Association of the Leukocyte Immunoglobulin-like Receptor A3 Gene With Neutrophil Activation and Disease Susceptibility in Adult-Onset Still’s Disease

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Objective. Adult-onset Still’s disease (AOSD) is a severe autoinflammatory disease. Neutrophil activation with enhanced neutrophil extracellular trap (NET) formation is involved in the pathogenesis of AOSD. Functional leukocyte immunoglobulin-like receptor A3 (LIR-A3; gene name LILRA3) has been reported to be associated with many autoimmune diseases. We aimed to investigate the association of LILRA3 with disease susceptibility and neutrophil activation in AOSD.

Methods. The LILRA3 deletion polymorphism and its tagging single-nucleotide polymorphism rs103294 were genotyped in 164 patients with AOSD and 305 healthy controls. The impact of LILRA3 on clinical features and messenger RNA expression was evaluated. Plasma levels of LIR-A3 were detected using enzyme-linked immunosorbent assay (ELISA), and the correlation between LIR-A3 plasma levels and disease activity and levels of circulating NET-DNA was investigated. LIR-A3–induced NETs were determined using PicoGreen double-stranded DNA dye and immunofluorescence analysis in human neutrophils and a neutrophil-like differentiated NB4 cell line transfected with LIR-B2 small interfering RNA.

Results. The findings from genotyping demonstrated that functional LILRA3 was a risk factor for AOSD (11% in AOSD patients versus 5.6% in healthy controls; odds ratio 2.089 [95% confidence interval 1.030–4.291], P = 0.034), and associated with leukocytosis (P = 0.039) and increased levels of circulating neutrophils (P = 0.027). Functional LILRA3 messenger RNA expression was higher in the peripheral blood mononuclear cells (P < 0.0001) and neutrophils (P < 0.001) of LILRA3+/− patients. Plasma levels of LIR-A3 were elevated in patients with AOSD (P < 0.0001) and correlated with disease activity indicators and levels of circulating NET–DNA complexes. Finally, enhanced NET formation was identified in neutrophils from healthy controls and patients with inactive AOSD after stimulation of the neutrophils with LIR-A3. Moreover, NET formation was impaired in NB4 cells after knockdown of LILRB2 gene expression.

Conclusion. Our study provides the first evidence that functional LILRA3 is a novel genetic risk factor for the development of AOSD and that functional LIR-A3 may play a pathogenic role by inducing formation of NETs.

INTRODUCTION

Adult-onset Still’s disease (AOSD) is a rare but clinically well-known multisystemic autoinflammatory disorder. It is typically characterized by a high spiking fever, evanescent skin rash, polyarthralgia, sore throat, leukocytosis, and hyperferritinemia. The etiology of AOSD is still elusive, though there is evidence that a complex interaction between genetic factors of disease...
susceptibility and environmental triggers contribute to the pathogenesis of AOSD (1). Associations of HLA antigens, including HLA class I and class II, with AOSD have been described in different ethnic groups (2). Our previous genome-wide association analyses indicated that both HLA class I and class II regions were susceptibility loci for AOSD in Chinese populations (3). The molecular mechanism of HLA class I and HLA class II in the pathogenesis of AOSD remains to be determined.

The leukocyte immunoglobulin-like receptor (LIR) family is a group of HLA class I receptors expressed mainly by monocytes and macrophages (4). LIRs are divided into activating receptors (LIR-As) and inhibitory receptors (LIR-Bs) (4). LIR-A3 is the only member of its family that exists as a soluble receptor due to a lack of a transmembrane domain and cytoplasmic tail. Interestingly, the LILRA3 gene, located in chromosome 19q13.4, is unique and the only LILR gene that shows genetic diversity (5). It exhibits a 6.7-kb deletion polymorphism by removing the first 6 of a total of 7 exons, the Ig-like domains of the gene, which produces a nonfunctional putative truncated form (6). The deletion occurs at an extremely higher frequency in Northeastern Asian populations (56–84%) than in European populations (17%) or African populations (10%) (7), with a frequency of 70–90% previously reported in one Chinese population (8).

Functional LILRA3 has been reported to be associated with susceptibility to and disease severity of many autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), primary Sjögren’s syndrome (primary SS), ankylosing spondylitis (AS), multiple sclerosis (MS), and Takayasu arteritis, among others (8–14). A higher frequency of functional LILRA3 has been observed in Chinese patients with RA, conferring greater risk for RA in male patients and a predisposition toward anti–citrullinated protein antibody–positive RA (10). The serum level of LIR-A3 is also significantly increased in RA patients and correlated with disease activity. Moreover, functional LILRA3 is defined as a factor of disease susceptibility in SLE and primary SS, and levels of LIR-A3 in both serum and CD14+ monocytes were significantly increased in SLE and correlated with disease activity (11,14). In addition, functional LILRA3 appears to be a strong genetic risk factor for susceptibility to AS, mainly in the Northern Han subpopulation, and typically confers an increase in the severity of disease activity (8). However, the association between LILRA3 and AOSD has not yet been illustrated.

