# Premenopausal Sexual Dimorphism in Lipopolysaccharide-Stimulated Production and Secretion of Tumor Necrosis Factor

GEORGE MOXLEY, ALAN G. STERN, PATRICIA CARLSON, ELOISE ESTRADA, JINFENG HAN, and LINDA L. BENSON

***ABSTRACT.*** ***Objective.*** To establish whether sexual dimorphism in tumor necrosis factor (TNF) concentration in lipopolysaccharide (LPS)-stimulated whole blood culture is related to menopausal status or hormone concentrations.

***Methods.*** Healthy volunteers (72 premenopausal female, 159 male, and 62 postmenopausal female) completed questionnaires and gave peripheral blood specimens for whole blood LPS-stimulated TNF assay and for selected hormone levels. TNFab microsatellite markers were genotyped.

***Results.*** Mean LPS-stimulated TNF level in the premenopausal female group was 18% lower than the postmenopausal female mean (1579 ± 913 pg/ml compared with 2257 ± 881 in the men and 1965 ± 950 in the postmenopausal women; $p < 0.0003$ and p 0.058, respectively). Analyzing a subset for which blood counts were obtained, mean stimulated TNF per monocyte was lower in the premenopausal female group than in the postmenopausal female group and appeared lower than in the male group (2.67 ± 1.96 pg/ml per $10^3$ monocytes vs 4.44 ± 2.16 and 3.60 ± 1.40; $p = 0.018$ and $p = 0.12$, respectively). Total plasma cortisol was higher in premenopausal women than men, and, in turn, higher in men than postmenopausal women (mean ± SD 16.1 ± 5.7, 12.2 ± 3.6, and 10.4 ± 4.3 µg/dl, respectively; $p < 0.05$ for each comparison). Using multiple linear regression to correct for covariates and TNF allelic effects, premenopausal status predicted TNF level independently from potential confounders or TNF genetic markers (covariate-adjusted decrement of 408 pg/ml; $p = 0.0241$). In the male group, total cortisol predicted lower TNF level (coefficient –67.5 pg/ml for each µg/dl cortisol; $p = 0.0006$ after stepwise selection), but total testosterone had no effect. In premenopausal women, LPS-stimulated TNF was not related to total estradiol, testosterone, or cortisol level.

***Conclusion.*** Premenopausal women had a lower mean whole blood LPS-stimulated TNF level than postmenopausal women, but there was no significant relation to total estradiol, testosterone, or cortisol levels in premenopausal women. (J Rheumatol 2004;31:686–94)

*Key Indexing Terms:*
SEX TUMOR NECROSIS FACTOR CORTISOL LIPOPOLYSACCHARIDE

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Ability to resist microbial invasion is based on diverse mechanisms including immune responses conceptually organized into innate immunity and acquired immunity. Differences and nuances controlling these functions may reveal clues to susceptibility for not only infectious diseases but also inflammatory and autoimmune diseases, such as multiple sclerosis (MS), idiopathic inflammatory bowel disease, and inflammatory rheumatic diseases. Innate or natural immunity is based on phagocytes and plasma proteins. For example, endotoxin binds to a plasma protein [lipopolysaccharide (LPS) binding protein] and the complex in turn adheres to monocyte cell surface receptors and incites an inflammatory reaction. This first line of defense is present before microbial exposure, is immediately active, does not change with repeated exposure, and is not specific for the invading foreign material. In contrast, adaptive or specific immunity develops after a delay of 4 to 7 days after

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exposure and develops specificity for the invading material; it stems from lymphocytes and immunoglobulins. Not all members of a species have the same immune functions, and in particular, female and male immune responses differ in type and degree[1,2]. One important sex-related distinction in innate immunity is reflected in LPS-stimulated tumor necrosis factor (TNF) production and secretion from whole blood cultures of healthy individuals[3]: premenopausal female subjects have a 30% lower mean LPS-stimulated TNF concentration than males. This sex difference represents a moderate to large statistical effect[4] and likely has a major biological influence. A similar percentage difference had a major consequence for meningococcal mortality: the mean LPS-stimulated TNF level in relatives of nonsurvivors was 33% lower than the corresponding mean in relatives of survivors[5]. Thus, males and females differ in not only acquired immunity but also this measure of innate immune response reflected in LPS-stimulated TNF level.

Sexual disparity in immune responses may underlie sex differences in susceptibility and phenotype. A prospective study of surgical sepsis showed that, despite similar severity in male and female groups, a female cohort had a better prognosis than did a male cohort[6]. Many autoimmune inflammatory diseases disproportionately affect females[7]. One may examine rheumatoid arthritis (RA), MS, and systemic lupus erythematosus (SLE) as examples. At all ages, female sex confers a 2- to 3-fold higher RA incidence, particularly under age 50[8]. The clinical phenotype of RA may differ according to sex: in comparison with a male RA subject group, a female RA group less frequently has erosive disease, rheumatoid nodules, and rheumatoid lung disease, and more often has sicca syndrome and reconstructive surgery[9]. Similar sexual dimorphism is present in MS[1]. From the RA protection associated with oral contraceptive use[8] and modest clinical benefit with hormone replacement[10], one might suspect hormonal effects. In a recent observational study, the interaction of age with the menopausal state predicted a major part of RA joint destruction and functional disability[11]. In SLE, observational data are consistent with early natural menopause fostering SLE susceptibility[12]. Some investigators have suggested that sex steroids, sexually dimorphic pituitary hormones (prolactin and growth hormone), or liver-derived insulin-like growth factor-1 may influence immune responses and autoimmune disease susceptibility[12]. Thus, sex and hormones may influence inflammatory disease incidence, phenotype, and clinical activity.

