

1 **Title: Vaccination-induced rapid protection against bacterial pneumonia via**  
2 **training alveolar macrophage in mice**

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23

24 **Abstract:**

25 Vaccination strategies for rapid protection against multidrug-resistant bacterial  
26 infection are very important, especially for hospitalized patients who have high risk of  
27 exposure to these bacteria. However, few such vaccination strategies exist due to a  
28 shortage of knowledge supporting their rapid effect. Here we demonstrated a single  
29 intranasal immunization of inactivated whole cell (IWC) of *Acinetobacter baumannii*  
30 elicits rapid protection against *A. baumannii*-infected pneumonia via training of innate  
31 immune response in *Rag1*<sup>-/-</sup> mice. Immunization-trained alveolar macrophages (AMs)  
32 showed enhanced TNF- $\alpha$  production upon restimulation. Adoptive transfer of  
33 immunization-trained AMs into naive mice mediated rapid protection against  
34 infection. Elevated TLR4 expression on vaccination-trained AMs contributed to rapid  
35 protection. Moreover, immunization-induced rapid protection was also seen in  
36 *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* pneumonia models, but not in  
37 *Staphylococcus aureus* and *Streptococcus pneumoniae* model. Our data reveal that a  
38 single intranasal immunization induces rapid and efficient protection against certain  
39 Gram-negative bacterial pneumonia via training AMs response, which highlights the  
40 importance and the possibility of harnessing trained immunity of AMs to design  
41 rapid-effecting vaccine.

42 **Keywords:** Multidrug-resistant bacteria, vaccine, rapid effect, alveolar macrophage,  
43 trained immunity, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella*  
44 *pneumoniae*

45

## 46 **Introduction**

47 The multidrug-resistant (MDR) bacteria, including *Acinetobacter baumannii*,  
48 *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and  
49 *Staphylococcus aureus*, pose a great threat to global public health (Tacconelli, 2017).  
50 Pneumonia caused by MDR bacteria is a major cause of morbidity and mortality,  
51 especially in hospitalized patients (Gonzalez-Villoria & Valverde-Garduno, 2016;  
52 Micek et al., 2015; Zilberberg, Nathanson, Sulham, Fan, & Shorr, 2016). The  
53 continuing spread of antimicrobial resistance has made treating MDR bacterial  
54 pneumonia extremely difficult. Vaccination has been proposed as a promising strategy  
55 for controlling MDR bacterial infections (Jansen, Knirsch, & Anderson, 2018;  
56 Rappuoli, Bloom, & Black, 2017; Williams, 2007). Current vaccination strategies  
57 usually require multiple injections weeks or months apart, which limit them to rapidly  
58 prevent infections for inpatients. However, hospitalized patients have an especially  
59 high risk for exposure to MDR bacteria (Pachon & McConnell, 2014). Therefore,  
60 rapid efficacy induced by vaccination is vital for vaccine development against MDR  
61 bacteria (Pachon & McConnell, 2014).

62 Induction of immunological memory is the central goal of vaccination.  
63 Immunological memory protects against infections by enabling a quicker and stronger  
64 immune response to a previously encountered antigen (Farber, Netea, Radbruch,  
65 Rajewsky, & Zinkernagel, 2016). Classically, immune memory is thought to be  
66 exclusively mediated by adaptive T and B cell responses. These responses are highly

67 specific to antigen, but take days or weeks to become effective. Another part of the  
68 immune system, innate immune response, provides an initial, relatively nonspecific  
69 response to infection within hours to days without immunological memory. However,  
70 in the past decade, evidence has emerged showing that innate immune cells such as  
71 monocytes, macrophage, and NK cells can also build long-term memory through  
72 epigenetic and metabolic reprogramming of cells. This memory termed “trained  
73 immunity” or “trained innate immunity,” produces hyperresponsiveness upon  
74 re-stimulation in these cells (Netea et al., 2016; Netea & Joosten, 2018; Netea,  
75 Quintin, & van der Meer, 2011). The rapidity of innate immune response leads us to  
76 speculate that trained innate immunity might effectively serve as the underlying  
77 mechanism for vaccination-induced rapid protection.

78 Here, we demonstrated that a single intranasal immunization of inactivated whole  
79 cell (IWC) induced rapid and efficient protection against certain Gram-negative  
80 bacterial pneumonia, which was dependent on trained innate immunity mediated by  
81 alveolar macrophages (AMs). These findings highlight the possibility to harness the  
82 trained immunity of AMs to design a vaccine with rapid efficacy against pulmonary  
83 infection.

84

## 85 **Results**

### 86 **Rapid protection against *A. baumannii* pneumonia by a single intranasal**

87 **vaccination.** Mice were immunized intranasally (i.n.) with an inactivated whole cell

88 (IWC) of *A. baumannii* and infected intratracheally (i.t.) with a lethal dose of *A.*

89 *baumannii* 2 days or 7 days later (Figure 1A). The control mice succumbed to the

90 infection, whereas all IWC-vaccinated mice survived when mice were challenged 2 days

91 or 7 days post immunization (Figure 1B, \*\*  $P < 0.01$  compared to control, log-rank test).

92 Consistent with the survival rate, bacterial burdens in lungs and blood of vaccinated mice

93 were significantly lower than those in the control group at 24 hours post infection (hpi) (

94 Figure 1C, \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , ordinary one-way ANOVA). Histopathology of

95 lung tissues showed reduced lung damage and decreased inflammatory cells infiltration

96 in IWC-immunized mice (Figure 1D). When challenging the mice at day 7 post

97 immunization, the pro-inflammatory cytokines of IL-6 in lungs and serum levels of IL-6

98 and TNF- $\alpha$  (Figure 1E and Figure 1-figure supplement 1, \*\*\*\*  $P < 0.0001$ , unpaired  $t$

99 test.) in IWC-vaccinated group were significantly lower than those in the control group at

100 24 hpi. Expression of chemokines *Cxcl1*, *Cxcl2*, *Cxcl5*, *Cxcl10*, and *Ccl2* were also

101 significantly reduced in the lungs of vaccinated mice at 24 hpi (Figure 1F, \*  $P < 0.05$ , \*\*

102  $P < 0.01$ , and \*\*\*\*  $P < 0.0001$ , unpaired  $t$  test.). Inflammatory cells in the lungs were

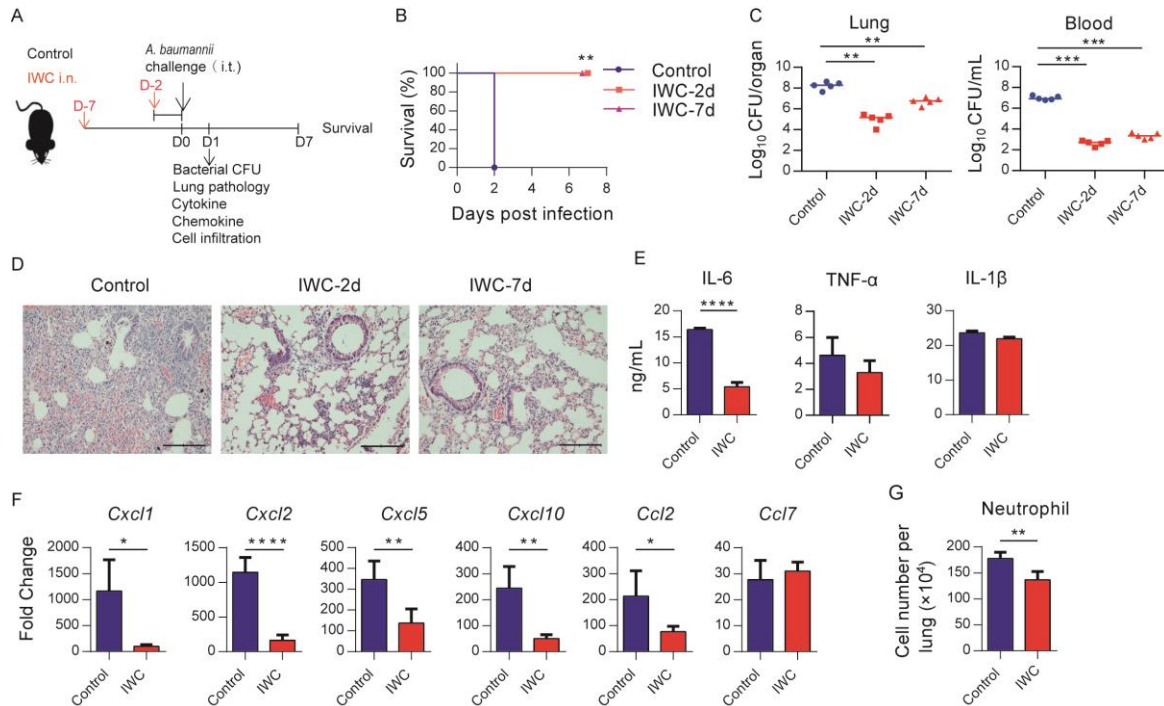
103 detected by the flow cytometry and gating strategy was shown in Figure 1-figure

104 supplement 1B. The results showed that the number of neutrophils in lungs of vaccinated

105 mice was significantly lower than that of control group at 24 hpi (Figure 1G and Figure

106 1-figure supplement 1C. \*\*  $P < 0.01$ , unpaired  $t$  test). Collectively, these findings  
 107 indicate a single intranasal immunization with IWC elicits rapid and complete protection  
 108 against pulmonary *A. baumannii* infection.