Neutrophil activation is also increasingly recognized as the major cause of amplified inflammation in AOSD (2). Some studies have reported the effects of LIRs on neutrophils. For example, inhibitory LIRs, such as LIR-B1/2/3, may provide a checkpoint for neutrophil activation and negatively modulate neutrophils (15,16). Nevertheless, LIR-A3 may act as a competitive antagonist for LIR-Bs (17). So, a link between LIR-A3 and the activation of neutrophils may exist. Neutrophil extracellular trap (NET) complexes are web-like structures released by neutrophils, and their formation (NETosis) is a special form of neutrophil activation (18). In a previous study, we demonstrated accelerated NET formation in AOSD (19). Given the potential correlation between LIR-A3 and neutrophil activation, we now aim to determine whether LIR-A3 can exert effects on NETosis.

In the present study, we were interested in the potential association between AOSD and LILRA3. We therefore investigated the association of functional LILRA3 with disease susceptibility in AOSD and explored its potential pathogenic mechanism by stimulating activation of neutrophils via NETosis.

**PATIENTS AND METHODS**

**Study population.** The first cohort in which LILRA3 genotyping was performed included 164 patients with AOSD and 305 healthy controls of Han Chinese ethnicity. The second cohort consisted of 128 patients with AOSD (84 with active AOSD and 44 with inactive AOSD) and 103 healthy controls. Clinical characteristics of the 2 cohorts are summarized in Supplementary Tables 1 and 2, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract.

All patients fulfilled the criteria for adult Still’s disease (20) after exclusion of those with infectious, neoplastic, and other autoimmune disorders. All healthy controls were age- and sex-matched volunteers with no history of autoimmune, rheumatic, or other diseases. Disease activity in AOSD was assessed using a modified Pouchot’s score (21). Patients were considered as having active AOSD if they had fever, arthralgia/arthritis, any suggestive skin lesions, and/or sore throat. The design of the study and the protocol in which biologic samples were obtained were approved by the Institutional Research Ethics Committee at Ruijin Hospital (2016–62) (Shanghai, China). The study was performed in accordance with the Declaration of Helsinki and the guidelines for good clinical practice. All study participants provided written informed consent.

**Genotyping of LILRA3 6.7-kb deletion and single-nucleotide polymorphism (SNP) rs103294.** Genotyping for the presence or absence of LILRA3 deletion was performed by sequence-specific polymerase chain reaction (PCR) from a previous study (see Supplementary Materials for detailed information on genotyping [http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract]) (11). SNP rs103294 was genotyped using the TaqMan genotyping assay as previously described (11).

**LILRA3 transcription quantification.** Peripheral blood mononuclear cells (PBMCs) from 32 patients with AOSD (LILRA3+/− [n = 6], LILRA3++ [n = 16], and LILRA3−− [n = 10]) and neutrophils from 24 patients with AOSD (LILRA33+/− [n = 3], LILRA33+− [n = 8], and LILRA33−− [n = 13]) were isolated to assess LILRA3 messenger RNA (mRNA) expression. Total RNA was extracted using TRIzol reagent (Takara) and complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit (Takara). Expression levels of mRNA were evaluated by quantitative reverse transcription–PCR (qRT-PCR) using SYBR Green as previously described.
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(Takara). Relative expression levels of mRNA were quantified using the following equation: amount of LILRA3 mRNA expression = $2^{-\Delta\Delta Ct}$, in which $\Delta Ct$ represents the difference in Ct for LILRA3 relative to GAPDH. Results were multiplied ×1,000. Primers for GAPDH and LILRA3 are listed in the Supplementary Materials, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract.

Detection of LIR-A3 levels in plasma by enzyme-linked immunosorbent assay (ELISA). Quantification of plasma levels of LIR-A3 in patients with AOSD and healthy controls was performed by ELISA (Cusabio) according to the manufacturer’s instructions. ELISA detected both functional and nonfunctional forms of LIR-A3.

Detection of interleukin-1β (IL-1β), IL-6, tumor necrosis factor (TNF), and IL-18. Serum levels of IL-1β, IL-6, IL-18, and TNF in AOSD patients were measured by Meso Scale Discovery electrochemiluminescence assay according to the manufacturer’s instructions.

Quantification of cell-free DNA and NET–DNA complexes in serum of AOSD patients. Cell-free DNA and NET–DNA complexes were quantified in the serum of 41 patients with AOSD using the Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit (Invitrogen) according to the manufacturer’s instructions.

