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TRIF-Dependent Innate Immune Activation Is Critical for Survival to Neonatal Gram-Negative Sepsis

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Current evidence suggests that neonatal immunity is functionally distinct from adults. Although TLR signaling through the adaptor protein, MyD88, has been shown to be critical for survival to sepsis in adults, little is known about the role of MyD88 or TRIF in neonatal sepsis. We demonstrate that TRIF^{-/-} but not MyD88^{-/-} neonates are highly susceptible to *Escherichia coli* peritonitis and bacteremia. This was associated with decreased innate immune recruitment and function. Importantly, we found that the reverse was true in adults that MyD88^{-/-} but not TRIF^{-/-} or wild-type adults are susceptible to *E. coli* peritonitis and bacteremia. In addition, we demonstrate that TRIF but not MyD88 signaling is critical for the TLR4 protective adjuvant effect we have previously demonstrated. These data suggest a differential requirement for the survival of neonates versus adults to Gram-negative infection, and that modulation of TRIF in neonates can be used to augment survival to neonatal sepsis. *The Journal of Immunology*, 2015, 194: 1169–1177.

One million newborn children die each year from sepsis or severe infection (1, 2). Mortality rates among these babies range from 10 to 40% depending on birth weight and age at onset of sepsis but are especially high in very low birth weight infants (3, 4). In addition, despite progress in outcomes with postpartum group B *Streptococcus* infections and sepsis, Gram-negative sepsis from *Escherichia coli* continues to be a significant problem in the neonatal intensive care units (5, 6).

It is now generally accepted that the functional status of neonatal innate and adaptive immunity may be one of the reasons why this population fares so poorly during infection (2). Numerous studies have examined the epidemiology of neonatal sepsis and the molecular markers associated with neonatal sepsis (3, 4, 7, 8), but few studies have characterized the underlying immunological responses and pathophysiology of neonatal sepsis. Although many studies in adult models of infection and sepsis exist, examining the relative requirements of innate immunity and potential modulators of in-

nate immune response, few studies have established these responses or mechanisms in the neonate and whether they are similar or different from in the adult.

Of these modulators of innate immune activation, TLR signaling is arguably one of the most important. TLR signaling proceeds through one of three different pathways: MyD88 alone, leading predominantly to the production of proinflammatory cytokines/chemokines, TRIF, alone leading to the production of both type I IFNs, as well as the production of proinflammatory cytokines and chemokines, or in the unique case of TLR4 in which both MyD88 and TRIF pathways are activated (9). Several adult studies have identified MyD88 signaling as critical for survival to Gram-negative infection or sepsis in adults as well as important for the production of reactive oxygen species (ROS) in neutrophils (10–14). The role of TRIF signaling in adult Gram-negative infection is somewhat controversial; the response to some Gram-negative infections such as *Burkholderia* infection is thought to be MyD88 dependent, whereas TRIF signaling was shown to be critical for survival to *Yersinia enterocolitica* (14, 15). The role of these TLR adaptor signaling proteins in the survival of the neonate to Gram-negative infection is unknown.

In this report, we demonstrate that TRIF^{-/-} neonates are more susceptible to *E. coli* sepsis compared with wild-type (WT) and MyD88^{-/-} neonates. This is associated with decreased recruitment of peritoneal neutrophils and macrophages at 12 h post-*E. coli* infection, decreased neutrophil and macrophage reactive oxygen species production, as well as increased peritoneal and blood bacterial counts in TRIF^{-/-} compared with WT neonates. Importantly, we also demonstrate that MyD88^{-/-}, but not TRIF^{-/-}, young adults were more susceptible to *E. coli* Gram-negative infection. In addition, we have previously shown that pretreatment of murine neonates with TLR4 ligand, LPS (16), is able to significantly improve survival to subsequent polymicrobial sepsis (2). Although this effect was associated with increased activation/recruitment of innate immune effector cells, the mechanism was unknown. In this report, we demonstrate that TRIF, but not MyD88 signaling, is critical for the observed TLR4 adjuvant effect.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ROS, reactive oxygen species; WT, wild-type.

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Materials and Methods

Mice

Six- to 8-wk-old male and female C57BL/6J (WT) and TRIF^{-/-} (C57BL/6J-Ticam1^{Lps2/J}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for adult breeders and adult experiments. MyD88^{-/-} mice (B6.129P2-Myd88^{tm1Defr}) were obtained from S. Akira (Osaka University, Osaka, Japan), initially maintained at Rhode Island Hospital and Brown University, and transferred to University of Florida for breeding and colony maintenance. IFN type 1 receptor null mice (B6.129S2-Ifnar1^{tm1Agt/J}; IFNAR^{-/-}) breeders were purchased from The Jackson Laboratory. These mice had been backcrossed onto a B6 background for six generations and inbred on the B6 background for an additional 12 generations. Adult mice were mated in a harem schema with one male to two females. Neonatal mice were studied between 4 and 7 d of age. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida, College of Medicine.

Cecal slurry model

Polymicrobial sepsis was induced as described previously (17). Briefly, cecal contents of adult C57BL/6J mice were suspended in 5% dextrose solution (D5W) at a final concentration of 80 mg/ml and injected i.p. into 5- to 7-d-old neonates. An LD₇₀ (1.3 mg fecal contents/g body weight) of cecal slurry, as determined from previous experiments, was used (17).

E. coli Gram-negative sepsis

After institutional review board permission was obtained to review patient records, a clinical isolate of *E. coli* was found from a blood culture drawn

from a neonate (<30 d of age) with *E. coli* urosepsis. This isolate was then subsequently serotyped as O11:H18 by Pennsylvania State University laboratories and used in the murine studies described in this study. *E. coli* O11:H18 was grown in trypticase soy agar broth and plates (Fisher Scientific). Neonatal C57BL/6 mice 5–7 days old received i.p. an LD₇₀ of *E. coli* (2×10^3 CFUs) in 50 μ l PBS. Total volume of injected solution was <50 μ l. Neonates were either followed for survival for 7 d or euthanized, and tissues were harvested at the time points indicated.

Colony forming unit calculation from blood and peritoneal washes

Blood harvested via intracardiac puncture or peritoneal washes were collected from neonates or adults, 24 h post-*E. coli* inoculation. Peritoneal washes were harvested from neonates or adults via i.p. injection of 500 μ l or 5 ml PBS at 4°C, respectively. Peritoneal washes and blood were then diluted and plated on blood agar plates (Fisher Scientific) (18–20).

Blockade of type 1 IFN signaling

To block type 1 IFN signaling, anti-IFNAR1 or isotype (100 μ g/animal) (mouse IgG₁) (Leinco Technologies, St. Louis, MO) was administered i.p. to neonatal C57BL/6J mice. Amount of Ab used was gleaned from Sotolongo et al. (15).

