skountzou et al., 2010), immune stimulating
57 complexes (ISCOMs) (Sjolander et al., 2001), or type-I interferons (Bracci et al., 2005)
58 have been developed to enhance the vaccine-specific nasal IgA response. While upper
59 respiratory tract contains commensal bacteria (Bassis et al., 2015; Clark, 2020),
60 intranasal administration of split vaccine alone was insufficient to induce the
61 vaccine-specific nasal IgA response (Ichinohe et al., 2005; Jangra et al., 2020),
62 suggesting that the amounts of commensal bacteria in upper respiratory tract are
63 insufficient to stimulate the vaccine-specific nasal IgA response.

64 A recent study has demonstrated that nasal mucosa-derived *Staphylococcus*
65 *epidermidis*, one of the most abundant colonizers of healthy human skin and mucosal
66 surface, suppressed influenza virus replication by stimulating IFN- λ production (Kim et
67 al., 2019). In addition, influenza virus-infected mice lacking both toll-like receptor 7
68 (TLR7) and mitochondrial antiviral signaling (MAVS) had elevated nasal bacterial
69 burdens, which resulted in death from pneumonia caused by secondary bacterial
70 infections (Pillai et al., 2016). In contrast to the role of nasal bacteria in innate antiviral
71 resistance to influenza virus infection or severity of the disease (Kim et al., 2019; Pillai
72 et al., 2016), it remains unclear whether nasal bacteria critically regulates the generation
73 of influenza virus-specific adaptive immune responses after infection or intranasal
74 vaccination. Here, we show that depletion of nasal bacteria by intranasal administration
75 of antibiotics enhanced the virus-specific nasal IgA and serum IgG response following
76 influenza virus infection. In addition, we found that lysozyme-induced disruption of
77 nasal bacteria or culturable oral bacteria from a healthy volunteer significantly enhanced
78 the vaccine-specific nasal IgA and serum IgG responses. Myd88-dependent signaling in
79 the hematopoietic compartment was required for adjuvant activity of intranasally
80 administered oral bacteria. Our findings here have identified a previously unappreciated
81 role for nasal bacteria in the induction of the virus-specific adaptive immune responses.

82 **Results**

83 **Depletion of nasal bacteria enhanced antibodies response to influenza virus**

84 **infection**

85 Gut commensal microbiota play a key role in innate and adaptive immune defense

86 against influenza virus infection (Abt et al., 2012; Bradley et al., 2019; Ichinohe et al.,

87 2011; Rosshart et al., 2017; Steed et al., 2017; Stefan, Kim, Iwasaki, & Kasper, 2020).

88 However, the role of oral or nasal bacteria in the induction of mucosal immune

89 responses following influenza virus infection remains unknown. To assess the effects of

90 oral or nasal bacteria in the induction of mucosal immune responses to influenza virus

91 infection, we treated mice intranasally with an antibiotic cocktail for five consecutive

92 days before influenza virus infection. This treatment resulted in significant reduction in

93 the numbers of culturable oral and nasal bacteria (**Supplementary Fig. 1**).

94 Antibiotic-treated mice were then infected intranasally with a mouse-adapted influenza

95 A virus strain A/Puerto Rico/8/1934 (PR8). Surprisingly, influenza virus-specific nasal

96 IgA and serum IgG levels were significantly elevated in the antibiotic-treated group

97 (**Fig. 1**). This led us to consider the possibility that depletion of commensal bacteria in

98 upper respiratory tract enhances influenza virus replication, resulting in enhancement of

99 the virus-specific antibody responses. However, depletion of commensal bacteria in

100 upper respiratory tract significantly reduced influenza virus replication at 2 days post
101 infection (**Supplementary Fig. 2A**). This is consistent with a previous report showing
102 that antibiotic treatment significantly reduce influenza virus replication at early time
103 point (Gopinath et al., 2018). In addition, the viral replication in upper respiratory tract
104 became comparable between antibiotics-treated and control groups at 3 and 5 days post
105 infection (**Supplementary Fig. 2B, C**). These data indicated that the levels of influenza
106 virus replication in upper respiratory tract is unlikely to account for increased the
107 virus-specific antibody responses in antibiotic-treated animals.

