A single nucleotide polymorphism of TRAF1 predicts the clinical response to anti-TNF treatment in Japanese patients with rheumatoid arthritis

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Abstract

Objective

Recent genome-wide association studies disclosed that several single nucleotide polymorphisms (SNPs), including tumour necrosis factor (TNF) receptor-associated factor 1 (TRAF1) (+16860A/G), are associated with the pathophysiology of rheumatoid arthritis (RA). We assessed the usefulness of TRAF1 genotyping as a genetic predictor of the response to anti-TNF treatment in Japanese RA patients.

Methods

TRAF1 (+16860A/G) was genotyped using the TaqMan SNP genotyping assay in 101 Japanese RA patients treated with anti-TNF drugs for >24 weeks. We retrospectively analysed the association between SNP and the clinical response to treatment. TRAF1 mRNA and protein expression was also evaluated in $CD4^+$, $CD8^+$, $CD14^+$, or $CD19^+$ cells from 25 healthy subjects using quantitative polymerase chain reaction and intracellular staining flow cytometry, respectively.

Results

No statistical difference in DAS28-ESR at baseline was observed between the patient groups with the AA, AG, or GG genotype. The GG genotype was more frequent in non-responders than in good or moderate responders [odds ratio (OR) 7.4, 95% confidence interval (CI) 1.5–37.5]. The non-responders possessed the G allele more frequently than the good or moderate responders (OR 3.5, 95% CI 1.4–9.0). TRAF1 protein expression increased significantly in $CD14^+$ monocytes from healthy subjects with the GG genotype compared with that in subjects with the AA or AG genotype.

Conclusion

TRAF1 (+16860A/G) may be useful for predicting the clinical response to anti-TNF treatment and may contribute to resistance to treatment in RA patients with the GG genotype by increasing the TRAF1 expression in circulating inflammatory cells.

Key words

rheumatoid arthritis, tumour necrosis factor, TRAF1, polymorphism

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Introduction

Rheumatoid arthritis (RA) is a progressive inflammatory disorder resulting in joint damage and disability (1). Abnormalities in circulating immune cells and inflammatory cytokines are known to be involved in the pathogenesis of RA (2). Recent genome-wide association studies have shown that several genetic factors, including single nucleotide polymorphisms (SNPs), are associated with the pathophysiology of RA and that genetic variants may contribute to 50–60% of the etiology of the disorder (3-5).

Conventional disease-modifying antirheumatic drugs (DMARDs) such as methotrexate (MTX) remain the standard treatment for RA, although efficacy and safety issues of these drugs and the heterogeneous nature of RA patients may necessitate additional treatment strategies (6). Increased understanding of the immunological processes associated with the pathophysiology of RA has led to the development of biological agents that target signal transduction molecules and proinflammatory cytokines responsible for inflammation and structural damage. Although the most commonly used biological agents in clinical practice, such as drugs against tumour necrosis factor (TNF), including infliximab (IFX), adalimumab (ADA), and etanercept (ETN), have excellent efficacy against RA, a substantial number of patients still show inadequate responses. Several clinical and genomic predictors of the response to anti-TNF treatments have been determined. It has been reported that the response to anti-TNF treatment is influenced by factors such as the level of disability at the onset of treatment as measured by the Health Assessment Questionnaire, current smoking, concurrent therapy with MTX, and autoantibody status, including rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibody (ACPA) (7-10). A number of studies have also evaluated the usefulness of genetic polymorphisms mainly in genes encoding TNF-α and TNF receptors as genetic predictors of the response to anti-TNF treatment (11-15). On the other hand, it has been reported that there is no association between the FcGRIIIa polymorphism and the response to anti-TNF treatments, although the FcGRIIIa polymorphism is shown to be associated with the development of RA (16). However, even when these factors are combined, the usefulness of these predictions still remains insufficient.

