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# Intergenerational protective anti-gut commensal immunoglobulin G originates in early life

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**Maternal immunoglobulins of the class G (IgGs) protect offspring from enteric infection, but when, where, and how these antibodies are physiologically generated and confer protection remains enigmatic. We found that circulating IgGs in adult mice preferentially bind early-life gut commensal bacteria over their own adult gut commensal bacteria. IgG-secreting plasma cells specific for early-life gut bacteria appear in the intestine soon after weaning, where they remain into adulthood. Manipulating exposure to gut bacteria or plasma cell development before, but not after, weaning reduced IgG-secreting plasma cells targeting early-life gut bacteria throughout life. Further, the development of this anti-gut commensal IgG response coincides with the early-life interval in which goblet cell–associated antigen passages (GAPs) are present in the colon. Offspring of dams "perturbed" by B cell ablation or reduced bacterial exposure in early life were more susceptible to enteric pathogen challenge. In contrast to current concepts, protective maternal IgGs targeted translocating gut commensals in the offspring, not the enteric pathogen. These early-life events affecting anti-commensal IgG production have intergenerational effects for protection of the offspring.**

anti-commensal | IgG | neonatal | infection | long-lived plasma cell

## Significance

This work describes the time-limited, early-life, physiologic development of protective maternal anti-gut commensal IgGs. The antibodies persist after weaning and are passed to the next generation. Perturbations during the mother's infancy lead to a loss of protection against infections of enteric origin in their offspring. These long-lasting plasma cells form in the intestinal immune compartment and provide passive protection to the offspring during enteric infections by preventing secondary systemic dissemination of gut commensals.

Passive humoral immunity is a crucial aspect of early-life protection because of immature adaptive immune responses in neonates. Class A and G immunoglobulins (Ig) are major effectors of this protection. Without transferred maternal IgA and IgG, neonatal mice have increased susceptibility to bacterial infections (1) and dysregulated gut mucosal immune and microbiota development (2). In neonatal mice, anti-gut commensal IgAs has been best studied, as it is the predominant Ig in maternal milk and the intestinal lumen (3, 4). In humans, studies of passive immune protection in infancy have focused on IgG responses to specific pathogens and vaccination strategies. However, recent data demonstrate that IgGs specific for gut commensal bacteria belong to the maternal milk repertoire and protect against early-life gut pathogens both luminally and systemically (1).

Humans acquire antibodies passively from placental transfer and, to a lesser extent, breast milk (5). In contrast, mice require constant maternal milk transfer (6) because of the shorter half-life of murine IgGs (7), vs. 14 to 29 d in humans (8). Anti-commensal IgGs are also found in humans (9), but the mechanisms underlying their generation in mice and humans, and how they protect from enteric infection, remain incompletely understood.

Systemic anti-commensal IgG activity is greatly diminished in germ-free mice, consistent with the requirement for gut bacteria to elicit these humoral responses. In conventional mice, the IgG response is complex, because both B1 (peripheral, present in the fetus) and B2 (classic adaptive, largely produced after birth) subsets of B-cells can generate plasma cells that contribute to the systemic Ig pool. While anti-commensal specificities of IgAs and IgGs can overlap, they originate in separate B cell lineages (10), suggesting the existence of independent mechanisms, compartments, and sources of IgA and IgG anti-commensal immunoglobulins.

The route and timing of exposure to gut commensal bacteria that elicit maternal anti-commensal IgG responses remain unclear. In view of the necessity of gut bacteria to be present to induce anti-commensal IgGs, we postulate that anti-commensal IgG-secreting cells and the circulating antibodies they produce originate by exposure to microbial antigens in the gut. Indeed, in adult mice, cells that produce IgGs are found in intestinal immune compartments in low frequency, and in bone marrow in higher frequency (2). Systemic immunization of adults with commensal bacteria induces antigen-specific IgGs that protect offspring against bacterial enteric pathogens (1), but systemic immunization likely does not recapitulate the physiologic events underlying anti-commensal IgGs generation. For example, systemic immunization with commensal bacteria elicits mainly $IgG_1$ in adult mice. High $IgG_1$ reactivity against commensals is mainly observed in immunodeficient mice such

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as Nos2$^{-/-}$ Cybb$^{-/-}$, Myd88$^{-/-}$ Ticam1$^{-/-}$ (11), IgA$^{-/-}$ (12, 13), or mice that experience intestinal breach from infection or inflammation (14). *Akkermansia muciniphila* is one of the few gut commensals under physiologic conditions that induces $IgG_1$ responses (15), but most physiologically generated anti-commensal responses belong to the $IgG_{2b}$ and $IgG_3$ subclasses (2).

In early life, the immune system and gut microbiota co-develop and are co-dependent. The "neonatal window of opportunity" (16) is a choreographed series of events, which includes the delivery to the immune compartment of microbial antigens through goblet cell–associated antigen passages (GAPs) (17). In mice prior to day of life (DOL) 10, colonic GAP formation is inhibited by high levels of epidermal growth factor (EGF) in the breastmilk. Beyond DOL~21, GAPs are inhibited by goblet cell intrinsic sensing of the abundant gut microbiota around the time of weaning and are maintained throughout life in normally housed mice (18). Early-life restricted colonic GAPs offer a unique time-limited opportunity for the developing immune system to sample intestinal commensals in the absence of overt inflammation or perturbation of the gut barrier, thus allowing multiple intestinal immune populations to be imprinted in a microbiota-dependent manner (19). If this opening is delayed or perturbed, immunity is impaired and altered into adulthood. The differing composition of infant vs. adult gut bacterial communities over time, the ability to develop memory phenotypes in early life, and the transient opening of colonic GAPs in infancy, suggests that physiologic anti-commensal IgGs could uniquely originate during this early-life period.

Here, we report that systemic anti-commensal IgGs in adult mice preferentially bind their earlier in life gut commensal bacteria over their current gut bacteria This suggests an interval in early life during which anti-commensal IgG responses are preferentially generated. We also find that the anti-commensal IgG response in the intestine depends on B cell maturation into plasma cells and the presence of colonic GAPs during an early-life period. Physiologic events in later life do not compensate for early-life perturbation by generating anti-infant commensal IgGs. IgGs targeting early-life gut commensals protect future offspring during infection with pathogenic bacteria. In contrast to current concepts, we find that protective maternal IgGs are not necessarily specific for the enteric pathogen, but rather display specificity to gut commensals that translocate during enteric infection. These data indicate that physiologic protective maternal B cell responses to gut microbiota occur during a finite interval in early life, to impact the future generation in mammalian species.

## Results

**Adult Anti-Commensal IgGs Have Preferential Specificity to Early-Life Gut Bacteria.** Mice receive maternal IgGs directed against bacteria of gut origin via the placenta in utero and from breastmilk after birth. These IgGs passively protect their recipient against pathogens before the offspring's adaptive immune system matures (1). Intraperitoneal immunization of adult germ-free mice with adult fixed commensal bacteria before pregnancy induces IgGs that passively protect the offspring (1), but how these anti-commensal antibodies arise physiologically is unclear. Moreover, the gut microbial community composition in early life drastically differs from that in adulthood, with the neonatal microbiota containing a limited set of commensals dominated by Lactobacillaceae (20–22). Thus, how dams, whose gut microbial community differs substantially from that of its offspring, physiologically generate passively protecting antibodies for their infants is unclear. We therefore asked whether anti-commensal IgGs in adults display specificity to early-life gut bacteria using longitudinally collected stools at two somewhat arbitrarily selected time points within the early-life interval, pre-weaning (2-wk-old) mice, weaning (3-wk-old), and adult (10-wk-old) mice (Fig. 1*A*). These samples were grown in aerobic and anaerobic conditions to obtain isolates for microbial flow cytometry (mFLOW) (*SI Appendix*, Fig. S1). Adult serum IgG preferentially bound to early-life gut bacteria over synchronous cognate adult gut microbiota (Fig. 1*B* and *SI Appendix*, Fig. S1*A*). mFLOW signal was absent in samples incubated with Rag$^{-/-}$ serum (B and T cell deficient) or no serum (*SI Appendix*, Fig. S1*B*). IgG binding to outgrowths of stool from 2-wk-old and adult mice was comparable to fresh stools of age-matched Rag$^{-/-}$ mice (*SI Appendix*, Fig. S1*C*), indicating that culture did not affect the overall level of serum IgG binding to commensal bacteria. Commensals from outgrowths showed high overlap with freshly obtained gut microbiota at 2 wk (86%) and lesser overlap at 3 wk and in adulthood (*SI Appendix*, Fig. S2 *A* and *B*) indicating that culture did not substantially change the composition of gut microbes from early-life mice. The relative abundance of the genera did vary between outgrowth and fresh samples (*SI Appendix*, Fig. S2*C*), but fresh bacteria in stool and bacteria in outgrowths had the same average predicted ability to bind IgGs (*SI Appendix*, Fig. S2*D*) as indicated by the genera identified by IgG$^{+}$ sorted bacteria sequencing (*SI Appendix*, Fig. S7). Note that we used outgrowths to obviate concerns about signal from pre-bound IgGs from maternal milk (23) in early-life samples, and theoretical epitope masking by IgAs.

