Defective macrophage function in neonates and its impact on unresponsiveness of neonates to polysaccharide antigens

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Abstract: Neonates do not respond to thymus-independent (TI) antigens (Ag), making them vulnerable to infection with encapsulated bacteria. The antibody (Ab) response of adult and neonatal B cells to TI Ag requires certain cytokines, which are provided by T cells or macrophages (MΦ). Lipopolysaccharide (LPS) failed to induce neonatal MΦ to produce interleukin (IL)-1β and tumor necrosis factor α (TNF-α) mRNA and to secrete IL-1β, IL-12, and TNF-α. However, LPS induced neonates to secrete some IL-6 and three- to fivefold more IL-10 than adults. Accordingly, adding adult but not neonatal MΦ could restore the response of purified adult B cells to trinitrophenol (TNP)–LPS, a TI Ag. Increased IL-10 is causally related to decreased IL-1β and IL-6 production, as IL-10$^{-/-}$ neonatal MΦ responded to LPS by secreting more IL-1β and IL-6 than wild-type (WT) neonatal MΦ. When cultures were supplemented with a neutralizing Ab to IL-10, WT neonatal MΦ secreted increased amounts of IL-6 and allowed neonatal MΦ to promote adult B cells to mount an Ab response against TNP–LPS. Thus, neonates do not respond to TI Ag as a result of the inability of neonatal MΦ to secrete cytokines, such as IL-1β and IL-6, probably as a result of an excess production of IL-10. This dysregulated cytokine secretion by neonatal MΦ may be a result of a reduction in expression of Toll-like receptor-2 (TLR-2) and TLR-4 and CD14. *J. Leukoc. Biol.* 75: 982–994; 2004.

Key words: *B lymphocytes · cytokines · LPS · suppression*

INTRODUCTION

The immune system of the newborn human, as well as mice and a number of other species, is not as mature as that of older children and adults, leading to a diminished capacity to generate vigorous immune responses. This results in reduced immune responses to viral infections, inability to clear fungal pathogens such as *Pneumocystis carinii*, and in increased susceptibility to infection with encapsulated bacteria, such as *Streptococcus pneumoniae* and *Hemophilus influenzae*. Neonatal immune responses appear to be dominated by T helper cell type 2 (Th2) responses [1, 2]. This is in part a result of reduced numbers of dendritic cells (DC) as well as defects in secretion of cytokines such as interleukin (IL)-12 by the neonatal DC [3]. The delayed clearance of *P. carinii* has been found to be a result of defects in the function of phagocytic cells such as the DC and alveolar macrophages (MΦ) from the neonate [4]. The increased incidence of infections with capsule-containing bacteria such as *H. influenzae* and *S. pneumoniae* is a result of an absence of antibody (Ab) response to the capsular polysaccharide (PS), a major protective antigen (Ag) for these organisms [5]. The spleen is important for immune response to these organisms, as splenectomized patients and animals are unresponsive to PS Ag from such bacteria [6, 7]. Although part of the defect in Ab responses to bacterial PS Ag is B cell immaturity [8], the possibility that neonatal MΦ may be defective in providing accessory function to B cells responsive to PS Ag has not been investigated thus far.

The responses to PS Ag have been classified as thymus-independent (TI), as they induce Ab responses from B cells in the absence of major histocompatibility complex (MHC) class II-restricted T cell help. TI Ag are divided into two types, based on whether or not they induce immune responses in CBA/N or neonatal wild-type (WT) mice. CBA/N mice, which have an X-linked, recessive, immune defect (Xid), and neonatal WT mice respond to TI-1 Ag but not to TI-2 Ag [8, 9]. Another difference is that at high doses, TI-1 Ag, unlike TI-2 Ag, become mitogenic and invoke a polyclonal response from B cells [5, 10]. In this study, we show that in low cell-density cultures, the TI-1 Ag are also unable to elicit an immune response from neonates.

Although TI Ag stimulate B cells without cognate T cell–B cell interactions, the responses of adult B cells to TI Ag are dependent on the presence of other cells, such as T cells and MΦ. Thus, rigorous depletion of T cells and MΦ will virtually eliminate the response of adult B cells to TI-1 and TI-2 Ag. This response can be reconstituted by supplementing highly purified B cells with purified adult MΦ or MΦ-derived cytokines, such as IL-1β and IL-6, suggesting that a function of MΦ in the TI Ag response is to secrete B cell-stimulatory cytokines. Thus, MΦ are an integral cellular component of response of splenic cells to TI Ag [5, 10–13].

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As a result of neonatal splenic B cells being of an immature phenotype upon cross-linking of the Ag receptor, they remain unresponsive or undergo apoptosis. Thus, it is not surprising that the focus until now has been on the immature nature of B cells as the main reason for the hyporesponsiveness of neonates to TI-2 Ag [5, 14, 15]. Despite this widely held view, we have demonstrated clearly that when supplemented with IL-1β and IL-6 or with oligodeoxynucleotides containing the immunostimulatory CpG motif, neonatal B cells could be induced to produce a robust, adult-like response to TI-2 stimuli [11, 16]. Similarly, neonatal B cells have been shown to respond to anti-immunoglobulin (Ig) coupled to high molecular weight dextran, a polyclonal TI-2 Ag, in the presence of CD40L, IL-4, and IL-5 [14]. However, when we found that neonatal spleen cells were defective in the production of IL-1β, we began to examine the ability of neonatal MΦ to produce cytokines that are known to affect B cell function.

Although lipopolysaccharide (LPS) has been characterized as a TI-1 Ag, which by definition induces an Ag-specific Ab response in neonates, it is well known that the newborn have an increased susceptibility for septic infections. This appears to be due to a reduction in secretion of cytokines such as tumor necrosis factor α (TNF-α), IL-1β, and IL-6 in neonates as compared with adults, and this is believed to play a major role in their susceptibility to bacterial infections [17, 18]. Such differences in cytokine levels in neonatal and adult humans is controversial but has not yet been reported for mice. Since it is known that the B cell response to trinitrophenol (TNP)–LPS, a TI Ag, is dependent on MΦ-derived cytokines [12, 19], we determined if splenic MΦ from neonatal mice are also defective in their response to TNP–LPS.

We hypothesized that in neonatal mice, the failure to elicit TI responses was in large part a result of defects in MΦ and that the B cells can be induced to respond to TI Ag in the presence of cytokines derived from these cells. In this study, we show for the first time that MΦ from neonatal mice, although present in numbers comparable with those in the adult, are qualitatively different from adult MΦ in that they are defective in secretion of a variety of cytokines. Consequently, they have an intrinsic inability to reconstitute the response of purified, adult B cells to TNP–LPS, a model TI-1 Ag. In particular, neonatal MΦ are defective in the secretion of IL-1β, IL-6, IL-12, and TNF-α, cytokines that have a profound influence on B cell activation. The defect in the production of some of the cytokines is a result of suppression by IL-10, whose production is elevated in the neonate. Thus, secretion of IL-1β and IL-6 but not IL-12 was restored in neonatal MΦ from IL-10$^{-/-}$ mice or by treatment of WT MΦ with anti-IL-10. Furthermore, inhibition of IL-10 partially restored TI-2 Ag responses as well as cytokine production in WT neonatal spleen cells. The defects in cytokine production by neonatal MΦ may be related to defects in the expression of Toll-like receptor (TLR)-2, TLR-4, and CD14, key mediators of MΦ activation by bacteria.

MATERIALS AND METHODS

Mice and reagents

BALB/c mice, 9–12 days and 3–4 months old, were purchased from the National Institutes of Health (Bethesda, MD) or from Harlan Sprague Dawley (Indianapolis, IN). C57BL/10 and breeding pairs of IL-10$^{-/-}$ mice on a C57BL/10 background were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in specific, pathogen-free conditions in microisolator cages in our Association for Accreditation of Laboratory Animal Care-approved facilities. For each experiment, at least two to three adults and one litter of neonatal mice were used. Phenol-extracted LPS and gel-purified TNP–LPS (*Escherichia coli* 0111:B4) were obtained from Sigma Chemical Co. (St. Louis, MO). The polyclonal goat anti-mouse IL-10-neutralizing Ab and the control goat IgG were obtained from R&D Systems (Minneapolis, MN). The scavenger receptor class A (SR-A) was detected by the rat monoclonal IgG_{2b}, Ab, 2F8. Fluorochrome-conjugated Ab to F4/80 and Mac-1 (CD11b) were purchased from Caltag (Burlingame, CA), and those to CD14, MHC II, and CD86 were obtained from Becton Dickinson (Franklin Lakes, NJ). The Ab to TLR-2 (Clone 6C2), TLR-4 (Clone MTS510), and CD16/32 (Clone 93) were obtained from eBioscience (San Diego, CA).

