***spd1672*, a novel in vivo-induced gene, affects inflammatory response in a murine model of *Streptococcus pneumoniae* infection**

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2017-0662.R1
Manuscript Type:	Article
Date Submitted by the Author:	16-Feb-2018
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Is the invited manuscript for consideration in a Special Issue? :	N/A
Keyword:	<i>Streptococcus pneumoniae</i> , in vivo-induced gene, <i>spd1672</i> , AKT, STAT3

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1 **Title Page**

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3 **in a murine model of *Streptococcus pneumoniae* infection**

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35 Abstract

36 *spd1672*, a novel *Streptococcus pneumoniae* (*S. pn*) gene induced in vivo,
37 has been identified to contribute to the virulence of *S. pn*; however, the
38 role of *spd1672* during host innate immune reaction against *S. pn*
39 infection is unknown. In the present study, mice were infected with wild
40 type D39 and mutant D39 Δ *spd1672* strains. Compared to the
41 D39-infected mice, reduced bacterial load and attenuated inflammatory
42 response were observed in the D39 Δ *spd1672*-treated mice. The levels of
43 proinflammatory cytokines, including those of IFN- γ , TNF- α , and IL-1 β ,
44 in the blood of D39 Δ *spd1672*-infected mice were lower than that in the
45 D39-infected group. Additionally, attenuated activation of STAT3 and
46 AKT was observed in the D39 Δ *spd1672*-infected mice. In conclusion,
47 our data indicated that *spd1672* expression modulates the release of
48 proinflammatory cytokines, and AKT-STAT3 signaling appears to
49 participate in the process. In conclusion, the present study extends our
50 understanding of the role of an in vivo-induced gene in *S. pn*-host
51 interaction.

52 **Keywords:** *Streptococcus pneumoniae*; in vivo-induced gene; *spd1672*;
53 AKT; STAT3

54

55 **Introduction**

56 *Streptococcus pneumoniae* (*S. pn*) is a conditionally pathogenic
57 bacterium causing meningitis, otitis media, and septicemia (Mitchell and
58 Mitchell 2010). Currently, *S. pn* is one of the three leading causes of
59 death in children aged less than five years worldwide (Liu et al. 2015),
60 and it is still the most common bacterial cause of community-acquired
61 pneumonia (Feldman and Anderson 2016). Therefore, identification of
62 novel virulence factors and knowledge of the potential mechanisms
63 underlying host-*S. pn* interactions are urgently required.

64 *S. pn* possesses numerous virulence factors, such as capsule, cell
65 wall (CW), pneumolysin (Kadioglu et al. 2008). Virulence factors are
66 essential for *S. pn* invasion, adaptation, and evasion of host defenses. In
67 addition, they also act as pathogen-associated molecular patterns
68 (PAMPs), which are recognized by pattern recognition receptors to
69 initiate the host's innate immune response (Calbo and Garau 2010). For
70 instance, CW could be internalized by epithelial and endothelial cells in
71 an actin-dependent pathway or platelet-activating factor
72 receptor-dependent uptake pathway (Loh et al. 2017). Moreover, it is
73 recognized by Toll-like receptor 2, which induces the host inflammatory
74 response (Loh et al. 2017).

75 Upon invading the host, some bacterial genes are expressed or
76 upregulated in vivo for adaption to the host environment, and the proteins
77 encoded by these genes are possibly related to virulence factors (Chiang
78 et al. 1999). In our previous study, we used the in vivo expression
79 technology (Mahan et al. 1993) and differential fluorescence induction
80 (Marra 2002) to identify *spd1672* a novel in vivo-induced gene (Meng et
81 al. 2008). The mutant strain D39 Δ *spd1672* was constructed by allelic
82 replacement with the *erm* cassette and long flanking homology
83 polymerase chain reaction. The *spd1672* deletion reduced the synthesis of
84 teichoic acid (Wu et al. 2014a) and *S. pn* showed attenuated virulence
85 (Xu et al. 2015a). The *S. pn* strain SPY1 is a capsule-negative mutant
86 harboring the D39 Δ *spd1672* mutation. In addition to the *spd1672* deletion,
87 several mutations outside *spd1672* were identified by whole genome
88 sequencing. As a potential live attenuated pneumococcus vaccine, SPY1
89 was capable of eliciting serotype-independent protection against
90 pneumococcal infections (Wu et al. 2014b; Xu et al. 2014) and inducing
91 humoral and Th2-Th17-Regulatory T cell immunity (Xu et al. 2015b).
92 These preliminary observations indicated that *spd1672* deficiency
93 possibly affects mice immune response induced by *S. pn*; however, the
94 interaction between *S. pn* and host innate immunity in murine pneumonia
95 models has not yet been fully elucidated.

