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1 # The serogroup B meningococcal outer membrane vesicle-based
2 vaccine 4CMenB induces cross-species protection against
3 *Neisseria gonorrhoeae*

4
5 Isabelle Leduc[1†], Kristie L. Connolly[1†], Afrin Begum[1], Knashka Underwood[1], Nazia Rahman,
6 Stephen Darnell[1], Jacqueline T. Balthazar[2], William M. Shafer[2], Andrew N. Macintyre[3], Gregory
7 D. Sempowski[3], and Ann E. Jerse[1*]
8
9
10 [1]Department of Microbiology and Immunology, F. Edward Hèbert School of Medicine, Uniformed
11 Services University, Bethesda, Maryland, United States of America
12
13 [2]Department of Microbiology and Immunology and The Emory Antibiotic Resistance Center,
14 Emory University School of Medicine, Atlanta, Georgia, United States of America
15
16 [3]Duke Human Vaccine Institute, Duke University School of Medicine, Durham, North Carolina,
17 United States of America
18
19
20 *Corresponding author
21 Email: ann.jerse1@usuhs.edu (AEJ)
22
23
24 [†]These authors contributed equally to the work.
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28 # Abstract
29

30 There is a pressing need for a gonorrhea vaccine due to the high disease burden
31 associated with gonococcal infections globally and the rapid evolution of antibiotic resistance in
32 *Neisseria gonorrhoeae* (*Ng*). Current gonorrhea vaccine research is in the stages of antigen
33 discovery and the identification of protective immune responses, and no vaccine has been tested
34 in clinical trials in over 30 years. Recently, however, it was reported in a retrospective case-control
35 study that vaccination of humans with a serogroup B *Neisseria meningitidis* (*Nm*) outer membrane
36 vesicle (OMV) vaccine (MeNZB) was associated with reduced rates of gonorrhea. Here we
37 directly tested the hypothesis that *Nm* OMVs induce cross-protection against gonorrhea in a well-
38 characterized female mouse model of *Ng* genital tract infection. We found that immunization with
39 the licensed *Nm* OMV-based vaccine 4CMenB (Bexsero®) significantly accelerated clearance
40 and reduced the *Ng* bacterial burden compared to administration of alum or PBS. High titers of
41 serum IgG1 and IgG2a and vaginal IgG1 that cross-reacted with *Ng* OMVs were induced by
42 vaccination via either the subcutaneous or intraperitoneal routes, and a 4-fold increase in the
43 serum bactericidal$_{50}$ titers was detected against the challenge strain. Antibodies from vaccinated
44 mice recognized several surface proteins in a diverse collection of *Ng* strains, including PilQ,
45 BamA, MtrE, PorB, and Opa, and 4CMenB-induced antibodies bound PilQ and MtrE in native
46 form on the surface of viable bacteria. In contrast, the antibodies were only cross-reactive against
47 lipooligosaccharide species from a few *Ng* strains. Our findings directly support epidemiological
48 evidence that *Nm* OMVs confer cross-species protection against *Ng* and implicate several *Ng*
49 surface antigens as potentially protective targets. This work also validates the murine infection
50 model as a relevant experimental system for investigating mechanisms of vaccine-mediated
51 protection against gonorrhea.

52

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53 ## Author summary
54

55 Over 78 million *Neisseria gonorrhoeae (Ng)* infections occur globally each year and control
56 of gonorrhea through vaccination is challenged by a lack of strong evidence that immunity to
57 gonorrhea is possible. This contention was recently challenged by epidemiological evidence
58 suggesting that an outer membrane vesicle (OMV) vaccine from the related species *Neisseria*
59 *meningitidis* (*Nm*) protected humans against gonorrhea. Here we provide experimental evidence
60 in support of this hypothesis by demonstrating that a licensed, modified version of this *Nm* OMV-
61 based vaccine accelerates clearance of *Ng* in a mouse infection model. These results confirm the
62 possibility cross-species protection and are important in that they support the biological feasibility
63 of vaccine-induced immunity against gonorrhea. We also showed that several *Ng* outer
64 membrane proteins are recognized by antisera from vaccinated mice that may be protective
65 targets of the vaccine. Additionally, our demonstration that a vaccine that may reduce the risk of
66 gonorrhea in humans protects mice against *Ng*, a highly host-restricted pathogen, validates the
67 mouse model as a potentially useful tool for examining mechanisms of protection, which could be
68 exploited in the development of other candidate gonorrhea vaccines.

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69 ## Introduction
70

71 An estimated 78 million new gonorrheal infections occur each year worldwide [1] and rates
72 are rising globally, with a 67% increase in reported infections in the U.S. between 2014 and 2018
73 [2]. Caused by the Gram-negative bacterium *Neisseria gonorrhoeae (Ng)*, gonorrhea is
74 associated with significant morbidity and mortality that disproportionately affects women and
75 newborns. Ascending lower urogenital tract infection can occur in both sexes to cause epididymis,
76 endometritis and salpingitis, but is more frequent in females. *Ng* pelvic inflammatory disease can
77 be asymptomatic or acute, and is associated with ectopic pregnancy, infertility and chronic pelvic
78 pain. Disseminated gonococcal infection can occur in either gender [3]. Transmission of
79 gonorrhea to neonates from infected mothers can cause acute neonatal conjunctivitis [4] and
80 there is a clear association between maternal gonorrhea, low-birth weight and pre-mature delivery
81 [5]. The impact of gonorrhea on human health is amplified by its role in increasing both
82 transmission and susceptibility to the human immunodeficiency virus (HIV) [6, 7].

83 Gonorrhea is classified as an urgent public health threat due to decreasing susceptibility
84 to the last remaining reliable monotherapy for gonorrhea, the extended-spectrum cephalosporins.
85 Dual therapy with high-dose ceftriaxone and azithromycin is currently recommended for empirical
86 treatment of gonorrhea in many countries. However, *Ng* susceptibility to these antibiotics
87 continues to decrease world-wide [8], and alarmingly, treatment failures due to strains that are
88 resistant to these antibiotics have been reported [9, 10]. New antibiotics are under development
89 [11, 12]; however, the call for a gonorrhea vaccine has been reinvigorated by the evolutionary
90 success of the gonococcus in outrunning public health efforts to contain it through antibiotic
91 therapy [13, 14].

92 Early vaccine research was challenged by the discovery that several *Ng* surface
93 molecules are phase or antigenically variable. There was also no animal model other than
94 chimpanzees for analyzing host responses and systematic testing of immunogens, and two

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95 published clinical trials using a killed whole cell vaccine [15] or purified pili [16] were unsuccessful
96 despite earlier small in-house studies that showed protection from urethral challenge in human
97 male volunteers [17]. Since this time, several conserved and semi-conserved vaccine antigens
98 that elicit bactericidal antibodies or inhibit target function have been identified, some of which
99 show protection in a well-characterized mouse genital tract infection model [13]. How well the
100 mouse model predicts vaccine efficacy in humans is not known, however, due to the strict host-
101 specificity of *Ng* and a lack of information on correlates of immune protection in humans. There
102 is little immunity to natural infection in humans and mice [18], and there is growing evidence that
103 the adaptive response to *Ng* infection is suppressed. As recently reviewed by Lovett and Duncan
104 [19], human humoral immune responses to *Ng* to infection are modest at best and the analysis
105 thereof is complicated by pre-existing antibodies to carbohydrate and protein surface antigens
106 that are induced by commensal *Neisseria sp*., although antibodies to some antigens are increased
107 by infection [20]. Human cellular responses to *Ng* infection are less well studied, but appear to be
108 driven by a Th17 pro-inflammatory response. Th1 responses, in contrast, appear suppressed [20],
109 and several pathways that result in reduced antigen presentation and, or inhibition of T cell
110 responses to *Ng* have been identified using human immune cells and experimentally infected
111 mice [21-25].

112 Recent epidemiological evidence, however, suggests immunity to gonorrhea can be
113 achieved in humans through vaccination with outer membrane vesicles (OMVs) of the related
114 species, *Neisseria meningitidis* (*Nm*). In this cross-sectional study, vaccination of individuals with
115 the serogroup B meningococcal vaccine MeNZB, which consisted of OMVs from an endemic New
116 Zealand strain, was associated with a reduced rate of gonorrhea in adolescents and adults aged
117 15-30 years old [26]. Using cases of chlamydia as a control, the estimated effectiveness of this
118 meningococcal vaccine against gonorrhea was predicted to be 31%. These data are the first
119 controlled evidence in humans in over 40 years that vaccine-induced protection against gonorrhea

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120 is possible. A similar finding was suggested by epidemiological studies on *Nm* OMV vaccines in
121 Cuba and Norway [27].

122 To directly test the hypothesis that *Nm* OMV-based vaccines induce cross-species
123 protection against *Ng*, here we evaluated the *in vivo* efficacy of the licensed 4CMenB ("4
124 Component Meningitis B"; Bexsero®) vaccine in a female mouse model of *Ng* lower genital tract
125 infection. 4CMenB consists of *Nm* OMVs from the *Nm* strain used in the MenNZB vaccine and
126 five recombinant *Nm* proteins [28], only one of which, the neisserial heparin-binding antigen
127 (NHBP), is a feasible vaccine target for gonorrhea [29, 30]. Our results show that 4CMenB
128 significantly reduces the *Ng* bioburden, accelerates clearance of infection, and induces antibodies
129 that recognize several *Ng* proteins, at least three of which are promising vaccine targets. These
130 findings are consistent with epidemiological data that suggest cross-species protection against
131 gonorrhea is possible and validate the gonorrhea mouse model as a useful experimental system
132 for studying vaccine-mediated correlates of protection against this human disease.
133

134 ## Results
135

136 ### Optimization of the immunization regimen to induce serum and vaginal antibodies

137 The recommended dosing regimen for 4CMenB in humans is two 500 µL doses given
138 intramuscularly, four weeks apart. As a preliminary step for mouse immunization/challenge
139 studies, we immunized BALB/c mice with 20, 125, or 250 µL of the 4CMenB vaccine on days 1
140 and 28 by the subcutaneous (SC) or intraperitoneal (IP) routes to assess safety and
141 immunogenicity. A dose-response in serum IgG1 titers against the 4CMenB vaccine components
142 was detected by ELISA in IP- and SC-immunized mice (Fig S1A), and IgG2a titers were higher in
143 mice given 100 µL or 250 µL compared to 20 µL (Fig S1B). This dose response was mirrored in
144 Western blots using anti-IgG secondary antibody against whole-cell lysates of *Nm* and six

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145 different strains *Ng* (Fig S1C). Serum from control mice given PBS or Alum adjuvant alone did
146 not recognize any *Nm* or *Ng* proteins. No adverse effects were observed following IP injection.
147 Nodules formed at the injection site in SC-immunized mice for all doses given, which resolved
148 over time.

149 The MenNZB human epidemiology study reported by Petousis-Harris, et *al.* [26] was
150 based on subjects who received a 3-dose regimen separated by one month. Upon demonstrating
151 that the 250 µL dose of 4CMenB was well-tolerated and induced the highest serum antibody titers,
152 we added a third immunization in subsequent mouse immunization/challenge experiments. Mice
153 were given 250 µL of the formulated vaccine three times by IP or SC injection; controls received
154 Alum or PBS (IP). A 3-week interval between immunizations was used to avoid increasing the
155 age of the mice before challenge, which can reduce susceptibly to *Ng*. Significantly higher titers
156 of *Ng*-specific serum total Ig, IgG1, and IgG2a ($p < 0.004$) but not IgA were detected in SC- and
157 IP-immunized mice against *Ng* OMVs compared to control groups that received Alum or PBS on
158 day 52 (Fig 1A-D). Total Ig, IgG1 and IgG2a titers were further elevated by the third immunization
159 in both the IP- and SC-immunized groups (day 31 versus day 52) ($p \leq 0.05$). The IgG1/IgG2a ratio
160 was significantly lower for IP-immunized mice on day 31, but similar to SC-immunized mice on
161 day 52 (Fig 1E) due to a marked increase in IgG2a titers in the SC-immunized group after the
162 third immunization (Fig 1C). Vaginal total Ig and IgG1 ($p < 0.0001$), but not IgG2a or IgA were
163 significantly elevated in vaginal washes collected after the second immunization compared to
164 control groups in IP-immunized mice, but not SC-immunized mice (Fig 1F-I). Vaginal washes
165 were not collected after the third immunization to avoid altering the vaginal microenvironment
166 before bacterial challenge and cessation of the LeBoot effect, which would increase the number
167 of mice in the undesired stages of the estrous cycle at the time of challenge [31]. We conclude
168 that a half human-dose of 4CMenB is well-tolerated in mice and that a dosing regimen similar to
169 that used in the New Zealand study elicits systemic and mucosal humoral immune responses that
170 are cross-reactive against *Ng*.