It has been recently reported that SNPs in the TNF receptor-associated factor 1 (TRAF1) gene are associated with the pathophysiology of RA in Asians, Caucasians, and the North Africa population (17, 18). In particular, TRAF1 (+16860A/G) has been shown to be associated with RA susceptibility. TRAF1 binds several protein kinases and adaptor proteins and possesses multiple functions in signalling networks through the TNF receptor superfamily (19). This suggests that the TRAF1 polymorphism may be associated with the pathophysiology of RA as a consequence of modulation of TNF signalling. This study assessed the usefulness of TRAF1 (+16860A/G) genotyping as a novel genetic predictor of the response to anti-TNF treatments in Japanese patients with RA. The study also examined the underlying mechanism of the association between TRAF1 polymorphisms and the clinical response to anti-TNF treatment.

Materials and methods

Patients and healthy subjects

A total of 364 unrelated Japanese adult patients with RA treated at Keio University Hospital were reviewed retrospectively using the database of the SAKURA study, a single center cohort study on RA. The patients eligibility for the study was based on the following criteria: treated for more than 24 weeks with anti-TNF drugs such as IFX, ADA, and ETN, as the first biological agent; started anti-TNF treatment between July 2009 and June 2012; and had available complete medical records. All patients enrolled in the study fulfilled the American College of Rheumatology 1987 revised criteria for RA. The clinical response to anti-TNF treatments was based on the EULAR response criteria (20) and was evaluated using the relative change in disease activity score in 28 joints (DAS28-

ESR) from baseline to 24 weeks. Patients with a good or moderate response were defined as responders, while patients with no response were classified as non-responders. To evaluate the expression level of TRAF1, 25 healthy subjects with the AA (n=10), AG (n=10), or GG (n=5) genotype were also recruited. All the samples from the patients and healthy subjects were obtained after they provided written informed consent to participate in the study. The study protocol was approved by the Institutional Review Board of Keio University.

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood samples using Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) density-gradient centrifugation. In some experiments, $CD4^+$, $CD8^+$, $CD14^+$, or $CD19^+$ cells were separated from the PBMCs obtained from healthy subjects with either the AA, AG, or GG genotype using magnetic cell sorting column separation (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. All these sorted fractions consistently had >90% purity, as assessed by flow cytometric analysis.

TRAF1 (+16860) SNP genotyping

Genomic DNA was extracted from the PBMCs using the QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. TRAF1 (+16860A/G) was determined using a TaqMan Real-Time PCR System and TaqMan SNP Genotyping Assay C_29005978_10 (Applied Biosystems, Inc., Foster City, CA, USA) according to the manufacturer's instructions.

Analysis of mRNA expression for TRAF1

The mRNA expression for TRAF1 was examined using the reverse transcription (RT)-polymerase chain reaction (PCR) as described previously with some modifications (21). In brief, total RNA was extracted from $CD4^+$, $CD8^+$, $CD14^+$, or $CD19^+$ cells using the RNeasy Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesised from total RNA using avian myeloblastosis virus reverse transcriptase (Takara, Kyoto, Japan) with oligo-dT priming. The cDNA was then subjected to a quantitative TaqMan Real-Time PCR System and TaqMan Gene Expression Assay Hs01090170_m1 (Applied Biosystems). The expression levels of the TRAF1 gene were normalised to the expression level of GAPDH.

Intracellular staining of TRAF1 by flow cytometry

The protein expression level of TRAF1 was evaluated using flow cytometry to detect intracellular staining in combination with staining for CD4, CD8, CD14, or CD19. In brief, the PBMCs were stained with fluorescein-conjugated anti-CD4 (clone 13B8.2; Beckman-Coulter, Fullerton, CA, USA), anti-CD8 (clone B9.11; Beckman-Coulter), anti-CD14 (clone RMO52; Beckman-Coulter), or anti-CD19 monoclonal antibody (clone SJ25C1; Becton Dickinson, San Jose, CA, USA). The cells were then permeabilised and fixed using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (Becton Dickinson), followed by incubation with fluorescein-conjugated anti-TRAF1 monoclonal antibody (clone H-3; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were analysed on a FACS® Calibur Flow Cytometer (Becton Dickinson) using CellQuest software. The TRAF1 expression level was quantified as the mean fluorescence index (MFI), calculated as the ratio of cells treated with anti-TRAF1 antibody to those treated with isotype-matched control antibody.