108

109 **Lysozyme-induced disruption of nasal bacteria enhances antibody responses
110 induced by intranasal vaccination**

111 Thus, we next examined the possibility that antibiotic-induced disruption of nasal
112 bacteria releases pathogen-associated molecular patterns, which may act as adjuvants to
113 enhance the virus-specific antibody responses. To assess the possibility that disruption
114 of nasal bacteria acts as adjuvant for intranasal influenza vaccine, we immunized mice
115 intranasally with influenza virus hemagglutinin (HA) protein and lysozyme to disrupt
116 nasal bacteria. We used poly(I:C) adjuvant as a positive control (Ichinohe et al., 2005).
117 Strikingly, we found that intranasal immunization with HA and lysozyme significantly

118 enhanced the HA-specific nasal IgA and serum IgG responses (**Fig. 2**). While upper
119 respiratory tract contains commensal bacteria (Bassis et al., 2015; Clark, 2020),
120 intranasal administration of hemagglutinin (HA) vaccine alone was insufficient to
121 induce the HA-specific antibody responses (**Fig. 2**). Taken together, these results
122 suggest that disruption of nasal bacteria by intranasal administration of antibiotics or
123 lysozyme acts as adjuvant for intranasal influenza vaccine.

124

125 **Oral bacteria act as adjuvant for intranasal vaccine**

126 While upper respiratory tract contains commensal bacteria (Bassis et al., 2015; Clark,
127 2020), we found that relative amounts of 16S rRNA and culturable bacteria in nasal
128 mucosal surface were significantly lower than that in the oral cavity (**Supplementary**
129 **Fig. 3**). Thus, we next examine whether oral bacteria act as adjuvant for intranasal
130 vaccine. Intranasal vaccination with HA and culturable oral bacteria from mice or a
131 healthy volunteer significantly enhanced the HA-specific nasal IgA and serum IgG
132 responses (**Fig. 3A, B**). In addition, the oral bacteria from a healthy volunteer stimulated
133 the HA-specific nasal IgA and serum IgG responses in a dose-dependent manner (**Fig.**
134 **3C, D**). Next, we compared the ability of isolated bacterial strains from oral wash
135 sample of a healthy volunteer to stimulate the HA-specific antibody responses. To this

136 end, we immunized mice intranasally with HA and *streptococcus salivarius* (*S.*
137 *salivarius*), *streptococcus parasanguinis* (*S. parasanguinis*), or *streptococcus infantis* (*S.*
138 *infantis*). Mice immunized with HA and each isolated bacterial strain induced
139 comparable levels of the HA-specific nasal IgA and serum IgG responses (**Fig. 4**),
140 suggesting that adjuvant activity of the oral bacteria is unlikely to account for strain
141 specific.

142

143 **Myd88-dependent signaling in the hematopoietic compartment is required for
144 adjuvant activity of intranasally administered oral bacteria**

145 Next, we wished to determine the innate immune signaling through
146 pattern-recognition receptors required for adjuvant activity of the oral bacteria. To this
147 end, we immunized WT and MyD88-deficient mice intranasally with HA and culturable
148 oral bacteria from a healthy volunteer and measured the HA-specific nasal IgA and
149 serum IgG responses. The HA-specific nasal IgA and serum IgG responses were found
150 to be completely dependent on MyD88 (**Fig. 5A, B**). In addition, lysozyme-induced
151 disruption of nasal bacteria stimulated the HA-specific nasal IgA and serum IgG
152 responses in a MyD88-dependent manner (**Fig. 5C, D**). To determine the cellular
153 compartment responsible for adjuvant activity of oral bacteria, we generated bone

154 marrow (BM) chimeric mice in which only the hematopoietic (WT→MyD88^{+/−}) or the
155 stromal cells (MyD88^{+/−}→WT) expressed MyD88. After intranasal vaccination with HA
156 and oral bacteria, the HA-specific nasal IgA and serum IgG responses were significantly
157 reduced in MyD88^{+/−}→WT BM chimeric mice compared to WT→MyD88^{+/−} BM
158 chimeric mice (**Fig. 6**). These data indicate that MyD88-dependent signaling in the
159 hematopoietic, but not stromal, compartment is required for adjuvant activity of
160 intranasally administered oral bacteria.

