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IL-12, TNF-α, and Hormonal Changes during Late Pregnancy and Early Postpartum: Implications for Autoimmune Disease Activity during These Times

ILIA J. ELENKOV*, RONALD L. WILDER*, VLADIMIR K. BAKALOV, AMREY A. LINK, MARIANA A. DIMITROV, SCOTT FISHER, MARIANNA CRANE, KEITH S. KANIK, AND GEORGE P. CHROUSOS

Arthritis and Rheumatism Branch (I.J.E., R.L.W., M.C., K.S.K.), National Institute of Arthritis and Musculoskeletal and Skin Diseases, Pediatric and Reproductive Endocrinology Branch (I.J.E., A.A.L., S.F., G.P.C.), National Institute of Child Health and Human Development, Developmental Endocrinology Branch (V.K.B.), National Institute of Child Health and Human Development, and Laboratory of Experimental and Computational Biology (M.D.), National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Clinical observations indicate that some autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, frequently remit during pregnancy but exacerbate, or have their onset, in the postpartum period. The immune basis for these phenomena is poorly understood. Recently, excessive production of IL-12 and TNF-α was causally linked to rheumatoid arthritis and multiple sclerosis. We studied 18 women with normal pregnancies in their third trimester and during the early postpartum period. We report that during the third trimester pregnancy, *ex vivo* monocytic IL-12 production was about 3-fold and TNF-α production was approximately 40% lower than postpartum values. At the same time, urinary cortisol and norepinephrine excretion and serum levels of 1,25-dihydroxyvitamin were 2- to 3-fold higher than postpartum values. As shown previously, these hormones can directly suppress IL-12 and TNF-α production by monocytes/macrophages *in vitro*. We suggest that a cortisol-, norepinephrine-, and 1,25-dihydroxyvitamin-induced inhibition and subsequent rebound of IL-12 and TNF-α production may represent a major mechanism by which pregnancy and postpartum alter the course of or susceptibility to various autoimmune disorders. (*J Clin Endocrinol Metab* 86: 4933–4938, 2001)

IL-12, PRODUCED by antigen-presenting cells, is a major inducer of T helper 1 (Th1) responses by stimulating Th1 lymphocyte proliferation and differentiation and by inducing interferon (IFN)-γ production from natural killer and T cells (1, 2). Antigen-presenting cell-derived IL-12 and TNF-α, in concert with Th1 cell-derived IFN-γ, stimulate the activity of T cytotoxic and natural killer cells, and monocytes/macrophages, *i.e.* the major components of cellular immunity. IL-12 and TNF-α are considered major proinflammatory cytokines because they stimulate the synthesis of nitric oxide and other inflammatory mediators that drive chronic delayed-type inflammatory responses (2). On the other hand, the antiinflammatory cytokine IL-10 produced by monocytes/macrophages and Th2 cells promotes humoral immunity and inhibits monocyte/macrophage activation and the production of proinflammatory cytokines (1). Excessive production of IL-12 and TNF-α and a deficit of IL-10 appears to play a key role in the inflammatory activity and the tissue damage observed in organ-specific autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (MS) (3–5). Moreover, excessive IL-12 production is the pivotal factor in the proliferation and differentiation of pathogenic autoreactive Th1 effector cells in the experimental models of these diseases (6).

Some autoimmune diseases, such as RA and MS, often remit during pregnancy, particularly in the third trimester, but have an exacerbation or their onset during the postpartum period (7–10). The risk of developing new onset RA during pregnancy, compared with nonpregnancy, is decreased by about 70%. In contrast, the risk of developing RA is markedly increased in the postpartum period, particularly the first 3 months (odds ratio of 5.6 overall and 10.8 after first pregnancy) (10). Moreover, a substantial fraction (20–30%) of premenopausal onset RA develops within 1 yr of pregnancy (R. Wilder, unpublished observations). In women with multiple sclerosis, the rate of relapse declines during pregnancy, especially in the third trimester, increases during the first 3 months postpartum, and then returns to the prepregnancy rate (8). Although documented extensively, these observations remain poorly understood.

