ARTHRITIS & RHEUMATISM
Vol. 46, No. 1, January 2002, pp 250–258
DOI 10.1002/art.10064

Published by Wiley-Liss, Inc.

Sexual Dimorphism in Innate Immunity

George Moxley,[1] Danielle Posthuma,[2] Patricia Carlson,[3] Eloise Estrada,[3] Jinfeng Han,[1] Linda L. Benson,[1] and Michael C. Neale[4]

***Objective*. To establish whether variation in innate immunity, as measured by the level of tumor necrosis factor (TNF) in lipopolysaccharide (LPS)–stimulated whole-blood culture, is related to sex or HLA.**

***Methods*. Normal volunteers (72 women, 159 men) completed questionnaires and donated peripheral blood specimens. Blood samples were exposed to LPS in a 4-hour in vitro culture, and supernatants were then tested by sandwich-type immunoassay measuring TNF levels. Statistical techniques included multivariate analysis and maximal-likelihood modeling of allelic effects.**

***Results*. Both male and female groups showed substantial within-group variation (coefficient of variation 59.1% for women, 40.3% for men). However, the mean ± SD LPS-stimulated TNF level in the female group was nearly 30% lower than in the male group (1,556 ± 919 pg/ml versus 2,203 ± 889 pg/ml; $P < 0.0001$, unadjusted for covariates). Sex was independent of any microsatellite marker allele of TNF (covariate-adjusted increment of 785 pg/ml from female to male sex; $P < 0.0001$). In multivariate modeling of the female group, the LPS-stimulated TNF level was not independently influenced by menstrual cycle phase, oral contraceptive use, or plasma estradiol level. Allelic modeling showed that significant TNFab microsatellite allelic effects existed ($P = 0.002$ versus model omitting allelic effects). The female group showed a significantly downward deviation from mean TNF level with TNFa4b5 (−903 pg/ml deviation from the overall mean) and an upward deviation with TNFa10b4 (598 pg/ml). The male group showed significantly higher-than-mean levels with TNFa1b5 (909 pg/ml), TNFa5b7 (1,191 pg/ml), and TNFa6b5 (332 pg/ml). Thus, the two sex groups differed in which of their TNFab marker alleles showed significant deviations from the overall mean.**

***Conclusion*. Female subjects have a nearly 30% lower innate immune response, stemming largely from influence independent of the HLA-region TNF locus and without further independent variation stemming from plasma estrogen level.**

Immunity, the ability to resist microbial invasion, is a paramount function based on diverse mechanisms. Host defense may stem from physical barriers, phagocytes, natural killer cells, and various plasma effector molecules, such as the complement system. Innate or natural immunity, a method of host defense represented among most multicellular living things, is based on phagocytes and plasma proteins. For example, endotoxin binds to a plasma protein (lipopolysaccharide [LPS]–binding protein, or LBP), and the complex in turn binds 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. Another kind of immunity found only in vertebrates is adaptive or specific immunity. This kind of host defense develops after a delay of 4–7 days after exposure and develops specificity for the invading material; it stems from specific lymphocytes and immunoglobulins.

Immune responses are sexually dimorphic in type and degree (for review, see refs. 1 and 2). Overall,

Supported by the NIH (grant U54-HD-28934 from the National Institute of Child Health and Human Development) as part of the Specialized Cooperative Centers Program in Reproduction Research, a Department of Veterans Affairs Merit award, Mid-Career Clinical Investigator award AR-K24-02131, and the Grace Branch Moore–Arthritis Foundation professorship from the Medical College of Virginia Foundation. Dr. Posthuma's work was supported by the Netherlands Organization for Scientific Research (NWO, travel fund R 56-454) and the Simons Stichting (travel fund).

[1]George Moxley, MD, Jinfeng Han, BSN, Linda L. Benson, MPH: Hunter Holmes McGuire Veterans Affairs Medical Center, and Virginia Commonwealth University, Richmond; [2]Danielle Posthuma, MS: Vrije Universiteit Amsterdam, The Netherlands; [3]Patricia Carlson, PhD, Eloise Estrada, BS: Hunter Holmes McGuire Veterans Affairs Medical Center, Richmond; [4]Michael C. Neale, PhD: Virginia Commonwealth University, Richmond.

Address correspondence and reprint requests to George Moxley, MD, Box 111M, McGuire VAMC, 1201 Broad Rock Boulevard, Richmond, VA 23249. E-mail: gmoxley@hsc.vcu.edu.

Submitted for publication June 7, 2001; accepted in revised form September 3, 2001.

female experimental animals are more likely to develop a Th1-type response after exposure to an immunogen or infectious agent, except during pregnancy, when a Th2 response supercedes. In humans, the immune response also differs by degree: women develop more vigorous immunity with higher antibody levels and stronger cell-mediated immunity. Some 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 (1).

Rheumatoid arthritis (RA) and a plethora of other autoimmune inflammatory disorders occur predominantly in women (3). The clinical phenotype of RA may differ according to sex: compared with a group of male subjects with RA, a group of female subjects with RA less frequently had erosive disease, rheumatoid nodules, and rheumatoid lung disease, and more often had reconstructive surgery for RA joint damage and sicca syndrome (4). Exactly why these sex biases occur is unknown.

RA is a complex genetic disease. It shows familial aggregation, increased twin concordance in monozygotic twins compared with dizygotic twins, and diminished penetrance, but no recognizable Mendelian pattern of inheritance (5). The genetic basis of RA probably involves several disease genes, of which at least two are HLA-region disease susceptibility loci, one recognized through association with shared-epitope DRB1 alleles and the other by HLA class III markers (6–13). Recent genome-wide and candidate-gene studies to detect genetic loci linked with RA have generally confirmed HLA linkage and implicated several genomic intervals distant from HLA (12,14–16). Because of high utility stemming from degree of polymorphism, the microsatellites near the TNF gene have generally been used to represent the TNF region. These include microsatellite $(GT)_n$ and $(GA)_n$ repeats ~3.5 kb upstream to the lymphotoxin-alpha gene LTA (TNFa and TNFb, respectively) that are, in turn, near the TNF gene; the TNFa and TNFb markers form TNFab haplotypes like TNFa2-TNFb1, abbreviated TNFa2b1 (17,18). The TNFab microsatellite markers have been found to be RA susceptibility factors independent of the HLA–DRB1 region (6,7,10,12,13). Sex-related bias applies to RA heredity; sex influences association of HLA markers with RA (19,20).

