Hyper innate responses in neonates lead to increased morbidity and mortality after infection

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Neonates suffer high morbidity and mortality in infection, presumably because of the lack of a fully developed adaptive and innate immune system. Evidence of poor innate responses in neonates has been shown by using a model that sensitizes the host to Toll-like receptor (TLR)-mediated inflammation with D-galactosamine (D-GalN). However, we show that neonatal mice demonstrate much stronger inflammatory responses than adult mice in response to LPS stimulation, and such hypersensitivity extends to other TLR agonists including actual viral infection. Our study reveals that the ensuing inflammatory reaction after D-GalN sensitization reflects preferential toxicity of D-GalN to adult liver cells, rather than accurately reflecting the TLR response to LPS. We show further that an uncontrolled proinflammatory innate response due to inadequate T cells makes neonates more vulnerable to TLR agonists or viral infection. Remarkably, through transfer of T cells into neonates or depletion of T cells in adult mice, we show that T cells are sufficient and necessary to control the early inflammatory response to LPS. Therefore, neonates might suffer from the unleashed innate responses caused by an insufficient number of T cells, which leads to increased morbidity and mortality.

Toll-like receptors (TLRs) are essential in innate immune cell recognition of invading microorganisms and transduction of signals to adaptive immune cells to orchestrate pathogen clearance over time (1, 2). However, excessive innate immune activation of proinflammatory cytokines can be harmful, leading to microcirculatory dysfunction, tissue damage, shock, or even death of the host (3–6). Our recent finding suggests that conventional T cells play a critical role in tempering such unleashed inflammatory responses by innate cells in adult mice (7). The interplay between adaptive and innate immune system in the neonate, however, has not been defined.

Complete maturation of the immune response occurs gradually after birth. The neonatal immune response has been shown to be quantitatively and qualitatively distinct from that of adults, rendering neonates more susceptible to infection (8, 9). Whether and how the innate immune response in neonates contributes to their susceptibility to infection or TLR stimulation is still controversial. In human, it has been reported that mononuclear cells from cord blood secrete lower amounts of TNF than those from healthy adults in response to TLR agonists (10–13), whereas other studies draw opposite conclusions (14–16). Results from studies in mice also conflict because neonates produce either lower (17, 18) or higher (19) levels of TNF compared with adults. The most consistent observation, however, is that the ratio of IL-6 to TNF is higher, whereas IFN-γ is defective in neonates (10, 11, 20). However, whether and how these changes of proinflammatory cytokines will have any impact *in vivo* in response to infection or TLRs stimulation has not been well defined.

The adaptive response in neonates is characterized by defects in T helper 1 (Th1) cell-polarizing cytokines (8, 21). Furthermore, T cells in neonatal mice are 1–2 logs fewer than in adult mice (21, 22). If large numbers of naïve T cells are required to temper the early innate responses to protect the hosts from harmful "cytokine storm" (7), a lack of sufficient T cells in neonates may result in stronger innate responses. To clarify whether innate cells in neonates are more or less sensitive to TLR stimulation, we investigate the status of innate cells of neonates in response to TLR stimulation both *in vitro* and *in vivo*. We report in the present work that neonatal mice are much more susceptible to various TLR stimuli and viral infection. In contrast to earlier studies that suggest a reduced innate response in neonates, we demonstrate that the neonatal proinflammatory response is quite robust. In fact, because of the low number of T cells in the periphery, neonatal mice could more likely undergo a lethal, uncontrolled innate immune response to strong TLR stimulation or severe viral infection.