96 In this study, we observed impeded systemic bacterial dissemination,
97 attenuated inflammatory response, and decreased release of IFN- γ ,
98 TNF- α , and IL-1 β in mice infected with D39 Δ *spd1672* strain. Activation
99 of AKT and STAT3 may modulate the secretion of inflammatory
100 cytokines. In conclusion, studying the effect of in vivo-induced gene
101 *spd1672* on mice innate immune response would contribute to our
102 understanding of host-*S. pn* interaction.

103 **Materials and methods**

104 **Mice**

105 Female Balb/c mice (6 to 8 weeks) were obtained from the animal
106 center of Chongqing Medical University, Chongqing, China. Mice were
107 kept under specific-pathogen-free conditions at the animal facilities of
108 Chongqing Medical University during the time of the experiments. All
109 the animal experiments were done in accordance with the guidelines of
110 the Institutional Animal Care and Use Committee of Chongqing Medical
111 University.

112 **Bacterial strain growth conditions and culture**

113 *S. pn* strain NCTC7466 (D39, serotype 2) was obtained from the
114 National Collection of Type Cultures (NCTC; London, United Kingdom).

115 *S. pn* strain were grown either on Columbia sheep blood agar plates or in
116 casein-based medium with yeast extract (C+Y medium) at 37°C in 5%
117 CO₂. Growth in medium was assessed by measuring the OD at 600 nm
118 with spectrophotometer. Bacterial colony-forming units (CFU) were
119 calculated by plating serial dilutions of lung homogenate suspensions,
120 spleen homogenate suspensions, or blood onto Columbia sheep blood
121 agar plates and incubated for 18 h at 37°C with 5% CO₂

122 **Intranasal infection**

123 Mice were anesthetized with 1.5% pentobarbital and each was
124 inoculated intranasally with 30 µl sterile PBS containing 1×10⁸ CFU D39
125 or D39Δ*spd1672*. 48 h post challenge, mice were sacrificed and lung
126 homogenate, whole blood, and splenic homogenate were collected for the
127 following experiments.

128 **Histological analysis**

129 Lung tissues of mice from control group and infection group were
130 fixed by immersion in 10% neutral buffered formalin and embedded in
131 paraffin. And then 4-µm tissue sections were cut and stained with
132 hematoxylin and eosin (H&E) according to a standard protocol and
133 examined under light microscopy.

134 **Flow cytometric analysis of neutrophil and macrophage cell** 135 **percentages**

136 Blood was obtained via aspiration after right heart puncture, cells
137 were pelleted at 1,200 g for 10 minutes and resuspended in PBS, then
138 incubated with purified anti-Fc receptor blocking antibody
139 (antiCD16/CD32; eBioscience) before the addition of the specific
140 antibodies. A combination of fluorescein isothiocyanate
141 (FITC)-conjugated anti-Ly-6G antibody (BD Pharmingen, San Diego,
142 CA, USA) and allophycocyanin (APC)-conjugated anti-CD-11b antibody
143 (BD Pharmingen, San Diego, CA, USA) was used for neutrophil and
144 macrophage detection. The relative percentages were analyzed using a
145 Becton Dickinson FACSCalibur flow cytometer.

146 **Cytokines analysis**

147 Blood was collected, and serum was prepared as the same before.
148 Lung tissues were homogenized and centrifuged at 3,000 g for 20 min at
149 4°C. The supernatant was used for the determination of cytokines.
150 Cytokines (IFN- γ , TNF- α , IL-1 β , IL-6, and IL-17A) in the serum and
151 lung homogenate were quantitated by ELISA according to the
152 manufacturer's protocol (Biolegend, San Diego, USA). Adequate

153 dilutions of the serum and lung homogenate were necessary if the
154 concentration was beyond the detection range.

155 **Immunohistochemistry**

156 Mice were euthanized by an injection of pentobarbital sodium.
157 Lungs were inflation-fixed with 4% paraformaldehyde for 1 minute and
158 immersed in the same fixative. Tissues was fixed for 15 to 24 h at 4°C
159 and processed according to standard methods for paraffin-embedded
160 blocks. Immunohistochemistry was performed on 5-µm-thick sections
161 using antibodies generated to AKT, STAT3 and MAPK (Cell Signaling
162 Technology, Danvers, MA, USA).