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171

172 **4CMenB-immunized mice clear *Ng* infection significantly faster and have a reduced**
173 **bioburden following vaginal challenge**

174 To assess the protective efficacy of 4CMenB against *Ng*, we challenged 4CMenB-
175 immunized and control mice with *Ng* strain F62 three weeks after the third immunization and
176 quantitatively cultured vaginal swabs for *Ng* over seven days. In combined data from two
177 independent experiments, IP-immunized mice exhibited a significantly faster clearance rate (p
178 ≤ 0.0001) (Fig 2A) and lower bioburden compared to control groups given PBS or Alum alone (p
179 < 0.05 and ≤ 0.01, respectively) (Fig 2B and 2C). Data for each individual experiment, which also
180 showed significantly faster clearance for both immunized groups compared to control mice, are
181 shown in Fig S2A and S2C. The bioburden in the IP-immunized group was significantly lower than
182 that of the Alum only group in both experiments, while the difference in the bioburden in SC-
183 immunized mice compared to Alum was significant only in the repeat experiment ($p < 0.0001$)
184 (Fig S2B and S2D). Combined data from the two experiments showed that 70% and 88% of mice
185 given 4CMenB by the SC and IP routes, respectively, cleared infection by day 7 compared to 25-
186 30% of mice given alum or PBS (Fig 2D).

187 A peak vaginal PMN influx beginning on day 4 post-bacterial challenge was observed in
188 all groups, and there was no difference in percentage of PMNs among experimental groups over
189 time (Fig 3S). We also evaluated complement-dependent bactericidal activity of pooled sera from
190 each group against *Ng* strain F62, the serum-sensitive challenge strain, and against the serum-
191 resistant strain FA1090, using normal human serum as the complement source. The bactericidal_{50}
192 titers were 1:480 and 1:240, respectively, which were 4-fold greater than that of pooled serum
193 from the unimmunized group (Fig 3). We conclude that 4CMenB reproducibly accelerates
194 clearance of *Ng* from the murine genital tract and lowers the bioburden over time and that
195 opsonophagocytosis and complement-mediated bacteriolysis maybe contribute to the protection.

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196

197 **4CMenB-Induced serum and vaginal antibodies cross-react with several *Ng* OMV**
198 **proteins but not with *Ng* LOS species in a majority of strains**

199 To examine the cross-reactivity of 4CMenB-induced antibodies against *Ng* surface
200 proteins, we conducted western immunoblots against OMVs from the challenge strain and five
201 other *Ng* strains that are geographically and temporally distinct in their isolation. Pooled antisera
202 from mice immunized twice with the 250 µL dose by either the IP or SC routes (250IP or 250SC,
203 respectively) recognized four prominent bands in fractionated OMV preparations all six strains: a
204 high molecular weight (HMW) band > 220 kD, a doublet with bands of apparent molecular weight
205 of 97 and 94 kDa, and a 55 kDa band (Fig 4). Several low intensity bands between 26 and 36
206 kDa were also recognized in several of the strains. Reactivity of the 250SC antiserum was weaker
207 than the 250IP antiserum, which likely reflects the lower titers of this antiserum. Serum from PBS-
208 or Alum-treated control mice did not recognize any *Ng* proteins (data not shown). Consistent with
209 ELISA data, serum reactivity as assessed by band intensity was increased by a third immunization
210 (Fig 4A), and additional bands were recognized including several bands in the 30-35 kDa range.
211 A similar recognition pattern was observed on blots incubated with pooled vaginal washes and
212 sera collected 10 days after the third immunization from immunized but unchallenged mice
213 followed by anti-mouse IgG or anti-mouse IgA (Fig. 4B; compare lanes 1 and 2 with lane 5 in
214 each blot). These results also show that while vaginal titers were low after the second
215 immunization as measured by ELISA (Fig 1), vaccine-induced vaginal antibodies were readily
216 detectable by immunoblot after a third immunization.

217 We also examined the reactivity of the immune serum against *Ng* lipooligosaccharide
218 (LOS) based on the report that a significant percentage of the bactericidal activity induced by an
219 *Nm* OMV vaccine was directed towards *Nm* LOS [32]. Neisserial LOS is a branched structure that
220 consists of oligosaccharide extensions from the core oligosaccharide called the α- and β-chains.

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221 An additional extension called the γ-chain is present in some strains [33]. Different LOS species
222 can be produced within a strain due to phase variable expression of the glycosyltransferase genes
223 *lgtA*, *lgtC*, *lgtD* and *lgtG*, which results in different lengths of the oligosaccharide chains [34, 35].
224 To test the cross-reactivity of 4CMenB antisera against *Ng* LOS, we fractionated crude LOS
225 extracts by gel electrophoresis from four laboratory and thirteen clinical *Ng* isolates isolated
226 between 1991 and 2019. Gels were stained or electroblotted to filters and incubated with
227 monoclonal antibodies (3F11, 4C4 and 2C7) that recognize known epitopes within *Ng* LOS [36],
228 or with the 4CMenB antiserum. All of the strains produced an LOS that bound one or more of
229 monoclonal antibodies (Fig 5A). In contrast, the 4CMenB antiserum did not recognize the LOS in
230 thirteen of the seventeen strains tested. The remaining four strains (MS11 and three clinical
231 isolates LGB24, NMCSD322 and NMCSD6364) produced one or two LOS species that cross-
232 reacted with the 4CMenB antiserum (Fig 5B). The lack of recognition of LOS in some strains could
233 potentially be explained by the reactive LOS epitope being phase variable. Schneider *et al.* [37]
234 demonstrated that long-chain LOS species are selected during urethral infection in men and Rice
235 and colleagues have shown that the phase variable 2C7 LOS epitope is expressed among a
236 majority of clinical isolates [36]. Therefore, to test investigate whether the anti-4CMenB-reactive
237 LOS epitope is perhaps phase variable and selected *in vivo*, we infected mice with strain H041.
238 No 4CMenB-reactive LOS species were detected in LOS preps from pooled vaginal H041 isolates
239 cultured on days 2 and 5 post-inoculation (Fig 5C). We conclude that while cross-reactive
240 antibodies to *Ng* LOS epitopes are induced by 4CMenB, the epitopes do not appear to be shared
241 by a majority of *Ng* strains.

242

243 **4CMenB induces antibodies against promising *Ng* vaccine targets**

244 To identify the proteins recognized by 4CMenB-induced antisera, we fractionated OMVs
245 from *Ng* strain F62 on two separate gels. One was stained with a G-250 Coomassie stain for

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246 mass spectrometry analysis, while the other was used for Western blotting with the 250IP
247 antiserum. The blot and gel were aligned and the reactive bands identified by molecular weight
248 and band intensity. Bands indicated by the numbered arrows (Fig 6A), which correspond to the
249 most intensely recognized bands in the Western blot (Fig. 6B), were submitted for mass
250 spectrometry analysis (Table 1). The HMW band at the top of the gel was identified as PilQ,
251 which is a protein that forms a dodecamer through which gonococcal pili extend [38]. Mass
252 spectrometry analysis identified two potential proteins in band 2 (97 kDa): an elongation factor
253 and a phosphoenolpyruvate. Band 3 (94 kDa) also contained 2 potential proteins: BamA, an
254 Omp85 homologue involved in the biogenesis of OM proteins (OMPs) [39], and a
255 methyltransferase. Band 4 (55 kDa) was identified as MtrE, the OM channel of the three different
256 gonococcal active efflux pump systems [40]. The 36 kDa protein (band 5) was identified as PorB,
257 and the 32 kDa band (band 6), as Opa. In summary, we identified eight proteins from six cross-
258 reactive bands, five of which are known surface-exposed *Ng* antigens.

259

260 **Table 1. Identification of protein bands recognized by 4CMenB antisera as determined by**
261 **mass spectrometry**

Sample (band)	Protein name	Database Accession ID[a]	MW (Da)	Peptide Count[b]	MS & MS/MS Score[c]	Peptide sequenced ion score[d]	Scoring Threshold[e]
1	Type IV pilus biogenesis and competence protein PilQ	Q5FAD2	77903	9	556	482	55
2	Elongation factor G	B4RQX2	77124	12	852	722	55
2	Phosphoenolpyruvate synthase	KLS49216	87167	10	666	557	55
3	Outer membrane protein assembly factor BamA	Q5F5W8	87888	16	617	481	55
3	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	Q5F863	85030	6	227	204	55
4	Multidrug transporter MtrE	Q5F726	50382	10	452	317	55
5	Porin (PorB)	YP_208842	35516	16	969	814	55
6	PII/Opa	Q51014	31429	10	670	568	55

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263 [a]For the protein sequence, search this ID at "http://www.ncbi.nlm.nih.gov", under the parameter
264 to "Protein"; [b]Number of observed peptides matching the theoretical digest of the identified
265 protein; [c]Combined score of the quality of the peptide-mass fingerprint match and MS/MS peptide
266 fragment ion matches (if MS/MS data was generated); [d]Score of the quality of MS/MS peptide
267 fragment ion matches only (if MS/MS data was generated); [e]Significant score threshold. A hit with
268 an "MS & MS/MS score" or an "Ion score" above this value is considered a significant
269 identification. $p<0.05$ for the given species database. Only database search hits with "MS and
270 MSMS scores" above this value are reported.
271

272 **4CMenB-induced antibodies bind native PilQ and MtrE on the surface of viable**
273 **gonococci**

274 We next performed immunoprecipitations using 4CMenB-induced mouse antisera and live
275 FA1090 and MS11 bacteria. Antigen-antibody complexes were solubilized in detergent, retrieved
276 using protein A/G agarose, and subjected to non-denaturing Western blotting with the 250IP
277 antiserum. Whole cell lysates and OMVs were run in parallel and exhibited the same reactive
278 band pattern shown in Figs 4A and 6B. Serum from the alum-alone group pulled down non-
279 specific proteins smaller than 30 kDa that did not align with bands in lanes containing whole cell
280 lysates or OMVs. In contrast, the 250IP antiserum pulled down two proteins from both strains: a
281 HMW protein, possibly PilQ, and a ~ 55 kDa protein (Fig 7). We hypothesized the 55 kD protein
282 was MtrE based on the greater intensity of this band in western blots against OMVs from strains
283 MS11, H041 and F89, which carry one or more *mtr* mutations that cause increased production of
284 the MtrCDE efflux pump (Fig 4A).

