EXTENDED REPORT

Differential association of polymorphisms in the TNFα region with psoriatic arthritis but not psoriasis

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Objective: To investigate the potential association of tumour necrosis factor α (TNFα) microsatellite and promoter alleles with psoriatic arthritis (PsA).

Methods: DNA from 89 white patients with PsA, 65 patients with psoriasis, and 99 healthy white controls was investigated for two TNFα promoter (–238 and –308) and three microsatellite polymorphisms (TNFa, c, and d). Patients had previously been studied by serology for HLA class I antigens and by sequence-specific polymerase chain reaction for DRB1* alleles. In addition, TNFα production of Ficoll separated peripheral blood mononuclear cells (PBMC) into culture supernatants after stimulation with lipopolysaccharide, αCD3 antibodies, phytohaemagglutinin, and streptococcal superantigen C was determined.

Results: A significant, HLA class I independent increase of the TNFa6c1d3 haplotype was found in the group with PsA but not among patients with psoriasis (32% *v* 8%, pc<0.008; relative risk (RR)=5.3). In addition, patients with PsA showed a marked decrease of the TNF308A promoter allele (6% *v* 18%; pc<0.008; RR=3.5) compared with healthy controls, which was independent of the increased frequency of the –238A polymorphism in this group. PBMC from patients with PsA secreted significantly less TNFα than cells from patients without arthritis. In particular, the TNFa6 microsatellite was associated with decreased TNFα production.

Conclusion: These data indicate that allelic variations at the TNFα locus influence susceptibility to PsA. Decreased production of TNFα is at least in part genetically determined and might be related to the development of arthritis. However, the association of the TNF308G allele with the disease also points to other disease related haplotypes with still unknown susceptibility genes.

Psoriatic arthritis (PsA) is defined as rheumatoid factor negative inflammatory arthritis in the presence of psoriasis.[1] It develops in 7–42% of patients with psoriasis,[1] giving the disease an overall prevalence of 0.3% in the German group.[2] PsA is closely related to the other seronegative spondyloarthropathies, including ankylosing spondylitis (AS), undifferentiated spondyloarthropathies, reactive arthritis, and spondyloarthropathies in inflammatory bowel diseases. Several studies have shown that genes, particularly of the major histocompatibility complex (MHC) on chromosome 6, confer susceptibility to psoriasis and PsA[1 3 4] and influence disease progression.[5] The HLA antigens B13, B16 (B38, B39), B57, Cw6, and DR7 have been associated with psoriasis with and without arthritis.[1 4] Apart from HLA-B27, which is strongly associated with spondyloarthropathy in patients with psoriasis, no specific immunogenetic predisposition to joint disease has been reported in patients with psoriasis. Although it is possible that the reported HLA class I associations are of primary pathogenetic importance because these molecules are critical to antigen presentation and thus fundamental to the CD8+ T cell response, they might also be markers for other closely linked genes on the short arm of chromosome 6 only.

Tumour necrosis factor alpha (TNFα) is one of the key cytokines in the development of psoriasis and PsA. It is encoded within the HLA class III region 250 kb centromeric to the HLA class genes.[6] The TNF locus is 12 kb in length and contains several polymorphic areas (fig 1) including five microsatellites[7 8] and several promoter polymorphisms, of which the two G to A transitions at position –308[9] and –238[10] are the most common in white populations. We and others have previously reported an association of a polymorphism in the TNFα promoter with psoriasis and PsA.[11 12] Specific MHC class II alleles[13] and genetic polymorphisms[14–16] at the TNFα locus have been correlated with differences in TNFα production.[15–17] In this study we determined TNF microsatellite allele frequencies at three loci in white patients with psoriasis, PsA, and in ethnically matched controls. In addition we studied the influence of these TNFα microsatellites on TNFα production of peripheral blood mononuclear cells (PBMC) in patients with psoriasis after stimulation with different mitogens and streptococcal superantigen. Our results identify a combination of TNF microsatellite alleles that is associated with PsA and low TNFα production.

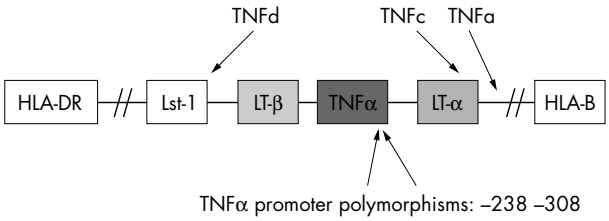

Figure 1 Location of three TNFα microsatellite (TNFa, c, and d) and two promoter polymorphisms in the MHC class III region on chromosome 6.

