Molecular mechanisms underlying anti-inflammatory phenotype of neonatal splenic macrophages

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Abstract: Neonatal humans and rodents are susceptible to infection with encapsulated bacteria as a result of an inability to make antibodies to capsular polysaccharides. This is partly a result of decreased production of proinflammatory cytokines by splenic macrophages (MΦ) from neonates. In this study, we show that when stimulated with a variety of agonists to TLR2, -4, and -9, neonatal MΦ make less proinflammatory cytokines and more IL-10 than adult MΦ. IL-10 appears to have a role in the decreased proinflammatory cytokine production, as neonatal MΦ treated with anti-IL-10 receptor antibody or from IL-10$^{-/-}$ mice produced levels of proinflammatory cytokines at a level comparable with that produced by adult MΦ. A microarray analysis of RNA from resting and LPS-stimulated MΦ from neonatal and adult mice showed that expression of a large number of genes encoding cytokines, chemokines, and their receptors was decreased dramatically in the neonatal MΦ, although some cytokines, including IL-10 and IL-16, were enhanced. Several genes in the TLR signaling pathway leading to NF-κB activation were down-regulated, which may account for the decreased chemokine and cytokine synthesis. It is surprising that p38α MAPK, known to be required for TLR-induced cytokine secretion, was enhanced in the neonatal MΦ. Our studies with the p38 MAPK inhibitor SB203580 suggested that excess p38 MAPK activity can be inhibitory for TLR2-, -4-, and -9-induced production of proinflammatory cytokines but not IL-10. The anti-inflammatory phenotype of the neonatal Mϕ may be unique to the developing organism, although it compromises the neonate's ability to respond to encapsulated bacteria. *J. Leukoc. Biol.* 82: 403–416; 2007.

Key Words: *TLR · LPS · CpG · cytokines · p38 MAPK · microarray*

INTRODUCTION

Young children, ranging from 6 months to 2 years of age, have an increased susceptibility to infection with *Streptococcus pneumoniae*, resulting in 1 million children dying every year worldwide as a result of pneumococcal infections [1]. The antibody production against the capsular polysaccharides is defective in neonates [2]. This response is critical for opsonophagocytosis and killing of the bacteria by granulocytes. The cellular basis of the inability of neonates to mount this antibody response has not been understood completely. Although neonatal B cells are unlike adult B cells in being immature and thus, unable to respond to polysaccharide antigens, we demonstrated that neonatal B cells can be induced to respond to such polysaccharide antigens when supplemented with IL-1 and -6, cytokines produced by macrophages (MΦ) [3]. Our previous studies have established a role for MΦ in antibody responses to polysaccharide antigens. In a mixed culture experiment, we found that B cells from adult mice failed to respond to polysaccharide antigens when MΦ from neonatal mice were provided, whereas they responded well when MΦ from adult mice were provided [4]. This was the first demonstration that MΦ from neonates were defective in their ability to help polysaccharide-responsive B cells.

Our results led us to investigate further whether splenic MΦ from neonates have similar defects in their response to other microbial-derived molecules. It is now clear that several microbial products use a series of evolutionary, well-conserved receptors, which belong to the family of TLRs [5, 6]. Presently, the number of TLR molecules ranges from 11 to 15, depending on the species. They bind a variety of bacterial- and viral-derived agonists such as peptidoglycan (PGN), lipoteichoic acid, flagellin, lipopeptides, single- and double-stranded RNA, unmethylated DNA containing CpG sequences, as well as host-derived heat shock proteins. This list of agonists is presently in the 30s, but it has been growing constantly. The TLR agonists use a conserved, intracellular signaling pathway leading to activation of NF-κB, MAPKs, and IFN regulatory factor 3 (IRF3), the ultimate target varying with each TLR [7, 8]. We focused on TLR2, a receptor shown to be involved in immune responses to *S. pneumoniae* as well as to *Listeria monocytogenes*; TLR4, important for response to LPS from Gram-negative bacteria; and TLR9, which recognizes unmethylated DNA from bacteria [9–13]. Although the TLRs differ in the adaptor molecules used for signal transduction, a majority of them uses

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the MyD88 molecule to activate IL-1 receptor (IL-1R)-associated kinases (IRAKs), which in turn, leads to activation of TNF receptor (TNFR)-associated factor 6 (TRAF6), feeding into the NF-κB and MAPK pathways [5].

MAPKs comprise four subfamilies, which have activation cascades, using distinct upstream kinases; these are the ERK1/2, ERK5 (also known as BMK1 and Mapk7), JNKs, and p38 MAPK. Each kinase is activated by distinct pathways and transmits signals independently or coordinately. MAPKs are crucial for a wide range of basic biological processes, including control of the inflammatory response, cell cycle, cell differentiation, and apoptosis [14, 15]. The first member of the p38 MAPK subfamily to be identified was p38α, a 38-kDa protein, which is phosphorylated on serine, threonine, and tyrosine residues in MΦ stimulated with LPS. In all, there are four p38 MAPK isoforms, each encoded by a separate gene: p38α and p38β MAPK are 74% identical at the amino acid level and are expressed widely. They have in common in vitro substrates such as myelin basic protein (MBP), activating transcription factor 2 (ATF2), and MAPK-activated protein kinase 2 (MAPKAPK2) but differ with regard to activity. Expression of p38γ MAPK, which has 63% amino acid identity with p38α, is largely restricted to skeletal muscle. Unlike p38α and p38β MAPK, p38γ MAPK can phosphorylate MBP but not ATF2 or MAPKAPK2. p38δ has 61% amino acid identity with p38α and is found mainly in the testes, pancreas, and small intestine, as well as in CD4+ T cells; it phosphorylates MBP and ATF2 robustly but not MAPKAPK2 [15]. SB203580, a pyridinyl imidazole compound, is the best-studied inhibitor of p38 MAPK. It inhibits activity of p38α and p38β specifically but not p38δ MAPK. SB203580 does not prevent p38 MAPK activation by upstream kinases but prevents the phosphorylation of the downstream substrate MAPKAPK2 [15, 16]. Recently, we showed that LPS-induced IL-10 secretion by MΦ is extremely sensitive to p38 MAPK inhibitors [17].

Our previous study indicated that splenic MΦ from neonates, when stimulated via TLR4, produced reduced amounts of the proinflammatory cytokines IL-1, IL-6, TNF-α, and IL-12 p40 and increased anti-inflammatory cytokine IL-10 in comparison with those from adult mice [18]. IL-10 has been shown to suppress TLR4-induced, proinflammatory cytokine production by MΦ in adult mice [19, 20]. Previously, we showed that in the neonate, the increased production of IL-10 had a role in the reduced production of TLR4-induced, proinflammatory cytokines [18]. In this study, we asked if the TLR4-induced cytokine dysregulation in neonates also extends to other TLRs. We also investigated if there was a widespread defect in TLR-induced responses in the neonatal MΦ and if in particular, p38 MAPK has a critical role in the cytokine dysregulation (increased anti-inflammatory IL-10) in the neonate. Three different agonists were used to stimulate the TLR2 pathway. To determine if differences in the MyD88 pathway are key to the neonatal cytokine secretion phenotype, we used CpG oligodeoxynucleotides (ODN) to stimulate TLR9, a receptor known to use the MyD88 pathway exclusively for signal transduction [21]. The results from these studies are likely to have a significant impact on the development of better pneumococcal polysaccharide vaccines.

MATERIALS AND METHODS

Mice

Neonatal (7–10 days old) and adult (3–4 months old) BALB/c and BL/6 mice were obtained from Harlan (Indianapolis IN, USA), and breeding pairs of IL-10 knockout mice on the BL/6 background were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). The mice were maintained in the animal facility at the Department of Laboratory Animal Research (University of Kentucky, Lexington, KY, USA) on a 12:12-h light:dark cycle and were given food and water ad libitum. All protocols were implemented in accordance with the National Institutes of Health guidelines (Bethesda, MD, USA) and approved by the University of Kentucky Institutional Animal Care and Use Committee (Lexington, KY, USA).

Reagents

Fixed *S. pneumoniae* was a gift from Dr. Beth A. Garvy (University of Kentucky). TLR agonists, heat-killed *L. monocytogenes* (HKLM), and CpG 1826 ODN were obtained from Invivogen (San Diego, CA, USA). Other TLR agonists, PGN and LPS, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The polyclonal goat anti-mouse, IL-10-neutralizing antibody was obtained from R&D Systems (Minneapolis MN, USA), and the anti-IL-10R (Clone 1B1.3a) was obtained from BD Biosciences (San Diego, CA, USA). Antibodies to p38 MAPK and β-actin (Clone AC-15) were obtained from Cell Signaling Technologies (Danvers, MA, USA) and Sigma Chemical Co., respectively. The p38 MAPK inhibitor SB203580 was obtained from EMD Biosciences (San Diego, CA, USA).

Cell preparation

CD11b-positive cells were purified from spleens of mice using CD11b antibody-coupled magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purified cells were found to be routinely 90–95% CD11b-positive when tested by flow cytometry. For each experiment, the CD11b-positive cells were pooled from two adult mice and one litter of neonatal mice. However, for the microarray study, the CD11b-positive cells were pooled from 10 adult mice and two litters of neonatal mice. As neutrophils also express CD11b, we have now introduced another magnetic bead isolation step prior to CD11b purification. Spleen cells were stained initially with a cocktail of FITC-conjugated antibodies against CD5, B220, and Ly-6G (BD Biosciences). FITC +ve cells were then separated from FITC –ve cells using anti-FITC-coupled magnetic beads (Miltenyi Biotec). The negative cells were purified further to obtain MΦ using the CD11b microbeads as explained above. Although the protocol has been changed, we have not observed any difference in phenotype of cells purified using CD11b alone or in conjunction with depleting the FITC +ve cells. The newer protocol was used in the later experiments (see Figs. 6C–8).