Considering the low diversity of early-life gut microbial community pre-weaning and their low frequency or absence in adulthood we next attempted to establish whether these anti-commensal IgGs develop in early life and persisted in adults by quantifying IgG-antibody secreting cells (ASCs) using ELISpot of total cell isolates from immune compartments. The gut microbiota is dynamic in early life and impacts immune development on multiple levels. Thus, controlling immune exposure to the gut commensal bacteria is complex, and manipulating early-life intestinal commensals could have multiple off-target effects given the central role of the microbiota in normal immune development (16). We therefore complemented these studies with a time-limited pre-weaning exposure of dietary ovalbumin (OVA), which is transferred from mothers to their pups via breastmilk (16, 24) to confirm that IgGs specific for luminal antigens can be generated during early life and persist into adulthood. Early-life exposure to dietary OVA induces long-lasting OVA-specific T regulatory cells (Tregs) originating in the colon, similar to gut bacteria-specific Tregs generated at this time in life (24). Thus, while the events of immune encounter with dietary and commensal antigens may differ, early-life dietary OVA is a tractable model to determine whether time-limited luminal antigen exposure in early life can induce a long-lived IgG response. Adult mice have ASCs in the small intestine, colon (2), bone marrow (25), and spleen (26); we therefore focused on these compartments to determine where gut luminal-antigen-specific IgG-ASCs reside.

Because IgG-producing plasma cells develop several weeks after antigen exposure, we quantified the IgG-secreting cells in these compartments by ELISpot in young adult mice at 4 and 5 wk of age. Total IgG-ASCs were present at appreciable densities at 5 wk of age in the intestinal immune compartment (Fig. 1*C*). Total IgG-ASC counts were highest in the spleen, followed by the small intestine lamina propria, with lower and comparable values in the colon lamina propria and bone marrow (*SI Appendix*, Fig. S3*A*). We next sought to localize where anti-OVA IgG-ASCs arose when OVA was encountered only in a pre-weaning period. Like the total IgG-ASCs, OVA-specific IgG-ASCs were found in all compartments but were significantly enriched in the colonic lamina propria at 5 wk (Fig. 1*D*). To measure IgG responses to gut commensals we prepared sonicates from fecal outgrowths collected pre-weaning, weaning, and fecal pellets at 5 wk of age and performed ELISpots.

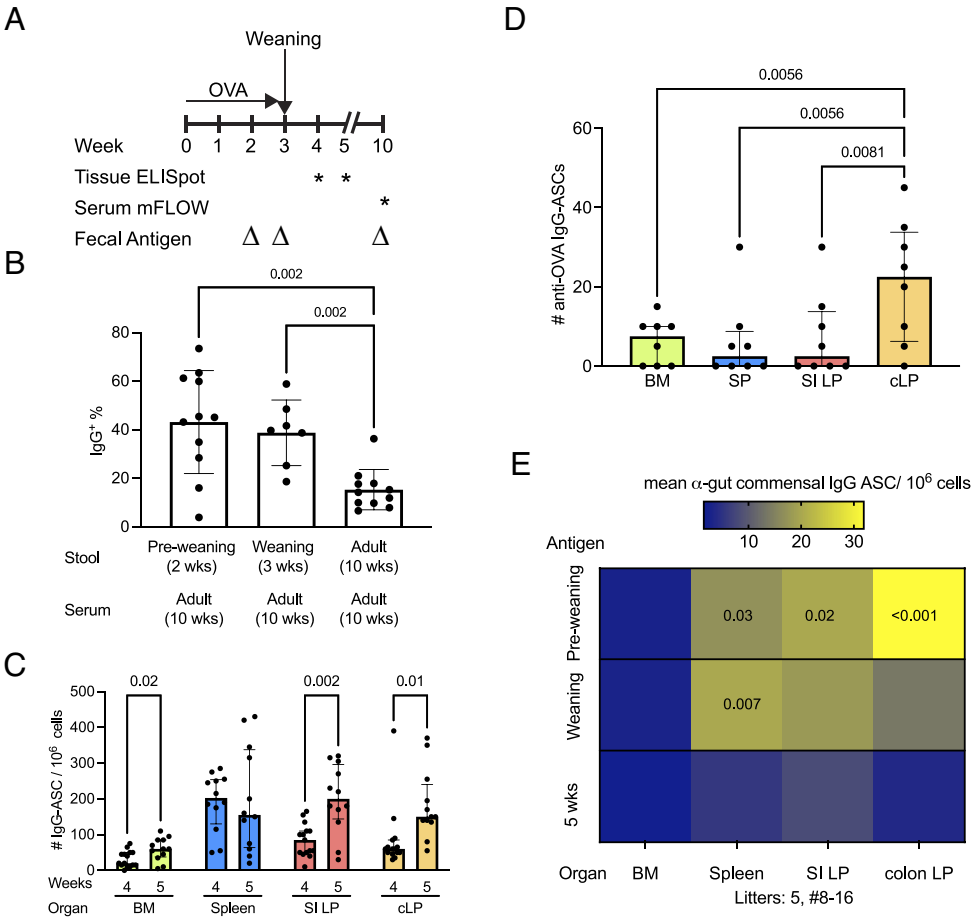


**Fig. 1.** Systemic and tissue-specific anti-commensal IgG-ASCs occurrence over time and organ. (*A*) Schematic of dietary OVA administration and time points for serum (mFLOW), tissue (ELISpot), and gut microbial antigen sampling time points used for detecting anti-commensal/luminal antigen IgG responses. Experiment endpoints are indicated with an asterisk (*) and sampling of gut microbiota for antigen source with a delta (Δ). (*B*) mFLOW of gut commensals incubated with cognate adult serum show significant increased binding to early-life commensals compared to corresponding adult gut commensals. Average with SD from five independent litters compared by mixed effect analysis with multiple comparison correction. Gating strategy and no serum controls shown in *SI Appendix*, Fig. S1 *A* and *B*. (*C*) IgG-ASC detected by ELISpot were significantly different by age ($P < 0.001$) and organ ($P < 0.001$) by mixed-effect analysis and significantly increased in in all organs at 5 wk of age except the spleen by uncorrected Fisher's LSD test. Data from five independent litters. (*D*) OVA specific IgG-ASC appear at 5 wk of age in the colonic lamina propria in mice given dietary OVA from birth to weaning. Data from three independent litters. Mixed-effect analysis between 4 and 5 wk showed a significant effect for time ($P = 0.0047$) with FDR corrected comparison between organs at 5 wk of age. (*E*) Gut commensal specific IgG-ASC at 5 wk of age predominantly recognize the pre-weaning (2 wk) and weaning (3 wk) gut microbiota and are largely found in the colonic lamina propria. Anti-commensal responses at 5 wk significantly differed by organ ($P < 0.001$) and antigen ($P < 0.001$) by two-way ANOVA with Fisher's LSD test for each antigen. Heatmap represents average IgG-ASCs count per unsorted $10^6$ cells per organ detected by ELISpot. Raw data for anti-commensal responses are available in *SI Appendix*, Fig. S3*C*.

Similar to anti-OVA IgG ASCs, commensal-specific IgG-ASCs were predominantly found in the colonic lamina propria at 5 wk of age (Fig. 1*E*). Specifically, pre-weaning commensals elicited the greatest binding of colonic IgG-ASCs, although not significantly greater than weaning commensals, whereas IgG-ASCs demonstrated reduced specificity for synchronous adult microbiota (Fig. 1*E* and *SI Appendix*, Fig. S3*B*). For simplicity in comparing multiple organs and antigen sources simultaneously, we depict the mean frequencies of IgG-ASCs for each condition in heatmaps (Fig. 1*E*) with individual datapoints underlying the heatmap displayed in *SI Appendix*, Fig. S3*C*. Similar to the mFLOW data with systemic IgG (Fig. 1*B*), the reactivity of IgG-ASCs to gut commensals depended on the age at which the gut bacteria were collected. Thus, physiologic anti-commensal IgGs predominantly arise in the colon and are preferentially directed against early-life gut microbiota.

