Murine Neonates Infected with Yersinia enterocolitica Develop Rapid and Robust Proinflammatory Responses in Intestinal Lymphoid Tissues

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Neonatal animals are generally very susceptible to infection with bacterial pathogens. However, we recently reported that neonatal mice are highly resistant to orogastric infection with Yersinia enterocolitica. Here, we show that proinflammatory responses greatly exceeding those in adults arise very rapidly in the mesenteric lymph nodes (MLN) of neonates. High-level induction of proinflammatory gene expression occurred in the neonatal MLN as early as 18 h postinfection. Marked innate phagocyte recruitment was subsequently detected at 24 h postinfection. Enzyme-linked immunosorbent spot assay (ELISPOT) analyses indicated that enhanced inflammation in neonatal MLN is contributed to, in part, by an increased frequency of proinflammatory cytokine-secreting cells. Moreover, both CD11b+ and CD11b− cell populations appeared to play a role in proinflammatory gene expression. The level of inflammation in neonatal MLN was also dependent on key bacterial components. Y. enterocolitica lacking the virulence plasmid failed to induce innate phagocyte recruitment. In contrast, tumor necrosis factor alpha (TNF-α) protein expression and neutrophil recruitment were strikingly higher in neonatal MLN after infection with a yopP-deficient strain than with wild-type Y. enterocolitica, whereas only modest increases occurred in adults. This hyperinflammatory response was associated with greater colonization of the spleen and higher mortality in neonates, while there was no difference in mortality among adults. This model highlights the dynamic levels of inflammation in the intestinal lymphoid tissues and reveals the protective (wild-type strain) versus harmful (yopP-deficient strain) consequences of inflammation in neonates. Moreover, these results reveal that the neonatal intestinal lymphoid tissues have great potential to rapidly mobilize innate components in response to infection with bacterial enteropathogens.
sion of proinflammatory gene expression compared to that of adults (20). This is associated with neonate-specific gastritis, higher viral loads, and delayed viral clearance in neonates (19, 21). These studies have provided information on the relationship between inflammation and heightened sensitivity to infection in neonates. However, relatively little is known about intestinal inflammation in a setting of protection against infection in neonates. Recently, we have discovered that 7-day-old neonatal mice are as resistant to the Gram-negative enteropathogen Yersinia enterocolitica as adults (22, 23). Notably, infected neonates mobilize high frequencies of innate phagocytes which are essential for protection against peripheral dissemination of the bacteria. Thus, this system provides a unique model for studying protective innate immunity elicited by a gastric pathogen in early life.

In this report, we compare early inflammatory processes in neonates and adults orogastrically infected with Y. enterocolitica. We found that recruitment of innate phagocytes to the mesenteric lymph nodes (MLN) of neonates was extremely rapid, occurring between 18 and 24 h postinfection with Y. enterocolitica. This rapid and robust inflammation met or exceeded that of adults at all time points examined. Accordingly, increased proinflammatory gene expression occurred in the MLN of infected neonates prior to inflammatory cell infiltration, providing a possible mechanism for rapid and enhanced innate phagocyte recruitment in neonates. This enhanced response appears to be at least partially due to higher proportions of responding cells, since greater frequencies of neonatal MLN cells than adult MLN cells produced interleukin 6 (IL-6) protein after stimulation with lipopolysaccharide (LPS) in vitro. Interestingly, experiments with bacterial mutants revealed that the neonatal MLN has an even greater capacity for innate inflammation. Infection of neonates with the Y. enterocoliticaΔyopP strain elicited substantially enhanced tumor necrosis factor alpha (TNF-α) protein expression in the MLN and greater recruitment of innate phagocytes than infection with wild-type Y. enterocolitica. The hyperinflammatory response to the Y. enterocoliticaΔyopP strain in neonates was associated with greater dissemination of the bacteria to systemic organs and increased mortality. In contrast, infection of neonates with a strain cured of the virulence plasmid encoding the type 3 secretion system (TSSS) and anti-host effector Yops did not induce significant recruitment of innate phagocytes to the MLN. Hence, all together, we have three unique models in which (i) neonates mount a moderate, protective inflammatory response to an intestinal pathogen (wild-type strain), (ii) neonates mount a hyperinflammatory, pathogenic inflammatory response to an intestinal pathogen (ΔyopP strain), and (iii) neonates clear an attenuated intestinal pathogen in the absence of inflammation (virulence plasmid cured). These unique models reveal that the neonatal intestinal lymphoid tissues maintain a high degree of plasticity in inflammatory responses to microbial pathogens.

MATERIALS AND METHODS

Bacterial strains. Wild-type high-virulence Y. enterocolitica A127/90 serotype 0:8 biotype 1B (wild-type Y. enterocolitica), Y. enterocolitica A127/90 serotype 0:8 biotype 1BΔyopP (Y. enterocolitica ΔyopP strain) (both provided by G. R. Cornelis, Universität Basel, Basel, Switzerland), and plasmid-cured A127/90 serotype 0:8 biotype 1B were used in this study. To cure wild-type Y. enterocolitica of the virulence plasmid, wild-type Y. enterocolitica was subjected to multiple rounds of selection on magnesium oxalate agar plates (24).