Cell culture

Cells were cultured at 37°C in 5% CO_2 in growth medium consisting of an equal volume of IMDM and Ham's F12 and supplemented as described previously [3, 22, 23]. As we have seen previously that optimal IL-6 production required 5–10% FBS (data not shown), the growth medium was supplemented routinely with 10% FBS. For the microarray study, MΦ (10×10^6) were cultured in triplicate at a density of 1×10^6/ml. Cultures were stimulated with 1 μg/ml LPS or left unstimulated for 6 h. To generate supernatants to measure cytokines, cells were cultured in duplicate with 0.25×10^6 cells per 1 ml and stimulated with CpG ODN (1 μM) or PGN (1 μg/ml). A titration of LPS indicated that the optimal dose for neonatal MΦ was 1 μg/ml [18], and this concentration was used in this study. LPS-induced cytokine production in MΦ peaked by 24 h (data not shown), so cytokine production was determined after 1 day of stimulation. For Western blotting, 2×10^6 cells were cultured for 15 min in 0.5 ml medium, and lysates containing an equivalent of 1×10^6 cells were loaded in each lane.

RNA isolation

The cells were spun down in the plate, the growth medium was removed by pipetting, and the cultures were incubated for 5 min in TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Total RNA was extracted under RNase-free conditions. The total RNA was purified further using the Qiagen

RNeasy mini kit, according to the manufacturer's protocol. Total RNA yield and purity were assessed with a spectrophotometer and with the Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA); all samples had two sharp peaks corresponding to 18S and 28S RNA on the Bioanalyzer electropherograms. From each sample, 4 μg total RNA was used for the amplification and labeling reactions. The RNA was transcribed into cRNA by the microarray facility and was hybridized to the whole mouse genome chips (Affymetrix Mouse Genome 430 2.0) using one chip for RNA from each culture.

Statistical data analysis

To evaluate the significance of the differences among the averages in Western blots, ELISA, and PCR assays, Student's *t*-test was used. To understand better the impact of LPS on gene regulation in neonates, a microarray study was performed. The comprehensive analysis investigated the effect of LPS in MΦ from three age groups: neonate, adult, and aged adult mice. The Affymetrix Mouse Genome 430 2.0 has 45,101 probe sets, and those with absent calls across all 18 chips and unannotated genes were removed to reduce the multiple-testing problem. These steps resulted in 19,209 probe sets. Three by two ANOVA tests were performed to identify differentially expressed genes as described previously [17]. Briefly, for each probe set, the model $y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk}$ was applied, where y_{ijk} is the log-transformed expression level of the *k*th chip in the *i*th age and the *j*th LPS. Furthermore, μ represents the grand mean expression, α_i is the effect as a result of age, β_j is the effect as a result of LPS, γ_{ij} is the interaction effect between age and LPS, and ε_{ijk} is an error term, which is assumed to be distributed normally with mean 0 and variance σ^2. Applying an overall *P* value of <0.01, ANOVA analysis indicated that 12,785 out of the 19,209 probe sets were regulated significantly for age, treatment, or both. The data associated with the six chips for aged mice were removed, as the focus of this study was the comparison of neonatal versus adult mice. The study investigating the properties of MΦ from aged adult has been published already [17]. To find the genes with biological significance, we applied the intensity, fold-change, and pair-wise *P* values as additional filters. Probe sets with mean intensities <200 across all four treatments (i.e., neonate medium and LPS and adult medium and LPS); fold changes, which were less than twofold [i.e., (neonate+LPS) vs. (adult+LPS)$\geq$0.5 and $\leq$2.0]; or a pair-wise *P* value $\geq$0.01 [i.e., (neonate+LPS) vs. (adult+LPS)] were not analyzed further. Application of these filters resulted in 4392 probe sets. As a large number of genes are detected by multiple probe sets, the number of unique genes regulated by LPS was reduced further to 2925.

Cytokine analysis

MΦ (0.25×10^6) were cultured in duplicate for 1 day. Various cytokines in the supernatant were estimated in duplicate using ELISA. IL-12 p40, IL-10, and TNF-α were estimated with OptEIA kits (PharMingen, San Diego, CA, USA). IL-6 was measured with a matched-pair antibody set (Clones MP5-20F3 and MP5-32C11) from BD Biosciences. The ODs were read on a Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT, USA). Results are presented as mean ± SE of duplicate measurements for duplicate cultures.

Western blotting

Approximately 2.5×10^6 MΦ were cultured per 500 μl. After allowing the cells to rest for at least 90 min, the cells were stimulated with LPS for 15 min. Cells were lysed in the plate using the lysis buffer provided by Cell Signaling Technologies. An aliquot of the lysate was subjected to SDS-PAGE and Western blot analysis. The blots were analyzed by probing the membrane using various primary antibodies followed by HRP-conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The blots were developed with the Pico chemiluminescence substrate (Pierce Biotechnology, Rockford, IL, USA) and exposed to Kodak X-Omat film, which was then scanned by a Kodak Image Station 2000RT (Eastman Kodak, New Haven, CT, USA). For reprobing, membranes were stripped using a solution containing 62.5 mM Tris-HCl, 2% SDS, and 100 mM β-ME at 65°C for 5 min. The relative integrated OD of the protein bands was estimated using the Kodak Image Station. Band intensities were normalized by dividing the intensity of phosphorylated protein by that of total protein or β-actin. These values were normalized further to adult (see Fig. 6C), adult medium [phospho (p)-p38/total p38], or adult + LPS (p-p38/β-actin; see Fig. 6D).

RESULTS

TLR2-induced cytokine production is different in neonates and adult MΦ

We have shown previously that MΦ from neonatal mice (9–12 days old), upon stimulation with LPS, the agonist to TLR4, produced reduced amounts of proinflammatory cytokines, such as IL-1β, -6, and -12 and TNF-α, in comparison with MΦ from adult mice [18]. However, MΦ from neonates produced more IL-10 than MΦ from adult mice. We were interested to know if the signaling via other TLRs led to similar changes in cytokine production in neonates. We measured the production of cytokines upon stimulating MΦ via the other TLRs. Previously, we had performed studies with 1- to 3-day-old neonatal mice and obtained results similar to those with the 7- to 10-day-old mice [3]. Mosier et al. [24] also showed that mice, until at least 2 weeks of age, were compromised in their response to polysaccharide antigen. Moreover, Holladay and Smialowicz [25] compared the murine and human neonates for a variety of immune system functions and found that neonatal mice were more immature than human neonates at early stages of development, such as in the fetus and immediately after birth. Therefore, we used 7- to 10-day-old neonates in this study. Neonatal mice are susceptible to infection with *S. pneumoniae* [26, 27]. As *S. pneumoniae*, like other Gram-positive bacteria, contains one or more agonists for TLR2 on its surface [10, 11], we stimulated MΦ with various doses of paraformaldehyde-fixed *S. pneumoniae*. At a dose of 1×10^5 cfu, MΦ from adult BALB/c mice responded and produced IL-6, but MΦ from neonatal mice did not secrete any IL-6 (**Fig. 1A**). At the higher dose of 1×10^6 cfu, MΦ from neonatal mice now produced IL-6, but it was significantly ($P<0.01$) lower than that produced by MΦ from adult mice. However, at 1×10^6 cfu, MΦ from neonatal mice produced significantly more IL-10 than adult MΦ (Fig. 1B).

PGN, which was described initially to be an agonist for TLR2, is present on the surface of Gram-positive bacteria [28]. When MΦ from neonatal mice were stimulated with PGN, they secreted reduced amounts of IL-6 (**Fig. 2A**). The decrease in proinflammatory cytokine production was not restricted to IL-6, as IL-12 p40 production was also reduced in the neonate (data not shown). Furthermore, just as seen with whole *S. pneumoniae*, PGN induced a significantly higher level of IL-10 production from MΦ from neonatal mice (Fig. 2B).

Thus, MΦ from neonates, when stimulated with the TLR2 agonists, *S. pneumoniae* and PGN, secrete more IL-10 and less IL-6 than MΦ from adult mice.

Effect of neutralizing IL-10 on cytokine production

We also tested the ability of HKLM, a well-characterized, specific agonist for TLR2, to induce cytokine secretion [12, 13]. MΦ from neonatal, wild-type BL/6 mice, unlike those from adult mice, produced little IL-6 and IL-12 p40 (**Fig. 3, A** and **B**) and reduced amounts of TNF-α (Fig. 3C). However, as seen with other TLR agonists, HKLM induced MΦ from WT neonates to produce higher levels of IL-10 than those from wild-type, adult mice (Fig. 3D). Our previous study showed that neutralization of IL-10 production in cultures of MΦ from

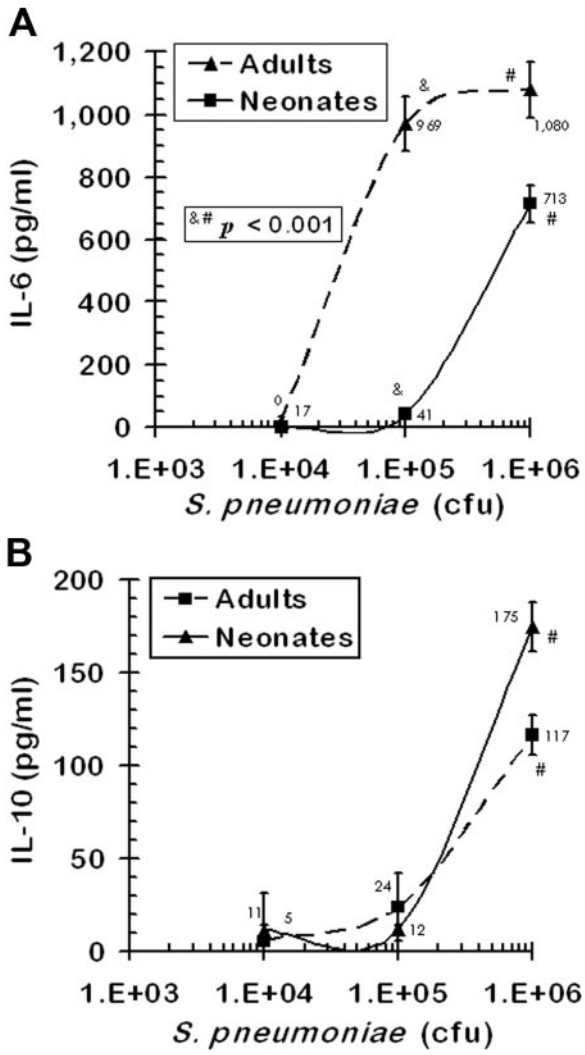

Fig. 1. Neonatal MΦ have an altered pattern of cytokine secretion upon stimulation with *S. pneumoniae*. MΦ from neonatal and adult BALB/c mice were cultured for 1 day with various doses of fixed *S. pneumoniae*. Culture supernatants were collected and assayed by ELISA for IL-6 (A) and IL-10 (B). The averages indicated by the same symbol are statistically, significantly different ($P<0.001$). This experiment is representative of two independent experiments.

