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Tumor Necrosis Factor/Cachectin and Interleukin-1 Secretion by Cord Blood Monocytes from Premature and Term Neonates

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ABSTRACT. Blood monocytes produce two well-defined cytokines, tumor necrosis factor/cachectin (TNF) and Il-1, that have multiple immunologic and inflammatory functions. This study examined the secretion of these cytokines by cord blood monocytes from preterm and term neonates stimulated with or without lipopolysaccharide (LPS). Seventeen samples (eight preterm, nine term) were collected. Supernatants of monocytes were assayed for activities of IL-1 (mitogenesis for mouse thymocytes) and TNF (cytotoxicity for L929 cells). IL-1 and TNF activities were not significant in supernatants of unstimulated monocytes from either preterm or term infants. IL-1 secretion by LPS-stimulated monocytes from term and preterm neonates was comparable to IL-1 activity by monocytes from adults, but TNF activity by LPS-stimulated monocytes from preterm neonates was significantly less than that from monocytes of either term or adult groups ($p < 0.05$). TNF activity in supernatants of LPS-stimulated monocytes was neutralized 100% by monoclonal antibody to TNF-α; IL-1 activity was neutralized 80% by polyclonal antiserum to IL-1-β. Given the multifactorial biologic activities of TNF, the decreased secretion of TNF by monocytes from preterm neonates may be significant in describing mechanisms for the increased susceptibility of the preterm neonate to infection. (*Pediatr Res* 25:342–346, 1989)

Abbreviations

TNF, tumor necrosis factor
LPS, lipopolysaccharide

The immunologic competence of the neonate has been an area of concern both for the clinician and the researcher because neonates have an increased risk for sepsis and often do not manifest the typical signs, such as fever, that are seen in older children (1). Most studies of the immune and inflammatory response from neonates have examined the functions of lymphocytes and neutrophils. For example, lymphocytes from neonates respond adequately to mitogens and antigens (2–4), but lymphokine production is variable (2, 5). Polymorphonuclear cells of neonates are similar to those of adults with regard to phagocytosis and bactericidal activity against certain microorganisms, but adherence and migration are deficient (6, 7).

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Mononuclear phagocytes such as blood monocytes and tissue macrophages produce mediators of fever and sepsis (8), are capable of phagocytosis and bactericidal activity, and regulate the immune response (6, 8–10). Blood monocytes are derived from a granulocyte-macrophage progenitor cell (11) and probably first appear in the circulation during the 20th–22nd wk of gestation (10). Effector functions of monocytes obtained from neonates such as phagocytosis and bactericidal activity are comparable to those of monocytes from adults but chemotaxis is decreased (10).

IL-1 and TNF, also known as cachectin, are two well-defined cytokines produced by mononuclear phagocytes responsible for induction of fever and possibly cachexia (8, 12). IL-1 and TNF are distinct proteins with mol wt of 17 kD (8). Both are produced by blood monocytes and tissue macrophages when stimulated with bacterial LPS. The production of these cytokines, however, alters as blood monocytes differentiate into tissue macrophages (13, 14). Cord blood monocytes from neonates are capable of producing both IL-1 (15, 16) and TNF (17) activities, and the levels expressed are similar to those of blood monocytes from adults. The effect of gestational age on expression of IL-1 and TNF by neonatal monocytes, however, has not been reported and is the subject of this study.

MATERIALS AND METHODS

Preparation of monocytes. Seventeen blood samples were obtained from the umbilical cord and fetal side of the placenta of premature and term infants approximately 5 to 10 min after delivery. The samples were collected in heparinized syringes (20 U heparin/mL blood). Peripheral blood was also obtained by venipuncture of healthy adult volunteers, ages 20–40 y. This study was approved by the Institutional Review Board of University Hospitals of Cleveland.