Mice. C57BL/6 and B cell-deficient mice (μMT, B6.1292-Igh-6tm1lcnj) (The Jackson Laboratory, Bar Harbor, ME) were purchased from The Jackson Laboratory, CD4−/− mice were generously provided by R. Levy (University of Miami Miller School of Medicine, Miami, FL). MyD88−/−, TLR4−/−, and TLR9−/− mice were generously provided by M. Abreu (University of Miami Miller School of Medicine, Miami, FL). All mice were bred and housed under barrier conditions in the Division of Veterinary Resources of the University of Miami Miller School of Medicine. Mice were regularly screened for specific common pathogens. Adult mice (6 to 10 weeks of age) and neonatal mice (7 days of age) were used in experiments. All mice were handled in compliance with the Institutional Animal Care and Use Committee (IACUC) of University of Miami Miller School of Medicine, Miami, FL.

Animal infections. Bacterial frozen stocks (22) were washed twice with Hank’s balanced salt solution (HBSS; Gibco, Grand Island, NY) and diluted to the desired concentration with 0.1% blue food coloring (McCormick, Baltimore, MD). Neonates and adults were inoculated with 5 × 107 CFU, unless otherwise indicated. Adults were inoculated orogastrically with a 22-gauge, round-tipped feeding needle (Fine Science Tools, Foster City, CA) attached to a 1-ml syringe (Becton, Dickinson, Franklin Lakes, NJ). Neonates were inoculated orogastrically with PE-10 tubing (polyethylene tubing with an outside diameter of 0.61 mm; Clay Adams, Sparks, MD) attached to a 30-gauge needle and Hamilton syringe (19). The actual administered dose was determined by plating serial dilutions of the suspensions on Luria broth (LB) plates and incubating for 48 h at 27°C.

Bacterial enumeration from organs of infected mice. To measure Y. enterocolitica titers in the entire small intestine with contents, the small intestine was excised from pyleor to cecum and placed into HBSS. Tissues were weighed and homogenized in 4 to 6 ml HBSS using a Seward Biomaster 80 stomacher (Brinkman, Westbury, NY) for 4 min at high speed. To measure small intestine mucosa-associated Y. enterocolitica titers, the small intestine contents were flushed with HBSS prior to homogenization. Individual mesenteric lymph nodes were homogenized in 400 μl (neonates) or 500 μl (adults) of HBSS using a VWR disposable pellet mixer with a cordless motor (VWR International). Y. enterocolitica titers were enumerated by plating dilutions of homogenates on Difco Versinia selective agar base plates (Becton, Dickinson, Sparks, MD).

Cell staining, antibodies, and flow cytometry analysis. Individual MLN from infected neonates and adults, and Peyer’s patches (PP) from infected adults, were harvested and placed in cold HBSS containing 1% calf serum (Gibco), 10 mM HEPES (Gibco), and 4 mM sodium azide. Age-matched uninfected animals were used as controls. Cell suspensions were prepared by mincing tissues with scissors and pressing cells through wire mesh with a 74-μm pore size (Compass Wire, Westville, NJ). Cell types were distinguished by incubating in mouse Fc block (CD16/CD32; BD Pharmingen, San Diego, CA) for 5 min on ice, followed by a 30-min incubation with phycoerythrin-conjugated anti-CD11b (BD Pharmingen) and fluorescein isothiocyanate-conjugated anti-Ly6G (BD Pharmingen) and fluorescein isothiocyanate-conjugated anti-Ly6G (BD Pharmingen). Samples were run on a Becton, Dickinson LSR II flow cytometer and analyzed with FlowJo flow cytometry analysis software.

Wright-Giemsa staining of Ly6G−CD11b− neutrophils. Ly6G−CD11b− cells were sorted from pools of MLN from neonates 48 h postinfection with the Y. enterocolitica ΔyopP strain by using a Becton, Dickinson FACSAria. Sorted Ly6G−CD11b− cells were spun onto positively charged slides (VWR, West Chester, PA) by using a cytocentrifuge. Slides were allowed to dry and were fixed in 100% methanol. Slides were allowed to dry and submerged in Wright stain (Sigma, St. Louis, MO) for 4 min, followed by Sorenson buffer (0.15 M Na2PO4, 0.15 M KH2PO4, pH 6.5) for 4 min. Slides were then stained with Giemsa stain (Sigma) for 6 min and washed with distilled water. Images were taken with a Leitz Laborlux S microscope at ×63 magnification.

