Repertoire of naturally acquired maternal antibodies transferred to infants for protection against shigellosis

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Abstract

Shigella is the second leading cause of diarrheal diseases, accounting for >200,000 infections and >50,000 deaths in children under 5 years of age worldwide. The incidence of *Shigella*-induced diarrhea is relatively low during the first year of life and increases substantially (reaching its peak) between 11 to 24 months of age. This epidemiological trend hints to an early protective immunity of maternal origin and an increase in disease incidence when maternal immunity wanes. The magnitude, type, antigenic diversity and anti-microbial activity of maternal antibodies transferred via placenta that can prevent shigellosis during early infancy are not known. To address this knowledge gap, *Shigella*-specific antibodies directed against the lipopolysaccharide (LPS) and virulent factors (IpaB, IpaC, IpaD, IpaH and VirG) and antibody mediated serum bactericidal (SBA) and opsonophagocytic killing antibody (OPKA) activity were measured in maternal and cord blood sera from a longitudinal cohort of mother-infant pairs living in rural Malawi. Protein-specific IgG (very high levels) and *Shigella* LPS were detected in maternal and cord blood sera; efficiency of placental transfer was 100% and 60%, respectively and was associated with IgG subclass distribution (protein-specific IgG1 > LPS-specific IgG2). In contrast, SBA and OPKA activity in cord blood was substantially lower as compared to maternal serum and varied among *Shigella* serotypes. LPS was identified as a target of SBA and OPKA activity. Maternal sera had remarkably elevated *Shigella flexneri* 2a LPS IgM indicative of recent exposure. Our study revealed a broad repertoire of maternally acquired antibodies in infants living in a *Shigella*-endemic region and highlights the abundance of protein-specific antibodies and their likely contribution to disease prevention during the first months of life. These results contribute new knowledge on maternal infant immunity and target antigens that can inform the development of vaccines or therapeutics that can extend protection after maternal immunity wanes.

47 **Introduction**

48 *Shigella* spp. are major contributors of the global diarrheal disease burden, accounting for more
49 than 250 million cases and 200,000 deaths annually (1, 2). The most affected are children under
50 5 years of age living in low- and middle-income countries (LMIC) (2, 3). Though usually self-
51 limiting, repeated bouts of disease result in debilitating sequalae including malnutrition, growth
52 stunting, and deficits in immune and cognitive development (3, 4). The preeminence of multidrug
53 resistant *Shigella* strains globally makes the development of vaccines and therapeutics a
54 compelling priority (5). Because the burden of disease disproportionately affects young children,
55 a clear understanding of the elements and immune mechanisms that can protect this group is
56 necessary to inform the development of efficacious vaccines or prophylaxes.

57 Most of what is known to date about *Shigella* immunity has been learned from infections in adults.
58 Individuals living in endemic regions acquire natural immunity from repeated exposure (6-9).
59 While there is no definitive immune correlate of protection against shigellosis, serum IgG against
60 the *Shigella* surface-exposed lipopolysaccharide (LPS) has been associated with reduced risk of
61 infection with serotype matching strains in early field trials [reviewed in Ref (10)]. We have
62 reported a strong correlation between serum IgG specific for the *Shigella* invasion plasmid antigen
63 (Ipa) B and the virulence protein, VirG (IcsA), and reduced risk of infection in a controlled human
64 infection model (CHIM) study (11). In the same experimentally infected adult volunteers,
65 complement-mediated serum bactericidal (SBA) and opsonophagocytic killing (OPKA) were
66 identified as functional attributes of *Shigella*-specific antibodies associated with clinical protection
67 (11).

68 Children living in endemic regions produce serum LPS- and Ipa-specific IgG in response to
69 *Shigella* infection, and the magnitude of these responses increases progressively through
70 adulthood (6, 9, 12, 13). Multiple surveillance studies have reported consistently that the rate of
71 *Shigella* infection is relatively low during the first months of life, but gradually increases and

72 reaches its peak during the second year of life (14, 15). The shielding of young infants from
73 *Shigella*-induced diarrhea (while they still suffer from other enteric infections such as rotavirus),
74 hints to a putative pathogen-specific protection afforded by maternal immunity (antibodies
75 transferred via placenta and the immune components of breast milk (16)). Studies of
76 transplacental antibody transfer against other pathogens have shown that this process—termed
77 placental "sieving" (17)—is regulated and selective, antigen-dependent (18, 19), and favors
78 transfer of antibodies with specific biophysical features that render them most functional in the
79 context of the immature neonatal immune system (17). Information on antigen-specificity,
80 magnitude, subclass distribution and function of *Shigella* antibodies in mothers and infants and
81 the process of placental transfer is lacking. Here, we characterized the specificity and anti-
82 microbial function of *Shigella*-specific antibodies in mothers and their infants at birth in a
83 longitudinal cohort from rural Malawi. The magnitude of serum IgG (and IgG subclasses) specific
84 for *S. flexneri* 2a LPS, *S. sonnei* LPS, IpaB, IpaC, IpaD, IpaH, and VirG were determined. SBA
85 and OPKA levels and the target antigen mediating complement- and phagocytic-effector functions
86 were investigated. Finally, correlative analysis and comparison with protective thresholds were
87 conducted to identify unique features and the potential anti-microbial activity in vivo of *Shigella*
88 antibodies in mother-infant pairs.

89

90 **Results**

91

92 **Cohort characteristics**

93 This study utilized a mother-infant cohort from a malaria surveillance study in Malawi. Participants
94 were enrolled from the rural villages in Chikwawa and in the southern region of Malawi. Out of
95 108 mother and infant pairs enrolled, 63 mother-infant pairs were analyzable (Figure 1). Cord
96 blood was collected at birth. Maternal blood was obtained at recruitment within 3 months of

97 delivery. Characteristics of the cohort are summarized in Table 1. Mean age of the mothers was
98 26.8 years (17-43 years). Median maternal parity was 3 (0-8). Among the infants, 36 (57%) were
99 female and 5 (8%) had a low birth weight (less than 2.5kg). As for the season of birth, 21 (33%)
100 were born during the rainy season (November – April).

101

102 ***Shigella* antigen-specific IgG in mothers and their newborns**

103 Naturally acquired *Shigella*-specific antibodies were determined in paired maternal and cord blood
104 sera. The antigenic repertoire analysis was focused on *S. flexneri* 2a and *S. sonnei*, as these
105 strains had been attributed the highest incidence of moderate-to-severe diarrhea (MSD) in <5-
106 year-old children (37.8% and 13.5%, respectively) in Malawi-neighboring Mozambique by the
107 Global Enteric Multicenter Study (GEMS) (20); precise information on *Shigella* prevalence in
108 Malawi is not available. *S. flexneri* 2a and *S. sonnei* LPS-specific IgG titers in maternal sera were
109 significantly higher as compared to those in cord blood (Figure 2A). The cord to maternal *S.*
110 *flexneri* 2a and *S. sonnei* LPS IgG transfer ratio were 0.60 and 0.70 respectively (Figure 2B, Table
111 2), indicating low transplacental sieving efficiency of LPS IgG.

