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Angiotensin receptor-binding molecule in leukocytes in association with the systemic and leukocyte inflammatory profile

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ABSTRACT

Background and aims: The components of the renin-angiotensin system in leukocytes is involved in the pathophysiology of non-communicable diseases (NCDs), including hypertension, atherosclerosis and chronic kidney disease. Angiotensin II type 1 receptor (AT1R)-associated protein (ATRAP) is an AT1R-specific binding protein, and is able to inhibit the pathological activation of AT1R signaling in certain animal models of NCDs. The aim of the present study was to investigate the expression and regulation of ATRAP in leukocytes.

Methods: Human leukocyte *ATRAP* mRNA was measured with droplet digital polymerase chain reaction system, and analyzed in relation to the clinical variables. We also examined the leukocyte cytokines mRNA in bone-marrow ATRAP-deficient and wild-type chimeric mice after injection of low-dose lipopolysaccharide.

Results: The *ATRAP* mRNA was abundantly expressed in leukocytes, predominantly granulocytes and monocytes, of healthy subjects. In 86 outpatients with NCDs, leukocyte *ATRAP* mRNA levels correlated positively with granulocyte and monocyte counts and serum C-reactive protein levels. These positive relationships remained significant even after adjustment. Furthermore, the leukocyte *ATRAP* mRNA was significantly associated with the interleukin-1β, tumor necrosis factor-α and monocyte chemotactic protein-1 mRNA levels in leukocytes of NCDs patients. In addition, the leukocyte interleukin-1β mRNA level was significantly upregulated in bone marrow ATRAP-deficient chimeric mice in comparison to wild-type chimeric mice after injection of lipopolysaccharide.

Abbreviations: ABI, ankle-brachial index; ACE, angiotensin-converting enzyme; ATRAP, angiotensin II type 1 receptor-associated protein; AT1R, angiotensin II type 1 receptor; BM-KO, bone marrow ATRAP-deficient chimeric mice; baPWV, brachial-ankle pulse wave velocity; BM-WT, bone marrow wild-type chimeric mice; CKD, chronic kidney disease; ddPCR, reverse transcription droplet digital polymerase chain reaction; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; NCDs, Non-communicable diseases; (P)RR, (pro) renin receptor; RAS, renin-angiotensin system; RT-qPCR, real-time quantitative reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α; UACR, urinary albumin-to-creatinine ratio; UUO, unilateral ureteral obstruction.

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Conclusions: These results suggest that leukocyte ATRAP is an emerging marker capable of reflecting the systemic and leukocyte inflammatory profile, and plays a role as an anti-inflammatory factor in the pathophysiology of NCDs.

1. Introduction

Non-communicable diseases (NCDs), including hypertension, diabetes, atherosclerosis and chronic kidney disease (CKD), are major health burdens and cause significant mortality and morbidity worldwide. Recent studies have shown that chronic low-grade inflammation via dysregulation of the immune system may play a role in the pathogenesis of NCDs [1–5]. In addition, excessive activation of the renin-angiotensin system (RAS) in local tissue may mediate the development and progression of NCDs, at least in part, by provoking dysregulation of the immune system [6–8].

The existence of functional RAS with its components expression has been proposed in leukocytes and the immune system [9, 10]. In animal studies, angiotensin II type 1 receptor (AT1R) signaling in mouse bone marrow-derived cells and leukocytes affected the inflammatory status and differentiation of immune cells and was implicated in the pathogenesis of angiotensin II-induced hypertension [11,12], subsequent renal injuries [12,13] and unilateral ureteral obstruction (UUO)-induced renal fibrosis [14, 15]. Other previous studies have shown that the activation of angiotensin-converting enzyme (ACE) and renin in bone marrow-derived cells was associated with the development of atherosclerosis [16,17]. Furthermore, exaggerated activation of human leukocyte RAS components, including ACE and (pro) renin receptor ([P]RR), has also been implicated in the pathophysiology of NCDs, as has the RAS in other local tissues and organs [18,19].

We previously identified AT1R-associated protein (ATRAP) as a specific binding protein to AT1R and as a promoting molecule of AT1R internalization [20–26]. In several animal models of NCDs, we showed that the enhancement of local ATRAP expression, such as in the heart, vasculature and kidney, ameliorated tissue injury, probably through the inhibition of hyperactivation of the local tissue AT1R signaling [22–24]. Furthermore, the results of our studies showed that ATRAP deficiency lead to blood pressure elevation in a remnant kidney model and insulin resistance induced by a high-fat diet, concomitant with enhancement of inflammation markers [25, 26]. However, the expression and significance of ATRAP in leukocytes in the physiology and pathophysiology of NCDs have not been evaluated.

Therefore, in the present study, we first examined the ATRAP expression in human leukocytes of healthy subjects. We next analyzed possible relevant clinical factors affecting ATRAP expression in leukocytes of patients with NCDs. Furthermore, we examined the possible effect of ATRAP downregulation on the inflammatory profile of leukocytes in animals after low-dose lipopolysaccharide (LPS) injection, a model of low-grade inflammation in patients with NCDs [27,28], using bone marrow ATRAP-deficient chimeric mice.

