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ORIGINAL ARTICLE

The role of infection in the development of non-valvular atrial fibrillation: Up-regulation of Toll-like receptor 2 expression levels on monocytes

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Summary Many studies have suggested that inflammation may participate in the pathogenesis of non-valvular atrial fibrillation (AF). However, it has been unknown by exposure to what the inflammation is caused. Recently, we reported that Toll-like receptor 2 (TLR2) level on monocytes was significantly up-regulated in viral and bacterial infections, but not in non-infectious inflammatory states. Our purpose was to test the hypothesis that expression of TLR2 levels may be up-regulated in patients with non-valvular AF. A total of 48 consecutive patients with non-valvular AF who were hospitalized for catheter ablation were enrolled in this study. TLR2 levels were assayed by using flow-cytometric analysis and compared with volunteers in sinus rhythm (control group, $n = 24$). Additionally, C-reactive protein (CRP) and interleukin-6 (IL-6) levels were assayed, and the left atrial volume indexes (LAVI) in the non-valvular AF group were measured. The results demonstrated that TLR2 levels in the non-valvular AF group were significantly higher than in the control group (median, 4682 vs. 3866 sites/cell; $P < 0.01$). Moreover, non-valvular AF patients had significantly higher IL-6 levels than controls. However, there was no significant difference in CRP levels between the two groups. It was observed in 44 AF patients, in

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whom pulmonary vein isolation was confirmed to be successful, that the LAVI significantly diminished 1 month after ablation (median, 33.6 vs. 29.5 ml/m^2; $P < 0.001$), but not the TLR2 and IL-6 levels. Our results implied that an infectious inflammation may participate in the pathogenesis of non-valvular AF.

Introduction

Atrial fibrillation (AF), the most commonly encountered arrhythmia in clinical practice, is associated with substantial morbidity and mortality. AF is generally categorized into valvular and non-valvular AF/lone AF, depending on the presence of valvular heart disease. Atrial biopsies of non-valvular AF patients provided the evidence that inflammation may participate in the pathogenesis [1], and the reflecting markers in sera have been explored. In this context, it has been controversial whether non-valvular AF patients have higher CRP levels compared with controls in sinus rhythm [2—7]. On the other hand, Toll-like receptor (TLR) family members, which are key regulators of both innate and acquired immune responses, have been studied not only in the immunological response [8—10], but also in the clinical implications [11—13]. In this study, based on our previous finding that TLR2 expression levels on monocytes are up-regulated in infectious diseases [14,15], we tested how the expression level of TLR2 is modulated in patients with non-valvular AF, compared with volunteers in sinus rhythm (control group). In addition, we measured plasma interleukin-6 (IL-6) levels and amino-terminal propeptide of type III procollagen (PIIINP) levels in both groups. PIIINP is a collagen III synthesis marker, and if fibroid degeneration occurs in the atrium of patients with non-valvular AF, we hypothesized that it might be reflected in PIIINP levels. Subsequently, we evaluated TLR2 expression levels and the left atrial volume index (LAVI) before and 1 month after successful catheter ablation in the non-valvular AF group.

