

Original Article

Alteration of circulating innate lymphoid cells in patients with atherosclerotic cerebral infarction

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Abstract: Innate lymphoid cells (ILCs) are associated with innate immunity and tissue remodeling. However, the changes in ILCs and their role in acute cerebral infarction (ACI) remain unexplored. This study aimed to examine the expression of ILCs in patients with ACI and explore the mechanism underlying changes in ILCs induced by the atherosclerotic factor oxidized low-density lipoprotein (ox-LDL). The levels of ILC1, ILC2, and ILC3 in the blood of patients with ACI and controls were examined at the time of admission. The correlation of serum levels of ox-LDL and inflammatory biomarkers with the level of ILCs and the effects of ox-LDL on ILCs *in vitro* were analyzed. Our results showed that the levels of ILC1 increased while the levels of ILC2 decreased in patients with ACI compared with controls. Serum levels of ox-LDL, LDL-C, and biochemical biomarkers correlated positively with the levels of ILC1 and ILC1/ILC2 ratio but negatively with the levels of ILC2. The *in vitro* incubation of peripheral blood mononuclear cells (PBMC) with ox-LDL resulted in an elevation of the levels of ILC1s and a marked reduction in the levels of ILC2s in a dose-dependent manner. ILC1s and ILC2s were more susceptible to ox-LDL-mediated alterations in patients with ACI than in controls. Furthermore, the expression of Interleukin 18 (IL-18), IL-33 and IL-23 in PBMCs was detected by real-time PCR, which showed the change trends of related key cytokines were highly consistent with the variation of ILC subsets. These results suggested that the levels of ILC1s and ILC2s appeared to be a novel, sensitive indicator for diagnosing ACI. Ox-LDL directly affected ILC1s and ILC2s, thus contributing to the alternations of ILC1 and ILC2 and occurrence of ACI.

Keywords: Atherosclerosis, biomarkers, cerebral infarction, innate lymphoid cells, oxidized low-density lipoprotein

Introduction

Acute cerebral infarction (ACI) is a disease with high rates of incidence, mortality, and disability worldwide and is a serious threat to human health. Atherosclerosis (AS) is a chronic inflammatory disease that is an important risk factor for ACI in which immune cells, particularly innate immunity, are crucial in atherogenesis [1, 2].

ACI occurs because of plaque erosion or rupture, and lymphocytes are crucial in this pro-

cess [3]. AS and plaque rupture are the major causes of ACI [4]. Innate lymphoid cells (ILCs) have recently emerged as important effector cells in both protective immunity against pathogens and immune/inflammatory diseases [5, 6]. ILCs have three subsets and secrete a distinct group of cytokines. The pattern of cytokine production corresponds approximately to that of its T-cell counterpart: ILC1s secrete Th1-related cytokines, ILC2s secrete Th2-related cytokines, and ILC3s secrete Th17-related cytokines. However, the expression and roles of ILCs in ACI are unknown. Oxidized low-density

Table 1. The sequences of the primer pairs used for cDNA quantitation of real-time PCR

Primer	Sequence (5'-3')
IL-18-F	CCTGGAATCAGATTACTTTGGC
IL-18-R	TATGGTCCGGGTGCATTA
IL-33-F	AGGTGACGGTGTGATGGT
IL-33-R	CTGGTCTGGCAGTGGTTT
IL-23-F	CTGTGGGCCAGCTTCATGC
IL-23-R	TGGAGGCTGCGAAGGATTT
GAPDH-F	AATCCCATCACCATCTTCCA
GAPDH-R	AAATGAGCCCCAGCCTTCT

lipoprotein (ox-LDL) is a key contributor to the initiation and development of AS and leads to endothelial dysfunction and plaque destabilization through multiple mechanisms [7, 8]. Whether ox-LDL has direct effects on ILCs is also not clear.

The present study aimed to examine the expression of ILCs and the mechanisms underlying the elevation of ILCs in ACI.

Materials and methods

Human subjects

The present study was approved by the Medical Research Ethics Committee of Anhui Provincial Hospital, and all patients gave written informed consent prior to enrollment.