2. Materials and methods

2.1. Setting and participants

For the analysis of ATRAP expression in each leukocyte fraction from healthy subjects, volunteers without any diagnosis of a chronic disease were recruited to the present study. For the analysis of clinical factors relevant to ATRAP expression in leukocytes from patients with NCDs, consecutive patients who visited the outpatient clinic at Yokohama City University Hospital, Yokohama, Japan, from April 2015 to March 2016, were recruited. The exclusion criteria were patients who (i) were aged under 19 years, (ii) had a history of corticosteroid or immunosuppressive therapy within the past 3 months, (iii) had been diagnosed with a hematological disease, (iv) were receiving renal replacement therapies and (v) had undergone any adjustments of prescribed medications for hypertension, diabetes or dyslipidemia in the past month.

Healthy subjects and patients with NCDs provided written informed consent for participation. This study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the ethics review board of Yokohama City University Hospital (No. A150122002) and was entered into the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR, www.umin.ac.jp/ctr/index/htm/, UMIN000016846).

2.2. Separation of leukocyte fractions

Approximately 20 mL of whole blood was collected in heparin-coated tubes and incubated with hemolysis solution (BD Pharm-Lyse; BD Biosciences, San Jose, CA) for 10 min at room temperature. After being washed, cells were incubated with 10% human AB serum (Access Biologicals, Vista, CA) for 20 min at 4 °C [29, 30]. For surface marker staining, cells were incubated for 30 min at 4 °C with appropriately diluted antibodies. Granulocytes were separated with FITC-*anti*-CD66b and PE-*anti*-CD16 (Biolegend, San Diego, CA). Monocytes and B- and T-lymphocytes were fractionated with FITC-*anti*-CD19, PE-*anti*-CD14, and APC/Cy7-antiCD3 (Biolegend). Granulocytes, monocytes, B-lymphocytes and T-lymphocytes were defined as CD66b+/CD16+, CD14+/CD3-/CD19-, CD14-/CD3-/CD19+ and CD14-/CD3+/CD19- cells, respectively. 7-aminoactinomycin D (Sigma-Aldrich, St. Louis, MO) was used to exclude dead cells. Cell fractions were separated on a BD FACSAria II (BD Biosciences). Total RNA was extracted using the RNeasy Micro Kit (QIAGEN, Germantown, MD), and cDNA was synthesized from 28.5 ng of total RNA using the PrimeScript RT reagent Kit (Takara-Bio, Shiga, Japan).

2.3. Gene expression analyses by reverse transcription droplet digital polymerase chain reaction and real-time quantitative reverse transcription PCR

Approximately 2.5 mL of whole blood was collected in PAX gene Blood RNA Tubes (Nippon Becton Dickinson Company, Tokyo, Japan). Total leukocyte RNA was extracted using the PAX gene Blood RNA Kit (QIAGEN) for reverse transcription, and leukocyte cDNA was synthesized from 0.25 μg of total RNA using the SuperScript III First-Strand System (Invitrogen, Carlsbad, CA).

Recent developments in the quantitative analysis of gene expression by reverse transcription droplet digital polymerase chain reaction (ddPCR) might provide an opportunity to reduce the quantitative variability seen using real-time quantitative reverse transcription PCR (RT-qPCR), particularly in human samples of

small quantity [31, 32]. Therefore, in the present study, to analyze gene expression, we employed the ddPCR method (QX200 Droplet Digital PCR system; Bio-Rad, Hercules, CA) by incubating the reverse transcription product with ddPCR Supermix for Probes (Bio-Rad) and specific TaqMan probes (Applied Biosystems, Foster City, CA). The absolute levels of the target gene were expressed as the number of copies per microgram of RNA.

Gene expression was also determined by RT-qPCR (ABI PRISM 7000 Sequence Detection System; Life Technologies, Carlsbad, CA), in which the reverse transcription product was incubated with qPCR Mastermix Plus Low ROX (NIPPON GENE, Tokyo, Japan) and specific TaqMan probes (Applied Biosystems), as described [22]. In the RT-qPCR analysis, the expression was normalized to 18S ribosomal RNA. The TaqMan probe IDs are listed in Supplementary Table 1.

2.4. Clinical variables

Clinical information was obtained from the data collected at a single visit to our outpatient clinic. Hypertension was defined as office blood pressure >140/90 mmHg or receiving medical treatment for hypertension. Diabetes mellitus was defined as an HbA1c value $\geq$ 6.5% (NGSP) or receiving medical treatment for diabetes mellitus. Dyslipidemia was defined as LDL-cholesterol >140 mg/dL, triglyceride >150 mg/dL, HDL <40 mg/dL for males and <50 mg/dL for females [33], or use of lipid-lowering medications, including statins and ezetimibe. The estimated glomerular filtration rate (eGFR) was calculated using a modified three-variable equation for GFR in Japanese patients [34]: $eGFR = 194 \times age^{-0.287} \times serum\ creatinine\ level^{-1.094}$ ($\times 0.739$, if female). Urinary excretion was determined by a spot urine analysis and was standardized to urinary creatinine concentrations. CKD was defined as urinary albumin-to-creatinine ratio (UACR) $\geq$ 30 mg/gCr or eGFR < 60 mL/min/1.73 m^2. The ankle-brachial index (ABI) and brachial-ankle pulse wave velocity (baPWV) were measured as described [35, 36].

