1 **An Enterobacterial Common Antigen Mutant of *Salmonella enterica* serovar Typhimurium**
2 **as a Vaccine Candidate**

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24 **ABSTRACT**

25 Due to increasing rates of invasive *Salmonella enterica* serovar Typhimurium infection, there is a
26 need for an effective vaccine to prevent this disease. Previous studies showed that a mutation in
27 the first gene of the Enterobacterial common antigen biosynthetic pathway, *wecA*, resulted in
28 attenuation of *S. Typhimurium* in a murine model of salmonellosis. Furthermore, immunization with
29 a *wecA*⁻ strain protected against lethal challenge with the parental wild type *S. Typhimurium* strain.
30 Herein, we examined whether the *S. Typhimurium wecA*⁻ strain could also provide cross-protection
31 against non-parental strains of *S. Typhimurium* and *S. Enteritidis*. We found that intraperitoneal
32 immunization (IP) with *S. Typhimurium* SL1344 *wecA*⁻ resulted in a significant increase in survival
33 compared to control mice for all *Salmonella* challenge strains tested. Oral immunization with
34 SL1344 *wecA*⁻ also resulted in increased survival; however, protection was less significant than
35 with intraperitoneal immunization. The increase in survival of SL1344 *wecA*⁻ immunized mice was
36 associated with a *Salmonella*-specific IgG antibody response. Furthermore, analysis of sera from
37 IP and orally immunized animals revealed cross-reactive antibodies to numerous *Salmonella*
38 isolates. Functional analysis of antibodies found within the sera from IP immunized animals
39 revealed agglutination and opsonophagocytic activity against all tested O:4 *Salmonella* serovars.
40 Together these results indicate that immunization with a *S. Typhimurium wecA*⁻ strain confers
41 protection against lethal challenge with wild type *S. Typhimurium* and *S. Enteritidis* and that
42 immunization correlates with functional antibody production.

43

44 **Keywords:** *Salmonella* Typhimurium, *Salmonella* Enteritidis, vaccine, live-attenuated, antibody,
45 *wecA*

46 **ABBREVIATIONS**

47 ECA: Enterobacterial common antigen; gm: gentamicin; IP: intraperitoneal; LVS: live-vaccine
48 strain; MTD: Mean time to death; NTS: Non-typhoidal salmonellae; ODU: OD unit/mL; slgA:
49 secretory IgA; T3SS: Type III secretion system

50

51 **INTRODUCTION**

52 Non-typhoidal salmonellae (NTS), the causative agents of gastroenteritis, are transmitted to
53 humans through ingestion of contaminated meat, eggs, and processed food (Feasey *et al.*, 2012).
54 Although NTS infections can be caused by over 2,500 serovars of *S. enterica* (Grimont and Weill,
55 2007; Jones *et al.*, 2008), the majority of NTS cases are caused by *S. Typhimurium* and *S.*
56 Enteritidis (Reddy *et al.*, 2010). Together NTS are responsible for over 93.8 million cases of
57 gastroenteritis worldwide and 155,000 deaths annually (Majowicz *et al.*, 2010). In the United
58 States, the overall incidence of *Salmonella* infection has remained steady ((CDC), 2014); however,
59 a rise in the incidence of invasive NTS disease has occurred (Chimalizeni *et al.*, 2010). In
60 developed countries the most vulnerable patient populations for invasive NTS infections are
61 children under the age of 3 months, the elderly, and those who are immunocompromised; 45 to
62 50% of these infections are attributed to *S. Typhimurium* and *S. Enteritidis* (Parry *et al.*, 2013;
63 Tennant *et al.*, 2011). While the overall mortality rate associated with NTS infections in developed
64 countries is less than 2%, approximately 7% of these NTS infections are invasive; 5% of these are
65 then fatal (Chimalizeni *et al.*, 2010; Feasey *et al.*, 2012; Tennant *et al.*, 2011).

66 NTS-mediated disease causes an estimated 2.5 million cases of disease in less developed
67 regions of the world, such as sub-Saharan Africa, with approximately 4,100 deaths per year
68 (Feasey *et al.*, 2012). In the sub-Saharan African population, individuals that succumb to NTS
69 infection are mainly children younger than 3 years, individuals infected with malaria, and HIV-

70 infected adults (Feasey *et al.*, 2012; Morpeth *et al.*, 2009). Mortality rates for invasive NTS
71 infections in that region range from 4.4-27% for children and from 22-47% for adults (Morpeth *et*
72 *al.*, 2009). The increased invasiveness of NTS in sub-Saharan Africa has been linked to the
73 emergence of a more virulent clade of *S. Typhimurium* known as ST313 (Feasey *et al.*, 2012;
74 Kingsley *et al.*, 2009).

75 To date, only two *Salmonella* vaccines have been approved for human use and are targeted
76 against *S. Typhi* for typhoid fever (Engels *et al.*, 1998; Fraser *et al.*, 2007; Khan *et al.*, 2010).
77 However, due to a rise in antibiotic resistance of NTS (Feasey *et al.*, 2012; Lunguya *et al.*, 2013),
78 the emergence of the highly antibiotic resistant and virulent ST313 strain in sub-Saharan Africa
79 (Kingsley *et al.*, 2009; Okoro *et al.*, 2012), and the increasing severity of NTS-mediated disease in
80 developed countries, a pressing need exists for the development of an effective vaccine to prevent
81 NTS infection. Vaccine strategies for NTS have included: 1) live-attenuation of housekeeping
82 genes or genes that are important for essential biosynthetic processes such as Δaro (Hormaeche
83 *et al.*, 1990, 1991), Δdam (Heithoff *et al.*, 2001), $\Delta crp/\Delta cdt$ (Zhang *et al.*, 1999; Zhang *et al.*,
84 1997b), $\Delta relA/\Delta spoT$ (Na *et al.*, 2006), or $\Delta guaA/\Delta clpP/\Delta fliD$ (Tennant *et al.*, 2011); 2) conjugate
85 vaccines consisting of conjugation of the lipopolysaccharide (LPS)-derived serovar-specific O-
86 polysaccharide to protein (Jazani *et al.*, 2005; Simon *et al.*, 2011b; Simon *et al.*, 2013; Svenson *et*
87 *al.*, 1979; Watson *et al.*, 1992); and 3) subunit vaccines utilizing peptides from the type 3 secretion
88 system (T3SS) (Kurtz *et al.*, 2014), fimbriae (Pang *et al.*, 2013), and flagellin (Bobat *et al.*, 2011).
89 Nevertheless, despite years of research no licensed human NTS vaccines currently exist. The lack
90 of success in the development of such vaccines is due in part to unwanted reactogenicity and/or
91 poor immunogenicity of vaccine candidates tested to date (Tennant *et al.*, 2011).

