

# 1 *Bacillus subtilis* spore induces efficient generation of 2 memory T cells via ICAM-1 expression on dendritic cells

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## 9 **Abstract**

10 The intestinal mucosa is the primary exposure and entry site of infectious  
11 organisms. Tissue-resident memory T cells (Trms) is an important first line of defense  
12 against infection in mucosal tissues, their function in intestinal immunization remains  
13 to be investigated. Here, we reported that the levels of local mucosal and systemic  
14 immune responses were enhanced through oral immunization with H9N2 whole  
15 inactivated virus (H9N2 WIV) plus spore. Subsequently, H9N2 WIV plus spore led to  
16 the generation of CD103<sup>+</sup>CD69<sup>+</sup> Trms, which was independent of circulating T cells  
17 during the immune period. Meanwhile, we also found that *Bacillus subtilis* spore can  
18 stimulate Acrp30 expression in 3T3-L1 adipocytes. Moreover, adipocyte supernatant  
19 or spore also upregulated intercellular adhesion molecule-1 (ICAM-1) expression on  
20 dendritic cells (DCs) ( $P<0.01$ ). Furthermore, the proportion of HA-tetramer<sup>+</sup> cells was  
21 severely curtailed when ICAM-1 expression was suppressed, which was also  
22 dependent on HA-loaded DCs. Taken together, our data demonstrated that spore  
23 promoted the immune response by stimulating Trms, which were associated with  
24 activation of ICAM-1 in DCs.

## 25 **Author summary**

26 Taken together, *Bacillus subtilis* spore combined with H9N2 WIV enhanced the  
27 mucosal antibody response and induced efficient intestinal-resident memory T cells.  
28 Then we demonstrated that spore can induce memory T cell formation through an  
29 ICAM-1-mediated contact of a dendritic cell (DC)-derived mechanism. Further, our  
30 findings indicated that Acrp30 from adipocytes induced by spore might increase  
31 ICAM-1 expression on DCs, which might provide new insight into the significance of  
32 adipocyte metabolism related molecules in regulating immunological memory T cells.

33

## 34 **Introduction**

35 H9N2 subtype avian influenza virus, a low-pathogenicity avian influenza (AIV),  
36 has become endemic and pose a significant threaten to human and animal [1, 2],  
37 which can replicate in avian guts and spread by fecal-oral transmission [3, 4].  
38 Mucosal immune is an effective way to block the infectious organisms through  
39 eliciting memory T cells against pathogens. Hence, acquisition of the protective  
40 immune responses at mucosal sites is a priority vaccination strategy to prevent  
41 pathogenesis. Although expert in eliciting immune responses, oral immunization with  
42 vaccination also induces poor mucosal effect when immunized with inactivated virus  
43 alone [5, 6]. Thus, urgent vaccine development involves inducing protective immune  
44 responses against potential pathogens on mucosal surfaces, suggesting a critical need  
45 for more efficacious adjuvants to improve vaccine potency. *Bacillus subtilis* spore,  
46 acts as an adjuvant, can strongly induce immune responses against pathogen,  
47 especially in modulating intestinal mucosal immunity through evoking tissue-resident  
48 memory T cell (Trm) [7-9].

49 Previous studies on *Bacillus subtilis* spore found that it can stimulate the  
50 secretion of cytokines as innate immune signaling, which is indispensable for efficient  
51 induction of adaptive immune responses during primary immunization [10]. Recent  
52 study found that mucosal immunization with Spore-FP1 increased CD69<sup>+</sup>CD103<sup>+</sup> Trm  
53 in the lung parenchyma [11]. Meanwhile, our previous study also confirmed *Bacillus*  
54 *subtilis* spore, as advantages of mucosal delivery, could regulate memory T cells in  
55 the intestine of piglets [12]. As we known, T cells is important for cell immunity,  
56 while vaccine-mediated intestinal T cell responses reveal a requirement for the  
57 addition of adjuvant for evoking a robust Trm response[13]. Recent reports have  
58 demonstrated that the expression patterns of lymph node homing receptors CCR7 and  
59 CD62L are closely related to the functional status of central memory T cells (Tcms)  
60 and effector memory T cells (Tems) [14]. Moreover, Trms mediate rapid clearance of  
61 and heterosubtypic protection against secondary IAV infections in mice [15, 16].  
62 Further analyses have revealed that Trms were also detected in the intestinal mucosa,  
63 leading to tissue-specific influences [17-19]. Until recently, Trms, which express high  
64 levels of C-type lectin CD69 and low levels of the sphingosine-1-phosphate (S1P)  
65 receptor S1PR1, are thought to be phenotypically and functionally distinct from  
66 circulating memory T cells [20]. Furthermore, establishment of long-term and resident  
67 memory depends on the maintenance of CD103 and CD69 expression in T cells [21].  
68 Thus, the features and processes of memory cells are involved in the retention and  
69 persistence of T cells in mucosal tissue, thereby promoting long-term protection for  
70 viral clearance [19].

71 Our study provided further insight into the potential immunopotentiator ability of  
72 *Bacillus subtilis* spore to assist PEDV WIV in the potentiation of immunity by  
73 upregulating memory T cells via oral immunization in piglets [12]. However, the  
74 specific mechanism in memory cell formation remains to be further studied. Previous  
75 studies suggest that intercellular adhesion molecule-1 (ICAM-1) is critical for  
76 establishing memory T cells following acute infection [22]. In addition, a substantial  
77 number of liver-resident memory populations are regulated by LFA-1-ICAM-1  
78 interactions following LCMV immunization [23]. Lipid metabolism related molecules

79 play important role in regulating ICAM-1 expression [24]. Hence, our study try to  
80 illustrated the underline mechanism of whether *Bacillus subtilis* spore induce memory  
81 T cell formation through activating ICAM-1, as well as inducing Acrp30.

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## 89 Results

### 90 Spore recruited and activated DCs

91 DCs are essential for the generation of T-cell immunity after mucosal immunization  
92 [25]. To investigate whether spore had the capacity to recruit submucosal DCs to form  
93 transepithelial dendrites (TEDs) for viral capture, we assessed TED formation *in vitro*  
94 and *in vivo*. Initially, in a DC/epithelial cell (EC) coculture system (Fig. 1A), we  
95 observed spore, but not medium, induced DCs to form TEDs across ECs at 30 min in  
96 cross-sectional images (Fig. 1B). Then, Ligated loop experiments at 0.5 h after spore  
97 administration found DCs were apparently gathering to the lamina propria of ECs,  
98 which were significantly increased by spore compared with the control (Fig. 1C).  
99 Moreover, we found spore had the powerful capacity to increase the expression of  
100 CD40 and CD80 (CD80:  $P < 0.01$ , CD40:  $P < 0.01$ ) (Fig. 1D) in coculture system,  
101 compared with medium alone. Furthermore, the release of proinflammatory cytokines  
102 (IL-1 $\beta$ , TNF- $\alpha$ ) stimulated by spore, which also indicated the functional maturation of  
103 DCs (Fig. 1F) ( $P < 0.01$ ). By the way, our result also observed spore significantly  
104 increased the length of DCs (Fig. 1E).

105

### 106 Fig 1. Spore activated dendritic cells *in vitro* and *in vivo*.

107 (A) Schematic of the experiment used to study activation of DCs in the DC/EC  
108 coculture system. (B) In the coculture system, medium (a, b) and spore (c, d) were  
109 incubated on the apical side of the Caco-2 monolayer for 0.5 h. The filters were  
110 processed for immunofluorescence staining and observed using CLSM. A  
111 three-dimensional (3D) rendering of representative fields was obtained with ZEN2012  
112 software. Submucosal DCs (CD11c, red) caused dendrites (white arrow) to creep  
113 through the tight junctions (TJs) of ECs (ZO-1, green) in response to spore but not  
114 medium. Scale bars = 50  $\mu\text{m}$ . (C) Ligated loops of mice were injected with spore *in*  
115 *vivo*, and intestines were isolated after 1 h and then processed for  
116 immunofluorescence staining. Cryosections stained with anti-CD11c antibody (red)  
117 and 4', 6-diamidino-2-phenylindole (DAPI; blue) were observed under a confocal  
118 microscope. TEDs are indicated by arrows. Scale bars = 10  $\mu\text{m}$ . (D) In the coculture  
119 system, DCs were stimulated for 24 h with LPS (100 ng/ml) or spore ( $10^6$ ,  $10^7$ , and  
120  $10^8$  CFU/ml), and surface molecule expression on gated viable cells was measured by  
121 flow cytometry on gated viable cells. The phenotypic expression levels of CD40 and  
122 CD80 on DCs were analyzed by FACS. (E) DCs were treated with medium and spore  
123 separately for 24 h, and the morphology of DC dendrites was observed by microscopy.  
124 Scale bar = 20  $\mu\text{m}$ . (F) Secretion of interleukin (IL)-1 $\beta$  and TNF- $\alpha$  in culture  
125 supernatants was measured by ELISA. The results are expressed as the mean  $\pm$  SEM.  
126 Significance was tested against the unstimulated control by one-way ANOVA, \* $P <$   
127 0.05, \*\* $P <$  0.01. One representative result of three similar independent experiments  
128 is shown.

129

### 130 Spore facilitated H9N2 WIV to enhance H9N2-specific antibodies and induce T 131 cell proliferation

132 Local secretion of IgA antibodies is the most important characteristic mediating oral  
133 adaptive immunity and mucosal protection. As shown in the immunization schedule,  
134 vaccine induced antibody responses were analyzed in the serum and mucosal fluids at  
135 different time points (Fig. 2A). Spore facilitated H9N2 WIV in enhancing the  
136 intestinal IgA response after oral immunization in mice (Fig. 2C). Similar changes in  
137 IgA levels in lung wash samples were also observed at 7 d, 35 d and 49 d (Fig. 2B) ( $P$   
138  $< 0.01$ ), suggesting a marked effect of spore on mucosal responses in the lower  
139 respiratory tract. In addition, a trend reflecting increased levels of H9N2-specific IgG  
140 induced by spore plus H9N2 WIV was observed. Spore was able to significantly  
141 enhance the levels of IgG in the serum compared with PBS. As shown by the results,  
142 the levels of IgG at 21 d, 35 d and 49 d (Fig. 2D) ( $P < 0.01$ ), IgG1 at 35 d (Fig. 2E) ( $P$   
143  $< 0.01$ ) and IgG2a at 21 d, 35 d and 49 d (Fig. 2F) ( $P < 0.01$ ) induced by H9N2 WIV  
144 plus spore were significantly higher than the levels induced by H9N2 WIV alone. In  
145 addition, serum collected from different groups of mice at 21 d and 49 d showed a  
146 powerful ability to inhibit hemagglutination against 4-HA units of H9N2 compared  
147 with antigen alone (Fig. 2I). We isolated lymphocytes from the spleen and mesenteric  
148 lymph node (MLN) 21 d post-immunization, and cells were restimulated with H9N2  
149 WIV *in vitro*. We found that the proliferative index in MLNs was markedly increased  
150 in the spore plus H9N2 WIV group compared with that in the antigen-alone group  
151 (Fig. 2G) ( $P < 0.01$ ). Similarly, the proliferative index in the spleen was increased  
152 (Fig. 2H) ( $P < 0.05$ ), reflecting effective induction of systemic and local immune  
153 responses in mice.

154

## 155 **Fig 2. Spore facilitated H9N2 WIV to enhance antigen-specific antibodies.**

156 (A) Schematic of oral immunization and the sampling schedule of intestinal fluids,  
157 lung wash fluids, serum, mesenteric lymph nodes (MLNs) and the spleen. H9N2 WIV  
158 (20  $\mu\text{g}$ ) and spore ( $10^8$  CFU/ml) were orally administered to each mouse. Primary and  
159 secondary immunizations were performed at 0 d and 7 d, respectively. Booster  
160 immunizations were administered at 42 d. The details of the immunization schedule  
161 indicate the time point of immunization (black arrows above the line). Sampling is  
162 indicated by the time points of the serum, intestinal fluid and lung washing buffer  
163 collection arrows (below the line). (B-F) H9N2 specific IgA and IgG antibodies in  
164 mice post-immunization were determined by ELISA. Antigen-specific serum (D) IgG  
165 titers, (E) IgG1 titers, (F) IgG2a titers, and (B) mucosa IgA titers in intestinal wash  
166 and (C) lung wash fluids were detected at different time points. The asterisks indicate  
167 significant differences between H9N2 WIV plus spore and H9N2 WIV alone. (G and  
168 H) MLNs (G) and splenic (H) lymphocytes from immunized mice were isolated and  
169 restimulated with H9N2 WIV (10  $\mu\text{g}/\text{ml}$ ) *in vitro*. The proliferative response was  
170 detected by a CCK8 assay. (I) Hemagglutination inhibition (HI) titers were detected at  
171 21 d and 49d. The results are expressed as the mean  $\pm$ SEM.  $P$  values  $< 0.05$  were  
172 considered to be statistically significant ( $*P < 0.05$ ,  $**P < 0.01$ ) (n=6).

173

## 174 **Spore-adjuvanted immunization induced CD69<sup>+</sup>CD103<sup>+</sup> Trms in intestinal tissue**

175 To further evaluate whether spore could induce Trm formation *in vivo*, we performed

176 oral immunization in mice with PBS, H9N2 WIV alone or H9N2 WIV combined with  
177 spore. In the present study, spore-adjuvant immunization significantly upregulated the  
178 expression levels of the Tcm surface makers CD62L and CCR7 in blood at 7 d after  
179 primary immunization (S1 Fig. A) ( $P < 0.01$ ). Nevertheless, no significant difference  
180 was observed at 45 d (S1 Fig. C). Recently, Trms were found in several tissues,  
181 including the intestinal mucosa [26] and lung [27]. Since spore could not cause the  
182 development of Tcms in blood following immunization, we next examined presence  
183 and proportion of Trms in the intestine. Two surface markers, CD103 and CD69, have  
184 been considered in distinguishing Trms from other memory T cells [28-30]. Intestinal  
185 tissues were harvested from immunized animals, and CD3-positive cells were then  
186 assessed for the expression of the tissue retention markers CD69 and CD103 at 7 d, 14  
187 d and 45 d (Fig. 3A). No effect on the frequency of CD69<sup>+</sup> CD103<sup>+</sup> cells among CD3<sup>+</sup>  
188 T cells was observed at 7 d (Fig. 3B). Notably, flow cytometry analysis showed that  
189 spore plus H9N2 WIV induced 21.5% CD69<sup>+</sup>CD103<sup>+</sup> Trms at 14 d (Fig. 3C) and  
190 49.1% CD69<sup>+</sup>CD103<sup>+</sup> Trms at 45 d (Fig. 3D). Furthermore, the expression of IFN- $\gamma$ <sup>+</sup>  
191 T cells markedly increased at 45 d (Fig. 3E) after H9N2 WIV restimulated IMALs.  
192 These data supported the capacity of a mucosal vaccine to induce substantial T cell  
193 responses after oral immunization.

194

195 **Fig 3. The frequency of CD69<sup>+</sup>CD103<sup>+</sup> Trms after oral immunization in the**  
196 **intestinal tract.**

197 (A) Schematic experimental design to examine the frequency of Trms after oral  
198 immunization with different vaccines. (B-D) The frequency of Trms (CD3<sup>+</sup> CD69<sup>+</sup>  
199 CD103<sup>+</sup>) was detected in the intestinal mucosa at 7 d (B), 14 d (C) and 45 d (D) after  
200 priming immunization. A gating strategy was applied in this study to determine the  
201 memory cell phenotype of CD3<sup>+</sup> T cells according to CD69 and CD103 from in  
202 intestinal tissue. (E) IFN- $\gamma$  expression in CD3<sup>+</sup> T cells from immunized mice was  
203 detected by FACS following H9N2 WIV recall. Data are represented as the mean  $\pm$   
204 SEM (n=6). \* $P < 0.05$ , \*\* $P < 0.01$ . One representative of two similar independent  
205 experiments was shown.

