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Production of Lymphotoxin and Tumor Necrosis Factor by Human Neonatal Mononuclear Cells

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ABSTRACT. Lymphotoxin (LT) and tumor necrosis factor (TNF) are cytokines with many common biologic effects including antiviral activity and induction of fever and the acute phase response; despite common effects, they are molecularly distinct. Because neonates are unduly susceptible to viral infection and frequently fail to mount a febrile response to infection, we hypothesized that neonatal cells would produce less LT and TNF than adult cells. We analyzed LT and TNF production by blood mononuclear cells and purified T cells using Northern blot analysis to detect specific messenger ribonucleic acid and specific assays to detect LT and TNF protein in culture supernatants. Compared to LT, TNF messenger ribonucleic acid and protein were produced more rapidly both by total mononuclear cells and by T cells in response to mitogen stimulation. Although there was intersubject variability, adult and neonatal mononuclear cells and T cells ($n = 6$) produced similar amounts of LT and TNF messenger ribonucleic acid and protein with similar kinetics. In experiments with phytohemagglutinin-stimulated mononuclear cells from ten additional subjects, supernatant LT was somewhat greater in neonatal cultures (neonatal = 62.8 ± 60.5, adult = 13.2 ± 10.7 units/ml, $p < 0.05$), and TNF was somewhat greater in adult cultures (neonatal = 708 ± 429, adult = 1987 ± 392 pg/ml, $p < 0.01$) at 24 h; results at 48 h and 72 h were similar. Thus, neonatal MC produced as much or more LT than did adult MC. Although the decreased production of TNF by neonatal MC was statistically significant, these cells did produce substantial amounts of this cytokine. Because the reduction in TNF production by neonatal MC in response to nonspecific stimuli was modest, it is unlikely to be the principal mechanism but may contribute to the diminished febrile response and increased susceptibility to infection of the human neonate. (*Pediatr Res* 24:717–722, 1988)

Abbreviations

LT, lymphotoxin
rLT, recombinant lymphotoxin
TNF, tumor necrosis factor
rTNF, recombinant tumor necrosis factor
IFN-γ, interferon-gamma
IL-2, interleukin-2
mRNA, messenger ribonucleic acid
MC, mononuclear cells
HBSS, Hanks' balanced salt solution
LPS, lipopolysaccharide
PMA, phorbol myristate acetate
PHA, phytohemagglutinin
FCS, fetal calf serum
Con A, concanavalin A
OPD, ortho-phenylenediamine

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LT and TNF are cytokines with minimal molecular homology but multiple common biologic activities, including antitumor and antiviral effects and mediation of fever and the acute phase response to infection (1–3). These effects are potentiated by (IFN-γ. TNF, also called TNF-α, is principally a monocyte/macrophage product (4–6) but is also produced by natural killer cells (7–9) and T cells (9). LT, also called TNF-β, is primarily a product of T lymphocytes (10, 11); though the cDNA for LT was initially cloned from a B-cell lymphoblastoid cell line (11), it is not produced by purified B cells (12), monocytes (10), or natural killer cells (8). We and others have previously reported diminished production of IFN-γ by mitogen-stimulated neonatal T cells compared to adult T cells (13–17), and we have shown that this is associated with diminished production of IFN-γ mRNA but normal production of IL2 and IL2 receptor proteins and their cognate mRNA (13, 14). A previous study reported decreased LT activity in the supernatants of mitogen-stimulated neonatal MC as compared with adult peripheral blood MC (18). However, the assay used in that study would have measured LT, TNF-α, and perhaps other lymphokines, including IFN-γ. TNF and LT have been recently cloned and characterized (6, 10), allowing a more specific analysis of their production.

In this study, we examined the hypothesis that neonatal MC and T cells produce less LT and TNF after mitogenic stimulation than do adult MC and T cells. We studied the accumulation of LT and TNF in supernatants of stimulated adult and neonatal cord blood MC and T cell preparations and also measured the accumulation of the specific mRNA for LT, TNF, and IFN-γ by Northern blot analysis.

