

Natural killer cell activity and frequency of killer cell immunoglobulin-like receptors in children with different forms of juvenile idiopathic arthritis

Juan Zhou, Xuemei Tang, Yuan Ding, Yunfei An & Xiaodong Zhao

Ministry of Education Key Laboratory of Child Development and Disorders, Key Laboratory of Pediatrics in Chongqing, Chongqing International Science and Technology Cooperation Center for Child Development and Disorders, Children's Hospital of Chongqing Medical University, Chongqing, China

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Keywords

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Correspondence

Juan Zhou, Department of Immunology, Children's Hospital of Chongqing Medical University, NO.136, Zhongshan 2nd Road, Yuzhong District, Chongqing, China
Tel.: 86 23 6362 1847
Fax: 86 23 6362 2874
E-mail: zhoujuan1112@gmail.com

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Abstract

Background: Juvenile idiopathic arthritis (JIA) has three major onset types with widely varying clinical features: systemic, polyarticular and pauciarticular. We assessed natural killer (NK) cell function and killer cell immunoglobulin-like receptor (KIR) genotypes in patients with different JIA subtypes.

Methods: Peripheral blood samples from 72 children with active JIA (systemic, 25; polyarticular, 24; pauciarticular, 23) and 25 controls were used for flow cytometric assessments of NK cell count, cytotoxicity, perforin, granzyme B, interferon (IFN)- γ and tumour necrosis factor (TNF)- α . Samples from 220 children with JIA (systemic, 84; polyarticular, 72; pauciarticular, 64) and 150 controls were used for *KIR2DS2*, *KIR2DS4*, *KIR3DS1*, *KIR2DL1*, *KIR2DL2*, *KIR2DL3* and *KIR3DL1* typing by polymerase chain reaction with sequence-specific oligonucleotide probes.

Results: Compared with the controls, the patients with systemic JIA showed lower NK cell counts, cytotoxicity and perforin and granzyme B expression ($p < 0.05$), while the patients with pauci- and polyarticular JIA showed higher perforin and granzyme B expression ($p < 0.05$). NK cells produced higher level of TNF- α while lower level of IFN- γ in the pauci- and polyarticular JIA groups than in the systemic JIA group ($p < 0.05$). No significant differences in KIR gene frequencies were found between the JIA subgroups and healthy controls, except for the positive frequency and locus frequency of *KIR2DS4*, which were lower in the systemic JIA group.

Conclusions: Compared with poly- and pauciarticular JIA, systemic JIA is associated with decreased NK cell function, more IFN- γ and less TNF- α secretion of NK cell and lower *KIR2DS4* frequency.

Juvenile idiopathic arthritis (JIA) is a chronic inflammatory disorder with diverse clinical symptoms, both at onset and during the course of the disease. There are at least three major onset types: pauciarticular, polyarticular and systemic. The systemic form is the most distinct clinical subtype of JIA with its markedly extra-articular features of spiking fevers, while the joint disease of pauci- and polyarticular JIA usually overshadows the general symptomatology. Macrophage activation syndrome (MAS), a severe complication of autoimmune diseases and most commonly occurring in systemic JIA (1), is considered to be an acquired form of haemophagocytic lymphohistiocytosis (HLH), which is characterized by diminished natural killer (NK) cell function (2, 3). The reason for the immense variation in clinical features between different

subtypes of JIA is unknown, and we hypothesized that NK cells abnormalities might play a role.

NK cell activation is governed by signalling from a repertoire of activating and inhibitory receptors expressed on the cell surface. The killer cell immunoglobulin-like receptors (KIRs), an NK cell receptor family member, comprise a variable and highly polymorphic multigene family of receptors that includes inhibitory and activating receptors. Recently, KIRs have been found to have associations with autoimmune diseases such as scleroderma, systemic sclerosis and systemic lupus erythematosus (4). However, contradictory roles of KIR genes in the pathogenesis of rheumatoid arthritis (RA) have been reported in different studies based on various ethnic groups (5–7). Moreover, no association between KIRs and JIA

has been reported. The main purpose of this study was to investigate NK cell activity and the polymorphisms of *KIRs* in patients with different subtypes of JIA.