206

207 **Spore-adjuvanted immunization induced HA-specific Trms in intestinal tissue**

208 We also investigated antigen-specific Trms after mucosal immunization. To detect  
209 whether FTY720 treatment could inhibit lymphocyte circulation, FTY720 was  
210 administered to inhibit the circulation of T cells six weeks after the primary  
211 vaccination, as illustrated in Figure 4A. In addition, mice were injected i.v. with  
212 anti-CD45-FITC antibodies 10 min prior to harvesting blood and intestinal Peyer's  
213 patches (PPs). The vast majority (> 99.9%) of the intestine failed to be stained by  
214 anti-CD45 antibodies after treatment with FTY720 (S2 Fig). Interestingly, the  
215 influenza HA-specific T cells following mucosal immunization were significantly  
216 increased in H9N2 WIV plus spore-treated mice and were not altered by FTY720  
217 treatment (Fig. 4C and D) ( $P < 0.05$ ). Thus, we can speculate that these memory cells  
218 also show a bias toward tissue residency. Consistent with the flow cytometry results,  
219 we also observed accumulation of influenza HA-specific cells in the intestinal tract,



220 and imaging using microscopy showed that at 5 weeks after oral immunization,  
221 allophycocyanin (APC)-tetramer<sup>+</sup> T cells were readily detectable in the PPs of the  
222 ileum (Fig. 4B). Together, these results demonstrated that mucosal immunization with  
223 H9N2 WIV plus spore can generate influenza-specific T cells in the intestinal tract.

224

#### 225 **Fig 4. Evidence of HA-specific T cells after oral immunization in intestinal tract.**

226 (A) BALB/c mice were immunized as described previously, and lymphocytes from  
227 the intestine were analyzed by HA-specific tetramer staining at 45 d to detect the  
228 effect of FTY720 treatment on T cells. Five weeks after immunization, immunized  
229 mice were treated with FTY720, 1 mg/kg by i.p. daily for 10 d. (B-D) APC-tetramer<sup>+</sup>  
230 T cell populations were analyzed and compared with those of immunized mice treated  
231 with H9N2 WIV plus spore but not treated with FTY720 by FACS (C, D) and  
232 confocal microscopy (B). The scale bar represents 50  $\mu$ m. Data are represented as the  
233 mean  $\pm$  SEM (n=6). \* $P$  < 0.05, \*\* $P$  < 0.01. One representative result of two similar  
234 independent experiments is shown.

235

#### 236 **Spore upregulated ICAM-1 expression after mucosal immunization**

237 Upon revealing the activation of DCs, we then assumed molecules that could be  
238 regulated in the intestinal microenvironment. To this end, a proteome profiler array  
239 analysis was performed to measure 110 proteins from the lysates of the intestinal tract  
240 at 7 d or 45 d after oral immunization of mice with PBS or spore (Fig. 5B). In  
241 particular, ICAM-1 and adiponectin/Acrp30 was relatively upregulated in intestinal  
242 tissue after spore immunization compared with PBS treatment (Fig. 5C). Based on  
243 these considerations, we focused on ICAM-1 and its potential action on DCs. High  
244 expression of ICAM-1 was also verified in intestinal tissue by western blot and  
245 immunohistochemical staining (Fig. 5D and 5E) ( $P$  < 0.01).

246

#### 247 **Fig 5. ICAM-1 expression in the intestinal tract after oral administration of** 248 **spore.**

249 (A) Schematic experimental design of oral immunization with PBS or spore and the  
250 sampling schedule of intestinal tissue and lymphocytes. (B) Gray value intensity was  
251 detected utilizing chemiluminescence and membranes can be assessed for protein  
252 levels. Intensity is shown in a pseudocolor scale (from low [blue] to high [red]). (C)  
253 Mouse intestinal whole-tissue lysate was analyzed by an XL mouse antibody array.  
254 The solid black circles indicate proteins secreted by mice. (D) Western blot analysis  
255 revealed the time-dependent upregulation of ICAM-1 in the ileum tissue following  
256 immunization with spore at 7 d and 45 d. Equal proteins loading was confirmed using  
257 the house-keeping gene  $\beta$ -actin. (E) Induction of ICAM-1 expression was confirmed  
258 by immunohistochemistry (IHC) staining in the ileum. The scale bar represents 20  $\mu$ m.  
259 Data are represented as the mean  $\pm$  SEM (n=6). \* $P$  < 0.05, \*\* $P$  < 0.01. One  
260 representative of three similar independent experiments is shown.

261

#### 262 **ICAM-1 expression on DCs increased after oral spore treatment**

263 Previously, it has been showed that upregulation of ICAM-1 on APC could regulate

264 the generation of central memory cells [31]. According to previous results, we showed  
265 that ICAM-1 expression was significantly increased in DCs following spore treatment  
266 ( $10^6$ ,  $10^7$ ,  $10^8$  CFU/ml) depending on the concentration (Fig. 6A and B) ( $P < 0.01$ ).  
267 Moreover, this finding suggested that the effect coincided with the increased levels of  
268 ICAM-1 measured by qPCR and IF (Fig. 6C and D) ( $P < 0.01$ ). Our current results  
269 implied that spore first stimulated DC recruitment to the LP *in vivo* (Fig. 1) and then  
270 activated ICAM-1 molecule to further stimulate T cells. To further define whether this  
271 phenomenon was reproducible *in vivo*, we performed FACS for ICAM-1 expression  
272 in CD11c<sup>+</sup> DCs. We noted that ICAM-1 was upregulated in the intestinal submucosal  
273 DCs of mice after immunization with spore plus H9N2 WIV (Fig. 6E). Furthermore,  
274 the number of ICAM-1<sup>+</sup> DCs (CD11c<sup>+</sup>) was detected in the intestine using the double  
275 fluorescence staining method (Fig. 6F). In brief, ICAM-1 expression was notably  
276 increased on DCs after spore stimulation *in vitro* and *in vivo*.

277

### 278 **Fig 6. ICAM-1 expression on DCs was increased after stimulation with spore.**

279 DCs were treated with spore ( $10^6$  CFU/ml,  $10^7$  CFU/ml and  $10^8$  CFU/ml) or spore  
280 plus A-205804 (10  $\mu$ M) for 24 h. (A, B) ICAM-1 expression was detected on DCs  
281 after spore treatment by FACS. (C) ICAM-1 mRNA expression was measured by  
282 RT-qPCR and confocal microscopy. (D) ICAM-1 protein expression was detected by  
283 immunofluorescence after DCs were incubated with medium, spore or spore plus  
284 ICAM-1 inhibitor A-205804 for 24 h. The scale bar represents 20  $\mu$ m. (E) Cells were  
285 collected from the intestinal tract, and the MFI of ICAM-1 gated from CD11c<sup>+</sup> DCs  
286 was detected by FACS. (G) ICAM-1<sup>+</sup> CD11c<sup>+</sup> double positive cells in the intestine  
287 were strong positivity stained by immunofluorescent staining. The scale bar  
288 represents 20  $\mu$ m. Data are represented as the mean  $\pm$  SEM (n=6). \* $P < 0.05$ , \*\* $P <$   
289 0.01. One representative result of three similar independent experiments is show

290

### 291 **Spore stimulated Acrp30 expression in 3T3-L1 Adipocytes**

292 Since we have demonstrated that spore could enhance the Acrp30 level in intestine  
293 (Fig. 5A). In order to determine stimulation effects of spore treatment on  
294 differentiated 3T3-L1 cells, we performed the induction culture assay of adipocytes.  
295 Initially, cells displayed a fibroblast phenotype. Then, cell morphology changed and  
296 cells accumulated lipid droplets internally during the process of differentiation. After  
297 12 days, when the adipocytes were mature, almost the entire cell volume was stained  
298 red by red oil (Fig. 7A). We subsequently determined the effects of spore on Acrp30  
299 protein expression. Spore at  $10^6$  CFU/ml was sufficient to elicit up-regulation of the  
300 Acrp30. Treatment of  $10^7$  CFU/ml of spore led to a 2-fold increase in the Acrp30  
301 protein amount in comparison with the control without spore (Fig. 7B). The extent of  
302 spore-induced increase in protein levels was positively associated with the  
303 concentrations of spore, indicating that there is a dose-dependent effect of spore on  
304 the increase of adiponectin proteins. We also investigated whether Acrp30 induced by  
305 spore could increase ICAM-1 expression on DCs. The cultural supernatant from  
306 differentiated 3T3-L1 cells treated by spore or PBS could stimulate the ICAM-1  
307 expression on DCs (Fig. 1C and D). Our data demonstrated that spore effectively



308 up-regulated the expression of the adiponectin and enhanced ICAM-1 expression on  
309 DCs.

310

311 **Fig 7. Secretion of Acrp30 from adipocyte induced by Spore increased ICAM-1**  
312 **expression on DCs.**

313 (A) 3T3-L1 cells were differentiated into adipocytes according to the differentiation  
314 protocol. Phase contrast images of 3T3-L1 cells from 0 day induction (pre-adipocyte)  
315 to ten days post-induction (adipocyte). Triglyceride staining of 3T3-L1 cells with Oil  
316 Red O. (B) Differentiated 3T3-L1 cells were treated with spore for 24 h. Western Blot  
317 assay was performed to detect the levels of adiponectin in the cells. The bar graph  
318 represents quantification of the relative protein levels of adiponectin. (C-D) DCs were  
319 treated with medium from Differentiated 3T3-L1 treated with spore for 24 h. Flow  
320 cytometry analysis was performed with anti-ICAM-1-FITC staining. A representative  
321 blot is shown in the upper panel. Data are represented as the mean  $\pm$  SEM. \* $P < 0.05$ ,  
322 \*\* $P < 0.01$ . One representative of three similar independent experiments is shown.  
323 Bars: 25  $\mu$ m.

324

325 **ICAM-1-dependent DCs induced the generation of HA-specific T cells**

326 Pretreatment with spore and/or S-205804 suppressed the ICAM-1 expression of DCs  
327 or anti-ICAM-1 neutralizing antibody, and the CD44<sup>+</sup> CD69<sup>+</sup> phenotype markers of T  
328 cells were detected by FACS. As expected, flow cytometry analysis showed  
329 significantly increased levels of the CD44<sup>+</sup>CD69<sup>+</sup> phenotype markers of Trms in DCs  
330 with spore treatment, and the treatment of ICAM-1 inhibitor A-205804 suppressed the  
331 CD44<sup>+</sup>CD69<sup>+</sup> phenotype induced by spore (Fig. 8A and B)( $P < 0.01$ ).

332 To assess whether HA-tetramer<sup>+</sup> specific T cells persisted in the intestine at 6  
333 weeks after immunization, antigen-loaded DCs with or without ICAM-1 inhibitor  
334 were incubated with IMALs isolated from immunized mice at 37°C for 3 d (Fig. 8D).  
335 Flow cytometric results showed that spore plus H9N2 WIV induced more  
336 HA-tetramer<sup>+</sup> specific cells than H9N2 WIV alone at the present of antigen-pulsed  
337 DCs (Fig. 3E and F). Taken together, these results indicated that DCs partially require  
338 the expression of ICAM-1 for the generation of antigen-specific Trms, which were  
339 altered in the presence of ICAM-1 inhibitor added in DCs.

340

341 **Fig 8. ICAM-1 dependent in DCs increased HA-specific Trm formation.**

342 (A) DCs were pretreated with spore and/or A204804 and then co-incubated with  
343 sorted CD3<sup>+</sup> T cells from wild-type mice for 3 d. (B, C) Gated T cells were analyzed  
344 for the surface markers of memory cells CD44 and CD69 by FACS. (D) Six weeks  
345 after the primary vaccination, the intestines were dissected to prepare lymphocytes.  
346 BMDCs ( $5 \times 10^5$  cells/well) were stimulated with HA<sub>518-526</sub> (IYSTVASSL) peptide  
347 (10  $\mu$ l) overnight. Antigen-pulsed DCs were used as APCs to stimulate IMALs ( $1 \times$   
348  $10^6$  cells/well) for 5 d. (E, F) Frequency of APC-tetramer<sup>+</sup> T cells was detected by  
349 flow cytometry. Representative flow cytometry results and graphs for statistical  
350 analysis are shown. A representative result of two similar independent experiments is  
351 shown. Data are presented as the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

352

353 **Fig 9. Overview of generating memory T cell generations after mucosal**  
354 **vaccination.**

355 Upon mucosal vaccination, DCs were stimulated with H9N2 WIV plus spore and  
356 then migrated to the draining lymph nodes and stimulated naïve T cells. Tcms  
357 recirculated between the blood and lymphoid organs or entered peripheral tissues.  
358 Vaccination also activated DCs to express ICAM-1 cytokines as well as LFA-1  
359 binding to ICAM-1 on DCs. In addition, Acrp30 induced by 3T3-L1 differentiated  
360 adipocyte with the treatment of spore might generate the ICAM-1 expression on DCs.  
361 Then, ICAM-1 expression on DCs could upregulate proportion of antigen-specific  
362 Trms, which was altered after treatment of ICAM-1 inhibitor. The surface markers of  
363 Trms, such as CD69 and CD103, were upregulated after mucosal immunity. Local  
364 reactivation of mucosal Trm formation was induced by the ICAM-1 molecule, which  
365 triggered DCs recruitment and cytokine expression. The existence and maintenance of  
366 Trm subsets in intestine could accelerate pathogen clearance.

## 368 Discussion

369 Oral immunization is beneficial for eliciting mucosal immune responses against  
370 pathogens that invade through a mucosal surface [32]. Here, we used a mucosal  
371 immunization strategy known as “prime-second-boost” and dissected the multifaceted  
372 adaptive immune mechanisms after mucosal immunization with spore plus H9N2  
373 WIV. This strategy may represent an ideal platform for immunological protection and  
374 lead to robust antibody responses to generate long-lasting immunological memory.

375 This platform has numerous advantages as spore survived well and favorably  
376 stimulated DCs. Additionally, APCs such as DCs result in the generation of different  
377 fates for T cells as distinct populations of memory T cells in the absence of antigen  
378 [33]. Recent results prove that potent activation signatures in macrophages and bone  
379 marrow DCs with *Bacillus subtilis* spore treatment are accompanied by increased  
380 expression levels of the maturation markers CD40 and MHC classes I and II on DCs  
381 [34]. Moreover, the recruitment of DCs, particularly CD103<sup>+</sup> DCs, promotes CD103  
382 expression on immune cells and is essential for the efficient induction of Trms [35].  
383 Subsequently, we focused on the innate immune system. Here, we performed  
384 experiments using a DC/EC coculture system and showed that spore played important  
385 roles in facilitating the delivery of DCs across intestinal mucosal barriers at an early  
386 stage of mucosal immunity and induced DC maturation, including the secretion of  
387 IL-1 $\beta$ . In accordance, mucosal expression of IL-1 $\beta$  is a sufficient and crucial mediator  
388 of Trm formation [36].

389 ICAM-1 expression is known to play a crucial role in the proper generation of T  
390 cell memory responses by APCs [37]. Furthermore, we observed that ICAM-1 had a  
391 much stronger impact on the initiation of mucosal memory T cells. Thus, the  
392 difference in mucosal T cell numbers and function between the immunized animals in  
393 the different groups likely reflects the response to spore-induced recruitment signals  
394 in the intestine. In this study, we identified ICAM-1 as a critical regulator of DCs in  
395 inducing memory T cell information. When the expression of ICAM-1 was inhibited,  
396 the memory T cell phenotype markers CD44 and CD69 were observed less frequently  
397 than in the regular DC group. Current studies have shown that potential KLRG1-CD8<sup>+</sup>  
398 Trms precursors with increased expression of CD69 can be isolated from the intestine,  
399 which remain the most reliable markers for intestinal-resident T cells in mice [16, 38].  
400 A previous model predicted that ICAM-1 is required to augment the priming process,  
401 likely by promoting the recruitment of naïve T cells, prolonging cell-cell interactions,  
402 facilitating cytokine signaling, and permitting the differentiation of memory T cell  
403 precursors [22]. However, mucosal cytokines such as IL-1 $\beta$  induced by spore were  
404 not sufficient to induce differentiation of antigen-experienced T cells into Trms,  
405 which indicated the requirement for ICAM-1 upregulation on DCs in mucosal tissues.  
406 Our findings indicated that Acrp30 from adipocytes induced by spore increased  
407 ICAM-1 expression on DCs, which might provide new insight into the significance of  
408 adipocyte metabolism related molecules in regulating immunological memory T cells.

