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Maternal immunization confers protection to the offspring against an attaching and effacing pathogen through delivery of IgG in breast milk

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SUMMARY

Infants are more susceptible to enteric infections due to immature immune systems and impaired colonization resistance mediated by the microbiota. Maternal antibodies can provide immunity, with maternal vaccination offering a protective strategy. We find that oral infection of adult females with the enteric pathogen *Citrobacter rodentium* protects dams and offspring against oral challenge. Parenteral immunization of dams with heat-inactivated *C. rodentium* reduces pathogen loads and mortality in offspring but not mothers. IgG, but not IgA or IgM, transferred through breast milk to the intestinal lumen of suckling offspring coats the pathogen and reduces intestinal colonization. Protective IgG largely recognizes virulence factors encoded within the locus of enterocyte effacement (LEE) pathogenicity island, including the adhesin Intimin and T3SS filament EspA, which are major antigens conferring protection. Thus, pathogen-specific IgG in breast milk induced during maternal infection or immunization protects neonates against infection with an attaching and effacing pathogen.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

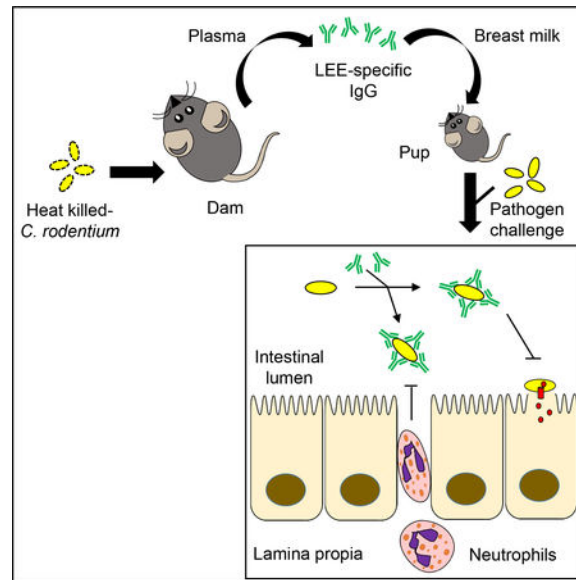
G.C.-F. and G.N. conceived and designed the experiments. G.C.-F. conducted most of the experiments. K.S. and Y.W. performed flow cytometry and phagocytosis experiments. M.Z. and N.I. provided key materials and/or scientific advice. J.H. and V.M.-A. provided technical help with the experiments. G.C.-F. and G.N. analyzed all the data and wrote the manuscript with contributions from all authors.

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DECLARATION OF INTERESTS

The authors declare no competing financial interests.

Caballero-Flores et al. demonstrate that maternal immunization with heat inactivated *C. rodentium* or surface pathogen antigens protects neonatal mice against pathogen oral challenge. Protection was mediated by the delivery of pathogen-specific IgG through breast milk which coated the pathogen, increased its phagocytosis and decreased epithelial attachment in the gut.



Keywords

Maternal vaccination; enteric infection; *C. rodentium*; EPEC; IgG; LEE-virulence factors

INTRODUCTION

Enteric and diarrheal diseases are important causes of childhood death worldwide, leading to nearly 1 million deaths per year in children under 5 years old, and ranking as the second cause of death in this age group (Lanata et al., 2013; Croxen et al., 2013). The increased susceptibility of newborns and infants to enteric infections has been ascribed to immaturity of their immune system and impaired colonization resistance conferred by the neonatal microbiota (Simon et al., 2015; Kim, et al., 2017). Thus, neonates rely, at least in part, on maternal antibodies for immunity against enteric infection by pathogens that often gain access to the gastrointestinal tract (PrabhuDas et al., 2011; Kollmann et al., 2012). For this reason, maternal vaccination has emerged as a valuable tool in covering the gap between birth and the maturation of the immune system, since it represents a safe and effective strategy to transfer protective maternal antibodies to the offspring during pregnancy (Jennewein et al., 2017; Riccardo et al., 2017).

There are currently various vaccines recommended for use on all pregnant women, such as those against Pertussis, Tetanus and Influenza, whereas others including those against Hepatitis, Pneumococcus, Meningococcus, Smallpox, Varicella and Rubella are only indicated for pregnant women with specific risk factors (Riccardo et al., 2017). However, even though maternal vaccination has proven itself as a safe and effective strategy for

prevention of life-threatening infections in neonates, there are no licensed maternal vaccines for protection against bacterial enteric pathogens, including Enteropathogenic *Escherichia coli* (EPEC), *Vibrio cholerae* and *Shigella*, which remain important causes of childhood death worldwide (Czerkinsky and Holmgren, 2015; Riccardo et al., 2017). EPEC, a food- and water-borne non-invasive enteric pathogen, is a common cause of diarrheal deaths in newborns and infants (Lanata et al., 2013; Kotloff et al., 2013), whereas infections in adults are rarely observed (Croxen et al., 2013). EPEC attaches to and colonizes the intestinal tract by inducing characteristic “attaching and effacing” (A/E) lesions on the intestinal epithelium (Kaper et al., 2004; Mundy et al., 2005). The A/E phenotype is conferred by the locus of enterocyte effacement (LEE), a pathogenicity island encoding a specialized type III secretion system (T3SS), as well as translocators, secreted effector proteins, bacterial adhesins and Ler, the global activator of all LEE genes (Frankel and Philips, 2008; Gaytán et al., 2016). Unfortunately, antibiotic therapy for EPEC infection is not effective and may worsen disease (Croxen et al., 2013). Thus, there is an urgent need for novel therapies to prevent or treat infections by this pathogen, particularly in infants and young children.

Citrobacter rodentium, a mouse pathogen that also harbors the LEE and induces A/E intestinal lesions, has been extensively used as a model for studying the pathogenesis of EPEC infection in humans (Law et al., 2013; Collins et al., 2014). Previous studies have demonstrated that *C. rodentium*-specific IgG antibodies, but not IgA or IgM, are required for pathogen clearance and host survival in adult mice (Bry and Brenner, 2004; Maaser et al., 2004; Kamada et al., 2015). In contrast, the role of maternal IgG in neonatal host defense against *C. rodentium* or other enteric pathogens remains poorly understood. During pregnancy, maternal IgG is transported through the placenta to the fetus by the neonatal Fc receptor (FcRn) that also regulates the stability of plasma IgG and mediates the neonatal uptake of maternally derived IgG from ingested milk into the small intestine (Rath et al., 2013; Pyzik et al., 2015).

In this study, we examined the mechanism by which maternal antibodies protect the offspring against A/E pathogens during the neonatal period. We show that maternal pathogen-specific IgG, but not IgA or IgM, plays a critical role in neonatal protection, and that protective IgG is mainly delivered through breast milk into the neonatal intestinal lumen. Furthermore, we observed that maternal protective IgG was produced against LEE virulence factors and identified LEE-encoded Intimin and EspA as major antigens for maternal vaccination and protection of the offspring.

RESULTS

Oral infection with *C. rodentium*, but not parenteral immunization, confers protection against secondary pathogen challenge in adult mice

Initial experiments showed that oral infection with *C. rodentium*, but not intraperitoneal (IP) immunization with heat-inactivated bacteria, conferred full protection in adult mice against pathogen colonization after a second oral challenge (Figure 1A), even though both routes of immunization induced similar levels of pathogen-specific IgG antibodies in serum that were largely induced against Ler-regulated virulence proteins (Figure 1B and C). In contrast, fecal IgG1, IgG2b, and total IgG specific for *C. rodentium* were observed only after oral infection,

but not IP immunization (Figure 1D). The increased amount of *C. rodentium*-reactive IgG observed in the feces after oral infection, compared with that obtained after IP immunization, was maintained on day 2 and 7 after a second oral pathogen challenge (Figure S1A and B). Likewise, oral infection but not IP immunization, induced fecal *C. rodentium*-specific IgA that was also observed after a second pathogen challenge (Figure S1C and D). These results indicate that protective immunity against enteric infection is induced after oral infection but not IP immunization in adult mice, which correlates with the presence of pathogen-specific immunoglobulins in the intestinal lumen.

Parenteral maternal immunization reduces pathogen loads and intestinal inflammation in the offspring after oral challenge

To determine whether oral infection and/or IP immunization of adult females confer protection to the offspring against *C. rodentium*, we first optimized a lethal infection model in neonatal mice by inoculating 18-day old pups with different doses of *C. rodentium*. We used 5×10^8 colony-forming units (CFU), a dose previously used for inoculation of adult mice (Maaser et al., 2004), as well as inocula containing 10-fold and 100-fold less bacteria (5×10^7 and 5×10^6 CFU, respectively). As expected, all adult mice survived after infection with the highest dose of 5×10^8 CFU, while 100% of the pups succumbed to infection with both 5×10^8 and 5×10^7 bacteria, and only ~ 50% of the pups survived after 5×10^6 -challenge (Figure 1E). After challenging the offspring of both orally and IP inoculated dams, we found that both routes of maternal immunization protected the offspring against oral challenge with 5×10^8 CFU whereas the same dose resulted in lethality in age-matched naïve pups (Figure 1F). These data indicate that maternal immunization via both the oral and parenteral routes is effective in protecting neonates against enteric infection. Because maternal immunization with heat-killed *C. rodentium*, hereinafter referred to as “HK-Cr”, promoted offspring survival in the absence of infection in the dam (Figure 1F), we further studied the protective phenotype in the offspring of such maternally immunized pups after challenge with the pathogen. Consistently with results shown in Figure 1F, HKCr maternal immunization led to ~90% survival of pups after challenge with 5×10^8 CFU of *C. rodentium*, while all pups from naïve dams succumbed to this pathogen dose (Figure 2A). These results correlated with lower pathogen loads in feces, liver and spleen (Figure 2B to D), as well as reduced inflammation and pathology scores in the intestine of the maternally immunized pups compared to naïve mice (Figure 2E and F). In contrast, HKCr treatment did not reduce pathogen loads or intestinal pathology in the immunized dams after pathogen challenge when compared to naïve mice (Figure S2A to E). These results confirm the previous observations that parenteral HK-Cr immunization does not protect adult mice against enteric *C. rodentium* infection, but it is effective in transferring passive immunity to the offspring through maternal immunization.