Cell enrichment and culture

Spleen B cells were purified by negative selection using the magnetic cell sorter (MACS) B cell enrichment microbeads from Miltenyi Biotec (Bergisch Gladbachuor, Germany). MΦ were purified by overnight adherence on tissue-culture dishes followed by collection of adherent cells by scraping in the presence of 2 mM EDTA. The enriched MΦ were routinely found to be 40–60% Mac-1$^+$. The numbers of CD11c$^+$ DC were at the level of detection. For some experiments, the MΦ were further enriched by positive selection using CD11b MACS microbeads. For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, >95% pure MΦ were isolated by sorting on a flow cytometer (MoFlo, Cytomation, Fort Collins, CO). For in vitro immunization, cultures were set up in 1 ml Iscove's DMEM + Ham's F-12 (IF-12) nutrient media + 10% fetal bovine serum in 48-well plates (Costar, Cambridge, MA) with TNP–LPS (1 μg/ml) and 50,000 MΦ and/or 125,000 B cells/well for 4 days in 5% CO_2 and at 37°C [7]. The number of IgM anti-TNP-secreting cells was determined using a glass-slide version of the Ab-forming cell (AFC) assay as described earlier [11].

Cytokine analysis

MΦ prepared by adherence (1×10^6) or further enriched with CD11b MACS microbeads (0.25×10^6) were cultured in duplicates for 1 day in medium or in 1 μg/ml LPS. Various cytokines in the supernatant were estimated in duplicate using enzyme-linked immunosorbent assay (ELISA). IL-12, IL-10, and TNF-α were estimated with OptEIA kits (PharMingen, San Diego, CA) and IL-1β, with the Quantikine M ELISA kit (R&D Systems). IL-6 was measured with a matched-pair Ab set (Clones MP5-20F3 and MP5-32C11) from PharMingen. The optical densities were read on an HTS 7000 (Perkin Elmer, Norwalk, CT) or a Molecular Devices' (Sunnyvale, CA) plate reader. Results are presented as mean of four measurements ± SD.

mRNA analysis

MΦ (2×10^6), enriched by sorting on a MoFlo cytometer (Cytomation), were cultured in duplicate with or without LPS for 6 h; the cells were harvested by incubating with 2 mM EDTA. RNA*later* (Qiagen, Valencia, CA) was added to the cells to stabilize the RNA, which was then extracted and purified using the RNeasy mini kit (Qiagen). RT-PCR was performed using the Superscript first-strand synthesis system, recombinant *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA), and a Perkin Elmer DNA thermal cycler 480. The gene-specific primers used were obtained from IDT (Coralville, IA). The sequences of the primers used for TNF-α were 5′-ATg AgC ACA gAA AgC ATg ATC (forward) and 5′-TAC Agg CTT gTC ACT CgA ATT (reverse) and for IL-1β, 5′-CAg gAT gAg gAC ATC AgC ACC (forward) and 5′-CTC TgC AgA CTC AAA CTC CAC (reverse). The PCR fragments were amplified for 30 cycles and then resolved on a 12.5% polyacrylamide gel, and the bands were visualized by staining with SYBR Gold (Molecular Probes, Eugene, OR) and quantified using a Fujifilm (Tokyo, Japan) FLA-2000 scanner. Note that the number of cycles was chosen on the basis of preliminary studies to identify conditions that were subsaturating so that quantitative comparisons between samples could be established. The data were expressed as a ratio of message-of-interest to actin message.

Flow cytometry

MΦ ($0.25–1.00\times10^6$) were incubated with 1 μg Ab for 30 min on ice. The cells were then washed with Hanks' balanced saline solution (+0.1% bovine serum albumin). Where necessary, the cells were further incubated with dye (fluorescein isothiocyanate, phycoerythrin, or Quantum Red)-conjugated streptavidin or secondary Ab for 20 min. The cells were then washed and fixed in 1% paraformaldehyde (Sigma Chemical Co.) and analyzed using a FACS-Calibur (BD Instruments, San Jose, CA).

Statistical analysis

Statistical significance of differences between averages of neonatal and adult responses was determined by the Student's *t*-test. We considered the two samples to be compared of equal variance and determined the significance for a two-tailed distribution.

RESULTS

Neonatal MΦ are unable to secrete IL-1β, IL-12, or TNF-α

Neonates, unlike adults, fail to elicit a B cell response to TI Ag. Previously, we have shown that cytokines such as IL-1β, which is reduced in neonatal mice, enabled neonatal spleen cells to respond to TNP–Ficoll, a TI Ag [7, 11]. To determine if the failure of neonates to respond to TI Ag was a result of a deficiency in the production of cytokines by neonatal MΦ, we stimulated splenic MΦ with LPS, which is not only a TI Ag but is also a potent stimulator of MΦ [8, 20]. The culture supernatants were collected, and the levels of IL-1β, IL-12, and TNF-α were measured. These cytokines are produced by MΦ upon stimulation with LPS and have previously been implicated to be directly or indirectly involved in TI responses [7, 11, 19, 21–25].

When stimulated with LPS, MΦ from adults, as seen previously [26], increased their secretion of IL-1β, IL-6, and TNF-α (**Fig. 1**). However, LPS stimulation induced neonatal MΦ to produce about five times less IL-1β and eight times less IL-12 than adult cultures (Fig. 1A and B). Within 1 day of stimulation with LPS, adult MΦ produced up to 3000 pg/ml TNF-α, and the amount secreted by neonatal MΦ (114 pg/ml) was ~26-fold less than the adult amount (Fig. 1C). Even after a further day of culturing with LPS, neonatal MΦ could still not produce any additional IL-12 or TNF-α, and conversely, adult MΦ produced a robust amount of IL-12 as early as 9 h (data not shown).