This study was cross-sectional and blinded. Patients at Anhui Provincial Hospital were examined between March 2017 and June 2017. Group 1 consisted of 40 male and 23 female patients with ACI (mean age: 61.9 ± 9.8 years). The diagnosis was based on a modification of the TOAST criteria based on the clinical, radiographic, and diagnostic information available [9]. Group 2 consisted of controls (38 male and 22 female; mean age, 57.6 ± 10.5 years), who were selected based on recent angiography showing normal carotid arteries. No evident differences in age were reported between the two groups.

No patient was treated with anti-inflammatory drugs and/or immunosuppressive agents. None had subarachnoid, extradural, or subdural hemorrhage; brain abscess; surgery; trauma; thromboembolism; disseminated intravascular coagulation; advanced liver disease; renal failure; malignant disease; other inflammatory diseases; or chronic immune-mediated disorders.

Blood samples

Peripheral blood (PB; 5-10 mL) was collected from all patients, in a fasting state, on the morning following admission. The time interval between symptom onset and blood sampling was less than 24 h in all cases. All samples were treated with sodium heparin and examined within 4 h. Peripheral blood mononuclear cells (PBMCs) were prepared using the Ficoll density gradient for flow cytometry (FCM) and reverse transcription-polymerase chain reaction (RT-PCR). Serum was obtained after centrifugation and stored at -80°C until further use.

Detection of ILCs by flow cytometry

For analyzing regulatory T cells, cell surface staining was performed using fluorescein isothiocyanate-conjugated anti-CD45 (HI30 clone, Biolegend, CA, USA); phycoerythrin (PE)-conjugated anti-CD3 (B1.49.9 clone, Biolegend); PE-anti-CD19, CD20, CD14, CD16, CD56, CD11b, CD11c, CD123, and FcγRIa; PerCP-Cy5.5-anti-CD127; PE-cy7-conjugated anti-CD294 (CRTH2, BM16 clone, Biolegend); Brilliant Violet 421 (BV421)-anti-CD117, Alexa Fluor 700-anti-CD161, and APC anti-CD336 (NKp44, Biolegend); and appropriate isotype controls for 20 min at room temperature in the dark, followed by washing with phosphate-buffered saline solution. Stained cells were assessed by FCM using an FACS Aria II flow cytometer with BD FACSDiva Software (Becton Dickinson, CA, USA). The levels of ILC1 (CD161⁺CD294⁻), ILC2 (CD294⁺), and ILC3 (CD117⁺CD294⁻) were expressed as a percentage of CD45⁺Lin⁻CD127⁺ cells by sequential gating on lymphocytes, CD45⁺, and Lin⁻CD127⁺ cells. One million CD45⁺ cells were collected in each sample.

Real-time PCR

Total RNA was extracted with TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The amount and purity of the obtained RNA was determined by measurements of the optical density (OD) at 260 and 280 nm. Reverse transcription was performed with RNA PCR Kit (TransGen Biotech, Switzerland). Gene expression levels of cytokines were determined with SYBR Green Master Mix (Invitrogen) on an ABI Prism 7500 sequence Detection System (Applied Biosystems, USA). For each sample, mRNA expression level was normalized to the

Table 2. Patient characteristics

Characteristic	Control (n = 63)	ACI (n = 60)
Age	57.6 ± 10.5	61.9 ± 9.8
Gender (male/female)	40/23	38/22
Hypertension, n (%)	25 (39.7)	29 (48.3)
Smoking rate, n (%)	20 (31.7)	22 (36.7)
FBG (mmol/L)	4.94 ± 0.39	6.61 ± 1.03 [▲]
TC (mmol/L)	4.83 ± 0.62	5.32 ± 0.58 [*]
TG (mmol/L)	1.42 ± 0.65	1.73 ± 0.86
HDL-C (mmol/L)	1.38 ± 0.59	1.09 ± 0.35
LDL-C (mmol/L)	2.76 ± 0.63	3.12 ± 0.48 [*]
VLDL-C (mmol/L)	0.83 ± 0.26	1.01 ± 0.57

Values are expressed as mean ± SD or number. ACI, Acute cerebral infarction; FBG, fasting blood glucose; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, total triglyceride; VLDL-C, very-low-density lipoprotein-cholesterol. ^{*}*P* < 0.05 versus control; [▲]*P* < 0.01 versus control.

level of GAPDH housekeeping genes. The primers were purchased from Sangon Biotech. See the primer pairs in **Table 1**.