112 Maternal and cord IgG levels against five different *Shigella* virulent factors: IpaB, IpaC, IpaD,
113 IpaH, and VirG and their placental transfer efficiency were also determined (Figure 2A and 2B).
114 High levels of circulating IgG specific for all protein antigens were detected in both maternal and
115 cord blood sera, which far surpassed the levels of IgG against LPS (Figure 2A). Likewise,
116 placental transfer of protein-specific maternal IgG was more efficient than the transfer of LPS-
117 specific IgG; mean transfer ratios were: 1.10, 1.53, 0.98, 0.99 and 1.01 for anti-IpaB, -IpaC, -IpaD,
118 -IpaH, and -VirG antibodies, respectively (Figure 2B and Table 2). Despite differences in transfer
119 efficiency between LPS- and protein-specific antibody titers, there was a significant and positive
120 linear correlation between maternal and cord blood IgG levels for all antigens, implicating a
121 distinct regulated transport (Figure 2C). A comparative analysis of maternal antibody specificity

122 (including all *Shigella* antigens tested) revealed positive associations on the basis of antigenic
123 target (protein or LPS). Protein-specific antibody levels were generally highly and positively
124 associated (r > 0.5) as were *S. flexneri* 2a and *S. sonnei* LPS IgG titers (Pearson's r > 0.6) (Figure
125 2D). Antibodies against *S. flexneri* 2a and *S. sonnei* LPS are not expected to be cross-reactive
126 (7, 21, 22). Therefore, the observed association between the two suggests that most mothers had
127 been exposed and responded similarly to both serotypes. Correlations between protein- and LPS-
128 specific antibody titers were generally lower (Pearson's r < 0.4), except for the association
129 between IpaC- and both *S. flexneri* 2a and *S. sonnei* LPS IgG (Figure 2D). These associations
130 were mirrored in the antibody responses in the infant serum, confirming the selective and
131 regulated principles of placental antibody transfer (Figure 2D). Geometric mean titers (GMT),
132 mean transfer ratios, and standard deviations (SD) between maternal and cord serum titers are
133 summarized in Table 2.

134

135 ***Shigella* protein- and LPS-specific IgG subclass placental transfer**

136 Placental transport of maternal antibodies is primarily mediated through binding to the neonatal
137 Fc receptor (FcRn) expressed in syncytiotrophoblast (23, 24). Qualitative differences in the Fc
138 structure, such as in the human IgG subclasses, can influence FcRn binding and placental
139 transfer (25). We therefore explored IgG subclass distribution of *Shigella*-specific antibodies as a
140 contributor to the observed differences in IgG transfer efficiency.

141 As with total IgG titers, protein-specific IgG subclasses had common features, which differed from
142 those against LPS. In both the mothers and their infants, IgG1 was the most abundant subclass
143 against all protein antigens, followed by IgG2 and IgG3. In contrast, IgG2 was the most abundant
144 subclass against both *S. flexneri* 2a and *S. sonnei* LPS. IgG4 titers were generally the lowest for
145 all antigens tested (Figure 3A). For protein antigens, IgG1 and IgG4 had the highest cord-
146 blood:maternal mean transfer ratios (1.05-1.27 and 1.29-1.68, respectively, Figure 3B and C).

147 For LPS antigens, IgG1 also exhibited the highest mean transfer ratios: 1.3 and 1.58 for *S. flexneri*
148 2a and *S. sonnei,* respectively (Figure 3B). Mean transfer ratios for LPS IgG2 were lower,
149 regardless of the serotype. The predominance of maternal LPS-IgG2 and the lower transfer
150 efficiency of IgG2 explains the low levels of LPS IgG in the cord blood despite their abundance in
151 maternal circulation. The mean transfer ratios for antigen-specific IgG compared to IgG
152 subclasses are represented in a heatmap (Figure 3C).

153

154 **Functional capacity of placentally transferred *Shigella*-specific antibodies**

155 In addition to antibody specificity through direct binding, we examined the functional capacity of
156 maternal and placentally-acquired antibodies to render complement-dependent bactericidal and
157 opsonophagocytic activity. SBA and OPKA activity was detected in both maternal and newborn
158 sera. Maternal SBA and OPKA titers against *S. flexneri* 2a were significantly higher as compared
159 to those against *S. sonnei* (GMT 26,944 compared to 306, respectively). Maternal SBA and OPKA
160 titers specific for *S. flexneri* 2a were also significantly higher in maternal sera as compared to
161 those of cord blood (Figure 4A and 4B); the mean transfer ratios were 0.16 and 0.18, respectively
162 (Figure 4A and B, and Table 3). In contrast, SBA and OPKA titers against *S. sonnei* in maternal
163 serum and in infant serum were comparable (Figure 4A); the mean transfer ratios were 1.29 and
164 0.82 (Figure 4B and Table 3). It was noticed that while maternal and cord blood functional antibody
165 titers against *S. sonnei* were strongly correlated, those against *S. flexneri* 2a were not (Figure 4C
166 and D). The discrepancy in functional antibody titer against *S. flexneri* 2a between mothers and
167 infants prompted us to investigate the specificity and type of antibodies involved in bactericidal
168 and opsonophagocytic killing.

169

170 **Specificity of maternally acquired functional antibodies**

171 Mouse monoclonal antibodies specific for *Shigella* LPS were reported to have bactericidal activity
172 (21). Several studies have reported increases in SBA titers in response to *Shigella*
173 polysaccharide-based vaccine candidates in adult volunteers (26-28). LPS is therefore presumed
174 to be the main antigenic target of antibody-mediated shigellacidal activity. We probed the antigen-
175 specificity of the SBA activity in our cohort by evaluating complement-dependent *Shigella* killing
176 in sera that had been pre-incubated with increasing amounts of IpaB or LPS to sequester specific
177 antibodies; efficiency of antibody removal was demonstrated by a decrease of ELISA binding
178 signal (Figure 5A left). Depletion of *S. flexneri* 2a and *S. sonnei* LPS antibodies from both maternal
179 and cord blood serum resulted in a proportional (dose-responsive) reduction of bactericidal
180 activity that was serotype-specific (Figure 5A). Depletion of IpaB-specific antibodies had no effect
181 on *S. flexneri* 2a killing. These results confirm the LPS-specificity of the maternal and infant SBA
182 antibodies.

183 Having identified LPS as molecular target of antibody function, we compared LPS IgG and SBA
184 titers in both maternal and infant serum. A positive and significant correlation was observed
185 between maternal *S. sonnei* LPS IgG and SBA (Pearson's r = 0.686) but the same was not true
186 for *S. flexneri* 2a (Pearson's r = 0.281) (Figure 5B). However, in the infants, LPS IgG and SBA
187 titers were positively and significantly correlated for both *S. flexneri* 2a (Pearson's r = 0.614) and
188 *S. sonnei* (Pearson's r = 0.707) (Figure 5F). These results hinted that another component,
189 different from IgG, was contributing to maternal *S. flexneri* 2a SBA but was not sieved through
190 the placenta.

191 IgM is a strong activator of complement that could account for the excess maternal *S. flexneri* 2a
192 SBA and OPKA observed. LPS-specific IgM titers against both *S. flexneri* 2a and *S. sonnei* LPS
193 were detected in maternal serum and in a handful of cord blood samples by ELISA (Figure 5C).
194 While similar in the infants, there was substantially higher IgM against *S. flexneri* 2a as compared
195 to *S. sonnei* LPS (mean of 263 EU/mL compared to 99 EU/mL) in maternal blood (Figure 5C).

196 Depletion of maternal IgM greatly diminished *S. flexneri* 2a SBA (Figure 5D). *S. flexneri* 2a SBA
197 titers measured in the IgM-depleted maternal sera were strongly associated with maternal LPS-
198 specific IgG (Pearson's r = 0.64); this was in contrast to the limited correlation observed when
199 SBA was measured in intact (IgM- and IgG-containing) sera (Figure 5E). These results attribute
200 complement dependent *Shigella* killing activity to both LPS-specific circulating IgG and IgM. In
201 the correlation analyses, maternal *S. flexneri* 2a SBA was more closely associated with LPS IgM
202 (likely due to its abundance in sera) while *S. sonnei* SBA was mostly associated with LPS IgG
203 (Figure 5F). Associations were also calculated for SBA and LPS-specific IgG, IgG1-4 and IgM for
204 both strains in maternal and cord blood. IgG2 (the predominant LPS-specific antibody) was the
205 subclass most associated with *S. sonnei* and *S. flexneri* 2a SBA in cord blood serum (Figure 5F).
206 IgG1 against *S. sonnei* LPS was equally associated with SBA in the infants.