Peripheral blood mononuclear cells were separated from whole blood by sedimentation on Ficoll-Hypaque (Pharmacia, Inc., Piscataway, NJ) gradients for 45 min at room temperature. The cells were then washed three times in RPMI 1640 (Whittaker MA Bioproducts, Walkersville, MD) culture medium containing 2 mM L-glutamine (Grand Island Biological Co., Grand Island, NY), 50 U/mL penicillin (E. R. Squibb & Sons, Princeton, NJ) and 5 μg/mL gentamicin (Whittaker). Culture medium supplemented with glutamine and antibiotics will be referred to as supplemented medium. To isolate monocytes from total mononuclear cells, 10×10^6 cells/mL complete medium plus 10% heat-inactivated pooled human serum were added to 100 × 20 mm plastic Petri dishes precoated for 10 min at 37°C with 0.5 mL of heat-inactivated pooled human serum. The cells were allowed to adhere to the plates for 1–2 h at 37°C. The plates then were vortexed for 10 s. Nonadherent cells, which were predominantly lymphocytes, were washed from the plates using supple-

mented medium plus 10% heat-inactivated FCS (HyClone, Logan, UT). Cold (4°C) Hanks' balanced salt solution without calcium and magnesium (Grand Island Biological Co.) was added to the adherent cells. The cells were incubated at 4°C for 30 min and then removed by gently scraping the plates with a rubber policeman and washing with Hanks' balanced salt solution. Cells were counted with trypan blue to assess viability.

Generation of supernatants containing IL-1 and TNF activities. The adherent cells were sedimented, resuspended in supplemented medium, and counted using a hemacytometer. The adherent cell preparation will be referred to as monocytes on the basis of their staining characteristics as described under "Results." Monocytes were incubated on 24-well plastic plates (Grand Island Biological Co.) at 10^6 cells/mL in supplemented medium plus 2% pooled human serum for 24 h at 37°C under two conditions: no stimulus and 10 μg/mL LPS (*Escherichia coli*, Difco Laboratories, Inc., Detroit, MI). In preliminary experiments, this concentration of LPS and period of incubation were found to be optimal for stimulation of cytokine production as previously reported (14). The supernatants were collected and aliquots frozen at −20°C to be assayed for IL-1 and TNF activities. Assays were performed an average of 14 d after culture.

Assay for IL-1 activity. The IL-1 activity of the supernatants was measured as direct mitogenesis for mouse thymocytes as described previously (18) and modified (19). Thymocytes were obtained from 8- to 12-wk-old female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) which are an LPS-resistant strain. Mice were killed by ether anesthesia and thymus glands removed using aseptic technique. The tissue was ground through a wire sieve, washed twice, and resuspended in supplemented medium plus 5×10^{-5} M 2-mercaptoethanol. An IL-1 standard was prepared from pooled samples of supernatants of monocytes collected from healthy adults, ages 20–30, and stimulated with LPS (10 μg/mL) in the presence of indomethacin (1 μg/mL) for 24 h. Samples and the IL-1 standard were serially diluted in triplicate into flat-bottomed microtiter plate wells (Becton Dickinson & Co., Oxnard, CA) at 1:4–1:256 using 0.1 mL/dilution. Mouse thymocytes were suspended in supplemented medium plus 5×10^{-5} M 2-mercaptoethanol plus 20% FCS at 15×10^6 cells/mL, and 0.1 mL was added to each well. The plates were incubated for 72 h at 37°C in 5% CO_2. The wells were then pulsed with 1.0 μCi methyl-^{3}H-thymidine (sp act 6.7 Ci/mmol) (ICN Radiochemicals, Irvine, CA) and incubated for 6 h at 37°C before harvesting onto glass filter paper discs. The ^{3}H-thymidine content/well was assayed by liquid scintillation spectrophotometry. IL-1 activity was calculated by modified probit analysis (20) and expressed as U/mL, which was the equivalent of U/10^6 cells as supernatants were generated at 10^6 cells/mL. The IL-1 standard reference cpm was arbitrarily assigned an activity of 100 U/mL. The results of the dilutions of the standard and supernatant samples were used to plot regression lines. The sample dilution yielding 50% of the maximal cpm obtained with the standard preparation was determined from the regression lines and the following equation used to convert the resulting titers into units: Reciprocal titer of sample at 50% of maximal cpm of standard/Reciprocal titer of standard at 50% of maximal cpm × 100 = U/ml sample.