Measurement of ox-LDL, blood biochemistry, and biomarkers

The serum levels of ox-LDL were examined by enzyme-linked immunosorbent assay (ELISA) and measured at 450 nm on a Biocell HT1 ELISA microplate reader (ox-LDL ELISA kits from Uscn Life, TX, USA). The minimal detectable level was 4.5 µg/L for ox-LDL. The intra- and interassay coefficients of variation for all ELISAs were less than 5%. All samples were measured in duplicate.

Blood sugar, lipids, and homocysteine (Hcy) levels were determined by the enzymatic method. Lipoprotein (a) [Lp(a)] and C-reactive protein (CRP) levels were measured by the immunoturbidimetric method. All assays were conducted on an Olympus AU2700 biochemical autoanalyzer (Olympus, Japan).

Effects of ox-LDL on ILCs

Preparation of LDL and ox-LDL: Blood for lipoprotein isolation was collected from controls after 12-h fasting. LDL was isolated by the ultracentrifugation of serum, as previously described [10]. The LDL oxidation assay was performed as previously described [11], and the lipid hydroperoxide content was then determined using the FOX assay [12].

In vitro experiments with Ox-LDL

Human PBMCs (1.0×10^6 cells/mL) from controls (*n* = 5) were incubated with a culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum), including various levels of ox-LDL (0, 1, 5, 10, 50, and 100 µg/mL) for 24 h *in vitro*. After incubation, the level of ILC was measured, as described earlier.

PBMCs (*n* = 6 from each group) were incubated with 10 µg/mL ox-LDL for 24 h *in vitro*, and the level of ILC was measured using FCM to compare the effects of ox-LDL on ILCs from patients with ACI and controls.

Statistical analysis

Values were expressed as the mean ± standard deviation. Data were analyzed using statistical software (SPSS 13.0; LEAD Technologies, Inc., IL, USA). Statistical significance for the differences in the groups was assessed using a two-tailed *t* test. Spearman's correlation was used as a test of correlation between two continuous variables. All the *P* values less than 0.05 were considered statistically significant.

Results

Patients and controls

No significant differences were reported in age, gender, hypertension, smoking rate, total triglyceride, and levels of high-density lipoprotein-cholesterol and very-low-density lipoprotein-cholesterol between the two groups. However, fasting blood glucose, total cholesterol, and low-density lipoprotein-cholesterol (LDL-C) levels were significantly higher in patients with ACI than in the controls (*P* < 0.05 and *P* < 0.01, respectively, **Table 2**).

Identification of ILC1, ILC2, and ILC3 by FCM in human PB

Compared with T cells, ILCs do not express antigen receptors or undergo clonal selection and expansion. Instead, ILCs react promptly to signals from infected or injured tissues and produce cytokines, which direct an early immune response. According to the expression of CD-294 (CRTH2) and CD117, CD127⁺ ILC can be divided into three subsets: ILC1 (CD117⁺CD-161⁺CD294⁻), ILC2 (CD294⁺), and ILC3 (CD294⁻CD117⁺) cells (**Figure 1A**).