Methods

Patients and controls

A total of 220 children with JIA and 150 age- and gender-matched healthy controls were involved in this study from 2006 to 2013, and the case series was non-consecutive. The diagnosis and classification of JIA were established on the criteria of the International League of Associations for Rheumatology (8). All patients and controls were of the Han ethnicity and without episode of MAS or HLH. Informed consent approved by the medical ethics committee of Chongqing Children's Hospital was obtained from the participants before sampling. The main clinical characteristics of the patients are summarized in Table 1. All patients had not received anti-interleukin therapy. All blood samples were used for KIR genes typing. Among all the patients with JIA, 72 patients (systemic, 25; polyarticular, 24; pauciarticular, 23; 33 girls, 39 boys; ages, 2–14 yr) were newly diagnosed without receiving steroid, methotrexate or tumour necrosis factor (TNF)-blocking agents therapy before sampling. These 72 JIA patients with active arthritis or systemic disease and 25 healthy controls were used for NK cell counts and NK cell cytotoxicity, perforin, granzyme B and intracellular cytokine assays.

NK cell count

Heparinized whole blood samples were surface-stained with anti-human CD3-allophycocyanin (APC; Beijing 4ABiotech, Beijing, China) and anti-human CD56-phycoerythrin (PE)/Cy4 (Biolegend, San Diego, CA, USA). The red cells were then lysed with FACSlyse (BD Biosciences, San Jose, CA, USA) and washed. The resultant white cell pellets were resuspended

and analysed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The NK cell population was defined as CD3⁻CD56⁺ cells.

Cytotoxicity analysis

NK cell cytotoxicity was assayed using procedures similar to those described previously (9, 10). In brief, 10⁶/ml NK-sensitive K562 cells were labelled with 10 µl of 30 mM 3, 3'-diiododecylcarbocyanine perchlorate (DiO; Sigma, St. Louis, MO, USA) at 37°C in 5% CO₂ for 20 min. Peripheral blood mononuclear cells (PBMCs) from heparinized blood were isolated with Ficoll-Paque. PBMCs (effector cells) were mixed with K562 cells (target cells) at an effector:target cell ratio of 5:1 in 2 ml Roswell Park Memorial Institute (RPMI) 1640 medium. After the addition of 10 µl of 10 mg/100 ml propidium iodide (PI; Sigma, St. Louis, MO, USA), the cell mixtures were incubated for 2 h at 37°C in 5% CO₂, and then analysed with flow cytometer. Controls consisted of target cells only plus PI and effector cells only plus PI were used to detect spontaneous lysis and non-viable effector cells, respectively. The percentage lysis was calculated as previously described (10).

Perforin and granzyme B expression

The PBMC samples were surface-stained with anti-human CD3-APC and anti-human CD56-PE/Cy4 for 30 min at 4°C, fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA, USA). The cells were stained with anti-human perforin-PE (eBioscience, San Diego, CA, USA) and anti-human granzyme B-fluorescein isothiocyanate (FITC; Biolegend) at 4°C for 30 min in the dark, and then assessed by flow cytometry. Perforin-positive or granzyme B-positive regions were set using isotype-matched negative control samples, and the percentage of cells positive for each gate was reported.

Table 1 Clinical characteristics of patients with juvenile idiopathic arthritis

	Systemic JIA	Polyarticular JIA	Pauciarticular JIA
Male/Female	36/48	40/32	36/28
Age (yr)	7.32 ± 4.08	9.64 ± 3.87	10.26 ± 4.62
Disease duration (yr)	3.15 ± 1.47	4.32 ± 1.33	2.08 ± 0.87
No. of patients with new diagnosis	25	24	23
No. of patients with active arthritis	12	40	26
No. of joints with active arthritis*	3.27 ± 2.23	9.61 ± 3.42	2.71 ± 1.37
No. of patients with active systemic disease	41	19	1
ESR (mm/h)	48.79 ± 22.21	34.64 ± 16.47	27.46 ± 13.88
DAS28	3.17 ± 1.41	3.51 ± 1.68	2.64 ± 1.03
No. receiving steroid	27	12	4
No. receiving methotrexate	38	42	36
No. receiving NSAIDs	48	43	38
No. receiving TNF-blocking agent	7	8	0

ESR, erythrocyte sedimentation rate; DAS28, disease activity score assessing 28 joints; NSAIDs, non-steroidal anti-inflammatory drugs; TNF, tumour necrosis factor.