Methods

Patient population

Forty-eight patients with non-valvular AF, who underwent catheter ablation for drug-refractory paroxysmal AF in our hospital, were enrolled. This group consisted of 39 males and 9 females, ranging from 30 to 72 years old (mean = 54 years). The patients with non-valvular AF that were indicated for catheter ablation had a left atrial diameter <45 mm. Forty patients were taking medications, including anti-inflammatory drugs, such as angiotensin-converting enzyme inhibitors (ACE-I), angiotensin II receptor blockers (ARB), and statins, and eight patients were not taking medication (Table 1). In the non-valvular AF group, blood samples were taken before and 1 month after catheter ablation. In this study, the ablation was defined to be successful with two criteria: (1) patients were free from palpitations during the first month after ablation; (2) the absence of AF was confirmed by checking the electrocardiogram and 24-h Holter monitor a month after ablation. Consequently, 44 out of 48 AF patients were judged as successful ablation cases. Alternatively, 25 out of 48 AF patients offered blood samples not only from peripherals but also from left atria just before ablation. Volunteers ($n = 24$) in sinus rhythm were confirmed to be infection-free for at least 1 month at the time of blood sampling and did not have a fever within a week from the time the blood sample was drawn. They were categorized as the control group, which consisted of 19 males and 5 females, ranging from 30 to 72 years old (mean = 49 years). The control group was age- and sex-matched with the non-valvular AF group. Sixteen out of 24 volunteers were under medical treatment for hypertension, hyperlipidemia, and diabetes mellitus. Patients were excluded with organic disorders responsible for AF, such as valvular heart disease, congestive heart failure (NYHA class II or greater), recent acute coronary events/revascularization, thyroid disease, and patients with systemic inflammatory diseases including infection, collagen diseases, malignancies, renal failure, and hepatic failure. Informed consent was obtained from all patients and volunteers, which was in accordance with a protocol approved by the Kagoshima University Ethics Committee. Ten milliliters of peripheral blood were taken from each patient with a heparinized blood collecting tube.

Flow-cytometer analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by density gradient centrifugation using Ficoll-Paque plus

Table 1 Patient population.

	Control ($n = 24$)	Non-valvular AF ($n = 48$)	P-Value
Age (years)	49	54	0.06
Male/female, *n* (%)	19/5 (79%)	39/9 (81%)	0.72
Hypertension, *n* (%)	8 (33%)	20 (42%)	0.18
Hyperlipidemia, *n* (%)	5 (20%)	15 (31%)	0.07
Diabetes mellitus, *n* (%)	3 (13%)	9 (19%)	0.24
Medication			
ACE-I, *n* (%)	2 (8%)	4 (8%)	1.00
ARB, *n* (%)	4 (16%)	11 (23%)	0.10
Statin, *n* (%)	3 (13%)	8 (17%)	0.06

ACE-I: angiotensin-converting enzyme inhibitors; ARB: angiotensin II receptor blockers. The number in parentheses shows the percentage of male patients, the disease prevalence, and the patient ratio taking the medication.

liquid (Amersham Bioscience, GE Healthcare, Amersham, UK). To assess the expression levels of TLR2 on monocytes, PBMCs were divided into three tubes and stained in parallel with PE-labeled anti-TLR2 monoclonal antibody (mAb) (clone T2.1; eBIOSCIENCE, CA, USA), anti-CD14 mAb (clone M5E2; eBIOSCIENCE), or control mouse IgG2a (eBM2a; eBIOSCIENCE). The stained cells were analyzed on a flow cytometer FACS Calibur using CellQuest software (Becton-Dickinson Biosciences, CA, USA). In this study, we employed a single color flow cytometric analysis to avoid problems of interference between fluorescence dyes. For each donor, monocytes were first gated according to the forward/side scatter properties and CD14 staining. Subsequently, the same gate setting for monocytes was applied to the analysis of TLR2- and control-stained PBMC samples. The mean fluorescence intensity (MFI) value for just TLR2 was obtained by subtracting the control staining MFI value from the TLR2 staining MFI value.

Quantification of TLR2 levels

TLR2 expression levels on monocyte were numerically represented as previously described in order to avoid assay-to-assay variation caused by daily fluctuation in the performance of flow cytometers [14,15]. In this assay system, inter-assay variation was within acceptable levels (CV < 6.8%). In brief, a mixture of our developed TLR2-coupled standard beads, which consists of 4 types of beads carrying 4 different numbers of recombinant TLR2 molecules, was stained in parallel with PBMCs under the same experimental conditions in each assay. A calibration curve was then obtained by plotting MFI values of the standard beads. Using the calibration curve, the TLR2 expression level on monocytes was converted to the number of antibody-binding sites per cell.

Assessment of left atrial volume index

The LAVI was assessed by echocardiography using Simpson's method [16] before and 1 month after ablation in non-valvular AF patients.