92 Our group previously characterized *S. Typhimurium* TML and SL1344 strains that contained
93 a mutation in the *wecA* gene, which encodes a key component in Enterobacterial common antigen
94 (ECA) biosynthesis (Gilbreath *et al.*, 2012). ECA is a cell-surface glycolipid produced by members
95 of the Enterobacteriaceae family that has been shown to play a role in virulence in several
96 bacterial pathogens, including *S. Typhimurium* (Chaudhuri *et al.*, 2009; Monack *et al.*, 2004;
97 Ramos-Morales *et al.*, 2003; Valtonen *et al.*, 1976), *Escherichia coli* O157:H7 (Barua *et al.*, 2002),
98 *Yersinia* sp. (Zhang *et al.*, 1997a), and *Haemophilus ducreyi* (Banks *et al.*, 2008). Through studies
99 utilizing *Salmonella*, it was found that ECA is devoid of endotoxin-like activity (Mayer and Schmidt,
100 1979), absence of ECA results in bile salt sensitivity (Ramos-Morales *et al.*, 2003), and anti-ECA
101 antibodies do not passively protect against salmonellosis (Albertson *et al.*, 2003). Furthermore,
102 ECA deficient *S. Typhimurium* (*wecA*⁻) are attenuated *in vivo* (Chaudhuri *et al.*, 2009; Gilbreath *et*
103 *al.*, 2012; Valtonen *et al.*, 1976), retain expression of wild type (WT) O-4 antigen and LPS profiles,
104 persistently colonize the spleen and liver of mice at low levels, and induce a protective immune
105 response when used as a live-attenuated vaccine (Gilbreath *et al.*, 2012). The studies described
106 herein determined whether a *S. Typhimurium* *wecA* mutant could induce immunity against non-
107 parental *S. Typhimurium* strains or against non-Typhimurium serovars of *S. enterica*. Furthermore,
108 the mechanism behind the observed protective immunity was investigated.

109

110

111 MATERIALS AND METHODS

112 **Bacterial strains, eukaryotic cells, and growth conditions.** Bacterial strains and plasmids used
113 in the study are listed in Supplementary Table 1. Bacterial stocks were maintained in Trypticase
114 Soy Broth (TSB; BD Biosciences; Franklin Lakes, NJ) containing 40% glycerol at -80°C. Bacteria
115 were routinely cultured in TSB or TSB containing 20 µg/ml gentamicin (gm) where applicable at
116 37°C.

117 J774A.1 murine macrophages (ATCC TIB-67) (kindly provided by Anthony T. Maurelli,
118 Uniformed Services University) were cultured in Dulbecco's modified Eagle medium (DMEM)
119 containing 4.5 g/L D-glucose, 584g/L L-glutamine, and 110 mg/L sodium pyruvate (Quality
120 Biological, Inc.; Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco; Grand
121 Island, NY) at 37°C in 5% CO₂ as directed by ATCC specifications.

122

123 **Mice.** All animal protocols were approved by the Uniformed Services University of the Health
124 Sciences Animal Care and Use Committee (Protocol number MIC-11-815) and were conducted in
125 accordance with guidelines established by the National Institutes of Health. Five-seven week old
126 female C57BL/6 or BALB/c mice (Jackson laboratories; Bar Harbor, ME) were used as indicated
127 below.

128

129 **Immunization and lethal *Salmonella* challenge.** Prior to immunization, SL1344 *wecA*⁻ was
130 cultured for 12-18 hr in TSB, diluted 1:100 in TSB, and grown to an optical density at 600 nm
131 (OD₆₀₀) of 0.5-0.9. The culture was then diluted to make a suspension of 0.5 OD unit/mL (ODU) in
132 isotonic saline, which was then further diluted as needed for the respective immunization doses.
133 Doses given by intraperitoneal injection (IP) and oral gavage were inoculated in a volume of 0.2

134 mL. To investigate the ability of SL1344 *wecA*⁻ to protect against lethal *Salmonella* challenge, mice
135 were immunized with 1.3×10^6 CFU of SL1344 *wecA*⁻ by oral gavage or with 8.0×10^2 CFU IP 28
136 days prior to lethal challenge. Control mice were mock-immunized with 0.2 ml isotonic saline by
137 oral gavage or IP injection. For oral immunization only, mice were fasted for 4 hr prior to and 1 hr
138 post-inoculation. Exact SL1344 *wecA*⁻ doses were determined via serial dilution and plating
139 following immunization. Mice were monitored for 28 days post-immunization for mortality or onset
140 of severe disease symptoms.

141 For lethal challenge studies, WT SL1344, WT TML, or WT *S. Enteritidis* R11 (kindly
142 provided by Raphael Simon and Sharon Tennant, University of Maryland School of Medicine;
143 Baltimore, MD) were grown in TSB and processed as described above. To determine if SL1344
144 *wecA*⁻ could protect against WT strains of *S. Typhimurium*, C57BL/6 mice were challenged with 5.4
145 $\times 10^5$ or 9.0×10^7 CFU of WT SL1344 (reported oral LD₅₀ is $\sim 1.0 \times 10^5$ to 1.0×10^6 CFU)
146 (Humphreys *et al.*, 2003; Monack *et al.*, 2000) or with 3.4×10^4 or 7.0×10^5 CFU of WT TML
147 (reported LD₅₀ is 4.0×10^4 CFU) (O'Brien *et al.*, 1984) by oral gavage. To evaluate whether
148 immunization with the *wecA* mutant strain could protect against infection with *S. Enteritidis* R11,
149 C57BL/6 mice were challenged with 6.6×10^2 or 2.2×10^5 CFU of the R11 strain by IP injection (IP
150 LD₅₀ is less than 10 CFU) (R. Simon, personal communication). BALB/c mice were challenged with
151 2.2×10^6 or 2.0×10^6 CFU of the R11 strain by oral gavage (reported oral LD₅₀ is 2.0×10^4 CFU)
152 (Tennant *et al.*, 2011) to determine the efficacy of immunization with SL1344 *wecA*⁻ against oral
153 infection. Mice were monitored daily for 31 days for mortality following all challenges and all
154 animals that displayed extreme signs of morbidity, including hunched posture and shallow
155 breathing, were euthanized.

156

157 **Fecal pellet and sera collection.** Between days 25-27 post-immunization, mice were separated
158 into individual cages and approximately 0.1 g of fecal pellet material/mouse was collected for
159 secretory IgA (sIgA) analysis by ELISA. After harvesting, fecal pellets were frozen at -80°C until
160 processed. Fecal pellets were thawed in 500 µl fecal extraction buffer (10% goat serum (Sigma-
161 Aldrich; St. Louis, MO), 0.1% kathon (SUPELCO; St. Louis, MO) in phosphate buffered saline
162 (PBS)) and vortexed for 30 min to homogenize the pellet material. Fecal homogenates were then
163 centrifuged at 14,000 rpm for 10 min. The supernatant from each sample was collected and
164 transferred to a new tube and frozen at -80°C.

165 To analyze pre-challenge sera for the presence of Ig and IgG antibodies, blood was
166 collected between days 25-27 post-immunization from each individual mouse via the tail-vein. To
167 evaluate the presence of antibodies post-challenge, blood was collected via cardiac puncture from
168 all surviving mice on day 31 for mice challenged with *Salmonella*. Following collection, the blood
169 was allowed to coagulate at room temperature for 1 hr. The samples were then centrifuged at
170 14,000 rpm for 10 min, and the sera was transferred to a new tube and frozen at -80°C.