2.5. Animals, generation of bone marrow chimeric mice and low-dose LPS injection

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals and was reviewed and approved by the Animal Studies Committee of Yokohama City University. Male C57BL/6 wild-type mice were purchased from Charles River Laboratories. We used ATRAP-knockout mice on a C57BL/6 background, which had previously been generated using a targeted gene disruption strategy, as described previously [25, 26].

Low-dose LPS (0.25 mg/kg, L6529, Sigma-Aldrich) was intraperitoneally injected into male C57BL/6 wild-type mice (in a single dose) under isoflurane anesthesia [28]. The mice were then sacrificed at 4, 24 and 72 h after the injection of LPS to collect blood samples. Blood samples from mice of the same age that had not been injected with LPS were used as baseline control group.

Recipient male wild-type mice 8 weeks of age were lethally irradiated with 9.5 Gy. Bone marrow cells were harvested by flushing the femurs and tibias from donor wild-type mice and donor ATRAP-knockout mice. Within 24 h after irradiation, the recipient mice were injected via the tail vein with 1×10^6 bone marrow cells suspended in 0.4 mL RPMI1640 (Nacalai Tesque, Kyoto, Japan) from either wild-type or ATRAP-knockout mouse donors. The bone marrow genotype was confirmed by examination of the *ATRAP* gene expression in peripheral leukocytes using PCR, as previously described [37]. The primer sequences used are shown in Supplementary Table 1.

Eight weeks after bone marrow transplantation, low-dose LPS (0.25 mg/kg, L6529, Sigma-Aldrich) was intraperitoneally injected into recipient mice on 3 consecutive days under isoflurane anesthesia [28]. The recipient mice were then sacrificed at 4 h after the last LPS injection to collect blood samples.

Total leukocyte RNA was extracted using the Leukocyte RNA Purification Kit (Norgen Biotek, Thorold, Canada). Leukocyte cDNA was synthesized, and gene levels were measured using RT-qPCR as described above. The TaqMan probe IDs are listed in Supplementary Table 1.

2.6. Statistical analyses

Continuous variables were presented as mean ± standard deviation. For comparisons between two groups, Student's *t*-test or Mann-Whitney *U* test was used as appropriate. Correlations between two variables were evaluated by Spearman's correlation, because the expression of leukocyte RAS components showed a non-Gaussian distribution. Absolute levels of leukocyte ATRAP were analyzed by linear regression using the forced entry method to determine the effects of other clinical variables. The explanatory variables were those that were considered clinically relevant. Data were analyzed using the SPSS software program, ver. 23 (IBM Inc., Armonk, NY) and GraphPad PRISM 5J (GraphPad Software, La Jolla, CA). *p*-values <0.05 were considered to be statistically significant.

3. Results

3.1. Quantitative analyses of ATRAP expression in human leukocytes from healthy subjects by ddPCR

We first analyzed the endogenous *ATRAP* mRNA expression in human leukocytes from healthy subjects by ddPCR and confirmed the close positive correlation between absolute ATRAP levels measured using ddPCR and the relative expression of *ATRAP* to *18S* ribosomal RNA as measured by RT-qPCR ($r = 0.967$, $p < 0.0001$, Supplementary Fig. 1).

We next analyzed the expression of ATRAP in each leukocyte fraction from four healthy subjects. The results showed that *ATRAP* mRNA expression was more abundant in granulocytes and monocytes than in B- and T-lymphocytes (Fig. 1).

3.2. Analyses of clinical factors potentially influencing the ATRAP expression in leukocytes from patients with NCDs

Characteristics of the study participants including 86 patients with NCDs are summarized in Table 1. With regard to their medical histories, 82 (95%) had hypertension, 24 (28%) had diabetes, 65 (76%) had dyslipidemia, 54 (63%) had CKD and 13 (15%) had cardiovascular disease. The blood pressure was well controlled with antihypertensive agents, the mean number of medications of which was 1.8 ± 1.2. Of these patients, urinalyses were performed in 85 (99%), and indices of arterial stiffness, including ABI and baPWV, were measured in 58 (67%).