Determination of NET formation. Neutrophils were isolated from AOSD patients and healthy controls, and NET formation was quantified as previously described (19). Neutrophils were cultured in 96-well plates for 3.5 hours in the absence or presence of LIR-A3 (Sino Biological) or 20 mM of phorbol myristate acetate (PMA), and PicoGreen was used to detect total DNA according to the manufacturer’s instruction. Myeloperoxidase (MPO) activity assessment and fluorescence microscopy were also applied in the determination of NET formation (see Supplementary Materials for details on MPO and fluorescence microscopy, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract). In some experiments, 25 nM of diphenyleneiodonium chloride (DPI) was added for 30 minutes and subsequently activated with LIR-A3. NETs induced by LIR-A3 in sera from AOSD patients with different LILRA3 genotypes were also evaluated (Supplementary Materials [http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract]).

NB4 cell transfection and differentiation. NB4 cells, which are obtained from a human acute myeloblastic leukemia cell line, were transfected using lentivirus LIRB2-RNAi interference (Shanghai Genechem Co.) according to the manufacturer’s guidelines. To differentiate NB4 cells into a neutrophil-like state, transfected NB4 cells were treated with 2.5 μM all-trans retinoic acid (ATRA) (Sigma-Aldrich) for 3–5 days.

Statistical analysis. All data were analyzed using SPSS version 22 (SPSS Inc.). Quantitative data are expressed as the mean ± SD. Data with Gaussian distribution were analyzed by unpaired t-test or one-way analysis of variance (ANOVA), whereas nonparametric data were assessed by Mann-Whitney U test or Wilcoxon’s rank sum test. Results are expressed as the odds ratio (OR) with 95% confidence intervals (95% CI). P values less than 0.05 were considered significant.

| Table 1. Association of LILRA3 deletion polymorphism or tagging SNP rs103294 with susceptibility to AOSD* |
|---------------------------------------------------------------|
| Patients with AOSD (n = 164) | Healthy controls (n = 305) | OR (95% CI) | P |
| LILRA3 Allele | | | |
| − | 220 (67.1) | 454 (74.4) | 1.429 (1.063–1.908) | 0.017 |
| + | 108 (32.9) | 156 (25.6) | | |
| Genotype | | | |
| +/− and −/− | 146 (89.0) | 288 (94.4) | 2.089 (1.030–4.291) | 0.034 |
| +/+ | 18 (11.0) | 17 (5.6) | | |
| rs103294 Allele | | | |
| T | 202 (69.7) | 428 (77.0) | 1.457 (1.055–1.995) | 0.020 |
| C | 88 (30.3) | 128 (23.0) | | |
| Genotype | | | |
| CT+TT | 131 (90.3) | 265 (95.3) | 2.179 (1.040–4.650) | 0.047 |
| CC | 14 (9.7) | 13 (4.7) | | |

*Values are the number (%). For the polymorphisms in LILRA3, “−” indicates a 6.7-kb deletion in the LILRA3 gene, while “+” indicates a nondeletion in the gene. Genotypes are shown as heterozygous (+/−) or homozygous (−/− or +/+). SNP = single-nucleotide polymorphism; AOSD = adult-onset Still’s disease; OR = odds ratio; 95% CI = 95% confidence interval.
RESULTS

Functional LILRA3 as a risk factor for AOSD. We first compared the distribution of LILRA3 genotypes in 164 patients with AOSD and 305 healthy controls to determine whether functional LILRA3 might be a susceptibility factor for AOSD. Results showed an increased frequency of functional LILRA3 in AOSD patients compared with healthy controls (11% versus 5.6%, respectively; OR 2.089 [95% CI 1.030–4.291], \(P = 0.034\)) (Table 1). At the allele level, a significant association was also observed (32.9% versus 25.6%; OR 1.429 [95% CI 1.063–1.908], \(P = 0.017\)) (Table 1). To confirm this association, we also genotyped rs103294, a SNP reported to be in strong linkage disequilibrium with LILRA3 (\(r^2 = 0.83\)) (22). Consistent with earlier findings, a significant association between SNP rs103294 and AOSD susceptibility was also found, with the genotype model showing that 9.7% of patients with AOSD versus 4.7% of healthy controls carried rs103294 (OR for association 2.179 [95% CI 1.040–4.650], \(P = 0.047\)) and the allele model showing that 30.3% of patients with AOSD versus 23% of healthy controls carried rs103294 (OR for association 1.457 [95% CI 1.055–1.995], \(P = 0.020\)) (Table 1).

Association of functional LILRA3 with leukocytosis and neutrophilia in AOSD. We investigated the association of functional LILRA3 with clinical characteristics and laboratory values in AOSD. All clinical data on AOSD patients applied in this part of the analysis were recorded during disease onset or flare. After assessing the proportion of functional LILRA3 and rs103294 in patients with different clinical manifestations by logistic regression, myalgia was the only condition found to have a significant association with rs103294 (\(P = 0.034\)) (Table 2).