161

162 **Oral bacteria-combined intranasal vaccine protects from influenza virus and**
163 **SARS-CoV-2 infection**

164 Finally, we examined protective effects of intranasal vaccination with oral
165 bacteria-adjuvanted vaccine against influenza virus and SARS-CoV-2 infection. To this
166 end, we immunized mice intranasally with quadrivalent influenza HA vaccine
167 containing A/California/7/2009 HA together with culturable oral bacteria or lysozyme.
168 Two weeks after the second vaccination, we challenged vaccinated mice intranasally
169 with a heterologous A/Narita/1/2009 (pdm09) strain (**Fig. 7**). Mice immunized with HA
170 vaccine adjuvanted with oral bacteria or lysozyme significantly reduced the virus titer
171 compared to control mice immunized with the HA vaccine alone (**Fig. 7**). We next

172 assessed protective effects of intranasal vaccination with oral bacteria-adjuvanted
173 SARS-CoV-2 spike protein against SARS-CoV-2 infection in Syrian hamsters. To this
174 end, we immunized hamsters intranasally with a recombinant SARS-CoV-2 spike
175 protein and culturable oral bacteria from a healthy volunteer. We immunized hamsters
176 subcutaneously with the spike protein alone as a control. We
177 Both the spike- and the virus-specific serum IgG levels were significantly elevated in
178 immunized hamsters (**Fig. 8A, B**). In addition, immunized hamsters significantly
179 reduced the virus titer compared to naïve animals following high-dose (2×10^6 pfu of
180 SARS-CoV-2) challenge (**Fig. 8C**). These data collectively indicated that disruption of
181 nasal bacteria or intranasal administration of oral bacteria compensate inability of nasal
182 bacteria to generate protective adaptive immunity to intranasally administered split
183 vaccines.

184

185

186 **Discussion**

187 The innate immune system, the first line of defense against pathogens, utilizes pattern
188 recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs).

189 The recognition of influenza virus by PRRs plays a key role not only in limiting virus
190 replication at early stages of infection, but also in initiating the virus-specific adaptive
191 immune responses. In addition, previous studies have demonstrated that gut commensal
192 microbiota play a key role in innate and adaptive immune defense against influenza

193 virus infection (Abt et al., 2012; Bradley et al., 2019; Ichinohe et al., 2011; Rosshart et
194 al., 2017; Steed et al., 2017; Stefan et al., 2020). Further, recent studies have indicated

195 the roles of nasal bacteria in innate antiviral resistance to influenza virus infection or
196 severity of the diseases (Kim et al., 2019; Pillai et al., 2016). However, it remains
197 unclear whether nasal bacteria critically regulates the generation of influenza

198 virus-specific adaptive immune responses after influenza virus infection. In this study,
199 we demonstrated that depletion of commensal bacteria in upper respiratory tract by
200 intranasal administration of antibiotics enhanced the virus-specific antibodies response

201 following influenza virus infection. Surprisingly, depletion of nasal bacteria by
202 intranasal administration of antibiotics before influenza virus infection significantly

203 reduced the virus titer at 2 days post infection. This is consistent with a previous report

204 showing that antibiotic treatment significantly reduce influenza virus replication at 6
205 hours post infection (Gopinath et al., 2018). Intranasal application of antibiotics
206 suppressed influenza virus replication through at least two possible mechanisms. First,
207 intranasal administration of antibiotics enhances host resistance to influenza virus
208 infection in a microbiota-independent manner (Gopinath et al., 2018). Second,
209 disruption of nasal bacteria by intranasal antibiotic treatment may release PAMPs from
210 the antibiotic-killed bacteria, which stimulate innate antiviral immune responses to
211 suppress influenza virus replication (Matsuo et al., 2000). After 3 and 5 days post
212 infection, the viral replication in upper respiratory tract became comparable between
213 antibiotic-treated and control groups, indicating that the levels of influenza virus
214 replication in upper respiratory tract is unlikely to account for increased levels of the
215 virus-specific antibodies response in antibiotic-treated mice.