To examine the clinical variables associated with leukocyte ATRAP expression, we compared the clinical characteristics of patients with lower and higher levels of leukocyte ATRAP gene expression, as measured by the ddPCR system (Table 1). The median ATRAP level in our cohort was 2,042,000 copies/μg RNA, and patients were stratified into two groups according to this value. The age, granulocyte and monocyte counts and serum high-sensitivity C-reactive protein (hsCRP) levels were significantly higher in patients with higher leukocyte ATRAP expression. These patients also tended to have more severe renal injury, as evidenced by a lower eGFR and higher UACR and urinary tubule marker levels, including β2 microglobulin, N-acetyl-β-D-glucosaminidase and liver-type

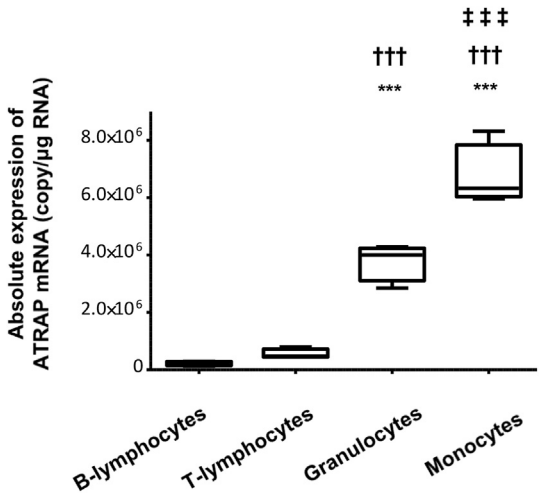

Fig. 1. *ATRAP* expression in each leukocyte fraction from healthy volunteers (N = 4). One-way ANOVA and Bonferroni analyses. ***$p < 0.001$ *vs.* B-lymphocytes. †††$p < 0.001$ *vs.* T-lymphocytes. ‡‡‡$p < 0.001$ *vs.* granulocytes.

fatty acid-binding protein. In contrast, the blood pressure, glucose-lipid metabolism status and indices of arterial stiffness were comparable between these groups.

Since these results strongly suggested that the leukocyte ATRAP expression was associated with age, the inflammatory status and parameters of renal injury, we analyzed the absolute leukocyte ATRAP expression levels by a multiple linear regression analysis using these clinical variables and other clinical variables, including BMI and smoking status, which are important and modifiable factors in the management of NCDs (Table 2). In the univariate analysis, age, granulocyte count, monocyte count and hsCRP correlated significantly with the absolute ATRAP levels. In the multivariable models, granulocyte count, monocyte count and hsCRP were associated with the absolute leukocyte ATRAP levels, independent of other clinical variables.

Because ATRAP expression was correlated with inflammatory parameters, especially hsCRP, we examined the relationship between hsCRP and the expression of other RAS components (ACE and [P]RR) in leukocytes (Fig. 2A–C). In contrast to leukocyte ATRAP, ACE and (P)RR expression in leukocytes did not correlate

Table 1
Patient characteristics.

Variable		All patients (N = 86)	Low *ATRAP* mRNA (N = 43)	High *ATRAP* mRNA (N = 43)	*p*-value
Age	years	63 ± 15	60 ± 15	66 ± 14	0.039
Sex; male	n (%)	53 (62)	24 (56)	29 (67)	0.270
Body mass index	kg/m^2	24.9 ± 4.4	25.6 ± 4.9	24.2 ± 3.8	0.133
Current smoker	n (%)	15 (17)	7 (16)	8 (19)	0.658
Medication					
ACE–I/ARB	n (%)	54 (63)	26 (61)	28 (65)	0.657
α–blocker	n (%)	5 (6)	2 (5)	3 (7)	0.647
β–blocker	n (%)	11 (13)	4 (9)	7 (16)	0.336
CCB	n (%)	59 (69)	28 (65)	32 (74)	0.350
Diuretics	n (%)	22 (26)	11 (26)	11 (26)	1.000
Insulin	n (%)	5 (6)	1 (2)	4 (9)	0.169
OHA	n (%)	16 (19)	7 (16)	9 (21)	0.582
Statin	n (%)	30 (35)	18 (42)	12 (28)	0.177
Ezetimibe	n (%)	10 (12)	3 (7)	7 (16)	0.181
Blood pressure and renal function					
SBP	mm Hg	140 ± 19	139 ± 19	141 ± 19	0.578
DBP	mm Hg	82 ± 12	83 ± 12	81 ± 12	0.594
eGFR	mL/min/1.73m^2	61 ± 25	67 ± 24	55 ± 25	0.068
eGFR <60 mL/min/1.73 m^2	n (%)	39 (45)	17 (40)	22 (51)	0.386
Uric acid	mg/dL	6.0 ± 1.4	6.2 ± 1.3	5.8 ± 1.4	0.387
Glucose and lipid metabolism					
Glucose	mg/dL	119 ± 26	120 ± 28	117 ± 23	0.904
HbA1c	%	6.1 ± 0.9	6.1 ± 0.9	6.0 ± 1.0	0.649
Triglyceride	mg/dL	176 ± 145	206 ± 186	146 ± 77	0.110
HDL-cholesterol	mg/dL	58 ± 17	58 ± 17	59 ± 17	0.896
LDL-cholesterol	mg/dL	112 ± 40	116 ± 46	107 ± 33	0.339
CBC and inflammation-related					
Leukocyte count	/μL	6510 ± 1723	5942 ± 1512	7079 ± 1750	0.001
Granulocyte count	/μL	3909 ± 1346	3358 ± 1109	4474 ± 1344	<0.001
Monocyte count	/μL	478 ± 182	411 ± 147	545 ± 190	0.001
Lymphocyte count	/μL	1838 ± 562	1885 ± 553	1790 ± 575	0.453
hsCRP	mg/dL	0.37 ± 1.00	0.18 ± 0.50	0.56 ± 1.30	0.006
Variable		All patients (N = 85)	Low ATRAP mRNA (N = 42)	High ATRAP mRNA (N = 43)	*p*-value
Urinalysis					
UACR	mg/gCr	287 ± 714	233 ± 733	339 ± 700	0.067
β2 microglobulin	μg/gCr	5486 ± 21897	1075 ± 2177	9771 ± 30271	0.142
NAG	U/gCr	8.8 ± 7.0	7.4 ± 5.9	10.0 ± 7.9	0.124
L–FABP	μg/gCr	7.2 ± 12.8	4.4 ± 9.8	8.3 ± 14.2	0.285
Variable		All patients (N = 58)	Low ATRAP mRNA (N = 29)	High ATRAP mRNA (N = 29)	*p*-value
Arterial stiffness					
ABI		1.13 ± 0.10	1.13 ± 0.11	1.14 ± 0.08	0.994
baPWV	m/sec	1676 ± 421	1685 ± 444	1667 ± 403	0.926

