Rapid CD8+ Function Is Critical for Protection of Neonatal Mice from an Extracellular Bacterial Enteropathogen

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Both human and murine neonates are characteristically highly susceptible to bacterial infections. However, we recently discovered that neonatal mice are surprisingly highly resistant to oral infection with *Yersinia enterocolitica*. This resistance was linked with activation of both innate and adaptive responses, involving innate phagocytes, CD4+ cells, and B cells. We have now extended these studies and found that CD8+ cells also contribute importantly to neonatal protection from *Y. enterocolitica*. Strikingly, neonatal CD8+ cells in the mesenteric lymph nodes (MLN) are rapidly mobilized, increasing in proportion, number, and IFNγ production as early as 48 h post infection. This early activation appears to be critical for protection since B2m$^{-/-}$ neonates are significantly more susceptible than wt neonates to primary *Y. enterocolitica* infection. In the absence of CD8+ cells, *Y. enterocolitica* rapidly disseminated to peripheral tissues. Within 48 h of infection, both the spleens and livers of B2m$^{-/-}$, but not wt, neonates became heavily colonized, likely leading to their deaths from sepsis. In contrast to primary infection, CD8+ cells were dispensable for the generation of immunological memory protective against secondary infection. These results indicate that CD8+ cells in the neonatal MLN contribute importantly to protection against an extracellular bacterial enteropathogen but, notably, they appear to act during the early innate phase of the immune response.

Keywords: neonatal, enteropathogens, CD8+ T cells, immunity, innate, IFN-gamma

INTRODUCTION

Neonates and infants are commonly highly susceptible to infectious diseases. Many of these diseases are caused by bacterial pathogens, which are largely naturally acquired by oral ingestion. Because of the many similarities in immunity in early life between mice and humans (1, 2), neonatal mice provide a reasonably faithful and experimentally convenient model system for studying infection with bacterial enteropathogens. Indeed, as in humans, mouse neonates are very sensitive to oral infection with a number of bacterial enteropathogens, including *Salmonella typhimurium* (3, 4), *Helicobacter pylori* (5–7), *Shigella flexneri* (8–10), *Vibrio cholera* (11, 12), and the Enteropathogenic *E. coli*-related *Citrobacter rodentium* (13–16). Often, these susceptibilities are linked to quantitative or qualitative

differences in neonatal and adult responses involving both the innate and adaptive gastrointestinal immune systems.

In contrast to all other descriptions of infections in neonatal mice, our laboratory found that 7-day-old murine neonates are highly resistant to orogastric infection with the extracellular enteropathogen *Yersinia enterocolitica* (17). *Y. enterocolitica* disseminates to the mesenteric lymph nodes (MLN), and analyses of immune function in that site revealed robust responses involving both the innate and adaptive arms of immunity (18, 19). Proinflammatory cytokine gene expression was highly induced, and innate phagocytes infiltrated the MLN in high numbers. Mature or supra-mature adaptive responses were also detected. We demonstrated that CD4 cell Th1 and Th17 function were both critical for protection of neonates and serum antibody responses of similar magnitude and avidity to those in adults were observed. Finally, and strikingly, neonates developed protective immunity against subsequent exposure as adults to a lethal dose of the bacterium.

These results indicated that protective responses against *Y. enterocolitica* in early life involve multiple innate and adaptive cell types. In these initial experiments, we largely ignored CD8 cells since these cells are more commonly associated with viral or intracellular bacterial infections. However, in the course of these studies, we noted that a large proportion of CD8 cells in the MLN of uninfected neonates expressed the proliferative antigen Ki67. Upon infection, CD8$^+$, but not CD4$^+$ cells, increased rapidly in proportion and IFNγ production. B2m$^{-/-}$ neonates were more susceptible to infection, compared with wt neonates, indicating that CD8$^+$ cells were required for survival of primary infection. The susceptibility of B2m$^{-/-}$ neonates was linked to the early dissemination of the bacteria to peripheral organs. Last, although required for primary infection, CD8$^+$ cells were dispensable for survival of secondary infection. These results indicate that neonatal CD8$^+$ cells may play an important early, innate-like role in survival to primary infection with *Y. enterocolitica* but they are not necessary for the development of protective memory in neonates.

MATERIALS AND METHODS

Mice
Adult C57BL/6 and B2m-deficient mice (B6.129P2-B2m$^{tm1-Unc}$/J) were purchased from Jackson Laboratories. 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 (6–10 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 the University of Miami Miller School of Medicine, Miami, FL, USA.