Pretreatment with a TLR4 agonist (bacterial LPS)

Neonatal C57BL/6 mice 4–6 d old received ip administration of LPS (16) (1 μ g/g body weight; ultrapure via ion exchange chromatography *E. coli* O26:B6) (Sigma-Aldrich) in physiologic saline 24 h before challenge with

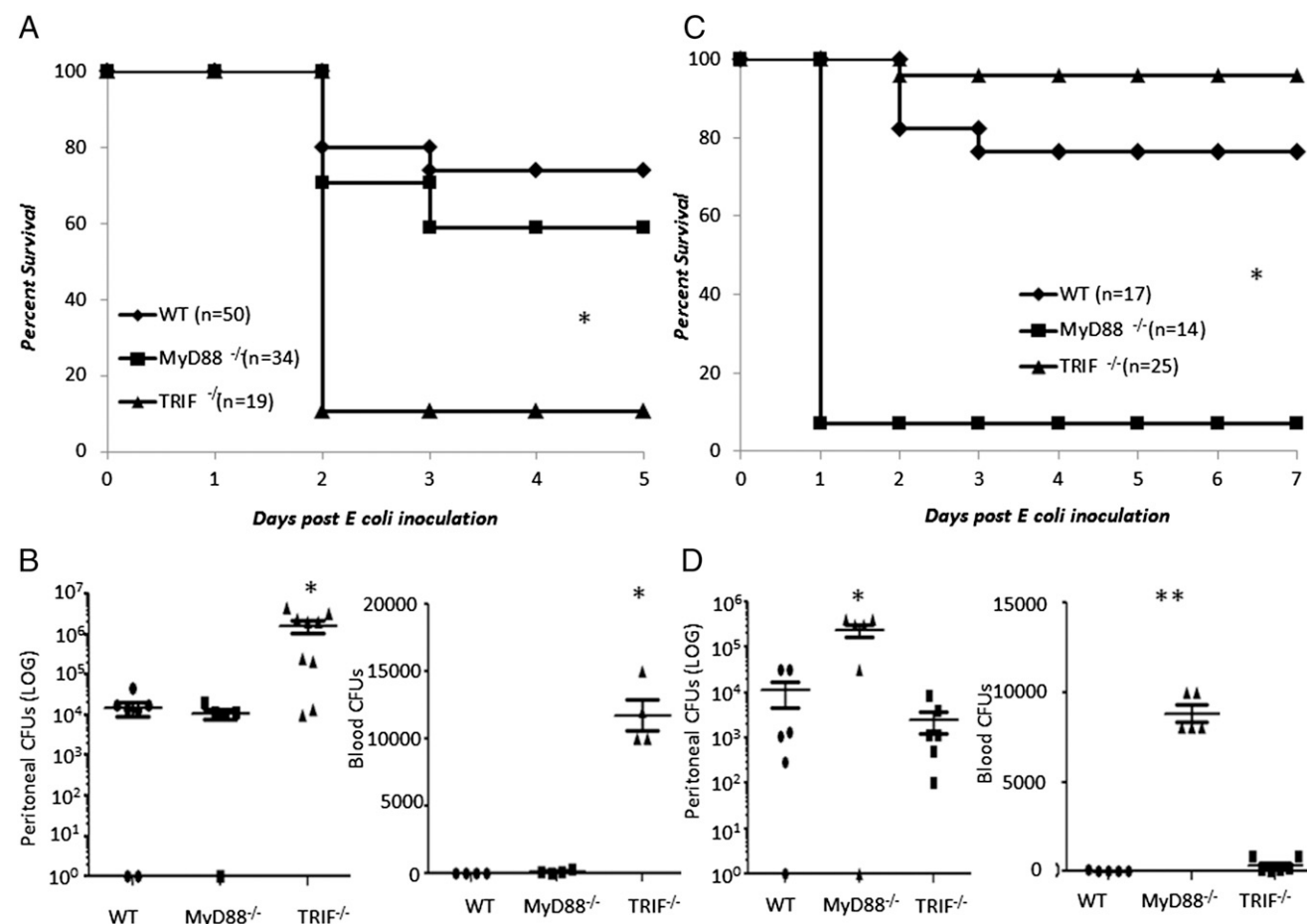


FIGURE 1. TRIF^{-/-} neonates, but not adults, are more susceptible to gram negative infection compared with MyD88^{-/-} or WT neonates. (A) WT, TRIF^{-/-}, and MyD88^{-/-} neonates were inoculated i.p. with *E. coli* and subsequently followed for survival (Fisher's exact; **p* < 0.001). (B) Peritoneal washes or blood were harvested 24 h post-*E. coli* inoculation and plated on blood agar, and CFUs were counted (*n* = 5–7 animals/group). (Data were analyzed before log transformation by both one-way ANOVA and Kruskal–Wallis ANOVA; in both cases, data were significantly different; **p* < 0.05.) (C) Six- to 12-wk-old WT, TRIF^{-/-}, and MyD88^{-/-} were inoculated i.p. with *E. coli* and followed for survival (Fisher's exact; **p* < 0.001). (D) Peritoneal washes and blood were collected from MyD88^{-/-}, TRIF^{-/-}, and WT adults 24 h post-*E. coli* infection and plated on blood agar, and CFUs were counted (data were analyzed before log transformation by both one way ANOVA and Kruskal–Wallis ANOVA; *n* = 5 animals/group; **p* < 0.009, ***p* < 0.001).

cecal slurry. Control animals received saline only i.p. 24 h before cecal slurry challenge. Total volume of injected solution was $<50 \mu\text{l}$.

Peritoneal, sera, and media cytokine concentrations

Blood and peritoneal washes were harvested from neonates at the time points described following LPS, cecal slurry, or *E. coli* administration. As reports have shown that heparin can bind to CXCL10 and possibly reduce its detection in blood, serum was used for these assays (21, 22). Blood was collected via intracardiac puncture and centrifuged to collect the supernatant (serum). To collect peritoneal washes, the peritoneal cavity was instilled with 500 μl cold PBS (Cellgro, Manassas, VA). The peritoneal cavity was then opened over a sterile tissue culture dish, and the peritoneal wash was collected. Peritoneal washes were then centrifuged to remove debris, and the supernatants were stored at -80°C . Medium was collected from in vitro LPS stimulation experiments and centrifuged to remove cellular debris. The supernatant was then used for cytokine detection assays. Sera, peritoneal washes, and media were then measured for IL-1 β , IL-6, IL-10, IL-12p70, KC, IFN- γ , TNF- α , MCP-1, MIP-1 α , and CXCL10 concentrations via Multiplex Luminex Assay (Milttenyi Biotec, Auburn, CA).

Flow cytometry

Single-cell suspensions were characterized using anti-Ly6G allophycocyanin, anti-CD11b-PECy7, and/or anti-F4/80 allophycocyanin-Alex Fluor 750 (eBioscience, San Diego, CA). All Abs were purchased from BD Biosciences (Franklin Lakes, NJ) unless otherwise indicated. Samples were acquired and analyzed on an LSR II flow cytometer (BD Biosciences) and analyzed via FACSDiva (BD Biosciences) or FloJo (Tree Star, Ashland, OR) software. At least 1×10^4 live cells (SYTOX Blue $^-$; Invitrogen, Carlsbad, CA) were collected for analysis.