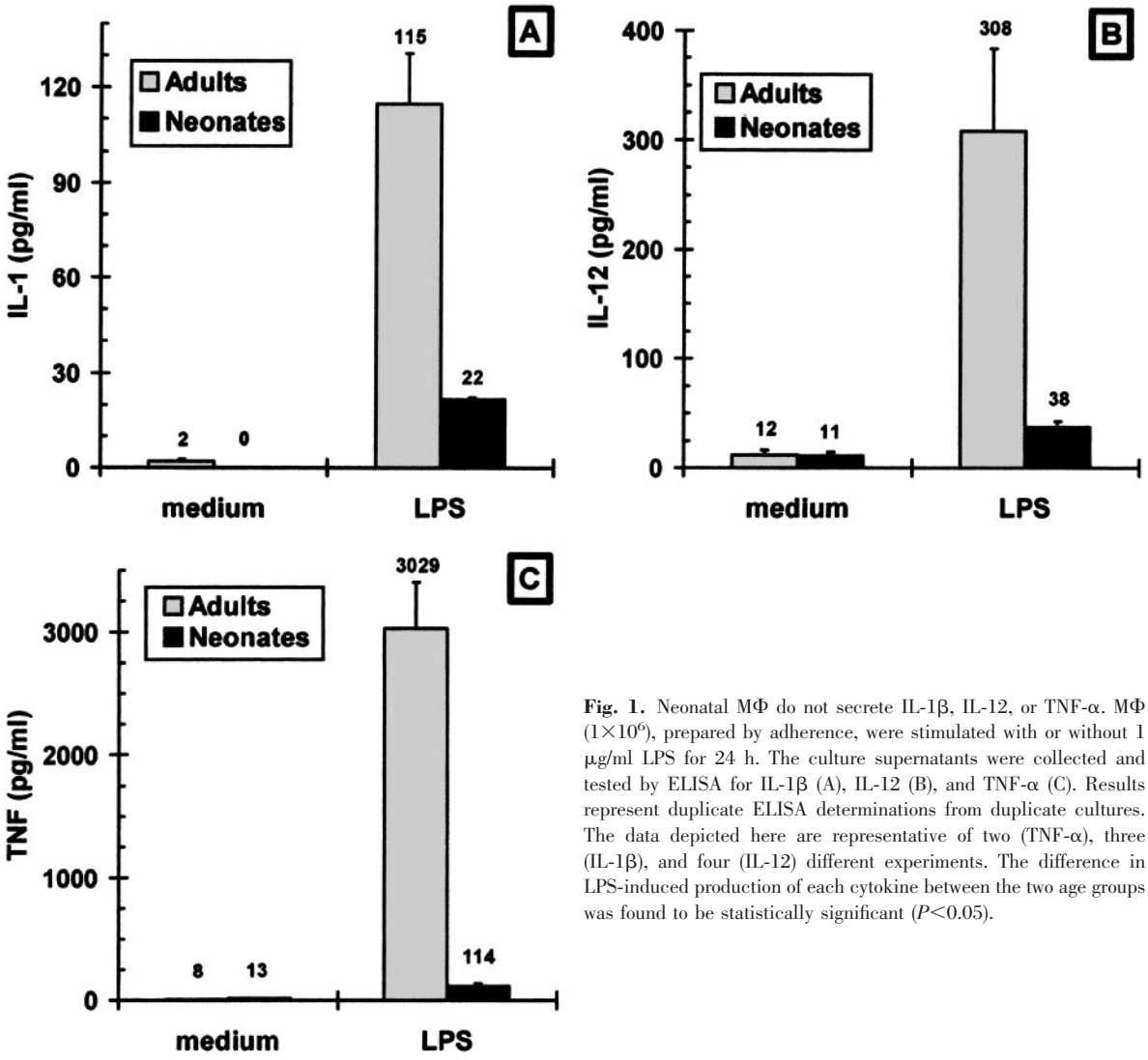

Fig. 1. Neonatal MΦ do not secrete IL-1β, IL-12, or TNF-α. MΦ (1×10^6), prepared by adherence, were stimulated with or without 1 μg/ml LPS for 24 h. The culture supernatants were collected and tested by ELISA for IL-1β (A), IL-12 (B), and TNF-α (C). Results represent duplicate ELISA determinations from duplicate cultures. The data depicted here are representative of two (TNF-α), three (IL-1β), and four (IL-12) different experiments. The difference in LPS-induced production of each cytokine between the two age groups was found to be statistically significant ($P<0.05$).

Neonatal MΦ secrete less IL-6 and more IL-10 than adult MΦ

Were neonatal MΦ compromised in their ability to secrete only these three key B cell-stimulatory cytokines (i.e., IL-1β, IL-12, and TNF-α), or was there a general defect in the response of neonatal cells to LPS? To address this, we measured other LPS-induced cytokines, such as IL-6, which plays an important role in the regulation of immune responses [27]. Upon stimulation with LPS, MΦ from both age groups produced IL-6, and adult MΦ produced about twice as much as neonatal MΦ (**Fig. 2A**). On average, neonatal MΦ produced only 42 ± 17% ($P<0.001$) of the amount secreted by adult MΦ (data not shown). Decreasing the concentration of LPS resulted in a dose-dependent decrease in IL-6 production from MΦ of both ages (Fig. 2B), indicating that the IL-6 defect is not a result of a suppression caused by too high a concentration of LPS. Increasing the concentration of LPS to 10 μg/ml also did not overcome this defect (data not shown). Furthermore, neonatal MΦ did not secrete as much as IL-6 as adult MΦ, even when a highly purified preparation of LPS in the form of TNP–LPS was used as a stimulant (Fig. 2A). Thus, the IL-6 synthetic machinery in neonatal MΦ is also compromised, however not as severely as those involved in the synthesis of IL-1β, IL-12, and TNF-α.

We also tested the production of IL-10, as it suppresses activity of MΦ by inhibiting the LPS-induced secretion of cytokines such as IL-1β, IL-6, IL-12, and TNF-α [28, 29] and promoting B cell survival and Ig secretion [28]. IL-10 was produced by neonatal and adult MΦ, but the neonatal MΦ consistently produced more (two- to three-fold) IL-10 than adults when stimulated with LPS (Fig. 2C, data not shown). This is in accordance with a recent report that showed a slight, but significant, increase in IL-10 production by neonatal MΦ compared with adult MΦ in the mouse listeriosis model [30]. The ability of neonatal MΦ to secrete IL-6 and IL-10 indicated that neonatal MΦ were indeed viable and responded to LPS, although very differently from adult MΦ.

Analysis by flow cytometry showed that the adherent cell preparation of MΦ contained a substantial proportion of B cells (data not shown). To test if this B cell contamination could interfere with the response of MΦ, we further enriched the MΦ preparation by positive selection with CD11b microbeads and then stimulated the cells with LPS. Analysis of cytokines secreted by these highly purified MΦ demonstrated that they produced significantly less IL-6 and increased IL-10 in the neonate compared with adults (Fig. 2D and E), in complete agreement with the results presented in Figures 1 and 2, A–C. This indicates that the differential pattern of cytokine secretion seen with adherent MΦ preparations (i.e., neonatal MΦ make no IL-1β, IL-12, TNF-α, reduced IL-6, and excess IL-10) is most likely intrinsic to MΦ and is not influenced by nor caused by the contaminating B cells.

IL-1β and TNF-α production suppressed at mRNA level in neonatal MΦ

As there are instances where IL-1β and TNF-α are regulated at the level of secretion and transcription [26, 31, 32], we were interested to see if the production of these cytokines in neonatal MΦ was blocked at the level of transcription. Unstimulated adult and neonatal MΦ (fluorescein-activated cell sorter, sorted on the basis of CD11b expression) were stimulated with LPS, and levels of IL-1β and TNF-α mRNA were measured by RT-PCR. To control for differences in the RNA content of different stimulation groups, actin transcripts were measured by RT-PCR and were used to normalize the cytokine mRNA. Stimulating with LPS caused an increase in the steady-state levels of IL-1β and TNF-α mRNA in adults but not in neonates (**Fig. 3A** and **B**, respectively). Thus, the cytokine defect in neonatal MΦ appears to be a result of a lack of sufficient transcripts for IL-1β and TNF-α.

Neonatal MΦ have reduced ability to support B cell TI Ag responses

To determine the functional impact of this cytokine deficiency in neonatal MΦ, we measured Ab responses to TNP–LPS, a TI Ag known to require MΦ for optimal Ab responses [12]. Neonates (9–12 days old) made Ab responses similar in magnitude to those of adults (3–4 months old) in high cell-density cultures, but at low cell densities, their responses to TNP–LPS were dramatically reduced (**Fig. 4A**). We further investigated to see if these reduced TI Ag responses in the neonate could be a result of functional defects of neonatal MΦ. Despite the well-known dependence of TI Ag on MΦ, few studies have compared directly the ability of neonatal and adult MΦ to restore TI response from purified B cells.

Previously, it had been shown that under limiting culture conditions, responses of highly purified, adult B cells to TNP–LPS were dependent on the presence of MΦ [12], implying that although TNP–LPS is a TI Ag, its ability to elicit an AFC response from B cells requires help from MΦ. We therefore purified B cells from adult mice and stimulated them in vitro with TNP–LPS in the presence of various doses of MΦ-enriched, splenic adherent cells from adult or neonatal mice. Accordingly, purified adult B cells (125×10^3 per culture) did not produce an AFC response to a highly purified preparation of TNP–LPS in the absence of MΦ (Fig. 4B). Supplementing adult B cells with 50,000 adult MΦ enabled adult B cells to respond to TNP–LPS. However, when the same number of neonatal MΦ was used instead, the adult B cell response was equal to that of the background (i.e., response without any MΦ; Fig. 4B). Even doubling the number of neonatal MΦ to 100,000 per culture did not have any effect on the B cell response (data not shown). Although the MΦ preparations do contain some B cells (ca., 20,000), the numbers of B cells present are too small to have any meaningful impact on the response of the purified adult B cells (125,000; data not shown). Thus, neonatal MΦ are defective in helping B cells respond to TI Ag responses.