**Physiologic Generation of Anti-Commensal IgG Responses Is Largely Restricted to an Early-Life Period.** Anti-commensal IgG responses to commensals in the adult intestinal tract in humans and mice are mainly observed after intestinal breach from infection or inflammation (14). We determined that anti-commensal IgGs from adults preferentially bound to gut bacterial taxa that are more prevalent in early life. These taxa might have special capacities to elicit IgGs and/or the response might be limited to a specific interval when these taxa are more prevalent. We therefore sought to identify when these anti-commensal IgG responses arise and if this was limited to a specific time in life.

Antibodies are secreted by short-lived plasmablasts and long-lived plasma cells. Plasmablasts are generated at first antigen exposure, or later through re-exposure of memory B-cells to the same antigen (27). Long-lived plasma cells can secrete antibodies and persist for years in the bone marrow and small intestine. To determine whether the generation of anti-commensal IgGs is restricted to a defined period in early life, we depleted all B-cells (including memory B-cells) before or after weaning using αCD20 (28). This effectively removes naïve and memory B-cells but retains plasma cells (Fig. 2*A* schematic and *SI Appendix*, Fig. S4*A*). Specifically, plasma cells, which do not express CD20, that are generated before αCD20 treatment, will remain the only pre-existing source of IgG-ASC immediately after αCD20 depletion (28), which can be complemented by plasmablasts and plasma cells that are newly formed upon recovery from transient depletion. This allows us to determine

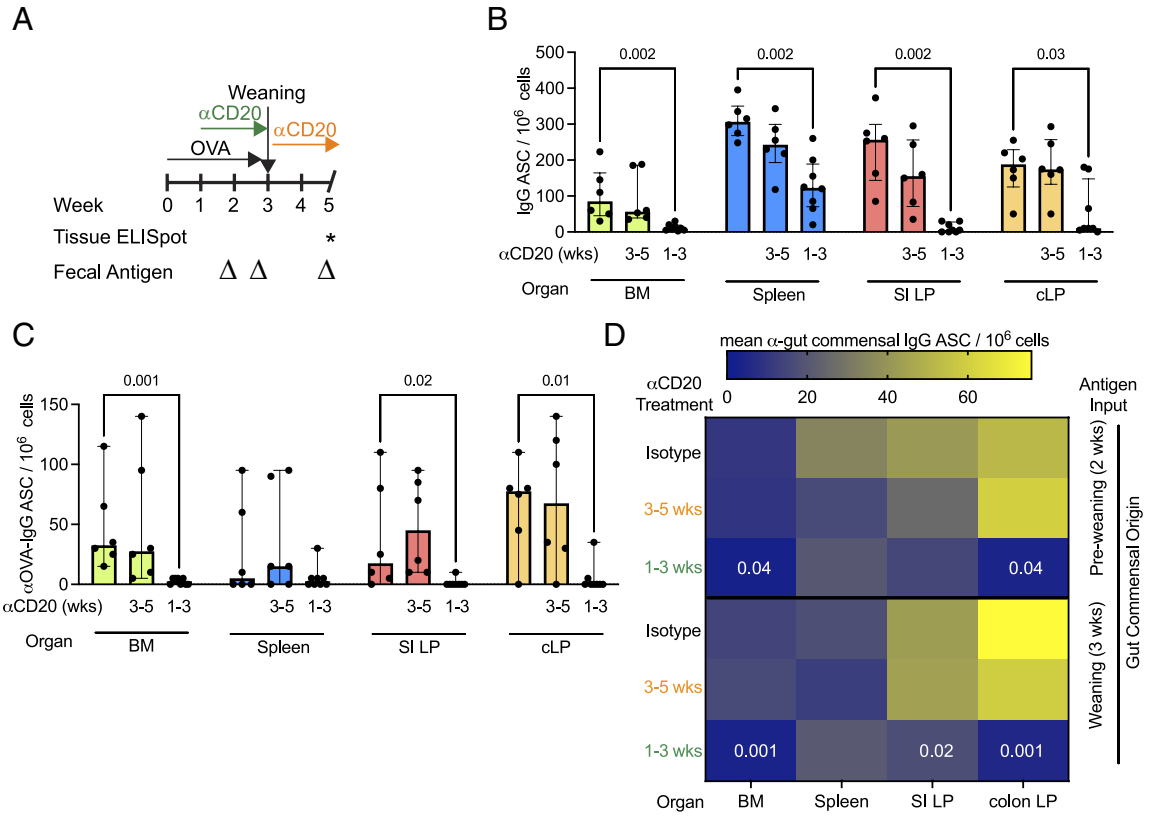


**Fig. 2.** Long-lived anti-commensal IgG plasma cells are generated pre-weaning. (*A*) Schematic of the tissue sampling (ELISpot) and fecal antigen used for detecting anti-commensal IgG responses following B cell depletion (αCD20) pre- or post-weaning. (*B*) Animals with αCD20 B cell depletion post weaning (3 to 5 wk) have the same IgG-ASCs numbers in all organs. Animals with αCD20 B cell depletion pre-weaning (1 to 3 wk) have significantly reduced levels of total IgG-ASCs in all organs. (*C*) Only αCD20 B cell depletion pre-weaning (1 to 3 wk) but not post weaning (3 to 5 wk) causes a loss of OVA-specific IgG responses in the intestinal lamina propria, and bone marrow of mice given dietary OVA as in Fig. 1. (*D*) Animals with αCD20 B cell depletion post-weaning (3 to 5 wk) have unchanged levels of anti-commensal IgG-ASC at 5 wk of age whereas αCD20 B cell depletion pre-weaning (1 to 3 wk) results in a loss of commensal specific IgG responses at 5 wk of age. ELISpot cell count represents average from three independent litters per organ with littermate controls (total #6 to 8). Effect of αCD20 treatments within each organ was determined by FDR corrected multiple Mann–Whitney *U* test for each antigen source. Raw data displaying bar plots with median and interquartile range shown in *SI Appendix*, Fig. S4*B*.

redundancy between plasma cells formed in early life vs. post-weaning in anti-commensal/luminal antigen IgGs responses.

IgG responses were quantified using ELISpots, as opposed to serum IgG responses, to avoid confounding effects from residual endogenous or maternal circulating or tissue IgGs. Transient B cell depletion (αCD20 treatment) post-weaning did not significantly diminish total IgG-ASCs in the small intestinal and colonic lamina propria or spleen (Fig. 2*B*), suggesting that long-lived plasma cells generated pre-weaning represent a substantial proportion of the IgG secreting cells at this age. Animals in which B-cells were depleted before weaning sustained significant depletion of total IgG-ASCs in both intestinal compartments, complete depletion in the bone marrow, and partial depletion in the spleen (Fig. 2*B*), indicating that B cell responses that generate IgG-ASCs in the gut develop mainly in early life. Short or long-term (2 mo) αCD20 treatment in adult immunized mice does not affect pre-developed serum antibody levels as they maintain antigen-specific plasma cells in the bone marrow and spleen (29). We therefore interpreted the lack of IgG-ASC responses after early-life αCD20 treatment, to be mainly due to a lack of formation of long-lived plasma cells. However, we cannot exclude that plasma cells formed in the mucosal compartment in early life have a shorter half-life and might not persist beyond the treatment period.

The lack of IgG-ASCs after pre-weaning B cell depletion prompted us to explore the effects on luminal antigen-specific IgG responses. We again administered dietary OVA to dams and pups from birth to weaning, treated mice with αCD20 before or after weaning and evaluated the presence of anti-OVA specific IgG-ASCs. Indeed, anti-OVA IgG-ASCs were impacted by the pre-weaning αCD20 treatment, but not post-weaning (Fig. 2*C*), indicating exposure to luminal antigen in early life can induce a long-lasting IgG response. Similarly, while colonic early-life anti-commensal IgG-ASCs were significantly diminished by depleting B-cells before weaning, this effect was not seen post-weaning (Fig. 2*D* and *SI Appendix*, Fig. S4*B*). Taken together, these data suggest the existence of an early-life interval during which the host physiologically generates a substantial proportion of memory anti-commensal IgG-secreting plasma cells to luminal antigens, which persist into adulthood.