109 **Figure 1**



110  
 111 **Figure 1. Rapid protection against *A. baumannii* pneumonia by a single**  
 112 **intranasal vaccination.** (A) Schematic diagram of the experimental procedure.  
 113 C57BL/6 mice were immunized intranasally (i.n.) with inactivated whole cell (IWC) of  
 114 *A. baumannii* and challenged intratracheally (i.t.) with *A. baumannii* at day 2 (IWC-2d)  
 115 or day7 (IWC-7d) after immunization (n=5/group). (B) Survival of mice was recorded  
 116 for 7 days. \*\* $P < 0.01$  determined by log-rank test. (C) Bacterial burdens in lungs and  
 117 blood at 24 hour post infection (hpi) was determined. Each plot represents one mouse.  
 118 The line indicates the median of the data. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  evaluated by  
 119 ordinary one-way ANOVA followed by Tukey's multiple comparisons test. (D)  
 120 Representative histopathologic images of lungs at 24 hpi. Scale bars: 100  $\mu$ m. (E-G)  
 121 IWC-immunized mice were challenged at day 7 and were sacrificed at 24 hpi. (E) Levels  
 122 of inflammatory cytokines in the lungs were detected by ELISA. (F) Transcriptional  
 123 levels of chemokines in the lungs were detected by real-time PCR. (G) Numbers of  
 124 neutrophils in the lungs were detected by flow cytometry. Data are mean  $\pm$  SD. n=4-5  
 125 mice/group. For (E) to (G), \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*\*  $P < 0.0001$ , determined by  
 126 two-tail unpaired  $t$  test. Data are representative of at least two independent experiments.  
 127 **Figure supplement 1. Intranasal IWC vaccination provides rapid protection**  
 128 **against *A. baumannii* infection.**

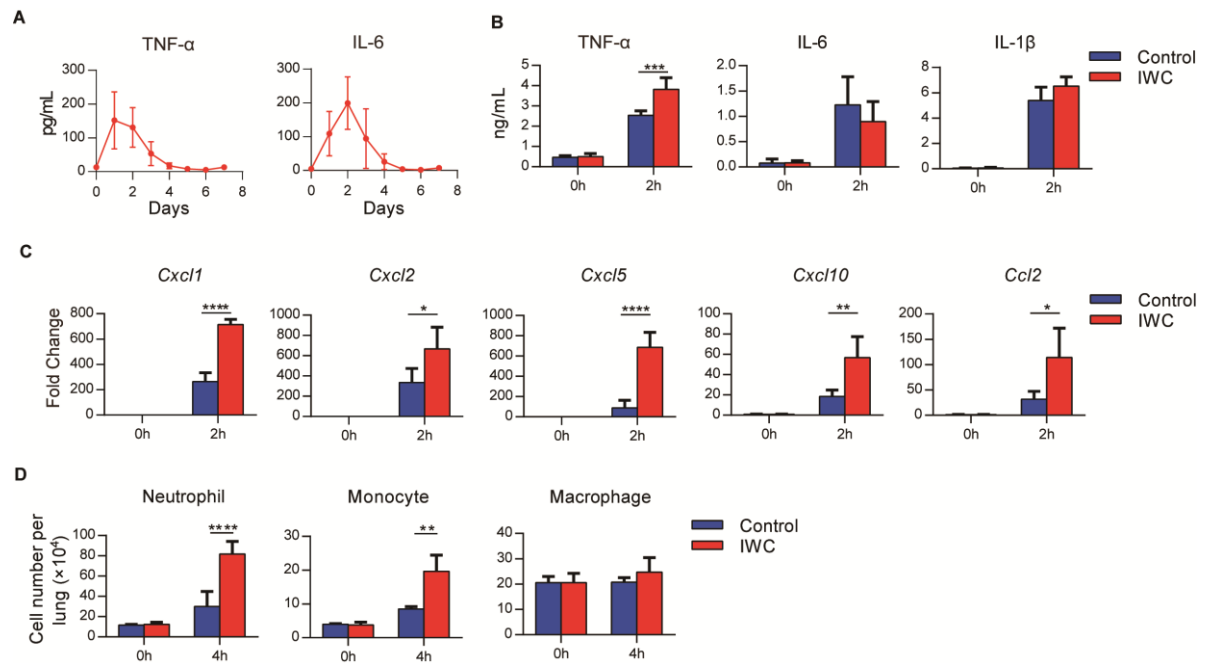
129 **Rapid immune memory induced by a single intranasal vaccination.**

130 Immunological memory is defined as functionally enhanced, quicker, and more  
131 effective response to pathogens that have been encountered previously. This is the  
132 basis of successful vaccines against subsequent infections. To assess whether the  
133 IWC-induced rapid protection is a result of immunological memory or a result of the  
134 persistent activation of innate immune responses, we measured the dynamic immune  
135 response after immunization of IWC from day 0 to day 7. In response to intranasal  
136 immunization of IWC, levels of TNF- $\alpha$  and IL-6 in lungs increased from day 1 to day  
137 4 and completely declined to baseline by day 5 (Figure 2A), indicating that the host  
138 response rapidly primed and rest 5 days later. When we challenged the mice with *A.*  
139 *baumannii* on day 7 after vaccination and assessed the cytokine levels in lungs early  
140 at 2 hpi, we found that TNF- $\alpha$  but not IL-6 and IL-1 $\beta$  levels was significantly higher  
141 from IWC-immunized mice than those from control mice (Figure 2B, \*\*\*,  $P < 0.001$ ,  
142 ordinary two-way ANOVA). Further, mRNA levels of *Cxcl1*, *Cxcl2*, *Cxcl5*, *Cxcl10*,  
143 and *Ccl2* were higher in vaccinated mice than in control mice at early 2 hpi (Figure  
144 2C, \* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ , ordinary two-way ANOVA). Meanwhile,  
145 consistent with the increased chemokine expression, vaccinated mice had significantly  
146 higher numbers of neutrophils and monocytes in their lungs than did control mice at 4  
147 hpi (Figure 2D, \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ , ordinary two-way ANOVA). These  
148 results indicate IWC immunization induces quicker, enhanced responses upon *A.*  
149 *baumannii* challenge on day 7 after vaccination. Vaccination-induced protection



150 against *A. baumannii* on day 7 after vaccination is an enhanced recall response of  
151 immune response.

152 **Figure 2**



153

154 **Figure 2. Rapid immune memory induced by a single intranasal vaccination. (A)**

155 Dynamic responses of TNF- $\alpha$  and IL-6 in the lungs of *A. baumannii* IWC-immunized

156 mice (n = 3 per timepoint). **(B-D)** IWC-immunized mice were challenged i.t. with *A.*

157 *baumannii* at day 7 after immunization. **(B)** Levels of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  at 0 h

158 and 2 hpi in the lungs were measured by ELISA. **(C)** Transcriptional levels of

159 chemokines in the lungs at 0 h and 2 hpi were assessed by real-time PCR. **(D)** Numbers

160 of neutrophils, monocytes, and macrophages in the lungs of mice were determined by

161 flow cytometry. Data are presented as mean  $\pm$  SD. (n=3-4 mice/group). \* $P < 0.05$ ; \*\* $P$

162  $< 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ordinary two-way ANOVA. Data are

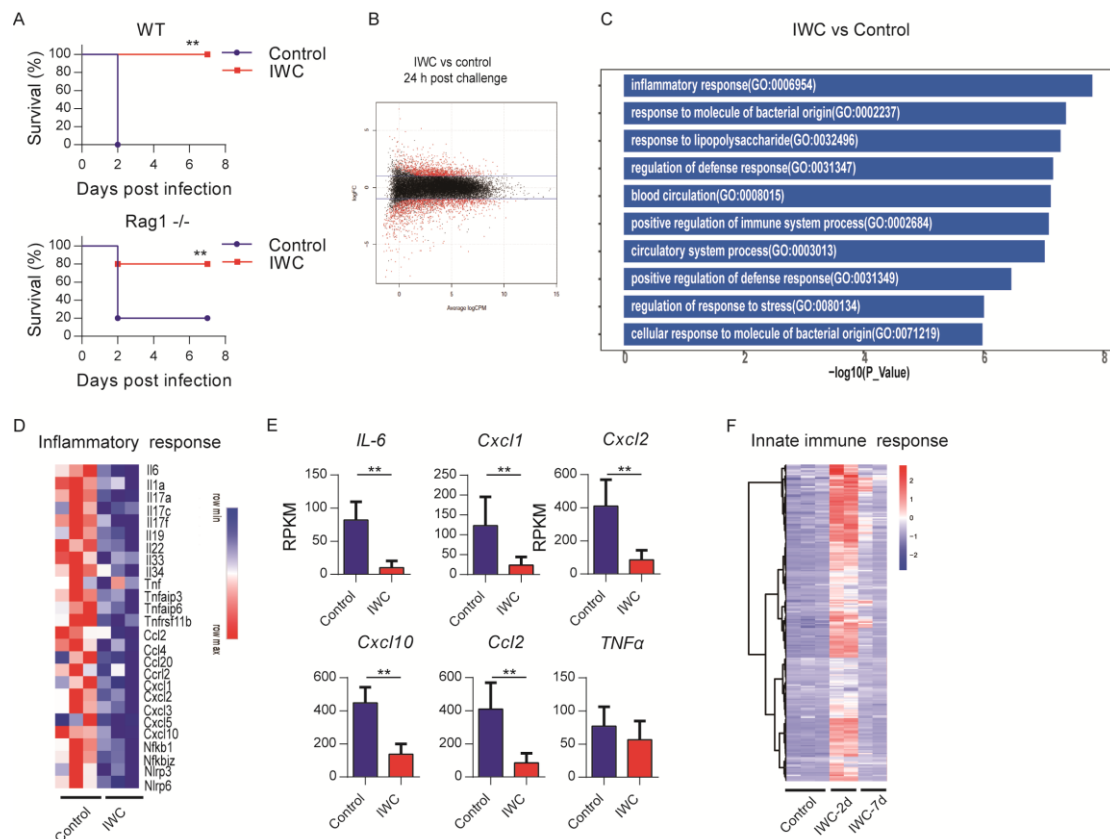
163 representative of two independent experiments.