Statistical analysis

Statistical power was calculated using IBM SPSS Statics, version 18 (International Business Machines Corporation, Armonk, NY, USA). Differences were considered significant if $p<0.05$. Continuous values were expressed as the mean ± standard deviation (SD). Baseline characteristics were compared across TRAF1 (+16860A/G) genotypes using the Kruskal–Wallis test for continuous data and the 2-tailed Yates chi square test or Fisher's test for qualitative variables. The associations between the EULAR response at 24 weeks and explanatory variables, including TRAF1 (+16860A/G) genotypes, patient and disease characteristics, and concurrent treatments at baseline, were analysed by the 2-tailed Yates chi square test, Fisher's test, or univariate logistic regressions. Significant variables in the univariate analyses were then entered into a forced entry multivariate model. The results were expressed as the odds ratios (ORs) and 95% confidence interval (CI). The association between the TRAF1 (+16860A/G) genotypes and TRAF1 expression level were examined by the Kruskal-Wallis test, followed by the non-parametric Mann-Whitney U-test between the 2 groups.

Results

Clinical characteristics of the RA patients

Of the 364 patients, 116 were treated with anti-TNF drugs as the first biological agent, with 112 continuing this treatment for more than 24 weeks. Of these 112 patients, 11 did not have full clinical information, leaving a total of 101 patients enrolled in the study (Fig. 1). The demographic and clinical characteristics of these patients are summarised in Table I. The age (Mean ± SD) of the patients was 56±16 years, 85% were female, 81% were RF-positive, and 77% were ACPA-positive. The proportion of patients treated with IFX, ETN, and ADA as the first biological agent was 65%, 22%, and 13%, respectively. During the anti-TNF treatment, 89% of patients also received MTX. There was no difference in the clinical baseline characteristics of the RA patients grouped according to their TRAF1 (+16860) genotype (AA, AG, or GG).

Association between a TRAF1 SNP and clinical response to anti-TNF treatment

The frequencies of the TRAF1 (+16860A/G) genotypes in the patients were AA 50%, AG 42%, and GG 8%. This genotype distribution was consistent with the Hardy-Weinberg equilibrium and similar to the HapMap-JPT (Japanese) frequencies [AA 57%, AG

