Toll-like receptor 4 gene polymorphisms in polymyalgia rheumatica and elderly-onset rheumatoid arthritis

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Abstract

Objective

*Coding variants in TLR4 gene have been reported to be associated with inflammatory diseases. The aim of this study was to determine whether two of these polymorphisms (*Asp299Gly *and* Thr399Ile*) of TLR4 contribute to the genetic background of polymyalgia rheumatica (PMR) and elderly-onset rheumatoid arthritis (EORA). Furthermore, we have attempted to correlate the functional consequences of these polymorphisms.*

Methods

164 patients with PMR, 93 with EORA and 126 unrelated age-matched controls were genotyped. The TLR4 genotypes were determined using allele-specific primers and restriction fragment length polymorphism analysis. Association of genotypes and alleles with disease susceptibility and disease phenotypes were studied. TLR4 expression was assessed on PBMCs by flow cytometry and TLR4 function was assessed by stimulating PBMCs in vitro with LPS.

Results

No significant difference in allele frequency or genotype between patients with elderly-onset inflammatory conditions and controls was observed. The Thr399Ile *CC genotype was associated with a higher cumulative dose of corticosteroids in patients with PMR (p=0.031). We found no association with TLR4 expression on B cells, T cells or monocytes or a distinct phenotype of TLR4 response with the* Asp299Gly *or* Thr399Ile *genotypes.*

Conclusion

These results do not support the association of these TLR4 variants with two age-related inflammatory conditions. The value of determining Thr399Ile *genotypes for disease prognosis in PMR should be confirmed in different populations.*

Key words

aging, elderly-onset rheumatoid arthritis, innate immunity, polymyalgia rheumatica, toll-like receptor 4

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Introduction

Accumulating evidence has demonstrated that activation of innate immunity is a requisite to induce acquired immunity (1). One of the most important elements of innate immunity, Toll-like receptors (TLRs), play an essential role in the activation and regulation of the innate and acquired immune responses through the recognition of specific molecular patterns of pathogens and endogenous peptides (2). TLR activation has been implicated in the development of autoimmunity and chronic inflammation (1, 2).

Besides their role in the immune response, there are several facts supporting the study of TLRs in patients with polymyalgia rheumatica (PMR). First, TLR4 polymorphisms have been associated with the development of GCA (3), another age-restricted clinical syndrome related to PMR. Although with conflicting results, TLR4 polymorphisms have been associated with infectious diseases (4), a factor related with the pathogenesis of PMR (5). Furthermore, two non-synonymous polymorphisms of TLR4 (*Asp299Gly and Thr399Ile*) have been reported to alter the function of the receptor (6) and related with the development of chronic inflamatory conditions (7, 8).

Accumulating evidence points also to a role of TLRs in the pathogenesis of rheumatoid arthritis (RA). TLRs are highly expressed in RA synovium, and cells from patients with RA produce higher amounts of inflammatory mediators upon TLR-mediated activation (9, 10). It has been also suggested that the polymorphism *Asp299Gly* in TLR4 may interfere with treatment response to a single DMARD in early RA (11). Furthermore, the involvement of TLRs in arthritis has been also substantiated in experimental models of arthritis (12). Although the association of the TLR4 (*Asp299Gly and Thr399Ile*) polymorphisms with RA remains controversial (13-16), it has not been studied specifically in patients with elderly-onset rheumatoid arthritis (EORA).

The aim of this study was to determine whether two of these polymorphisms in TLR4 (*Asp299Gly and Thr399Ile*) are associated with susceptibility and clinical features in PMR, and also susceptibility to another age-restricted condition like EORA. We also attempted to correlate the functional consequences of these polymorphisms.

Material and methods

Study subjects

The present study included 164 patients with PMR, 93 with EORA, and 126 age-matched healthy controls (Table I). Patients with PMR were diagnosed according to the criteria proposed by Chuang *et al*. (17). Patients with RA had to satisfy the ACR 1987 revised criteria for RA (18). Patients with RA were considered as EORA if the age at symptoms onset was higher than 60 years. All the patients and controls gave signed informed consent, and the study was approved by the regional ethics committee.