207

208 **Comparisons with protective titers**

209 Finally, to place the mother-infant antigen-specific and functional antibody titers determined in this
210 study in the context of protective immunity, we compared serological outcomes in the dyad with
211 those measured in individuals who remained healthy or had only mild disease when challenged
212 with wild type *S. flexneri* 2a in a CHIM study (11). IpaB and *S. flexneri* 2a LPS-IgG titers in
213 maternal serum and cord blood were significantly higher than those found in adult American
214 volunteers that remained healthy post experimental oral challenge (Figure 6A). Likewise, maternal
215 *S. flexneri* SBA and OPKA titers were similar or higher than those of the same protected
216 individuals. In contrast, the functional SBA and OPKA antibodies in the infants were significantly
217 lower than those of volunteers clinically protected against experimental *Shigella* infection (Figure
218 6B).

219

220 **Discussion**

221 Through lifelong exposure, adults living in endemic regions develop natural immunity against
222 *Shigella*, which has been attributed to antibodies against serotype specific LPS (10). The
223 incidence of moderate to severe diarrhea attributable to *Shigella* progressively increases after the
224 first year of life, reaching its peak in young children 24 to 59 months of age. Although they
225 experience other diarrheal diseases, young infants are shielded from *Shigella* dysentery
226 presumably through maternal immunity acquired via placenta or breast milk (16). The exact
227 elements that prevent infection in these children, the contribution of antibodies specific for LPS or
228 for other bacterial antigens, and the antimicrobial mechanisms involved are not known. An
229 understanding of the particular components of this shielding immunity is important as new
230 candidate vaccines targeted for the susceptible infant and toddler groups continue to advance in
231 the clinical pathway. To identify maternally derived humoral immune components that may
232 contribute to protection of young infants during the first months of life, we characterized the
233 repertoire of *Shigella*-specific antibodies in a cohort of Malawian mothers and their infants around
234 the time of birth.

235 Serum IgG specific for both *S. flexneri* 2a LPS and *S. sonnei* LPS were detected in maternal and
236 in cord blood sera, although their placental transfer efficiency was moderate, between 0.6 and
237 0.7. Two previous studies of transplacental transfer of antibodies against *S. flexneri* 2a and *S.*
238 *sonnei* LPS in Israel (29) and against *S. sonnei* LPS in Vietnam (30), also found high levels of
239 IgG specific for *Shigella* LPS in maternal sera. The mothers in our cohort had clearly been
240 exposed to both *S. flexneri* 2a and *S. sonnei*, which is consistent with the reported serotype
241 prevalence in countries neighboring Malawi (20), and other endemic regions (29, 31, 32). The
242 study in Vietnam reported a much higher median transfer ratio (1.33) for *S. sonnei* LPS IgG (30)
243 than was observed in our cohort, which may reflect regional differences in seroprevalence due to
244 *Shigella* circulation (and possibly increased proportion of LPS-specific IgG1).

245 An abundance of serum IgG against *Shigella* type 3 secretion system proteins IpaB, IpaC, IpaD,
246 IpaH, and the virulence factor, VirG were observed in maternal and infant circulation, with levels
247 being substantially higher than those of antibodies specific for LPS. Such a detailed serological
248 interrogation of protein specific antibodies had been hindered by the difficulty in obtaining *Shigella*
249 antigens of high quality and in sufficient yield; classical literature that report Ipa antibody analyses
250 in endemic regions typically relied on crude and undefined protein extracts (9, 12). Different from
251 antibodies to LPS, protein-specific antibodies were efficiently transferred to the newborns; cord
252 blood IgG titers were similar or even higher than those in maternal sera for all the proteins
253 examined. Consistent with our findings, a reduced placental transfer of LPS compared to protein
254 antibodies has been reported for other pathogens, such as *Haemophilus influenzae*, *Neisseria*
255 *meningitidis*, and *Streptococcus pneumoniae* (33-35). Despite the differences in magnitude, we
256 observed that maternal and infant antibody levels were correlated for all antigens, which confirms
257 the regulated and selective nature of transplacental transfer in a process that is antigen/antibody
258 dependent. *Shigella*-specific placental antibody sieving was distinctly linked to IgG subclass.
259 While protein-specific antibodies were primarily IgG1 (followed by IgG2, IgG3 and IgG4), *S.*
260 *flexneri* 2a and *S. sonnei* LPS-specific IgG contained mainly of IgG2 (followed by IgG1, IgG3 and
261 IgG4). The superior levels of protein-specific IgG1 in infant blood as compared to LPS-specific
262 IgG2 is consistent with the hierarchy of receptor-mediated IgG subclass transport based on affinity
263 to FcRn and other placental Fc receptors (34-36). Given their distinct functional attributes, the IgG
264 subclass profile available to the infants will determine the anti-microbial capacity of humoral
265 immunity early in life (1, 23, 43).

266 SBA and OPKA titers have been correlated with clinical protection in adults experimentally
267 infected with virulent *S. flexneri* 2a (11). We confirmed *Shigella* LPS as a target antigen for *S.*
268 *flexneri* 2a SBA activity in both maternal and infant sera. Consistently, LPS IgG (and particularly
269 IgG2) was strongly correlated with SBA activity for both *S. flexneri* 2a and *S. sonnei*. Interestingly,

270 IgG2, which makes the bulk of LPS-IgG, is a poor complement activator. Notwithstanding, IgG2
271 is known to mediate complement-dependent killing of *Haemophilus influenzae* type b, albeit not
272 as efficiently as IgG1 (37). On the other hand, LPS IgG2 may activate an alternate pathway when
273 epitope densities are high (38), which is likely the case for a surface-exposed target like LPS.
274 IgG2 can act in a complement independent manner as has been shown with opsonophagocytic
275 activity against *S. pneumonia* (39), which is consistent with OPKA activity observed in our study.

276 An unexpectedly enhanced SBA and OPKA activity against *S. flexneri* 2a was observed in the
277 mothers (but not in the infants) from our cohort that was linked to high levels of IgM (a potent
278 activator of complement that is not placentally transferred). A dissimilar functional activity between
279 maternal and infant human sera, also due to maternal IgM, had been shown against *E. coli* and
280 *Salmonella* (40, 41). Though mothers in our cohort had comparable levels of LPS IgG against
281 both serotypes, maternal LPS-specific IgM against *S. flexneri* 2a was markedly higher resulting
282 in heightened SBA activity. Differences in IgM levels likely reflect frequency of exposures and
283 strain circulation, suggesting, in our study, a higher prevalence of *S. flexneri* 2a as compared to
284 *S. sonnei* in the Blantyre, Malawi region. A handful of infant samples had detectable IgM against
285 LPS from both serotypes. IgM against environmental and vaccine antigens has been reported in
286 cord blood from infants in LMIC countries but not in those from industrialized nations. The origin
287 of this IgM is unclear and presumed to reflect environmental factors, such as intrauterine
288 infections that affect placenta integrity (42, 43), and non-specific natural antibodies (42, 44).
289 Maternal SBA and OPKA against both serotypes were strongly associated implying shared
290 antibody contribution to microbial killing.