163 **Statistical analysis**

164 All datas were expressed as the mean $\pm$ SD. Means were compared
165 between groups by using analysis of Student's *t*-test or Mann-Whitney
166 test. The program GraphPad Prism 6 was used for the analysis. A value of
167 $P < 0.05$ was considered statistically significant.

168

169 **Results**

170 **Systemic bacterial dissemination was impeded following**
171 **D39 Δ *spd1672* infection**

172 To investigate the effect of *spd1672* deletion on mice innate
173 immunity after *S. pn* invasion, Balb/c mice were intranasally infected
174 with 1×10^8 CFU each of the D39 and D39 Δ *spd1672* strains, and
175 bacterial load in the lung (Fig. 1A), whole blood (Fig. 1B), and spleen
176 (Fig. 1C) were measured at different time points. Compared to the D39
177 infection, the D39 Δ *spd1672* infection reduced bacterial burden and
178 attenuated bacterial dissemination from the lung to blood and spleen,
179 which suggested that pulmonary colonization of D39 Δ *spd1672* and
180 systemic bacterial dissemination were impeded.

181 **Pulmonary injury and inflammatory response were reduced in**
182 **D39 Δ *spd1672*-infected mice**

183 To further investigate the host damage caused by D39 Δ *spd1672*,
184 pathological changes in the lung tissue were evaluated 48 h post-infection.
185 Light microscopy images of the lung tissue revealed significant
186 differences among the control and infected groups. Inflammatory cell
187 infiltration was notably higher in the lung of the D39-infected mice than
188 in the control group. Destruction of the alveolar structure was observed in
189 the alveolar and interstitial tissues with severe swelling and bleeding in

190 the D39-infected mice. Slight changes in histological features were
191 observed in the lung tissues of the D39 Δ *spd1672*-infected group (Fig.
192 2A). A significant percentage of neutrophils and macrophages were also
193 detected in the blood (Fig. 2B). Neutrophil numbers increased when mice
194 were infected with D39, whereas their percentage decreased from 89.343 ± 2.701
195 to 68.643 ± 3.013 in mice infected with D39 Δ *spd1672*. The
196 relative percentage of macrophages was slightly lower in mice infected
197 with D39 Δ *spd1672* (4.223 ± 0.710) than with D39 (5.216 ± 1.167);
198 however, this was not statistically significant (Fig. 2C). Based on these
199 results, we concluded that the pulmonary inflammatory response was
200 highly attenuated in mice infected with the *spd1672* mutant.

201 **Reduced production of inflammatory cytokines in mice during** 202 **D39 Δ *spd1672* infection**

203 A series of immune responses is elicited after bacteria infect a host.
204 We analyzed cytokine expression to estimate the *S. pn*-induced
205 inflammatory response in mice. Compared to mice infected with D39, a
206 markedly reduced level of IFN- γ was detected in the whole blood and
207 lung homogenate of mice treated with D39 Δ *spd1672* (Fig. 3A, 3F).
208 Similarly, reduced production of TNF- α and IL-1 β was observed in the
209 blood when *spd1672* was deleted (Fig. 3B, 3C); however, this reduction

was not observed in the lung homogenate when mice were infected with the mutant strain (Fig. 3G, 3H). There were no significant differences in IL-6 levels (Fig. 3D, 3E) and IL-17A secretion (Fig. 3I, 3G) between D39- and D39 Δ *spd1672*-infected mice. These results confirmed that the release of inflammatory cytokines was modulated by *spd1672*.

***spd1672* deletion decreased the activation of STAT3 and AKT**

Regulation of inflammation and immunity is a characteristic feature of STAT3 signaling. Recent evidence showed that phosphorylated STAT3 is observed in the lungs of mice with bacterial or viral pneumonia. To further examine whether *spd1672* deletion influenced STAT3 and AKT, we investigated the levels of STAT3 and AKT in lung tissues. Immunohistochemical studies showed increased activation of STAT3 and AKT in D39-infected mice (Fig. 4, middle panel), whereas *spd1672* deletion hindered the upregulation (Fig. 4, right panel). Simultaneously, there was no significant difference in MAPK expression between the two infected groups. These results indicated that the AKT-STAT3 signaling pathway might regulate the interaction between the D39 Δ *spd1672* mutant and the host.