Disease course was only ascertained in patients with PMR. Relapse was defined as the new appearance of typical clinical symptoms with increased acute-phase reactants or if symptoms disappeared with the increase of steroid dose. For the analysis of some variables such as relapses, duration of corticosteroid therapy, and accumulated dose of prednisone, only those patients with a follow-up ≥2 years were included.

Genotyping TLR4 gene

Genomic DNA was extracted from 5 ml of whole blood using DNAzol method according to the manufacturer's instructions (Invitrogen; Carlsbad, CA). Genotyping of the TLR4 *Asp299Gly* (*+896 A/G*; rs4986790) and TLR4 *Thr399Ile* (*+1196 C/T*; rs4986791) polymorphisms were performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as described by Lorenz *et al*. (19).

TLR4 protein expression in PBMCs

Cell surface expression of TLR4 was assessed on distinct PBMCs subpopulations (T cells, B cells and monocytes) by flow cytometry as previously described (20). Expression of TLRs was analysed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) as mean fluorescence intensity (MFI).

TLR4 function assessment in circulating monocytes

TLR4 function was assessed by measuring intracellular cytokine production (IL-1β, TNF-α, IL-6) by monocytes after *in vitro* stimulation with LPS (InvivoGen, San Diego, CA) (20). Levels of intracellular cytokine–producing monocytes were analysed using Cell Quest Pro Software (BD Biosciences).

Statistical analysis

All the statistical analysis of data was carried out using SPSS 12.0 (Chicago, IL). Arlequin v3 software was used to determine the Hardy-Weinberg equilibrium and haplotype analysis. The strength of the association between PMR or EORA and alleles or genotypes of the TLR4 gene was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either chi-square or Fisher's exact test analysis. For expression and functional studies, the statistical comparisons of data between PMR or EORA and controls were performed using the Mann-Whitney U-test. Differences were considered significant when *p*-values were <0.05.

Results

Association between TLR4 gene polymorphisms and disease susceptibility in PMR and EORA

The study population was found to be in Hardy-Weinberg equilibrium for the TLR4 gene polymorphisms (Table II). The distribution of the alleles and genotypes for these two polymorphisms was not significantly different between patients and controls (Tables III and IV). Furthermore, we observed no significant differences between PMR and EORA patients (Table V). Likewise, the haplotype analysis revealed that none of the haplotypes were significantly associated with the disease risk (Table VI).

TLR4 gene polymorphisms are associated with disease severity in PMR patients

The severity of PMR was addressed by analysing the presence of at least one relapse, number of relapses, duration of corticosteroid treatment and cumulative

Table I. Main demographic and clinical characteristics in patients with polymyalgia rheumatica (PMR) and elderly-onset rheumatoid arthritis (EORA) and healthy controls.

	Control	PMR	EORA
Number	126	164	93
Age (mean ± SD)	74.3 ± 11.0	73.1 ± 7.7	70.1 ± 7.4
Sex (% females)	68.3	61.3	63.4
Time to diagnosis (months)	–	3.2 ± 2.6	3.2 ± 2.2
PMR symptoms (%)	–	100	31.2
Pre-treatment ESR (mean ± SD mm/1 hr)	–	56.46 ± 30.2	64.58 ± 32.9
Pre-treatment CRP (mean ± SD mg/dl)	0.27 ± 0.2	4.74 ± 5.18	6.42 ± 5.3
RF (Ul/ml) (mean ± SD)	–	–	266.3 ± 468.3
ACCP (mean ± SD)	–	–	900.8 ± 629.5

PMR: polymyalgia rheumatica; EORA: elderly-onset rheumatoid arthritis; SD: standard deviation; ESR: erytrocyte sedimentation rate; CRP: C reactive protein; RF: rheumatoid factor; ACCP; anti-cyclic citrulline peptide.

Table II. Hardy-Weinberg *p*-value distribution of TLR4 gene in patients and controls.

SNP	Observed Heterozygosity	Expected Heterozygosity	*p*-value
EORA			
TLR4 +1196C/T	0.08602	0.08277	1.00000
TLR4 +896A/G	0.18280	0.16699	1.00000
PMR			
TLR4 +1196C/T	0.11585	0.10948	1.00000
TLR4 +896A/G	0.19512	0.17662	0.37317
Controls			
TLR4 +1196C/T	0.10317	0.09824	0.31840
TLR4 +896A/G	0.15873	0.15999	1.00000

EORA: elderly-onset of rheumatoid arthritis; PMR: polymyalgia rheumatica.