409 To investigate immunological antibodies associated with mucosal responses, we  
410 first evaluated antigen-specific antibody production in serum and mucosal fluid. We

411 found that mice immunized with spore plus H9N2 WIV produced more specific IgG  
412 in the serum and IgA in the mucosal fluid compared with mice treated with H9N2  
413 WIV alone. A similar trend was observed for lymphocyte proliferation after recalling  
414 antigens. Furthermore, we demonstrated that SF produced by *Bacillus subtilis* spore  
415 also induced more antibodies through the Th2 response, thus differing from spore.  
416 Overall, these findings may reflect a detection limitation in our assays or at least some  
417 potential metabolites of *Bacillus subtilis* spore as active components of mucosal  
418 immune enhancement.

419 Here, spore was able to induce a dramatically larger percentage of proliferating  
420 lymphocytes, indicating either a higher frequency of memory cells or cells with a  
421 higher proliferative capacity at the very least. Along with conventional T-cell  
422 activation signatures, we also observed a striking accumulation of gross  
423 CD69<sup>+</sup>CD103<sup>+</sup> Trms in intestinal tissue after immunization with spore plus H9N2  
424 WIV. Factors necessary for the establishment of Trms may play critical roles in this  
425 process and are not well understood. Recent vaccine studies have demonstrated that  
426 mucosal administration of antigen is important for the establishment of localized T  
427 cell responses [16]. In an optimally immunized individual, Trms were more protective  
428 than circulating memory cells based on their location and function [39]. Regarding the  
429 reason why no Trms are directed against spores themselves, spores may act as a  
430 mammalian commensal agent [40], thus suppressing the mobilization of Tefs that  
431 would lead to their clearance.

## 432 **Methods**

### 433 **Animals and ethics statement**

434 This study was approved by the Ethics Committee of Animal Experiments center of  
435 Nanjing Agricultural University. All animal studies were approved by the Institutional  
436 Animal Care and Use Committee of Nanjing Agricultural University  
437 (SYXK-2017-0007), and followed the National Institutes of Health guidelines for the  
438 performance of animal experiments. Specific pathogen-free C57BL/6 (4 to 6 weeks)  
439 and Balb/c (6 to 8 weeks) mice were obtained from Comparative Medical Center of  
440 Yangzhou University (Jiangsu, China). All animals were conducted at an animal  
441 facility under pathogen free conditions.

### 443 **Vaccine preparation**

444 The influenza A/Duck/Nan Jing/01/1999 H9N2 virus was generously provided by the  
445 Jiangsu Academy of Agricultural Sciences [41]. The H9N2 virus was purified using a  
446 discontinuous sucrose density gradient. H9N2 WIV is normally inactivated via  
447 incubation at 56°C for 0.5 h to achieve a complete loss of infectivity. The *Bacillus*  
448 *subtilis* SQR9 strain was kindly supplied by Professor Shen of Nanjing Agricultural  
449 University [42].

### 451 **Immunogenicity study**

452 Six-week-old Balb/c mice were orally immunized with H9N2 WIV (20 µg) alone or  
453 in combination with spore (10<sup>7</sup> CFU) three times (at 0, 7 and 42 d). The mice were

454 euthanized, and samples were collected at one-week intervals after the primary  
455 immunization. The levels of specific IgA in intestinal lavage fluid and specific IgG,  
456 IgG1, and IgG2a in serum were detected by ELISA. In brief, H9N2 WIV (2 µg/ml)  
457 antigens were coated onto a plate overnight, followed by blocking for 2 h with PBST  
458 containing 3% BSA. Intestinal lavage fluid and serum were diluted in PBS with 0.1%  
459 BSA and added to the plate in triplicate for 1.5 h at 37°C. Following five washes,  
460 HRP-conjugated rabbit anti-mouse IgG was incubated on the plate for 1 h. OD<sub>450</sub>  
461 values were read on a Tecan plate reader at 450 nm absorbance. A hemagglutination  
462 inhibition (HI) test was performed according to a previously described procedure [43].  
463

#### 464 **Intestinal mucosa associated lymphocyte (IMAL) isolation**

465 IMALs were isolated as described previously [44]. In brief, the intestine was opened  
466 longitudinally after the removal of residual mesenteric fat tissue. The tissue was then  
467 dissected into pieces and thoroughly washed with ice-cold PBS followed by digestion  
468 in 0.5 mg/ml collagenase D (Sigma), 0.5 mg/ml DNase I (Roche) and 50 U/ml dispase  
469 (Sigma) in Dulbecco's phosphate buffered saline (DPBS) containing 5 mM EDTA,  
470 4% fetal calf serum, and 100 µg/ml penicillin/streptomycin for 30 min at 37°C with  
471 slow rotation (100 rpm). After incubation, cells were collected and passed through a  
472 70-µm strainer (BD Biosciences) and washed once with cold RPMI-1640. Then, the  
473 cells were resuspended in 6 ml of the 30% fraction of a Percoll gradient and overlaid  
474 on 6 ml of the 70% fraction in a 15-ml Falcon tube. Percoll gradient separation was  
475 performed by centrifugation at 300 g for 20 min. IMALs were collected at the  
476 interphase of the Percoll gradient, washed once, and resuspended in cold RPMI-1640  
477 with 5% FBS. The cells were used immediately for experiments.  
478

#### 479 **General flow cytometry and cell sorting**

480 For most experiments, cells were first stained with an Fc receptor blocker (1:20  
481 dilution; eBioscience). For surface staining, cells were then stained with a mix of  
482 fluorescent antibodies in flow cytometry buffer for 30-45 min at 4°C for 0.5 h per the  
483 manufacturer's guidelines. For Trm FACS, cells were stained with CD3-APC  
484 (145-2C11, eBioscience), CD103-FITC (2E7, eBioscience) and CD69-PE (H1.2F3,  
485 eBioscience) separately. For Tcms in blood, cells in 50 µl blood were stained with  
486 CD3-percp-cy5.5 (1452C11, Miltenyi Biotec), CD62L-APC (REA828, Miltenyi  
487 Biotec) and CCR7-PE (REA685, Miltenyi Biotec). Then, the whole blood was filtered  
488 through a 70-µm cell strainer, and the suspensions were incubated with an ammonium  
489 chloride potassium lysis buffer for 30 min at RT. For intracellular staining, the cells  
490 were incubated with 50 ng/ml phorbol myristate acetate (PMA; Sigma), 750 ng/ml  
491 ionomycin (Sigma), and 10 µg/ml brefeldin A (Invitrogen) in a cell culture incubator  
492 at 37°C for 5 h. After surface staining, the cells were resuspended in fixation and  
493 permeabilization solution (BD Biosciences) for 45 min at 4°C. Consistent with  
494 previous reports, the signature cytokines, interleukin (IL)-4 and IFN-γ, were measured  
495 on a BD FACS Verse and analyzed with FlowJo v.10.

496 For the lymphocyte enrichment assay, naïve CD4 or CD3 T cells were purified by  
497 negative selection similar to previously described methods [45]. Briefly, single-cell



498 suspensions of lymph node (LN) or enteric cells were incubated with the following  
499 dilutions provided for each. For staining, a 100 µl volume of the antibody cocktail  
500 (BD Biosciences) was used per tissue sample from one mouse. Resuspended cells  
501 were incubated in an antibody cocktail for 15-30 min at 4°C in the dark. After  
502 washing the cells with 10 ml of PBS, the mixture was passed over a magnet following  
503 the manufacturer's instructions. The purity of the flow-through fraction was routinely  
504 > 90%.

505

### 506 **Adipocyte differentiation culture**

507 Mouse 3T3-L1 pre-adipocyte cells were cultured and differentiated as previously  
508 described [46]. Briefly, 3T3-L1 were grown in regular medium (high-glucose  
509 Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS  
510 containing 1% penicillin and streptomycin). About  $2 \times 10^5$  cells were seeded on  
511 12-well plates and grown to full confluence for 4 days. Then the cells were subjected  
512 to the first differentiation medium (DMEM supplemented 10% FBS, 0.5 mM  
513 3-isobutyl-1-methylxanthine, 1 µM dexamethasone and 10 µg/ml insulin) starting on  
514 day 0 after confluence. After 2 days of induction, the medium was replaced with only  
515 insulin in DMEM with 10% FBS for an additional 2 days. Two days later, the cells  
516 were grown in regular medium for an additional 8 days and the medium was replaced  
517 every 2 days. Isobutyl-1-methylxanthine, dexamethasone and insulin were obtained  
518 from Sigma-Aldrich. 3T3-L1 cells were obtained from professor Yang of Nanjing  
519 Agriculture University. In this study, the medium was taken from 3T3-L1 adipocytes  
520 treated with spore varying from  $10^6$  and  $10^7$  CFU/ml for 24 h. Then cell culture  
521 supernatant from adipocyte was added to DCs for another 24 h.

522

### 523 **FTY720 treatments and tetramer staining**

524 To inhibit circulation of memory T cells, FTY720 (Sigma) 1 mg/kg in PBS was  
525 administered intraperitoneally (i.p.), daily for 10 d. In addition, to assess the  
526 protective efficacy of the vaccines, the mice were immunized with the same vaccine.  
527 Intravascular staining was performed by injecting mice i.v. with FITC-conjugated  
528 anti-mouse CD45 antibody (5 µg) for 8-10 min before being euthanized. After  
529 immunization and FTY720 treatment, intestinal tissue was collected and collagenase  
530 digested, and cells were isolated for flow cytometric analysis as described previously  
531 [47]. The cells were stained with fluorochrome-conjugated antibodies or influenza  
532 HA-specific (HA<sub>518-526</sub>) H-2Kd tetramer-IYSTVASSL (MBL) reagent and analyzed  
533 using a flow cytometer (BD Biosciences). BMDCs ( $10^5$  cells) were incubated  
534 overnight in the presence of 10 µg/ml of influenza HA<sub>518-526</sub> (IYSTVASSL) peptide  
535 (Genscript). IMALs were isolated from treated mice and A205804 was added to the  
536 plate for 5 d. The cells were stained with anti-CD3 for 20 min and HA<sub>518-526</sub><sup>+</sup> tetramer  
537 for an hour.

538

### 539 **DC/EC coculture system**

540 DCs were generated from 4 to 6-week-old C57BL/6 mice using our previous method  
541 [48]. Briefly, bone marrow was extracted from the tibias and femurs of C57BL/6 mice

542 with RPMI 1640. Then, the cells were suspended in complete medium (RPMI 1640  
543 supplemented with 10% heat-inactivated FBS, 1% PenStrep), 10 ng/ml interleukin-4  
544 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF). After  
545 culture for approximately 60 h, the medium was lightly discarded to detach  
546 non-adherent granulocytes. Then clusters were harvested and subcultured overnight to  
547 remove adherent cells at 5 d. Non-adherent cells were collected at 6 d and used in  
548 subsequent studies. Caco-2 cells were seeded on the upper side of ThinCert  
549 membrane inserts (pore size, 3  $\mu\text{m}$ ) (Greiner Bio-One, Germany) in a 24-well plate  
550 overnight. The cells were maintained for 6 to 10 d until steady-state transepithelial  
551 electrical resistance of 300  $\Omega\cdot\text{cm}^2$  was achieved [25]. In the coculture system, the  
552 filters were turned upside down, and then, DCs ( $5 \times 10^5$  cells/ml) were cultured on the  
553 basolateral side of ECs for 4 h to let the cells attach to the filter. The filters were then  
554 turned right side up and placed into 24-well plates. The cells were incubated with  
555 different treatments with spore ( $10^7$  CFU/ml), or lipopolysaccharide (LPS) (1  $\mu\text{g}/\text{ml}$ )  
556 for 24 h from the apical side. The filters and cells were fixed with 4%  
557 paraformaldehyde (PFA) for 15 min and processed for confocal microscopy. In  
558 addition, DCs were collected for phenotype assays and basolateral supernatants were  
559 collected for cytokine secretion assays.

560

#### 561 **Ligated loop experiments**

562 Mice were anesthetized with chloral hydrate (350 mg/kg body weight, intramuscular  
563 injection). The terminal ileal or jejunal ligated loop was injected with spore ( $10^8$   
564 CFU/ml) or the same volume of PBS (0.01 M), and the intestines were removed after  
565 0.5 h, optimal cutting temperature (OCT) (Tissue Freezing Medium, Sakura, Torrance,  
566 CA) and cut into 8  $\mu\text{m}$  for immunofluorescence assays, as described below.

567

#### 568 **Mouse cytokine array by a proteome profiler**

569 Intestinal tissues from mice treated with PBS or spore for 7 d and 45 d were lysed  
570 with cell lysis buffer (R&D Systems) supplemented with 1% 0.2 mM  
571 phenylmethylsulfonyl fluoride (PMSF) at 4°C for 30 min. The protein concentration  
572 was detected with a protein bicinchoninic acid (BCA) kit (Thermo Fisher Scientific).  
573 Samples were analyzed with a mouse XL cytokine array kit (R&D Systems),  
574 according to the manufacturer's instructions [49]. Immunospots were captured with  
575 an Odyssey Fc Imager (LI-COR), and data were analyzed with ImageJ software.

576

#### 577 **Histology and immunohistochemistry**

578 Immunohistochemistry detection was performed with the SABC kit (Boster  
579 Bioscience). Intrinsic peroxidase in samples was inactivated using 3% hydrogen  
580 peroxide after antigen retrieval was performed with buffer. Tissue sections were  
581 incubated with primary antibodies against ICAM-1 (1:200; Abcam) overnight at 4°C.  
582 Subsequently, the sections were incubated with biotinylated goat anti-mouse IgG as  
583 the secondary antibody. After staining with DAB, images were captured using a  
584 digital camera (Leica-DM4000B).

585

586 **Immunofluorescence (IF)**

587 The fixed filters were permeabilized in 0.5% Triton X-100 in PBS for 5 min and  
588 blocked with 5% bovine serum albumin (BSA) in PBS for 2 h. Then the filters were  
589 stained with primary antibodies Armenian hamster anti-CD11c (N418) and rabbit  
590 anti-ICAM-1 (1A29, Abcam) overnight at 4°C, followed by incubation with  
591 secondary antibodies for 2 h at room temperature. For the *in vivo* model, cryosections  
592 were treated as described above. The filters were identified using confocal laser  
593 scanning microscopy (CLSM) (LSM 710; Zeiss, Oberkochen, Germany).  
594 Cross-sectional images were observed by z-axis views and analyzed using Zeiss ZEN  
595 2012 and Adobe Photoshop CC (Adobe, San Jose, CA).

596

597 **Quantitative RT-PCR (qRT-PCR)**

598 Total RNA from intestinal tissues was prepared using Trizol reagent (Takara, JPN)  
599 following the manufacturer's guidelines and reverse-transcribed using a PrimeScript  
600 RT reagent Kit (Takara, JPN) according to the manufacturer's instructions. QPCR  
601 was performed for triplicate samples using a SYBR Green qPCR Kit (Takara, JPN) by  
602 the Applied Biosystems™ QuantStudio™ 6 standard Real-Time PCR System  
603 (Thermo Fisher Scientific). The housekeeping genes  $\beta$ -actin was routinely used as  
604 internal controls. The primers used in this study were as follows: for  $\beta$ -actin,  
605 5'-AAGTGTGACGTTGACATCCG-3', rev 5'-GATCCACATCTGCTGGAAG-3';  
606 for ICAM-1, 5'-TCACCAGGAATGTGTACCTGAC-3', rev  
607 5'-GGCTTGTCCTTGAGTTTTATGG-3'.