neonatal BALB/c mice allowed an increase in TLR4-induced IL-6 production by neonates [18]. Did IL-10 also modulate production of proinflammatory cytokines induced by other TLRs? We used splenic MΦ from neonatal IL-$10^{-/-}$ mice and neonatal and adult, BL/6 wild-type mice. MΦ from IL-$10^{-/-}$ neonatal mice secreted IL-6, IL-12 p40, and TNF-α in amounts comparable with that of MΦ from adult, wild-type BL/6 mice (Fig. 3, A–C). As expected, no IL-10 was produced by the IL-10 knockout MΦ (data not shown).

TLR9 senses single-stranded DNA and uses a signaling pathway similar to other TLRs [29]. To assess if the pattern of the TLR9-induced cytokine production was similar to that seen with TLR2 and -4, we stimulated the cells with CpG ODN, which resulted in MΦ from wild-type BL/6 neonates producing the inflammatory cytokines, IL-6 and IL-12 p40, at reduced levels when compared with those produced by MΦ from wild-type, BL/6 adult mice (**Fig. 4, A** and **B**). Just as we saw with TLR2, IL-10 has a role in this neonatal cytokine phenotype, as TLR9-stimulated MΦ from IL-10 knockout neonatal mice produced IL-6 at a level comparable with that of MΦ from WT BL/6 adults (Fig. 4A).

As IL-10 knockout mice could have developmental defects, which may affect MΦ function, we also tested the role of IL-10 in wild-type, neonatal MΦ by supplementing these cultures with an antibody to the extracellular region of the IL-10R (i.e., CD210). Clone 1B1.3a is a neutralizing antibody, which blocks the binding of IL-10 to its receptor [30]. When the effect of IL-10 was neutralized with anti-IL-10R, the MΦ from neonatal, wild-type BL/6 mice secreted IL-6 and IL-12 p40 at levels comparable with that seen with MΦ from adult BL/6 mice not treated with anti-IL-10R antibody (**Fig. 5, A** and **B**). Neutralizing IL-10 also enhanced the level of TNF-α from MΦ from neonatal mice. However, the level remained considerably lower than that of adult mice (Fig. 5C). Neutralizing IL-10 also enhanced the secretion of these cytokines by MΦ from adult mice (data not shown). The anti-IL-10R antibody also en-

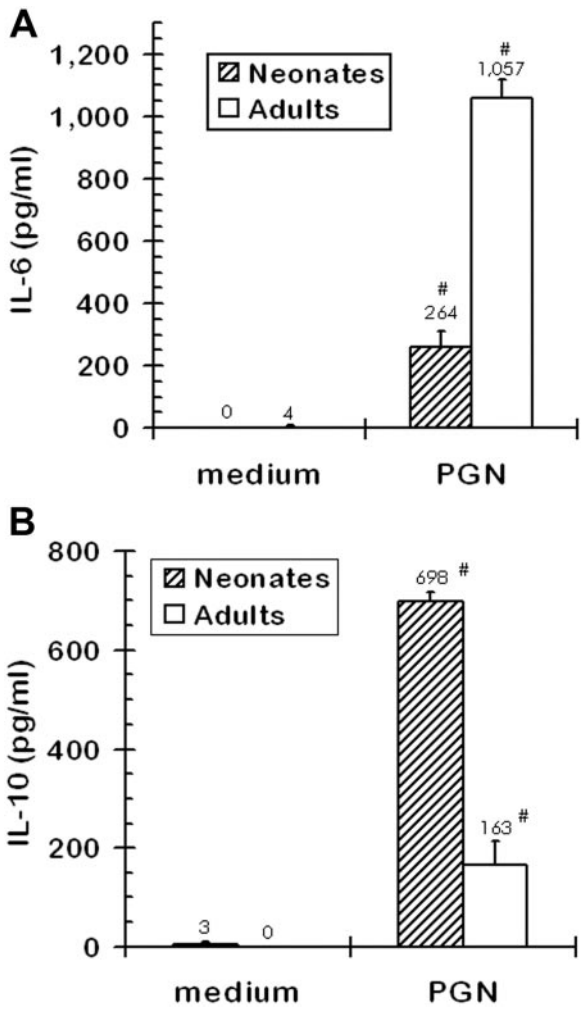

Fig. 2. Neonatal MΦ have an altered pattern of PGN-induced cytokine secretion. MΦ from neonatal and adult BALB/c mice were cultured for 1 day, with or without PGN (1 μg/ml). Culture supernatants were collected and assayed by ELISA for IL-6 (A) and IL-10 (B). The averages indicated by the same symbol are statistically, significantly different ($P<0.001$). This experiment is representative of two independent experiments.

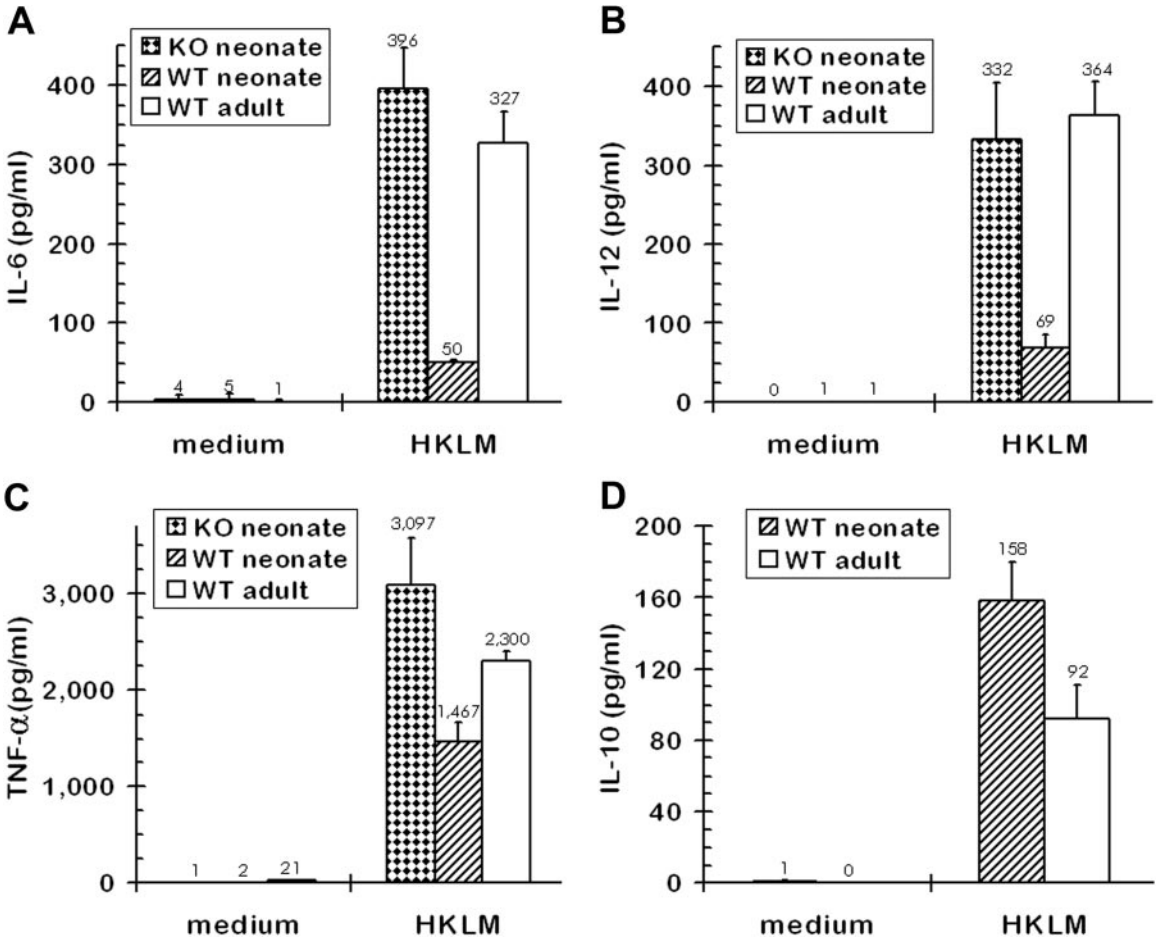

Fig. 3. MΦ from IL-10$^{-/-}$ neonatal mice are not defective in HKLM-induced cytokine production. Cultures of MΦ from IL-10$^{-/-}$ neonates [knockout (KO)]; wild-type (WT), control C57BL/6 neonates; and adults were stimulated with or without HKLM (10^8 cells/ml) for 1 day. Supernatants were then collected, and IL-6 (A), IL-12 p40 (B), TNF-α (C), and IL-10 (D) were measured by ELISA. This experiment is representative of two (D) or three (A–C) independent experiments.

hanced proinflammatory cytokine (i.e., IL-6, IL-12 p40, and TNF-α) production dramatically by neonatal MΦ upon stimulation via TLR2 or -4 (data not shown).

Thus, in the absence of IL-10, MΦ from neonatal mice secrete TLR2- or -9-induced, proinflammatory cytokines at a level comparable with MΦ from adult mice.