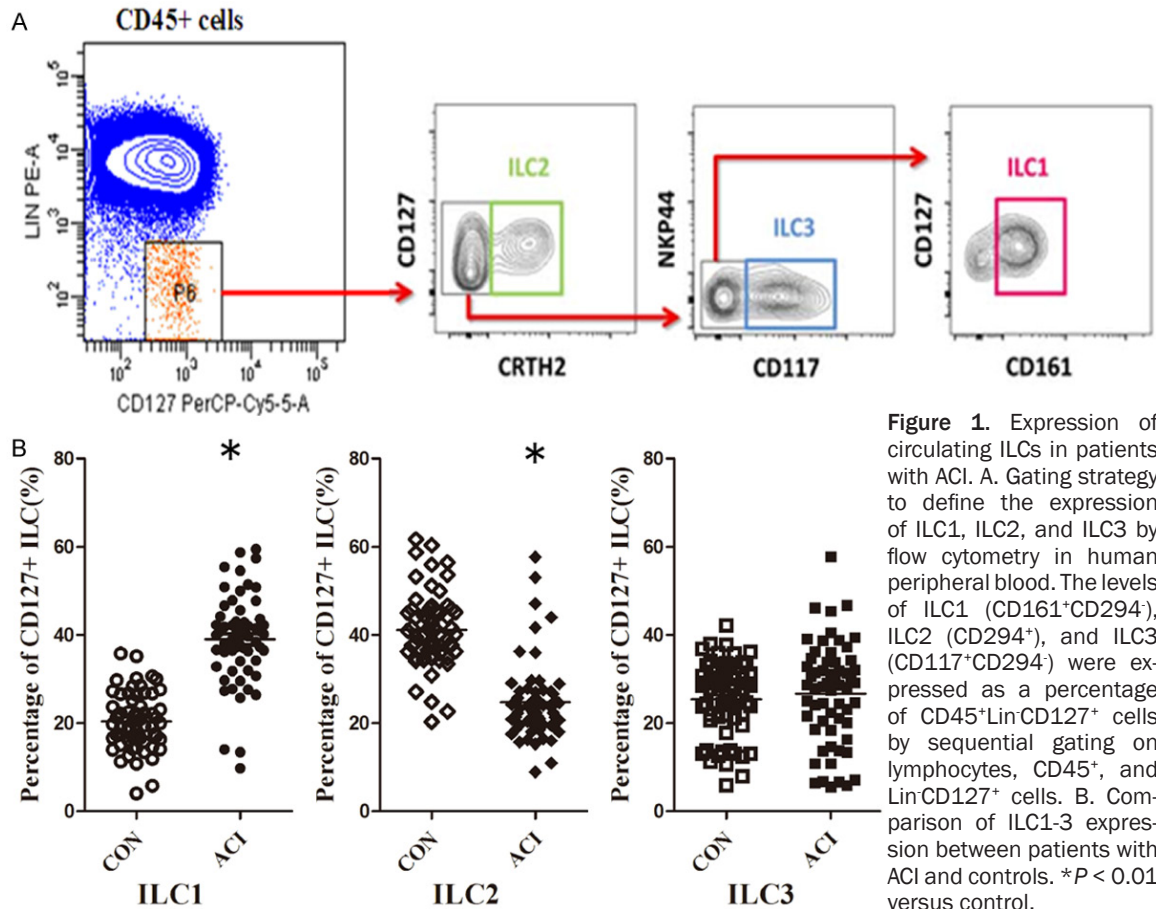


Table 3. Serum levels of cytokines and inflammatory biomarkers in the two groups

	Control (n = 63)	ACI (n = 60)
ox-LDL (μg/L)	315.90 ± 42.70	451.80 ± 69.23 [▲]
Hcy (μmol/L)	8.40 ± 2.85	19.87 ± 3.12 [▲]
Lp(a) (pg/L)	108.92 ± 72.17	231.64 ± 145.21 [#]
CRP (mg/L)	2.91 ± 1.64	54.53 ± 37.92 [▲]

ACI, Acute cerebral infarction; CRP, C-reactive protein; Hcy, homocysteine; Lp(a), lipoprotein (a); ox-LDL, oxidized low-density lipoprotein. [#]*P* < 0.05 versus the control group; [▲]*P* < 0.01 versus the control group.

Decrease in levels of ILC2s and increase in levels of ILC1s in patients with ACI

The level of ILC1 was significantly higher in patients with ACI (39.01 ± 10.06%) than in controls (20.42 ± 6.53%; *P* < 0.01) (Figure 1B). The level of ILC2 was markedly lower in patients with ACI (24.72 ± 8.98%) than in controls (41.13 ± 8.29%; *P* < 0.01). However, no significant difference was observed in the level of ILC3 between patients with ACI (26.69 ± 11.43%)

and controls (25.42 ± 8.36%; *P* > 0.05; Figure 1B).

In addition, the ILC1/ILC2 ratio was markedly higher in patients with ACI (1.72 ± 0.35) compared with controls (0.58 ± 0.24, *P* < 0.01).

Serum levels of ox-LDL and biochemical markers in patients with ACI

The levels of ox-LDL, Hcy, Lp(a), and CRP increased significantly in the ACI group compared with the control group (*P* < 0.05 and *P* < 0.01, respectively; Table 3).