285 To confirm that the two identified proteins are PilQ and MtrE, we included single isogenic
286 mutants that lack expression of either protein, and the results confirmed the identity of the two
287 immunoprecipitated proteins as PilQ and MtrE. The HMW band but not the 55-kDa protein was

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288 absent from the sample in which mutant strain FA1090*pilQ* was incubated with the antisera.
289 Inversely, immunoprecipitation with mutant MS11*mtrE*- did not yield a band around 55 kDa, but
290 did retain binding to the HMW protein PilQ. We conclude that 4CMenB induces antibodies
291 recognize the native conformation of *Ng* PilQ and MtrE on the gonococcal surface.
292

293 **Discussion**
294
295 The pathogenic *Neisseria* are human-specific pathogens that differ in the capacity to
296 cause life-threatening septicemia and meningitis (*Nm*) and nonulcerative sexually transmitted
297 infections of the urogenital tract that can ascend to cause damage to the upper reproductive tract
298 (*Ng*). The only reservoir for these pathogens is human pharyngeal, genital and rectal mucosae
299 where the bacteria reside extracellularly and within an intracellular niche [41, 42]. *Nm*, unlike *Ng*,
300 produces a complex polysaccharide capsule that is critical for invasive disease, and vaccines that
301 target the capsules of four of the five most prevalent capsular serogroups A, C, W135 and Y have
302 been effectively used for decades. This approach is not successful against serogroup B *Nm* due
303 to the α2-8-linked polysialic acid composition of the serogroup B capsule, which mimics α2-8-
304 sialylated human glycoproteins [43]. A recent advance in biomedical research was the
305 development of two licensed vaccines that prevent serogroup B *Nm* invasive disease, one
306 consisting of purified lipoprotein subunits, rLP2086 (Trumenba®, Pfizer), and the other, 4CMenB
307 (Bexsero®, GSK) [43]. The 4CMenB vaccine was proceeded by *Nm* OMV vaccines that were
308 tailor-made against endemic serogroup B strains in New Zealand, Brazil, Cuba and Norway [27,
309 43].
310 Like vaccine development for serogroup B *Nm*, gonorrhea vaccine research has focused
311 on conserved outer membrane proteins, *Ng* outer membrane vesicles, and in one case, the 2C7
312 oligosaccharide epitope within *Ng* LOS [18]. Vaccine development for gonorrhea is more
313 complicated, however, by a lack of defined immune correlates of protection. Meningococcal

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314 vaccine development is guided by serum bactericidal activity, which was first demonstrated as
315 the main correlate of protection against *Nm* invasive disease in a classic case-control study at
316 Fort Ord, California in the 1960s [44]. Natural history studies for gonorrhea, in contrast, have led
317 only to an association between antibodies against the restriction modifiable protein (Rmp) and
318 increased susceptibility to infection, and in high-risk women, antibodies to porin and Opa proteins
319 as being associated with reduced risk of *Ng* upper reproductive tract infection [18]. The lack of
320 clear correlates of protection and the absence of immunity to reinfection have challenged the
321 possibility of a gonorrhea vaccine.

322 It is in this context that the reported reduced risk of gonorrhea in subjects immunized with
323 an *Nm* OMV vaccine [26] may herald a breakthrough for gonorrhea vaccine development. In
324 support of this epidemiological evidence, we demonstrated that 4CMenB reproducibly
325 accelerated *Ng* clearance and lowered the bioburden of *Ng* in a well-characterized mouse model
326 of genital tract infection. 4CMenB induced vaginal and serum IgG1, IgG2a and IgA when
327 administered subcutaneously that cross-react with several *Ng* OMV proteins expressed by six
328 different *Ng* strains. The dosing regimen we used was similar to that of the MenNZB vaccine given
329 to the subjects in the retrospective case-control epidemiological study [26]. These data are direct
330 evidence of cross-species protection and suggest female mice may reproduce vaccine-induced
331 mechanisms that protect humans against gonorrhea.

332 Known host-restrictions that limit the capacity of mice to mimic human neisserial infections
333 have been extensively reviewed [18]. Restricted factors include receptors for several neisserial
334 colonization or invasion ligands that mediate adherence to and, or uptake by human cells,
335 including the type IV pili, the Opa proteins, and in *Ng*, the pili/PorB/C3b complex. In experimental
336 murine infection, *Ng* is seen adherent to vaginal epithelial cells, in cervical tissue and within the
337 lamina propria, which is presumably mediated by other *Ng* colonization ligands for which species
338 specificity has not been defined [45, 46] or that are not host-restricted [47]. Acquisition of iron
339 from lactoferrin (LF) and transferrin (TF), and recently, zinc from calprotectin [48], is also host-

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340 restricted. These restrictions limit the ability to fully evaluate the efficacy of vaccines that induce
341 antibodies that block colonization or nutrient uptake in the mouse model, although mice that are
342 transgenic for the human carcinoemybryonic antigen cellular adherence molecules (CEACAMs),
343 the major Opa protein receptors and hTF could be used [49-51]. Restrictions in soluble negative
344 regulators of the complement cascade, factor H (fH) and C4b-binding protein (C4BP), also exist
345 and are especially important to consider when testing vaccines that clear *Ng* infection through
346 bactericidal and opsonophagocytic activity. Transgenic hFH and hC4BP mice made for this
347 purpose and not used in our study, were recently utilized by Rice and colleagues to more
348 rigorously test the *in vivo* efficacy of immunotherapeutic strategies against *Ng* [52].

349 While unable to fully mimic human neisserial infections, animal models provide a
350 physiologically relevant and immunologically intact system for testing vaccine-induced immune
351 responses against infection. Meningococcal vaccine development is aided by the use of mice or
352 rabbits to test whether candidate vaccines induce bactericidal antibodies against *Nm* or cause
353 adverse effects [53, 54], and improved mouse and infant rat bacteremia models have been used
354 to measure the efficacy of candidate *Nm* vaccines in eliminating *Nm* from the bloodstream [50,
355 55, 56]. Early gonorrhea vaccine studies used chimpanzees, which do not have all the host
356 restrictions found in other animal species, and human male subjects [18]. Chimpanzees are no
357 longer used for gonorrhea research, however a human urethritis model is still available [57] and
358 is the most relevant model for studying vaccine efficacy against *Ng* urethral infection.

359 Currently, the estradiol-treated mouse model is the only animal model for studying
360 gonorrhea vaccine efficacy in females, where the majority of morbidity and mortality associated
361 with gonorrhea occurs. This model is also used to systematically screen antigens, immunization
362 regimens, and adjuvants, and to analyze host responses [18]. Similarities between human and
363 experimental murine infection include the fact that mice, like humans, produce a transient and
364 unremarkable humoral response to *Ng* infection and can be reinfected with the same strain. *Ng*
365 induces the Th17 pathway in both humans [19, 20] and mice [58, 59], which leads to recruitment

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366 of PMNs to the infection site. Similar to that reported for human cervical infections, hormonally
367 driven, cyclical fluctuations in *Ng* colonization load and selection for Opa protein phase variants
368 occurs over the course of murine infection [60]. *Ng* is seen within murine PMNs and importantly,
369 *Ng* mutants that are more or less susceptible to killing by human PMNs or cationic antimicrobial
370 peptides *in vitro* have a similar phenotype when tested against murine PMNs and cathelicidins,
371 and are more fit or attenuated compared to the wild-type strain, respectively, during murine
372 infection [60, 61].

373 We also showed that the 4CMenB vaccine induced serum bactericidal activity and high
374 titers of serum IgG1 and IgG2a that cross-reacted with *Ng* OMVs. Vaccine-induced vaginal IgA
375 was readily detected by immunoblot. Recruitment of PMNs to the infection site occurred in all
376 groups, which could enable opsonophagocytic killing of *Ng* in the presence of specific antibody.
377 Serum bactericidal activity was also detected that could mediate protection through complement-
378 mediated bacteriolysis. Detailed investigation of the mechanism of protection have thus far only
379 been reported for one candidate *Ng* OMV vaccine [62] and the 2C7 vaccine [36], both of which
380 induced Th1 responses and bactericidal antibodies. The demonstration by Russell and colleagues
381 that a vaginally applied Th1-inducing cytokine adjuvant clears *Ng* infection in mice and induces a
382 specific adaptive response and memory humoral response also suggests Th1 responses are
383 protective [63]. Whether this is true for human infection is not known. The importance of
384 bactericidal activity in clearing *Ng* mucosal infections, is also not known and may be vaccine-
385 specific. Passive protection studies with bactericidal monoclonal antibody against the 2C7 epitope
386 clearly showed antibodies were sufficient for vaccine-mediated clearance [36]; however, eleven
387 other promising purified protein subunit vaccines that induced high-titered bactericidal activity
388 against *Ng* have been tested by our laboratory over the years that did not show protective efficacy
389 in the gonorrhea mouse model (A.E. Jerse, unpublished data in collaboration with others). Further
390 investigation of the immune responses induced by 4CMenB in mice and vaccinated humans
391 should define mechanisms of vaccine-mediated protection. The use of 4CMenB as a

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392 commercially available positive control will also strengthen the protocol for screening of candidate
393 gonorrhea vaccine antigens in the mouse model.

394 Recently, the proteome of the 4CMenB vaccine was defined and shown to contain 461
395 proteins, of which 60 proteins were predicted to be inner membrane or periplasmic and 36 were
396 predicted to be in the outer membrane or extracellular [64]. Others identified twenty-two *Nm*
397 proteins as comprising >90% of the 4CMenB proteome, twenty of which have homologs in *Ng,*
398 These investigators also showed that post-vaccinated serum from 4CMenB-immunized humans
399 recognized several bands in fractionated *Ng* OMVs by immunoblot [30]. In our study, the
400 4CMenB-induced antisera recognized several denatured OMV proteins in a panel of diverse *Ng*
401 strains, which is consistent with the OMV portion of the 4CMenB vaccine generating a robust
402 cross-reactive response. Using mass spectrometry, we identified eight cross-reactive proteins,
403 including MtrE, PilQ and BamA, which are promising vaccine candidates [18]. Recognition of LOS
404 species was less consistent, with only ca 25% of strains expressing LOS species that were
405 reactive with anti-4CMenB serum. We also demonstrated that 4CMenB-induced antibodies bound
406 native PilQ and MtrE at the surface of viable *Ng*.

407 The 94% amino acid identity (S1 Table) between the *Nm* and *Ng* homologs identified in
408 this report is consistent with the cross-reactivity that we observed. Importantly, the residues
409 encompassing the two short surface-exposed loops of the MtrE monomer (residues 92-99 and
410 299-311 [65]), which are highly conserved among *Ng* strains, are identical to those from MtrE
411 expressed by *Nm* strain MC58 (data not shown). MtrE is the outer membrane channel of the
412 MtrCDE, FarABMtrE and MacABMtrE efflux pumps, which expel antibiotics and host-derived
413 antimicrobial compounds [66]. The importance of the MtrCDE active efflux pump in protecting *Ng*
414 against host innate effectors has been demonstrated in the mouse model [67]. Antisera directed
415 against the two surface-exposed MtrE loops could target *Ng* for complement-mediated
416 bacteriolysis and opsonophagocytosis, and may possibly impair efflux pump function to increase
417 *Ng* susceptibility to host innate effectors. The PilQ protein is critical for pilus secretion [68] and

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418 mutations in PilQ are associated with increased entry of heme and antimicrobial compounds [69]
419 and enhanced resistance to cephalosporin [70]. Amino acids 406 to 770 of *Nm* PilQ were shown
420 to be a promising vaccine target, and are 94% identical with the same region of *Ng* PilQ (strain
421 FA1090) [71]. BamA is a surface-exposed outer membrane belonging to the Omp85 family [72,
422 73]. The essential role of BamA in outer membrane protein biogenesis suggests it may be a
423 highly effective vaccine target as it is present in cell envelopes and OMVs, surface-exposed, and
424 is well-conserved among clinical *Ng* isolates [39].