Bacterial Infections
Wild-type high-virulence *Y. enterocolitica* A127/90 serotype 0:8 biotype IB was originally provided by G. R. Cornelis (Universität Basel, Basel, Switzerland). For infection, bacterial frozen stocks (17) were washed twice with Hank's Balanced Salt Solution (HBSS, Gibco, Grand Island, NY, USA), diluted to the desired concentration, and inoculated with the indicated doses. Adults were inoculated orogastrically with a 22-gauge, round-tipped feeding needle (Fine Science Tools, Foster City, CA, USA) attached to a 1-ml syringe (Becton Dickinson, Franklin Lakes, NJ, USA). Neonates were inoculated orogastrically with PE-10 tubing (polyethylene tubing with an outside diameter of 0.61 mm) (Clay Adams, Sparks, MD, USA) attached to a 30-gauge needle and Hamilton syringe (20). The actual administered dose was determined by plating serial dilutions of the suspensions on Luria Broth plates and incubating for 48 h at 27°C.

Cell Staining, Antibodies, and Flow Cytometry Analysis
Individual MLN from neonates and adults were harvested and placed in cold HBSS containing 1% calf serum (Gibco), 10 mM HEPES (Gibco), and 4 mM sodium azide. Cell suspensions were prepared by mincing tissues with scissors and pressing cells through wire mesh with 74 µm pore size (Compass Wire, Westville, NJ, USA). Cells were incubated in mouse Fc Block (CD16/CD32; BD Pharmingen, San Diego, CA, USA) for 5 min on ice, followed by a 30-min incubation with fluorochrome-conjugated antibodies specific for CD4, CD8, Ki67, or TCRαβ (BD Pharmingen). For intracellular cytokine staining, cells were activated with 50 ng/ml PMA and 0.5 µM ionomycin in the presence of 5 µg/ml brefeldin A, fixed and permeabilized, and stained with fluorochrome-conjugated anti-IFNγ (BD Pharmingen). Samples were run on a Becton Dickinson LSR II flow cytometer and analyzed with FlowJo flow cytometry analysis software.

Bacterial Enumeration from Organs of Infected Mice
To measure *Y. enterocolitica* titers, tissues were weighed and homogenized in HBSS using a Seward Biomaster 80 Stomacher (Brinkman, Westbury, NY, USA) for 4 min at high speed. For small intestine mucosa-associated *Y. enterocolitica* titers, the small intestine contents were flushed with HBSS prior to homogenization. Individual MLN were homogenized in 400 µl (neonates) or 500 µl (adults) of HBSS using a VWR disposable pellet mixer with cordless motor (VWR International). *Y. enterocolitica* titers were enumerated by plating dilutions of homogenates on Difco Yersinia Selective Agar Base plates (Becton Dickinson, Sparks, MD, USA).

Statistical Analyses
All experiments were performed at least two times. Statistical tests were performed using GraphPad Prism software, as follows: unpaired *t* test for the Ki67/CD4/CD8/IFNγ staining experiments; Mann–Whitney test for the bacterial colonization experiments; Log-rank (Mantel–Cox) test for the survival experiments. The significant threshold was $P \leq 0.05$.

RESULTS

Rapid Responses of Neonatal MLN CD8+ Cells following Oral *Y. enterocolitica* Infection

In the course of characterizing immune responses to *Y. enterocolitica* infection, we compared MLN cells from uninfected neonates and adults for expression of the proliferation antigen Ki67. Approximately 10% of both CD4+ and CD8+ cells in adult MLN expressed the Ki67 antigen. A much greater proportion of both T cell types were Ki67+ in neonatal MLN (**Figures 1A,B**). This is consistent with previous studies in which neonatal T cells in mouse spleen and cord blood were found to be spontaneously proliferating at a higher rate than in adults ([21], [22]). However, the frequency of proliferating cells in the MLN, especially among the CD8+ population, is markedly higher than in blood or spleen.