Table 2. Association of LILRA3 deletion polymorphism with clinical manifestations of AOSD*

| Genotype | Healthy controls | OR (95% CI) |
|----------|-----------------|-------------|
| rs1032924 | rs1032924 | rs1032924 |
| TT + CT, no. (%) | 265 (95.3) | 0.986 (0.116–8.368) |
| CC, no. (%) | 13 (4.7) | 0.313 (0.089–1.103) |
| rs103294 | rs103294 | rs103294 |
| TT + CT, no. (%) | 122 (91.0) | 372 (117–1.185) |
| CC, no. (%) | 12 (9.0) | 27 (9.0) |

* For the genotypes, “−” indicates a 6.7-kb deletion in the LILRA3 gene, while “+” indicates a nondeletion in the gene. Genotypes are shown as heterozygous (+/−) or homozygous (−/− or +/-). See Table 1 for definitions.
† \(P = 0.034\).
When comparing laboratory test results among different LILRA3 genotype subgroups in AOSD patients, we observed significantly higher leukocyte counts (P = 0.039) and neutrophil counts (P = 0.027) as well as an increased erythrocyte sedimentation rate (ESR) (P = 0.038) and increased C-reactive protein (CRP) level (P = 0.040) in patients with homozygous functional LILRA3 than in carriers of nonfunctional LILRA3 (Figures 1A–D). These findings provide a basis for the relationship between LIR-A3 and neutrophil activation. Ferritin levels and the modified Pouchot’s score of systemic disease in AOSD patients were not correlated with the presence of functional LILRA3 (data not shown).

**Association of LILRA3 variation with its mRNA expression.** We detected the levels of LIR-A3 mRNA expression in PBMCs from 32 AOSD patients (LILRA3+/+ [n = 6], LILRA3+/− [n = 16], and LILRA3−/− [n = 10]) and neutrophils from 24 patients with AOSD (LILRA3+/+ [n = 3], LILRA3+/− [n = 8], and LILRA3−/− [n = 13]). We also compared LILRA3 mRNA expression from PBMCs and neutrophils obtained from 14 patients with AOSD who were paired for analysis. Expression of messenger RNA for LILRA3 in both PBMCs and neutrophils was significantly increased among AOSD patients who were homozygous for functional LILRA3 compared with carriers of nonfunctional LILRA3 (Figure 1E). We also demonstrated that LILRA3 mRNA expression was higher in neutrophils than in PBMCs from paired individuals (Supplementary Figure 1, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract).

**Elevated plasma levels of LIR-A3 in AOSD, especially active AOSD.** Plasma levels of LIR-A3 in 128 patients with AOSD and 103 healthy controls were assessed by ELISA. Significantly higher plasma levels of LIR-A3 in patients with AOSD compared to healthy controls were observed (mean ± SD 611.3 ± 84.41 ng/ml versus 0.6705 ± 0.06732 ng/ml, respectively; P < 0.0001) (Figure 2A).

We next investigated whether LIR-A3 could assess disease activity in patients with AOSD. Patients with AOSD were divided into subgroups of active disease and inactive disease. Significantly elevated levels of LIR-A3 were detected in patients with active AOSD compared to patients with inactive AOSD (880.0 ± 117.6 ng/ml versus 98.43 ± 30.75 ng/ml, respectively; P < 0.0001) (Figure 2B), whereas no difference in LIR-A3 levels was found between patients with inactive AOSD and healthy controls.

We also compared plasma levels of LIR-A3 before and after treatment in 9 patients with AOSD. During this serial follow-up, LIR-A3 levels significantly decreased after treatment (P = 0.0323) (Figure 2C). Thus, we speculate that LIR-A3 levels in the plasma could serve as a biomarker of disease activity. Correlation analysis

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**Figure 1.** Association of LILRA3 deletion polymorphism with clinical laboratory findings and LILRA3 mRNA expression. A–D. White blood cell (WBC) counts (A), neutrophil counts (B), erythrocyte sedimentation rate (ESR) (C), and C-reactive protein (CRP) levels (D) in patients with adult-onset Still’s disease (AOSD) who are carriers of homozygous functional LILRA3 (+/+; n = 6), LILRA3+/− (n = 16), and LILRA3−/− (n = 10) and neutrophils from 24 patients with AOSD (LILRA3+/+ [n = 3], LILRA3+/− [n = 8], and LILRA3−/− [n = 13]). We also compared LILRA3 mRNA expression from PBMCs and neutrophils obtained from 14 patients with AOSD who were paired for analysis. Expression of messenger RNA for LILRA3 in both PBMCs and neutrophils was significantly increased among AOSD patients who were homozygous for functional LILRA3 compared with carriers of nonfunctional LILRA3 (Figure 1E). We also demonstrated that LILRA3 mRNA expression was higher in neutrophils than in PBMCs from paired individuals (Supplementary Figure 1, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract).
also showed that LIR-A3 levels were positively associated with the modified Pouchot's score of systemic disease ($r = 0.642$, $P < 0.0001$) (Figure 2D). Moreover, correlation of LIR-A3 levels with laboratory values was assessed using Spearman's correlation test, and LIR-A3 levels were found to be positively correlated with all meaningful laboratory values, including the white blood cell count ($r = 0.1985$, $P = 0.029$), neutrophil count ($r = 0.0297$, $P = 0.0279$), ESR ($r = 0.2989$, $P = 0.0011$), CRP level ($r = 0.4064$, $P < 0.0001$), ferritin level ($r = 0.6184$, $P < 0.0001$), and levels of alanine aminotransferase ($r = 0.2717$, $P = 0.0036$) and aspartate aminotransferase ($r = 0.5447$, $P < 0.0001$) (Figure 2D and Supplementary Figure 3 [http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract]), which we attribute to the fact that the ELISA detected both functional and nonfunctional forms of LIR-A3. However, even though nonfunctional LILRA3 carriers had increased plasma levels of LIR-A3, which was mainly composed of nonfunctional LIR-A3, their inflammation indicators, including leukocyte counts, neutrophil counts, ESR, and CRP levels, were still lower than that observed in LILRA3$^{++}$ patients.