608

609 **Western blot assay**

610 The cells were lysed with RIPA buffer containing a 1% protease inhibitor cocktail on  
611 ice for 20 min. After removing debris by centrifugation at 4°C, supernatant protein  
612 was collected, and the total concentration was determined by a BCA protein assay kit.  
613 Protein was separated by electrophoresis on 10% sodium-dodecyl sulfate  
614 polyacrylamide gels (SDS-PAGE) and transferred to a polyvinylidene difluoride  
615 (PVDF) membrane. Mouse anti-ICAM-1 (1A29, Abcam), anti-Acrp30 (PA1-054,  
616 Thermo Fisher) and anti- $\beta$ -actin (4D3, bioworld) were used to assess ICAM-1 and  
617 Acrp30 expression. Western blot images were visualized using an Image Reader  
618 Tanon-5200 imaging system.

619

620 **Statistical analysis**

621 Results were shown as mean  $\pm$  SEM. Student's t-test was employed to determine that  
622 between two groups and One-way analysis of variance (ANOVA) with Dunnett's test  
623 were performed with SPSS among multiple groups. The statistical analysis was  
624 performed using FlowJo v10, Microsoft Excel 2010 and Graph Pad Prism 7 Software.  
625 The asterisks indicate significant differences between H9N2 WIV plus spore and  
626 H9N2 WIV. P values  $< 0.05$  were considered to be statistically significant (\*  $P < 0.05$ ,  
627 \*\* $P < 0.01$ ).

628

629

631

## 632 **Acknowledgments**

633 We thank for Penghao Zhang and Yuchen Li performed most of the immunization  
634 and in vitro immunogenicity experiments. We also thank for Jian Lin and Qinghua Yu  
635 conceived the study and revised the manuscript. Finally, we thank for Lulu Huang  
636 study conception and design, data analysis and interpretation, manuscript writing,  
637 final approval of the manuscript.

638

## 639 **Funding**

640 This work was supported by the National Natural Science Foundation of China  
641 (31772777) and the Fundamental Research Funds for the Central Universities  
642 (JCQY201906). This work was also supported by a project funded by the Priority  
643 Academic Program Development of Jiangsu Higher Education Institutions (PAPD)  
644 and the Jiangsu Natural Science Foundation--Excellent Youth Foundation  
645 (BK20190077). And this work was also supported by 2018YFD0500600 from the  
646 National Key Research and Development Program of China.

647

## 648 **Abbreviations**

649 BMDCs: Bone marrow derived dendritic cells, AIV: avian influenza virus, Tcms:  
650 central memory T cells, Trms: Tissue-resident memory T cells, Tems: effector  
651 memory T cells, SIP: sphingosine-1-phosphate, TEDs: transepithelial dendrites, EC:  
652 epithelial cell, GM-CSF: granulocyte colony-stimulating factor, MHC-II: major  
653 histocompatibility complex class II, FACS: Fluorescence Activated Cell Sorter.

654

## 655 **Conflict of Interest**

656 The authors declare no competing financial interests.

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833 **Key Resources Tables**

Reagent or Resources	Source	Clone
anti-mouse CD11c-APC	eBioscience	N418
anti-mouse CD40-PE	eBioscience	1C10
anti-mouse CD80-FITC	eBioscience	16-10A1
anti-mouse CD86-PE	eBioscience	GL1
anti-mouse MHC class II I-A-FITC	eBioscience	NIMR-4
anti-mouse CD3e-APC	eBioscience	145-2C11
anti-mouse CD3-percp-cy5.5 (Clone: SH2.1)	Miltenyi	145-2C11
anti-mouse CD62L-APC	Miltenyi	REA828
anti-mouse CCR7-PE	Miltenyi	REA685
Recombinant murine GM-CSF	Peptotech	Cat#214-14-204G
Recombinant murine IL-4	Peptotech	Cat#315-03-204G
anti-mouse CD69-PE	eBioscience	2E7
anti-mouse CD103-FITC	eBioscience	M290
anti-mouse INF- $\gamma$ -PE	eBioscience	XMG1.2
anti-mouse CD44-FITC	eBioscience	IM7
rabbit anti-mouse ICAM-1	Abcam	EPR22161-284
rabbit anti-mouse Acrp30	Thermo Fisher	PA1-054
rabbit anti-mouse $\beta$ -actin	Bioworld	4D3
influenza HA518–526 (IYSTVASSL) peptide	Genscript	N/A
HA-specific (HA518–526)	MBL	N/A
H-2Kd tetramer-IYSTVASSL		

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844 **Supporting information**

845 **S1 Fig Spore plus H9N2 WIV generated abundant CD62L<sup>+</sup>CCR7<sup>+</sup> cells during**  
846 **the early immunization period.**

847 The effect of spore on Tcms in the blood was detected by FACS. (A-D) The  
848 frequencies of Tcms (CD3<sup>+</sup> CD62L<sup>+</sup> CCR7<sup>+</sup>) were detected in the blood at 7 d (A, C)  
849 and 45 d (B, D) after priming immunization. A gating strategy of live cells and  
850 lymphocytes was applied, followed by gating for CD3<sup>+</sup> cells and determination of the  
851 memory cell phenotype according to CCR7 and CD62L expression. The results are  
852 expressed as the mean ± SEM (n=6). \**P* < 0.05, \*\**P* < 0.01. One representative of  
853 three similar independent experiments is shown.