We investigated the sexually dimorphic LPS-stimulated TNF production and secretion test by comparing previous measurements in premenopausal female and male subjects with those from a postmenopausal female panel, and by measuring total plasma estradiol, testosterone, and cortisol values.

## MATERIALS AND METHODS

This study was approved by the respective institutional review boards from Virginia Commonwealth University and McGuire VAMC and all volunteer subjects gave informed consent. We identified 293 normal healthy volunteers from the university and VAMC communities (159 men, 72 premenopausal women, and 62 postmenopausal women). Postmenopausal status was defined by duration 13 months or longer; no subject had surgical menopause. Demographic characteristics are listed in Table 1. Subjects completed a short questionnaire and underwent peripheral venipuncture during a period from 8 AM to 10 AM. Blood was introduced into a heparin tube and a potassium EDTA tube. The questionnaire addressed demographic information about age and self-reported ethnicity, listing of medications [nonsteroidal antiinflammatory drugs (NSAID), oral corticosteroids, or others], illnesses, and exercise within the previous 72 h. It is known that oral corticosteroids and exercise are expected to reduce the innate immunity measure employed[13,14], sleep disturbance reduces TNF[15], and aspirin (and by inference, some other NSAID) may increase TNF concentrations[16]. We had noted the potential influence of cigarette smoking to reduce the LPS-stimulated TNF production and secretion measure employed in this study[17] and asked about current smoking history in a portion of the subjects. Automated blood counts and differential were done using a commercial cell counter (Coulter Gen-S). [In pilot studies of 36 individuals, absolute CD14-positive mononuclear cell number (by flow-cytometric enumeration) correlated strongly with automated absolute monocyte count (Pearson and Spearman correlation coefficients 0.876 and 0.880, respectively, data not shown).]

*LPS-stimulated whole blood culture and TNF immunoassay.* We used a whole blood stimulation procedure followed by TNF-α ELISA as described[18]. The whole blood stimulation involves diluting heparinized blood with an equal volume of RPMI 1640 plus glutamine (Life Technologies, Rockville, MD, USA), adding a dilution of LPS (Sigma, St. Louis, MO, USA), culturing for 4 h (5% $CO_2$, 37°C), and harvesting supernatant for later immunoassay. The TNF-α sandwich immunoassay used a coating monoclonal antibody directed toward TNF (R&D Systems, Minneapolis, MN, USA), then a dilution of culture supernatant, then a second biotinylated mouse monoclonal anti-TNF (R&D Systems), then streptavidin-peroxidase, then a solution of TMB substrate, and finally a stop solution (0.25 M sulfuric acid). Optical density was measured at 450 nm using an automated spectrophotometer. Maximal TNF production occurred with LPS 1000 ng/ml. Technical details of storage, assay, and medication effects are published[3]. This *in vitro* assay of innate immunity is known to correlate with survival or death from infections like serious meningococcal illnesses[5].

*DNA purification and TNFab genotyping.* The potassium EDTA tube was used for DNA purification using the salting-out method (PureGene kit; Gentra, Minneapolis, MN, USA). TNF microsatellites TNFa and TNFb are adjacent loci located 6 kb telomeric to the TNFA locus (NCBI accession number Z15026)[19,20]. Genotypes were assigned by polymerase chain reaction (PCR)[19] followed by sequencing-type gel electrophoresis and silver staining; controls included PCR products from templates derived from cell lines of known TNF genotype[21,22].

*Hormone assays.* Plasma or diluted plasma was used to measure concentrations of cortisol, testosterone, and estradiol by commercial enzyme immunoassays (Active® Estradiol EIA, Active® Cortisol EIA, and Active® Testosterone EIA, all from Diagnostic Systems Laboratories, Webster, TX, USA). We have reported estradiol levels for 51 premenopausal women[3], and we confirmed previously obtained levels using identical samples (n = 3). Each kit measures competitive inhibition of binding of an enzyme-labeled antigen to a specific antibody by comparison with known standards. Measured levels for each kit's internal controls were close to the nominal values: low testosterone control (nominal 0.7 ng/ml) measured 0.61 ± 0.10 (mean ± SD), high testosterone (5 ng/ml) 4.9 ± 1.1 (5 assays); low estradiol control (250 pg/ml) measured 251 pg/ml, high estradiol control (1000 pg/ml) 919 pg/ml (one assay); and low cortisol control (nominal 4.0 µg/dl)