Passive immunity is transferred from the mother to the offspring mainly through breast milk

In mice, maternal antibody transfer occurs during pregnancy and after birth through the placenta and breast milk, respectively (Hurley and Theil, 2011; Ma et al., 2018). To determine whether protective maternal antibodies are transferred to the offspring pre- or postnatally, we performed cross-fostering experiments in which 1- to 2-day old pups from

HK-Cr immunized mothers were cross-fostered and nursed by naïve dams (prenatal antibody transfer), while newly born pups from such naïve mothers were nursed by immunized dams (postnatal antibody transfer) (Figure 3A). We observed that ~60% of pups receiving *C. rodentium*-specific antibodies postnatally survived pathogen challenge (Figure 3B), while only ~10% of pups that received maternal antibodies prenatally, and none of the naïve (non-cross fostered) mice, survived the infection (Figure 3B). The increased survival observed in the postnatal group correlated with reduced pathogen loads and pathology scores compared to the prenatal and naïve groups (Figure 3C to F). These results also correlated with increased detection of *C. rodentium*-specific IgG antibodies in breast milk of HK-Cr immunized mothers compared to milk samples of naïve dams as detected by immunoblotting (Figure 3G). As previously observed in the serum of infected mice (Figure 1C), pathogen-specific IgG antibodies in breast milk were mainly produced against Ler-dependent virulence factors (Figure 3G). Altogether, these data suggest that postnatal transfer of maternal antibodies through breast milk is critical for protection of the offspring, whereas prenatal transfer during pregnancy confers only a slight degree of protection to the offspring.

Maternal IgG, but not IgA or IgM, are required for protection of the offspring

We next tested the role of maternal IgG, IgA and IgM in protecting the offspring against *C. rodentium* infection. In these experiments, we performed maternal HK-Cr immunization experiments in mutant mice lacking IgA (*Igha*^{-/-}), FcRn (*Fcgrt*^{-/-}) or pIgR (*Pigr*^{-/-}), the transporter of secretory IgA and IgM. We found that HK-Cr immunization of IgA-deficient dams resulted in protection of the corresponding *Igha*^{-/-} offspring against oral pathogen challenge (Figure 4A), which correlated with reduced pathogen loads in feces, liver and spleen (Figure 4B to D), as well as lower intestinal pathology scores compared to naïve *Igha*^{-/-} pups that succumbed to infection (Figure 4E and Figure S3). Likewise, immunization of pIgR-deficient females also protected the *Pigr*^{-/-} offspring against pathogen challenge compared to naïve *Pigr*^{-/-} pups (Figure 4F to J and Figure S3). As expected, we did not detect *C. rodentium*-specific IgA in breast milk from *Igha*^{-/-} immunized dams, whereas IgA and IgM were highly reduced in samples from *Pigr*^{-/-} mice compared to WT dams (Figure S4B and C). The levels of anti-pathogen IgM in breast milk were increased in *Igha*^{-/-} mice perhaps reflecting a compensatory response (Harriman et al., 1999; Furuya et al., 2013). Additionally, we did not observe significant differences in susceptibility of naïve *Igha*^{-/-} or *Pigr*^{-/-} pups compared to naïve wild-type (WT) pups (compare Figure 2A with Figure 4A and F). Altogether, these results indicate that maternal IgA and IgM do not contribute significantly to protection of the offspring. In contrast, both maternally immunized and naïve *Fcgrt*^{-/-} pups from FcRn-deficient dams succumbed to infection (Figure 4K), and showed comparable pathogen loads in feces, liver and spleen (Figure 4L to N), as well as comparable intestinal pathology scores after *C. rodentium* challenge (Figure 4O and Figure S3). FcRn mediates the transfer of maternal IgG across the placenta and neonatal intestine, as well as regulates plasma IgG levels by protecting IgG from catabolism (Israel et al., 1995; Ghetie et al., 1996; Roopenian et al., 2003). However, its role in IgG transfer from plasma into breast milk remains controversial (Israel et al., 1995; Cianga et al., 1999; Ma et al., 2018). Although we found a reduction of total anti-*C. rodentium* IgG in breast milk of *Fcgrt*^{-/-} dams compared to WT mice after HKCr immunization, the difference was not significant

compared to the other experimental groups (Figure S4A). However, immunoblotting analysis confirmed that *C. rodentium*-specific IgG antibodies, including those against Ler-dependent virulence factors, were reduced in breast milk of immunized *Fcgrt*^{-/-} dams compared to WT mice (Figure S4D). We observed a similar reduction of pathogen-specific IgG in the serum of such *Fcgrt*^{-/-} mice after immunization (Figure S4E), indicating that FcRn is required for a robust IgG response after immunization, as reported for other foreign antigens (Roopenian et al., 2003; Cervenak, et al., 2011). Consistently, we found that *Fcgrt*^{+/-} heterozygous pups from HK-Cr immunized *Fcgrt*^{-/-} mothers succumbed to pathogen challenge and showed comparable pathogen burdens than naïve or maternally immunized *Fcgrt*^{-/-} pups (Figure S5A to D). Altogether, these results indicate that an efficient IgG, but not IgA or IgM, immune response in the dam is important for protection of the offspring after maternal immunization.

Maternal IgG coats *C. rodentium*, promotes phagocytosis of virulent bacteria and reduces pathogen attachment to intestinal mucosa

To determine the mechanism by which maternal IgG confers protection to the offspring, we first evaluated whether maternal *C. rodentium*-specific IgG can coat the pathogen both *in vitro* and during the infection. We found that >90% of *C. rodentium* were coated by IgG after incubation *in vitro* with milk samples from HK-Cr immunized dams, whereas incubation with milk from naïve mothers did not lead to pathogen coating (Figure 5A). These results correlated with increased levels (~50%) of pathogen-IgG coating in the intestinal lumen of maternally immunized pups compared to naïve pups (<1% coating) after oral challenge with the pathogen (Figure 5B). Because Ler-dependent factors are targeted by maternal IgG (Figure 3G), we tested next whether pathogen coating by IgG in breast milk promotes selective phagocytosis of Ler-positive (WT) *C. rodentium* by neutrophils, which have been shown to be involved in intestinal pathogen eradication in adult mice (Kamada et al., 2015). We found that incubation of the pathogen with milk samples from HK-Cr immunized dams increased selectively the intracellular loads of Ler-expressing bacteria about seven-fold compared to incubation with naïve milk (Figure 5C). To evaluate whether maternal immunization increases the phagocytosis of Ler-expressing *C. rodentium* *in vivo*, WT and *ler*-deficient *C. rodentium* were co-injected in the neutrophil-rich peritoneal cavity of naïve and maternally immunized pups. There were increased intracellular loads of WT *C. rodentium*, but not *ler* mutant bacteria, in neutrophils harvested from the peritoneum of maternally immunized pups compared to naïve pups (Figure 5D). We also observed reduced pathogen attachment to the intestinal mucosa of maternally immunized pups compared to naïve pups after oral infection, both by using a *C. rodentium* luciferase reporter strain (Figure 5E), and by counting the number of mucosa-attached bacteria (Figure 5F). Collectively, these results suggest that maternal IgG opsonizes virulent *C. rodentium*, increases its phagocytosis by neutrophils and reduces pathogen attachment to the intestinal epithelium during the neonatal period.

LEE virulence factors are required to confer passive immunity against *C. rodentium*

Our results suggest that Ler-dependent LEE virulence factors are required for an efficient antibody response against *C. rodentium* in the immunized dams (Figure 1C and 3G). Thus, we determined whether expression of the LEE-encoded T3SS is required to confer passive

immunity to the offspring after maternal immunization. To test this, we immunized adult females with WT or *Jer* HK-Cr (that does not express LEE). We found that maternal immunization with the WT strain, but not the *Jer* mutant, conferred protection to the offspring against oral pathogen challenge (Figure 6A) that was associated with lower pathogen loads in feces, liver and spleen (Figure 6B to D), as well as reduced pathology scores in the intestine (Figure 6E). These results indicate that LEE-encoded virulence factors are the main *C. rodentium* antigens that promote production of protective IgG antibodies after maternal immunization.

Maternal vaccination with purified LEE antigens confers protection against *C. rodentium*

To identify LEE virulence factors that promote the production of protective IgG maternal antibodies for the offspring, we purified two LEE-encoded antigens: a non-fimbrial adhesin (Intimin) and a component of the T3SS filament (EspA), which are both exposed on the bacterial surface of the pathogen (Gaytán et al., 2016). We found that both proteins were recognized by IgG in the serum and milk of HK-Cr immunized dams, but not by the serum of dams immunized with the *Jer* mutant (Figure 7A). Vaccination of adult females with purified Intimin in the presence of the adjuvant Alum elicited mainly antigen-specific IgG1 antibodies, while EspA immunization induced IgG1, IgG2b and IgG3 (Figure 7B). In contrast, HK-Cr immunization elicited IgG1, IgG2b and IgG3 against both Intimin and EspA albeit at lower levels compared to single protein vaccinations (Figure S6A and B). Both Intimin- and EspA-maternal vaccinations promoted higher survival rates in the offspring (Figure 7C) that correlated with reduced pathogen loads (Figure 7D to F), and pathology scores (Figure 7G) compared to pups maternally vaccinated with ovalbumin (OVA), an irrelevant protein used as a negative control. However, the level of protection observed with Intimin- and EspA-maternal vaccinations was lower than that observed with HK-Cr immunization (see Figure 2), which might be due to the differential IgG responses against the pathogen elicited by both immunization approaches (Figure S6C). Likewise, maternal vaccination with purified EspB, a pore component of the T3SS, or a triple vaccine comprising Intimin, EspA and EspB, conferred similar levels of protection as Intimin and EspA single immunizations (Figure S7A to E). These results indicate that maternal vaccination with single LEE-antigens confers effective but partial protection to the offspring against *C. rodentium*.