The third trimester of pregnancy and the early postpartum period are also known to be associated with abrupt changes of several hormones, including in tandem increases and decreases, respectively, of E2, progesterone, cortisol, and 1,25-dihydroxyvitamin D_3 (9, 11). Recently, we and others demonstrated that cortisol, catecholamines (norepinephrine and epinephrine), and 1,25-dihydroxyvitamin D_3 are potent inhibitors of IL-12 and TNF-α production by monocytes/macrophages *ex vivo* and *in vitro* (12–16). We hypothesized that during late pregnancy the increase of these hormones, and their rapid decline in the early postpartum period, may induce opposite changes in both IL-12 and TNF-α production (17). Therefore, we examined the production of IL-12 and TNF-α after lipopolysaccharide (LPS) stimulation of whole

Abbreviations: IFN, Interferon; LPS, lipopolysaccharide; MS, multiple sclerosis; NE, norepinephrine; RA, rheumatoid arthritis; Th, T helper.

blood cultures *ex vivo* and measured the levels of E2, progesterone, cortisol, 1,25-dihydroxyvitamin D_3, and catecholamines in women during gestation wk 33–36 and 3–6 wk after delivery.

Materials and Methods

Subjects

Eighteen healthy pregnant women between the ages of 20 and 40 yr and 18 age-matched, healthy, nonpregnant women participated in the study, which was approved by the institutional review board of the NIH. The pregnant women underwent testing during gestation wk 33–36 and 3–6 wk after delivery. They were enrolled in the study with the approval of their obstetricians. We screened each participant at the NIH Clinical Center by history, physical examination, and routine laboratory tests. All signed informed consents. The controls had their tests during the early and mid follicular phases of their menstrual cycle (d 3–8). All participants abstained from taking medications (except prenatal vitamins and iron supplements in pregnancy) during the week before the study. Blood specimens for hormone measurements were drawn after 1 h of rest between 1300 and 1400 h. Urine samples were collected for two 24-h periods during the preceding 2 d to measure free cortisol and catecholamine excretion rates.

Whole blood cultures

Ex vivo whole blood cytokine production assays were performed as described elsewhere (12). Blood was drawn into sodium-heparin-containing sterile tubes (Vacutainer, Becton Dickinson and Co., Lincoln Park, NJ) and processed within 45 min. The blood, diluted 1:5 with RPMI 1640 (supplemented with 1% glutamine and 50 μg/ml gentamicin) with no added exogenous serum, was divided into aliquots (1.0 ml) in 24-well cell culture plates (Costar, Cambridge, MA). To induce cytokine production, bacterial LPS was added at 1 μg/ml final concentration, and the samples were incubated in 5% CO_2 at 37 C for 18 h. After incubation, the blood was centrifuged, and the supernatant plasma was separated and stored in polypropylene tubes at −70 C until assayed.

The whole blood *ex vivo* cytokine assay, which has recently found favor elsewhere (18), has several advantages. This method avoids the isolation of leukocytes from whole blood that may cause activation and artifactual differences not present *in vivo*. The method also preserves the "natural environment" (including hormones) of cytokine-producing cells. Importantly, in comparison to methods using isolated peripheral blood mononuclear cells, the whole blood assay also shows less intraindividual variation. Less than 15% intraindividual variation of whole blood cytokine production is reported when subjects are sampled over time (18, 19) (Elenkov, I. J., R. L. Wilder, and G. P. Chrousos, unpublished observations). This contrasts with the wide (but stable) range of IL-12, TNF-α, and IL-10 secretion levels seen across healthy individuals (interindividual variation) (18–21), demonstrating that this test forms a good basis for the study of genetically or hormonally defined variation.

Monocytes/macrophages are the main IL-12-, TNF-α-, and IL-10-producing cells in LPS-stimulated whole blood (22). In view of the observed changes of monocyte/macrophage numbers during pregnancy (see *Results*), the whole blood cytokine production was corrected for monocyte/macrophage counts (pg per 10^6 monocytes/macrophages).