ACE–I, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blockade; OHA, oral hypoglycemic agent; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL, high density lipoprotein; LDL, low density lipoprotein; CBC, complete blood count; hsCRP, serum high sensitivity C–reactive protein; UACR, urinary albumin–to–creatinine ratio; NAG, N–acetyl–β–D-glucosaminidase–to–creatinine ratio; L–FABP, urinary liver–type fatty acid–binding protein–to–creatinine ratio; ABI, ankle brachial pressure index; baPWV, brachial–ankle pulse wave velocity.

Table 2
Multiple linear regression analysis of absolute levels of ATRAP as measured using ddPCR.

Variable	Univariate		Multivariate					
	r	*p*-value	Model 1		Model 2		Model 3	
			Standardized β	*p*-value	Standardized β	*p*-value	Standardized β	*p*-value
Age (years)	0.235	0.030	0.211	0.072	0.199	0.124	0.207	0.121
Sex; male	0.158	0.146	0.113	0.210	0.070	0.506	0.151	0.144
Body mass index (kg/m^2)	−0.128	0.244	−0.224	0.031	−0.110	0.310	−0.038	0.731
Current smoking (yes)	−0.016	0.884	0.061	0.521	0.049	0.639	0.032	0.768
eGFR (mL/min/1.73 m^2)	−0.138	0.204	0.052	0.664	0.030	0.822	0.063	0.651
UACR (mg/gCr)	0.198	0.069	−0.044	0.661	−0.026	0.812	0.004	0.975
Granulocyte count (/μL)	0.517	<0.001	0.437	<0.001	–	–	–	–
Monocyte count (/μL)	0.446	<0.001	–	–	0.237	0.036	–	–
Lymphocyte count (/μL)	−0.147	0.195	–	–	–	–	−0.104	0.327
hsCRP (mg/dL)	0.243	0.024	0.454	<0.001	0.446	<0.001	0.509	<0.001
			$R^2 = 0.447$		$R^2 = 0.323$		$R^2 = 0.286$	

ATRAP, angiotensin II type 1 receptor–associated protein; ddPCR, droplet digital polymerase chain reaction; eGFR, estimated glomerular filtration rate; UACR, urinary albumin-to-creatinine ratio; hsCRP; serum high-sensitivity C-reactive protein; β, standardized β.

significantly with hsCRP. The leukocyte *ATRAP* expression was closely correlated with the expression of interleukin-1β (*IL-1β*, r = 0.791, $p < 0.001$), tumor necrosis factor-α (*TNF-α*, r = 0.728, $p < 0.001$) and monocyte chemotactic protein-1 (*MCP-1*, r = 0.544, $p < 0.001$) in leukocytes in our patients (Fig. 2D–F).

3.3. Analyses of the possible effects of ATRAP downregulation on the inflammatory profile of leukocytes after LPS injection using bone marrow ATRAP-deficient chimeric mice

To further investigate the relationship between leukocyte ATRAP expression and inflammation, we next examined the effects of the injection of low-dose LPS on leukocyte ATRAP and inflammatory cytokine expression in wild-type mice. The injection of LPS enhanced the expression of both leukocyte ATRAP and IL-1β mRNA in wild-type mice (Fig. 3A). These findings indicate that leukocyte ATRAP expression is associated with inflammation in both humans and mice.