Results

Neonatal Mice Are Paradoxically Hypersusceptible to LPS and Other TLR Stimulation. It is hard to predict whether neonates suffer from overzealous inflammatory response during severe infection in clinical studies, partly owing to inconsistent readouts of neonatal response to TLR agonists. We therefore used a high-dose LPS model, which is a more clinically relevant recapitulation of sepsis, to assess TLR response in neonatal mice. In contrast to previous reports that the innate immune response is impaired in neonates, proinflammatory cytokine levels were much higher in neonatal than in adult mice in response to a high dose of LPS alone (Fig. 1*A*). To analyze susceptibility to other TLR stimulation, neonatal and adult mice were administered the TLR3 stimulus poly(I:C), and again, neonatal mice produced much more vigorous inflammatory responses (Fig. 1*B*). Similarly, higher cytokine production could be detected in neonates than in adults after murine hepatitis virus (MHV)-A59 infection [supporting information (SI) Fig. S1]. To confirm this, splenocytes from neonatal and adult mice were isolated and incubated with various TLR stimuli *in vitro*. As expected, cytokine production in response to LPS, poly(I:C), or MHV-A59 stimulation was much greater by neonatal than adult splenocytes (Fig. 1*C*).

To address whether this dramatic increase in cytokine production, suggestive of a cytokine storm, had any bearing on survival, we analyzed the responses to LPS in mice of various age groups. Under the same LPS dose (10 mg/kg), neonates (days 1 and 7 after birth) died within 24–48 h, whereas all 2- and 10-week-old mice survived (Fig. 2*A*). This increased lethality in neonatal mice was not limited to LPS (TLR4) but was also observed in poly(I:C) (TLR3) treatment (Fig. S2). Neonatal

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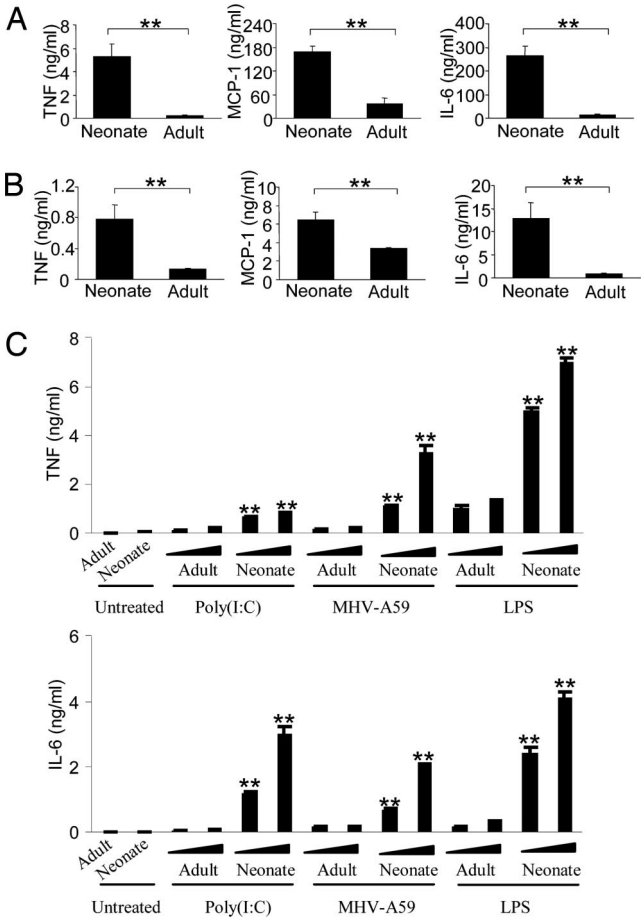

Fig. 1. Neonatal mice produce stronger inflammatory responses to high-dose LPS and other TLR stimulation. (*A* and *B*) Neonatal and adult mice were administered 10 mg/kg LPS (*A*) or 40 mg/kg poly(I:C) (*B*), and serum proinflammatory cytokines were detected at 6 h after injection (n = 3–5). (*C*) Splenocytes (1×10^6 and 2×10^6) from neonatal and adult mice were stimulated with 50 μg/ml poly(I:C), 5×10^5 pfu MHV-A59, or 100 ng/ml LPS for 20 h, and the culture supernatants were harvested and analyzed for detection of cytokines. **, $P < 0.01$ by t test. Data are presented as mean values (±SEM).