Discussion

230 In this study, we showed that infection with *spd1672*-deficient *S. pn*
231 D39 Δ *spd1672* attenuated systemic bacterial dissemination, alleviated
232 pulmonary injury, and reduced secretion of inflammatory cytokines in
233 mice. AKT-STAT3 signaling might be involved in this process.

234 *spd1672*, an in vivo-induced gene of *S. pn*, is involved in teichoic
235 acid synthesis (Wu et al. 2014a) and is related to the virulence of *S. pn*
236 (Jian et al. 2015). As the major component of *S. pn* CW, teichoic acid
237 performs crucial functions in cell envelope formation, cell division and
238 protection against antimicrobial peptides and antibiotics (van der Es et al.
239 2017). Notably, either as a surface antigen with species specificity or as a
240 PAMP, teichoic acid plays an important role in bacterial adhesion to host
241 cells, development of inflammation, and activation of immune response
242 (Weidenmaier and Peschel 2008). In our previous study, we constructed a
243 potential live attenuated pneumococcus vaccine SPY1 based on
244 D39 Δ *spd1672* and confirmed that SPY1 is capable of inducing protective
245 immune response against pneumococcal infections in mice (Gao et al.
246 2016; Wu et al. 2014a; Xu et al. 2015b). Based on these findings, we
247 speculated whether defective synthesis of *S. pn* teichoic acid caused by
248 *spd1672* deletion might subsequently affect the interaction between *S. pn*
249 and host innate immune response.

250 Bacterial load was determined to investigate D39 Δ *spd1672*
251 dissemination in mice after *spd1672* deletion. After 48 h of intranasal
252 infection, decreased pulmonary bacterial load was observed in the
253 D39 Δ *spd1672* infected-mice than in the D39 infected-mice, which was
254 consistent with the previous observation that intraperitoneal injection
255 with D39 Δ *spd1672* showed a lower blood bacterial load (Jian et al. 2015).
256 Furthermore, the bacterial loads in the whole blood and spleen were
257 lower following D39 Δ *spd1672* infection than after D39 infection,
258 suggesting that *spd1672* deficiency contributed to reduced bacterial
259 colonization and dissemination.

260 *S. pn* invasion of host induces a systemic inflammatory response. As
261 the first line of defense against infection, the host innate immune system
262 constitutes the earliest response to invading microbes. Neutrophils, a
263 crucial component of the innate immune system, are the major innate
264 immune cells responsible for tissue damage to the host (Selders et al.
265 2017). In present study, hematoxylin-eosin staining of the D39-infected
266 mice lung tissues showed distinct inflammation and abundant neutrophil
267 infiltration around the perivascular areas and bronchiole cavities, whereas
268 the degree of inflammatory response stimulated by D39 Δ *spd1672* was
269 slightly weaker. These results suggested that *spd1672* may enhance local
270 inflammatory response by recruiting neutrophil infiltration and thereby

271 aggravate pulmonary damage. Nevertheless, the macrophage percentage
272 in the lung did not differ significantly between D39 Δ *spd1672*-infected
273 and D39-infected mice, which is possibly because *spd1672* deficiency
274 does not influence the local recruitment of macrophages at the site of
275 infection.

276 Compared to the D39-infected mice, downregulation of IFN- γ ,
277 TNF- α , and IL-1 β were observed in the blood of
278 D39 Δ *spd1672*-stimulated mice; however, only lower IFN- γ level was
279 observed in the lung homogenate. The levels of IL-6 and IL-17A were
280 not significantly different in mice infected with D39 and D39 Δ *spd1672*.
281 Since D39 Δ *spd1672* reduced the secretion of IFN- γ , TNF- α , and IL-1 β in
282 mice, we speculate that the *spd1672*-encoded protein may be a PAMP,
283 which is recognized by corresponding receptors to subsequently initiate a
284 signaling cascade and induce the release of inflammatory cytokines
285 during pneumococcal infection. In a follow-up study, we intend to purify
286 the *spd1672*-encoded protein and determine whether it acts as a PAMP
287 and the underlying mechanisms.