164 **Vaccination-induced rapid protection is dependent on trained innate immunity.** To  
165 further explore the potential mechanism of rapid effect of vaccination, *Rag1*<sup>-/-</sup> mice  
166 (which lack mature T and B cells) were immunized i.n. with IWC and then challenged  
167 with a lethal dose of *A. baumannii* to determine which part of immune response is  
168 responsible for rapid protection. Results showed IWC provided rapid and effective  
169 protection both in WT and *Rag1*<sup>-/-</sup> mice at day 7 after immunization (Figure 3A, \*\*  $P <$   
170 0.01 compared to control, log-rank test. There is no significant difference between WT  
171 and *Rag1*<sup>-/-</sup> mice in terms of survival of the IWC-immunized group (Figure 3A,  $P=0.30$ ,  
172 log-rank test). RNA sequencing analyses (RNA-seq) of lung tissue at day 7 after  
173 immunization and 24 h after *A. baumannii* challenge revealed significant differentially  
174 expressed genes (DEGs) between transcriptional profiles of vaccinated *Rag1*<sup>-/-</sup> mice and  
175 those of control *Rag1*<sup>-/-</sup> mice (Figure 3B and Figure 3-figure supplement 1A). There  
176 were a total of 2401 DEGs between these two groups; 1084 upregulated genes and  
177 1317 downregulated in the vaccinated mice (Figure 3-figure supplement 1B). Gene  
178 ontology (GO) analysis of DEGs revealed that genes associated with inflammatory  
179 response, response to molecule of bacterial origin, and response to liposaccharide were  
180 significantly downregulated in vaccinated mice at 24 hpi (Figure 3C and Figure  
181 3-figure supplement 1C). The expression of inflammation related genes including *Il6*,  
182 *Cxcl1*, *Cxcl2*, *Cxcl10*, and *Ccl2* were notably lower in vaccinated-*Rag1*<sup>-/-</sup> mice than  
183 those in control mice at 24 hpi (Figure 3D and E and Figure 3-figure supplement 1D,  
184 \*\* $P < 0.01$ , unpaired  $t$  test). These data indicate that IWC immunization induces rapid

185 protection in *RagI*<sup>-/-</sup> mice, highlighting the role of innate immune response in  
186 vaccination-induced rapid protection. We also analyzed the dynamic transcriptional  
187 response to IWC immunization in *RagI*<sup>-/-</sup> mice and found that the innate immune  
188 response was activated at day 2 and rested at day 7 (Figure 3F), which has the similar  
189 pattern to that in control mice (Figure 2). Upon *A. baumannii* challenge at day 7 after  
190 immunization, the immunized mice exhibited different responses to those unimmunized  
191 mice (Figure 3-figure supplement 1E). These results indicate IWC immunization  
192 induces a trained feature of innate immune response, which is critical for  
193 vaccination-induced rapid protection.

194

195 **Figure 3**



196

197 **Figure 3. Trained innate immunity mediates vaccination-induced rapid**  
 198 **protection. (A)** Survival of WT and *Rag1*<sup>-/-</sup> mice immunized i.n. with IWC or PBS,  
 199 challenged i.t by lethal *A. baumannii* 7 days later (n=5/group for WT mice, n=10  
 200 /group for *Rag1*<sup>-/-</sup> mice). \*\* *P* < 0.01 compared to control calculated by log-rank test.  
 201 Data are representative of two independent experiments. (B) MA plot of the DEGs of  
 202 IWC-immunized mice vs control mice at 24 hpi. X-axis represents average  
 203 counts-per-million (logCPM) and Y-axis represents log fold-changes (logFC) in  
 204 IWC-immunized mice vs control mice. The blue line is the threshold, logFC > 1  
 205 means upregulation and logFC < -1 means downregulation. (C) Top 10 GO  
 206 enrichment terms of downregulated DEGs in the IWC-immunized group at 24 hpi. (D)  
 207 Heatmap of DEGs related to inflammatory response was shown. False discovery rate  
 208 (FDR) < 0.05. (E) Reads per kilobase per million reads (RPKM) of inflammatory and  
 209 chemokine genes. Data are mean ± SD. \*\**P* < 0.01 determined by two-tailed unpaired  
 210 *t* test. (F) The heatmap of innate immune response related genes (GO\_0045087) of  
 211 lung samples from control, IWC-immunized at day 2 (IWC-2d), and day 7 (IWC-7d)  
 212 in *Rag1*<sup>-/-</sup> mice.

213 **Figure supplement 1. Vaccination-induced protection in *Rag1*<sup>-/-</sup> mice.**

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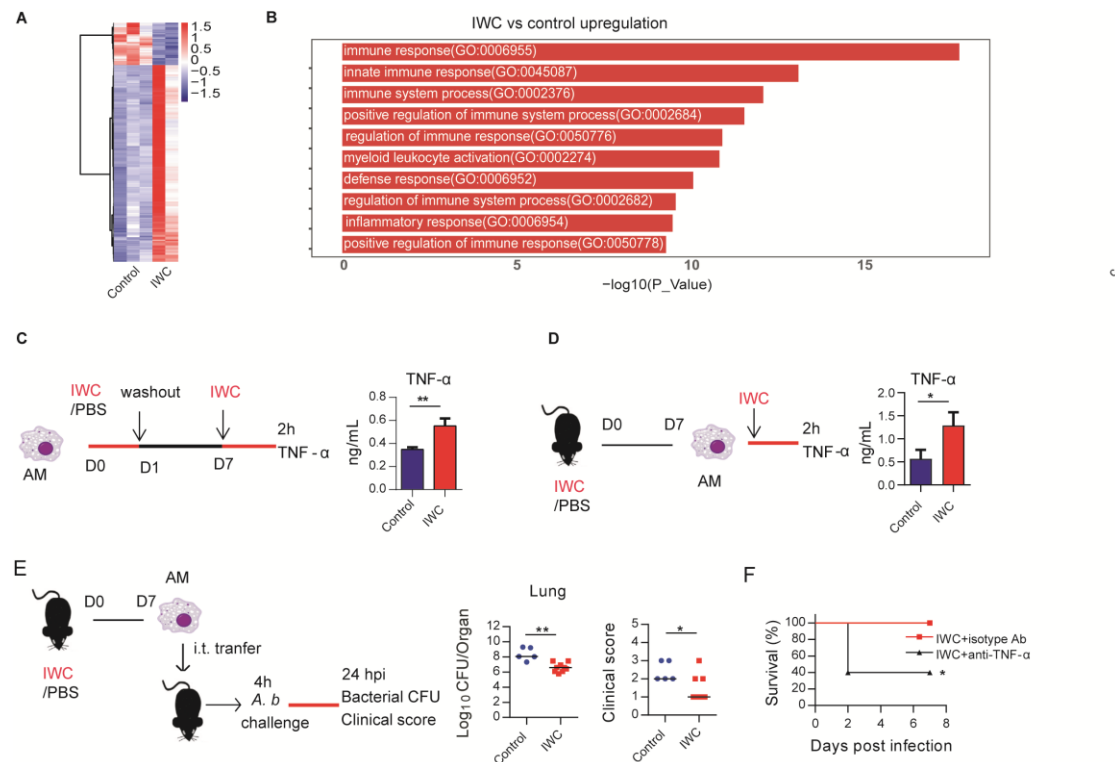
215 **Trained immunity of alveolar macrophages mediates vaccination-induced rapid**  
216 **protection.** Further, we analyzed the transcriptome change induced by vaccination at  
217 day 7 to identify DEGs associated with trained innate immunity. RNA-seq data  
218 showed a total of 308 DEGs in lungs of IWC-vaccinated *Rag1*<sup>-/-</sup> mice at day 7 (Figure  
219 4A and Figure 4-figure supplement 1A). The upregulated 253 DEGs were enriched to  
220 myeloid leukocyte activation (Figure 4B) and these genes were enriched to  
221 macrophage-associated genes (Figure 4-figure supplement 1B). So, we reasoned that  
222 alveolar macrophages (AMs), the predominant patrol myeloid cells in airways, might  
223 play a key role in vaccination-induced rapid protection. To test this hypothesis, we  
224 established a trained immunity model of AMs by stimulation with IWC *in vitro*.  
225 IWC-trained AMs induced an enhanced TNF- $\alpha$  production upon restimulation with  
226 IWC 7 days later. This result indicates that IWC trains AMs directly (Figure 4C,  $P <$   
227 0.01, unpaired  $t$  test.). AMs from IWC-immunized or control mice at day 7 were  
228 sorted from bronchoalveolar lavage fluid (BALF) using CD11c<sup>+</sup> microbeads. Flow  
229 cytometry analysis with anti-CD11c and anti-F4/80 confirmed the purity of AMs was  
230 greater than 95% (Figure 4- figure supplement 1C). Sorted AMs were stimulated with  
231 IWC *ex vivo* for 2 h. TNF- $\alpha$  production in vaccinated AMs after restimulation was  
232 significantly higher than that in control AMs (Figure 4D, \* $P <$  0.05, unpaired  $t$  test.).  
233 Thus, AMs could be trained by IWC with functional reprogramming, showing  
234 increased TNF- $\alpha$  production to a previously encountered stimulus. Further, the  
235 purified AMs from *A. baumannii* IWC-immunized or control mice at day 7 were

236 adoptively transferred into the airway of naïve mice by direct intra-tracheal  
237 instillation. Upon *A. baumannii* challenge, the lungs of mice that had received transfer  
238 of IWC-primed AMs had significantly lower bacterial burdens with alleviated clinical  
239 scores (Figure 4E, \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , Mann-Whitney U test)). Further treatment  
240 of IWC-immunized mice with anti-TNF- $\alpha$  antibody before *A. baumannii* challenge  
241 resulted in reduced protection (Figure 4F, \*,  $P < 0.05$ , log-rank test.). These results  
242 indicate that vaccination-trained AMs mediate rapid protection against infection via  
243 enhanced TNF- $\alpha$  production.

244

245

246 Figure 4



247

248 **Figure 4. Trained immunity of AMs mediates rapid protection induced by**  
 249 **vaccination.** (A) Heatmap of DEGs in lungs of IWC-immunized and control *Rag1*<sup>-/-</sup>  
 250 mice at day 7 after immunization. (B) Top 10 GO terms of upregulated DEGs in  
 251 IWC-immunized group at day 7. (C) *In vitro* model of IWC-trained AMs. (D)  
 252 C57BL/6 mice were immunized with IWC and recall responses of trained AMs to  
 253 IWC were evaluated *ex vivo* by detection of TNF- $\alpha$  production at 2 h after stimulation.  
 254 For C and D, Data are mean  $\pm$  SD. n=3. \**P* < 0.05, \*\**P* < 0.01, two-tail unpaired *t* test.  
 255 (E) Schema of evaluating roles of AMs in BALF of PBS or IWC-immunized  
 256 C57BL/6 mice. Bacterial burdens and clinical scores at 24 hpi were measured (n=5-9).  
 257 The line represents the median. \* *P* < 0.05, \*\**P* < 0.01 determined by Mann-Whitney  
 258 U test. (F) WT mice were immunized with IWC for 7 days. Mice were treated  
 259 intraperitoneally with anti-TNF- $\alpha$  antibody or isotype control then were challenge  
 260 with lethal *A. baumannii* 1 hour later. The survival of mice was monitored (n = 5). \*,  
 261 *P* < 0.05, calculated by log-rank test. Data are representative of two independent  
 262 experiments.