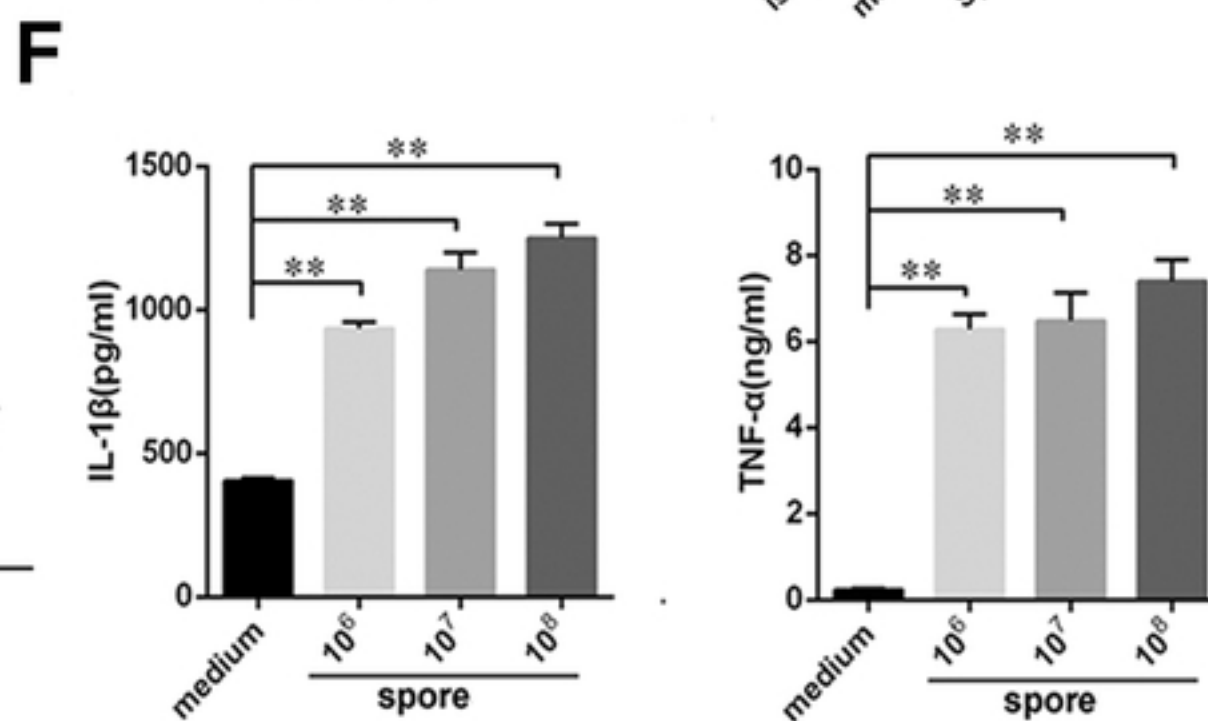
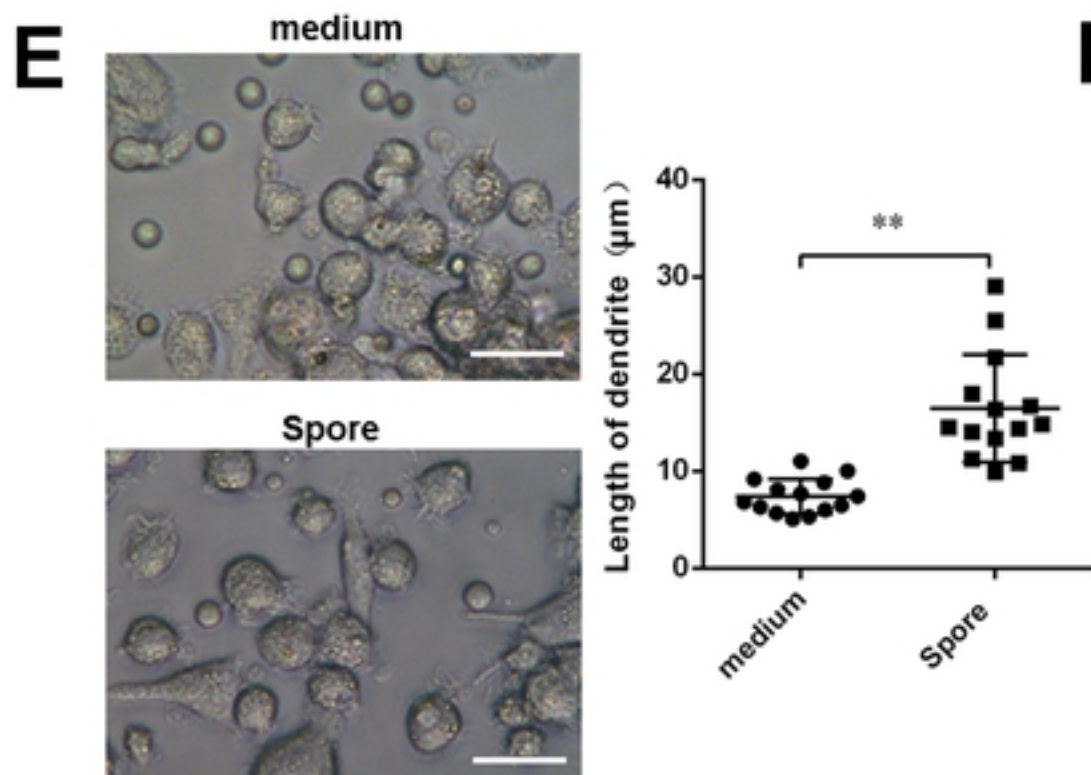
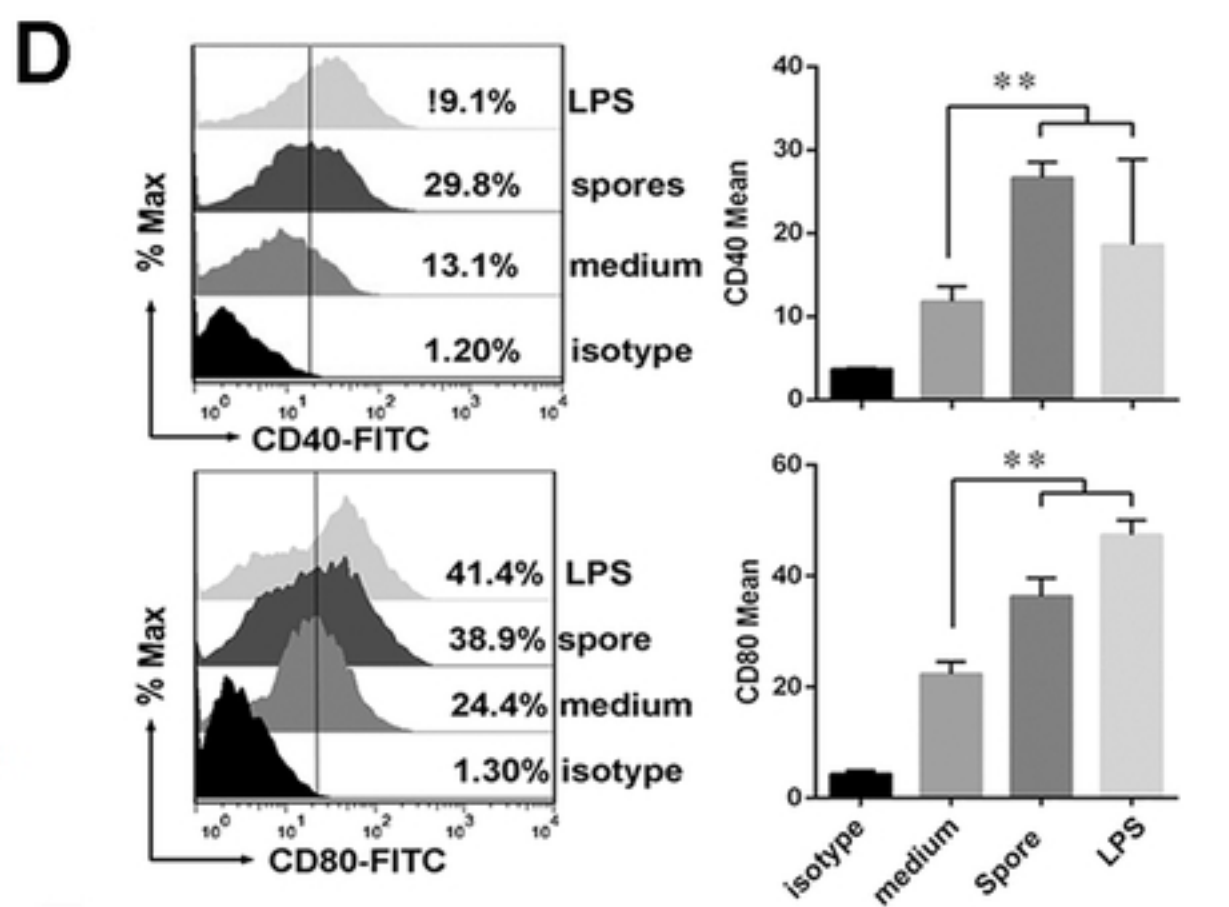
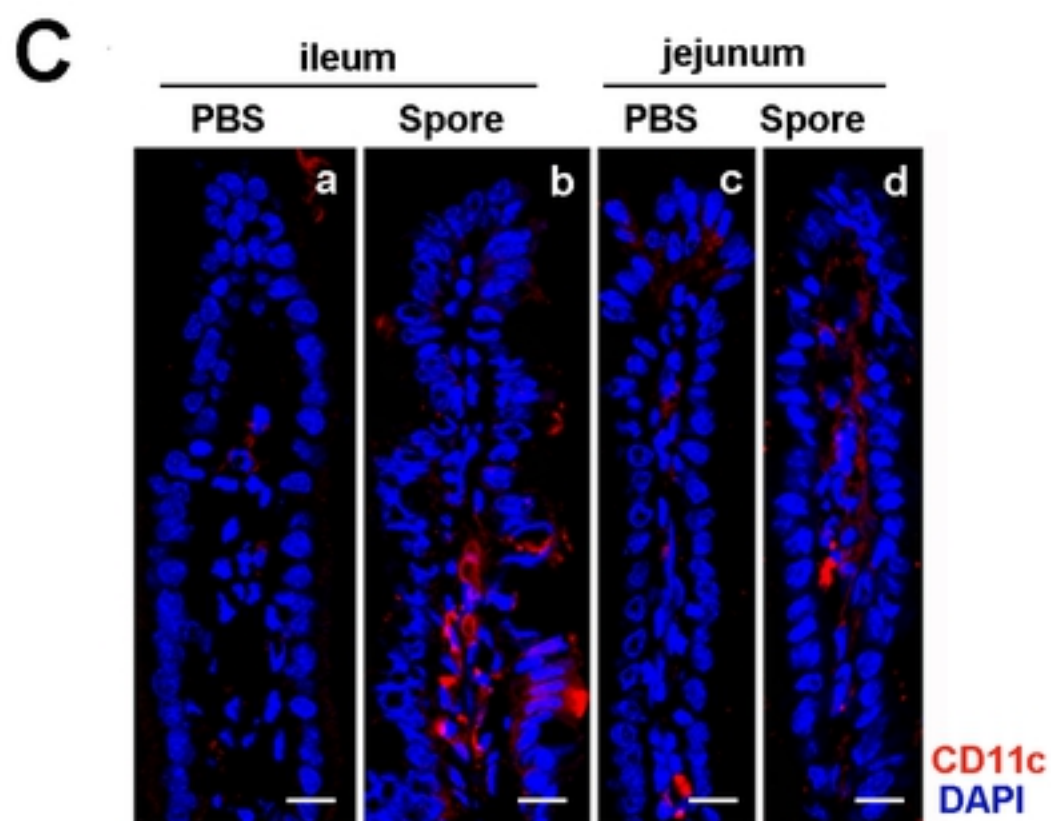
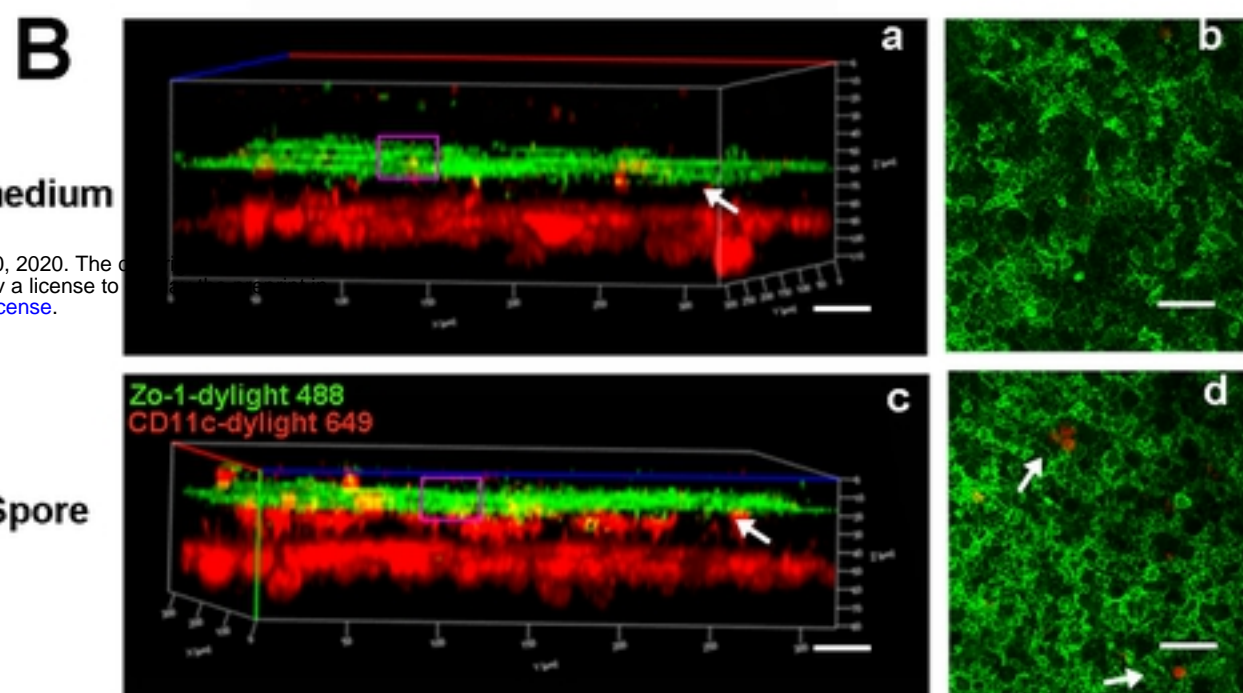
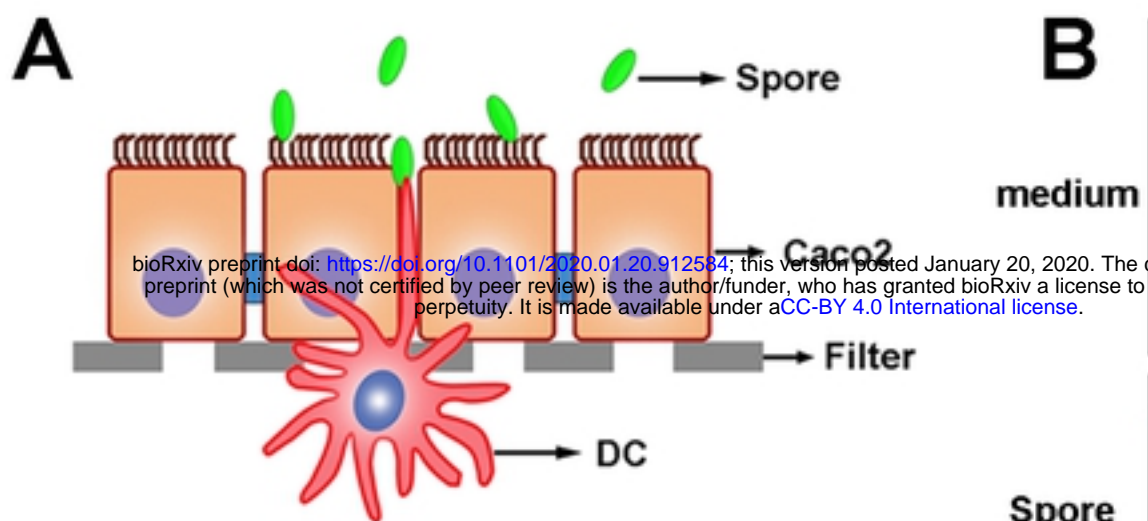
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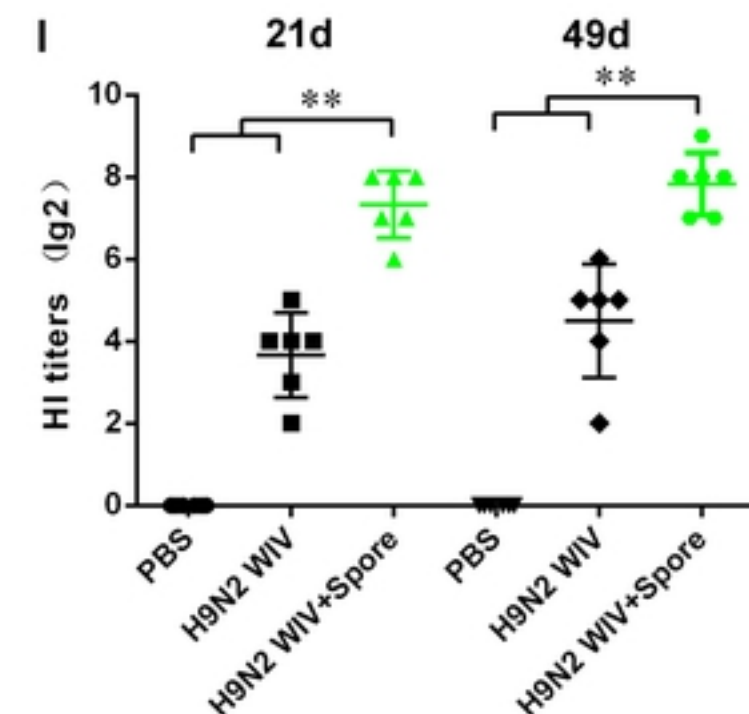
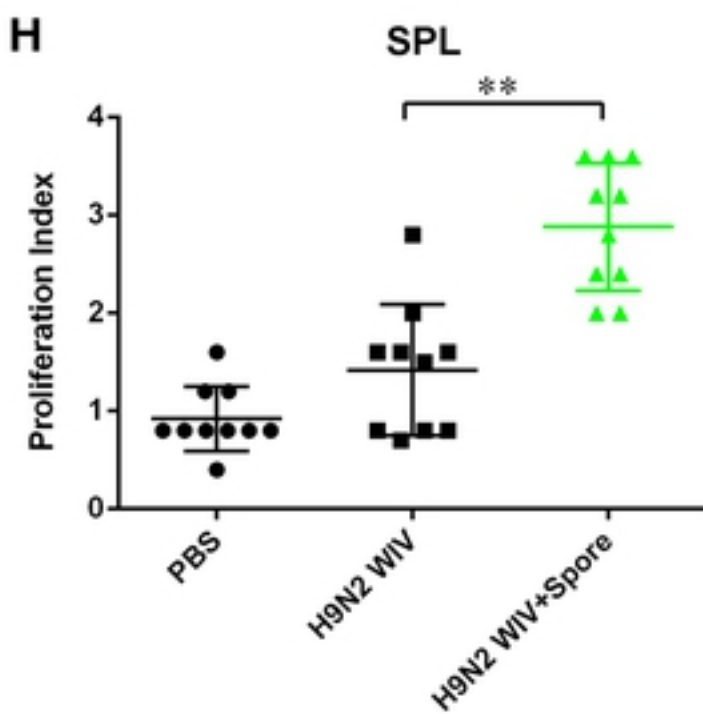
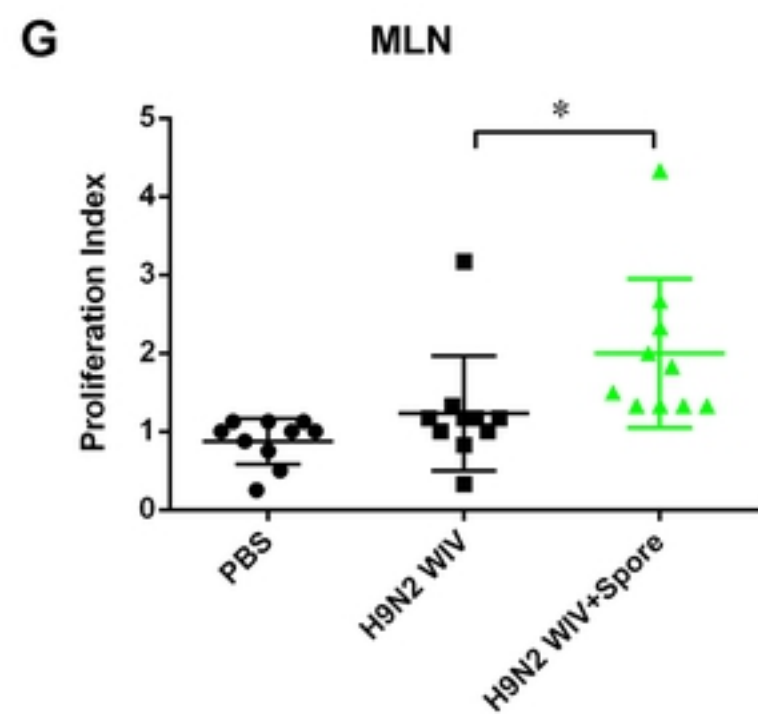
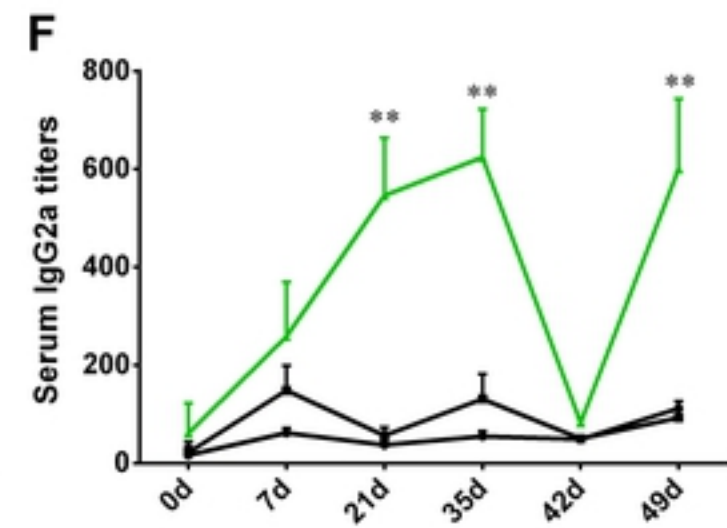
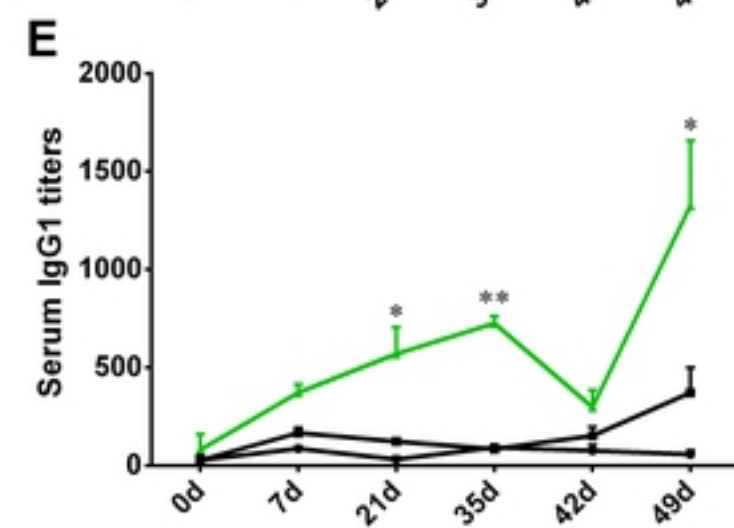
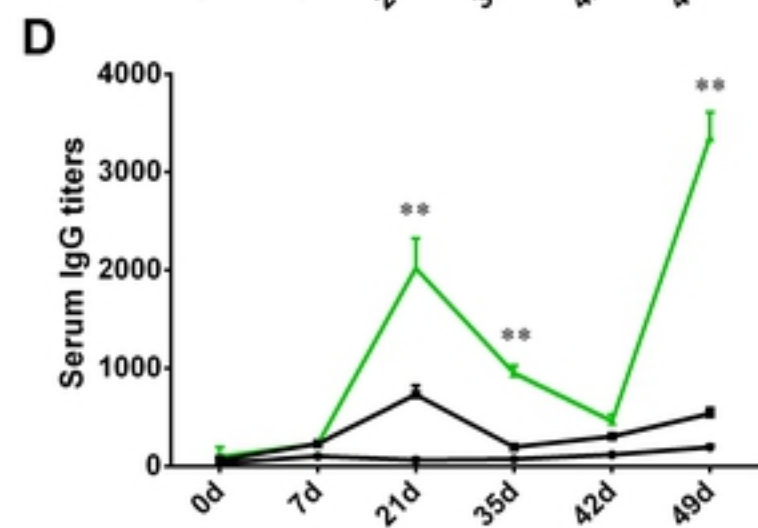
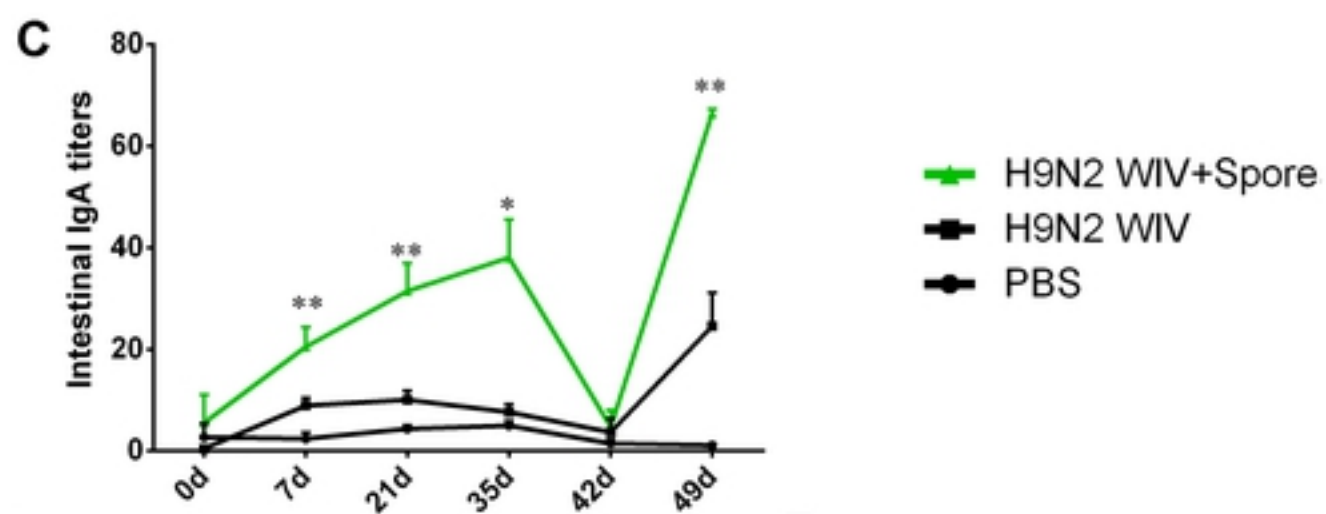
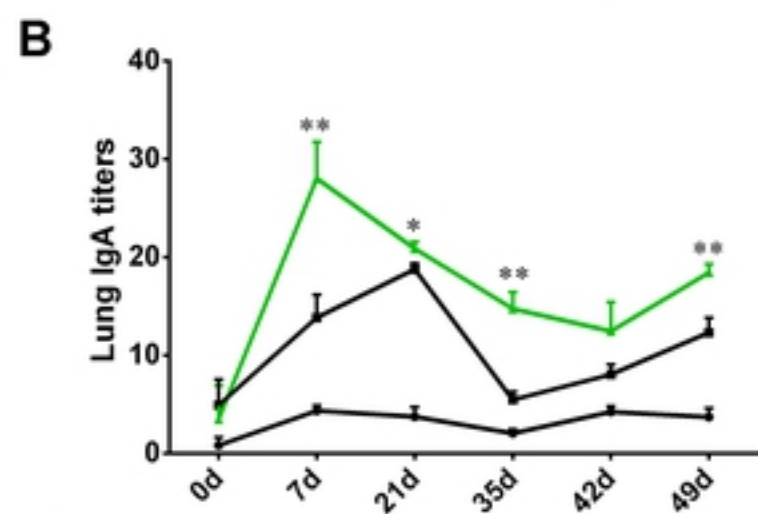
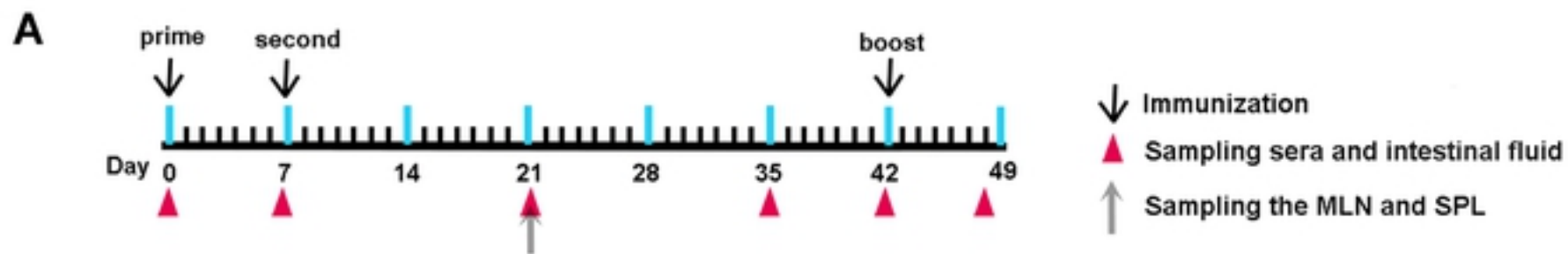
855 **S2 Fig Evaluation of tissue-resident T cells with a combination of FTY720**  
856 **treatment and IV staining.**