**Early-Life Anti-Commensal IgG Responses Depend on Colonic Goblet Cell–Associated Antigen Passages.** The above data indicate that the encounter of the immune system with gut commensal bacterial antigens to induce long-lived IgGs primarily occurs in the pre-weaning period. A unique aspect of the early-life intestinal tract is the presence of colonic goblet cell–associated antigen passages (GAPs), which deliver commensal bacteria and dietary antigens to generate antigen-specific T cell responses (19). The timing of colonic GAP opening is tightly controlled by epidermal growth factor (EGF) in maternal breast milk, which inhibits colonic GAPs before day of life (DOL) 10, and the increasing gut microbial load, which inhibits colonic GAPs starting at weaning (DOL 21) and throughout adulthood (30). These events define a period in early life (DOL10 to 21) in which the immune system encounters

antigens from the colonic lumen, while in adulthood luminal antigen sampling in the absence of barrier breach is primarily by microfold cells overlying the follicle-associated epithelium (31), a few GAPs present in the distal colon, and GAPs in the small intestine (18).

Considering the role of colonic GAPs in delivering antigens for specific T cell responses in early life and the temporal correlation of the presence of colonic GAPs in early life with the period in which physiologic anti-commensal IgGs are generated, we sought a potential role for colonic GAPs in the development of these antibodies. Accordingly, we gavaged EGF between DOL10 and 21, which inhibits colonic GAP formation and T cell responses to luminal antigens in the colon (30) (Fig. 3*A* schematic). While the inhibition of colonic GAPs was associated with an expansion of total IgG-ASCs in the intestinal immune compartments (*SI Appendix*, Fig. S5*A*), the antigen-specific IgG response to dietary OVA administered pre-weaning was significantly less in the colon and trended downward in the bone marrow. These findings are consistent with a role for colonic GAPs in the induction of IgG responses to luminal antigens in early life (Fig. 3*B*). This period coincides with the presence of vacuolated fetal enterocytes in the distal small intestine that have the capacity to take up macromolecules (32). However, T cell responses to luminal antigens in the pre-weaning interval are largely restricted to the colon and delivered by colonic GAPs (17). Consistent with a role for colonic GAPs in generating IgG responses to luminal antigens, EGF did not affect the development of vacuolated fetal enterocytes (*SI Appendix*, Fig. S5*C*). Like dietary OVA, blockade of colonic GAPs by EGF treatment reduced the anti-commensal IgG response to early-life microbial antigens in all compartments except the spleen for the pre-weaning, but not to cognate adult bacteria (Fig. 3 *C* and *D*). The incomplete reduction in IgG-ASC responses in the spleen might be due to an incomplete blocking of antigen uptake by EGF or to the relative paucity of anti-pre-weaning commensal IgG-ASCs in the spleen at this time in life. Based on these data, we conclude that colonic GAPs support the development of long-lived IgG responses to early-life commensal antigens and suggest that IgG responses to commensal antigens generated in later life may occur by other mechanisms. Using these strategies to block the development of anti-commensal IgGs, we evaluated whether anti-commensal IgGs formed in early life are required for passive protection of the infant.

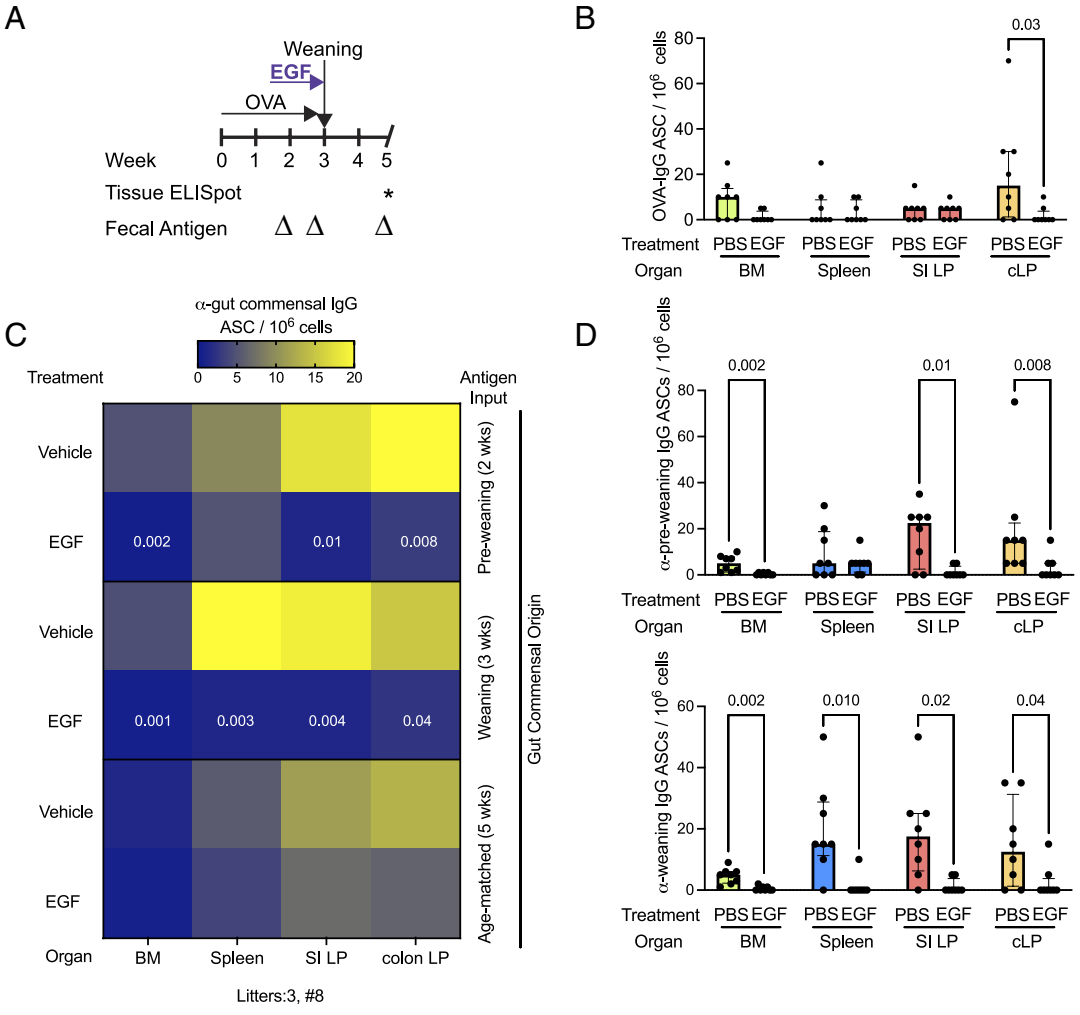


**Fig. 3.** Anti-commensal/luminal antigen IgG-ASC formation is reduced when colonic GAPs are inhibited preweaning. (*A*) Schematic of the tissue sampling (ELISpot) and fecal antigen used for detecting anti-commensal IgG responses of mice receiving EGF from DOL 10 to 21 and OVA. (*B*) OVA-specific IgG-ASC (ELISpot) in the colonic lamina propria were significantly abrogated in 5-wk-old mice that received EGF from DOL 10 to 21. (*C*) Heatmap of average gut commensal specific IgG-ASC (ELISpot) displayed by organ, fecal antigen source, and treatment demonstrating abrogation of anti-commensal IgG responses following EGF treatment from DOL 10 to 21. (*D*) Raw data related to (*C*) demonstrating abrogation of responses to pre-weaning and weaning commensals following EGF treatment from DOL 10 to 21 compared to vehicle control treated animals at 5 wk of age. Response to adult commensals was not impacted in animals exposed to EGF; raw data for responses to fecal antigen from 5-wk-old mice shown in *SI Appendix*, Fig. S5*B*. Data from at least three independent litters with a total of eight mice per treatment and organ represented as median with interquartile range. Significance was assessed by multiple Mann–Whitney *U* test within each organ.