Table III. Distribution of TLR4 (+1196C/T and +896A/G) genotype, allele frequency and allele carriage frequency in patients with PMR and controls.

Genotype	PMR (n=164)	Controls (n=126)	*p*-value	OR (95% CI)
TLR4 +1196C/T (db SNP ID rs4986791) polymorphism				
Genotype frequency				
CC	145/164 (88.4)	113/126 (89.7)	–	–
CT	19/164 (11.6)	12/126 (9.5)	0.7021	1.24 (0.58–2.67)
TT	00/164 (0.0)	01/126 (0.8)	–	–
Allele frequency				
C	309/328 (94.2)	238/252 (94.4)	–	–
T	19/328 (5.8)	14/252 (5.6)	0.903	1.04 (0.51–2.13)
Allele carriage frequency				
C allele carriage	164/164 (100.0)	125/126 (99.2)	–	–
T allele carriage	19/164 (11.6)	13/126 (10.3)	0.8506	1.14 (0.53–2.41)
TLR4 +896A/G (db SNP ID rs4986790) polymorphism				
Genotype frequency				
AA	132/164 (80.5)	103/126 (81.7)	–	–
AG	32/164 (19.5)	22/126 (17.5)	0.7612	1.15 (0.62–2.09)
GG	00/164 (0.0)	01/126 (0.8)	–	–
Allele frequency				
A	296/328 (90.2)	228/252 (90.5)	–	–
G	32/328 (9.8)	24/252 (9.5)	0.925	1.03 (0.58–1.79)
Allele carriage frequency				
A allele carriage	164/164 (100.0)	125/126 (99.2)	–	–
G allele carriage	32/164 (19.5)	23/126 (18.3)	0.8801	1.08 (0.59–1.97)

PMR: polymyalgia rheumatica; OR: odds ratio; CI: confidence interval.

Table IV. Distribution of TLR4 (+1196C/T and +896A/G) genotype, allele frequency and allele carriage frequency in patients with EORA and controls.

Genotype	EORA (n=93)	Controls (n=126)	*p*-value	OR (95% CI)
TLR4 +1196C/T (db SNP ID rs4986791) polymorphism				
Genotype frequency				
CC	85/93 (91.4)	113/126 (89.7)	–	–
CT	08/93 (8.6)	12/126 (9.5)	1.0000	0.89 (0.35–2.28)
TT	00/93 (0.0)	01/126 (0.8)	–	–
Allele frequency				
C	178/186 (95.7)	238/252 (94.4)	–	–
T	08/186 (4.3)	14/252 (5.6)	0.6604	0.76 (0.31–1.86)
Allele carriage frequency				
C allele carriage	93/93 (100.0)	125/126 (99.2)	–	–
T allele carriage	08/93 (8.6)	13/126 (10.3)	0.817	0.82 (0.33–2.06)
TLR4 +896A/G (db SNP ID rs4986790) polymorphism				
Genotype frequency				
AA	76/93 (81.7)	103/126 (81.7)	–	–
AG	17/93 (18.3)	22/126 (17.5)	1.0000	1.06 (0.52–2.12)
GG	00/93 (0.0)	01/126 (0.8)	–	–
Allele frequency				
A	169/186 (90.9)	228/252 (90.5)	–	–
G	17/186 (9.1)	24/252 (9.5)	0.892	0.96 (0.50–1.84)
Allele carriage frequency				
A allele carriage	93/93 (100.0)	125/126 (99.2)	–	–
G allele carriage	17/93 (18.3)	23/126 (18.3)	1.0000	1.01 (0.50–2.00)

EORA: elderly-onset rheumatoid arthritis; OR: odds ratio: CI: confidence interval.

Table V. Distribution of TLR4 (+1196C/T and +896A/G) genotype, allele frequency and allele carriage frequency in patients with PMR and EORA.