Abbreviations: AS, ankylosing spondylitis; LPS, lipopolysaccharide; MHC, major histocompatibility complex; OR, odds ratio; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RR, relative risk; TNFα, tumour necrosis factor α

PATIENTS AND METHODS

Patients

Eighty nine white patients with PsA (49 patients from the Department of Rheumatology, University Hospital Ghent, 40 patients from the First Medical Department, Johannes Gutenberg University Mainz) and 65 white patients with type I psoriasis from the Department of Dermatology and the First Medical Department, Johannes Gutenberg University Mainz, University Clinic of Mainz, were recruited for this study. PsA was defined as a seronegative inflammatory arthritis, associated with psoriasis.[1] All patients with PsA were seen by a rheumatologist. Forty two (47%) patients with PsA were female and 47 male (53%), with a mean age of 47.8 years (range 27–82). Forty three (48%) patients had polyarticular, 24 (27%) oligoarticular joint disease, and in 22 (25%) patients the disease affected mainly the spine. Joint disease was confirmed by scintigraphy, radiography, or magnetic resonance imaging, where appropriate. The group with psoriasis included 22 (34%) female and 43 male patients (66%), with a mean age of 43.7 years (range 20–79). None of the patients chosen for assessment of TNFα production by PBMC were receiving immunosuppressive treatment, such as corticosteroids, methotrexate, cyclosporin A, or UVA treatment at the time of recruitment. Patients did not have any other relevant disease nor did they show any signs of streptococcal infections at the time of recruitment.

The healthy control group consisted of 99 unrelated, healthy white subjects from the Mainz area (mean age 38.3 years (range 23–91)). HLA class I and DRB1* associations in these patients have been published previously.[18] Patients from Ghent showed a similar allele distribution. To investigate TNF microsatellite haplotypes we established the segregation of haplotypes in five different families.

Detection of TNFα promoter polymorphisms

The polymorphisms at positions –238 and –308 were studied as previously described.[19]

TNF microsatellites

After polymerase chain reaction (PCR) amplification TNFa, TNFc, and TNFd microsatellite alleles were determined by automated fragment length analysis on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Data analysis was done with Genetic Analyzer Software—310 Genescan 2.1 (table 1).

Fluorescent labelled 5′ oligonucleotide primers were synthesised, TAMRA for TNFa and JOE for TNFc and d (Bio Source, Belgium) according to the published sequences.[19 20]

Target DNA (10 ng) was amplified in 30 µl reaction mixtures containing 0.4 µM each primer, 85 µM dCTP, dATP, dTTP, and dGTP (Roth, Karlsruhe, Germany). One unit Taq polymerase (Gibco, Karlsruhe, Germany) was used for the microsatellite reactions and $MgCl_2$ was kept at 1.5 mM using 10×PCR buffer (Gibco). The following cell lines were obtained from the Tissue Typing Workshop panel as standards for the microsatellite reactions: Vavy (TNFa2, c1, d1), IBW9 (TNFa4, c2, d5), and HOM2 (TNFa6, c1, d3). Cell lines were maintained in RPMI 1640 culture medium supplemented with 10% fetal calf serum, penicillin, and streptomycin.

PCR for TNFa and TNFc started with a denaturation step (94°C, four minutes) followed by six cycles of a touchdown protocol (94°C denaturation 30 seconds, annealing 67°C to 62°C, Δ −1°C, each cycle one minute and 72°C one minute) and by 23 cycles (94°C 30 seconds, 62°C one minute, 72°C one minute), and a final extension at 72°C for seven minutes. For TNFd an initial denaturation step (94°C, four minutes) was followed by 30 cycles of 94°C 30 seconds, 55°C one minute, 72°C one minute, and a final extension at 72°C for seven minutes.

In our study group 14 alleles were detected at the TNFa locus, two at TNFc, and seven at TNFd alleles.