Tumor necrosis factor (TNF) may play a role in RA risk and pathogenesis. TNF is an HLA class III region cytokine encoded by a locus between those for the second component of complement and HLA–B. TNF may be critical for RA pathogenesis, directly mediating cartilage and bone damage and amplifying effects from other proinflammatory cytokines, such as interleukin-1 (IL-1) and granulocyte–macrophage colony-stimulating factor (21,22). Experimental therapies directed toward TNF, such as chimeric monoclonal antibodies or soluble TNF receptors, appear to have remarkable benefit in RA subjects (23–25).

LPS-stimulated TNF production is an in vitro assay of innate immunity. This measure has marked interindividual variation (26,27) stemming in part from genetic factors. Westendorp and colleagues found in 1997 that the LPS-stimulated TNF level had a broad-sense heritability of 0.60 in a Dutch population (28). Jacob and colleagues noted interindividual variation in the mitogen-stimulated TNF level that was specific for the stimulus used; although the response level was not the same with all stimuli, high producers with one stimulus were high producers with another, and low producers with one stimulus were low producers with another (29). The TNF production level was related to HLA haplotype (29). This in vitro measure of innate immunity also has substantial pathogenetic implications. The families of survivors of meningococcal infections had a profile of higher levels of LPS-stimulated TNF and lower levels of LPS-stimulated IL-10 than did families of those who did not survive meningococcal infections (28).

Therefore, to investigate sex and HLA influences on innate immunity, we examined an in vitro assay, LPS-stimulated whole-blood culture with subsequent TNF immunoassay on culture supernatant, for influences stemming from sex and the HLA-region microsatellite marker, TNFab.

PATIENTS AND METHODS

Subjects. This study was approved by the respective Institutional Review Boards of Virginia Commonwealth University and the Hunter Holmes McGuire Veterans Affairs Medical Center (VAMC). We identified 231 normal volunteers (159 men, 72 women) from the Virginia Commonwealth University and McGuire VAMC communities. Demographic characteristics are listed in Table 1. Following informed consent, volunteers completed a short questionnaire and underwent peripheral blood venipuncture from 8:00 AM to 10:00 AM. Blood was introduced into a heparin tube and into a potassium EDTA tube. The questionnaire included demographic information, including age and self-reported ethnicity, listing of medications (nonsteroidal antiinflammatory drugs [NSAIDs], oral corticosteroids, or others), and illnesses or exercise within the previous 72 hours. Cigarette smoking influences the innate immunity measure employed in this study (30), but of samples from 59 men and 53 women, only 6 subjects gave this history.

Table 1. Variables used in multiple linear regression models*

Variable	Women (n = 72)†	Men (n = 159)‡
Continuous		
TNF level, mean ± SD pg/ml	1,556 ± 919	2,203 ± 889
Age, mean ± SD years	30.9 ± 8.2	30.4 ± 8.7
Sleep, mean ± SD hours	6.9 ± 1.2	7.0 ± 1.1
Estradiol, mean ± SD pg/ml§	34.6 ± 34.9	NA
Categorical¶		
NSAIDs	31/41	36/123
Oral corticosteroids	1/71	1/158
Illness	11/61	11/148
Menstrual cycle phase, no. in late luteal phase/no. in early follicular phase	29/39	NA
Oral contraceptive use, no. currently using/no. not using	33/39	NA
Exercise	35/37	59/23

* TNF = tumor necrosis factor; NA = not applicable; NSAIDs = nonsteroidal antiinflammatory drugs.
† Includes 55 whites, 8 African Americans, 6 Asians, and 3 of other ethnic origin.
‡ Includes 118 whites, 9 African Americans, 23 Asians, and 9 of other ethnic origin.
§ n = 51 in this group.
¶ Except for menstrual cycle and oral contraceptives, values are the number of subjects who have/number who have not, during the previous 72 hours, taken NSAIDs or oral corticosteroids, experienced an illness, or engaged in exercise. Sums less than n reflect missing data.

Automated blood counts and differential were done using a commercial cell counter (Gen-S; Coulter, Hialeah, FL).

LPS-stimulated whole-blood culture and TNF immunoassay. We used a whole-blood stimulation procedure followed by TNF enzyme-linked immunoassay as developed by van der Linden and colleagues at Leiden (31). The whole-blood stimulation involves diluting heparinized blood with an equal volume of RPMI 1640 plus glutamine (Invitrogen, Carlsbad, CA), adding a dilution of LPS (Sigma, St. Louis, MO), culturing for 4 hours (5% CO_2, 37°C), and harvesting supernatant for later immunoassay. Maximal TNF production occurred with 1,000 ng/ml LPS. We stored the supernatants at –70°C; despite these conditions, immunoreactive TNF levels declined 8% over a 1-month period and 27% over 3 months. Addition of protease inhibitors or glycerol or silanization of storage tubes did not prevent this decline. With 1, 2, and 3 freeze–thaw cycles, the decrements of detectable TNF were 10%, 17%, and 27%. To correct for storage-related decline, we adopted the strategies of aliquoting supernatants for –70°C storage, storing an internal control (with known TNF level) with each batch of unknowns, and testing an aliquot within 2 weeks.

The sandwich immunoassay used a coating monoclonal antibody directed toward TNF (R&D Systems, Minneapolis, MN), followed by a dilution of culture supernatant, then by a second biotinylated mouse monoclonal anti-TNF antibody (R&D Systems), then by streptavidin–peroxidase, then by a solution of tetramethylbenzidine substrate, and finally by a stop solution (0.25*M* sulfuric acid). Optical density was measured at 450 nm using an automated spectrophotometer (Tecan Spectra Rainbow, Durham, NC). We also optimized the TNF immunoassay to maximize sensitivity. We examined the influence of NaCl added to the supernatant just before immunoassay and found that a final NaCl concentration of 0.225*M* led to a 16% increment in TNF detected; we reasoned that this condition might have released TNF from binding proteins. The intraassay variation for the whole-blood stimulation plus immunoassay was 3.7%, and the interassay variation over 3 months was 8.2%, both of which were acceptable.