Levels of ILC1 and ILC2 and ILC1/ILC2 ratio correlated with the levels of ox-LDL, LDL-C, and biochemical markers

Notably, the levels of ILC1 and ILC2 and ILC1/ILC2 ratio showed a significant correlation with the levels of ox-LDL, LDL-C, and biochemical markers in the two groups. The level of ILC1 positively correlated with the serum levels of ox-LDL, LDL-C, Hcy, Lp(a), and CRP (*P* < 0.01

Alteration of circulating ILCs in ACI patients

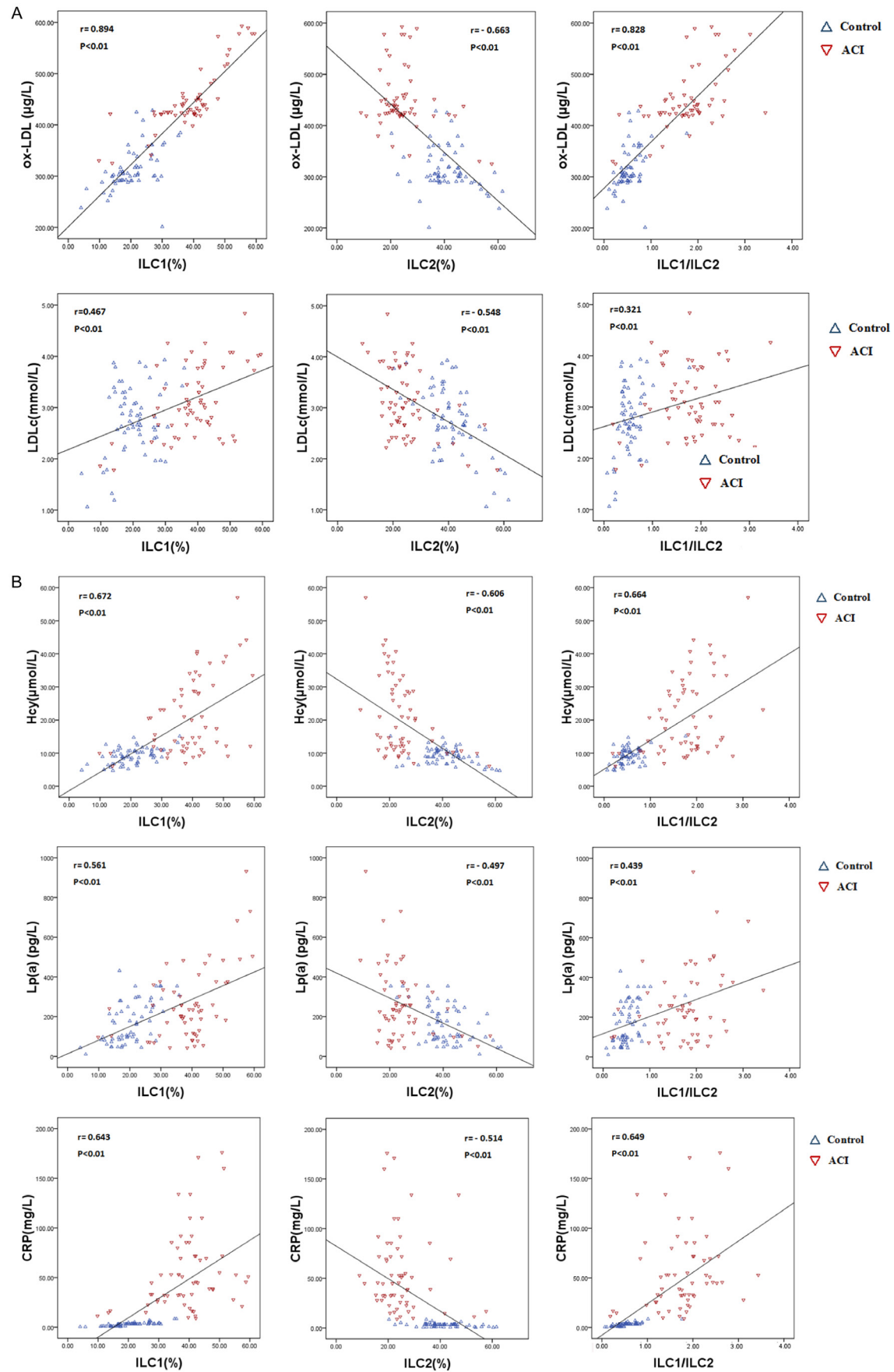


Figure 2. Spearman correlation of the levels of ILC1 and ILC2, and ILC1/ILC2 ratio with the levels of ox-LDL, LDL-C, and biochemical markers. A. Spearman correlation of the levels of ILC1 and ILC2 and ILC1/ILC2 ratio with the levels of ox-LDL and LDL-C. B. Spearman correlation of the levels of ILC1 and ILC2 and ILC1/ILC2 ratio with the levels of biochemical markers.