The substantial endogenous proliferation of CD8+ cells in uninfected neonates indicated that this population may pre-exist in a state poised for rapid responsiveness to invasive intestinal pathogens. Indeed, within 24 h of infection with *Y. enterocolitica*, there was a significant increase in the proportion of neonatal CD8+ cells while percentages of neonatal CD4+ and adult CD4+ and CD8+ did not increase (**Figure 1C**). The absolute numbers showed a similar pattern, although neonatal CD4+ cells also increased by fivefold while CD8+ cell numbers increased eightfold. Neither population increased in absolute numbers in adults.
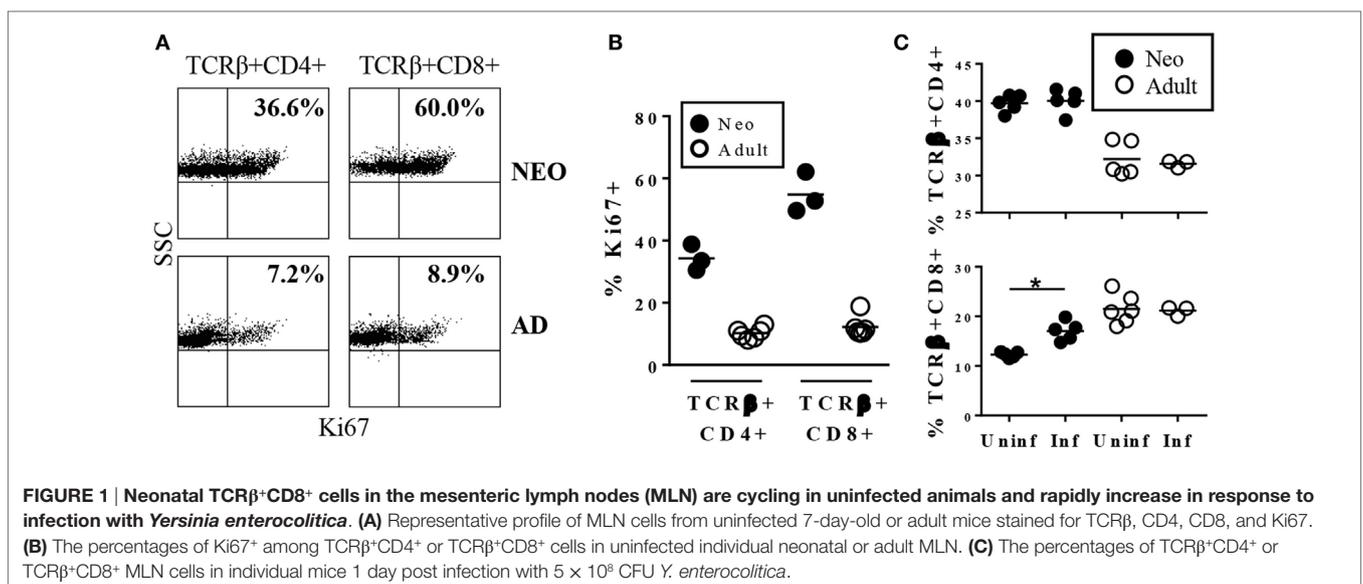
These results indicated that the neonatal CD8+ population was able to expand rapidly in response to *Y. enterocolitica* infection. The next question was whether this expansion was accompanied by effector function. A prominent effector function of CD8+ cells is the production of IFNγ. Our previous studies had shown that IFNγ is required for survival of neonates to *Y. enterocolitica* infection ([18]) and that IFNγ production by CD4+ cells was an important component of protection. However, IFNγ-producing