The effects of common over-the-counter and prescription medications and diurnal variation are also important. We examined medication effects: a single 650-mg dose of aspirin had little effect within 4 hours, and a single 10-mg dose of prednisone gave 98% suppression of TNF within 2 hours. Inducible TNF levels increased slightly from 8:00 AM to 11:00 AM (~18%); thus, morning sampling during this period does increase variation slightly. We examined one male internal control by obtaining 21 different blood samples over a period of 15 months to examine interassay plus biologic variation (including NSAID, minor illness, and aerobic exercise effects), and the mean ± SD TNF level was 2,823 ± 533 pg/ml (coefficient of variation 18.9%).

DNA purification and TNFab genotyping. The potassium EDTA tube was used for DNA purification using the salting-out method (PureGene kit; Gentra Systems, Minneapolis, MN). TNF microsatellites TNFa and TNFb are adjacent loci 6 kb telomeric to the TNFA locus (National Center for Biotechnology Information accession no. Z15026) (17,18). Genotypes were assigned by polymerase chain reaction (PCR) (17) followed by sequencing-type gel electrophoresis and silver staining; controls included PCR products from templates derived from cell lines of known TNF genotype (32,33). We first typed for TNFa and TNFb microsatellite genotypes. Then, by amplifying the entire TNFab region, we derived the TNFab haplotype from the known TNFa and TNFb genotypes and the TNFab product size (33). For example, in an individual with TNF genotypes a2,a6 and b1,b5 and TNFab product lengths of 202 bp and 214 bp, we reasoned that the TNFab length was consistent with haplotypes TNFa2b1 (202 bp) and TNFa6b5 (214 bp), not a2b5 (206 bp) and a6b1 (210 bp). Each TNFab microsatellite haplotype was treated as an allele for further analyses.

Statistical analysis. We used SAS System for Windows (Release 8.01; SAS Institute, Cary, NC) to develop models to predict the LPS-stimulated whole-blood TNF level (pg/ml) in normal volunteers based on sex and TNFab markers (each coded for the number of alleles: 0, 1, or 2), as adjusted for confounders (age in years, race, NSAIDs within the previous 3 days, oral corticosteroids within the previous 3 days, duration of sleep the prior night in hours, or illness within the previous 3 days). For example, a simplified equation for all subjects is as follows: TNF = 2,615 + (261 × NSAIDs) + (612 × sex) + (–551 × illness) + (–142 × sleep) + (–573 × TNFa4b5) + (–381 × TNFa5b5) + (903 × TNFa5b7).

Here, the dependent or response variable is the LPS-stimulated whole-blood TNF level, and the explanatory variables are sex, 3 TNFab markers, and 3 potential confounding variables (NSAIDs, illness, and hours of sleep). The numbers in front of the explanatory variables are regression coefficients and are interpreted as follows: if NSAIDs, illness, sleep, TNFa4b5, TNFa5b5, and TNFa5b7 are held constant, then the LPS-stimulated TNF level increases by ~612 pg/ml for male

Table 2. Multivariate model for predicting lipopolysaccharide-stimulated TNF level*

	No. of alleles†						
	Female	Male	Coefficient β	SE β	95% CI	*P*	T score
Intercept			2,130	538	1,043, 3,217	<0.0001	3.96
Age			3.5	7.8	−12, 19	0.6504	0.45
African American			−489	249	−992, 14	0.0512	−1.96
Asian			−156	182	−524, 212	0.3937	−0.85
NSAIDs			267	143	−22, 556	0.0628	1.87
Steroids			−87	648	−1,396, 1,222	0.8928	−0.13
Illness			−477	211	−903, −51	0.0252	−2.26
Sleep			−177	53	−284, −70	0.0011	−3.32
Sex			**785**	**169**	**444, 1,126**	**<0.0001**	**4.63**
TNFa1b5	1	6	−1,602	888	−3,396, 192	0.0703	−1.80
TNFa2b1	10	43	259	181	−107, 625	0.1532	1.43
TNFa2b3	13	24	192	204	−220, 604	0.3490	0.94
TNFa2b5	3	12	−67	271	−614, 480	0.8047	−0.25
TNFa4b5	**8**	**11**	**−285**	**259**	**−808, 238**	**0.2720**	**−1.10**
TNFa5b5	2	20	−145	228	−606, 316	0.5248	−0.64
TNFa5b7	**1**	**5**	**1,234**	**394**	**438, 2,030**	**0.0020**	**3.13**
TNFa6b5	13	37	426	189	44, 808	0.0251	2.26
TNFa7b4	4	30	295	195	−99, 689	0.1309	1.52
TNFa10b4	19	45	849	259	326, 1,372	0.0012	3.28
TNFa11b4	21	48	326	180	−38, 690	0.0713	1.81
TNFa12b4	1	9	217	318	−425, 859	0.4963	0.68
Sex by TNFa10b4			−706	261	−1,233, −179	0.0074	−2.71
Sex by TNFa1b5			**2,604**	**948**	**689, 4,519**	**0.0066**	**2.75**

* Variables selected as significant by stepwise linear regression are shown in bold. The model has 22 degrees of freedom, an error of 188, and a corrected total of 210; $r^2 = 0.3013$, $F = 3.68$, $P < 0.0001$. Coefficient β represents the increment in TNF level for each unit of the independent variables listed in the Table, SE β is the standard error of β, and 95% CI represents the 95% confidence interval for β. The obtained 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. See Table 1 for other definitions.

† The number of alleles represents those among 53 women or 159 men; the remaining alleles were grouped as "infrequents" (see Figure 3).

sex over that for female sex. (Please note that this model represents a simplified explanatory illustration; the best-fitting models are incorporated into the Results.)

We analyzed by using multiple linear regression modeling methods to verify the association between the LPS-stimulated whole-blood TNF level and the explanatory variables of sex and TNFab markers, while controlling for confounders (as listed above). The independent variables used in the full model include those listed in Table 2. 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 (SAS System for Windows; SAS Institute); those variables selected by stepwise linear regression are shown in bold in Table 2. Potential explanatory variables were examined and found to have no significant collinearity by PROC GLM (SAS System for Windows; SAS Institute); sex did have some trend toward interaction with TNFa1b5 ($P = 0.0237$) and TNFa10b4 ($P = 0.0259$). We found no outliers in data for men or women.