Measurement of IL-6 and PIIINP levels

IL-6 (pg/ml) was measured by an enzyme immunoassay method and PIIINP level (U/ml) was measured by an immunoradioassay method.

Statistics

Given two data sets, we assessed whether two samples of observations came from the same distribution. Since the distributions of all unpaired two groups compared in this study were not normal and/or homoscedastic, a Mann—Whitney U-test was performed. When we compared the paired two groups, a paired Student's t-test or a Wilcoxon signed-rank test was applied according to whether the distributions were normal and homoscedastic or not. Data are expressed as the mean value ± standard deviation (S.D.) or as the median value (25—75th percentile) according to the statistical processing. Disease prevalence, gender, and patients ratio taking medication were compared between the control and the non-valvular AF groups with a chi-square test. All differences were considered significant at $P < 0.05$. All statistical analyses were performed with Excel Statistics 2006 for Windows® (Social Survey Research Information Co., Tokyo, Japan).

Results

TLR2 levels in non-valvular AF patients

As shown in Table 1, the number of patients in the non-valvular AF group with hypertension,

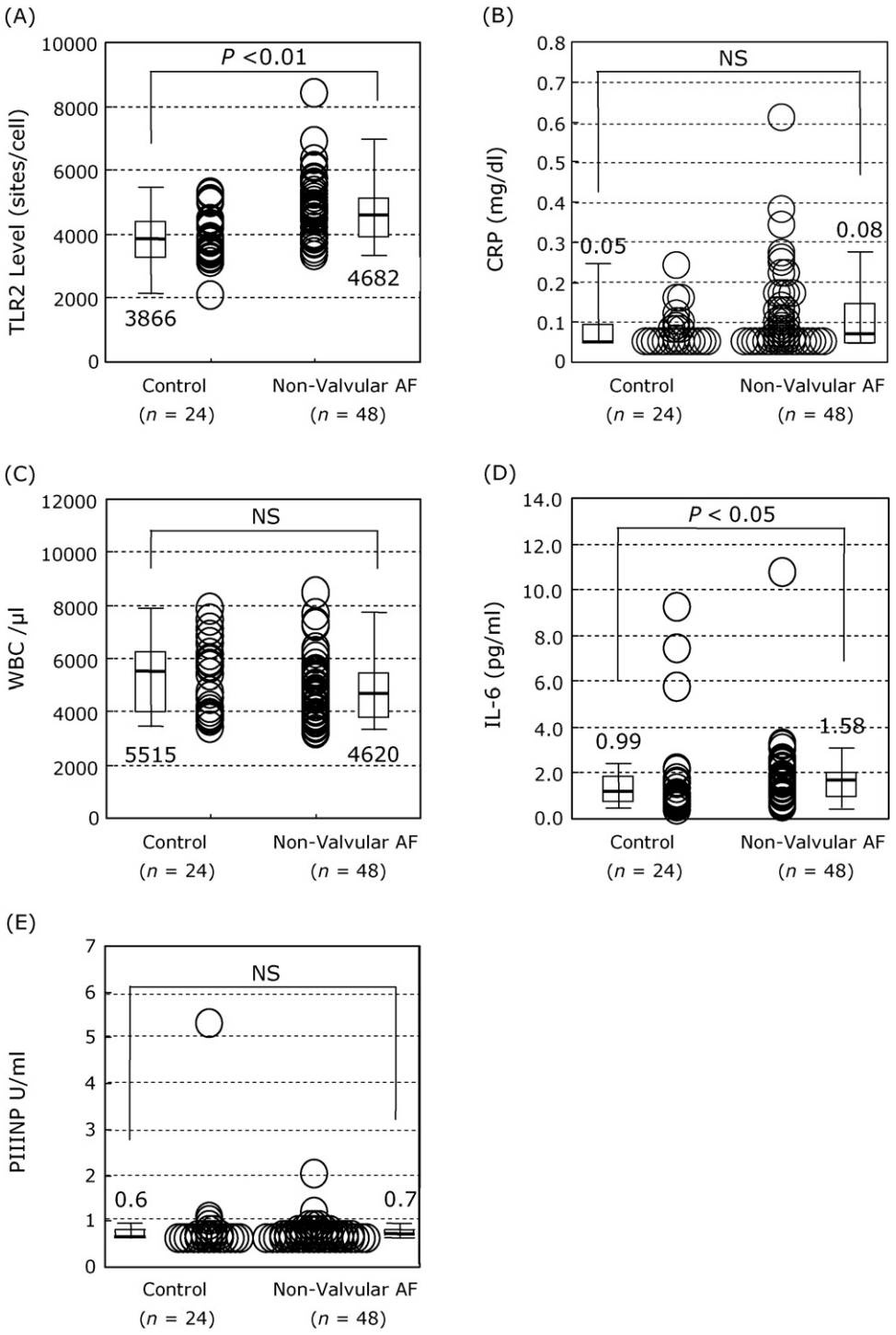

Figure 1 Comparison between the control group ($n = 24$) and non-valvular AF group ($n = 48$). TLR2 levels were compared between the two groups. Circles represent individual TLR2 expression values (A). CRP levels were compared between the two groups. Circles represent individual CRP levels (B). WBC values were compared between the two groups. Circles represent individual WBC values (C). IL-6 levels were compared between the two groups. Circles represent individual IL-6 levels (D). PIIINP levels were compared between the two groups. Circles represent individual CRP levels (E). Box plot and the horizontal bar show the interquartile range and median value, respectively. The whiskers extend to at most 1.5 times the box width (the interquartile range) from either or both ends of the box. *P*-Value estimates were based on the Mann—Whitney *U*-test (A—E).