171

172 **ELISA analysis of serum and fecal antibodies.** For determination of anti-*Salmonella* Ig and IgG
173 antibody responses, liquid overnight cultures of SL1344 *wecA*⁻ were pelleted and re-suspended in
174 PBS to a density of approximately 1 ODU/mL. Immulon 4HBX (Thermo Scientific; Waltham, MA)
175 ELISA plates were coated with 50 µl of the 1 ODU/mL suspension of SL1344 *wecA*⁻ and were
176 incubated overnight at 4°C, followed by blocking with PBS containing 10% FBS (Gibco) for 1 hr at
177 room temperature. Serum samples were diluted in PBS containing 0.05% tween (PBST) starting at
178 1:20 followed by 1:4 serial dilutions. Serially diluted serum was added in duplicate (50 µL) to the
179 ELISA plates and incubated overnight at 4°C. Ig was detected for 1 hr at room temperature using

180 50 μ L of a 1:2000 dilution of biotin labeled goat anti-mouse Ig primary (Cat# 553999; Lot 16325;
181 BD Biosciences) followed by 50 μ L of a 1:3000 dilution of a Streptavidin-AKP secondary (Cat#
182 554065; Lot 08661; BD Biosciences) in PBST. IgG antibodies were detected using a 1:750 dilution
183 of AKP-conjugated rabbit anti-mouse IgG (Cat# 401262; Lot D00096130; Calbiochem, Billerica,
184 MA) in PBST for 1 hr at room temperature. For analysis of anti-*Salmonella* sIgA from fecal pellet
185 supernatants, plates were coated as indicated above and incubated overnight at 4⁰C; plates were
186 then blocked with 200 μ L of PBS containing 1% bovine serum albumin (BSA; Calbiochem) and
187 incubated at room temperature for 2 hrs. Undiluted fecal pellet supernatants (50 μ L) were added in
188 duplicate to ELISA plates and incubated overnight at 4⁰C. sIgA was then detected for 1 hr at room
189 temperature with 50 μ L of a 1:1000 dilution of rat anti-mouse IgA-Biotin (Clone 11-44-2; Lot I4212-
190 ZA02; Southern Biotech; Birmingham, AL) followed by a 1:2000 dilution of Streptavidin-AKP
191 secondary (Cat# 554065; Lot 08661; BD Biosciences) in PBST. All antibodies were detected
192 following incubation with 100 μ L 4-nitrophenyl phosphate (PNP; Sigma-Aldrich) in TM buffer (0.5M
193 Tris-base, 300 μ M MgCl₂, pH 9.8) at a concentration of 1 mg PNP/mL TM buffer. ELISA plates
194 were read at an absorbance of 405 nm on a SpectraMax M2 plate reader (Molecular Devices;
195 Downingtown, PA). Serum Ig and IgG titers were calculated following determination of a standard
196 curve and are reported as titer units. sIgA titers are reported as OD units.

197

198 **Analysis of antibody functionality.** Fifteen animals per group were immunized with 2.0×10^6
199 CFU of SL1344 *wecA*⁻ by oral gavage, with 8.2×10^2 CFU of SL1344 *wecA*⁻ IP, or mock-immunized
200 with isotonic saline as described above. Twenty-eight days post-immunization, blood was collected
201 from each individual mouse via cardiac puncture and allowed to coagulate at room temperature for
202 1 hr. The samples were then centrifuged at 14,000 rpm for 10 min, and the sera was pooled for

203 each immunization group and frozen at -80°C until use.

204 **i) Cross-reactive antibody identification following immunization with SL1344 *wecA*⁻.** To
205 determine if immunization with SL1344 *wecA*⁻ induced the production of cross-reactive antibodies
206 to various *Salmonella* strains, overnight cultures of *S. Typhimurium* strains SL1344, SL1344 *wecA*⁻
207 , and TML, along with *S. Enteritidis* R11, *S. Stanleyville* J65, *S. Dublin* (kindly provided by Raphael
208 Simon and Sharon Tennant; University of Maryland School of Medicine), and *S. Javiana* (kindly
209 provided by Carol Sandt; Pennsylvania Department of Health; Harrisburg, PA) were pelleted and
210 resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% SDS, 10%
211 glycerol, and 1 Mini-Complete EDTA-Free Proteinase Inhibitor Cocktail tablet (Roche; Indianapolis,
212 IN)). Protein concentration was analyzed using the BCA protein assay (Pierce; Rockford, IL). Equal
213 amounts of protein were electrophoresed in quadruplicate on 8% Bis-Tris gels (Novex by Life
214 Technologies; Grand Island, NY) and transferred to nitrocellulose membranes, which were blocked
215 with PBST containing 5% BSA (Calbiochem). Membranes were probed with a 1:500 dilution of
216 sera in PBST pooled from animals as described above that were immunized orally with SL1344
217 *wecA*⁻ or IP with SL1344 *wecA*⁻. Membranes were also probed with a 1:500 dilution of sera pooled
218 from animals mock-immunized as described above. Antibodies were detected with a 1:6700
219 dilution of an anti-mouse HRP secondary (SC-2055; Lot F2413; Santa Cruz Biotechnology, Inc.,
220 Dallas, TX), ECL Western Blotting Substrate (Pierce), and an ImageQuant LAS4000 Luminescent
221 Image Analyzer (GE Healthcare; Laurel, MD) using ImageQuant LAS4000 Software (GE
222 Healthcare).

223 **ii) Serum agglutination.** To measure the presence of functional antibody from immunized
224 animals, *S. Typhimurium* strains SL1344, SL1344 *wecA*⁻, and TML, along with *S. Enteritidis* R11,
225 *S. Stanleyville* J65, and *S. Dublin* that had been cultured overnight on LB Lennox plates were

226 mixed with 20 μ l PBS on glass slides and combined with 5-15 μ l of undiluted sera from IP
227 immunized animals for 5-10 minutes. As a control for serotype, all *Salmonella* strains were also
228 incubated with either O4 (Cat#23839, Lot#876E-H20; Statens Serum Institute, Denmark) or O9
229 (Cat# 228181, Lot# 4206902; BD Biosciences) diagnostic antisera. Agglutination was determined
230 by microscopy.

231 **iii) Opsonophagocytic uptake.** To measure additional functional antibody levels from immunized
232 animals, serum-mediated uptake by macrophages was examined by an opsonophagocytic assay
233 as described by (Tennant *et al.*, 2011). Briefly, J774A.1 mouse macrophages were seeded at $1 \times$
234 10^5 for 2 days in 24-well plates (Costar, Corning Inc.; Corning, NY). Cells were then washed with
235 sterile PBS and replaced with culture medium. Overnight *Salmonella* cultures were diluted 1:1000
236 in TSB, except for *S. Enteritidis* R11, which was diluted 1:300, and incubated at 37°C until an
237 OD₆₀₀ of 0.2. Ninety microliters from a 1:20 dilution of the 0.2 OD₆₀₀ cultures was incubated for 20
238 min at room temperature with 10 μ l of PBS, heat-inactivated serum (heat inactivated at 55°C for 20
239 min), or O4 (SSI) or O9 (BD Biosciences) diagnostic antisera. Twenty microliters of the cell/sera
240 suspension was then added to the J774A.1 cells, and incubated for 1 hour at 37°C, 5% CO₂. The
241 cells were next washed once with sterile PBS and incubated for 1 hour at 37°C, 5% CO₂ with
242 medium containing 100 μ g/ml gm to kill any extracellular bacteria. Following gm treatment, the
243 cells were washed three times in sterile PBS and lysed in 1 ml of 0.5% (wt./vol) Triton X-100 in
244 PBS. Intracellular bacteria were enumerated in duplicate for each strain from two-three
245 independent experiments. Percent opsonization was calculated by dividing the number of
246 intracellular bacteria by the inoculum and multiplying by 100.