291 The underlying premise for dissecting the humoral immune profile against *Shigella* in mothers
292 and very young infants living in endemic regions is the epidemiological evidence of the lowest risk
293 of infection in this group. Maternal *S. flexneri* 2a LPS-IgG, SBA and OPKA titers were comparable
294 or higher than those observed in North American volunteers who remained healthy following

295 challenge with wild-type *S. flexneri* 2a organisms (11). The functional antibody activity in the
296 infants was noticeably below the threshold of clinical protected adults. In contrast, serum IgG
297 against protective target antigens IpaB and VirG in mother and infant sera were well above those
298 detected in the clinically protected challenged volunteers (11). The low levels of LPS-specific IgG
299 and their limited functional capacity (SBA and OPKA) in cord blood, along with high levels of
300 maternal protein-specific IgG and its efficient transfer, argue in favor of a more prominent role of
301 antibodies against *Shigella* virulence antigens in preventing *Shigella* infection than originally
302 appreciated. Similarly, others observed that naturally acquired maternal antibodies against
303 pneumococcal proteins, unlike anti-polysaccharide antibodies, were associated with protection
304 against nasal carriage in infants during the first 3 months of life (45). Further studies are warranted
305 to dissect the mechanisms by which such antibodies block microbial infection (not captured by
306 our traditional assays) and to corroborate their disease protective capacity in humans.

307 A *Shigella* vaccine that is efficacious in children under 3 years of age, the most vulnerable target
308 group, would make a major public health impact. The limited efficacy of a clinically-advanced O-
309 polysaccharide based vaccine candidate in young children (46, 47) has been linked with young
310 children's hypo-responsiveness to *Shigella* LPS (and likely impaired bactericidal/phagocytic
311 antibody activity). Our results showing the abundance of protein-specific antibodies in groups
312 naturally immune (low-risk group) to *Shigella* and the ease with which children respond to protein-
313 based immunization highlight the prospect of a *Shigella* protein-based vaccine approach. Purified
314 IpaB and IpaD (48-50) or a formulation containing IpaB and IpaC (Invaplex, (51)) have been
315 shown to prevent *Shigella* infection in preclinical studies. A vaccine combining multiple conserved
316 *Shigella* proteins would not only be likely broadly protective, but also effective, targeting multiple
317 mechanisms important for *Shigella* invasion and virulence.

318 One limitation in our study was that maternal serum was obtained within 3 months of birth. While
319 some antibody features may change after birth, we did not find significant differences in maternal

320 titers determined in sera collected prior to or after delivery. The limited sample size may have also
321 precluded more extensive and complex statistical analyses. It would be important to conduct
322 similar analyses of the antibody repertoire beyond birth and through the first 3 years of life to
323 better understand trends of disease and immune acquisition, and to investigate age-specific
324 antibody mediated antimicrobial functions using age-relevant immune cells to recreate elements
325 that would operate in vivo (17).

326 In summary, we have demonstrated, for the first time, the efficient placental transfer of maternal
327 antibodies against *Shigella* protein antigens and their availability at high levels to the infant at birth
328 along with the less efficient transfer of LPS-specific IgG with bactericidal and opsonophagocytic
329 killing activity. Our results define the maternally-acquired protective antibody repertoire available
330 to infants at birth and suggest a larger role for protein-specific immunity than has previously been
331 appreciated. Revisiting the concept of a protein-based vaccine that would target these antigens
332 either alone or in conjunction with *Shigella* LPS is warranted. Finally, these findings also
333 emphasize the need to better understand strain-specific immunity in young children to inform
334 preventive strategies.

335

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347

348

349 ## Methods

350 ### Study population and sample collection

351 Our study population consisted of mothers and infants recruited between January and November
352 2016 at Mfera Health Clinic, on the outskirts of Blantyre in Malawi. Healthy pregnant women who
353 were HIV seronegative were enrolled either before birth (screening) or during delivery. Infants
354 were enrolled at birth. Baseline information on mother and the infant's health was obtained during
355 the first hours post-delivery. Other information such as village, baseline health info, and current
356 physical complaints was also collected during the visit. Umbilical cord blood was collected at
357 delivery at the Mfera Health Clinic. Venous maternal blood was obtained at enrollment, either
358 during screening before birth, at a well-child visit (week 1, 6 or 10) or at 3 months. Samples were
359 frozen and shipped to the University of Maryland School of Medicine in Baltimore for analysis.
360 This study was approved by the Institutional Review Board of University of Maryland School of
361 Medicine, and the College of Medicine Research and Ethics Committee (COMREC) at the College
362 of Medicine in Malawi. All participating mothers provided written informed consent for themselves
363 and their infants.

364 ### Antigen-specific antibody analysis

365 *Shigella* antigens IpaB, IpaC, IpaD, *S. flexneri* 2a LPS and *S. sonnei* LPS were obtained from
366 Walter Reade Army Institute of Research (WRAIR). The N-terminal domain of VirG was
367 expressed and purified inhouse in an *E. coli* expression system (Chitra STS et al, unpublished).
368 The C-terminal domain of IpaH was expressed and purified using a cell-free expression system
369 (Neeraj Kapoor, Vaxcyte, Inc. personal communication). Antigen-specific serum IgG titers were

370 measured by ELISA as previously described (52). Briefly, Immulon 2HB plates (Thermo Scientific,
371 Waltham MA) were coated with IpaB, IpaC, IpaD and IpaH at 0.1µg/mL in PBS, and VirG, *S.*
372 *flexneri* 2a LPS and *S. sonnei* LPS at 5µg/mL in carbonate buffer, pH 9.6. Plates were incubated
373 for 3h at 37°C and blocked at 4°C overnight in PBS containing 10% w/v non-fat dry milk (NFDM).
374 Sera diluted in PBS containing 10% NFDM and 0.05% Tween-20 (PBS-T) were added, and the
375 plates incubated at 37°C for 1h. Plates were incubated with HRP-labeled goat IgG specific for
376 human IgG (Jackson Immuno Research, West Grove, PA) for another 1h at 37°C. Plates were
377 washed 6 times with PBS-T following every incubation step. Tetramethylbenzidine (TMB; KPL,
378 Gaithersburg, MD) was added as substrate for 15 min in the dark with shaking, and the reaction
379 was stopped by adding 1M phosphoric acid (Millipore Sigma, Burlington, MA). Endpoint titers
380 were calculated as the inverse serum dilution that resulted in an absorbance value at 450 nm of
381 0.2 above background and were reported as the ELISA units/mL.
382 Antigen-specific IgG subclasses were measured using *Shigella* multiplex assay using the
383 MesoScale Diagnostic platform (MSD, Rockville, MD). Assays are run in the same way as the
384 antigen-specific ELISAs, with a few exceptions: 1. There is no antigen-coating step as the
385 antigens are pre-printed on MSD plates. 2. After the serum incubation step, the plates were
386 incubated with biotinylated anti-IgG subclass antibodies (SouthernBiotech, Birmingham, AL) plus
387 the SULFOTag-STREP (MSD) for another 1h at 37°C. 3. Binding was then detected using MSD
388 GOLD Read buffer. The plates were read using an MSD sector imager, model 2400 and data
389 analyzed by the MSD workbench software provided by the manufacturer. The ECL signal (minus
390 background from blank) for each sample (diluted at 1:100 in PBS containing 10% NFDM) was
391 reported.
392 **Functional assays**
393 SBA. The SBA assay was performed as previously described (53). SBA titers were determined
394 by Opsotiter (53) as the reciprocal of the serum dilution that produced 50% bacterial killing as
395 determined by Reed-Muench regression analysis. The provisional reference serum sample,