**Disrupting the Development of Early-Life Anti-Commensal IgG Responses in Dams Makes the Offspring More Susceptible to Enteric Infection in Early Life.** Maternal anti-microbial IgGs, delivered trans-placentally and/or via breast milk, protect offspring from enteric and systemic infections in the earliest days and weeks of life (1). After demonstrating above that anti-commensal IgG specific for early-life gut bacteria can be generated by the infant, we next asked whether these antibodies could protect the offspring of these mice by passive transfer to the next generation. Contrary to current concepts that IgGs are transferred from the bloodstream to breast milk (23), breast milk IgG (but not IgA) concentrations were reduced in dams that had been perturbed in early life despite comparable systemic IgG levels (*SI Appendix*, Fig. S6 *A–C*). Interestingly serum reactivity toward early-life commensals was also reduced in dams perturbed in early life (*SI Appendix*, Fig. S6*D*), indicating that systemic total IgG concentrations do not necessarily reflect anti-commensal activity. This could also partially explain the reduced concentrations of IgG in the breast milk of dams perturbed in early life. Based on this information, we raised to adulthood female mice in which EGF and B cell depletion treatments hindered early-life development of anti-commensal IgG responses, mating them with unmanipulated male mice. Then, we exposed their offspring to a previously published enteric infection model (1) in which maternal IgGs are protective (Schematic Fig. 4*A*). This approach allowed us to ask whether anti-commensal IgGs generated in early life, as opposed to those generated later in life, are required for passive protection of the offspring.

Offspring of manipulated and unmanipulated dams were challenged orally with nalidixic acid-resistant enterotoxigenic *Escherichia coli* (ETEC) O138 (nal$^r$ O138) on DOL5-6, which causes small intestinal inflammation and damage. Pups of dams manipulated in early life had greater small intestinal burden of the nal$^r$ O138 ETEC than those born to non-perturbed dams (Fig. 4*B*). Pups from unmanipulated mice had high infective burden shortly after infection but were able to clear it in the small intestine, but not the colon by day 3 compared to pups from manipulated dams (*SI Appendix*, Fig. S6*E*). The ETEC nal$^r$ O138 remained in the small intestine, and rarely disseminated in any of the treatment groups (Fig. 4*B*), which mirrors the clinical presentation of enterotoxigenic *E. coli* infections in humans. Adult wild-type mice did not have ETEC nal$^r$ O138 specific antibodies by mFLOW or anti-labile toxin (LT) antibodies by ELISA, unless they were immunized by intraperitoneal inoculation of heat-killed or sonicated ETEC nal$^r$ O138 (Fig. 4*C* and *SI Appendix*, Fig. S6*G*). Therefore, the difference in intestinal ETEC nal$^r$ O138 burden could not be explained by direct effects of IgGs on the ETEC. More likely, this difference reflects indirect effects of maternal IgG on the early-life intestinal microbiota**.** In addition, IgA binding

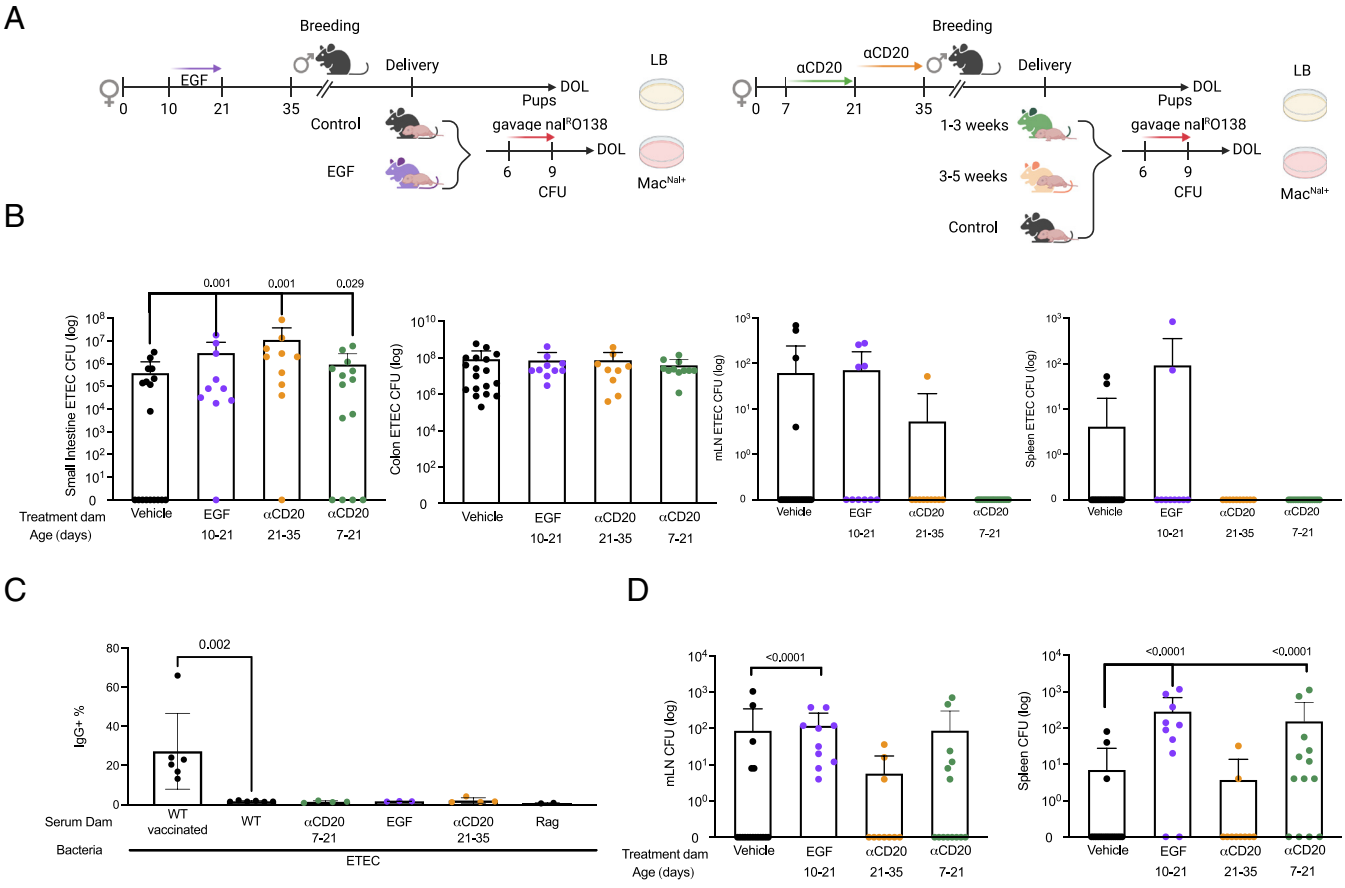


**Fig. 4.** Early-life perturbations of anti-commensal IgG response in dams limits pup ability to control systemic dissemination of commensal bacteria in an ETEC infection model. (*A*) Schematic of the generational effect experiments. Female pups treated with EGF (DOL 10 to 21) to inhibit colonic GAPs, or αCD20 (1 to 3 wk) to deplete B-cells in early life were subsequently bred to control untreated males and their pups exposed to ETEC nal$^R$O138 on DOL 5-6. (*B*) Pups from dams perturbed in early life have increased colonization with ETEC in the small intestine, but not the colon (limit of detection 10 CFU). Systemic dissemination of ETEC was limited in all conditions (limit of detection 4 CFU). (*C*) mFLOW demonstrates only animals vaccinated with heat-inactivated or sonicated ETEC nal$^R$O138 i.p. were reactive to ETEC nal$^R$O138 isolated from the small intestine of neonatal mice. None of the dams independent of treatment had reactivity toward ETEC nal$^R$O138. Rag$^{-/-}$ serum used as a negative control for IgG anti-ETEC nal$^R$O138 binding. Gating strategy is provided in *SI Appendix*, Fig. S6*F*. Mean with SD compared by one-way ANOVA with FDR-corrected multiple comparison. (*D*) Pups from dams perturbed in early life have increased frequency of systemic dissemination of non-ETEC bacteria upon intestinal ETEC infection. Treated animals were compared to vehicle control by the binomial distribution test.

to ETEC was minimal in all treatment groups (*SI Appendix*, Fig. S6*H*). In healthy adults, intestinal damage caused by a pathogen or chronic inflammation can lead to systemic dissemination of intestinal commensals and anti-commensal IgG responses (14). Neonates on the other hand depend on IgGs provided by dams during enteric infection. Indeed, pups reared by dams perturbed in early life experienced extensive non-ETEC dissemination to the spleen compared to pups reared by unperturbed dams (Fig. 4*D*). We observed a significant increase in non-ETEC bacteria in the mesenteric lymph nodes of the pups from dams treated with EGF and a trend toward increased dissemination of commensals in pups from dams treated with αCD20 on DOL7-21 (Fig. 4*D*).