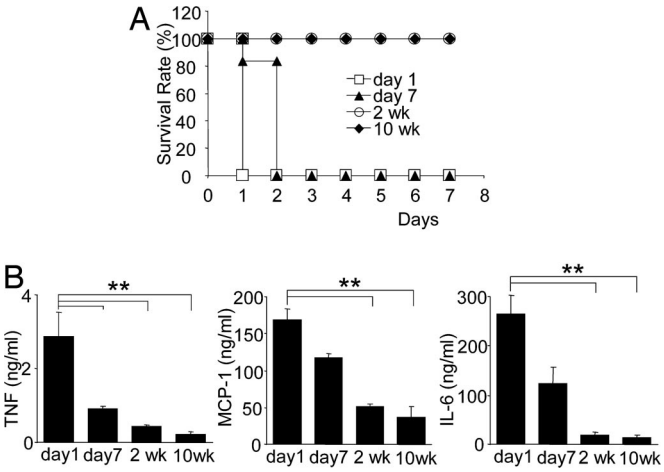

Fig. 2. Age-dependent sensitivity to LPS alone. Mice of different ages (1-d-, 7-d-, 2-week-, and 10-week-old; n = 6–10 for each group) were injected with 10 mg/kg LPS. Survival was monitored for 7 d (*A*) and serum inflammatory cytokines were detected at 6 h after injection (*B*). **, $P < 0.01$ by t test. Data are presented as mean values (±SEM).

mice were also more sensitive to live-virus infection than adult mice, because the latter survived well an MHV-A59 virus challenge at doses absolutely lethal to newborns (Fig. S3). Surprisingly, the inflammatory cytokines (TNF, MCP-1, and IL-6) in serum after LPS treatment gradually decreased as the age of the mice increased, demonstrating an age-dependent characteristic of the sensitivity to LPS (Fig. 2*B*). Collectively, it is likely that the sensitivity to TLR stimulation is age-dependent, and neonatal mice are definitely more sensitive to MHV infection or TLR stimulation.

Neonatal Mice Are More Resistant to LPS/D-GalN Challenge than Adult Mice. The increased morbidity and mortality characteristic of neonatal infection is thought to be caused by underdeveloped innate and adaptive immunity. A recent study indicates that neonates are more resistant to a lower dose of LPS than adult mice after being sensitized by D-GalN, presumably because of an impaired innate response in the newborn host (18). The LPS plus D-GalN system is widely used to study LPS-induced liver injury mediated by macrophages (23–25). Consistently, LPS/D-GalN treatment caused all adult mice to die within 12 h, whereas newborns survived (Fig. 3*A*). Severe liver injury by LPS/D-GalN could be a lethal factor, because serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), a functional readout for liver damage, increased drastically in adult mice in contrast to the slight increase in neonatal mice at 6 h after treatment (Fig. 3*B*). Serum levels of TNF, MCP-1, and IL-6 were more pronounced in adult than neonatal mice (Fig. 3*C*), agreeing overall with the surge of ALT/AST. We were intrigued by the seemingly conflicting results to our observation that neonatal innate responses were greater than those by adults after infection or TLR stimulation. To synthesize a possible explanation for the opposing outcomes, we delved further into the role of D-GalN in the low-dose LPS model used by others.

D-GalN Induced More Severe Hepatotoxicity in Adult Mice than in Neonatal Mice. We reasoned that the different outcomes of LPS/D-GalN and LPS models might reside in D-GalN, a sensitizer of liver damage and lethality induced by endotoxin, superantigen, lipoprotein, or bacteria (26). Because the toxic effects of D-GalN in hosts of different ages have not been well defined, we analyzed the hepatotoxicity of D-GalN in neonatal and adult mice. D-GalN inflicts liver injury by increasing toxic intermediary metabolites of uracil nucleotides in liver, which eventually leads to the depletion of hepatic glycogen (27, 28). As we expected, D-GalN treatment caused significant hepatic glycogen decrease in adult mice, whereas it maintained a rather constant level in neonatal mice (Fig. 3*D*). Furthermore, D-GalN *per se* led to a remarkable increase of ALT and AST in adults, whereas ALT and AST in neonates showed minimal increase (Fig. 3*E*). Histological study also depicted more severe liver pathology by D-GalN in adult than in neonatal mice (Fig. 3*F*). Therefore, D-GalN in the low-LPS model might serve as a key that reverses the age-dependent response to TLRs stimulation, as we observed in the high-dose LPS model. Therefore, one should be cautious in using D-GalN as a sensitizer for other immune stimuli because it may disguise the underlying TLR signaling rather than an erroneous hepatotoxicity by D-GalN. Having reconciled the different outcomes and having confidence in the consistency between the response to high-dose LPS and actual infection, we further investigated the mechanisms by which neonates are more vulnerable to TLR-mediated death.