857 (A, B) Vascular T cells were completely depleted by FTY720 treatment and stained  
858 by CD45-FITC I.V. staining. Schematic of vaccination, which was performed as  
859 described previously. Briefly, the immune-suppressive agent FTY720 (0.5 mg/kg/day)  
860 was administered intraperitoneally (i.p.) for 10 sequential days to deplete circulating  
861 lymphocytes at 6 weeks after the primary vaccination. On the following day,  
862 anti-CD45 mAb (FITC-labeled, 2.5 µg/mouse) was injected into the orbital vein to  
863 stain vascular leukocytes (IV staining) 10 min before euthanasia. Intestinal and  
864 peripheral blood leukocytes were collected to validate the efficacy of FTY720  
865 administration and IV staining.

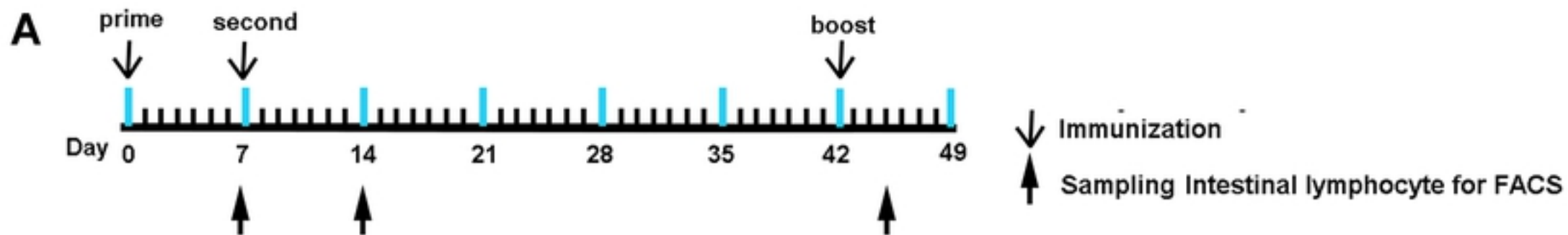
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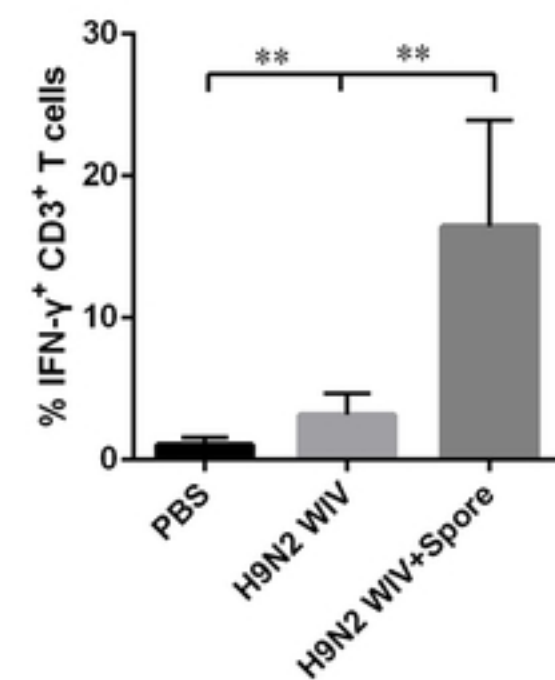
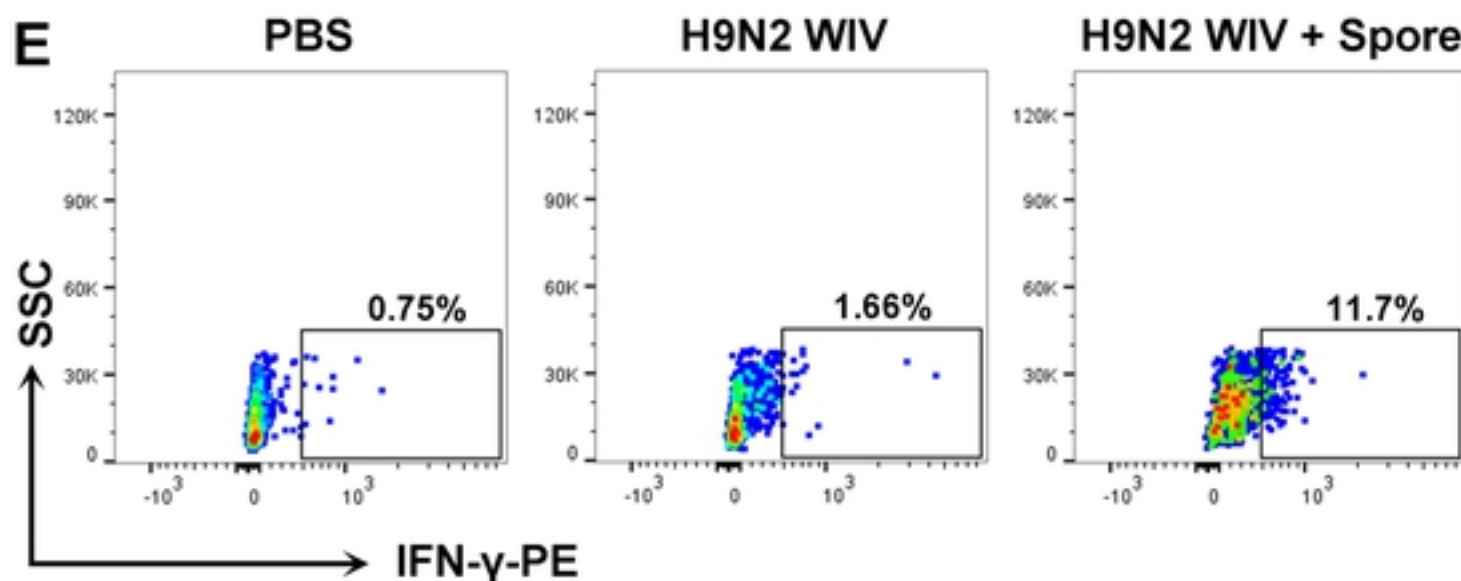
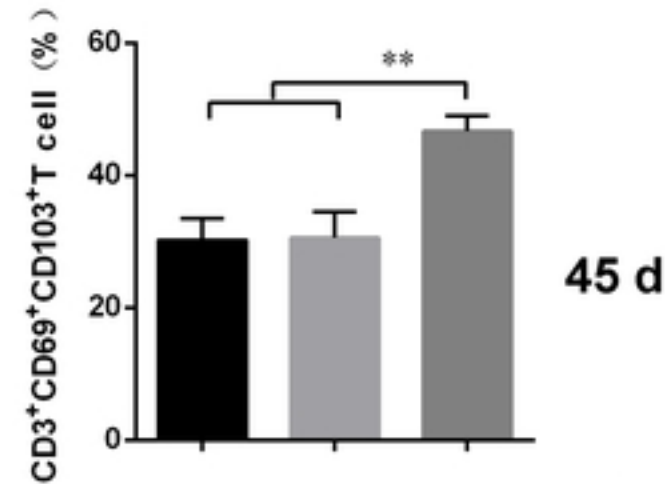
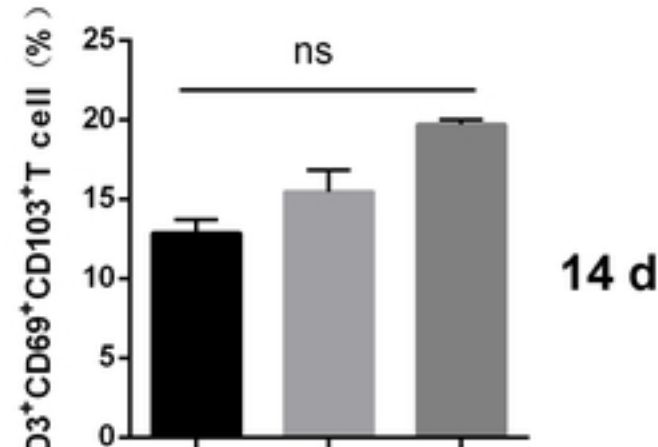
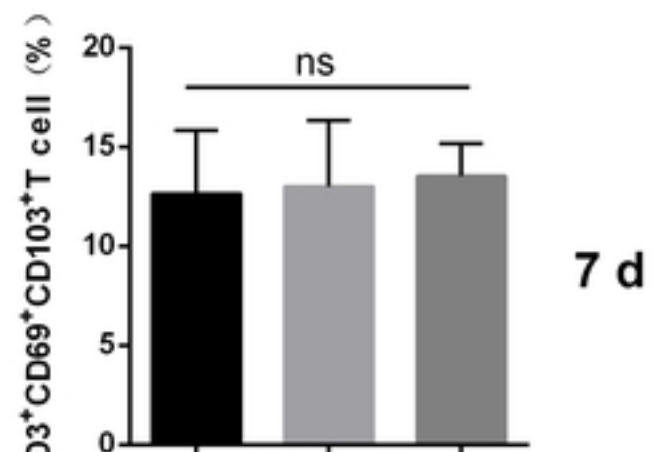
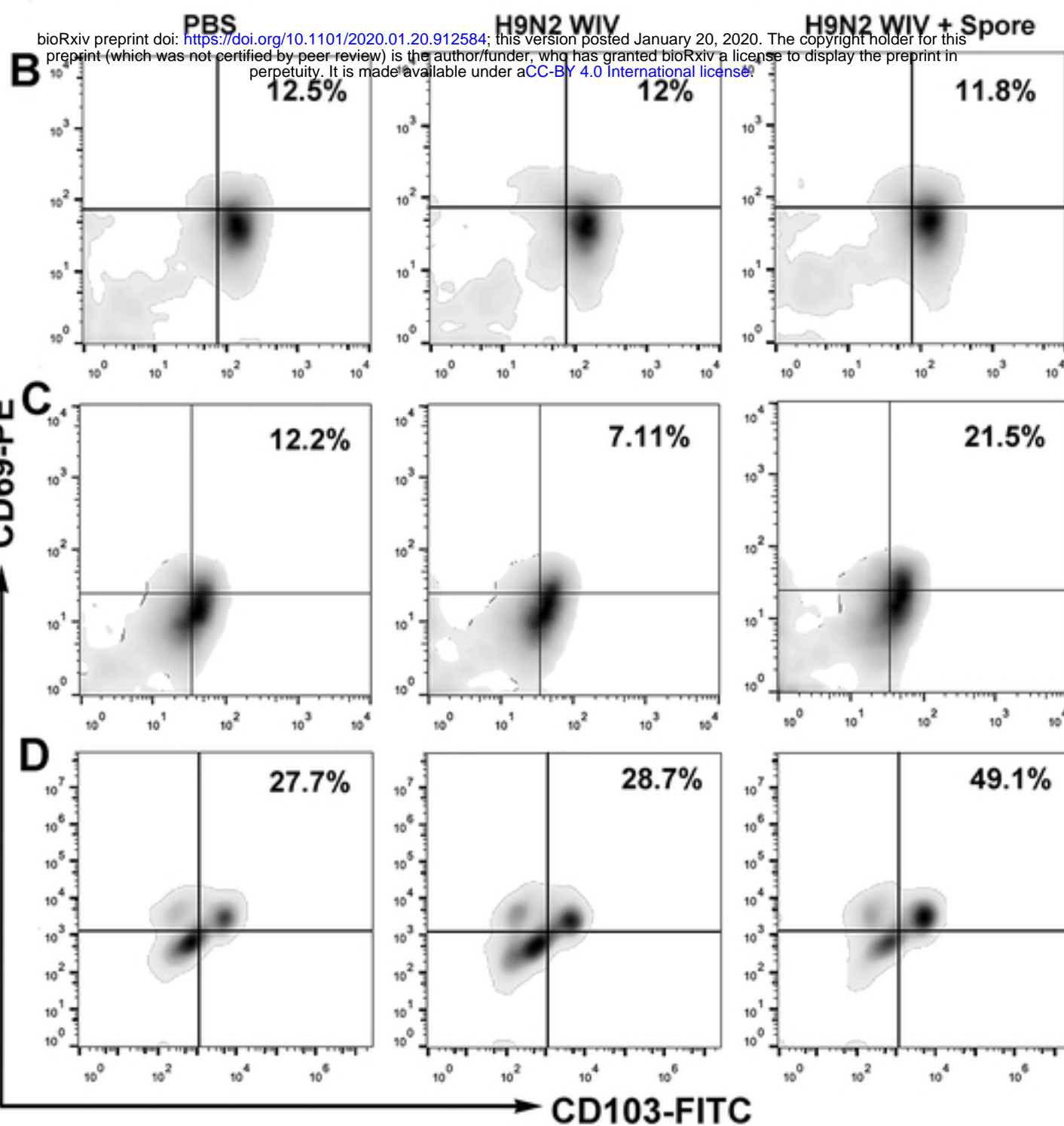