247 **Data analysis and statistics.** All survival curves and graphs were created using GraphPad Prism
248 6 (GraphPad Software, Inc.; La Jolla, California). Differences in percent survival were determined

249 by Kaplan-Meier analysis using SPSS Statistics Version 20 (IBM; Armonk, NY). Mean time to
250 death (MTD) was calculated as described in (Gilbreath *et al.*, 2012). A one-way analysis of
251 variance (ANOVA) was used to evaluate differences in ELISA antibody titers and a two-way
252 ANOVA was used to evaluate differences in opsonophagocytic uptake (GraphPad Software, Inc.;
253 La Jolla, CA). Multiple comparisons were then analyzed by a Tukey's multiple comparison test with
254 a single pooled variance (GraphPad Software, Inc.). Differences in antibody titers between non-
255 survivors and survivors were evaluated using an unpaired Student's *t*-test with Welch's correction
256 (GraphPad Software, Inc.). Differences in pre-challenge vs. terminal antibody titers were
257 determined using a paired Student's *t*-test for groups of 3 or more animals (GraphPad Software,
258 Inc.). For all data, a two-sided α level set at 0.05 determined significance. All ELISA data were
259 displayed as the geometric mean $\pm$ 95% confidence interval (Bland and Altman, 1996). The
260 percent of opsonophagocytic uptake is displayed as mean $\pm$ the standard error of the mean (SEM).

261

262 **RESULTS**

263 **A *wecA* mutant strain protects against lethal parental *S. Typhimurium* challenge.** Our
264 previous studies showed that oral and IP immunization with the TML *wecA*⁻ strain, or oral
265 immunization with the *S. Typhimurium* SL1344 *wecA*⁻ strain provided protection against lethal
266 challenge with the WT parental strain (Gilbreath *et al.*, 2012). *S. Typhimurium* SL1344, a virulent
267 mouse strain originally isolated from cattle, has been widely utilized as a model of *Salmonella*
268 pathogenesis and in vaccine development (Qi *et al.*, 1996; Wray and Sojka, 1978). Therefore, to
269 confirm and expand on our previous results, mice were orally immunized with SL1344 *wecA*⁻ and
270 then orally challenged with WT SL1344. As expected, oral immunization protected against lethal
271 challenge with WT SL1344 compared to mock-immunized control mice (Table 1). Since our

272 previous SL1344 *wecA*⁻ immunization studies only used an oral immunization route, we next
273 sought to determine if IP immunization with SL1344 *wecA*⁻ also conferred protection against WT
274 SL1344 challenge. As such, mice were immunized IP with SL1344 *wecA*⁻ and then challenged with
275 WT SL1344. Following oral challenge, all mice mock-immunized IP succumbed to infection,
276 whereas 87.5% of mice receiving the SL1344 *wecA*⁻ IP immunization survived (Table I). To test the
277 boundaries of this protection, we next immunized mice orally or IP with SL1344 *wecA*⁻ followed by
278 challenge with an increased dose of WT SL1344. As previously observed, there was a significant
279 increase in the survival rate of the immunized mice compared to the mock-immunized group for
280 both routes of immunization (Table 1). *En masse*, these results confirm and extend our previous
281 studies that indicate that oral immunization with SL1344 *wecA*⁻ provides protection against oral
282 challenge with WT SL1344. Furthermore, protection against WT SL1344 challenge could be
283 extended to mice immunized IP with SL1344 *wecA*⁻. Finally, significant levels of protection were
284 still evident even when the challenge dose was dramatically increased.

285

286 **SL1344 *wecA*⁻ protects against *S. Typhimurium* strain TML.** To determine if immunization with
287 the SL1344 *wecA* mutant could provide cross-protection against lethal challenge with a non-
288 parental serogroup O:4 *S. Typhimurium* strain, we selected *S. Typhimurium* TML for heterologous
289 challenge. *S. Typhimurium* TML is a clinical isolate from a patient with gastroenteritis, which is
290 known to invade and to induce fluid secretion from rabbit ileal loops, and to be virulent in a mouse
291 model of salmonellosis (Giannella *et al.*, 1973). TML was specifically selected to test the
292 boundaries of protection, since the reported LD₅₀ for TML in a mouse model of salmonellosis is
293 less than that of WT SL1344 (Humphreys *et al.*, 2003; Monack *et al.*, 2000; O'Brien *et al.*, 1984).
294 For these studies, mice were immunized orally or IP with the SL1344 *wecA* mutant followed by oral

challenge with *S. Typhimurium* strain TML (Table 1). All immunized mice survived lethal challenge with WT TML as compared to the mock-immunized controls (Table 1). Based on the low mortality rate of the oral mock-immunized animals, the increase in survival with SL1344 *wecA*⁻ immunization was only statistically significant for the IP immunized group (Table 1).

Since a large number of the mice mock-immunized orally and IP survived TML challenge, we repeated this experiment increasing the TML challenge dose. At this challenge dose, all control mice succumbed to infection. Furthermore, there was a significant difference in survival between the immunized animals compared to the mock-immunized animals (Table 1). Together, these results indicate that SL1344 *wecA*⁻ can protect against lethal challenge by a non-parental *S. Typhimurium* strain.

A *wecA* mutant strain of *S. Typhimurium* cross-protects against lethal *S. Enteritidis* infection. Due to the rise in invasive NTS infections that can be attributed to both *S. Typhimurium* and *S. Enteritidis* (Parry *et al.*, 2013; Tennant *et al.*, 2011), the use of a live-attenuated *S. Typhimurium* vaccine that could also protect against serogroup O:9 *S. Enteritidis* infection would be beneficial. For these studies, a clinically relevant *S. Enteritidis* isolate, R11, was chosen; R11 was isolated from a blood culture in Mali (Tennant *et al.*, 2010). To determine if the *S. Typhimurium* SL1344 *wecA*⁻ strain could protect against lethal challenge with *S. Enteritidis* R11, mice were immunized orally or IP with SL1344 *wecA*⁻ and challenged IP with *S. Enteritidis* R11 (Table 2). Of the mice that received oral immunization with SL1344 *wecA*⁻, the survival rate was 11.1%; though low, this percent survival was significantly higher than the mock-immunized controls (Table 2). Furthermore, the survival rate and level of significance was higher for mice that received IP immunization with SL1344 *wecA*⁻ as compared to the IP mock-immunized control group (Table 2).

318 As the *S. Enteritidis* R11 IP LD₅₀ is less than 10 CFU (R. Simon, personal communication),
319 and because we saw some protection at lower challenge doses, we sought to determine if SL1344
320 *wecA*⁻ could protect against a substantially increased R11 IP challenge dose. For this experiment,
321 mice were immunized orally or IP and challenged IP with R11 at dose that was 22,000 times
322 greater than the IP LD₅₀ (Table 2). During this experiment, only mice immunized IP with SL1344
323 *wecA*⁻ survived the increased challenge dose compared to mock-immunized controls (Table 2).
324 Together, these data indicate that oral and IP immunization with SL1344 *wecA*⁻ can provide cross-
325 protection against low challenge doses with serogroup D *S. Enteritidis* R11. Moreover, at challenge
326 doses substantially greater than the IP LD₅₀, IP immunization with SL1344 *wecA*⁻ provided cross-
327 protection against *S. Enteritidis* R11 infection.