Neonatal MΦ are of mature phenotype

To test if this cytokine and functional deficiency could be attributed to the level of maturity of neonatal MΦ, we measured the expression of a number of key MΦ-surface Ag. There was no difference between neonates and adults in the proportions of MΦ-expressing MHC II, CD86, or SR-A. Neonatal MΦ expressed similar amounts of CD86 and SR-A but exhibited a

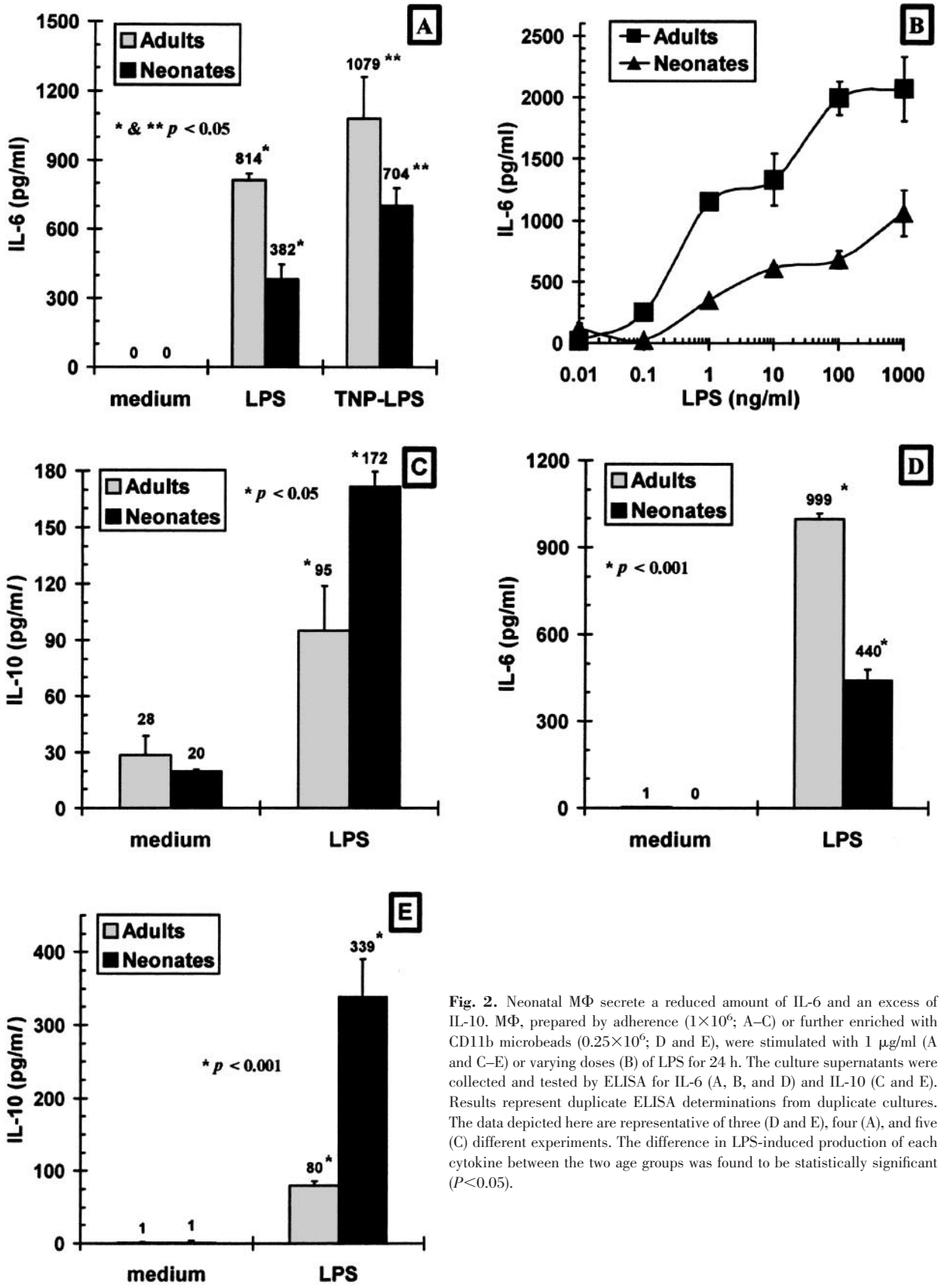

Fig. 2. Neonatal MΦ secrete a reduced amount of IL-6 and an excess of IL-10. MΦ, prepared by adherence (1×10^6; A–C) or further enriched with CD11b microbeads (0.25×10^6; D and E), were stimulated with 1 μg/ml (A and C–E) or varying doses (B) of LPS for 24 h. The culture supernatants were collected and tested by ELISA for IL-6 (A, B, and D) and IL-10 (C and E). Results represent duplicate ELISA determinations from duplicate cultures. The data depicted here are representative of three (D and E), four (A), and five (C) different experiments. The difference in LPS-induced production of each cytokine between the two age groups was found to be statistically significant ($P<0.05$).

considerably higher density of MHC II (**Table 1**). There was no difference between the two age groups in the proportions of cells expressing Mac-1, but neonatal cells had a considerably higher level of Mac-1 on their cell surface (Table 1). A higher proportion of neonatal MΦ expressed F4/80, and these positive cells had a much higher amount of F4/80 on their cell surface. As F4/80 and Mac-1 are considered to be markers for mature MΦ [33], our results indicate that neonatal MΦ are of a mature

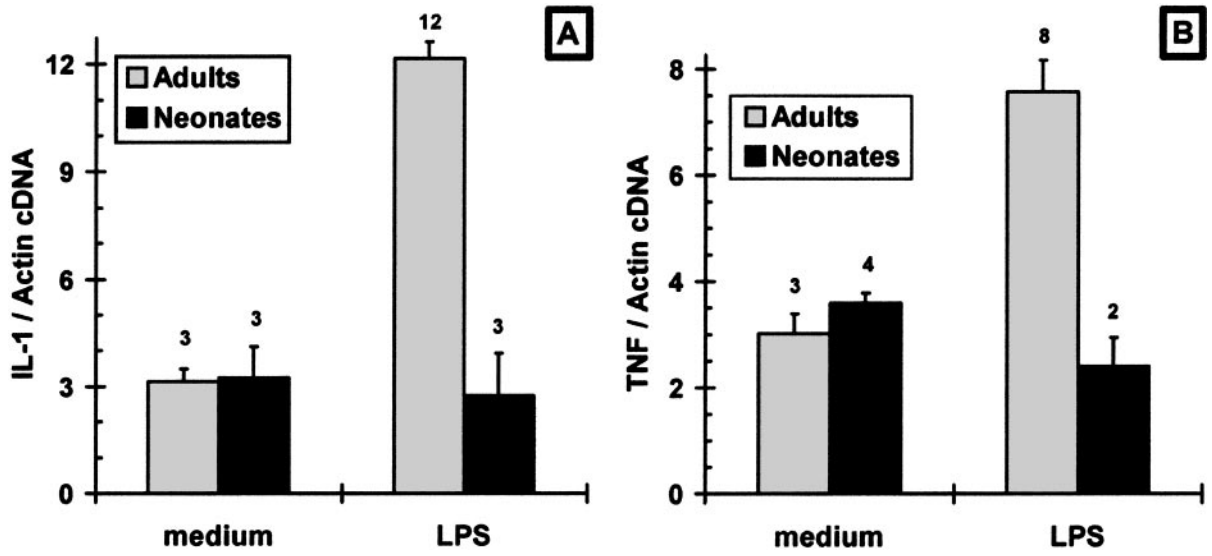

Fig. 3. Neonatal MΦ are unable to up-regulate IL-1β and TNF-α mRNA upon LPS stimulation. CD11b$^+$ MΦ were enriched by flow cytometry and stimulated for 6 h with 10 μg/ml LPS. The cDNA was transcribed from the extracted RNA. The levels of cDNA for IL-1β (A) and TNF-α (B) were determined by semiquantitative PCR. Results represent duplicate determinations of cDNA from duplicate cultures. The differences between adult and neonatal MΦ stimulated with LPS were statistically significant ($P<0.05$).

phenotype. Also, we determined the levels of expression of MOMA-1 and MOMA-2, markers for metallophilic MΦ and marginal zone MΦ, respectively, because of their importance for TI Ag responses [33, 34]. However, we did not see significant differences in staining patterns between neonatal and adult marginal-zone, resident MΦ (data not shown). In summary, neonatal MΦ expressed comparable or higher amounts of key surface Ag but yet, were functionally immature, as they were unable to respond appropriately to LPS stimulation or provide adequate help to B cells to respond to TI Ag (Figs. 1–4).