To analyze the causal relationships between leukocyte ATRAP expression and the inflammatory profile, we examined the expression of several genes in leukocytes in bone marrow chimeric mice after LPS injection. We confirmed the replacement of the bone

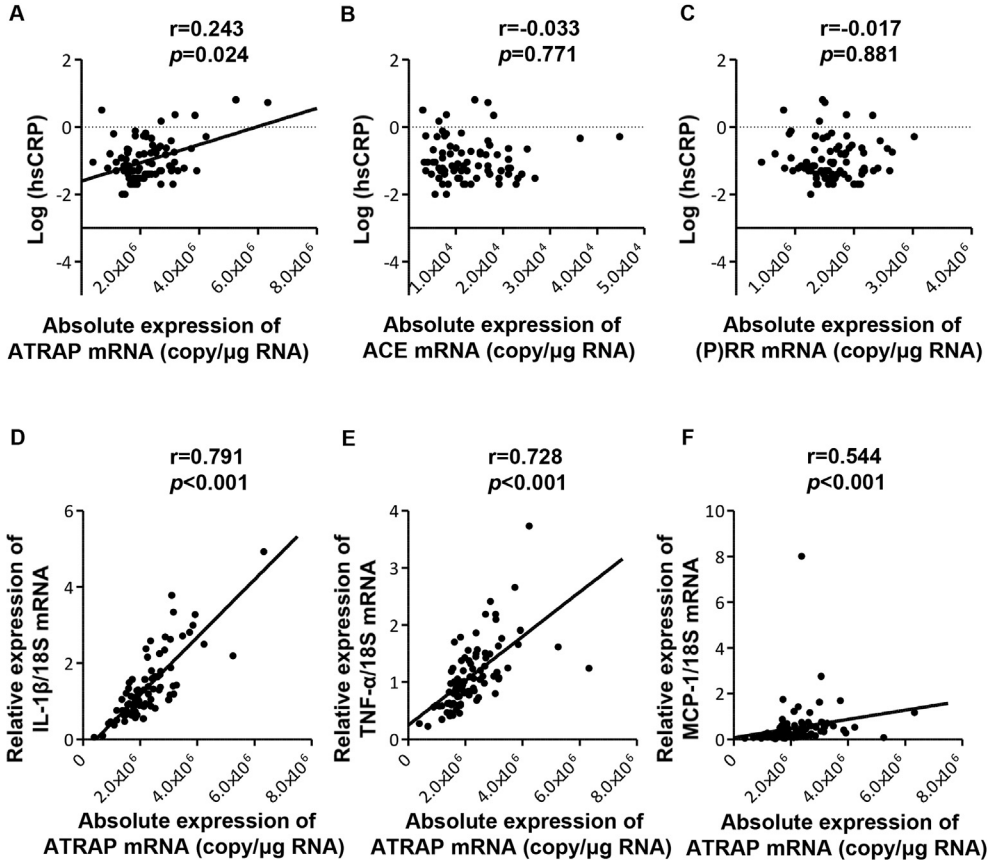

Fig. 2. Correlations between leukocyte RAS components and inflammatory status.
(A-C) Relationship between hsCRP and absolute levels of leukocytic (A) ATRAP, (B) ACE and (C) (P)RR in 86 patients with NCDs. (D-F) Relationship between absolute levels of leukocytic *ATRAP* and relative expression of leukocytic (D) *IL-1β*, (E) *TNF-α* and (F) *MCP-1* in 86 patients with NCDs. Spearman's correlation analysis.