Table 1 Primers used for TNFα microsatellite typing

Primer	5′ - 3′
TNFa FOR	GCC TCT AGA TTT CAT CCA GCC ACA
TNFa REV	CCT CTC TCC CCT GCA ACA CAC A
TNFc FOR	GGG AGG TCT GTC TTC CGC CG
TNFc REV	CGT TCA GGT GGT GTC ATG GG
TNFd FOR	CTG TCA TTC CAC TAT CGC AAG G
TNFd REV	GGA GTT CCT GCT CTG AGG AG

TNFα ELISA

Thirty two patients with psoriasis and 15 with PsA were randomly selected for determination of TNFα production by PBMC. In addition, in 43 healthy controls the association between certain TNF microsatellite alleles and TNFα production by PBMC was studied. All studies were done using lipopolysaccharide (LPS) free reagents and tubes. PBMC were isolated from freshly taken heparinised blood by standard Ficoll-Hypaque gradient centrifugation. All experiments were performed in duplicate in 96 well microtitre plates (Nunc, Roskilde, Denmark) with 10^5 mononuclear cells in 100 µl assay medium per well. The following mitogens and antigens were added: LPS (Sigma, Deisenhofen, Germany) 10 ng/ml, anti-CD3 monoclonal antibodies (αCD3) 1 µg/ml, phytohaemagglutinin (Difco Labs, Detroit, USA) 1 µg/ml, and streptococcal superantigen SPEC 0.25 µg/ml. Cells were incubated for 24 hours at 37°C and 5% CO_2 in a humidified atmosphere. TNFα production was measured in supernatants using a Pharmin-Gen TNFα test kit (Pharmin-Gen, Hamburg, Germany)

Statistical analysis

Comparisons of TNF microsatellite allele frequencies in patients with PsA, psoriasis, and in controls were tested by χ^2 test with Yates's correction. TNF microsatellite 3 locus haplotypes were derived from previous group analyses, family studies, and their existence as haplotypes in homozygous cell lines.[7 8] In addition, associations between loci were estimated by constructing a series of 2×2 tables which were then analysed by the χ^2 test. The probability obtained was corrected for multiple comparisons (pc) according to the number of alleles observed. Odds ratio (OR) and relative risks (RR) associated with a particular allele were calculated using a χ^2 distribution: $OR=(n1\times n4)/(n2\times n3)$, where n1 is the number of patients with allele x, n2 is the number of controls with allele x, and n3 and n4 are the corresponding proportions of subjects in patient and control groups not carrying allele x.[21] In each group the allele distribution was checked for deviations from Hardy-Weinberg equilibrium using an exact test.[22] Associations between alleles ($p<0.05$) were considered significant for $pc<0.05$. Wilcoxon and Kruskal-Wallis tests were used for statistical analysis of enzyme linked immunosorbent assay (ELISA) data.

RESULTS

Microsatellite distribution

Table 2 shows the allele frequencies at the three microsatellite loci. The TNFa locus is highly polymorphic with 14 different alleles in our homogeneous German/Belgium group. Allele frequencies are similar to those described previously for other Western European groups.[8 19 23] Patients with PsA from Ghent and Mainz showed an identical distribution of TNF microsatellites at the three loci. There was an increase of the d4 allele in the psoriasis group ($p<0.04$) and of the a6 allele in PsA

Table 2 TNF microsatellite allele frequencies at three loci in patients with psoriasis (Ps), psoriatic arthritis (PsA), and in ethnically matched healthy controls

	TNFa			TNFc			TNFd		
Allele	Ps	PsA	Control	Ps	PsA	Control	Ps	PsA	Control
1	0	0.017	0.017	0.567	0.657	0.703	0.048	0.118	0.098
2	0.328	0.333	0.25	0.433	0.343	0.297	0.016	0.026	0.029
3	0	0.005	0.006				0.427	0.447	0.494
4	0.067	0.033	0.085				0.444	0.283	0.259
5	0.089	0.033	0.068				0.065	0.138	0.08
6	0.089	0.177	0.119				0	0.013	0.023
7	0.119	0.066	0.063				0	0	0.017
8	0.007	0.017	0.006						
9	0.022	0.061	0.006						
10	0.149	0.117	0.176						
11	0.157	0.089	0.170						
12	0.007	0.011	0						
13	0.03	0.033	0.034						
14	0	0.005	0						

compared with patients with psoriasis, which lost significance after correction for the number of investigated alleles.