263 **Figure supplement 1. Transcriptional difference at day 7 after immunization.**

264 **Contribution of higher TLR4 expression on trained AMs to rapid protection.**

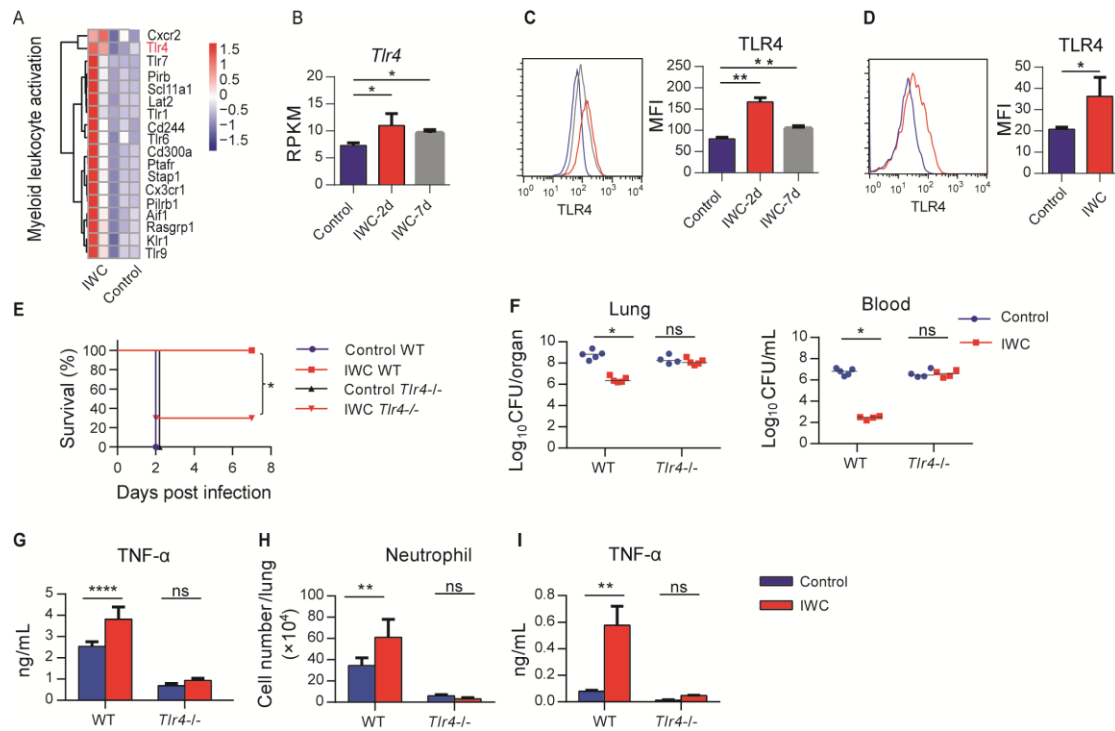
265 RNA-seq reveals that genes related to myeloid leukocyte activation including TLRs  
266 were significantly upregulated on day 7 after *A. baumannii* IWC immunization in  
267 *Rag1*<sup>-/-</sup> mice (Figure 5A and Figure 5-figure supplement 1). These results suggest that  
268 surface molecules associated with cell activation might be markers for trained AMs.  
269 Since TLR4 plays an important role in host recognition of Gram-negative bacteria, we  
270 hypothesized that IWC-trained AMs with elevated TLR4 expression might be more  
271 sensitive for second recall activation and enhanced function. RAN-seq data showed  
272 that lung TLR4 transcript significantly increased in response to IWC immunization at  
273 day 2 and day 7 (Figure 5B, \**P* < 0.05, one-way ANOVA). The elevated TLR4  
274 expression on BALF AMs at day 2 or day 7 after IWC immunization was also  
275 confirmed by flow cytometry (Figure 5C, \*\**P* < 0.01, one-way ANOVA). We also  
276 found TLR4 expression on AMs was elevated at day 7 after IWC training *in vitro*  
277 (Figure 5D, \**P* < 0.05, unpaired *t* test). Further, we found that the rapid protective  
278 effect of IWC-vaccination was significantly reduced in *Tlr4*<sup>-/-</sup> mice than in WT mice  
279 (Figure 5E, \* *P*<0.05, log-rank test). Accordingly, IWC-vaccination could not reduce  
280 bacterial burdens in lungs and blood in *Tlr4*<sup>-/-</sup> mice upon *A. baumannii* challenge  
281 (Figure 5F, \**P* < 0.05, ordinary two-way ANOVA). IWC-immunization induced rapid  
282 TNF- $\alpha$  expression at 2 hpi and neutrophil infiltration at 4 hour post *A. baumannii*  
283 challenge were dismissed in *Tlr4*<sup>-/-</sup> mice (Figure 5G and 5H, \*\*, *P* < 0.01, \*\*\*\*, *P* <  
284 0.0001, ordinary two-way ANOVA). In addition, we found that IWC-priming AMs



285 from *Tlr4*<sup>-/-</sup> mice significantly lost TNF- $\alpha$  secretion in response to IWC restimulation  
286 *ex vivo* (Figure 5I). These results suggest TLR4 signaling is vital for IWC-trained  
287 AMs. Taken together, these results suggest that up-regulation of TLR4 expression on  
288 trained AMs plays an important role in vaccination-induced rapid protection.

289

290 Figure 5



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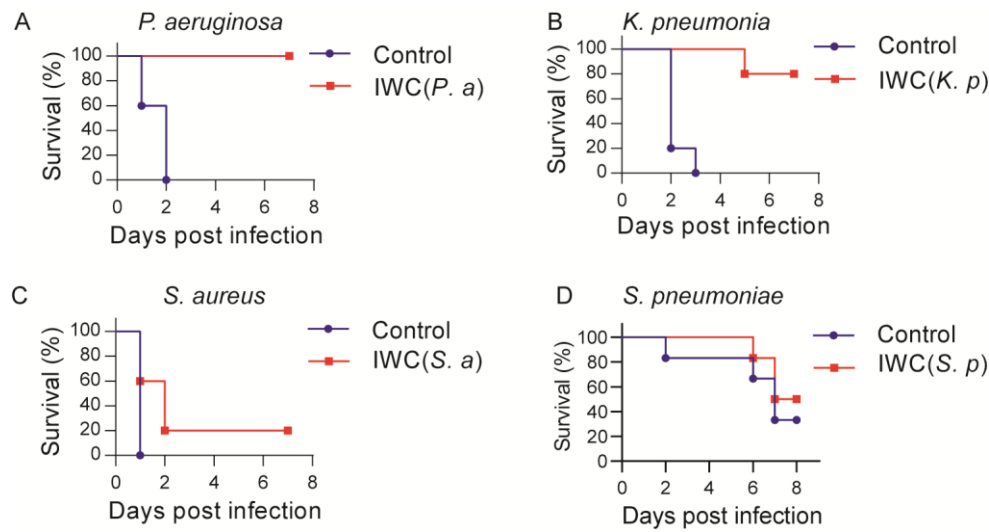
292 **Figure 5. Higher TLR4 expression on IWC-trained AMs mediates rapid**  
 293 **protection.** (A) Heatmap of DEGs associated with myeloid leukocyte activation at  
 294 day 7 after *A. baumannii* IWC immunization in *Rag1*<sup>-/-</sup> mice. (B) RPKM of TLR4 in  
 295 lungs on day 2 and day 7 after IWC immunization. (C) Representative histogram of  
 296 TLR4 expression and mean fluorescence index (MFI) of TLR4 on AMs in BALF on  
 297 day 2 (red line) and day 7 (grey line) after IWC immunization or control (blue line).  
 298 n=3. For B and C, \**P* < 0.05, \*\**P* < 0.01, evaluated by ordinary one-way ANOVA. (D)  
 299 Representative histogram of TLR4 expression and MFI of TLR4 on AMs after IWC  
 300 stimulation *in vitro* (red line) for 7 days. n=4. Data are mean ± SD. \**P* < 0.05,  
 301 determined by two-tailed unpaired *t* test. (E-H) *Tlr4*<sup>-/-</sup> and WT mice were immunized  
 302 i.n. with IWC and challenged with *A. baumannii* 7 days later. (n=5-10 mice/  
 303 group). (E) Survival curve, (F) Bacterial burdens at 24 hpi, (G) TNF-α in lungs at 2 hpi, and  
 304 (H) Neutrophil infiltration in lungs at 4 hpi. n=4-5 mice. (I) TNF-α levels in 2 h  
 305 culture supernatants of *ex vivo* IWC-stimulated AMs from 7-day vaccinated WT or  
 306 *Tlr4*<sup>-/-</sup> mice. For survival, *P* value was calculated by log-rank test. From (F) to (I), \**P*  
 307 < 0.05, \*\**P* < 0.01, \*\*\*\*, *P* < 0.0001, ns, not significant, compared by ordinary  
 308 two-way ANOVA. In (F), the line means median. Data are representative of at least  
 309 two independent experiments.