To identify the translocating bacteria and their potential origin, we compared full-length 16S rRNA Sanger sequencing of isolates from the spleen to the early-life commensal bacterial species bound by serum IgGs from unmanipulated adult mice identified by full-length PacBio 16S rRNA sequencing (*SI Appendix*, Fig. S7 *A* and *B* and Dataset S1). We considered a taxon to be IgG bound if it was present in the $IgG^+$ fraction of sorting regardless of its presence or absence in the $IgG^-$ fraction. Of note, we observed some taxa that were present only in the $IgG^+$ fraction or the $IgG^-$ fraction as well as some present in both fractions, which might be related to the abundance of the taxa and antibody in each condition. In each case, the translocating isolates from pups reared by manipulated dams (purple and green leaves of the phylogeny tree; *SI Appendix*, Fig. S7*A*) were closely related or the same species as IgGs bound commensals (gray leaves on the phylogeny tree; *SI Appendix*, Fig. S7*A*). Lack of identical matches between Sanger and PacBio 16srRNA sequencing could be due to multiple copies of the 16sRNA gene being present within a genome that can have single nucleotide polymorphisms (SNPs). This could lead to a Sanger consensus sequence that varies from the individual amplicon sequence variants (ASVs) obtained by PacBio, or increased frequency of sequencing errors introducing spurious SNPs observed in longer reads. However, the majority of splenic isolates mapped to the same species as bacteria bound by adult serum IgG (Dataset S2). As the experiments were performed in a "closed" environment in one in-house bred colony these commensals are most likely not only the same species, but also the same strain. Nonetheless, if the strains minimally differed, the IgGs would likely have specificity for both.

Most splenic isolates belonged to the *Staphylococcus* genus, which has multiple species that cannot be resolved solely by 16SrRNA reads (33). BLASTn of the splenic isolates and $IgG^+$ sorted bacteria 16SrRNA matched both *S. xylosus* and *S. saprophyticus. S. saprophyticus* is rarely found in mice prompting us to hypothesize that the isolates were most likely *S. xylosus*. Species attribution was confirmed by mapping shallow shotgun reads from the early-life intestinal community to a *S. xylosus*–specific genomic region (34) (*SI Appendix*, Fig. S8). We therefore conclude that dams treated with EGF or αCD20 pre-weaning are deficient in anti-commensal IgGs, and that this deficiency enables dissemination of *S. xylosus* following ETEC $nal^r$ O138-induced intestinal injury in their offspring. We would like to note that while IgGs bind to a broad range of genera of early-life commensals, only a subset were identified in spleens during ETEC infection in the absence of these IgGs. It is not clear what the role of these other anti-commensal IgGs might be if the bacteria they target are not able to disseminate systemically after intestinal breach. Nonetheless, our data substantiate the importance of IgGs in dams that had been elicited by gut commensal bacteria in early life in controlling dissemination of gut bacteria in their offspring following pathogen challenge.

## Discussion

Early-life interaction between the host's immune system and intestinal bacteria is a critical process in mammalian biology (16). This interaction occurs during an interval characterized by rapid changes in the content, quantity, and diversity of gut microbial populations and accelerating host adaptive immunity and can confer lasting protection from pathogens (30, 35). However, a major question remains: How does the immune system learn to tolerate gut commensal bacteria while maintaining its ability to recognize and neutralize bacteria that escape the intestinal milieu? Studies have largely focused on the role of acquired (from breast milk) IgA in shaping and controlling intestinal microbial populations in early life (30, 35). IgA binds to commensals and pathogens and can hinder, though in some circumstances facilitate, colonization of the gut (36). ASCs that produce IgAs can be generated before weaning as well as during adulthood (36–39), providing multiple opportunities for this class of antibodies to respond to gut bacterial antigens.

In contrast, we know little about the origin of anti-commensal IgGs, despite data from mice (2) and humans (9) that these antibodies provide important protection from early-life enteric and systemic infections (1). Interestingly these IgGs overlap in specificity with IgAs, but their origin differs, and only bind a subset of gut commensals (2).

Anti-commensal IgGs generated by parenteral immunization of adults protect offspring from enteric infections because of their cross-reactivity to enteric pathogens. Systemic vs. mucosal exposure of commensals might result in responses to different antigens of the same bacteria as well as inducing a predominant $IgG_1$ response. Vujkovic-Cvijin et al. demonstrated that adult mice and humans mount new anti-commensal IgG responses mainly after systemic exposure following intestinal injury or inflammation (14). Increased anti-commensal IgG responses were also observed in IgA deficient humans and mice, which also have intestinal barrier disfunction. While these observations suggest that barrier breach could be a precursor to anti-commensal IgG production in adults, these studies are not exhaustive and other settings and taxa might induce anti-commensal IgG responses in adults. Considering the importance of these immunoglobulins in protecting the offspring during enteric infection, evolutionarily this process would be more likely to be dependent on coordinated physiologic events rather than on serendipitous intestinal breach.

Our data suggest that protective physiological anti-commensal IgGs are largely generated during a defined early-life interval. Protection by maternal anti-commensal IgG is conferred to offspring via reactivity to commensals that translocate during enteric infection. How broadly this paradigm extends to other anti-commensal IgGs, enteric infections, and other settings remains to be determined. In addition, the composition of breastmilk IgG changes during lactation allowing for multiple mechanisms to be at play depending on the infection model used. Our findings are, however, concordant with the original description of anti-commensal IgG activity that shows greater reactivity toward early-life gut commensals incubated with age-matched serum compared to adult serum and cognate microbial community (2). Interestingly, a recent publication describing a pediatric gut community (PedsCom) showed that colonizing adult germ-free dams with this community compared to conventional adult specific pathogen–free (SPF) intestinal content resulted in higher serum $IgG_1$ and $IgG_{2c}$ levels in their offspring (22). These mice were "locked in" with an immature microbiota, but only the IgA binding to commensals appeared to be affected. Continuous presence of early-life commensal in adulthood did not increase $IgG_1$ binding of commensals despite increase in total concentration of this

subtype. These data are concordant with our findings that early-life commensals need to be sensed by the gut immune system at a specific time point to elicit protective anti-commensal IgG responses.

The colonic immune compartment appears to be particularly sensitive to disruptions during the "neonatal window of opportunity," no doubt partly because of the time-limited presence of colonic GAPs (17, 19). These structures support the delivery of luminal antigens to generate antigen-specific adaptive immune responses. The early-life formation of gut commensal bacteria–specific $T_{regs}$ (17) as well the anti-commensal IgG responses shown here are rendered possible by the time-limited presence of colonic GAPs. Interestingly both the $T_{regs}$ (17, 35) and the anti-commensal IgG-ASCs supported by colonic GAPs in early life share the property of being long lived.

The small intestine is considered to be a more dynamic immune compartment than the colon in adulthood. Small intestinal IgG responses to luminal antigens in the adult initially reside locally, though the bone marrow provides the ability to maintain long-term antibody production (25) in SPF mice. In early life, small intestinal enterocytes take up macromolecules by fluid-phase endocytosis (32) and GAPs are closed due to the high levels of EGF in the breast milk (17). EGF has been reported to improve intestinal permeability, by increasing small intestinal occludin and claudin-3 expression (40). Therefore, we cannot exclude that the effects of EGF on luminal antigen exposure are not also mediated by impacting intestinal permeability during early life.

The biomass and richness of the microbial community in the small intestine varies less before and after weaning than does the colon (16). Despite a more consistent microbial community, almost half of small intestinal and bone marrow IgA-ASCs in adulthood are formed in the same early-life interval as shown by time-stamping (41). Both populations react to commensals despite being clonally distinct. Similar to IgA-ASCs, we did find early-life imprinted anti-commensal IgG responses in the small intestine, but to a lesser extent than in the colon. Additional studies focused on the small intestine are required to determine the contribution of these early-life-induced Ig-ASCs in maternal protection, as migration from the small intestine to the mammary gland is well established for IgA-ASCs.

We found that long-lived plasma cells are a reservoir of physiological anti-commensal IgGs that are induced pre-weaning, and that αCD20 depletion post-weaning did not abrogate the anti-commensal IgG-ASCs against early-life commensals. While we cannot exclude the possibility that memory B cells also contribute to the anti-commensal IgG response generated in early life, the post-weaning B cell depletion suggests that long-lived plasma cells are the major source of these IgGs that provide protection to offspring. This finding further indicates that immune memory in the colon generated in early life is maintained by mechanisms different from the more dynamic small intestinal immune responses generated later in life. Surprisingly, we observed that early-life perturbations affected dam's IgG concentrations in the breastmilk, but not in the circulation, challenging the concept that systemic IgG is the sole source for breastmilk IgG and suggesting additional sources of these IgG pools.