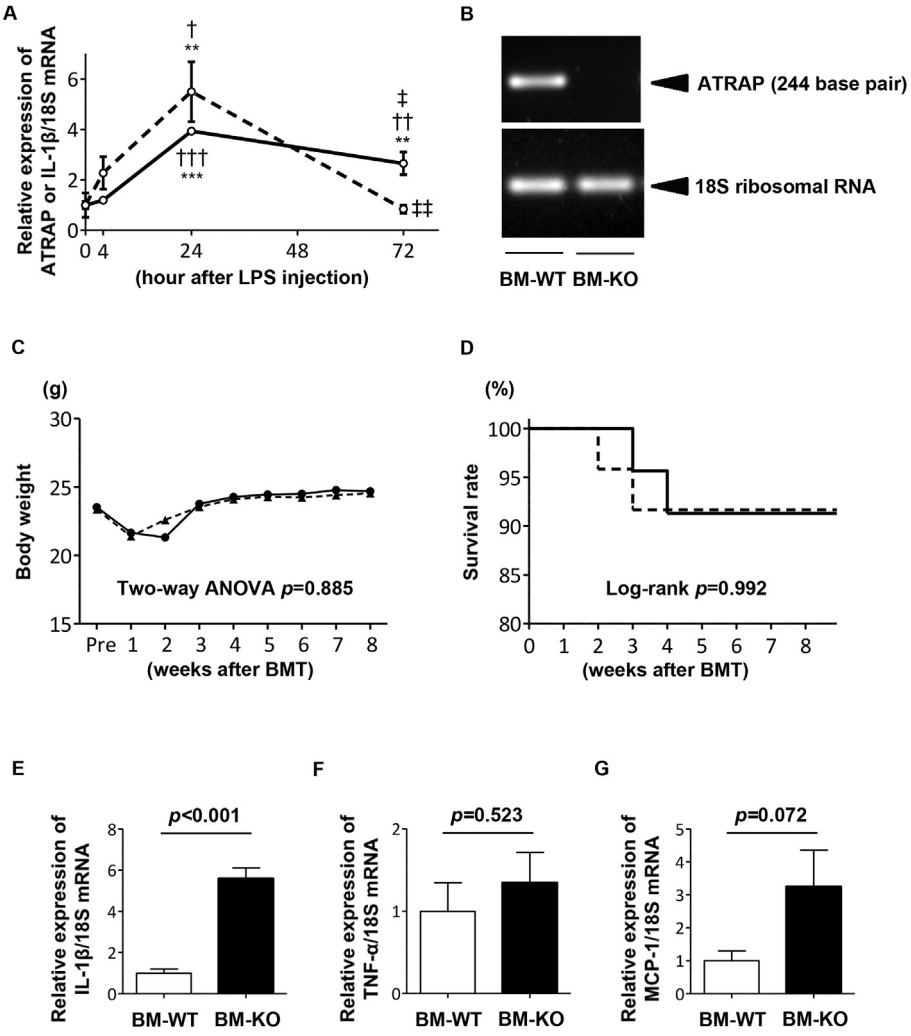

Fig. 3. A mouse model of inflammation induced by the injection of low-dose LPS.
(A) Time course of gene expression of *ATRAP* and *IL-1β* in leukocyte after injection of a single dose of LPS (0.25 mg/kg). The solid and dotted lines indicate the *ATRAP* and *IL-1β* gene expression, respectively. Each mRNA level is normalized to each baseline value. N = 4 in each time point. One-way ANOVA and Bonferroni analyses. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* Baseline control. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ *vs.* 4 h $^{\ddagger}p < 0.05$, $^{\ddagger\ddagger}p < 0.01$ *vs.* 24 h. (B) Representative *ATRAP* and *18S* ribosomal RNA gene expression in peripheral leukocytes from BM-WT and BM-KO mice before low-dose LPS injection. (C) Change in body weight after bone marrow transplantation. The solid and dotted lines indicate the body weight in BM-WT and BM-KO mice, respectively. Two-way ANOVA. (D) Survival analysis after bone marrow transplantation. The solid and dotted lines indicate the survival rate in BM-WT and BM-KO mice, respectively. Log-rank test. (E-G) Leukocyte gene expression of inflammatory cytokines and chemokines in bone marrow chimeric mice after low-dose LPS injection. (E) *IL-1β*, (F) *TNF-α* and (G) *MCP-1*. N = 4 to 5 in each group, unpaired-*t* test. BM-WT, bone marrow wild-type chimeric mice; BM-KO, bone marrow ATRAP-deficient chimeric mice.

marrow genotype by an examination for the ATRAP gene expression of peripheral leukocytes before LPS injection (Fig. 3B). There was no significant difference in the survival rate or body weight gain between bone marrow wild-type chimeric mice (BM-WT) and bone marrow ATRAP-deficient chimeric mice (BM-KO) after bone marrow transplantation (Fig. 3C and D). After low-dose LPS injection, the leukocyte expression of IL-1β was higher in BM-KO than in BM-WT mice (N = 4 to 5 in each group, $p < 0.001$), as was the expression of MCP-1 ($p = .072$, Fig. 3B–D). These results indicated that the low-dose LPS-mediated upregulation of leukocyte IL-1β was exacerbated in bone marrow ATRAP-deficient chimeric mice in comparison to wild-type chimeric mice.

4. Discussion

Recent developments in the field of quantitative analysis of gene expression by ddPCR may provide the opportunity to reduce the quantitative variability currently seen using RT-qPCR analyses and obtain absolute values instead of relative levels, particularly in human samples of small quantity [31, 32]. Quantifying absolute levels is particularly useful in the clinical setting, allowing for the establishment of cut-offs for predicting the clinical endpoints and the observation of the trajectory of clinical markers without any reference samples for calibration. In the present study, precisely analyzing the gene expression in human leukocytes was crucial. A major advantage of this study was that we measured the absolute levels of genes using the ddPCR method.

We measured leukocyte *ATRAP* mRNA level, but not the protein level. With respect to the *in vivo* regulation of ATRAP expression, we previously reported that a high-salt diet reduced the renal mRNA and protein levels of ATRAP, and accelerated the progression of hypertensive kidney injury in Dahl salt-sensitive rats [38]. We also showed that chronic angiotensin II infusion reduced the mRNA and protein levels of ATRAP in the heart and kidneys of mice, and accelerated the progression of hypertension and cardiac hypertrophy [22, 39]. In UUO-treated mice, renal mRNA and protein levels of ATRAP were decreased as renal fibrosis progressed [40]. Another group also reported that ATRAP mRNA and protein levels decreased

in the injured femoral artery of mice [41]. On the other hand, it was reported that bortezomib, a proteasome inhibitor, could affect the ATRAP protein level in a post-translational manner [42]. These results indicate that *ATRAP* mRNA level closely reflects ATRAP protein level except under specific conditions, such as proteasome inhibitor treatment.