MATERIALS AND METHODS

Reagents. HBSS, L-glutamine, penicillin, and streptomycin were obtained from Gibco (Grand Island Biological Co., Grand Island, NY). RPMI 1640 containing 25 mM HEPES buffer was obtained from Whittaker MA Bioproducts (Walkersville, MD) or Cellgro (Dulles International Airport, Washington, DC) and contained <0.3 EU LPS/ml by Limulus amebocyte lysate assay (Pyrotell Associates of Cape Cod, Inc., Woods Hole, MA). Ficoll-Hypaque and Con A were obtained from Pharmacia Fine Chem-

icals (Piscataway, NJ). PMA was obtained from Sigma Chemical Co. (St. Louis, MO). Actinomycin D was obtained from Calbiochem (La Jolla, CA). L-929 fibroblast cells, mycoplasma-free by the Kaplan method (19), were supplied by Dr. Gale Granger (University of California, Irvine, CA). PHA-P was obtained from Burroughs-Wellcome (Research Triangle Park, NC) and PHA-M was obtained from Difco (Detroit, MI).

Human recombinant LT (spec. act. 1.2×10^8 U/mg), human recombinant TNF (spec. act. 5×10^7 U/mg), rabbit polyclonal anti-LT antibody (spec. act. 2.9×10^7 neutralizing U/ml), rabbit polyclonal anti-TNF antibody (5.95×10^5 neutralizing U/ml), mouse monoclonal anti-TNF antibody (specific activity 6×10^6 neutralizing U/mg), and horseradish peroxidase conjugated mouse monoclonal anti-TNF antibody were supplied by Genentech, Inc. (South San Francisco, CA).

Cell preparations. MC were isolated from peripheral blood of healthy adult donors or umbilical cord blood of healthy term neonates by Ficoll-Hypaque density gradient centrifugation as previously described (20), washed twice in HBSS, resuspended in RPMI 1640 containing 2 mM L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin, and 5% human AB serum (medium), and counted. Purified T cells were prepared by treatment of MC with T cell Lymphokwik as specified by the manufacturer (One Lambda, Los Angeles, CA). The compositions of the MC and T cell preparations were assessed by indirect immunofluorescence using mAbs recognizing the indicated antigens: 9.6-CD2 (T and NK cells); 64.1-CD3 (T cells); 2H7-Bp32 (B cells); 5F1-CDW14 (monocytes); 1G10-CDW15 (granulocytes); M21-IgG1 murine myeloma protein (negative control), and Fc1-CD16 (NK cells). 9.6, 64.1, and 2H7 were obtained from Genetic Systems (Seattle, WA); 5F1 and 1G10 were provided by Dr. I. Bernstein (Fred Hutchinson Cancer Research Center, Seattle, WA). Fc1 was provided by E. Clark (University of Washington, Seattle, WA). M21 was purchased from Litton Bionetics (Kensington, MD). MC and T cell preparations were >95% viable by the trypan blue exclusion method. T cell preparations were >95% positive with 9.6, >85% positive with 64.1, and <1% positive with M21, 2H7, 5F1, and 1G10. In the T cell preparations, 8–20% of cells were positive with Fc1.

Induction and assay of lymphokines. For the studies in which the kinetics of TNF and LT protein and mRNA accumulation were evaluated in parallel, blood MC and T cells were cultured in conical 15-ml polypropylene tubes at 5×10^6/ml and stimulated with 25 μg/ml Con A with or without 50 ng/ml PMA. For the other studies, blood MC were cultured at 1×10^6/ml in microtiter wells (because fewer cells were required) and stimulated with PHA-P (5 μg/ml), PHA-M (1:40) or PMA (10 ng/ml). After the indicated incubation time, supernatants were collected, frozen at −70° C, and later assayed for LT and TNF.

TNF was assayed by a capture ELISA technique, using polyclonal rabbit anti-rTNF as coating antibody and murine monoclonal anti-rTNF conjugated to horseradish peroxidase as the second antibody. OPD was used as the substrate, and the reaction was stopped with 4.5 N H_2SO_4. Absorbance at 492 nm (405 nm reference beam) was determined on a microplate reader. rTNF was used as a standard in each assay.