Increased Production of Proinflammatory Cytokines to LPS Stimulation Is Related to Low Numbers of T Cells in Neonates. With the high-dose LPS or infection model, our recent data show that

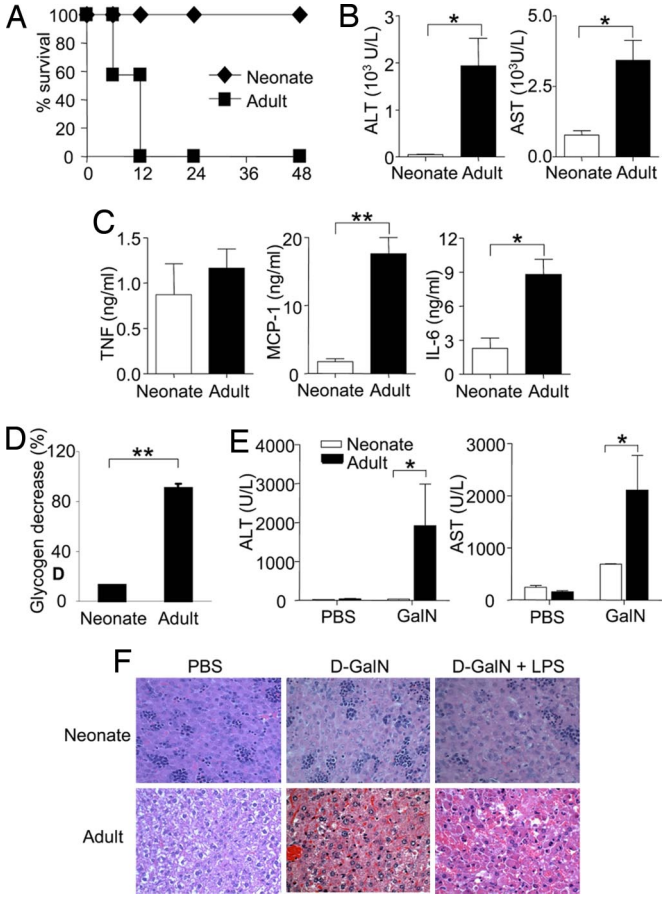

Fig. 3. Neonatal mice are insensitive to low-dose LPS challenge, and D-GalN induces more severe hepatotoxicity in adult mice. (*A*) Mortality curves of neonatal (♦, $n = 6$) and adult (■, $n = 7$) mice after injection with 0.5 mg/kg LPS and 0.35 g/kg D-GalN. (*B*) Serum ALT and AST were detected 6 h after injection. (*C*) Serum proinflammatory cytokines (TNF, MCP-1, and IL-6) were detected 2 h after injection ($n = 5$–6). (*D*) Neonatal (day 7) and adult mice were injected with 0.35 g/kg D-GalN, and glycogen in liver was detected at 6 h after injection. Data were presented by percentage of glycogen decrease after D-GalN treatment ($n = 4$–5). (*E*) Neonatal (day 7) and adult mice were injected with 3 g/kg D-GalN, and serum ALT and AST were detected at 24 h after injection ($n = 4$–5). (*F*) H&E staining of formalin-fixed sections of livers from neonates or adults treated with PBS, D-GalN, or D-Gal/LPS. *, $P < 0.05$; **, $P < 0.01$ by *t* test. Data are presented as mean values (±SEM).