In the absence of IL-10, neonatal MΦ produce adult levels of IL-1β and IL-6

IL-10 has previously been shown to inhibit the synthesis of IL-1β, IL-12, and TNF-α and reduce the production of IL-6 in LPS-activated MΦ [29, 35]. Accordingly, we found that at concentrations detected in LPS-induced, neonatal MΦ cultures, IL-10 inhibited secretion of IL-6, IL-1β, IL-12, and TNF-α by adult MΦ (data not shown). To investigate whether IL-10 is one of the causes of the defect in the cytokine production in neonatal MΦ or whether its perturbation is

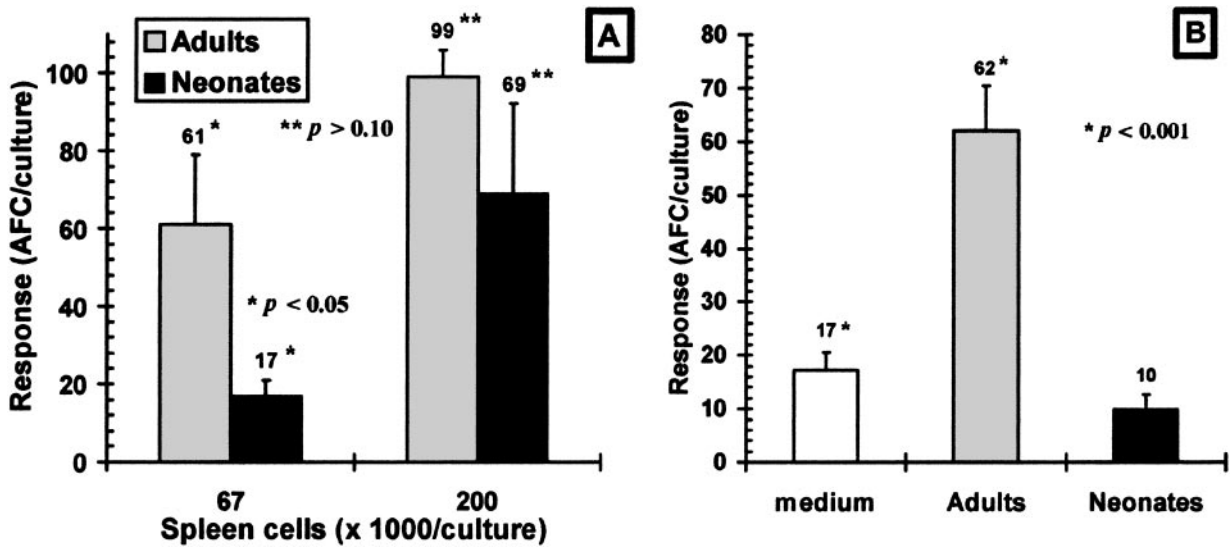

Fig. 4. Unseparated neonatal spleen cells do not respond to TNP–LPS, and neonatal MΦ cannot reconstitute the response of adult B cells to TNP–LPS. The Ab responses were determined by the AFC assay on the fourth day after culturing with 1 μg/ml TNP–LPS. (A) Two doses of unseparated spleen cells. (B) Purified adult B cells (125,000) were cultured with 50,000 MΦ from neonates or adults. Results are representative of three other experiments. The differences between the adult and neonates were statistically significant ($P<0.05$) except in the high cell-density cultures (A).

TABLE 1. Neonatal MΦ Have Reduced Amounts of Cell-Surface CD14 and TLR-4

	Neonates		Adults	
Marker	% +ve	MFI	% +ve	MFI
MHC II	54	582	58	346
CD86	13	118	10	110
SR-A	15	122	12	123
Mac-1	57	393	53	227
F4/80	56	1044	33	411
CD14	27	131	16	607

MΦ enriched by adherence were stained with the appropriate Ab and then subjected to flow cytometry. MFI, Mean fluorescence intensity. Representative results from one of two experiments are shown.

actually a result of the defect, we assessed the LPS-induced cytokine pattern in neonatal IL-10$^{-/-}$ mice. First, we measured these cytokines in C57BL/10 neonates and adults, as our IL-10$^{-/-}$ mice were in the BL/10 background. Like BALB/c, the BL/10 neonates also produced less IL-1, IL-12, and IL-6 and more IL-10 than adults (**Fig. 5**). The TNF-α production was also less in BL/10 neonates compared with adults, but the reduction was not as high as in the BALB/c neonates. When stimulated with LPS, neonatal MΦ from IL-10$^{-/-}$ mice consistently produced comparable amounts or slightly more IL-6 and IL-1β than the IL-10$^{-/-}$ adults (**Fig. 6A** and **B**), in contrast to neonatal WT MΦ, which consistently produced less IL-1 and IL-6 than the WT adult (Figs. 1A and 2B, and 5A and D). The small differences between the IL-10$^{-/-}$ adult and neonates were not statistically significant ($P>0.05$). However, even in the absence of IL-10, neonatal MΦ were unable to secrete adult levels of TNF or IL-12, in response to LPS (Fig. 6C and D). This is rather surprising, as it is well-documented that IL-10 also suppresses LPS-induced production of IL-12 and TNF-α in adult MΦ [29, 35]. This would imply that the suppression of LPS-induced IL-12 and TNF in neonates is more complex than the suppression of IL-1β and IL-6. The restoration of IL-1 and IL-6 is critical for B cell responses, as these two cytokines together can enable highly purified neonatal or adult B cells to respond to TI Ag [11].

Neutralization of IL-10 increases IL-6 production and enhances response to TI Ag in WT neonates

As the altered cytokine phenotype in the IL-10$^{-/-}$ mice could be a result of developmental defects from the lack of IL-10, we determined if eliminating IL-10 in WT cultures would induce WT neonates to secrete cytokines at adult levels. Indeed, neutralizing IL-10 with Ab, induced neonatal WT MΦ to secrete increased amounts of IL-6 (1898±31 pg/ml) in response to LPS stimulation (**Fig. 7A**), which compared favorably with the IL-6 levels produced by the adult MΦ (1844±44 pg/ml) in the same experiment. As IL-1β and IL-6 are crucial for the B cell response to TI Ag [11], we predicted that the alleviation of the suppressive effects of IL-10 would allow the neonatal MΦ to provide adequate support to adult B cells to respond to TNP–LPS. To test this hypothesis, we stimulated purified B cells from WT adult mice with TNP–LPS in the presence of WT neonatal MΦ and anti-IL-10 and then measured the Ag-specific Ab response by the plaque-forming cell assay 4 days later. Purified WT adult B cell response was enhanced by supplementing the cultures with adult but not neonatal MΦ (Fig. 7B) as seen previously (Fig. 4B). However, when cultures of adult B cells and neonatal MΦ were supplemented with a neutralizing Ab to IL-10, this resulted in a TNP–LPS response comparable with that seen with adult MΦ. This would suggest that the inability of neonatal MΦ to provide adequate help to B cells to respond to TI Ag is partly a result of their nature to produce excess amounts of IL-10 (Fig. 2C and E).

As upon LPS stimulation, IL-10$^{-/-}$ neonatal MΦ did not produce any IL-12, we then supplemented cultures with this cytokine to see if it would overcome the unresponsiveness. IL-12 did not enable neonatal MΦ to help adult B cells to respond to TNP–LPS (Fig. 7B) and when added together with anti-IL-10, did not enhance the response seen with only anti-IL-10 (data not shown). Thus, although neutralization of IL-10 does not lead to neonatal MΦ producing IL-12 upon stimulation with TNP–LPS (Fig. 6D), it appears that the inability of neonatal MΦ to make IL-12 is not one of the reasons why they are unable to help B cells to respond to TI Ag.

Neonatal MΦ have reduced amounts of CD14 and TLR-2 and TLR-4

This then led us to examine the expression of the receptor complexes such as CD14 and TLRs involved in the recognition and activation by LPS [36]. Although the proportion of CD14$^+$ cells in the neonate was higher than the adult MΦ, the neonatal MΦ had a much lower level (fivefold less) of surface expression of CD14 (Table 1). Initially, we probed CD11b$^+$ MΦ with Clone MTS510, an Ab that detects the TLR-4/myeloid differentiation protein-2 complex [37], which is a small protein secreted by MΦ that appears to be physically associated with TLR-4 and enhances the ability of LPS to activate cells via TLR-4 [37, 38]. A significantly fewer number of freshly prepared neonatal MΦ have TLR-4 (46% vs. 67%; **Fig. 8A**). Moreover, neonatal TLR-4$^+$ MΦ also have a reduced amount of surface TLR-4. CD11b$^+$ MΦ were also analyzed for TLR-2 expression using Clone 6C2. Unstimulated, neonatal CD11b$^+$ MΦ were also compromised in expressing TLR-2 (14% in neonates vs. 38% in adults; Fig. 8B).

Reduction of CD14 and TLR-2 and -4 proteins on the cell surface might partially explain why neonatal MΦ have a defective response to LPS. The fact that two cytokines, IL-6 and IL-10, are produced by neonatal, splenic accessory cells in substantial amounts, and three other cytokines are dramatically reduced suggests that the decreased expression of CD14 and TLR molecules may have differential effects on intracellular signaling pathways.