LT activity was determined by a modification of the assay described in Aggarwal *et al.* (4) using Actinomycin-D (1 μg/ml)-treated mouse L-929 fibroblasts. Appropriate serial dilutions of each supernatant were assayed for LT in parallel after preincubation for 1 h with 5 neutralizing units of anti-TNF mAb with or without 10 neutralizing units of anti-LT polyclonal antibody. After incubation at 37° C for 18 h, samples were decanted, monolayers were washed twice with phosphate-buffered saline, and residual cells were stained with crystal violet, washed, and dried. The crystal violet was eluted, and the optical density of the eluate was determined at 550 nm (405 nm reference beam). Optical density of L-929 cells incubated with medium alone represented 0% lysis, and cells treated with 3 M guanidine hydrochloride 100% lysis. One unit LT or TNF activity is that producing 50% lysis. LT activity was defined as that activity not neutralized by preincubation with the anti-TNF antibody; more than 90% of total lytic activity was neutralized by preincubation with a combination of the anti-TNF and anti-LT antibodies. rTNF and rLT were run as standards in each assay, and were completely neutralized by anti-TNF mAb and anti-LT polyclonal Ab, respectively.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from MC or T cells by the guanidinium isothiocyanate/cesium chloride method and quantitated spectrophotometrically as previously described (14). Integrity of the RNA was confirmed by electrophoresis of 1 μg in 1% agarose gels and staining with ethidium bromide. For blots, 5 μg RNA was electrophoresed in 2.2M formaldehyde-1% agarose gels, transferred to Nytran (Schleicher & Schuell, Keene, NH), and baked at 80° C for 90 min as described (21). Blots were hybridized with ^{32}P-labeled RNA probes transcribed from the following subclones in pGEM vectors (Promega Biotec, Madison, WI): LT, the 940 bp *Eco*RI cDNA fragment (11); TNF, the 800 bp *Eco*RI cDNA fragment (6); and IFN-γ, nucleotides 266-860 of clone 52 (22). These cDNA were originally provided by P. Gray (Genentech, Inc.). After hybridization, blots were washed at 63° C with 6 × SSC, 0.1% SDS for 30 minutes then with 0.1 × SSC, 0.1% sodium dodecyl sulfate for 30 min, then autoradiographed at −80° C. In some cases, previously probed Nytran filters were stripped by boiling in 20 mM Tris pH 8.5, 2 mM EDTA, 0.1% sodium dodecyl sulfate for 15 min, dried, and then reprobed.

Statistics. Data are expressed as mean ± one SD. Statistical analysis was performed by a two-tailed Student's *t* test, and comparison groups were considered significantly different if $p < 0.05$.

RESULTS

Secretion of LT and TNF by blood MC and T cells. We studied the kinetics of secretion of LT and TNF by adult and neonatal (cord) MC and T cell preparations after stimulation with Con A (MC) or Con A plus PMA (MC and T cells). Results from four representative experiments of six are shown in Table 1. LT activity was undetectable in unstimulated adult and neonatal MC and T cell supernatants. After stimulation, LT was detectable at 4–8 h and continued to increase thereafter, peaking at 24–48 h. TNF was undetectable or present in very small amounts in unstimulated adult and neonatal MC and T cell supernatants, rose rapidly after stimulation, and usually peaked by 16–24 h. In these representative experiments, there was considerable subject-to-subject variation for both adult and neonatal cell preparations, but, overall, adult and neonatal MC and T cells secreted similar amounts of LT and TNF with similar kinetics.

Because of the variability noted in the kinetic experiments, we also studied the secretion of LT and TNF by 10 adult and neonatal MC preparations after stimulation with either PHA or PMA (Table 2). With PHA as the stimulus, neonatal MC secreted approximately twice as much LT as adult MC, but the difference was significant only at 24 h. PMA alone was a poor stimulus for LT production. In contrast, with PHA as the stimulus, adult MC secreted approximately twice as much TNF as neonatal MC, and the difference was significant at 24, 48, and 72 h. With PMA as the stimulus, adult MC also produced more TNF than neonatal MC, but this was significant only at 24 h. In these experiments, LT secretion was greatest at 72 h, whereas TNF secretion was maximal at 24–48 h.

LT and TNF mRNA accumulation by blood MC and T cells. To analyze LT and TNF production by an independent and specific method, we examined the accumulation of specific mRNAs for LT and TNF by Northern blot analysis. Initial experiments revealed that Con A plus PMA resulted in the greatest accumulation of LT and TNF mRNA, so this combination was used to examine the kinetics of LT and TNF mRNA accumulation in adult and neonatal (cord) MC (Fig. 1). For

Table 1. *Kinetics of secretion of LT and TNF by adult and neonatal (cord) blood MC**

LT (U/ml)