310 **Figure Supplement 1. Upregulated differentially expressed genes at day 7 after *A.***  
 311 **IWC immunization in *Rag1*<sup>-/-</sup> mice.**

312

313 **Vaccination-induced rapid protection against *P. aeruginosa* and *K. pneumoniae***  
314 **infection.** Further, we tested whether intranasal vaccination could induce rapid  
315 protection in other bacterial pneumonia models. We immunized mice i.n. with IWC of  
316 *P. aeruginosa* (IWC(*P.a*)), *K. pneumoniae* (IWC (*K.p*)), *S. aureus* (IWC(*S.a*)) or *S.*  
317 *pneumoniae* (IWC(*S.p*)) and challenged with the same bacteria 7 days after  
318 immunization. Rapid and efficient protection after intranasal immunization was also  
319 observed in *P. aeruginosa* (Figure 6A) and *K. pneumoniae* infected pneumonia  
320 models (Figure 6B). However, immunization could not induce effective protection  
321 against *S. aureus* (Figure 6C) and *S. pneumoniae* (Figure 6D). Since *A. baumannii*, *P.*  
322 *aeruginosa*, and *K. pneumoniae* are Gram-negative bacteria and *S. aureus* and *S.*  
323 *pneumoniae* are Gram positive bacteria, we reasoned that intranasal  
324 vaccination-induced rapid protection might be an effective way to protect certain  
325 Gram negative bacterial pneumonia.  
326

327 **Figure 6**



328

329 **Figure 6. A rapid protection induced by intranasal vaccination against other**

330 **bacteria.** C57BL/6 mice were immunized i.n. with IWC of *P. aeruginosa* (IWC(*P.a*))

331 (A), *K. pneumoniae* (IWC(*K.p*)) (B), *S. aureus* (IWC(*S.a*)) (C), or *S. pneumoniae*

332 (IWC(*S.p*)) (D) and were challenged i.t. with the same species of bacteria 7 days later.

333 The survival rates were monitored for 7 days. n=5-10 mice/group. Data are

334 representative of at least two independent experiments.

335

336

337 **Discussion**

338 The challenge of MDR bacterial infection highlights the urgent need to develop  
339 rapid-acting vaccine. Currently, only some vector-based virus vaccines are reported to  
340 be able to elicit rapid protection by a single dose, such as Ebola and Zika virus  
341 vaccines (Marzi et al., 2015; Pardi et al., 2017; Wong, 2019). Here, we showed a  
342 single intranasal immunization of IWC of *A. baumannii* elicits a very rapid and  
343 complete protection against *A. baumannii* infection 2 or 7 days after vaccination,  
344 supported by 100% survival, reduced bacterial burdens, alleviated lung injury, and  
345 reduced inflammatory cytokines expression after challenge (Figure 1). What's more,  
346 the vaccination-induced rapid protection is also observed in *P. aeruginosa* and *K.*  
347 *pneumoniae*-infected pneumonia models but not in *S. aureus* and *S.*  
348 *pneumoniae*-infected pneumonia models. Our study suggests the vaccination-induced  
349 rapid protection might be a common phenomenon in certain Gram negative bacterial  
350 infection, which is critical to protect MDR bacterial pneumonia for inpatients.

351 The development of immunological memory by vaccination is a central goal in  
352 fighting against infections. *A. baumannii* IWC-induced fast protection leads us to  
353 suspect whether it is a result of sustained and activated immune response elicited by  
354 vaccination. Dynamic response to intranasal immunization of IWC of *A. baumannii*  
355 shows that host response rapidly undergoes the priming, resting, and results in  
356 memory stage 5 days later (Figure 2A and 3F). The innate immune response 2 days  
357 post vaccination might be an activated innate immune response reflected by increase

358 IL-6 and TNF- $\alpha$  response to vaccination (Figure 2A), which provides the host  
359 resistance to *A. baumannii* infection. The host response to vaccination completely  
360 recovered to baseline level at day 5 post vaccination. However, IWC still induces  
361 protection against *A. baumannii* infections at day 7 after immunization, which  
362 represents a recall response to vaccination. Upon reexposure to the *A. baumannii* 7  
363 days after vaccination, IWC-vaccinated mice recalls a rapid, heightened TNF- $\alpha$   
364 secretion and chemokine production at 2 h post challenge and subsequently increased  
365 neutrophils infiltration earlier at 4 h post challenge in lungs of vaccinated mice  
366 (Figure 2B-D). It's well known that neutrophil, macrophage, and monocytes play  
367 essential roles in host defense against *A. baumannii* infection (Qiu et al., 2012; van  
368 Faassen et al., 2007). So, vaccination-induced rapid recall responses to infection lead  
369 to a rapid elimination of bacteria, thereby limit the uncontrolled inflammation at 24  
370 hpi in vaccinated mice, eventually prevent lung damage and efficiently improve the  
371 survival of mice.

372 For mechanisms study, we highlight the role of trained innate immunity in  
373 vaccination-induced rapid protection. Traditionally, immune memory is thought to be  
374 an exclusive feature of adaptive immune response of T cells and B cells, which is  
375 harnessed to design the vaccine extensively, whereas the role of innate immune  
376 response to vaccination is recognized as modulation of adaptive immunity. Recently,  
377 trained immunity has been proposed to describe the enhanced immune response of  
378 innate cells to second stimuli via epigenetic, metabolic, and functional reprogramming

379 by initial stimulation (Mulder, Ochando, Joosten, Fayad, & Netea, 2019; Netea et al.,  
380 2016). Our study in *Rag1*<sup>-/-</sup> mice (which lack mature T and B cells) further showed  
381 innate immune responses can be trained by vaccination to mediate rapid protection  
382 (Figure 3). The rapidity of innate response with the trained feature enables it as a good  
383 target to design the vaccine to induce rapid protective response against MDR bacteria.

384 AMs are the predominant cells in the airway mucosa and play important roles for  
385 infection controls (Hussell & Bell, 2014). The embryonic origin and the ability of  
386 self-renewal in steady state of AMs make them be able to store immune memory  
387 (Hussell & Bell, 2014). In this study, we found that AMs could be trained by IWC of  
388 *A. baumannii* with functional reprogramming, showing increased TNF- $\alpha$  production  
389 upon restimulation with the same IWC. More importantly, adoptive transfer of *A.*  
390 *baumannii* IWC-trained AMs into naive recipient mice enhanced the bacteria  
391 clearance after lethal challenge with *A. baumannii* (Figure 4), confirming IWC-trained  
392 AMs mediate rapid protection. It has been reported that AMs could be trained to gain  
393 the memory phenotype in respiratory viral infection models, which is dependent on  
394 IFN- $\gamma$  production from effector CD8<sup>+</sup> T cells (Yao et al., 2018). However, in our  
395 vaccination-trained model, trained AMs is independent on adaptive T cells, since AMs  
396 could be trained by IWC directly *in vitro* (Figure 4C) and vaccination-induced  
397 protection is still efficient in *Rag1*<sup>-/-</sup> mice (Figure 3A). It indicates the different  
398 mechanisms of AMs training are involved in our models. Although the different  
399 mechanisms are involved, our data along with the data of virus-primed AMs both

400 show that AMs could be trained, which can be manipulated to combat MDR bacterial  
401 pneumonia and also the respiratory virus infection.

402 As for how AMs are trained, a range of pattern recognition receptors (PRRs),  
403 including Toll like receptors (TLRs), nucleotide-binding oligomerization  
404 domain-containing protein 2 (NOD2), and dectin-1 might be engaged to promote  
405 trained immunity. Bacille Calmette-Guérin (BCG) and its main component muramyl  
406 dipeptide induce trained immunity through NOD2-ligand (Kleinnijenhuis et al., 2012)  
407 and  $\beta$ -glucan through dectin-1 receptor (Quintin et al., 2012). Some studies have  
408 implicated TLRs are upregulated to BCG or  $\beta$ -glucan training (Kleinnijenhuis et al.,  
409 2012; Quintin et al., 2012), however how TLRs are involved in trained immunity is  
410 not clear. In this study, we demonstrate that TLR4 is elevated on IWC-trained AMs.  
411 Enhanced TNF- $\alpha$  production of vaccine-primed AMs upon restimulation is impaired  
412 in *Tlr4*<sup>-/-</sup> mice, which results in reduced protective effect of vaccination (Figure 5).  
413 These data suggest that elevated TLR4 expression on AMs might be a trained marker,  
414 which can sense the pathogen-associated molecular pattern more efficiently and  
415 results in rapid activation of trained AMs compared to naïve AMs. TNF- $\alpha$  is one of  
416 the main cytokines which has been thoroughly used as a functional cytokine marker  
417 indicating of trained immunity along with IL-6 and IL-1 $\beta$  (Arts et al., 2018). In this  
418 study, we found that vaccination-trained immune response produces heightened  
419 TNF- $\alpha$ , but not IL-6 and IL-1 $\beta$  when encounter with the infection (Figure 2B). In  
420 addition, blocking TNF- $\alpha$  with specific antibody before challenge significantly



421 reduces vaccine-induced rapid protection (Figure 4F). These data collectively indicate  
422 that enhanced production of TNF- $\alpha$  from AMs is a functional indicator of trained AMs  
423 and responsible for vaccine-elicited rapid protection.

424 The limitation of this study is that we couldn't test the vaccination-induced rapid  
425 protection in more MDR bacterial pneumonia models, due to the lack of more  
426 bacterial pneumonia models in our hands. Also, our study also leaves many open  
427 questions, such as how AMs are trained by vaccination, what ligand-receptor pairs are  
428 responsible for training AM, what molecular mechanism is involved. Further  
429 investigation is needed to better understand the trained immunity of AMs, which in  
430 turn will pave the way for improved vaccine design.

431 In summary, in this study we demonstrate that intranasal immunization of IWC of  
432 certain bacteria induces a rapid and sufficient protection against lethal respiratory  
433 infection through inducing trained immunity of AMs. Our study highlights the  
434 importance and the possibility of harnessing trained immunity of AMs to design rapid  
435 effecting vaccine. Even for long-lasting effect of vaccine, exploiting the classical  
436 adaptive memory and trained innate immunity in an integrated fashion seems  
437 plausible for a potential good design of vaccination strategies against bacterial  
438 infection.

439

## 440 **Materials and Methods**

### 441 **Experimental design and ethical approval**

442 This study was designed to determine whether intranasal immunization of IWC could  
443 elicit rapid protection against bacterial pneumonia and explore the underlying  
444 mechanisms. For animal studies, 6 to 8 weeks old, female C57BL/6, *Rag1*<sup>-/-</sup>, and  
445 *Tlr4*<sup>-/-</sup> mice were used. The animal protocols adhered to the National Institutes of  
446 Health Guide for the Care and Use of Laboratory Animals and were approved by the  
447 Institutional Animal Care and Treatment Committee of West China Hospital, Sichuan  
448 University (Approval No. 2019190A). A MS Excel randomization tool was used to  
449 randomize the mice to different treatment groups. To assess the protection efficacy of  
450 IWC, survival rate, clinical score, bacterial burdens, and lung histopathology of mice  
451 were monitored after infection. RNA-seq, *ex vivo* and *in vitro* AMs stimulation model  
452 and adoptive transfer of AMs were performed to investigate the mechanisms  
453 underlying the rapid protection. The animal studies were not blinded. The group sizes  
454 for survival varied from 5 to 10 in the different studies and 3 to 5 for analysis of  
455 immune response. All experiments were conducted at least 2 times independently,  
456 which was indicated in figure legends.