425 In summary, the demonstration that a licensed *Nm* OMV-based vaccine accelerates *Ng* in
426 clearance in a genital tract infection mouse model is direct evidence that cross neisserial species
427 protection may be an effective vaccine strategy for gonorrhea. Whether this approach would
428 protect against *Ng* rectal or pharyngeal infections, which are very common, is not known and in
429 the absence of animal or human challenge models for these infections, this question must be
430 solely addressed by epidemiological or clinical trials. Future detailed immunological studies in
431 mice, which can be experimentally manipulated to directly test hypothesized mechanisms of
432 protection, combined with clinical research studies on 4CMenB-vaccinated humans should reveal
433 new and important information on how to combat this ancient, highly successful pathogen.
434

435 ## Materials and Methods

436 ### Bacterial strains and culture conditions

437 *Ng* strains used in this study are listed in Table 2. Supplemented GC agar (Difco) was
438 used to routinely propagate *Ng* as described [74]. GC-VNCTS agar [GC agar with vancomycin,
439 colistin, nystatin, trimethoprim (VCNTS supplement; Difco) and 100 µg/ml streptomycin (Sm)] and
440 heart infusion agar (HIA) were used to isolate *Ng* and facultatively anaerobic commensal flora,
441 respectively, from murine vaginal swabs [75].
442

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443 **Table 2. Bacterial strains used in this study**
444

Strain	Source	Location, Date	Reference(s)
N. meningitidis			
MC58	blood	UK, 1983	ATCC [76]
***N. gonorrhoeae* (laboratory strains)**			
F62	urogenital	Atlanta, GA, 1962	[77]
FA1090	cervical (DGI)	Chapel Hill, NC, 1983	[78]
FA6140*pilQ*2	*pilQ* mutant of FA1090		[79]
MS11	cervical	Mt. Sinai, NY, 1972	[80]
DW3-MS11	*mtrE* mutant of MS11		[81]
FA19	DGI	Copenhagen, 1959	[82]
***N. gonorrhoeae* (clinical isolates)**			
H041	pharyngeal	Japan, 2009	[83]
F89	urethral	France, 2011	[84]
LGB20[a]	urogenital	Baltimore, MD	[85]
LGB24[a]	urogenital	Baltimore, MD	[85]
WAMC 7720[b]	urethral	Fort Bragg, NC, 2014	USU GC Isolate Reference Lab and Repository [86]
WAMC 7749[b]	urethral	Fort Bragg, NC, 2014	As above
NMCP 4856[b]	urine	Portsmouth, VA, 2017	As above
NMCP 9542[b]	urine	Portsmouth, VA, 2017	As above
SAMMC 7363	urine	San Antonio, TX, 2016	As above
MAMC 3183	urethral	Tacoma, WA, 2015	As above
MAMC 3668	urine	Tacoma, WA, 2019	As above
NMCSD 3277	urine	San Diego, CA, 2016	As above
NMCSD 6364	urethral	San Diego, CA, 2014	As above

445
446 PorB phenotype determined by Phadebact Monoclonal GC Assay (MKL Diagnostics). All strains are PorB1b
447 except FA19, which is PorB1a. [a]LGB20 and LGB24 were collected between 1991 and 1994 and were kindly
448 provided by Dr. Margaret Bash, CBER/FDA. [b]WAMC isolates 7720 and 7749 were isolated 6 months apart

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449 in 2014 (November and April, respectively) and NMCP isolates 4856 and 9542 were isolated 3 months
450 apart in 2017 (March and June, respectively).
451
452

453 **Immunizations and challenge experiments.**

454 Four-week-old female BALB/c mice (Charles River; NCI Frederick strain of inbred
455 BALB/cAnNCr mice, strain code 555) were used in these studies. In pilot immunization studies,
456 groups of 5 mice each were immunized with 20, 100 or 250 µL of 4CMenB (GSK) by the
457 intraperitoneal (IP) or subcutaneous (SQ) routes on days 0 and 28. Two independent
458 immunization and challenge experiments were conducted. For these experiments, 250 µL of the
459 vaccine were given IP or SC on days 0, 21 and 42. Control mice received PBS or alum in the form
460 of Alhydrogel (InVivogen) diluted in PBS (n = 20-25 mice/group). Venous blood was collected on
461 days 31 and 52; vaginal washes were collected on day 31. Three weeks after the final
462 immunization, mice in the anestrus or the diestrus stage of the reproductive cycle were implanted
463 subcutaneously with a 21-day slow-release 17β-estradiol pellet (Innovative Research of America)
464 and treated with antibiotics to suppress overgrowth of potentially inhibitory flora as described [60].
465 Two days after pellet implantation, mice were inoculated vaginally with 10^6 colony-forming units
466 (CFU) of *Ng* strain F62. Vaginal swabs were quantitatively cultured for *Ng* on 7 consecutive days
467 post-challenge and used to prepare stained smears to examine the influx of vaginal
468 polymorphonuclear leukocytes (PMNs) [75].
469

470 **Enzyme-linked immunosorbent assay (ELISA) and western blots**

471 Serum or vaginal total Ig, IgG1, IgG2a and IgA were measured as endpoint titers as
472 determined by standard ELISA [87]. Microtiter plates were coated with 20 µL/well of a 1:5 dilution
473 of the formulated Bexsero vaccine in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5, or with 4 µg/ml of
474 OMV from *Ng* strain F62. OMVs were isolated from supernatants from late-logarithmic phase

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475 cultures that were centrifuged for 1 hour at 100,000 x g at 4 °C. Pellets were resuspended in 1
476 mL of PBS. Protein concentration was determined by the BCA protein assay (Thermo Scientific).
477 For whole-cell lysates (WCL) (total cellular proteins), bacteria from agar plates or mid-logarithmic
478 phase cultures were centrifuged and the bacterial pellets suspended to an OD_{600} = 0.5. One
479 milliliter of this suspension was mixed with 60 µL of Laemmli sample buffer. For western blots,
480 WCL (4 µL) or 20 µg of OMV were subjected to sodium dodecyl sulfate polyacrylamide gel
481 electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, stained with Ponceau S,
482 and blocked overnight with 0.5% Tween20 in PBS. Membranes were incubated with pooled
483 antisera or vaginal washes from each experimental group diluted in block and washed three times
484 with 0.05% Tween 20 in PBS. Secondary antibody was horseradish peroxidase (HRP)-conjugated
485 anti-mouse IgG or IgA and a chemiluminescence HRP was used as substrate (GE Healthcare).
486 Apparent molecular weight of bands was determined using a standard curve generated from
487 molecular weight markers (r^2=0.9916; http://www.bio-
488 rad.com/webroot/web/pdf/lsr/literature/Bulletin_6210.pdf). For western blots with LOS, proteinase
489 K-treated bacterial extracts were generated as described [88] without the phenol treatment step,
490 separated on 16% tricine gels (Novex) and probed with 1:10,000 dilutions of pooled serum from
491 immunized and control mice followed by HRP-conjugated anti-mouse IgG as above.
492

493 Immunoprecipitation and mass spectrometry

494 Two milliliters of a *Ng* suspension (OD_{600} = 1) prepared from a mid-logarithmic phase Gc
495 broth culture were mixed with 30 µL of antisera for 20 minute at room temperature. Cell pellets
496 were washed once with GCB and solubilized in 2% Zwittergent 3,14 (EMD Millipore) in PBS for
497 one hour at 37°C. The solubilized suspension was centrifuged for 10 minutes at 2,0000 x g, the
498 supernatant mixed with protein A/G resin (ExAlpha Biologicals) for two hours at 4°C with mixing,
499 and the resin washed three times with 0.5% Zwittergent 3,14 in PBS, and once with PBS alone.

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500 The resin was suspended in 50 µL Laemmli sample buffer without β-mercaptoethanol and
501 subjected to SDS-PAGE for Western blotting; a duplicate gel was run in parallel and stained with
502 Coomassie G-250. Bands from the stained gel were submitted to the Michael Hooker Proteomics
503 Center at the University of North Carolina at Chapel Hill for trypsin digest and identification using
504 mass spectrometry. Accession ID numbers of proteins described in this report are disclosed in
505 Table 1 and Supp. Table 1. Alignment of amino acid sequences was performed using ClustalW.
506

507 **Bactericidal assay**

508 A modification of a previously described bactericidal assay [89] was used to test the
509 bactericidal activity of serum from immunized mice. Pooled sera from immunized and control mice
510 were heated at 56°C for 30 min and serially diluted 1:2 in minimal essential medium (MEM) (1:30
511 – 1:960). Fifty microliters of each dilution were pipetted into wells of a 96-well microtiter plate. Fifty
512 microliters of an MEM suspension containing 100-400 CFU of the target strain were added to the
513 wells and to a well containing 50 µl of MEM alone. After 5 minutes incubation at RT, 50 µl of
514 pooled normal human serum (NHS) (PelFreeze) were added to each well (final concentration
515 10%) and the plate was incubated for 55 minutes at 37ºC in 5% CO_2. Fifty microliters of GC broth
516 were then added, mixed, and 50 µl aliquots were cultured in duplicate on GC agar and incubated
517 overnight. The antiserum dilution that gave 50% recovery compared to wells without antiserum
518 was defined as the $bactericidal_{50}$ titer. Wells containing heat-inactivated NHS were tested in
519 parallel to measure complement-independent loss of bacterial viability during the assay; no
520 appreciable loss was detected in any experiment. The assay was performed against each strain
521 in two or three independent experiments.
522

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523 **Statistical analysis**

524 ELISA titers were compared by a Kruskal-Wallis test with Dunn's multiple comparison. For
525 challenge experiments, the percentage of mice with positive cultures at each time point was
526 plotted for each experimental group as a Kaplan Meier curve and analyzed by the Log Rank test.
527 The number of CFU recovered from vaginal swabs over time was compared by repeated
528 measures ANOVA with Bonferroni correction. The area under the curve (AUC) was calculated for
529 each individual mouse by determining the AUC across the 7 culture time points that was above
530 the limit of detection (20 CFU/mL). Differences between AUC and percentage of vaginal PMNs
531 were compared using a Kruskal-Wallis test with Dunn's multiple comparison. Statistical analyses
532 were performed using the software Prism (GraphPad Software, La Jolla, CA). Raw data used for
533 statistical analysis of ELISA, *in vivo* efficacy testing, and bactericidal assays have been published
534 [90].
535

536 **Animal ethics statement**

537 All animal experiments were conducted at the Uniformed Services University according to
538 guidelines established by the Association for the Assessment and Accreditation of Laboratory
539 Animal Care using a protocol approved by the University's Institutional Animal Care and Use
540 Committee.
541

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543

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551

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942 # Figure captions

943 **Fig 1. Serum and vaginal antibody titers against F62 OMVs from 4CMenB-immunized mice.**
944 Groups of 25 BALB/c mice were immunized three times, three weeks apart with 250 µl or 4CMenB
945 by the IP or SC routes or with PBS or Alum (IP route). Serum and vaginal antibody titers on day
946 31 and day 52 (ten days after the 2nd and 3rd immunization, respectively) against F62 OMVs were
947 measured by ELISA. Shown are serum **(A)** total Ig, **(B)** IgG1, **(C)** IgG2a, **(D)** IgA and **(E)**
948 IgG1/IgG2a ratios for on days 31 and 52 (left column). Vaginal **(F)** total Ig, **(G)** IgG1, **(H)** IgG2a
949 and **(I)** IgA on day 31 are shown in the right column. No difference was found in any sample or Ig
950 tested between control animals receiving PBS and those receiving alum only. *, p<0.05; **,
951 p<0.01; ***, p<0.0001. Results from the repeat experiment were similar.

952

953 **Fig 2. 4CMenB has *in vivo* efficacy against *Ng*.** Mice were immunized three weeks apart with
954 250-µL doses of 4CMenB by the IP (blue) or SC (red) route or with PBS (black) or alum (purple)
955 by the IP route and challenged with *Ng* strain F62 three weeks after the final immunization. Shown
956 are the combined data from two independent trials (total n = 38-41 mice/group). **(A)** Percentage
957 of culture-positive mice over time; **(B)** Average CFU per ml of a single vaginal swab suspension;
958 **(C)** total bioburden over 7 days expressed as area under the curve; **(D)** Percentage of mice that
959 cleared infection by day 7 post-challenge. *, p < 05; **, p < 0.01; ***, p < 0.0001.