Mx modeling of allelic effects. Mx (written by MCN; available at no charge online at http://www.vipbg.vcu.edu/mxgui) is a combination of a matrix algebra interpreter and a numerical optimizer. It enables exploration of matrix algebra through a variety of operations and functions. There are many built-in fit functions to enable structural equation modeling and other types of statistical modeling of data. It offers the fitting functions found in commercial software such as LISREL, LISCOMP, EQS, and CALIS, along with facilities for maximum likelihood estimation of parameters from missing data structures, under normal theory. Complex "nonstandard" models are easy to specify. For further general applicability, Mx allows users to define their own fit functions, and optimization may be performed subject to linear and nonlinear equality or boundary constraints. In the present study, Mx was used to provide maximum likelihood fit for TNFab marker allelic effects to actual data in sex-stratified analyses; infrequent marker alleles were combined into a single category. To assess statistical significance, the modeling examined reduction in model fit when specific marker alleles were dropped. The potential confounders of age, self-reported race, medications, sleep, and illness were incorporated into the models.

RESULTS

Demographic features. There were 159 male subjects and 72 female subjects, of whom 19 women were not typed for TNFab microsatellites. The male and female subject groups did not differ significantly in some potential confounders (hours of sleep, age, frequency of corticosteroid use, or race) (Table 1). The male group showed a trend toward reporting illness less frequently

($P = 0.0543$, by Fisher's exact test) and using NSAIDs less frequently ($P = 0.003$, by Fisher's exact test).

LPS-stimulated TNF level influenced by sex. Both male and female groups showed substantial within-group variation (coefficient of variation 59.1% for women, 40.3% for men). However, the mean ± SD LPS-stimulated TNF level in the female group was nearly 30% lower than in the male group (1,556 ± 919 pg/ml versus 2,203 ± 889 pg/ml; $P < 0.0001$, by Wilcoxon 2-sample test). The possibility that this stemmed from a difference in peripheral blood leukocyte count was examined in a subset of 31 men and 14 women; there was no statistically significant difference in mean ± SD absolute monocyte count (49.3 ± 17.2 monocytes/mm^3 for women versus 49.6 ± 11.3 monocytes/mm^3 for men; $P = 0.455$, by Wilcoxon 2-sample test) or lymphocyte count (233.5 ± 95.8 lymphocytes/mm^3 for women versus 183.3 ± 54.3 lymphocytes/mm^3 for men; $P = 0.167$, by Wilcoxon 2-sample test).

In both male and female groups, the LPS-stimulated TNF level correlated with the absolute monocyte count (Pearson's r = 0.382 and Pearson's r = 0.582, respectively, $P < 0.05$ for each). There was also a correlation with the absolute lymphocyte count in men and a trend in women (Pearson's r = 0.386, $P < 0.05$ and Pearson's r = 0.491, P not significant, respectively). Regardless of this, however, the mean ± SD LPS-stimulated TNF level was lower in the female group, whether expressed per 10^3 leukocytes (198.7 ± 144.3 pg/ml versus 329 ± 138 pg/ml; $P = 0.006$), per 10^3 monocytes (26.6 ± 19.6 pg/ml versus 49.6 ± 11.2 pg/ml; $P = 0.041$), or per 10^3 lymphocytes (5.6 ± 4.0 pg/ml versus 10.0 ± 4.3 pg/ml; $P = 0.003$) (all by Wilcoxon 2-sample test). To address whether differences in protease activity might account for the female group–related decrement, we incubated cell-free supernatants at 37°C for periods of up to 2 hours, but there was no decrease in TNF detected. Thus, the lower value in the female group did not solely reflect an influence on cell populations or induced proteases; women produced lower quantities of TNF.

TNFab microsatellite marker genotyping. The scatter plots of data for both men and women showed substantial overlap among the values represented for all alleles (Figures 1 and 2). The frequency distribution of TNFab markers did not differ significantly between male and female groups ($\chi^2 = 14.5$, 8 degrees of freedom [df], $P = 0.182$).

TNF level predicted by sex independently of TNFab (Table 2). Multivariate analyses allowed detection of whether sex was independent of TNFab markers

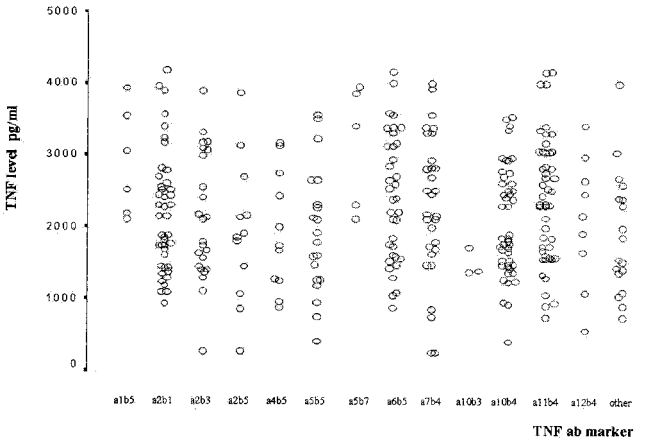

Figure 1. Lipopolysaccharide-stimulated tumor necrosis factor (TNF) levels represented among 159 healthy normal male subjects. Each circle represents a value for an individual, with the specific TNFab marker indicated on the bottom of the figure. Heterozygotes are represented as two circles, one for each TNFab marker, while homozygotes are represented as two circles for a single TNFab marker. Overlapping values are represented as overlapping circles.

while controlling for the potential confounders of age, race, medications, recent illness, or sleep. The results are shown in Table 2. Sex was a statistically significant independent variable predictive of the LPS-stimulated TNF level ($P < 0.0001$); when all other potential confounders and independent variables were held constant, female sex led to a predicted TNF level slightly <800 pg/ml lower than male sex (Table 2). In sex-stratified