Microarray analysis

A microarray analysis was performed to determine if the MΦ from the neonates were also compromised in the production of other cytokines. Splenic MΦ were obtained from two litters of neonatal mice and 10 adult BALB/c mice and then stimulated, with or without LPS for 6 h. We determined that upon stimulation with LPS, there were 1690 genes, which were expressed significantly ($P<0.01$) lower in neonatal MΦ [(neonate+LPS)/(adult+LPS)≤0.5]. Conversely, there were 1235 genes, which were expressed at least twofold higher in neonatal MΦ.

Using the Database for Annotation, Visualization and Integrated Discovery (DAVID), we determined that categories of genes involved in critical biological processes, such as immune cell activation, protein biosynthesis, immune response, antigen processing, and cytokine biosynthesis, were significantly ($P\leq1\times10^{-4}$) enriched in the group of genes, which were expressed at a reduced level in the neonates (**Table 1**) [31]. The list of genes expressed at increased levels in neonates after stimulation with LPS was enriched significantly ($P\leq1\times10^{-4}$) in a number of gene categories, such as cell cycle, lipid metabolism, and biogenesis.

To see if the difference in cytokine production was restricted to only IL-6, -10, and -12 p40 and TNF-α, we focused on the LPS-induced expression levels of messages for cytokines, chemokines, and their receptors. The levels of mRNA for a number of LPS-induced interleukins, chemokines, IFNs, and members of the TNF family were reduced significantly in MΦ from neonatal mice in comparison with those from adults (**Table 2**). Important genes, which had reduced expression in neonates, included IL-1α, -6, and -12 (*Il1a*, *Il6*, *Il12a*, and *Il12b*). Our ELISA assays measuring the protein levels of these cytokines (Figs. 1–5) serve as a validation of the microarray data. The mRNA of a number of proinflammatory cytokines, such as CXCL1, -10, -11, and -16 and CCL2 and -24 (*Cxcl1*, *-10*, *-11*, and *-16* and *Ccl2* and *-24*) [32], was also reduced in neonates. However, the transcripts for a few genes, such as *Il16* and *Il1f6* (interleukins), *Ccl6* and *Cklf* (chemokines), *Il18rap*, *Il17ra*, *Ccr1*, and *Ccr2* (receptors for interleukins and chemokines), and *Tnfrsf19l* and *Tnfsf13b* (TNF family), were increased substantially in MΦ from neonates.

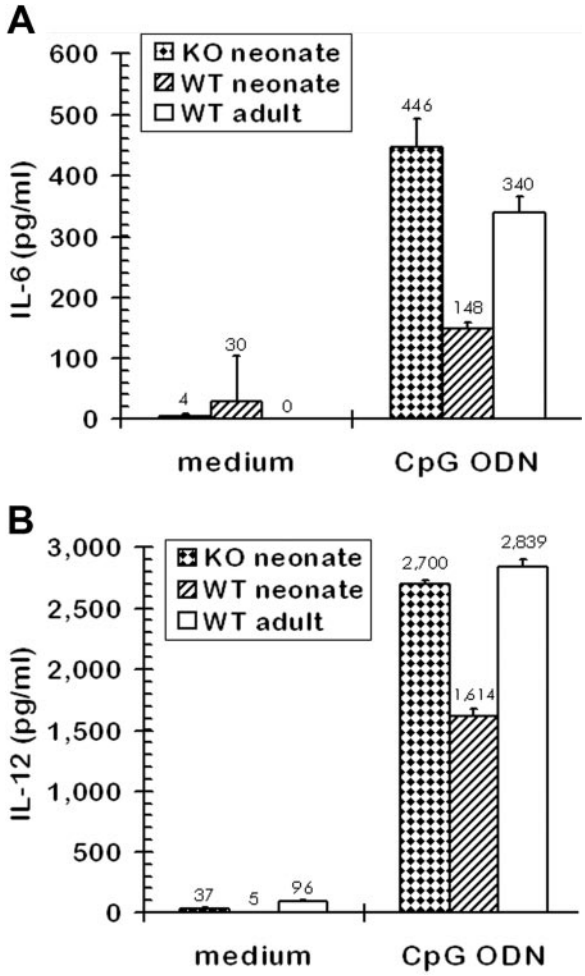

Fig. 4. MΦ from IL-10$^{-/-}$ neonatal mice are not defective in CpG ODN-induced cytokine production. Cultures of MΦ from IL-10$^{-/-}$ neonates and wild-type, control C57BL/6 neonates and adults were stimulated with or without CpG ODN (1 μM) for 1 day. Supernatants were then collected, and IL-6 (A) and IL-12 p40 (B) were measured by ELISA. The averages indicated by the same symbol are statistically, significantly different. This experiment is representative of three independent experiments.

The current microarray study indicated that the expression of a number of TLRs was reduced significantly in unstimulated MΦ from neonatal mice, particularly Tlr1, -4, and -6 (**Table 3**). We have shown previously a reduction in expression of TLR4 at the protein level, which correlates well with the microarray data. The TLRs, upon ligation, use a complex series of molecules to convey the signal to the nucleus [21, 33]. The microarray data indicate that upon stimulation via TLR4, there was a reduction in the expression of a number of key adaptor and interacting proteins (e.g., *Peli1 and Traf6*), MAPK pathway and transcription factors (e.g., *Mapk9 and Irf1*), and NF-κB pathway (e.g., *Nfkb2*; **Table 4**). Not all molecules involved in TLR signaling were reduced in MΦ from neonatal mice. Some molecules (e.g., *Myd88*) were present at levels comparable with that seen in MΦ from adult mice, and others, such as *Irak3* (negative regulator of TLR signaling [34]) and *Mapk14* (MAPK pathways), were present in neonatal mice at significantly increased levels.

Thus, the microarray analysis indicates that the messages of a number of critical, proinflammatory cytokines, chemokines, and TLRs are reduced in MΦ from neonatal mice. However, the messages of a few cytokines and chemokines were also increased.

Neonates have increased amounts of p38 MAPK

As MΦ from neonates have substantially increased amounts of mRNA for p38α MAPK and p38δ MAPK (Table 4 and **Fig. 6, A** and **B**), we determined if this was also true at the protein level. Indeed, when normalized to β-actin, neonates had about four times the amount of p38 MAPK protein as adult MΦ (Fig. 6C). As p38 MAPK has to be phosphorylated to be active [35], we also probed lysates from neonatal and adult MΦ for the active, phosphorylated form of p38 MAPK (Fig. 6D). Upon stimulation with LPS, neonatal MΦ had more p-p38 (3.1 vs. 2.1) when normalized to total p38. As neonates had approximately four- to fivefold more total p38 MAPK, the actual amount of p-p38 per neonatal MΦ was increased substantially. In fact, when p-p38 MAPK was normalized to β-actin, neonatal MΦ had about four times more p-p38 MAPK than adult MΦ (data not shown).

To determine if the increased production of p38 MAPK in neonates could be responsible for the imbalance in cytokine production in this age group, we stimulated MΦ from neonates with LPS in the presence of varying amounts of SB203580, a well-known inhibitor of p38 MAPK. As functional p38 MAPK levels were reduced by the drug, the levels of secreted IL-10 decreased, and by 1 μM SB203580, there was little or no secretion of IL-10 (**Fig. 7A**). However, during this titration, as the levels of functional p38 MAPK decreased, there was an increase in the amount of IL-6 and IL-12 p40 secretion, which reached a peak by 2 μM SB203580. At this optimal dose of the drug, the level of secretion of the two proinflammatory cytokines was nearly threefold of that seen with only LPS (i.e., 0 μM SB203580). At higher concentrations of SB203580, the levels of IL-6 and IL-12 p40 decrease. However, this decrease was not a result of any nonspecific toxicity of the drug, as the viability of the cells at the highest dose of SB203580 used was still ~70%, compared with 80% at 2 μM SB203580, a concentration that enhanced IL-12 p40 production (data not shown).

In the absence of IL-10, there was an enhancement of the production of proinflammatory cytokines (Figs. 3–5) [18, 20, 36, 37]. To determine if the enhancement of IL-6 and IL-12 p40 during the titration of SB203580 was actually a result of a loss of IL-10, we stimulated MΦ from neonates in the presence of anti-IL-10R and SB203580 (Fig. 7B). α-IL-10R and SB203580 alone enhanced the production of IL-6 and IL-12 p40 ($P<0.001$). Inhibiting p38 MAPK in the absence of IL-10 signaling led to a further enhancement of IL-6 and IL-12 p40 (cf., Treatments 3 and 5 in Fig. 7B). It is interesting that although α-IL-10R and SB203580 have opposite effects on LPS-induced IL-10 production, when both reagents were added together, the result was the same as that seen with SB203580 alone.