Cytokine assays

IL-12 p70, TNF-α, and IL-10 were measured using ELISA employing the multiple antibody sandwich principle (Quantikine, R&D Systems, Inc., Minneapolis, MN). IL-12 p70 ELISA recognizes specifically the biologically active IL-12 heterodimer without cross-reactivity with the individual subunits of the dimer (p35 and p40). The detection limits of the IL-12 p70 and the high sensitivity IL-12 p70 ELISA were 7.5 and 0.5 pg/ml, respectively, whereas they were 15.0 and 2.0 pg/ml for the TNF-α and IL-10 ELISA. The quality control parameters of these ELISAs were as follows: intraassay coefficient of variation (CV), 1.1–1.5%; interassay CV, 3.3–7.1%. Plates were read by a microplate reader (model 550, Bio-Rad Laboratories, Inc., Richmond, CA), and absorbance was transformed to cytokine concentration (pg/ml) using a standard curve computed by Microplate Manager III (Macintosh Data Analysis Software, Bio-Rad Laboratories, Inc.).

Hormonal measurements

E2 was measured by RIA after extraction and LH20 column chromatography. Intraassay CV was 4.5%, and interassay CV was 11%. Normal values for the follicular phase are 0.38–367 nmol/l. Progesterone was measured by RIA after extraction with hexane. Interassay CV was 6.7%, and intraassay CV was 4.5%. Normal values for the follicular phase are 0.003–0.03 nmol/l. 1,25-Dihydroxyvitamin D_3 was measured by cartridge extraction and RRA. Intraassay and interassay CVs were 10%. Normal values are 53–161 pmol/l (Mayo Clinical Laboratories, Rochester, MN). Twenty-four-hour urinary excretion of free cortisol was measured after extraction by chemiluminescent competitive protein binding assay. Intraassay and interassay CVs were 4.4%. Normal values are 66–298 nmol/24 h (Mayo Clinic Laboratories). Twenty-four-hour urinary excretion of epinephrine and norepinephrine (NE) were measured by HPLC with electrochemical detection. Intraassay and interassay CVs were 3.5 and 4.0, respectively. Normal values are 0–109 and 89–473 nmol/24 h, respectively (Mayo Clinic Laboratories).

Data analysis

All data are presented as means ± SE. ANOVA was done with Statistica (version 5.5, StatSoft, Inc., Tulsa, OK). Pregnancy and postpartum values of the same individuals were compared by repeated measures ANOVA. Values from healthy age-matched control subjects were compared with those of pregnancy and postpartum subjects using one-way ANOVA.

Results

Blood count changes during pregnancy and postpartum

Pregnancy was associated with an increase of white blood cell counts compared with healthy, nonpregnant controls and the postpartum state ($8.8 \pm 0.5 \times 10^3$ mm^3 *vs.* $6.0 \pm 0.3 \times 10^3$ mm^3, both in controls and postpartum; $P < 0.001$). This was attributable to a significant increase of polymorphonuclear leukocytes and monocytes (mean, $6.6 \pm 0.5 \times 10^3/\mu$l and $0.62 \pm 0.04 \times 10^3/\mu$l, respectively) during pregnancy compared with healthy matched nonpregnant controls (mean, $3.3 \pm 0.3 \times 10^3/\mu$l and $0.38 \pm 0.03 \times 10^3/\mu$l, respectively; $P < 0.001$) and the postpartum state (mean, $3.4 \pm 0.2 \times 10^3/\mu$l and $0.4 \pm 0.02 \times 10^3/\mu$l, respectively; $P < 0.001$). Pregnancy was also associated with a moderate but significant decrease ($P < 0.05$) of lymphocytes and eosinophil counts (mean, $1.8 \pm 0.1 \times 10^3/\mu$l and $0.09 \pm 0.01 \times 10^3/\mu$l, respectively) compared with control age-matched nonpregnant women (mean, $2.2 \pm 0.1 \times 10^3/\mu$l and $0.16 \pm 0.03 \times 10^3/\mu$l, respectively).