DISCUSSION

The results presented here have demonstrated that neonatal MΦ are defective in the production of a variety of proinflammatory cytokines upon stimulation with the potent MΦ activator LPS. Specifically, we find that neonatal MΦ produce re-

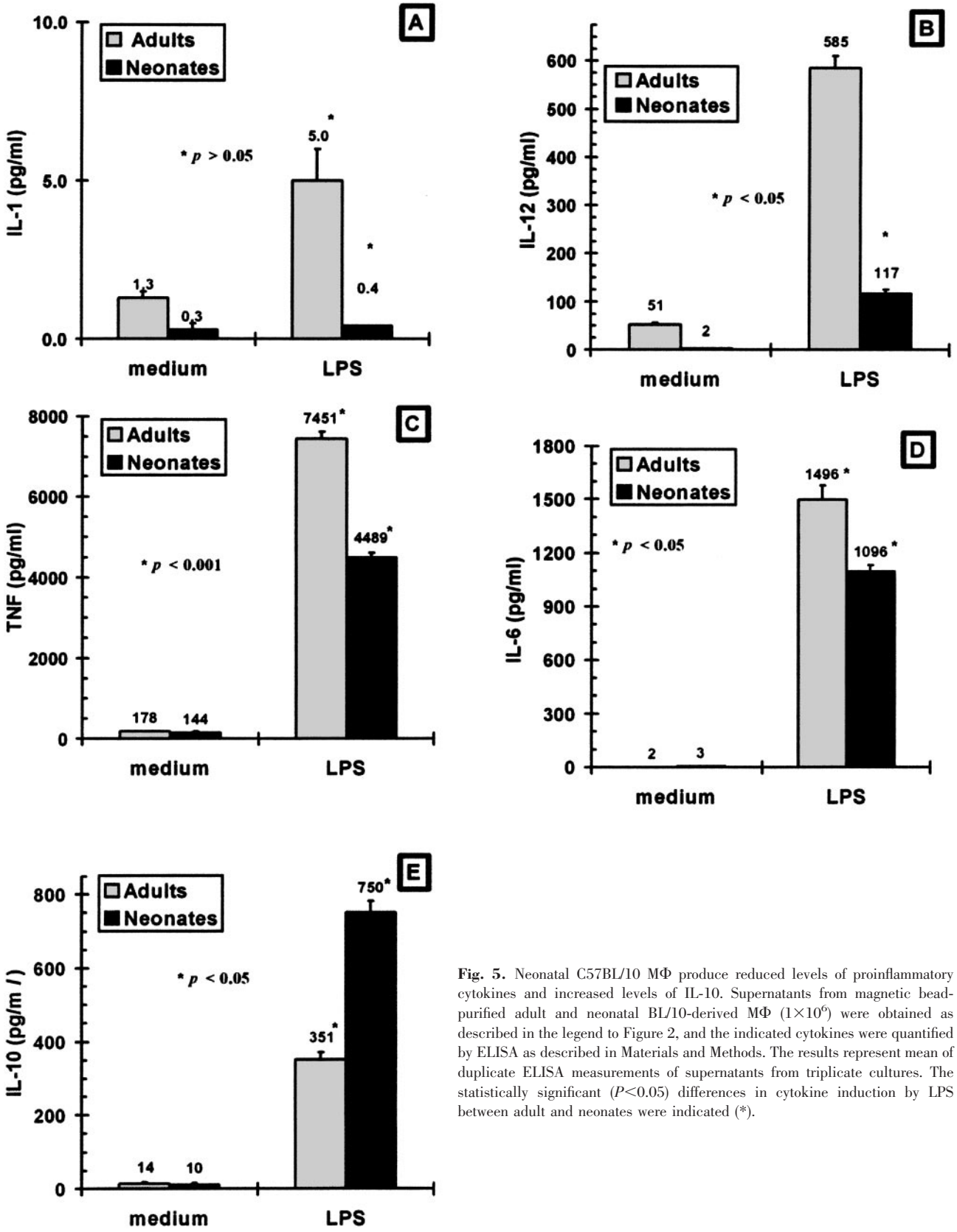

Fig. 5. Neonatal C57BL/10 MΦ produce reduced levels of proinflammatory cytokines and increased levels of IL-10. Supernatants from magnetic bead-purified adult and neonatal BL/10-derived MΦ (1×10^6) were obtained as described in the legend to Figure 2, and the indicated cytokines were quantified by ELISA as described in Materials and Methods. The results represent mean of duplicate ELISA measurements of supernatants from triplicate cultures. The statistically significant ($P<0.05$) differences in cytokine induction by LPS between adult and neonates were indicated (*).

duced levels of cytokines that stimulate B cells, such as IL-1β, IL-6, IL-12, and TNF-α, all of which are proinflammatory cytokines. The defect in production of these cytokines appears to be at the level of mRNA, as demonstrated by RT-PCR analysis. Furthermore, we show that the defective production of these proinflammatory cytokines is in part a result of an increase in IL-10 production in the neonate, as the production of IL-1β and IL-6 is restored to almost adult levels in neonatal MΦ from IL-$10^{-/-}$ mice or by treating WT neonatal MΦ cultures with anti-IL-10 Ab.