### 457 **Mice**

458 Female C57BL/6 mice were purchased from Beijing HFK Bioscience Limited  
459 Company (Beijing, China). *Rag1* gene knockout mice (*Rag1*<sup>-/-</sup>,  
460 B6.129S7-*Rag1*<sup>tm1Mom/J</sup>), TLR4 gene knockout mice (*Tlr4*<sup>-/-</sup>, C57BL/10ScNjNju) and

461 control WT were purchased from Model Animal Research Center of Nanjing  
462 University. The mice were kept under specific pathogen-free conditions.

### 463 **Bacterial strains**

464 *A. baumannii* strain LAC-4 was kindly provided by Professor Chen (Harris et al.,  
465 2013). *P. aeruginosa* strain XN-1, *K. pneumoniae* strain YBQ and *S. pneumoniae* was  
466 isolated from Chongqing southwest hospital. *S. aureus* strain KM-22 was isolated  
467 from the Second Affiliated Hospital of Kunming Medical University. The bacteria  
468 were grown in tryptone soy broth (*A. baumannii*), luria bertani broth (*P. aeruginosa*,  
469 and *K. pneumoniae*), mueller-hinton broth (*S. aureus*), or blood agar plate (*S.*  
470 *pneumoniae*) at 37°C. At mid-log-phase, bacteria were collected and suspended in  
471 phosphate buffer saline (PBS). Fresh bacteria were used to infect the mice. For  
472 inactivated whole cells (IWC) preparation, fresh bacteria were fixed with 4%  
473 paraformaldehyde.

### 474 **Intranasal immunization and pneumonia model**

475 Mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (62.5  
476 mg/kg of body weight) and then immunized intranasally (i.n.) with IWC ( $1 \times 10^8$  CFUs  
477 in 20  $\mu$ l PBS) or PBS as a control. Mice were infected with a lethal dose of bacteria  
478 intratracheally (i.t.) through mouth via a soft-end needle under direct visualization to  
479 establish pneumonia model (Gu et al., 2018). The survival rate, clinical score, bacteria  
480 burdens, and lung pathology were evaluated as described previously (Gu et al., 2018).  
481 The lethal doses for different bacteria are as follow:  $2 \times 10^7$  CFUs for *A. baumannii*;

482  $1 \times 10^7$  CFUs for *P. aeruginosa*;  $5 \times 10^7$  CFUs for *S. aureus*;  $2 \times 10^6$  CFUs for *K.*  
483 *pneumoniae*; or  $1 \times 10^7$  CFUs for *S. pneumoniae*.

#### 484 **ELISA**

485 TNF- $\alpha$ , IL-6, and IL-1 $\beta$  concentrations in serum, lung homogenates, and cell culture  
486 supernatants were detected using mouse TNF- $\alpha$  ELISA kit, mouse IL-6 ELISA kit,  
487 and mouse IL-1 $\beta$  ELISA kit (eBioscience, San Diego, CA, USA) following the  
488 manufacturer's instructions.

#### 489 **Real-time PCR**

490 Total RNA of lungs was extracted by RNA iso Plus (Takara Biotechnology, Dalian,  
491 China) and reverse transcribed to cDNA with PrimeScript™ RT reagent Kit (Takara  
492 Biotechnology). Gene expression was detected using SYBR green Premix (Takara  
493 Biotechnology) on CFX96 real-time PCR detection machine (Bio-Rad, Hercules, CA,  
494 USA) with specific primers listed in Supplementary Table S1. The  $\Delta\Delta C_t$  method was  
495 used to calculate the relative gene expression with  $\beta$ -actin as the housekeep-gene.

#### 496 **Preparation of BALF and lung cell suspension**

497 Cells were obtained from bronchoalveolar lavage fluid (BALF) as described (Gu et al.,  
498 2018). Perfused lungs were cut into small pieces and digested with 1 mg/mL  
499 collagenase D (Sigma-Aldrich, St. Louis, MO, USA) and 100  $\mu$ g/mL DNAase (Sigma)  
500 at 37°C for 60 min. Cell suspension was prepared by crushing and filtering the  
501 digested tissue through a 70  $\mu$ m cell strainer (BD Biosciences, New Jersey, USA) and  
502 the cell numbers were counted by Countess II Automated Cell Counter (Thermo

503 Fisher Scientific, MA, USA)

#### 504 **Flow cytometry**

505 Cell suspensions were blocked with rat serum then stained with  
506 fluorophore-conjugated specific or isotype control antibodies in the dark at 4 °C for  
507 30 min. The antibodies were as follows: CD45-PE/Cy7 (30-F11),  
508 CD11b-PerCP/Cy5.5 (M1/70), F4/80-APC (BM8), Ly-6C-PE (HK1.4), Ly6G-FITC  
509 (1A8), CD11c-PE (N418) from Biolegend (San Diego, CA, USA) and TLR4-PE  
510 (UT41) from Invitrogen (Carlsbad, CA, USA). Labeled cells were run on a BD  
511 FACSCanto™ II flow cytometer (BD Biosciences) and analyzed with FlowJo (BD  
512 Biosciences). AMs were defined as CD45<sup>+</sup>CD11b<sup>-</sup>F4/80<sup>+</sup>; monocytes were defined as  
513 CD45<sup>+</sup>CD11b<sup>+</sup> Ly6C<sup>+</sup>; Neutrophils were defined as CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>hi</sup>. The cell  
514 numbers of each cell types were calculated with total cell number multiplied by the  
515 cell percentage. AMs in BALF were identified as CD11c<sup>+</sup> F4/80<sup>+</sup> cells and TLR4  
516 expression on AMs were detected by flow cytometry and expressed as mean  
517 fluorescence index (MFI).

#### 518 **RNA sequencing (RNA-seq)**

519 Tissue samples from lungs were sent to Wuhan Seqhealth Co., Ltd. (Wuhan, China)  
520 for RNA-seq. Briefly, Total RNAs were extracted from lung samples using TRIzol  
521 (Invitrogen) and DNA was digested by DNaseI after RNA extraction. A260/A280 was  
522 examined with Nanodrop™ One spectrophotometer (Thermo Fisher Scientific) to  
523 determine RNA quality. RNA Integrity was confirmed by 1.5% agarose gel

524 electrophoresis. Qualified RNAs were finally quantified by Qubit3.0 with Qubit™  
525 RNA Broad Range Assay kit (Life Technologies, Carlsbad, CA, USA). Total RNAs (2  
526 µg) were used for to prepare sequencing library using KC-Total RNA-seq Library  
527 Prep Kit for Illumina® (Wuhan Seqhealth Co., Ltd. Wuhan, China) following the  
528 manufacturer's instruction. PCR products corresponding to 200-500 bps were  
529 enriched, quantified and finally sequenced on Hiseq X 10 sequencer (Illumina).

### 530 **Analysis of RNA-seq data**

531 Raw data of sequencing were cleaned using Trimmomatic software. The clean reads  
532 after quality control were mapped to the mouse genome GRCm38 with STAR  
533 software (version 2.5.3a). The reads counts for each gene were calculated using  
534 FeatureCounts (version1.5.1) and expressed as RPKM (reads per kilobase per million  
535 reads). EdgeR package were used to identify the differentially expressed genes by  
536 statistics with an adjusted *P* value < 0.05 and fold change > 1.5. Gene Ontology (GO)  
537 and (Kyoto Encyclopedia of Genes and Genomes) KEGG enrichment was done with  
538 Kobas (Version 2.1.1). Hierarchical clustering and heatmaps were drawing using  
539 pheatmap R package and MA-plot was drawn using EdgeR package.

### 540 ***In vitro* AMs training model**

541 BALF cells were cultured in DMEM (containing 10% FBS and 1%  
542 penicillin/streptomycin) in plate for 1 h and non-adherent cells were discarded and  
543 remaining AMs were stimulated with *A. baumannii* IWC  
544 (Multiplicity of infection (MOI) =1) for 24 h, then washout and rest for 6 days. AMs

545 were restimulated with IWC (MOI=1) at day 7 and TNF- $\alpha$  in supernatants at 2 h after  
546 restimulation was detected by ELISA. In some experiments, AMs were collected at  
547 day 7 to detect TLR4 expression with PE-anti TLR4 antibody (UT41, Invitrogen) by  
548 flow cytometry. The expression of TLR4 is showed as MFI.

#### 549 **Magnetic-activated cell sorting (MACS)**

550 AMs from BALF were sorted by positive selection with an anti-mouse CD11c  
551 Microbeads kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to  
552 manufacturer's instruction. Sorted cells were stained with PE-anti-mouse CD11c  
553 antibody (N418) and F4/80-APC (BM8) and analyzed by flow cytometry to check the  
554 purity.

#### 555 **Stimulation of AMs *ex vivo***

556 Mice were immunized i.n. with IWC or PBS as control. AMs (CD11c<sup>+</sup>) sorted by  
557 MACS from BALF at day 7 were stimulated *ex vivo* with IWC (MOI=1) for 2 h and  
558 supernatant were collected for measuring TNF- $\alpha$  using ELISA.

#### 559 **Adoptive transfer of AMs**

560 AMs (CD11c<sup>+</sup>) from BALF of IWC-immunized or control mice at day 7 after  
561 immunization were sorted by MACS as described above (purity >95%). Donor AMs  
562 ( $5 \times 10^4$ ) were i.t. transferred into the airways of recipient mice. Recipient mice were  
563 challenged with *A. baumannii* LAC-4 ( $2 \times 10^7$ ) at 4 h after transfer and bacterial  
564 burdens in lungs and clinical score were detected 24 hpi.

#### 565 **Blocking TNF- $\alpha$ *In vivo***

566 For neutralizing TNF- $\alpha$ , mice were treated with 200  $\mu$ g anti-mouse TNF- $\alpha$  (XT3.11  
567 clone, BioXcell, West Lebanon, NH) or rat IgG1 isotype antibody intraperitoneally 1  
568 h before infection, the survival was recorded for 7 days.