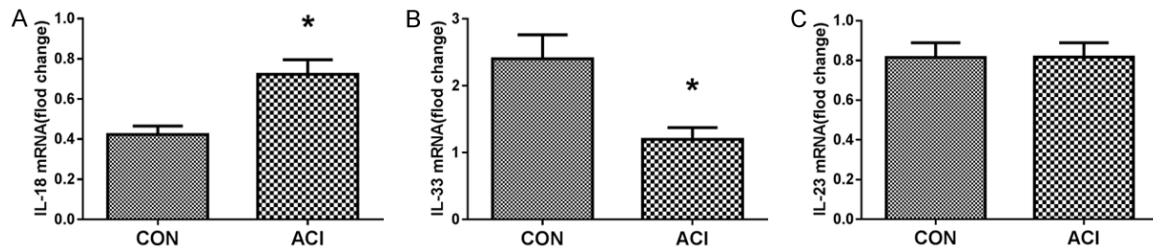


Figure 3. Expression of Interleukin 18 (IL-18), IL-33 and IL-23 in PBMCs from ACI patients and health control. The mRNA levels of cytokines were assessed by RT-PCR subjected to GAPDH. IL-18 mRNA levels in PBMCs were significantly higher in ACI patients than in control subjects (A, $P < 0.01$), IL-33 mRNA levels were markedly lower in ACI patients than in control subjects (B, $P < 0.01$). There were no significant differences between ACI and controls for IL-23 ($P > 0.05$, C). (n = 10 each group * $P < 0.01$ vs. Control).

and $r = 0.894, 0.467, 0.672, 0.561$, and 0.643 , respectively), while the level of ILC2 negatively correlated with the serum levels of five markers ($P < 0.01$ and $r = -0.663, -0.548, -0.606, -0.497$, and -0.514 , respectively). In addition, the ILC1/ILC2 ratio also positively correlated with the levels of ox-LDL, LDL-C, Hcy, Lp(a), and CRP ($P < 0.01$ and $r = 0.828, 0.321, 0.664, 0.439$, and 0.649 , respectively, **Figure 2A** and **2B**).

Expression of Interleukin 18 (IL-18), IL-33 and IL-23 in PBMCs from ACI

IL-18 mRNA levels in PBMCs were significantly higher in ACI patients than in control subjects (**Figure 3A**, $P < 0.01$), while IL-33 mRNA levels were markedly lower in ACI patients than in control subjects (**Figure 3B**, $P < 0.01$). With respect to IL-23 mRNA levels, there were no significant differences between ACI and control groups ($P > 0.05$, **Figure 3C**, n = 10 for each group).

Effects of ox-LDL on ILCs in vitro

The effects of various levels of ox-LDL on the number of ILCs in controls were examined. The level of ILC2 decreased while the level of ILC1 increased with increased levels of ox-LDL. However, no difference was observed in the level of ILC3 in cultured PBMCs derived from the ox-LDL-treated controls (**Figure 4A**).

The sensitivity of the ox-LDL-mediated decrease in ILC2s and increase in ILC1s in patients with

ACI and controls were investigated. Significant changes were noted in the number of ILC1s and ILC2s in ox-LDL-treated patients with ACI (approximately 39.2% reduction in the levels of ILC2s and 41.7% increase in the levels of ILC1s) than in controls (approximately 19.3% reduction in the levels of ILC2s and 28.7% increase in the levels of ILC1s) (**Figure 4B** and **4C**). However, no difference was observed in the numbers of whole ILC cells in cultured PBMCs derived from controls and ox-LDL-treated patients with ACI.

Discussion

ILCs are recently discovered immune cells that display a similar subdivision as $CD4^+$ T cells: T-bet-regulated ILC1, GATA3-regulated ILC2, and ROR γ t-regulated ILC3, similar to T helper (Th)1, Th2, and Th17 cells, respectively. Unlike T cells, the activation of cytokine production by ILCs is not contingent upon an antigenic determinant (ILCs do not express T-cell receptor); rather, it is induced by cytokine signals produced by myeloid or epithelial cells [13].