Psoriatic arthritis is associated with the a6c1d3 haplotype

TNF allelic haplotypes have been established in homozygous cell lines and have been derived from group based studies.[7 8 19 20 24] We formally showed haplotype segregation in five families (two families shown in fig 2). In addition, unambiguous haplotype assignment was possible in 17/76 (22%) patients with PsA because of homozygosity (for example, homozygous for c1d3 in an a6 positive patient). We performed a haplotype analysis of the four most common European haplotypes[20] a2c2d4, a6c1d3, a11c1d3, and a10c1d3 in our patients (table 3). There was a highly significant increase of the a6c1d3 haplotype in patients with PsA compared with the psoriasis group (32% *v* 8%, respectively; pc<0.008). This increase was independent of HLA-B27, the only HLA antigen significantly increased in the PsA group compared with patients with psoriasis (2/56 (4%) HLA-B27 negative patients with psoriasis *v* 14/66 (21%) HLA-B27 negative patients with PsA; p<0.01). The a6c1d3 haplotype was not linked to any of the investigated promoter polymorphisms. Patients with psoriasis as well as those with PsA showed an enrichment of the a2c2d4 haplotype. This three locus haplotype is part of the ancestral haplotype TNF238A/B57/Cw6 and significance of this association was lost when B57/cw6 negative patients were analysed (data not shown).

Association of TNF308G with PsA but not with psoriasis

In accordance with our previously published results we observed a highly significant association of the TNF238A polymorphism with psoriasis and PsA (table 4). This effect was independent of HLA-B27. In addition, we observed a highly significant decrease of the 308A allele in the PsA group. The 238A and the 308A exchange in the TNF promoter never occur on the same chromosome. Therefore increased frequencies of the –238A variant automatically lead to a decreased 308A frequency. However, the decrease of the 308A allele remained significant after exclusion of 238A haplotypes from the analysis (7% in PsA compared with 18% in controls; pc<0.008) (table 4).

Decreased TNFα production in PsA

PBMC from patients with PsA secreted significantly less TNFα after stimulation with αCD3 antibodies than PBMC from the psoriasis group (fig 3). The other mitogens also led to a decreased TNFα production in patients with PsA compared with those with psoriasis, but differences did not reach statistical significance.

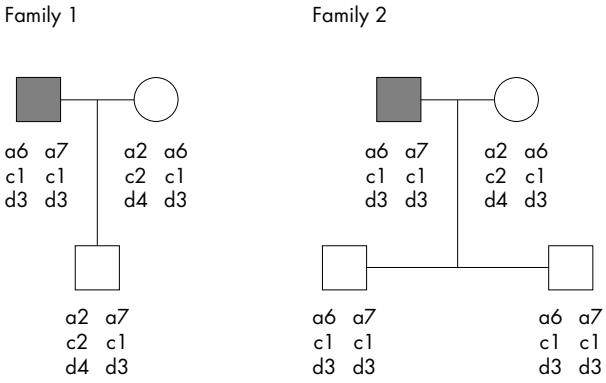

Figure 2 Pedigrees of two families with members affected by PsA. The allelic combination TNFa6c1d3 was transmitted from parent to sibling as a complete haplotype. Squares represent male subjects, circles female subjects, and shaded figures represent family members with PsA.

TNFa6 associated with decreased TNFα production

The association of TNF microsatellites with TNFα production by PBMC was studied in 43 healthy controls. There was no association of low or high TNFα production with any TNFc or TNFd allele. In contrast, PBMC from subjects carrying the TNFa6 allele produced significantly less TNF than carriers of other alleles like a4 and a7 (fig 4).

DISCUSSION

PsA has been associated with a number of HLA antigens including B13, B27, B38, B39, B57, Cw6, and DR7. Apart from B27 most of these antigens have also been associated with psoriasis and it is unclear which factors predispose patients to

Table 3 Distribution of locus 3 TNF haplotypes

TNFacd haplotype	Psoriasis (n=62) No (%)	PsA (n=76) No (%)
a2c2d4	33 (53)	34 (45)
a6c1d3	5 (8)	24 (32)*
a10c1d3	16 (26)	13 (17)
a11c1d3	9 (15)	14 (18)

*PsA *v* psoriasis pc<0.01, χ^2=10.0, RR=5.3.