Isolation of blood, bone marrow, and splenocytes for phenotypic analysis

Blood was obtained via intracardiac puncture of isoflurane anesthetized neonatal mice into a heparinized syringe. Spleens were harvested from neonates and then dissociated through a 70- μm sterile filter. To harvest bone marrow, both femurs and tibias from individual animals were collected and flushed with PBS (Cellgro). Blood, splenocyte, and bone mar-

row suspensions were then subjected to erythrocyte lysis using ammonium chloride lysis solution. Cell suspensions were then stained for neutrophil (Ly6G $^+$,CD11b $^+$) or macrophage (F4/80 $^+$,CD11b $^+$) cell surface markers and analyzed via flow cytometry. A representative scattergram for staining of neutrophil (Ly6G $^+$,CD11b $^+$) or macrophage (F4/80 $^+$,CD11b $^+$) cell surface markers from neonatal splenocytes is presented in Supplemental Fig. 1.

Harvest of peritoneal washes

Peritoneal cell isolation was carried out in a similar manner as described previously (2). Because peritoneal lavages from individual animals contained relatively few cells, peritoneal washes from three to five animals were pooled for analysis and considered to be one sample. For each time point or condition analyzed, a total of three to four peritoneal samples were collected per experiment. To determine absolute numbers of innate immune effector cells, the percentage of macrophages or neutrophils within the total sample population was determined via flow cytometry, multiplied by the total sample cell number, and then divided by the total number of mice in the pooled sample. Absolute numbers represent total macrophages or neutrophils per mouse.

Isolation of splenic macrophages and neutrophils for functional analyses

Spleens were disassociated through a 70- μm sterile filter into RPMI 1640 medium (Cellgro) with 10% heat-inactivated low endotoxin FBS 0.5% and penicillin/streptomycin (Cellgro). Suspensions were then overlaid on top of a Histopaque 1119 (Sigma-Aldrich) density gradient and centrifuged. Mixed cell suspensions were then washed with media alone, and individual cell populations were assayed for phagocytosis or reactive oxygen species (23) production by flow cytometry.

Reduction of cytochrome c assay

In HBSS (Sigma-Aldrich) containing 145 $\mu\text{mol/l}$ cytochrome c (Sigma-Aldrich), 1×10^5 cells isolated from peritoneal washes of WT or TRIF $^{-/-}$ neonates 24 h post-*E. coli* infection were stimulated with or without PMA (98 nmol/l) (Sigma-Aldrich) at 37°C . Reduction of cytochrome c was measured at 550 nm at 1-min intervals for 45 min. The maximal rate of reduction (V_{max}) was calculated by linear regression analysis. Purified

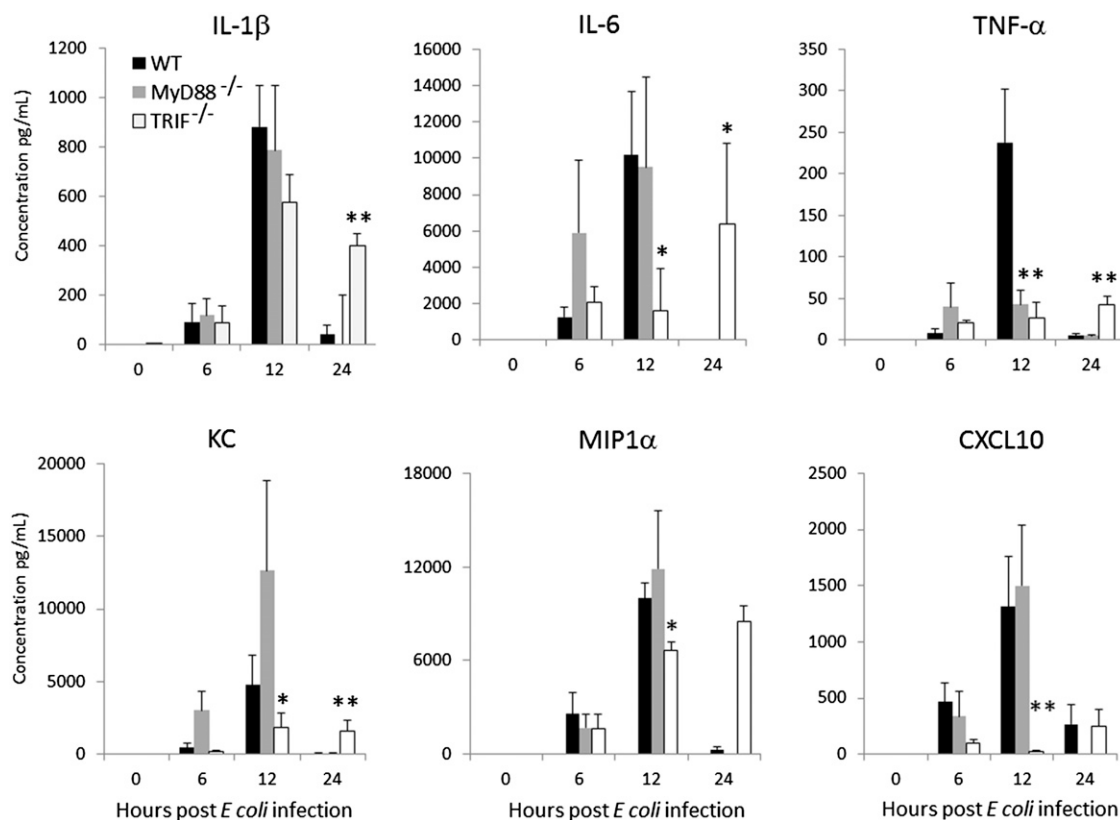


FIGURE 2. Decreased peritoneal inflammatory mediators in TRIF $^{-/-}$ neonates compared with MyD88 $^{-/-}$ or WT neonates. Peritoneal washes were obtained from TRIF $^{-/-}$, MyD88 $^{-/-}$, and WT neonates at 0, 6, 12, and 24 h post-*E. coli* administration and assayed for concentration (values represent mean $\pm$ SD; data were analyzed by either one way ANOVA or Kruskal–Wallis ANOVA; $n = 4$ –5 animals/group; * $p < 0.05$, ** $p < 0.001$).

superoxide dismutase 1 (0.5 U/ml) was used to confirm specificity of superoxide production.

In vitro stimulation with recombinant CXCL10

Splenocytes isolated by Histopaque 1119 (Sigma-Aldrich) gradient were stimulated with or without recombinant murine CXCL10 at 100 ng/ml for 4 h (R&D Systems, Minneapolis, MN). After incubation with CXCL10, cells were washed with PBS and subsequently assayed for phagocytic function or ROS production and phenotypically characterized via flow cytometry.