To our knowledge, this is the first study to examine the expression and distribution of ATRAP in leukocytes and its clinical relevance in patients with NCDs. Here, we demonstrated for the first time that ATRAP was abundantly expressed in human leukocytes, predominantly in monocytes and granulocytes, from healthy subjects. We also showed that leukocyte ATRAP expression positively correlated with inflammatory parameters, such as the granulocyte and monocyte count, hsCRP and proinflammatory cytokine and chemokine levels in leukocytes of patients with NCDs. Furthermore, low-dose LPS enhanced leukocyte *ATRAP* and *IL-1β* gene expression in wild-type mice. In addition, after injection of low-dose LPS, the level of leukocyte IL-1β in bone marrow ATRAP-deficient chimeric mice was significantly upregulated in comparison to control wild-type chimeric mice, a model of low-grade inflammation in patients with NCDs [27]. These results suggest that ATRAP expression in leukocytes is closely linked to systemic inflammation and the leukocyte inflammatory status and that there is likely a compensatory upregulation of leukocyte ATRAP expression to improve the inflammatory profile in response to pathological stimuli in patients with NCDs.

RAS in local tissues is reportedly associated with the inflammatory status [8, 43]. The serum hsCRP level is a well-established inflammatory marker that predicts the incidence and progression of NCDs, including hypertension [44], cardiovascular diseases [45, 46] and CKD [47]. However, previous studies on the relationship between leukocyte expression of RAS components and hsCRP have reported inconsistent findings [48–50]. A study reported that expression of RAS components in dendritic cells from patients with or without coronary artery disease correlated significantly with hsCRP levels [48]. Furthermore, peak AT1R and ACE levels in T-lymphocytes after angiotensin II stimulation were associated with hsCRP in normotensive and hypertensive patients [49]. In contrast, leukocyte AT1R expression did not correlate with hsCRP in healthy subjects and CKD patients in other study [50]. Although these inconsistent results might be due to differences in patient populations, medication status and methods for measuring RAS components in leukocytes, no studies have yet identified any RAS component that consistently reflects the circulating hsCRP levels and systemic inflammatory profile. Based on these previous findings and the results of the present study, we propose that leukocyte ATRAP is an emerging marker capable of reflecting the systemic and leukocyte inflammatory profile.

The proinflammatory cytokines and chemokines, including IL-1β, TNF-α and MCP-1, mediate acute and chronic inflammation and play a role in the development of hypertension [2, 51–53], cardiovascular diseases [4, 54] and renal injury [55, 56] in animal models and humans. Concerning the possible link between leukocyte RAS components and these proinflammatory cytokines, MCP-1 and IL-1β were significantly upregulated in the kidney in angiotensin II-infused chimeric mice that lacked AT1R in the bone marrow compared with control animals [12]. In addition, macrophage-specific AT1R deficiency exacerbated UUO-induced renal fibrosis through the activation of the IL-1 receptor pathway [15]. The results of the present study, which used bone marrow-transplanted mice, showed that leukocyte IL-1β expression was significantly upregulated in bone marrow ATRAP-deficient mice, suggesting that leukocyte ATRAP level increases to mitigate inflammation.

However, no significant associations between leukocyte ATRAP and blood pressure, diabetes or atherosclerosis were observed, probably because the NCDs patients in the present study were being treated for cardiovascular risk factors, including hypertension, diabetes and dyslipidemia, which were well controlled. The causal relationships between leukocyte ATRAP and the incidence of NCDs and subsequent target organ injuries need to be further examined in a future prospective study, with investigations to determine the functional significance of leukocyte ATRAP at the molecular level.

The present study has several limitations. First, we are not able to clearly state the advantage of the measurement of leukocyte *ATRAP* mRNA in comparison to the measurement of established inflammatory markers, such as hsCRP, in the clinical setting. To clarify this issue, we will examine the clinical significance of the absolute leukocyte *ATRAP* mRNA level, as measured by ddPCR, with regard to the mortality and/or cardiovascular outcome in a future longitudinal study. Second, it is not clear whether human serum, as a blocking solution for Fc receptor, affects leukocyte gene expression in experiments in which leukocytes are sorted using flow cytometry.

In conclusion, we herein showed that leukocyte ATRAP expression was linked to systemic inflammation and leukocyte inflammatory profile in patients with NCDs. Furthermore, ATRAP deficiency in the bone marrow of mice resulted in the upregulation of leukocyte IL-1β after injection of low-dose LPS, as a model of low-grade inflammation in patients with NCDs. These results strongly support leukocyte ATRAP as an emerging marker that closely reflects systemic and leukocyte inflammatory status and further suggest that leukocyte ATRAP plays a role as a possible anti-inflammatory factor in the pathophysiology of NCDs. Further studies are needed to determine the functional significance of leukocyte ATRAP in NCDs.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

K.H., H.W., A.Y., T.T. and K.T. designed research; K.A., K.U., S.H., R.K., K.O., S.K., M.O., S.M., T.I. and H.N. recruited the patients; K.H., D.K., W.K. and M.M. performed experiments; K.H., K.A. and N.T. analyzed the data; K.H., H.W., K.A., T.Y. and K.T. wrote the manuscript; H.W., K.A. and K.T. had primary responsibility for final content. All authors have reviewed the final manuscript and approved the submission to this journal.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2018.01.013.

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