ANALYSIS OF TUMOR NECROSIS FACTOR -ALPHA(TNF-α) SYNTHESIS IN PATIENTS WITH RHEUMATOID ARTHRITIS (RA)

By

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ABSTRACT

Rheumatoid arthritis (RA) is one of the most common types of arthritis worldwide, especially in the elderly. A wide range of cytokines and inflammatory mediators are expressed in the joints in RA. However, TNF-α is placed at the heart of the inflammatory process in RA. The aim of this work was to compare spontaneous and LPS induced TNF-α production between early and late RA patients which affect the treatment regimen, and to detect the association of TNF-α –308A/G polymorphism in Egyptian patients with RA using PCR-RFLP.Our results shows that LPS induced TNF-α production is high in late RA patients (disease duration > 1 year) not early patients (disease duration < 1 year). Also this study found no association between TNF-α –308A/G polymorphism in Egyptian patients with RA.

Key Indexing Terms:

Lipopolysaccharide, tumor necrosis factor, peripheral blood mononuclear cells, rheumatoid arthritis, 308(a/g) tnf-α polyorphism.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease with 1% prevalence in the industrialized world. It comprises a syndrome of pain, stiffness, and symmetrical synovitis (inflammation of the synovial membrane) of diarthrodial joints (freely moveable joints such as the knee) that leads to articular destruction, functional decline, and substantial comorbidity in the cardiovascular, neurologic, and metabolic systems **(Brennan and McInnes, 2008).** Disease onset generally occurs between 30 and 55 years of age, and women are affected more often than men (**Donahue, et al., 2008).**

RA is one of the most common types of arthritis worldwide, especially in the elderly. They not only cause pain and physical disability that lead to a substantial consumption of health resources, but also lead to productivity losses and early retirement **(Xie, 2008).**

A wide range of cytokines and other inflammatory mediators are expressed in the inflamed joints in RA. However, TNF-α is placed at the heart of the inflammatory process in RA **(Macías et al., 2005).** There is considerable evidence implicating TNF-α as a major contributor to the pathogenesis of chronic inflammatory diseases. TNF-α has been shown to have pro-inflammatory properties by stimulating the production of prostaglandin E, collagenase as well as other cytokines such as IL-1 and IL-6. Increased levels of TNF-α expression have been observed in the synovial fluid and in the tissue from rheumatoid arthritis patients **(Gao-yun et al., 2010).**

Rheumatoid arthritis is a heterogeneous disease where both genetic and environmental factors have important roles in pathogenesis.http://www.ncbi.nlm.nih.gov/pubmed/9236671 A genetic contribution to the development of rheumatoid arthritis is estimated to account for about 30% of the disease risk. Many genes contribute in the development of the disease, genes for cytokines that play a key role in rheumatoid arthritis pathogenesis are claimed to play an important role in the disease pathogenesis. Among them, IL -1 and TNF-α are central mediators of joint inflammation and

destruction in rheumatoid arthritis (**Kazkaz et al., 2007**).

Several single-nucleotide polymorphisms (SNPs) have been identified in the human TNF-α gene promoter. Several TNF-α promoter SNPs had been detected-238, -308, -376, -857, -863, -1031 **(Waldron-Lynch et al., 2001).** The polymorphism at position -308 has been implicated in the regulation of TNF-α transcription. The TNF-α promoter polymorphism at -308 involves a biallelic single-base pair transition from G to A. Generating two different allele *TNF1* (G/G) and *TNF2* allele which may be heterozygous (G/A) or homozugous (A/A) **((Surianoetal., 2005**). Reports regarding the effect of -308 position polymorphism on the susceptibility and severity of RA are contraversal

The aim of this study wastoanalyze the capacity of TNF-α production by PBMC stimulated with LPS in patients with early RA (disease duration < 1 yr) and those with long-standing RA (> 1 yr) and to detect the association of -308 TNF-α promoter gene polymorphism and RA.

SUBJECTS AND METHODS

This study was performed in Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, during the period from December 2008 to December 2009.