DISCUSSION

Maternal vaccination has proven to be an effective strategy for prevention of life-threatening infections in neonates and infants. Although various maternal vaccines are currently available against pertussis, neonatal tetanus and influenza (Riccardo et al., 2017), no licensed maternal vaccines against enteric bacterial pathogens such as EPEC are available despite the fact that enteric bacterial infection remains a major cause of childhood mortality worldwide. In this study, we report that maternal immunization with HK-Cr or pathogen-specific purified surface proteins confers protection to the offspring but not to the dam against enteric infection with the pathogen. We found that oral infection, but not parenteral immunization, with *C. rodentium* protected adult mice against a subsequent oral challenge with the pathogen. Although such a difference in protection could be potentially explained

by different antibody responses elicited by oral and parenteral routes of immunization, we observed similar levels of anti-*C. rodentium* IgG antibodies in the serum of both groups of animals. However, fecal pathogen-specific IgG was only detected after oral infection, providing a plausible mechanism to account for the differential protective effect of oral vs IP immunization. Previous studies have demonstrated that natural (oral) infection by various pathogens confers protection against disease caused by subsequent infection with the same pathogen (Czerkinsky and Jan Holmgren, 2015). This long-lasting immunological memory relies on the generation of B memory (BM) cells in the germinal centers of intestinal Peyer's patches and lymphoid follicles, these BM cells express gut homing properties and differentiate into antibody-secreting plasma cells that produce mucosal antibodies in the gut (Pasetti et al., 2011; Czerkinsky and Holmgren, 2015). In contrast, systemic antibodies, such as those produced after IP immunization, are unable to reach the intestinal lumen under homeostatic conditions since most of the intestine is not permeable to serum antibodies in the absence of intestinal damage (Czerkinsky and Holmgren, 2015). This may explain why parenteral immunization did not confer protection against oral challenge with *C. rodentium* in adult mice because IP immunization did not induce the presence of IgG against the pathogen in the gut. Thus, plasma antibodies elicited via the parenteral route might be primarily effective against enteric pathogens that cross the epithelial barrier. Consistently, parenteral immunization with murein lipoprotein, an outer membrane protein of Gram-negative bacteria, conferred protection against systemic infections by *E. coli* and *Salmonella* through IgG-coating of the pathogen to promote its killing by phagocytes (Zeng et al., 2016). Although *C. rodentium*-specific IgA was also observed in the feces after oral infection, but not IP immunization, orally infected IgA-deficient mice develop effective protective immunity against a secondary challenge with *C. rodentium* (Maaser et al., 2004). Thus, IgA is not important for the development of protective immunity against the pathogen. Altogether, these data suggest that luminal *C. rodentium*-specific IgG, but not IgA, generated during oral infection is essential to confer protection against reinfection with the pathogen.

Although secretory IgA and IgM can confer protection against various enteric pathogens by preventing colonization and/or replication of virulent bacteria (Boullier et al., 2009; Brandtzaeg, 2009), we found that genetic deficiency of IgA, or the reduction of passive transfer of maternal IgA and IgM through breast milk in pIgR-deficient mice, did not have any effect in protection of the offspring against pathogen challenge. In contrast, the absence of the neonatal IgG-specific transporter FcRn abolished the protection conferred by maternal immunization due to an impaired production and/or maintenance of IgG in the dams after immunization. Consistently, in addition to its role in regulating IgG stability and the passive transfer of maternal IgG to the offspring, FcRn is also important for the generation of a potent IgG response against foreign antigens in adult mice (Roopenian et al., 2003; Cervenak et al., 2011). Additionally, a previous study using transgenic mice constitutively expressing FcRn, whose natural expression is turned off after weaning in rodents (Yoshida et al., 2006), showed that selective expression of FcRn in the intestinal epithelium of adult mice was associated with increased secretion of IgG into the lumen and reduced susceptibility against *C. rodentium* (Yoshida et al., 2006). Altogether, these observations

highlight the essential role of IgG and its transporter FcRn in protection against *C. rodentium* infection in both adult and neonatal mice.

Several studies have addressed the role of maternal immunization in generation of passive immunity in the offspring against enteric pathogens such as *Shigella* (Mitra et al., 2012), Enterotoxigenic *E. coli* (ETEC) (Luiz et al., 2015) and Enterohemorrhagic *E. coli* (EHEC) (Dean-Nystrom et al., 2002; Rabinovitz et al., 2016). However, the protective immunoglobulin, the route of delivery, and the mechanism by which maternal immunization conferred protection to the offspring were not determined in such studies. Additionally, in the case of EHEC, the lack of a lethal and/or natural model of infection resulted in only subtle differences in susceptibility between the immunized and control groups (Dean-Nystrom et al., 2002; Rabinovitz et al., 2016). In this study, we showed that maternal pathogen-specific IgG delivered through breast milk confers protection to the offspring and demonstrated that protective IgG coats the pathogen in the intestinal lumen of the suckling pup, thus reducing its attachment to the mucosa and preventing epithelial damage.

Our studies indicate that LEE-specific antigens are essential for protection of the offspring against *C. rodentium* infection, since immunization with a Ler mutant that does not express LEE virulence factors did not confer protection to the offspring. We identified EspA, Intimin and EspB, three extracellular components of the T3SS encoded by LEE (Gaytán et al., 2016), as major antigens to confer passive immunity in the offspring after maternal immunization. Notably, antibodies reactive to these T3SS components have been detected in serum (Jenkins et al., 2000; Li et al., 2000; Martinez et al., 1999) and milk samples (Manjarrez-Hernandez et al., 2000; Gavilanes-Parra et al., 2013; Altman et al., 2017) of patients infected with EPEC. The observation that maternal vaccination with single LEE-antigens (Intimin, EspA or EspB) or their combination conferred similar levels of protection in the offspring suggests that antibodies against these antigens might act through a similar mechanism to confer protection. Such antibodies could act by promoting pathogen opsonization and killing by neutrophils in the intestine as previously reported for adult mice (Kamada et al., 2015) and/or by interfering with the intimate binding of the pathogen to the intestinal epithelium promoted by these LEE proteins (Deng et al., 2004; Gaytán et al., 2016). Because protection obtained with single protein vaccinations was lower than that observed after HK-Cr immunizations, it is also possible that naturally folded LEE antigens, and/or additional LEE proteins, are required for the induction of an optimal IgG response and full protection after maternal vaccination. In addition, we cannot rule out the possibility that non-LEE factors may contribute to the full protective response conferred by LEE antigens, for example, by acting as natural adjuvants.

In summary, our results provide insights into the mechanism by which maternal antibodies, in particular IgG in breast milk, protect mice from enteric infection in neonates, a population highly susceptible to diarrheal disease, including EPEC infection. These results may lead to the development of better approaches to treat enteric infections during the neonatal period including the delivery of protective IgG to the intestine of infants via breast milk after maternal vaccination and/or through enriched formula containing pathogen-specific IgG antibodies.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCING SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Gabriel Nunez (gabriel.nunez@umich.edu)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—The WT and FcRn-deficient (*Fcgrt*^{-/-}) mice were purchased from The Jackson Laboratory. The IgA-deficient (*Igha*^{-/-}) mice were provided by Dr. Bernard Arulanandam (UT Health San Antonio; Harriman et al., 1999). The pIgR-deficient (*Pigr*^{-/-}) mice were provided by Dr. Christiane E. Wobus (University of Michigan; Uren et al., 2003). All mice were in the C57BL/6 background and bred/kept under the same specific-pathogen-free (SPF) conditions in our mouse Facility at the University of Michigan. Adult females (6–8-week old) and neonatal mice (18–19-day old, males and females) were used for immunization and/or pathogen challenge. All animal studies were approved by the University of Michigan Committee on the Care and Use of Animals.

Bacterial strains—The kanamycin-resistant (Kan^R) *C. rodentium* strain DBS120 (pCRP1::Tn5) was originally provided by Dr. David Schauer (Schauer and Falkow, 1993). The isogenic *C. rodentium* *ler* mutant (Kan^R), the ampicillin-resistant (Amp^R) *C. rodentium-lux*, and the chloramphenicol-resistant (Chl^R) *C. rodentium*-GFP reporter strains have been previously described (Deng et al., 2004; Bergstrom et al., 2010; Kamada et al., 2015). The *E. coli* strain BL21 was purchased from New England Biolabs. All bacteria were routinely grown in Luria-Bertani broth (LB, Invitrogen) or Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific). Media were supplemented with Kan (50 µg/ml), Amp (200 µg/ml) or Chl (35 µg/ml) as required.

METHOD DETAILS

Mouse immunization and infection.—For HK-Cr immunizations, bacteria were grown in DMEM (5% CO₂, 37°C, static) and concentrated to ~5×10⁸ CFU/ml in phosphate buffered saline (PBS 1X, Thermo Fisher Scientific). Bacterial suspensions were then heat-inactivated at 75°C for 45–60 min, and 0.2 ml were IP injected into adult females. After two weeks, a second HK-Cr injection was administered. For protein immunizations, 50 µg of the purified proteins were mixed 1:1 (v/v) with Imject Alum (Thermo Fisher Scientific) and IP injected into adult females. A second protein/Alum injection, and a final antigen immunization (100 µg) without adjuvant were administered at 2-week intervals. For both HK-Cr and protein immunizations, the females were mated with naïve males two weeks after last immunization, and the resulting offspring was orally challenged with *C. rodentium* as described below.