960

961 **Fig 3. Antisera from 4CMenB-immunized mice is bactericidal against a serum-sensitive**
962 **and a serum-resistant *Ng* strain**. Serial dilutions of pooled serum from mice vaccinated with
963 250 µl of 4CMenB (blue lines) or Alum alone (black lines) by the IP route were incubated with 10^4
964 CFU of the challenge strain F62 or strain FA1090 in microtiter plates as described in the Materials
965 and Methods. After 5 min, NHS or heat-inactivated (HI)-NHS (final concentration, 10%) was
966 added. After 45 min incubation at 37ºC, the number of viable *Ng* in each well was determined by

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967 duplicate culture on GC agar. Data are expressed as the number of CFU from wells incubated
968 with test serum divided by the number recovered from wells containing PBS instead of test serum,
969 X times 100. Solid lines indicate NHS was used as the complement source; dotted lines represent
970 data from wells tested in parallel with HI-NHS. The dotted line at 50% survival was drawn to
971 identify the bactericidal$_{50}$ titers.
972
973 **Fig 4. Serum and vaginal antibodies from 4CMenB-immunized mice recognize *Ng* outer**
974 **membrane proteins by western immunoblot. (A)** Pooled antisera from mice immunized with
975 250 µL of 4CMenB by the IP (250IP, upper panels) or SC (250SC, lower panels) route were tested
976 against OMVs (app. 20 µg per lane) from 7 different *Ng* strains fractionated on 4-20% Tris-glycine
977 gels by western blot (1:10,000 dilution of primary antisera) followed by secondary anti-mouse IgG-
978 HRP. A boosting effect is observed when comparing the band intensities for serum collected after
979 2 and 3 immunizations. **(B)** Pooled vaginal washes from immunized or control mice collected after
980 the third immunization tested against OMVs from the F62 challenge strain by western blot (1:100
981 dilution), followed by secondary anti-mouse IgG-HRP or anti-mouse IgA-HRP. Pooled vaginal
982 washes were from mice given: (1) SC250; (2) IP250; (3) PBS; (4) Alum only. (5) Results using
983 250IP mouse serum (1: 10,000) for comparison. The band recognition pattern was similar for blots
984 incubated with serum (lanes 5) or vaginal washes (lanes 1-4), and vaginal washes from IP-
985 immunized mice were more strongly reactive than from SC-immunized mice. All lanes were
986 equally loaded, as determined by Ponceau S staining. Shown are representative results from at
987 least 2 separate experiments with identical results.
988
989 **Fig 5. 4CMenB antiserum recognizes *Ng* LOS in a minority of strains.** Proteinase K-treated
990 bacterial extracts from 4 laboratory strains and 13 clinical isolates were resolved on 16% Tricine
991 gels and stained with (A) silver stain (top panel) or (B) Emerald green (top panels), or
992 electroblotted and probed with the following: (A) Mabs 3F11, 4C4 and 2C7, which recognize *Ng*

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993 LOS epitopes (bottom panels). Note that FA19 1986 is a variant of FA19 (panel B) that has a
994 phase-off *lgtA* gene that results in truncation of the LOS to a single 3.6 kDa species [91]. (B)
995 Pooled IP250 4CMenB antisera (bottom panels). The doublets in LGB-24 and NMCSD 3277 and
996 single LOS species in MS11 and NMCSD 6364 that were recognized by the antiserum are distinct
997 from the LOS species identified by the Mabs shown in Panel A. (C) Emerald green-stained (upper
998 panel) LOS from *Ng* strain H041 and LGB-24 (positive control) used to inoculate mice (Inoc) and
999 from vaginal cultures collected on days 2 and 5 of infection. No change in the LOS species or
1000 4CMenB reactivity was observed during infection by these strains.
1001
1002 **Fig 6. PilQ, MtrE, porin and OpA are recognized by 4CMenB antisera.** OMV (app. 20 μg) from
1003 6 *Ng* strains (Table 2) were subjected to SDS-PAGE on a 4-20% Tris-glycine gel and (A) stained
1004 with sypro ruby or (B) transferred to PVDF for western blot with the 250IP antiserum. The stained
1005 gel was aligned with the Western blot, and corresponding bands were digested and analyzed by
1006 mass spectrometry. The numbers indicated with arrows on each panel correspond to the same
1007 numbers on the Western blots except for bands 5 and 6, which were excised from a different gel
1008 but are indicated on the western based on the banding patterns. Proteins identified are described
1009 in Table 1. Among the proteins identified, known surface-exposed outer membrane proteins are:
1010 (1) PilQ, (2) BamA, (4) MtrE, (5) PorB, and (6) Opa.
1011
1012 **Fig 7. 4CMenB -induced antibodies bind PilQ and MtrE at the surface of viable *Ng* FA1090**
1013 **and MS11 bacteria.** Immunoprecipitations were performed with wild-type strains FA1090 and
1014 MS11 and their isogenic *pilQ* and *mtrE* mutants using antisera from mice immunized with 250 μL
1015 of 4CMenB via the IP route (250IP) or given alum only (negative control). Bacterial components
1016 bound by 4CMenB-induced antisera were subjected to SDS-PAGE (non-denaturing conditions,
1017 4-20% Tris-glycine) and Western blotting with 4CMenB 250IP antiserum. WCLs, total cellular
1018 proteins; OMVs, outer membrane vesicles; *pilQ*-, FA1090Δ*pilQ*; *mtrE*-, MS11Δ*mtrE*. Data shown

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1019 are representative of at least 2 separate experiments with identical results. The wide band around
1020 200 kDa corresponds to the antibodies within the test antisera that are present in the antigen-
1021 antibody complexes and pulled down with the protein A/G agarose.

1022

1023 ## Supporting Information
1024

1025 **S1 Fig. Pilot dose response immunization study with the 4CMenB vaccine.** Groups of 5
1026 BALB/c mice were given 20, 100 or 250 µl of the formulated vaccine on days 1 and 28 by the IP
1027 or SC routes. (A, B) Serum IgG1 and IgG2a titers against the formulated 4CMenB vaccine 10
1028 days after the second immunization. A dose response is shown for serum IgG1 in both IP- and
1029 SC-immunized mice. (C) Serum reactivity against whole cell lysates of *Nm* strain MC58 and of 6
1030 different *Ng* strains using anti-mouse IgG secondary antibody shows a similar dose response
1031 based on differences band intensity. A nonparametric test, the Kruskal Wallis test with Dunn's
1032 multiple comparison, was used to analyze ELISA data due to the low sample size. **, $p < 0.01$.

1033

1034 **S2 Fig. 4CMenB significantly accelerated *Ng* clearance and reduced the *Ng* colonization**
1035 **load in two independent experiments.** In each experiment, mice were immunized three weeks
1036 apart with 250-µl doses of 4CMenB by the IP (blue) or SC (red) route or given PBS (black) or
1037 alum ([urple) by the IP route (n = 25 or 20 mice per group in experiments 1 and 2, respectively).
1038 Three weeks after the final immunization, mice in the diestrus stage or anestrus were treated with
1039 17β-estradiol and antibiotics and challenged with *Ng* strain F62 as described in the Materials and
1040 Methods. (A, B) Percentage of culture-positive mice over time and average CFU per ml of a single
1041 vaginal swab suspension, respectively for experiment 1 (n = 20-23 mice/group); (C, D)
1042 Percentage of culture-positive mice over time and average CFU per ml of a single vaginal swab
1043 suspension, respectively for experiment 2 (n = 18-19 mice/group).

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1044 *, $p < 0.05$, **$p < 0.01$, *** $p < 0.0001$.

1045

1046 **S3 Fig. A similar vaginal PMN influx occurred in all experimental groups**. Vaginal smears
1047 collected on each culture day following bacterial challenge were stained with Hemacolor Stain
1048 (Sigma), and the percent of PMNs among 100 vaginal cells was determined by cytological
1049 differentiation using a light microscopy. An increase in the percentage of PMNs occurred between
1050 days 4-7 as is characteristic of this model, with no statistical difference between the groups. The
1051 median percent PMNs is shown for each time-point.

1052

1053 **S1 Table. Amino acid identity between proteins of *N. meningitidis* MC58 and *N.***
1054 ***gonorrhoeae* FA1090**