hyperlipidemia, and diabetes mellitus was 20, 15, and 9, respectively. Coronary angiography confirmed that no patient had coronary artery disease (≥75% coronary arterial stenosis). The prevalence of hypertension, hyperlipidemia, and diabetes mellitus was similar in the control group (Table 1).

The expression levels of TLR2 in the non-valvular AF group were significantly higher than in the control group as shown in Fig. 1A [median (25—75th percentile), 4682 (3925—5260) vs. 3866 (3455—4421) sites/cell, $P < 0.01$]. However, there was no significant difference in CRP level and WBC [median (25—75th percentile), 0.08 (0.05—0.15) vs. 0.05 (0.05—0.09) mg/dl, $P = 0.074$ and 4620 (3793—5390) vs. 5515 (3980—6218) μl^{-1}, $P = 0.068$, respectively] between these two groups (Fig. 1B and C). In this study, the normal range of CRP level and WBC in men and women was set as 0.05—0.5 mg/dl, 3500—9700 μl^{-1}, and 3500—9300 μl^{-1}, respectively. Next, we examined IL-6 and PIIINP concentrations. Here, normal ranges of IL-6 and PIIINP levels were 0.17—9.96 pg/ml and 0.6—1.0 U/ml, respectively. There was a significant difference in IL-6 level [median (25—75th percentile), 1.58 (1.09—1.97) vs. 0.99 (0.65—1.84) pg/ml, $P < 0.05$] between the non-valvular AF and the control groups, but not in PIIINP level [median (25—75th percentile), 0.7 (0.6—0.7) vs. 0.6 (0.6—0.7) U/ml, $P = 0.75$] (Fig. 1D and E).

Correlation between TLR2 levels and CRP/IL-6 levels

We examined the statistical correlation between TLR2 level and CRP/IL-6 level of individual non-valvular AF patients ($n = 48$) enrolled in this study. TLR2 levels did not correlate with CRP levels ($r = -0.026$, $P = 0.09$). Hence, TLR2 levels on monocytes might serve as a new biological marker of inflammation independent of CRP levels. Next, the correlation between TLR2 levels and IL-6 levels was examined (Fig. 2B). Contrary to the findings shown in Fig. 2A, TLR2 levels were well correlated with IL-6 levels ($r = 0.54$, $P < 0.001$) in the AF patients.