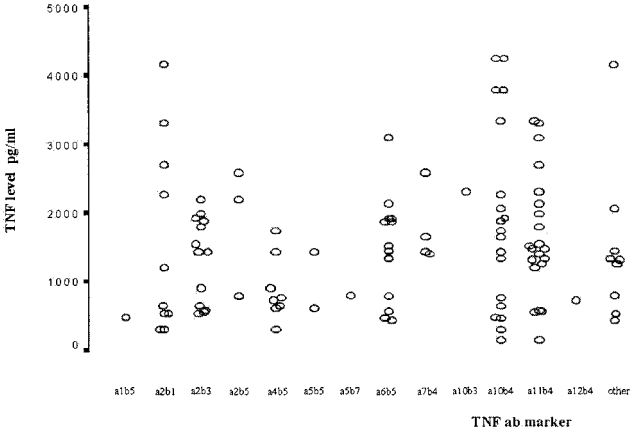

Figure 2. Lipopolysaccharide-stimulated TNF levels represented among 72 healthy normal female subjects. Each circle represents a value for an individual, with the specific TNFab marker indicated on the bottom of the figure. Heterozygotes are represented as two circles, one for each TNFab marker, while homozygotes are represented as two circles for a single TNFab marker. Overlapping values are represented as overlapping circles. See Figure 1 for definitions.

linear regression analyses with stepwise selection with main effects only, the female model included TNFa1b5, TNFa4b5, and TNFa10b4 with P values of <0.05. The corresponding male model selected TNFa1b5, TNFa5b7, TNFa6b5, and TNFa11b4. However, the TNFa1b5 coefficients for women and men differed: the coefficient for women was highly negative (mean ± SE −2,466 ± 963 pg/ml), the corresponding coefficient for men was positive (mean ± SE 1,102 ± 400 pg/ml), and the two confidence intervals did not overlap.

Stepwise selection employing the model using men and women together, as outlined in Table 2, selected only sex ($P < 0.0001$ in the reduced model), TNFa4b5 ($P = 0.0131$), TNFa5b7 ($P = 0.0233$), and sex by TNFa1b5 ($P = 0.0396$), but not the interaction term of sex by TNFa10b4. The r^2 represented in Table 2 is the fraction of all TNF variation accounted for by this model, 30%; thus, much variation remains uncaptured by the included variables.

TNFab marker allelic effects on LPS-stimulated TNF level. Modeling of allelic effects for each TNFab marker allele allowed testing of significant deviations from the overall means for the female or the male groups while controlling for the covariates of age, race, medications, illness, and sleep. Dominance effects did not fit as well as additive model effects. The overall modeling showed that there were significant TNFab marker allelic effects (comparison of full model versus model with no TNFab allelic effects: $\chi^2 = 44.8$, 19 df, $P = 0.002$). With the model showing the best fit, allowing different allelic means for each sex group, the female group showed negative deviation from the mean for TNFa4b5 and positive deviation with TNFa10b4 (Table 3 and Figure 3). For the male group, higher-than-mean levels were found with TNFa1b5, TNFa5b7, and TNFa6b5 (Table 3 and Figure 3).

The multiple linear regression model represented in Table 2 showed a significant coefficient for an interaction term of sex with TNFa1b5 and sex with TNFa10b4, but stepwise selection removed the sex with TNFa10b4 variable. Thus, both regression and modeling of allelic effects suggested that some TNFab marker effects may differ between male and female groups. In allelic effects models, covariates accounted for only a small fraction of variation (8% in women, 2% in men), while TNFab marker allele effects conferred substantially more of the variation (23% in the female group, 17% in the male group). Thus, while the sex influence on the LPS-stimulated TNF level is largely exerted outside TNFab, TNFab itself does account for substantial variation, with different TNFab marker alleles conferring statistically significant deviations from the mean for each sex group.

Table 3. Influence of individual TNFab marker alleles on lipopolysaccharide-stimulated TNF level*

Allelic effects	Deviation from mean, pg/ml†	95% CI
Female‡		
Infrequent alleles	44.3	−95.7, 186.8
TNFa2b1	37.4	−522.5, 622.9
TNFa2b3	−440.2	−996.2, 133.1
TNFa4b5	**−903.0**	**−1,606.4, −152.9**
TNFa6b5	−157.4	−648.6, 350.0
TNFa10b4	**597.9**	**135.0, 1,083.3**
TNFa11b4	289.1	−178.4, 772.2
Male‡		
Infrequent alleles	−122.8	−300.9, 55.6
TNFa1b5	**908.6**	**247.5, 1,595.2**
TNFa2b1	128.3	−155.4, 420.3
TNFa2b3	111.5	−260.9, 494.5
TNFa2b5	−383.8	−890.5, 145.7
TNFa4b5	−277.2	−823.3, 296.5
TNFa5b5	−297.6	−684.2, 104.7
TNFa5b7	**1,191.3**	**473.4, 1,962.2**
TNFa6b5	**332.1**	**4.9, 670.4**
TNFa7b4	205.4	−108.3, 529.0
TNFa10b3	−401.4	−1,362.4, 640.3
TNFa10b4	9.1	−279.0, 305.5
TNFa11b4	241.0	−48.5, 540.4
TNFa12b4	74.7	−487.6, 671.2

* Comparison of full model versus model with no TNFab allelic effects: $\chi^2 = 44.8$, 19 degrees of freedom, $P = 0.002$. Effects with P values less than 0.05 are shown in bold. 95% CI = 95% confidence interval (see Table 1 for other definitions).
† Grand mean for women = 2,792.2 + (−838.4 × Asian race) + (−164.9 × hours of sleep) + (−378.2 × illness). Grand mean for men = 2,513.5 + (6.25 × years of age) + (−99.9 × hours of sleep).
‡ Eight percent of variation among women was accounted for by Asian race, age, and sleep; 23% was accounted for by TNFab allelic effects. Two percent of variation among men was accounted for by age and sleep; 17% was accounted for by TNFab allelic effects.

Consideration of estrogen effects. The possibility that TNF measures reflected estrogen effects was examined by obtaining blood samples from 10 normal female volunteers twice during a menstrual cycle. There was no trend toward lower or higher levels during the luteal phase of the menstrual cycle. A subset of 51 female subjects had estradiol levels measured. TNF did not correlate significantly with the plasma estradiol level (Pearson's r = 0.148, $P = 0.30$). Using multivariate analytic methods (similar to those reflected in Table 2, but including only TNFa1b5, TNFa4b5, TNFa10b4, and TNFa11b4), estradiol levels and variables representing oral contraceptive use and menstrual cycle phase did not influence the TNF level ($P = 0.266$, $P = 0.870$, and $P = 0.404$, respectively; data not shown) or interact with TNFab marker alleles (data not shown).