Functional analysis of splenic macrophages and neutrophils or peritoneal macrophages or neutrophils

For phagocytosis assays, 1×10^5 splenocytes isolated via density gradient or peritoneal cell suspensions were incubated with 1×10^5 fluorescent polystyrene microspheres (Fluospheres; Invitrogen) for 30 min at 37°C. Cells were then stained and analyzed by flow cytometry, as described above. After cells were gated on either neutrophil (Ly-6G⁺, CD11b⁺) or macrophage (F4/80⁺, CD11b⁺) populations, FITC⁺ cells were considered to be phagocytic cells. To assay for ROS production, 2×10^6 splenocytes isolated via density gradient were first stained for cell surface markers and labeled with dihydrorhodamine 1,2,3 (Invitrogen). Cells were then stimulated with 1 μ M PMA (Sigma-Aldrich) at 37°C, and aliquots were evaluated by flow cytometry at various points over a 30-min period using a LSR II flow cytometer (BD Biosciences). A minimum of 1×10^4 live, non-debris cells were collected for analysis.

Statistical analyses

Continuous variables were tested for normality and equality of variances. Differences in survival were determined by Fisher's exact test, whereas differences in continuous variables among groups were evaluated by either one-way ANOVA with either Dunn's or Tukey posthoc analysis or Student *t* test. In those cases where normality failed, either a Mann-Whitney *U* test using the Hodges-Lehmann estimator or a Kruskal-Wallis ANOVA on ranks was also performed. In the latter case, a Dunn's post hoc analysis was performed. However, nonparametric tests are not without their limits. In particular, for small sample sizes the power of the Mann-Whitney *U* versus the Student *t* test is considerably less. In all cases, however, significance was determined at the 95% confidence level.

Results

TRIF^{-/-} neonates, but not adults, are susceptible to Gram-negative sepsis

Though the loss of MyD88 has been associated with susceptibility to Gram-negative and polymicrobial infection in adults, the role of MyD88 in neonatal gram negative infection and sepsis is unclear (13, 14, 24, 25). To address this, TRIF^{-/-}, MyD88^{-/-}, and WT neonates were challenged with 1×10^3 (LD₂₀₋₃₀) CFUs of an *E. coli* isolate (serotype O11:H18) from a neonate with urosepsis and followed for survival. As demonstrated in Fig. 1A, TRIF^{-/-} neonates were more susceptible to *E. coli* challenge compared with both MyD88^{-/-} and WT neonates. This was associated with increased peritoneal and blood CFUs (Fig. 1B). To see if previous observations regarding the adult requirement for MyD88 for survival to Gram-negative infection held true in our model system, we administered 1×10^6 *E. coli* (LD₂₀₋₃₀) ip into adult MyD88^{-/-}, TRIF^{-/-}, and WT mice. Not surprisingly, MyD88^{-/-} adults were dramatically more susceptible to *E. coli* challenge compared with TRIF^{-/-} or WT adults (Fig. 1C). This increased susceptibility of MyD88^{-/-} adults to *E. coli* was associated with increased bacteremia and peritoneal bacterial counts (Fig. 1D). These data suggest intrinsic differences in the TLR signaling adaptor protein pathways that are important for the clearance of Gram-negative infection between neonates and adults.

*TRIF^{-/-} neonates produce decreased local inflammatory cytokines and chemokines early, and increased inflammatory cytokines and chemokines late in response to *E. coli* Gram-negative sepsis*

As the downstream products of TLR signaling are inflammatory mediators, we next examined whether there was any difference in

the production of key cytokines or chemokines in TRIF^{-/-} neonates compared with WT or MyD88^{-/-} neonates that could potentially explain this increased susceptibility to gram negative sepsis. Peritoneal washes from TRIF^{-/-}, MyD88^{-/-}, and WT neonates were harvested at 0, 6, 12, and 24 h post-*E. coli* administration and assayed for the concentration of IL-10, IL-12, IFN- γ , IL-1 β , TNF- α , IL-6, CXCL10, MCP-1, MIP-1 α , and KC via Luminex multiplex cytokine arrays. There were significant reductions in the peritoneal concentration of IL-6, KC, and MIP-1 α , and CXCL10 in TRIF^{-/-} neonates compared with MyD88^{-/-} or WT neonates at 12 h following *E. coli* inoculation (Fig. 2). There were also differences in TNF α concentrations between both MyD88^{-/-} and TRIF^{-/-} neonates compared with WT neonates in response to *E. coli* infection. Concentrations of several cytokines (IL-1 β , IL-6, MIP-1 α , and TNF- α) were significantly elevated in the TRIF^{-/-} neonates but not the WT or the MyD88^{-/-} at 24 h. These increased concentrations were associated with markedly increased bacterial counts seen in the blood and peritoneal fluid in TRIF^{-/-} mice at 24 h (Fig. 1B) and dramatic mortality over the next 24 h.

Increased susceptibility of neonates to Gram-negative infection is not dependent on type 1 IFNs

A recent report by Sotolongo et al. (15) demonstrated that the loss of type 1 IFNs increased the susceptibility of adults to Gram-negative infection and was associated with defects in innate immune phagocytosis. To address whether type 1 IFNs played a role in the survival of neonates to *E. coli*, two approaches were used. First, we administered anti-IFNAR1 or isotype Ab to neonatal mice 6 h before *E. coli* challenge and then subsequently followed them for survival.

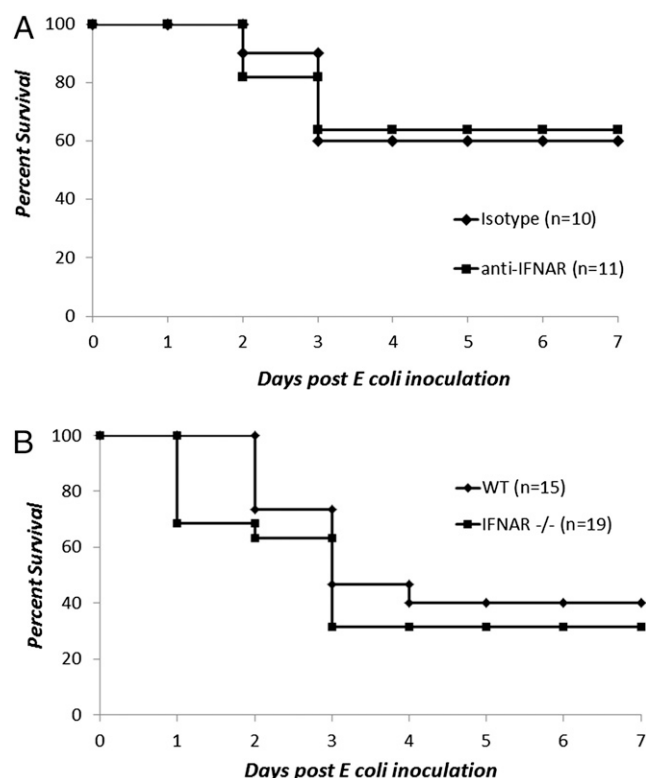


FIGURE 3. Susceptibility of neonates to gram negative infection is not dependent on type 1 IFN signaling. (A) Neonatal mice were administered either anti-IFNAR1 or isotype Ab i.p. 6 h before *E. coli* challenge and followed for 5–7 d for survival (Fisher's exact; no significant differences in survival were noted). (B) Neonatal WT or IFNAR1^{-/-} mice were challenged with *E. coli* and followed for 5–7 d for survival (Fisher's exact; no significant differences in survival were noted).