Decrease of IL-12 and TNF-α production during pregnancy

During pregnancy, whole blood IL-12 production was decreased 2-fold compared with the postpartum period (61.0 ± 10.5 *vs.* 120.7 ± 31.8 pg/ml, respectively). When corrected for monocyte count, the decrease of IL-12 production was more pronounced (>3 fold; Table 1). The individual changes of IL-12 production corrected for monocyte count in 18 women during pregnancy and their follow-up in the postpartum period are shown in Fig. 1. During pregnancy, 15 of the women had lower IL-12 production than postpartum. Of interest, we found a large interindividual variation of the "effect of pregnancy" on IL-12 production, *i.e.* 5 individuals

TABLE 1. Summary of LPS-induced IL-12 and TNF-α production *ex vivo* and hormone levels in 18 subjects during the third trimester of pregnancy and 3–6 wk after delivery and in 18 healthy age-matched nonpregnant women

Cytokine or Hormone	Nonpregnant age-matched controls	Pregnancy	Postpartum	Controls *vs.* pregnancy	Pregnancy *vs.* postpartum	Controls *vs.* postpartum
IL-12	142.1 ± 60.4	106.8 ± 21.4	340.1 ± 88.5	NS	<0.01	NS
TNF-α	5803.2 ± 971.6	5648.8 ± 484.5	7829.9 ± 870.4	NS	0.07	NS
24-h urinary free cortisol	109.1 ± 12.2	380.1 ± 31.9	134.2 ± 16.9	<0.001	<0.001	NS
24-h NE	184.9 ± 10.7	285.2 ± 17.4	132.6 ± 10.2	<0.001	<0.001	<0.01
25-dihydroxy-vitamin D	57.4 ± 6.3	87.4 ± 5.8	78.4 ± 4.6	<0.001	NS	<0.05
1,25-dihydroxy-vitamin D	111.9 ± 7.3	260.0 ± 20.6	94.1 ± 5.9	<0.0001	<0.0001	NS
E2	246.6 ± 29.2	53816.1 ± 11547.6	231.5 ± 151.9	<0.001	<0.001	NS
Progesterone	1.6 ± 0.4	380.1 ± 48.4	1.1 ± 0.4	<0.001	<0.001	NS

Data are presented as means ± SE. Cytokine production is corrected for monocyte number (pg/10^6 monocytes). Hormone levels are expressed as nmol/L, except for 1,25-dihydroxy-vitamin D, which is expressed in pmol/L; UFC (urinary free-cortisol) and urinary NE are expressed as nmol/24 h.

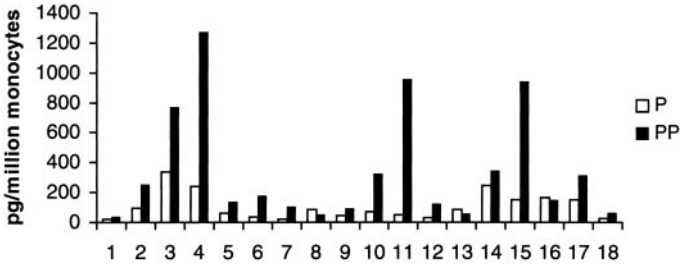

FIG. 1. Individual changes of IL-12 production by whole blood cultures stimulated with bacterial LPS *ex vivo* in 18 subjects during the third trimester of pregnancy and 3–6 wk after delivery. Cytokine production is corrected for monocyte number (pg/10^6 monocytes). P, Pregnancy; PP, postpartum.

had dramatic changes of IL-12 production, whereas others had moderate or minimal changes.

The production of TNF-α per monocyte was decreased by 40% during pregnancy compared with the postpartum period and narrowly failed to reach statistical significance ($P = 0.07$; Table 1). However, we observed similar levels of TNF-α production in pregnant *vs.* nonpregnant women (Table 1). There were no significant differences of LPS-induced IL-10 production by monocytes among the control, pregnant, and postpartum groups (data not shown).

Normal pregnancy is characterized by marked hormonal changes

The 24-h urinary cortisol excretion was increased about 4-fold during pregnancy compared with the nonpregnant state (Table 1), and in all cases it exceeded the upper limit of the reference values. After delivery, the cortisol excretion returned to normal levels. No changes in 24-h urinary excretion of epinephrine and dopamine were observed (data not shown), but we found that the 24-h urinary NE excretion was significantly increased during pregnancy and returned to baseline or lower levels in the postpartum period (Table 1).

As expected, the serum levels of E2 and progesterone were markedly increased during pregnancy. Postpartum, the ovarian hormone levels returned to normal follicular phase levels (Table 1). During pregnancy, plasma 25-hydroxyvitamin D_3 was increased by 50%, whereas 1,25-dihydroxyvitamin D_3 increased more than 2-fold (Table 1).