*Calculated on the basis of patients with active arthritis.

Intracellular IFN- γ and TNF- α

PBMCs were suspended at a density of 2×10^6 cells/ml in complete RPMI 1640 medium. The cultures were stimulated with phorbol myristate acetate (50 ng/ml) plus ionomycin (1 μ M) in the presence of monensin (500 ng/ml; all from Alexis Biochemicals, San Diego, CA, USA) at 37°C under 5% CO₂ for 5 h. The cells were stained with anti-human CD3-APC and anti-human CD56-PE/Cy4 for 30 min at 4°C, then fixed, permeabilized with Cytofix/Cytoperm and stained with anti-human IFN- γ -PE and anti-human TNF- α -FITC (all from eBioscience) at 4°C for 30 min in the dark. Isotype controls were used to enable correct compensation and confirm antibody specificity. Afterwards, the cells were washed and assessed by flow cytometry. Analysis of the samples consisted of first gating on CD3⁺CD56⁺ cells, followed by an examination of intracellular IFN- γ or TNF- α production presented as positive percentages of cell populations.

KIR gene typing

KIRs were typed by the polymerase chain reaction with sequence-specific oligonucleotide probe (PCR-SSP) method described earlier (11). A salting-out method was used to extract DNA from whole blood samples. Genomic DNA was used as the template, and *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DS2*, *KIR2DS4*, *KIR3DL1*, *KIR3DS1* and *GAPDH* (internal control) were examined using the primer pairs listed previously (11). The PCR mixtures contained 50 ng DNA, 1 U Taq polymerase (Takara Biotechnology, Dalian, China), 5 $\times$ TaqTM DNA polymerase buffer, 1.0 mM each of dNTP, and forward and reverse primers (1 μ M each) in a total volume of 25 μ L. PCR was performed under the following conditions: 60 s at 94°C for one cycle followed by 30 cycles of 10 s at 94°C, 30 s at annealing temperatures of 61–65°C (65°C for *KIR2DL1*, *KIR2DL3*, *KIR2DS2*, *KIR3DL1*, *KIR3DS1*; 63°C for *KIR2DL2*; 61°C for *KIR2DS4*) and extension for 60 or 90 s at 68°C (60 s for *KIR2DL2*, *KIR2DL3*, *KIR3DL1*, *KIR3DS1*; 90 s for *KIR2DL1*, *KIR2DS2*, *KIR2DS4*). Each experiment included negative control and internal control. The PCR products were analysed on a 1.5% agarose gel after electrophoresis. KIR gene locus frequencies were estimated by the formula: locus frequency = $1 - (1 - f)^{1/2}$, where f is the observed frequency of the KIR gene tested for in a population by direct counting.

Statistical analysis

Data were expressed as the mean and standard deviation (SD) and analysed with Statistical Package for the Social Sciences for Windows, version 11.0 (SPSS Inc, Chicago, IL, USA). The analysis of variance (ANOVA) among groups followed by the Student–Newman–Keuls test was used to compare NK cell count, cytotoxicity, perforin/granzyme B expression and intracellular cytokine level between the patient and control groups. Differences in KIR gene frequencies among populations were compared by the chi-square test. A two-sided p value of <0.05 was considered statistically significant.