Exp.	Subject	Cell type	Stimulus	Time after stimulation (h) 0	4	8	16	24	48
1	Adult 1	MC	Con A	0	56	71	128	172	500
	Neonate 1	MC	Con A	0	49	52	15	76	104
2	Adult 2	MC	Con A + PMA	0	ND	33	10	195	115
	Neonate 2	MC	Con A + PMA	ND†	ND	80	40	105	ND
3	Adult 3	T cells	Con A + PMA	0	ND	35	35	175	690
	Neonate 3	T cells	Con A + PMA	0	ND	ND	ND	810	1255
4	Adult 4	T cells	Con A + PMA	0	ND	15	ND	705	1056
	Neonate 4	T cells	Con A + PMA	0	ND	89	ND	1367	1330

TNF (pg/ml)

Exp.	Subject	Cell type	Stimulus	Time after stimulation (h) 0	4	8	16	24	48
1	Adult 1	MC	Con A	78	1188	1300	1334	1741	1006
	Neonate 1	MC	Con A	0	914	1447	957	862	406
2	Adult 2	MC	Con A + PMA	ND	ND	5098	10690	9850	ND
	Neonate 2	MC	Con A + PMA	ND	ND	1353	2869	2973	ND
3	Adult 3	T cells	Con A + PMA	0	2082	7076	9473	10726	10593
	Neonate 3	T cells	Con A + PMA	0	ND	ND	8248	13650	13885
4	Adult 4	T cells	Con A + PMA	0	ND	7605	ND	18180	16230
	Neonate 4	T cells	Con A + PMA	0	ND	4036	ND	11812	7733

* Cells were cultured at 5×10^6/ml in 15-ml polypropylene tubes and stimulated with Con A (25 μg/ml) with or without PMA (50 ng/ml) for the indicated interval. Supernatants were assayed as described in "Materials and Methods."

† ND, not determined.

Table 2. *Secretion of LT and TNF by adult and neonatal (cord) blood MC**

LT (U/ml)

Subject	Stimulus	Time after stimulus (h) 24	48	72
Adult	PHA	13.2 ± 10.7†	168.3 ± 154.4	238.2 ± 183.4
Neonatal	PHA	62.8 ± 60.5†	283.6 ± 279.6	433.6 ± 706.5
Adult	PMA	10.0 ± 4.8†	33.4 ± 25.1	41.8 ± 39.2†
Neonatal	PMA	5.2 ± 3.1†	51.9 ± 143.1	7.2 ± 5.1†

TNF (pg/ml)

	Stimulus	Time after stimulus (h) 24	48	72
Adult	PHA	1986.6 ± 391.6‡	2160.2 ± 519.3†	1186.9 ± 956.9†
Neonate	PHA	708.3 ± 428.7‡	995.6 ± 838.7†	598.1 ± 390.1†
Adult	PMA	3227.2 ± 1938.0†	1797.6 ± 1313.3	2240.7 ± 2217.9
Neonate	PMA	774.1 ± 897.1‡	1070.7 ± 637.9	888.4 ± 348.2

* Cells were cultured at 1×10^6/ml in microtiter wells and stimulated with PHA-P (5 μg/ml) or PMA (10 ng/ml) for the indicated interval. Results are means ± SD of 10 experiments each. Supernatants were assayed as described in "Materials and Methods."

† $p < 0.05$.

‡ $p < 0.01$.

comparison, accumulation of IFN-γ mRNA was studied in parallel.

LT mRNA was not detectable in unstimulated adult and neonatal MC. After stimulation, LT mRNA was detectable by 4 h, peaked by 8–24 h, and remained elevated at 48 h. TNF mRNA was just detectable in unstimulated adult and neonatal MC, rose rapidly after stimulation, peaked by 8 h, and returned to baseline levels by 24–48 h. In this experiment, adult MC contained slightly more LT and TNF mRNA than neonatal MC, whereas in other experiments neonatal MC contained somewhat more LT and TNF mRNA than adult MC (not shown). In contrast, IFN-γ accumulation was dramatically reduced in neonatal MC in all experiments, as previously reported (14).

Results of Northern blot analyses of T cell preparations paralleled those of MC (Fig. 2). Neonatal T cells produced as much or more LT and TNF mRNA as did adult T cells. IFN-γ mRNA accumulation was greatly diminished in neonatal T cell preparations as compared with adult T cells.