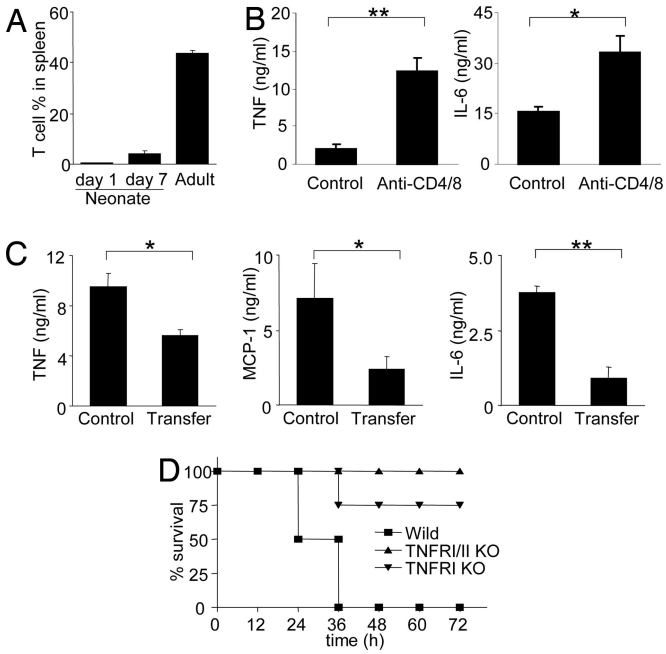

Fig. 4. Higher production of proinflammatory cytokines during LPS stimulation is related to low numbers of T cells in neonates. (*A*) The percentages of T cells in the spleens of neonatal and adult mice ($n = 3$). (*B*) TNF and IL-6 in serum of WT mice and mice depleted of T cells were detected at 2 and 6 h, respectively, after 10 mg/kg LPS injection ($n = 4$; **, $P < 0.01$). (*C*) Neonatal mice were adoptively transferred with 3×10^7 T cells purified from adult mice. Cytokines were detected 2 h after 10 mg/kg LPS ($n = 3$–4; *, $P < 0.05$; **, $P < 0.01$). Data are presented as mean values (±SEM). (*D*) Lethal dose of LPS (20 mg/kg) was injected to WT, TNFRI KO, or TNFRI/II KO neonatal mice. Survival was recorded for 7 d.

adult mice deficient in T cells are far more sensitive than WT adult mice to unleashed proinflammatory cytokine response (7). We therefore speculated that the enhanced innate response to LPS in neonatal mice may result from their insufficient numbers of T cells. As indicated by the percentage of total splenic lymphocytes, the T cell number was much lower in neonates than in adults (Fig. 4*A*). To confirm that hypersusceptibility to LPS was attributable to insufficient T cell numbers, we first used CD4- and CD8-specific antibodies to deplete T cells in adult mice, and the depleted mice produced more inflammatory cytokines than the control mice upon LPS treatment (Fig. 4*B*). To assess whether T cells are sufficient to control the inflammatory responses in neonates, we adoptively transferred T cells isolated from adult mice into neonatal mice and found that the reconstituted mice produced lower levels of proinflammatory cytokines than control mice (Fig. 4*C*). Therefore, T cells are essential and sufficient to control the innate responses to LPS stimulation in immunocompromised hosts such as neonates. Furthermore, to determine whether TNF is critical to LPS-mediated lethality, neonatal WT, TNFRI$^{-/-}$, or TNFRI/II$^{-/-}$ mice were administered LPS. Neonatal mice of either TNFRI$^{-/-}$ or TNFRI/II$^{-/-}$ background survived LPS challenge, whereas WT neonatal mice died in 1–2 d (Fig. 4*D*). Therefore, it is possible that high levels of TNF in neonates after LPS challenge may contribute to their increased death rates in response to TLR stimulation or infection.

T Cells Control the Production of Inflammatory Cytokines by Neonatal Splenocytes upon TLRs Stimulation. To further validate the role of T cells in suppression of the cytokine surge in neonates upon TLR stimulation, we next isolated splenocytes from neonatal mice and determined levels of cytokine production in response to LPS, poly(I:C), or MHV-A59. Impressively, cytokine production by neonatal splenocytes was mostly controlled by the addition of adult T cells (Fig. 5*A*). To explore whether T cells from neonatal mice were qualitatively or functionally different from adult T cells in tempering innate responses, we next compared the T cells from neonatal and adult mice in this system. Neonatal splenocytes were divided into non-T and T cell populations. Stimulation of non-T cells with LPS in the presence of equal numbers of neonatal or adult T cells showed that both could suppress cytokine production with the same efficiency (Fig. 5*B*). Therefore, it is likely that the much lower number of T cells in neonates accounts for the unrestrained innate response to TLR stimulation, which leads to higher susceptibility to TLR stimulation and acute infection.