Real-time RT-PCR. Individual neonatal and adult MLN were harvested 18 h postinfection, flash frozen, and stored at −80°C until extraction of RNA. Homogenization and total RNA extraction from individual
MLN were performed with a VWR disposable pellet mixer in conjunction with the RNeasy microkit (Qiagen, Valencia, CA). cDNA was synthesized using the Omniscript reverse transcription (RT) kit (Qiagen) with added oligo(dT)16 primers (Applied Biosystems). Real-time PCR was performed on an Applied Biosystems 7300 real-time PCR system using TaqMan gene expression assay primer/probe sets (Applied Biosystems) for mouse Il6 (assay identifier [ID] Mm00446190_m1), Tnfa (assay ID Mm00443258_m1), Cxcl1 (assay ID Mm00433859_m1), Il17a (assay ID Mm00439619_m1), Ifig (assay ID Mm00817786_m1), and Il10 (assay ID Mm00439614_m1). Mouse gapdh was used as an endogenous control (catalog no. 4352923E; Life Technologies). Data were analyzed using the threshold cycle (ΔΔCt) method.

CD11b^+ cell depletion. Single-cell suspensions of pools of MLN from 10 to 14 neonates 18 h postinfection were prepared as described above in “Cell staining, antibodies, and flow cytometry analysis.” CD11b^+ and CD11b^- cells were fractionated using magnetically activated cell sorting (MACS) separation (Miltenyi Biotec). Aliquots of each fraction were stained with CD11b-PE (Pharmingen) and analyzed by flow cytometry to confirm depletion. The remaining cells were stored at −80°C until RNA extraction and real-time RT-PCR as described above in “Real-time RT-PCR.”

IL-6 ELISA. Protein homogenates from pools of neonatal MLN and individual adult MLN 24 h postinfection were prepared as a VWR disposable pellet mixer and lysis buffer (20 mm Tris-HCl [pH 8], 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.25% deoxycholate, 1% NP-40, 1X protease inhibitor cocktail [Roche]). Pools of at least three neonatal MLN were homogenized in 250 µl, and 1 or 2 adult MLN were homogenized in 400 µl. Protein quantification was determined by QuantiPro bicinchoninic acid (BCA) assay (Sigma). Enzyme-linked immunosorbent assay (ELISA) was performed as previously described in reference 23 with the following modifications. Briefly, Costar 96-well high-binding polystyrene plates (Corning Inc., Corning, NY) were coated overnight at 4°C with 0.05 µg per well of purified anti-IL-6 antibody (BD Pharmingen). Wells were loaded with 50 µl of MLN homogenate or IL-6 standard (R&D Systems) and incubated overnight at 4°C. After wells were washed, 0.05 µg per well of biotinylated anti-IL-6 antibody (BD Pharmingen) was used as the detecting antibody. A total of 100 µl of a 1:2,000 dilution of peroxi-dase-conjugated streptavidin (Jackson Immunoresearch) was added to each well and incubated for 30 min at room temperature. After the wells were washed three times with wash buffer, 100 µl 3',5',3',5'-tetramethylbenzidine (TMB) substrate solution (Invitrogen) was added and incubated 3 to 5 min before the reaction was stopped with 0.1 M sulfuric acid.

TNF-α immunofluorescence. Neonatal and adult MLN were extracted uninfected and 48 h after infection with 2 × 10^7 CFU wild-type Y. enterocolitica and the Y. enterocolitica ΔTnfα strain and plated in 4% paraformaldehyde in phosphate-buffered saline (PBS; Gibco) for 24 h at room temperature. Tissues were washed twice for 15 min in PBS, transferred to 20% sucrose in PBS, and incubated for 24 h at 4°C. Tissues were then flash frozen in cryomolds (Tissue-Tek, Torrance, CA) with tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) and stored at −80°C. Sections of 10 µm were cut using a Leica CM 1900 cryostat and applied to VWR Superfrost Plus microslides (VWR). Sections were washed with PBS and permeabilized with PBS containing 0.4% Triton X-100. Sections were blocked with 5% normal goat serum in PBS containing 0.4% Triton X-100 for 1 h at room temperature and incubated overnight at 4°C in a humidifying chamber with rabbit polyclonal anti-mouse TNF-α (Santa Cruz Biotechnology), diluted 1:50 in PBS containing 5% normal goat serum and 0.1% Triton X-100. Sections were washed and incubated for 1 h at room temperature with goat anti-rabbit Alexa Flour 488 (Invitrogen), diluted 1:750 in PBS containing 0.1% Triton X-100. Sections were then washed and incubated at room temperature for 10 min with Hoechst stain (Roche). Sections were washed and mounted with a coverslip using Gel Mount (Biomedica). Images were acquired using a Zeiss Axiovert 200 M fluorescence microscope at ×63 magnification, equipped with AxioVision software.