#### 569 **Statistical analyses**

570 Bacterial burdens and clinical score data were expressed as median. Other bar graph  
571 data were presented as means  $\pm$  SD. Survival data were compared by log-rank test.  
572 For data more than 2 groups, data were evaluated by ordinary one-way ANOVA  
573 followed by Tukey's multiple comparisons test. Data of two samples with normal  
574 distribution were compared by two-tail unpaired  $t$  test. Mann-Whitney U test was  
575 used for comparing data of non-normal distribution (bacterial burdens and clinical  
576 score). For grouped data, statistical significance was evaluated by ordinary two-way  
577 ANOVA. The software GraphPad Prism version 6.0 was used for all statistical  
578 analyses. All comparisons used a two-sided  $\alpha$  of 0.05 for significance testing and  $P <$   
579 0.05 was considered significant. The specific statistical methods were indicated in the  
580 figure legends.

#### 581 **Data availability:**

582 Raw data files for RNAseq have been deposited in the NCBI Gene Expression  
583 Omnibus under accession number GEO: GSE141729.

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591 **Competing interests:** The authors declare that no competing interests exist.

592

593

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665

666 **Supplemental Information**

667 Figure 1-figure supplement 1. Intranasal IWC vaccination provides rapid protection

668 against *A. baumannii* infection.

669 Figure 3-figure supplement 1. Vaccination-induced protection in *Rag1*<sup>-/-</sup> mice.

670 Figure 4-figure supplement 1. Transcriptional difference at day 7 after immunization.

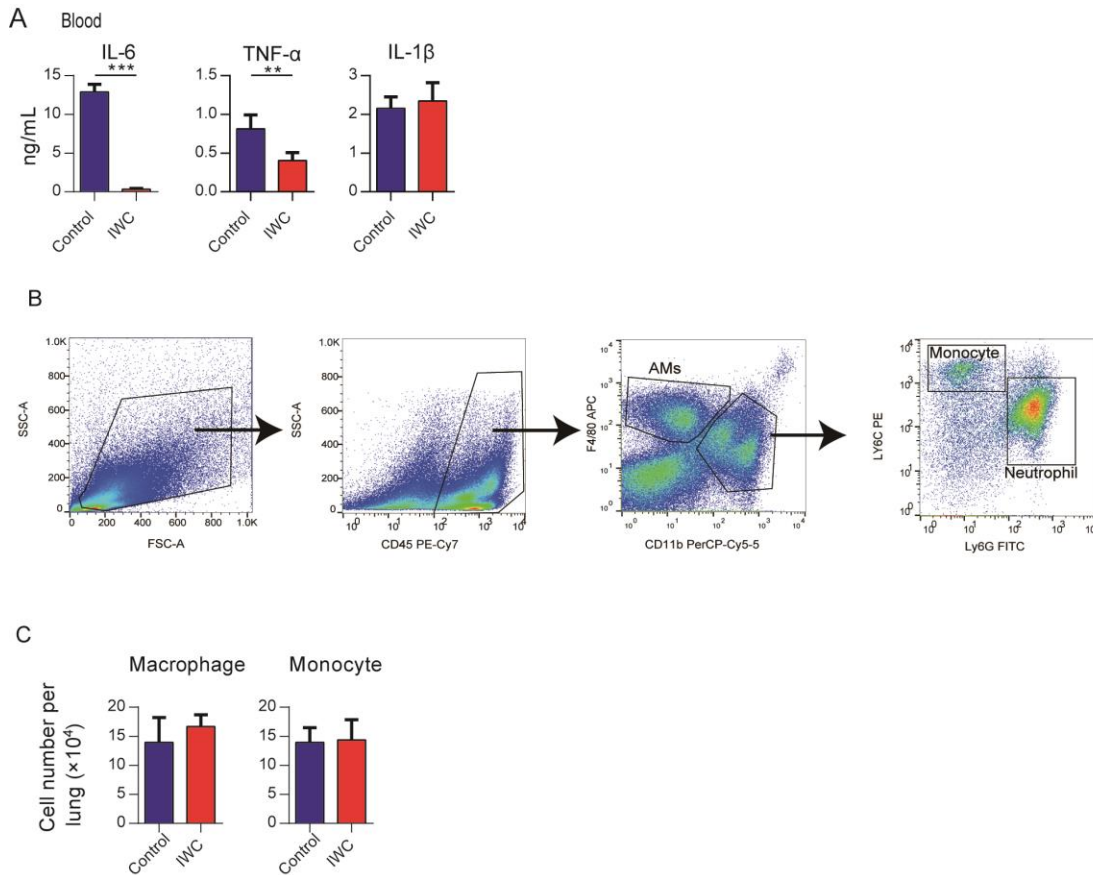
671 Figure 5-figure supplement 1. Upregulated differentially expressed genes at day 7

672 after *A. IWC* immunization in *Rag1*<sup>-/-</sup> mice.

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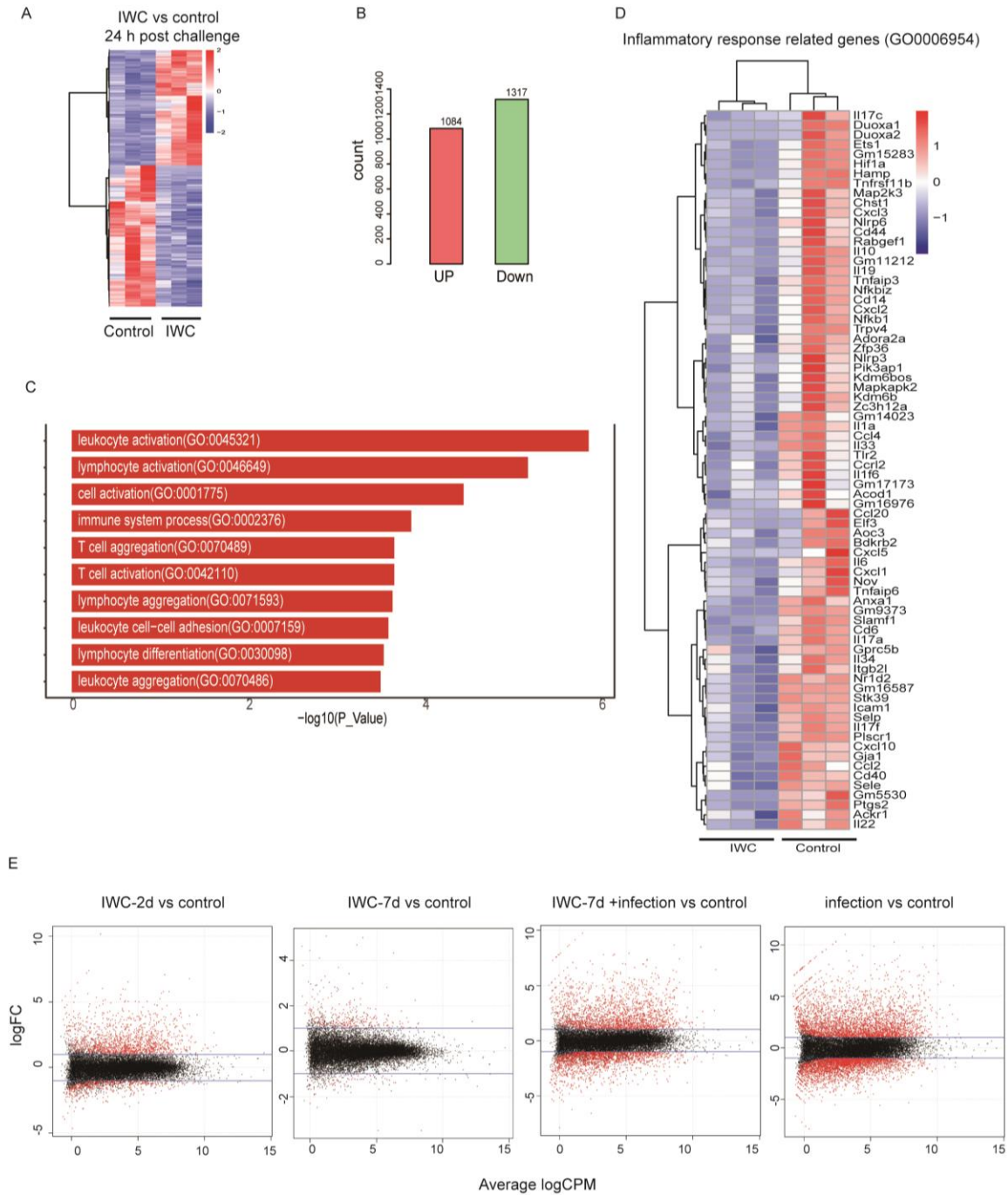
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Figure 1-figure supplement 1



**Figure 1-figure supplement 1. Intranasal IWC vaccination provides rapid protection against *A. baumannii* infection.** (A) IWC-immunized mice were challenged at day 7 and levels of inflammatory cytokines in blood at 24 hpi were detected by ELISA. (B) Gating strategy used in this study for detecting neutrophil, monocyte and alveolar macrophages in lungs. AMs were defined as CD45<sup>+</sup>CD11b<sup>-</sup>F4/80<sup>+</sup>; monocytes were defined as CD45<sup>+</sup>CD11b<sup>+</sup> Ly6C<sup>+</sup>; Neutrophils were defined as CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>hi</sup>. (C) Numbers of monocytes and macrophages in lungs at 24 hpi were detected by flow cytometry. Data are mean  $\pm$  SD. *P* value was determined by unpaired *t* test. \*\* *P* < 0.01, \*\*\* *P* < 0.001. Data are representative of two independent experiments (n =4- 5 mice/group).