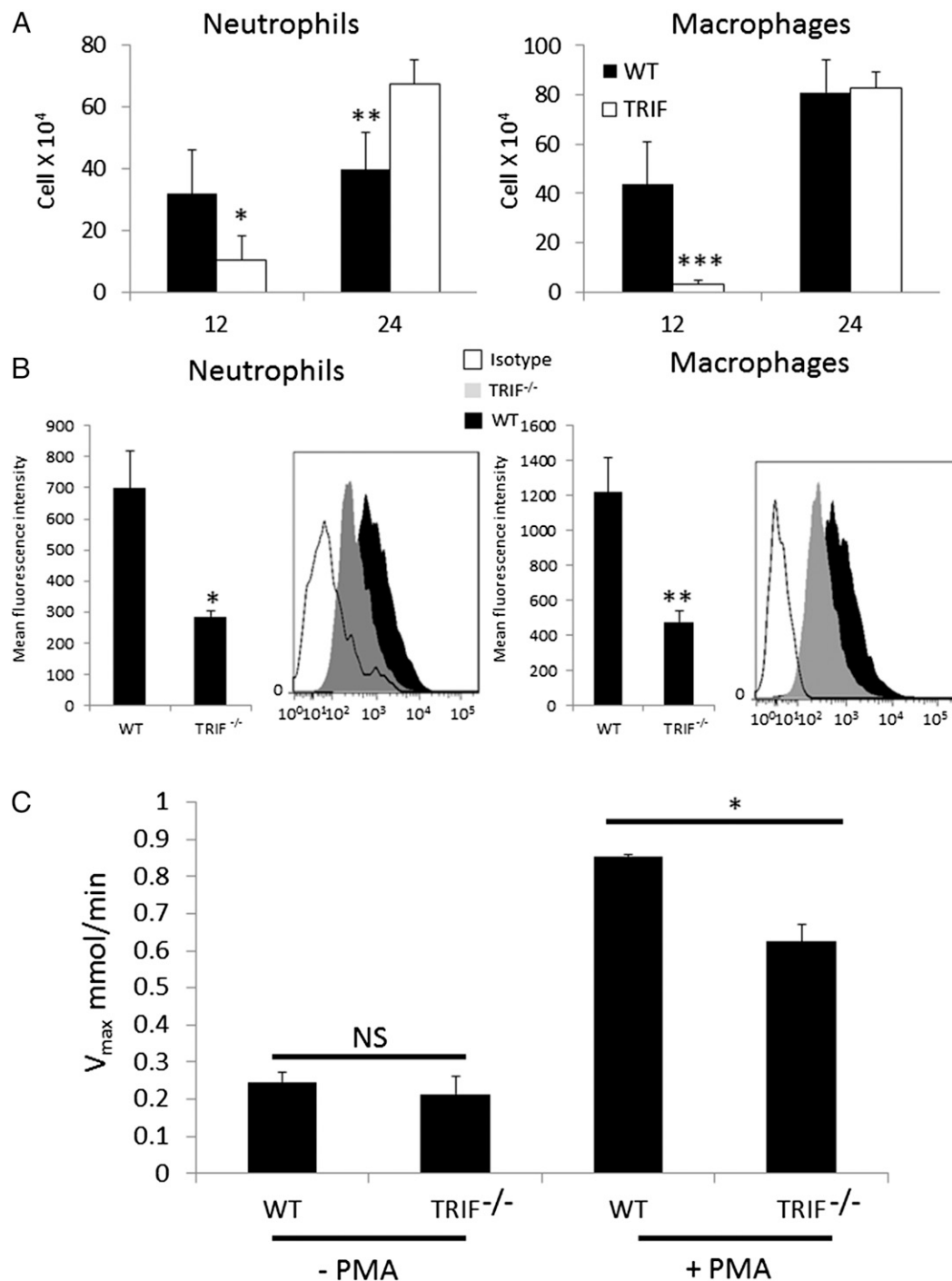


FIGURE 4. Despite increased numbers and phagocytic peritoneal neutrophils and macrophages, TRIF^{-/-} neonates have decreased ROS production 24 h post-*E. coli* infection. **(A)** Peritoneal washes were harvested from WT and TRIF^{-/-} neonates at 12 and 24 h post-*E. coli* inoculation and assayed for the presence of neutrophils and macrophages via flow cytometry (values represent mean $\pm$ SD; Student *t* test or Mann-Whitney *U* test; *n* = 4–5 animals/group; **p* < 0.04, ***p* < 0.01, ****p* < 0.001). **(B)** Peritoneal washes from WT and TRIF^{-/-} neonates 24 h post-*E. coli* were phenotyped for the presence of neutrophils and macrophages and subsequently stained for DHR 123 expression via flow cytometry (values represent mean $\pm$ SD; *n* = 4–5 animals/group; **p* = 0.016, ***p* < 0.001). **(C)** Twenty-four hours following *E. coli* infection, peritoneal washes from WT or TRIF^{-/-} were isolated and stimulated with or without PMA. Maximal rate of cytochrome c reduction (*V*_{max}) calculated (values represent mean $\pm$ SD; *n* = 5 animals/group; Student *t* test **p* < 0.001).

No difference in survival was noted between isotype and anti-IFNAR Ab mice suggesting that the increased susceptibility of TRIF^{-/-} neonates was type 1 IFN independent (Fig. 3A). Second, we repeated the studies in IFN type 1 receptor knockout mice (IFNAR^{-/-}) and WT controls. Both groups of mice were administered an LD₅₀ of *E. coli* and survival was evaluated over the next seven days. Once again, no difference in survival was noted (Fig. 3B).

Decreased early recruitment of peritoneal neutrophils and macrophages and decreased late ROS production in TRIF^{-/-} neonates in response to *E. coli* neonatal sepsis

To understand why TRIF^{-/-} neonates were so susceptible to *E. coli* sepsis compared with WT neonates, we harvested peritoneal washes from TRIF^{-/-} and WT neonates at 12 and 24 h post-*E. coli* infection. TRIF^{-/-} neonates had significantly fewer peritoneal neutrophils

and macrophages compared with WT neonates at 12 h post-*E. coli* administration (Fig. 4A). A representative scattergram of splenic neutrophil (Ly6G⁺,CD11b⁺) or macrophage (Ly6G⁻,F4/80⁺,CD11b⁺) populations is presented in Supplemental Fig. 1. Although no differences existed in the phagocytic function of these innate immune effector cells, TRIF^{-/-} neonatal neutrophils and macrophages produced significantly less ROS than WT neonates at 24 h post-*E. coli* infection at baseline (Fig. 4B). To further support this, we performed a cytochrome *c* reduction assay on peritoneal washes isolated from neonates 24 h following *E. coli*. TRIF^{-/-} mice had significantly slower reduction of cytochrome *c*, and as a corollary decreased ROS production, compared with peritoneal washes isolated from WT neonates (Fig. 4C) ($p < 0.001$). Taken together, these data suggest that TRIF^{-/-} neonates have impaired innate immune neutrophil and macrophage function compared with WT neonates that subsequently impairs clearance of Gram-negative infection.