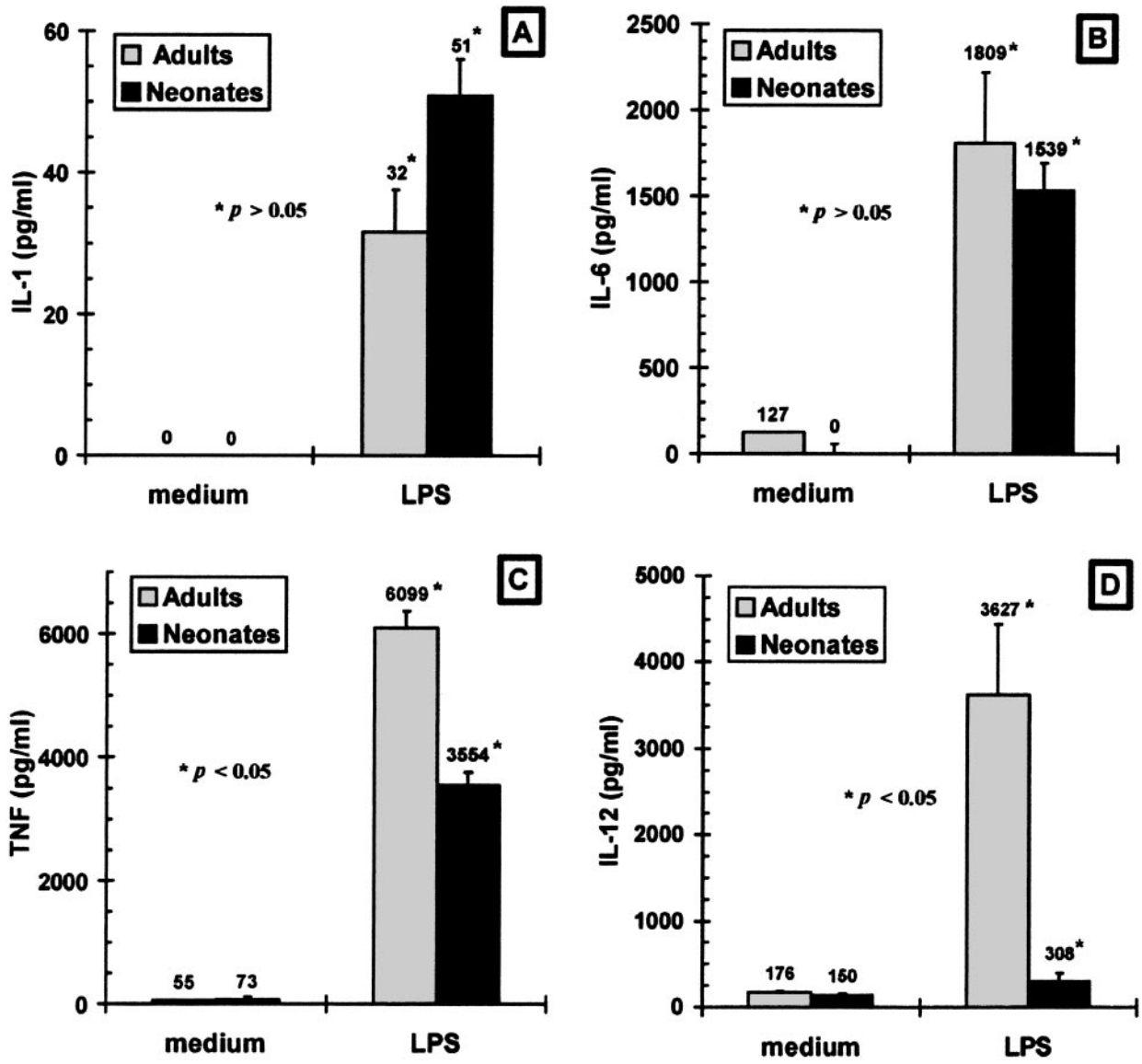

Fig. 6. Neonatal IL-10$^{-/-}$ MΦ produce adult levels of IL-1β and IL-6 but not IL-12. MΦ (1×10^6), prepared by adherence, were stimulated with or without 1 μg/ml LPS for 24 h. The culture supernatants were collected and tested by ELISA for IL-1β (A), IL-6 (B), TNF-α (C), and IL-12 (D). The differences between adult and neonate were not statistically significant in panels A and B but were significant in panels C and D ($P<0.05$). Differences between cells cultured with medium versus LPS are significant ($P<0.05$; A–D). Results are representative of two (D) or three (A–C) experiments.

This study has also clearly established that MΦ are necessary for B cells to respond to TI Ag. We previously showed that neonates cannot mount an Ab response to a TI Ag, unless supplemented with exogenous IL-1β [11]. This study draws these pieces of the puzzle together. Here, we show for the first time that the one major reason for the unresponsiveness of neonates to TI Ag is a result of a defect in MΦ function in neonates, which can be overcome by supplementing cultures with anti-IL-10. The effect of IL-10 appears to be primarily on neonatal MΦ but not on B cells, as it does not suppress TI Ag response of neonatal spleen cells supplemented with exogenous IL-1β (data not shown). Taken together, these results show that neonatal unresponsiveness to TI Ag is a result of defective function of MΦ in addition to B cell immaturity.

The defective cytokine production by neonatal MΦ is not unique to LPS stimulation. We recently found that neonatal MΦ produced less of IL-6 and more of IL-10 than adult MΦ when stimulated with the TLR-2 ligands, fixed *S. pneumoniae* bacteria, or peptidoglycan (data not shown). Furthermore, the functional deficiency of neonatal MΦ to help TI Ag-specific Ab responses is also not unique to TNP–LPS, as we observed similar defects with neonatal MΦ when TNP–Ficoll, another TI-Ag, was used to stimulate purified, adult B cells. Also the TNP–Ficoll response of neonatal splenocytes was enhanced by the use of the neutralizing Ab to IL-10 (data not shown).

What is the basis of cytokine dysregulation in the neonatal MΦ? This did not appear to be a result of the immaturity of the neonatal MΦ, as the phenotypic markers such as Mac-1, F4/80, MHC class II, and SR-A are present in similar or greater levels than adult MΦ. Also the numbers of MΦ expressing these markers are again slightly more in the neonate than in the adult. However, neonatal Mϕ express reduced

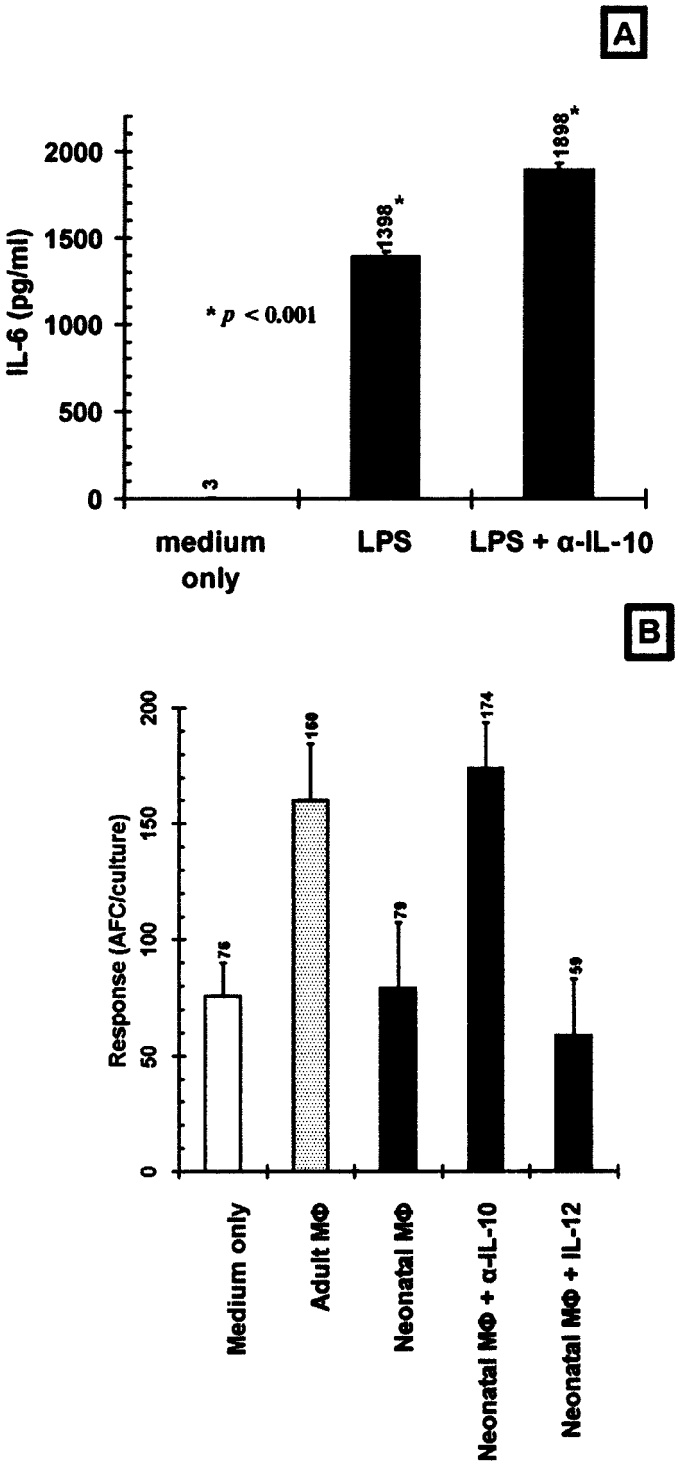

Fig. 7. Neutralization of IL-10 restores LPS-induced IL-6 production and B cell response to TNP–LPS. (A) MΦ (1×10^6) were stimulated with 1 μg/ml LPS for 24 h in the presence of 0.625 μg/ml neutralizing Ab to IL-10 (α-IL-10). The culture supernatants were collected and tested by ELISA for IL-6. Results represent duplicate ELISA determinations from duplicate cultures. The difference in LPS-induced production of IL-6 in the absence and presence of the of anti-IL-10 Ab was statistically significant ($P<0.001$). Control cultures received normal goat IgG + LPS. (B) Purified adult B cells (125,000) were cultured with 50,000 purified MΦ from neonates or adults. Where necessary, cultures were also supplemented with 0.625 μg/ml anti-IL-10 (α-IL-10) or 30 pg/ml IL-12. The effect of anti-IL-10 was statistically significant ($P<0.005$). The data depicted here are the mean ± SE from two independent experiments.

levels of important receptors such as CD14, TLR-2, and TLR-4, which are involved in recognition and binding of LPS and *S. pneumoniae*. The TLR signaling is mediated by the adaptor protein MyD88, as well as by translation initiation region (TIR) domain-containing adapter protein and TIR domain-containing adapter-inducing interferon-β [39]. The MyD88 pathway involves activation of IL-1 receptor-activating kinase and its association with TNF receptor-associated factor-6, and this results in activation of mitogen-activated protein kinases and nuclear factor (NF)-κB transcription factor, which has been shown to play a crucial role in MΦ production of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. Decrease in these cytokines but not of IL-10 is consistent with the finding that NF-κB is not required for production of IL-10 in response to several mitogenic stimuli [40]. Recently, transcription of IL-10 mRNA has been shown to depend on another transcription factor, specificity protein-1 (Sp1), which is constitutively active in MΦ [41]. Presently, it is unclear if Sp1 is up-regulated in neonatal MΦ. Furthermore, IL-10 has been shown to selectively and rapidly inhibit nuclear translocation of NF-κB in a dose-dependent manner [42]. The fact that the TLR defect affects production of IL-1β but not IL-10 is consistent with the recent findings that TLR can activate MΦ in NF-κB-dependent and -independent pathways [43]. The excess production of IL-10 in the neonate may lead to an increase in suppressor of cytokine signaling-3, which is known to block the transcription of a number of cytokine genes [44].