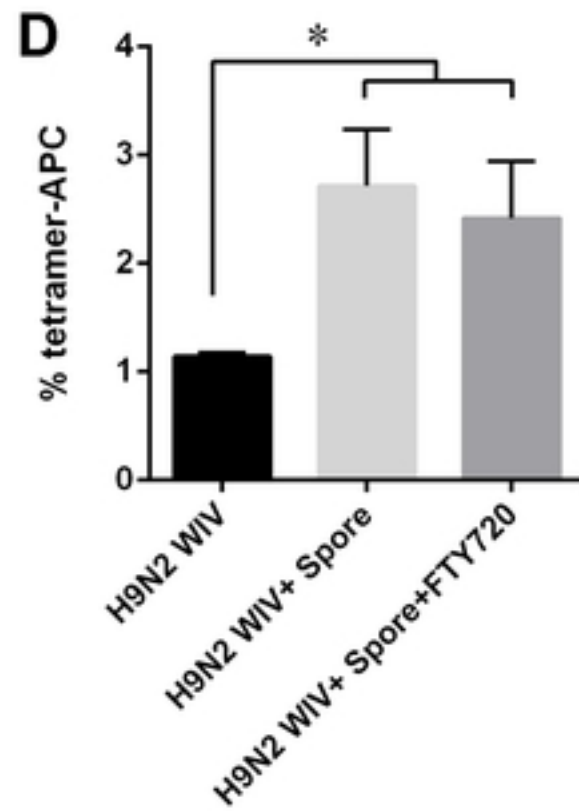
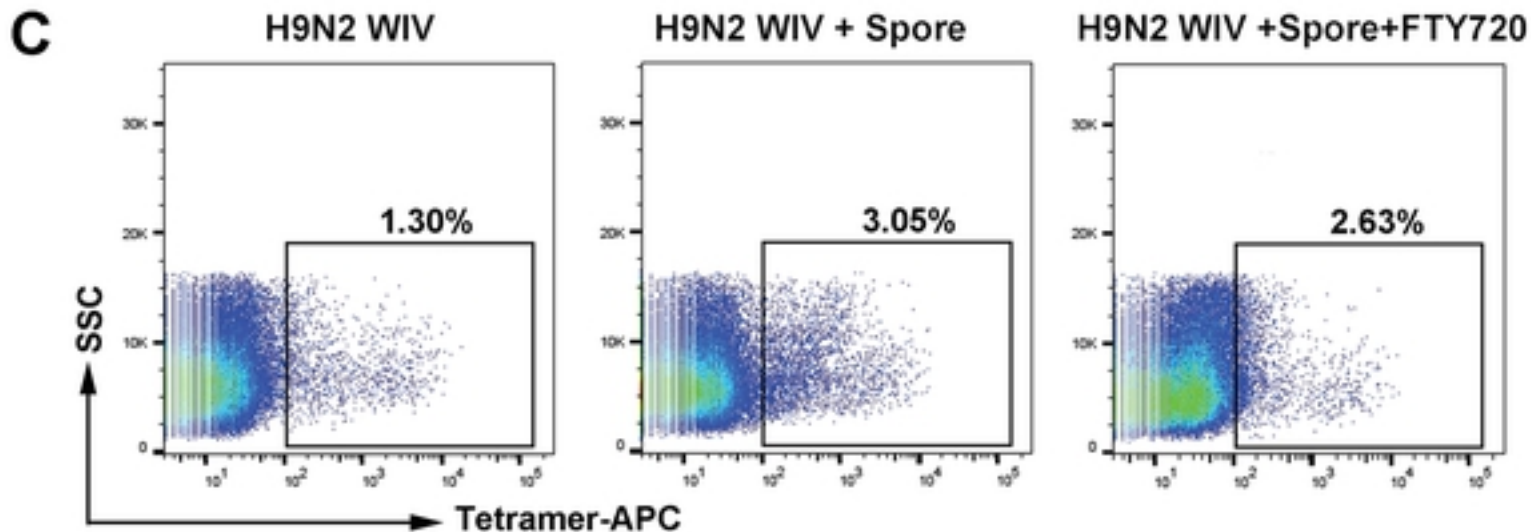
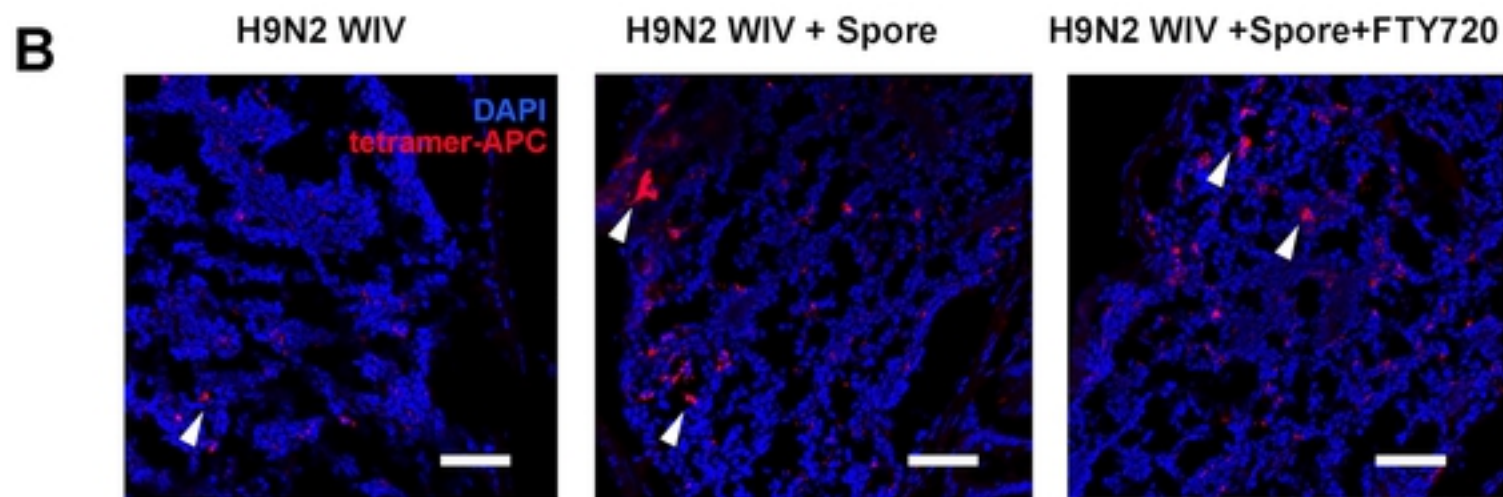
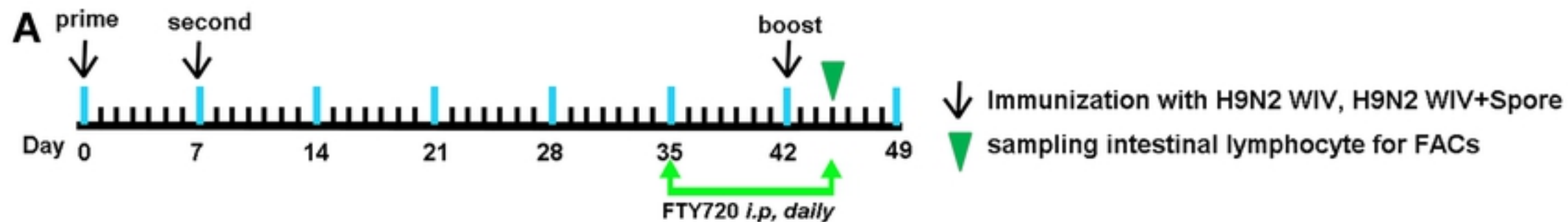




Gate on CD3<sup>+</sup>T cells

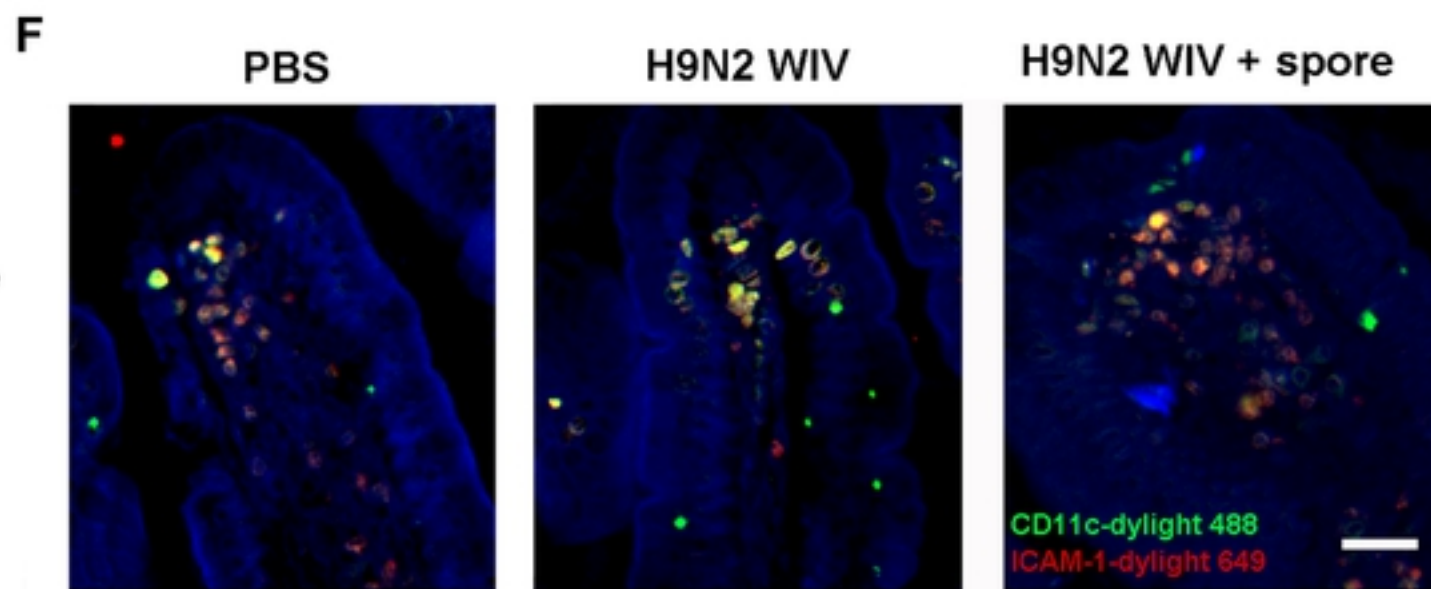
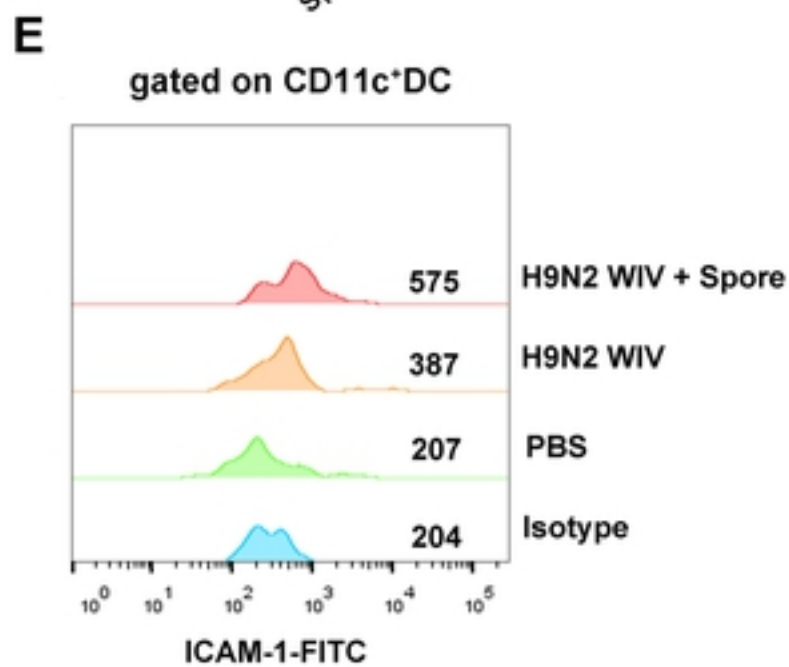
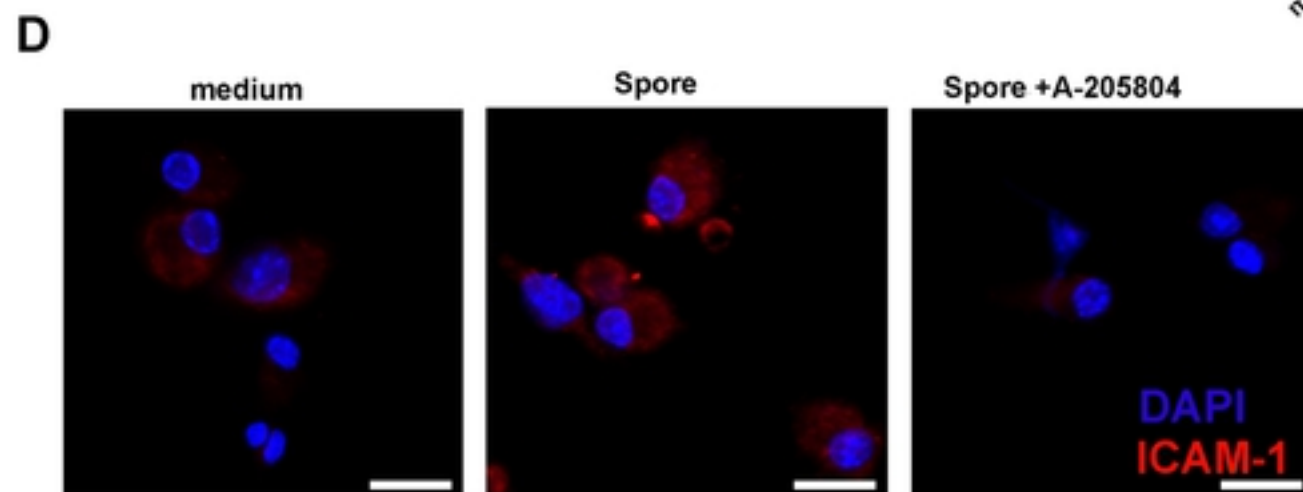
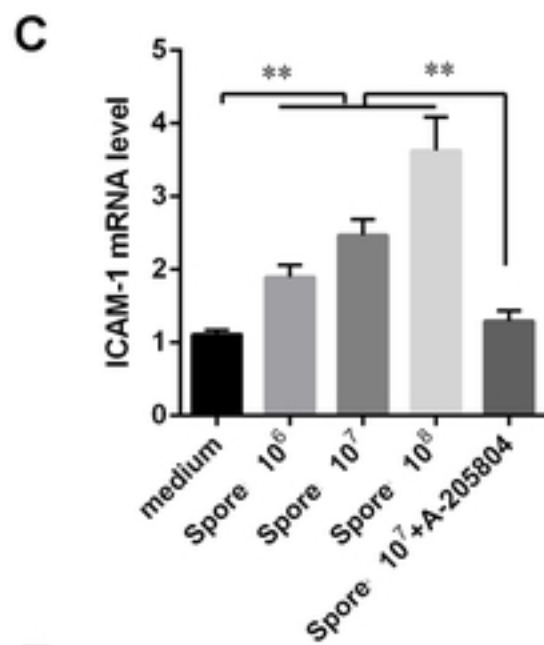
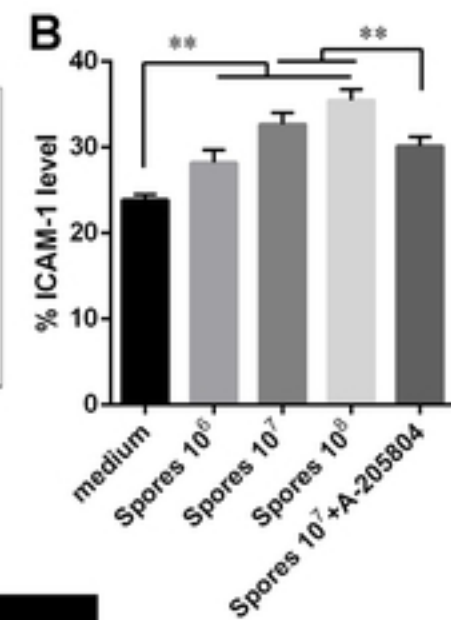
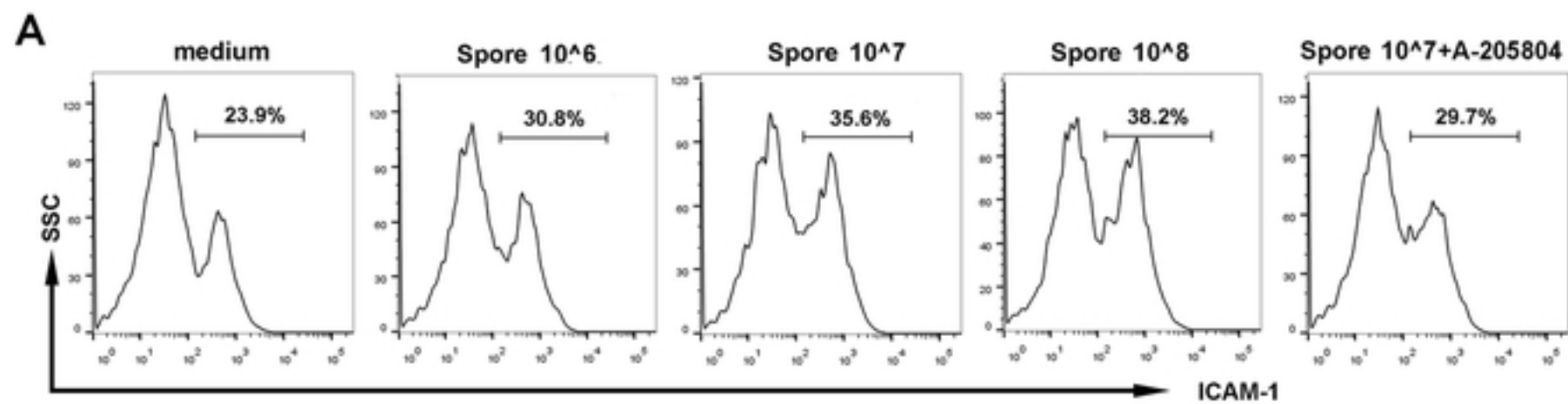