Follow-up data on TLR2, IL-6 levels, and LA volume index

All patients in the non-valvular AF group were treated with radiofrequency catheter ablation to attempt to achieve complete four pulmonary vein isolation. Therefore, the left atrial diameter of all patients with non-valvular AF in this study was less than 45 mm. As a consequence, the overall LAVI of patients enrolled in this study was

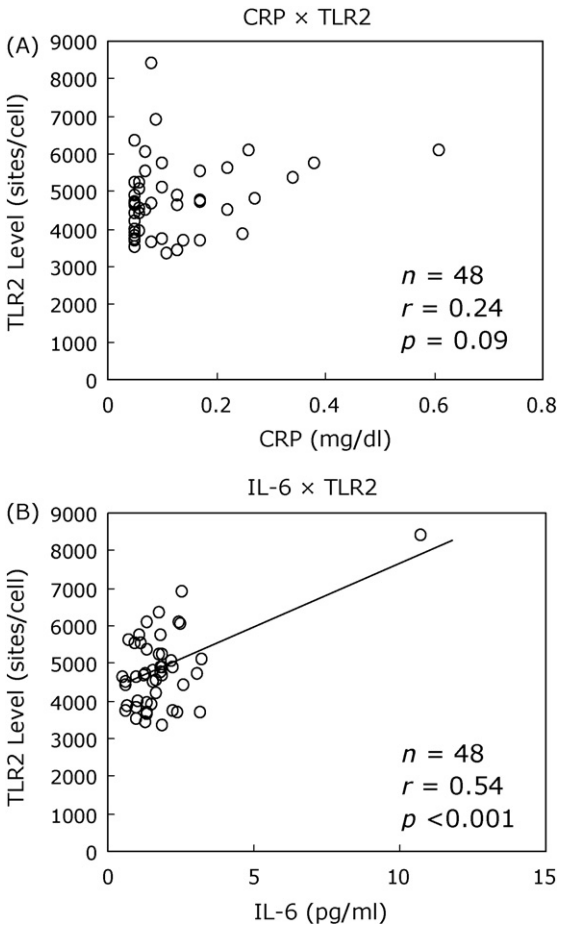

Figure 2 Correlation of TLR2 levels with CRP levels/IL-6 levels. The correlation is shown between the TLR2 value (*Y*-axis) and the CRP level (*X*-axis) at the same time point in each non-valvular AF patient (A). The correlation is shown between the TLR2 value (*Y*-axis) and IL-6 level (*X*-axis) at the same time point in each non-valvular AF patient (B). Number of patients (*n*), index of correlation (*r*) and *P*-value (*P*) are indicated in the correlation charts. Dots represent individual cases.

relatively small [median (25—75th percentile), 33.4 (29.5—38.2) ml/m^2]. We carried out catheter ablation for these patients with non-valvular AF. One month later, 44 out of 48 patients were confirmed to be successful in the treatment, and then the LAVI was assessed. As shown in Fig. 3A, the LAVI observed before ablation was significantly reduced 1 month after ablation [median (25—75th percentile), 33.6 (29.6—38.2) vs. 29.5 (24.0—35.0) ml/m^2, $P < 0.001$]. As for 4 patients with recurrence of AF, the individual LAVI did not change before and after ablation. In contrast, as shown in Fig. 3B and C, the TLR2 expression levels and IL-6 levels observed before ablation were not significantly altered 1 month after ablation [median (25—75th percentile), 4698 (4144—5302)