396 Korean QC19, was ran with each assay to normalize and reduce variability between assays.
397 Korean QC19 was assigned a titer = 28000 for *S. flexneri* 2a and 1100 for *S sonnei* (53). The
398 lowest dilution tested was 1:200 so that the lowest titer is 100.
399 OPKA. OPKA was also performed as previously described (11) with some modifications. Briefly,
400 10 µL of target bacteria (~10^4 CFU/mL) was opsonized by mixing with 20µL of a heat-inactivated,
401 serially diluted test sample in a well of a round-bottom microtiter plate and incubated for 15min at
402 37°C in room air, with shaking. As with the SBA, control wells had bacteria, baby rabbit
403 complement (BRC), and buffer only; no test sample was added to these wells. 10µL of baby rabbit
404 complement (10% final concentration), mixed with 60µL of 10^5 dimethylformamide (DMF)-
405 differentiated HL-60 cells (ATCC CCL-240) were then added to the reaction mixture (for a 100µL
406 total volume). Following a 45 min incubation at 37°C, 5% CO_2, 10 µL from each well was spotted
407 on LB agar. The agar plates were incubated overnight at 29°C for *S. flexneri* 2a and 26°C for *S.*
408 *sonnei*. The percentage of bacteria that were phagocytosed and killed per well was determined
409 measuring the colony counts and determining the titer as was done for the SBA (above).
410 Standardization of the *Shigella* OPKA has not yet been done, but the Korean QC19 was run with
411 each assay and was found to have an average titer of 15574 for *S. flexneri* 2a and 288 for *S*
412 *sonnei*. The lowest dilution tested was 1:200 so that the lowest titer is 100.
413 Antibody depletion. (i) LPS and IpaB-specific antibodies were sequestered by incubating serum
414 samples into ELISA plates coated with serial dilutions of antigen (0 - 50µg/mL) as described above
415 for 3 h at 37°C with shaking. The depleted serum sample was then tested for SBA activity as
416 described above. (ii) IgM was removed by incubating serum samples with 50mM of beta-
417 mercaptoethanol for 1 h at 37°C. The IgM-depleted serum was the further diluted and used in the
418 SBA reaction as described above.
419 **Statistical analysis**
420 Geometric mean titers (GMT) were calculated for antigen-specific IgG in maternal and cord blood
421 sera. Comparisons of titers between maternal and cord samples, or between antibody levels

422 against different antigens, were measured by paired t-test or one-way analysis of variance
423 (ANOVA) with Tukey's post-test correction. Placental transfer ratios were assessed as a ratio of
424 cord blood divided by maternal antibody titers. Associations between maternal and cord blood
425 titers were calculated using Pearson's correlation. A two-way ANOVA with Tukey's post-test
426 correction was used to compare transfer ratios between IgG subclasses. All statistical analysis
427 was conducted using GraphPad Prism 9.

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Tables

Table 1. Baseline Characteristics of 63 Participating Mother-Infant Pairs

Characteristic	Value
Maternal characteristics	
Age, y, median (range)	26 (17-43)
Parity, median (range)	3 (0-8)
Infant characteristics	
Female sex (%)	36 (57%)
Birth weight, kg, median (range)	3.1 (1.5-4.3)
< 2.5 kg (low birth weight), n (%)	5 (8%)
Twin births, n (%)	3(4.8%)
Born during rainy season (November- April), n (%)	21 (33%)

Table 2. *Shigella* antigen-specific titers

Antigen	Cord blood titer (EU/mL) GMT (range)	Maternal serum titer (EU/mL) GMT (range)	Transfer ratio Mean (range)
S. flexneri 2a LPS	1,752 (120-26,789)	3,491 (153-123,844)	0.60 (0.05-2.20)
S. sonnei LPS	1,264 (45-19,002)	2,109 (135-32,727)	0.70 (0.23-3.38)
IpaB	77,134 (7,274-419,898)	73,737 (6,818-324,773)	1.10 (0.27-2.00)
IpaC	21,856 (279-125,268)	21,732 (292-127,040)	1.06 (0.34-2.02)
IpaD	9,761 (84-92,804)	10,653 (81-112,576)	0.98 (0.26-2.08)
IpaH	19,890 (1,224-122,206)	21,156 (1,067-145,110)	1.00 (0.21-1.98)
VirG	14,840 (3,038-63,877)	15,726 (2,003-75,273)	1.01 (0.29-3.71)

588

Table 3. *Shigella* functional antibody (SBA and OPKA) titers

Antigen	Cord blood GMT (range)	Maternal serum GMT (range)	Transfer ratio Mean (range)
S. flexneri 2a SBA	1,227 (200-113,628)	44,076 (6,889-1,278,483)	0.16 (0.00-2.78)
S. flexneri 2a OPKA	895 (100-178,424)	26,944 (2,770-596,279)	0.18 (0.00-1.77)
S. sonnei SBA	832 (200-13,536)	1,017 (200-17,916)	1.29 (0.19-13.61)
S. sonnei OPKA	245 (100-2,468)	357 (100-2,460)	0.78 (0.18-3.03)

589

590 ## Figure Legends

591 **Figure 1. Flowchart showing selection of paired maternal-infant samples**

592 **Figure 2. *Shigella*-specific maternal antibody repertoire and placental transfer efficiency**.
593 (A) IgG against *Shigella* LPS and protein antigens in maternal (M) and cord blood (CB) sera.
594 Symbols represent individual titers. Asterisks indicate statistically significant differences between
595 M and CB titers as determined by paired t test. * $P < 0.05$, **** $P < 0.0001$. (B) Placental transfer
596 ratios (CB titer/M titer) for antibodies against each antigen. Whiskers show minimum and
597 maximum values. Line is at ratio = 1. (C) Associations between maternal and cord blood titers for
598 each antigen. Pearson's r and *P* values are shown within each graph. (D) Heatmaps showing
599 association between the antibody titers against each antigen in maternal serum (left) and in cord
600 blood serum (right). Pearson correlation, r, is shown in each box.

601 **Figure 3. IgG subclass distribution of maternal and infant placentally acquired antibodies**.
602 (A) ECL signal for IgG1, IgG2, IgG3 and IgG4 against *Shigella* antigens measured in serum from
603 mothers (M) and cord blood (CB) diluted at 1:100. Symbols represent individual titers. (B) Mean
604 placental transfer ratios (CB titer/M titer) for subclass. Dashed line is at ratio = 1. (C) Heatmap
605 representing transfer ratios of total IgG and IgG subclasses against *Shigella* antigens. Asterisks
606 indicate statistically significant differences as determined by Paired t test. *, $P < 0.05$, **, $P < 0.01$,

607 ***, $P < 0.001$, ****, $P < 0.0001$. For the bar graphs, significance was evaluated using a two-way
608 ANOVA with a Tukey's post-test correction.

609 **Figure 4. Maternal and infant placentally acquired functional antibodies against *Shigella*.**
610 (A) Serum bactericidal antibody (SBA) and (B) opsonophagocytic killing antibody (OPKA) titers
611 measured in serum from mothers (M) and cord blood (CB) (left graphs). Data represent individual
612 titers. Placental transfer ratios (CB titer/M titer) are shown on the graphs on the right. Data
613 represent individual titers. Asterisks indicate statistically significant differences between M and
614 CB titers as determined by one-way ANOVA with a Tukey's post-test correction (** $P<0.01$, ***
615 $P<0.001$, **** $P<0.0001$). Whiskers indicate minimum and maximum values. Line is at ratio = 1.
616 (C) and (D) Associations between maternal and cord blood SBA and OPKA titers, respectively.
617 Pearson's r and *P* values are shown within each graph.