Significantly higher levels of LIR-A3 were observed in patients with AOSD with nearly all of the classic symptoms included in the Pouchot's score of systemic disease, except for pleuritis, hepatomegaly, and myalgia (Table 3). The results above suggest that LIR-A3 is correlated with the disease activity level in patients with AOSD and might play a role in the pathogenesis of the disease.

As plasma levels of LIR-A3 in different LILRA3 genotypes were compared, it was found that the LIR-A3 protein was detected in both deletion and nondeletion carriers in the AOSD subgroup. Plasma levels of LIR-A3 were even higher in LILRA3$^{++}$ and LILRA3$^{-/-}$ patients than in LILRA3$^{++}$ patients (Supplementary Figure 3 [http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract]), which we attribute to the fact that the ELISA detected both functional and nonfunctional forms of LIR-A3. However, even though nonfunctional LILRA3 carriers had increased plasma levels of LIR-A3, which was mainly composed of nonfunctional LIR-A3, their inflammation indicators, including leukocyte counts, neutrophil counts, ESR, and CRP levels, were still lower than that observed in LILRA3$^{++}$ and LILRA3$^{-/-}$ patients.
Table 3. Comparison of plasma levels of LIR-A3 according to presence versus absence of clinical manifestations in patients with adult-onset Still’s disease

| Manifestation | No. of patients | LIR-A3 plasma level, ng/ml | P    |
|---------------|----------------|---------------------------|------|
| Fever         |                |                           |      |
| Positive      | 61             | 975.1 ± 136.3             | <0.0001 |
| Negative      | 67             | 280.1 ± 85.6              |      |
| Skin rash     |                |                           |      |
| Positive      | 63             | 1,000.3 ± 147.1           | <0.0001 |
| Negative      | 65             | 234.3 ± 54.9              |      |
| Sore throat   |                |                           |      |
| Positive      | 47             | 983.6 ± 165.4             | <0.0001 |
| Negative      | 81             | 395.3 ± 84.6              |      |
| Arthralgia    |                |                           |      |
| Positive      | 56             | 860.4 ± 140.6             | 0.0087 |
| Negative      | 72             | 417.6 ± 97.7              |      |
| Pleuritis     |                |                           |      |
| Positive      | 18             | 820.7 ± 219.5             | 0.3176 |
| Negative      | 110            | 577.1 ± 91.4              |      |
| Pneumonia     |                |                           |      |
| Positive      | 29             | 1,255.3 ± 222.2           | 0.0001 |
| Negative      | 99             | 422.7 ± 78.8              |      |
| Pericarditis  |                |                           |      |
| Positive      | 17             | 1,149.7 ± 260.5           | 0.0120 |
| Negative      | 11             | 528.2 ± 86.7              |      |
| Hepatomegaly  |                |                           |      |
| Positive      | 6              | 885.9 ± 588.6             | 0.4729 |
| Negative      | 122            | 597.8 ± 84.3              |      |
| Splenomegaly  |                |                           |      |
| Positive      | 36             | 1,117.9 ± 193.9           | <0.0001 |
| Negative      | 92             | 413.1 ± 81.5              |      |
| Lymphadenopathy |            |                           |      |
| Positive      | 68             | 939.2 ± 138.5             | <0.0001 |
| Negative      | 60             | 239.7 ± 60.2              |      |
| Myalgia       |                |                           |      |
| Positive      | 24             | 849.7 ± 219.8             | 0.1759 |
| Negative      | 104            | 556.3 ± 90.4              |      |

* Values are the mean ± SD plasma levels of leukocyte immunoglobulin-like receptor A3 (LIR-A3).