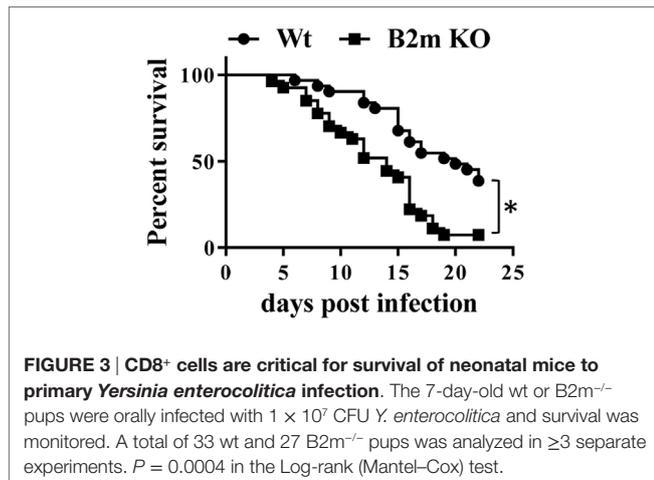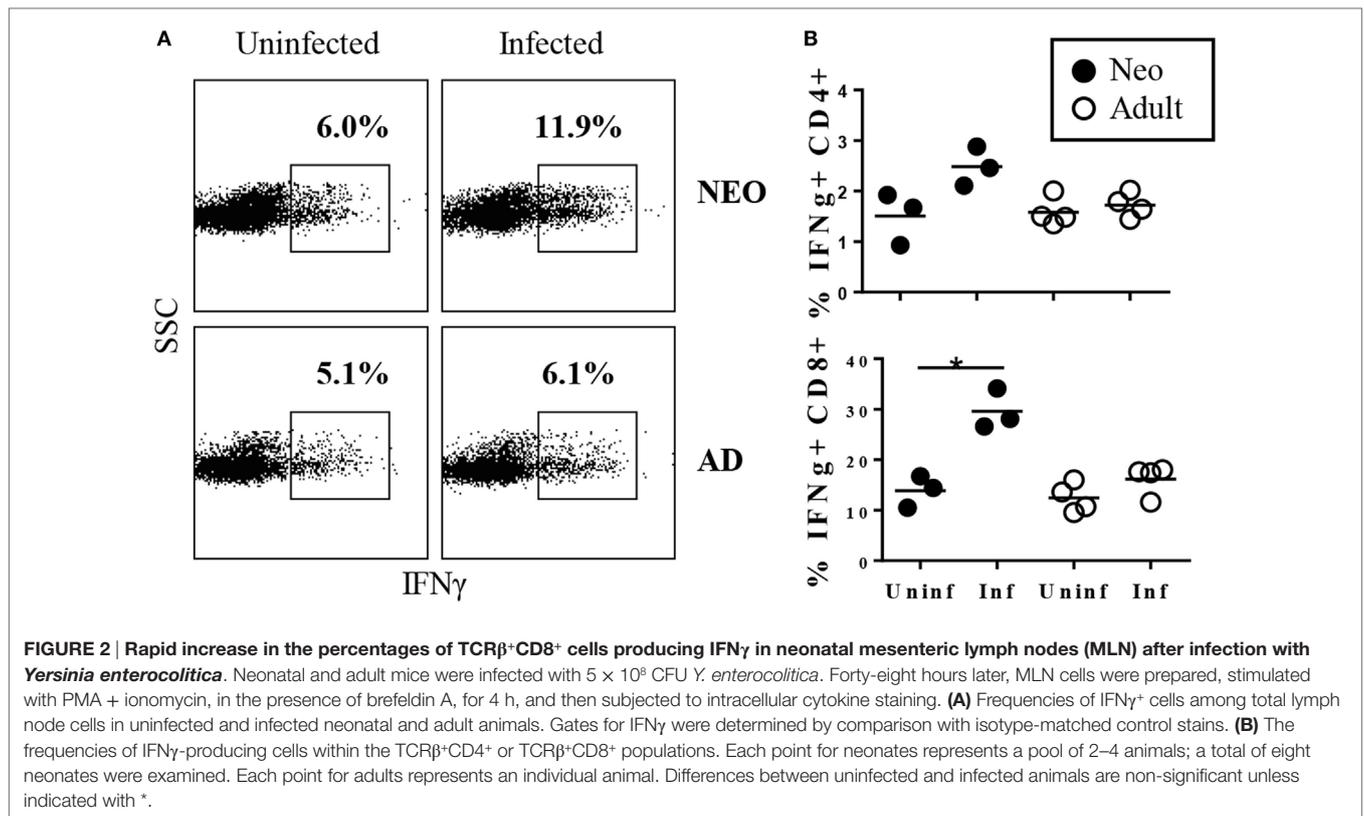
CD4+ cells most likely act in the later stages of primary infection, during the adaptive phase of the response, since death in CD4-deficient neonates is delayed until ≥15 days post infection. In striking contrast, IFNγ-deficient neonates die rapidly, within 7 days of infection. Together, these results suggest that IFNγ may provide important protection both early and late post infection. Thus, we proposed that early activated CD8+ cells may be a rapid source of IFNγ production. We first examined early IFNγ production among total MLN cells and found an increase in total cytokine-producing cells in neonates, but not adults (**Figure 2A**). Indeed, a significant increase in IFNγ-production was selectively observed in the neonatal CD8+ population (**Figure 2B**). Thus, *Y. enterocolitica* infection of neonates is characterized by the rapid mobilization of CD8+ MLN populations with IFNγ-producing effector function.