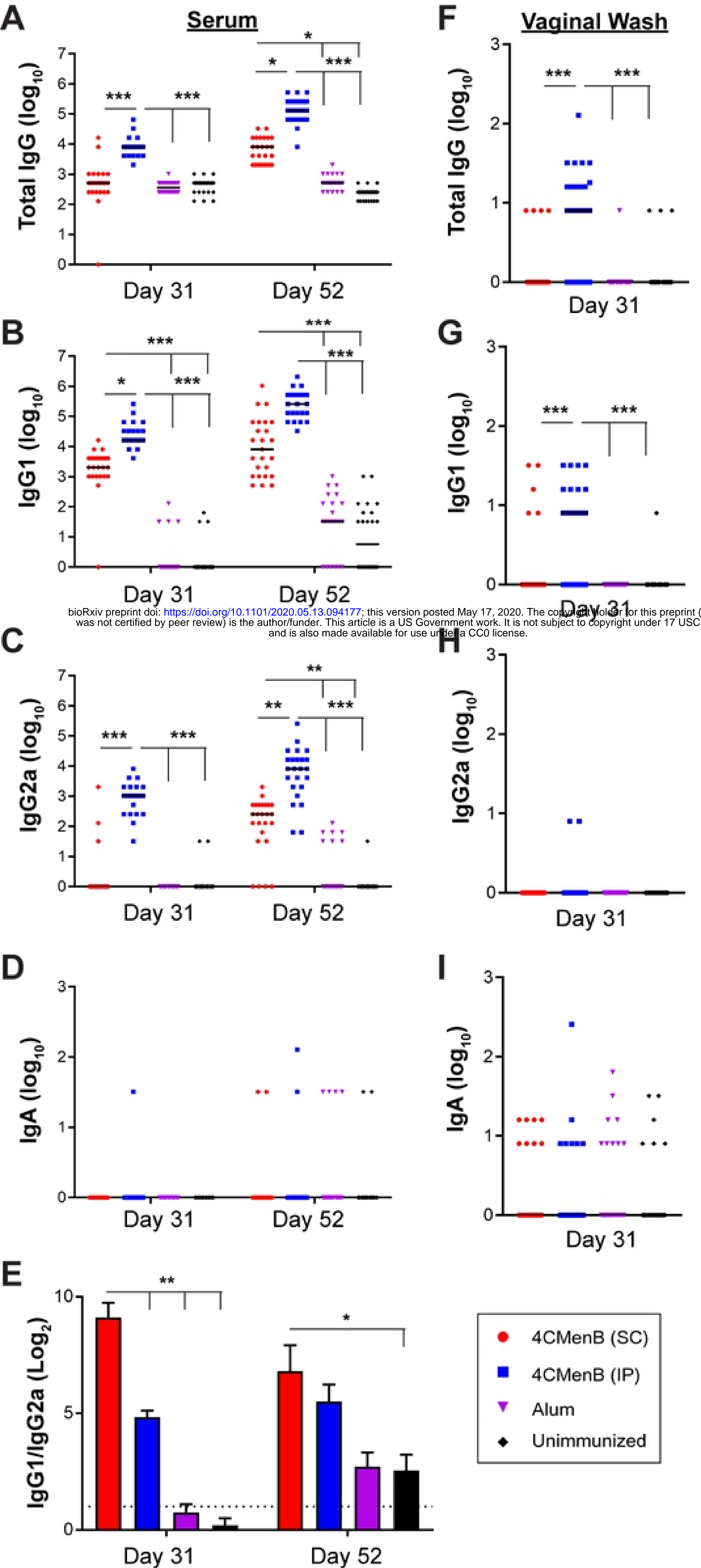

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Figure 1

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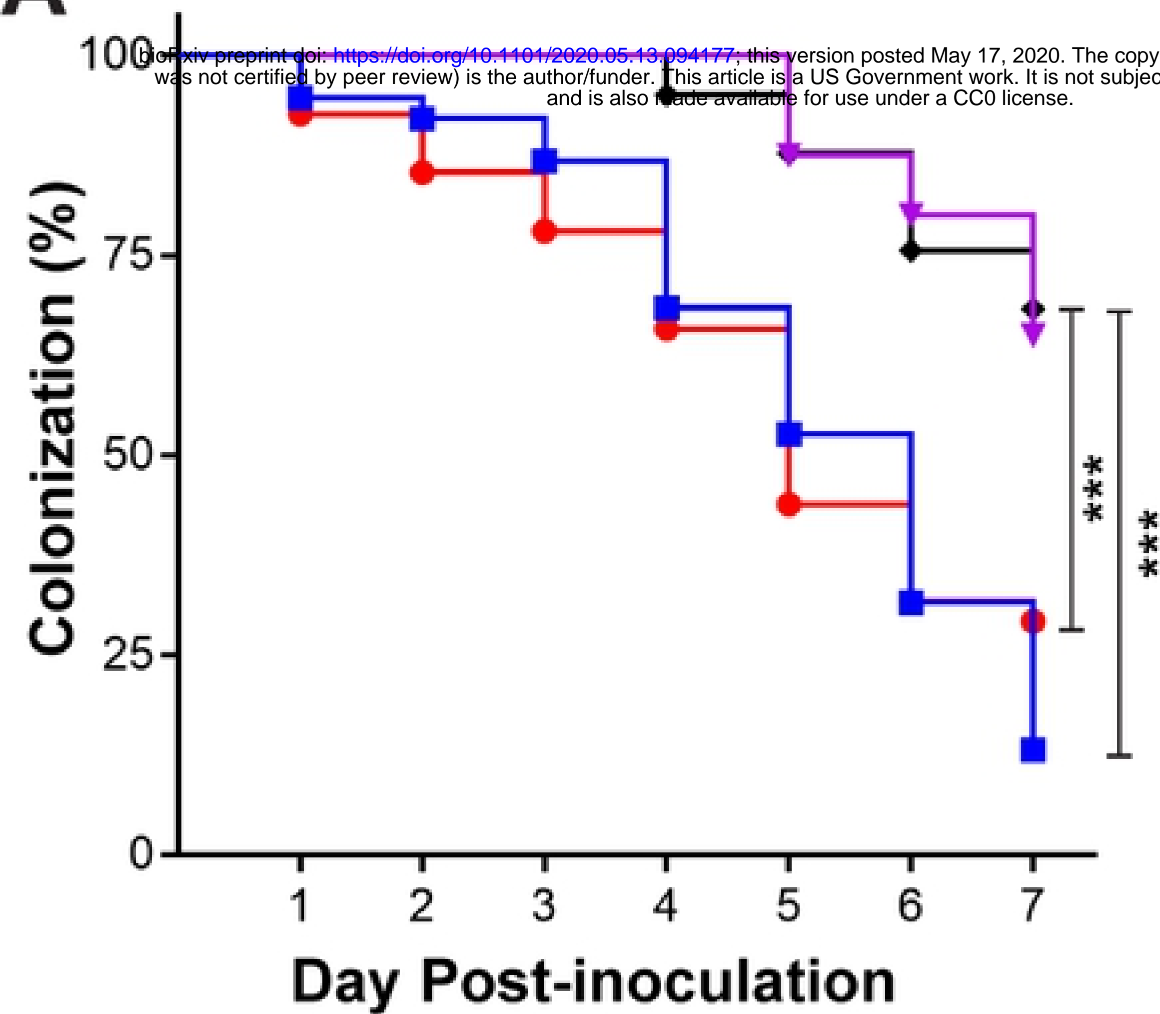
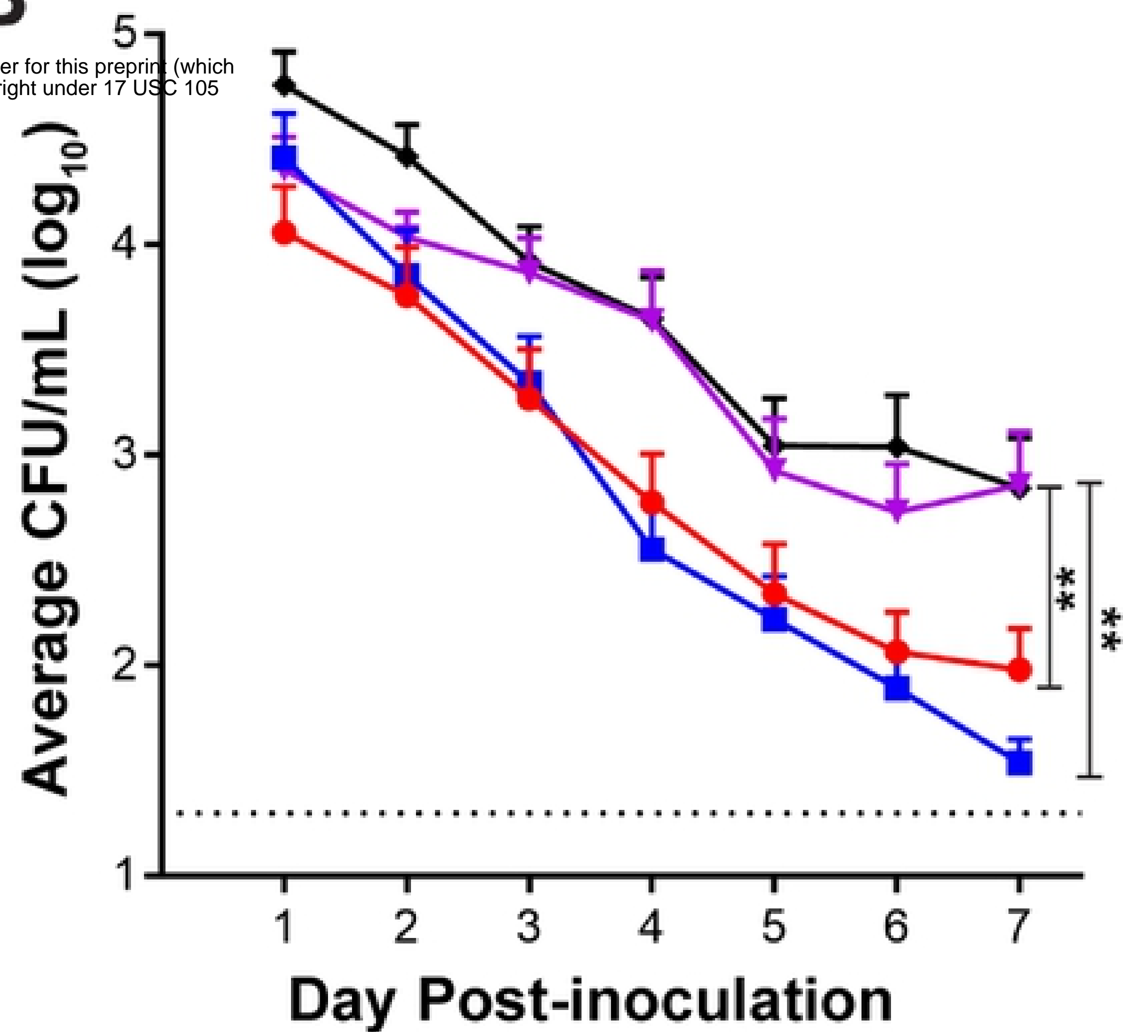
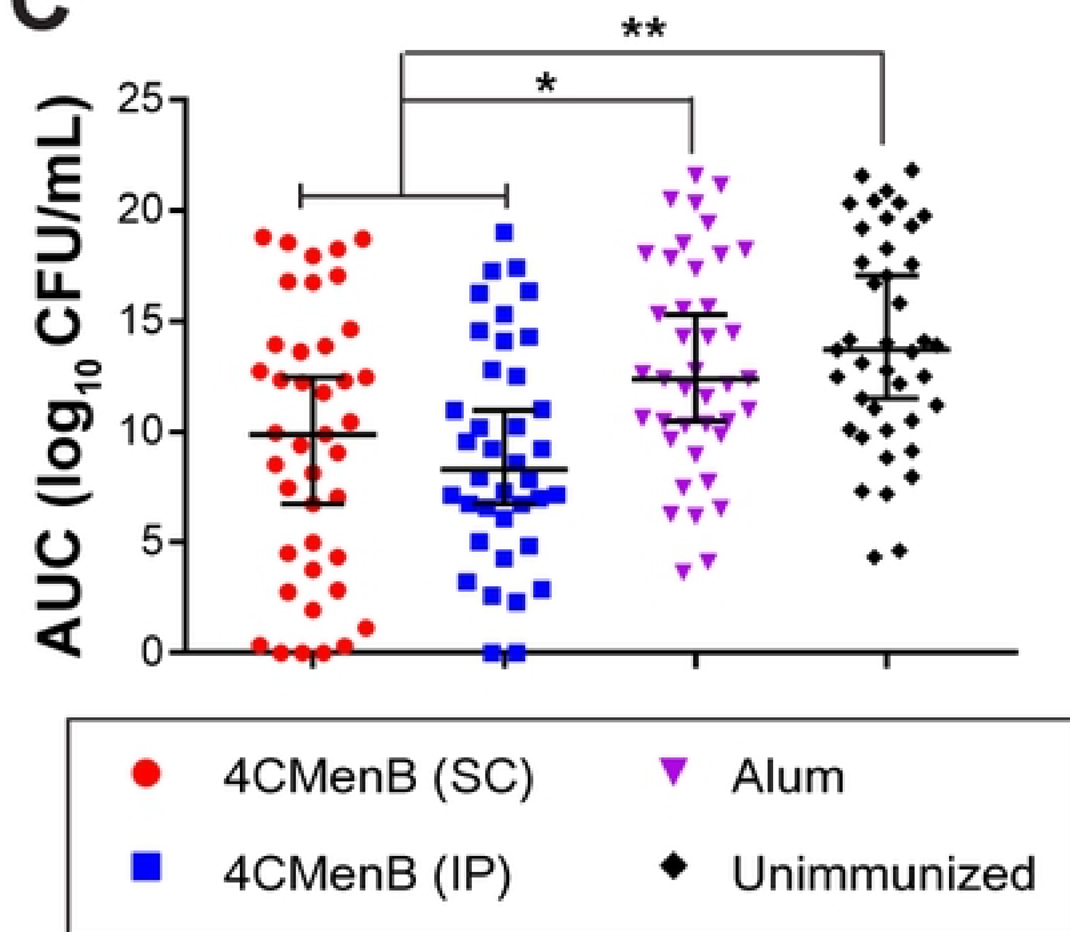