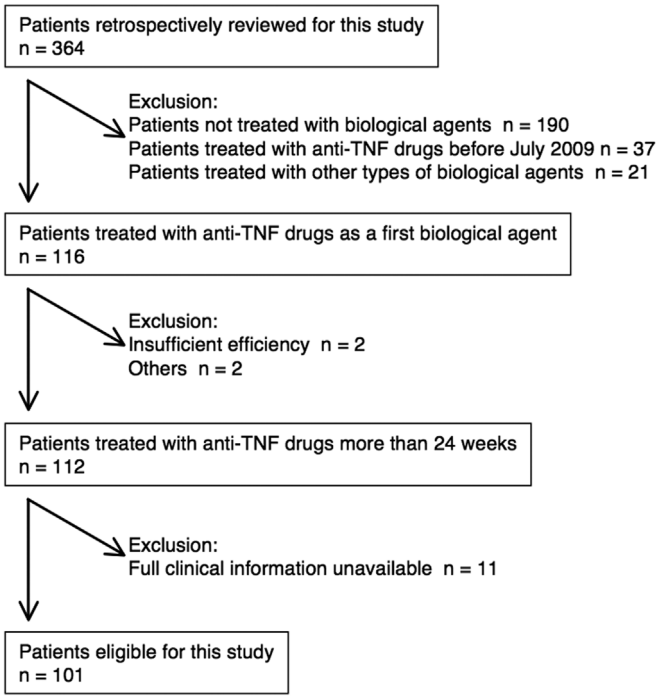

Fig. 1. Flow diagram of the study selection process. Patients eligible for the study were selected using the following criteria: treated for more than 24 weeks with anti-TNF drugs such as IFX, ADA, and ETN as a first biological agent; started anti-TNF treatment between July 2009 and June 2012; and had available complete medical records. Of the 364 RA patients followed at Keio University Hospital, 101 fulfilled these criteria, with their data being analysed in the study.

Table I. Baseline characteristics of the 101 patients with RA treated with anti-TNF agents*.

	Total	Genotype			*p* value**
	n=101	AA n=51	AG n=42	GG n=8	
Mean age (SD)	55.5 (15.7)	55.6 (15.4)	55.9 (16.5)	52.4 (14.2)	0.82
Female, n. (%)	86 (85.1)	42 (82.4)	36 (85.7)	8 (100.0)	0.42
DAS28-ESR, mean (SD)	4.9 (1.1)	5.0 (1.1)	4.9 (1.0)	4.4 (0.7)	0.39
SDAI, mean (SD)	21.1 (10.6)	21.6 (11.9)	21.3 (9.7)	17.1 (5.5)	0.48
RF positive, n. (%)	82 (81.2)	38 (74.5)	37 (88.1)	7 (87.5)	0.22
ACPA positive, n. (%)	78 (77.2)	37 (72.5)	35 (83.3)	6 (75.0)	0.46
Concurrent treatments					
Methotrexate, n. (%)	90 (89.1)	44 (86.3)	39 (92.9)	7 (87.5)	0.59
Other DMARDs, n. (%)	29 (28.7)	15 (29.4)	12 (28.6)	2 (25.0)	0.97
Prednisolone, n. (%)	18 (17.8)	8 (15.7)	7 (16.7)	3 (37.5)	0.32
Anti-TNF treatments					
Infliximab, n. (%)	66 (65.3)	34 (66.7)	30 (71.4)	4 (50.0)	0.75
Etanercept, n. (%)	22 (21.8)	12 (23.5)	8 (19.0)	2 (25.0)	
Adalimumab, n. (%)	13 (12.9)	5 (9.8)	6 (14.3)	2 (25.0)	

*RA: rheumatoid arthritis; TNF: tumour necrosis factor; SD: standard deviation; DAS-28: disease activity score in 28-joint count; SDAI: simplified disease activity index; RF: rheumatoid factor; ACPA: anti-cyclic citrullinated peptide antibody; DMARDs: disease-modifying anti-rheumatic drugs.
**p-values were calculated using the 2-tailed Yates chi square test or Fisher's test for dichotomous variables or the Kruskal-Wallis test for continuous variables.

38%, and GG 5%] and previous reports on other Asian populations (17, 22).

In the 101 RA patients who received anti-TNF treatment for 24 weeks, 63 (62.4%), 28 (27.7%), and 10 (9.9%) patients achieved a good, moderate, or no response, respectively. A summary of the EULAR response in the patients is shown in Figure 2. The non-responders to anti-TNF treatment were mainly patients with the GG genotype than those with the AA or AG genotype (37.5% *vs*. 7.5%, p=0.031, OR 7.4, 95% CI 1.5–37.5). On the other hand, the responders to anti-TNF treatments were mainly patients with the AA genotype than those with the AG or GG genotypes (96.1% *vs*. 84.0%, p=0.051, OR 4.7, 95% CI 0.9–23.2). The absolute change in DAS28-ESR from baseline to 24 weeks after initiation of anti-TNF treatment tended to decrease in patients with the GG genotype compared with that in patients with the AA or AG genotype (p=0.058) (data not shown). According to the allele frequency analysis, the non-responders to anti-TNF treatments more frequently possessed the G allele than the responders (55.0% *vs*. 25.8%, p=0.006, OR 3.5, 95% CI 1.4–9.0).