Neonatal CD8+ Cells Are Essential for Survival to Primary Infection and for Preventing Early Systemic Dissemination of *Y. enterocolitica*

Our results thus far indicated that neonates infected with *Y. enterocolitca* mount early CD8+ responses. To test whether CD8+ cells were essential for protection against infection, survival of wt and B2m$^{-/-}$ neonates to a threshold lethal dose of *Y. enterocolitica* was compared. As shown in **Figure 3**, B2m$^{-/-}$ neonates were more susceptible to lethal infection and, notably, most deaths occurred prior to 14 days post infection—i.e., well before the time that CD4$^{-/-}$ neonates begin to succumb ([18]). In these experiments, we used female and male neonates indiscriminately but subset analyses showed that there were no differences in survival of male or female neonates in either wt or B2m$^{-/-}$ neonates. Therefore, CD8+ cells appear to play an important role in the survival of neonates, regardless of gender.

Yersinia enterocolitica replicates in the small intestines and routinely invades to the MLN but only systemically disseminates

FIGURE 1 | Neonatal TCRβ+CD8+ cells in the mesenteric lymph nodes (MLN) are cycling in uninfected animals and rapidly increase in response to infection with *Yersinia enterocolitica*. (A) Representative profile of MLN cells from uninfected 7-day-old or adult mice stained for TCRβ, CD4, CD8, and Ki67. **(B)** The percentages of Ki67+ among TCRβ+CD4+ or TCRβ+CD8+ cells in uninfected individual neonatal or adult MLN. **(C)** The percentages of TCRβ+CD4+ or TCRβ+CD8+ MLN cells in individual mice 1 day post infection with 5 × 10⁸ CFU *Y. enterocolitica*.

FIGURE 2 | Rapid increase in the percentages of TCRβ+CD8+ cells producing IFNγ in neonatal mesenteric lymph nodes (MLN) after infection with *Yersinia enterocolitica*. Neonatal and adult mice were infected with 5×10^8 CFU *Y. enterocolitica*. Forty-eight hours later, MLN cells were prepared, stimulated with PMA + ionomycin, in the presence of brefeldin A, for 4 h, and then subjected to intracellular cytokine staining. **(A)** Frequencies of IFNγ+ cells among total lymph node cells in uninfected and infected neonatal and adult animals. Gates for IFNγ were determined by comparison with isotype-matched control stains. **(B)** The frequencies of IFNγ-producing cells within the TCRβ+CD4+ or TCRβ+CD8+ populations. Each point for neonates represents a pool of 2–4 animals; a total of eight neonates were examined. Each point for adults represents an individual animal. Differences between uninfected and infected animals are non-significant unless indicated with *.

FIGURE 3 | CD8+ cells are critical for survival of neonatal mice to primary *Yersinia enterocolitica* **infection**. The 7-day-old wt or B2m−/− pups were orally infected with 1×10^7 CFU *Y. enterocolitica* and survival was monitored. A total of 33 wt and 27 B2m−/− pups was analyzed in ≥3 separate experiments. *P* = 0.0004 in the Log-rank (Mantel–Cox) test.