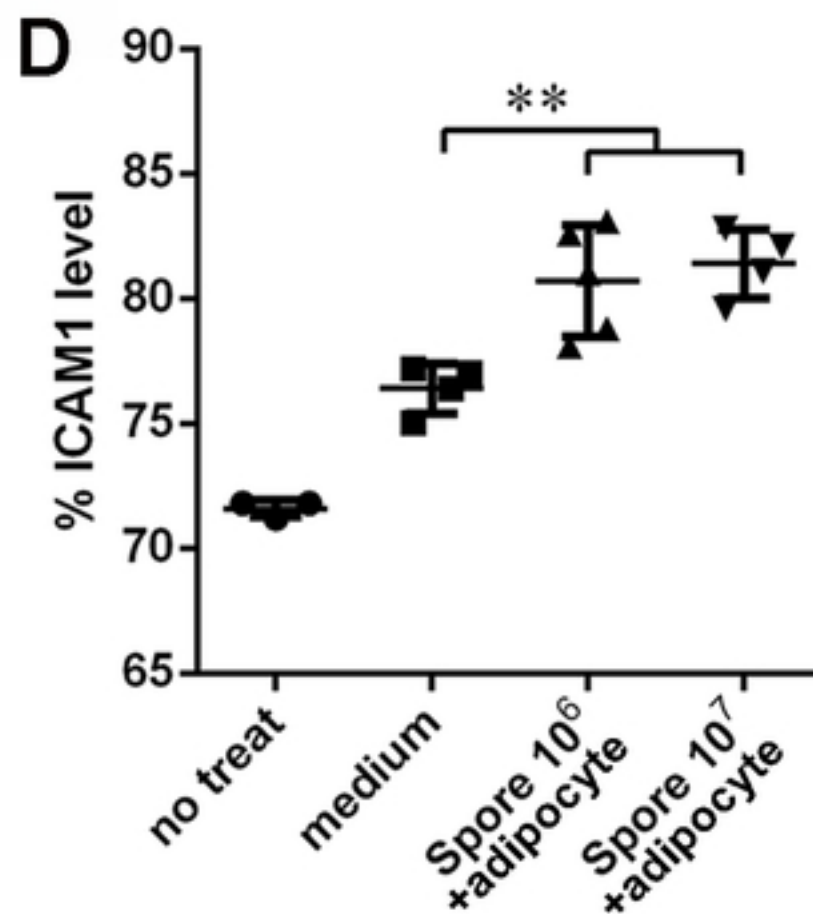
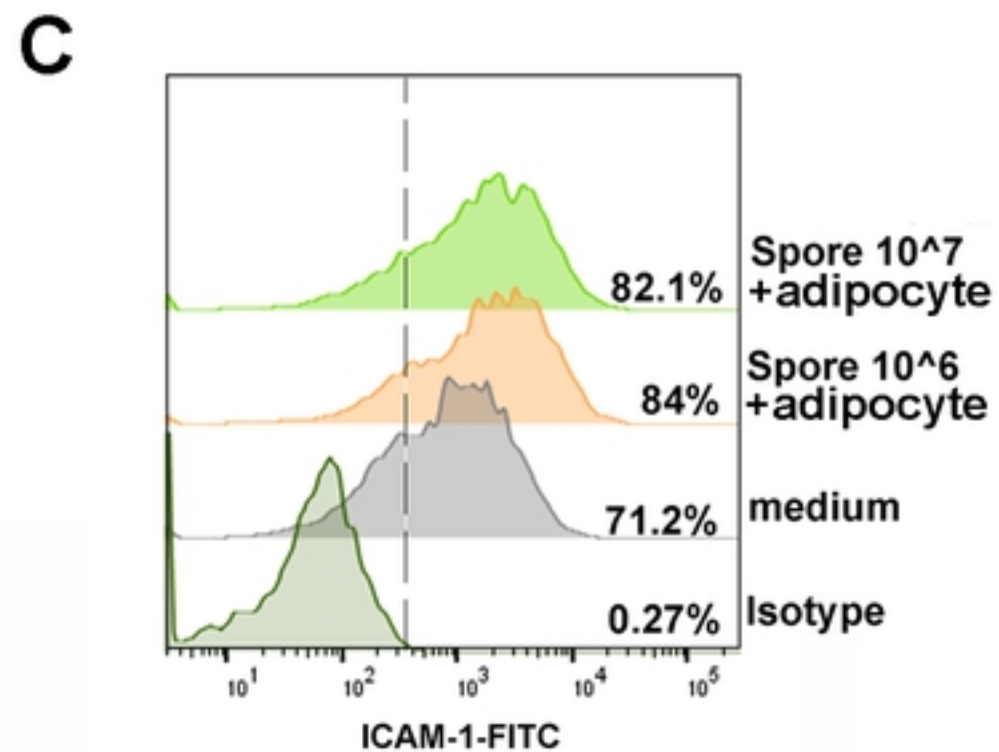
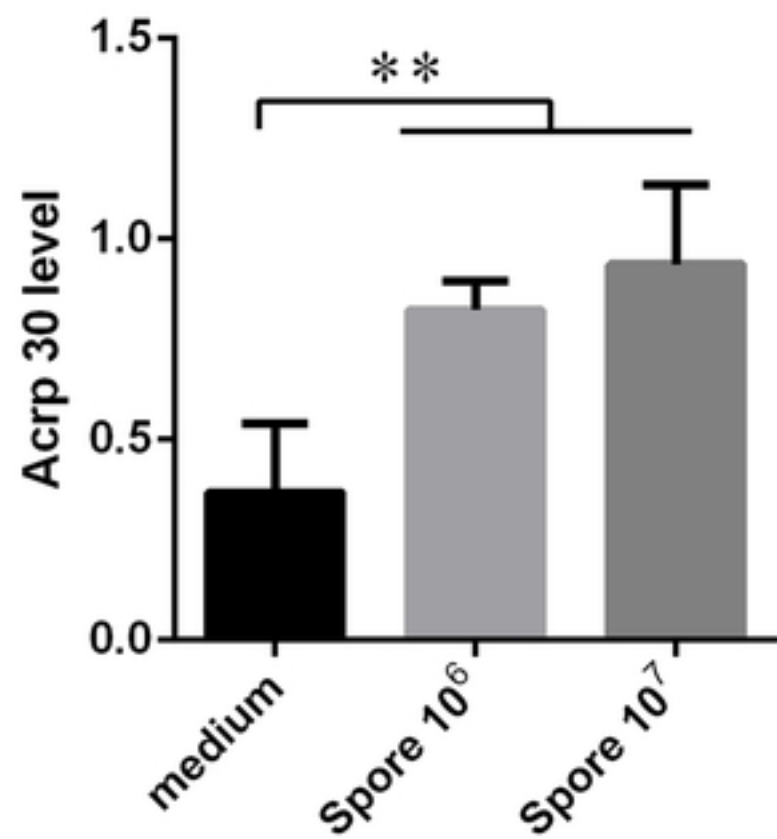
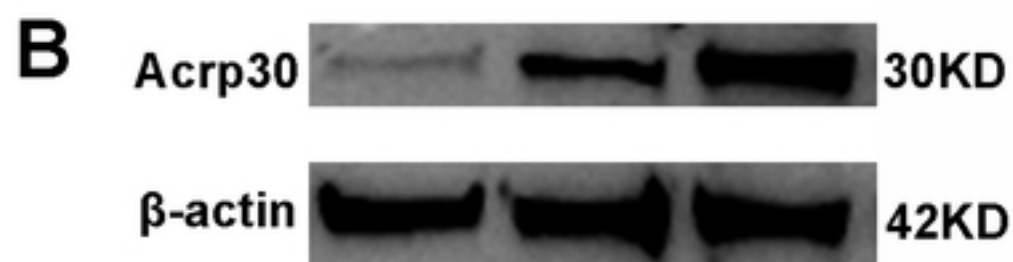
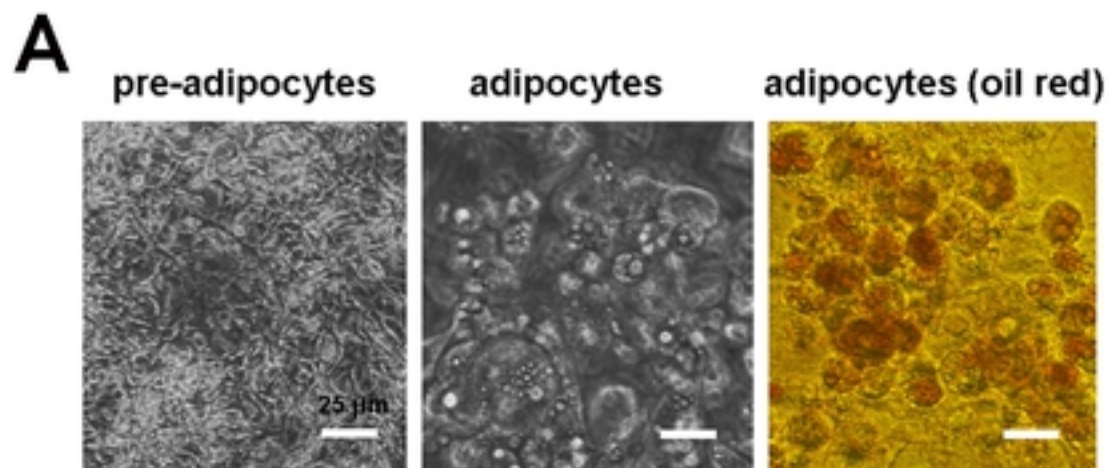
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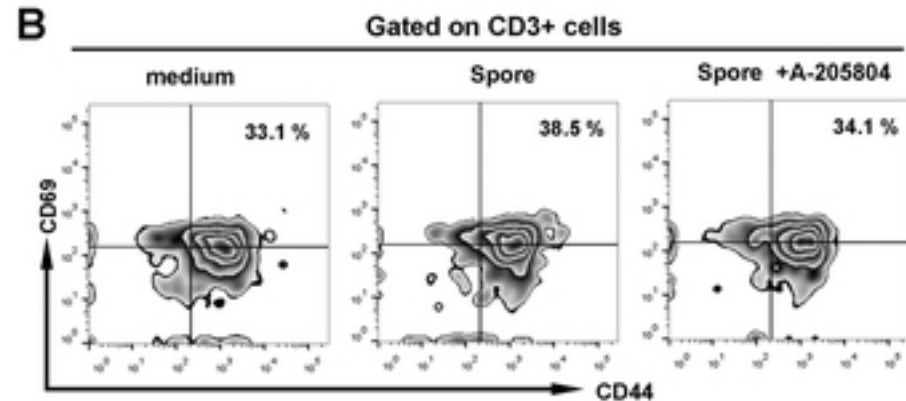
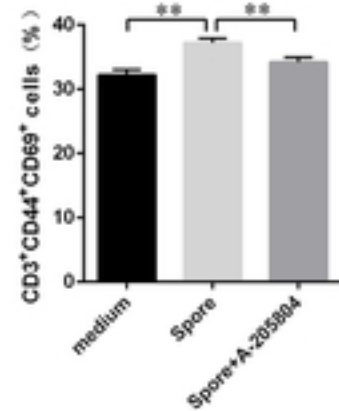
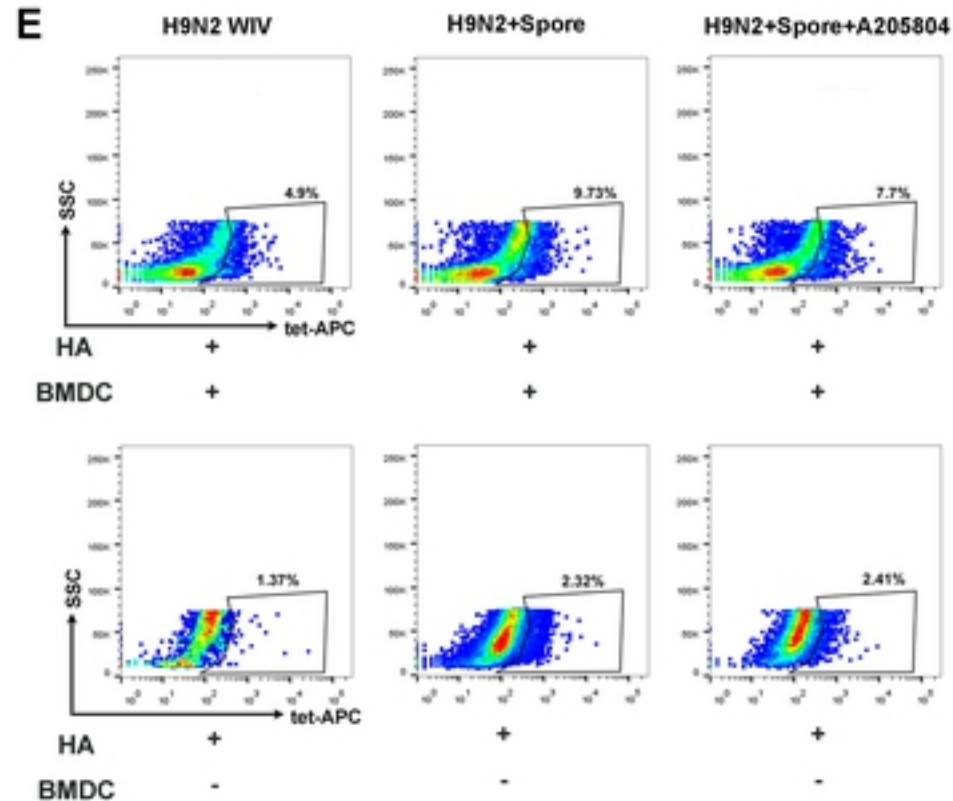










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