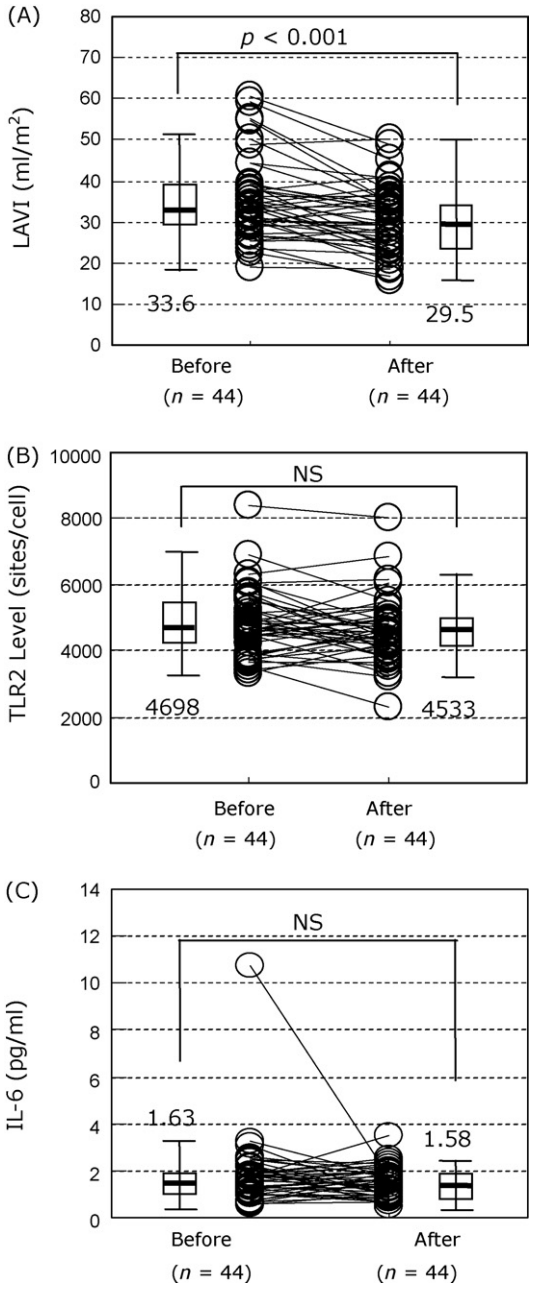

Figure 3 Comparison of the LAVI, TLR2 level, and IL-6 level in the patients judged as successful pulmonary vein isolation ($n = 44$) before and after ablation. Circles connected by a line show the LAVI in the same individual before and after ablation (A). Circles connected by a line show the TLR2 level in the same individual before and after ablation (B). Circles connected by a line show the IL-6 level in the same individual before and after ablation (C). Box plot and the horizontal bar show the interquartile range and median value, respectively. The whiskers extend to at most 1.5 times the box width (the interquartile range) from either or both ends of the box. *P*-Value estimates were based on the Wilcoxon signed-rank test.

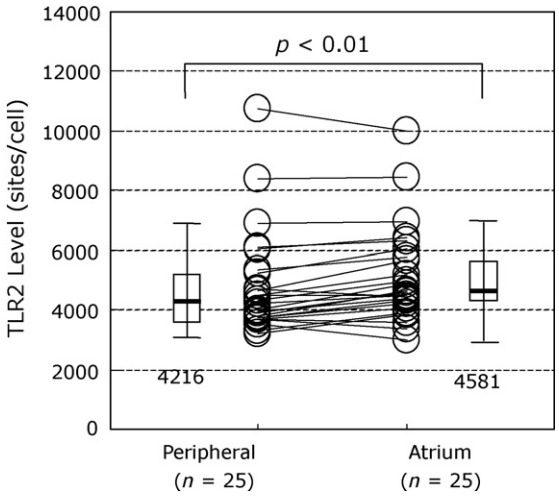

Figure 4 Comparison of TLR2 levels in the patients ($n = 25$) between monocytes from left atrium and from peripheral. Circles connected by a line show the TLR2 levels on monocytes taken from left atrium and peripheral at the same time in the same individual. Box plot and the horizontal bar show the interquartile range and median value, respectively. The whiskers extend to at most 1.5 times the box width (the interquartile range) from either or both ends of the box. *P*-Value estimates were based on the Wilcoxon signed-rank test.

vs. 4533 (4089—4950) sites/cell, $P = 0.15$, median (25—75th percentile), 1.63 (1.09—1.97) vs. 1.58 (0.95—1.94) pg/ml, $P = 0.43$, respectively]. Moreover, compared to before ablation CRP, WBC, and PIIINP levels did not change 1 month after ablation [mean ± S.D., 0.11 ± 0.11 vs. 0.10 ± 0.09 mg/dl, $P = 0.26$, median (25—75th percentile), 4620 (3793—5308) vs. 4765 (3975—5403) μl^{-1}, $P = 0.48$, 0.7 (0.6—0.7) vs. 0.6 (0.6—0.7) U/ml, $P = 0.06$, respectively]. These results are summarized in Table 2.