328 As salmonellosis is typically acquired via oral ingestion (Simon *et al.*, 2011a), we next
329 sought to determine if SL1344 *wecA*⁻ could protect against oral challenge with *S. Enteritidis* R11.
330 For these experiments, mice were immunized with SL1344 *wecA*⁻ and then orally challenged with
331 *S. Enteritidis* R11 (Table 2). As shown in Table 2, mice that were immunized orally with SL1344
332 *wecA*⁻ survived challenge with *S. Enteritidis* R11, whereas none of the mock-immunized animals
333 survived. When mice were immunized IP with a low does of SL1344 *wecA*⁻, there was no
334 difference in survival when compared to the mock-immunized controls. To determine if these
335 results were due to the low immunization dose, the experiment was repeated with increased oral
336 and IP SL1344 *wecA*⁻ immunization doses; the increased vaccination doses resulted in a
337 statistically significant difference in the survival rate when compared to the mock-immunized
338 controls (Table 2). Taken together, these results show that immunization with SL1344 *wecA*⁻ cross-
339 protects against lethal IP and oral *S. Enteritidis* R11 challenge. Furthermore, consistent with the

340 data displayed in Table 1, the data suggest that IP immunization provides better protection than
341 oral immunization.

342

343 **Protection conferred by SL1344 *wecA*⁻ correlates with antibody titer.** To determine if an
344 antibody-mediated immune response could be responsible for the protection conferred by
345 immunization with SL1344 *wecA*⁻, we assayed sera collected from the animals described in the first
346 set of immunization experiments (Tables 1 and 2). Serum titers were analyzed based on
347 immunization dose groups; therefore, the data include mice that were ultimately challenged orally
348 with WT SL1344 or TML, or IP with *S. Enteritidis* R11; those animals were immunized at the same
349 time. There was a statistically significant increase in serum Ig and IgG antibody titers in mice that
350 were immunized with SL1344 *wecA*⁻ as compared to the respective mock-immunized controls for
351 both routes of immunization (Fig. 1A and 1B). Furthermore, mice immunized IP with SL1344 *wecA*⁻
352 had significantly higher titers of Ig and IgG as compared to the orally immunized mice. To
353 determine if pre-challenge antibodies correlated with overall survival, serum Ig and IgG titers were
354 grouped based on animal survival status following challenge with WT SL1344, TML, or R11
355 (Tables 1 and 2; Fig. 1C-D). These groupings are irrespective of the immunization dose/route and
356 also included mice that survived challenge following mock-immunization. When evaluated in this
357 manner, mice that survived challenge exhibited a significant increase in pre-challenge serum Ig
358 titers; while there was an increase in pre-challenge serum IgG in mice that survived lethal
359 challenge, the increase failed to reach statistical significance (Fig. 1C, 1D). The levels of serum Ig
360 and IgG in mice that survived lethal *Salmonella* challenge were further increased compared to pre-
361 challenge titer levels; however the increase was only significant for mice challenged with *S.*
362 Typhimurium strains SL1344 and TML (Fig. 1E-F). The increase in antibody titers may suggest

initiation of a memory immune response upon challenge. As observed in the analysis of pre-challenge sera, mice that were immunized IP had the highest Ig and IgG titers (Fig. 1E-F).

slgA found in the gut mucosa is known to be protective against *Salmonella* infection since it inhibits bacterial adherence to the epithelial cell surface (Holmgren and Czerkinsky, 2005). In order to determine the role of slgA in protection conferred by immunization with SL1344 *wecA*⁻, fecal pellets were analyzed by ELISA. As shown in Fig. 1G, slgA titers were significantly higher in the SL1344 *wecA*⁻ IP immunized group as compared to the mock-immunized control (Fig. 1G), and to the SL1344 *wecA*⁻ oral immunization group. Thus the levels of fecal slgA correlate with the levels of Ig and IgG observed in Figs. 1A and 1B.

To verify and extend these ELISA results, similar assays were conducted on sera from animals immunized in the second set of survival experiments (Tables 1 and 2; Fig. S1). Overall the results were reproducible between the two experiments. Of note in the repeat experiment, the increase in serum Ig and IgG antibodies in orally immunized mice compared to the mock-immunized controls failed to reach significance (Fig. S1A-B) and IgG titers were significantly increased in animals that survived challenge (Fig. S1D).

Finally, we evaluated antibody titers in mice that were immunized with SL1344 *wecA*⁻ and then challenged orally with *S. Enteritidis* R11 (Fig. 2). Overall, similar trends in antibody titers were observed in this experiment when compared to the experiments described above; however, the increase in IgG antibody titers in mice immunized orally with SL1344 *wecA*⁻ was not significant compared to the respective mock-immunized control animals or compared to IP immunized animals (Fig. 2B). Furthermore, the increase in terminal Ig titers in IP immunized mice compared to pre-challenge titers was not significant (Fig. 2E). Lastly, there was a significant increase in slgA

385 titers in mice immunized orally with SL1344 *wecA*⁻ as compared to mock-immunized controls (Fig.
386 2E). Similar results were obtained in a repeat experiment (Fig. S2).

387 Taken *en masse*, these results indicate that 1) immunization with SL1344 *wecA*⁻ induces an
388 antibody mediated immune response; 2) pre-challenge antibody titers correlate with survival
389 following lethal challenge with *S. Typhimurium* or *S. Enteritidis*; and 3) the increase in serum
390 antibody titers following challenge suggest the presence of a memory antibody response.

391

392 **Analysis of antibody cross-reactivity and functionality.** Previous studies have shown that
393 immunization with attenuated *Salmonella* strains can produce cross-reactive antibodies that react
394 to different *Salmonella* serovars (Beal *et al.*, 2006; Dehghani *et al.*, 2013; Matulova *et al.*, 2013).
395 To determine if the antibodies generated from oral and IP immunization with *S. Typhimurium*
396 SL1344 *wecA*⁻ were cross-reactive, lysates of *S. Typhimurium* strains SL1344, SL1344 *wecA*⁻ and
397 TML and *S. Enteritidis* R11 were probed with immune sera or sera from the mock-immunized
398 counterparts (Fig. 3 and S3). Sera from mice mock-immunized orally or IP reacted minimally to the
399 *Salmonella* lysates (Fig. S3). However, sera from animals immunized both orally (Fig. 3A) and IP
400 (Fig. 3B) with SL1344 *wecA*⁻, reacted strongly with three bands (20 kDa, 30 kDa, and slightly
401 above 60 kDa) from all strains. Additionally, other protein bands reacted with less intensity (Fig. 3).
402 To determine if the antibodies in the immune sera would cross-react against additional *Salmonella*
403 serovars, lysates were also used from *S. Stanleyville* J65 (O:4), *S. Dublin* R17 (O:9), and *S.*
404 *Javiana* (O:9); these strains were chosen due to their clinical relevance. *S. Stanleyville* J65 and *S.*
405 *Dublin* R17 are clinical isolates from blood cultures taken in Mali (Tennant *et al.*, 2010). *S. Javiana*,
406 now one of the top four serovars found in the US, is a blood isolate from a *Salmonella* outbreak in
407 2004 ((CDC), 2005, 2013). As was observed for *S. Typhimurium* and *S. Enteritidis* lysates, *S.*

Stanleyville J65, S. Dublin R17, and S. Javiana lysates reacted with immune sera from orally and IP immunized mice and not with sera from mock-immunized mice (Fig. 3 and Fig. S3). Together, these results indicate that antibodies generated from immunization with SL1344 *wecA*⁻ react with *Salmonella* strains across serovars O:4 and O:9.