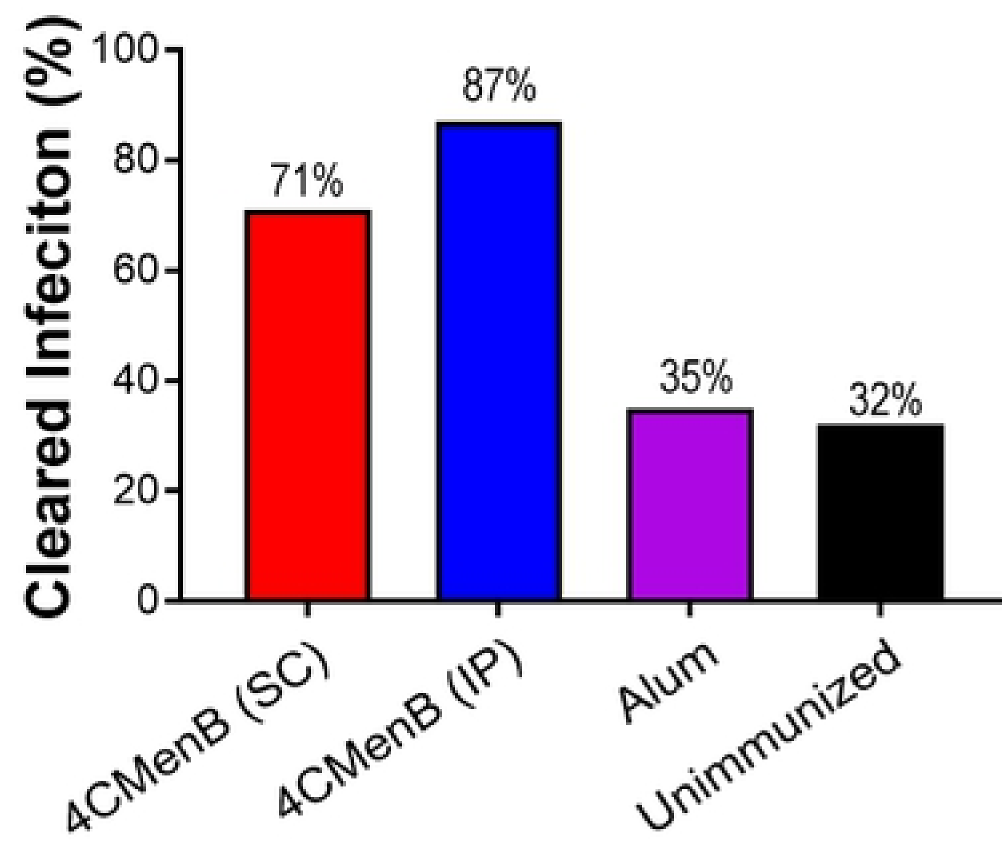
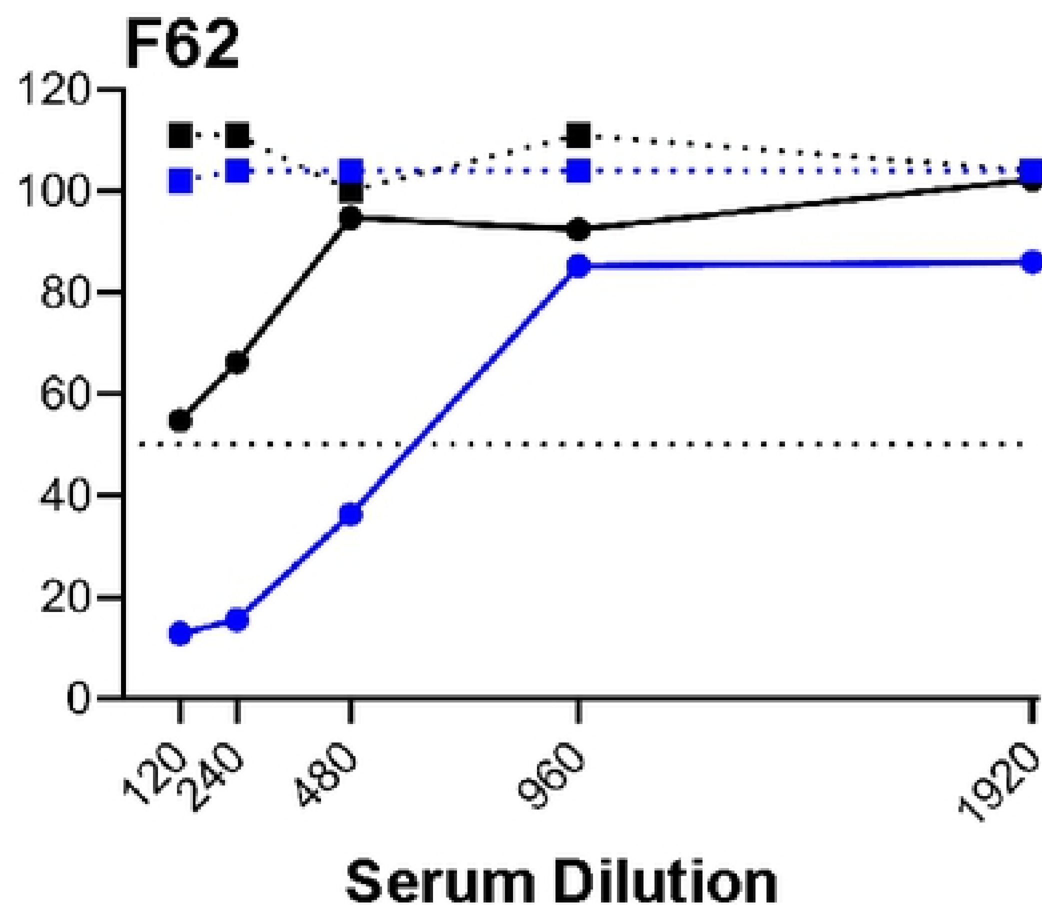
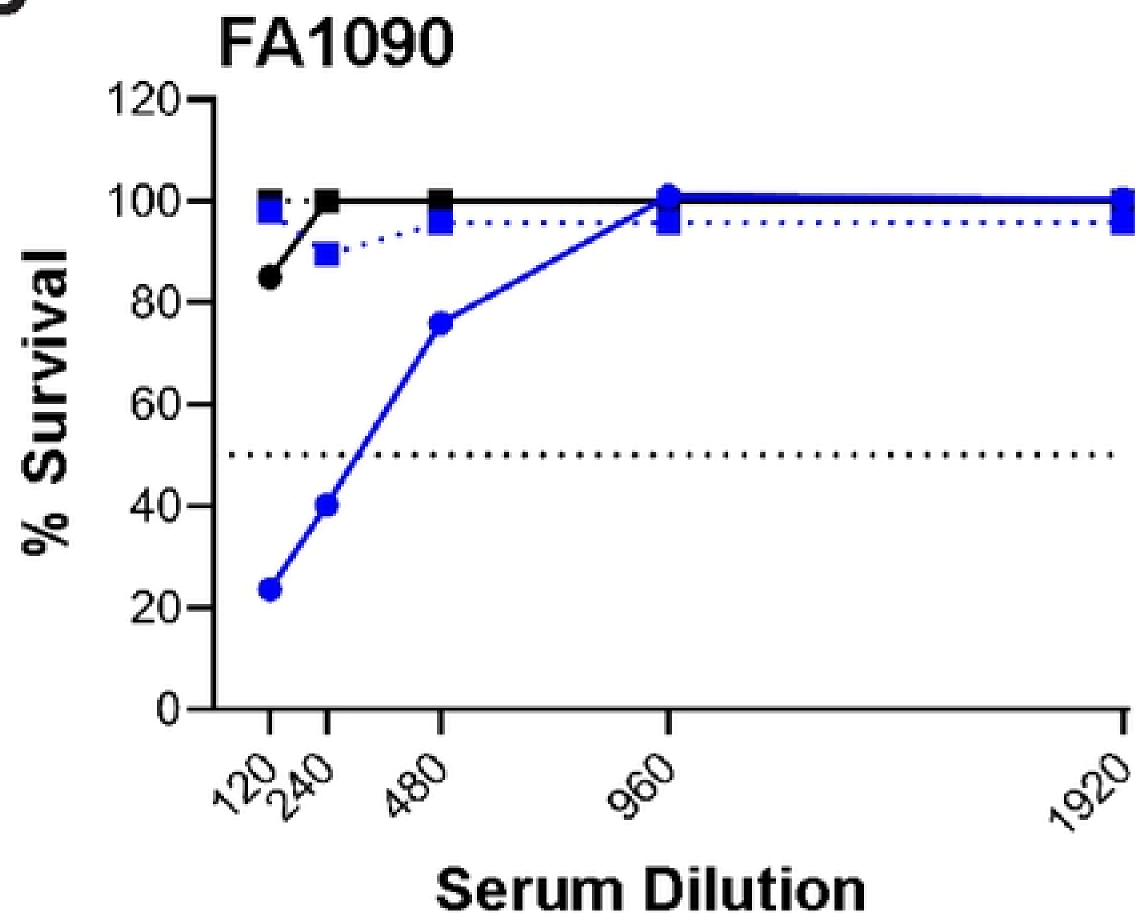