Cytokine production and hormone levels in a single individual before, during, and after pregnancy

We had the opportunity to follow the cytokine and hormonal changes of one woman (subject 4 in Fig. 1) as nonpregnant, during pregnancy, and postpartum (Fig. 2). She had a substantial decline in IL-12 production during pregnancy compared with the nonpregnant state. Three weeks after delivery, when cortisol, NE, E2, progesterone, and 1,25-dihydroxyvitamin D_3 returned to prepregnancy levels or lower, there was a notable rebound of LPS-induced IL-12 and TNF-α production.

E2 and progesterone do not affect IL-12, TNF-α, and IL-10 production

No data are available regarding whether E2 and progesterone are able to modulate the production of IL-12 by monocytes/macrophages. The significant changes of E2 and progesterone during pregnancy prompted us to study their direct effects in our assay system. Neither E2 nor progesterone at 10^{-5} to 10^{-11} M modulated the production of IL-12, TNF-α, or IL-10 in the LPS-stimulated human whole blood from five normal, nonpregnant individuals and three pregnant individuals (data not shown).

Discussion

We demonstrated that during late pregnancy, compared with the postpartum period, the capacity of monocytes to produce IL-12 was reduced more than 3-fold, whereas the capacity for TNF-α production was reduced by ~40%. The decreased production of these proinflammatory cytokines was paralleled by significant increases of cortisol, NE, 1,25-dihydroxyvitamin D_3, E2, and progesterone.

The pregnant women also had lower LPS-induced IL-12 production compared with age-matched controls, although the difference did not reach statistical significance (Table 1). The lack of significance in this case may reflect the large interindividual variability of monocytic IL-12 production across healthy individuals (Table 1). However, we observed a clear suppression of IL-12 production during pregnancy and a rebound in the postpartum when we followed a single individual through the nonpregnant, pregnant, and postpartum states (Fig. 2). Thus, because of the substantial interindividual variability of IL-12 production, larger and more