Patients included in the study (table 1): Patients included in the study were diagnosed according to the criteria for the classification of rheumatoid arthritis determined by The American College of Rheumatology**(Tehlirian and Bathon, 2008)**. Patients were classified in to two groups. Patients with early disease activity < 12 months. This group included 1 male and 14 female with age range from 24 to 62 years with the mean age (40.8±12.3).Patients with late disease activity >12 months. This group included 2 males and 13 females with age range from 30 to 59 years with mean age (45.9±9.3).Healthy donors**:**Blood samples were obtained from 15 healthy donors from the Medical Microbiology and Immunology Department. None of the participants reported any history ofacute or chronic medical problems.This group included 3 males and 12 females with age ranged from 30 to 52 years with mean age (38.53±7.7).

Table 1: Subjects included in the study

	Control group	Early RA patients	Late RA patients
Total number	15	15	15
Male	12	14	13
Male	3	1	2
Age (mean/range)±SD	38.53 ± 7.7 (30-52)	40.8 ± 12.3 (22-60)	45.9 ± 9.3 (29-58)

Separation,culture and stimulation of PBMC using LPS: Separation of PBMCs was done using Ficoll-Hypaquesolution of specified density (1.077) and according to

the protocol mentioned by **(Nilsson et al., 2008).**To stimulate TNF-α production**,**100ul of PBMCs (conc. 1x1000, 000/ml) were recovered into the supplemented RPMI media containing 10% fetal calf serum (FCS)(Sigma Chemical Co., St. Louis) and 1% streptomycin/penicillin. and incubated with 100ul of 1ng/ml LPS in each of 96 wells tissue culture plate. The final volume ratio between cell suspension and LPS from (*E.coli* serotype 055:B5,Sigma Chemical Co., St. Louis) was (1:1)**.** For negative control, 100ul of 1x1000, 000/ml PBMCs was added to the culture media without LPS, to detect spontaneous TNF-α production. The cell culture plate was incubated at 37°C in a 5% CO_2 humidified incubator for 24 hours. After 24h incubation, the supernatant was collected and kept frozen at -20°C**.** Frozen supernatants were analyzed for TNF-α by ELISA techniques with commercially available kits

Quantitative detection of the TNF-α production by ELISA. The Human TNF-α ELISA kit (**BioSource International, Camarillo, CA, USA; sensitivity: 3pg/ml)**was used for the quantitative measurement of human TNF-α in cell culture supernatants. This assay employs an antibody specific for human TNF-α coated on a 96-well plate. Standards and samples were pipetted into the wells and TNF-α present in a sample was then bound to the wells by the immobilized antibody. The wells were washed and then biotinylated anti human TNF-α antibody was added. After washing away unbound biotinylated antibody, Hourse radish peroxidase (HRP)-conjugated streptavidin was pipetted to the wells. The wells are again washed, a Tetramethylbenzidine (TMB) substrate solution is added to the wells and color develops in proportion to the amount of TNF-α bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Whole blood DNA extraction Using AxyPrep Blood Genomic DNA Miniprep Kit (**Axygen bioscience, USA).** This method is based on the efficient release of genomic DNA from anti-coagulated whole blood by a special cell lysis and heme/protein precipitation buffer (Buffer AP1) coupled with the selective adsorption of the genomic DNA to a special Axyprep column. The purified genomic DNA is eluted in a low salt Tris buffer containing 0,5mM EDTA, which enhances DNA solubility and helps to protect the high molecular weight DNA against subsequent nuclease degradation. Blood genomic DNA is directly isolated from white blood cell component of the whole blood.

PCR amplification:PCR reactions were all performed using *Taq* PCR Master Mix Kit, which was supplied in 20ul reaction form by (BIORON, Germany). This gives a final concentration (1X) of 1 units *Taq* DNA Polymerase, 250µM of each dNTP, 1.5 mM $MgCl_2$, 10 mMTris-HCl (pH 9) and 30 mMKCl. It was stored at -20˚C till its use. Primer sequencespair specificfor detection of The TNF-α -308 (G/A) polymorphism in the promoter of the TNF-αgene was determined by: -308 Forward primer(5' AGG CAA TAG GTT TTG AGG GCC AT 3') and -308 Reverse (5' TCC TCCCTG CTC CGA TTC CG 3') The temperature cycles were 1 min at 94°C for denaturation,45 s at the 56°C annealing temperature of each primer, and 45 s at 72°C for

elongation for 35 cycles in (Biomerta Ltd, Germany, thermal cycler)96. Amplified products werevisualized on a 1.5 or 2% agarose gel with ethidium bromide (1 mg/ml) staining (**Moukoko et al.,2003**).