618 **Figure 5. *Shigella* LPS-specific antibodies exhibit bactericidal activity.** (A) Depletion of IpaB-
619 or LPS-specific antibodies (left panel) and percent killing of *S. flexneri* 2a in a bactericidal assay
620 using antibody-depleted sera (right panel). Darker-shaded symbols represent maternal sera and
621 lighter-shaded symbols represent infant sera. (B) Correlations between maternal LPS IgG and
622 SBA for *S. flexneri* 2a (left panel) or *S. sonnei* (right panel). (C) Mean IgM titers (bars) against *S.*
623 *flexneri* 2a and *S. sonnei* LPS in maternal (M) and cord blood (CB) sera. Symbols represent
624 individual titers. Maternal and cord blood titers were compared by one-way ANOVA with a Tukey's
625 post-test correction (****, $P < 0.0001$). (D) Mean SBA titers (bars) in maternal serum before (Total)
626 or after IgM depletion (IgM depleted). SBA titers in the two groups were compared by paired t test
627 (**, $P < 0.01$). Symbols represent individual titers. (E) Associations between *S. flexneri* 2a LPS
628 IgG and SBA titers before (filled circle) and after IgM depletion (open circles). Pearson's r values
629 are shown within the graph. (F) Heatmap showing associations (Pearson's r) between SBA and
630 Total IgG, IgG subclasses and IgM against LPS.

631 **Figure 6. Comparative analysis of *Shigella* antigen-specific and functional antibody titers**
632 **in the mother-infant dyad in relation to clinical protection.** *Shigella* antigen-specific (A) and
633 *S. flexneri* 2a functional (B) antibody titers measured side-by-side in pre-challenge serum from
634 North American individuals (black squares, protected) that were either healthy or had mild disease
635 after wild-type *Shigella* challenge and in maternal (M) or cord blood (CB) sera from the Malawi
636 cohort. Data represent individual titers. Differences between groups was determined by paired t-
637 test (ns $P>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

638

639

Figure 1

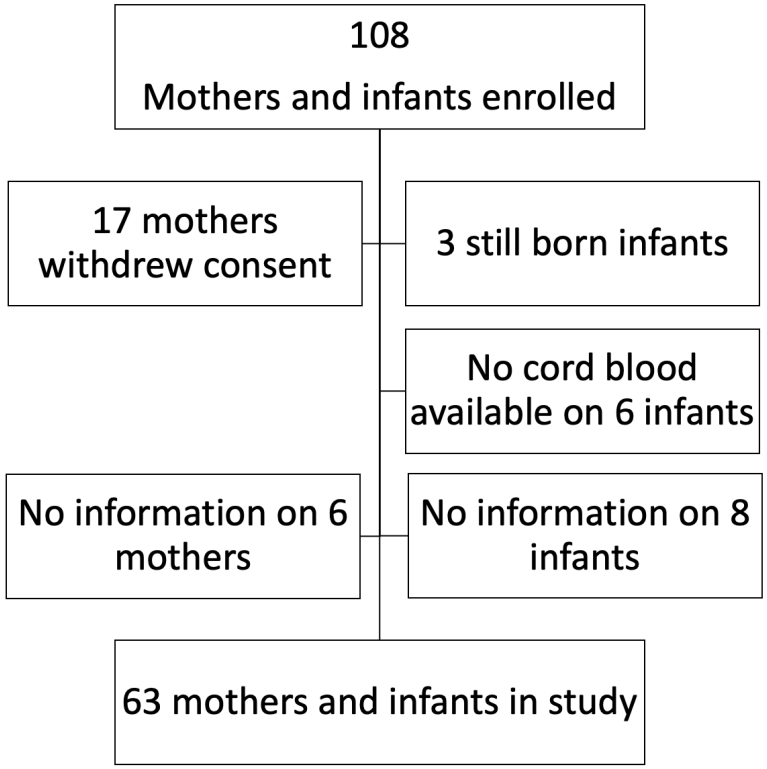

Figure 1. Flowchart showing selection of paired maternal-infant samples

Figure 2

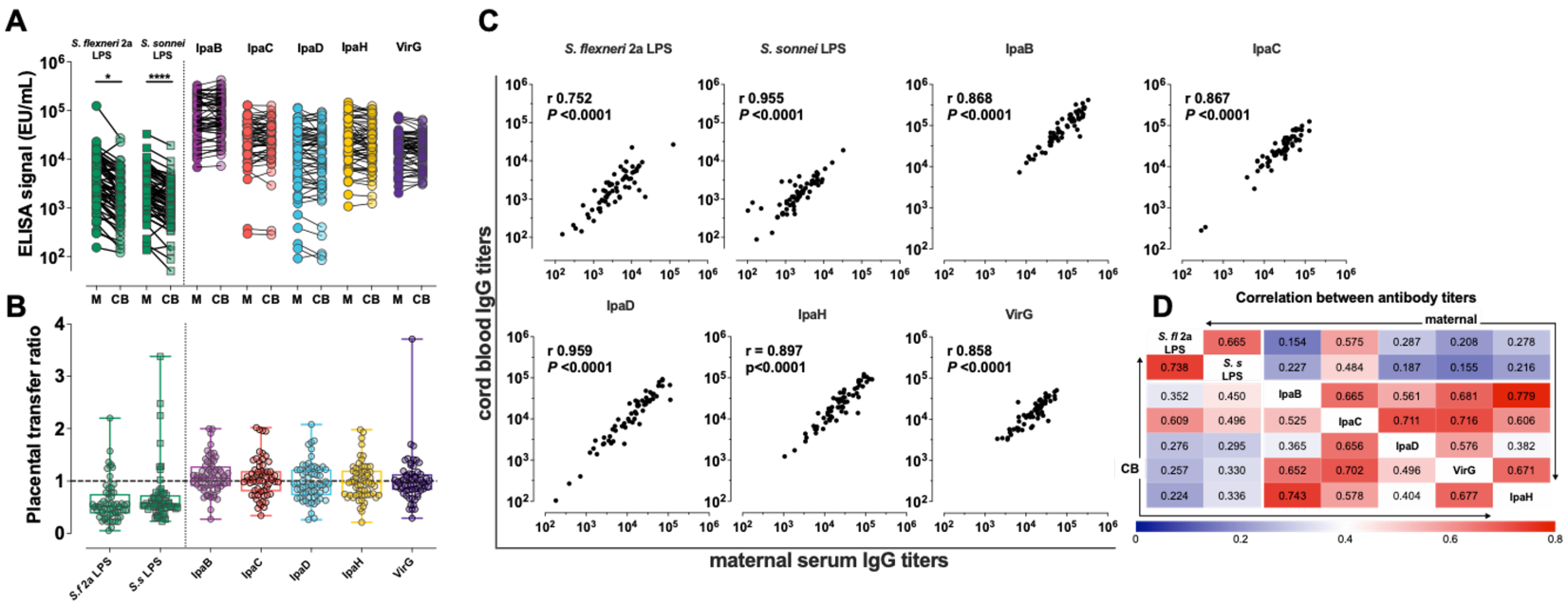

Figure 2. *Shigella*-specific maternal antibody repertoire and placental transfer efficiency. (A) IgG against *Shigella* LPS and protein antigens in maternal (M) and cord blood (CB) sera. Symbols represent individual titers. Asterisks indicate statistically significant differences between M and CB titers as determined by paired t test. * $P < 0.05$, **** $P < 0.0001$. (B) Placental transfer ratios (CB titer/M titer) for antibodies against each antigen. Whiskers show minimum and maximum values. Line is at ratio = 1. (C) Associations between maternal and cord blood titers for each antigen. Pearson's r and *P* values are shown within each graph. (D) Heatmaps showing association between the antibody titers against each antigen in maternal serum (left) and in cord blood serum (right). Pearson correlation, r, is shown in each box.