Results

The percentages and absolute counts of NK cells in different groups are shown in Fig. 1. The patients with systemic JIA had significantly lower proportion ($2.38 \pm 0.95\%$ vs. $10.34 \pm 3.89\%$, $p = 0.01$) and absolute number ($202 \pm 48/\mu$ L vs. $527 \pm 210/\mu$ L, $p = 0.02$) of NK cells than healthy controls. Although the mean NK cell counts in the patients with polyarticular and pauciarticular JIA were lower than those in the controls, the differences were not significant ($p > 0.05$). In NK cell function assays, the cytotoxicity of NK cells was lower in the polyarticular JIA ($15.11 \pm 4.32\%$) and pauciarticular JIA ($13.39 \pm 6.27\%$) groups than in the control group ($17.32 \pm 4.87\%$), but the differences were not statistically significant ($p > 0.05$). However, the patients with systemic JIA showed significantly lower NK cell activity ($6.78 \pm 3.46\%$, $p = 0.02$) than the controls (Fig 2). The cytotoxicity is closely related to cellular perforin and granzyme B; we therefore assessed perforin and granzyme B expression in NK cells. In the systemic JIA, perforin ($60.34 \pm 15.72\%$ vs. $75.63 \pm 11.33\%$, $p = 0.04$) and granzyme B ($52.48 \pm 18.27\%$ vs. $72.66 \pm 10.28\%$, $p = 0.03$) expressions were severely decreased than those in the controls. In contrast, the perforin and granzyme B expressions in NK cells were higher in the pauciarticular JIA ($p = 0.04$) and polyarticular JIA ($p = 0.04$) than in the controls (Fig 3).

The profiles of NK cell-mediated cytokine production in the patients with JIA and controls are shown in Fig. 4. The IFN- γ ($p = 0.03$) and TNF- α ($p = 0.02$) levels in the NK cells of the various JIA groups were significantly higher than those of the healthy controls. But the TNF- α level in NK cells was higher in the pauciarticular JIA ($11.24 \pm 3.38\%$, $p = 0.04$) and polyarticular JIA ($10.69 \pm 3.56\%$, $p = 0.04$) than in the systemic JIA ($8.35 \pm 2.64\%$). In contrast, the intracellular IFN- γ level was

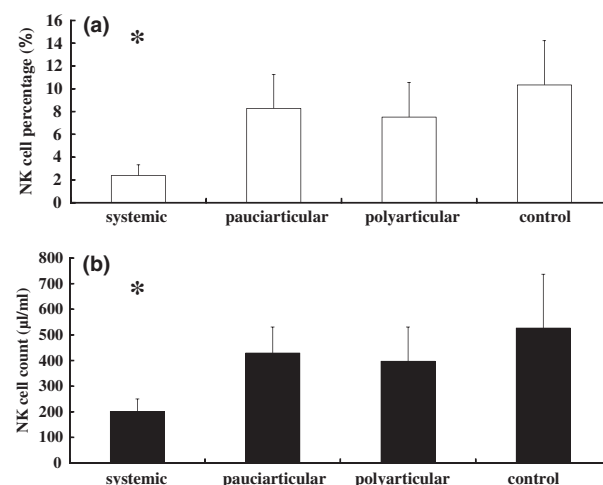


Figure 1 Natural killer (NK) cell counts in children with juvenile idiopathic arthritis (JIA) and healthy controls. Relative frequency (a) and absolute number (b) of peripheral NK cells were counted by flow cytometry. Statistically significant differences between each patient group and the healthy controls are shown as * $p < 0.05$.

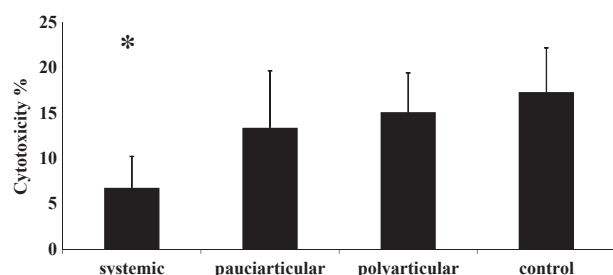


Figure 2 NK cell cytotoxicity in children with JIA and healthy controls. Assays were prepared with a peripheral blood mononuclear cell (PBMC) to NK-sensitive K562 cell (target cells) ratio of 5:1. Statistically significant differences between the patient groups and healthy controls are shown as * $p < 0.05$.