Treatment of PCR product with *BSP19*I restriction enzyme: To detect polymorphism at -308 TNF-α promoter. The components were added in the following order in 20µl tube reaction: 1ul of 10X SE

buffer, 17 ul of DNA (obtained from 20ul reaction tube of PCR), 1ul Bsp19I(SibEnzyme, Russia), and 1 ul BSA.The components were mixed gently, spin down briefly, and then incubated at 37◦C for 2 hours. Digestion products wereanalyzed on 2% agarose gel stained with ethidiumbromide solution (1 mg/ml). After digestion with restriction enzyme and subsequent electrophoresis, two alleles of TNF-308 can be revealed: *TNF1* (G/G genotype -fragments of 87 bp and 20 bp), *TNF2* (G/A genotype -fragments of 107 bp, 87 pb and 20 bp- A/A genotype fragment of 107 bp).

Statistical analysis as done using t-test and chi square **(SPSS 16 software).**

RESULTS & DISCUSSION

Our results**(table 2)**show comparable low level of spontaneous TNF-α production in all three groups. There is no statistically significant difference in spontaneous TNF-α production in control, early or late RA patients groups. These results agreed with the result obtained by **Muller et al.(1998), Xia et al.(1998), Cuenca et al. (2003),Fabris et al.(2005)** who foundno statistically significant difference in spontaneous TNF-α production between control and patients groups.

Table (2): Differences in spontaneous TNF-α production in RA patients and healthy controls "concentrations are in (pg/ml)"

	Control (Median\ Range)	Early RA patients (Median\ Range)	Late RA patients (Median\ Range)	Kruskal Wallis test	P
Spontaneous TNF-α production	28.0 (10-41)	26.0 (12-50)	25 (9-56)	0.72	0.69

This tablecompares between spontaneous TNF –α production in RA patients and control group and shows that the medians of spontaneous TNF-α production in was slightly high in control group (28) than early (26) and late patient group (25). However,these differences show no statistically significant difference between them (P= 0.69).

When we compared between LPS- induced TNF- α production in RA patient and healthy control we found that**(table 3),** there were no difference in LPS-induced TNF –α production in early and control group. However, there was significant increase in LPS-induced TNF –α production in late group when this group was compared with either control group or early patient group.

Table (3): Differences in LPS-induced TNF-α concentration (pg/ml) in RA patients and healthy controls

	Control (Median\ Range)	Early RA patients (Median\ Range)	Late RA patients (Median\ Range)	Kruskal Wallis test	P
LPS-induced	63 (24-119)	80 (29-120)	978 (618-1649)	29.9	0.000

The table shows that the median of LPS –induced TNF-α production in late RA patients was significantly higher (978) than median of LPS –induced TNF-α production in control group(63) and early RA patient groups (80). So, LPS –induced TNF –α production in late RA patients was significantly higher than LPS –induced TNF-α production in control group and early RA patient groups.

These results agreed with**Fabris et al., 2005**, who found that patients with early RA (disease duration < 1 yr) belonged mainly to the low TNF-α producer subgroup, whereas patients with long-standing RA (> 1 yr) were prevalently high TNF-α producers. Also these result were supported by **de Paz et al., 2010** who concluded that Patients at onset of RA present fewer alterations in cytokine levels than those with longer disease duration.

This was not the same result obtained by **Cuenca et al., 2003**, who found that the spontaneous and LPS-induced TNF-α levelsdid not differ significantly between RA patients and healthycontrols. This difference can be explained by the fact that studies that classify patients in to early and late groups can found the difference between both early and late patient group from one side and late patient group and control group from other side.

In this study**(table 4),** the homozygous ***TNF1***(G/G) allele was present in (73.3%) of controls, (66.7%) of early RA patients and 60% of late RA patients,while the heterozygous ***TNF2*** (G/A) allele was foundin (26.7%) of the control group, (33.3%) in the early RA patients and (33.3%) in the late RA patients.The homozygous ***TNF2*** (A/A) allele was detected in only one RA late patient (2.2%). These results regarding the Egyptian population showed that there is no statistically significant difference between the distribution of *TNF1* and *TNF2* alleles between control, early and late patient groups.