The present study found a significant increase in the level of ILC1 and a significant decline in the level of ILC2 in patients with ACI compared with controls (a novel finding). Furthermore, a significant positive correlation of the level of ILC1 and a negative correlation of the level of ILC2 with the level of serum ox-LDL were found. These results indicated that the levels of ILC1 and ILC2 in PB were associated with the onset of ACI.

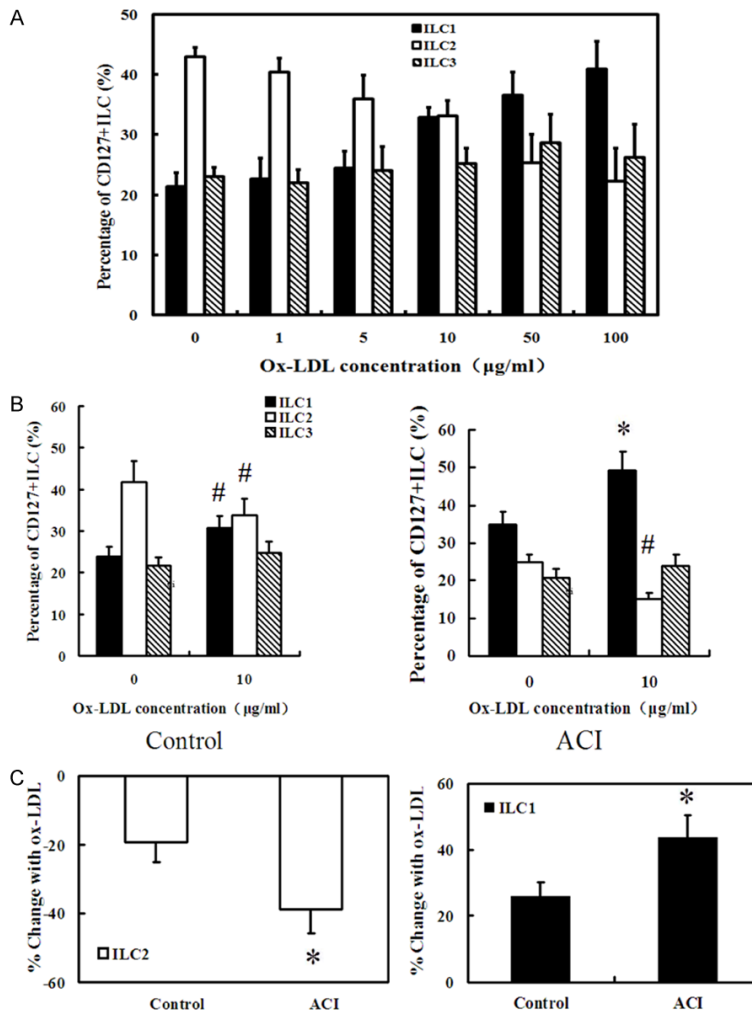


Figure 4. Effects of ox-LDL on the levels of ILCs from controls and patients with ACI *in vitro*. ox-LDL induces a more evident alteration of ILCs from ACI patients. A. A co-culture assay was performed, as described in the Methods section. PBMCs from the control group were incubated with different levels of ox-LDL for 24 h. B. PBMCs from the control and ACI groups were incubated with 10 µg/mL ox-LDL for 24 h. # $P < 0.05$ versus the ox-LDL-untreated group; * $P < 0.01$ versus the ox-LDL-untreated group. C. The average change in ILC levels in the two groups after a 24-h co-culture with 10 µg/mL ox-LDL. * $P < 0.01$ versus the ox-LDL-untreated group.

Only a few studies directly explored the change and role of ILCs in AS. The activation of ILC2s was reported to be associated with a reduced number of atherosclerotic plaques in experimental AS [14]. As a subset of ILCs, natural killer cells were depleted in apoE^{-/-} mice, resulting in a reduction in AS [15]. However, the mechanism underlying the protective role of ILCs in AS requires investigate. The present study showed that the level of ILC1 was significantly higher while the level of ILC2 was significantly lower in patients with ACI than in controls (both $P < 0.01$), suggesting that ILC1 was

atherogenic while ILC2 was atheroprotective.