Besides IL-10, transforming growth factor (TGF)-β might also play a role in the dysregulated production of cytokines by neonatal MΦ. This cytokine is induced in MΦ by LPS and exhibits identical effects on target cells [45–47]. This may explain our finding that deletion of the IL-10 gene alone restored production of IL-1β and IL-6 but has only modest effects on TI Ag responses. Also, TGF-β may contribute to the IL-12 defect in the neonates, as production of this cytokine is not restored in the IL-$10^{-/-}$ mice. Furthermore, as TGF-β enhances the amount of LPS-induced IL-10 [46], this might account for the increased presence of IL-10 in activated, neonatal MΦ cultures from WT mice.

Although TI Ag are not as numerous or as well-studied as the better known thymus-dependent (TD) Ag, they span a diverse range of molecules, such as PS, liposomes, phosphorylcholine, and poly(I:C). The main feature of these diverse molecules that allows them to activate B cells without the requirement for MHC-restricted help is that they are all large, rigid molecules with repeating determinants that allow efficient cross-linking of the B cell receptors [8]. A few years back, it was shown that IgM production in response to polio virus or to a recombinant vaccinia virus expressing the neutralizing determinant of vesicular stomatitis virus behaved like classical TI-2 responses, in that Xid mice, which have a mutation in Btk, were unresponsive to these viruses [48, 49]. This is not surprising, as these viral proteins are large molecules with repeating Ag epitopes. These viruses also initiate TD responses, but as the TI-2 responses peak earlier, the TI-2 responses provide the host with valuable time to reduce the viral load [50]. However, it has not yet been shown if MΦ play a role in the TI responses initiated by viral proteins, although one could speculate that B cells specific for viral TI Ag should be just as

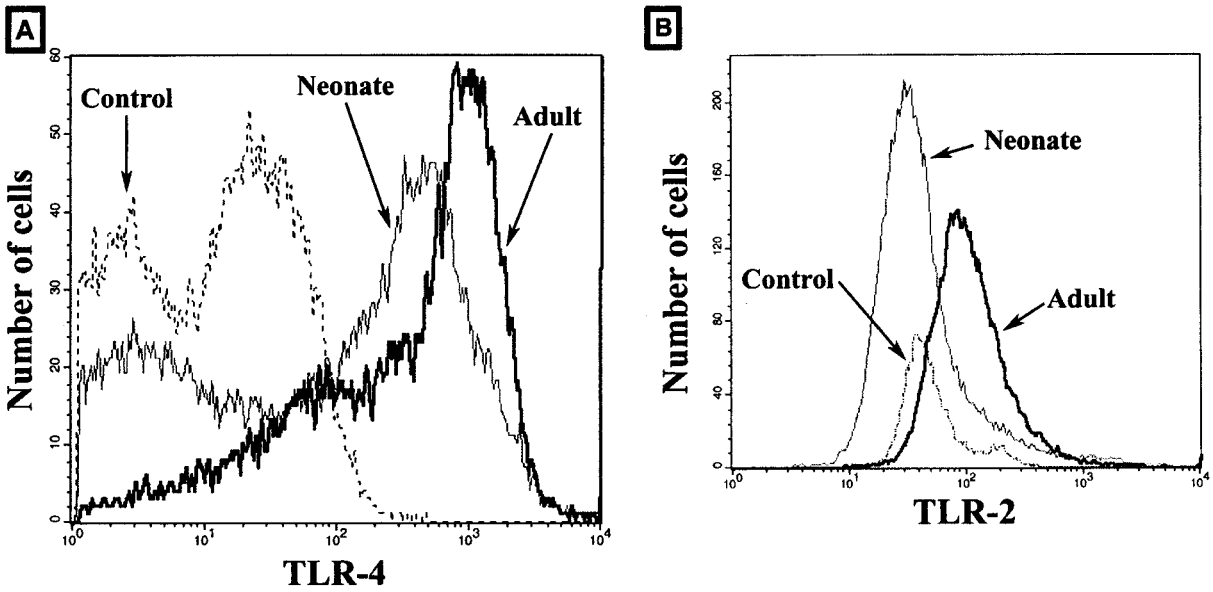

Fig. 8. Neonatal MΦ express reduced amounts of surface TLR-2 and TLR-4 protein. MΦ were enriched by adherence and stained with anti-CD11b and anti-TLR-4 or anti-TLR-2. The staining pattern of CD11b-gated cells for TLR-4 (A) and TLR-2 (B) is depicted. Results are representative of two independent experiments. All samples were blocked with anti-CD16/CD32 before staining. Bold lines, Adult; thin lines, neonate; dashed lines, isotype-control Ig.

dependent on MΦ as those B cells for other traditional TI Ag. Similarly, it is not known if the TI responses to vesicular stomatitis virus are defective in neonates.

This is the first distinct demonstration of profound defects in neonatal MΦ, which contribute to the neonatal unresponsiveness to TI Ag-like TNP–LPS, TNP–Ficoll, and pneumococcal PS. The ability of neonates to respond well to TI-1 Ag under some conditions may relate to the ability of TI-1 Ag to induce a direct, mitogenic response in B cells as well as a result of their need for very small numbers of MΦ [9]. It was surprising that production of a variety of proinflammatory cytokines is reduced in neonates, considering the fact that newborns are highly susceptible to septic shock. However, in vivo, MΦ from different tissues, such as liver Kupffer cells, may participate in the septic response, and these studies have not examined MΦ from individual tissues, such as the spleen. It is interesting that Farver and Kobzik [51] show that lung MΦ from neonatal rats are functionally immature. Murine alveolar MΦ were also found to be immature by Garvy and Qureshi [4].

The neonatal MΦ defect contributing to the defective TI Ag responses is similar to a MΦ defect in aged mice, leading to their defective responses to pneumococcal PS Ag reported by us previously [52]. In this system, we also found that young-adult MΦ or IL-1β were able to restore pneumococcal PS response from aged mouse B cells. There is, however, a difference between the two ages. Aged, splenic MΦ had a quantitative defect in providing accessory function. Whereas, as no amount of neonatal MΦ could help B cells to respond to TI Ag, the defect in the neonate appears to be qualitative. The differences between the two will become clearer once we measure cytokine production and TLR levels in aged, splenic MΦ.

The neonatal immune system has been characterized as being unresponsive to a number of classes of Ag and being predisposed toward Th2 responses. Initial studies implicated T and B cells [5, 15, 53, 54]. As these cells in the neonates were functionally immature, they were unable to respond appropriately to Ag or would actually undergo apoptosis upon Ag encounter. More recent studies though have focused on the role of accessory cells in inducing tolerance in neonatal immunity. These defects have been interpreted to be in part a result of a reduced number of DC [2, 53, 54]. Moreover, even these few DC are not fully functional. One study, using TD–PS conjugate vaccines, shows that neonatal DC were severely defective in presenting TD epitopes to Ag-specific T cell clones [55]. The profound defects in MΦ production of IL-1β and IL-12 suggest that the hyporesponsiveness of neonates is not only a result of a defect in B cells and DC but also may be a result of a qualitative defect in MΦ.

However, the MΦ defect in neonates is rather complex. IL-10 plays a critical role in their unresponsiveness; nevertheless, it is not the sole determining factor. Upon stimulation with LPS, neonatal IL-$10^{-/-}$ MΦ behaved like adult MΦ in that they secreted adult levels of IL-1β and IL-6; however, they, like WT neonates, still had significantly reduced amounts of TLR-4 and CD14, molecules critical for LPS binding and signal transduction (data not shown). Thus, the IL-10-induced suppression of LPS-induced cytokine secretion in neonates is downstream of TLR-4 and CD14.

This study suggests that approaches that involve improving the immunogenicity of PS vaccines in the neonates will have to involve directly activating MΦ. Suppressing endogenous IL-10 will help redress the imbalance and induce a response to TI Ag. However, to be able to induce neonates to respond in an adult-like manner, further study in this area is required, particularly to understand why neonates are defective in CD14, TLR-4, and IL-12. As MΦ are also a key player in TD responses, by acting as professional antigen-presenting cells, their immaturity will surely affect at least the efficacy of some TD vaccines.

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