For oral *C. rodentium* challenge, bacteria were grown in LB broth with shaking at 37°C until cultures reached an optical density at 600 nm (O.D.₆₀₀) of ~1.0. Bacterial cultures were then used to prepare bacterial suspensions containing ~5×10⁹, 5×10⁸ or 5×10⁷ CFU/ml in PBS 1X and 0.1 ml were administered by oral gavage into adult or neonatal mice. Pathogen counts in feces were determined at different time points after infection through serial

dilution plating. Some mice were sacrificed between day 7 and 10 after infection to determine pathogen loads in liver and spleen, and to process colonic and cecal tissues for Haemotoxylin and Eosin (H&E) staining, whereas the rest of the mice were used to monitor mouse survival.

Histological scoring—The pathology score was evaluated blindly by one pathologist using a previously described scoring system (Chen et al., 2008). Briefly, severity of inflammation (0= none, 1= mild, 2= moderate, 3= severe), level of involvement (0= none, 1= mucosa, 2= mucosa and submucosa, 3= transmural), and extent of epithelial/crypt damage (0= none, 1= basal 1/3, 2= basal 2/3, 3= crypt loss, 4= crypt and surface epithelial destruction) were determined. Each variable was then multiplied by a factor reflecting the percentage of the cecum/colon involved (0–25%, 26–50%, 51–75%, 76–100%), and then summed to obtain the overall score.

Protein purifications—The full nucleotide coding regions of EspA and EspB were amplified using the sets of primers pQE60-EspA and pQE60-EspB, respectively (see Key Resources Table), and then cloned into the plasmid pQE60 (QIAGEN). The plasmid p6his-Intb385 containing the DNA region encoding the extracellular C-terminal 385 amino acids of Intimin- β (6xHis-tagged-Intimin- β ₃₈₅) cloned into the vector pQE32 was kindly provided by Alison D. O'Brien (Sinclair and O'Brien, 2004). The recombinant plasmids were transformed into *E. coli* BL21 containing the plasmid pREP4 (QIAGEN) and protein overexpression was induced by adding 1mM isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich) into the bacterial cultures. Recombinant proteins were purified under denaturing conditions using nickel nitrilotriacetic acid (Ni-NTA) agarose (QIAGEN), and then dialyzed in PBS 1X. Protein concentration was determined using the Quick Start Bradford Protein Assay (Bio-Rad) and the purity of the samples was analyzed by electrophoresis into 12% SDS-PAGE. Aliquots of the proteins were stored at -20°C until use.

Milk collection from mice—Milk collection was performed as previously reported (Gómez-Gallego, et al., 2013). Briefly, adult female mice with nursing litters were separated from their pups for 4–6 h to allow accumulation of milk in the mammary glands. Dams were then anesthetized using 2% isoflurane (VetOne), and 2–8 units per mouse of oxytocin (VetOne) was administered intraperitoneally between the left and right inguinal nipples to induce milk flow. Samples were collected using an electric human breast pump that was modified to accommodate mouse nipples and to handle small liquid volumes (Gómez-Gallego, et al., 2013). When no more milk was recovered from various nipples, the milking machine was stopped, and the milk stored at -20°C until use.

Immunoblotting and ELISA—For detection of pathogen-specific IgG by immunoblotting, bacterial lysates of WT and *ler C. rodentium*, or the indicated purified proteins, were separated by electrophoresis into 12% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore). Blots were incubated overnight at 4°C with mouse serum (1:10,000 dilution) or milk (1:1,000) in blocking buffer (PBS 1X, 0.1% Tween 20, 5% dry milk). Membranes were washed with PBS-T buffer (PBS 1X, 0.1% Tween), and then

incubated for 1 h at room temperature with peroxidase rabbit anti-mouse IgG (1:10,000; Jackson ImmunoResearch) in blocking buffer. Bound IgG was detected with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) following the manufacturer's instructions.

For measurement of antigen-specific antibodies by ELISA, 96-well plates were coated with a suspension containing $\sim 1 \times 10^7$ CFU of HK-Cr, or 1 μ g of purified protein, in PBS 1X. Wells were blocked with PBS-A buffer (PBS 1X, 2% bovine serum albumin), and incubated overnight at 4°C with mouse serum (1:1,000 to 1:10,000), milk (1:100 to 1:1,000) or luminal contents (0.1 to 0.01 μ g/ml) in PBS-A. Wells were washed with PBS-T, and then incubated 1 h with goat alkaline phosphatase (AP)-conjugated anti-mouse IgG, IgA or IgM (1:500; SouthernBiotech). Binding of specific IgG subtypes or IgA was detected by measuring the O.D. at 405 nm after adding p-nitrophenyl phosphate (pNPP) substrate (SouthernBiotech).

Cross-fostering experiments—Naïve or HK-Cr immunized females were pair-housed with naïve males at the same day (two weeks after last immunization of the treated group). Once pregnancy was noticed, the male was removed from the cage and the pregnant females were monitored daily for delivery. Age matched (1- to 2-day old) newly born pups were then reciprocally cross-fostered between naïve and HK-Cr immunized dams. Pups were left undisturbed with the recipient mother until they were 18- to 19-day old, and then challenged with *C. rodentium* as described above. Age matched naïve (non-cross-fostered) pups were used as controls in these experiments.

Neutrophil phagocytosis assays—For the *in vitro* phagocytosis assays, 1×10^6 CFU of WT *C. rodentium* (Chl^R) and *ler* mutant (Kan^R), previously grown in DMEM to induce expression of virulence factors, were mixed 1:1 and incubated with milk from naïve or HK-Cr immunized dams for 30 min at room temperature. Bacterial mixtures were then incubated (30 min, 37°C, 5% CO₂) with 1×10^6 neutrophil cells harvested from the peritoneum of naïve mice previously treated with 4% thioglycolate broth (Sigma-Aldrich) to induce neutrophil recruitment in the peritoneal cavity. Neutrophils-bacteria mixtures were then treated with gentamycin (10 μ g/ml) for 30 min to kill extracellular bacteria. Cells were washed with PBS 1X to remove the excess of gentamicin in the media, and then permeabilized with 0.1% triton-X100. The number of intracellular bacteria was determined by plating serial dilutions of the permeabilized cell samples in LB agar plus Chl or Kan. For the *in vivo* phagocytosis assays, 1×10^6 CFU of the WT and *ler* strains were co-injected into the neutrophil-rich peritoneal cavity of naïve or maternally immunized pups, previously treated with 4% thioglycolate. After 30 min, neutrophils were harvested from the peritoneal cavity and the number of intracellular bacteria was determined as described above.

Measurement of mucosa-associated bacteria—For detection of mucosa-attached pathogen, naïve or maternally immunized pups were infected with $\sim 5 \times 10^8$ CFU of the luciferase reporter strain *C. rodentium-lux* and cecal/colonic tissue were harvested four days after infection. To remove non-adherent bacteria, intestinal tissues were flushed with PBS 1X using an oral gavage needle, and further washed by vortex in a conical tube containing sterile PBS 1X. Washed tissues were immediately placed into the light-tight chamber of a CCD *in vivo* imaging system (IVIS 200, PerkinElmer) and bacterial bioluminescence in the

tissue was captured using the software Living Image (PerkinElmer). Both control and treatment groups were analyzed at the same time and using the same software settings. After capturing the images, ceca and colons were separately homogenized in sterile PBS 1X (0.1 mg tissue/ μ l buffer), and serial dilutions of the tissue homogenates were plated on LB agar plus Amp to determine the total number of attached bacteria.

IgG coating of luminal *C. rodentium*—For IgG coating of *C. rodentium in vivo*, naïve and maternally immunized pups were infected with the reporter strain *C. rodentium*-GFP. Two to four hours after infection, gastrointestinal (GI) contents were collected, resuspended in PBS 1X and filtrated sequentially through 100 μ m, 70 μ m and 40 μ m strainers (Falcon). The filtrated luminal contents (cell pellets) were then washed with FACS buffer (PBS1 X, 1% BSA, 0.1% NaN₃) and stained with biotin rat anti-mouse IgG (eBiosciences). Samples were washed with FACS buffer and then incubated with APC-streptavidin (Biolegend). After a second washing, Immunoglobulin bound GFP⁺-bacteria were detected by Flow Cytometry using FACSCalibur or FACSCanto II (BD Biosciences). For IgG coating of *C. rodentium in vitro*, bacteria were cultured in DMEM, incubated for 30 min with milk samples from naïve or HK-Cr immunized dams, and then stained and analyzed by Flow Cytometry as described above.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software). Differences between two groups were evaluated using Student's t test (parametric) or Mann-Whitney U test (non-parametric). For multiple comparisons, statistical analyses were performed using one-way ANOVA (parametric) or Kruskal-Wallis test (non-parametric), and then Bonferroni test for parametric samples, or Dunn's test for non-parametric samples as post hoc tests. Differences at or below $p < 0.05$ were considered significant. If not otherwise stated, data are shown as mean \pm standard deviation (SD). Information regarding exact number of animals ($n = "X"$), experimental replication, dispersion measures, and statistical tests used are described in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS:

- Maternal immunization confers protection to offspring against *C. rodentium* infection.
- Maternal IgG in breast milk, but not IgA or IgM, is required for neonatal protection.
- Maternal IgG coats the pathogen and increases its engulfment by neutrophils.
- LEE-specific antibodies are required to confer protection in the offspring.