Wilmore et al showed the importance of anti-commensal immunoglobulins in protecting against poly-microbial sepsis by demonstrating that adult mice with intestinal injury upregulated IgA responses to prevent bacterial dissemination (42). Protection was due to the presence of specific gut commensals that induce IgA responses as seen by co-housing experiments prior to sepsis. However, neonatal mice are impaired at mounting IgA or IgG responses and these responses could take weeks to mature, so in this system, maternally transferred IgGs are important to provide protection against disseminating bacteria. Contrary to prevailing concepts, we did not find that physiologically generated anti-commensal IgGs reacted to the pathogen. Instead, antibodies present before the pathogen is introduced react to disseminating gut bacteria. Most bacteria disseminating to the neonatal spleen in this model were *S. xylosus,* an organism typically found on the skin of mammals, but which has been isolated from early-life mouse intestines (11, 22). *S. xylosus* is expanded in the intestine of immunosuppressed mice (43) and contains multiple virulence genes (44). Further, adult SPF mice can harbor $IgG_{2b}$ directed against *Staphylococci* (11). Thus, in retrospect, passive protection via IgG responses to *S. xylosus* might be expected.

The intestinal microbial community in early life is less diverse than in later life, and often dominated by few taxa (20–22), thereby limiting the opportunistic pathogen pool compared to an adult setting. We were compelled to use bacterial outgrowths from early-life interval, which could potentially exclude some bacteria with high reactivity. However, comparison of outgrowth to fresh microbial communities showed >80% overlap at the 2-wk time point (*SI Appendix*, Fig. S2). In addition, all splenic isolates identified clustered with IgG-bound commensals from outgrowths (*SI Appendix*, Fig. S7). Our data suggest that disruption of IgG production in the early life of the dam reduces immunocompetency in the subsequent generation of neonates by depriving them of maternally transferred anti-commensal IgGs preventing opportunistic dissemination by *S. xylosus.* Because gut bacteria often cause human neonatal sepsis (45), maternally derived IgGs likely play a similar role after intestinal barrier breach in neonatal humans.

In summary, early life is an interval during which mammals assemble a crucial repertoire of anti-commensal IgGs and simultaneously assemble gut microbial communities. We find that these antibodies persist into adulthood, even though the immunizing bacteria are either transient or become profoundly less abundant members of the gut microbiota in later life. While anti-commensal IgGs can be generated in adulthood by mucosal injury or intraperitoneal immunization (15), such processes are not representative of physiologic mechanisms. Our data suggest that these early-life-generated immunoglobulins protect the next generation from early-life bloodstream infections from gut residing bacteria. This concept of inherited protection has two important theoretical and practical implications. First, our findings introduce the concept that vertically transmitted passive immunity carries with it precision protection for the newborn, based on the mother's exposure during her infancy. Second, practices that disrupt microbial antigen exposures in infancy, such as antibiotics, might attenuate the immune response to early-life gut commensals, but the consequences of altering the immune repertoire in the infant might not be apparent until the offspring of that infant are exposed to intestinal pathobionts among the commensal gut bacterial communities. Future studies will be needed to determine the capacity of a mother's IgG to offer intergenerational protection in these settings, particularly if this process applies to humans.

## Materials and Methods

**Study Design.** All mouse experiments were approved by the Washington University School of Medicine in St. Louis Institutional Animal Care and Use Committee. Mice were housed at temperatures ranging from 19 to 24 °C and humidity ranging from 30 to 70% with a 12 h light/dark cycle. No mice underwent other procedures before experiments were performed. Both male and female mice were used in these studies. Mice were fed a standard chow diet (Cat: 5058) and received water through an automated watering system, except when exposed to ovalbumin (Sigma, A5253) water. Ovalbumin water was changed twice a week. C57BL/6 (JAX:000664) mice were bred in house for all experiments. Serum from cheek bleeds from B6.129S6-$Rag2^{tm1Fwa}$ (TAC:RAGN12) was used for mFLOW controls.

**Early-Life Perturbations.** At birth, dams were placed on ovalbumin water (10 g/L) until weaning at 21 d of life. At 10 d of life, animals received EGF (Shenandoah, 200-53) by feeding (1 μg/10 μL) daily in PBS until weaning (17). For αCD20 (Invivogen, mcd20-mab10-10) treatment, animals were injected intraperitoneally weekly with 10 mg/kg body weight of αCD20, or vehicle control either from 7 to 21 d or 21 to 35 d (28). Littermates were separated by gender until used for breeding, ELISpot, or mFLOW analysis was performed at ages indicated in the result section. Stool collection occurred at 2 and 3 wk of age and one day prior to end point. Collected stool was kept at −20 °C until needed.

**Elispot.** ELISpots were performed as previously described (46) with adaptations for microbial antigens. Commensal antigens were obtained for plate outgrowth on LB with agar (BD, 244610, 214010) (aerobic) and BHI (Sigma, 53286) (anaerobic) of 2- and 3-wk-old cognate microbiota to permit sufficient microbial content. Plate outgrowths were resuspended in molecular grade water. Adult bacteria were directly obtained from fresh stool. Fresh stool was resuspended in 5 mM N-acetyl cysteine (Sigma, A7250) and debris was left to settle. Supernatant was centrifuged for 3 min at 100 g to remove further debris. Supernatant was centrifuged (3 min, 3,000 g) to pellet bacteria. The pellet was resuspended in water and all bacterial slurries were sonicated for 10 min prior to coating of ELISpot plates. For OVA, we coated the plates with 100 ng per well of albumin from chicken egg white (lyophilized powder, ≥98% (agarose gel electrophoresis) from Sigma-Aldrich (AA5503). Organs were collected and processed as described (35) at 4 or 5 wk. Plated cells were treated as previously described (46). Plates were imaged with CTL-Immunospot S6 Universal Analyzer and counted with spot counter in Fiji v2.3.0/1.53f. Each ELISpot was performed on four animals at a time and repeated to include at least three independent litters for each treatment condition. For EGF-treated animals, same-age litters were used as controls as littermates could not be used due to the oral treatment of the pups.

**Flow Cytometry.** B cell depletion was confirmed by staining isolated cells from all four compartments with CD19-PerCp-Cy5.5, B220-AF780, and Dump (CD4-v450, CD8-BV421, F4/80-e450, Ter119-v450) for 20 min on ice in FACS buffer [0.1% BSA (Sigma-Aldrich, A-3059), 1 mg/mL Human Ig (Innovative Research Inc, IR-HU-GF-818)]. Cells were washed with cold PBS and acquired on AttuneNxT. Data were analyzed with FlowJo v10 (RRID:SCR_008520). All antibodies used are available in *SI Appendix*, Table S1.

**Immunofluorescence.** At 10 d of life, animals received EGF (Shenandoah, 200-53) by feeding (1 μg/10 μL) daily in PBS until DOL14 (17). At DOL 14, animals were anesthetized with sub-cutaneous ketamine and the abdominal cavity was opened to inject the small intestine with 10-kDa tetramethylrhodamine-dextran (Invitrogen, D1868) (10 mg/mL). After 45 min, animals were killed and intestines were fixed with 10% buffered formalin overnight. Tissues were placed in 30% sucrose overnight prior to OCT (4585 Fisher Scientific) embedding and cryosectioning at 6 μm. Sections were stained with DAPI and mounted with permanent VectaMount (H-5000, Vector) prior to imaging with Keyence Fluorescence Microscope BZ-X800 at 20×.

**mFLOW.** The mFLOW protocol was adapted from ref. 47. Bacteria were incubated with PBS and 10% goat serum (Southern Biotech, 0060-01) for 10 min on ice. Bacteria were centrifuged (6,000 g, 3 min) and washed in FACS buffer (1% BSA in PBS). Bacteria were centrifuged and resuspended in FACS buffer and incubated with mouse serum at 1:50 for 30 min on ice. Bacteria were centrifuged again and washed with FACS buffer prior to incubation with 1:200 IgG-PE or IgA-APC in FACS Buffer for 30 min on ice. Bacteria were washed twice with FACS Buffer and resuspended in 200 μL of PBS with DAPI (Roche, 10236276001) 1:1,000 or SYTO BC 1:10,000 (Invitrogen, S34855). Bacteria were acquired on the AttuneNxT (Thermo Fisher), and data were analyzed in FlowJo by comparing to no serum and Rag serum control mice. Samples were from three independent litters and microbiota was matched to the corresponding serum from the same animal. Gating strategy is provided in *SI Appendix*, Fig. S1*A* for commensals and *SI Appendix*, Fig. S6*F* for ETEC IgG and IgA responses.