Deletion of TLR signaling does not affect survival to polymicrobial neonatal sepsis

The data above suggests that TRIF^{-/-} neonatal mice are more susceptible to classic Gram-negative infections such as *E. coli*; however, this does not address the response in a mixed polymicrobial infection that exist in conditions such as necrotizing enterocolitis, an important cause of mortality in premature infants (26). Therefore, to address this, we used a cecal slurry model of peritonitis and sepsis to generate polymicrobial "hit," previously reported by our group (2, 18). Surprisingly, no differences in survival were seen between TRIF^{-/-}, MyD88^{-/-}, or WT animals in response to either an LD₄₀ or LD₇₀ of cecal slurry (Fig. 5).

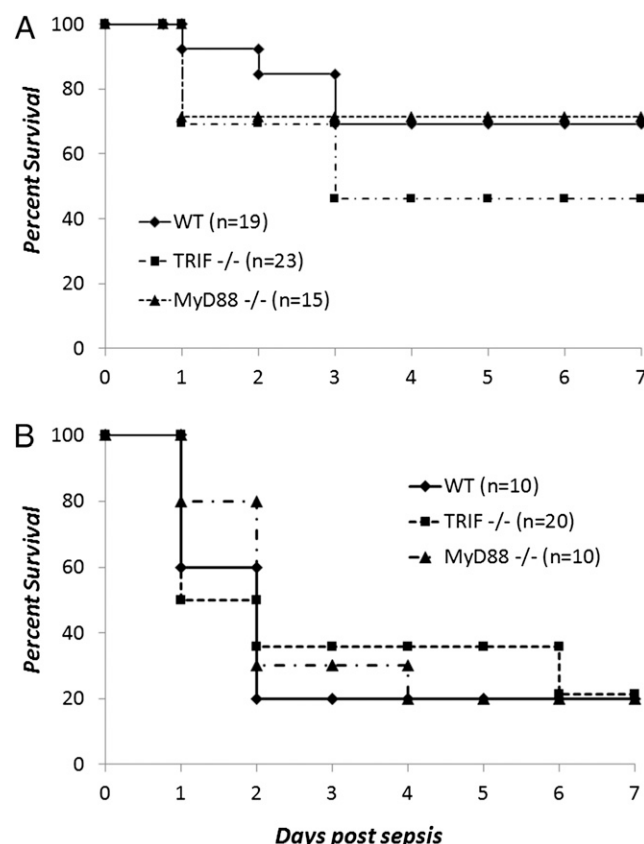


FIGURE 5. Deletion of TLR signaling does not affect survival to polymicrobial neonatal sepsis. WT, TRIF^{-/-}, or MyD88^{-/-} neonates were treated with either an LD₄₀ (A) or an LD₇₀ (B) dose of cecal slurry and then followed for 7 d for survival (Fisher's exact NS).

These data suggest there are other signaling pathways that are able to compensate for the lack of TLR signaling following polymicrobial infection.

TRIF^{-/-} neonates are not protected against polymicrobial sepsis in response to TLR4 adjuvant protection

We have previously shown that WT neonates pretreated with LPS are protected against polymicrobial sepsis compared with neonates pretreated with saline (2). Despite the data above, demonstrating that there is no difference in mortality in response to polymicrobial sepsis, this does not address the adjuvant effect of LPS in this setting. To address this, WT, TRIF^{-/-}, and MyD88^{-/-} neonates were pretreated with LPS (1 μ g/g body weight) 24 h before polymicrobial sepsis challenge, and subsequently followed for survival. As demonstrated in Fig. 6A, in contrast to both MyD88^{-/-} and WT neonates, TRIF^{-/-} neonates were not protected against cecal slurry-induced polymicrobial sepsis. These data suggests that the loss of TRIF not only impacts the inflammatory responses and functional maturity of innate immune effectors cells recruited to the peritoneum, but also is critical for the TLR4 adjuvant protective effect that we have demonstrated previously (2).

Both the phagocytic function and production of neutrophil extracellular traps have been demonstrated to be decreased or impaired in neonatal neutrophils relative to adults (2, 27, 28). To investigate whether TLR signaling through TRIF- or MyD88-dependent pathways played any role in the neonatal neutrophil function, splenocytes were harvested from neonates and stimulated with LPS (100 ng/ml) for 24 h. Following staining for neutrophil cell surface markers, cell suspensions were then either

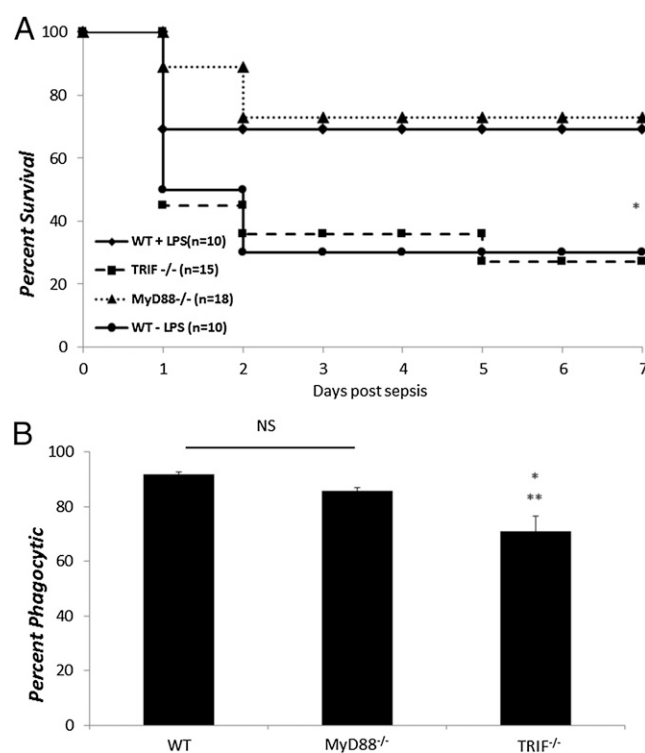


FIGURE 6. TRIF^{-/-} neonates are not protected against polymicrobial sepsis with TLR4 agonist pretreatment. (A) WT, TRIF^{-/-}, and MyD88^{-/-} neonates were treated with either LPS or saline 24 h before cecal slurry challenge and followed for survival (Fisher's exact $*p < 0.01$). (B) Phagocytic uptake of polystyrene FITC-labeled beads in neutrophils following 24 h stimulation with LPS (values represent mean $\pm$ SD; one-way ANOVA; $n = 4$ –5 animals/group; $*p < 0.001$, $**p < 0.004$).

incubated with either FITC-labeled polystyrene beads or evaluated for ROS production via phorbol ester stimulation. Although no differences were noted between neutrophils from MyD88^{-/-}, TRIF^{-/-}, and WT neonates with regards to ROS respiratory burst following PMA stimulation (data not shown), neutrophils from TRIF^{-/-} animals had significantly decreased phagocytic function relative to neutrophils from both MyD88^{-/-} and WT animals in response to LPS ($p < 0.001$) (Fig. 6B).