To investigate whether the TRAF1 (+16860A/G) polymorphism was an independent factor of the clinical response to anti-TNF treatment, explanatory variables including the TRAF1 (+16860A/G) genotype, clinical characteristics, and concurrent treatment were analysed in univariate and multivariate analyses (Table II). The univariate analysis showed that the GG genotype and prednisolone treatment (50% *vs*. 14.3%, p=0.015, OR 6.0, 95% CI 1.5–23.7) were more frequent in the non-responders than in the responders. Multivariate analysis confirmed that the GG genotype was independently associated with no response to anti-TNF treatment (p<0.001, OR 16.9, 95% CI 6.7–41.7).

Association between a TRAF1 SNP and gene expression levels of TRAF1

We next investigated the potential association between TRAF1 (+16860A/G) and the expression levels of TRAF1 using $CD4^+$, $CD8^+$, $CD14^+$, or $CD19^+$ cells obtained from healthy subjects. Quantitative RT-PCR showed that there was no significant difference in the expression levels of mRNA for TRAF1 in healthy subjects with either the AA, AG, or GG genotype (data not shown). However, flow cytometry showed that the protein expression levels of TRAF1 increased significantly in $CD14^+$ cells with the GG genotype compared with those in the cells with the AA or AG genotype (p=0.044) (Fig. 3). This finding indicates that subjects with the GG genotype have increased expression of TRAF1 in circulating monocytes.

Discussion

The present study examined the clinical features of 101 RA patients who received 24 weeks of anti-TNF treatment. We found that a TRAF1 (+16860A/G)

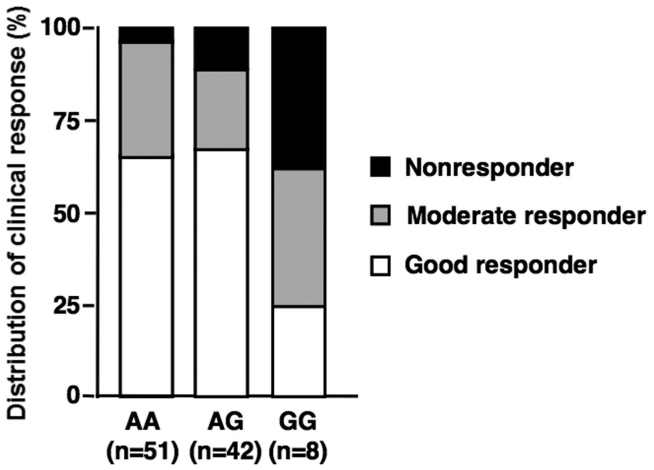

Fig. 2. Distributions of the EULAR response to anti-TNF treatment in the 101 patients with RA.

Table II. Association between the clinical response to anti-TNF treatment and explanatory variables in the univariate and multivariate analyses*.

	Univariate analysis**		Multivariate analysis	
Variable	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)
GG genotype	0.031	7.4 (1.5–37.5)	<0.001	16.9 (6.7–41.7)
Age	0.695	1.0 (1.0–1.1)		
Females	0.351	1.1 (1.0–1.2)		
DAS28-ESR at baseline	0.791	0.9 (0.5–1.7)		
SDAI at baseline	0.824	1.0 (0.9–1.1)		
Positive RF	0.201	0.8 (0.7–1.9)		
Positive ACPA	0.448	2.9 (0.3–23.9)		
Methotrexate	1.000	1.1 (0.1–9.7)		
Prednisolone	0.015	6.0 (1.5–23.7)	0.073	3.3 (0.9–14.3)

*TNF: tumour necrosis factor; DAS-28: disease activity score in 28-joint count; SDAI: simplified disease activity index; RF: rheumatoid factor; ACPA: anti-cyclic citrullinated peptide antibody.
**_p_-values were calculated using the 2-tailed Yates chi square test or Fisher's test for dichotomous variables or the logistic regression test for continuous variables.