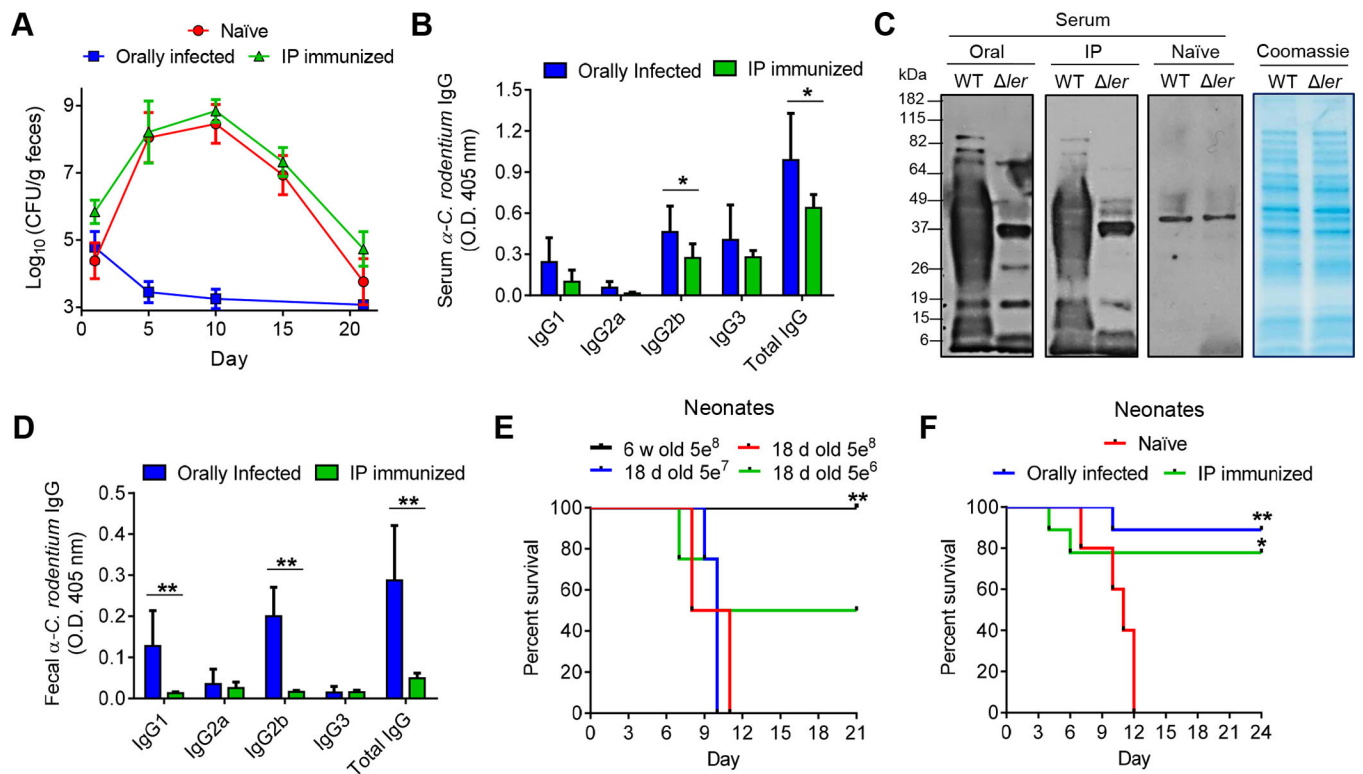


Figure 1. Parenteral immunization protects the offspring, but not the dam, against pathogen oral challenge

(A) Adult C57BL/6 mice were orally- or IP-inoculated with alive or heat-inactivated *C. rodentium*, respectively. After one month, mice were challenged orally with 5×10^8 CFU and bacterial counts in feces were determined on the indicated days after infection. Naïve, untreated animals. (B) Serum α -*C. rodentium* IgG subtypes in orally infected or IP immunized adult mice determined by ELISA two weeks after inoculation (n= 4 mice per group). (C) Immunoblotting of *C. rodentium* WT and Δ *ler* bacterial lysates using serum from orally infected or IP immunized adult mice. Proteins recognized by antibodies in serum were revealed with HRP-anti-mouse IgG. SDS-PAGE gel stained with Coomassie blue is shown to reveal total amounts of cell lysates loaded. (D) Fecal α -*C. rodentium* IgG subtypes in orally infected or IP immunized adult mice determined by ELISA two weeks after inoculation (n= 4 mice per group). (E) Adult mice (6-week old) and pups (18-day old) were orally infected with 5×10^8 , 5×10^7 or 5×10^6 CFU and mouse survival was determined over the indicated time after infection. (F) 18-day old pups from orally infected or IP immunized mothers were challenged orally with 5×10^8 CFU of *C. rodentium* and survival rates were determined. Naïve, pups from untreated dams. Representative results from two independent experiments are shown. Data are represented as mean \pm SD (A-B, and D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Mann-Whitney U test (B and D) or log rank test (E and F, compared to control groups indicated in red). Only statistically significant differences are shown. See also Figure S1.

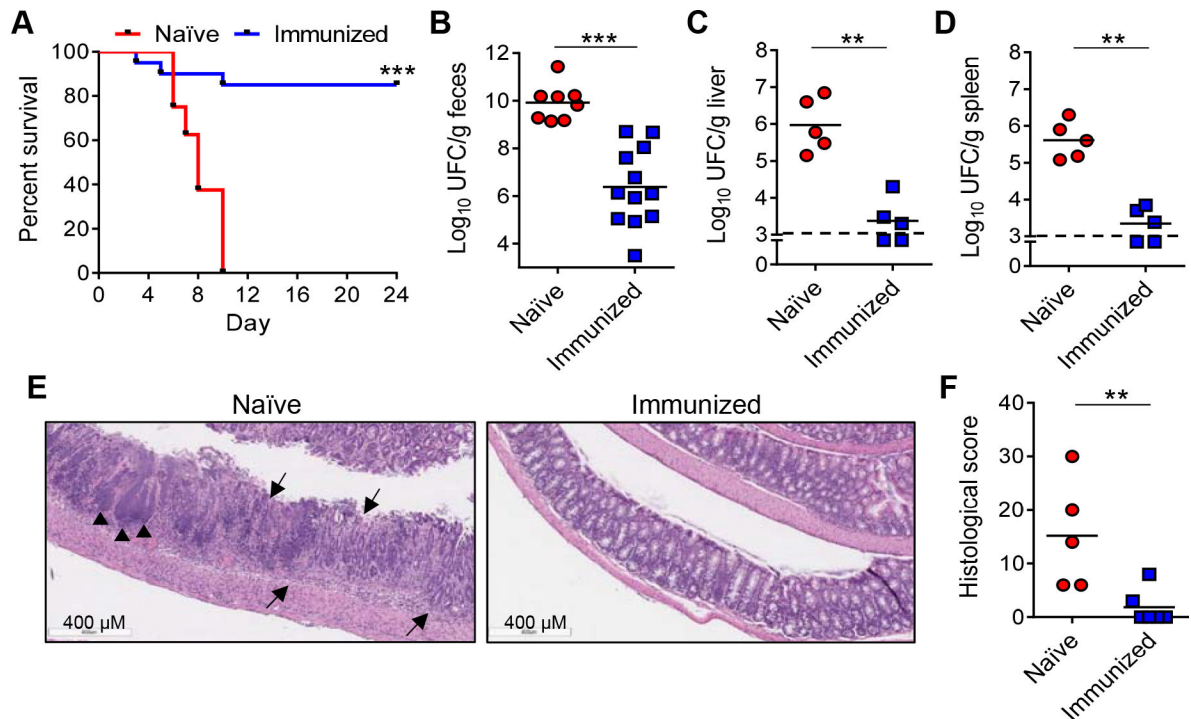


Figure 2. Parenteral maternal immunization reduces pathogen loads and intestinal inflammation in the offspring after oral challenge

(A) 18-day old pups from naïve or HK-Cr immunized mothers were challenged orally with 5×10^8 CFU of *C. rodentium* and mouse survival was monitored at the indicated days post-infection (dpi). (B-D) Pathogen load in feces at 5 dpi (B), liver (C) and spleen (D) (7 dpi), in pups from naïve and HK-Cr immunized mothers after *C. rodentium* challenge. (E-F) Representative H&E staining sections (E) and pathology scores (F) of colonic tissue harvested from pathogen challenged pups depicted in B-D. Arrows indicate areas with marked epithelial damage and inflammation and arrowheads pathogen foci in the mucosa. Scale bars, 400 μm . Data show representative (E) or combined (A-D, and F) results from at least two independent experiments. Each dot represents one mouse (B-D, and F). Dotted line indicates the limit of detection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by log rank test (A), unpaired two-tailed T- test (B) or Mann-Whitney U test (C-D, and F). See also Figure S2.