Assay for TNF activity. The TNF activity of the supernatants was assessed as direct cytotoxicity to L-929 fibroblasts using a modification of the Flick method (21). A supernatant containing TNF activity was obtained from a tumor cell line denoted D-1 (R. S. Wallis, Case Western Reserve University, Cleveland, OH) and was used as a positive control (22). L-929 fibroblasts were cultured for 24 h at 2.5×10^4/well in 0.2 mL Eagle's minimal essential medium (M.A. Bioproducts) supplemented with L-glutamine 2 mM, gentamicin 100 μg/mL, HEPES 25 mM, nonessential amino acids 1%, pyruvate 2 mM and FCS 20%. By microscopic examination, the cells were in confluent growth in flat-bottomed culture plate wells (Becton Dickinson). Triplicates of dilutions of supernatants and standard at 1:2–1:2048 were added in 0.1 mL aliquots to the L-929 cells. Actinomycin D (Calbiochem, LaJolla, CA) (10 μg/ml) was added to inhibit further fibroblast growth. The plates were incubated at 37°C for 20–24 h. Freshly prepared 0.033% neutral red (K & K Laboratories Inc., Plainview, NY) was added to each well and incubated for 1 h, allowing the remaining live fibroblasts to take up the stain. The plates were washed twice with PBS (Sigma Chemical Co., St. Louis, MO) at 37°C. A mixture of 50% 0.1 M sodium phosphate and 50% ethanol was then added to each well to lyse the remaining live cells and allow mixing of the neutral red in solution. The plates were then read with an ELISA spectrophotometer (MR580, Dynatech Laboratories, Inc., Alexandria, VA) at 570 nm. Units of TNF activity were calculated by modified probit analysis, as described above, using the maximal OD of the D-1 sample as the standard in the equation. Upper limits of activity considered to be detected in this assay was 1000 U/mL.

Statistical analysis. Statistical analysis was performed using the unpaired 2-tailed *t* test and simple linear regression.

RESULTS

The demographic data on the study population are shown in Table 1. Term gestation was considered to be greater than 37 wk. The average gestational age was 8 wk greater in term than in preterm neonates. There were no infants or mothers with culture-proven sepsis in either of the two groups.

The cytologic characteristics of the adherent cell or monocyte-enriched populations were examined first (Table 2). Cytocentrifuge preparations were made of the adherent cells and stained with Wright's stain or subjected to cytochemical analysis for peroxidase activity. Wright's stain was used to differentiate monocytes, polymorphonuclear leukocytes, and lymphocytes on the basis of their morphology. Peroxidase activity identifies most monocytes (23). There were no major differences between the term and preterm groups in the cell populations by Wright's stain or peroxidase activity (Table 2). Preparations from three adults were examined and showed 85% positive peroxidase activity, comparable to the neonatal preparations. Viability of the

Table 1. *Demographic data of study population*

	Preterm ($n = 8$)	Term ($n = 9$)
Gestational age (wk)	31.6 ± 2.5*	39.6 ± 1.3*
	Range 28–35	Range 38–42
Wt (g)	1730 ± 572*	3475 ± 692*
	Range 940–2520	Range 2410–4850
Sex	3 female	4 female
	5 male	5 male
Delivery†	6 SVD	4 SVD
	2 C/S	5 C/S
O_2 requirement	8	0

* Data are expressed as mean ± SD.

† SVD = spontaneous vaginal delivery; C/S = cesarean section.

Table 2. *Cytologic and cytochemical characteristics of adherent cells from preterm and term neonates**

	Adherent cells from neonates (mean % ± SD)	
	Preterm	Term
Wright's	Monocytes 90 ± 2	Monocytes 91 ± 4
	Lymphocytes 10 ± 2	Lymphocytes 8 ± 3
	Polymorphonuclear leukocytes < 1	Polymorphonuclear leukocytes < 1
Peroxidase positive	83 ± 7	85 ± 4

* Adherent cells from preterm and term neonates were prepared by cytocentrifugation using 3×10^5 cells/slide.

monocyte-enriched adherent cells as assessed by exclusion of trypan blue was >90% for monocytes from each group.

The ability of cord blood monocytes from neonates to express TNF and IL-1 is illustrated in Figure 1 and Figure 2. Unstimulated monocytes secreted negligible levels of IL-1 and TNF activity. LPS-stimulated monocytes from neonates secreted significant levels of IL-1 and TNF activities as compared to unstimulated monocytes. To confirm that the observed bioactivities could be attributed to immunoreactive IL-1 and TNF, supernatants containing these activities were incubated with or without neutralizing antibodies (Table 3). Polyclonal antiserum to IL-1-β neutralized 80% of the IL-1 activity. Antibody to TNF-α neutralized all of the TNF activity. Thus, neonatal monocytes were capable of expressing both bioactive and immunoreactive IL-1 and TNF.