To establish definitively that the p38 MAPK inhibitor effects on proinflammatory cytokine production are independent of IL-10, we studied its effect on cytokine production by IL-10 knockout MΦ. The enhancement of IL-12 p40 and IL-6 was similar in MΦ from IL-10 knockout and BL/6 wild-type mice

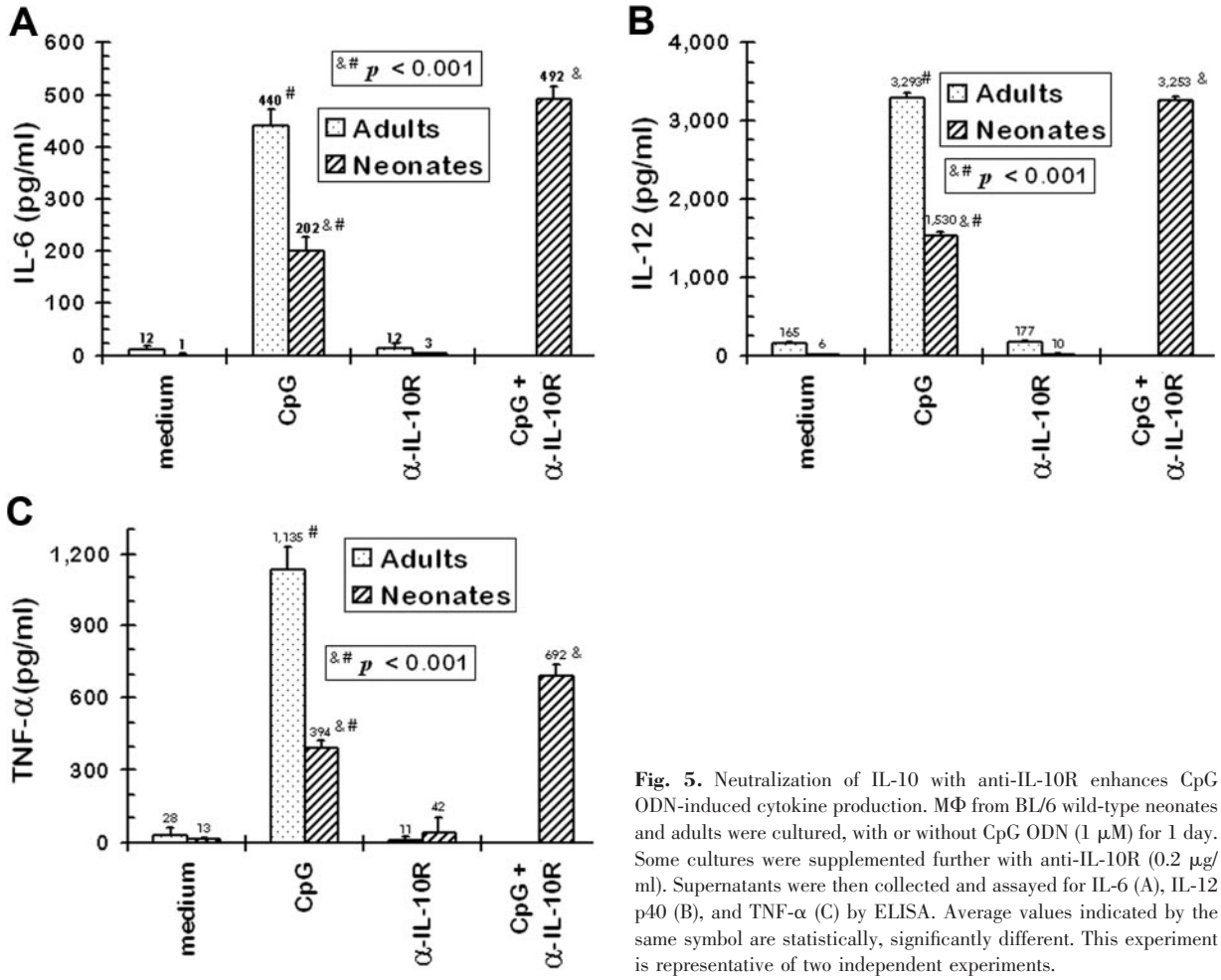

Fig. 5. Neutralization of IL-10 with anti-IL-10R enhances CpG ODN-induced cytokine production. MΦ from BL/6 wild-type neonates and adults were cultured, with or without CpG ODN (1 μM) for 1 day. Some cultures were supplemented further with anti-IL-10R (0.2 μg/ml). Supernatants were then collected and assayed for IL-6 (A), IL-12 p40 (B), and TNF-α (C) by ELISA. Average values indicated by the same symbol are statistically, significantly different. This experiment is representative of two independent experiments.

TABLE 1. Categories of Genes Expressed at Different Levels in Neonatal MΦ Compared with Adult MΦ

Gene categories reduced in neonates

Term	Examples	Number of genes	*P* value
immune cell activation	*Tlr1, Il15, Cd40, Cd80, Traf6*	52	2E-14
protein biosynthesis	*Psen2, Rpl27, Stat5a*	109	1E-12
immune response	*Cxcl2, Cxcl10, Nos2, Ccl2*	147	2E-12
programmed cell death	*Fasl, Casp1, Casp7, Card4*	94	2E-10
response to biotic stimulus	*Gpr68, Tbk1, Socs5, Ifi47*	165	5E-09
antigen processing	*B2m, H2-DMb2, Tap2*	23	1E-06
protein folding	*Hspd1, Cct4, Canx, Hspb1*	40	7E-05
cytokine biosynthesis	*Ifng, Il12b, Il6*	17	1E-04

Gene categories increased in neonates

Term	Examples	Number of genes	*P* value
cell cycle	*Ccnb1, Ccna2, Ccnd3, Cdc2a*	125	2E-29
lipid metabolism	*Plcb4, Mgll, Lpl, Alox5*	81	6E-12
protein modification	*Srpk2, Usp46, Smurf2, Ube2c*	167	9E-12
organelle organization and biogenesis	*Tuba8, Kif1b, Parp4, Hmgb1*	114	9E-10
cellular carbohydrate metabolism	*Fbp1, Hexb, Gyg, Ldhc*	40	6E-06
vesicle-mediated transport	*Vamp5, Lyst, Flna, Rab3d*	43	1E-04

Genes expressing significantly different amounts in neonatal MΦ (compared with adult MΦ) after stimulation with LPS were subdivided into gene categories using DAVID 2006. Categories relevant to the study, which was enriched significantly, are depicted.

TABLE 2. Neonatal MΦ Produce Altered Amounts of Transcripts to a Number of Cytokines and Chemokines

Category	Reduced in neonates		Increased in neonates	
Interleukin	*Il1a*	*Il15*	*Il1f6*	
	Il12a	*Il23a*	*Il16*	
	Il12b	*Il6*		
Interleukin receptor	*Il1rn*	*Il2rb*	*Il17ra*	*Il6ra*
	Il10rb	*Il21r*	*Il18rap*	*Il8rb*
	Il15ra	*Il7r*	*Il28ra*	
	Il2ra		*Il4ra*	
Chemokine	*Ccl2*	*Cxcl13*	*Ccl6*	
	Ccl24	*Cxcl16*	*Cklf*	
	Cxcl1	*Cxcl2*		
	Cxcl10	*Cxcl9*		
	Cxcl11	*Mif*		
Chemokine receptor	*Blr1*	*Ccr7*	*Ccr1*	
	Ccr5	*Ccrl2*	*Ccr2*	
	Ccr6	*Cxcr6*	*Cyfip2*	
Interferon	*Ifng*			
	Ifnz			
TNF family	*Tnfrsf13c* (BAFF-R)	*Tnfsf14* (LIGHT)	*Tnfrsf19l* (RELT)	
	Tnfrsf18 (GITR)	*Tnfsf4* (OX-40L)	*Tnfrsf21* (DR6)	
	Tnfrsf9 (Cd137)	*Tnfsf7* (CD70)	*Tnfsf13b* (BAFF)	
	Tnfsf12-tnfsf13	*Tnfsf9* (CD137L)		
BMP and TGF-β family	*Bmp2*	*Inhba*		
	Gdf15			

Data generated from microarray analysis. The genes depicted in this table meet the criteria of a minimum intensity of at least 200 and have a pair-wise P value of less than 0.002. A fold-change criteria of (neonate+LPS)/(adult+LPS) of ≤0.5 (lower in neonates) and ≥2.0 (higher in neonates) were applied. BMP, Bone morphogenetic protein.

(**Fig. 8, A** and **B**). As seen previously, inhibition of p38 MAPK by SB203580 also suppressed IL-10 production by MΦ from BL/6 wild-type mice (Fig. 8B). We then questioned if p38 MAPK activity can regulate TLR2- and -9-induced cytokine secretion in a manner similar to its effect on TLR4 signaling. Once again, partial inhibition of p38 MAPK also enhanced production of IL-12 p40 and IL-6 when neonatal MΦ were stimulated via TLR2 and -9 (**Table 5**). Moreover, as with TLR4, higher doses of SB203580 inhibited IL-12 p40 and IL-6 secretion induced by TLR2 and -9 (data not shown). Incubation with 2 μM SB203580 reduced TLR2-induced IL-10 but not that induced by TLR9, suggesting that IL-10 induction may be regulated differently in TLR2/4 versus TLR9 pathways (Table 5).

TABLE 3. MΦ from Neonatal Mice Have Reduced Expression of a Number of TLRs

Gene	Symbol	Neonate/Adult	Pair-wise P
Toll-like receptor 1	***Tlr1***	0.3	9.E-11
Toll-like receptor 2	***Tlr2***	0.5	2.E-08
Toll-like receptor 3	*Tlr3*	0.3	9.E-06
Toll-like receptor 4	***Tlr4***	0.3	4.E-13
Toll-like receptor 5	*Tlr5*	1.4	9.E-02
Toll-like receptor 6	***Tlr6***	0.3	4.E-11
Toll-like receptor 7	***Tlr7***	0.4	6.E-12
Toll-like receptor 8	*Tlr8*	0.7	8.E-03
Toll-like receptor 9	*Tlr9*	0.8	3.E-01
Toll-like receptor 12	*Tlr12*	0.9	8.E-01
Toll-like receptor 13	***Tlr13***	0.7	3.E-05

Data generated from microarray analysis. The third column represents the ratio of hybridization intensity of any gene in unstimulated MΦ from neonates to that in adults. The fourth column depicts the corresponding, pair-wise P value. Those genes that meet the criteria of a minimum intensity of at least 200 and a pair-wise $P < 0.05$ are depicted with symbols in bold font.

This study demonstrates that MΦ from neonatal mice secrete reduced amounts of proinflammatory cytokines upon stimulation via various TLRs, which could be reversed in the absence of IL-10. A microarray analysis showed that the mRNA of a large number of cytokines, chemokines, and TLR signaling components was reduced in neonates. However, some molecules were increased in neonates, such as p38 MAPK. Inhibiting p38 MAPK increased proinflammatory cytokine production.