RESULTS

Rapid and enhanced innate phagocyte infiltration in neonatal mice infected orogastrically with Y. enterocolitica. Our previous study (22) demonstrated elevated innate cell inflammation in neonatal compared to adult MLN 3 days after infection with Y. enterocolitica. To determine how early this inflammatory response is established, we examined innate phagocyte recruitment 18, 24, and 48 h postinfection with 5 × 10^6 CFU of the high-virulence strain A127/90 (biotype IB, serotype O:8) (wild-type Y. enterocolitica). Inflammatory cell infiltrate was observed in the MLN of both neonates and adults as early as 24 h postinfection (Fig. 1A). However, the percentages of both the neutrophil (Ly6G^-CD11b^-) and monocyte (Ly6G^-CD11b+) populations were significantly increased in neonates compared to adults at both 24 and 48 h postinfection. Importantly, this inflammatory response was not elicited by the physical procedure since mock-infected neonates did not show an increase in innate phagocytes (see Fig. S1A in the supplemental material). Microscopic inspection of the Ly6G^-CD11b^- population confirmed that these cells have a polymorphonuclear morphology typical of neutrophils (see Fig. S1B).

For our experiments, we use neonates 7 days of age because they are considered to most closely represent human newborns immunologically (25). Six- to 10-week-old adult C57BL/6 mice have approximately five times the body mass of a 7-day-old neonate. It could be argued that the reduced inflammatory responses in adults are due to the relatively smaller infective dose for their size. To test this, we infected adult mice with 2.5 × 10^6 CFU Y. enterocolitica, i.e., five times the dose given previously, to account for the adults’ greater body mass. We found that there was no significant increase in neutrophil or monocyte cell recruitment to the adult MLN at the higher dose compared to our standard dose. However, we did observe a
significant increase in recruitment to the adult Peyer’s patches (PP) at the higher dose, which reached percentages similar to those observed in the neonatal MLN (Fig. 1B).

Similar levels of invasion of *Y. enterocolitica* to the MLN occur in neonatal and adult mice early after infection. One possible explanation for the enhanced inflammatory response of infected neonates is that *Y. enterocolitica* may colonize the neonatal tissues to a greater extent than in adults. To test this hypothesis, we evaluated *Y. enterocolitica* titers in the MLN and intestines early after infection. Evaluation of *Y. enterocolitica* titers in the small intestine containing fecal contents 18 h postinfection revealed a greater bacterial load in the neonatal intestine than in the adult intestine (Fig. 2). To determine the level of tissue-associated *Y. enterocolitica*, the intestines were flushed free of contents before *Y. enterocolitica* titers were enumerated. It was found that neonatal and adult intestines contained similar levels of mucosa-associated *Y. enterocolitica*. *Y. enterocolitica* titers were also found to be similar in the MLN of neonates and adults at 18 and 48 h postinfection (Fig. 2). Therefore, invasion and colonization of the intestinal tissues appear to occur equivalently in neonates and adults.

*Y. enterocolitica* infection induces exaggerated early proinflammatory gene expression in the neonatal MLN compared to that in adults. To examine early inflammatory events that may lead to enhanced innate phagocyte recruitment to the MLN after *Y. enterocolitica* infection, we evaluated cytokine and chemokine RNA expression in the MLN by real-time RT-PCR. An early time point of 18 h postinfection was initially chosen because no cellular infiltrate was yet detectable at this time point (Fig. 1A) and *Y. enterocolitica* titers were similar between neonates and adults (Fig. 2). Therefore, target genes detected at 18 h postinfection would be expressed by endogenous cells initially responding to *Y. enterocolitica* infection. We found that neonates expressed significantly higher levels of the proinflammatory cytokine genes *Il6*, *Cxcl1*, *Il17a*, and *Ifng* than adults at this time point (Fig. 3). Expression of most of the proinflammatory cytokine genes remained elevated compared to that for adults at 24 and 48 h postinfection. Notably,
at no time point did the expression of these genes in adults exceed that of neonates. Importantly, enhanced IL-6 protein expression in the neonatal MLN compared to that in adults correlated with enhanced mRNA expression (see Fig. S2 in the supplemental material). In contrast, there was no difference in the expression of the anti-inflammatory cytokine gene Il10 between neonates and adults, and no induction of Il10 gene expression was observed following infection (Fig. 3).
Distinct cell populations are responsible for inflammation in neonates. To identify the cell populations within the neonatal MLN responsible for inflammation after infection with *Y. enterocolitica*, we used neonatal targeted-gene-knockout mice. B and CD4 T cells are the most prevalent cell types in the MLN, so we used B cell-deficient (μMT) and CD4−/− neonatal mice. Inflammation in μMT and CD4−/− mice was shown to be at levels similar to that observed in wild-type neonates 24 h postinfection, indicating that B cells and CD4+ cells are not required for inflammation in neonates early after infection with *Y. enterocolitica* (Fig. 4A). Although we did not evaluate inflammation in B cell-deficient or CD4−/− adults, we expect similar results.