Genotype	PMR (n=164)	EORA (n=93)	*p*-value	OR (95% CI)
TLR4 +1196C/T (db SNP ID rs4986791) polymorphism				
Genotype frequency				
CC	145/164 (88.4)	85/93 (91.4)	–	–
CT	19/164 (11.6)	08/93 (8.6)	0.5297	1.39 (0.58–3.32)
TT	00/164 (0.0)	00/93 (0.0)	–	–
Allele frequency				
C	309/328 (94.2)	178/186 (95.7)		
T	19/328 (5.8)	08/186 (4.3)	0.466	1.37 (0.59–3.19)
Allele carriage frequency				
C allele carriage	164/164 (100.0)	93/93 (100.0)	–	–
T allele carriage	19/164 (11.6)	08/93 (8.6)	0.5297	1.39 (0.58–3.32)
TLR4 +896A/G (db SNP ID rs4986790) polymorphism				
Genotype frequency				
AA	132/164 (80.5)	76/93 (81.7)	–	–
AG	32/164 (19.5)	17/93 (18.3)	0.8698	1.08 (0.56–2.08)
GG	00/164 (0.0)	00/93 (0.0)	–	–
Allele frequency				
A	296/328 (90.2)	169/186 (90.9)		
G	32/328 (9.8)	17/186 (9.1)	0.419	1.08 (0.60–1.99)
Allele carriage frequency				
A allele carriage	164/164 (100.0)	93/93 (100.0)	–	–
G allele carriage	32/164 (19.5)	17/93 (18.3)	0.8698	1.08 (0.56–2.08)

PMR: polymyalgia rheumatica; EORA: elderly-onset rheumatoid arthritis; OR: odds ratio: CI: confidence interval.

prednisone dose. The *Thr399Ile* CC genotype was associated with a higher cumulative dose of prednisone in patients with PMR (5.6±4.8 *vs.* 2.1±0.5 gr; p=0.031). This was mainly due to a trend in longer prednisone treatment (41.8±39.3 *vs.* 16.0±10.4 months; p=0.10) and a higher number of relapses (0.9±1.3 *vs.* 0.0±0.0; p=0.07) for this genotype.

TLR4 gene polymorphisms are not associated with expression and function of TLR4 in active PMR and EORA patients and controls

Although the results are probably limited by the sample size, we did not find an association with TLR4 expression on PBMCs (Fig. 1) or a distinct phenotype of TLR4 response with the *Asp299Gly* or *Thr399Ile* genotypes in neither patients and controls (Fig. 2).

Discussion

Two co-segregating SNPs (*+896 A/G* and *+1196 C/T*) within the gene encoding TLR4 have been characterised and studied in several inflammatory conditions (7, 8, 14), including some age-restricted disorders such as GCA (3, 20, 21). In the present work, we expanded the study of TLR4 gene polymorphisms in susceptibility or disease severity to PMR and EORA, two clinical syndromes that affect elderly individuals. It is well-known that PMR is a clinical syndrome closely related with GCA (17), and it has been also suggested that PMR and EORA, especially the seronegative forms, have much in common (22). No significant difference in allele frequency or genotype between patients with elderly-onset inflammatory conditions and controls was observed in this study. To the best of our knowledge, a large sample of patients with PMR has not been previously reported for these polymorphisms.

Although a genetic influence in the pathogenesis of PMR does exist, there is still a need to find genetic markers that may predict the severity of disease (23, 24). It has been previously suggested that the *-174 G/C* promoter IL-6 polymorphism is associated with persistently elevated levels of IL-6 and a higher risk of developing relapse/recurrence (25). Here, we have shown that

Table VI. Haplotype distribution of TLR4 +1196C/T and +896A/G among PMR, EORA and healthy control groups.

Haplotypes	EORA (n=93)	Controls (n=126)	*p*-value	OR (95% CI)
TLR4 +1196C/T and +896A/G				
C/A	169/186 (90.9%)	228/252 (90.5%)	1.0000	1.05 (0.54-2.01)
C/G	09/186 (4.8%)	10/252 (3.9%)	0.8131	1.21 (0.48-3.09)
T/A	00/186 (0.0%)	00/252 (0.0%)	–	–
T/G	08/186 (4.3%)	14/252 (5.6%)	0.6604	0.76 (0.31-1.86)
Haplotype	PMR (n=164)	Controls (n=126)	*p*-value	OR (95% CI)
TLR4 +1196C/T and +896A/G				
C/A	294/328 (89.6%)	228/252 (90.5%)	0.7813	0.91 (0.52-1.58)
C/G	15 /328 (4.6%)	10/252 (3.9%)	0.8376	1.16 (0.51-2.62)
T/A	02/328 (0.6%)	00/252 (0.0%)	–	–
T/G	17/328 (5.2%)	14/252 (5.6%)	0.8543	0.92 (0.44-1.92)