Table 4 Frequencies of TNF promoter polymorphisms in HLA-B27 positive (B27+) and B27 negative (B27–) patients. In addition distribution of the 308 promoter polymorphism was studied after exclusion of 238A positive haplotypes (238G)

	B27–			B27+			238G+		
	Controls (n=184)	Psoriasis (n=106)	PsA (n=128)	Controls (n=14)	Psoriasis (n=14)	PsA (n=46)	Controls (n=180)	Psoriasis (n=78)	PsA (n=104)
308G	153(83)	98(93)	121(95)*	13(93)	13(93)	44(96)	149(83)	70(90)	97(93)§
308A	31(17)	8(7)	7(5)	1(7)	1(7)	2(4)	31(17)	8(10)	7(7)
238G	180(98)	78(74)	104(81)	11(79)	14(100)	40(87)			
238A	4(2)	28(26)†	24(19)‡	3(21)	0	6(13)			

RR, relative risk; OR, odds ratio; 95% CI, 95% confidence interval.
*PsA *v* controls, pc<0.008, χ^2=8.1, OR=3.5 (95% CI 1.5 to 8.2); RR=1.14 (95% CI 1.1 to 1.23); †psoriasis *v* controls, pc<0.00001, χ^2=37.8, OR=16.2 (95% CI 5.5 to 47.6); RR=2.9 (95% CI 2.3 to 3.6); ‡PsA *v* controls, pc<0.00001, χ^2=22.9, OR=10.4 (95% CI 3.4 to 30.2); RR=1.13 (95% CI 1.05 to 1.2); §PsA *v* controls, pc<0.008, χ^2=8.2, OR=2.7 (95% CI 1.5 to 6.8); RR=2.3 (95% CI 1.9 to 3.2).

the development of joint inflammation. In this extended analysis of TNFα promoter and microsatellite polymorphisms we found a strong, HLA-B27 independent association of the TNFa6c1d3 haplotype with PsA. Interestingly, PBMC from patients with PsA secreted less TNFα than PBMC from patients with psoriasis. The a6 allele was associated with decreased secretion of TNFα, as has been reported in an earlier study.[14] This raises the intriguing possibility that low TNFα secretion in patients with PsA is genetically determined. We have recently shown that the TNF238A promoter variant, which is associated with psoriasis and PsA, decreases transcriptional activity of the TNFα promoter and consequently reduces production of TNFα by PBMC.[25] The association of the a6 allele with low TNFα production is independent of the TNF238A exchange, as this promoter variant is part of the ancestral haplotype TNF238A/a2/c2/d4/B57/Cw6 which was found with the highest frequency in the psoriasis group. These results are surprising, particularly in the light of recent therapeutic trials with soluble TNF receptor, which have shown dramatic responses in patients with PsA.[26] However, these data do not question the crucial role of TNFα in the regulation of articular inflammation.

TNF microsatellites have been investigated in other rheumatic diseases like rheumatoid arthritis (RA),[27–29] AS,[30] and reactive arthritis.[31] Interestingly, two studies in RA reported an association of the TNFa6 allele with susceptibility[27] and severity[28] of RA. However, these results remain controversial in the light of a third study that reported an association of the a11 allele with severe disease.[29] TNFa6 was also found to be associated with reactive arthritis in HLA-B27 positive subjects,[31] whereas patients with AS did not show any differences in TNFa allele distribution in comparison with HLA-B27 positive controls.[30]

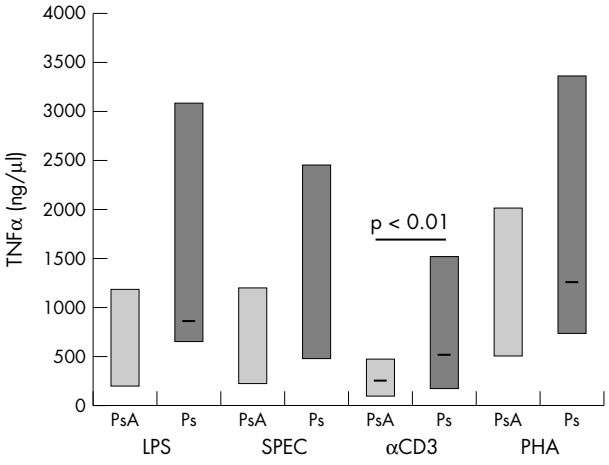

Figure 3 Production of TNFα by peripheral blood mononuclear cells from patients with psoriasis (Ps; n=32) and patients with PsA (n=15) after stimulation with LPS, αCD3 monoclonal antibodies, phytohaemagglutinin (PHA), and streptococcal superantigen C (SPEC). None of the patients tested received immunosuppressive treatment, such as, corticosteroids, methotrexate, cyclosporin A, or UVA treatment at the time of recruitment. Microsatellite frequencies among tested patients with PsA were: a2 (n=7), a4 (n=2), a5 (n=1), a6 (n=7), a7 (n=3), a9 (n=2), a10 (n=5), a11 (n=1), a13 (n=1), and a14 (n=1).