The ability of cord blood monocytes from preterm and term neonates to secrete IL-1 was compared. The IL-1 activity secreted by LPS-stimulated monocytes was comparable between the two groups (Fig. 1).

Expression of TNF activity by cord blood monocytes from preterm and term infants was compared next (Fig. 2). In contrast to the comparable levels of IL-1 activity, LPS-stimulated monocytes from preterm infants secreted significantly less TNF activity than did stimulated monocytes from term infants. TNF activity secreted by LPS-stimulated monocytes from preterm infants was significantly less than TNF activity secreted by monocytes from adults (Fig. 3). TNF activity secreted by monocytes from term infants was not significantly less than TNF activity expressed by monocytes from adults. When term and preterm groups were considered together as one total neonatal group, however, the TNF activity expressed was significantly less than TNF activity expressed by monocytes from adults (total neonatal 329 U/mL, $n = 17$, adult 878 U/mL, $n = 6$). The association of TNF expression by cord blood monocytes with gestational age studied was analyzed further by linear regression (Fig. 4). Levels of TNF

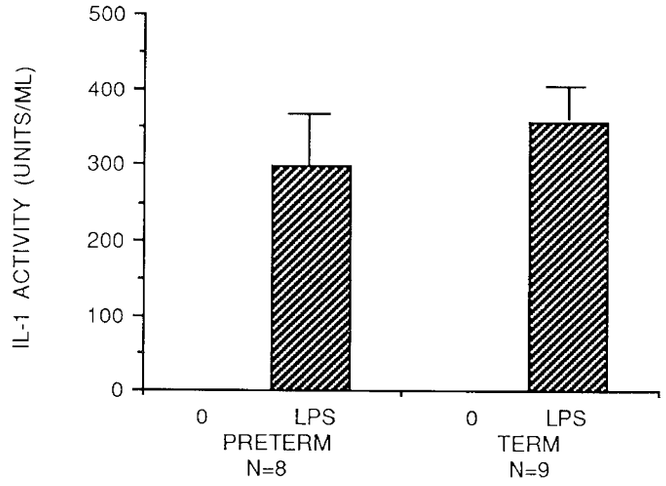

Fig. 1. IL-1 activity by preterm and term cord blood monocytes. Cord blood monocytes from preterm and term neonates were either unstimulated (0) or stimulated with LPS (10 μg/mL) for 24 h. Supernatants were assayed for IL-1 activity. Data are expressed as mean U/ml ± SEM.

Table 3. *Neutralization of LPS-stimulated IL-1 and TNF activities of term cord blood monocytes**

LPS	Antibody†	TNF Activity	IL-1 Activity
—	—	U/ml	U/ml
—	—	16	0
+	—	413	43
+	OKT3	315	88
+	anti TNF	0	—
+	anti IL-1	—	9

* Cord blood monocytes were incubated at 10^6 cells/mL in supplemented medium plus 2% pooled human serum for 24 h with or without LPS (10 μg/mL).

† Supernatants were collected and incubated for 1 h at 37°C with or without monoclonal antibody to TNF-α (Olympus Corp., Lake Success, NY) at a concentration of antibody which neutralized 6000 U/mL recombinant TNF-α (gift from Genentech, Inc., South San Francisco, CA) activity or with neutralizing polyclonal antibody to IL-1 β (Cistron, Pine Brook, NJ) at a concentration of antibody which neutralized 350 U/mL recombinant IL-1β. Control supernatants were incubated with OKT3, an irrelevant MAb of the same IgG_1 subclass (Ortho-mune, Raritan, NJ) at a concentration of 55 μg/mL in the IL-1 assay and 70 μg/mL in the TNF assay.