Innate cells are a likely source of early proinflammatory gene expression. Therefore, we compared proportions of innate cells in the MLN of uninfected neonatal and adult C57BL/6 mice and found that Ly6G+ CD11b+ neutrophils are enhanced 18-fold in uninfected neonatal MLN compared to in adults. Additionally, Ly6G−CD11b+ cells are also increased 2-fold in uninfected neonatal MLN compared to adults (see Fig. S3 in the supplemental material). Because of the markedly enhanced proportions of total CD11b+ cells in the MLN of neonates at basal levels, we investigated their role in proinflammatory gene expression early after *Y. enterocolitica* infection, prior to inflammatory cell infiltration. Total MLN cells were harvested from neonatal mice 18 h postinfection, and CD11b+ cells were depleted with MACS beads (Fig. 4B). RNA from the whole fraction and the CD11b-depleted fraction was evaluated for gene expression by real-time RT-PCR. These experiments indicated that CD11b+ cells are important for expression of *Il6* but not *Il17a* or *Ifng* (Fig. 4C).

Greater frequencies of responding cells in the neonatal MLN than in adults potentially contribute to enhanced proinflammatory gene expression. As previously described, we observed a greater percentage of innate cells in the neonatal MLN than in adults (see Fig. S3 in the supplemental material). To test the hypothesis that an increased percentage of cells in the neonatal MLN compared to that in adults could be responding to *Y. enterocolitica* infection, we performed cytokine ELISPOT analysis of neonatal and adult MLN cells stimulated with LPS *in vitro*. We found that significantly greater frequencies of neonatal than adult MLN cells produced IL-6 protein (Fig. 5A). Additionally, we evaluated TNF-α protein expression in the MLN of neonates and adults *in situ* after infection with wild-type *Y. enterocolitica* and YopP-deficient *Y. enterocolitica* (Fig. 5B). As expected, we found enhanced TNF-α immunostaining in the MLN of neonates compared to that in adults 48 h after infection with wild-type *Y. enterocolitica*. We also observed enhanced TNF-α staining in both neonates and adults after infection with YopP-deficient *Y. enterocolitica* compared to that after infection with the wild type. However, as with wild-type *Y. enterocolitica*, TNF-α levels were strikingly increased in the neonatal MLN compared to those in adults after infection with YopP-deficient *Y. enterocolitica*.

MyD88 is required for early inflammation in neonates after infection with *Y. enterocolitica*. To test the potential role of NF-κB signaling in neonates after infection with *Y. enterocolitica*, we evaluated bacterial burden and inflammation in wild-type and YopP-deficient *Y. enterocolitica* strains and stained by immunohistochemistry for TNF-α and YopP. As expected, we observed elevated TNF-α staining in both neonates and adults treated or stimulated *in vitro* for 16 h with 100 ng/ml LPS, and the frequency of IL-6-expressing cells was evaluated by ELISPOT. *P* ≤ 0.05. (B) Neonatal and adult MLN were harvested 48 h postinfection with 2 × 10^8 CFU of the Y. enterocolitica ΔyopP strain and stained by immunohistochemistry for TNF-α. TNF-α is indicated by Alexa Fluor 488 (green), and Hoechst was used as a nuclear counterstain (blue). Representative images are shown at ×63 magnification.

FIG 4 Distinct cell populations are responsible for inflammation in neonates. (A) Neither CD4+ cells nor B cells are required for initiating inflammation in response to *Y. enterocolitica* infection in neonates. Neonatal wild-type C57BL/6 (wt) (●), μMT (▲), and CD4−/− (○) mice were infected orogastrically with 5 × 10^8 CFU *Y. enterocolitica*, and the MLN were evaluated for neutrophil recruitment by flow cytometry 24 h postinfection. Open symbols represent uninfected animals. (B) Representative flow cytometry profiles showing efficiency of CD11b+ cell depletion. (C) Neonatal MLN cells were depleted of CD11b+ cells by MACS separation, and RNA was evaluated for gene expression in the whole fraction (●) and CD11b-depleted (○) fraction 18 h postinfection. The left y axis corresponds to *Il6*, *Il17a*, and *Il10*. The right y axis corresponds to *Ifng*. Data are expressed relative to the lowest expressor. ns, not significant; *, *P* ≤ 0.05.

FIG 5 Greater frequencies of cells in the MLN of neonates potentially contribute to enhanced proinflammatory gene expression after *Y. enterocolitica* infection. (A) MLN cells from uninfected neonates or adults were either untreated or stimulated *in vitro* for 16 h with 100 ng/ml LPS, and the frequency of IL-6-expressing cells was evaluated by ELISPOT. *P* ≤ 0.05. (B) Neonatal and adult MLN were harvested 48 h postinfection with 2 × 10^8 CFU of the *Y. enterocolitica* ΔyopP strain and stained by immunohistochemistry for TNF-α. TNF-α is indicated by Alexa Fluor 488 (green), and Hoechst was used as a nuclear counterstain (blue). Representative images are shown at ×63 magnification.
MyD88-deficient neonatal mice. Innate phagocyte recruitment to the MLN was evaluated by flow cytometry 24 h postinfection. As expected, *Y. enterocolitica* infection of wild-type neonates induced inflammatory cell recruitment to the MLN at 24 h postinfection (Fig. 6A). In contrast, MyD88-deficient neonates recruited neither neutrophils nor Ly6G−CD11b+ cells to the MLN at this time point. We also evaluated bacterial burden in the MLN and small intestines to see how bacterial titers correlated with inflammation. We found that wild-type and MyD88-deficient neonates had similar bacterial burdens in the small intestines; however, MyD88-deficient neonates had significantly higher titers in the MLN 18 h postinfection (Fig. 6B). Additionally, we found reduced gene expression of several proinflammatory cytokines and chemokines in the MLN of MyD88-deficient neonates (Fig. 6C), despite the higher bacterial burden.