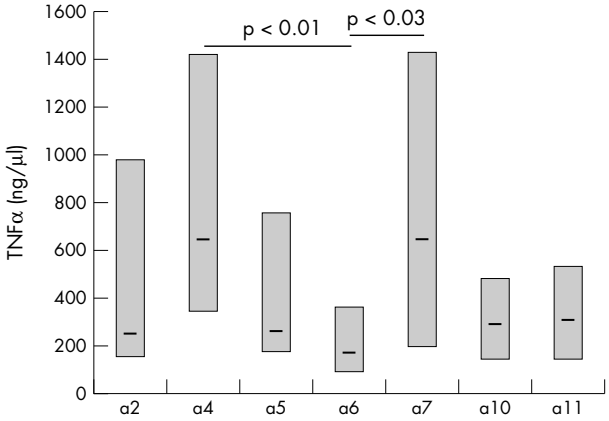

Figure 4 Secretion of TNFα into culture supernatants by PBMC from healthy controls (n=43) after stimulation with αCD3 monoclonal antibodies. Microsatellite frequencies among tested controls were: a2 (n=19), a4 (n=9), a5 (n=8), a6 (n=7), a7 (n=5), a10 (n=19), and a11 (n=15).

Increasing evidence shows that the pattern of cytokine secretion influences the course of spondyloarthropathies. A Th2 cytokine pattern (low TNFα, low interferon γ, and high interleukin 10) dominates in the joints of patients with reactive arthritis.[32] Low TNFα secretion by PBMC in these patients has been associated with a chronic disease course,[33] suggesting that diminished TNFα production might reflect a state of relative immunodeficiency which contributes to bacterial persistence,[33] as has been shown in animal models for *Yersinia enterocolitica*[34] and *Chlamydia trachomatis*[35] infections. Both viral[36], bacterial[36] and, in particular, streptococcal[37] infections have been proposed as causative agents in PsA. Although the importance of bacterial persistence for the development of PsA has not been shown formally, there is indirect evidence for this. Thus the increased incidence of PsA in untreated HIV infected subjects[38] and increased IgG antibody levels against *Streptococcus pyogenes* M protein in patients with PsA[37] but not in those with psoriasis indicate the possibility of infectious disease triggers.

In addition, the increased frequency of the TNF308G allele among patients with PsA suggests an independent predisposition to the development of arthritic complications. Interestingly, an association of this promoter allele has been reported in German[39 40] and Scottish[30] patients with AS, although this association was not found in Spanish[41] and British[40] AS cohorts. Conflicting results have been reported for the functional consequences of the TNF308A variant. Increased transcriptional activity of the TNF308A allele[15] and increased TNFα production of PBMC carrying this allele have been described.[16 17] However, findings of other groups, including our own, suggest that this allele has no significant effect on TNFα production.[25 42 43] The conflicting associations in ethnically different AS groups are probably explained by variations in linkage disequilibrium. In British white subjects strong linkage disequilibrium is present between B27 and DR1,[44] whereas in German patients B27 is linked to DR4.[45] However, in patients with PsA we found only a very mild increase of the B27 allele, and the association of the TNF308G allele with PsA persisted after elimination of B27 positive subjects. These findings suggest an arthritis predisposing effect of another gene in linkage disequilibrium with the TNF308G allele. A Spanish study recently reported a psoriasis independent association of a MICA triplet repeat polymorphism in the transmembrane region with PsA,[46] probably another marker for PsA associated MHC haplotypes.

Owing to the relatively small number of patients with PsA in this study, the results should be confirmed in an independent investigation. However, they show a psoriasis independent association of PsA with the a6c1d3 haplotype and decreased production of TNFα by PBMC of patients with PsA. Although genetically determined, low TNFα production may be important for the development of arthritis in patients with psoriasis.

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