To determine if these antibodies possessed functional activity, pooled immune sera from animals immunized IP with SL1344 *wecA*⁻ was tested for the ability to mediate agglutination of the various O:4 and O:9 *Salmonella* strains. Sera from IP immunized animals but not mock-immunized animals was able to mediate agglutination of the O:4 strains but not the O:9 strains (data not shown). As a control, all *Salmonella* strains were also tested for agglutination with diagnostic antisera. The O4 and O9 strains were only agglutinated with the O4 and O9 antisera, respectively (data not shown). Next, pooled immune sera from IP immunized animals was tested for the ability to mediate opsonophagocytic uptake of the strains by murine macrophages. As shown in Figure 4, incubation with sera from mice immunized IP with SL1344 *wecA*⁻ resulted in significant increases in percent opsonization as compared to the saline and mock-immunized sera controls for all serovar O:4 strains. However, there was no increase in opsonization compared to the saline or mock-immunized sera controls for serovar O:9 strains (Fig. 4). Furthermore, *Salmonella* strains preincubated with the O4 and O9 diagnostic antisera were only phagocytosed upon addition of the antisera directed against the specific O-antigen of each of the strains (Fig. S4). Together, these data indicate that sera from *wecA*⁻ immunized mice produces functional antibodies that aide in opsonization of *Salmonella* from the same serovar, but not from other serovars. Thus, while antibodies produced from immunization with SL1344 *wecA*⁻ are able to cross-react with other *Salmonella* serovars (Fig. 3) the ability of the antibodies to mediate agglutination and

opsonophagocytic activity is only observed against strains of the same serovar (Fig. 4 and data not shown).

DISCUSSION

Due to the increases in prevalence and associated morbidity and mortality of NTS infections, and the absence of a licensed NTS vaccine approved for human use, a pressing need for vaccine development exists. In the present series of studies we explored whether a *wecA* mutant could provide protective immunity against non-parental *Salmonella* and show the following: 1) either oral or IP immunization with SL1344 *wecA*⁻ provides protection against parental SL1344 (Table 1), highlighting the reproducibility of our previous work with the oral vaccine and introducing IP immunization as a second and more effective route of protection; 2) either oral or IP immunization with SL1344 *wecA*⁻ provides protection against the non-parental *S. Typhimurium* TML strain (Table 1); 3) IP immunization with SL1344 *wecA*⁻ provides protection against *S. Enteritidis* IP and oral challenge (Table 2); and 4) protection against parental and non-*Typhimurium* challenge correlates with post-immunization anti-*S. Typhimurium* antibody titers (Fig. 1, 2, S1, and S2). Furthermore, antibodies contained within immune sera are cross-reactive across serovars O:4 and O:9 (Fig. 3), and are able to mediate agglutination and opsonization of serovar O:4 *Salmonella* strains (Fig. 4 and data not shown).

A major challenge in *Salmonella* vaccine design is the ability to provide cross-protection against the numerous O-antigen serotypes, of which there are more than 26 (Grimont and Weill, 2007). The heterologous protection studies described herein suggest that the *wecA* mutant might be an appropriate candidate for further development as a cross-protecting live-vaccine strain (LVS). Indeed, our results are comparable to prior studies that showed that immunization with live-attenuated *S. Typhimurium* or *S. Enteritidis* could induce protection against heterologous challenge

with *S. Enteritidis*, *S. Typhimurium*, or in some studies, *S. Dublin* (Collins, 1968; Heithoff *et al.*, 2001). In terms of cross-protection, we do note that elucidation of a true specific heterologous protection versus a nonspecific generalized immune activation that is also protective is complicated. Indeed, previous studies have shown that persistently colonizing LVS can induce a generalized long-lived immune response (Srinivasan *et al.*, 2004) that can mediate protection from subsequent lethal *Salmonella* challenge (Hormaeche *et al.*, 1991; Hormaeche *et al.*, 1996) as well as to unrelated bacterial pathogens. While we cannot completely rule out this possibility, we do note the following data that support true specific cross-protection: 1) experiments conducted to determine if vaccination with the *S. Typhimurium wecA* mutant could provide nonspecific protection against an unrelated bacterial pathogen (*Pseudomonas aeruginosa*) showed no protection (data not shown) and 2) protection demonstrated in this study strongly correlate with a *Salmonella*-specific antibody response (Fig. 1, 2, S1 and S2). Based on these data, we believe that the *wecA* mutant provides true specific cross-protection and future work will seek to determine if this cross-protection can be extended to additional *Salmonella* serotypes.

Protection against *Salmonella* in the mouse model is known to be mediated by both humoral and cell-mediated responses (Griffin and McSorley, 2011; Kotton and Hohmann, 2004; Oliveira *et al.*, 2012). While we did not investigate the role of cell-mediated immunity in the present study, we did find that efficacy of the *wecA*⁻ LVS correlated with the production of Ig, IgG, and sIgA antibodies in the murine model of salmonellosis. Additionally, we found that the antibodies produced upon immunization with the SL1344 *wecA* mutant were cross-reactive to whole cell lysates across *Salmonella* serovars O:4 and O:9; however, these antibodies were only able to mediate agglutination and opsonization of serovar O:4 strains by murine macrophages. (Liang-Takasaki *et al.*, 1982; Makela *et al.*, 1973). Members of the O:4 and O:9 *Salmonella* serovars all

476 contain a tetrasaccharide repeat in their LPS, where a dideoxyhexose sugar is linked to a
477 conserved trisaccharide backbone (Fierer and Guiney, 2001). For members of the O:4 serovar, the
478 presence of the dideoxyhexose sugar abequose has been linked to increased C3-bacterial binding,
479 resulting in increased phagocytosis when compared to tyvelose, the dideoxyhexose sugar found in
480 O:9 serovars (Fierer and Guiney, 2001; Liang-Takasaki *et al.*, 1982). Furthermore, the presence of
481 long antigenic polysaccharides found on the LPS of O:9 strains such as *S. Enteritidis* are known to
482 activate the alternative complement pathway and influence bacterial uptake inversely proportional
483 to virulence (Liang-Takasaki *et al.*, 1982). The difference in virulence between *Salmonella* strains
484 is presumably due to differences in bacterial clearance due to phagocytosis, as antibody induction
485 between strains with the different dideoxyhexose sugars does not vary (Liang-Takasaki *et al.*,
486 1982; Makela *et al.*, 1973). Therefore, the differences in cross-protection that occur independently
487 of differences in LPS recognition in our studies may be due to differences in the sugar residues
488 between serovars, which lead to decreased agglutination and opsonization of the O:9 strains used
489 in this study. These results are supported by the results of control experiments where the O9
490 *Salmonella* strains were not opsonized for phagocytosis by macrophages after preincubation with
491 O4 antisera (Fig. S4).