Figure 2





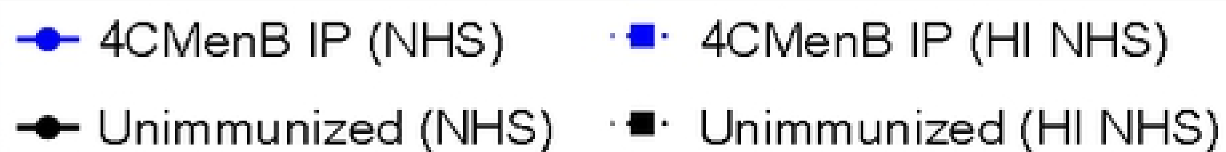


Figure 3

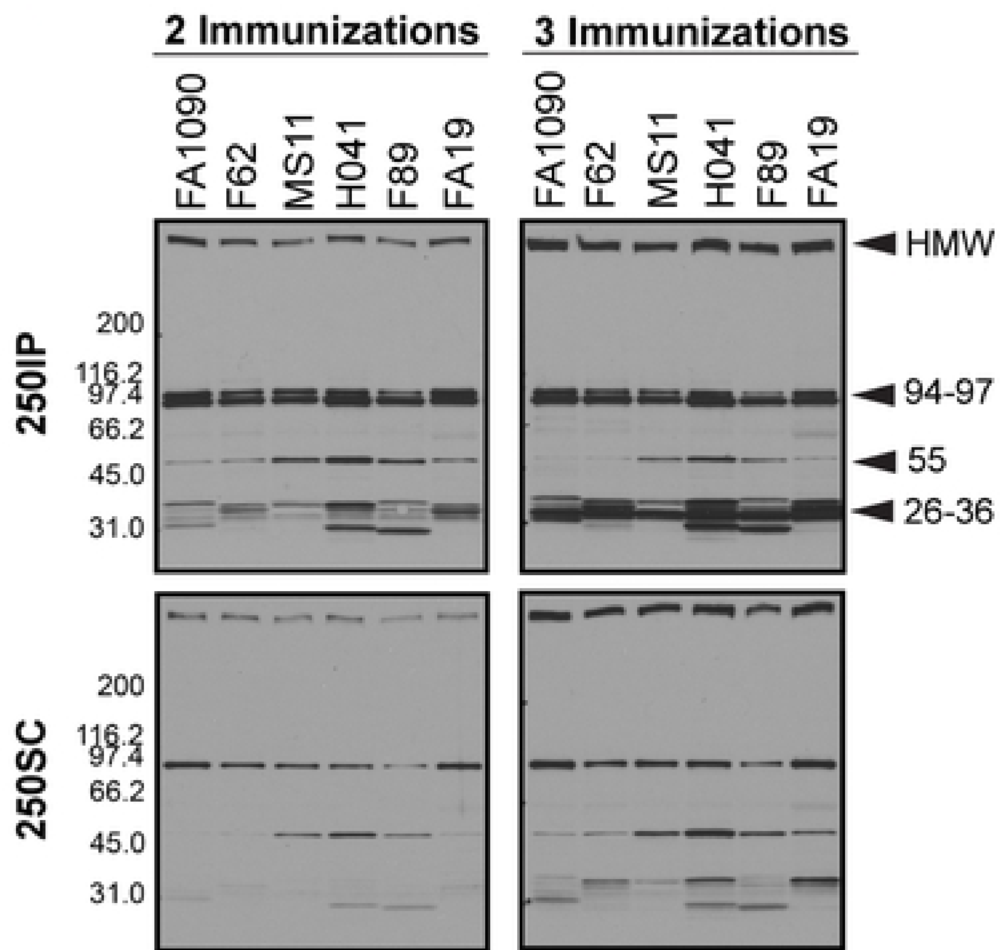


Figure 4

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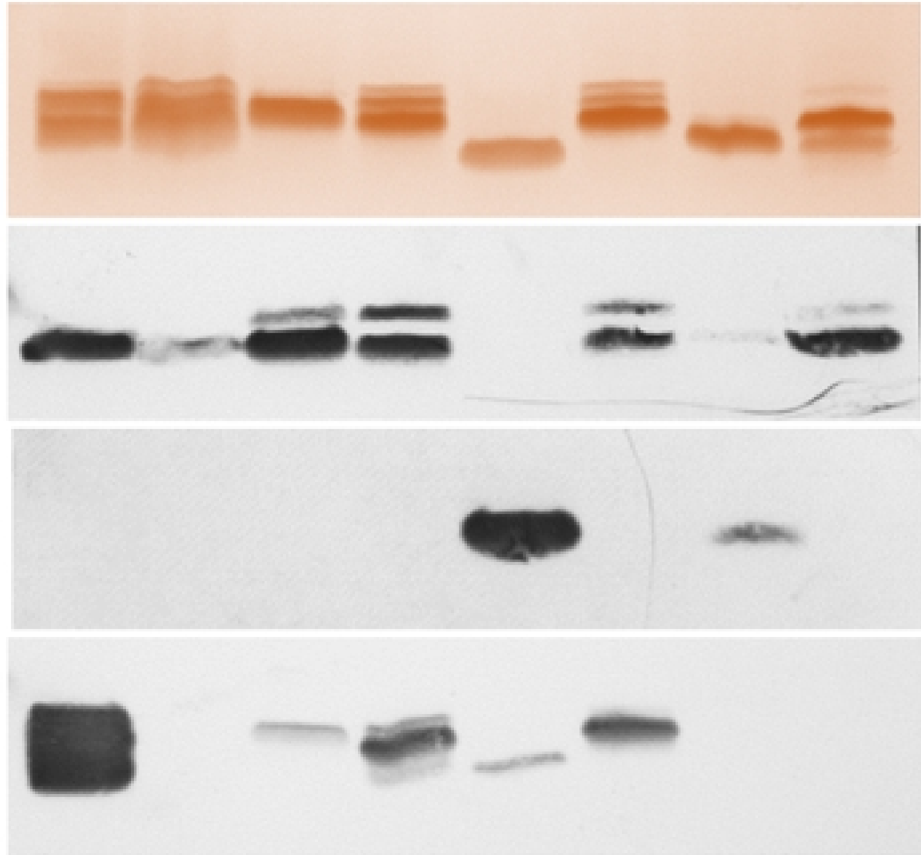

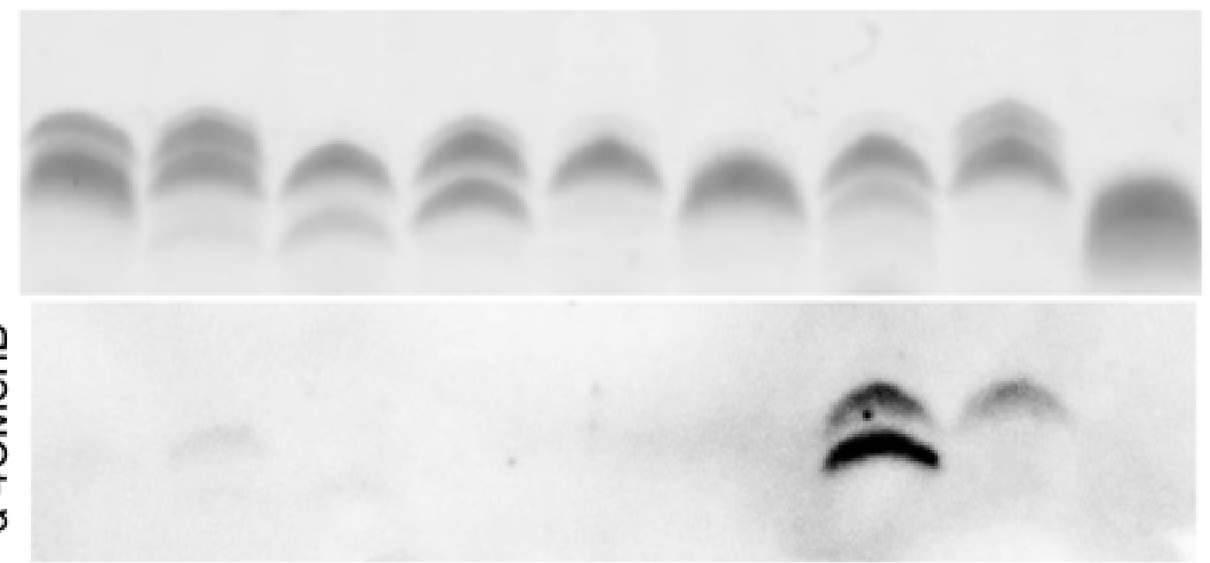

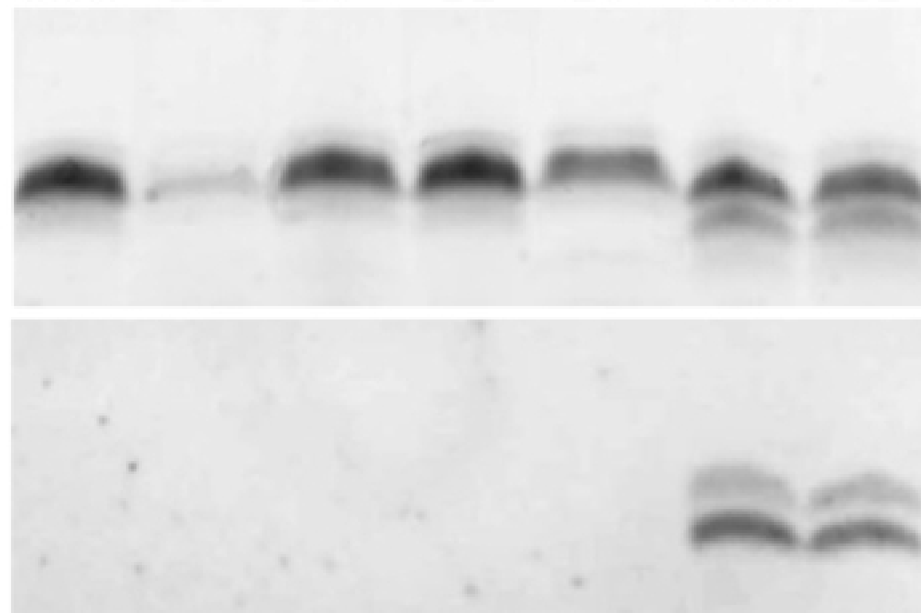

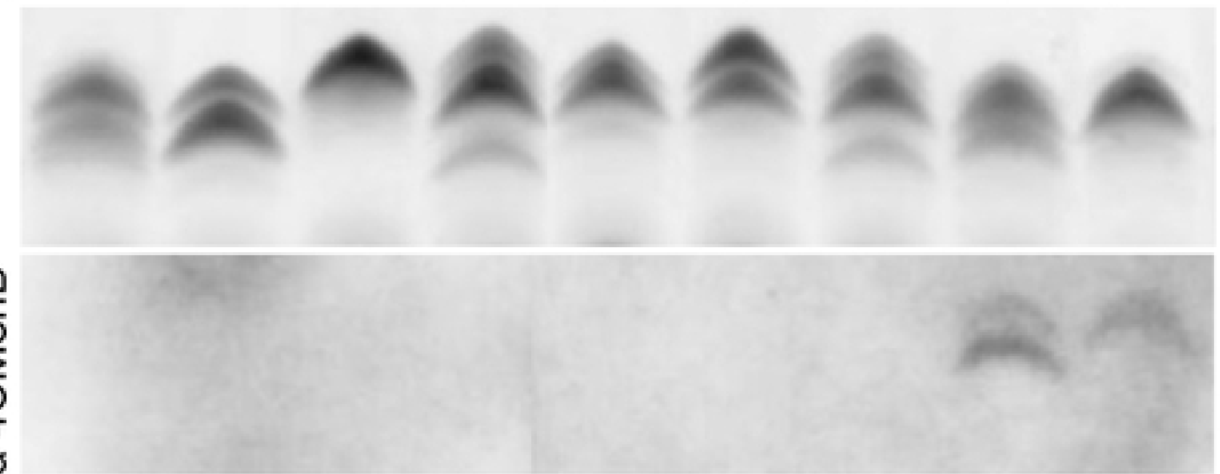

Figure 5

A

FA1090 F62 MS11 H041 F89 FA19

205
116.2
97.4
66.2
45.0
31.0
21.5
14.4

1
2
3
4

SR

B

FA1090 F62 MS11 H041 F89 FA19

1 PilQ
2 BamA
4 MtrE
5 PorB
6 Opa

250IP

Figure 6

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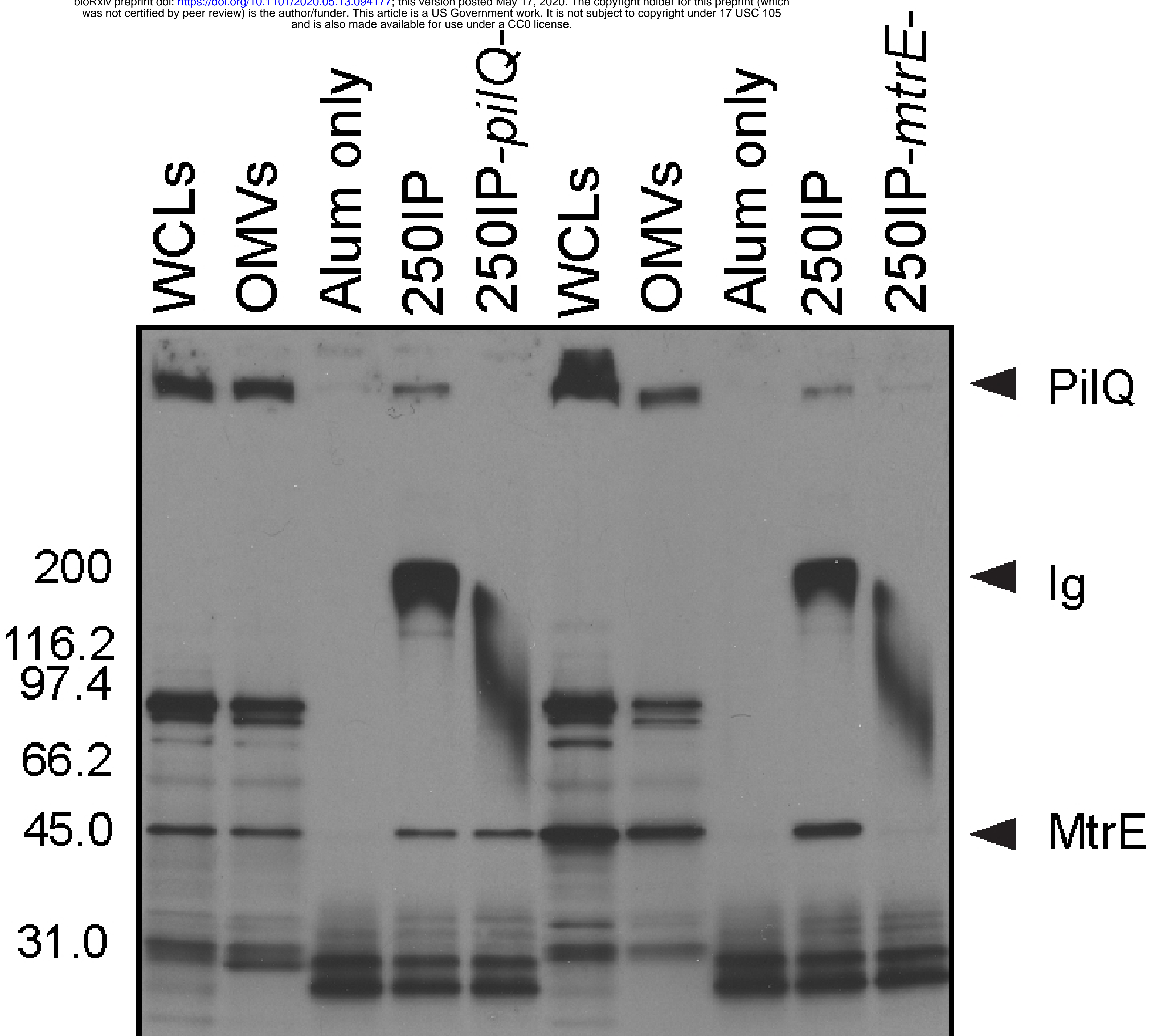

Figure 7