One of the reasons why neutrophil phagocytic function may be impaired in TRIF^{-/-} neonates may be due to poor inflammatory mediator production, which can have a paracrine effect on innate immune effector function (18, 29). To assess whether the inflammatory cytokine production by inflammatory cell populations

was also impaired in TRIF^{-/-} neonates, media supernatant from neonatal splenocytes stimulated with LPS (100 ng/ml) for 24 h and assayed via Luminex for the concentration of cyto/chemokines, including IL-1 β , TNF- α , IFN- γ , and CXCL10. Although both MyD88^{-/-} and TRIF^{-/-} produced significantly less IL-6, TRIF^{-/-} neonates produced significantly less IL-1 β and CXCL10 (Fig. 7A). These data suggest that deletion of TRIF but not MyD88 in the neonate significantly impairs both neutrophil phagocytic function and cellular inflammatory responses to LPS.

To determine whether this observed decrease in cytokine/chemokine production is recapitulated in vivo, WT, MyD88^{-/-} and TRIF^{-/-} neonates were injected with LPS at a dose of 1 μ g/g body weight, and serum was harvested at 0, 6, and 24 h postinjection.

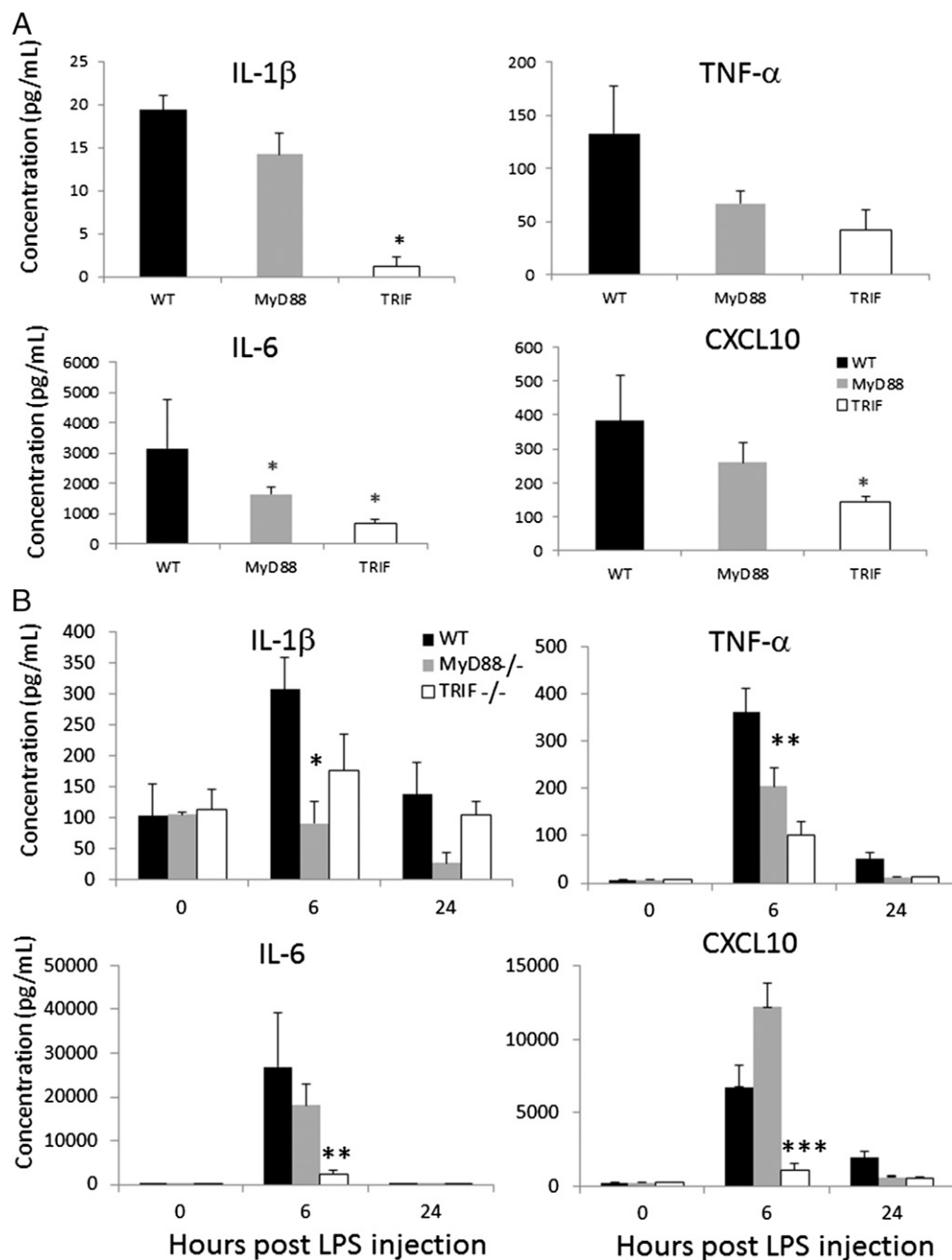


FIGURE 7. (A) Cytokine production from splenocytes isolated from WT, TRIF^{-/-}, and MyD88^{-/-} neonates following 24 h stimulation in vitro with LPS (100 ng/ml) ($n = 3-4$ animals/group) (* $p < 0.05$). (B) Sera cytokine concentrations from WT, TRIF^{-/-}, and MyD88^{-/-} neonates at 0, 6, and 24 h post-LPS administration (values represent mean $\pm$ SD; data were analyzed by either one way ANOVA or Kruskal-Wallis ANOVA; $n = 4-5$ animals/group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.024$).

Interestingly, in contrast to the in vitro data, MyD88^{-/-} neonates produced significantly less IL-1 β and TNF- α compared with WT neonates, whereas TRIF^{-/-} neonates produced significantly less TNF α , IL-6, and CXCL10 (Fig. 7B). These data further support the data above and suggest that TRIF plays a critical role in the endotoxin-mediated inflammatory responses in the neonate.

Reversal of poor phagocytic function of TRIF^{-/-} neonatal granulocytes in the presence of CXCL10

We have previously demonstrated that CXCL10 is important for neonatal and adult neutrophil phagocytosis. Because CXCL10 was significantly decreased following both in vitro stimulation and in vivo administration of LPS in TRIF^{-/-} but not MyD88^{-/-} or WT neonates and TRIF^{-/-} granulocytes had significantly decreased phagocytic function compared with WT or MyD88^{-/-} neonates following LPS stimulation, we treated TRIF^{-/-} and WT neonatal splenocytes with or without 100 ng/ml recombinant murine CXCL10 in the presence of LPS. As demonstrated in Fig. 8, incubation of TRIF^{-/-} splenocytes with CXCL10 partially but significantly reversed the phagocytic function of TRIF^{-/-} neutrophils, 84% with CXCL10 versus 70% without CXCL10, following LPS stimulation ($p < 0.05$). No differences were noted between MyD88^{-/-} and WT neutrophil phagocytic function in the presence of LPS (see Fig. 8). Although we were not able to demonstrate complete reversal of the phagocytic defect in TRIF^{-/-} mice via CXCL10, these data support the importance of TRIF signaling and the production of TRIF-dependent inflammatory mediators in innate immune function.