216 Since the primary targets of influenza virus are the nasal epithelial cells in upper
217 respiratory tract, it is beneficial to induce the virus-specific nasal IgA antibody at the
218 nasal mucosal epithelium. However, intranasal vaccination with split-virus vaccine
219 alone is often insufficient to elicit proper immune responses at the upper respiratory
220 tract. Therefore, adjuvants are required for a given vaccine to induce the
221 vaccine-specific nasal IgA response. In developing intranasal vaccines, cholera toxin

222 (CT) and *Escherichia coli* heat-labile toxin (LT) have been used as adjuvant to enhance
223 nasal immune response (S. I. Tamura & Kurata, 2000). Although CT and LT are
224 effective adjuvants to enhance mucosal immune responses including secretory IgA
225 responses, they have some side effects in humans, including Bell's palsy and nasal
226 discharge (Mutsch et al., 2004). Therefore, several adjuvants that are as effective as CT
227 or LT and are also safe for human use have been developed for clinical application with
228 intranasal influenza vaccine (Ainai et al., 2010; Bracci et al., 2005; Ichinohe et al.,
229 2005; Sjolander et al., 2001; Skountzou et al., 2010; Spinner et al., 2015). In this study,
230 we show that intranasal vaccination with influenza virus HA vaccine and culturable oral
231 bacteria from a healthy human volunteer induced significant levels of the
232 vaccine-specific nasal IgA and serum IgG responses in a dose-dependent manner. All
233 commensal bacterial strains tested, including *S. salivarius*, *S. parasanguinis*, or *S.*
234 *infantis*, induced comparable levels of the HA-specific nasal IgA and serum IgG
235 responses, suggesting that adjuvant activity of the oral bacteria is unlikely to account for
236 strain specific. In addition to culturable oral bacteria from a healthy human volunteer,
237 we demonstrated that disruption of nasal bacteria by lysozyme induced significant
238 levels of the vaccine-specific antibodies response. Although relative amounts of nasal
239 bacteria were significantly lower than that in the oral cavity, disruption of nasal bacteria

240 by lysozyme could rescue the inability of nasal bacteria to generate the vaccine-specific
241 antibodies response. In mice, nasal commensal microbiota are predominantly composed
242 of gram-positive bacteria including *Lactobacillus spp.*, *Bacillus spp.*, *Staphylococcus*
243 *spp.*, and *Streptococcus spp.* (Ichinohe et al., 2011). In addition, *Lactobacillus spp.* were
244 found to contain higher amounts of double-stranded RNA than the pathogenic bacteria
245 (Kawashima et al., 2013). Since activation of TLRs by different PAMPs such as
246 poly(I:C) and zymosan synergistically enhanced the nasal IgA response to intranasally
247 administered influenza virus HA vaccine (Ainai et al., 2010), disruption of nasal
248 bacteria could stimulate different TLRs to enhance the vaccine-specific antibodies
249 response. Most TLRs signal through the adaptor protein MyD88 (Kawai & Akira, 2010;
250 Medzhitov, 2001). Although nasal epithelial cells express various TLRs (Tengroth et al.,
251 2014; van Tongeren et al., 2015), deficiency of MyD88 in stromal compartment did not
252 significantly affect the levels of nasal IgA and serum IgG responses following intranasal
253 vaccination with influenza virus HA and culturable oral bacteria. Instead,
254 MyD88-dependent signaling in the hematopoietic cells were required for adjuvant
255 activity of intranasally administered oral bacteria. These data are consistent with
256 previous studies showing that both TLR-induced dendritic cell maturation and B-cell

257 activation are required for optimal antibody responses to T-dependent antigens (Iwasaki
258 & Medzhitov, 2015; Pasare & Medzhitov, 2005).

259 In summary, our study demonstrated the effects of commensal microbiota in upper
260 respiratory tract in the induction of the virus-specific adaptive immune responses after

261 influenza virus infection or intranasal vaccination. Our data indicated that disruption of

262 nasal bacteria by lysozyme or supplementation of oral bacteria from a healthy volunteer

263 enhanced nasal IgA and serum IgG antibodies response to intranasally administered

264 influenza virus HA or SARS-CoV-2 S proteins. Although the vaccinated animals

265 significantly reduced the virus titer compared to unadjuvanted group or naïve animals

266 following high-dose of influenza virus or SARS-CoV-2 challenge, further studies are

267 needed to establish the safety and efficacy of this vaccination method in an additional

268 animal model such as nonhuman primate.