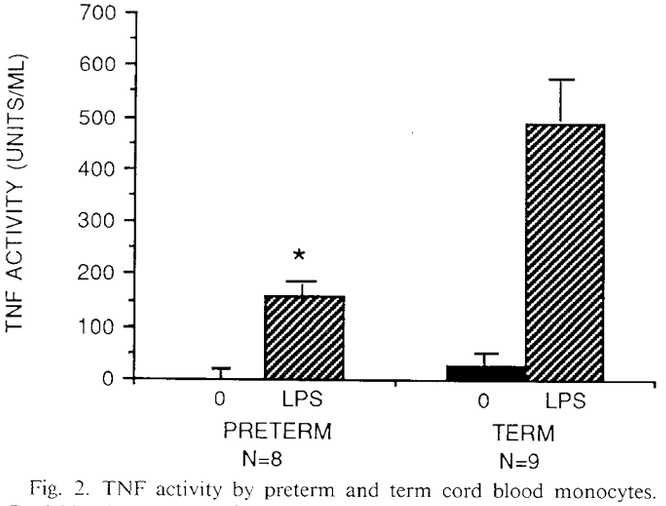

Fig. 2. TNF activity by preterm and term cord blood monocytes. Cord blood monocytes from preterm and term neonates were either unstimulated (0) or stimulated with LPS (10 μg/mL) for 24 h. Supernatants were assayed for TNF activity. Data are expressed as mean U/ml ± SEM. LPS-stimulated monocytes from preterm neonates (*) expressed significantly less TNF activity than did LPS-stimulated monocytes from term infants ($p < 0.05$).

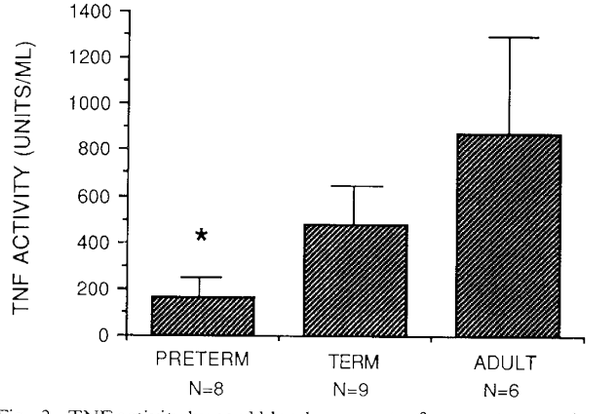

Fig. 3. TNF activity by cord blood monocytes from preterm and term neonatal samples and from adult blood monocytes. Cord blood monocytes from preterm and term groups were stimulated with LPS (10 μg/mL) and supernatants compared for TNF activity. Blood monocytes from adults were also stimulated with LPS (10 μg/mL) and supernatants assayed for TNF activity and compared to TNF activities of monocytes from the neonatal groups. Data are expressed as mean U/ml ± SEM. As depicted by *, TNF activity by LPS-stimulated monocytes from preterm infants was significantly less than TNF activity by monocytes from adults ($p < 0.05$).

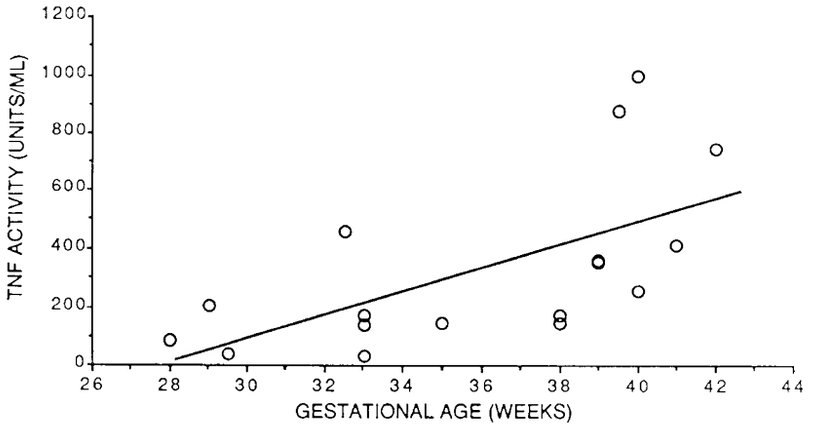

Fig. 4. Simple regression analysis of gestational age and TNF activity: $r = 0.624$, $p = 0.007$, ($y = 40.326 \times -1116.62$). This correlation applies to only those gestational ages studied.

activity by cord blood monocytes correlated significantly with gestational age.