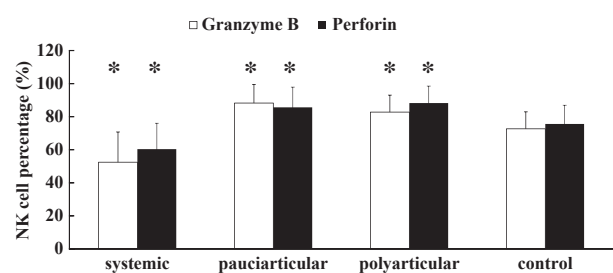


Figure 3 The perforin and granzyme B expression in children with JIA and healthy controls. Percentages of CD3⁺CD56⁺Perforin⁺ cell and CD3⁺CD56⁺Granzyme B⁺ cell assessed using flow cytometry. Statistically significant differences between each patient group and the healthy controls are shown as * $p < 0.05$.

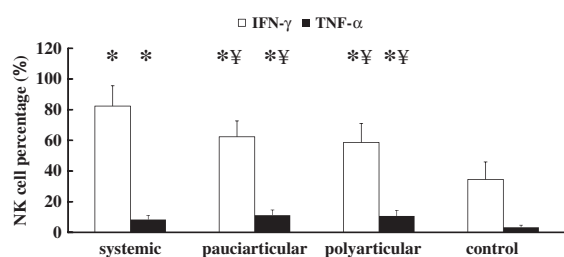


Figure 4 IFN- γ and TNF- α production by NK cells. Percentages of CD3⁺CD56⁺IFN- γ ⁺ cell and CD3⁺CD56⁺TNF- α ⁺ cell in children with active JIA and healthy controls, as assessed using flow cytometry. Statistically significant differences are shown as * $p < 0.05$ vs. healthy subjects, # $p < 0.05$ vs. patients with systemic JIA.

higher in the systemic JIA group ($82.34 \pm 13.29\%$) than in the pauciarticular JIA ($62.37 \pm 10.31\%$, $p = 0.04$) and polyarticular JIA groups ($58.65 \pm 12.38\%$, $p = 0.03$). All differences were significant.

To determine whether the NK cell abnormalities in patients with JIA were associated with changes in NK cell receptor, we assessed the variation in KIR gene expression in the JIA children and controls. Seven groups of activating and

inhibitory KIRs were assayed by PCR-SSP method. The positive internal control *GAPDH* of all samples indicated the credible DNA samples. The positive frequency (f) and locus frequency of KIRs in the patients with JIA and controls are given in Table 2. No significant differences in KIR gene frequencies were found among the JIA subgroups and the controls, except for the positive and locus frequencies of *KIR2DS4* ($p = 0.03$).

Discussion

NK cells play a considerable role in the innate and adaptive immunity, and they have been found to cause autoimmune diseases by damaging normal cells directly or through interaction with other cells (12, 13). Both protective and detrimental roles of NK cells in RA have been reported (14, 15), and the role of NK cells in JIA is being increasingly recognized. In this study, we found that NK cell counts and cytotoxicity were decreased in patients with systemic JIA, which is consistent with the results of previous studies in other autoimmune conditions (16, 17). Interestingly, we found the peripheral NK cell expressions of perforin and granzyme B were severely diminished in children with systemic JIA, but increased in children with poly- or pauciarticular JIA. Other researchers also found that NK cells from patients with systemic JIA expressed less intracellular perforin than those from patients with polyarticular JIA or controls, which could give a reason why systemic JIA might be complicated by MAS (18). We consider that NK cell dysfunction may be a feature that distinguishes systemic JIA from other clinical forms of JIA. The decreased NK cell population and reduced expressions of perforin and granzyme B in patients with systemic JIA caused these patients to have low NK cell cytotoxicity. Recent studies have suggested that perforin- and granzyme B-dependent cytotoxicity was involved in the initiation of tissue damage in RA and collagen-induced arthritis, which is an approved animal model of RA (19–22). The increased peripheral NK cell expressions of perforin and granzyme B in pauci- and polyarticular JIA might contribute to the tissue damage in the joints. The low levels of perforin and granzyme B in systemic JIA may contribute to the dysfunction of immunoregulation by NK cells in these patients. Thus, perforin and granzyme B have been shown to have dual, apparently contradictory roles in JIA inflammation. On one hand, they could promote tissue damage by direct cytotoxicity through CD8⁺ T cells and NK cells. On the other hand, they might provide peripheral tolerance by their immune-regulatory function.