Comparison in TLR2 levels between two samples from left atrium and from peripheral

Several studies reported that some cytokines are capable of up-regulating TLR2 expression on monocytes [17,18]. Simultaneously, the secretion of these cytokines from inflammatory tissue will prime monocyte chemotaxis. We hypothesized that blood sampling site may be near to the focus of inflammation in accordance with the level of up-regulated TLR2 expression. So, we examined the difference in TLR2 level between paired samples collected at the same time from left atrium and from peripheral. The result is shown in Fig. 4. TLR2 level on monocytes from left atrium was significantly higher than that from peripheral [median (25—75th percentile),

Table 2 Follow-up study of patients with non-valvular AF (successful PV isolation, $n = 44$).

	Before ablation	One month after ablation	*P*-Value
WBC (μl^{-1})	4620 (3793—5308)	4785 (3975—5403)	0.48
CRP (mg/dl)	0.11 ± 0.11	0.10 ± 0.09	0.26
TLR2 (sites/cell)	4698 (4144—5302)	4533 (4089—4950)	0.15
IL-6 (pg/ml)	1.63 (1.09—1.97)	1.58 (0.95—1.94)	0.43
PIIINP (U/ml)	0.7 (0.6—0.7)	0.6 (0.6—0.7)	0.06
LAVI (ml/m^2)	33.6 (29.6—38.2)	29.5 (24.0—35.0)	<0.001

4581 (4247—5782) vs. 4216 (3728—5225) sites/cell, $P < 0.01$].

Discussion

Recently, we reported up-regulation of TLR2 expression levels on monocytes in infectious inflammatory diseases including viral infections, but not in non-infectious inflammatory diseases [14,15]. In the present study, we showed that non-valvular AF patients had higher TLR2 levels on monocytes and IL-6 levels than control patients in sinus rhythm. In addition, we showed that there was no significant difference in CRP and PIIINP levels in ablation-indicated AF patients compared to control patients. Subsequently, in 44 patients with non-valvular AF in whom pulmonary vein isolation was confirmed to be successful, TLR2 and IL-6 levels were also higher 1 month after ablation, whereas the LAVI was significantly decreased.

Frustaci et al. previously documented that abnormal atrial histology and inflammation was observed in endomyocardial biopsy specimens in all patients with non-valvular AF, whereas biopsy specimens in all patients with Wolff—Parkinson—White syndrome were normal. The type of abnormality varied: hypertrophy with vascular degeneration of the atrial myocytes, lymphomononuclear infiltration with necrosis of the adjacent myocytes, and nonspecific patchy fibrosis [19]. In addition, it has been reported that several anti-inflammatory and anti-oxidant agents have favorable effects in some types of AF [20—23]. Hence, an inflammation is likely to be cited as a cause for the pathogenesis of non-valvular AF.

Although there is consistent evidence that inflammation occurs in the atrium of non-valvular and paroxysmal/persistent AF patients, the elevation of inflammatory markers, such as CRP and IL-6 levels, in non-valvular AF is controversial. Ellinor et al. reported that there was no significant difference in high sensitivity (hs)-CRP between non-valvular AF patients and controls [6]. Gedikli et al. also found that there was no significant difference in hs-CRP and IL-6 between non-valvular AF patients and controls [7]. In contrast, Chung et al. showed that lone atrial tachyarrhythmia patients had higher CRP than controls [3], and Hatzinikolau-Kotsakou et al. reported that the first paroxysmal episode of non-valvular AF is associated with elevated hs-CRP levels [4]. They suggested that hs-CRP may be a marker for inflammatory states that may promote perpetuation and/or initiation of non-valvular AF. Although our study was carried out in non-valvular AF patients indicated for ablation (left atrial diameter <45 mm), a statistically significant elevation of IL-6 levels was found in the AF group compared to the control group, but not CRP levels.