The *Y. enterocolitica* ΔyopP strain elicits profound neutrophil recruitment to the neonatal MLN compared to the adult MLN 48 h postinfection. *Yersinia* species contain a virulence plasmid encoding a T3SS and anti-host effector Yops that are injected into host cells (26). YopP, in particular, downregulates inflammatory responses in adult target host cells in vitro (27). Given that adults fail to recruit innate phagocytes to the MLN to the level of neonates, we reasoned that YopP may be more efficient in downregulating inflammation in adults. To test this hypothesis, we compared inflammation in neonatal and adult C57BL/6 mice 24 and 48 h postinfection with wild-type *Y. enterocolitica* and the *Y. enterocolitica* ΔyopP strain. Neonates and adults infected with the *Y. enterocolitica* ΔyopP strain recruited neutrophils to the MLN to an average of 16.7% of total cells, compared to only 3.2% after wild-type bacterial infection (Fig. 7A). Neutrophilia was also enhanced in the MLN of adult mice after infection with the *Y. enterocolitica* ΔyopP strain compared to infection with the wild type. However, neutrophilia in *Y. enterocolitica* ΔyopP strain-infected adult MLN reached an average of only 2.3%. Representative flow profiles are shown in Fig. 7B. Similar to adults, neonates were colonized to comparable levels by wild-type *Y. enterocolitica* and the *Y. enterocolitica* ΔyopP strain (Fig. 7C). Therefore, the massive inflammation observed in neonates infected with the *Y. enterocolitica* ΔyopP strain doesn’t appear to be due to enhanced bacterial colonization.

High-level inflammation in the neonatal MLN after infection with the *Y. enterocolitica* ΔyopP strain is associated with enhanced mortality and bacterial dissemination to systemic tissues. We next determined the effect of YopP on host survival in neonatal and adult mice. Neonatal mice infected with the *Y. enterocolitica* ΔyopP strain succumbed to the infection at a higher rate and overall significantly greater mortality than neonates infected with wild-type *Y. enterocolitica*. However, neither the survival kinetics nor mortality differed in adult mice (Fig. 8A and B). Colonizations of the intestine and MLN were similar between neonates infected with wild-type *Y. enterocolitica* and the *Y. enterocolitica* ΔyopP strain (Fig. 8C). However, the high-level inflammation (Fig. 7A) and enhanced mortality (Fig. 8B) was associated with increased dissemination of the *Y. enterocolitica* ΔyopP strain to the spleens of neonatal mice compared to that of the wild type (Fig. 8C).
The T3SS and/or Yops is required to elicit inflammation in neonates. Y. enterocolitica cured of the virulence plasmid induces greater levels of proinflammatory cytokines and chemokines than wild-type Y. enterocolitica in bone marrow-derived dendritic cells in vitro (28). To investigate the potential role of the virulence plasmid in eliciting the robust inflammation in the neonatal MLN in vivo, we compared infection with wild-type Y. enterocolitica and plasmid-cured Y. enterocolitica. First we evaluated the ability of plasmid-cured Y. enterocolitica to induce recruitment of neutrophils to the MLN of neonates. In contrast to wild-type Y. enterocolitica, plasmid-cured Y. enterocolitica failed to elicit neutrophil recruitment to the neonatal MLN 24 h postinfection (Fig. 9A). To determine if the virulence plasmid is required for expression of proinflammatory cytokine genes, we evaluated Il6, Il17a, and Ifng RNA expression 18 h postinfection. Similar to neonates infected with wild-type Y. enterocolitica, Il6 and Ifng expression was induced after infection with plasmid-cured Y. enterocolitica (Fig. 9B). Strikingly, however, expression of the neutrophil-recruiting cytokine Il17a was significantly and markedly lower in neonates infected with the plasmid-cured strain than in those infected with the wild type. Determination of Yersinia titers in the MLN showed that plasmid-cured Y. enterocolitica colonized the neonatal MLN to the same levels as wild-type Y. enterocolitica (Fig. 9C).