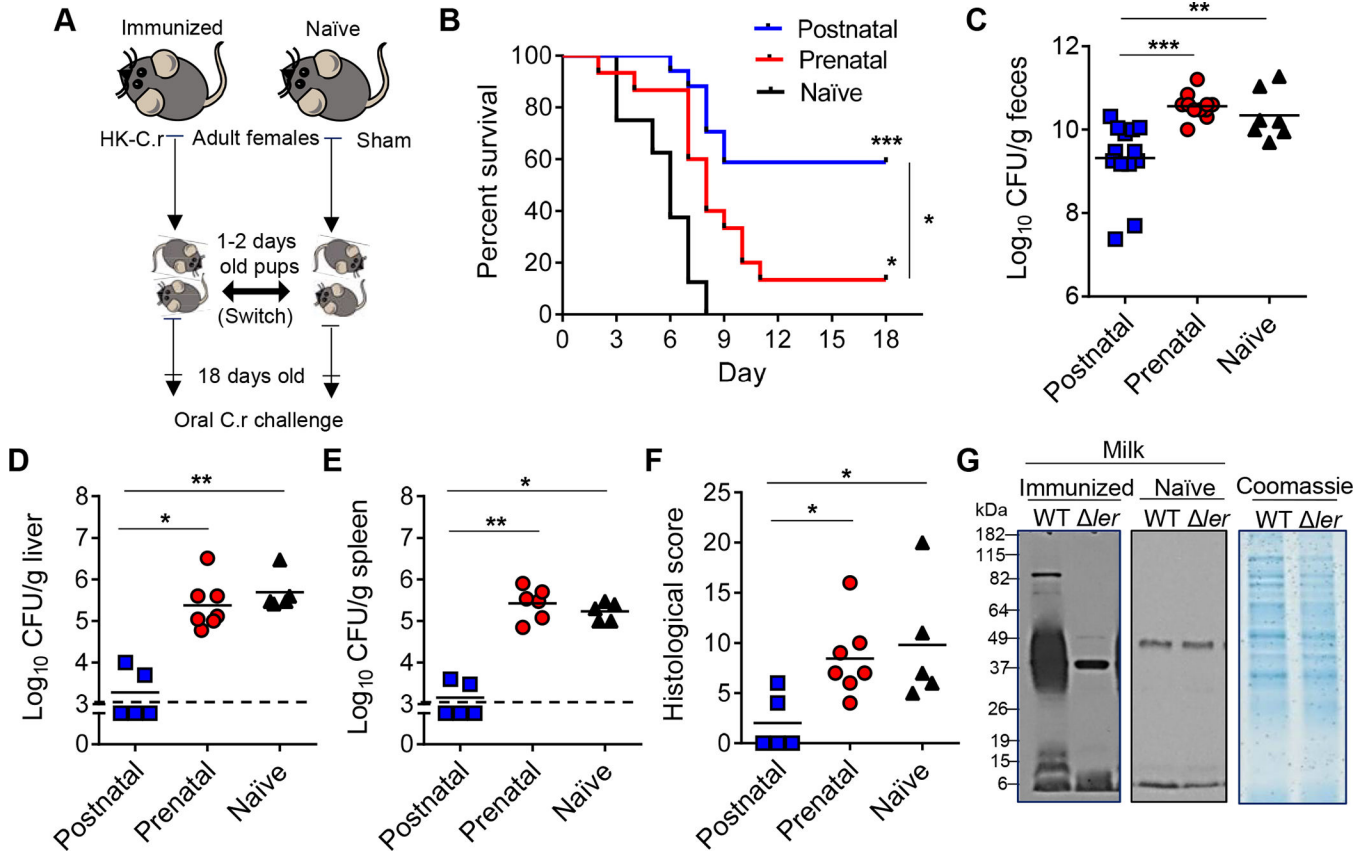


Figure 3. Passive immunity is transferred from the mother to the offspring mainly through breast milk.

(A) Experimental design. Newborn pups from naïve and HK-Cr immunized dams were cross-fostered and nursed by the indicated recipient mother. Pups were then challenged with 5×10^8 CFU of *C. rodentium* when they were 18-day old. (B) Survival rates of the challenged pups over the indicated days post-infection (dpi). Prenatal, pups from immunized mothers nursed by naïve dams; Postnatal, pups from naïve mothers nursed by immunized dams; Naïve, non-fostered pups from untreated mothers. (C-E) Pathogen load in feces at 5 dpi (C), liver (D) and spleen (E) (7 dpi) of the pathogen challenged pups. (F) Pathology scores of cecal and colonic tissues from pups depicted in B-E. (G) Immunoblotting of *C. rodentium* WT and Δler bacterial lysates with breast milk from naïve or HK-Cr immunized mothers. Proteins recognized by antibodies in milk were revealed with HRP-anti-mouse IgG. SDS-PAGE gel stained with Coomassie blue is shown to reveal total amounts of cell lysates loaded. Data show representative (G) or combined (B-F) results from at least two independent experiments. Each dot represents one mouse (C-F). Dotted line indicates the limit of detection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by log rank test (B), one-way ANOVA (C) or Kruskal-Wallis test (D-F). Only statistically significant differences are indicated.

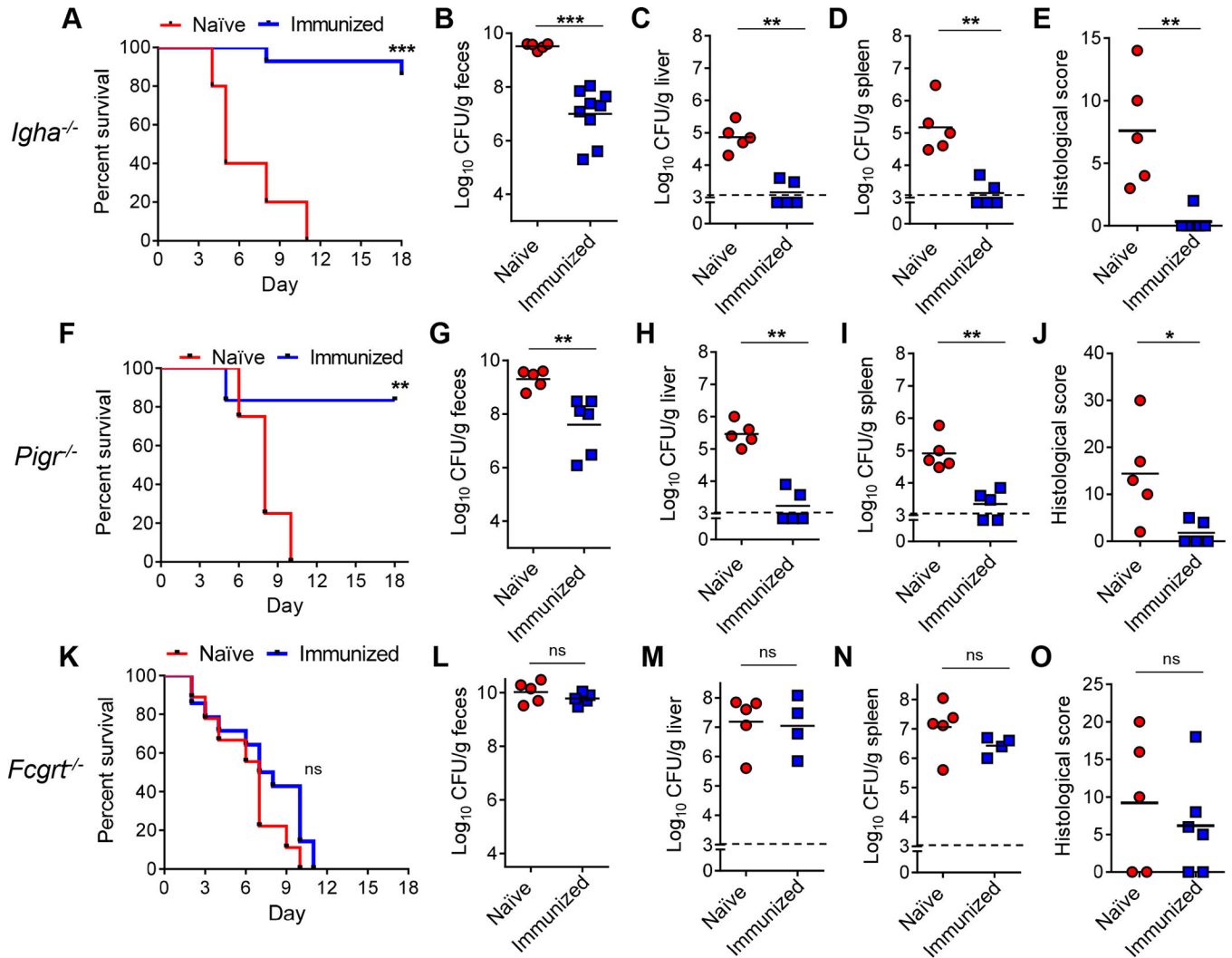


Figure 4. Maternal IgG, but not IgA or IgM, is required for protection of the offspring. (A) 18-day old pups from naïve or HK-Cr immunized IgA-deficient (*Igha*^{-/-}) mothers were challenged with 5×10⁸ CFU of *C. rodentium* and survival rates were determined over the indicated days post-infection (dpi). (B-D) Pathogen load in feces at 5 dpi (B), liver (C) and spleen (D) (7 dpi) in pups from naïve and HK-Cr immunized *Igha*^{-/-} mothers after pathogen challenge. (E) Pathology scores of cecal and colonic tissue harvested from infected pups depicted in B-D. (F) Pups from naïve or HK-Cr immunized pIgR-deficient (*Pigr*^{-/-}) mothers were challenged and mouse survival recorded as in A). (G-I) Pathogen load in feces at 5 dpi (G), liver (H) and spleen (I) (7 dpi), in pups from naïve and HK-Cr immunized *Pigr*^{-/-} mothers after *C. rodentium* challenge. (J) Pathology scores of cecal and colonic tissue harvested from infected pups depicted in G-I. (K) Pups from naïve or HK-Cr immunized FcRn^{-/-}-deficient (*Fcgrt*^{-/-}) mothers were challenged and mouse survival recorded as in A). (L-N) Pathogen load in feces at 5 dpi (L), liver (M) and spleen (N) (7 dpi), in pups from naïve and HK-Cr immunized *Fcgrt*^{-/-} mothers after *C. rodentium* challenge. (O) Pathology scores of cecal and colonic tissue harvested from infected pups depicted in L-N. Data represent combined results from two independent experiments. Each dot represents one

mouse (B-E, G-J, L-N). Dotted line indicates the limit of detection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by log rank test (A, F and K) and Mann-Whitney U test (BE, G-J, L-O). ns, not significant. See also Figures S3–5.