269

270

271 **Materials and methods**

272 **Mice**

273 Age- and sex-matched Balb/c mice obtained from Japan SLC, Inc. were used as WT
274 controls. MyD88-deficient Balb/c mice were a gift from T. Taniguchi. All animal
275 experiments were performed in accordance with the University of Tokyo's Regulations
276 for Animal Care and Use, which were approved by the Animal Experiment Committee
277 of the Institute of Medical Science, the University of Tokyo (approval number PA17–
278 69).

279

280 **Cells**

281 Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential
282 medium (E-MEM; Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS),
283 penicillin (100 U/ml), and streptomycin (100 µg/ml). VeroE6 cells stably expressing
284 transmembrane protease serine 2 (VeroE6/TMPRSS2; JCRB Cell Bank 1819) were
285 maintained in Dulbecco's modified Eagle's medium (DMEM) low glucose
286 (Cat#08456-65; Nacalai Tesque) supplemented with 10% FBS, penicillin (100 U/ml),
287 streptomycin (100 µg/ml), and G418 (1mg/ml) (Matsuyama et al., 2020).

288

289 **Depletion of nasal bacteria *in vivo***

290 The antibiotic cocktail consisted of ampicillin sodium salt (1 g/L), neomycin sulfate
291 (1 g/L), metronidazole (1 g/L), vancomycin hydrochloride (0.5 g/L), gentamicin (10
292 mg/L), penicillin (100 U/ml), streptomycin (100 U/ml), and amphotericin B (0.25 mg/L)
293 (Moriyama & Ichinohe, 2019). For intranasal treatment, mice were anaesthetized and 5
294 μl of antibiotic was administered dropwise into each nostril using a pipette tip. All
295 antibiotics with the exception of vancomycin hydrochloride were obtained from Nacalai
296 Tesque. Vancomycin hydrochloride was obtained from Duchefa Biochemie.

297

298 **Virus infection**

299 WT A/Puerto Rico/8/34 (A/PR8) and A/Narita/1/09 (pdm09) influenza viruses were
300 grown in allantoic cavities of 10-d-old fertile chicken egg at 35 °C for 2 d (Moriyama et
301 al., 2020). Viral titer was quantified by a standard plaque assay using MDCK cells and
302 viral stock was stored at -80 °C (Moriyama, Koshiba, & Ichinohe, 2019). For intranasal
303 infection, mice were fully anesthetized by i.p. injection of pentobarbital sodium
304 (Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) and then infected by
305 intranasal application of 30 μl of virus suspension (1,000 pfu of A/PR8 or pdm09 in
306 PBS). This procedure leads to upper and lower respiratory tract infection (Moriyama &

307 Ichinohe, 2019).

308 SARS-CoV-2 (a gift from Y. Kawaoka) was amplified on VeroE6/TMPRSS2 cells

309 and stored at -80 °C until use. The infectious titer was determined by a standard plaque

310 assay using VeroE6/TMPRSS2 cells, as described previously (Imai et al., 2020). For

311 intranasal infection, one-month-old female Syrian hamsters (Japan SLC Inc.) were fully

312 anesthetized by i.p. injection of pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku

313 Co., Ltd., Tokyo, Japan) and then infected intranasally with 2×10^6 pfu (in 100 µL) of

314 SARS-CoV-2. All experiments with SARS-CoV-2 were performed in enhanced

315 biosafety level 3 (BSL-3) containment laboratories at the University of Tokyo, in

316 accordance with the institutional biosafety operating procedures.

317

318 **Vaccination**

319 For intranasal infection, mice were fully anesthetized by i.p. injection of pentobarbital

320 sodium (Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) and then infected

321 intranasally by dropping 2 µl of PBS containing 1,000 pfu of A/PR8 into the nostril.