Figure 3

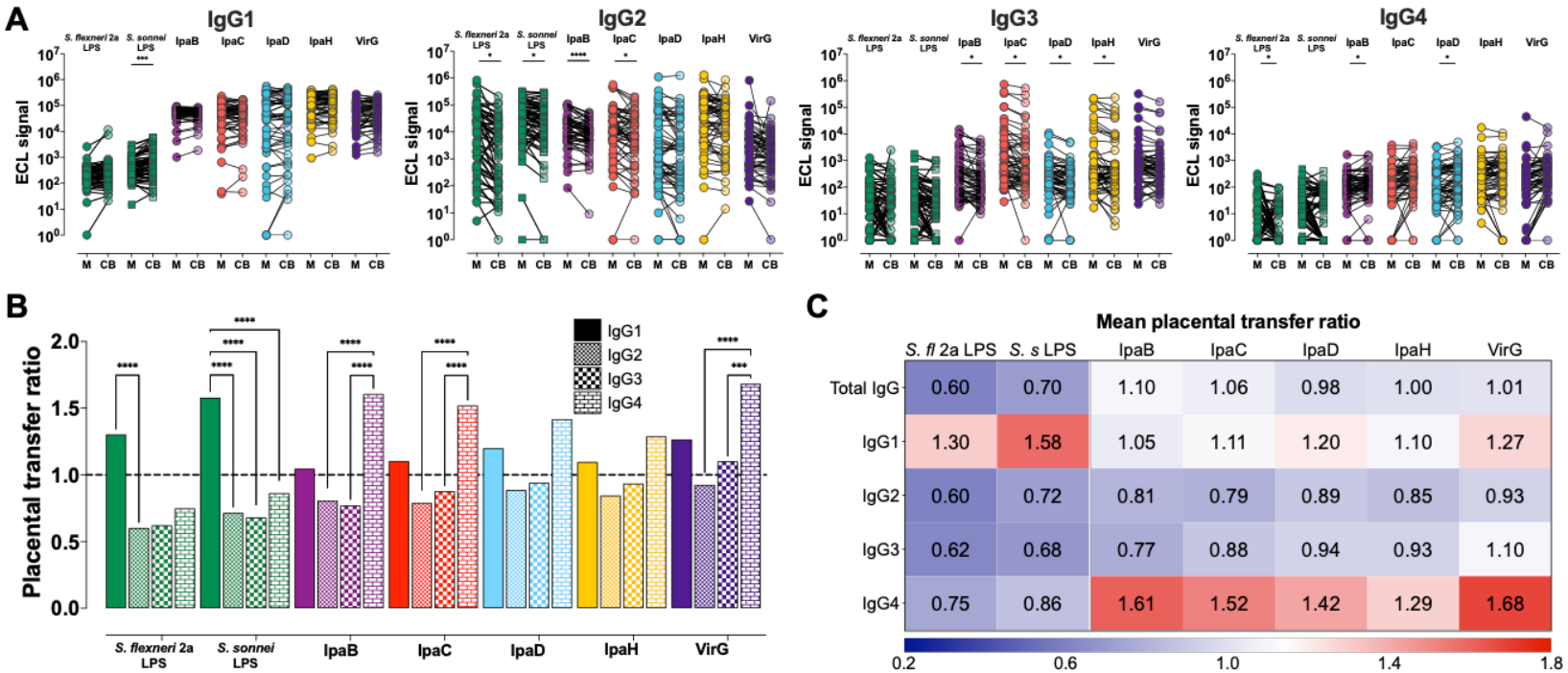

Figure 3. IgG subclass distribution of maternal and infant placentally acquired antibodies. (A) ECL signal for IgG1, IgG2, IgG3 and IgG4 against *Shigella* antigens measured in serum from mothers (M) and cord blood (CB) diluted at 1:100. Symbols represent individual titers. (B) Mean placental transfer ratios (CB titer/M titer) for subclass. Dashed line is at ratio = 1. (C) Heatmap representing transfer ratios of total IgG and IgG subclasses against *Shigella* antigens. Asterisks indicate statistically significant differences as determined by Paired t test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$. For the bar graphs, significance was evaluated using a two-way ANOVA with a Tukey's post-test correction.

Figure 4

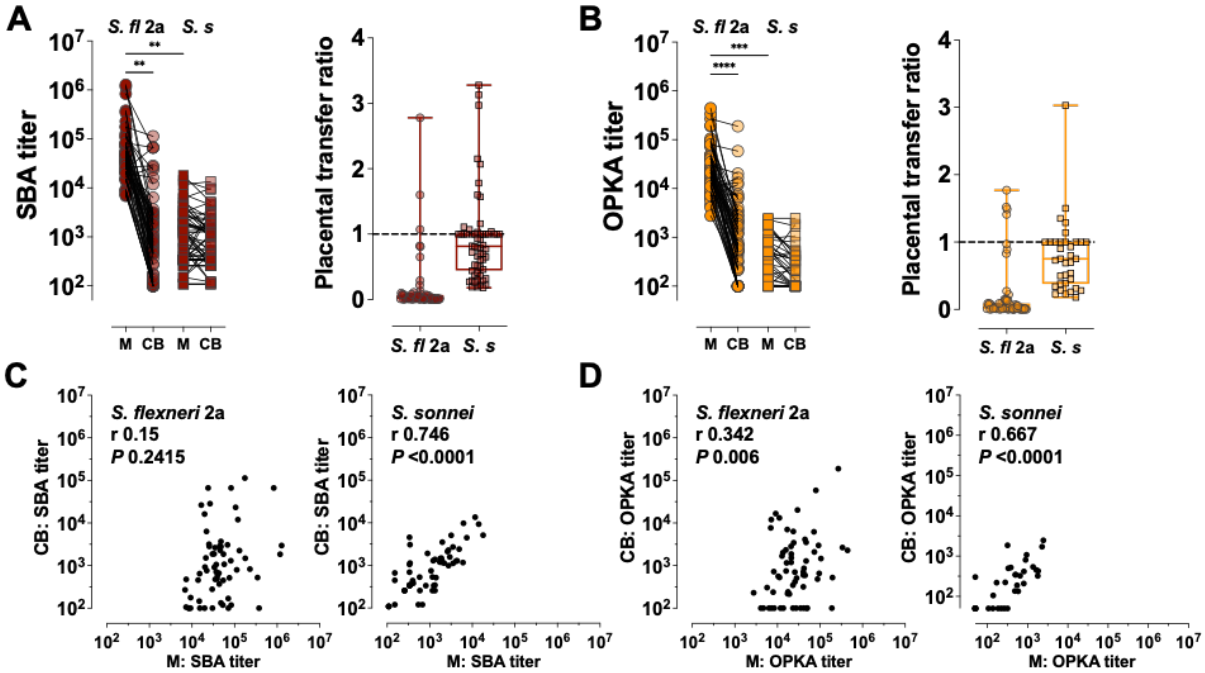

Figure 4. Maternal and infant placentally acquired functional antibodies against *Shigella*. (A) Serum bactericidal antibody (SBA) and (B) opsonophagocytic killing antibody (OPKA) titers measured in serum from mothers (M) and cord blood (CB) (left graphs). Data represent individual titers. Placental transfer ratios (CB titer/M titer) are shown on the graphs on the right. Data represent individual titers. Asterisks indicate statistically significant differences between M and CB titers as determined by one-way ANOVA with a Tukey's post-test correction (** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). Whiskers indicate minimum and maximum values. Line is at ratio = 1. (C) and (D) Associations between maternal and cord blood SBA and OPKA titers, respectively. Pearson's r and P values are shown within each graph.