if the dose of infection is very high in wt animals or if animals are immunocompromised (23). Significant systemic dissemination most likely leads to death *via* septic shock. To investigate the potential importance of CD8+ cells in confining *Y. enterocolitica* to the intestinal tissues, we compared early colonization of intestinal and systemic organs in infected wt and B2m−/− neonates. 48 h post infection, colonization levels of the small intestines and MLN were similar in wt and B2m−/− neonates (**Figures 4A,B**). However, at this relatively early time point, both the liver and
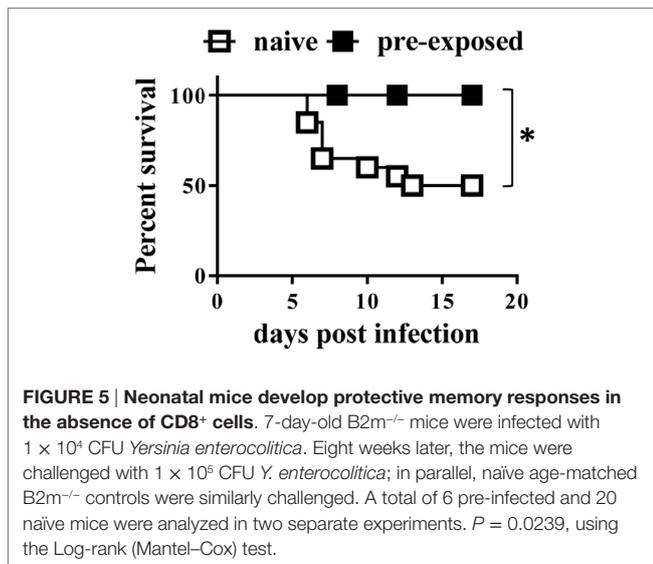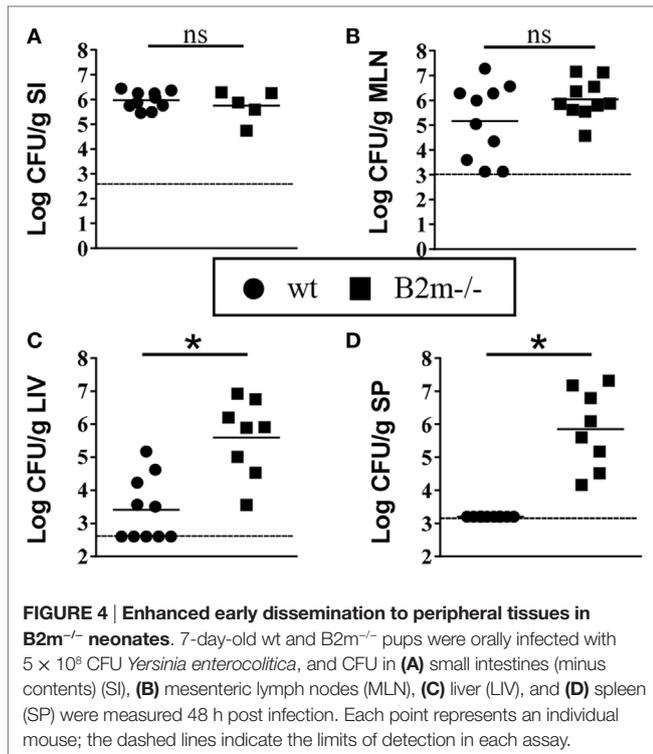
spleen showed massive bacterial infiltration only in the B2m−/− neonates (**Figures 4C,D**). These results indicate that the early activation of CD8+ cells in the neonatal MLN may be critical for preventing the systemic spread of *Y. enterocolitica*.

Protective Memory Responses Are Generated in Neonates in the Absence of CD8+ Cells

In adult mice, CD8+ cells appear to be essential for protection against secondary infection with the closely related bacterium *Yersinia pseudotuberculosis* (24). To test whether this was also the case in neonates, B2m−/− neonates were infected with a sublethal dose (1×10^4 CFU) of *Y. enterocolitica*. Uninfected littermates were kept as control mice. Eight weeks later, all mice were challenged with 1×10^5 CFU of *Y. enterocolitica*, and survival was monitored. At this challenge dose, approximately 50% of the control, previously uninfected, mice survived (**Figure 5**). However, 100% of the B2m−/− mice previously infected as neonates survived the infection. Therefore, CD8+ cells are not required for either the development of immunological memory in neonates or for its manifestation in response to secondary challenge.

DISCUSSION

The results presented here demonstrate that CD8+ cells are critical for protection of neonates against oral exposure to an

FIGURE 4 | **Enhanced early dissemination to peripheral tissues in B2m$^{-/-}$ neonates.** 7-day-old wt and B2m$^{-/-}$ pups were orally infected with 5×10^8 CFU *Yersinia enterocolitica*, and CFU in **(A)** small intestines (minus contents) (SI), **(B)** mesenteric lymph nodes (MLN), **(C)** liver (LIV), and **(D)** spleen (SP) were measured 48 h post infection. Each point represents an individual mouse; the dashed lines indicate the limits of detection in each assay.