Our RT-PCR results of cytokine levels from ACI and controls suggested that the alterations of ILC subgroups may affect the progression and outcome of ACI. With the increase of ILC1 expression, high levels of inflammatory factors, such as IFN- γ and TNF- α , were secreted, which can cause inflammatory tissue damage and promote ACI development. In contrast, the levels of ILC2 decreased with the production of ILC2-related cytokines, such as IL-5, IL-10 and IL-13 reduced, which lead to the ability of ILC2 to inhibit ACI occurrence was depressed.

The roles of ILC3- and ILC3-associated cytokines on AS have been inconsistent, and the presence of ILC3s in murine and human atherosclerotic lesions remains uncertain. ILC3s produce a range of cytokines, including Th17. The pro-inflammatory properties of IL-17 in AS have been reported [16, 17]. Other studies have indicated the genetic deletion of IL-17 in the AS model resulting in reduced atherosclerotic lesions [18]. However, the study by Taleb et al. showed decreased AS after IL-17 treatment and increased atherosclerotic lesions after blocking

anti-IL-17 antibody injections [19]. The present study also showed no significant change in the level of ILC3, and its mechanism remains to be further explored.

The mechanisms underlying the changes of ILCs in the patients with ACI are not clear. In the ACI, necrotic neurons release damage-associated molecular patterns (DAMPs), and these molecules release into brain and peripheral blood and activate monocytes/macrophages or myeloid cells via pattern-recognition receptors and inflammasomes [20]. The activated mono-

cytes/macrophages or myeloid cells contribute to enhance early inflammatory process via the release of proinflammatory cytokines to activate innate immune cells such as ILCs. It is reported that IL-18 is able to induce the production of ILC1s and the secretion of IFN- γ , and IL-23 is able to generate and activate ILC3s, while IL-33 can promote the formation and function of ILC2s [21, 22]. Our research found that, at the occurrence of ACI, the expression of IL-18 mRNA in PBMCs was significantly increased while the expression of IL-23 mRNA was decreased markedly as compared to control group. Interestingly, there was no significant difference in the expression level of IL-23 mRNA between the 2 groups. The change trends of related key cytokines were highly consistent with the variation of ILC subsets. The results indicated that increase of above key cytokines from monocytes/macrophages or myeloid cells in PBMCs could be the reason for the alteration of ILC subsets at the occurrence of ACI.

The possible mechanisms underlying the alteration of ILC1 and ILC2 to ACI were correlated with the secretion of inflammatory cytokines as observed in this study. However, further studies may require for understand the mechanisms of ACI and ILCs. Ox-LDL, a key pathogenic factor for the development of AS, was found to have an effect on the levels of ILC1s and ILC2s *in vitro*.

Serum LDL-C is one of the established risk factors for AS and coronary artery disease [23]. After oxidation, LDL becomes more toxic and plays a key role in AS development [24]. Ox-LDL is reported to be a pathogenic factor in AS. The levels of ox-LDL have a high correlation with the levels of ILC1 and ILC2, indicating that the level of ox-LDL is linked to the variation of levels of ILCs in ACI. Certainly, this study found that ox-LDL directly induced the alternation of ILC1s and ILC2s. This might be a novel mechanism involved in increasing the risk of plaque rupture in AS and the occurrence of ACI through ox-LDL.

Biochemical markers related to AS are crucial in the diagnosis and management of patients with ACI. Ox-LDL is considered an instrumental factor that promotes atherogenesis and ACI occurrence [25]. Hcy, which is relevant to carotid plaque occurrence, is an important biomarker for ACI [26]. Lp(a) is associated with inflammatory reactions, which are considered to be

an important factor for plaque instability [27]. CRP is a well-known inflammatory biomarker that reflects proinflammatory responses [28]. In the present study, the level of ILC2 negatively correlated with the serum levels of ox-LDL, CRP, Lp(a), and Hcy, while the level of ILC1 and ILC1/ILC2 ratio positively correlated with the serum levels of the four markers. The levels of ILC1 and ILC2 or ILC1/ILC2 ratio may be considered an indicator in diagnosing ACI.

In conclusion, a significant alteration of ILC1 and ILC2 occurred in patients with ACI. The levels of ILC1s and ILC2s appeared to be a new and sensitive indicator for the early diagnosis of ACI. Ox-LDL had a direct effect on the alteration of ILC1s and ILC2s, contributing to the occurrence of ACI.

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Disclosure of conflict of interest

None.

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