DISCUSSION

We had reported previously that MΦ from neonatal mice produced less proinflammatory cytokines and more of the anti-inflammatory cytokine IL-10 than MΦ from adult mice upon stimulation with LPS, an agonist for TLR4. Furthermore, when IL-10 was neutralized, this caused an increase in the production of proinflammatory cytokines to levels produced by adults [18]. The current study was initiated to determine if the inability of MΦ from neonates to respond appropriately was restricted to TLR4 only or if signaling via other TLRs was likewise also affected. MΦ play a critical role in the successful host immune response against *S. pneumoniae*, and this organism, like other Gram-positive bacteria, stimulates host immune cells via TLR2 [10, 11]. Therefore, we initially investigated the production of cytokines by splenic MΦ upon stimulation via TLR2. Second, we wanted to determine if IL-10 had a role in

TABLE 4. Expression of Key Signaling Components of the TLR and Nucleotide-Binding Oligomerization Domain (Nod) Pathways

Gene	Symbol	Alias	Neonate/Adult	Pair-wise *P*
Adaptors & interacting proteins in TLR & Nod pathways				
caspase recruitment domain family, member 6	*Card6*		2.2	2.E-03
interleukin-1 receptor-associated kinase 3	*Irak3*		4.6	8.E-15
interleukin-1 receptor-associated kinase 4	*Irak4*		2.7	1.E-08
lymphocyte antigen 86	*Ly86*	MD-1	0.2	8.E-09
lymphocyte antigen 96	*Ly96*	MD-2	0.4	2.E-08
myeloid differentiation primary response gene 88	*Myd88*		1.0	1.E-01
nucleotide-binding oligomerization domain containing 1	*Nod1*	Card4	0.3	3.E-11
pellino 1	*Peli1*		0.5	1.E-07
receptor (TNFRSF)-interacting serine-threonine kinase 2	*Ripk2*		0.2	1.E-16
Tnf receptor-associated factor 6	*Traf6*		0.5	3.E-07
Toll-IL-1 receptor (TIR) domain-containing adaptor protein	*Tirap*		3.7	8.E-07
ubiquitin-conjugating enzyme E2N	*Ube2n*		0.5	8.E-05
MAPK pathway & transcription factors				
FBJ osteosarcoma oncogene	*Fos*	c-fos	2.9	7.E-06
interferon regulatory factor 1	*Irf1*		0.1	8.E-16
Jun oncogene	*Jun*		0.3	6.E-09
mitogen-activated protein kinase 13	*Mapk13*	p38δ MAPK	70.4	9.E-09
mitogen-activated protein kinase 14	*Mapk14*	p38α MAPK	7.2	8.E-13
mitogen-activated protein kinase 3	*Mapk3*	Erk-1	3.4	1.E-12
mitogen-activated protein kinase 7	*Mapk7*	Erk5-T	2.1	5.E-06
mitogen-activated protein kinase 9	*Mapk9*	Jnk-2	0.5	2.E-08
mitogen-activated protein kinase binding protein 1	*Mapkbp1*		0.4	3.E-07
mitogen-activated protein kinase 4	*Mapk4*	p63 MAPK	5.9	3.E-05
mitogen-activated protein kinase 6	*Mapk6*	Prkm6	0.3	2.E-12
TANK-binding kinase 1	*Tbk1*		0.5	1.E-10
NF-κB pathway				
I-κB like protein	*Nfkbil1*	Def-7	2.7	2.E-06
IκBβ	*Nfkbib*		0.5	7.E-13
IKBE	*Nfkbie*		0.3	1.E-12
inhibitor of κB kinase δ	*Ikbkg*	IKKγ	0.5	1.E-10
mitogen-activated protien kinase kinase kinase 14	*Map3k14*	Nik	0.5	2.E-03
NF-κB2, p49/p100	*Nfkb2*		0.5	3.E-07

Data generated from microarray analysis. The third column represents the ratio of hybridization intensity of any gene in LPS-stimulated MΦ from neonates to that in adults. The fourth column depicts the corresponding pair-wise *P* value. The genes depicted in this table meet the criteria of a minimum intensity of at least 200. TNFRSF, TNFR superfamily.

the production of proinflammatory cytokines when MΦ were stimulated by TLRs other than TLR4. When splenic MΦ from neonates were stimulated with fixed *S. pneumoniae*, they produced reduced amounts of IL-6, a cytokine known to modulate B cell responses leading to antibody production [3, 38, 39]. It is interesting that these bacteria also induced neonatal MΦ to secrete more IL-10 than adult MΦ, a cytokine shown previously by us and others to down-regulate proinflammatory cytokine production by TLR4-induced MΦ [19, 20]. We also confirmed this finding with HKLM, another TLR2 agonist [12, 13, 17, 18]. Furthermore, the cytokine secretion pattern induced by TLR9 signaling (i.e., decreased proinflammatory cytokines and increased IL-10 in the neonate in comparison with the adult) was nearly identical to that seen with TLR4 and -2 agonists, suggesting that this is a fundamental property of the neonatal MΦ. Based on responses to three different TLRs, it appears that the neonatal, splenic MΦ are fundamentally different from adult MΦ in that they have a greater predominance of an anti-inflammatory phenotype, as the relative ratio of IL-10 to other proinflammatory cytokines (IL-12 p40, IL-6, and TNF-α) is more in the neonate. The IL-12 p 40 ELISA measures the p40 subunit, which is common to IL-12 and IL-23 [40]. Consequently, we have now switched over to measuring the p35:p40 heterodimer of IL-12. When stimulated with LPS, neonatal MΦ make little IL-12 p70 (10±6 pg/ml with medium only to 25±14 with LPS) compared with adult MΦ (0±0 pg/ml with medium only to 106±39 with LPS). Second, 2 μM SB203580 also strongly enhances the production of LPS-induced IL-12 p70. Although TNF and IL-12 are clearly proinflammatory cytokines, there are some differing views about IL-6. Despite its production during early stages of inflammation, it is better defined as a resolution factor, which balances pro- and anti-inflammatory outcomes to further the immunological response, and IL-6 activity appears to be critical for the effective management of acute inflammation [41]. Hence, the deficiency in the neonate may not be simple to characterize as pro- or anti-inflammatory, but there is a dysregulation in the balance of cytokines produced. Despite the controversy about the role of IL-6, its production by the TLR pathway in MΦ is negatively regulated by IL-10 [4, 36, 37, 42].

Although *S. pneumoniae* stimulates host cells via TLR2, it also contains pneumolysin, which is a TLR4 agonist [11, 43]. To confirm that the TLR2-induced cytokine pattern was different in neonates, we repeated the study with PGN, a well-studied agonist for TLR2 [28]. The PGN used in this study was obtained from the Gram-positive *Staphylococcus aureus*. Just as

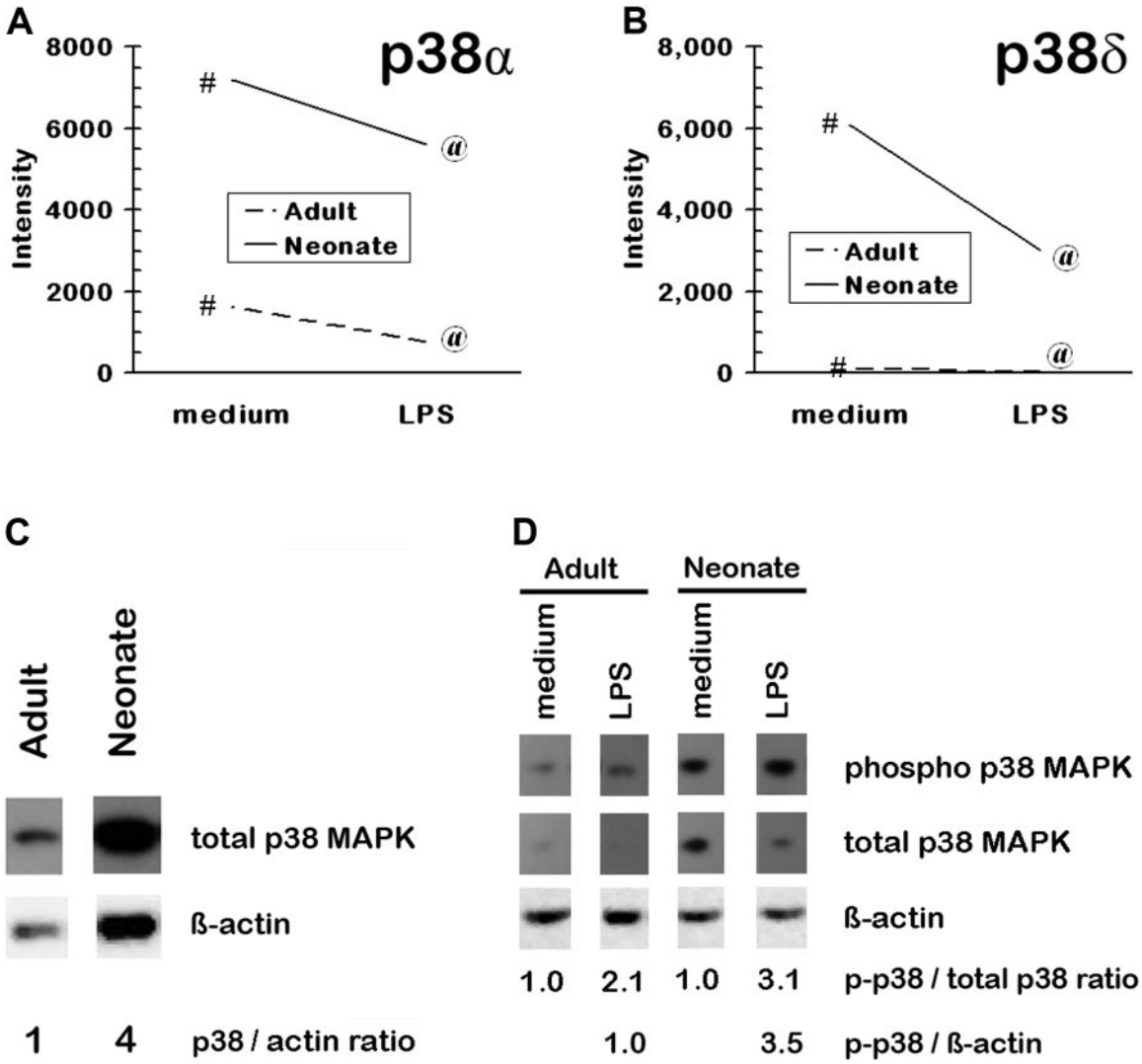

Fig. 6. MΦ from neonatal mice have increased amounts of p38 MAPK. RNA from MΦ from BALB/c, wild-type neonates and adults was subjected to microarray analysis. Graphs depict mean hybridization signals for p38α (A) and p38δ (B) MAPK for MΦ incubated with or without 1 μg/ml LPS for 6 h. Averages identified by the same symbol are statistically different ($P<0.001$). A Western blot was performed on the lysates derived from MΦ from adult and neonatal mice, cultured with or without LPS for 15 min (C and D), and each panel represents a separate experiment. The intensities of the p38 MAPK bands (total and phospho) were normalized to the corresponding bands for total p38 MAPK and β-actin, and the ratios are indicated below the blots.