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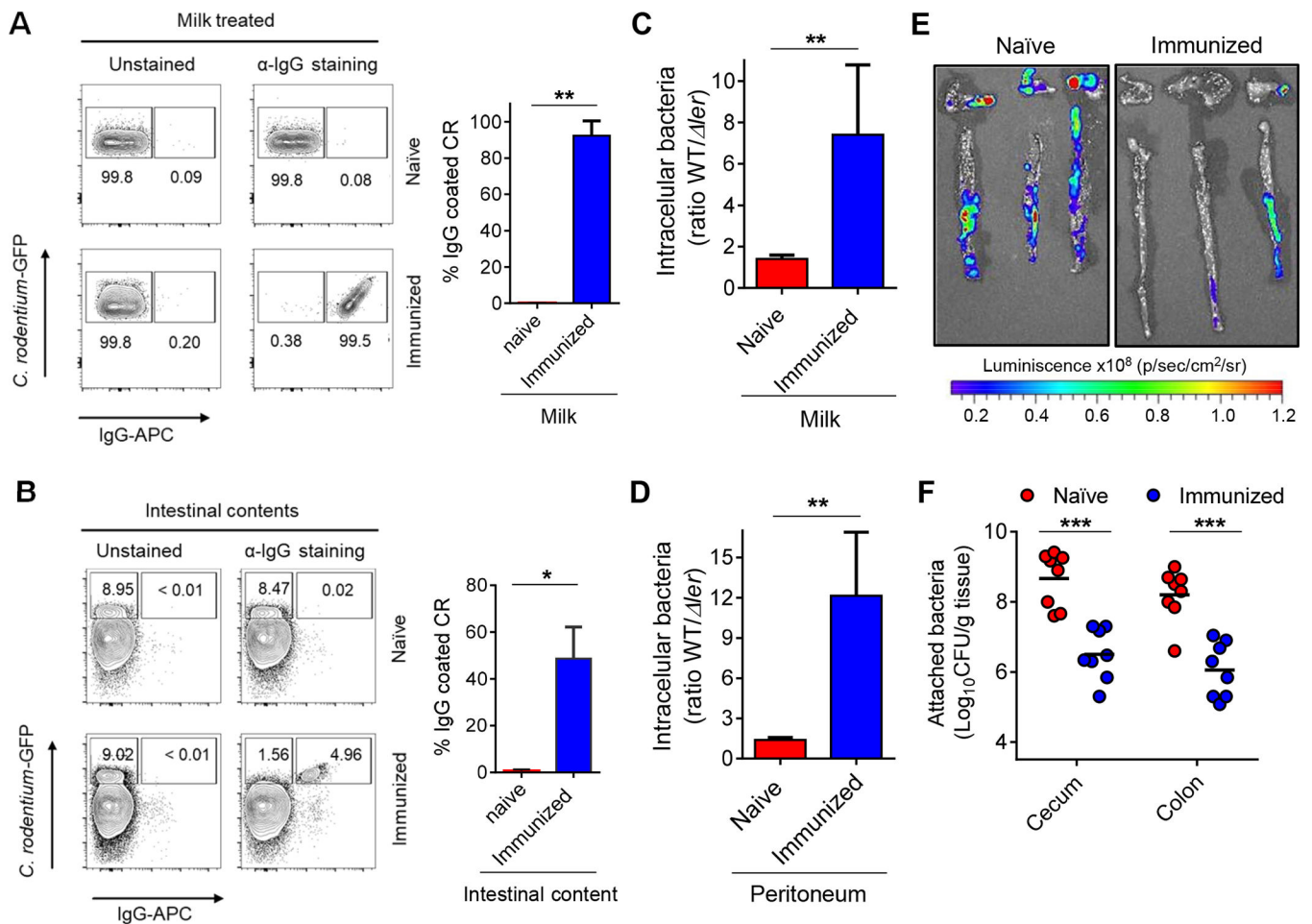


Figure 5. Maternal IgG coats *C. rodentium* in the intestine of the offspring, promotes phagocytosis of virulent bacteria, and reduces pathogen attachment.

(A) IgG coating of *C. rodentium*-GFP incubated *in vitro* with breast milk from naïve or HK-Cr immunized mothers. Left panel, representative flow cytometry profiles; right panel, percentage of IgG-coated *C. rodentium*-GFP (naïve, n = 4 mice; immunized, n = 5 mice). (B) IgG coating of *C. rodentium*-GFP in intestinal contents of infected pups from naïve or HKCr immunized mothers. Left panel, representative flow cytometry profiles; right panel, percentage of IgG-coated *C. rodentium*-GFP (naïve, n = 4 mice; immunized, n = 5 mice). (C) Neutrophil phagocytosis assays *in vitro*. WT/ Δ ler bacterial mixtures (1:1) were treated with breast milk from naïve or HK-Cr immunized dams and incubated with neutrophils harvested from the peritoneal cavity of naïve mice. Ratios (WT/ Δ ler) of intracellular bacteria in gentamicin-treated neutrophils are shown (naïve, n = 5 mice; immunized, n = 6 mice). (D) Neutrophil phagocytosis assays *in vivo*. WT/ Δ ler mixtures (1:1) were IP-injected into the neutrophil-rich peritoneal cavity of pups from naïve or HK-Cr immunized mothers. Ratios (WT/ Δ ler) of intracellular bacteria in gentamicin-treated neutrophils are shown (naïve, n = 6 mice; immunized, n = 6 mice). (E-F) Levels of *C. rodentium* attached to intestinal epithelium. Pups from naïve or HK-Cr immunized mothers were orally inoculated with 5×10^8 CFU of a *C. rodentium*-lux reporter strain. Cecal and colonic tissues were harvested, washed to remove non-adherent bacteria, and used to assess bacterial bioluminescence (E)

and total bacteria in tissue homogenates (F). Data show representative (A and B, left panels; and E) or combined (A and B, right panels; C, D and F) results from two independent experiments. Data are represented as mean \pm SD (A-B, right panels; C and D). Each dot represents one mouse (F). * $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$ by Mann-Whitney U test (A to D) or unpaired two-tailed T-test (F).

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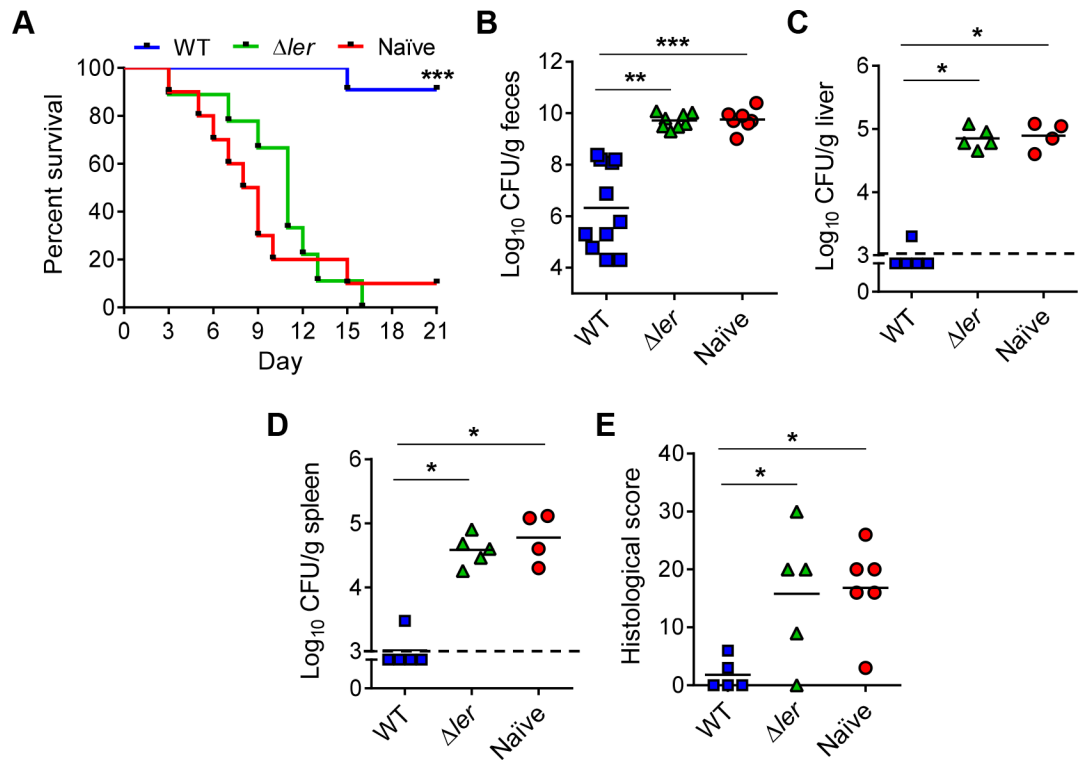


Figure 6. LEE virulence factors are required to confer passive immunity against *C. rodentium* (A) 18-day old pups from mothers immunized with WT or *ler* HK-Cr were challenged with 5×10^8 CFU of *C. rodentium* and mouse survival was determined over the indicated days post-infection (dpi). Naïve, pups from untreated dams. (B-D) Pathogen load in feces at 5 dpi (B), liver (C) and spleen (D) (7 dpi) in pups from naïve and HK-Cr immunized mothers after *C. rodentium* challenge. (E) Pathology scores of cecal and colonic tissue harvested from infected pups depicted in B-D. Data represent combined results from two independent experiments. Each dot represents one mouse (B-E). Dotted line indicates the limit of detection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by log rank test (A, compared to naïve group), one-way ANOVA (B) or Kruskal-Wallis test (C-E). Only statistically significant differences are indicated.