Discussion

In response to infection, there is a delicate balance between allowing the efficient immune response to target the pathogen and preventing widespread overactivation. In immunocompetent adults, the various arms of the immune system carefully

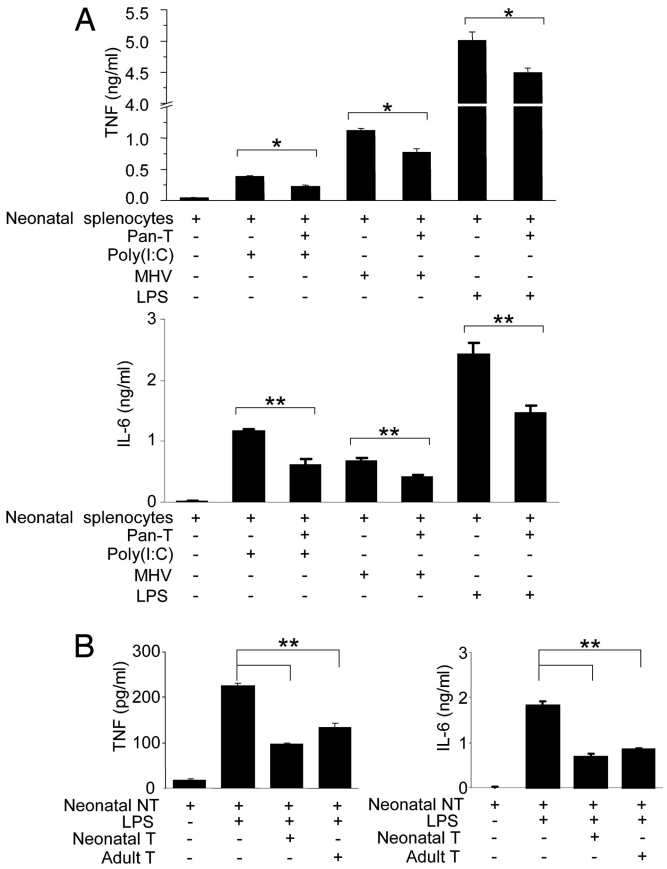

Fig. 5. T cells restrict inflammatory cytokine production by neonatal splenocytes. (*A*) Neonatal splenocytes (1×10^6) were stimulated with 50 μg/ml poly (I:C), 5×10^5 pfu MHV-A59, or 100 ng/ml LPS in the presence or absence of 2×10^6 pan-T cells. (*B*) Neonatal splenocytes (1×10^6) were stimulated with 100 ng/ml LPS in the presence or absence of 2×10^6 T cells purified from neonatal or adult mice. The culture supernatants were harvested 20 h after coculture and analyzed with Cytometric Bead Array (CBA) (BD Biosciences) for detection of cytokines. *, $P < 0.05$; **, $P < 0.01$ by *t* test. Data are presented as mean values (±SEM).

orchestrate this equilibrium. However, in neonates, the immune system has yet to fully mature, potentially leaving newborns susceptible to either infection or inappropriate activation. Lacking a fully developed adaptive immune system, newborns must rely on the innate immune response for protection against pathogens (29, 30). However, intrinsic deficiencies in the capability of neonatal innate immunity lead to inefficient clearance of pathogens (9). On the other hand, the idea that proinflammatory cytokines are produced by innate cells of newborns is quite controversial, with either impaired (10, 11, 31) or elevated (14–16) TNF production in human newborns being reported. The difficulties in comparison of *in vitro* and *in vivo* results and the age differences of studied newborn babies may account for some of these different outcomes.