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*Table 1*. Variables among subjects*.

| | 72 Premenopausal Women | | 62 Postmenopausal Women | | 159 Men | |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD |
| Continuous variables | | | | | | |
| TNF, pg/ml** | **1579** | **913** | 1965 | 950 | **2257** | **881** |
| TNF per $10^3$ monocytes, pg/ml (subset 14) | **2.67** | **1.96** | **4.44** | **2.16** | 3.60 | 1.40 |
| TNF per $10^6$ leukocytes, pg/ml (subset 14) | **199** | **144** | **362** | **179** | **328** | **136** |
| Age, yrs | 30.9 | 8.2 | 54.0 | 8.0 | 30.4 | 8.7 |
| Sleep, hours | 6.9 | 1.3 | 7.3 | 1.3 | 7.0 | 1.1 |
| Exercise hours in previous 72 hours | 0.68 | 0.95 | **0.52** | **0.80** | **0.94** | **1.10** |
| Total plasma cortisol, µg/dl (subset 70) | **16.1** | **5.7** | **10.4** | **4.3** | **12.2** | **3.6** |
| Total plasma estradiol, pg/ml (subset 51) | **34.6** | **34.9** | **19.0** | **49.3** | Not done | Not done |
| Total plasma testosterone, ng/ml (subset 51)† | **1.9** | **1.2** | Not done | Not done | **11.2** | **5.2** |
| White blood cell count (x $10^6$/ml) (subset 14) | 6.6 | 1.8 | 6.3 | 2.0 | 5.5 | 1.4 |
| Monocyte count (x $10^3$/ml) (subset 14) | 493 | 172 | 510 | 164 | 493 | 112 |
| Categorical variables | | | | | | |
| Ethnic group, code†† (n) | 1 (55), 2 (8), 3 (6), 4 (3) | | **1 (45), 2 (16), 3 (1), 4 (0)** | | **1 (118), 2 (9), 3 (23), 4 (9)** | |
| NSAID (Code 1, any within previous 72 hours; Code 0, none) | Code 1, **32** | Code 0, **40** | Code 1, **30** | Code 0, **32** | Code 1, **36** | Code 0, **123** |
| Oral corticosteroids (1, any within 72 hours; 0, none) | 3 | 69 | 1 | 61 | 3 | 156 |
| Illness (1, any within 72 hours; 0, none) | 12 | 60 | **12** | **50** | **11** | **148** |
| Oral contraceptive use (1, present; 0, not at present) | 33 | 39 | | | | |
| Hormone replacement therapy (1, present; 0, not at present) | | | 31 | 31 | | |
| Exercise (1, any within previous 72 hours; 0, none) | 33 | 39 | **24** | **38** | **36** | **23** |
| Cigarette smoking | 3 | 50 | 7 | 55 | 3 | 56 |

* Significant statistical differences among and between groups are shown in bold type. For frequency data, this reflects an overall difference ($p = 0.05$ by Fisher's exact test followed by pairwise comparison with $p < 0.05$ after correction for numbers of comparisons). For continuous variables, the significant differences are obtained by Kuskal-Wallis statistic corresponding to $p < 0.05$ followed by Wilcoxon test $p < 0.05$ (corrected for number of comparisons). ** TNF values differ slightly from previous report[3], reflecting substitution of average rather than single values for 19 female subjects and correction of data entry errors for 25 male values. † Testosterone values we obtained are somewhat higher than reference female and male norms (< 1 ng/dl and 3–10 ng/ml)[58]. If one multiplies the measured levels by a calculated calibration factor derived from an external control (see Methods) (0.59, representing the quotient of 7.5 and 12.8), the levels (1.1 ± 0.7 for females and 6.2 ± 3.1 for males) approach those reported[58]. †† 1: Caucasian, 2: African American, 3: Asian, 4: other. Sums totaling less than number of total subjects represent missing data.

measured 4.4 ± 1.1 µg/dl, high cortisol (nominal 20 µg/dl) measured 20.4 ± 1.8 µg/dl (6 assays). With a commercially available external control (Bio-Rad Liquichek Immunoassay Plus Control Level 2; Bio-Rad, Hercules, CA, USA), the values were: testosterone nominal 7.5 ng/ml with 12.8 ± 0.9 measured, estradiol nominal 498 pg/ml with 154 measured, and cortisol nominal 22 µg/dl with 23.5 ± 2.2 measured.