Table (4): Genotype distribution of the -308 TNF promoter polymorphism in RA patients and healthy controls.

TNF -308 genotype	Controls [*n* (%)]	Early RA patients [*n* (%)]	Late RA patients [*n* (%)]	Total	X^2	P
TNF1 (G/G)	11 (73.3%)	10 (66.7%)	9 (60%)	30 (66.7%)	2.34	0.67
TNF2 (G/A)	4 (26.7%)	5 (33.3%)	5 (33.3%)	14 (31.1%)		
TNF2 (A/A)	0 (0.0)	0 (0.0)	1 (6.7%)	1 (2.2%)		

This table shows that the distribution of *TNF1* (G/G) genotype was in Controls 11 (73.3%),in Early RA patients 10 (66.7%) and in Late RA patients 9 (60%). The distribution of *TNF2* (G/A) genotype was in Controls 4 (26.7%), in Early RA patients 5 (33.3%), and in Late RA patients 5 (33.3%), while *TNF2* (A/A) was found only in one patient (6.7%)in Late RA patients. However, the distribution of TNF1 and TNF2 genotypes between the early, control and late patients groups does not significantly differ.

These results match with the results of **Cuenca et al., 2003**, **Majetschak et al., 2005Kazkaz et al., 2007**who studied the -308 **TNF**- α polymorphism in patientsand controls and reported no statistically significant difference between the distribution of *TNF1* and *TNF2* alleles between control, and patient groups.

Whenthe relation between spontaneous TNF-α production and -308 TNF-α promoter polymorphism**(table 5)**in RA patients and healthy controls was investigated, no association between the polymorphism and spontaneous TNF-α production was found.

Table (5): Differences in spontaneous TNF-α concentrations (pg/ml) according to -308 TNF-α promoter polymorphism in RA patients and healthy controls

	Control		Early RA patients		Late RA patients	
	TNF1	*TNF2*	*TNF1*	*TNF2*	*TNF1*	*TNF2*
Spontaneous TNF-α production	25 (10-41)	29.5 (16-35)	28.5 (12-50)	26 (16-30)	25 (10-43)	32 (9-56)
Mann Whitney U	0.58		0.67		1.34	
P	0.57		0.51		0.51	

This table shows that there are no differences in spontaneous TNF-α concentration (pg/ml) according to -308 TNF-α promoter polymorphism in early and late RA patients and healthy control (P= 0.57, 0.51,) respectively. So, there is no association between the polymorphism and spontaneous TNF-α production.

And, whenthe relation between LPS-induced TNF-α production and -308 TNF-α promoter polymorphism**(table 6)**in RA patients and healthy controlswas investigated, no association between the polymorphism and LPS-induced TNF-α production was found.

Table (6): Differences in LPS-induced TNF-α concentrations (pg/ml) according to -308 TNF-α promoter polymorphism in RA patients and healthy controls

	Control		Early RA patients		Late RA patients	
	TNF1	*TNF2*	*TNF1*	*TNF2*	*TNF1*	*TNF2*
LPS-induced	62 (24-98)	88 (27-119)	79 (29-120)	96 (73-111)	978 (618-1649)	916 (687-1190)
Mann Whitney	1.17		1.10		0.067	
P	0.28		0.31		1.00	

This table shows that there are no differences in LPS-induced TNF-α concentrations (pg/ml) according to -308 TNF-α promoter polymorphism in RA

patients and healthy controls (P= 0.28, 0.31and 1.00) respectively. So, there is no association between the polymorphism and LPS-induced TNF-α production.

These results are similar to the results obtained by **EL Kaijzel et al.(2001);Cuenca et al.(2003),**and**Reneseset al.(2009)**who studied the -308 TNF-αpolymorphism in RA patientsand controls and indicated a lack of associationbetween -308 TNF-α promoter polymorphism and susceptibility toRA.

Yen et al., 2010 who investigated the association of TNF-α promoter polymorphisms with RA in Taiwan not only denied the relationshipbetween TNF promoter polymorphisms and the clinical manifestations of patients with RA, but also reported that*TNF2* (G/A) genotypeitself or a neighboring gene may be a protective factor for the development of RA in the HLA-DR4 negative population in Taiwan.