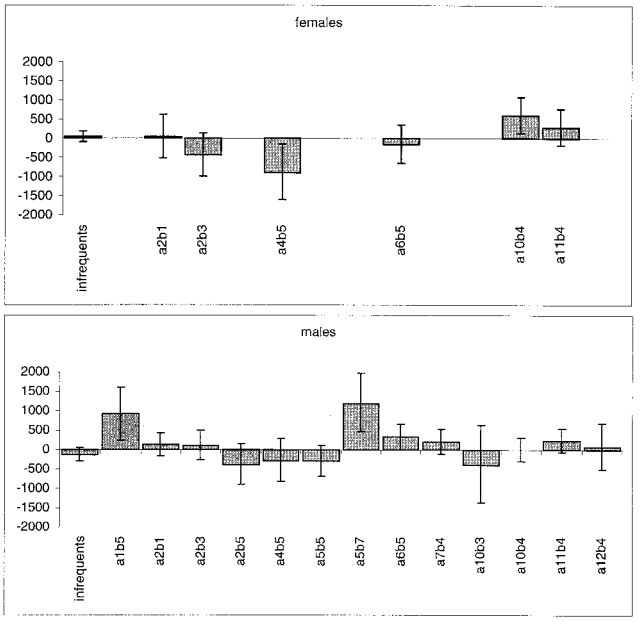

Figure 3. Deviations from mean allelic values, with standard error bars, for each TNFab marker as derived from the best-fitting Mx model (see Patients and Methods). Equations used to derive grand means for male and female subjects are represented in Table 3. See Figure 1 for definitions.

DISCUSSION

Taken as a whole, this in vitro measure of innate immunity, the LPS-stimulated TNF level, varies remarkably. Some detectable variation stems from the HLA region (as reflected in TNFab microsatellite marker alleles), a large fraction is environmental/random variation (as reflected in the repeated measures of an internal control), and another large fraction is associated with sex and is not directly related to estradiol levels among premenopausal women. Judging from the model represented in Table 2, the sum of TNFab markers, sex, interaction terms, and the potential confounders (age, race, NSAID use, corticosteroid use, illness, and sleep) accounts for only 30% of all LPS-stimulated TNF variation. Thus, while substantial nongenetic variation exists (as shown by the 19% coefficient of variation in the internal control), much variation is not accounted for, particularly in female subjects. One can only speculate about sources of nongenetic variation at this time, but exposure to ultraviolet radiation, unreported minor illnesses, environmental exposures, and seasonal variations may bear examination as potential sources. Examining why variation occurs may help explain differences in the threshold for disease or differences in clinical phenotype and potentially might address differences in therapeutic responses.

It is not known why the female group had lower levels of LPS-stimulated TNF. One possibility is an influence on the hypothalamic–pituitary–adrenal axis. In previous studies of physiologic corticosteroid doses administered to normal volunteers, LPS-stimulated TNF levels were suppressed (34). Also, LPS-stimulated TNF levels were lower in morning samples than in afternoon samples, a pattern inverse to that of cortisol levels (34). While our morning sampling minimized this diurnal variation, this finding suggests that any variation in plasma cortisol level, whether genetic, sex-influenced, or nongenetic, may affect this measure of innate immunity. There exists a sexual dimorphism in cortisol response to endogenous adrenocorticotropic hormone (ACTH); although equivalent daily cortisol secretion is attained, female subjects have lower ACTH secretion levels, suggesting more response from the female adrenal cortex (35). Whether the sex-related difference in LPS-stimulated TNF reflects such an endocrine dimorphism remains to be determined.

Another potential explanation is a sexual dimorphism influencing LPS-stimulated signaling. One might speculate that the LPS-signaling pathway molecules (such as LBP, MD-2, CD14, Toll-like receptor 4, myeloid differentiation primary response gene 88, or IL-1 receptor–associated kinase 1) might be influenced by sexually dimorphic hormones, or that such hormones influence TNF messenger RNA stability factors. Yet other explanations might lie in TNF signaling pathways.

That women differ from men in their LPS-stimulated TNF level is not an entirely novel finding. Jacob and colleagues had noted that premenopausal women had more variation than men upon repeated sampling (29), while postmenopausal female subjects seemed to have less variation and a mean level more nearly like that of male subjects. Their study did not find a sex-related difference in level, but the magnitude of difference found in the present study would not have been discerned with the number of previously examined subjects (29). The present study adds the observations of magnitude of difference and apparent absence of relation to menstrual cycle phase, oral contraceptive use, or estradiol level in female subjects.

This degree of variation may be important. Experimental animals genetically engineered to lack a functional TNF pathway, say, by knocking out one or both TNF receptors, have a propensity toward infections with *Listeria monocytogenes*, and those overexpressing TNF may develop an ileitis similar to Crohn's disease

and also inflammatory arthritis (for review, see ref. 36). In humans, cerebral malaria is associated with high TNF levels and with a TNF promoter allele, −308A, known to occur on the TNFa2b3 haplotype (37). Survival of male subjects with surgery-related sepsis may be influenced by a TNF-region polymorphic marker (38).

This study is only the latest of several showing variation of TNF level stemming from HLA-region influences (for a recent review, see ref. 39), but it does have the advantages of larger numbers and model-fitting analyses. Santamaria and colleagues found high in vitro secretion levels with DR2/DR3 heterozygotes and low levels with DR3/DR4 heterozygotes (40). Jacob and colleagues found stable interindividual differences in stimulated TNF production that were associated with HLA–DR serologic markers: high levels with DR3 or DR4 and low levels with DR2 (29). In 87 Dutch male subjects, Pociot and colleagues found that the TNF level reflected HLA influence: high TNF levels were associated with TNFa2, DR3, and DR4, and low levels with TNFa6, DR2, and DR5 (41). These effects were not discernable in our male study subjects, but the previous studies did not correct for covariates or fit models of allelic effects. Garcia-Merino and colleagues found that the TNFab marker a2b3 on the ancestral B8–DR3 haplotype was associated with higher TNF secretion levels than was the TNFa11b4 marker on a B7–DR2 haplotype (42). Investigators at Leiden have described up to 4-fold differences in stimulated TNF levels among families that could not be attributed to TNF promoter polymorphism differences (43); there was a slightly lower TNF level associated with the –238GA promoter genotype.