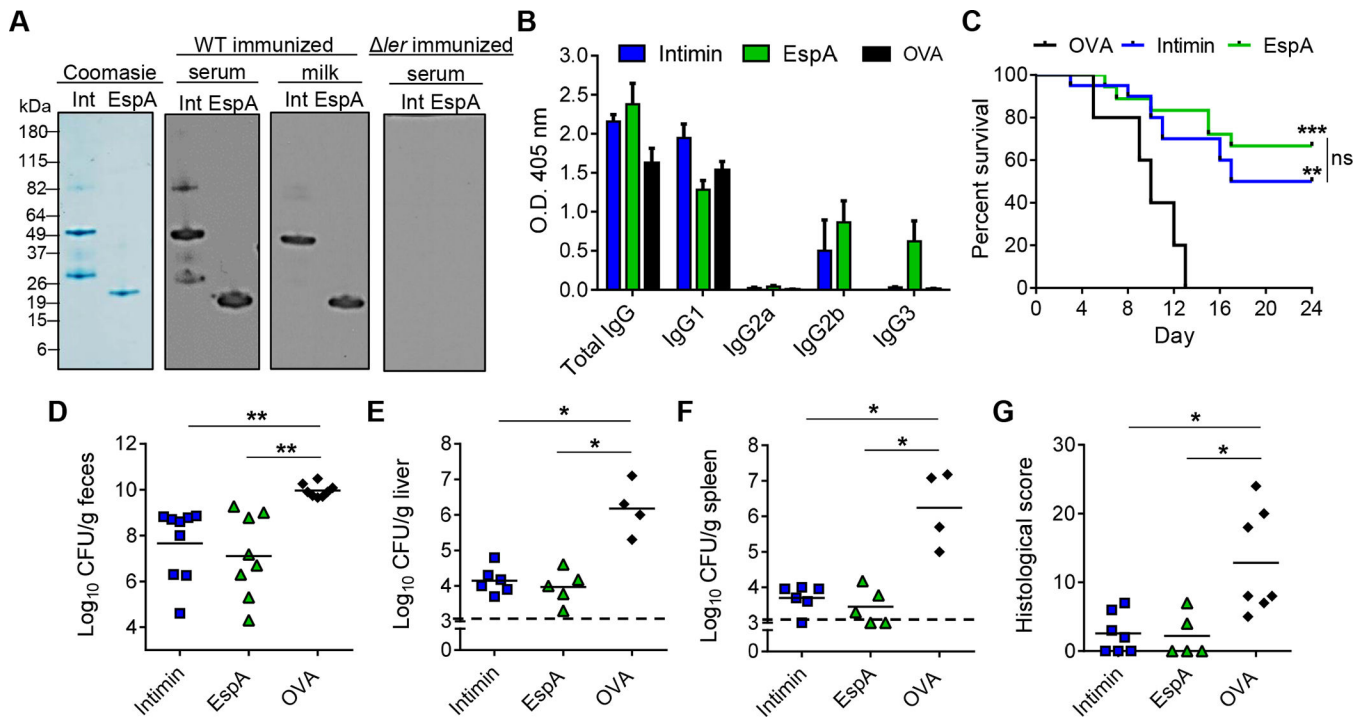


Figure 7. Maternal vaccination with purified LEE antigens confers protection to the offspring against *C. rodentium*.

(A) Immunoblotting of purified Intimin and EspA using serum or breast milk from mothers immunized with WT or *ler*HK-Cr. Proteins recognized by antibodies in serum and milk were revealed with HRP-anti-mouse IgG. SDS-PAGE gel stained with Coomassie blue is shown to reveal total amounts of protein loaded. (B) Antigen-specific IgG subtypes determined by ELISA using serum from dams immunized with the indicated proteins. OVA, Ovalbumin. (C) 19-days old pups from dams immunized with the indicated proteins were challenged with 5×10^7 CFU of *C. rodentium* and survival rates were determined over the indicated days post-infection (dpi). (D-F) Pathogen load in feces at 5 dpi (D), liver (E) and spleen (F) (7 dpi) in the pathogen challenged pups. (G) Pathology scores of cecal and colonic tissue harvested from the infected pups depicted in D-F. Data show representative (A and B) or combined (C-G) results from two independent experiments. Each dot represents one mouse (D-G). Dotted line indicates the limit of detection. * $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$ by log rank test (C, compared to OVA group), one-way ANOVA (D and G) or Kruskal-Wallis test (E and F). Only statistically significant differences are indicated. See also Figures S6–7.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotin Rat anti-Mouse IgG1	BD Biosciences	Cat# 553441; RRID: AB_394861
Biotin Rat anti-Mouse IgG2a	BD Biosciences	Cat# 553388; RRID: AB_394826
Biotin Rat anti-Mouse IgG2b	BD Biosciences	Cat# 553393; RRID: AB_394831
Biotin Rat anti-Mouse IgG3	BD Biosciences	Cat# 553401; RRID: AB_394838
Goat anti-Mouse IgA, Human ads-AP	SouthernBiotech	Cat# 1040-04; RRID: AB_619826
Goat anti-Mouse IgG1, Human ads-AP	SouthernBiotech	Cat# 1070-04
Goat anti-Mouse IgG2a, Human ads-AP	SouthernBiotech	Cat# 1080-04; RRID: AB_2692322
Goat anti-Mouse IgG2b, Human ads-AP	SouthernBiotech	Cat# 1090-04; RRID: AB_619828
Goat anti-Mouse IgG3, Human ads-AP	SouthernBiotech	Cat# 1100-04
Goat anti-Mouse IgM, Human ads-AP	SouthernBiotech	Cat# 1020-04; RRID: AB_619829
Peroxidase AffiniPure Rabbit Anti-Mouse IgG, F(ab') ₂ fragment specific	Jackson ImmunoResearch	Cat# 315-035-047; RRID: AB_2340068
Bacterial and Virus Strains		
<i>Citrobacter rodentium</i> DBS120 (pCRP1::Tn5)	Dr. David Schauer (Schauer and Falkow, 1993)	N/A
<i>Citrobacter rodentium</i> DBS100 <i>ler</i>	Dr. José Luis Puente (Deng et al., 2004)	N/A
<i>Citrobacter rodentium</i> DBS100- <i>lux</i>	Dr. Bruce A. Vallance (Bergstrom et al., 2010)	N/A
<i>Citrobacter rodentium</i> DBS100-GFP	Dr. Bruce A. Vallance (Bergstrom et al., 2010)	N/A
<i>Escherichia coli</i> BL21	New England Biolabs	Cat# C2530H
Chemicals, Peptides, and Recombinant Proteins		
APC-streptavidin	Biolegend	Cat#: 405207
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	Cat#: 11995065
EspA-6xHis-tagged	This paper	N/A
EspB-6xHis-tagged	This paper	N/A
Fluriso (Isoflurane)	VetOne	Cat#: 501017
Gentamicin	Thermo Fisher Scientific	Cat#: 15710064
Imject Alum	Thermo Fisher Scientific	Cat#: 77161
Ni-NTA Agarose	QIAGEN	Cat#: 30210
NP-Ovalbumin	Biosearch Technologies	Cat#: N-5051
Oxytocin	VetOne	Cat#: 501013
pNPP Substrate, Tablets	SouthernBiotech	Cat#: 0201-01
Thyoglicolate Broth	Sigma-Aldrich	Cat# 70157
6xHis-tagged-Intimin-β ₃₈₅	This paper & Sinclair and O'Brien, 2004	N/A
Critical Commercial Assays		
Quick Start Bradford	Bio-Rad	Cat#: 500-0205

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Protein Assay		
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific	Cat#: 32106
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J: WT	The Jackson Laboratory	Cat#: 000664; RRID: IMSR_JAX:000664
Mouse: FcRn-deficient (<i>Fcgr1^{-/-}</i>): B6.129X1- <i>Fcgr1^{m1Dcr}</i>	The Jackson Laboratory	Cat#: 003982; RRID: IMSR_JAX:003982
Mouse: IgA-deficient (<i>Igha1^{-/-}</i>): C.129S7(B6)- <i>Igh-2^{m1Grh}</i>	Dr. Bernard Arulanandam (Harriman et al., 1999)	Cat#: 031020-MU; RRID: MMRRC_031020-MU
Mouse: pIgR-deficient (<i>Pigr1^{-/-}</i>): B6.129P2- <i>Pigr1^{m1Fejo}</i>	Dr. Christiane E. Wobus (Uren et al., 2003).	Cat#: 030988-MU; RRID: MMRRC_030988-MU
Oligonucleotides		
pQE60-EspA-F: GGGTTACCCATGGATACATCAACTATG	This paper	N/A
pQE60-EspA-R: TATCTCGGATCCCTTGGCAATGGGTATTGCTG	This paper	N/A
pQE60-EspB-F: CGAGACCATGGATACTATCGATTATAACAATGC	This paper	N/A
pQE60-EspB-R: AATGTTGGATCCCCCTGCTAAACGAGCCGATTG	This paper	N/A
Recombinant DNA		
Plasmid pQE60	QIAGEN	Cat#: 32903
Plasmid pQE60-EspA	This paper	N/A
Plasmid pQE60-EspB	This paper	N/A
Plasmid pREP4	QIAGEN	N/A
Plasmid p6his-Intb385	Dr. Alison D. O'Brien (Sinclair and O'Brien, 2004)	N/A
Software and Algorithms		
GraphPad Prism version 7	GraphPad Software	https://www.graphpad.com/
Living Image Software	PerkinElmer	http://www.perkinelmer.com/lab-products-and-services/resources/in-vivo