322 The quadrivalent inactivated influenza vaccine (split-product virus vaccines,

323 hemagglutinin [HA] vaccine) prepared for the 2015–2016 season and including

324 A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2),

325 B/Phuket/3073/2013, and B/Texas/2/2013 were purchased from Kaketsuken
326 (Kumamoto, Japan). Mice were immunized by intranasal administration of the
327 quadrivalent HA vaccine containing 150 ng of each HA with or without 5 µg of
328 lipopolysaccharide (LPS; InvivoGen), 5 µg of poly(I:C) (InvivoGen), 250 µg of
329 lysozyme (Thermo Fisher Scientific), or 1 mg of culturable oral bacteria from a healthy
330 volunteer.

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

335

336 **Clinical specimens**

337 Oral and nasal washes were collected from a healthy volunteer by rinsing the mouth
338 with 50 ml of saline or washing the nasal cavity with 50 ml of saline using a syringe.
339 The research protocol was approved by the Research Ethics Review Committee of the
340 Institute of Medical Science, the University of Tokyo (approval number 2019-42-1121).
341 For preparation of oral bacteria adjuvant, oral wash samples were grown in brain heart
342 infusion broth (BD 237500) at 37 °C overnight, washed repeatedly, and resuspended in

343 PBS (200 µg/ml).

344

345 **Bacterial recovery and identification**

346 Oral and nasal washes were collected from a healthy volunteer as described above.

347 Aliquots of 100µl of serial 10-fold dilution of the oral and nasal wash were inoculated

348 into brain heart infusion agar plates (BD 252109). After incubation at 37 °C overnight

349 under the aerobic conditions, the bacterial colonies were grown in brain heart infusion

350 broth (BD 237500) at 37 °C overnight. Bacterial DNA was isolated as described

351 previously (Moriyama & Ichinohe, 2019). A 300-bp portion of the 16S rRNA was

352 amplified by PCR using specific primer pairs of 515F

353 (5'-GTGCCAGCMGCCGCGTAA-3') and 806R

354 (5'-GGACTACHVGGGTWTCTAAT-3'), purified (Qiagen), sequenced, and the

355 sequence compared by Blast analysis to known bacterial sequences.

356

357 **Bone marrow chimera**

358 Bone marrow chimeras were generated as described (Pang, Ichinohe, & Iwasaki,

359 2013). WT and MyD88-deficient mice were γ -irradiated with 6 Gy, then were

360 reconstituted with 5×10^6 bone marrow cells of the appropriate genotype via i.v.

361 injection and allowed to recover for 8 weeks before vaccination.

362

363 **Measurement of virus titers**

364 For measurement of influenza virus titer, bronchoalveolar (BAL) fluid was collected

365 by washing the trachea and lungs of mice twice by injecting a total of 2 ml PBS

366 containing 0.1% bovine serum albumin (BSA). The virus titer was measured as follows:

367 aliquots of 200 μ l of serial 10-fold dilution of the BAL fluid by PBS containing 0.1%

368 BSA were inoculated into MDCK cells in 6-well plates. After 1 hour of incubation,

369 cells were washed with PBS thoroughly and overlaid with 2 ml of agar medium.

370 For measurement of SARS-CoV-2 titer, BAL fluid was collected by washing the

371 trachea and lungs of hamsters twice by injecting a total of 2 ml DMEM containing 5%

372 FBS. The virus titer was measured as follows: aliquots of 200 μ l of serial 10-fold

373 dilution of the BAL fluid by DMEM containing 5% FBS were inoculated into

374 VeroE6/TMPRSS2 cells in 6-well plates. After 1 hour of incubation, cells were washed

375 with PBS thoroughly and overlaid with 2 ml of agar medium. The number of plaques in

376 each well was counted 2 days after inoculation.

377

378 **Enzyme-linked immunosorbent assay (ELISA)**

379 Serum and nasal wash were collected from the immunized mice for measurement of
380 the PR8- or HA-specific nasal IgA and serum IgG antibodies. Nasal wash was collected
381 by washing the nasopharynx three times by injecting a total of 1 ml PBS containing
382 0.1% BSA. The levels of the PR8- or HA-specific nasal IgA and serum IgG antibodies
383 were determined by ELISA as described previously (Moriyama & Ichinohe, 2019).
384 Standards for PR8- or HA-reactive IgA and IgG antibody titration were prepared from
385 the nasal wash or serum of the virus-infected or vaccinated mice, and expressed as the
386 same arbitrary units (160-unit). The antibody titers of unknown specimens were
387 determined from the standard regression curve constructed by two fold serial dilution of
388 the 160-unit standard for each assay.