Discussion

Many studies have focused on the role of MyD88 in response to polymicrobial or Gram-negative sepsis in adults (10–14, 24, 25). Furthermore, these studies have linked or suggested a critical role for TLR4-MyD88 signaling in the survival of adult mice to both polymicrobial and Gram-negative sepsis. Although Akira's group has identified the importance of MyD88 and TRIF signaling in the lethality of adults to endotoxemia, again the loss of MyD88 appears to offer more protection to lethal endotoxin challenge in young adults than the loss of TRIF in these studies (30, 31). TLR signaling in neonates, however, is not as well characterized, although a recent study by Andrade et al. (32) has demonstrated that blockade of TLR2 signaling following group B *Streptococcus* infection improves neutrophil recruitment and improves survival to Gram-positive neonatal sepsis.

Our data suggest that TLR4-TRIF signaling in the neonate, in contrast to the young adult, is critical for response to TLR4 agonists in neonates. We demonstrate that the loss of TRIF in neonates impairs the early production of several inflammatory cytokines and chemokines in response to *E. coli* infection. Mortality is markedly increased in these animals and is associated with systemic bacteremia and an ongoing, exaggerated late inflammatory cytokine response. In addition, following either in vitro stimulation with LPS or in vivo administration, our data show a reduced phagocytic function in neutrophils from TRIF^{-/-} mice compared with neutrophils from WT or MyD88^{-/-} animals. Previous work from our laboratory has also demonstrated a critical role for CXCL10 in the phagocytosis and survival of neonates to polymicrobial challenge (18). In the current study, we show that the loss of TRIF in neonates impairs both in vitro and in vivo CXCL10 production as well as the phagocytic response of neutrophils to LPS. Furthermore, in the current study, we also demonstrate that TRIF^{-/-} neonates are not protected following LPS adjuvant pretreatment to polymicrobial sepsis. Similarly, our recent work shows that if the CXCL10 response to LPS is blocked, the protective adjuvant ef-

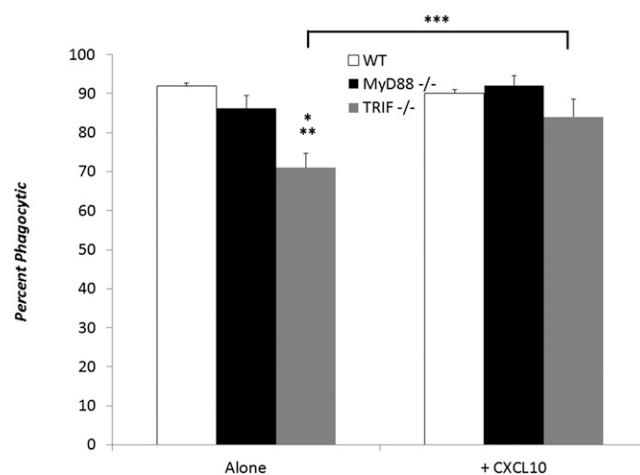


FIGURE 8. Splenocytes from WT, MyD88^{-/-}, or TRIF^{-/-} neonates were stimulated with LPS (100 ng/ml). TRIF^{-/-} splenocytes were also either stimulated with or without CXCL10 (100 ng/ml) (values represent mean $\pm$ SD; data were analyzed by either one-way ANOVA; $n = 4$ –5 animals/group; *WT versus TRIF^{-/-} $p < 0.01$, **MyD88^{-/-} versus TRIF^{-/-} $p < 0.05$, Student t test ***TRIF^{-/-} versus TRIF^{-/-} + CXCL10 $p < 0.05$).

fect of LPS is lost in response to polymicrobial sepsis (18). These findings suggest a novel critical role for a TRIF-dependent inflammation in response to LPS and Gram-negative infection. Importantly, we also demonstrate that the same TRIF signaling is not as critical in adults and suggest a reliance of neonates on TRIF signaling in contrast to adults who appear to be more dependent on MyD88 for response and survival to gram negative infection.

Although analysis of cytokines at individual time points can be difficult to interpret, we also attempted to compare the cytokine production of WT, TRIF^{-/-}, and MyD88^{-/-} neonates at 0, 12, and 24 h post-*E. coli* inoculation. Of the presented cytokines/chemokines, IL-1 β , TNF- α , KC, and MIP-1 α , were all found to be significantly ($p < 0.05$) higher in the TRIF knockout animals compared with either MyD88^{-/-} or WT animals at 24 h post-*E. coli* inoculation (Fig. 2). In addition, we found that the numbers of neutrophils were significantly greater in the TRIF knockout animals compared with WT animals at 24 h post-*E. coli* infection (Fig. 4A). However, as shown in Fig. 1C, TRIF^{-/-} neonates have significantly more bacterial CFUs than either WT or MyD88^{-/-} animals at 24 h post-*E. coli* inoculation. We postulate that the increases in peritoneal neutrophils and cytokines/chemokines that were measured are more a reflection of the overwhelming *E. coli*-dependent sepsis that is occurring in the TRIF^{-/-} animals at 24 h postinoculation compared with the MyD88^{-/-} or WT animals.

Although a recent report by Sotolongo et al. (15) has demonstrated that TRIF signaling is important for the adult survival to Gram-negative infection, it is important to note that their model of infection used the Gram-negative enteropathogen *Y. enterocolitica*, not *E. coli*, which may indeed have different immunopathological processes and requirements for clearance despite both being Gram-negative organisms. In addition, susceptibility of MyD88^{-/-} adults to Gram-negative infection was not examined in the paper but was rather inferred from data investigating phagocytic function of MyD88^{-/-}, TRIF^{-/-}, TLR4^{-/-}, and WT macrophages. Finally, the authors stressed and demonstrated the importance of TRIF-dependent type I IFN production in the survival of adult mice to Gram-negative infection. In a previous paper, we have demonstrated that loss of type I IFN signaling is not important for survival of neonates to Gram-negative infection (2). Although it was in a polymicrobial model of neonatal sepsis, most of the enteric organisms

within the cecum that are administered through cecal slurry challenge are Gram negative (>99%).

These data highlight important differences between the neonatal and adult responses to bacterial products and gram negative infection. Previous data have suggested that neonates rely more heavily on a functioning innate immune system for protective immunity than do young adults. This paper moves that observation forward and, to our knowledge, demonstrates for the first time the novel finding that TRIF but not MyD88 signaling is essential for innate immune activation and is required for survival of neonates to Gram-negative infection. This is important because much of the focus on early signaling pathways of innate immunity to microbial infection has been on MyD88, as it is the primary adaptor protein responsible for downstream signaling for the majority of TLRs (30). In addition, these data suggest an ontological difference between adults and neonates that reflect differential requirement for TRIF-dependent inflammation during the neonatal period and redirect focus on therapeutics designed around TRIF-dependent inflammation and innate immune activation to improve outcome in these particularly susceptible patients.

Disclosures

The authors have no financial conflicts of interest.

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