(Figures 1A–D). Furthermore, the plasma level of LIR-A3 in each LILRA3 genotype consistently increased concomitantly with the active disease stage and was correlated with various disease indicators. Therefore, we hypothesize that increased plasma level of nonfunctional LIR-A3 might not play a pathogenic role in AOSD.

Association of LIR-A3 levels with inflammatory cytokine levels. Levels of proinflammatory cytokines such as IL-1β, IL-18, and TNF are typically elevated in AOSD and considered critical players in its disease course. To assess the relationships between LIR-A3 and inflammatory conditions in AOSD, the correlation between plasma levels of LIR-A3 and the levels of various cytokines, including IL-1β, IL-6, IL-18, and TNF, were analyzed by Spearman’s correlation test. Positive correlations were demonstrated between LIR-A3 levels and concentrations of IL-1β (r = 0.3267, P = 0.0019), IL-6 (r = 0.2568, P = 0.0120), IL-18 (r = 0.8051, P < 0.0001), and TNF (r = 0.4376, P < 0.0001) (Figure 2E). Taken together, these data indicate that LIR-A3 may play an important role in inflammatory conditions in AOSD.

Association of LIR-A3 levels with NET components in the serum of patients with AOSD. The potential role of LIR-A3 on NET generation was also investigated. Cell-free DNA, an indirect marker, and NET–DNA complexes in the sera of 21 patients with active AOSD and 20 patients with inactive AOSD were measured. Clinical characteristics of these patients are listed in Supplementary Table 3, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract. Levels of LIR-A3 were positively correlated with levels of cell-free DNA (r = 0.4129, P = 0.0073), MPO–DNA (r = 0.3173, P = 0.0458), and citrullinated histone 3–DNA (r = 0.3229, P = 0.0395) complexes (Figure 3A), suggesting the potential link between LIR-A3 and NETosis in AOSD.

Neutrophil activation by LIR-A3 and formation of NETs. To further determine whether LIR-A3 could mediate NETosis, we isolated peripheral blood neutrophils from patients with AOSD and healthy controls for the analysis of NET release. First, we stimulated neutrophils from 3 healthy controls with different concentrations of LIR-A3 (0.5 μg/ml, 2.5 μg/ml, and 5.5 μg/ml) so that we could choose the most appropriate concentration of stimulating factor. Twenty nM of PMA, a potent NET activator, was used as a positive control. As a result, 500 ng/ml of LIR-A3 was found to be the most effective concentration in stimulating NETosis (P = 0.042) (Supplementary Figure 4A, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract). We then detected the level of NET release by neutrophils from patients with active AOSD, patients with inactive AOSD, and healthy controls (all n = 5) following stimulation with LIR-A3 (500 ng/ml). At baseline, neutrophils from both patients with active AOSD and patients with inactive AOSD displayed significantly enhanced NET formation compared to healthy controls (P = 0.003 and P = 0.0212, respectively) (Figure 3B), and neutrophils from patients with active AOSD exhibited significantly augmented NET formation when compared to patients with inactive AOSD (P = 0.0483). With LIR-A3 treatment, enhanced NET formation of neutrophils from healthy controls (P = 0.0014) and patients with inactive AOSD (P = 0.0127) was observed, but not in neutrophils from patients with active AOSD (P = 0.3061) (Figure 3B), which might be attributed to the existing high level of LIR-A3 stimulation in the plasma of patients with active AOSD. Consistently, immunofluorescence analysis revealed similar findings. Immunofluorescence analysis showed that LIR-A3–stimulated neutrophils from healthy controls and patients with inactive AOSD showed significantly increased NET formation (P = 0.0122 and P = 0.0193, respectively), whereas in patients with active AOSD, the stimulating effect was weaker (P = 0.1059) (Figure 3C). We also determined the level of MPO activity in detached NETs and found that it significantly
increased after stimulation with 500 ng/ml of LIR-A3 (Supplementary Figure 4B, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract).

We stimulated neutrophils from 9 healthy controls with LIR-A3 in the presence of DPI, a NADPH oxidase inhibitor to block reactive oxygen species (ROS). Our results revealed that DPI could significantly suppress LIR-A3–induced elevation of dsDNA (Supplementary Figure 4C). These results further support our hypothesis that LIR-A3 contributes to AOSD pathogenesis by inducing NET formation.