SNP in intron 3 of the TRAF1 gene was significantly associated with the clinical response to anti-TNF treatment and that the GG genotype was more frequent in patients who did not respond to treatment. The G allele was also associated with increased expression levels of TRAF1 in circulating $CD14^+$ monocytes from healthy subjects.

Although the role of TRAF1 in the TNF-α signalling network has not been fully elucidated, TRAF1 mainly inhibits TNF-α-mediated signalling through the TNF receptor II (18). Furthermore, TRAF1 knockout mice are hypersensitive to TNF-induced stimulation through the NF-κB and JNK pathways (23). In addition, several studies have shown that $CD14^+$ monocytes play an important role in the pathophysiology of RA (24-28). Of note, TNF-α is known to increase bone resorption by monocyte-derived osteoclasts, resulting in joint inflammation and damage in RA (28). These earlier reports and our results suggest that TNF-α-mediated stimulation is possibly inhibited by increased TRAF1 expression in circulating monocytes from subjects with the G allele and that other inflammatory cytokines such as IL-6 and IL-1, but not TNF-α, play a pivotal role in the pathophysiology of RA in patients with the GG genotype rather than those with the AA or AG genotypes. Therefore, RA patients with the GG genotype may be refractory to anti-TNF treatment. Recently, several studies demonstrated the association between SNPs for candidate genes encoding for several cytokines including TNF, and bone erosion evaluated by musculoskeletal ultrasound (MSUS) (29). Since TRAF1 is also involved in the TNF-mediated signalling pathway, the association between the three TRAF1 genotypes and MSUS-detected bone erosion should be evaluated in future.

The TRAF1 (+16860A/G) SNP (rs7021206) has been reported to be in linkage disequilibrium with other SNPs associated with RA susceptibility, such as rs2416806, rs2900180, and rs3761847 (17, 30). Although further experiments are necessary to determine which of these SNPs directly affects TRAF1 expression, the present study is the first to report an association between SNPs within the TRAF1 gene and expression levels of TRAF1.

Autoantibody status, including RF and ACPA, and concurrent therapy with MTX are associated with the response to anti-TNF treatment (7-10). However, these associations were not detected in our study. The lower OR in previous studies and the small number of samples in this study may explain this absence of a statistically significant difference.

There were 2 methodological limitations in our study. First, the patients were treated with structurally different types of anti-TNF drugs, including IFX, ADA, and ETN. IFX and ADA are both monoclonal antibodies specifically reactive to TNF-α, the first being a chimeric human–murine antibody and the latter a fully human antibody. On the other hand, ETN is a fusion protein consisting of the extracellular domain of the p75 TNF receptor and the hinge and Fc domains of human IgG_1 (31). However, the 3 drugs have a similar mechanism of action and their efficacy was comparable in our study. When only RA patients treated with IFX were analysed, the association between the TRAF1 (+16860A/G) SNP and clinical response was consistent (data not shown). Second, only patients treated with anti-TNF drugs for more than 24 weeks were selected for this study. This was necessary as the primary endpoint of the study was assessment of the clinical response after 24 weeks of treatment. Some patients had discontinued or changed treatment prior to the 24-week follow-up because of inefficiency