492 When the sugars of the O-antigen across serovars and the high protein cross-reactivity are
493 taken into consideration, it is possible that both LPS and other surface exposed antigens are
494 required to mediate cross-serovar protection against *Salmonella* (MacLennan *et al.*, 2010;
495 Trebicka *et al.*, 2013). Indeed, serum from individuals with NTS contained antibodies to *Salmonella*
496 outer membrane proteins (MacLennan *et al.*, 2010), while prolonged mouse survival was
497 associated with a small fraction of surface exposed antigens (Barat *et al.*, 2012). In thinking about
498 the role of LPS-specific antibodies, patient sera showed complement-dependent, antibody-

mediated bactericidal activity against *S. Typhimurium* or NTS infection; this bactericidal activity increased with increasing age and was associated with LPS or the O-antigen (MacLennan *et al.*, 2008; Trebicka *et al.*, 2013). Another study utilizing mouse and rabbit models of invasive *S. Typhimurium* infection showed that bactericidal antibodies were mainly active against the O-antigen (Rondini *et al.*, 2013). Lastly, both typhoid vaccines were found to stimulate a humoral immune response against NTS strains that share similarities in the O-antigen with *S. Typhi*; *S. Typhimurium* and *S. Enteritidis* share the O-12 antigen with *S. Typhi* (Kantele *et al.*, 2012; Pakkanen *et al.*, 2013). Thus, while internal *Salmonella* antigens may offer protection against infection, LPS and outer membrane protein-specific antibody responses may also be protective. As such, since removal of ECA from the bacterial surface removes a major component of the surface exposed glycolipid, it is tempting to speculate that loss of ECA “unmasks” a surface exposed protein or an immunogenic epitope on LPS, which is then targeted in such a way as to yield a protective antibody response. Clearly, further study will be required to identify the source of antigen specificity against SL1344 *wecA*.

In the present study, we observed that the humoral response to IP immunization was greater and more effective at providing protection from lethal challenge than oral immunization. Despite the elevated protection seen with IP immunization (Simon *et al.*, 2011a), clearly oral immunizations are preferred due to the ease of administration, cost effectiveness, and potential stability (Kotton and Hohmann, 2004). However, a clear disadvantage to mucosal immunization is that generation of a strong sIgA response has been difficult to obtain in most cases; our sIgA ELISA results for mice receiving oral immunization are consistent with this observation (Fig. 1, 2, S1, S2). As a result, most immunizations are still administered parentally (Holmgren and Czerkinsky, 2005; Petrovsky and Aguilar, 2004). In order to increase efficacy of the oral vaccine,

522 increasing the vaccination dose could be beneficial; we previously showed that protection and
523 attenuation induced by the *wecA*⁻ LVS varied by vaccination and challenge doses (Gilbreath *et al.*,
524 2012). However, we note that when mice were vaccinated with greater than 2.0×10^6 CFU of the
525 *wecA* mutant, we observed neurological symptoms (spinning, head tilt, and unidirectional motion)
526 in a small subset of mice that was consistent with meningitis (data not shown). Similar neurological
527 symptoms were previously observed in mice challenged with 10^6 - 10^8 CFU of WT SL1344
528 (Wickham *et al.*, 2007) or greater than 10^8 CFU of a SL1344 *crp*⁻ strain (Zhang *et al.*, 1997b). The
529 ability of the bacteria to cause meningitis required the T3SS; no neurological side effects were
530 seen in mice infected with T3SS negative *Salmonella* (Wickham *et al.*, 2007). Identification of the
531 highly conserved antigens associated with the *wecA*⁻ LVS that are responsible for serovar cross-
532 protection may lead to the development of a subunit or conjugate vaccine that could protect across
533 serovars. Furthermore, it may be possible to the use *wecA* mutant strain as a background for
534 introduction of additional mutations that will further attenuate the strain but still mediate vaccine
535 efficacy.

536 In conclusion, we have shown that SL1344 *wecA*⁻ offers protection against lethal *S.*
537 Typhimurium and *S. Enteritidis* challenge. While we have shown that protection offered by both IP
538 and oral immunization involves a robust antibody response, further studies will be required to
539 identify the antigen responsible for the antibody-mediated response. Finally, studies designed to
540 analyze the role of cell-mediated immunity in SL1344 *wecA*⁻ protection will help to elucidate the
541 role of T-cells in the development of protective immunity against NTS.

542

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733 **FIGURE LEGENDS**

734 **Figure 1. SL1344 *wecA*⁻ immunization induces a humoral immune response that correlates**
735 **with protection against oral SL1344, oral TML or IP *S. Enteritidis* R11 challenge.** Pre-
736 challenge sera was collected 25-27 days post-immunization from mice that were immunized orally
737 with 1.2×10^6 CFU or IP with 7.2×10^2 CFU SL1344 *wecA*⁻. The sera was then analyzed for *S.*
738 Typhimurium-specific **(A)** Ig or **(B)** IgG antibodies by ELISA (ANOVA; overall *P* value: *P*<0.0001 for
739 Ig and IgG). Pre-challenge antibody titers were grouped together based on animal survivor status
740 for **(C)** Ig and **(D)** IgG to determine if pre-challenge antibody titers corresponded with survival
741 (unpaired Student's *t*-test); data includes animals that survived challenge following mock-
742 immunization. *Salmonella*-specific **(E)** Ig or **(F)** IgG antibody titers from mice surviving lethal oral
743 challenge with *S. Typhimurium* strains SL1344 or TML, or IP challenge with *S. Enteritidis* R11 were
744 compared to pre-challenge antibody titers for detection of a memory immune response (paired
745 Student's *t*-test). Finally, fecal pellets collected 25-27 days post-immunization were analyzed by
746 ELISA for **(G)** sIgA (ANOVA; overall *P* value: *P*<0.0001). While oral immunization induced a
747 modest Ig, IgG, and sIgA response, IP immunization induced a much greater response to the
748 *Salmonella* LVS SL1344 *wecA*⁻. The presence of pre-challenge antibodies correlated with survival
749 of the mice post-challenge. **P**-pre-challenge sera, **T**-terminal sera, **SL**-WT SL1344. ELISA data are
750 presented as the geometric mean + 95% confidence interval. **NS**-not significant, **P*<0.05,
751 ***P*<0.0001, # statistical analysis could not be determined due to an insufficient number of animals,
752 and no error bar indicates a single survivor. No bar indicates a value less than 1.

753

754 **Figure 2. Immunization with SL1344 *wecA*⁻ induces Ig, IgG, and sIgA antibodies that protect**
755 **against oral *S. Enteritidis* challenge.** Pre-challenge sera was collected and analyzed as

described in Fig. 1 from mice that were immunized orally with 3.0×10^5 CFU or IP with 1.0×10^2 CFU SL1344 *wecA*⁻. The sera was again analyzed for *S. Typhimurium*-specific (A) Ig or (B) IgG antibodies by ELISA (ANOVA; overall *P* value: *P*<0.0001 for Ig and IgG). To determine if pre-challenge antibody titers corresponded with survival, antibody titers were grouped together based on animal survivor status, including mock-immunized animals for (C) Ig and (D) IgG (unpaired Student's *t*-test). Terminal (E) Ig and (F) IgG antibody titers were compared to pre-challenge titers for determination of a memory antibody response to *Salmonella* (paired Student's *t*-test). (G) sIgA antibody titers were analyzed from fecal pellets (ANOVA; overall *P* value: *P*<0.0001). As observed in Fig. 1, oral immunization induced a modest Ig, IgG, and sIgA response, while IP immunization induced a much greater response to the *Salmonella* LVS SL1344 *wecA*⁻. The presence of Ig and IgG antibodies correlated with survival status post-challenge. **P**-pre-challenge sera, **T**-terminal sera. ELISA data are presented as the geometric mean + 95% confidence interval. **NS**-not significant, **P*<0.05. No bar indicates a value less than 1.