FIGURE 5 | **Neonatal mice develop protective memory responses in the absence of CD8$^+$ cells.** 7-day-old B2m$^{-/-}$ mice were infected with 1×10^4 CFU *Yersinia enterocolitica*. Eight weeks later, the mice were challenged with 1×10^5 CFU *Y. enterocolitica*; in parallel, naïve age-matched B2m$^{-/-}$ controls were similarly challenged. A total of 6 pre-infected and 20 naïve mice were analyzed in two separate experiments. $P = 0.0239$, using the Log-rank (Mantel–Cox) test.

extracellular bacterial enteropathogen. The protective effects appear to manifest early after infection since CD8$^+$ cells in the MLN selectively increase within 24 h of infection and over ¼ of the cells are producing IFNγ just 48 h post infection. Moreover, CD8$^+$ cells appear to be important for the early containment of bacteria within the intestines; the spleens and livers of B2m$^{-/-}$ neonates were heavily colonized by bacteria as early as 48 h post infection. Therefore, unlike in their typical adaptive role, neonatal CD8$^+$ cells in intestinal lymphoid tissues act during the

early, innate phase of the response, providing an important rapid antibacterial function.

CD8$^+$ cell function in neonates has been mostly studied in response to infection with viruses, including influenza virus (25, 26), herpes simplex virus (27–29), respiratory syncytial virus (30), cytomegalovirus (31, 32), lymphocytic choriomeningitis virus (33), adenovirus (34), and Cas-Br-E murine leukemia virus (35–37). Most often, these viruses have been introduced either systemically or through a pulmonary route. Common, although not exclusive, observations are that the CD8 cell response is delayed relative to that of adults, lytic and/or IFNγ-secreting activities are diminished, the repertoire appears to be limited, and neonatal CD8$^+$ memory responses are poor. In all cases, however, neonatal CD8$^+$ cell activity was measured during the adaptive phase of the response. There are far fewer studies on neonatal CD8 cell function in response to bacterial infection and those are limited to systemic infection of neonates with the intracellular pathogen *Listeria monocytogenes* (38, 39). Thus, we believe that the neonatal response to *Y. enterocolitica* represents the first description of neonatal CD8$^+$ cells acting in a protective manner during the innate phase of the response against an extracellular bacterial enteropathogen.

Although CD8 cells had not previously been implicated in enterobacterial infection in neonates, a role for these cells in adult *Yersinia* infection has been described (24). Like our observations in neonates, it was reported that oral infection with *Y. pseudo-tuberculosis* led to increased colonization of peripheral tissues in B2m$^{-/-}$ adults. However, we found rapid (≤48 h) increased colonization in neonatal B2m$^{-/-}$ mice whereas the increase in adults was not detected until 14 days post inoculation, during the adaptive phase of immunity. In addition, we found that CD8 cells were not required for protective memory responses whereas in adults, anti-CD8 treatment greatly compromised memory responses. Overall, while CD8 cells may contribute to immunity against *Yersinia* infection in both neonates and adults, the roles these cells play and when they act differ in early life and adulthood.

The mechanisms underlying the early responses of neonatal CD8 cells in *Yersinia enterocolitica* infection are not yet fully understood. One finding that may provide some insight is the observation that >50% of neonatal MLN CD8$^+$ cells express proliferating antigens in uninfected, resting animals. Due to the lymphopenic state of neonates, homeostatic proliferation of both CD4$^+$ and CD8$^+$ cells has been previously described in neonatal spleen (21) and in human cord blood (22). In both cases, as we see here, a greater proportion of CD8$^+$ cells are cycling relative to CD4$^+$ cells. However, the percentages of CD8$^+$ cells in cycle in the MLN is approximately fivefold higher than that in the blood or spleen, perhaps due to the proximity of ongoing colonization by the commensal microbiota (40). Proliferation in lymphopenic hosts leads to the capacity for rapid induction of IFNγ expression (41, 42)—in our case, the IFNγ appears to be elicited upon exposure to bacterial enteropathogen. In that regard, human neonatal CD8$^+$ cells have been shown to rapidly respond to TLR2 or TLR5 stimulation with increased proliferation and cytokine production (43), and

it is possible that murine neonatal CD8^{+} cells in the MLN are similarly responding to PAMPs expressed by *Y. enterocolitica*. Thus, neonatal CD8 cells may perform both adaptive functions, in response to virus or intracellular bacteria, and innate functions when exposed to extracellular pathogens, especially at mucosal sites.

AUTHOR CONTRIBUTIONS

DS performed many of the experiments in partial fulfillment of his doctoral degree. He contributed significantly to the intellectual development of the overall project and the manuscript. BA, the principal investigator, oversaw the scholarly and technical progress of the project and wrote the manuscript.

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