DISCUSSION

Our data indicate that neonatal blood MC and T cells produce and secrete both LT and TNF in response to mitogens. It is likely that some of the TNF produced by blood MC under our conditions was derived from monocytes. However, because monocytes secrete less TNF in response to Con A or PHA than do lymphocytes (9), do not secrete LT (9, 12, 23), and comprised less than 20% of the total cells in MC preparations, most of the TNF and LT was probably derived from T cells and NK cells. This is supported by experiments with T cell enriched, monocyte-de-

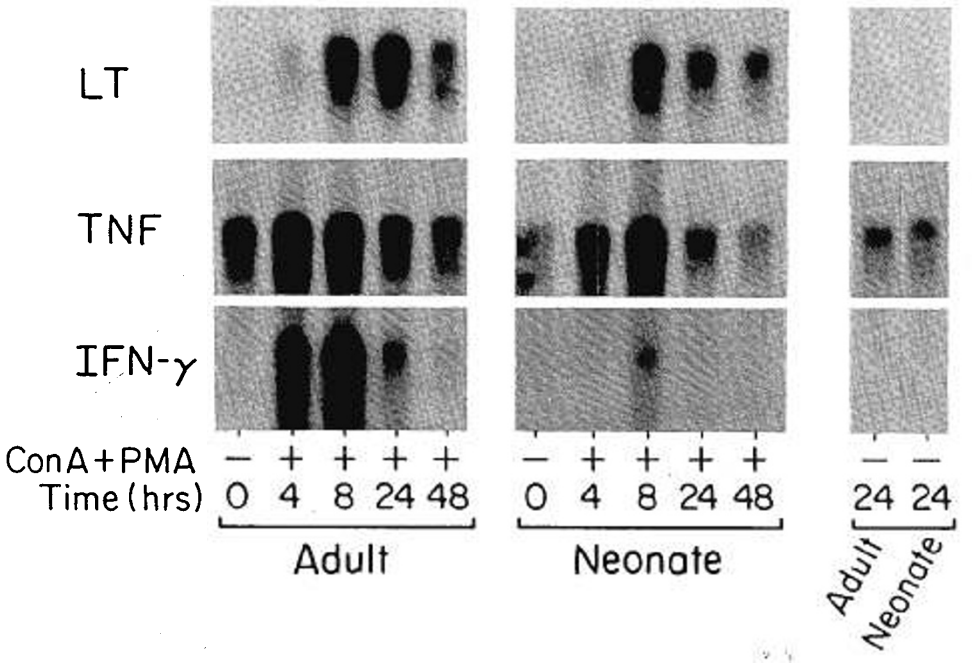

Fig. 1. Northern blot analysis of blood mononuclear cell (MC) RNA. Cells were cultured at 5×10^6/ml, and stimulated with Con A plus PMA as described in the Materials and Methods. RNA was isolated from MC at the indicated times. In each lane, 5 μg of total RNA was electrophoresed.

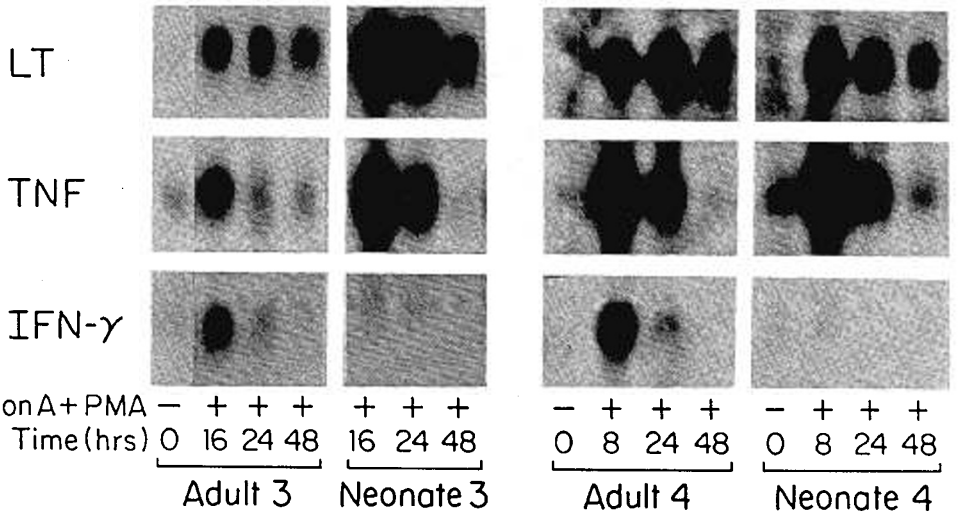

Fig. 2. Northern blot analysis of T cell preparations. T cells were cultured at 5×10^6/ml and stimulated with Con A plus PMA as described in the "Materials and Methods." RNA was isolated from T cells at the indicated times. In each lane, 5 μg of total RNA was electrophoresed.