Figure 3. Antibodies from SL1344 *wecA*⁻ immunized animals cross-react across *Salmonella* O:4 and O:9 serovars. Bacterial lysates from serovar O:4 strains (*S. Typhimurium* SL1344, TML and SL1344 *wecA*⁻, and *S. Stanleyville* J65) and serovar O:9 strains (*S. Enteritidis* R11, *S. Dublin* R17, and *S. Javiana*) were probed with sera from animals immunized (A) orally with 2.0×10^6 CFU of SL1344 *wecA*⁻ or (B) IP with 8.2×10^2 CFU of SL1344 *wecA*⁻. Numerous cross-reactive bands were apparent across serovars with both oral and IP immune sera.

Figure 4. Serum antibodies from IP SL1344 *wecA*⁻ immunized animals mediate opsonophagocytic uptake of serovar O:4 *Salmonella* strains. *S. Typhimurium* strains SL1344,

779 TML and SL1344 *wecA*⁻, *S. Enteritidis* R11, *S. Stanleyville* J65, *S. Dublin* R17, and *S. Javiana*
780 were pre-incubated with immune sera from mice immunized IP with 8.2×10^2 CFU of SL1344
781 *wecA*⁻ prior to incubation with murine macrophage J774A.1 cells. Bacteria were also incubated
782 with sera from mock-immunized mice, or with saline alone as controls. The percent opsonization
783 was determined by comparing the number of opsonized bacteria to the inoculum. Data are
784 presented as the mean +SEM for three independent experiments. * $P < 0.05$ compared to saline and
785 sera: saline controls.

786 **Table 1. IP immunization confers cross-protection against non-parental *S. Typhimurium* challenge**

Immunization				Oral <i>S. Typhimurium</i> Challenge					
Strain	Route	Dose	No. Survived/total	Strain	Dose	No. Survived/total	MTD ^a	% Survival	Log-Rank (P-value)
Mock	Oral	NA	8/8	SL1344	5.4 x 10 ⁵	4/8	9.8	50	0.025
SL1344 <i>wecA</i> ⁻	Oral	1.2 x 10 ⁶	8/8	SL1344	5.4 x 10 ⁵	8/8	NA	100	
Mock	IP	NA	8/8	SL1344	5.4 x 10 ⁵	0/8	10.1	0	<0.0001
SL1344 <i>wecA</i> ⁻	IP	7.2 x 10 ²	8/8	SL1344	5.4 x 10 ⁵	7/8	NA ^b	87.5	
Mock	Oral	NA	8/8	SL1344	9.0 x 10 ⁷	0/8	9.8	0	0.004
SL1344 <i>wecA</i> ⁻	Oral	1.5 x 10 ⁶	8/8	SL1344	9.0 x 10 ⁷	5/8	10.7	62.5	
Mock	IP	NA	8/8	SL1344	9.0 x 10 ⁷	0/8	9.1	0	<0.0001
SL1344 <i>wecA</i> ⁻	IP	6.0 x 10 ²	5/9	SL1344	9.0 x 10 ⁷	5/5	NA	100	
Mock	Oral	NA	8/8	TML	3.4 x 10 ⁴	6/8	11.5	75	0.143
SL1344 <i>wecA</i> ⁻	Oral	1.2 x 10 ⁶	8/9	TML	3.4 x 10 ⁴	8/8	NA	100	
Mock	IP	NA	8/8	TML	3.4 x 10 ⁴	2/8	9.2	25	0.002
SL1344 <i>wecA</i> ⁻	IP	7.2 x 10 ²	8/8	TML	3.4 x 10 ⁴	8/8	NA	100	
Mock	Oral	NA	8/8	TML	7.0 x 10 ⁵	0/8	12.0	0	0.004
SL1344 <i>wecA</i> ⁻	Oral	1.5 x 10 ⁶	8/8	TML	7.0 x 10 ⁵	6/8	9.0	75	
Mock	IP	NA	8/8	TML	7.0 x 10 ⁵	0/8	11.3	0	<0.0001
SL1344 <i>wecA</i> ⁻	IP	6.0 x 10 ²	7/8	TML	7.0 x 10 ⁵	7/7	NA	100	

787

788 NA-Not applicable; ^aMTD-mean time to death in days; ^bOne mouse death at day 7

789 **Table 2. Immunization with SL1344 *wecA*⁻ cross-protects against lethal IP and oral *S. Enteritidis* R11 Challenge**

Immunization				S. Enteritidis Challenge					
Strain	Route	Dose	No. Survived/total	Route	Dose	No. Survived/total	MTD ^a	% Survival	Log-Rank (P-value)
Mock	Oral	NA	8/8	IP	6.6 x 10 ²	0/8	5.4	0	0.04
SL1344 <i>wecA</i> ⁻	Oral	1.5 x 10 ⁶	9/10	IP	6.6 x 10 ²	1/9	6.6	11.1	
Mock	IP	NA	8/8	IP	6.6 x 10 ²	0/8	6.0	0	<0.0001
SL1344 <i>wecA</i> ⁻	IP	6.0 x 10 ²	7/9 ^b	IP	6.6 x 10 ²	3/7	20.8	42.9	
Mock	Oral	NA	8/8	IP	2.2 x 10 ⁵	0/8	3.6	0	0.086
SL1344 <i>wecA</i> ⁻	Oral	1.2 x 10 ⁶	9/9	IP	2.2 x 10 ⁵	0/9	5.0	0	
Mock	IP	NA	8/8	IP	2.2 x 10 ⁵	0/8	3.8	0	<0.0001
SL1344 <i>wecA</i> ⁻	IP	7.2 x 10 ²	7/10 ^c	IP	2.2 x 10 ⁵	3/7	13.5	42.9	
Mock	Oral	NA	10/10	Oral	2.2 x 10 ⁶	0/10	11.6	0	0.001
SL1344 <i>wecA</i> ⁻	Oral	3.0 x 10 ⁵	10/10	Oral	2.2 x 10 ⁶	8/10	9.5	80	
Mock	IP	NA	10/10	Oral	2.2 x 10 ⁶	3/10	11.1	30	0.236
SL1344 <i>wecA</i> ⁻	IP	1.0 x 10 ²	9/10	Oral	2.2 x 10 ⁶	5/9	14.3	55.6	
Mock	Oral	NA	10/10	Oral	2.0 x 10 ⁶	1/10	9.9	10	0.007
SL1344 <i>wecA</i> ⁻	Oral	1.3 x 10 ⁶	10/10	Oral	2.0 x 10 ⁶	5/10	16.2	50	
Mock	IP	NA	10/10	Oral	2.0 x 10 ⁶	1/10	12.1	10	0.001
SL1344 <i>wecA</i> ⁻	IP	8.8 x 10 ²	8/10	Oral	2.0 x 10 ⁶	6/8	23.0	75	

790 NA-Not applicable; ^aMTD-mean time to death in days; ^bOne mouse succumbed post-tail-vein bleeds; ^cTwo mice succumbed

791 post-tail-vein bleeds

Figure 1

Figure 2

Figure 3

Figure 4