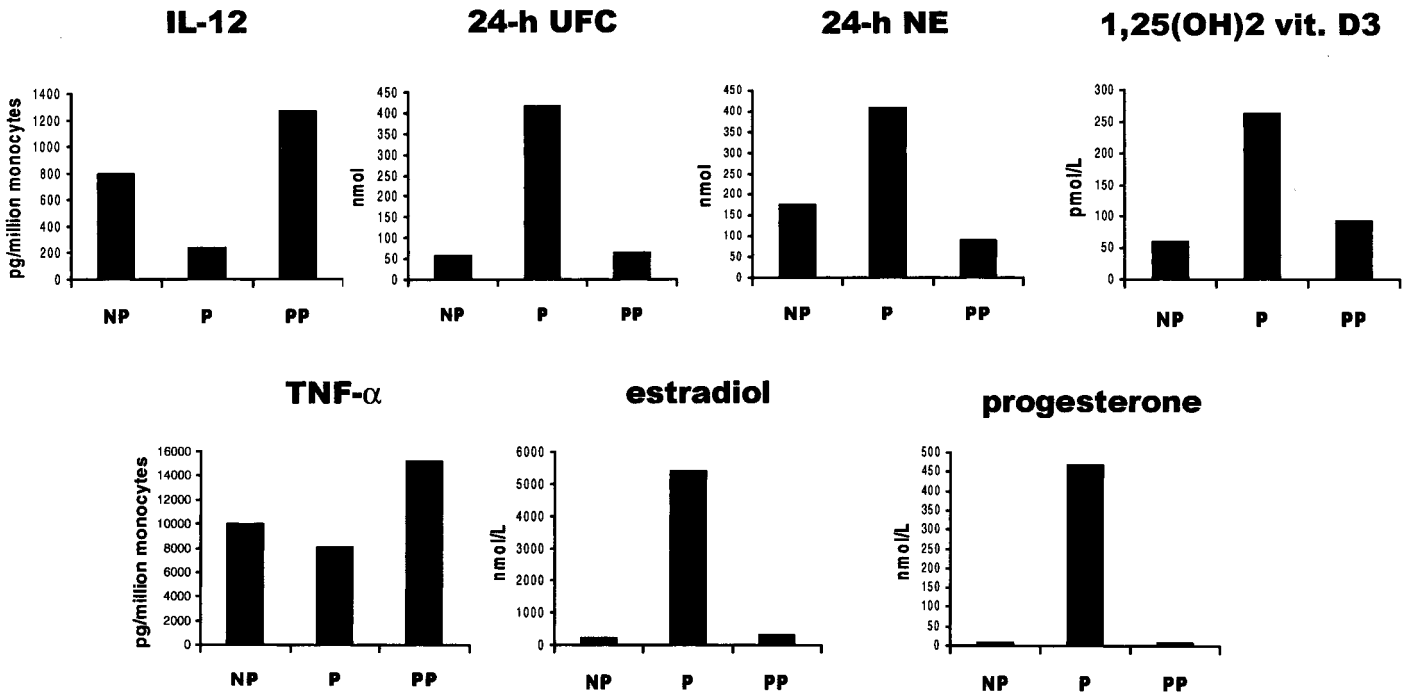

FIG. 2. Proinflammatory cytokine and hormonal changes in subject 4 (see Fig. 1) in the nonpregnant, pregnant, and postpartum states. Cytokine production is corrected for monocyte number (pg/10^6 monocytes), and hormone levels are expressed as nmol/l, except for 1,25-dihydroxyvitamin D_3, which is expressed as pmol/l. NP, Nonpregnant state; P, pregnancy; PP, postpartum; UFC, urinary free cortisol.

extended longitudinal studies are needed to address the differences between pregnancy and the nonpregnacy state.

Like others (23), we did not observe a difference of TNF-α production in pregnant *vs.* nonpregnant women. This might reflect the same factors described above for IL-12 production and an increase of soluble TNF receptors p55 and p75 in the third trimester of pregnancy, documented by Russell *et al.* (23). We did not find significant differences of LPS-induced IL-10 production by monocytes among the control, pregnant, and postpartum groups. Previous studies have shown increased production of IL-10 by peripheral blood mononuclear cells and placenta during pregnancy (24, 25). This discrepancy with our observations most likely reflects methodological differences. In the LPS-stimulated whole blood assay used by us, the primary source of IL-10 is the monocyte (22), whereas in the isolated peripheral blood mononuclear cell assay, which involves stimulation by mitogens, the primary source of IL-10 most likely is the lymphocyte.

The substantially increased urinary free cortisol excretion during the third trimester of pregnancy that returned to normal levels 3 wk after delivery indicates that late pregnancy is a state of adrenocortical activation, probably caused by the large amounts of CRH secreted by the placenta (11). We also found a significant increase of 24-h NE urinary excretion during pregnancy with a return to low normal levels in the postpartum period. This is consistent with observations in pregnant rats, in which a more than 2-fold increase of 24-h urinary excretion of NE has been described (26). Further studies are needed to elucidate whether these observations are linked to increased sympathetic nerve activity and/or reduced NE uptake during pregnancy, although, most likely, both take place (see also below).

The moderate increase of serum 25-hydroxyvitamin D_3 during pregnancy probably reflects the increased levels of serum vitamin D-binding proteins (27, 28). Like Seely *et al.* (28), we observed a more than 2-fold increase of the highly regulated serum 1,25-dihydroxyvitamin D_3 in the third trimester of pregnancy. These changes most likely result from increased conversion of 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3 in the human placenta, in addition to the increase of vitamin D-binding proteins (27, 28).

Recent evidence indicates that glucocorticoids, NE, and 1,25-dihydroxyvitamin D_3 potently inhibit the production of IL-12 and TNF-α by human monocytes/macrophages *in vitro* and *ex vivo* (12–16, 29). These hormones also inhibit the production of IL-2 and IFN-γ by Th1 cells (15, 29). In contrast, glucocorticoids do not affect the production of IL-10 by monocytes, but they potentiate IL-10 and IL-4 production by Th2 cells (12, 29, 30). Thus, the observed hormonal changes during pregnancy may explain the inhibition of monocytic IL-12 and TNF-α production. Furthermore, because IL-12 is extremely potent in enhancing IFN-γ and inhibiting IL-4 synthesis by T cells, the inhibition of IL-12 production may represent an important mechanism by which these hormones mediate a Th2 shift during pregnancy (Fig. 3).