**DISCUSSION**

We recently discovered that murine neonates are highly resistant to orogastric infection with the Gram-negative enteropathogen Y. enterocolitica (22). Here, we report that this resistance is associated with the rapid and vigorous mobilization of the intestinal innate immune system. The expression of proinflammatory, but not anti-inflammatory, cytokine genes was markedly induced in the neonatal MLN shortly after infection. Strikingly, the expression levels in neonates greatly exceeded those seen in infected adults. Elevated proinflammatory gene expression was quickly followed by enhanced innate phagocyte recruitment to the MLN of neonates compared to that to the MLN of adults. Neither CD4⁺ nor B cells were required for inflammation in the neonatal MLN; however, MyD88 signaling and CD11b⁺ cells appeared to play a role. Although MyD88 was shown to be critical in eliciting inflammation in neonates, neither Toll-like receptor 4 (TLR4) nor TLR9 were required (see Fig. S4 in the supplemental material). Because Y. enterocolitica contains ligands for multiple TLRs, it is possible that compensation by other TLRs is sufficient to elicit inflammation in the absence of TLR4 or TLR9. Bacterial components were also critical in eliciting high-level inflammation in the neonatal MLN. The virulence plasmid of Y. enterocolitica was required for selected proinflammatory gene expression and neutrophil recruitment to the neonatal MLN. TNF-α protein expression and neutrophil recruitment were markedly enhanced in the neonatal MLN after infection with the ΔyopP strain, while only a modest increase occurred in adults. Strikingly, the ΔyopP strain caused significantly earlier and greater mortality than wild-type Y. enterocolitica in neonates, while there was no difference in mortality in adults. The enhanced mortality of neonates infected with the Y. enterocolitica ΔyopP strain coincided with a significant increase in dissemination to the neonatal spleen. These models suggest that...
the level of intestinal inflammation in neonates is critical in determining whether protection or pathology occurs following infection with microbial pathogens.

In the majority of our experiments, we infected neonates and adults with the same dose of bacteria. Therefore, a decreased infective dose per gram in adults due to greater body weight could potentially account for the weaker inflammatory response. However, when we infected adults with 5 times the dose of *Y. enterocolitica*, to normalize for increased body weight, there was no significant increase in neutrophil recruitment to the MLN. There was a marked increase in neutrophilia of the PP of adults at the higher dose. This is consistent with previous reports of immune activation in the PP of infected adults (29). An attractive hypothesis is that the main site of innate immune system activation in infected adults is the intestine proper, whereas the main site in neonates is the MLN. This may be important, because the MLN drains all the intestinal tissues and therefore is poised to combat intestinal pathogens.

It is important to note that we cannot directly compare parameters of infection in neonatal and adult PP in the intestine proper, because neonatal PP are too small to identify for macroscopic dissection. Nonetheless, there are experimental observations that allow the prediction that the neonatal intestine proper would be poorly responsive to *Y. enterocolitica* infection. First, we have found that proinflammatory gene expression is poorly induced in the small intestine, relative to the MLN, of infected neonates (unpublished data). Second, Rhee et al. (16) described neonatal intestinal epithelial cells to have reduced expression of gamma interferon (IFN-γ)-inducible genes compared to that in adults. Accordingly, neonates mount poor intestinal inflammatory responses to *S. Typhimurium* compared to adults, as exemplified by reduced *Tnfα* gene expression and neutrophil recruitment to the cecum (16). Third, it has recently been shown that the intestinal epithelia are transiently hyporesponsive to endotoxin during commensal colonization after birth (30). Thus, the neonatal MLN may be hyperresponsive to infection as a compensatory mechanism for a hyporesponsive intestinal environment that is developing homeostasis with commensal bacteria.

Our results are consistent with earlier reports (31, 32) that the MLN of neonatal goats and sheep respond to TLR ligands in *vitro* with greater inflammatory responses than adults. The observation that strong inflammatory responses in the neonatal MLN exposed to TLR ligands and live infectious organisms are conserved among many species suggests that the MLN is an important site for host defense to gastrointestinal infection in early life. An important question that arises from these results is, "What are the mechanisms leading to much greater inflammation in infected neonates than in adults?" The neonatal MLN in both mice (see Fig. S3 in the supplemental material) and sheep (31) contain higher frequencies of endogenous innate cells, which have the potential to respond to bacterial infections with inflammatory signals. Therefore, a larger responding innate population may contribute to the increased inflammation observed in neonates after infection with *Y. enterocolitica*. Indeed, we show that a greater frequency of neonatal than adult MLN cells respond to LPS *in vitro* by IL-6 protein secretion (Fig. 5A). It is also possible that neonatal MLN cells express greater levels of proinflammatory proteins per cell than adults. We are currently addressing this question by intracellular cytokine staining. However, the adaptive immune system may also play a role. Zhao et al. (33) showed that adoptive transfer of adult T cells into neonates inhibited inflammatory responses to LPS. The implication is that neonates lack adaptive components that downregulate innate responses. Together, both the greater percentage of innate cells and reduced number of adaptive cells in neonates may contribute to high-level innate inflammation after *Y. enterocolitica* infection.