EORA: elderly-onset of rheumatoid arthritis; PMR: polymyalgia rheumatica.

the *Thr399Ile* CC genotype was associated with a significant higher cumulative dose of prednisone in patients with PMR and this was mainly due to a trend in longer prednisone treatment and a higher number of relapses. The value of determining *Thr399Ile* genotypes for disease prognosis in PMR should be confirmed in different populations.

Accumulating evidence points to a role of TLRs in the pathogenesis of RA (9, 10, 12). As stated above, the association of these two polymorphisms with RA remains controversial (13-16). Our results did not support the association of EORA with these two polymorphisms. Furthermore, we did not find any association in patients with young-onset RA (n=79) or in the whole group of RA (data not shown). Although we were not able to check for the clinical characteristics and disease severity of EORA, previous studies did not find association between these variables and the presence of these polymorphisms (14, 16).

The reasoning behind the proposed involvement of TLR4 gene polymorphisms in susceptibility or disease severity is that they may influence the receptor function (6). Therefore, we investigated whether the TLR4 variants have functional consequences for TLR4 expression and TLR4-mediated cytokine production by $CD14^+$ cells carrying the TLR4 variants, and observed that in both, patients and healthy controls, there was no relationship with the genotype of the TLR4 gene. Our results are in agreement with most studies done on stimulated whole blood and PBMCs where no distinct phenotype was found for these TLR4 gene polymorphisms (6, 20).

In conclusion, our results do not support the association of these TLR4 variants with susceptibility of PMR and EORA. The value of determining *Thr399Ile* genotype for disease prognosis in PMR should be confirmed in different populations. Furthermore, we found no association with TLR4 expression on PBMCs or a distinct phenotype of TLR4 response with the *Asp299Gly* or *Thr399Ile* genotypes.

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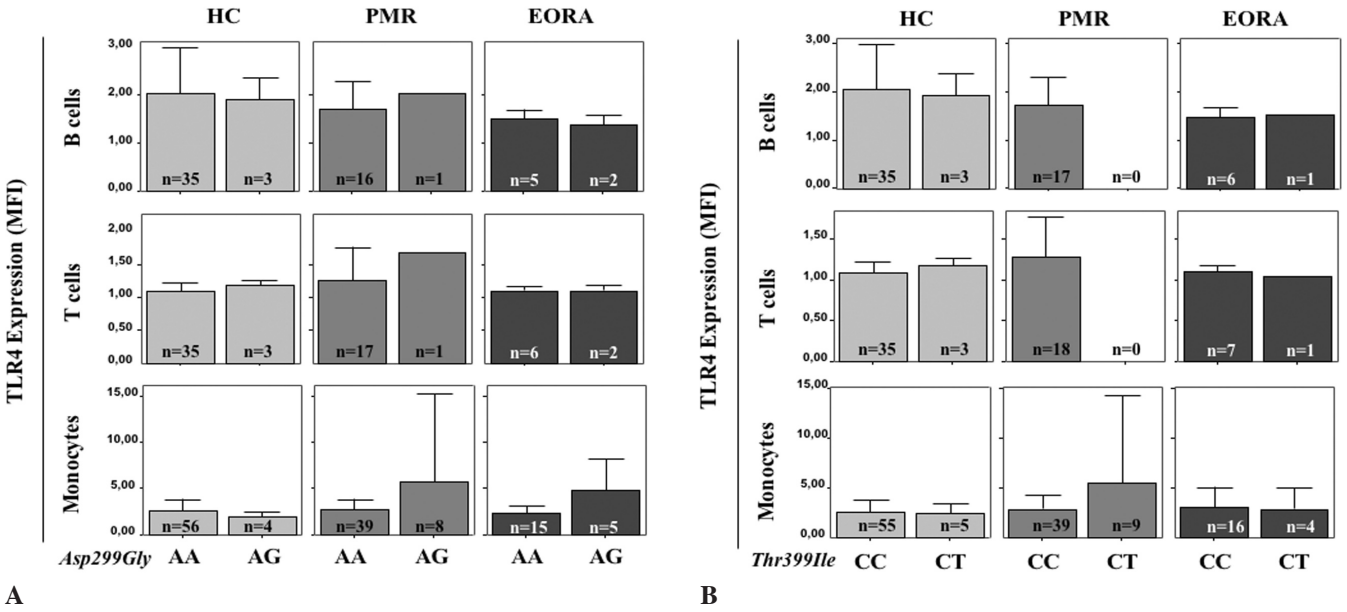