Figure 3-figure supplement 1



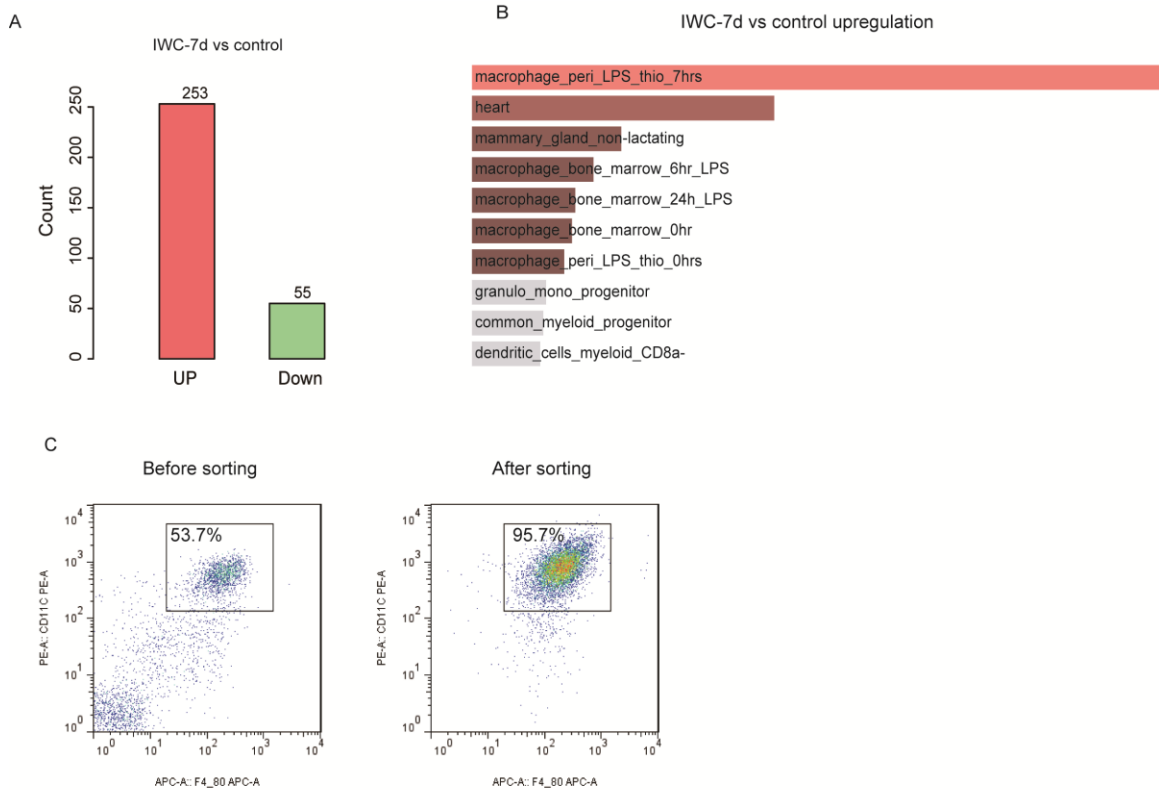
**Figure 3-figure supplement 1. Vaccination-induced protection in *Rag1*<sup>-/-</sup> mice. (A-D)**

*Rag1*<sup>-/-</sup> mice were immunized with IWC of *A. baumannii* or PBS as control and were challenged with *A. baumannii* ( $2 \times 10^7$  CFU/mice) 7 days later and lungs were processed



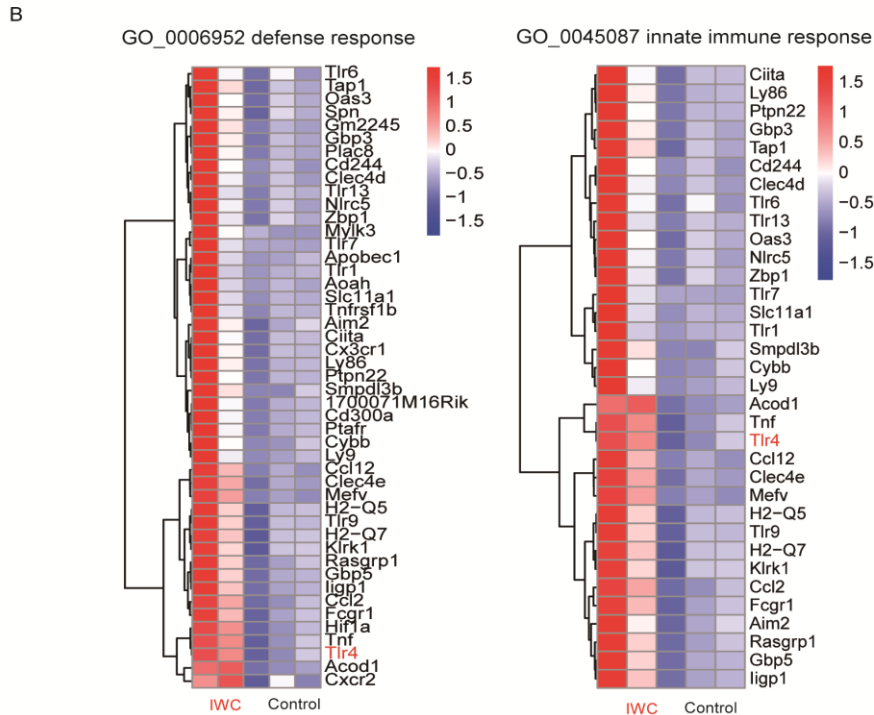
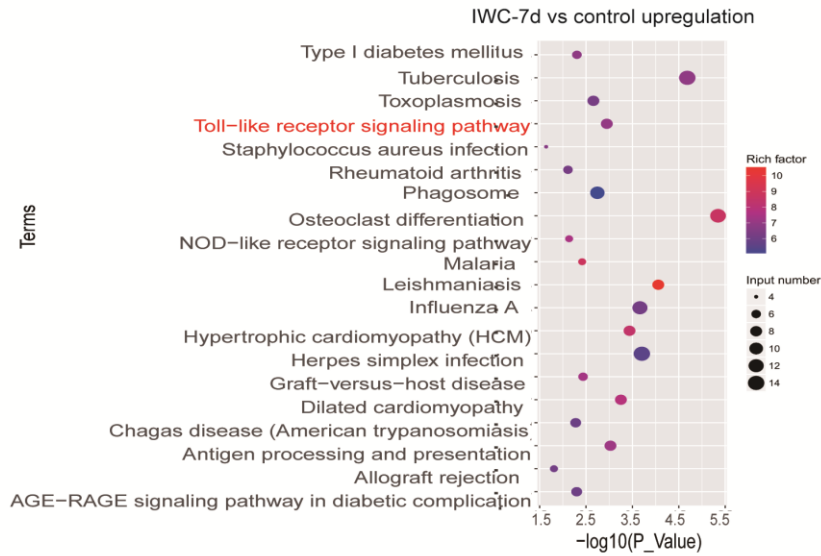
for RNAseq at 24 hpi. n=3. **(A)** The heatmap of 2401 DEGs in lungs of 7 days immunized and control *RagI*<sup>-/-</sup> mice after *A. baumannii* challenge at 24 hpi (n = 3 biological replicates, false discovery rate (FDR) < 0.05). Red indicates increased expression; blue indicates decreased expression. **(B)** Numbers of upregulated and downregulated DEGs of IWC-immunized vs control mice 24 hpi. **(C)** Top 10 GO terms of upregulated DEGs of IWC-immunized group compared to control group at 24 hpi. **(D)** Heatmap of DEGs related to inflammatory response (GO0006954) was shown. False discovery rate (FDR) < 0.05. **(E)** Lung samples from control, IWC-immunized *RagI*<sup>-/-</sup> mice at day 2 (IWC-2d), day 7 (IWC-7d), and IWC immunized mice (7 day) at 24 hours after challenge with *A. baumannii* (IWC-7d+infection) and control mice at 24 h after challenge (infection) were processed for RNA-seq. MA plot of DEGs in each treatment group compared with that in control group.

Figure 4-figure supplement 1



**Figure 4-figure supplement 1. Transcriptional difference at day 7 after immunization.** (A, B) *Rag1*<sup>-/-</sup> mice were immunized i.n. with IWC of *A. baumannii* or PBS as control, at day 7 gene expression in lungs from IWC immunized mice (IWC-7d) or control mice were assessed by RNA-seq. (A) Numbers of upregulated and downregulated DEGs of lungs in IWC-7d and control mice. (B) Top 10 mouse gene atlas terms of upregulated genes in lungs of IWC-7d vs control mice analyzed by Enrichr (<https://amp.pharm.mssm.edu/Enrichr/>) (1, 2). (C) Representative flow cytometry of CD11c<sup>+</sup> cells from vaccinated BALF before and after MACS sorting. CD11c<sup>+</sup> cells are also F4/80<sup>+</sup>, representing AMs and the purity of AMs after sorting are above 95%.

Figure 5-supplement Figure 1  
A



**Figure 5-figure supplement 1. Upregulated differentially expressed genes at day 7 after *A. IWC* immunization in *Rag1*<sup>-/-</sup> mice. (A) Top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) terms of upregulated DEGs in IWC-7d vs control mice ( $P < 0.05$ ). (B) Heatmap of DEGs related to defense response (GO: 0006952) and innate immune response (GO0045087).**

**Table S1. Primers used in real-time PCR.**

Primer name	Primer sequence
CXCL1-Forward	5'-ATGGCTGGGATTCACCTCAA-3'
CXCL1-Reverse	5'-AGTGTGGCTATGACTTCGGT-3'
CXCL2- Forward	5'-AGGGCGGTCAAAAAGTTTGC-3'
CXCL2- Reverse	5'-CAGGTACGATCCAGGCTTCC-3'
CXCL5- Forward	5'-TGGCATTCTGTGCTGTTC-3'
CXCL5- Reverse	5'-CACCTCCAAATTAGCGATCAA-3'
CXCL10- Forward	5'-ATCATCCCTGCGAGCCTATCCT-3'
CXCL10- Reverse	5'-GACCTTTTTTGGCTAAACGCTTTC-3'
CCL2- Forward	5'-TTAAAACCTGGATCGGAACCAA-3'
CCL2- Reverse	5'-GCATTAGCTTCAGATTTACGGGT-3'
CCL7- Forward	5'-CCACCATGAGGATCTCTGC-3'
CCL7- Reverse	5'-TTGACATAG CAGCATGTGGAT-3'
$\beta$ -actin- Forward	5'-GGCTGTATTCCCCTCCATCG-3'
$\beta$ -actin- Reverse	5'-CCAGTTGGTAACAATGCCATGT-3'