To investigate the cell types responsible for eliciting robust early inflammation in neonates, we evaluated the role of CD11b⁺ cells in proinflammatory gene expression. We found that CD11b⁺ cells were important in *Il6* gene expression. These results are in line with the recent report by DePaolo et al. (34), in which they described IL-6 production by MLN dendritic cells stimulated *in vitro* with *Y. enterocolitica* lysates. IL-6 is an important early proinflammatory cytokine that activates endothelium to express adhesion molecules, cytokines, and chemokines. In addition, IL-6 increases myelopoiesis and increases the level of circulating neutrophils (35). However, CD11b-negative cells may also play an important role in inflammation. Notably, IL-17 and IFN-γ expression was not reduced in CD11b-depleted populations (Fig. 4C). IL-17 in particular may play a major role in this setting. Infection of neonates with plasmid-cured *Y. enterocolitica* resulted in reduced expression of *Il17a* gene expression compared to that of the wild type, despite comparable *Yersinia* colonization of the MLN (Fig. 9B and C). The reduction in *Il17a* gene expression was associated with negligible recruitment of neutrophils to the neonatal MLN (Fig. 9A). These results suggest that the T3SS and/or secreted Yops may be particularly important for eliciting *Il17a* gene expression and neutrophil recruitment to the neonatal MLN early after infection. Because of the neutrophil recruit-
ing properties of IL-17 (36), we are specifically interested in cell types expressing this cytokine. Although Th17 development is delayed in neonates (37), some provocative candidates could be innate-like lymphoid cells, such as LTi (reviewed in references 38 and 39) and fetal-derived γδ T cells (40, 41).

The secreted effector YopP prevents NF-κB activation by acetylating IkB kinase beta (IKKB) and thereby preventing the IKK complex from promoting IkBα degradation (27). In vitro, YopP induces apoptosis and markedly represses proinflammatory cytokine expression (28). Importantly, this is the first report of the ΔyopP mutant elicting enhanced inflammatory responses in vivo in the intestinal lymphoid tissues as would be expected by the in vitro literature. These results complement reports that *Y. pestis* YopJ, the equivalent of YopP in *Y. enterocolitica*, downregulates TNF-α serum levels in a rat model of buphonic plague (42). Furthermore, based on the in vitro findings, the expectation was that *Y. enterocolitica* lacking yopP would have greatly reduced virulence in vivo. In adult mice, however, the ΔyopP strain is not or only slightly attenuated (our results) (43, 44). Strikingly, we have made the novel finding that the ΔyopP strain is more virulent in neonatal mice compared to the wild type, as exemplified by enhanced dissemination of the *Y. enterocolitica* ΔyopP strain to the spleen and consequently rapid mortality (Fig. 8). Excessive proinflammatory protein expression and neutrophilia in neonates infected with the ΔyopP strain may contribute to the increased susceptibility. Indeed, innate phagocytes were increased at least 5-fold in neonates infected with the *Y. enterocolitica* ΔyopP strain compared to that in those infected with wild-type *Y. enterocolitica*, despite similar bacterial loads in the MLN (Fig. 7). Additionally, TNF-α protein was strikingly enhanced in the MLN of neonates infected with the *Y. enterocolitica* ΔyopP strain compared to those infected with the wild type (Fig. 5). Because the enhanced inflammation in ΔyopP infection was delayed until 48 h postinfection, YopP may primarily be acting to downregulate inflammation in the recruited inflammatory cells as opposed to the endogenous cells. These results point to a possible therapeutic activity of YopP in treating established inflammation in the pediatric population.

The TLR adaptor molecule MyD88 was shown to be required for early proinflammatory cytokine expression and innate phagocyte recruitment to the neonatal MLN (Fig. 6). IL-1 and IL-18 signaling is also mediated by MyD88. These proinflammatory cytokines are initially translated in proform and require the assembly and activation of the inflammasome, a molecular platform which cleaves them into their active form (45). Indeed, various inflammasomes have been implicated in *Yersinia* infection (46–49). It is therefore possible that the MyD88-dependent effects we observe in neonates after *Y. enterocolitica* infection are associated with inflammasome activity.

Overall, our results provide further insight into the complex interplay between pathogen, host, and intestinal inflammation in early life. We have shown that the neonatal MLN is a highly active site for innate inflammation in *Y. enterocolitica* infection and that the potential for high-level inflammation in the neonatal MLN can reach pathological conditions under certain circumstances. It is clear that the mechanisms of inflammation are complex. However, our novel models of neonatal *Y. enterocolitica* infection will provide useful tools in further elucidating the mechanisms and consequences of inflammation and infection in the intestinal lymphoid tissues in early life.

**ACKNOWLEDGMENTS**

We thank Patty Guevara, University of Miami, for expert technical assistance. We also thank Guy R. Cornelis, Universitat Basel (Basel, Switzerland), for providing the *Y. enterocolitica* ΔyopP strain.

This work was supported by the National Institute of Allergy and Infectious Diseases (grant number 5R21AI083461, NIAID, to B.A.; grant number R01AI095255 to M.F.) and the Department of Microbiology and Immunology, University of Miami Miller School of Medicine.

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