**Etec Infection.** Ten milliliter of an overnight culture of enterotoxigenic *E. coli* O138 (ST-LT, Tarr group) was plated on MacConkey agar (BD, 212123) plates containing nalidixic acid (8 mg/L) (Sigma, N4382-25G) to select a spontaneously resistant colony. This nalidixic acid-resistant isolate ($nal^r$ O138) was grown overnight in LB media with nalidixic acid (8 mg/L) and stored at −80 °C in 15% glycerol. For challenges, we inoculated from frozen stock an overnight culture of $nal^R$ O138, and on the morning of the experiments, we inoculated 1:100 in LB with nalidixic acid (8 mg/L) to achieve exponential growth phase, at an $OD_{600}$ of 0.5 to 0.7 (Nanodrop, Thermo Scientific). Bacteria were centrifuged (3,000 g, 2 min) and resuspended at 2 $10^8$/mL in PBS. Mice were gavaged with 50 μL of bacterial slurry with a PICU line catheter (PICC-NATE 1.9 FRENCH REF Utah Medical Products, 1180968). The outgrowth was streaked on MacConkey nalidixic acid (8 mg/L) plates to confirm the identity of the inoculum. Mice were monitored for three days and then killed for organ collection. Experiments were repeated with at least three independent litters for each treatment condition. Spleen, mesenteric lymph nodes, colon, and distal small intestine (1 cm) were collected aseptically and homogenized with Bullet Blender (Next Advance) in 200 μL of LB with sterile ceramic beads for 2 min. Homogenates were plated on MacConkey nalidixic (8 mg/L) and LB agar plates, with serial dilutions for intestinal homogenates. The colony forming units of ETEC, and total commensals were determined, and if present, splenic colonies were restreaked on the same conditions for full-length 16S sequencing. 16SrRNA gene was amplified from splenic isolates with the 7F (AGAGTTTGATYMTGGCTCAG) and 1510R (ACGGYTACCTTGTTACGACTT) primers with a KAPA polymerase (Roche, 07958935001). The sequences were cleaned with a PCR column clean-up (Roche, 04983055001) and sent out for Sanger sequencing at Azenta. Serum and stomach contents were collected to quantify maternal IgG.

**Elisa.** In-house ELISA was performed as previously described (46). Stomach contents of pups were resuspended in PBS at 100 μL per 10 mg of content. Stomach content was serially diluted starting at 1:100, 1:500 for IgG or IgA quantification in diluent buffer (1% BSA in wash buffer). For serum from dams or pups, samples were diluted 1:500 to 1:20,000. Plates were detected at 405 nm with FLOUStar Omega Plate (BMG BioTech) reader. Raw values were exported from Omega Analysis (BMG Labtech) and analyzed with GainData (Arigo Biolaboratories). Values were compared to the IgG (Southern Biotech, Cat# 0102-01) and IgA (Southern Biotech, 0106-01) standard curve. Serum and stomach contents from at least three independent litters or dams per experimental condition were assayed. For anti-LT ELISA, plates were coated with Ganglioside GM1 1 μg/mL in carbonate buffer [ 15 mM $Na_2CO_3$ (Sigma-Aldrich S5761), 35 mM $NaHCO_3$ (Sigma-Aldrich S7795), pH 9.6] over-night then washed three times with PBS 0.2% Tween-20 (Fisher Scientific BP337), blocked with 1% BSA for 1 h at 37 °C, incubated with purified LT 1 μg/mL for 1 h at 37 °C, washed three times with PBST, incubated for 1 h at 37 °C with diluted serum (1:500 to 1:10,000), washed five times, incubated with IgG-HRP (Jackson Immuno Laboratories) for 45 min at 37 °C, washed five times and detected with TMB substrate (R & D) and reaction blocked with 2N sulfuric acid. $OD_{450}$ was measured with FLOUStar Omega Plate (BMG BioTech) reader.

**Sequencing.** Bacteria from day of life 14 stool were incubated with serum from corresponding dam and stained as described in the mFLOW section. Upon gating for bacteria based on SYTO $BC^+$ events, an IgG gate was defined based on our negative control of bacteria incubated with $Rag^{-/-}$ serum (*SI Appendix*, Fig. S7*B*). Once the gates were established for each litter, we sorted $10^6$ $IgG^+$ and $IgG^-$ events with a FacsAriaII (BD Sciences) into phenol:chloroform:isopropanol (Invitrogen, 15593-01). IgG+ and $IgG^-$ negative events were further processed for full-length 16S gene sequencing using PacBio long reads. For analysis, we considered ASV as $IgG^+$ if falling into the $IgG^+$ gate regardless of their presence in the $IgG^-$ fraction. The experiment was performed with four independent litters. For outgrowth to fresh microbial content comparison, three independent litters with two animals each and four independent adult mice were selected. DNA was extracted the same way. After DNA extraction, phenol:chloroform:isopropanol salts were removed by the MagMax Microbiome Ultra Kit (Qiagen, A42354). The DNA quantity and quality was assessed with a nanodrop and Qubit high sensitivity dsDNA kit (Thermo Fisher, Q32851). The 16S gene was amplified with a KAPA polymerase (Roche, 07958935001), cleaned with AmpPure XP beads (Beckman Coulter, A63880) following the PacBio full-length 16SrRNA sequencing protocol with barcoded primers as provided in the manual. Barcoded samples were cleaned and pooled for library preparation and sequenced on a Sequel I PacBio sequencer (Azenta). For V4 16S amplification of outgrowth and fresh bacteria the Caporaso primers

515f/806r with custom barcodes were used. Pooled samples were gel-purified and sequenced on a MiSeq (Illumina). All software requiring *R* or command line used to analyze data was uploaded as precompiled Docker images on the university computer server cluster. Samples were demultiplexed and analyzed in *R* with the dada2 v1.22.0 (48) package for long read data in RStudio for PacBio and regular version for V4 16S. Taxonomic assignment and data visualization was performed in *R* v4.0.3 with phyloseq package v1.38.0 (49). Consensus sequence of Sanger sequenced splenic isolates was aligned with MAFFT v7.475 (50) to IgG bound bacteria 16S and reference genome 16S sequences and trimmed to overlapping region. Duplicated sequences within the reference and ASVs were removed with CD-HIT v4.8.1 (51). Remaining aligned sequences were analyzed with modeltest-ng v0.1.6 (52) to select appropriate evolutionary model. The GTR+I + G4 model was used with the –all options in RAxML-ng v. 0.9.0 to create a bootstrapped maximum likelihood tree (53). Tree was visualized and further annotated with Evolgenius V3 (54). Species assignment was confirmed with shallow shotgun sequencing obtained from early-life intestinal commensals. DNA was extracted as described above for $IgG^+$ bacteria and library preparation and sequencing were performed as previously described (55). The resulting demultiplexed reads were analyzed with METAPHLAN v3 (56) and based on taxonomic classification reads were mapped with Bowtie2 v2.4.1 (57) to reference genome of *Staphylococcus xylosus* to determine coverage. Coverage of the *S. xylosus*–specific region (34) was visualized with IGV v2.12.3. For comparison of outgrowth vs. fresh samples were merged by condition and collapsed to the genus level. Data were visualized with *R* package VennDiagram (58) and ggtree (59).

**Quantification and Statistical Analysis.** The number of animals and litters used for each condition is provided in each figure together with statistical tests used to infer significance. Nonparametric statistical approaches were used throughout the manuscript and false discovery rate correction (Benjamini Hochberg) was applied when appropriate. Heatmaps depict average cell count for ELISpots and bar graphs describe mean with SD with individual datapoints. Graphing and statistical analysis for all data except sequencing was performed in GraphPad Prism v10.0.2 (RRID:SCR_002798). Flow cytometry data were gated in FlowJo on DAPI or SYTO BC positive events followed by gating on IgG positive events to obtain percent of bacteria bound to IgG. Bacteria not incubated with serum and $Rag2^{-/-}$ serum controls were used to define gates. A binomial distribution test for ETEC infection model was performed in Excel. All *P*-values are two-tailed.

**Data, Materials, and Software Availability.** All study data are included in the article and/or supporting information.

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