On the other hand, **Waldron-Lynch et al.(2001),Suriano et al. (2005)** and**Khanna et al.(2006)**studied the association of TNF-α –308A/G polymorphism and found that *TNF2* (G/A) genotype polymorphism is associated with increasing the transcriptional activity of TNF-α gene and thus the susceptibility and severity of RA .

The controversy can be explained by a study done by **Cuenca et al., 2001** in which they conclude that there is a gradient in the distribution of the *TNF2* allele according to ethnicity; They have also hypothesized that populations bearing a higher proportion of the *TNF2* allele may have an increased predisposition toward or incidence of several inflammatory and autoimmune diseases, a latter study done by the same investigator regarding the same ethnic group of patient "Chileans" which they did not observe differences betweenspontaneous and LPS-stimulated TNF levelseither in RA patients or in controls when they were comparedaccording to -308 TNFgene promoter genotype **(Cuenca et al., 2003)**.

However, **Fonseca et al., 2007** suggested that both the -308GG and -308GA genotypes are implicated in increased RA severity. Although they suggest that *TNF-α* gene promoter polymorphisms influence the outcome of this chronic disease, they concluded that the value of genotyping RA patients in order to define their clinical course will remain unproven until a proper prospective evaluation of this cohort of patients validates this hypothesis**.**

This controversy can be explained by the fact that chromosome 6 is highly polymorphicand characterized by extensive linkage disequilibrium, the possibilityexists that TNF-α promoter polymorphism may be in some studies associated with other SNPs which are the real factors responsible for the difference in the susceptibility or the severity of RA, rather than being themselvesresponsible for the disease association **(Cuenca et al., 2003)**.

. Another explanation for the conflicting results may be due to differences in the cell lines assessed and the physiologic stimulators used to induce differential transcriptional activity (**Khanna et al, 2006).**

Regarding the differences in the cell line, monocytes and T cells that contain *TNF2* (A/A) promoter have a 2-3-fold greater transcriptional activity than the *TNF1* promoter (**Khanna et al, 2006).** In a study done by**kroeger et al., 2000**, it was found that a difference in *TNF1* and *TNF2* promoter activities was only observed in U937 and T-cells, and not in Raji (B cell line), HeLa (epithelial carcinoma cell line), HepG2 (hepatoma cell line) or THP-1 (monocyte), suggesting that cell-type specific transcription factors or modifications may be involved in the

formation of the -308 protein/DNA complex**.**

Regarding the physiological stimulators, when TNF and interferon gamma (IFN-gamma) were used, there were no differential promoter activity between *TNF1* and *TNF2*, however, other stimulators like LPS stimulates only the transcriptional activity of *TNF2*. While, phorbolmyristate acetate (PMA), retinoic acid, PMA plus LPS and PMA plus retinoic acid increase the transcriptional activity of both *TNF1* and *TNF2*. An understanding of the cell type and stimuli specific requirements for differential expression of the -308 polymorphism may help elucidate the role the TNF -308 polymorphism plays in diseases where elevated TNF levels are thought to be important **(kroeger et al., 2000).**

The conflicting results can be attributed to the differences in: ethnic groups on which the study was applied, as genetic factors affect the incidence of different alleles in certain population, the association of different -308 alleles other susceptibility loci that have an effect on TNF-α production, Adjustment of disease duration as our study and other studies demonstrate clearly that patients with RA must be classified according to the disease duration as early RA patient have a cytokine profile different than patients with long standing disease duration. The type of cells and the physiological stimuli used in triggering cells to measure the capability to produce cytokine.

Finally, this study conclude that TNF-α synthesis after LPS stimulation by PBMC from patients with early onset and long standing RA identified two main subsets of patients on the basis of their TNF-α-synthesizing behavior: a high-producer subset associated preferentially with long disease duration and a low producer subset associated mainly with short disease duration.

It also conclude that the -308 TNF-αpromoter polymorphism in the Egyptian population shows no differences between control and patient groups, and it is also not associatedwith increase production of TNF-α. Thus, other factors may be importantin determining the circulating levels of TNF-α in RA.

It is thus recommend that early starting of anti TNF-α therapy before marked changes in TNF-α profile occur and thus provide a better prognosis for the disease. We also recommend investigating TNF-α promoter polymorphisms more thourghly to detect the genetic factors affecting TNF-α production.

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