**Mediation of NETosis by functional LIR-A3 in neutrophils from patients with AOSD.** To determine whether LIR-A3 in plasma could mediate NETosis, we stimulated neutrophils from healthy controls with sera from 3 LILRA3+/+ patients with AOSD or 3 LILRA3−/− patients with AOSD before and after adsorption of LIR-A3 and detected NETosis mediated by the neutrophils. It was found that sera from patients homozygous for functional LIR-A3 had a stronger ability to induce NETs than sera from LILRA3−/− patients (Supplementary Figure 5, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract). Furthermore, NET generation mediated by sera from AOSD patients who were homozygous functional LILRA3 carriers was significantly reduced after absorption of LIR-A3, whereas no significant reduction of NET formation was found when the neutrophils were stimulated with absorbed sera from patients who were homozygous non-functional LILRA3 carriers (Figure 3D), suggesting that increased plasma levels of nonfunctional LILRA3 may not have the ability to induce NETs.
LILRA3-induced formation of NETs and its antagonistic effect on LIR-B2. We further investigated the mechanism of LIR-A3-mediated NET induction. It was acknowledged that LIR-A3 might act as a competitive antagonist in response to LIR-B1/LIR-B2. Accumulating evidence has shown that LIR-B2 has a role in neutrophil function. For instance, it was reported that LIR-B2 engagement could inhibit neutrophil phagocytosis and ROS production (23). In our previous study, we have found that enhanced NET formation in AOSD occurs in a ROS-dependent manner (19). So, we hypothesized that the NET-forming ability of LIR-A3 might be due to its antagonistic effect on LIR-B2. To this end, we applied RNAl to knock down the LILRB2 gene by transfecting lentivirus vector on the NB4 cell line, a human acute myeloblastic leukemia cell line, and the cells were then differentiated into a neutrophil-like stage using ATRA. Both PCR and Western blotting were performed to verify the efficacy of LILRB2 knockdown (Supplementary Figure 6, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41635/abstract). Compared with scrambled control small interfering RNA (siRNA)-transfected cells, ATRA-differentiated NB4 cells transfected with LILRB2 siRNA exhibited a significantly higher level of NET formation at baseline, and the ability of LIR-A3 to release NETs was impaired in NB4 cells after knockdown of LIRB2 gene expression. This finding indicates that the NET-forming ability of LIR-A3 may be due to its antagonistic effect in response to inhibitory receptors such as LIR-B2 (Figures 3E and F).

DISCUSSION

In recent years, many studies have shown functional LILRA3 to be a new susceptibility factor for autoimmune and autoinflammatory disease, including RA, SLE, primary SS, and AS in Han Chinese populations. To our knowledge, the present study is the first to undertake determining the genetic association of LILRA3 with AOSD. We identified that functional LILRA3 and its tagging SNP, rs103294, are closely associated with increased susceptibility to AOSD. The significant elevation of neutrophil count, ESR, and CRP level in AOSD patients who were homozygous for functional LILRA3 transcripts in function LILRA3 carriers, indicated a potential pathogenic role of functional LILRA3 in the regulation of AOSD susceptibility and inflammatory response. Furthermore, the potential role of functional LILRA3 to mediate neutrophil activation was confirmed by investigating the ability of LIR-A3 to form NETs. Thus, a new link between HLA antigens and neutrophil activation in the pathogenesis of autoinflammatory conditions was established.

Despite the well-illustrated association between LILRA3 and many autoimmune diseases, the underlying molecular mechanisms remain unclear. LIR-A3 binds both classic HLA-A*0201 and nonclassic HLA-G1 molecules, but with reduced affinities compared to members with similar sequences, such as LIR-B1 and LIR-B2 (17). LIR-A3 also preferentially binds to HLA-C free heavy chain (24). Since LIR-A3 is highly homologous to LIR-B1/LIR-B2 in the extracellular domains, it is predicted to act as an antagonist to these inhibitory receptors via shared ligands and regulate the immune response induced by these receptors. Furthermore, LIR-A3 facilitates immune response by stimulating the proliferation of cytotoxic T cells and natural killer cells and up-regulates a wide range of proinflammatory cytokines, including IL-1β, IL-6, and IL-8 (25). Our study identified a novel proinflammatory role of LIR-A3 in stimulating NETosis in patients with AOSD and healthy controls. The optimal concentration of stimulating factor (500 ng/ml) was around the median plasma level of LIR-A3 in our AOSD patients and has been previously reported to have the best effect on promoting gene expression of proinflammatory cytokines (26). Consistently, we found that the activating effect of LIR-A3 on NETosis was stronger in healthy controls and patients with inactive AOSD than in patients with active AOSD. One possible explanation might be that the plasma level of LIR-A3 in patients with active AOSD was already high enough to reach the best range of stimulating concentration. Further studies are needed to investigate the underlying mechanism of LIR-A3 that activates neutrophils to form NETs.

Neutrophils are of great importance in the pathogenesis of AOSD. One of the most studied aspects of neutrophils is their ability to form NETs that, coated with mitochondrial DNA, facilitate macrophage activation (19). The mechanism of how neutrophils are activated and form NETs in AOSD is still to be determined. We hypothesize that the NET-forming ability of LIR-A3 might be due to its antagonistic effect in response to inhibitory receptors like LIR-B1/LIR-B2. For example, LIR-B2 can inhibit Fc receptor IIa-mediated activation in human neutrophils, reduce neutrophil production of ROS, and suppress phagocytosis of pathogens (15).