288 IFN- γ , a Th1 cytokine, is an important stimulus for modulating
289 effective innate immune response against pathogens (Maher et al. 2007).
290 The biological effects of IFNs are primarily mediated via activation of the

291 JAK/STAT pathway (Majoros et al. 2017). Previously,
292 tyrosine-phosphorylated STAT3 was detected in the lungs of mice with
293 bacterial or viral pneumonia or acute inflammation (Gao et al. 2004;
294 Hokuto et al. 2004; Ikegami et al. 2008; Kida et al. 2008; Quinton et al.
295 2008; Severgnini et al. 2004). In this study, we observed an increased
296 production of IFN- γ both in the blood and lung of D39-infected mice.
297 Simultaneously, lung tissue immunohistochemistry confirmed that
298 activation of AKT and STAT3 was elevated during D39 infection. In the
299 absence of *spd1672* expression, the upregulation of IFN- γ and STAT3
300 was impeded, indicating that *spd1672* may facilitate cytokine release by
301 IFN- γ -induced activation of STAT3. A previous study showed that
302 MAPKs mediate the phosphorylation of serine residues in STATs
303 (Decker and Kovarik 2000), whereas our results indicate that MAPK
304 levels did not vary significantly between D39 and D39 Δ *spd1672*
305 infected-mice. The signaling pathway via which *spd1672* deficiency
306 affects mice innate immune response requires further investigation.

307 In summary, we reported for the first time that expression of
308 *spd1672*, a *S. pn* in vivo-induced gene, is vital for bacterial growth and
309 dissemination. *spd1672* deletion impeded pulmonary inflammatory
310 response and release of proinflammatory cytokines, which may be
311 associated with AKT-STAT3 signaling. Our observations provide a new

avenue for understanding host-pathogen interactions and might assist in
designing therapeutic strategies against streptococcal infection in the
future.

315

316 **Acknowledgments**

This work was supported by National Natural Science Foundation
grants of China (grant numbers 81460317 and 31500749); Chongqing
Natural Science Foundation (grant number cstc2015jcyjA10102).

320 **Conflict of interest**

The authors declare that they have no competing interest.

322

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424

425 Figure Captions

426 **Fig.1. Systemic bacterial dissemination was impeded following**
427 **D39 Δ *spd1672* infection.** Balb/c mice were anaesthetized with 1.5%
428 pentobarbital and intranasally infected with 30 μ l D39 and D39 Δ *spd1672*
429 containing 1×10^8 CFU. Different time post infection, bacterial load in
430 lung (A), blood (B) and spleen (C) of infected animals was determined by
431 seeding serial dilution of homogenized tissues on blood agar plate (n=6).
432 The data are expressed as the mean $\pm$ SD (n=3), results are representative
433 of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P <$
434 0.001 between the groups are indicated by horizontal lines.

435

436 **Fig.2. Pulmonary injury and inflammatory response were decreased**
437 **in D39 Δ *spd1672*-infected mice.** Mice were intranasal instillation of D39
438 and D39 Δ *spd1672* (1×10^8 CFU), after 48 h of infection, mice were

sacrificed. (A) Lungs were isolated and stained with Hematoxylin-eosin. Images are representative of staining observed in the lungs of mice within the group (n = 4 to 6 mice). (B, C) The percentage of neutrophils and macrophages in blood were measured by flow cytometry, the data are expressed as the mean $\pm$ SD (n=3), results are representative of three independent experiments. *** $P < 0.001$ between the groups is indicated by horizontal lines.

Fig.3. Reduced production of inflammatory factors in mice during D39 Δ *spd1672* infection. Balb/c mice (n=3) were intranasally instilled with 1×10^8 CFU of D39 or D39 Δ *spd1672*. 48 h after challenged, mice were sacrificed, the levels of IFN- γ , TNF- α , IL-1 β , IL-6, and IL-17A in whole blood and lung homogenate were assessed by ELISA. Data are the mean $\pm$ SD. The results shown are representative of three independent experiments. * $P < 0.05$ and *** $P < 0.001$ between the groups are indicated by horizontal lines.

Fig.4. *spd1672* deletion decreased the activation of STAT3 and AKT. Mice lungs were isolated after 48 h of intranasal instillation. The levels of STAT3, AKT, and MAPK in the infected mice lungs were determined via

459 immunohistochemistry. Images are representative of staining observed in
460 the lungs of mice within the group ($n=3$). Right panel, statistical analysis
461 of the mean IOD (Integral Optical Density) for STAT3, AKT, and MAPK
462 performed using Image-Pro Plus software. Data are the mean $\pm$ SD. The
463 results shown are representative of three independent experiments. $**P <$
464 0.01 and $***P < 0.001$ between the groups are indicated by horizontal
465 lines, ns, no significant.

466

Draft

Figure 1

Figure 2

Figure 3

Figure 4