DISCUSSION

This study demonstrates that LPS-stimulated cord blood monocytes from neonates produce and release biologically active IL-1 and TNF. Furthermore, as mitogenesis of thymocytes and killing of L929 fibroblasts were neutralized 80 and 100% by antibodies to IL-1-β and TNF-α, respectively, and were unaffected by the control antibody, the mitogenic and cytotoxic activities in supernatants of neonatal monocytes cannot be attributed to other mediators potentially present. In the current study, TNF production also was examined in cord blood monocytes as a function of gestational age. When the total neonatal group was stratified according to gestational age, it was found that term neonatal cord blood monocytes secreted levels of TNF activity comparable to those of adult blood monocytes but that preterm monocytes secreted significantly less TNF activity than did monocytes from adults.

Dinarello reported that expression of IL-1 activity by term neonatal cord blood monocytes was similar to that by adult blood monocytes (15). Wilmott *et al.* (16) found that production of IL-1 activity by LPS-stimulated preterm cord blood monocytes was similar to the levels of IL-1 activity secreted by adult peripheral blood monocytes. Our studies support and extend previous observations of IL-1 secretion by neonatal monocytes by demonstrating no significant alteration in the ability of cord blood monocytes to secrete IL-1 activity regardless of gestational age.

TNF is cytolytic and cytostatic to many but not all malignant cells both *in vitro* and *in vivo*. This cytotoxic property of TNF may not be relevant to the neonate as transformation of cells at this age is rare. Recently, Michie *et al.* (24) demonstrated that intravenous injection of LPS in humans is associated with an increase in TNF levels in the serum. Furthermore, mice passively immunized against TNF are protected against the effect of systemic LPS (25). TNF also is pyrogenic as is IL-1 (8). Together, these observations suggest that TNF may be an endogenous pyrogen and a mediator of responses to sepsis. TNF also has direct effects on phagocytes, for example stimulating complement receptors on neutrophils resulting in increased margination and phagocytic activity (26) Moreover, TNF and IL-1 have important interactions in that TNF stimulates monocyte production of IL-1 (8).

TNF is molecularly identical to cachectin (8, 12), which is capable of altering cellular metabolism by selectively suppressing the mRNA encoding anabolic proteins such as lipoprotein lipase in adipocytes (27, 28). In this capacity, TNF/cachectin has been implicated, but not proven, to be the agent responsible for cachexia in chronic inflammatory diseases (8). In terms of the cachectin activity of TNF, the relative deficiency of monocytes from preterm infants to produce TNF might be protective and help to preserve fetal growth. It would be of interest to determine TNF activity in monocytes from chronically stressed infants with poor growth who have prolonged oxygen requirements and bronchopulmonary dysplasia.

The significance of our findings that TNF is decreased in monocytes from premature infants is speculative. Preterm neonates, through a deficiency in elaboration of TNF by monocytes, may have blunted responses to infections. It should be noted, however, that other mediators such as IFN-γ may regulate TNF and IL-1 activity *in vivo* (8). This study does not address the TNF activities secreted by monocytes from preterm neonates who are already under stress, such as the small for gestational age (chronic stress) or the acutely infected infant. Such studies would be valuable adjuncts to this initial observation that monocytes from uninfected preterm infants show decreased TNF activity.

The differences in cytokine secretion also prompt questions concerning regulation of monocyte differentiation during maturation of the fetus. Induction of IL-1 and TNF are linked in some tissue macrophages such as peritoneal macrophages (29) but are not closely related events in rat bone-marrow-derived macrophages (30). Our study suggests that maturation also regulates TNF secretion as monocytes from premature infants produce less TNF than monocytes from term neonates or adults. In a somewhat parallel fashion, blood monocytes are the precursors of fully differentiated alveolar macrophages that express significantly more TNF than do blood monocytes (14). Furthermore, *in vitro* maturation of blood monocytes from adults led to increased expression of TNF upon stimulation with LPS (14). Therefore, a spectrum of expression of TNF appears to exist with monocytes from immature neonates producing less than monocytes from adults and tissue macrophages from adults producing more TNF. Functional maturation of monocytes occurring during gestation may explain the association between TNF expression and age. The growth factors and the molecular events involved in differentiation and maturation of mononuclear phagocytes during gestation and beyond and the confounding effects of acute and chronic stresses remain to be elucidated.

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