We did not demonstrate a direct effect of E2 or progesterone on the production of IL-12, TNF-α, or IL-10 by human monocytes *ex vivo*. However, estrogens may affect cytokine production indirectly by enhancing the activity of the stress system, *i.e.* via increases in the secretion of cortisol and catecholamines (11). In addition, estrogens are potent inhibitors of the extraneuronal uptake of NE (uptake 2) (31), which may also explain the increased NE excretion in pregnancy demonstrated in our study. Therefore, estrogens may amplify the IL-12/TNF-α-inhibitory and Th2-facilitatory activities of cor-

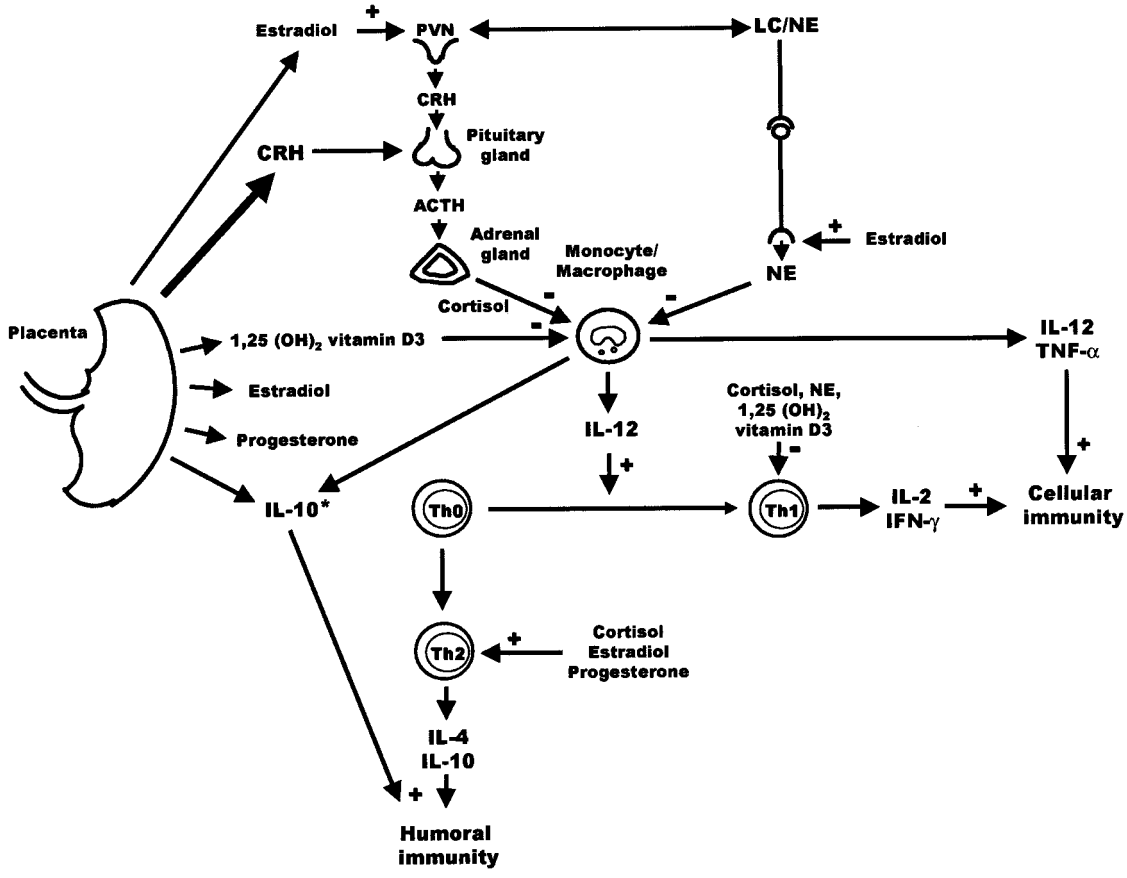

FIG. 3. A proposed simplified model of the role of different hormones in the regulation of innate and Th1 and Th2 cytokine profiles during pregnancy. Th1 cells primarily secrete IFN-γ and IL-2, which promote cellular immunity, whereas Th2 cells secrete primarily IL-4 and IL-10, which promote humoral immunity. During pregnancy, lymphocyte cytokine production is skewed toward the Th2 type: peripheral lymphocytes secrete less IFN-γ and IL-2 but more IL-4 and IL-10, particularly in the third trimester (24, 25). IL-12, a 75-kDa heterodimeric cytokine produced mostly by monocytes/macrophages, is a central inducer of Th1 responses and cell-mediated immunity by favoring Th1 cell proliferation and differentiation and by suppressing Th2 responses. Hypothalamic CRH stimulates the secretion of pituitary ACTH, which in turn triggers the secretion of cortisol from the adrenal cortex. During human pregnancy, the placenta is the major source of circulating CRH. The placenta also secretes IL-10 that may stimulate humoral and suppress cellular immunity. The sympathetic system innervates all peripheral tissues, including blood vessels and lymphoid organs. Upon activation, the sympathetic nerve terminals in these organs release NE locally and into the bloodstream. Cortisol, NE, 1,25-dihydroxyvitamin D_3, E2, and progesterone have multiple and divergent effects on the immune system. *Cortisol does not affect the production of IL-10 by monocytes/macrophages (see text). Note that *cortisol E2, and progesterone up-regulate IL-10 production by Th2 lymphocytes. In addition, E2 stimulates the activity of the CRH neurons and increases local NE concentrations by blocking its uptake. Thus, *in vivo*, E2 might amplify the effects of cortisol and NE. The net result of these complex hormonal effects is the suppression of IL-12 and TNF-α production, Th1 responses, and a Th2 shift. This hormonally induced Th2 shift may suppress Th1-related diseases such as RA and MS during pregnancy, whereas the rebound of IL-12 and TNF-α production and Th1 responses in the postpartum may facilitate the flares or the onset of these diseases. Note that several other factors, besides hormones (*e.g.