In its use of corresponding *in vivo* and *in vitro* systems, our study has provided yet another mechanism to understand why neonates are more susceptible to certain infections or TLR agonistic stimulation. We have shown that neonatal mice are fully capable of mounting much stronger inflammatory responses than adult mice to LPS, poly(I:C), and live-virus infection. This is consistent with previous observations in mice (19). More profoundly, human newborns also demonstrate an age-dependent decay of transient hyperresponsiveness to LPS, very reminiscent of our data in Fig. 2 (15). Moreover, newborns are characteristic for diminished IFN-γ, a T_H1-cell-polarizing cytokine, and monocyte-derived macrophages from neonates are hyporesponsive to activation by IFN-γ (32). In accord with this notion, we also observed that the serum IFN-γ level is much lower in neonatal mice than adults in response to TLR stimulation including virus infection (data not shown). Finally, our study demonstrates that TNF is responsible for LPS-induced lethality in neonatal mice (Fig. 4*D*). Consequently, we conclude that neonatal mice have elevated inflammatory responses to TLR3 and TLR4 stimulation compared with adults, which contributes to the high morbidity and mortality in infection among neonates.

It is noteworthy that, contrary to our findings, a recent study shows that the defective inflammatory response of neonatal mice due to increased IL-10 production by a B1 subset of B cells may increase their survival rate upon stimulation by various TLR agonists (18). The major difference between the studies is the use of D-GalN. D-GalN not only increases toxic metabolites of uracil nucleotides in liver cells but also sensitizes liver cells to TNF (26). Because D-GalN is more toxic in adult mouse liver (Fig. 3), in agreement with the results in rats (27, 33), and D-GalN makes adult mice more sensitive to a wide range of LPS doses (0.1–4 mg/kg) than neonates (data not shown), we reason that the age-dependency of D-GalN hepatotoxicity should be subtracted before comparison of inflammatory response to TLR agonists in neonates and adults.

Although neonatal immunity has been investigated compartmentally, most studies have not synthesized the interplay between the innate and adaptive responses. We recently proposed that sufficient naïve T cells are required to temper the early innate responses to protect the hosts from harmful "cytokine storm" (7). Therefore, whether a deficit in sheer numbers or the relative immature functionality of adaptive immune cells may affect the innate immune response in neonates becomes an intriguing puzzle. In the present study, we have observed that the quantity of T cells, which increases in an age-dependent fashion, is negatively correlated with proinflammatory cytokine production and death rate during TLRs stimulation. In fact, the overzealous inflammatory response in neonatal innate cells may directly contribute to their severe immunopathology and high mortality.

Unlike neonatal mice, human newborns have considerable numbers of immune cells in the peripheral blood at birth (34). However, the T cell proportion is smaller than that in adults, especially in infants who are small for their gestational age (SGA) (35–37). Furthermore, there is a steady enlargement of lymphoid tissues during the first 12 years of postnatal life, indicating that lymphocytes gradually fill in lymphoid tissues (38). Therefore, it is not surprising to observe that the up-regulation of various cytokines (IL-6, IL-10, TNF, and IFN-γ) shows a reverse correlation with age in human newborns (15). Although this case of human study exactly agrees with our observation in mice, more studies are needed to determine whether the observation of T cell regulation of innate response in neonatal mice can be extended to human newborns. Extensive clinical evidence already suggests that excessive cytokines in newborns might contribute to mortality and morbidity in postnatal infection. In neonatal sepsis, overwhelming amounts of TNF and IL-6 are detected in serum (39, 40). Necrotizing enterocolitis, an intestinal disease that occurs frequently in preterm newborns, is thought to be due to a robust intestinal epithelial inflammatory responses to bacterial infection (9). Sudden infant death syndrome (SIDS) is another newborn-related disease, and both infections in the 2 weeks before death occur in >40% of SIDS infants and increased IL-6 in the cerebrospinal fluid have been reported (41–43).