389

390 **Quantification and statistical analysis**

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

395

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405

406 **Competing interests statement**

407 The authors declare no competing financial interests.

408

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559

560 **Figure legends**

561 **Figure 1. Disruption of nasal bacteria enhances the virus-specific antibody**

562 **responses following influenza virus infection.**

563 **(A and B)** Mice were inoculated intranasally with an antibiotic cocktail (Abx) for 5

564 consecutive days. Two days later, mice were intranasally infected with 1,000 pfu of

565 A/PR8 virus. The nasal wash and serum were collected at 4 weeks p.i., and the

566 virus-specific nasal IgA and serum IgG titers were determined by ELISA. Open circles

567 indicate values for individual mice. The data are from three independent experiments

568 (mean \pm SEM). * $P < 0.05$ and *** $P < 0.001$; (one-way ANOVA and Tukey's test).

569

570 **Figure 2. Disruption of nasal bacteria induces the HA-specific antibody responses**

571 **after intranasal vaccination.**

572 **(A and B)** Mice were immunized intranasally with quadrivalent HA vaccine with or

573 without poly(I:C) or lysozyme twice in a 3-week interval. Two weeks later, the nasal

574 wash and serum were collected and the HA-specific nasal IgA and serum IgG titers

575 were determined by ELISA. Open circles indicate values for individual mice. The data

576 are from three independent experiments (mean \pm SEM). ** $P < 0.01$ and *** $P < 0.001$;

577 (one-way ANOVA and Tukey's test).

578

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

580 **(A and B)** Mice were immunized intranasally with quadrivalent HA vaccine with or
581 without LPS, poly(I:C), or culturable oral bacteria from mice or a healthy volunteer
582 twice in a 3-week interval. Two weeks later, the nasal wash and serum were collected
583 and the HA-specific nasal IgA and serum IgG titers were determined by ELISA. **(C and**
584 **D)** Mice were immunized intranasally with quadrivalent HA vaccine with or without
585 indicated amounts of oral bacteria from a healthy volunteer twice in a 3-week interval.
586 Two weeks later, the nasal wash and serum were collected and the HA-specific nasal
587 IgA and serum IgG titers were determined by ELISA. Open circles indicate values for
588 individual mice. The data are from two independent experiments (mean \pm SEM). * P <
589 0.05, ** P < 0.01 and *** P < 0.001; (one-way ANOVA and Tukey's test).

590

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

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

596 serum IgG titers were determined by ELISA. Open circles indicate values for individual
597 mice. The data are from two independent experiments (mean \pm SEM). * P < 0.05, ** P <
598 0.01 and *** P < 0.001; (one-way ANOVA and Tukey's test).