in the case with whole bacteria, MΦ from neonatal mice responded by producing increased amounts of IL-10 and reduced amounts of IL-6. TLR2 forms heterophilic dimers with TLR1 or -6, both of which are structurally related to TLR2 [6]. Thus, there is a possibility that the differences in MΦ signaling via TLR2 between neonates and adults could be a result of differences in signaling via one of the partner TLRs or both. As it is now known that TLR1 and -6 functionally associate with TLR2 and discriminate between diacyl or triacyl lipopeptides [6], these lipoproteins will be useful in determining if the TLR2 signaling defect in neonatal MΦ involves one or both TLR heterodimers. As shown by the microarray data expression of TLR2, TLR1 as well as TLR6 are decreased by two- to three-fold in the neonatal MΦ (Table 3).

It has been reported recently that highly purified preparations of PGN ceased to stimulate mammalian cells via TLR2 [44, 45]. This would imply that the previous requirement for TLR2 seen for stimulation via PGN was a result of contaminating lipoproteins. It is now believed that PGN is actually an agonist for Nod proteins [44, 45]. Our microarray data show that neonatal MΦ have a significantly reduced amount of message to Nod1 (Table 4). Nod1 and Nod2 are involved in detecting PGN but require distinct motifs to achieve sensing [46]. Inadvertently, by using PGN as an agonist for TLR2, we have discovered that the unique cytokine pattern induced by TLR2 signaling is also seen when the NOD1/2 pathway is used.

Using IL-10 knockout mice or neutralizing the effect of IL-10 with antibodies, we showed that the increased production of IL-10 by TLR4-signaled neonatal MΦ was in part responsible for the reduced, proinflammatory cytokine production. It is interesting that neutralizing IL-10 also allowed MΦ from neonatal mice to provide adequate support for adult B cells to produce polysaccharide-specific antibodies [18]. In this study, we see that the increased, TLR2-induced IL-10 production might also interfere with the production of proinflammatory cytokines by MΦ from neonatal mice. When

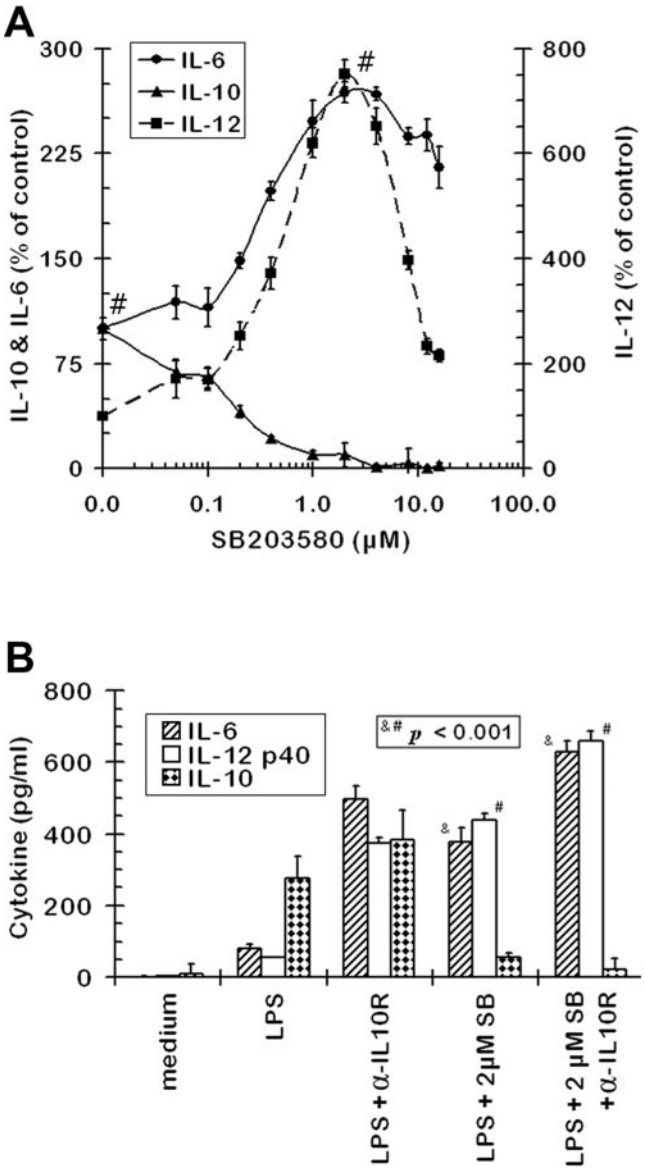

Fig. 7. Inhibition of p38 MAPK enhances proinflammatory cytokine production and suppresses IL-10 production in neonatal mice. MΦ cultures from neonatal, BALB/c, wild-type mice were stimulated with LPS (1 μg/ml) with various doses of SB203580 for 24 h. Cytokines were determined by ELISA (A). MΦ from neonatal, BALB/c wild-type mice were incubated with LPS for 24 h (B). Cultures were supplemented further with α-IL-10R (0.2 μg/ml) or SB203580 (SB). Cytokines were determined by ELISA. Averages identified by the same symbol are statistically different ($P<0.001$). This experiment is representative of two independent experiments.

splenic MΦ from neonatal IL-10 knockout mice were stimulated via TLR2, the production of proinflammatory cytokines was comparable with that produced by MΦ from adult, wild-type mice (Fig. 3).

Signaling via TLR9 also led to a reduced, proinflammatory cytokine production by MΦ from neonatal mice. However, unlike stimulation via TLR2 or -4, TLR9-induced MΦ from neonatal mice did not consistently produce more IL-10 than MΦ from adult mice (data not shown). Nevertheless, CpG-stimulated MΦ from IL-10 knockout neonates produced significantly more IL-6 and IL-12 p40 than wild-type neonates and amounts comparable with that of wild-type, adult MΦ. To be certain that the increased, TLR9-induced, proinflammatory cytokine production in IL-10 knockout, neonatal MΦ was not a result of a developmental defect, we supplemented cultures of wild-type MΦ with anti-IL-10R. Neutralizing IL-10 enhanced the production of TLR9-induced, proinflammatory cytokines, indicating that the increased, proinflammatory cytokine production by neonatal IL-10 knockout mice is actually a result of a loss of IL-10.

To determine if the production of other cytokines by MΦ from neonatal mice was also different and to investigate possible causes of this difference, we performed a microarray analysis on MΦ from neonatal and adult mice. This study indicates that the reduced production of proinflammatory cytokines by neonatal MΦ occurs, irrespective of whether the MΦ are stimulated via TLR2, -4, or -9. Therefore, we decided to stimulate the MΦ via TLR4 using LPS. The Expression Analysis Systematic Explorer analysis shows that in the list of gene transcripts, which were reduced in neonates, a number of categories of genes involved in immune response were enriched. A previous microarray analysis investigating the effect of LPS on MΦ from aged mice also showed that these same

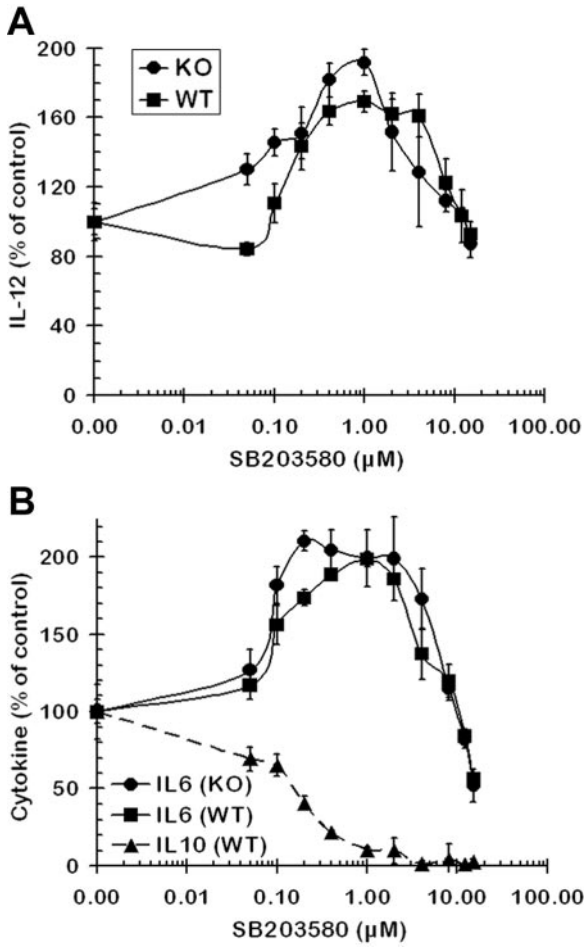

Fig. 8. Partial inhibition of IL-6 and -12 p40 by p38 MAPK occurs in the absence of IL-10. MΦ cultures from adult IL-10 knockout or wild-type BL/6 mice were stimulated with LPS (1 μg/ml) with various doses of SB203580 for 24 h. IL-12 p40 (A) and IL-6 and IL-10 (B) were determined by ELISA. This experiment is representative of two independent experiments.