To justify the aforementioned overreactive innate immune response in neonates, one must not ignore the qualitative difference between adult and neonatal T cells, even though

newborns can mount adult-like T cell immune responses under certain circumstances (21, 44, 45). First, most peripheral T cells in neonates are recent thymic emigrants (RTE) and significantly reduce in the adult peripheral T cell pool (46–49). RTE differ from resident naïve T cells and contribute to homeostatic regulation and diversity maintenance of the T cell repertoire (49, 50). In addition, peripheral T cells in both mouse and human newborns are a mixture differentiated from fetal and postnatal hematopoietic precursors (51–53), each lineage showing distinct characteristics (54, 55). Consequently, it is rather complex and intriguing to investigate whether RTE in neonates are functionally intact in the aspect of regulating inflammatory response. Our data show that, at least in our system, neonatal T cells have the same or even elevated ability to control TNF and IL-6 production in response to LPS as adult T cells (Fig. 5). Thus, we speculate that it is the insufficient number rather than the type/function of T cells in neonates that unleashes the inflammatory response to TLR stimulation. The increase in T cells might reflect an evolutionary adaptation for adult mammals to efficiently control both the divergent microbial intrusion and accompanying inflammation. Because different TLR agonists may activate distinctly innate and adaptive immune response *in vitro* or *in vivo* (18), further studies using other TLR stimuli or infections and better characterized T/innate cell interaction may help clarify the function of neonatal T cells.

When the neonatal immune system is considered to be immature, it is usually assumed that further development is needed in order for it to eradicate pathogens. We propose, however, that maturity of the immune response encompasses not only the ability to produce inflammatory cytokines but also the ability to regulate such response accordingly. In this respect, the neonatal immune system is indeed immature. Because neonates rely profoundly on the innate immune system, this biased inflammation may help improve their chances of clearing pathogens at the risk of excessive activation and death. It is therefore tempting to conclude that a sufficient number of T cells is vital for protecting neonates from the lethal inflammatory response of innate cells, a hypothesis that may provide insights into the high morbidity and mortality of newborns in response to infection.

Materials and Methods

Mice. C57BL/6 mice were purchased from Vital River, and TNFRI$^{-/-}$ and TNFR I/II$^{-/-}$ mice of C57BL/6 background were purchased from The Jackson Laboratory. All mice were maintained in animal facilities under specific pathogen-free conditions. Neonatal mice were 1 or 7 days old, and adult mice were 8–10 weeks old. The handling of mice and experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences.

Depletion and Adoptive Transfer of T Cells. For depletion of T cells, anti-CD4 and anti-CD8 antibodies were injected into adult mice i.p. at days 4 and 1 before LPS injection. For adoptive transfer of T cells to neonates, 3×10^7 T cells purified from adult mice were injected i.p. 60 h before LPS injection.

Isolation of Splenocytes and Non-T and Pan-T cells. Splenocytes were obtained, and non-T and pan-T cells (purity >95%) were isolated by magnetic activated cell sorting (MACS; Miltenyi Biotec) precisely as described (7).

LPS, poly(I:C), and MHV-A59 Stimulation *in Vitro* and *in Vivo*. MHV-A59 virus was prepared as previously described. LPS (*Escherichia coli* O111:B4), poly(I:C), and D-GalN were purchased from Sigma–Aldrich. Splenocytes from adult, neonatal, or T cell-depleted mice were stimulated with 100 ng/ml LPS, 50 μg/ml poly(I:C), or 5×10^5 pfu MHV-A59 in the presence or absence of T cells for 20 h before cytokine analysis as described (7). Suckling pups from each litter were randomly assigned to experimental groups, marked, and kept with the mother until completion of the experiments. Mice were injected i.p. with 0.5 mg/kg LPS mixed with 0.35 g/kg D-GalN, 10 or 20 mg/kg LPS alone, 40 mg/kg poly(I:C), or 2×10^3- 6×10^3 pfu/g MHV-A59.

Analysis of Liver Injury. The glycogen content of liver was estimated as described (56). Serum ALT and AST were measured as described (7). Liver pathology was assessed by H&E staining for necroinflammation by a pathologist blinded to the treatment groups.

Statistical Analysis. The Mann–Whitney test (for survival) and Student's *t* test were used. Error bars represent SEM. $P < 0.05$ was considered statistically significant.

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