Fig. 1. Toll-like receptor (TLR) 4 expression on subpopulations of PBMCs according to TLR4 genotypes at disease onset in patients with polymyalgia rheumatica (PMR), elderly-onset rheumatoid arthritis (EORA) and healthy controls (HC). Mean fluorescent intensity (MFI) expression of TLR4 in PBMC of patients with PMR, EORA and healthy controls HC.

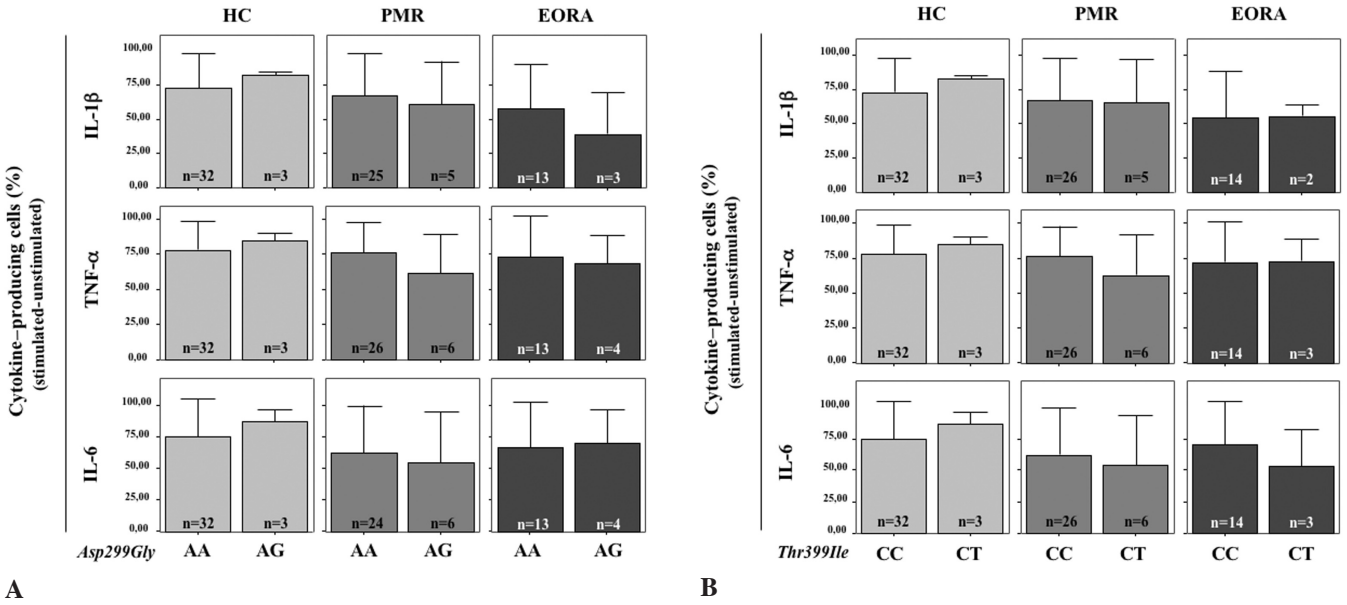

Fig. 2. Toll-like receptor (TLR) 4 function according to TLR4 genotypes at disease onset in patients with polymyalgia rheumatica (PMR), elderly-onset rheumatoid arthritis (EORA) and healthy controls (HC). TLR function was assessed by measuring intracellular proinflammatory cytokine production of circulating monocytes after TLR4 stimulation with specific agonist (LPS).

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