TABLE 5. Effect of SB203580 on TLR-Induced Cytokine Production from Neonatal MΦ

	IL-10		IL-12 p40		IL-6	
Stimulation	Medium	SB203580	Medium	SB203580	Medium	SB203580
none	4 ± 9	0 ± 0	0 ± 0	1 ± 2	1 ± 1	1 ± 1
TLR2	350 ± 16	72 ± 15	492 ± 37	1591 ± 73	26 ± 4	97 ± 16
TLR9	78 ± 4	96 ± 12	1964 ± 83	3229 ± 26	124 ± 23	347 ± 28
TLR4	205 ± 16	20 ± 18	70 ± 4	526 ± 18	471 ± 13	1265 ± 36

Duplicate neonatal MΦ cultures (250×10^3 each) were stimulated for 1 day with various TLR ligands. The supernatants were collected, aliquoted, and stored at –20°C. Cytokines were measured by ELISA, and each culture was determined in triplicate.

categories were reduced in aged MΦ (data not shown) [17]. This was not surprising, as aged MΦ also produced reduced amounts of proinflammatory cytokines and increased IL-10 upon stimulation via TLR. Just as we saw with aged MΦ, neonatal MΦ also express reduced amounts of *Ifng*, *Il1a*, *Il12a*, and *Il15* and increased amounts of *Il1f6* and *Il16* [17]. We then examined the expression of components of the TLR signaling pathway. Just as with aged MΦ, neonatal MΦ expressed increased amounts of *Mapk14* and *Irak3* [17]. It is important that expression of suppressor of cytokine signaling (SOCS) family members (1, 2, 3, 4, 5, and 7), known to down-regulate proinflammatory cytokine secretion and in part, mediate the inhibitory effects of IL-10, was reduced substantially (SOCS 1, 2, 3, 4, and 5) or unaltered (SOCS 6 and 7) in the neonate, ruling out these molecules as the reason for the altered cytokine pattern in the neonate (Table 1 and data not shown) [47–49]. Similarly, expression of IL-1RA, another inhibitor of expression of proinflammatory cytokines, was also reduced but not increased in the neonate (Table 2) [50, 51].

The microarray data indicate that the mRNAs for at least six TLRs were reduced in unstimulated, neonatal MΦ. Although mRNA levels do not necessarily reflect levels of proteins, we have shown previously that splenic MΦ from 9- to 12-day-old neonates have reduced surface expression of TLR2 and -4 proteins [18]. This observation, that neonates have reduced TLR expression, was also seen in humans [52]. However, other studies show that in neonatal humans, despite the impairment of TLR-mediated immunity, the amount of TLR protein expressed in neonatal cord blood cells is comparable with that seen in cells in adult peripheral blood [53, 54]. In our study, although signaling via TLR9 was compromised in MΦ from neonates, the level of TLR9 message was not significantly different in MΦ from the two age groups. This observation suggests that the cause of the different response in neonates probably lies at a common signaling defect rather than as a result of reduced levels of some TLRs. At the time we conducted the study, there were no good reagents to measure mouse TLR9. These antibodies are now available and will be used to validate our microarray data further. The TLRs, TLR2, -4, and -9, which we have studied so far, use the key upstream adaptor molecule MyD88 to transmit the signal from the receptor [21]. The defect in TLR signaling in MΦ from wild-type, neonatal mice could be a result of individual defects in TLR2, -4, and -9 signaling. However, it is more likely that it could be a result of a common pathway, which involves MyD88.

Although we use gel-purified LPS in this study, we have used phenol-extracted LPS previously, which had little bacterial lipoprotein carryover. We essentially obtained the same result. Further, as we are showing independently that a number of TLR2 agonists also elicit a pattern of cytokine secretion in neonates, which is different from adults, we think that the phenomenon we see with LPS indicates that neonates have a defect in TLR4 signaling. The study will be repeated with a highly purified LPS preparation to prove conclusively that TLR4 signaling in neonates is also compromised. In accordance with the concept of a common TLR signaling defect, the mRNA of a number of downstream mediators of TLR signaling, such as TRAF6, IRAK-1, IRF1, and NF-κB components, is down-regulated in neonatal MΦ. Also IRAK-M (*Irak3*), a key negative regulator, is enhanced in the neonate. This observation was later validated by Western blotting, which indicated that neonatal MΦ had significantly increased IRAK-M protein (data not shown). It is also interesting to note that c-Fos (*Fos*) and Erk, recently shown to inhibit IL-12 production in dendritic cells [55], are enhanced in the neonate. However, the situation is more complex, as certain key intermediates, such as Toll/IL-1R translation initiation region domain-containing adaptor protein and MAPKs, which enhance TLR signaling, are increased in the neonate. We are currently stimulating neonatal MΦ via TLR3, which does not use MyD88 to signal [21].

As it has clearly been established that p38α MAPK (*Mapk14*) plays a critical role in regulating TLR-induced cytokine production [5, 56–58], it was surprising that this key signaling molecule was highly enhanced in the neonate. Not only was the mRNA more, but also the protein as well as the active form of p38 MAPK were more in the neonate, validating the microarray data. However, there is at least one report indicating that in humans, the amount of functional p38 MAPK in neonates is actually reduced when compared with that in adults [52]. The difference in observation could be a result of a species difference and/or the fact that whereas we measured p-p38 MAPK using Western blotting, Sadeghi et al. [52] used flow cytometry. By inhibiting p38 MAPK activity partially with its specific inhibitor SB203580, we confirmed that p38 MAPK was required for TLR2- and -4-induced IL-10 production. It is interesting that low doses of the p38 MAPK inhibitor enhanced the proinflammatory cytokines, suggesting that high doses of p38 MAPK were somewhat inhibitory to proinflammatory cytokine production. However, consistent with the literature, p38 MAPK was required, even for proinflammatory cytokines, as higher doses of the inhibitor decreased their production. Although p38α and p38δ MAPKs were increased substantially in the neonates, p38α MAPK appears to be more important for

cytokine dysregulation, as SB203580 is not known to inhibit p38δ (and p38γ) isoforms [59, 60]. The increase in p38 MAPK in neonatal MΦ and the ability of SB203580 to enhance proinflammatory cytokines in the neonate are analogous to our recent observation with MΦ from the aged mice, which had an identical phenotype [17]. Moreover, both age groups have a similar defect in their inability to respond to pneumococcal bacteria, which is in part a result of defects in MΦ [18, 36], especially with regard to cytokines required for efficient B cell activation by polysaccharide antigens. It is intriguing that MΦ from the two ends of the age spectrum have similar cytokine secretion patterns and an elevation of p38 MAPK. This similarity between the age groups strengthens the concept that changes in p38 MAPK may be fundamental to the unique cytokine phenotype of the neonatal and the aged MΦ.

We have shown previously that neutralizing IL-10 enhances TLR4-induced, proinflammatory cytokine production [17, 18]. This led us to ask if the increase of proinflammatory cytokines seen at the low dose of SB203580 was a result of a decrease in IL-10. To test this hypothesis, we incubated MΦ with LPS + SB203580 or LPS + SB203580 + α-IL-10R. As the latter treatment enhanced proinflammatory cytokine production further, this would imply that the enhancement seen in neonates was independent of the loss of IL-10 production. However, the interpretation was complicated by the fact that α-IL-10R alone enhanced proinflammatory cytokine production. To simplify, we repeated the study with splenic MΦ from IL-10 knockout mice. We used adult mice, as we had shown previously that a partial inhibition of p38 MAPK also enhanced IL-12 p40 and IL-6 in MΦ from adult spleens (data not shown) [17]. Even in the absence of IL-10, reduction of p38 MAPK activity resulted in an increase in proinflammatory cytokine production. Thus, p38 MAPK suppresses proinflammatory cytokine production partially, independent of IL-10.

The present findings apply to the murine system and are supported by similar deficiencies in the human neonate. However, other species have to be examined carefully to make a broader generalization about the ability of TLRs to interact with agonists. As we see an age-related difference in the response of neonatal MΦ upon stimulation by at least three different TLRs, this would suggest that the "defect" in the TLR response in neonates is at the step of a critical signaling component common to all three TLRs.

In summary, we have identified four key parameters, which may account for the reduced, proinflammatory cytokine production by the neonatal MΦ. First, the levels of several TLRs are decreased, although TLR9 is an exception. Second, a number of signaling molecules in the MyD88 pathway are decreased. Third, negative regulators such as IRAK-M are increased in the neonate. Fourth, p38 MAPK activity is increased, which appears to have dual effects. It enhances IL-10, which can inhibit proinflammatory cytokine production. However, p38 MAPK suppresses proinflammatory cytokine production, independent of IL-10, and its effect is dominant, even when TLR9 expression was the same in the neonate and the aged. This suggests that p38 MAPK may be the key reason for the differences in the pattern of cytokines produced in the neonate and the adult. Presently, our observations are based on in vitro studies, and these properties of neonatal MΦ will have to be confirmed in vivo. We plan to reconstitute the MΦ defect by providing the limiting cytokines to boost the polysaccharide antibody responses of the neonate.

We have termed the distinct cytokine pattern of neonatal MΦ as a defect, as it correlates strongly with the inability of neonatal MΦ to support an antibody response to polysaccharide antigens [18]. As a similar phenotype is seen with three different TLRs, and there is a reduction in a variety of molecules required for TLR signaling, this attenuation of proinflammatory cytokine production by neonatal MΦ may be an important developmental program of the neonate rather than a defect. The anti-inflammatory phenotype may be beneficial to the neonate at a time when tissue growth and remodeling events are taking place at a rapid pace. The anti-inflammatory phenotype of the neonate might also be a residual extension of the fetal immune system, which is Th2-polarized to avoid alloimmune reaction with the mother [61]. Thus, the inability of the neonate to respond to infection with encapsulated bacteria may be the risk the organism takes for successful development.

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