*Statistical analyses.* We used the SAS System for Windows (Release 8.01, SAS Institute, Cary, NC, USA) for statistical analyses, including descriptive statistics (PROC MEANS and PROC FREQ), correlation (PROC CORR), and nonparametric assessment for differences among and between groups (PROC NPAR1WAY). We found no outliers in male or premenopausal female data. Outlier values, chiefly estradiol levels in some postmenopausal subjects, were addressed using nonparametric statistics. P values were corrected by multiplying by the number of comparisons, and p values reported here are so corrected. Wilcoxon 2-sample test results are denoted as Wilcoxon test. Because hormone measurements were done retrospectively, PROC REG was used to assess whether values showed evidence favoring storage-related decline. Estradiol and testosterone values showed no such decline, but because cortisol showed a small decrement, values were statistically adjusted upward by 0.42 µg/dl per year of storage. SAS was also used to assess association between LPS-stimulated whole blood TNF level (pg/ml) in healthy volunteers with independent variables such as sex, premenopausal or postmenopausal status, hormone levels, and TNFab markers (each coded for the number of alleles, 0, 1, or 2) while

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controlling for other potentially explanatory variables. The TNFab markers used were the most common and included TNFa1b5, TNFa2b1, TNFa2b3, TNFa2b5, TNFa4b5, TNFa5b5, TNFa5b7, TNFa6b5, TNFa7b4, TNFa10b4, and TNFa11b4; other markers were pooled as infrequents. The regression method adjusted for potential confounders such as age (years), race, NSAID within the previous 3 days, oral corticosteroids within the previous 3 days, duration of sleep the prior night (hours), or illness within the previous 3 days. These potential control variables we deemed to be of no particular interest for this study, and including them as variables in regression analyses has the effect of adjusting coefficients for other variables for the confounder influences[23]. For example, by including age as a control variable, the resulting model estimates a separate linear regression coefficient for the control variable age, and other independent variables accounting for variation are correspondingly adjusted for age effects. The method was also used to reduce the number of independent variables by stepwise selection. We confirmed the assumptions of multiple linear regression (linearity, equal variances, independence and normal distribution of TNF for each value of explanatory variables). The explanatory variables were evaluated by PROC REG; the covariates, variables selected by stepwise linear regression, and hormone levels are represented in Tables 3 and 4. Potential explanatory variables were examined and found to have no significant collinearity by PROC REG. PROC GLM showed some trend toward interaction of sex with TNFa10b4 ($p = 0.0137$) and TNFa4b5 with TNFa10b4 ($p = 0.008$) in all subjects taken together. We did not attempt to validate these regression models.

## RESULTS

*Demographic and potentially confounding features.* In addition to the 159 male and 72 premenopausal female subjects as described[3], we recruited 62 postmenopausal female subjects (Table 1). The 3 groups differed in frequency of reported exercise during the previous 72 hours, stemming from the contrast between males and postmenopausal females. Premenopausal females reported NSAID usage more frequently than did males, and postmenopausal females reported recent minor illness and NSAID usage more frequently than males. African American ethnicity was represented more frequently in the postmenopausal female group. White blood cell count showed a trend toward a difference among the 3 groups ($p = 0.052$), with the male mean tending to appear lower than the premenopausal female mean ($p = 0.07$). Peripheral blood monocyte count did not differ among the 3 groups (Kruskal-Wallis test 0.7, 2 df, $p = 0.715$).

*Hormone concentrations.* We had previously measured total plasma estradiol levels in a premenopausal subset, and this study extended the estradiol measurements to a postmenopausal subset. We also added cortisol and testosterone measurements in the subjects (subset numbers represented in Table 1). Total plasma cortisol levels differed among the 3 groups, with the premenopausal female mean levels higher than male and, in turn, male mean higher than postmenopausal mean levels (values listed in Table 1; Kruskal-Wallis test 37.1, 2 df, $p < 0.0001$, with Wilcoxon test for premenopausal female vs male 8790, $p < 0.0003$, and for male vs postmenopausal female 1421, $p = 0.02$). Total plasma cortisol levels in postmenopausal female subgroups either currently taking hormone replacement therapy or not currently on such therapy were $11.2 \pm 4.7$ and $9.4 \pm 3.8$ µg/dl, respectively (n = 31 each group, not significantly different). As expected, mean total estradiol levels were higher in the premenopausal female group than in postmenopausal females (Table 1; Wilcoxon test 674, $p = 0.0002$), and mean testosterone levels were higher in males than in premenopausal females (Wilcoxon test 1358, $p < 0.0001$). Male subjects over age 40 and postmenopausal female subjects over age 40 did not differ significantly in total plasma cortisol ($10.0 \pm 3.7$ vs $10.4 \pm 4.3$, reflecting subsets of n = 16 and 26, respectively).

*TNF concentrations.* LPS-stimulated TNF levels differed among the males, premenopausal females, and postmenopausal females (Table 1, Figure 1) (Kruskal-Wallis statistic 27.3, 2 df, $p < 0.0001$), with the overall difference among groups generated from the contrast between males and premenopausal females (Wilcoxon test 5904, $p < 0.0003$). There was a trend toward lower values in the premenopausal female group than in the postmenopausal group (Wilcoxon 4710, $p = 0.058$), but no difference between male and postmenopausal female groups (Wilcoxon statistic 5970, corrected $p = 0.1$). Using subsets for which monocyte counts were available, there was a difference in level among the 3 groups (Kruskal-Wallis 9.6, $p = 0.0083$). The mean male TNF pg/ml per $10^3$ monocytes showed a minimal trend toward difference with the corresponding premenopausal female mean (Wilcoxon test 242, $p = 0.12$), but power was limited because so few data points were available. The mean LPS-stimulated TNF in pg/ml per $10^3$ monocytes was significantly lower in the premenopausal female group than in the postmenopausal group (Wilcoxon test 278, corrected $p = 0.018$). LPS-stimulated TNF level expressed in relation to leukocyte count differed among the 3 groups (Kruskal-Wallis test 10.7, 2 df, $p = 0.005$), with the premenopausal female group mean lower