* antibodies, soluble cytokine receptors, etc.), that most likely are also involved in the modulation of Th1/Th2 balance during pregnancy and postpartum, are not discussed here. LC, Locus ceruleus; PVN, paraventricular nucleus.

tisol and NE *in vivo* (Fig. 3). Furthermore, progesterone and estrogens up-regulate the production of IL-4 and IL-10 by Th2 cells *in vitro* (32, 33). Thus, an increase of estrogens and progesterone may also facilitate a Th2 shift during pregnancy by directly stimulating the production of IL-4 and IL-10 by Th2 cells (Fig. 3). This is consistent with recent data documenting increased IL-4 and IL-10 production by lymphocytes and the placenta during the third trimester of pregnancy (24, 25).

In conclusion, we demonstrated that human third trimester pregnancy, compared with the early postpartum period, is characterized by a reduction of the monocytic production of the Th1 type/proinflammatory cytokines IL-12 and TNF-α and by an increase of the secretion of cortisol, NE, and 1,25-dihydroxyvitamin D_3. Postpartum, when these hormones return to normal or low normal levels, the removal of their inhibitory effects may induce a rebound of IL-12 and TNF-α production and a Th1 shift.

The changes of Th1 type/proinflammatory cytokine production observed in this study may provide new understanding of the clinical observations that Th1-related diseases such as RA and MS frequently remit during pregnancy but exacerbate or have their onset in the postpartum period. Our study also suggests that some individuals have exaggerated postpartum Th1 type/proinflammatory cytokine rebound, raising the question of the factors that control this phenomenon. These individuals could be at greater than average risk for developing or exacerbating already existing

autoimmune diseases. Thus, further studies of the role of neuroendocrine factors in the regulation of IL-12, TNF-α/IL-10, and Th1/Th2 balance may suggest novel diagnostic and therapeutic approaches for these diseases.

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Address all correspondence and requests for reprints to: Dr. Ilia J. Elenkov, Division of Rheumatology, Immunology, and Allergy, Georgetown University Medical Center, 3800 Reservoir Road NW, Washington, D.C. 20007-2197. E-mail: ije@gunet.georgetown.edu.

* These authors contributed equally to this work.

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