Thus, several groups of investigators have found HLA-region influences on stimulated TNF production, but the details vary and are probably related to differences in ancestral haplotypes according to the population studied. However, previous studies have addressed allelic effects either by comparing homozygous HLA genotypes (42) or by comparing subjects positive for one or another HLA haplotype (29), in effect partly ignoring the contribution of the second HLA haplotype. Model fitting allows better analyses of allelic effects, and from the present study, it appears that TNFab allelic effects are additive.

How LPS-stimulated TNF production as an in vitro measure of innate immunity relates to the genetics of RA remains to be clarified. The HLA region appears to contain at least two disease-risk genes, one encoded by shared-epitope DRB1 polypeptide chains (44) and the other within the class III region and recognized through independent association with markers such as TNFa2b1 in men and TNFa2b3 and TNFa6b5 in women (6,7,13). While some TNFab marker alleles caused significant deviations from the mean TNF level (Figure 3), from the present study one would not immediately discern a difference in LPS-stimulated TNF production for TNFa2b1 in men or for TNFa2b3 or TNFa6b5 in women. However, the present study does not directly address functional effects from ancestral HLA haplotypes (45) that might occur in disease-relevant tissues.

Thus, findings of this study are consistent with the notion that variation in this in vitro measure of innate immunity, LPS-stimulated TNF production, may be partitioned into substantial sex-related variation independent of HLA, HLA-region variation with some discernable superimposed sex-related effects, and variation due to unknown factors. While there is detectable HLA-related influence, the vast majority of variation stems from other sources, and some of this variation may influence the threshold for developing autoimmune disease.

ACKNOWLEDGMENT

The authors are indebted to the McGuire VAMC and Virginia Commonwealth University communities for recruiting and enlisting volunteer subjects.

REFERENCES

1. Whitacre CC, Reingold SC, O'Looney PA. A gender gap in autoimmunity. Science 1999;283:1277–8.
2. Whitacre CC, Blankenhorn E, Brinley FJ, Collier E, Duquette P, Fox H, et al. Sex differences in autoimmune disease: focus on multiple sclerosis [serial online]. 1999; Accessed October 15, 2001. URL: www.sciencemag.org/feature/data/983519.shl.
3. Beeson PB. Age and sex associations of 40 autoimmune diseases. Am J Med 1994;96:457–62.
4. Weyand CM, Schmidt D, Wagner U, Goronzy JJ. The influence of sex on the phenotype of rheumatoid arthritis. Arthritis Rheum 1998;41:817–22.
5. Silman A, Hochberg M. Rheumatoid arthritis. In: Silman A, Hochberg M, editors. Epidemiology of the rheumatic diseases. Oxford: Oxford University Press; 1993. p. 7–68.
6. Mulcahy B, Waldron-Lynch F, McDermott MF, Adams C, Amos CI, Zhu DK, et al. Genetic variability in the tumor necrosis factor-lymphotoxin region influences susceptibility to rheumatoid arthritis. Am J Hum Genet 1996;59:676–83.
7. Hajeer A, John S, Ollier WE, Silman AJ, Dawes P, Hassell A, et al. Tumor necrosis factor microsatellite haplotypes are different in male and female patients with RA. J Rheumatol 1997;24:217–9.
8. Ota M, Katsuyama Y, Kimura A, Tsuchiya K, Kondo M, Naruse T, et al. A second susceptibility gene for developing rheumatoid arthritis in the human MHC is localized within a 70-kb interval telomeric of the TNF genes in the HLA class III region. Genomics 2001;71:263–70.
9. Zanelli E, Jones G, Pascual M, Eerligh P, Van der Slik AR, Zwinderman AH, et al. The telomeric part of the HLA region predisposes to rheumatoid arthritis independently of the class II loci. Hum Immunol 2001;62:75–84.
10. Hajeer AH, Dababneh A, Makki RF, Thomson W, Poulton K, González-Gay MA, et al. Different gene loci within the HLA-DR