Our previous study has shown an elevated level of ROS and NET formation in AOSD. Enhanced NET formation occurs in an ROS-dependent manner in AOSD (19). The LIR-B2 inhibitory function may also affect other forms of neutrophil activation induced by pathogens or inflammation, including migration, cytokine production, and NETosis (15). LIR-A3 may interfere with this inhibitory function and in return amplify NETosis and inflammation. We found that LIR-A3–induced NET formation was impaired in NB4 cells following knockdown of LILRB2 gene. This may provide a new mechanism of how amplification of inflammation occurs in AOSD. Besides, infection, especially viral infection, has also been proved to trigger the initiation or relapse of AOSD, as demonstrated in our previous study (27). It is worth noting that LIR-A3 expression has been recently reported to be activated by Toll-like receptor 8, a sensor of non-self nucleic acids and viruses (26). So, virus infection may tend to trigger the pathogenic role of LIR-A3 on the basis of its genetic susceptibility in AOSD.

In a previous report, functional LILRA3 was found to be associated with leukopenia in SLE. In SLE, it is generally accepted that the main pathogenic immune cells are adaptive immune cells,
including B cells and T cells (28,29). However, abnormal innate immune cell subsets, such as neutrophils, have also been clearly identified in SLE. Neutropenia is a commonly observed symptom in SLE. The mechanisms of neutropenia in SLE may include cell removal driven by neutrophil-reactive autoantibody, autoantibodies neutralizing growth factors on neutrophils such as granulocyte colony-stimulating factor, enhanced neutrophil apoptosis and necrosis, and, possibly, cell death accompanied by NETosis (30).

To date, there is no direct evidence that has revealed the effect of LIR-A3 on neutrophils in SLE patients. LIR-A3 is able to promote the proliferation of T cells (31), which has a predominant pathogenic role in SLE, thus also contributing to neutropenia in SLE. We therefore assumed that the association of LIR-A3 with neutropenia in SLE is mainly caused by its effect on adaptive immune cells and autoimmunity.

Hallmarks of AOSD, an autoinflammatory disease mediated by innate immune cell activation, include neutrophil activation and neutrophilia (1,32). The mechanism of neutrophilia in AOSD is still to be determined. Bone marrow biopsy findings from AOSD patients have exhibited features of granulocytic hyperplasia (33). The number of neutrophils in blood is maintained at a constant level under resting conditions, and in response to inflammatory mediators, emergency myelopoiesis is rapidly switched on, leading to increased neutrophil counts. This can be found in some inflammatory diseases with enhanced NETs, including coronavirus disease 2019 (COVID-19), sepsis, and atherosclerosis. NETs can promote production of inflammatory mediators and further be enhanced by these mediators, leading to an uncontrollable, amplified inflammatory loop in COVID-19 and atherosclerosis (18,34,35).

In our previous study, it was found that NETs promote production of IL-1β, IL-18, and IL-6, which can further enhance NET release, leading to an amplified inflammatory milieu (36). In this study, we found that LIR-A3 could activate neutrophils to release NETs. Therefore, we hypothesized that LIR-A3–induced NETs can further promote amplified inflammatory response, which can enhance emergency myelopoiesis and thus amplify neutrophilia. Notably, neutrophils in both AOSD patients and SLE patients display enriched numbers of low-density granulocytes (LDGs) in the peripheral blood with an activated phenotype and elevated NETs (37–40). The association of LIR-A3 with LDG counts in SLE and AOSD may be interpreted as consistent findings. Taken together, these findings indicate that the mechanism of circulating neutrophil counts is complex and various in SLE and AOSD, and thus, the effect of LIR-A3 on neutrophil activation and NETosis would be comprehensively influenced by other factors, leading to different counts of neutrophils in SLE and AOSD.

One of the limitations of the present study was the modest size of our cohort and the absence of an association between LILRA3 genotype and main clinical manifestations like fever, skin rash, or arthralgia, which might be attributed to the universality of these manifestations in AOSD. Also, we have not yet directly compared the level of NETs in different LILRA3 genotypes because of the rare incidence of functional LILRA3+/- genotype as well as the various levels of disease activity among our patients.

In conclusion, our study provides the first evidence that functional LILRA3 is a new genetic susceptibility factor for AOSD. LIR-A3 has an impact on neutrophil count, ESR, and CRP levels in AOSD patients. Our study further demonstrates the role of LIR-A3 in the pathogenesis of AOSD by stimulating NET formation.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Hu had full access to all the study data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Yang, Hu.

Acquisition of data. M. Wang, M. Liu, Jia, Shi, Teng, H. Liu, Sun, Cheng, Ye, Su, Chi, T. Liu, Z. Wang, Wan, Meng, Ma, Yang.

Analysis and/or interpretation of data. M. Wang, M. Liu, Jia, Hu.

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