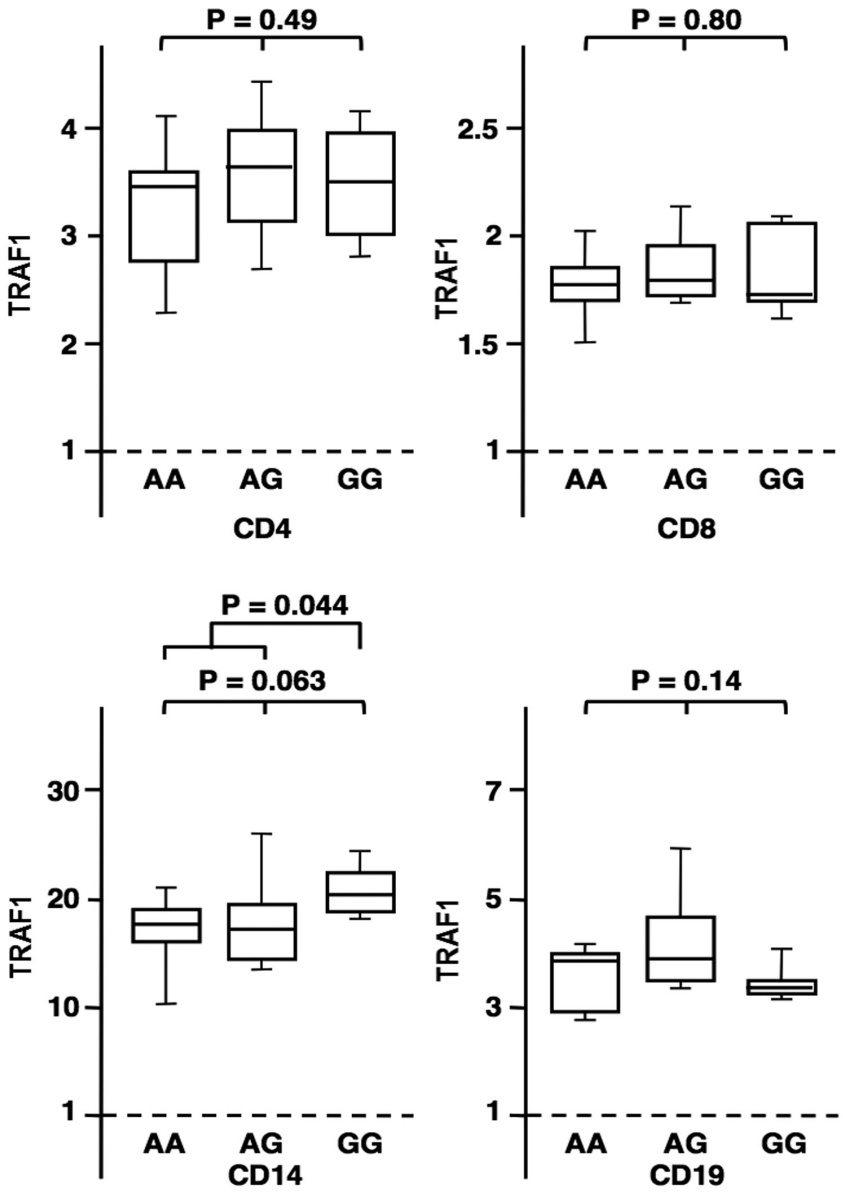

Fig. 3. Expression levels of TRAF1 in CD4+, CD8+, CD14+, or CD19+ cells. Expression levels of TRAF1 were evaluated in CD4+, CD8+, CD14+, or CD19+ cells from 25 healthy subjects with either the AA (n=10), AG (n=10), or GG (n=5) genotype by intracellular staining measured by flow cytometry. Differences in continuous variables were examined by the Kruskal-Wallis test, followed by the non-parametric Mann-Whitney U-test between the 2 groups.

of the therapy. It is therefore necessary to perform further prospective investigations including patients who withdraw from the study.

In conclusion, TRAF1 (+16860A/G) genotyping may be useful for predicting the clinical response to anti-TNF treatment. This polymorphism may contribute to resistance to anti-TNF treatments in RA patients with the GG genotype by increasing the expression of TRAF1 in circulating inflammatory cells.

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