Figure 5

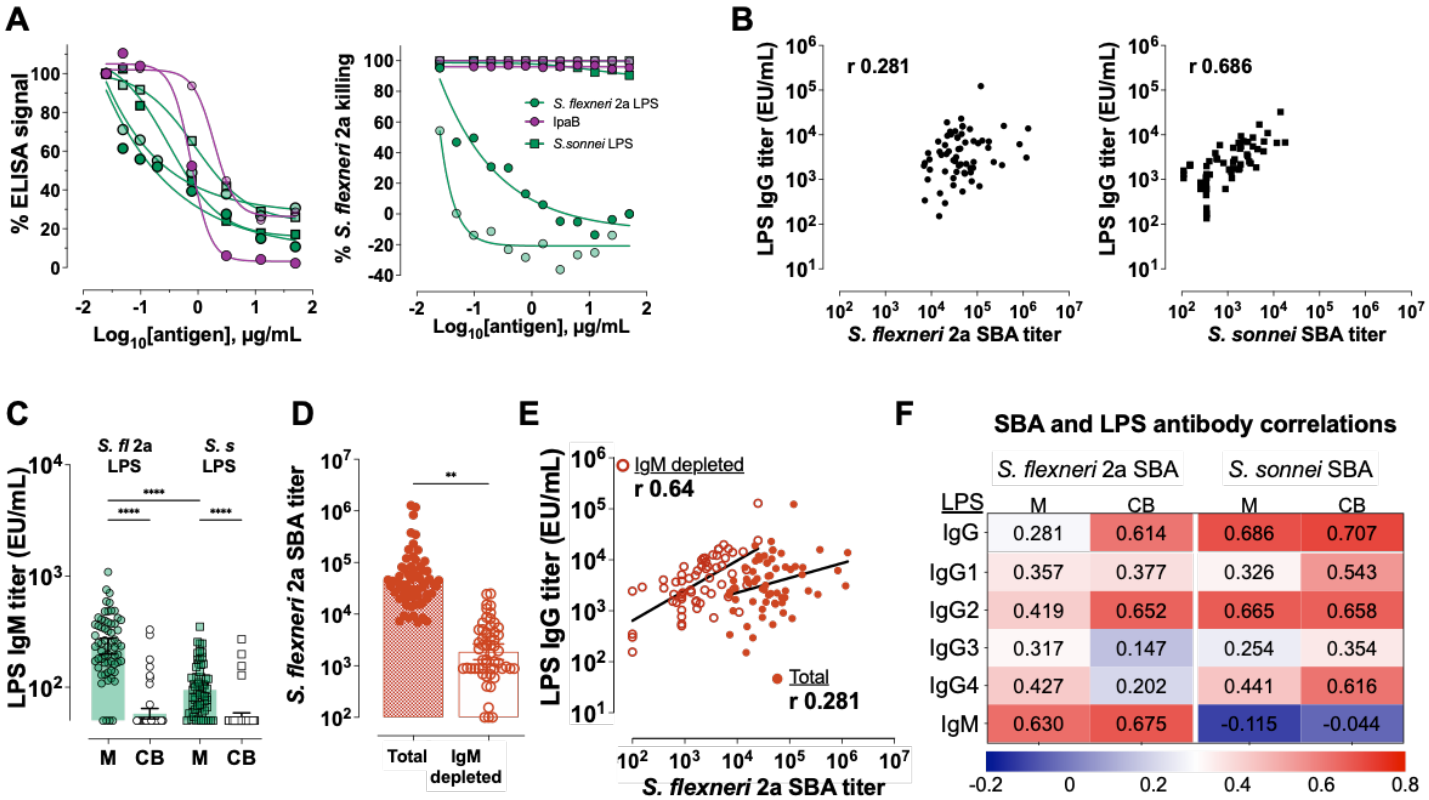

Figure 5. *Shigella* LPS-specific antibodies exhibit bactericidal activity. (A) Depletion of IpaB- or LPS-specific antibodies (left panel) and percent killing of *S. flexneri* 2a in a bactericidal assay using antibody-depleted sera (right panel). Darker-shaded symbols represent maternal sera and lighter-shaded symbols represent infant sera. (B) Correlations between maternal LPS IgG and SBA for *S. flexneri* 2a (left panel) or *S. sonnei* (right panel). (C) Mean IgM titers (bars) against *S. flexneri* 2a and *S. sonnei* LPS in maternal (M) and cord blood (CB) sera. Symbols represent individual titers. Maternal and cord blood titers were compared by one-way ANOVA with a Tukey's post-test correction (****, $P < 0.0001$). (D) Mean SBA titers (bars) in maternal serum before (Total) or after IgM depletion (IgM depleted). SBA titers in the two groups were compared by paired t test (**, $P < 0.01$). Symbols represent individual titers. (E) Associations between *S. flexneri* 2a LPS IgG and SBA titers before (filled circle) and after IgM depletion (open circles). Pearson's r values are shown within the graph. (F) Heatmap showing associations (Pearson's r) between SBA and Total IgG, IgG subclasses and IgM against LPS.

Figure 6

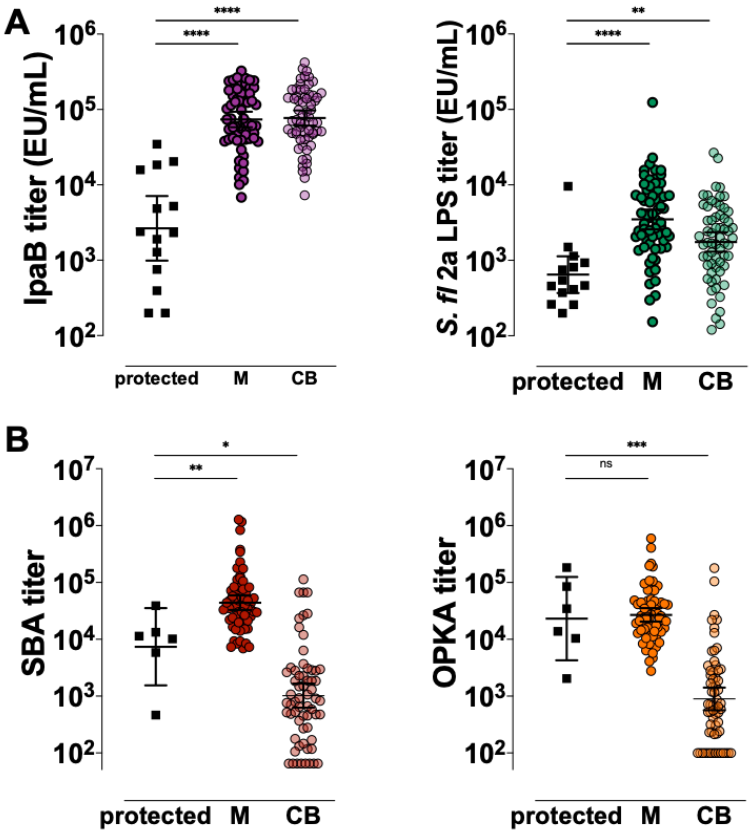

Figure 6. Comparative analysis of *Shigella* antigen-specific and functional antibody titers in the mother-infant dyad in relation to clinical protection. *Shigella* antigen-specific (A) and *S. flexneri* 2a functional (B) antibody titers measured side-by-side in pre-challenge serum from North American individuals (black squares, protected) that were either healthy or had mild disease after wild-type *Shigella* challenge and in maternal (M) or cord blood (CB) sera from the Malawi cohort. Data represent individual titers. Differences between groups was determined by paired t-test (ns $P>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).