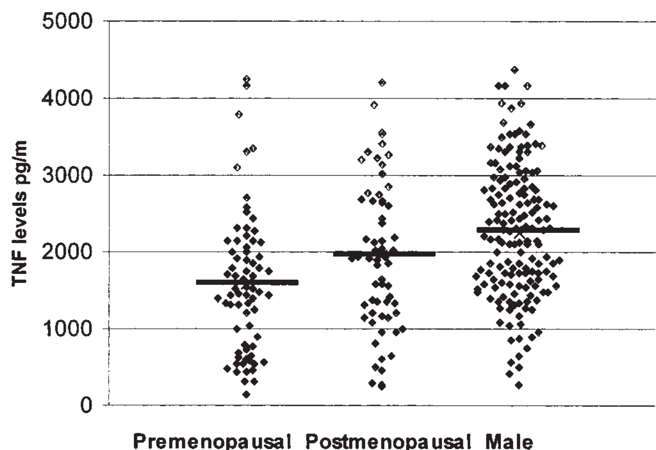


*Figure 1.* Scatterplot of LPS-stimulated whole blood TNF production and secretion (pg/ml); each point represents one subject. Lines denote means for the respective groups.

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than that of the male or postmenopausal female group (p = 0.02 and 0.018, respectively; Table 1). The mean LPS-stimulated TNF level did not differ significantly between the subgroups of postmenopausal females currently taking hormone replacement therapy or not currently on hormone replacement (1918 ± 884 and 2011 ± 1024 pg/ml, respectively); neither did the mean LPS-stimulated TNF per $10^3$ monocytes differ between the subgroups (4.3 ± 2.3 and 4.6 ± 2.1, respectively). TNF values among a subset of 14 premenopausal females aged 40 years and above were 1659 ± 750 pg/ml, apparently lower than those in 15 postmenopausal females aged 50 and younger (1884 ± 979 pg/ml) (difference not statistically significant). The subset of males over age 40 years had higher average TNF than the corresponding postmenopausal female group [2592 ± 752 pg/ml (n = 24) vs 1955 ± 950 pg/ml (n = 58); Wilcoxon test 1313, p = 0.01]. Thus, the sexual dimorphism is apparently present in the over-40 stratum.

*Correlations.* Male subjects showed a negative correlation of quite modest degree between LPS-stimulated TNF level and total plasma cortisol (Table 2) and no testosterone correlation with TNF. There was no significant correlation between TNF level and total plasma cortisol, estradiol, or testosterone levels in premenopausal females; however, the number of subjects studied achieves adequate power only for correlations defined as having a large effect size (for power 0.8, r = 0.4 or higher, or alternatively, r = –0.4 and lower)[4]. Among postmenopausal females, there was a modest positive correlation between total plasma estradiol and LPS-stimulated TNF level (Table 2); the postmenopausal subject numbers achieve adequate power for only strong correlations (power 0.8 for r values well above 0.5 or lower than –0.5 ). We expected TNF correlation with absolute monocyte count; it was statistically significant in male and premenopausal female groups but not in the postmenopausal group [Spearman r +0.35 (p = 0.05), + 0.61 (p = 0.02), and +0.20 (p = 0.18), respectively].

*Multiple linear regression analyses.* We developed models

*Table 2.* Correlation of LPS-stimulated whole blood TNF level with total plasma hormone levels (Spearman correlation coefficient r, n, p value).

| | Cortisol | Estradiol | Testosterone |
|---|---|---|---|
| Males | | | |
| r | –0.27 | — | 0.00 |
| n | 125 | — | 125 |
| p | 0.0025 | — | NS |
| Premenopausal females | | | |
| r | –0.06 | +0.15 | +0.24 |
| n | 70 | 51 | 51 |
| p | NS | NS | NS |
| Postmenopausal females | | | |
| r | +0.29 | +0.52 | — |
| n | 26 | 26 | — |
| p | NS | 0.01 | — |

to verify the relationship between LPS-stimulated TNF production and secretion from whole blood cultures to sex, premenopausal status, or hormone levels while controlling for potential confounders and for the independent allelic effects at the TNF locus. Using all subjects together, sex was a statistically significant independent predictor of TNF level (with all other variables remaining constant, coefficient –581 pg/ml for female sex, p < 0.0001), and sex remained in the stepwise-selected model (coefficient –552 pg/ml, p < 0.0001). Using stepwise selection on a model of all female subjects, premenopausal status was independent of all other variables, including age, in predicting TNF level (coefficient –408 pg/ml, p = 0.0257). A model incorporating only the subset of subjects over age 40 years did not fit well (F value 0.9, p = 0.5788), so age stratification was not possible.