and TNF regions are independently associated with susceptibility and severity in Spanish rheumatoid arthritis patients. Tissue Antigens 2000;55:319–25.
11. Singal DP, Li J, Zhu Y. HLA class III region and susceptibility to rheumatoid arthritis. Clin Exp Rheumatol 2000;18:485–91.
12. Martínez A, Fernández-Arquero M, Pascual-Salcedo D, Conjero L, Alves H, Balsa A, et al. Primary association of tumor necrosis factor-region genetic markers with susceptibility to rheumatoid arthritis. Arthritis Rheum 2000;43:1366–70.
13. Meyer JM, Han J, Moxley G. Tumor necrosis factor markers show sex-influenced association with rheumatoid arthritis. Arthritis Rheum 2001;44:286–95.
14. Cornelis F, Faure S, Martinez M, Prud'Homme JF, Fritz P, Dib C, et al. New susceptibility locus for rheumatoid arthritis suggested by a genome-wide linkage study. Proc Natl Acad Sci U S A 1998;95: 10746–50.
15. Jawaheer D, Seldin MF, Amos CI, Chen WV, Shigeta R, Monteiro J, et al. A genomewide screen in multiplex rheumatoid arthritis families suggests genetic overlap with other autoimmune diseases. Am J Hum Genet 2001;68:927–36.
16. Bali D, Gourley S, Kostyu DD, Goel N, Bruce I, Bell A, et al. Genetic analysis of multiplex rheumatoid arthritis families. Genes Immun 1999;1:28–36.
17. Jongeneel CV, Briant L, Udalova IA, Sevin A, Nedospasov SA, Cambon-Thomsen A. Extensive genetic polymorphism in the human tumor necrosis factor region and relation to extended HLA haplotypes. Proc Natl Acad Sci U S A 1991;88:9717–21.
18. Nedospasov SA, Udalova IA, Kuprash DV, Turetskaya RL. DNA sequence polymorphism at the human tumor necrosis factor (TNF) locus: numerous TNF/lymphotoxin alleles tagged by two closely linked microsatellites in the upstream region of the lymphotoxin (TNF-beta) gene. J Immunol 1991;147:1053–9.
19. Jaraquemada D, Ollier W, Awad J, Young A, Silman A, Roitt IM, et al. HLA and rheumatoid arthritis: a combined analysis of 440 British patients. Ann Rheum Dis 1986;45:627–36.
20. Meyer JM, Han J, Singh R, Moxley G. Sex influences on the penetrance of HLA shared-epitope genotypes for rheumatoid arthritis. Am J Hum Genet 1996;58:371–83.
21. Brennan FM, Maini RN, Feldmann M. TNF alpha: a pivotal role in rheumatoid arthritis? Br J Rheumatol 1992;31:293–8.
22. Arend WP, Dayer JM. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor α in rheumatoid arthritis. Arthritis Rheum 1995;38:151–60.
23. Elliott MJ, Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, et al. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. Lancet 1994;344:1105–10.
24. Moreland LW, Baumgartner SW, Schiff MH, Tindall EA, Fleischmann RM, Weaver AL, et al. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. N Engl J Med 1997;337:141–7.
25. Rankin EC, Choy EH, Kassimos D, Kingsley GH, Sopwith AM, Isenberg DA, et al. The therapeutic effects of an engineered human anti-tumour necrosis factor alpha antibody (CDP571) in rheumatoid arthritis. Br J Rheumatol 1995;34:334–42.
26. Molvig J, Baek L, Christensen P, Manogue KR, Vlassara H, Platz P, et al. Endotoxin-stimulated human monocyte secretion of interleukin 1, tumour necrosis factor alpha, and prostaglandin E2 shows stable interindividual differences. Scand J Immunol 1988;27:705–16.
27. Endres S, Cannon JG, Ghorbani R, Dempsey RA, Sisson SD, Lonnemann G, et al. In vitro production of IL 1 beta, IL 1 alpha, TNF and IL2 in healthy subjects: distribution, effect of cyclooxygenase inhibition and evidence of independent gene regulation. Eur J Immunol 1989;19:2327–33.
28. Westendorp RG, Langermans JA, Huizinga TW, Elouali AH, Verweij CL, Boomsma DI, et al. Genetic influence on cytokine production and fatal meningococcal disease. Lancet 1997;349:170–3.
29. Jacob CO, Fronek Z, Lewis GD, Koo M, Hansen JA, McDevitt HO. Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor alpha: relevance to genetic predisposition to systemic lupus erythematosus. Proc Natl Acad Sci U S A 1990;87:1233–7.
30. Yamaguchi E, Itoh A, Furuya K, Miyamoto H, Abe S, Kawakami Y. Release of tumor necrosis factor-alpha from human alveolar macrophages is decreased in smokers. Chest 1993;103:479–83.
31. Van der Linden MW, Huizinga TW, Stoeken DJ, Sturk A, Westendorp RG. Determination of tumour necrosis factor-alpha and interleukin-10 production in a whole blood stimulation system: assessment of laboratory error and individual variation. J Immunol Methods 1998;218:63–71.
32. Udalova IA, Nedospasov SA, Webb GC, Chaplin DD, Turetskaya RL. Highly informative typing of the human TNF locus using six adjacent polymorphic markers. Genomics 1993;16:180–6.
33. Monos DS, Kamoun M, Udalova IA, Csanky E, Cizman B, Turetskaya RL, et al. Genetic polymorphism of the human tumor necrosis factor region in insulin-dependent diabetes mellitus: linkage disequilibrium of TNFab microsatellite alleles with HLA haplotypes. Hum Immunol 1995;44:70–9.
34. DeRijk R, Michelson D, Karp B, Petrides J, Galliven E, Deuster P, et al. Exercise and circadian rhythm-induced variations in plasma cortisol differentially regulate interleukin-1 beta (IL-1 beta), IL-6, and tumor necrosis factor-alpha (TNF alpha) production in humans: high sensitivity of TNF alpha and resistance of IL-6. J Clin Endocrinol Metab 1997;82:2182–91.
35. Roelfsema F, van den Berg G, Frolich M, Veldhuis JD, van Eijk A, Buurman MM, et al. Sex-dependent alteration in cortisol response to endogenous adrenocorticotropin. J Clin Endocrinol Metab 1993;77:234–40.
36. Kollias G, Douni E, Kassiotis G, Kontoyiannis D. On the role of tumor necrosis factor and receptors in models of multiorgan failure, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. Immunol Rev 1999;169:175–94.
37. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature 1994;371:508–10.
38. Schroder J, Kahlke V, Book M, Stuber F. Gender differences in sepsis: genetically determined? Shock 2000;14:307–10.
39. Hajeer AH, Hutchinson IV. TNF-alpha gene polymorphism: clinical and biological implications. Microsc Res Tech 2000;50:216–28.
40. Santamaria P, Gehrz RC, Bryan MK, Barbosa JJ. Involvement of class II MHC molecules in the LPS-induction of IL-1/TNF secretions by human monocytes: quantitative differences at the polymorphic level. J Immunol 1989;143:913–22.
41. Pociot F, Briant L, Jongeneel CV, Molvig J, Worsaae H, Abbal M, et al. Association of tumor necrosis factor (TNF) and class II major histocompatibility complex alleles with the secretion of TNF-alpha and TNF-beta by human mononuclear cells: a possible link to insulin-dependent diabetes mellitus. Eur J Immunol 1993;23:224–31.
42. Garcia-Merino A, Alper CA, Usuku K, Marcus-Bagley D, Lincoln R, Awdeh Z, et al. Tumor necrosis factor (TNF) microsatellite haplotypes in relation to extended haplotypes, susceptibility to diseases associated with the major histocompatibility complex and TNF secretion. Hum Immunol 1996;50:11–21.
43. Bouma G, Crusius JB, Oudkerk PM, Kolkman JJ, von Blomberg BM, Kostense PJ, et al. Secretion of tumour necrosis factor alpha and lymphotoxin alpha in relation to polymorphisms in the TNF genes and HLA-DR alleles: relevance for inflammatory bowel disease. Scand J Immunol 1996;43:456–63.
44. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987;30:1205–13.
45. Degli-Esposti MA, Leaver AL, Christiansen FT, Witt CS, Abraham LJ, Dawkins RL. Ancestral haplotypes: conserved population MHC haplotypes. Hum Immunol 1992;34:242–52.