599

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

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

608

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

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

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

617

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

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

628

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

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

641

Figure 1

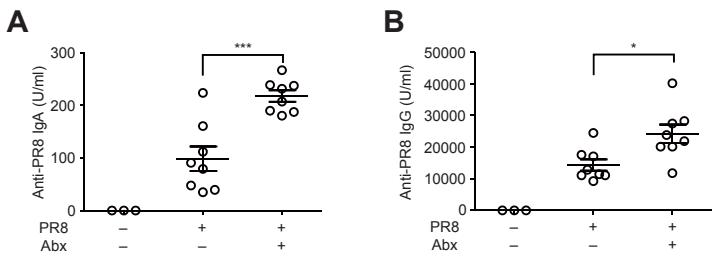


Figure 2

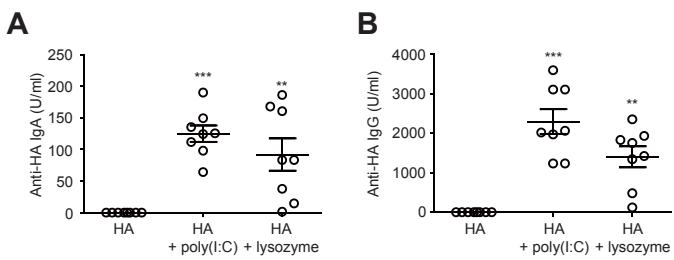


Figure 3

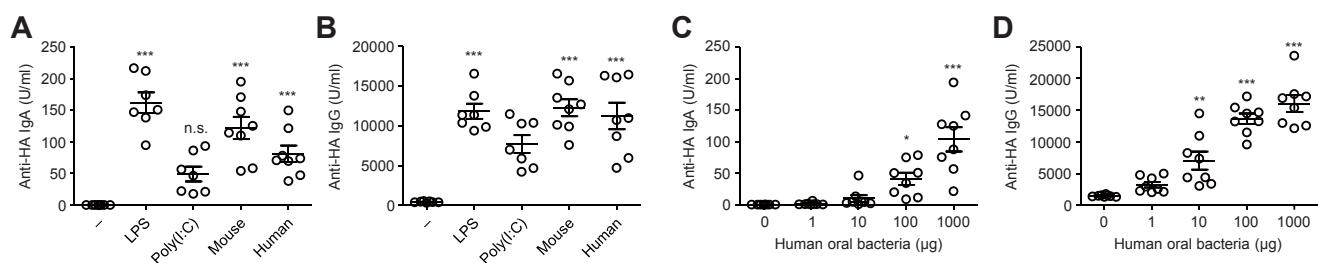


Figure 4

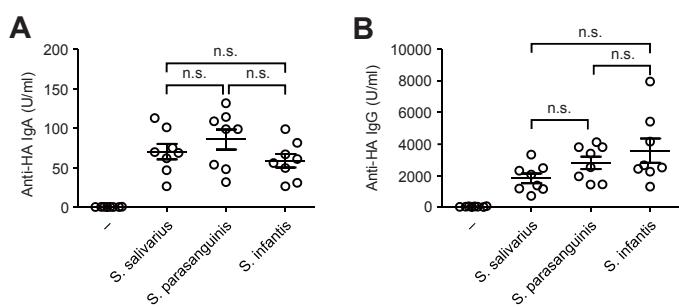


Figure 5

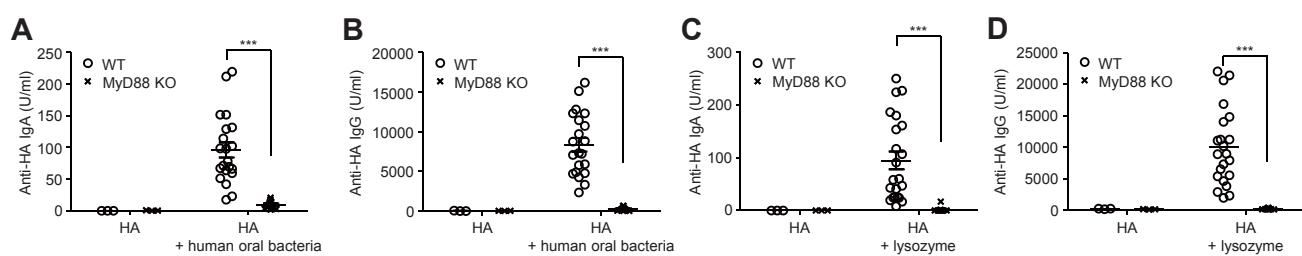


Figure 6

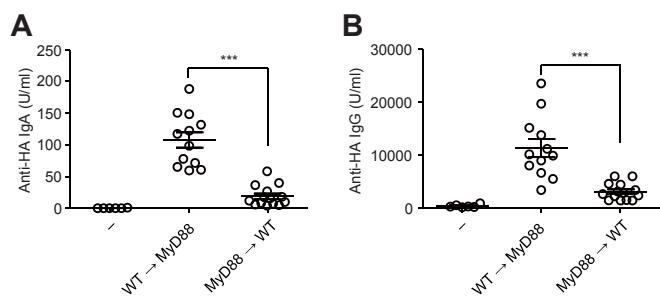


Figure 7

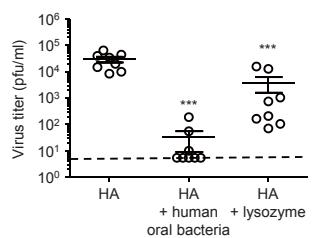


Figure 8