NK cells are the major source of IFN- γ , which is critically important for the systemic and local inflammation in JIA. TNF- α is a key cytokine in the pathogenesis of JIA and is closely related to significant damage and dysfunction of the involved joints. In this study, the NK cells in patients with pauci- and polyarticular JIA primarily secrete TNF- α , which accounts for the severe local arthritis overshadowing the general symptomatology in these patients. In contrast, the NK cells in children with systemic JIA produce mainly IFN- γ , which contributes to markedly febrile presentation with

Table 2 Distribution of KIR genes in patients with juvenile idiopathic arthritis (JIA) and control subjects

KIR	so-JIA (n = 84)			pa-JIA (n = 72)			po-JIA (n = 64)			Control (n = 150)			p
	n	f (%)	Locus frequency	n	f (%)	Locus frequency	n	f (%)	Locus frequency	n	f (%)	Locus frequency	
2DL1	72	85.71	0.62	58	80.56	0.56	52	77.22	0.47	129	86	0.63	0.67
2DL2	30	35.71	0.20	24	33.33	0.18	26	40.62	0.23	45	30	0.16	0.21
2DL3	68	80.95	0.56	64	88.89	0.67	50	78.13	0.53	123	82	0.58	0.52
2DS2	16	19.05	0.10	16	22.22	0.12	14	21.88	0.12	39	26	0.13	0.12
2DS4	40	47.62	0.28	46	63.89	0.40	44	68.75	0.44	120	80	0.55	0.03
3DL1	74	88.10	0.66	56	77.77	0.53	50	78.13	0.54	132	88	0.65	0.35
3DS1	26	30.95	0.17	30	41.67	0.24	22	34.38	0.19	48	32	0.18	0.20

n, number of subjects with a given KIR gene; f, frequency of the KIR gene tested for in the study population; locus frequency = $1 - (1 - f)^{1/2}$.

extra-articular features and rarely arthritis at the onset of the disease in systemic JIA. Thus, the different patterns of NK cell IFN- γ and TNF- α production in the three subgroups of JIA may at least partially account for the immense variation in the clinical features of different JIA subsets. This also might give a theoretical explanation of the rather weaker effect of anti-TNF treatment in patients with systemic JIA than in patients with polyarticular JIA (23, 24).

Then, we investigated whether any genetic background could account for the different NK cell functions in subgroups of JIA. Recently, mutations in familial HLH-associated genes such as the genes encoding perforin (*PRF1*) and Munc13-4 (*UNC13D*) have been reported to play a role in the development of MAS in patients with systemic JIA (25–27). In this study, we assayed the following activating and inhibitory KIRs: *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DS2*, *KIR2DS4*, *KIR3DL1* and *KIR3DS1* by PCR-SSP, and we found that both the positive frequency and locus frequency of *KIR2DS4* were lower in patients with systemic JIA than in patients with poly- or pauciarticular JIA and healthy controls. The frequencies of the other KIR genes in the total JIA group and in the three JIA subgroups did not significantly differ from those in the controls. Thus, the low expression of the activating receptor *KIR2DS4* was a characteristic feature of systemic JIA in our study and may contribute to the NK cell dysfunction in systemic JIA. Further studies involving KIR

gene assays in a large sample of children with JIA are necessary.

In conclusion, we found that NK cell function and KIR genetic polymorphisms differ among patients with pauciarticular, polyarticular and systemic JIA. NK cells from systemic JIA have lower cytotoxicity, decreased perforin and granzyme B, more IFN- γ and less TNF- α productions than those from pauciarticular and polyarticular JIA. KIR gene assays showed that patients with systemic JIA have lower positive frequency of the activating receptor *KIR2DS4*. These differences in NK cell functions and KIR genetic polymorphisms among the various forms of JIA may contribute to the immense variation in the clinical features of JIA and the increased incidence of MAS in patients with systemic JIA in comparison with those with pauci- or polyarticular JIA.

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