We used stepwise selection to derive a "best" subset of TNF allelic markers for either male or premenopausal female subjects, and then we examined total hormone levels in the context of the selected TNFab markers and potential confounders. The male model is illustrated in Table 3. In the context of TNFab microsatellite markers TNFa2b5 and TNFa7b4 and the potential confounders (age, ethnicity, NSAID, oral corticosteroids, minor illness, and sleep), total plasma testosterone did not predict TNF level, while total plasma cortisol did. For each increment of 1 µg/dl total plasma cortisol, the TNF level decreased 68 pg/ml, with statistical significance confirmed in a p value 0.0024. This is consistent with suspected suppressive effects from cortisol. A similar model with testosterone/cortisol ratio showed no independent effect (model not shown).

For the premenopausal female model, a similar approach was used. Despite a model that accounted for a larger fraction of total variation than did the male model [coefficient of multiple determination ($r^2$) 0.5270 vs 0.2275 for the male model; Table 4], neither cortisol nor testosterone nor estradiol independently influenced LPS-stimulated TNF level in the context of confounders and the TNF markers. The premenopausal females showed no independent effect of estradiol/cortisol, testosterone/cortisol, or estradiol/testosterone ratios on predicting TNF level (model not shown).

## DISCUSSION

Taken as a whole, our study extends the observation of sexual dimorphism in LPS-stimulated TNF production and secretion to show lower mean concentrations in premenopausal women than in postmenopausal women. There is also a sexual dimorphism in total plasma cortisol levels, with the premenopausal female mean higher than the mean of same-age males, and the male mean, in turn, higher than the postmenopausal female mean. Analyzed on a per-cell basis, the mean TNF production and secretion is lower in the premenopausal female group than in the postmenopausal female group. Because the blood cell counts were obtained on consecutively recruited subjects with total

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*Table 3*. Multiple linear regression model for males. Model df 11, error df 111, corrected total df 122. F = 2.97, p = 0.0017, $r^2 = 0.2275$. The model included covariates, plus TNFab variables remaining after stepwise selection, and selected hormone levels. Coefficient ß represents the increment in TNF level for each unit of the independent variables listed in the table; SE is the standard error of ß; 95% CI represents the confidence interval for ß; T score as noted. The coefficient of multiple determination $r^2$ represents the fraction of all TNF variation accounted for by this model, and the F ratio is an overall test of statistical significance for the multivariate model.

| | Alleles, n* | Coefficient ß | SE ß | 95% CI | p | T |
|---|---|---|---|---|---|---|
| Intercept | | 3818.0 | 742.6 | 2317.9 to 5318.1 | < 0.0001 | 5.14 |
| Age | | 12.4 | 10.8 | –9.4 to 34.2 | 0.2537 | 1.15 |
| African American | | 124.4 | 368.9 | –620.8 to 869.6 | 0.7365 | 0.34 |
| Asian | | –123.4 | 245.6 | –619.5 to 372.7 | 0.6163 | –0.50 |
| NSAID | | –62.2 | 178.2 | –422.2 to 297.8 | 0.7277 | –0.35 |
| Oral corticosteroids | | 73.0 | 479.5 | –895.6 to 1041.6 | 0.8792 | 0.15 |
| Illness | | –606.9 | 299.4 | –1211.7 to –2.1 | 0.0451 | –2.03 |
| Sleep | | –182.4 | 68.4 | –320.6 to –44.2 | 0.0088 | –2.67 |
| Testosterone | | 15.5 | 15.5 | –15.8 to 46.8 | 0.3186 | 1.00 |
| Cortisol | | –67.5 | 21.7 | –111.3 to –23.7 | 0.0024 | –3.11 |
| TNFa2b5 | 12 | –532.1 | 285.4 | –1108.6 to 44.4 | 0.0649 | –1.86 |
| TNFa7b4 | 30 | 285.6 | 162.2 | –42.0 to 613.2 | 0.0810 | 1.76 |

* Among 159 males.

*Table 4*. Multiple linear regression model for premenopausal females. Model df 14, error df 34, corrected total df 48, F = 2.71, p = 0.0089, $r^2 = 0.5270$. The model included covariates, plus TNFab variables remaining after stepwise selection, and selected hormone levels. Interpretation is analogous to that in Table 3.