pleted (<1%) preparations, which secreted at least as much TNF and LT and contained as much or more of their cognate mRNAs as did MC preparations under our conditions. In other studies, we have found that blood monocytes from adults and neonates secrete comparable amounts of TNF (24). Our T cell-enriched preparations were >95% CD2+; of these 85–90% were CD3+ T cells and 8–20% were CD16+ NK cells. However, lectins are poor activators of NK cells (25), and NK cells stimulated with PHA, phorbol ester, or both secrete much less TNF than do T cells or MC (9) and do not secrete LT (8, 9). We have found that adult T cells depleted of NK cells by monoclonal anti-CD16 antibody plus complement contain as much or more TNF and LT mRNA in response to Con A plus PMA as T cells or whole MC containing ~10% NK cells (data not shown). Thus, it is likely that our results largely reflect production of LT and TNF by T cells.

Although there was substantial variability, we found that neonatal MC produced approximately twice as much LT and approximately ½ as much TNF as adult MC. In contrast to this modest 2-fold lower TNF production. IFN-γ production by neonatal MC is less than 1/10 that of adult MC after mitogen stimulation (13). Although the number of experiments was more limited, results with T cells were similar except that TNF production by neonatal and adult T cells was comparable. Northern blot analyses also indicated that neonatal and adult MC and T cells produced similar amounts of LT and TNF mRNA, which contrasted with the markedly lower amount of IFN-γ mRNA. TNF protein and mRNA accumulated more rapidly than did LT protein and mRNA in both adult and neonatal cells.

These results differ somewhat from those of Eife *et al.* (18), who found that neonatal MC produced ~40% as much lymphotoxin as adult MC. These workers assayed PHA stimulated MC

supernatants for activity that inhibited ^{3}H-thymidine incorporation by a human hepatoma cell line; they referred to this activity as lymphotoxin. It is difficult to be certain which cytokines were measured by this assay, because these studies were performed before specific reagents were available to distinguish between these molecules. It is likely that the combined activity of LT, TNF, and IFN-γ [because human IFN-γ potentiates the cytotoxic effects of LT and TNF on human but not mouse cells (26)] and perhaps other cytokines were measured in their assay. We used a specific enzyme immunoassay to detect TNF and specific neutralization to determine LT bioactivity. We confirmed these results by measuring the accumulation of the specific mRNAs for LT and TNF. Thus, we are confident that the results obtained are specific for these molecules. It is possible that the diminished lytic activity of neonatal MC supernatants reported by Eife *et al.* (18) reflected the slightly lower production of TNF, the greatly diminished production of IFN-γ, or both. Other technical differences also may have contributed to the differences in findings.

TNF and LT have pleiotropic biologic effects in addition to their cytotoxic effects on tumor cells. These include the induction of acute phase protein synthesis (27) and fever (28), enhancement of granulocyte function (29–31) and myeloid differentiation (32), synergistic enhancement with IFN-γ of HLA class II expression (33) and of viral growth inhibition (34, 35). Compared to adults, neonates have deficiencies or immature responses of certain host defense mechanisms, among which are a diminished febrile response, abnormal granulocyte chemotaxis and limited marrow myeloid reserves with resultant decreased recruitment of granulocytes at sites of inflammation, and decreased expression of HLA class II molecules (reviewed in Refs. 36, 37). It is possible that the modest decrease in TNF production by neonatal MC after nonspecific stimulation contributes to these deficiencies. Other work in our laboratory has shown that freshly isolated neonatal and adult monocytes secrete similar amounts of TNF after stimulation; however, monocyte-derived and tissue macrophages from neonates secrete less TNF than adult macrophages, and TNF secretion by neonatal macrophages is not enhanced by the addition of IFN-γ as it is in adult macrophages (24). Taken together, these results suggest that the diminished production of TNF by neonatal T cells and macrophages may play a role in the decreased febrile response and the increased susceptibility to infection of the human neonate. Nevertheless, because TNF and LT share a common receptor and have nearly identical biologic activities (1, 3, 11, 29–31, 38–43), with rare exceptions (44, 45), the greater production of LT may in part counterbalance the decrease in TNF production under conditions in which the production of both cytokines is coordinately induced. Studies to assess the production of these cytokines *in vivo* will be required to address this possibility.

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