Our study is the first to demonstrate that patients with non-valvular AF have higher TLR2 expression levels compared to control patients. As for the medications in this study (Table 1), there was no statistical difference in each ratio of patients taking these drugs between the non-valvular AF and control groups. ACE-I, ARB, and statin, which are characterized as anti-inflammatory drugs [24], bring about depression of TLR2 expression levels [25—27]. Considering that patients taking medications in the non-valvular AF group are more than those in control group, these drugs seem not to cause the difference in TLR2 and IL-6 levels between these two groups.In our study, CRP level was not likely to reflect inflammation in the atrium of AF patients, and therefore it is believed that this gives some information about the characteristic of the inflammation. Myocarditis is most often induced by cardiotropic viruses and the clinical features are heterogeneous. Progression of myocarditis to its sequela, dilated cardiomyopathy (DCM), has been documented in 20% of cases and is pathogenically linked to chronic inflammation and viral persistence [28]. Interestingly, Sekiguchi et al. reported atrial myocarditis-like features in macrophage-infiltrated atrial endocardium during progression of paroxysmal/persistent AF in humans [1]. Our previous studies indicated that TLR2 level on monocytes has a characteristic to up-regulate

in some phases of bacterial infectious diseases and in viral infectious diseases, but not in non-infectious inflammatory diseases in the absence of any infectious complications. In particular, TLR2 expression level seems to be more sensitive to viral infections than CRP level [14,15]. We speculate that this is why TLR2 levels were significantly up-regulated in non-valvular AF patients, but not CRP levels.

As far as a result shown in Fig. 1, the possibility that the inflammation originates not from intracardia but from extracardia in non-valvular AF patients remains. Some cytokines will be secreted from inflammatory tissue, and prime the chemotaxis of monocytes. Therefore, we speculated that blood sampling site may be near to the focus of inflammation in accordance with the level of up-regulated TLR2 expression, and examined the difference in TLR2 level between paired samples from left atrium and peripheral in 25 non-valvular AF patients. The result demonstrated that TLR2 levels on monocyte from left atrium were significantly higher than those from peripheral (Fig. 4). Taken together, it is most likely that the focus of inflammation in non-valvular AF may be intracardia.

Alternatively, the LAVI significantly decreased 1 month after ablation in the patients judged as successful pulmonary vein isolation. Tops et al. previously reported a similar result [29]. In contrast, TLR2 expression levels and IL-6 levels remained elevated 1 month after ablation in the present study. Also, WBC, CRP, and PIIINP levels did not change. Consequently, we could not find any serological/biological marker to significantly change after ablation. Radiofrequency catheter ablation is a curative procedure that attempts to achieve complete four pulmonary vein isolation. This mechanical procedure is unlikely to suppress inflammation and the associated markers. The significant reduction of LAVI may be associated with the reverse remodeling of atrium due to alleviation of AF rather than the resolution of inflammation.

It is a limitation to examine the recurrence of AF in this study. Relapsed AF patients were too small to be statistically analyzed. Further study including a large number of refractory AF patients will be required.

In the present study, we examined the differences in serological/biological markers between the non-valvular AF and control groups. The result showed that TLR2 expression levels and IL-6 levels were significantly higher in non-valvular AF patients than in control patients. Subsequently, it was demonstrated that TLR2 levels were moderately correlated with IL-6 levels, but not with CRP levels. Furthermore, although the TLR2 expression levels and IL-6 levels in non-valvular AF remained elevated 1 month after successful ablation, it was demonstrated that the LAVI significantly diminished. In conclusion, our results suggest that an infectious inflammation, characterized by TLR2 up-regulation on monocytes, may participate in the pathogenesis of non-valvular AF.

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