| | Alleles, n* | Coefficient ß | SE ß | 95% CI | p | T |
|---|---|---|---|---|---|---|
| Intercept | | 2807.4 | 1285.5 | 210.7 to 5404.1 | 0.0360 | 2.18 |
| Age | | –18.6 | 23.1 | –65.3 to 28.1 | 0.4244 | –0.81 |
| African American | | –129.9 | 438.2 | –1,015.1 to 755.3 | 0.7688 | –0.30 |
| Asian | | –630.5 | 500.0 | –1,640.5 to 379.5 | 0.2159 | –1.26 |
| NSAID | | 806.1 | 283.8 | 232.8 to 1379.4 | 0.0076 | 2.84 |
| Oral corticosteroids | | –429.4 | 696.7 | –1,836.7 to 977.9 | 0.5417 | –0.62 |
| Illness | | –539.8 | 349.0 | –1,244.8 to 165.2 | 0.1312 | –1.55 |
| Sleep | | –167.1 | 106.2 | –381.6 to 47.4 | 0.1249 | –1.57 |
| Cortisol | | 13.1 | 27.8 | –43.1 to 69.3 | 0.6411 | 0.47 |
| Estradiol | | 4.7 | 4.3 | –4.0 to 13.4 | 0.2753 | 1.11 |
| Testosterone | | 10.4 | 119.1 | –230.2 to 251.0 | 0.9309 | 0.09 |
| TNFa1b5 | 1 | –2379.1 | 923.8 | –4,245.2 to –513.0 | 0.0145 | –2.58 |
| TNFa2b3 | 13 | –394.7 | 302.7 | –1,006.2 to 216.8 | 0.2011 | –1.30 |
| TNFa4b5 | 8 | –1185.5 | 397.7 | –1,988.9 to –382.1 | 0.0053 | –2.98 |
| TNFa10b4 | 19 | 575.4 | 249.8 | 70.8 TO 1080.0 | 0.0275 | 2.30 |

* Among 53 premenopausal females.

TNF values representative of the respective groups, there is no intrinsic reason to suspect biased ascertainment. The lower mean premenopausal female TNF concentration was not correlated with total plasma cortisol, estradiol, or testosterone in univariate analyses nor was it independently predicted by total hormone level in multiple linear regression analyses. While we grant that a more rigorous examination would compare surgically postmenopausal females with age-matched premenopausal females, age was included as a specific covariate in the multiple linear regression analyses but had an insignificant p value (p = 0.7183). For this reason, we do not believe the TNF level difference stems solely from age but rather from sexually dimorphic hormones differing between premenopausal females and males and largely disappearing at menopause. In males, LPS-stimulated TNF level was predicted by total plasma cortisol in a manner independent of confounders and TNF allelic effects.

The relationship of LPS-stimulated TNF production and secretion to hormones is obviously complex. The statistical relationship of total plasma cortisol to LPS-stimulated TNF level in men suggests that male cortisol levels may suppress TNF production and secretion. Apparent sexual dimorphism in corticosteroid metabolism has been noted previously[24-26]. Our study measured total plasma cortisol, including free and protein-bound forms. One previous study showed slightly

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lower morning cortisol levels in premenopausal females than in males[24], apparently in conflict with the results we obtained. We have no explanation for the difference, but the data are not comparable in all respects. The previous study used a slightly older subject group (men averaging 39.4 years, women 39.6 years), aggregated data from 7 centers, and unknown numbers of different cortisol assays[24]. Because our subjects were recruited from one center and the measurements were done in batch assays with a single method, our data have less potential for variation from ascertainment or technical factors. Other studies have shown sex differences in urinary cortisol values; these would be consistent with sexual dimorphism in cortisol effect[25-27]. The measurement of total plasma cortisol, both protein-bound and free, may explain some results of this study, and in particular, absence of cortisol effect in premenopausal females. Estrogen exposure may be accompanied by not only increased cortisol-binding globulin[28,29] but an increment in unbound cortisol[30]. In this context, we interpret our findings of higher mean total plasma cortisol in premenopausal females than in males, and the trend toward a higher mean level in postmenopausal females undergoing current hormone replacement than in those not on hormone replacement, as largely consistent with estrogen-associated increase in cortisol-binding globulin. One could speculate that estrogen's influences on cortisol-binding globulin might so obscure a statistical relationship that total plasma cortisol would correlate less than would other measures of cortisol metabolism more reflective of cortisol effect, say, urinary cortisol or free cortisol.

Next, regarding estrogen, we showed that total plasma estradiol did not influence premenopausal female TNF in a statistically significant manner. Because estrogen's influence may be exerted in other ways, like a biphasic or threshold effect, or based on cyclical variation, or unbound hormone, or free hormone ratio, or even effects mediated via other hormones, our data do not exclude estrogen influence. One must note that TNF transcriptional effects differ among cell types, so one cannot extrapolate from one cell line, like monocytic cells, to hepatoma or B-lymphoblastoid cells or even pre-monocytic cell lines[31]. Under some culture conditions, estrogen is known to have biphasic effects on TNF production: when T-lymphocyte clones were exposed to estradiol, lower levels stimulated, and higher levels inhibited[32]. As with cortisol, estradiol may be unbound or bound to plasma proteins. We measured total estradiol, including unbound and that bound to sex hormone-binding globulin. Sex hormone-binding globulin level is stimulated by estrogens and reduced by androgens, insulin, and other factors[28,33]. Accordingly, premenopausal women would represent a population in which it might be difficult to show a linear relationship of LPS-stimulated TNF level to total estradiol because of cyclical variation in sex hormone-binding globulin and thus unbound hormone. However, our results indicate that if estrogen does influence premenopausal LPS-stimulated TNF concentration, it does so in a manner more complicated than simply based on total estradiol levels.

How steroid hormones affect TNF control is important because one or more may account for the sexual dimorphism in LPS-stimulated TNF production. First, glucocorticoids appear to suppress TNF production and secretion from blood cells[13,34,35]. In human cell lines, diminished transcription is the primary mechanism, but in murine cell lines, interaction with the 3' AU-rich untranslated region blocks translation[36]. For sex steroid hormones, the literature shows conflict, ambiguity, or even silence. Studies disagree about differences in TNF production between follicular and luteal menstrual phases; one study found no menstrual variation in TNF production and secretion[37], another described lowered peripheral monocytes production during the luteal phase[38], yet another found an apparently paradoxical luteal phase increase in frequency of TNF-producing peripheral monocytes[37-39]. Increased spontaneous TNF production occurs after surgical menopause but not after simple hysterectomy; it returns to normal with estrogen replacement therapy[40], consistent with estrogen suppression. Studies of hormone effects on stimulated blood cells *in vitro* do not provide a clear picture, either. Ralston, *et al* found no estradiol effects on TNF production using cells from young men or premenopausal women. However, estradiol stimulated cells from older men but suppressed those from postmenopausal women[35]. Using a streptococcal stimulus, estradiol and progesterone increased TNF production from donors with low unstimulated TNF profile, but did not change TNF production in donors with high unstimulated TNF profile[41]. For androgens, the literature is sparse. LPS-stimulated TNF level was positively correlated with testosterone level in 9 male subjects[37], and testosterone exposure led to higher LPS-stimulated TNF production and secretion for cells derived from older men and postmenopausal women but not younger men or women[35]. One can reconcile these various reports by reasoning that estrogen effects, for example, must have counterbalancing interactions, with the direction of effect depending on which interaction is dominant in the cell or population examined.

We believe that understanding this sexual dimorphism in LPS-stimulated TNF production and secretion will provide important clues for autoimmune and inflammatory diseases. One might reason that, because TNF plays such an important role in RA pathogenesis and clinical inflammatory activity, a lower premenopausal female TNF concentration is inconsequential or even beneficial. One should not accept this assertion without further investigation. In conceptual terms, TNF has 2 opposite faces. One is proinflammatory, and that explains its role in clinical arthritis damage because TNF is critical for starting and controlling the cytokine cascade during inflammation[42]. TNF's opposite influence is

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immunosuppressive (as recently reviewed[43,44]). Whether TNF exerts its proinflammatory or immunosuppressive influence depends on tissue location, timing, and duration. Depending on when and how long TNF is given, it may worsen, mitigate, or prevent disease in animal models of lupus, diabetes, multiple sclerosis, or arthritis[45-49]. The most precise experimental control incorporated, and thus the clearest illustration to date, is represented in diabetes models[45,50-52]. With a tetracycline-controlled, islet cell-expressed TNF transgene, the duration of TNF-mediated inflammation determines autoimmune outcome. After 21 days of islet-TNF expression followed by TNF removal, diabetes never ensues; after 25-day expression, diabetes stemming from islet inflammation always follows[50]. In a viral diabetes model, TNF expressed early in disease pathogenesis enhances diabetes incidence; given late, it blocks the autoimmune process[45]. Clinicians have noted that TNF's immunosuppressive side is not simply a laboratory oddity. In humans, TNF blockade with biological products may worsen multiple sclerosis, precipitate demyelination, and lead to antinuclear, anti-DNA, and anticardiolipin antibodies[53-55]. Anti-TNF therapy may also reverse lymphocyte anergy[56,57]. When anti-TNF therapy leads to autoimmune phenomena, it suggests that TNF had held autoimmune features in check[43,44]. Thus, sexual dimorphism in TNF production and secretion may indicate differences in both proinflammatory and immunosuppressive effects. We submit that lower premenopausal female TNF concentrations make sense for features of clinical RA: higher female risk may arise from less immunosuppression to bridle autoimmunity during a critical period, fewer erosions may stem from lower proinflammatory effects during clinical illness[9], and menopause would worsen joint inflammation and destruction[11]. For SLE, a recent population-based case-control study found that natural menopause occurred earlier in women who subsequently developed SLE than in controls ($p < 0.001$), suggesting that early natural menopause increased SLE susceptibility[12]. Our observation of higher TNF concentrations in postmenopausal than in premenopausal women would be consistent with the notion that susceptibility may stem from augmented TNF response.

We have further defined the sexual dimorphism in LPS-stimulated TNF production and secretion by linking its relationship to premenopausal female status. While total plasma cortisol appears to influence male LPS-stimulated TNF concentration in a modest but statistically significant manner, female LPS-stimulated TNF concentrations are not affected by total plasma cortisol, estradiol, or testosterone. Nevertheless, the lower premenopausal female level of whole blood LPS-stimulated TNF production and secretion is likely related to hormonal effects that change at menopause. This lower level of LPS-stimulated TNF production and secretion may reflect mechanisms having a major influence on induction or severity of autoimmune disease.

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